

REVIEW

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Liver in infections: a single-cell and spatial transcriptomics perspective

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Abstract

The liver is an immune organ that plays a vital role in the detection, capture, and clearance of pathogens and foreign antigens that invade the human body. During acute and chronic infections, the liver transforms from a tolerant to an active immune state. The defence mechanism of the liver mainly depends on a complicated network of intrahepatic and translocated immune cells and non-immune cells. Therefore, a comprehensive liver cell atlas in both healthy and diseased states is needed for new therapeutic target development and disease intervention improvement. With the development of high-throughput single-cell technology, we can now decipher heterogeneity, differentiation, and intercellular communication at the single-cell level in sophisticated organs and complicated diseases. In this concise review, we aimed to summarise the advancement of emerging high-throughput single-cell technologies and re-define our understanding of liver function towards infections, including hepatitis B virus, hepatitis C virus, *Plasmodium*, schistosomiasis, endotoxemia, and corona virus disease 2019 (COVID-19). We also unravel previously unknown pathogenic pathways and disease mechanisms for the development of new therapeutic targets. As high-throughput single-cell technologies mature, their integration into spatial transcriptomics, multiomics, and clinical data analysis will aid in patient stratification and in developing effective treatment plans for patients with or without liver injury due to infectious diseases.

Keywords Liver, Infections, Single-cell technologies, Spatial transcriptome

Introduction

The mammalian liver is a complex organ consisting of diverse cell types that perform multiple physiological functions including digestion, synthesis, metabolism, and detoxification. It also acts as an immune organ and plays a crucial role in anti-infection, autoimmune stability, and

anti-tumour effects. In particular, the liver has a unique immunological advantage as its parenchymal and non-parenchymal cells can exert immune functions to participate in immunoregulation for maintaining homeostasis. As a fundamental immune organ, the liver receives blood directly through the portal vein draining from the peritoneum, gastrointestinal tract, pancreas, and spleen, while several classic immune sentinel surveillance sites, such as the lymph nodes and spleen, are omitted [1] (Fig. 1A). Under physiological circumstances, the liver is more heavily exposed to microorganisms and endotoxins than other organs or tissues owing to its unique anatomical location. Therefore, the liver usually maintains a tolerogenic immune state to prevent needless immune activation and excessive autoimmune responses [2, 3]. However, liver tolerance also allows viruses, parasites, and other microorganisms to persist chronically and

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cause long-term damage [4, 5]. The maintenance of the immunologically balanced state in the liver is essential for human health and survival. During acute and chronic infections, the liver transforms from a tolerant state to an active immune state and constitutes an important line of defence against invading microorganisms [6–9]. The defence mechanism of the liver depends mainly on a complex network of immune cells, including Kupffer cells (KCs), dendritic cells (DCs), neutrophils, natural killer (NK) cells, and B and T lymphocytes [10] (Fig. 1B). Furthermore, non-immune cells such as hepatocytes, cholangiocytes, hepatic stellate cells (HSCs), and liver sinusoidal endothelial cells (LSECs) contribute to immunity by recruiting proinflammatory immune cells (Fig. 1B) and simultaneously producing inflammatory cytokines such as acute-phase proteins, complement factors, and cell adhesion molecules [11–13]. Owing to the crucial role played by the liver in defence against harmful microorganisms, a comprehensive liver cell atlas in both healthy and diseased states is needed for new therapeutic target development and disease intervention improvement.

Conventionally, the liver landscape in a healthy or diseased state is studied using whole-tissue sequencing of a bulk and mixed population of cells within different zonation, which makes it difficult to interpret the uniqueness of individual cells and cell–cell crosstalk in maintaining cellular homeostasis [14]. With the development of high-throughput single-cell technology, we can now decipher heterogeneity, differentiation, and intercellular communication at the single-cell level in sophisticated organs and complicated diseases. For example, single-cell RNA sequencing (scRNA-seq) can characterise the complete transcriptome of a single cell on a massive scale. This novel technology usually involves tissue dissociation, single-cell capture, cell lysis, and messenger ribonucleic acid (mRNA) marked by cell barcodes (Fig. 2A, C). Following RNA capture, the next steps are reverse transcription and amplification, complementary deoxyribonucleic acid (cDNA) library construction, and sequencing using next-generation sequencing technology (Fig. 2C, D). Using the aforementioned cell barcode, the sequence reads are then navigated back to the original cells, producing complete transcriptome data for thousands of cells in a single experiment (Fig. 2). In many studies, scRNA-seq

has been used for better understanding of cellular and pathological mechanisms and gaining novel cellular and biomedical insights into disease pathology [15, 16].

To maintain liver homeostasis in a healthy state while remaining responsive to exogenous and endogenous stress, a highly organised and specialised structure of liver cells, including hormones, cytokines, metabolites, and microbial products, is needed to integrate local information at the molecular level. Liver research has embraced and benefited from innovative approaches (high-throughput single-cell technologies and multiomics), with several liver scRNA-seq studies being published in the past 5 years [17–23]. Recently, several reviews have summarised the application of single-cell techniques in various types of liver diseases, especially liver cancer [24], non-alcoholic fatty liver disease (NAFLD) [25], and fibrosis [6]. In our review, we elaborate on how emerging single-cell technologies strengthen our understanding of liver function during local and systemic infection, including the core tips of application schema in the liver, the single-cell atlas of the normal liver and diseased liver towards infection, clinical implications, and future directions. This concise review summarises recent findings and highlights new directions for the application of high-throughput single-cell and spatial technologies with multiomics to unravel previously unknown mechanisms for the advancement of new therapeutics targeting liver pathology in response to infections.

Technology

Advantages and disadvantages of scRNA-seq in liver research

RNA sequencing is increasingly being used to investigate phenotypes and deep driving mechanisms of liver pathologies. Whole-tissue RNA sequencing, also known as “bulk sequencing”, has been extensively used to identify major differences in the transcriptome between normal and diseased conditions [26, 27]. Bulk sequencing provides an average value of the molecular signal of every sample, which represents mixed RNA contents from different cells located within the same sample and is hence remarkably affected by cell type prevalence [28]. Therefore, bulk sequencing cannot be applied to investigate cell heterogeneity (i.e., cell subgroups among major cell

(See figure on next page.)

Fig. 1 Anatomical structure, composition, and function of the liver. **A** Arterial supply to the liver and portal venous drainage. **B** Anatomical structure and cellular composition of hepatic lobules. **C** Single-cell RNA sequencing has revealed gradients of gene expression along the three different zones of hepatic lobule in different cell lineages, enabling the differentiation of portal zone and central zone hepatocytes, LSECs, and HSCs. These markers are shown in Figure. **D** Main functions of the liver. *ALB* albumin, *ADAMTSL2* ADAMTS-like protein 2, *CYP2E1* cytochrome P450 2E1, *DLL4* Delta-like protein 4, *ECAD* E-cadherin, *EFNB2* ephrin-B2, *GSS* glutathione synthetase, *HSC* hepatic stellate cell, *ITGB3* integrin β 3, *KIT* mast/stem cell growth factor receptor Kit, *LSEC* liver sinusoidal endothelial cell, *NGFR* tumour necrosis factor receptor superfamily member 16, *Rspo3* R-spondin-3, *THBD* thrombomodulin, *WNT2* wingless-type MMTV integration site family member 2

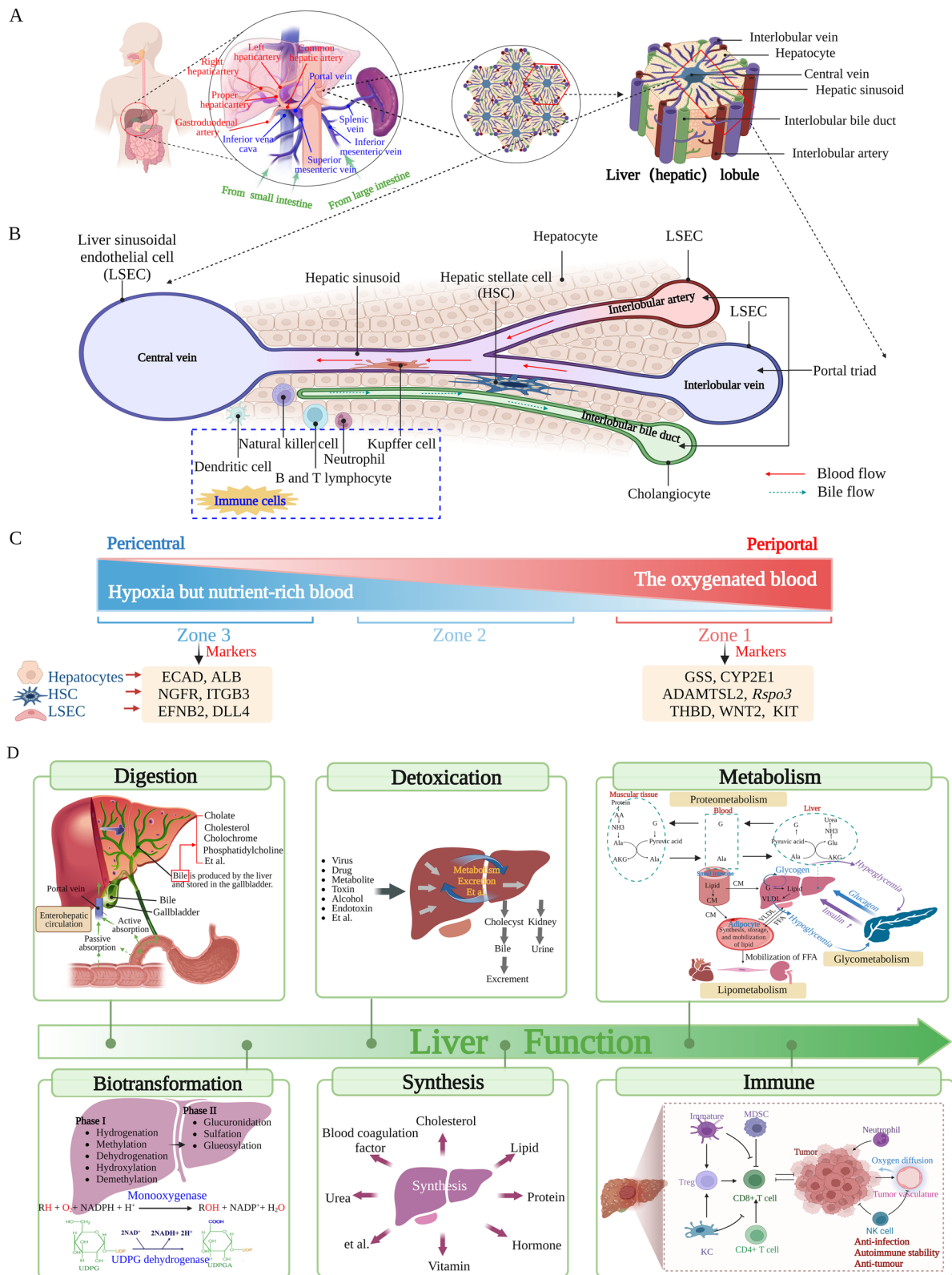


Fig. 1 (See legend on previous page.)

