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Review Article

The immune niche of the liver

Michael L. Cheng[1,2,*], **Diana Nakib**[1,3,*], **Catia T. Perciani**[1,2,3,‡] and **Sonya A. MacParland**[1,2,3,‡]

[1]Ajmera Family Transplant Centre, University Health Network, Toronto, ON, Canada; [2]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada; [3]Department of Immunology, University of Toronto, Toronto, ON, Canada

Correspondence: Sonya A. MacParland (s.macparland@utoronto.ca) or Catia T. Perciani (c.perciani@mail.utoronto.ca)

The liver is an essential organ that is critical for the removal of toxins, the production of proteins, and the maintenance of metabolic homeostasis. Behind each liver functional unit, termed lobules, hides a heterogeneous, complex, and well-orchestrated system. Despite parenchymal cells being most commonly associated with the liver's primary functionality, it has become clear that it is the immune niche of the liver that plays a central role in maintaining both local and systemic homeostasis by propagating hepatic inflammation and orchestrating its resolution. As such, the immunological processes that are at play in healthy and diseased livers are being investigated thoroughly in order to understand the underpinnings of inflammation and the potential avenues for restoring homeostasis. This review highlights recent advances in our understanding of the immune niche of the liver and provides perspectives for how the implementation of new transcriptomic, multimodal, and spatial technologies can uncover the heterogeneity, plasticity, and location of hepatic immune populations. Findings from these technologies will further our understanding of liver biology and create a new framework for the identification of therapeutic targets.

Background

The liver is the largest solid organ in the body and is responsible for performing several crucial biological functions such as metabolism, detoxification, and protein production [1]. Interestingly, the liver is also highly immune active [2] due to its function as a systemic barrier to gut-draining pathogens and toxins, in addition to its role in maintaining a local immunoregulatory environment that promotes tolerance to frequently encountered antigens. The liver's tolerogenic and immune-rich environment promotes local and systemic homeostasis, in addition to enabling the potential for hepatic regeneration. However, liver homeostasis can be disrupted by viral infections, drug and alcohol-induced liver injury, and autoimmunity. Chronic liver diseases leading to organ failure account for approximately 2 million global deaths annually [3]. The liver contains cells of the adaptive and innate immune systems that are recruited during both homeostasis and disease, however, much remains unknown about the individual and synergistic roles of immune cells in both the etiology and the combating of disease. To understand and treat hepatic diseases, we need to better grasp the constitution and function of the repertoire of cells that compose the organ, in addition to identifying their forms of communication, maintenance, plasticity, and origin. Using advanced tools to decipher the complex and highly regulated network that modulates the hepatic immune response during homeostasis and injury will be pivotal in understanding and targeting liver pathology and repair.

In this review, we discuss (**1**) the metabolic subspecialization of hepatic parenchymal cells, the origins of hepatic immune cells, and their impact on the transcriptional heterogeneity observed within cell populations; (**2**) the functional backbone that sustains the tolerogenic profile of a healthy liver, otherwise known as the hepatic immune microenvironment; (**3**) the importance and potential of utilizing single-cell transcriptomics techniques and spatial profiling methods to explore the liver, and (**4**) how next-generation sequencing techniques are allowing for the systematic interrogation of the cellular composition and functionality of healthy and diseased livers, in relation to altered gene expression signatures.

*These authors contributed equally to this work and should be considered first authors.
‡These authors contributed equally to this work and should be considered senior authors.

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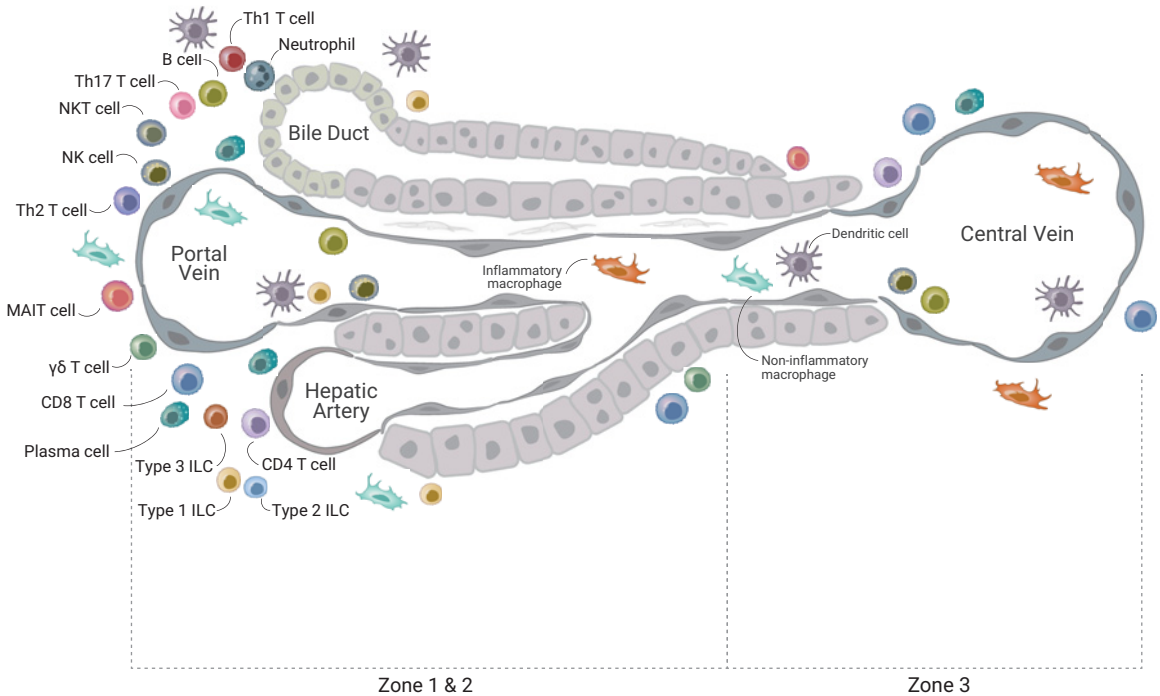

Figure 1. Immunological players in the liver

The liver lobule, the functional unit of the organ, is composed of a portal triad, a capillary network lined by hepatocytes, and a central vein. Zone 1 represents the periportal region, consisting of the portal triad that is made up of the hepatic artery, the hepatic portal vein, and the bile duct. Zone 2 represents the interzonal region, and Zone 3 consists of the pericentral region. The cellular composition of the liver can be categorized as parenchymal cells (hepatocytes) and non-parenchymal cells, including cholangiocytes, hepatic stellate cells, endothelial cells, and immune cells. Almost all types of immune cells can be found in the liver tissue. These immune cells include phagocytes (macrophages, DCs, and neutrophils) and lymphocytes (innate lymphoid cells, T cells, and B cells). Many of these populations currently lack confirmation and consensus regarding their location and definitive frequencies in the liver, thus most of their distribution remains speculative. Abbreviations: DC, dendritic cell; ILC, innate lymphoid cell (type 1, 2, and 3); MAIT, mucosal associated invariant T cell; NK cell, natural killer cell; NKT, natural killer T cell; Th1, type 1 helper T cell; Th2, type 2 helper T cell; Th17, type 17 helper T cell; Th22, type 22 helper T cell; Treg, regulatory T cell.

The liver

The functional unit of the liver, termed the hepatic lobule, is composed of parenchymal cells (hepatocytes) and cholangiocytes, lined with liver sinusoidal endothelial cells (LSECs). These hexagonal lobules contain liver sinusoids that extend from the central vein at the center of the lobule to the portal triads distributed at its vertices, which consist of branches of the hepatic artery, portal vein, and bile duct (Figure 1). With approximately 75% of its blood supplied by the portal vein, the liver is continuously exposed to gut-derived molecules, including pathogens, commensals, and food antigens. Sinusoidal capillaries significantly slow down the blood in the liver, subsequently contributing to effective immunosurveillance and the clearance of toxic metabolites from the blood [4]. Hepatocytes and cholangiocytes perform many of the classical liver functions and rely on non-parenchymal cells, such as LSECs, hepatic stellate cells (HSCs) and liver-resident immune cells, to help maintain a quiescent state [5–7] in response to persistent exposure to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). More recently, particular attention has been paid to the role that the hepatic immune niche has in liver homeostasis, inflammation and regeneration, and how new omics technologies can help uncover this interplay, a topic which is the main focus of this review. Figure 1 summarizes the types of immune cells that have been described in the human liver and the putative locations of these cells in reference to the hepatic sinusoid. Despite the significant advances in our understanding of the liver, studies have only just begun to tease out the distinct contributions and unique phenotypes of different liver-resident parenchymal and non-parenchymal cells. It is important to note that the presence and frequencies of these cell types of interest can vary across experiments as a result of inherent technological biases, differences in tissue processing protocols and bioinformatics pipelines.

The liver performs a vast number of functions, including protein synthesis, glucose and lipid metabolism (gluconeogenesis, glycogen storage, lipogenesis, fatty acid b-oxidation, and lipoprotein metabolism), detoxification, and bile production [1]. Subsequent metabolic products can directly or indirectly engage immunological signaling pathways and contribute to local responses, including function, proliferation, migration, and survival [8]. Hepatic functionality is intrinsically connected to microbial exposure, oxygen tension and nutrient supply, which varies as a gradient along the sinusoid, and contributes to the cellular heterogeneity observed in the liver. Hepatocytes, for example, are commonly segregated into three zones within the hepatic lobule. Hepatocytes close to the portal triad region (Zone 1, Z1, periportal) are the first to interact with gut-derived toxins and microbes, in addition to having access to blood enriched in nutrients and oxygen, and are marked by increased gluconeogenesis and β-oxidation [9]. However, cells close to the central vein areas (Zone 3, Z3, pericentral) are exposed to lower concentrations of nutrients and oxygen and are subsequently associated with detoxification, enhanced glycolysis, and lipogenesis [9]. As expected, the gradient of oxygen and nutrients is reflected in gene expression, with Z1 being enriched in genes that reflect innate inflammatory responses [10]. Not surprisingly, enzymes participating in metabolic processes, also follow a zonation pattern [9]. This metabolic subspecialization featured by the hepatic lobules has two main implications in the characterization of the cellular landscape of the liver, namely that (1) different cell populations that share similar gene expression signatures and pathways can work in collaboration; in addition to the fact that (2) cell populations can exhibit transcriptional heterogeneity depending on their position within the hepatic lobule. As expected, immune cells are an additional layer of complexity that shape the functional and transcriptional heterogeneity of the parenchyma within the liver. A clear understanding of immune cell identity, function, and zonation in hepatic health and disease is required to truly decipher the liver's complexity as an immune organ and to harness hepatic immune cells to promote homeostasis and regeneration.

The hepatic immune microenvironment

The liver is enriched with cells of the innate and adaptive immune systems that maintain tolerance in a homeostatic state and can mediate inflammation and hepatic injury in disease (Figure 1). Adaptive immune cells that are found in the liver include T cells, such as regulatory T cells (Tregs), cytotoxic T lymphocytes (CTLs) and Type 1, 2, 17 and 22 helper T cells (T_h1, T_h2, T_h17, and T_h22) [11–13], in addition to B cell and plasma cell populations. Tregs play a critical role in hepatic tolerance, with the expression of a range of immunoregulatory markers such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4), CD39, and lymphocyte-activation gene 3 (LAG-3). Tregs secrete IL-10 [14] which is a central mediator of the tolerogenic hepatic environment, due to its anti-inflammatory properties and role in limiting immunopathogenesis [15], in addition to being expressed and recognized by many immune and non-immune cells in the liver [16]. Hepatic Tregs also work in synergy with hepatic innate immune cells, such as liver-resident macrophages, known as Kupffer cells (KCs), to promote a local suppressive niche that prevents the development of murine CTL responses [17]. Hepatocytes and LSECs also contribute to the regulation of CTLs, by inducing clonal anergy or the deletion of effector T cells in the liver [18–22].

Cells of the innate immune system that are known to be liver-resident are KCs, natural killer (NK) cells, invariant NKT (iNKT) cells, mucosal-associated invariant T (MAIT) cells [23,24], dendritic cells (DCs), γδ T cells, and innate lymphoid cells (ILCs) [25–27]. Despite the fact that hepatic innate cells are educated in a tolerogenic environment during homeostasis, they participate in the clearance of commonly encountered PAMPs and DAMPs [28–31]. For example, KCs play a central role in maintaining liver homeostasis via phagocytosis, the clearance of dead and senescent cells, and tissue repair [2,6,32–36]. In mice, KCs also produce immunosuppressive metabolites such as prostaglandin E2 (PGE2) and IL-10 [37–39], besides expressing V-set and Ig domain-containing 4 (VSIG4) associated with the induction of tolerance in T and NKT cells [39]. Additionally, in murine models, IL-10 up-regulates programmed death ligand 1 (PD-L1) expression on KCs, which then provides an inhibitory signal to T cells and favors the development of Tregs, further sustaining an IL-10-enriched environment [38,40]. A hepatic environment enriched in macrophage colony-stimulating factor (M-CSF) and hepatocyte growth factor (HGF) favors the development of regulatory DCs ($IL\text{-}10^{+}IL\text{-}12^{-}$), further contributing to an anti-inflammatory murine liver [41,42]. Hepatic DCs exhibit a tolerogenic profile in mice, with the expression of PGE2, the up-regulation of indoleamine dioxygenase (IDO), IL-10, nucleotide-binding oligomerization domain-containing protein 2 (NOD2), and PD-L1 [43,44], in addition to the low expression of major histocompatibility complex (MHC) II and costimulatory molecules [43,45]. Human liver-resident NK cells (lrNK) hold an immature phenotype with low levels of pro-inflammatory cytokines and cytotoxic mediators in agreement with the tolerogenic environment of the liver [46]. Human lrNK cells can respond to increased interferon (IFN)-α (IFN-α) by up-regulating tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), Fas-L and NKG2D, leading to hepatocyte and T cell death [47,48]. Type 1 ILCs (ILC1) contribute

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to the hepatic immunoregulatory environment in mice through the expression of PD-1 and LAG-3, in addition to various other inhibitory receptors [49]. Meanwhile, type 2 ILCs (ILC2) in the murine liver display an immature and naive phenotype during homeostasis [50].

The cross-talk established between parenchymal and non-parenchymal hepatic cells is critical in producing a unified chain of command towards tolerance. Hepatocytes, cholangiocytes, LSECs, and HSCs contribute to hepatic immunological responses through various means, such as by secreting immunomodulatory signals, such as IL-10, transforming growth factor β (TGF-β), HGF, and HSC-derived retinoic acid (RA) in recognition of DAMPs and PAMPs [1,6,7,26,51–58]. LSECs and HSCs promote a tolerogenic hepatic environment through the secretion of IL-10, TGF-β and RA, in addition to the up-regulation of coinhibitory molecules in mice [7,19,52,59–63]. LSECs also exhibit potent endocytic capacity, antigen presentation, and the ability to up-regulate chemokine expression in a pro-inflammatory environment, subsequently contributing to leukocyte recruitment to the human liver [6]. It is quite evident that the liver does most of the heavylifting by actively participating in the removal of toxins and pathogens from circulation. This can be demonstrated by the non-inflammatory manner in which the concentration of LPS is significantly reduced between the portal blood and the peripheral venous blood [64,65]. Figure 2A,B summarizes the contributions of parenchymal and non-parenchymal cells in the induction and maintenance of liver homeostasis regarding both the immunoregulatory (Figure 2A) and pro-inflammatory (Figure 2B) processes. The nature of liver functionality requires an immunoregulatory and anti-inflammatory environment mediated by a complex network of interactions between hepatic tissue (hepatocytes, LSECs, HSCs, and cholangiocytes) and the immune niche of the liver (KCs, DCs, T cells, ILCs, and NKTs).