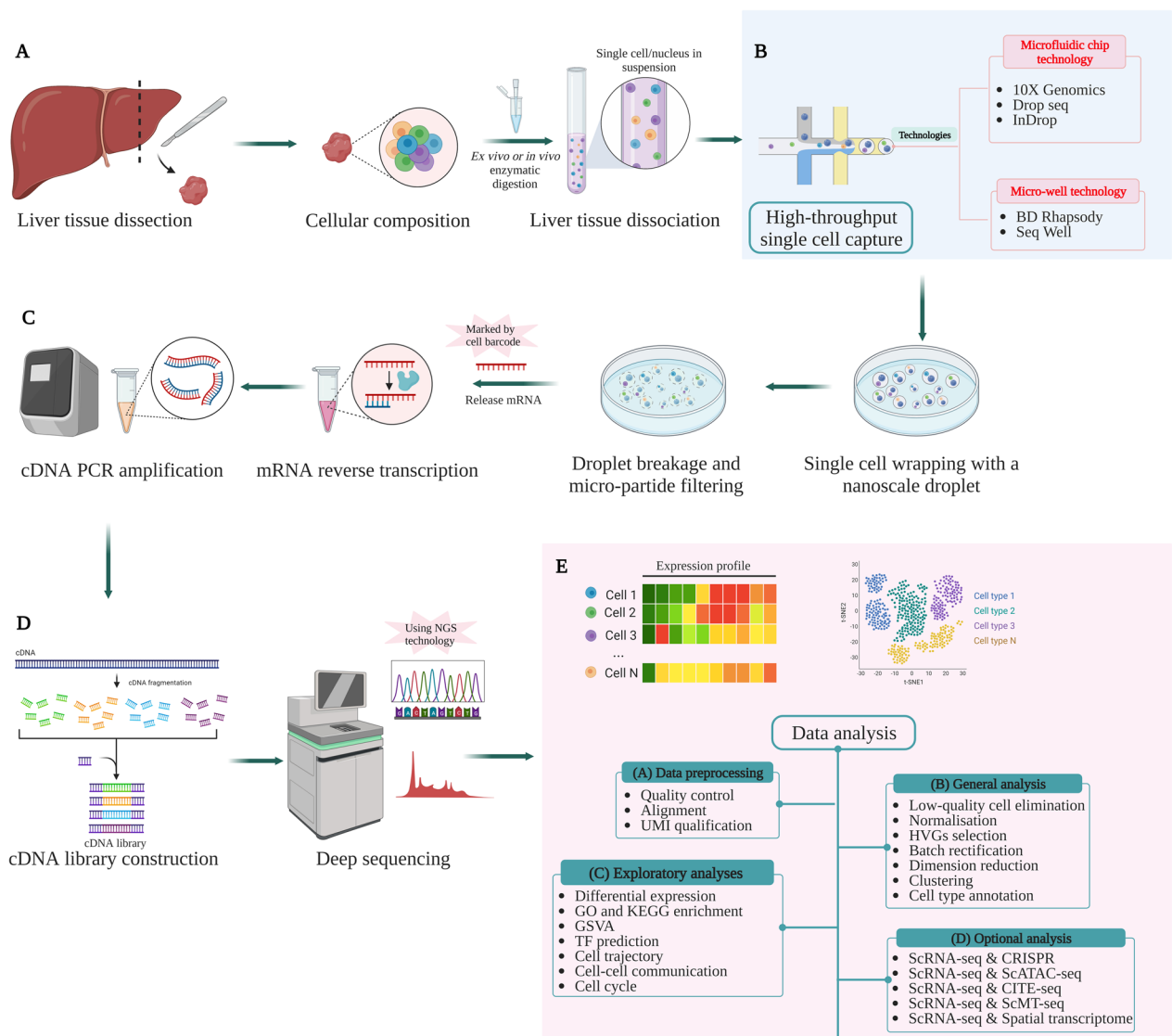


Fig. 2 A concise overview of scRNA-seq workflow. *CRISPR* clustered regularly interspaced short palindromic repeats, *CITE-seq* cellular indexing of transcriptomes and epitopes by sequencing, *GSVA* gene set variation analysis, *HVGs* highly variable genes, *PCR* polymerase chain reaction, *ScRNA-seq* single-cell RNA sequencing, *ScATAC-seq* single-cell assay for transposase-accessible chromatin using sequencing, *ScMT-seq* single-cell methylome and transcriptome sequencing, *UMI* unique molecular identifier

types), specific pathogenic cell culprits, and rare cell sub-populations or to dissect tumour clonal evolution and the microenvironment.

Owing to its high accuracy and specificity, scRNA-seq has become an ideal research tool for single cell study [29]. Unbiased high-throughput research can be performed with a minimum starting sample volume. ScRNA-seq can be used alongside multiomics to simultaneously analyse the genome, transcriptome, epigenome, proteome, and metabolome [30]. The functional state of a single cell can be evaluated to identify and discover novel cell types in an unbiased manner. It can also be used to

construct the differentiation track of the cell lineage and create a molecular map of the cell development lineage. Compared to traditional sequencing, scRNA-seq displays stronger discovery ability in new gene detection without knowing sequence information in advance and has higher sensitivity in rare mutations and transcript quantification [31]. Moreover, scRNA-seq avoids polymerase chain reaction (PCR) amplification bias. PCR amplification is required for sequencing in many cases; however, the limitation of amplification is that different nucleic acid sequences have different amplification degrees under the same conditions. Therefore, the abundance of

nucleic acid sequences after amplification differs from that before amplification. ScRNA-seq can avoid the PCR amplification preference because it calculates the actual value of the nucleic acid sequence before amplification rather than the relative abundance of the nucleic acid sequence after amplification.

However, scRNA-seq has several limitations. First, the need for cell viability and quality is high and essential for subsequent analysis, whereas obtaining qualified single-cell suspensions is sometimes difficult. ScRNA-seq has some inherent methodological defects, one of which is “artificial transcriptional stress responses” where tissue dissociation could induce the expression of stress genes, resulting in artificial changes in cellular transcriptome patterns, as confirmed by several experiments [32, 33]. Thus, the dissociation process should be carefully optimised to achieve maximum production without inducing biases [32]. Moreover, the dynamic nature of gene expression can lead to noise in scRNA-seq technologies, as the levels of multiple gene transcripts are typically in a constant state of flux [34].

Secondly, it should be noted that scRNA-seq is limited to detecting the expression at the RNA level, and cannot provide information on protein expression [35]. The conversion from RNA to protein involves a complex array of regulatory factors, including post-transcriptional modifications, RNA shearing, RNA stability, and protein degradation, which can result in discrepancies between RNA and protein levels. For instance, researchers in one study detected stimulator of interferon genes (STING) mRNA in hepatocytes, but did not detect STING proteins [36, 37]. This is particularly relevant in the liver, where endogenous signalling molecules can significantly impact liver immune tolerance, yet may not be revealed by scRNA-seq [38]. Therefore, to gain a comprehensive understanding of the transcriptome and proteome of individual cells, it is crucial to combine scRNA-seq with single-cell protein-based techniques, such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), cytometry by time-of-flight detection (CyTOF), and genetically encoded fluorescent probes.

Thirdly, compared with bulk sequencing, scRNA-seq is far more expensive. Although the experimental costs have been reduced to some extent due to the occurrence of high-throughput scRNA-seq workflows, it is still an expensive technique, costing up to nearly \$3000 per sample. Lastly, data analysis of scRNA-seq is time-consuming and requires professional expertise. The specialised knowledge required to conduct these bioinformatic analyses is not trivial. However, standardisation of single-cell analyses and the establishment of sequencing and analysis cores at major research institutes enable clinical biologists to bypass these obstacles step-by-step. Thus,

when designing single-cell sequencing experiments and workflows, several essential factors must be considered to obtain the most informative and credible data from each experiment. The advantages and disadvantages of scRNA-seq and bulk sequencing are compared in Fig. 3.

Moreover, the liver’s distinctive characteristics pose methodological challenges and impact scRNA-seq outcomes. Hepatocytes have an abundance of various enzymes, particularly RNA enzymes, which can degrade RNA and influence downstream analysis. Traditional dissociation methods usually employ mixed enzymes at 37 °C, a temperature at which the liver enzymes are highly active, which leads to transcriptional bias. However, the use of cold protease to dissociate tissues at low temperatures has been demonstrated to enhance cellular activity and significantly reduce gene artefact expression [33]. To further preserve RNA integrity, it is viable to add nuclease inhibitors, such as SUPERaseIn and EDTA, to the lysis buffer [39, 40]. Additionally, under high-fat conditions, the gene expression of liver cells may be significantly altered, resulting in the up- or downregulation of certain genes related to fat metabolism [41, 42]. This process may obscure signals from other cell types, thus introducing bias into single-cell transcriptome sequencing results. The use of degreasing reagents, such as Triton X-100, in the cell lysis process can effectively dissolve cells and remove lipids to ensure the acquisition of high-quality RNA [39, 43]. Lastly, hepatocytes are particularly rich in mitochondria, leading to elevated levels of mitochondrial RNA that may interfere with the identification of other RNAs. To address this issue, RNA sequencing data analysis can incorporate unique molecular identifier (UMI) technology to eliminate multi-copy RNA [44]. In essence, single-cell transcriptome technology encounters distinct obstacles when detecting liver tissue, necessitating meticulous procedures for sample collection, processing, separation, screening, and implementing specialised techniques to manage the liver’s high lipid and enzymatic properties, thereby ensuring the precision and dependability of sequencing outcomes.

From liver to single cell

Liver dissociation method

For a scRNA-seq experiment, a high quality, viable cell suspension of the liver needs to be obtained through tissue dissociation and cell isolation. Liver dissociation is challenging, and the conventional dissociation method usually causes low hepatic cell vitality and alterations in the genuine ratio and cell types. The basic structural units of the liver are complex in composition, with vigorous metabolism, and cell vitality rapidly decreases after isolation. To address this problem, a study examined the optimal method for retrieving the maximum possible

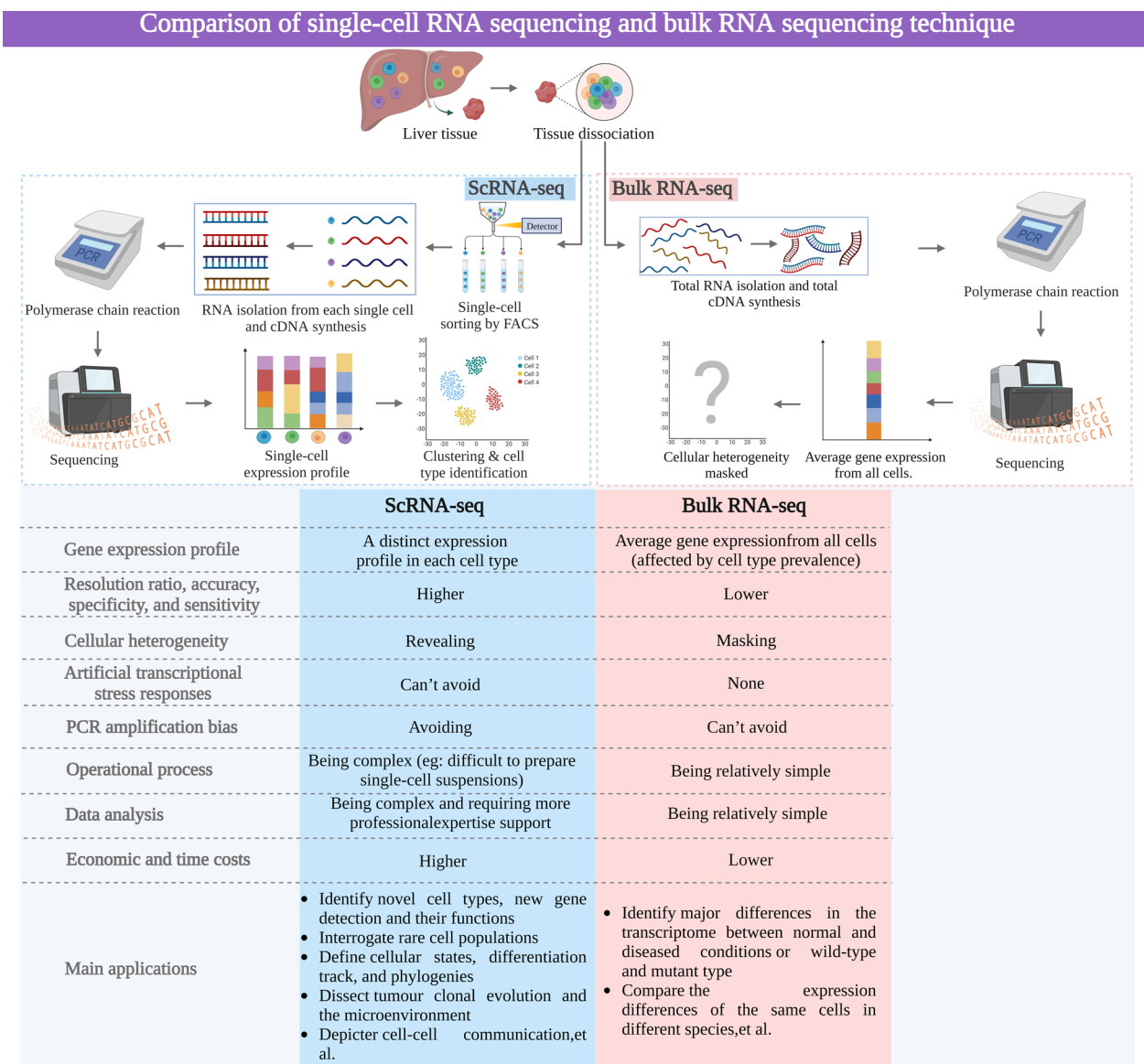


Fig. 3 Comparison of scRNA-seq and bulk RNA-seq in the liver. FACS fluorescence-activated cell sorting

number of high quality hepatic cells [41]. Hepatic cells were isolated via ex vivo or in vivo enzymatic digestion using murine livers. Briefly, for ex vivo digestion, mouse livers were isolated, cut into small pieces, and incubated with deoxyribonuclease (DNase) and collagenase A with gentle shaking (Fig. 4). For in vivo digestion, the livers were first perfused with an ethylene glycol tetraacetic acid (EGTA)-containing solution after retrograde cannulation, followed by perfusion with collagenase A. The liver was removed from the body, minced, and incubated with a combination of DNase and collagenase A. Regarding the number of genes/cells, no differences were observed between the two digestion methods. However,

there was a remarkable difference in the total cell numbers, cell types, and the cell percentages between the two digestion methods (Fig. 4). Notably, there is still an obvious discrepancy in the number and percentage of cells obtained from the digestion methods compared to that in the genuine liver [18, 45]. For example, parenchymal cells such as cholangiocytes which make up 3–5% of all liver cells [46] consist of only 199 (0.64%) of 8444 cells from the scRNA-seq map [41]. Thus, an optimal and unified method for retrieving all hepatic cells approximated to the genuine state is urgently required to start the scRNA-seq process and reduce bias in different research groups.

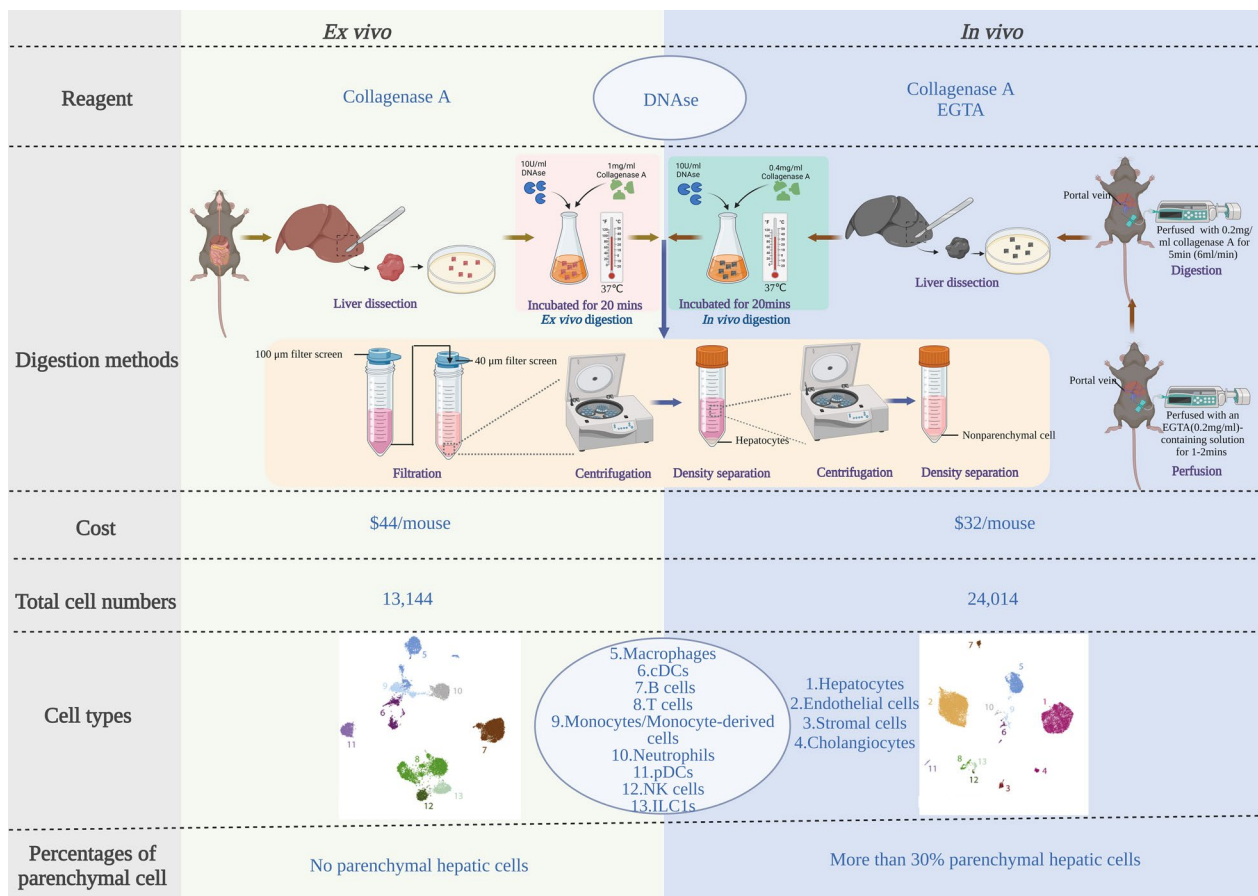


Fig. 4 Comparison of two different methods of liver dissociation: ex vivo and in vivo. This table is summarised from REF [41]. *DNase* deoxyribonuclease, *EGTA* ethylene glycol tetraacetic acid, *cDCs* conventional dendritic cells, *pDCs* plasmacytoid dendritic cells, *NK cells* natural killer cells, *ILC1s* type 1 innate lymphoid cells

Single-cell capture strategy

Before studying a single cell, we must first capture it reliably [47]. Currently, several vendors have provided technologies for single-cell capture. Early stage strategies include limiting dilution [48], fluorescence-activated cell sorting (FACS) [49], micromanipulation using an inverted microscope, and the use of motorised micromanipulation or laser microdissection [50]. Although every method has its advantages, their shortcomings are also evident, including the complex procedures, high capture cost, lack of suitability for micro samples, and susceptibility to errors during calculations [51]. In recent years, single-cell sequencing has entered has considerably progressed. There are two strategies for achieving high-throughput single-cell capture. One is microfluidic chip technology, represented by 10X Genomics [52], Drop seq [53] and inDrop [53]. The other is microwell technology, represented by BD Rhapsody [54] and Seq Well [55] (Fig. 2B). High-throughput microdroplet-based microfluidic technologies are used to capture single cells in oil

droplets through microfluidic chips, e.g., 10X chromium. The core idea is to assign different barcode sequences to different cells [52]. When building a library, nucleic acid molecules with the same barcode sequence are considered to be from the same cell; therefore, a library can be constructed for hundreds of cells at a time while distinguishing them smoothly [52]. Microwell plate technology involves depositing a single cell and magnetic bead into a micropore. The sequence structure of the magnetic bead is similar to that of 10X Genomics, and the poly A tail of free Mrna can be captured [56]. The advantages of the two methods are high cell flux, fast cycling, low cost, high cell capture efficiency, and simple operation of commercial instruments [56]. In summary, the selection of a single-cell capture strategy largely relies on the cell type of interest, its prevalence in the tissue or organ, and costs.

scRNA-seq data analysis

Bioinformational analysis of data is the central process for expanding the application scope of scRNA-seq in

the medical sciences. The pipeline for scRNA-seq data analysis primarily includes four consecutive analysis steps: data pre-processing, general analysis, exploratory analysis, and optional analysis [57]. According to the data source and sequencing platform, the basic formats of raw sequencing data for scRNA-seq are the FASTQ or BCL formats [58]. The pre-processing of raw data includes quality control, alignment, and UMI (transcript) qualification [44]. After data preparation, a general analysis is conducted, including low-quality cell elimination, normalisation, highly variable genes (HVGs) selection, batch rectification, dimension reduction, clustering, and cell type annotation [16]. Furthermore, various exploratory analyses are conducted based on the research purpose, including differential expression analysis, functional enrichment analyses such as gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment, gene set variation analysis (GSVA), transcription factor prediction, and cell trajectory, cell–cell communication, and cell cycle analyses [59]. In addition to exploratory analyses, many other significant issues require more attention and in-depth exploration. For instance, multidimensional analysis can be conducted by combining scRNA-seq and clustered regularly interspaced short palindromic repeats (CRISPR) screening [60]. Integrated analysis of scRNA-seq and multiomics can also be applied, including single-cell whole-genome sequencing [61], single-cell assay for transposase-accessible chromatin using sequencing (scATAC) [62], single-cell methylome and transcriptome sequencing (scMT) [63, 64], cellular indexing of transcriptomes and epitopes by sequencing [65, 66], single-microbe genomics [67], and spatial transcriptomics [68]. Application of conjoint analysis of these aforementioned techniques allows a better and deeper understanding of core biological and pathological processes and mechanisms, which opens a new avenue for the future development of single-cell technology. The major analysis workflow for scRNA-seq data is summarised in Fig. 2E. More details regarding each module can be found in other reviews [16].