Hepatic immunity during inflammation

Through the promotion of a regulatory milieu, the liver avoids the collateral damage that an inappropriate inflammatory response can induce in solid organs. However, when the liver's ability to successfully clear the commonly encountered antigens is surpassed, such as in the case of improperly metabolizing toxins or eliminating pathogens, it can break this tolerance and lead to liver inflammation. In such situations, liver-resident cells secrete factors to recruit circulating immune cells as part of the inflammatory response. Both parenchymal and non-parenchymal cells are able to act as professional antigen-presenting cells (APCs) by expressing immunological receptors such as scavenger receptors, Toll-like receptors (TLRs) and MHC class I and II, as well as non-classical MHC proteins contributing to an immunogenic state [1,6,26,51,54–56,58,66,67]. The mechanisms involved in triggering inflammation can vary according to the threat faced. Injured hepatocytes respond by secreting high mobility group protein B1 (HMGB1) and interleukin (IL) 33 (IL-33), as well as being critical for neutrophil recruitment to the murine liver [68]. IL-33 also stimulates hepatic ILC2 activation and proliferation in mice, in addition to up-regulating KLRG1 and CD25, and promoting the secretion of inflammatory cytokines, IL-5 and IL-13 [50]. Neutrophil infiltration aggravates local inflammatory processes due to their secretion of cytotoxic reactive oxygen and nitrogen species, IL-1β and TNF [69]. Neutrophils also up-regulate the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), in addition to promoting the release of chemokines [69–71]. Necrotic cells also release extracellular DNA and ATP, which can trigger TLR9, in addition to the NOD, LRR-, and pyrin domain-containing protein 3 (NLPR3) inflammasome complex in hepatic macrophages to induce a pro-inflammatory response [72,73]. In response to tumor-transformed or virally infected cells, the presence of IL-12 and IL-18 contributes to the activation of NK cells, which ultimately promotes the production of IFN-γ and perforin [74,75]. IL-17A/F and IL-22 secretion by murine hepatic ILC3s has been shown to be critical in the combatting of hepatic viral infections, given its role in priming T cells and promoting antimicrobial peptide production [76,77]. A pro-inflammatory environment within the liver can lead to the suppression of PD-L1 and the subsequent shift of T cells towards a T_h1 response [22]. Although the cellular microenvironment in the liver is tolerant to low levels of LPS [18], high levels of LPS can activate macrophages and HSCs, leading to the up-regulation of adhesion molecules, such as ICAM-1, as well as pro-inflammatory cytokines and chemokines [78].

Resolution of hepatic inflammation

After an inflammatory response, the liver initiates self-rescue mechanisms to limit tissue damage, such as through the phagocytosis of apoptotic bodies [79–81], autophagy [81–84], and an increased frequency of intrahepatic Tregs [85,86]. Resident macrophages contribute to this compensatory anti-inflammatory response by increasing the expression of PD-L1, reducing the levels of costimulatory molecules, and favoring the development of Tregs [87]. Importantly, liver-resident macrophages that originate from the yolk sac and fetal liver monocytes [88–91] are capable of self-renewal within the human liver [92]. However, in extraordinary circumstances, circulating monocytes

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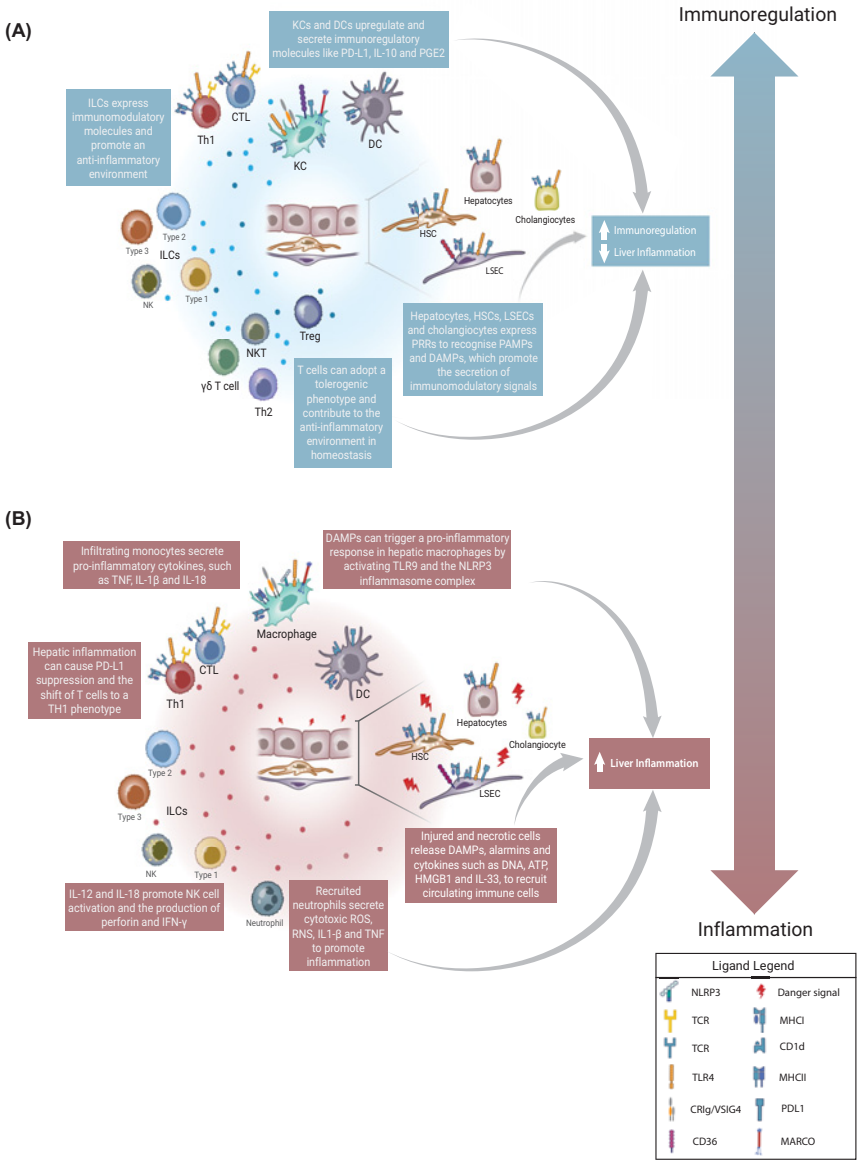

Figure 2. Immune niche of the liver at homeostasis

Depiction of the pro-inflammatory and anti-inflammatory processes involved in the maintenance of a homeostatic environment in the liver. (**A**) **Promotion of a tolerogenic and immunoregulatory hepatic environment.** Hepatocytes, HSCs, LSECs, and cholangiocytes express PRRs to detect PAMPs and DAMPs in the liver, which in turn promote the up-regulation and release of immunomodulatory molecules, in addition to promoting the expression of PD-L1, and the release of IL-10 and PGE2 from innate immune cells (KCs and DCs). The immunoregulatory environment induces ILCs, T cells, and NKT cells to adopt a tolerogenic phenotype to further promote an anti-inflammatory environment. (**B**) **Promotion of hepatic inflammation**. Injured parenchymal cells secrete DAMPs and alarmins that contribute to the recruitment of circulating immune cells. DAMPs can trigger TLR9 and the NLRP3 inflammasome complex in hepatic macrophages to induce a pro-inflammatory response. Infiltrating monocytes further promote the inflammatory environment and secrete inflammatory cytokines like TNF, IL-1β, and IL-18. Recruited neutrophils secrete cytotoxic reactive oxygen and nitrogen species, IL-1β and TNF. The IL-12- and IL-18-rich environment contributes to the activation of NK cells, which ultimately promotes the production of IFN-γ and perforin. A pro-inflammatory environment within the liver can lead to PD-L1 suppression and the subsequent shift of T cells towards a Th1 response. Abbreviations: CRIg, complement receptor of the immunoglobulin superfamily; CTL, cytotoxic lymphocyte; DAMP, danger-associated molecular pattern; HMGB1, high mobility group box protein 1; ILC, innate lymphoid cell (type 1, 2, and 3); IL, interleukin; MARCO, macrophage receptor with collagenous structure; NKT, natural killer T cell; NLRP3, NOD-, LRR-, and pyrin domain-containing protein 3; PD-L1, programmed death-ligand 1; RNS, reactive nitrogen species; ROS, reactive oxygen species; TCR, T-cell receptor; TLR9, toll-like receptor 9.

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have been shown in mice to engraft and replace typical liver-resident macrophages [93]. In the murine spleen, these monocyte-derived macrophages appear to originate from the myeloid progenitor committed to the macrophage/DC precursors (CX3CR1^{+}MDC) [94,95]. In later stages of murine fibrosis resolution, partially induced by phagocytosis [96], the monocyte-derived macrophages can undergo a phenotypic switch from profibrotic (CD11b^{hi} F4/80^{int} Ly-6C^{hi}) to scar-resolving (CD11b^{hi} F4/80^{int} Ly-6C^{lo}), as characterized by higher matrix metallopeptidases (MMP9, MMP12, MMP13) that clear additional extracellular matrix (ECM) [97]. This phenomenon has also been observed in other tissues, such as in murine muscle tissue [98], skin [99], and human lungs [100]. Recent work has highlighted the role of the immune microenvironment in directing either tissue repair or fibrosis [101–103]. For instance, distinct cues from the murine skeletal muscle microenvironment, such as IL-4 or IL-17, can favor macrophage polarization into clusters that are able to coordinate immune cells to be more pro-regenerative or pro-fibrotic, respectively [102]. Secretion of IL-6 by KCs in the liver is recognized as a potent hepatocyte mitogen that promotes hepatocyte proliferation and contributes to liver regeneration [104]. Additionally, Wnt signaling, HGF, epidermal growth factor (EGF), TGF-β, and nitric oxide synthase have also been shown to play a key role in liver regeneration [105–109]. Further understanding of how environmental cues prompt cells to phenotypically and transcriptionally replace lost or compromised liver-resident cells can contribute to the design of novel therapeutic approaches. However, certain pathological conditions, such as those associated with chronic inflammation, can impair the liver's ability to restore homeostasis and repair the tissue, ultimately leading to the development of end-stage liver diseases. Immunological mechanisms involved in liver diseases, such as cholestatic disease, viral hepatitis, non-alcoholic steatohepatitis (NASH), are reviewed by Heymann and Tacke [87]. Overall, it is evident that the tolerogenic environment maintained in the liver at homeostasis is a result of a complex web of interactions between parenchymal and non-parenchymal liver-resident cells that lead to the promotion of immunoregulatory immune cell phenotypes, and the expression of immunomodulatory cytokines, chemokines, and proteins. In circumstances where this balance is overthrown, a dramatic shift in the hepatic immune microenvironment occurs, wherein secreted cytokines and immune cells adopt a more pro-inflammatory phenotype, in an attempt to clear the aggravating pathogen or toxin. It is essential that this inflammation is controlled in a timely and effective manner because if not, the liver can be subject to a cycle of chronic inflammation, fibrosis, and scarring that incurs irreparable damage and increased risk of disease and cancer development [110].

Immune cell zonation in the liver

As mentioned above, the division of labor in the liver is highly zonated, and as such, the location of immune cells that support these functions within the liver is equally as important. It is accepted that immune cell localization is determined by interactions between surface receptors and the gradients of their chemokine ligands, in addition to adhesion molecule expression throughout the liver, such as C–C chemokine receptor type 6 (CCR6) binding to chemokine (C–C motif) ligand 20 (CCL20, also known as macrophage inflammatory protein 3, MIP-3a) and C–X–C chemokine receptor type 6 (CXCR6) binding to CXCL16 [111–114]. A recent study implicated pericellular matrix composition in the formation of hepatic chemokine gradients that subsequently influence myeloid and lymphoid cell localization [115]. This study also highlighted the importance of commensal bacteria in the stimulation of myeloid differentiation primary response 88 (MYD88) in LSECs, which promotes the enrichment of KCs and iNKT cells in the periportal region [115]. However, tissue-resident macrophages have been identified throughout the hepatic sinusoid [116–118] with macrophages in the periportal regions of the liver being more phagocytic and immunoregulatory, while macrophages in the central venous regions being more inflammatory [119,120]. Resident memory T (Trm) cells are documented to reside at non-liver sites that are prone to microbial re-exposure, however, the exact locations of Trm subsets within the liver are yet to be identified [121]. Interestingly, CD8^{+} Trm cells in the murine liver have been observed to occupy the vasculature compartments, as a means of participating in hepatic immunosurveillance [13]. Similarly, little is known about the definitive localization and role of B cells in healthy and diseased livers, although their presence is well-documented in humans [119,122] and mice [123]. However, CD20^{+} B cells have been shown to localize within hepatic sinusoids close to the portal tracts, while plasma cells were located in the connective tissue surrounding the portal tracts in humans [124]. DCs are thought to be located in the subcapsular region of the liver, between hepatocytes and concentrated in the periportal areas [125,126]. Liver-resident CD49a^{+} ILC1s are the major ILC subset found in the liver at homeostasis, and are found to reside close to DCs in the perivascular regions encompassing the portal triad in mice [127], but have yet to be examined in humans. Chemotactic stimuli, such as MIP-1a, RANTES and CXCL16 can direct the recruitment of lrNK cells (CD56^{bright}) to hepatic sinusoidal spaces [128]. iNKT cells can patrol the murine liver sinusoids until inflammatory signals trigger them to stop [129,130]. The full spectrum of the subsets encompassed within these cell populations, in addition to their frequencies, position, phenotype,

and function warrant further investigation. As described later, novel approaches are being developed to allow for thorough investigations of hepatic immune cell profiling, in order to provide a more accurate description of the landscape of the liver. Further understanding of the cellular and molecular bases of the healthy liver, in reference to the regulation of cell phenotype, function, reprogramming and self-renewal, by the hepatic immune microenvironment, will enable the design of novel therapeutic strategies against unmet liver diseases.

Single-cell profiling of the immune niche of the liver

Single-cell technologies have been rapidly expanding to uncover the cellular identity and spatial organization of immune and non-immune cells within organ tissues [131]. Nevertheless, several key questions concerning the localization, interactions, and identities of immune cells in the liver remain unanswered in the context of homeostasis and disease development. Spatial profiling, in its many forms, has contributed significantly to the elucidation of spatial hepatocyte heterogeneity, liver zonation [132], and murine lymphocyte migration [133,134]. Nevertheless, spatial profiling of the lymphoid and myeloid populations in the liver has been a significant challenge due to their lower frequencies and smaller cell size in comparison to parenchymal cells and non-immune non-parenchymal cells. Below, we briefly describe various transcriptomic, multimodal, and spatial profiling technologies that are guiding the continuing exploration of liver immunology regarding the identification, localization, and development of hepatic immune populations. These technologies can be divided into three categories, those that require prior enzymatic or detergent-based tissue dissociation (single-cell (scRNA-seq) and single-nucleus RNA sequencing (snRNA-seq), single-cell ATAC sequencing, multimodal technologies and flow and mass cytometry), those that are conducted on tissue-mounted slides (immunohistochemistry (IHC), single-molecule fluorescent *in situ* hybridization, laser capture microdissection (LCM), spatial transcriptomics, and imaging mass cytometry (IMC)), and those that are performed on a live animal (intravital live imaging). Figure 3 summarizes key single-cell-omics technologies, in addition to their current and potential applications within the context of the liver. A complete and comprehensive understanding of the cellular landscape of the immune niche of the liver will help identify immune signals that promote tolerance in the liver which could lead to new targets to allow for hepatic reprogramming towards homeostasis.