Single nucleus RNA sequencing (snRNA-seq) in liver analysis

The limitations of scRNA-seq include difficulty in tissue preservation and dissociation to obtain single-cell suspensions, inapplicability to sample types containing large cells, applicability to fresh tissue samples only, and artificial stress responses [69]. Recently, researchers have focused on snRNA-seq in liver research [18]. Notably, snRNA-seq can avoid the cell dissociation step by using detergents to directly release nuclei from intact cells [18]. Instead of sequencing all the Mrna in the cytoplasm, snRNA-seq captures only the transcripts in the

nucleus [70]. Thus, it is understandable that these differences affect the sensitivity of these methods in delineating the subtypes of the respective cell types. ScRNA-seq and snRNA-seq have been compared while conducting in liver analysis [41]. Typically, a lower number of genes/cells was yielded by snRNA-seq than by scRNA-seq. However, this did not prevent the identification of distinct cell types, as both scRNA-seq and snRNA-seq identified highly expressed genes in each population [41]. Nevertheless, gene expression was often higher in scRNA-seq than in snRNA-seq. Additionally, an expression pattern of digestion-associated and snRNA-seq-associated genes was observed across different cell types [41]. Despite the fact that snRNA-seq best recapitulated the cell frequencies observed in vivo, it was inferior to scRNA-seq in terms of genes/cell. In liver analysis, snRNA-seq might have advantages in detecting a greater number of cholangiocytes, hepatocytes, and mesenchymal cells using frozen liver tissues [19, 71]. Notably, snRNA-seq only captures Mrna in the nucleus, and hence, might lose important information on biological processes involved in RNA stability, Mrna processing, and metabolism. In addition, a comprehensive atlas of the intrahepatic immune landscape is essential for interpreting liver pathogenesis. However, plenty of important information regarding immune cells was lost from the snRNA-seq data. For example, none of the T cell or B-cell receptor components were detected in liver samples using snRNA-seq [18]. Therefore, it is recommended that studies investigate intrahepatic immune cell populations using scRNA-seq. As each approach has its strengths and weaknesses, the optimal method depends on the scientific questions being addressed.

Spatial transcriptomic techniques used in liver analysis

ScRNA-seq entails the dissociation of organs and tissues into single-cell suspensions, resulting in the complete loss of spatial information and the additional risk of undesirable transcriptional changes [32, 72]. The liver is a highly complex organ with distinct spatial properties. A deep understanding of spatial heterogeneity is vital for comprehending hepatic structure and function, both in homeostasis and disease. Previous studies have usually performed fluorescence in situ hybridisation [73], immunohistochemical staining, selective perfusion isolation, or laser capture microdissection to define unique transcriptional and protein patterns of perivenular and periportal hepatocytes in different regions of the liver using zonal “landmark genes” [74]. Owing to the limitations of methods available in the early stage, novel approaches are generalising the technique of spatial transcriptomics (ST) to eliminate dependency on landmark genes or nearby cells [75, 76] (Table 1). ST has unique advantages in obtaining

Table 1 Application of high-throughput single-cell and spatial technologies in the liver

Technologies	Molecular layer	Application	Ref.
ScRNA-seq	Transcriptomic	Applicable for fresh tissue samples only and artificial transcriptional stress responses	[69]
SnRNA-seq	Transcriptomic	Application to fresh and frozen samples, particularly those that are difficult to dissociate into single-cell suspensions. Can provide data on difficult to isolate cells with some loss of transcriptional depth and the cytoplasmic RNA fraction	[16]
Spatial transcriptomics	Transcriptomic	Used to eliminate dependency on landmark genes and adherent cells, and provide spatial annotation of structures	[75, 76]
Sc ATAC-seq	Epigenetic	Unbiased detection of epigenetic landscape across the human genome. Capture of early lineage-determining epigenetic features may allow for a higher resolution when identifying cell subsets than with transcriptomic data	[62]
Single-cell immune profiling	Transcriptomic and TCR sequencing	Enables annotation of invariant T-cells, tracking the expansion of T- and B-cells and the linking of antigen receptor sequences to lymphocyte transcriptome	[190]
Sc MT-seq	Multiomic: epigenetic and transcriptomic	Used to detect transcriptome, methylome, and single nucleotide polymorphism information within single cells to dissect the mechanisms of epigenetic gene regulation	[63, 64]
Sc CITE-seq	Multiomic: transcriptomic and proteomic	Used to provide detailed characterization of cellular phenotypes and be readily scale as the throughput of single-cell sequencing approaches increase	[65]
Single-microbe genomics	Genomic	Contribute high-throughput culture-free capabilities to investigate genomic blueprints of complex microbial communities with single-microbe resolution	[67]

ScRNA-seq single-cell RNA sequencing, *ScATAC-seq* single-cell assay for transposase-accessible chromatin using sequencing, *ScMT-seq* single-cell methylome and transcriptome sequencing, *snRNA-seq* single nuclear sequencing, *ScCITE-seq* single-cell cellular indexing of transcriptomes and epitopes by sequencing, *TCR* T cell receptor

high-resolution landscapes of spatial Mrna expression patterns across tissue sections and overcoming deficiencies related to tissue dissociation. Therefore, combining ST data from liver sections in their bona fide tissue location with previously recognised knowledge of liver zonation [77, 78] allows the spatial annotation of structures containing small mixtures of cells in the liver microenvironment (lobule) and liver macroenvironment (tissue). Additionally, ST application across whole liver sections can help identify novel structures that may play vital roles in the overall architecture of the liver, and which may be lost when using protocols that do not allow analysis of the structures in the spatial context.

General workflow for integrated analysis of scRNA-seq and ST data in the liver

The integrated analysis of single-cell techniques and ST can provide a deeper understanding of the function and interaction of liver cells in both physiological and pathological states, and are reviewed in detail in the following sections. The concise workflows combining scRNA-seq and ST techniques typically begin with establishing cell subtypes through dimensionality reduction and clustering of scRNA-seq data. Cell subpopulations are then localised through deconvolution and mapping [79, 80]. Spatial barcoding data are usually subjected to

deconvolution, while single-cell resolution spatial data (such as high-plex RNA imaging (HPRI) data) are typically subjected to mapping to localise scRNA-seq subpopulations. Next, algorithms that evaluate the spatial arrangement of localised subpopulations further assess the ligand–receptor interactions predicted from scRNA-seq data. Given that much cellular crosstalk, notably juxtacrine and paracrine communication, is spatially restricted, ST data are well suited to evaluate the reliability of the ligand–receptor interactions computed from scRNA-seq [81, 82]. Finally, the integrated results can be visualised through the creation of heat maps, scatter plots, and two-dimensional graphs, among other methods. For more information on each module, please refer to a recent review article published in “Nature Reviews Genetics”, which explores the attempts to integrate scRNA-seq with ST techniques, including the use of emerging integrated computational methods, and proposes effective ways to combine current methods [83].

Single cell and ST in a normal liver

The human liver is the largest organ of the body. It is made up of parenchymal cells, such as hepatocytes and cholangiocytes, that constitute 80% of the liver mass [84], and nonparenchymal cells, such as HSCs, LSECs, and recruited and tissue-resident immune cells [85].

scRNA-seq enables the simultaneous detection of parenchymal and nonparenchymal cells to generate an atlas in the normal liver, provides novel insights into the phenotypic zonation of hepatocytes, and improves our understanding of rare cell types, such as LSECs [73], cholangiocytes [73], and hepatic progenitor cells [86] (Fig. 1B). Additionally, the integration of scRNA-seq and ST can generate high-resolution maps of cell subpopulations in tissues. This combination can help us better understand the changes in cell types and gene expression in different regions of the liver.

A single-cell perspective of normal liver cell atlas

An important application of scRNA-seq is to generate a full-scale perspective of a normal liver cell atlas. Since 2018, several independent studies have performed single-cell analysis in healthy human livers at high-throughput resolution [15, 41, 59, 72, 73, 87–90]. MacParland et al. were the first to publish a study on the normal human liver utilising scRNA-seq technology [73]. Liver grafts were obtained from five neurologically deceased donors with systemic inflammation from brain death but normal liver histology, and 8444 liver cells were profiled. Twenty different cell populations were characterised, including hepatocytes, endothelial cells, cholangiocytes, HSCs, B cells, conventional and non-conventional T cells, and two distinct populations of resident macrophages [73]. Liver samples used to generate a normal liver cell atlas have also been acquired from individuals of both sexes across a wide range of age groups (from 21 to 65-years-old), and from those with diverse underlying medical conditions [72, 88, 90]

It is extremely difficult to obtain fresh, healthy human liver tissue of good quality in the field. Therefore, a recent study has compiled, integrated, and analysed available scRNA-seq data from 28 healthy human liver samples. Moreover, they have established an online cell browser that provides easy and open access to transcriptional data of a diverse range of annotated parenchymal and nonparenchymal cells [91]. The combined database includes 26 clusters of 36,188 human hepatic cells [91]. Notably, an R Shiny approach has been developed for interactive visualisation, and scientists can access the website (<http://liveratlas-vilarinholab.med.yale.edu>). This innovative approach offers a user-friendly experience and interactive visualisation of gene expression for each cell (sub) type, ranging from abundant to rare liver cell subpopulations. Furthermore, this pioneering web tool furnishes detailed resources on which and what proportion of cell (sub)types express a gene of interest.

In summary, using novel single-cell technologies, these studies provide new insights into the transcriptomic structure of the human liver in a physiological state,

revealing previously unattainable information that is highly valuable for the liver research community worldwide. However, there are still many aspects of liver biology that have barely been studied and several problems that need to be addressed in the future. First, the normal livers are obtained from patients with a variety of underlying medical conditions, in other words, they are not really “normal”. Additionally, the dissociation and handling of liver tissue are thought to induce changes in gene expression in liver cells. Finally, the dynamics of the normal liver atlas due to gender and age need to be elucidated more thoroughly.

Landscape of intercellular crosstalk

scRNA-seq has provided many innovative insights into cellular heterogeneity and function in liver physiology. However, pathological processes such as ischaemia, inflammation, anti-infection, fibrosis, regeneration, autoimmunity, and tumourigenesis are complicated, with a highly organised interactome between specific subgroups of multiple cell types that are likely to play crucial roles in disease progression. Therefore, to discover new and effective therapeutic targets, it is crucial to understand the complex interactions present in different liver diseases. Importantly, scRNA-seq data not only define the characterisation of different cell types but also provide information on which cell subgroups express specific genes of interest and to what extent. Accordingly, we can further analyse scRNA-seq data to study the underlying ligand–receptor interactions between distinct cell types, model the interactome, and highlight cellular and molecular mechanisms that might drive disease progression.

A number of bioinformatic methods have emerged to carry out interactome analysis on scRNA-seq data [92–94]. Unbiased CellPhone DB analysis [93] is an approach that infers inter- and para-cellular crosstalk from the combined expression of multi-subunit ligand–receptor complexes between cell clusters. To clarify the molecular mechanisms of an immune checkpoint inhibitor (ICIs)-related hepatitis, CellPhone DB analysis was carried out to identify the ligand–receptor communications between T cells and myeloids in the liver of an ICI-induced hepatitis murine model [95]. Significant ligand/receptor pairs with T cell activation cytokines TGFB, IL21, IL18, and IL15 were found between T cells and myeloids and between T cell subgroups [95]. The CellPhone DB algorithm has also been used to investigate the intra-scar activity of several pro-fibrogenic signalling pathways between endothelial cells, collagen-producing mesenchymal cells, and scar-associated macrophages in human fibrotic livers [90]. Notably, during fibrosis, high levels of the Notch ligands Delta-like ligand 4 (DLL4), Jagged 1 (JAG1), and Jagged 2 (JAG2) expressed by endothelial

cells can signal through Notch receptors expressed by mesenchymal cells [90]. Single-cell secretome gene analysis was performed on hepatocytes and nonparenchymal cells isolated from the livers of an NAFLD mouse model [42]. By combining scRNA-seq data with a ligand–receptor interactome database [96], a highly correlated signalling network among major hepatic cell types was generated, indicating potential paracrine and autocrine connectivity with a high speciality in the mammalian liver [42].

In summary, compared with traditional bulk RNA sequencing, scRNA-seq data analysis provides a sensitive approach for delineating precise and complicated cellular sources of ligands and receptors and elucidating the roadmap of intercellular communication within the mammalian liver. Future challenges include functional assessments of the predicted signalling pathways and the precise elucidation of their biological functions in health and disease.

Liver zonation and function based on a combination of spatial and single-cell transcriptomes

The hepatic lobules, a fundamental functional unit in the mammalian liver, take on a hexagonal shape and contain hepatocytes arranged in plates along the radial lobule axis, with the central and portal veins situated at either end [97, 98]. In accordance with this spatial variation, versatile functions of the liver vary non-uniformly inside the hepatic lobules along this porto-central axis, a phenomenon termed “zonation” [84, 99]. Therefore, according to different hepatic functions and anatomical locations, the hepatic lobule is classically divided into three different zones [75]. Zone 1 surrounds the portal tract where oxygenated blood from the hepatic arteries enters. Zone 3 encircles the central veins, which is hypoxic but receives a nutrient-rich blood supply from the gastrointestinal tract. Zone 2 is located between Zone 1 and Zone 3 (Fig. 1C).

Our knowledge of hepatic cell organisation within these zones was deepened by spatially resolved transcriptomic analysis using scRNA-seq. By combining scRNA-seq with single-molecule RNA fluorescence in situ hybridisation (smRNA-FISH), Halpern et al. performed spatially resolved RNA sequencing to study liver zonation in mice [100]. This study indicated that liver zonation was mainly determined by the oxygen gradient and classical WNT signalling [101], and also by rat sarcoma protein (RAS) signalling to a lesser extent. Intriguingly, a substantial number of spatially zoned genes were not predicted to be downstream targets of these signalling pathways, suggesting the need for the identification of many other molecular signalling pathways important for liver zonation identification. Notably, the mouse

interzonal hepatocyte marker genes identified are not applicable to human liver data [18, 73].

Building on the previous study [100], the concept of ST to dispel dependency on landmark genes or adherent cells was generalised by updated approaches. One of the current strategies is to fix MrnA molecules within the cell and introduce primers with barcode sequences, followed by rolling circle amplification (RCA) to increase the local copy number [102, 103]. Although a single-cell resolution is yet to be achieved, as recently reported by Sun et al., spatial transcriptomic techniques enable unbiased reconstruction of metabolic liver zonation [104]. Moreover, to highlight the spatiotemporal metabolic organisation of the liver, ST and scRNA-seq were performed in combination on individual liver cells obtained via perfusion from 10 ad libitum fed mice across the diurnal circadian cycle at different time points [22]. ST was also employed to resolve the spatial correlations of vascular parts involved in liver zonation and to explore previously unidentified structures in the mouse liver [20]. Tracking the expression of transcriptional markers associated with zonation along the lobular axis computationally enables the study of zonation gradients in physical positions and allows the prediction of vein-type identity according to the expression profiles of neighbouring spots. In addition, the presence of transcriptionally distinct structures, cluster 5, was discovered in liver tissues, consisting of a small number of spots with distinct spatial localisation, which express mesenchymal cell-marker genes such as *Vim* and *Gsn* [105]. This cluster has not been identified in previous transcriptomic studies, mainly owing to the cell rarity contributing to these structures [20].

In addition to MrnA, other cellular features in hepatic cells may have similar spatial heterogeneity, including proteins, metabolites, and regulatory molecules such as microRNAs (miRNAs). Understanding miRNA and protein space distribution are essential to deciphering the contributions of hepatic cells to liver development, metabolism, regeneration, fibrosis, infection, and cancer [106]. Itzkovitz et al. adopted “spatial sorting”, a generic method, to illustrate the zonation of hepatocyte proteins and miRNAs [104]. Their study provides a generalised spatial atlas of protein and miRNA zonation, identifying core hepatocyte-donated miRNAs, such as miR-30a-5p and miR-122-5p [104]. Through a combination of miRNA and target MrnA levels, they also identified potential regulatory interactions that could mediate the degradation of zoned MrnA. This provides an important resource for future studies because miRNAs are highly dynamic and zoned during disease progression of the liver, such as fibrosis, viral infection, and cancer [107–109]. Moreover, spatial identification of metabolic enzymes and signalling pathways using proteomics and in silico techniques

will definitely increase our knowledge of hepatotoxicity and can greatly improve the prediction accuracy of drug absorption, distribution, metabolism, and excretion [104, 110].

Therefore, ST approaches have profoundly improved our understanding of the functional specialisation of human livers and could be used to establish how this division of labour may be restored after disruptions caused by liver injury [72, 73]. For further anticipation, ST will greatly benefit studies addressing liver development, sexual dimorphisms of liver zonation, immunity, and general pathology in mammals, particularly humans.

Spatial and single-cell transcriptomics in the liver during infection

The liver is the largest immune organ of the human body. Because of acute or chronic infection, liver inflammation results in necrotic hepatocyte death [111]. Persistent hepatic damage leads to progressive fibrosis, disrupted liver architecture, cirrhosis, and subsequent tumorigenesis. The identification of single-cell processes throughout the course of infection is hindered by restricted access to human liver tissue longitudinally over time and, until recently, limited options for investigating rare and potentially disease-driving cell populations. However,

high-throughput spatial and single-cell technology has shed new light on the mechanisms underlying liver function during infection.

Hepatitis B virus (HBV) infection

HBV infection is a serious health and economic burden worldwide [112]. Currently, there is no radical cure for HBV infection because the virus remains in a latent state as stably inactivated covalently closed circular DNA (cccDNA) within host cells [113]. Therefore, shedding novel insights into the underlying mechanism for the maintenance of HBV cccDNA is of great significance in identifying new paths of HBV replication (Fig. 5). Using the scRNA-seq analysis, Hashimoto et al. characterised *DOCK11* and *DENND2A* as key genes linked to the preservation of HBV cccDNA in 2325 primary human hepatocytes infected with HBV [21]. The amounts of HBV DNA and cccDNA both decreased below the limit of detection under the depleted expression of these two genes, which revealed an important role of these genes in HBV maintenance in human hepatocytes [21] (Additional file 1: Fig. S1A). However, the underlying mechanism remains poorly understood and needs further exploration. Furthermore, to obtain an unbiased and full-scale landscape of intrahepatic immunological

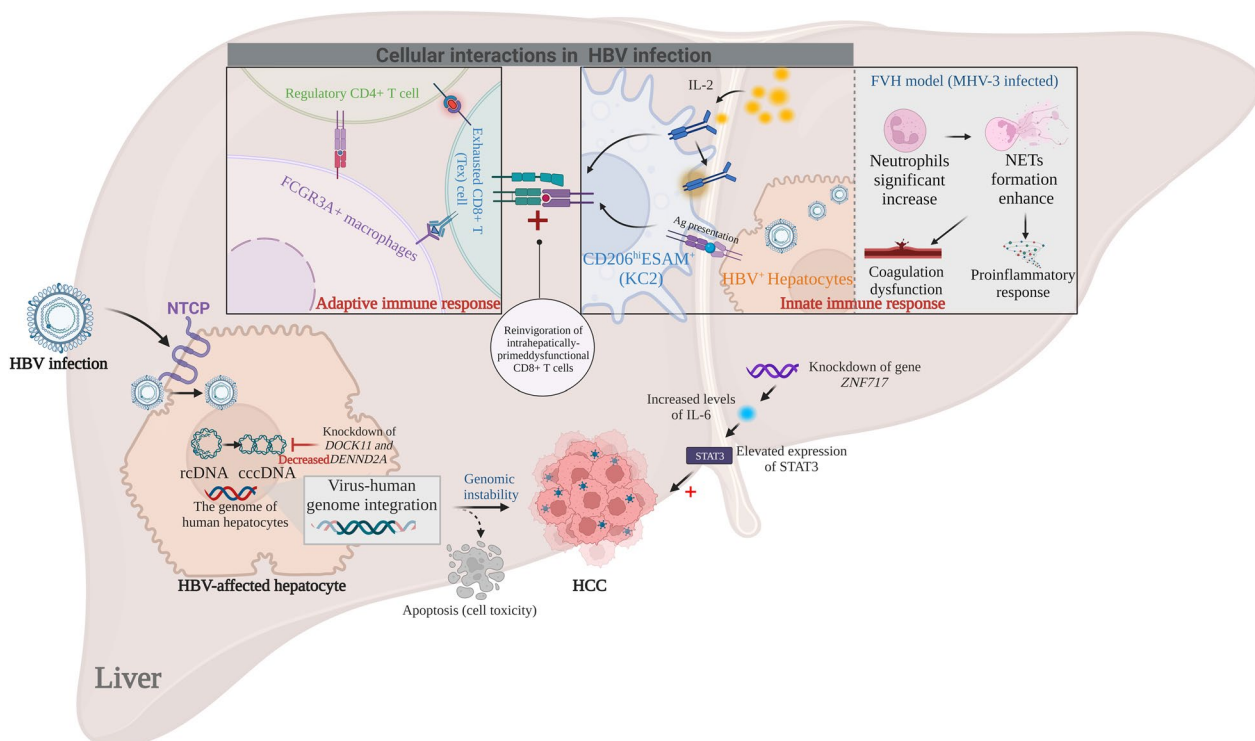


Fig. 5 Underlying mechanism in HBV infection using ScRNA-seq. cccDNA covalently closed circular DNA, FVH fulminant viral hepatitis, HCC hepatocellular carcinoma, HBV hepatitis B virus, IL-2 interleukin-2, KC Kupffer cell, MHV-3 murine hepatitis virus strain-3, NTCP Na⁺-taurocholate cotransporting polypeptide, NETs neutrophil extracellular traps, rcDNA relaxed circular DNA

characteristics and associations with disease status in HBV-infected patients, scRNA-seq was performed in the liver and blood during different stages of hepatitis B infection, including immune tolerance, immune activity, acute recovery, chronic resolved, and healthy controls [114] (Additional file 1: Fig. S1B). Diverse immune cell subgroups with different sources and cellular interactions have been observed in different disease statuses in HBV infection. This study highlighted the communication between exhausted CD8⁺ T (Tex) cells, regulatory CD4⁺ T cells, and FCGR3A⁺ macrophages. This communication contributes to immune failure during HBV maintenance through mediating HLA class I molecules together with their receptors such as leukocyte immunoglobulin-like receptor, and may be useful in guiding the progress of immunotherapy (Additional file 1: Fig. S1B). In addition to the adaptive immune response, the innate immune cells play a significant role in HBV infection. To delineate the function of KCs in HBV infection, live CD45⁺CD64⁺F4/80⁺ liver macrophages were sorted by flow cytometry from C57BL/6 mice and subjected to scRNA-seq analysis using the Smart-seq2 pipeline [23]. Two distinct clusters of KCs among liver-resident macrophages were identified, and the CD45⁺F4/80⁺CD11b^{int} TIM-4⁺CD206⁺ESAM⁺ cells were poised to respond to IL-2 treatment and cross-present viral antigens contained within circulating virions or hepatocytes. Therefore, specific subgroups of KCs were identified as regulating the signalling pathways that lead to the hepatocellular priming of HBV-specific CD8⁺ T cells, which acquired pathogenic effector and antiviral functions following exogenous IL-2 treatment (Additional file 1: Fig. S1C).