Flow cytometry and mass cytometry

Flow cytometry and fluorescence-activated cell sorting (FACS)-based approaches are effective and reliable tools to explore the immune microenvironment of the liver. As reviewed previously [135], this technology provides the ability to simultaneously analyze heterogeneous cell populations for multiple protein surface and intracellular markers. Characterizing and functionally validating rare populations in the liver is made possible through FACS, which enriches for populations identified by defined surface markers. Flow-based cell enrichment can complement characterization by additional technologies, such as single-cell RNA sequencing (scRNA-seq; discussed below) [136]. A challenge to FACS-based approaches is the tradeoff between maximizing the number of markers investigated, the subsequent number of fluorophores used, and avoiding the problem of spectral overlap. As solutions are being developed to solve this problem, mass cytometry has been a promising option that combines traditional flow cytometry practices and time-of-flight (TOF) mass spectrometry [137]. In mass cytometry, metal ions are conjugated to antibodies as opposed to fluorophores, therefore the challenges of cellular autofluorescence signals and spectral overlap are not encountered. However, given that the sample is destroyed during the process, sorting is not an option in mass cytometry. Additionally, as a result of forward and side scatters not being measured in mass cytometry, the removal of debris and dead cells is not as easy as in traditional methods, however, barcoding has been employed to ameliorate this [138]. In the context of the liver, mass cytometry has helped us uncover the immunological shifts within $CD8^+$ T cells in NASH and ILCs in human hepatocellular carcinoma (HCC) [139]. It is expected that mass cytometry, with spatial information incorporated in the form of IMC, as discussed later, will further expand our understanding of the immunological composition of the liver.

scRNA-seq

scRNA-seq (reviewed in Chen et al. [140]) is an unbiased approach which can be applied to describe the transcriptional landscape of tissues by profiling the genes expressed by individual cells resident in the organ. MacParland et al. [119] used a gentle liver cell dissociation protocol in combination with scRNA-seq and identified 20 discrete cell populations including immune and non-immune cells [119], corroborating the presumed heterogeneity of intrahepatic cell populations [29,141,142]. Importantly, this work revealed two subtypes of macrophages with inflammatory and non-inflammatory properties characterized by distinct markers. One of the non-inflammatory markers, macrophage

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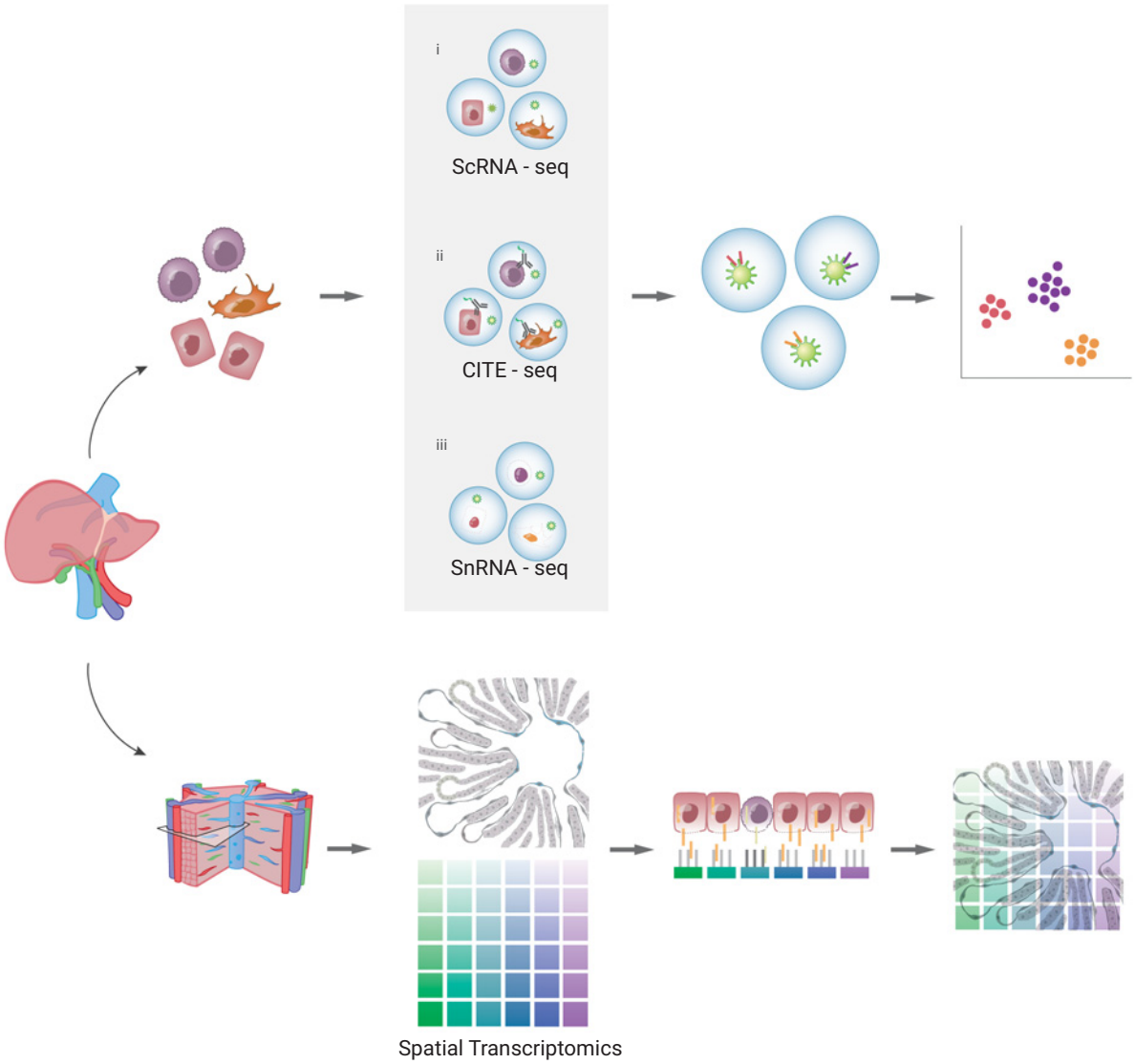

Figure 3. Novel technologies in the characterization of the cellular composition of the liver

The cellular heterogeneity of liver tissue can be deciphered as single cells (top) or whole tissue (bottom). (Top) Single-cell technologies transcriptomically categorize the sample at a single-cell level (scRNA-seq) and can be further enhanced by sorting nuclei instead of cells (snRNA-seq) or adding oligonucleotide-labeled antibodies to target surface antigens (CITE-seq). (Bottom) In spatial transcriptomics, freshly frozen tissue is attached to a chip, whose barcodes contain location information on a grid. Thus, mRNA transcripts released from the tissue after permeabilization can be traced back to its grid location, and therefore, the approximate location of the tissue. Abbreviation: CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing.

receptor with collagenous structure (MARCO), allowed for further investigation of the subset's immunological function and distribution in the liver via flow cytometry and IHC, respectively [119]. In a recent study exploring acute liver failure (ALF), Kolodziejczyk et al. [143] used scRNA-seq to highlight the activation of KCs, LSECs and HSCs during the progression of ALF, in addition to elucidating the potential upstream pro-inflammatory roles of MYC and the microbiome [143]. However, despite the significant insight and information that scRNA-seq provides on the liver, there are caveats to its usage that must be considered. For example, it is important to note that preparing samples for scRNA-seq and flow cytometry requires a certain degree of tissue dissociation and the isolation of single cells, subsequently resulting in the loss of the spatial context. Additionally, transcriptomics profiling alone is not able to fully distinguish between certain immune cell populations because environmental cues can influence different cell populations to possess similar transcriptional identities. For example, T and B cells are central players in adaptive

immunity and the promotion of local tolerogenicity, but their identity within the liver is not well described. Profiling T-cell receptors (TCRs) and B-cell receptors (BCRs) through V(D)J sequencing (reviewed in Minervina et al. [144]), can be employed to define various subtypes of lymphocytes with distinct roles in homeostasis and disease. For instance, MAIT cells are a subset of T cells with an αβ TCR characterized by a semi-invariant TCRα chain. TCR clonotyping allows for the examination of the frequency and transcriptional identity of these invariant T cells, given that they can be identified by the expression of known semi-invariant TCRs (*TRAV1-2/TRAJ12/20/3)*. Recently, TCR clonotyping has been used to investigate the relationship between T-cell activation and TCR sequence in the breast tumor microenvironment [145], in addition to discerning recombination bias during T cell development in the thymus [146], and the signatures that predict responses to anti-PDL1 therapy [147]. BCR clonotyping of HIV-infected individuals has been used to predict antigen specificity and to develop effective broadly neutralizing antibodies [148]. As scRNA-seq broadens its application to various tissues, an extensive TCR and BCR clonotyping of the liver will further characterize the tissue-resident T- and B-cell populations, as well as their infiltrating counterparts in hepatic disease states.

snRNA-seq

Despite scRNA-seq providing an unbiased approach to transcriptomically profile a sample, it has some intrinsic caveats. Since dissociation is required for this method, variations in this step can lead to dramatic differences in transcriptional stress responses, thereby introducing batch effects [149]. In order to overcome this obstacle, various nucleus-centric approaches of scRNA-seq such as sNuc-seq [150], Div-seq [150], and DroNC-seq [151] have been developed. These methods, referred to as snRNA-seq in this review, utilize droplet fluidics to isolate the nuclei from the sample prior to high-throughput sorting. By avoiding the need to dissociate the cells, as one would in scRNA-seq, snRNA-seq reduces dissociation bias, minimizes stress responses, and increases compatibility with frozen samples. For studies of the human liver, this reduced dissociation bias is especially important when comparing data from healthy and diseased samples, as some diseased cells may be more challenging to dissociate due to the presence of fibrosis. However, the lack of representation from cytoplasmic RNA is a major caveat to consider when employing snRNA-seq. An additional consideration is the fact that rare hepatic immune populations may not be sufficiently and accurately represented in snRNA-seq maps, as these maps will efficiently capture hepatocytes, at the cost of producing a less-enriched immune cell profile [152]. Therefore, while scRNA-seq allows for TCR clonotyping to further pass through TCR-possessing populations with similar transcriptional states, snRNA-seq excels in capturing the entirety of the single-cell map of the liver, given that hepatocytes comprise 80% of the liver by volume [153], which subsequently prevents the capturing and subclustering of immune cell populations [152]. However, in a similar manner to cell hashing in scRNA-seq, nucleus hashing has been introduced to snRNA-seq without influencing the recovered profiles of human brain cortex samples [154]. As a result of lowered batch effects and bias, snRNA-seq renders large-scale screening and longitudinal studies feasible without compromising the resolution of single-cell technologies [155].

Assay for transposase-accessible chromatin using sequencing: chromatin accessibility

In addition to reducing batch effects, single-nuclei technology allows for the enhanced investigation of chromatin accessibility, which is an important regulator of gene expression. Profiling chromatin accessibility has been a continuing endeavor, as it deepens our understanding of the functional and regulatory networks of cell populations [156]. The intersection between epigenetics and immunology has especially garnered interest in the quest to understand dysregulation-associated disease and defense against pathogens [157,158]. Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) is a technique that utilizes a mutated hyperactive Tn5 transposase to mark open chromatin regions by inserting DNA sequence adaptors, a process termed tagmentation [159,160]. After tagmentation, the number of reads is translated into chromatin openness. The application of ATAC-seq to explore liver regeneration [161], liver disease [162] and HCC [163] in mice, has uncovered key differentially expressed genes and transcription factors (TFs) that may possess therapeutic potential. Recently, the ATAC-seq protocol has been modified to measure chromatin accessibility at a single-cell level by sorting single nuclei [164]. This version of ATAC-seq, referred to as single-cell Assay for Transposase-Accessible Chromatin using sequencing (scATAC-seq), has been used to study the T-cell regulatory network [165–167], liver regeneration [168], and tumor heterogeneity [169]. scATAC-seq continues to be a tool utilized for the profiling of chromatin accessibility across mammalian tissues [170], and holds significant potential to define the epigenetic landscape of the human liver, in the context of homeostasis, trained immunity, cancer, and other hepatic diseases.

Clinical Science (2021) **135** 2445–2466
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Multimodal technologies

Next-generation multimodal approaches allow for in-depth investigations of tissues, wherein single cells can be defined in a range of forms, such as in terms of their proteome, transcriptome, epigenome, and metabolome. Profiling single cells through multiple means allows for the construction of a more complete understanding of the tissue in question, in addition to shedding light on distinct cell phases, subtypes, and receptor clonotypes. For example, one of the caveats of ascribing cell identity from gene expression profiles is that RNA expression does not directly translate to cell-surface protein expression, thus impairing the accurate identification of specific cell subtypes. To further describe complex cell populations, Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq) is able to overcome this limitation by combining immunophenotyping and the high-throughput nature of scRNA-seq [171]. CITE-seq has been utilized to investigate the immune populations of diseased murine livers [172], healthy and diseased human colon tissue [173], and tumor-afflicted human lung tissue [174], however, such an exploration of the human liver, both healthy and diseased, has not yet been undertaken. Moreover, CITE-seq has been shown to be amenable to modifications aimed at expanding its multiplexing potential, such as in the case of cell hashing [175], which allows for V(D)J profiling, as well as single-guide RNA (sgRNA) capture for CRISPR screening. This updated technique, named expanded CRISPR-compatible cellular indexing of transcriptomes and epitopes by sequencing (ECCITE-seq) (reviewed in Mimitou et al. [176]), demonstrates powerful adaptability to the ongoing evolution of single-cell technologies while expanding its limits beyond transcript RNAs [176].

Immunohistochemistry (IHC)

A classic method of spatial profiling is IHC, given that it provides a microscopy-mediated visualization of immunostained tissues. Various IHC techniques provide important information about the localization and presence of key proteins in a tissue, however, they do tend to be limited by the number of markers that can be visualized at once [177]. Additionally, given that the results of IHC are generally difficult to quantify [177,178], it is commonly employed to validate transcriptomics and proteomics data, in addition to providing spatial context to key expression profiles based on predetermined landmark genes. Zhang et al. [179] utilized scRNA-seq to investigate the interactions of immune cells in human HCC, and discovered resident LAMP3$^+$ DCs that express PD-L1. Upon validation with multicolor IHC, it was uncovered that PD-L1-expressing LAMP3$^+$ DCs were spatially juxtaposed to PD-1-expressing CD4$^+$ and CD8$^+$ T cells. LAMP3$^+$ DC gene signatures were also strongly associated with that of exhausted T cells, which suggests a potentially dysfunctional relationship between LAMP3$^+$ DCs and T cells in HCC [179]. Moreover, IHC has also been used to uncover the distribution of hepatic macrophage subsets, wherein CD68$^+$ MARCO$^+$ non-inflammatory macrophages were more enriched around the periportal region, in contrast with the central venous region [119]. The continued usage of IHC as a method for validating the results of transcriptomics analyses is extremely valuable, given the insight it provides into the expression and spatial distribution of surface proteins.