Indeed, scRNA-seq can provide abundant transcriptomic data to study molecular events and cell heterogeneity at the single-cell level. However, due to hereditary deficiency, high-expression genes in the library are sequenced more frequently, and rare genes can be easily ignored. Therefore, it is challenging to acquire transcript information from low-abundance or rare genes in the presence of highly abundant genes [115]. Tyrrell et al. developed a CRISPR-CRISPR-associated nuclease 9 (CRISPR-Cas9) assay to deplete high-abundance transcripts, resulting in preferential enrichment of low-abundance genes, such as HBV genes in human hepatocytes. By incorporating CRISPR-Cas9 with scRNA-seq in hepatocytes infected with HBV, the three most abundant transcripts were successfully depleted, which enabled selective enrichment of the HBV transcript and subsequent HBV RNA sequencing in more than 74% of the cells, compared to the detection of HBV RNA in only 0.6% of the cells through direct sequencing without CRISPR-mediated enrichment [116] (Additional file 1:

Fig. S1D). The technical improvement further enabled the study of HBV infection and interferon treatment of the HBV-infected Huh7.5-NTCP cell model.

The single-cell assay for transposase-accessible chromatin using sequencing (ScATAC-seq) is a novel technique that detects open regions and identifies regulatory regions of chromatin at the single-cell level, making it a broad region of single-cell high-throughput technologies [117]. The corresponding transcription factors or other regulatory proteins bind to open regions of chromatin and directly affect intracellular gene expression [118]. To better understand the HBV-associated cell types, different open regions of chromatin, and specific gene expression regulatory networks in patients with chronic hepatitis B (CHB), Dai et al. performed ScATAC-seq on 8016 peripheral blood mononuclear cells (PBMCs) derived from normal control (NC) individuals and CHB patients [119]. Specific leukocytic subgroups associated with CHB were found to be located in B-0 and T-3 clusters. Additionally, the potential mechanisms of the transcription factor (TF) motifs IRF2 and FOXC2, which are associated with B-0 and T-3, respectively, were elucidated in the occurrence of CHB (Additional file 1: Fig. S1D). Therefore, a more systematic and distinctive gene regulatory network was constructed by combining scRNA-seq and ScATAC-seq [119].

HBV can also cause fulminant viral hepatitis (FVH), a deadly disease that lacks an effective treatment, and its pathogenesis is not fully understood [120]. Immune response plays a crucial role in the pathological progression of liver failure, yet the entire immune micro-environment landscape of infected livers is important for effective strategy discovery [121]. Wang et al. constructed an scRNA-seq study of a mouse model of FVH due to murine hepatitis virus strain-3 (MHV-3) infection. ScRNA-seq revealed that neutrophils were the only innate immune cells exhibiting a significant increase in the liver in response to MHV-3 infection when the mice had severe systemic inflammation. Neutrophils extracellular traps (NETs) exacerbated liver damage by promoting fibrin deposition and inflammation, which were regulated by the FGL2-mucopolin 3-autophagy axis (Additional file 1: Fig. S1E). In summary, targeting NETs could offer a novel approach for treating FVH.

The underlying mechanisms of HBV-induced liver damage are multifarious; one of the well-known mechanisms is the HBV genome integration into the genome of human hepatocytes [122, 123]. Virus-human genome integration results in genomic instability and is a high-risk factor for the development of liver tumorigenesis (Fig. 5). In the context of hepatocellular carcinoma (HCC), the heterogeneity of HBV integration has been investigated using single-cell genome sequencing [124].

HBV integration was remarkably consistent in all cells of a single-nodular HCC with portal vein tumour thrombosis, supporting the notion that HBV integration is an early initiating event in hepatotumorigenesis. Moreover, a potential driver gene *ZNF717* was specifically identified in HCC. This gene exhibits a noteworthy mutation rate at both the single-cell and population levels, and acts as a tumour suppressor by modulating the IL-6/STAT3 pathway [124] (Fig. 5).

Hepatitis C virus (HCV) infection

Although HCV is the world's most common blood-borne virus, there is currently no vaccine to protect against it [125]. HCV persistence has been established in most infected patients, suggesting that it successfully evades innate and adaptive immune surveillance at multiple levels. Previous standard treatment depends on interferon (IFN)-based therapy; however, it has many adverse side effects and only 50% patients respond. Since 2015, highly effective direct-acting antiviral (DAA) therapy has revolutionised the treatment of chronic HCV infection, with standard cure rates exceeding 95% [126, 127]. Many studies have shown that, following IFN-free DAA treatment, both innate and adaptive immune homeostasis may be partially restored [127–129]. To clarify the role of DAA-mediated HCV eradication in global T cell immune function, Rosen et al. utilised scRNA-seq to characterise the transcriptome of circulating T cells before, during, and after the DAA-mediated HCV cure [130]. They revealed that rapid normalisation of IFN signalling was achieved in T cells with DAA therapy and was well maintained after therapy termination. Several T cell subtypes, including CD4 central memory and effector cells, CD8 effector memory and TEMRA cells, as well as Hi-interferon stimulated gene (ISG) populations, exhibit the most notable transcriptional alterations following this HCV cure. This study identified previously uncharacterised shifts in innate immune and interferon signalling within T cell subpopulations induced by DAA therapy in chronic HCV infection at different time points, providing an abundant data source for exploring the effects of DAA treatment on bulk T cells.

T-cell exhaustion may serve to limit excessive immunopathology during prolonged antigen stimulation; nevertheless, the potential outcomes of T cell exhaustion may include viral persistence and tumour progression [131–134]. In order to characterise the transcriptional profile, trajectory, and ultimate fate of these exhausted HCV-specific CD8+ T cells during and after the cessation of chronic antigen stimulation in the context of chronic HCV infection, Hofmann conducted scRNA-seq analysis in a well-defined cohort of patients receiving DAA therapy for chronic HCV infection [135]. It demonstrated

that memory-like T cells persisted while terminally exhausted T cells diminished after DAA-mediated HCV treatment, leading to memory polarisation of the overall HCV-specific CD8+ T cell response. However, a core signature of exhausted memory-like CD8+ T cells was still detectable, including HCV-specific CD8+ T cells targeting variant epitopes to a lesser extent (Fig. 6A). The aforementioned data delineated a fresh hallmark of T cell exhaustion, which persists as a chronic blemish in CD8+ T cells specific to HCV, even subsequent to the discontinuation of chronic antigenic stimulation. Consequently, in order to elicit complete effector potential, therapeutic targeting of these chronic blemishes, in addition to antigen withdrawal, is imperative.

In addition to adaptive immune response, myeloid cells from liver fine needle aspirates (FNAs) in HCV patients before and after DAA treatment were deeply profiled by scRNA-seq [136]. Subpopulations of liver neutrophils, classical monocytes, non-classical monocytes, macrophages, eosinophils, conventional dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), and mast cells were comprehensively characterised. Notably, the upregulation of MCM7+STMN1+ proliferating CD1c+cDCs and expected downregulation of ISGs were observed post-cure (Fig. 6A). Programmed cell death-ligand 1 (PD-L1)+ISG-high neutrophils and IDO1-high eosinophils were identified as being crucial for immunoregulatory during chronic infections (Fig. 6A). In addition, three recurring gene programmes, ISG, MHC-II, and S100 were shared by multiple cell types, providing immunotherapeutic insights into HCV infections.

Plasmodium infection

Malaria remains a grave global health concern, afflicting approximately 421 million individuals and resulting in 627,000 fatalities in the year 2020 [137]. *Plasmodium vivax* is responsible for the majority of non-African cases of malaria [137]. There is an obligatory developmental stage of all *Plasmodium* parasites that infect humans in the liver, where the parasite experiences asexual reproduction known as schizogony within a human hepatocyte before releasing thousands of merozoites into the blood [138]. In the life cycle of *P. vivax* in the liver, some parasites known as hypnozoites give up immediate division in hepatocytes and persist in the liver for days, weeks, months, or even years before initiating schizogony, resulting in a relapse of blood infection [139] (Fig. 6B). Therefore, eliminating *P. vivax* without therapy that directly targets hypnozoite reservoirs is challenging [140]. However, it remains unclear whether the mechanism regulating the formation and dormancy of hypnozoites is the same in different relapsing malarial parasites.

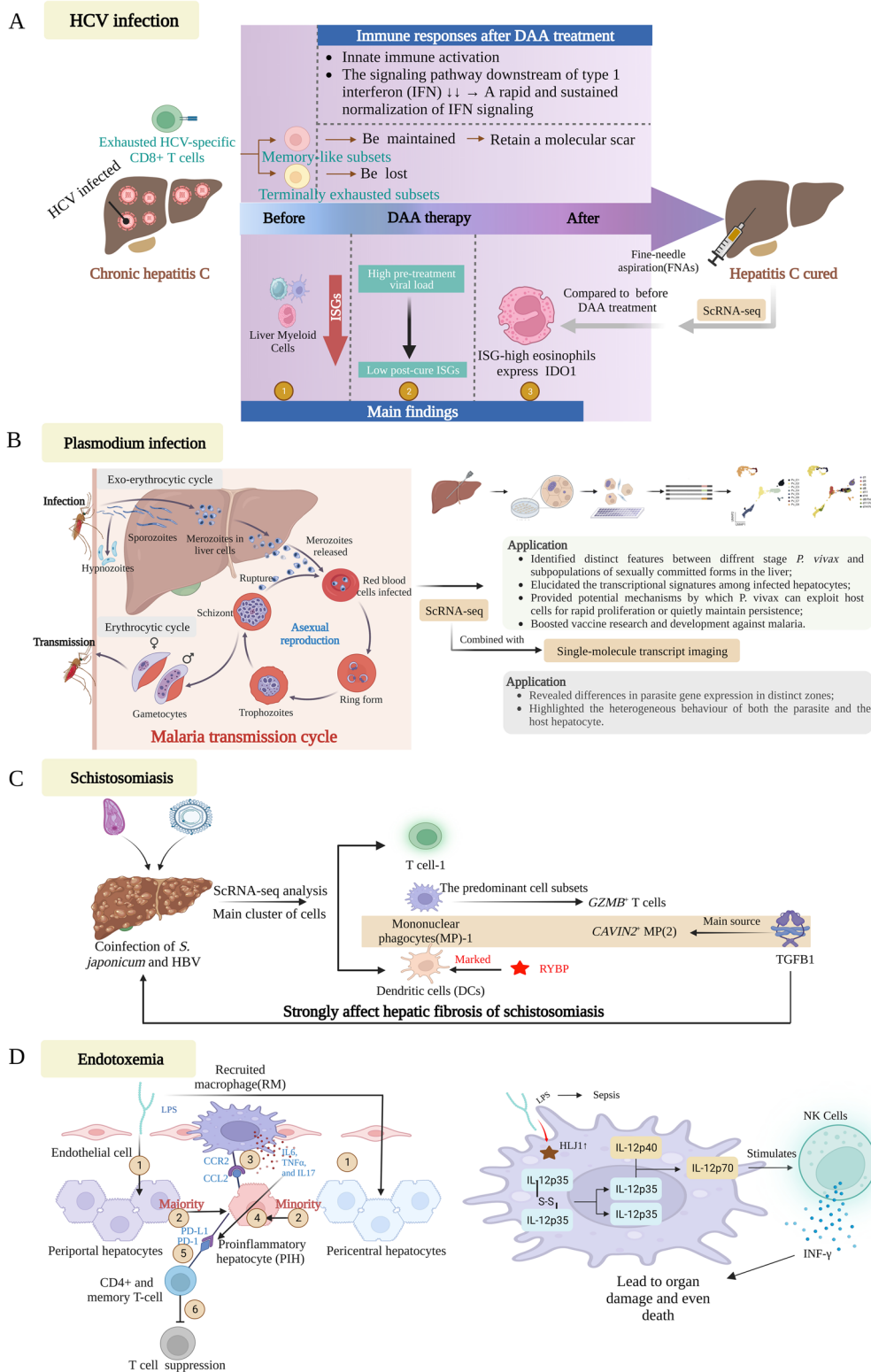


Fig. 6 Single-cell perspective of the liver in other infection diseases. DAA direct-acting antiviral agents, DCs dendritic cells, HCV hepatitis C virus, HLI1 human liver DnaJ-like protein, INF- γ interferon- γ , LPS lipopolysaccharide, MP mononuclear phagocytes, MPCC micropatterned co-cultures, NKs natural killer cells, P. vivax Plasmodium vivax, PIH proinflammatory hepatocyte, YBP ring1 and YY1 binding protein, RM recruited macrophage, TGFB1 transforming growth factor B1

Previously, transcriptome-wide profiling of the *Plasmodium* during liver stages was performed using traditional bulk RNA sequencing strategies [141]. Although informative, the study only provided insights into average transcriptional expression originating from either mixed (schizont and hypnozoite) or hypnozoite-only populations, instead of any transcriptional variations that might be present between individual parasites. Moreover, the potential impact of the parasite on host cells was not considered, impeding the investigation of host–pathogen interactions. These deficiencies emphasise the need to better understand the differences between individual plasmodia, as well as how the *Plasmodium* alters hepatic cells. Progress in these fields could facilitate the characterisation of parasite and host factors important for the development of the parasite liver stage.

To comprehensively examine *Plasmodium*-specific host interactions and potential responses in bystander hepatocytes, Dr. Sangeeta N. Bhatia conducted dual transcriptional profiling of *P. vivax* infection and surveyed host- and state-dependent gene expression patterns in both parasites and hepatocytes [142]. This research utilised a bioengineered human microliver platform to culture patient-derived *P. vivax*, generating a single-cell liver atlas of relapsing human malaria through scRNA-seq analysis. The study identified distinct features of early-, dormant-, mid-, and late-stage *P. vivax*, as well as subpopulations of sexually committed forms in the liver that were previously thought to emerge only during erythrocytic infection [143] (Fig. 6B). *P. vivax* infection inhibits the transcription of functional genes in key hepatocytes and induces an anti-*P. vivax* innate immune response, which includes the dysregulation of IFN and inflammatory signalling pathways. This study provides a foundation for understanding parasite–host interactions and sheds light on the biology of *P. vivax* dormancy and transmission. Moreover, the technology developed in this study can be applied to research other intracellular microorganisms, where low expression of pathogen transcripts or host contamination makes it difficult to perform single-cell studies.

At around the same time, Kyle et al. utilised the scRNA-seq platform of 10X Genomics to characterise the transcriptomic features of both the host and parasite in a *P. vivax* liver-stage model in vitro [144]. Differential gene expression was observed between replicating hypnozoites and schizonts, and previously unobservable variation by bulk sequencing was revealed between individual hypnozoites. Moreover, multiple host genes linked to energy metabolism and antioxidant stress were exclusively upregulated during hypnozoite infection in infected hepatocytes. This study not only elucidated the transcriptional signatures among infected hepatocytes but also

provided potential mechanisms by which *P. vivax* can exploit host cells for rapid proliferation or quietly maintain persistence (Fig. 6B), which can inform therapeutic targets against *P. vivax* liver-stage infection.

Previous ex vivo investigations have suggested that the rate of *Plasmodium* infection may differ among hepatocytes situated in distinct regions (pericentral and periportal) within the liver, owing to spatial heterogeneity of hepatocytes [145, 146]. To identify heterogeneous host and parasite responses, scRNA-seq and single-molecule transcript imaging were combined to characterise the host and parasite temporal expression programmes in a zonally controlled manner for the rodent malaria parasite *Plasmodium berghei* ANKA [147]. The study revealed differences in parasite gene expression in distinct zones, including potentially co-adaptive programmes related to iron and fatty acid metabolism (Fig. 6B). Parasites were found to develop more rapidly in the pericentral lobule zones, and a subpopulation of ‘abortive hepatocytes’ were identified periportally; these appeared predominantly with high parasite inoculum, upregulated immune recruitment, and key signalling programmes. This study provides an ideal application of combined spatial single-cell analysis for understanding the liver stage of *Plasmodium* infection at high spatial resolution and highlights the heterogeneous behaviour of both the parasite and the host hepatocyte [148].

Single-cell technology has also boosted vaccine research and development against malaria. Dr. Alexandra J. Spencer and her colleagues conducted an in-depth analysis of immune phenotyping and scRNA-seq on the kinetics of peripheral blood samples and thin needle puncture of liver tissue to investigate CD8+ tissue-resident memory (TRM) cells and their circulating counterparts in volunteers who received a novel malaria vaccine treatment called prime-target vaccination [149]. By exploring the heterogeneity among liver CD8+ TRM cells at the single-cell level, two main subpopulations were identified, each sharing expression profiles with blood T cells. This study uncovered the potential of liver TRM-like cells as a protected object by liver-stage malaria vaccines, especially those adopting a prime-target approach. Phase III clinical trials of liver-stage malaria vaccines are currently underway, and a reproducible correlation of protection would be particularly valuable (Fig. 6B).

Schistosomiasis

Schistosomiasis, whose pathogenesis remains unclear, is a widespread helminth disease that causes acute and chronic injury and poses a heavy social and economic burden on people worldwide [150–152]. Advanced schistosomiasis often progresses to liver fibrosis and cirrhosis.

To characterise the transcriptomic atlas of liver immune cells involved in schistosoma-associated fibrosis, scRNA-seq analysis was performed on liver samples from fibrotic patients coinfecting with *S. japonicum* (SJ) schistosoma and HBV, patients with HBV cirrhosis, and healthy controls [153]. Despite the small sample size, this study revealed that T cells, particularly granzyme B (GZMB)+ T cells, were a major subgroup of liver-resident immune cells and increased in the SJ group. A drastic upregulation was also observed in caveolae-associated protein 2 (CAVIN2)+ macrophages, characterised by the high expression of transforming growth factor B1 (TGFB1) (a master profibrotic gene) in the SJ group [134]. Additionally, in the SJ group, DCs using ring1 and YY1 binding protein (RYBP) as marker genes were the dominant cluster (Fig. 6C). Further analysis suggested that distinct signalling pathways, mainly associated with NK cell-mediated cytotoxicity and antigen processing and presentation, were activated in the SJ group. Although preliminary, this study revealed that liver-resident immune cells with unique cell-marker gene expression and specific signalling pathway activation might deepen our understanding of the mechanism of schistosoma-associated liver fibrosis (Fig. 6C).

Endotoxemia

In addition to its role in local infections, such as those by hepadna microorganisms, the liver also plays a crucial role in modulating immune defence during acute systemic infections [9, 154, 155]. The liver shifts from an immune tolerant to an immune active state, creating a formidable defence against invading microorganisms as systemic infection takes hold [9, 156]. This defence system relies on a complex network of immune and non-immune cells, including KCs, DCs, T and B lymphocytes, neutrophils, NK cells, hepatocytes, cholangiocytes, and LSECs, which recruit proinflammatory immune cells that produce acute-phase proteins, complement factors, inflammatory cytokines, and cell adhesion molecules to exert immune functions [10–13]. High-throughput single-cell transcriptomics enables us to decipher cell heterogeneity, differentiation, and cell–cell communication during the pathological process occurring in the liver during systemic inflammation. In a study conducted by Cai et al., the cellular landscape of hepatic cells at three different time stages in a “double-hit” endotoxemia mouse model was elucidated. Based on single-cell transcriptomic analyses, the study provided the first evidence that the phenotypic transition occurring in hepatocytes during endotoxemia plays a vital role in the recruitment of circulating monocytes to generate a recruited macrophage population, as well as in the inhibition of T lymphocytes through the CCL2-mediated pathway

and upregulation of PD-L1 [157]. This study enhances the comprehension of endotoxemia pathology through a liver-centric perspective and establishes a crucial basis for developing effective therapeutic strategies for acute infections. Human liver DnaJ-like protein (HLJ1), a molecular chaperone belonging to the heat shock protein 40 (HSP40) family, is a potential target for sepsis treatment. ScRNA-seq analysis elucidated the immune profile influenced by HLJ1 in a mouse model challenged with lipopolysaccharides (LPS). HLJ1 plays a critical role in regulating the IL-12/IFN- γ axis-dependent sepsis progression and may serve as a potential molecular target for novel antisepsis or immunomodulatory therapies [158] (Fig. 6D). Additionally, a mouse model of LPS-challenged endotoxemia was used to explore the zonation and spatial heterogeneity of innate immune function in the liver, revealing that NF-KB (p50) activation and c-reactive protein expression in response to endotoxemia are zone-specific [159]. However, this exploration was conducted using traditional RNAscope and immunohistochemistry methods instead of high-throughput spatial transcriptome analysis.