Single-molecule fluorescent *in situ* hybridization: integration of gene expression with tissue architecture

Single-cell technologies improve on traditional bulk RNA sequencing for their cell-by-cell approach and unbiased transcriptome-based clustering method without providing spatial context. However, RNA single-molecule fluorescent *in situ* hybridization (smFISH) is a high-throughput technique that detects RNA while preserving spatial information and tissue context. By targeting RNAs with fluorescently labeled DNA probes, smFISH visualizes individual transcripts with high detection efficiency and resolution [180,181]. This has allowed for the exploration of complex liver architecture, such as in terms of the zonation of hepatocytes and endothelial cells, which in turn alludes to the spatial distribution of immune cells [123,132]. A recent study conducted by Sharma et al. [182] utilized scRNA-seq to uncover the similarity between the highly nuanced and immunosuppressed cellular landscapes of the human fetal liver and HCC-afflicted adult livers. Within HCC tissue, two key tumor-associated macrophage (TAM) populations were identified; CD163$^+$ FOLR2$^+$ (TAM1) and CD162$^-$ SPP1$^+$ (TAM2). smFISH was employed to verify the scRNA-seq and FACS results, in addition to elucidating that the TAM1 and TAM2 populations were differentially localized within the tumor. Recently, multiplexed error-robust FISH (MERFISH) has also been developed to expand the profiling capacity of smFISH to thousands of RNA transcripts [183]. By contextualizing spatial transcriptional profiles with local tissue heterogeneity, smFISH helps untangle the interactions and organization that make up the immunological niche of the liver.

Laser capture microdissection

In order to more accurately explore the transcriptomics and proteomics expression profiles of immune cell types in healthy and diseased tissues, laser capture microdissection (LCM) can be employed for the isolation of key regions of interest, and individual cell populations [184]. LCM is ordinarily applied upstream of high-throughput characterization techniques and visualization experiments. In contrast with alternative biochemical techniques that sort cell populations, LCM does not subject the tissues to dissociative or digestive compounds, and thus allows for the isolation of intact niches and microenvironments for further assessment. However, due to the nature of LCM, it is difficult to capture cell types in isolation, as they are often captured in mixtures of multiple types of cells. Additionally, improper tissue preparation and staining protocols may severely reduce the ability to assess DNA, RNA and protein contents in tissues at the single-cell level [184].

In the context of the liver, LCM is widely used to investigate diseased hepatic tissue, such as in relation to infection by hepatitis C virus (HCV) [185] and hepatitis B virus (HBV) [186], in addition to HCC, cholangiocarcinoma (CCA) [116,187], and fibrosis [188]. However, a large proportion of these studies aim to microdissect hepatocytes, endothelial cells and HSCs, as opposed to immune cells, due to the difficulty of identifying and isolating them in the liver. One study by Gehring et al. [189] aimed to explore the role of KCs in mediating inflammation in bile duct ligation (BDL) mice, a common model for obstructive cholestatic injury [189]. They utilized LCM to isolate KCs from murine liver tissue, and then subjected them to reverse-transcription quantitative PCR (RT-qPCR) and photoimaging. The results indicated that KC-depleted and IL6-KO BDL mice experienced more severe liver necrosis in comparison to wildtype BDL mice. Additionally, *in vitro* stimulated KCs from BDL mice secreted increased levels of IL-6, in comparison to non-BDL mice. This suggests that KC production of IL-6 in BDL mice is protective against liver necrosis, which may potentially hold therapeutic potential for cholestatic injury in humans. Therefore, in the exploration of the spatial context of hepatic cell populations, LCM is particularly useful in the isolation of hepatic immune microenvironments, functional regions of interest, and diseased tissue, given the high-resolution and the lack of dissociative processes. Future studies in liver immunology may benefit from utilizing LCM to isolate and profile key regions of interest in healthy and disease hepatic tissue.

Intravital live imaging

Analogous to IHC and smFISH, a major limitation of LCM is the inability for researchers to observe the temporal nature of gene expression, in addition to that of the spatial dimension. An innovative solution to this problem is the usage of multiphoton microscopy or confocal laser-scanning intravital live imaging, otherwise known as intravital microscopy (IVM). Unlike the spatial profiling of tissues *ex vivo*, intravital imaging also allows for the visualization of cellular processes *in vivo*, wherein the complex interactions that make up natural environments remain intact [190]. Modern developments in IVM have rendered it possible to visualize spatiotemporal changes at the subcellular level, enabling observations at the resolution of distinct organelles.

Guidotti et al. utilized IVM to elucidate the mechanisms underlying the homing of effector T cells to HBV-infected murine livers. In contrast with other lymphoid organs, results indicated that $CD8^+$ T cell arrival to the liver is mediated by platelet aggregates that adhere to LSECs in a selectin-independent manner [133]. Through further investigation via IVM and additional genetic approaches, the authors uncovered the critical role of platelet-expressed CD44 and sinusoidal hyaluronan in the retention of migrating $CD8^+$ effector T cells to the liver [133]. However, intravital live imaging is limited to its usage in animal models and is therefore restrained in the extent to which it can be applied to human biology. In addition to this, a key limitation is the number of distinct fluorescent markers that can be visualized at once, given that IVM requires mice to be transgenic in fluorescent proteins located in cell types and tissues of interest. For example, through the usage of IVM, Wang and Kubes (2015) were able to identify a subset of peritoneal macrophages that infiltrate the liver upon sterile hepatic injury in mice [191]. To do so, they employed a range of visualization techniques, such as utilizing CX3CR1-GFP/CCR2-RFP reporter mice, in addition to tracking macrophages and cell damage through F4/80 antibodies and SYTOX green nucleic acid stain, respectively [191]. The authors also uncovered the critical role of peritoneal macrophages in hepatic tissue reparation, given that the revascularization and rehabilitation of liver sinusoids were delayed in mice with depleted peritoneal macrophages. When applied to the investigation of the liver in animal models, IVM can provide significant insight into the trafficking, proliferation, and interactions of immune cell populations during health and disease.

Spatial transcriptomics

Despite recent advances in RNA-FISH techniques, its multiplexing capability remains limited by the number of fluorophores available and the fact that the approach, by nature, is biased. The introduction of next-generation sequencing

Clinical Science (2021) **135** 2445–2466
https://doi.org/10.1042/CS20190654

allows for the unbiased exploration of entire transcriptomes with spatial information. By combining microscopy and RNA sequencing, Stahl et al. [192] developed *in situ* spatial transcriptomics, a method that provides transcriptomics profiling with respect to relevant histological morphology. This is made possible by laying a piece of frozen tissue upon a set of grids that contain positional barcodes within their sequencing probes (Figure 3). By contextualizing transcriptomics information at a spatial resolution, spatial transcriptomics illustrates a visual and quantitative snapshot of the sample tissue [192]. In the context of the liver, the spatial aspect of data is highly valuable, given the inherently organized hepatic architecture and specialized tissue microenvironments. Due to limited resolution, capturing rare cell types, smaller cells, and nuanced cell heterogeneity can be challenging. However, the resolution of *in situ* spatial transcriptomics has recently been optimized from 100 to 2 μm with the help of a split-and-pool approach and multiple hybridization rounds [193]. At this unprecedented resolution, *in situ* spatial transcriptomics paves new ways to incorporate the spatial dimension into emerging bioinformatics analyses and diagnostics.

Next-generation genomic technologies provide a lens through which we can better examine the immune niche of the liver. scRNA-seq, for instance, has improved our understanding of the immunological landscape of the liver in humans [119,122] and mice [123,132], in addition to T-cell infiltration during HCC [194], fetal liver hematopoiesis [136,195], fibrosis [196], and cancer [179]. However, in the past few years, scRNA-seq and several other genomic technologies have been workshopped to utilize bioinformatics to pseudospatially resolve scRNA-seq data across a two-dimensional space. For example, to reconstruct genome-wide human hepatic zonation patterns, Aizarani et al. [122] employed diffusion-pseudotime (DPT) analysis of scRNA-seq data, in combination with self-organizing maps, as a proxy for pseudospace [122]. This method depends on the assumption that there is a correlation between variability in gene expression and diversity in cell type [122]. In addition to this, the authors utilized IHC and immunofluorescence experiments to verify the inferred zonation patterns at the protein level. Alternatively, Halpern et al. combined the visualization sensitivity of smFISH and the transcriptomics profiling prowess of scRNA-seq to spatially resolve gene expression profiles across murine liver samples [132]. Through utilizing smFISH to visualize the expression of key landmark genes, the authors were able to map hepatocytes based on genome-wide expression profiles, in relation to zonation patterns. This method was also elaborated to explore the spatiotemporal gene expression patterns of hepatocytes within the murine liver, as a means of elucidating the relationship between the hepatic circadian rhythm and liver zonation [197]. Halpern et al. published another study utilizing paired scRNA-seq, wherein the known spatial context of one cell type is used to map the localization of alternative and neighboring cell types [123]. In this paper, they employed the spatially resolved scRNA-seq data of hepatocytes to map the location of liver endothelial cells, in addition to identifying several immune cell populations in the mouse liver, such as T cells, B cells, neutrophils, plasmacytoid DCs, and distinct populations of KCs and liver capsule macrophages [123].

There is significant potential for spatial transcriptomics to be utilized in the exploration of human liver immunology, particularly in terms of elucidating the identities and locations of immune cells during homeostasis and disease. Describing the differences in the transcriptional landscapes of immune cells within the spatial context of the liver will shed light on the molecular etiology of disease, and potential targets for treatment.

Imaging mass cytometry

Imaging mass cytometry (IMC) is a powerful tool that complements flow cytometry and mass cytometry and allows for highly multiplexed single-cell *in situ* protein analysis [198]. As an extension of mass cytometry, IMC harnesses metal isotope-conjugated antibodies and laser ablation to reconstruct high resolution images of a tissue section of interest [198]. In contrast with flow cytometry, the usage of metal isotope-conjugated antibodies in mass cytometry and IMC allows for the investigation of greater than 40 biological markers [199], because each metal isotope possesses its own unique non-overlapping detection peak, and background noise is minimal. When applied to the liver, IMC enables the identification of tissue-resident and infiltrating immune populations, as well as their spatial distribution relative to each other and parenchymal cell populations, such as in the case of HBV infection [200], HCC [201,202], and the biodistribution of chemotherapeutic agents [203]. IMC is also particularly relevant for clinical studies because the usage of paraffin-embedded tissues allows for a retrospective investigation of samples collected across time points. However, a major limitation of IMC is related to the rate of image acquisition and data generation, given that it is considerably slower than that of fluorescence slide scanning [204]. Additionally, similar to scRNA-seq, snRNA-seq and spatial transcriptomics, the computational portion of IMC data analysis can be particularly time-consuming and bereft of standardized protocols and workflows. As a result of this, there has been significant innovation in the development of informatics pipelines that allow for in-depth analysis, segmentation, and pixel classification [205] that build upon ilastik [206] and CellProfiler [207,208]. An example of this is iMaging mAss cyTometry mIcroscopy Single-cell

SegmEntation (MATISSE), which combines IMC and fluorescence microscopy to improve cell segmentation and the identification of immune cells within a tissue [209].

In a recent study by Popescu et al. [136], the authors used IMC to complement scRNA-seq, flow cytometry and alternative imaging methods in the investigation of fetal liver hematopoiesis [136]. The results from IMC shed light on a significant restructuring of liver architecture from 8 to 15 post-conception weeks in terms of hepatocyte organization, the presence of hematopoietic aggregates within the sinusoid, and the distribution of $CD1c^+$ DCs, B cells, and $CD68^+$ macrophages [136]. Alternatively, Bertocchi et al. [210] employed IMC to capture the role of *Escherichia coli* in disrupting the gut vascular barrier in the context of colorectal cancer, subsequently promoting the development of a pre-metastatic niche in the liver [210]. Overall, IMC enables the visualization and quantification of protein expression in complex tissues, subsequently allowing for a deeper understanding of rare cell types, cell–cell interactions, and the spatiotemporal changes in the immune cell populations of the liver.

Current limitations of single-cell technologies

Single-cell technologies and their advancement provide an expansive platform to characterize immune cell identity and plasticity, and as their application becomes increasingly ambitious, new roadblocks emerge. These techniques are expensive, labor-intensive, and the data analysis requires expert bioinformatics platforms and definitive cell annotations to be supported by biological validation. Functional confirmation of cell annotations and the characterization of heterogeneous cell populations relies on the ability to isolate the identified cells for characterization using *in vitro* immunological techniques, including multicolor flow cytometry and immunofluorescence microscopy (IFM) [211]. Conventional cell markers can fail to capture the diversity of hepatic cell populations and the ability to discriminate between tissue-resident and circulating cells [212]. The current single-cell approaches still demonstrate limited capture efficiency. This dropout problem means that weakly expressed genes may be missed, causing our interpretations and characterization to deviate from *in vivo* reality. Adaptability for different sample types also requires urgent attention for single-cell technologies, especially considering clinical applications, where sample types may be highly heterogeneous. To properly capture this heterogeneity, a sufficient number of biological samples must be analyzed, but due to the financial burden of these technologies, as well as limited access to fresh human tissue, too few samples are often investigated. One potential field of interest for the application of scRNA-seq is the investigation of host–pathogen interactions. While there have been promising leads in using scRNA-seq to explore transcriptional heterogeneity in pathogens, this area is limited by the availability of antibodies targeting conserved pathogen-associated epitopes. Moreover, the rapid advancement in spatial profiling technologies has allowed for the visualization of different tissues in various states across several organisms, but much has yet to be elucidated in terms of the spatial resolution of the subtypes, clonotypes, and transition states of hepatic immune populations. Current spatial transcriptomics technologies are varied and inconsistent, in addition to being limited in terms of their resolution. Due to the nature of hepatic immune populations, high resolution and high-throughput spatial transcriptomics technologies are necessary to visualize and characterize them accurately. As single-cell technologies extend their capability to cover beyond transcriptomic, spatial, epigenomic, and proteomic levels, further research is needed to amalgamate these findings into a coherent story. Not only will this endeavor require a standardized repository protocol, it will also challenge our notion of the boundary between cell identity and plasticity, in addition to cell phenotype and genetics.

Conclusions and future perspectives

The advancement in the understanding of the immune niche of the liver has accelerated from the scale of decades to years. Increased international efforts to make human samples available have allowed researchers to characterize hepatic tissue in a manner that is truly representative of the human liver, in health and disease. In addition to this, novel technologies are providing an opportunity to bridge the gap between the academic investigation of hepatic tissue and the optimization of targeted therapies. These technologies have also sparked a newfound appreciation for the origin, diversity and plasticity of immune cells. Over the years, the heterogeneity and plasticity of liver-resident immune cells have become apparent, and as a result, traditional phenotypic markers that have been employed to annotate immune populations are required to evolve to capture this diversity. It is essential to investigate the specific origins of individual hepatic populations and their nuanced differences, in order to truly harness and translate the regenerative capacity of these cells to treat liver disease. Key concepts to elucidate include discerning the cell types that proliferate locally and those that are recruited from a circulatory pool, the magnitude at which cell identity is fluid, and to what degree stem cells play a role in the regenerative process. As we unravel more intricate interactions between the hepatic immune microenvironment and the phenotypes of its constituents, it would be interesting to see where the boundaries between cell identity and plasticity truly lie.

Clinical Science (2021) **135** 2445–2466
https://doi.org/10.1042/CS20190654

Moreover, our understanding of the liver requires thorough characterization of its cellular composition, and while single-cell technologies produce transcriptomics snapshots of the tissue, CITE-seq, spatial transcriptomics and IMC provide additional protein-level and locational context that further annotate key cell populations that make up the immunological niche of the liver. After the investigation of healthy, fetal, and cancerous liver samples, the application of these powerful tools to other acute and chronic liver diseases will buttress the investigation of pathology-associated dysregulation. Techniques such as snRNA-seq make this endeavor possible by removing the bias introduced by dissociation. As these emerging techniques become increasingly prevalent, the standardization of common pipelines will help us build comprehensive repositories, robust meta-analyses, and most importantly, it will support the next paradigm shift that ultimately improves the development and execution of precision medicine and immunotherapy.

Data Availability

Data sharing is not applicable to the review.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

M.L.C., D.N., C.T.P. and S.A.M. conceived and drafted the original manuscript.

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Abbreviations

ATAC-seq, assay for transposase-accessible chromatin using sequencing; BCR, B-cell receptor; BDL, bile duct ligation; CITE-seq, Cellular Indexing of Transcriptomes and Epitopes by Sequencing; CTL, cytotoxic T lymphocyte; DAMP, damage-associated molecular pattern; DC, dendritic cell; DPT, diffusion-pseudotime; FACS, fluorescence-activated cell sorting; HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; HSC, hepatic stellate cell; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IHC, immunohistochemistry; IL, interleukin; ILC, innate lymphoid cell; IMC, imaging mass cytometry; iNKT cell, invariant NK T cell; IVM, intravital microscopy; KC, Kupffer cell; LAG-3, lymphocyte-activation gene 3; LCM, laser capture microdissection; lrNK, liver-resident NK cell; LSEC, liver sinusoidal endothelial cell; MAIT, mucosal-associated invariant T; MHC, major histocompatibility complex; MIP, macrophage inflammatory protein; MMP, matrix metallopeptidase; NASH, non-alcoholic steatohepatitis; NK cell, natural killer cell; PAMP, pathogen-associated molecular pattern; PD-L1, programmed death ligand 1; PGE2, prostaglandin E2; scATAC-seq, single-cell Assay for Transposase-Accessible Chromatin using sequencing; scRNA-seq, single-cell RNA sequencing; smFISH, single-molecule fluorescent *in situ* hybridization; snRNA-seq, single-nucleus RNA sequencing; TCR, T-cell receptor; TF, transcription factor; TGF-β, transforming growth factor β; T_h, helper T cell; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; Treg, regulatory T cell; VSIG4, V-set and Ig domain-containing 4.

References

1 Crispe, I.N. (2009) The liver as a lymphoid organ. *Annu. Rev. Immunol.* **27**, 147–163, https://doi.org/10.1146/annurev.immunol.021908.132629
2 Robinson, M.W., Harmon, C. and O'Farrelly, C. (2016) Liver immunology and its role in inflammation and homeostasis. *Cell Mol. Immunol.* **13**, 267–276, https://doi.org/10.1038/cmi.2016.3
3 Asrani, S.K., Devarbhavi, H., Eaton, J. and Kamath, P.S. (2019) Burden of liver diseases in the world. *J. Hepatol.* **70**, 151–171, https://doi.org/10.1016/j.jhep.2018.09.014
4 Oda, M., Yokomori, H. and Han, J.-Y. (2003) Regulatory mechanisms of hepatic microcirculation. *Clin. Hemorheol. Microcirc.* **29**, 167–182

5 Poisson, J., Lemoinne, S., Boulanger, C., Durand, F., Moreau, R., Valla, D. et al. (2017) Liver sinusoidal endothelial cells: Physiology and role in liver diseases. *J. Hepatol.* **66**, 212–227, https://doi.org/10.1016/j.jhep.2016.07.009
6 Shetty, S., Lalor, P.F. and Adams, D.H. (2018) Liver sinusoidal endothelial cells - gatekeepers of hepatic immunity. *Nat. Rev. Gastroenterol. Hepatol.* **15**, 555–567, https://doi.org/10.1038/s41575-018-0020-y
7 Weiskirchen, R. and Tacke, F. (2014) Cellular and molecular functions of hepatic stellate cells in inflammatory responses and liver immunology. *Hepatobiliary Surg. Nutr.* **3**, 344–363
8 Wawman, R.E., Bartlett, H. and Oo, Y.H. (2017) Regulatory T cell metabolism in the hepatic microenvironment. *Front. Immunol.* **8**, 1889, https://doi.org/10.3389/fimmu.2017.01889
9 Ben-Moshe, S. and Itzkovitz, S. (2019) Spatial heterogeneity in the mammalian liver. *Nat. Rev. Gastroenterol. Hepatol.* **16**, 395–410, https://doi.org/10.1038/s41575-019-0134-x
10 McEnerney, L., Duncan, K., Bang, B.-R., Elmasry, S., Li, M., Miki, T. et al. (2017) Dual modulation of human hepatic zonation via canonical and non-canonical Wnt pathways. *Exp. Mol. Med.* **49**, e413, https://doi.org/10.1038/emm.2017.226
11 Drescher, H.K., Bartsch, L.M., Weiskirchen, S. and Weiskirchen, R. (2020) Intrahepatic TH17/TReg cells in homeostasis and disease-it's all about the balance. *Front. Pharmacol.* **11**, 588436, https://doi.org/10.3389/fphar.2020.588436
12 Crispe, I.N. (2003) Hepatic T cells and liver tolerance. *Nat. Rev. Immunol.* **3**, 51–62, https://doi.org/10.1038/nri981
13 Steinert, E.M., Schenkel, J.M., Fraser, K.A., Beura, L.K., Manlove, L.S., Igyártó, B.Z. et al. (2015) Quantifying memory CD8 T cells reveals regionalization of immunosurveillance. *Cell* **161**, 737–749, https://doi.org/10.1016/j.cell.2015.03.031
14 Miyara, M. and Sakaguchi, S. (2007) Natural regulatory T cells: mechanisms of suppression. *Trends Mol. Med.* **13**, 108–116, https://doi.org/10.1016/j.molmed.2007.01.003
15 Saraiva, M. and O'Garra, A. (2010) The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* **10**, 170–181, https://doi.org/10.1038/nri2711
16 Zhang, L.-J. and Wang, X.-Z. (2006) Interleukin-10 and chronic liver disease. *World J. Gastroenterol.* **12**, 1681–1685, https://doi.org/10.3748/wjg.v12.i11.1681
17 Breous, E., Somanathan, S., Vandenberghe, L.H. and Wilson, J.M. (2009) Hepatic regulatory T cells and Kupffer cells are crucial mediators of systemic T cell tolerance to antigens targeting murine liver. *Hepatology* **50**, 612–621, https://doi.org/10.1002/hep.23043
18 Crispe, I.N. (2014) Immune tolerance in liver disease. *Hepatology* **60**, 2109–2117, https://doi.org/10.1002/hep.27254
19 Diehl, L., Schurich, A., Grochtmann, R., Hegenbarth, S., Chen, L. and Knolle, P.A. (2008) Tolerogenic maturation of liver sinusoidal endothelial cells promotes B7-homolog 1-dependent CD8+ T cell tolerance. *Hepatology* **47**, 296–305, https://doi.org/10.1002/hep.21965
20 Holz, L.E., Benseler, V., Bowen, D.G., Bouillet, P., Strasser, A., O'Reilly, L. et al. (2008) Intrahepatic murine CD8 T-cell activation associates with a distinct phenotype leading to Bim-dependent death. *Gastroenterology* **135**, 989–997, https://doi.org/10.1053/j.gastro.2008.05.078
21 Limmer, A., Ohl, J., Wingender, G., Berg, M., Jüngerkes, F., Schumak, B. et al. (2005) Cross-presentation of oral antigens by liver sinusoidal endothelial cells leads to CD8 T cell tolerance. *Eur. J. Immunol.* **35**, 2970–2981, https://doi.org/10.1002/eji.200526034
22 Schurich, A., Berg, M., Stabenow, D., Böttcher, J., Kern, M., Schild, H.-J. et al. (2010) Dynamic regulation of CD8 T cell tolerance induction by liver sinusoidal endothelial cells. *J. Immunol.* **184**, 4107–4114, https://doi.org/10.4049/jimmunol.0902580
23 Dusseaux, M., Martin, E., Serriari, N., Péguillet, I., Premel, V., Louis, D. et al. (2011) Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood* **117**, 1250–1259, https://doi.org/10.1182/blood-2010-08-303339
24 Doherty, D.G., Norris, S., Madrigal-Estebas, L., McEntee, G., Traynor, O., Hegarty, J.E. et al. (1999) The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. *J. Immunol.* **163**, 2314–2321
25 Forkel, M., Berglin, L., Kekäläinen, E., Carlsson, A., Svedin, E., Michaëlsson, J. et al. (2017) Composition and functionality of the intrahepatic innate lymphoid cell-compartment in human nonfibrotic and fibrotic livers. *Eur. J. Immunol.* **47**, 1280–1294, https://doi.org/10.1002/eji.201646890
26 Jeffery, H.C., van Wilgenburg, B., Kurioka, A., Parekh, K., Stirling, K., Roberts, S. et al. (2016) Biliary epithelium and liver B cells exposed to bacteria activate intrahepatic MAIT cells through MR1. *J. Hepatol.* **64**, 1118–1127, https://doi.org/10.1016/j.jhep.2015.12.017
27 Kenna, T., Golden-Mason, L., Norris, S., Hegarty, J.E., O'Farrelly, C. and Doherty, D.G. (2004) Distinct subpopulations of gamma delta T cells are present in normal and tumor-bearing human liver. *Clin. Immunol.* **113**, 56–63, https://doi.org/10.1016/j.clim.2004.05.003
28 Crosby, C.M. and Kronenberg, M. (2018) Tissue-specific functions of invariant natural killer T cells. *Nat. Rev. Immunol.* **18**, 559–574, https://doi.org/10.1038/s41577-018-0034-2
29 Engel, I., Seumois, G., Chavez, L., Samaniego-Castruita, D., White, B., Chawla, A. et al. (2016) Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nat. Immunol.* **17**, 728–739, https://doi.org/10.1038/ni.3437
30 Lee, Y.J., Starrett, G.J., Lee, S.T., Yang, R., Henzler, C.M., Jameson, S.C. et al. (2016) Lineage-specific effector signatures of invariant NKT cells are shared amongst $\gamma\delta$ T, innate lymphoid, and Th cells. *J. Immunol.* **197**, 1460–1470, https://doi.org/10.4049/jimmunol.1600643
31 Verykokakis, M. and Kee, B.L. (2018) Transcriptional and epigenetic regulation of innate-like T lymphocyte development. *Curr. Opin. Immunol.* **51**, 39–45, https://doi.org/10.1016/j.coi.2018.01.006
32 Bonnardel, J., T'Jonck, W., Gaublomme, D., Browaeys, R., Scott, C.L., Martens, L. et al. (2019) Stellate cells, hepatocytes, and endothelial cells imprint the kupffer cell identity on monocytes colonizing the liver macrophage niche. *Immunity* **51**, 638.e9–654.e9, https://doi.org/10.1016/j.immuni.2019.08.017
33 Ginhoux, F. and Guilliams, M. (2016) Tissue-resident macrophage ontogeny and homeostasis. *Immunity* **44**, 439–449, https://doi.org/10.1016/j.immuni.2016.02.024
34 Gordon, S., Plüddemann, A. and Martinez Estrada, F. (2014) Macrophage heterogeneity in tissues: phenotypic diversity and functions. *Immunol. Rev.* **262**, 36–55, https://doi.org/10.1111/imr.12223

35 Ju, C. and Tacke, F. (2016) Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. *Cell. Mol. Immunol.* **13**, 316–327, https://doi.org/10.1038/cmi.2015.104
36 Sakai, M., Troutman, T.D., Seidman, J.S., Ouyang, Z., Spann, N.J., Abe, Y. et al. (2019) Liver-derived signals sequentially reprogram myeloid enhancers to initiate and maintain Kupffer cell identity. *Immunity* **51**, 655.e8–670.e8, https://doi.org/10.1016/j.immuni.2019.09.002
37 Erhardt, A., Biburger, M., Papadopoulos, T. and Tiegs, G. (2007) IL-10, regulatory T cells, and Kupffer cells mediate tolerance in concanavalin A-induced liver injury in mice. *Hepatology* **45**, 475–485, https://doi.org/10.1002/hep.21498
38 Heymann, F., Peusquens, J., Ludwig-Portugall, I., Kohlhepp, M., Ergen, C., Niemietz, P. et al. (2015) Liver inflammation abrogates immunological tolerance induced by Kupffer cells. *Hepatology* **62**, 279–291, https://doi.org/10.1002/hep.27793
39 Jung, K., Kang, M., Park, C., Hyun Choi, Y., Jeon, Y., Park, S.-H. et al. (2012) Protective role of V-set and immunoglobulin domain-containing 4 expressed on kupffer cells during immune-mediated liver injury by inducing tolerance of liver T- and natural killer T-cells. *Hepatology* **56**, 1838–1848, https://doi.org/10.1002/hep.25906
40 Knolle, P., Schlaak, J., Uhrig, A., Kempf, P., Meyer zum Büschenfelde, K.H. and Gerken, G. (1995) Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. *J. Hepatol.* **22**, 226–229, https://doi.org/10.1016/0168-8278(95)80433-1
41 Li, G., Kim, Y.-J. and Broxmeyer, H.E. (2005) Macrophage colony-stimulating factor drives cord blood monocyte differentiation into IL-10(high)IL-12absent dendritic cells with tolerogenic potential. *J. Immunol.* **174**, 4706–4717, https://doi.org/10.4049/jimmunol.174.8.4706
42 Rutella, S., Bonanno, G., Procoli, A., Mariotti, A., de Ritis, D.G., Curti, A. et al. (2006) Hepatocyte growth factor favors monocyte differentiation into regulatory interleukin (IL)-10++IL-12low/neg accessory cells with dendritic-cell features. *Blood* **108**, 218–227, https://doi.org/10.1182/blood-2005-08-3141
43 Dou, L., Ono, Y., Chen, Y.-F., Thomson, A.W. and Chen, X.-P. (2018) Hepatic dendritic cells, the tolerogenic liver environment, and liver disease. *Semin. Liver Dis.* **38**, 170–180, https://doi.org/10.1055/s-0038-1646949
44 Raïch-Regué, D., Glancy, M. and Thomson, A.W. (2014) Regulatory dendritic cell therapy: from rodents to clinical application. *Immunol. Lett.* **161**, 216–221, https://doi.org/10.1016/j.imlet.2013.11.016
45 Lu, L., Woo, J., Rao, A.S., Li, Y., Watkins, S.C., Qian, S. et al. (1994) Propagation of dendritic cell progenitors from normal mouse liver using granulocyte/macrophage colony-stimulating factor and their maturational development in the presence of type-1 collagen. *J. Exp. Med.* **179**, 1823–1834, https://doi.org/10.1084/jem.179.6.1823
46 Stegmann, K.A., Robertson, F., Hansi, N., Gill, U., Pallant, C., Christophides, T. et al. (2016) CXCR6 marks a novel subset of T-bet(lo)Eomes(hi) natural killer cells residing in human liver. *Sci. Rep.* **6**, 26157, https://doi.org/10.1038/srep26157
47 Glässner, A., Eisenhardt, M., Krämer, B., Körner, C., Coenen, M., Sauerbruch, T. et al. (2012) NK cells from HCV-infected patients effectively induce apoptosis of activated primary human hepatic stellate cells in a TRAIL-, FasL- and NKG2D-dependent manner. *Lab. Invest.* **92**, 967–977, https://doi.org/10.1038/labinvest.2012.54
48 Peppa, D., Gill, U.S., Reynolds, G., Easom, N.J.W., Pallett, L.J., Schurich, A. et al. (2013) Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion. *J. Exp. Med.* **210**, 99–114, https://doi.org/10.1084/jem.20121172
49 Zhou, J., Peng, H., Li, K., Qu, K., Wang, B., Wu, Y. et al. (2019) Liver-resident NK cells control antiviral activity of hepatic T cells via the PD-1-PD-L1 axis. *Immunity* **50**, 403.e4–417.e4, https://doi.org/10.1016/j.immuni.2018.12.024
50 Steinmann, S., Schoedsack, M., Heinrich, F., Breda, P.C., Ochel, A., Tiegs, G. et al. (2020) Hepatic ILC2 activity is regulated by liver inflammation-induced cytokines and effector CD4+ T cells. *Sci. Rep.* **10**, 1071, https://doi.org/10.1038/s41598-020-57985-w
51 Bertolino, P., Trescol-Biémont, M.-C. and Rabourdin-Combe, C. (1998) Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival. *Eur. J. Immunol.* **28**, 221–236, https://doi.org/10.1002/(SICI)1521-4141(199801)28:01%3c221::AID-IMMU221%3e3.0.CO;2-F
52 Dunham, R.M., Thapa, M., Velazquez, V.M., Elrod, E.J., Denning, T.L., Pulendran, B. et al. (2013) Hepatic stellate cells preferentially induce Foxp3+ regulatory T cells by production of retinoic acid. *J. Immunol.* **190**, 2009–2016, https://doi.org/10.4049/jimmunol.1201937
53 Li, S., Hong, M., Tan, H.-Y., Wang, N. and Feng, Y. (2016) Insights into the role and interdependence of oxidative stress and inflammation in liver diseases. *Oxid. Med. Cell. Longev.* **2016**, 4234061, https://doi.org/10.1155/2016/4234061
54 Mehrfeld, C., Zenner, S., Kornek, M. and Lukacs-Kornek, V. (2018) The contribution of non-professional antigen-presenting cells to immunity and tolerance in the liver. *Front. Immunol.* **9**, 635, https://doi.org/10.3389/fimmu.2018.00635
55 Schrumpf, E., Tan, C., Karlsen, T.H., Sponheim, J., Björkström, N.K., Sundnes, O. et al. (2015) The biliary epithelium presents antigens to and activates natural killer T cells. *Hepatology* **62**, 1249–1259, https://doi.org/10.1002/hep.27840
56 Thomson, A.W. and Knolle, P.A. (2010) Antigen-presenting cell function in the tolerogenic liver environment. *Nat. Rev. Immunol.* **10**, 753–766, https://doi.org/10.1038/nri2858
57 Yu, M.-C., Chen, C.-H., Liang, X., Wang, L., Gandhi, C.R., Fung, J.J. et al. (2004) Inhibition of T-cell responses by hepatic stellate cells via B7-H1-mediated T-cell apoptosis in mice. *Hepatology* **40**, 1312–1321, https://doi.org/10.1002/hep.20488
58 Zeissig, S., Peuker, K., Iyer, S., Gensollen, T., Dougan, S.K., Olszak, T. et al. (2017) CD1d-Restricted pathways in hepatocytes control local natural killer T cell homeostasis and hepatic inflammation. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 10449–10454, https://doi.org/10.1073/pnas.1701428114
59 Carambia, A., Frenzel, C., Bruns, O.T., Schwinge, D., Reimer, R., Hohenberg, H. et al. (2013) Inhibition of inflammatory CD4 T cell activity by murine liver sinusoidal endothelial cells. *J. Hepatol.* **58**, 112–118, https://doi.org/10.1016/j.jhep.2012.09.008
60 Carambia, A., Freund, B., Schwinge, D., Heine, M., Laschtowitz, A., Huber, S. et al. (2014) TGF-β-dependent induction of $CD4^+CD25^+Foxp3^+$ Tregs by liver sinusoidal endothelial cells. *J. Hepatol.* **61**, 594–599, https://doi.org/10.1016/j.jhep.2014.04.027
61 Kruse, N., Neumann, K., Schrage, A., Derkow, K., Schott, E., Erben, U. et al. (2009) Priming of CD4+ T cells by liver sinusoidal endothelial cells induces CD25low forkhead box protein 3- regulatory T cells suppressing autoimmune hepatitis. *Hepatology* **50**, 1904–1913, https://doi.org/10.1002/hep.23191

62 Limmer, A., Ohl, J., Kurts, C., Ljunggren, H.G., Reiss, Y., Groettrup, M. et al. (2000) Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat. Med.* **6**, 1348–1354, https://doi.org/10.1038/82161
63 Neumann, K., Rudolph, C., Neumann, C., Janke, M., Amsen, D. and Scheffold, A. (2015) Liver sinusoidal endothelial cells induce immunosuppressive IL-10-producing Th1 cells via the Notch pathway. *Eur. J. Immunol.* **45**, 2008–2016, https://doi.org/10.1002/eji.201445346
64 Lumsden, A.B., Henderson, J.M. and Kutner, M.H. (1988) Endotoxin levels measured by a chromogenic assay in portal, hepatic and peripheral venous blood in patients with cirrhosis. *Hepatology* **8**, 232–236, https://doi.org/10.1002/hep.1840080207
65 Son, G., Kremer, M. and Hines, I.N. (2010) Contribution of gut bacteria to liver pathobiology. *Gastroenterol. Res. Pract.* **2010**, 453563, https://doi.org/10.1155/2010/453563
66 Yanagisawa, K., Yue, S., van der Vliet, H.J., Wang, R., Alatrakchi, N., Golden-Mason, L. et al. (2013) Ex vivo analysis of resident hepatic pro-inflammatory CD1d-reactive T cells and hepatocyte surface CD1d expression in hepatitis C. *J. Viral Hepat.* **20**, 556–565, https://doi.org/10.1111/jvh.12081
67 Zeissig, S., Murata, K., Sweet, L., Publicover, J., Hu, Z., Kaser, A. et al. (2012) Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity. *Nat. Med.* **18**, 1060–1068, https://doi.org/10.1038/nm.2811
68 Arshad, M.I., Patrat-Delon, S., Piquet-Pellorce, C., L'helgoualc'h, A., Rauch, M., Genet, V. et al. (2013) Pathogenic mouse hepatitis virus or poly(I: C) induce IL-33 in hepatocytes in murine models of hepatitis. *PLoS ONE* **8**, e74278, https://doi.org/10.1371/journal.pone.0074278
69 Marra, F. and Tacke, F. (2014) Roles for chemokines in liver disease. *Gastroenterology* **147**, 577.e1–594.e1, https://doi.org/10.1053/j.gastro.2014.06.043
70 Ramadori, G., Moriconi, F., Malik, I. and Dudas, J. (2008) Physiology and pathophysiology of liver inflammation, damage and repair. *J. Physiol. Pharmacol.* **59**, 107–117
71 Xu, R., Huang, H., Zhang, Z. and Wang, F.-S. (2014) The role of neutrophils in the development of liver diseases. *Cell. Mol. Immunol.* **11**, 224–231, https://doi.org/10.1038/cmi.2014.2
72 Imaeda, A.B., Watanabe, A., Sohail, M.A., Mahmood, S., Mohamadnejad, M., Sutterwala, F.S. et al. (2009) Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. *J. Clin. Invest.* **119**, 305–314
73 Martinon, F., Burns, K. and Tschopp, J. (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol. Cell* **10**, 417–426, https://doi.org/10.1016/S1097-2765(02)00599-3
74 Vivier, E., Raulet, D.H., Moretta, A., Caligiuri, M.A., Zitvogel, L., Lanier, L.L. et al. (2011) Innate or adaptive immunity? The example of natural killer cells. *Science* **331**, 44–49, https://doi.org/10.1126/science.1198687
75 Mikulak, J., Bruni, E., Oriolo, F., Di Vito, C. and Mavilio, D. (2019) Hepatic natural killer cells: organ-specific sentinels of liver immune homeostasis and physiopathology. *Front. Immunol.* **10**, 946, https://doi.org/10.3389/fimmu.2019.00946
76 Jie, Z., Liang, Y., Hou, L., Dong, C., Iwakura, Y., Soong, L. et al. (2014) Intrahepatic innate lymphoid cells secrete IL-17A and IL-17F that are crucial for T cell priming in viral infection. *J. Immunol.* **192**, 3289–3300, https://doi.org/10.4049/jimmunol.1303281
77 Ignacio, A., Breda, C.N.S. and Camara, N.O.S. (2017) Innate lymphoid cells in tissue homeostasis and diseases. *World J. Hepatol.* **9**, 979–989, https://doi.org/10.4254/wjh.v9.i23.979
78 Paik, Y.-H., Schwabe, R.F., Bataller, R., Russo, M.P., Jobin, C. and Brenner, D.A. (2003) Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology* **37**, 1043–1055, https://doi.org/10.1053/jhep.2003.50182
79 Canbay, A., Taimr, P., Torok, N., Higuchi, H., Friedman, S. and Gores, G.J. (2003) Apoptotic body engulfment by a human stellate cell line is profibrogenic. *Lab. Invest.* **83**, 655–663, https://doi.org/10.1097/01.LAB.0000069036.63405.5C
80 deCathelineau, A.M. and Henson, P.M. (2003) The final step in programmed cell death: phagocytes carry apoptotic cells to the grave. *Essays Biochem.* **39**, 105–117, https://doi.org/10.1042/bse0390105
81 Hernández-Gea, V., Ghiassi-Nejad, Z., Rozenfeld, R., Gordon, R., Fiel, M.I., Yue, Z. et al. (2012) Autophagy releases lipid that promotes fibrogenesis by activated hepatic stellate cells in mice and in human tissues. *Gastroenterology* **142**, 938–946, https://doi.org/10.1053/j.gastro.2011.12.044
82 Ilyas, G., Zhao, E., Liu, K., Lin, Y., Tesfa, L., Tanaka, K.E. et al. (2016) Macrophage autophagy limits acute toxic liver injury in mice through down regulation of interleukin-1β. *J. Hepatol.* **64**, 118–127, https://doi.org/10.1016/j.jhep.2015.08.019
83 Ruart, M., Chavarria, L., Campreciós, G., Suárez-Herrera, N., Montironi, C., Guixé-Muntet, S. et al. (2019) Impaired endothelial autophagy promotes liver fibrosis by aggravating the oxidative stress response during acute liver injury. *J. Hepatol.* **70**, 458–469, https://doi.org/10.1016/j.jhep.2018.10.015
84 Singh, R., Kaushik, S., Wang, Y., Xiang, Y., Novak, I., Komatsu, M. et al. (2009) Autophagy regulates lipid metabolism. *Nature* **458**, 1131–1135, https://doi.org/10.1038/nature07976
85 Jeffery, H.C., Braitch, M.K., Brown, S. and Oo, Y.H. (2016) Clinical potential of regulatory T cell therapy in liver diseases: an overview and current perspectives. *Front. Immunol.* **7**, 334, https://doi.org/10.3389/fimmu.2016.00334
86 Oo, Y.H., Weston, C.J., Lalor, P.F., Curbishley, S.M., Withers, D.R., Reynolds, G.M. et al. (2010) Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T cells in the inflamed human liver. *J. Immunol.* **184**, 2886–2898, https://doi.org/10.4049/jimmunol.0901216
87 Heymann, F. and Tacke, F. (2016) Immunology in the liver–from homeostasis to disease. *Nat. Rev. Gastroenterol. Hepatol.* **13**, 88–110, https://doi.org/10.1038/nrgastro.2015.200
88 Gomez Perdiguero, E., Klapproth, K., Schulz, C., Busch, K., Azzoni, E., Crozet, L. et al. (2015) Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature* **518**, 547–551, https://doi.org/10.1038/nature13989
89 Hoeffel, G., Chen, J., Lavin, Y., Low, D., Almeida, F.F., See, P. et al. (2015) C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity* **42**, 665–678, https://doi.org/10.1016/j.immuni.2015.03.011
90 Sheng, J., Ruedl, C. and Karjalainen, K. (2015) Most tissue-resident macrophages except microglia are derived from fetal hematopoietic stem cells. *Immunity* **43**, 382–393, https://doi.org/10.1016/j.immuni.2015.07.016

91 Yona, S., Kim, K.-W., Wolf, Y., Mildner, A., Varol, D., Breker, M. et al. (2013) Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* **38**, 79–91, https://doi.org/10.1016/j.immuni.2012.12.001
92 Pallett, L.J., Burton, A.R., Amin, O.E., Rodriguez-Tajes, S., Patel, A.A., Zakeri, N. et al. (2020) Longevity and replenishment of human liver-resident memory T cells and mononuclear phagocytes. *J. Exp. Med.* **217**, e20200050, https://doi.org/10.1084/jem.20200050
93 Scott, C.L., Zheng, F., De Baetselier, P., Martens, L., Saeys, Y., De Prijck, S. et al. (2016) Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nat. Commun.* **7**, 10321, https://doi.org/10.1038/ncomms10321
94 Auffray, C., Fogg, D.K., Narni-Mancinelli, E., Senechal, B., Trouillet, C., Saederup, N. et al. (2009) CX3CR1+ CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation. *J. Exp. Med.* **206**, 595–606, https://doi.org/10.1084/jem.20081385
95 Fogg, D.K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D.R. et al. (2006) A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* **311**, 83–87, https://doi.org/10.1126/science.1117729
96 Ramachandran, P., Pellicoro, A., Vernon, M.A., Boulter, L., Aucott, R.L., Ali, A. et al. (2012) Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc. Natl. Acad. Sci. U.S.A.* **109**, E3186–E3195, https://doi.org/10.1073/pnas.1119964109
97 Duffield, J.S., Forbes, S.J., Constandinou, C.M., Clay, S., Partolina, M., Vuthoori, S. et al. (2005) Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J. Clin. Invest.* **115**, 56–65, https://doi.org/10.1172/JCI200522675
98 Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A. et al. (2007) Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J. Exp. Med.* **204**, 1057–1069, https://doi.org/10.1084/jem.20070075
99 Rodero, M.P., Hodgson, S.S., Hollier, B., Combadiere, C. and Khosrotehrani, K. (2013) Reduced Il17a expression distinguishes a Ly6c(lo)MHCII(hi) macrophage population promoting wound healing. *J. Invest. Dermatol.* **133**, 783–792, https://doi.org/10.1038/jid.2012.368
100 Gibbons, M.A., MacKinnon, A.C., Ramachandran, P., Dhaliwal, K., Duffin, R., Phythian-Adams, A.T. et al. (2011) Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis. *Am. J. Respir. Crit. Care Med.* **184**, 569–581, https://doi.org/10.1164/rccm.201010-1719OC
101 Perciani, C.T. and MacParland, S.A. (2019) Lifting the veil on macrophage diversity in tissue regeneration and fibrosis. *Sci. Immunol.* **4**, https://doi.org/10.1126/sciimmunol.aaz0749
102 Sommerfeld, S.D., Cherry, C., Schwab, R.M., Chung, L., Maestas, D.R., Laffont, P. et al. (2019) Interleukin-36γ-producing macrophages drive IL-17-mediated fibrosis. *Sci. Immunol.* **4**, eaax4783, https://doi.org/10.1126/sciimmunol.aax4783
103 Sadtler, K., Estrellas, K., Allen, B.W., Wolf, M.T., Fan, H., Tam, A.J. et al. (2016) Developing a pro-regenerative biomaterial scaffold microenvironment requires T helper 2 cells. *Science* **352**, 366–370, https://doi.org/10.1126/science.aad9272
104 Schmidt-Arras, D. and Rose-John, S. (2016) IL-6 pathway in the liver: from physiopathology to therapy. *J. Hepatol.* **64**, 1403–1415, https://doi.org/10.1016/j.jhep.2016.02.004
105 Carnovale, C.E. and Ronco, M.T. (2012) Role of nitric oxide in liver regeneration. *Ann. Hepatol.* **11**, 636–647, https://doi.org/10.1016/S1665-2681(19)31436-X
106 Diehl, A.M. and Rai, R. (1996) Review: regulation of liver regeneration by pro-inflammatory cytokines. *J. Gastroenterol. Hepatol.* **11**, 466–470, https://doi.org/10.1111/j.1440-1746.1996.tb00292.x
107 Evarts, R.P., Hu, Z., Fujio, K., Marsden, E.R. and Thorgeirsson, S.S. (1993) Activation of hepatic stem cell compartment in the rat: role of transforming growth factor alpha, hepatocyte growth factor, and acidic fibroblast growth factor in early proliferation. *Cell Growth Differ.* **4**, 555–561
108 Huch, M., Dorrell, C., Boj, S.F., van Es, J.H., Li, V.S.W., van de Wetering, M. et al. (2013) In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature* **494**, 247–250, https://doi.org/10.1038/nature11826
109 Russell, J.O. and Monga, S.P. (2018) Wnt/β-catenin signaling in liver development, homeostasis, and pathobiology. *Annu. Rev. Pathol.* **13**, 351–378, https://doi.org/10.1146/annurev-pathol-020117-044010
110 Adler, M., Mayo, A., Zhou, X., Franklin, R.A., Meizlish, M.L., Medzhitov, R. et al. (2020) Principles of cell circuits for tissue repair and fibrosis. *iScience* **23**, 100841, https://doi.org/10.1016/j.isci.2020.100841
111 Heydtmann, M., Lalor, P.F., Eksteen, J.A., Hübscher, S.G., Briskin, M. and Adams, D.H. (2005) CXC chemokine ligand 16 promotes integrin-mediated adhesion of liver-infiltrating lymphocytes to cholangiocytes and hepatocytes within the inflamed human liver. *J. Immunol.* **174**, 1055–1062, https://doi.org/10.4049/jimmunol.174.2.1055
112 Oo, Y.H., Banz, V., Kavanagh, D., Liaskou, E., Withers, D.R., Humphreys, E. et al. (2012) CXCR3-dependent recruitment and CCR6-mediated positioning of Th-17 cells in the inflamed liver. *J. Hepatol.* **57**, 1044–1051, https://doi.org/10.1016/j.jhep.2012.07.008
113 Oo, Y.H., Shetty, S. and Adams, D.H. (2010) The role of chemokines in the recruitment of lymphocytes to the liver. *Dig. Dis.* **28**, 31–44, https://doi.org/10.1159/000282062
114 Wehr, A., Baeck, C., Heymann, F., Niemietz, P.M., Hammerich, L., Martin, C. et al. (2013) Chemokine receptor CXCR6-dependent hepatic NK T cell accumulation promotes inflammation and liver fibrosis. *J. Immunol.* **190**, 5226–5236, https://doi.org/10.4049/jimmunol.1202909
115 Gola, A., Dorrington, M.G., Speranza, E., Sala, C., Shih, R.M., Radtke, A.J. et al. (2021) Commensal-driven immune zonation of the liver promotes host defence. *Nature* **589**, 131–136, https://doi.org/10.1038/s41586-020-2977-2
116 Antoniades, C.G., Quaglia, A., Taams, L.S., Mitry, R.R., Hussain, M., Abeles, R. et al. (2012) Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans. *Hepatology* **56**, 735–746, https://doi.org/10.1002/hep.25657
117 Kinoshita, M., Uchida, T., Sato, A., Nakashima, M., Nakashima, H., Shono, S. et al. (2010) Characterization of two F4/80-positive Kupffer cell subsets by their function and phenotype in mice. *J. Hepatol.* **53**, 903–910, https://doi.org/10.1016/j.jhep.2010.04.037
118 Tomita, M., Yamamoto, K., Kobashi, H., Ohmoto, M. and Tsuji, T. (1994) Immunohistochemical phenotyping of liver macrophages in normal and diseased human liver. *Hepatology* **20**, 317–325, https://doi.org/10.1002/hep.1840200208

119 MacParland, S.A., Liu, J.C., Ma, X.-Z., Innes, B.T., Bartczak, A.M., Gage, B.K. et al. (2018) Single cell RNA sequencing of human liver reveals distinct intrahepatic macrophage populations. *Nat. Commun.* **9**, 4383, https://doi.org/10.1038/s41467-018-06318-7
120 Krenkel, O. and Tacke, F. (2017) Liver macrophages in tissue homeostasis and disease. *Nat. Rev. Immunol.* **17**, 306–321, https://doi.org/10.1038/nri.2017.11
121 Stamataki, Z. and Swadling, L. (2020) The liver as an immunological barrier redefined by single-cell analysis. *Immunology* **160**, 157–170, https://doi.org/10.1111/imm.13193
122 Aizarani, N., Saviano, A., Sagar, Mailly, L., Durand, S., Herman, J.S. et al. (2019) A human liver cell atlas reveals heterogeneity and epithelial progenitors. *Nature* **572**, 199–204, https://doi.org/10.1038/s41586-019-1373-2
123 Halpern, K.B., Shenhav, R., Massalha, H., Toth, B., Egozi, A., Massasa, E.E. et al. (2018) Paired-cell sequencing enables spatial gene expression mapping of liver endothelial cells. *Nat. Biotechnol.* **36**, 962–970, https://doi.org/10.1038/nbt.4231
124 Moro-Sibilot, L., Blanc, P., Taillardet, M., Bardel, E., Couillault, C., Boschetti, G. et al. (2016) Mouse and human liver contain immunoglobulin A-secreting cells originating from Peyer's patches and directed against intestinal antigens. *Gastroenterology* **151**, 311–323, https://doi.org/10.1053/j.gastro.2016.04.014
125 David, B.A., Rezende, R.M., Antunes, M.M., Santos, M.M., Freitas Lopes, M.A., Diniz, A.B. et al. (2016) Combination of mass cytometry and imaging analysis reveals origin, location, and functional repopulation of liver myeloid cells in mice. *Gastroenterology* **151**, 1176–1191, https://doi.org/10.1053/j.gastro.2016.08.024
126 Soysa, D.R. and Crispe, I.N. (2016) Subcapsular hepatic dendritic cells: hiding in plain sight. *Gastroenterology* **151**, 1065–1067, https://doi.org/10.1053/j.gastro.2016.10.030
127 Krueger, P.D., Narayanan, S., Surette, F.A., Brown, M.G., Sung, S.-S.J. and Hahn, Y.S. (2017) Murine liver-resident group 1 innate lymphoid cells regulate optimal priming of anti-viral CD8+ T cells. *J. Leukoc. Biol.* **101**, 329–338, https://doi.org/10.1189/jlb.3A0516-225R
128 Hudspeth, K., Donadon, M., Cimino, M., Pontarini, E., Tentorio, P., Preti, M. et al. (2016) Human liver-resident CD56(bright)/CD16(neg) NK cells are retained within hepatic sinusoids via the engagement of CCR5 and CXCR6 pathways. *J. Autoimmun.* **66**, 40–50, https://doi.org/10.1016/j.jaut.2015.08.011
129 Geissmann, F., Cameron, T.O., Sidobre, S., Manlongat, N., Kronenberg, M., Briskin, M.J. et al. (2005) Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. *PLoS Biol.* **3**, e113, https://doi.org/10.1371/journal.pbio.0030113
130 Velázquez, P., Cameron, T.O., Kinjo, Y., Nagarajan, N., Kronenberg, M. and Dustin, M.L. (2008) Cutting edge: activation by innate cytokines or microbial antigens can cause arrest of natural killer T cell patrolling of liver sinusoids. *J. Immunol.* **180**, 2024–2028, https://doi.org/10.4049/jimmunol.180.4.2024
131 Regev, A., Teichmann, S., Lander, E.S., Amit, I., Benoist, C., Birney, E. et al. (2017) The Human Cell Atlas. *eLife* **6**, e27041, https://doi.org/10.7554/eLife.27041
132 Halpern, K.B., Shenhav, R., Matcovitch-Natan, O., Toth, B., Lemze, D., Golan, M. et al. (2017) Single-cell spatial reconstruction reveals global division of labour in the mammalian liver. *Nature* **542**, 352–356, https://doi.org/10.1038/nature21065
133 Guidotti, L.G., Inverso, D., Sironi, L., Di Lucia, P., Fioravanti, J., Ganzer, L. et al. (2015) Immunosurveillance of the liver by intravascular effector CD8(+) T cells. *Cell* **161**, 486–500, https://doi.org/10.1016/j.cell.2015.03.005
134 Ramachandran, P., Matchett, K.P., Dobie, R., Wilson-Kanamori, J.R. and Henderson, N.C. (2020) Single-cell technologies in hepatology: new insights into liver biology and disease pathogenesis. *Nat. Rev. Gastroenterol. Hepatol.* **17**, 457–472, https://doi.org/10.1038/s41575-020-0304-x
135 McKinnon, K.M. (2018) Flow cytometry: an overview. *Curr. Protoc. Immunol.* **120**, 5, 1.1-5.1.11., https://doi.org/10.1002/cpim.40
136 Popescu, D.-M., Botting, R.A., Stephenson, E., Green, K., Webb, S., Jardine, L. et al. (2019) Decoding human fetal liver haematopoiesis. *Nature* **574**, 365–371, https://doi.org/10.1038/s41586-019-1652-y
137 Hartmann, F.J. and Bendall, S.C. (2020) Immune monitoring using mass cytometry and related high-dimensional imaging approaches. *Nat. Rev. Rheumatol.* **16**, 87–99, https://doi.org/10.1038/s41584-019-0338-z
138 Mei, H.E., Leipold, M.D., Schulz, A.R., Chester, C. and Maecker, H.T. (2015) Barcoding of live human peripheral blood mononuclear cells for multiplexed mass cytometry. *J. Immunol.* **194**, 2022–2031, https://doi.org/10.4049/jimmunol.1402661
139 Xu, X., Ye, L., Zhang, Q., Shen, H., Li, S., Zhang, X. et al. (2021) Group-2 innate lymphoid cells promote hepatocellular carcinoma progression via CXCL2-neutrophil induced immunosuppression. *Hepatology*, https://doi.org/10.1002/hep.31855
140 Chen, S., Lake, B.B. and Zhang, K. (2019) High-throughput sequencing of the transcriptome and chromatin accessibility in the same cell. *Nat. Biotechnol.* **37**, 1452–1457, https://doi.org/10.1038/s41587-019-0290-0
141 Kanno, N., LeSage, G., Glaser, S., Alvaro, D. and Alpini, G. (2000) Functional heterogeneity of the intrahepatic biliary epithelium. *Hepatology* **31**, 555–561, https://doi.org/10.1002/hep.510310302
142 Pepe-Mooney, B.J., Dill, M.T., Alemany, A., Ordovas-Montanes, J., Matsushita, Y., Rao, A. et al. (2019) Single-cell analysis of the liver epithelium reveals dynamic heterogeneity and an essential role for YAP in homeostasis and regeneration. *Cell Stem Cell* **25**, 23.e8–38.e8, https://doi.org/10.1016/j.stem.2019.04.004
143 Kolodziejczyk, A.A., Federici, S., Zmora, N., Mohapatra, G., Dori-Bachash, M., Hornstein, S. et al. (2020) Acute liver failure is regulated by MYC- and microbiome-dependent programs. *Nat. Med.* **26**, 1899–1911, https://doi.org/10.1038/s41591-020-1102-2
144 Minervina, A., Pogorelyy, M. and Mamedov, I. (2019) T-cell receptor and B-cell receptor repertoire profiling in adaptive immunity. *Transplant. Int.* **32**, 1111–1123, https://doi.org/10.1111/tri.13475
145 Azizi, E., Carr, A.J., Plitas, G., Cornish, A.E., Konopacki, C., Prabhakaran, S. et al. (2018) Single-cell map of diverse immune phenotypes in the breast tumor microenvironment. *Cell* **174**, 1293.e36–1308.e36, https://doi.org/10.1016/j.cell.2018.05.060
146 Park, J.-E., Botting, R.A., Domínguez Conde, C., Popescu, D.-M., Lavaert, M., Kunz, D.J. et al. (2020) A cell atlas of human thymic development defines T cell repertoire formation. *Science* **367**, 6480, https://doi.org/10.1126/science.aay3224 32079746

147 Wu, T.D., Madireddi, S., de Almeida, P.E., Banchereau, R., Chen, Y.-J.J., Chitre, A.S. et al. (2020) Peripheral T cell expansion predicts tumour infiltration and clinical response. *Nature* **579**, 274–278, https://doi.org/10.1038/s41586-020-2056-8
148 Setliff, I., Shiakolas, A.R., Pilewski, K.A., Murji, A.A., Mapengo, R.E., Janowska, K. et al. (2019) High-throughput mapping of B cell receptor sequences to antigen specificity. *Cell* **179**, 1636.e15–1646.e15, https://doi.org/10.1016/j.cell.2019.11.003
149 van den Brink, S.C., Sage, F., Vértesy, Á., Spanjaard, B., Peterson-Maduro, J., Baron, C.S. et al. (2017) Single-cell sequencing reveals dissociation-induced gene expression in tissue subpopulations. *Nat. Methods* **14**, 935–936, https://doi.org/10.1038/nmeth.4437
150 Habib, N., Li, Y., Heidenreich, M., Swiech, L., Avraham-Davidi, I., Trombetta, J.J. et al. (2016) Div-Seq: single-nucleus RNA-Seq reveals dynamics of rare adult newborn neurons. *Science* **353**, 925–928, https://doi.org/10.1126/science.aad7038
151 Habib, N., Avraham-Davidi, I., Basu, A., Burks, T., Shekhar, K., Hofree, M. et al. (2017) Massively parallel single-nucleus RNA-seq with DroNc-seq. *Nat. Methods* **14**, 955–958, https://doi.org/10.1038/nmeth.4407
152 Andrews, T.S., Atif, J., Liu, J.C., Perciani, C.T., Ma, X.-Z., Thoeni, C. et al. (2021) Single cell, single nucleus and spatial RNA sequencing of the human liver identifies hepatic stellate cell and cholangiocyte heterogeneity. *bioRxiv.* 436882, https://doi.org/10.1101/2021.03.27.436882
153 Kmieć, Z. (2001) Cooperation of liver cells in health and disease. *Adv. Anat. Embryol. Cell Biol.* **161**, 1–151 , https://doi.org/10.1007/978-3-642-56553-3
154 Gaublomme, J.T., Li, B., McCabe, C., Knecht, A., Yang, Y., Drokhlyansky, E. et al. (2019) Nuclei multiplexing with barcoded antibodies for single-nucleus genomics. *Nat. Commun.* **10**, 2907, https://doi.org/10.1038/s41467-019-10756-2
155 Slyper, M., Porter, C.B.M., Ashenberg, O., Waldman, J., Drokhlyansky, E., Wakiro, I. et al. (2020) A single-cell and single-nucleus RNA-Seq toolbox for fresh and frozen human tumors. *Nat. Med.* **26**, 792–802, https://doi.org/10.1038/s41591-020-0844-1
156 ENCODE Project Consortium (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature* **489**, 57–74, https://doi.org/10.1038/nature11247
157 Busslinger, M. and Tarakhovsky, A. (2014) Epigenetic control of immunity. *Cold Spring Harb. Perspect. Biol.* **6**, https://doi.org/10.1101/cshperspect.a024174
158 Zhang, Q. and Cao, X. (2019) Epigenetic regulation of the innate immune response to infection. *Nat. Rev. Immunol.* **19**, 417–432, https://doi.org/10.1038/s41577-019-0151-6
159 Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y. and Greenleaf, W.J. (2013) Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat. Methods* **10**, 1213–1218, https://doi.org/10.1038/nmeth.2688
160 Cusanovich, D.A., Daza, R., Adey, A., Pliner, H.A., Christiansen, L., Gunderson, K.L. et al. (2015) Multiplex single cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science* **348**, 910–914, https://doi.org/10.1126/science.aab1601
161 Wang, A.W., Wang, Y.J., Zahm, A.M., Morgan, A.R., Wangensteen, K.J. and Kaestner, K.H. (2020) The dynamic chromatin architecture of the regenerating liver. *Cell. Mol. Gastroenterol. Hepatol.* **9**, 121–143, https://doi.org/10.1016/j.jcmgh.2019.09.006
162 Winkler, M., Staniczek, T., Kürschner, S.W., Schmid, C.D., Schönhaber, H., Cordero, J. et al. (2021) Endothelial GATA4 controls liver fibrosis and regeneration by preventing a pathogenic switch in angiocrine signaling. *J. Hepatol.* **74**, 380–393, https://doi.org/10.1016/j.jhep.2020.08.033
163 Dechassa, M.L., Tryndyak, V., de Conti, A., Xiao, W., Beland, F.A. and Pogribny, I.P. (2018) Identification of chromatin-accessible domains in non-alcoholic steatohepatitis-derived hepatocellular carcinoma. *Mol. Carcinog.* **57**, 978–987, https://doi.org/10.1002/mc.22818
164 Buenrostro, J.D., Wu, B., Litzenburger, U.M., Ruff, D., Gonzales, M.L., Snyder, M.P. et al. (2015) Single-cell chromatin accessibility reveals principles of regulatory variation. *Nature* **523**, 486–490, https://doi.org/10.1038/nature14590
165 ElTanbouly, M.A., Zhao, Y., Nowak, E., Li, J., Schaafsma, E., Le Mercier, I. et al. (2020) VISTA is a checkpoint regulator for naïve T cell quiescence and peripheral tolerance. *Science* **367**, 6475, https://doi.org/10.1126/science.aay0524
166 Satpathy, A.T., Granja, J.M., Yost, K.E., Qi, Y., Meschi, F., McDermott, G.P. et al. (2019) Massively parallel single-cell chromatin landscapes of human immune cell development and intratumoral T cell exhaustion. *Nat. Biotechnol.* **37**, 925–936, https://doi.org/10.1038/s41587-019-0206-z
167 Wang, W., Fasolino, M., Cattau, B., Goldman, N., Kong, W., Frederick, M.A. et al. (2020) Joint profiling of chromatin accessibility and CAR-T integration site analysis at population and single-cell levels. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 5442–5452, https://doi.org/10.1073/pnas.1919259117
168 Chen, T., Oh, S., Gregory, S., Shen, X. and Diehl, A.M. (2020) Single-cell omics analysis reveals functional diversification of hepatocytes during liver regeneration. *JCI Insight* **5** (22), e141024, https://doi.org/10.1172/jci.insight.141024 33208554
169 Kim, G.B., Rincon Fernandez Pacheco, D., Saxon, D., Yang, A., Sabet, S., Dutra-Clarke, M. et al. (2019) Rapid generation of somatic mouse mosaics with locus-specific, stably integrated transgenic elements. *Cell* **179**, 251.e24–267.e24, https://doi.org/10.1016/j.cell.2019.08.013
170 Cusanovich, D.A., Hill, A.J., Aghamirzaie, D., Daza, R.M., Pliner, H.A., Berletch, J.B. et al. (2018) A single-cell atlas of in vivo mammalian chromatin accessibility. *Cell* **174**, 1309.e18–1324.e18, https://doi.org/10.1016/j.cell.2018.06.052
171 Stoeckius, M., Hafemeister, C., Stephenson, W., Houck-Loomis, B., Chattopadhyay, P.K., Swerdlow, H. et al. (2017) Simultaneous epitope and transcriptome measurement in single cells. *Nat. Methods* **14**, 865–868, https://doi.org/10.1038/nmeth.4380
172 Remmerie, A., Martens, L., Thoné, T., Castoldi, A., Seurinck, R., Pavie, B. et al. (2020) Osteopontin expression identifies a subset of recruited macrophages distinct from kupffer cells in the fatty liver. *Immunity* **53**, 641.e14–657.e14, https://doi.org/10.1016/j.immuni.2020.08.004
173 Corridoni, D., Antanaviciute, A., Gupta, T., Fawkner-Corbett, D., Aulicino, A., Jagielowicz, M. et al. (2020) Single-cell atlas of colonic CD8+ T cells in ulcerative colitis. *Nat. Med.* **26**, 1480–1490, https://doi.org/10.1038/s41591-020-1003-4
174 Leader, A.M., Grout, J.A., Chang, C., Maier, B., Tabachnikova, A., Walker, L. et al. (2020) CITEseq analysis of non-small-cell lung cancer lesions reveals an axis of immune cell activation associated with tumor antigen load and *TP53* mutations. *bioRxiv* 207605, https://doi.org/10.1101/2020.07.16.207605
175 Stoeckius, M., Zheng, S., Houck-Loomis, B., Hao, S., Yeung, B.Z., Mauck, W.M. et al. (2018) Cell Hashing with barcoded antibodies enables multiplexing and doublet detection for single cell genomics. *Genome Biol.* **19**, 224, https://doi.org/10.1186/s13059-018-1603-1

176 Mimitou, E.P., Cheng, A., Montalbano, A., Hao, S., Stoeckius, M., Legut, M. et al. (2019) Multiplexed detection of proteins, transcriptomes, clonotypes and CRISPR perturbations in single cells. *Nat. Methods* **16**, 409–412, https://doi.org/10.1038/s41592-019-0392-0
177 de Matos, L.L., Trufelli, D.C., de Matos, M.G.L. and da Silva Pinhal, M.A. (2010) Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomark Insights* **5**, 9–20, https://doi.org/10.4137/BMI.S2185
178 Taylor, C.R. and Levenson, R.M. (2006) Quantification of immunohistochemistry–issues concerning methods, utility and semiquantitative assessment II. *Histopathology* **49**, 411–424, https://doi.org/10.1111/j.1365-2559.2006.02513.x
179 Zhang, Q., He, Y., Luo, N., Patel, S.J., Han, Y., Gao, R. et al. (2019) Landscape and dynamics of single immune cells in hepatocellular carcinoma. *Cell* **179**, 829.e20–845.e20, https://doi.org/10.1016/j.cell.2019.10.003
180 Femino, A.M., Fay, F.S., Fogarty, K. and Singer, R.H. (1998) Visualization of single RNA transcripts in situ. *Science* **280**, 585–590, https://doi.org/10.1126/science.280.5363.585
181 Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A. and Tyagi, S. (2008) Imaging individual mRNA molecules using multiple singly labeled probes. *Nat. Methods* **5**, 877–879, https://doi.org/10.1038/nmeth.1253
182 Sharma, A., Seow, J.J.W., Dutertre, C.-A., Pai, R., Blériot, C., Mishra, A. et al. (2020) Onco-fetal reprogramming of endothelial cells drives immunosuppressive macrophages in hepatocellular carcinoma. *Cell* **183**, 377.e21–394.e21, https://doi.org/10.1016/j.cell.2020.08.040
183 Chen, K.H., Boettiger, A.N., Moffitt, J.R., Wang, S. and Zhuang, X. (2015) RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science* **348**, aaa6090, https://doi.org/10.1126/science.aaa6090
184 Aguilar-Bravo, B. and Sancho-Bru, P. (2019) Laser capture microdissection: techniques and applications in liver diseases. *Hepatol. Int.* **13**, 138–147, https://doi.org/10.1007/s12072-018-9917-3
185 Kandathil, A.J., Graw, F., Quinn, J., Hwang, H.S., Torbenson, M., Perelson, A.S. et al. (2013) Use of laser capture microdissection to map hepatitis C virus-positive hepatocytes in human liver. *Gastroenterology* **145**, 1404.e1–1413.e1, https://doi.org/10.1053/j.gastro.2013.08.034
186 Melis, M., Diaz, G., Kleiner, D.E., Zamboni, F., Kabat, J., Lai, J. et al. (2014) Viral expression and molecular profiling in liver tissue versus microdissected hepatocytes in hepatitis B virus-associated hepatocellular carcinoma. *J. Transl. Med.* **12**, 230, https://doi.org/10.1186/s12967-014-0230-1
187 Massalha, H., Bahar Halpern, K., Abu-Gazala, S., Jana, T., Massasa, E.E., Moor, A.E. et al. (2020) A single cell atlas of the human liver tumor microenvironment. *Mol. Syst. Biol.* **16**, e9682, https://doi.org/10.15252/msb.20209682
188 Munshaw, S., Hwang, H.S., Torbenson, M., Quinn, J., Hansen, K.D., Astemborski, J. et al. (2012) Laser captured hepatocytes show association of butyrylcholinesterase gene loss and fibrosis progression in hepatitis C-infected drug users. *Hepatology* **56**, 544–554, https://doi.org/10.1002/hep.25655
189 Gehring, S., Dickson, E.M., San Martin, M.E., van Rooijen, N., Papa, E.F., Harty, M.W. et al. (2006) Kupffer cells abrogate cholestatic liver injury in mice. *Gastroenterology* **130**, 810–822, https://doi.org/10.1053/j.gastro.2005.11.015
190 Pittet, M.J., Garris, C.S., Arlauckas, S.P. and Weissleder, R. (2018) Recording the wild lives of immune cells. *Sci. Immunol.* **3** (27), eaaq0491, https://doi.org/10.1126/sciimmunol.aaq0491
191 Wang, J. and Kubes, P. (2016) A reservoir of mature cavity macrophages that can rapidly invade visceral organs to affect tissue repair. *Cell* **165**, 668–678, https://doi.org/10.1016/j.cell.2016.03.009
192 Ståhl, P.L., Salmén, F., Vickovic, S., Lundmark, A., Navarro, J.F., Magnusson, J. et al. (2016) Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. *Science* **353**, 78–82, https://doi.org/10.1126/science.aaf2403
193 Vickovic, S., Eraslan, G., Salmén, F., Klughammer, J., Stenbeck, L., Schapiro, D. et al. (2019) High-definition spatial transcriptomics for in situ tissue profiling. *Nat. Methods* **16**, 987–990, https://doi.org/10.1038/s41592-019-0548-y
194 Zheng, C., Zheng, L., Yoo, J.-K., Guo, H., Zhang, Y., Guo, X. et al. (2017) Landscape of infiltrating T cells in liver cancer revealed by single-cell sequencing. *Cell* **169**, 1342.e16–1356.e16, https://doi.org/10.1016/j.cell.2017.05.035
195 Segal, J.M., Kent, D., Wesche, D.J., Ng, S.S., Serra, M., Oulès, B. et al. (2019) Single cell analysis of human foetal liver captures the transcriptional profile of hepatobiliary hybrid progenitors. *Nat. Commun.* **10**, 3350, https://doi.org/10.1038/s41467-019-11266-x
196 Ramachandran, P., Dobie, R., Wilson-Kanamori, J.R., Dora, E.F., Henderson, B.E.P., Luu, N.T. et al. (2019) Resolving the fibrotic niche of human liver cirrhosis at single-cell level. *Nature* **575**, 512–518, https://doi.org/10.1038/s41586-019-1631-3
197 Droin, C., Kholtei, J.E., Bahar Halpern, K., Hurni, C., Rozenberg, M., Muvkadi, S. et al. (2021) Space-time logic of liver gene expression at sub-lobular scale. *Nat. Metab.* **3**, 43–58, https://doi.org/10.1038/s42255-020-00323-1
198 Giesen, C., Wang, H.A.O., Schapiro, D., Zivanovic, N., Jacobs, A., Hattendorf, B. et al. (2014) Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. *Nat. Methods* **11**, 417–422, https://doi.org/10.1038/nmeth.2869
199 Baharlou, H., Canete, N.P., Cunningham, A.L., Harman, A.N. and Patrick, E. (2019) Mass cytometry imaging for the study of human diseases-applications and data analysis strategies. *Front. Immunol.* **10**, 2657, https://doi.org/10.3389/fimmu.2019.02657
200 Traum, D., Wang, Y.J., Schwarz, K.B., Schug, J., Wong, D.K., Janssen, H.L. et al. (2021) Highly multiplexed 2-dimensional imaging mass cytometry analysis of HBV-infected liver. *JCI Insight* **6** (7), e146883, https://doi.org/10.1172/jci.insight.146883
201 Ho, W.J., Zhu, Q., Durham, J., Popovic, A., Xavier, S., Leatherman, J. et al. (2021) Neoadjuvant cabozantinib and nivolumab convert locally advanced hepatocellular carcinoma into resectable disease with enhanced antitumor immunity. *Nat. Cancer* **2**, 891–903, https://doi.org/10.1038/s43018-021-00234-4
202 Sheng, J., Zhang, J., Wang, L., Tano, V., Tang, J., Wang, X. et al. (2021) Topological analysis of hepatocellular carcinoma tumour microenvironment based on imaging mass cytometry reveals cellular neighbourhood regulated reversely by macrophages with different ontogeny. *Gut*, https://doi.org/10.1136/gutjnl-2021-324339
203 Chang, Q., Ornatsky, O.I., Siddiqui, I., Straus, R., Baranov, V.I. and Hedley, D.W. (2016) Biodistribution of cisplatin revealed by imaging mass cytometry identifies extensive collagen binding in tumor and normal tissues. *Sci. Rep.* **6**, 36641, https://doi.org/10.1038/srep36641

Clinical Science (2021) **135** 2445–2466
https://doi.org/10.1042/CS20190654

204 Chang, Q., Ornatsky, O.I., Siddiqui, I., Loboda, A., Baranov, V.I. and Hedley, D.W. (2017) *Imaging Mass Cytometry. Cytometry A.* **91**, 160–169, https://doi.org/10.1002/cyto.a.23053 28160444

205 Schapiro, D., Jackson, H.W., Raghuraman, S., Fischer, J.R., Zanotelli, V.R.T., Schulz, D. et al. (2017) histoCAT: analysis of cell phenotypes and interactions in multiplex image cytometry data. *Nat. Methods* **14**, 873–876, https://doi.org/10.1038/nmeth.4391

206 Sommer, C., Straehle, C., Kothe, U. and Hamprecht, F.A. (2011) Ilastik: interactive learning and segmentation toolkit. *IEEE International Symposium on Biomedical Imaging: From Nano to Macro* 230–233, https://doi.org/10.1109/ISBI.2011.5872394

207 Dao, D., Fraser, A.N., Hung, J., Ljosa, V., Singh, S. and Carpenter, A.E. (2016) CellProfiler Analyst: interactive data exploration, analysis and classification of large biological image sets. *Bioinformatics* **32**, 3210–3212, https://doi.org/10.1093/bioinformatics/btw390

208 Carpenter, A.E., Jones, T.R., Lamprecht, M.R., Clarke, C., Kang, I.H., Friman, O. et al. (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* **7**, R100, https://doi.org/10.1186/gb-2006-7-10-r100

209 Baars, M.J.D., Sinha, N., Amini, M., Pieterman-Bos, A., van Dam, S., Ganpat, M.M.P. et al. (2021) MATISSE: a method for improved single cell segmentation in imaging mass cytometry. *BMC Biol.* **19**, 99, https://doi.org/10.1186/s12915-021-01043-y

210 Bertocchi, A., Carloni, S., Ravenda, P.S., Bertalot, G., Spadoni, I., Lo Cascio, A. et al. (2021) Gut vascular barrier impairment leads to intestinal bacteria dissemination and colorectal cancer metastasis to liver. *Cancer Cell* **39**, 708.e11–724.e11, https://doi.org/10.1016/j.ccell.2021.03.004

211 Strauss, O., Phillips, A., Ruggiero, K., Bartlett, A. and Dunbar, P.R. (2017) Immunofluorescence identifies distinct subsets of endothelial cells in the human liver. *Sci. Rep.* **7**, 44356, https://doi.org/10.1038/srep44356

212 Eckert, C., Klein, N., Kornek, M. and Lukacs-Kornek, V. (2015) The complex myeloid network of the liver with diverse functional capacity at steady state and in inflammation. *Front. Immunol.* **6**, 179, https://doi.org/10.3389/fimmu.2015.00179