COVID-19

Since December 2019, the corona virus disease 2019 (COVID-19) and its pathogen, a novel coronavirus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), have rapidly spread worldwide [160]. The essential factors for SARS-CoV-2 invasion into host cells are angiotensin-converting enzyme 2 (ACE2), a receptor binding to the spike glycoprotein (S) of the virus, and type II transmembrane serine protease (TMPRSS2), which mediates membrane fusion vital to release the viral contents into the infected cell cytosol [161]. Thus, the underlying mechanism of COVID-19-induced organ injury in the human body is related to these two factors (Fig. 7A). Although the lungs are the most frequently affected organ in COVID-19, SARS-CoV-2 can also cause damage to other organs, such as the brain, gut, and liver. Recent studies have shown that liver abnormalities have been observed in more than 50% of patients with COVID-19; in 20% of the patients, the abnormalities subsequently progressed to liver injury [162–166] (Fig. 7B). Comprehensive single-cell/spatial organ atlases of COVID-19 have been generated from various types of patient samples, including livers, using available scRNA-seq and spatial data [166–169]. By performing scRNA-seq and spatial transcriptomic profiling of livers from 17 COVID-19 patients, hepatocytes were identified as positive for SARS-CoV-2 RNA [169]. Further integrated analysis and comparisons with healthy controls revealed massive changes in cellular composition and gene expression in livers affected by COVID-19, mainly in those exhibiting

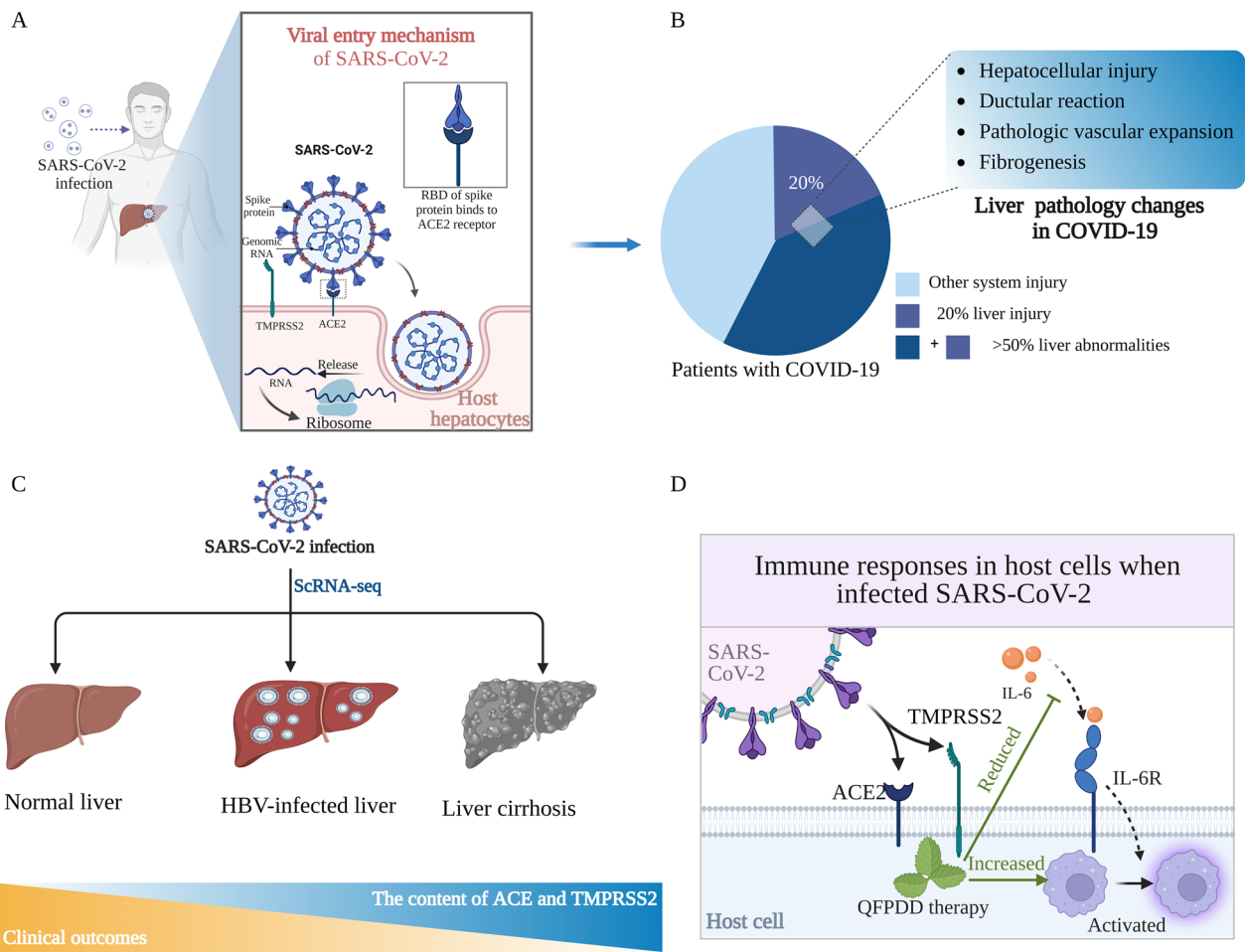


Fig. 7 Single-cell perspective of the liver in COVID-19. ACE2 angiotensin-converting enzyme 2, COVID-19 Corona Virus Disease 2019, IL-6 interleukin 6, IL-6R interleukin 6 receptor, QFPDD Qing-Fei-Pai-Du decoction, SARS-CoV-2 Severe Acute Respiratory Syndrome Coronavirus 2, ScRNA-seq single-cell RNA sequencing, TMPRSS2 transmembrane protease serine 2

hepatocellular injury, ductular reactions, pathologic vascular expansion, and fibrogenesis (Fig. 7B). This human atlas is fundamental to the investigation and understanding of the liver physiology and pathology in COVID-19 [169]. ACE2 has also been reported to be significantly expressed in liver cholangiocytes, indicating that the liver might be a target organ for SARS-CoV-2 [170]. However, the limitation of the study was its small size (only one liver dataset was analysed), and it did not focus on liver tissue specifically or reported only on ACE2 expression without considering TMPRSS2 expression [171]. Notably, although the percentage of ACE-TMPRSS2 co-expressing hepatocytes was extremely low in the two datasets containing representative hepatocytes [72, 73], it was not entirely absent. Given that the human liver is believed to comprise tens of billions of hepatocytes [172], this extremely low percentage could still endanger millions of hepatocytes.

To further elucidate the specific hepatic cell type implicated in COVID-19 pathophysiology, scRNA-seq analysis was conducted on five representative liver tissue types, including healthy, human foetal, cirrhotic, tumour, and precancerous, comprising over 300,000 single cells. In liver cirrhosis, a population of trophoblast cell surface antigen 2 (TROP2)⁺ liver progenitors was identified as a potential target for viral ingress, suggesting that viral infection of TROP2⁺ progenitors may significantly impair liver regeneration in cirrhotic patients [173]. Moreover, a study revealed that cirrhotic livers exhibited a higher number of ACE2⁺ and TMPRSS2⁺ cells than healthy livers, while HBV-infected livers had the lowest number of ACE2⁺ and TMPRSS2⁺ cells [160] (Fig. 7C). This finding may account for the worse clinical outcomes observed in COVID-19 patients with cirrhosis compared to those with viral hepatitis [160]. Using liver organoids derived from human-induced pluripotent stem cells to

recapitulate liver pathology following SARS-CoV-2 exposure, robust transcriptomic changes were characterised, and IL-6 signalling was identified as a potential mechanism for liver-mediated activation of circulating macrophages [174] (Fig. 7D).

Clinical studies on potential therapies for COVID-19 are currently in full swing [175, 176]. In China, traditional Chinese medicine (TCM) has played a preventive and therapeutic role in COVID-19 and has been recommended by the latest edition of the COVID-19 guidelines in lightly, moderately, and severely infected patients [177]. The combination of TCM and Western medicine has been shown to reduce inflammation and mitigate multi-organ damage [178]. In a murine model of pneumonia, the therapeutic mechanism of a typical TCM, Qing-Fei-Pai-Du decoction (QFPDD), was systematically investigated using scRNA-seq. The results demonstrated that QFPDD therapy increased peripheral blood lymphocytes (CD4+ and CD8+ T cells, B cells) in mice, reduced the levels of inflammatory cytokines such as IL-6, TNF- α , and IFN- γ , and regulated dysfunctional liver metabolism [179] (Fig. 7D).

The limitation of existing studies is that they cannot determine the impact of vaccination and only reflect on the very early lineages of the virus, as all samples were analysed during the pandemic. To gain more insight into the identity of cell types at risk of SARS-CoV-2 infection, and to uncover potential mechanisms for prospective target identification, CITE-seq that uses antibodies against ACE2 and TMPRSS2 could prove to be a valuable tool.

Clinical implications

In addition to mapping the healthy and diseased liver to elucidate cellular drivers of liver pathogenesis in infection, single-cell transcriptomics provide an opportunity to enhance the effectiveness of treatment modalities that target the liver or provide additional value for diagnosis and prognosis (Additional file 1: Fig. S2). Prognostic modelling of tumour cell stemness and intratumoural heterogeneity signatures will be relevant in the future for patient stratification of treatments, particularly in liver cancer [180, 181]. Moreover, these unbiased data allow the characterisation of immune features for the molecular classification of patients with liver infection through immunophenotyping, adopting the strategy employed in HCC [182, 183] (Additional file 1: Fig. S2). Deeper comprehension of diseased liver tissue using single-cell multiomics has highlighted diagnostic biomarkers and gene signatures, pathogenic cell subgroups, and potential targetable signalling pathways [184, 185]. To realise this potential, further investigation into preclinical lineage-tracing and gene manipulation research with clinical

trials is required to identify druggable targets for future therapeutic applications.

High-throughput single-cell technology has been used in various clinical settings. Single-cell genomics has already been applied to track disease trajectories before and after HCV therapy and tumour immunotherapy, highlighting future clinical applications as a precision medicine approach [130, 135, 186] (Additional file 1: Fig. S2). Moreover, these studies were aimed at investigating whether antiviral therapy facilitates the restoration of T cell responsiveness. Single-cell genomics can also be applied to evaluate the therapeutic efficacy and disease progression in clinical trials of a variety of liver diseases to generate frameworks for genetic, epigenetic, transcriptional, protein, and cellular landscape-based therapy strategies [187] (Additional file 1: Fig. S2). For instance, novel mechanisms and biomarkers of drug resistance have been identified to stratify patients and guide personalised therapeutic decisions via the application of scRNA-seq in clinical trials of multiple myeloma patient cohorts [188, 189]. In the future, patient-specific single-cell analysis of liver biopsies can reveal opportunities for personalised immunology by aiding the evaluation of patient outcomes and treatment effects. The extensive clinical application of single-cell technologies will revolutionise our capacity to track efficacy and tailor treatment strategies for understudied liver pathologies.

Future directions

In the era of precision medicine, higher-resolution data are needed to identify heterogeneous tissues and complicated diseases, such as microorganism infections. Currently, technologies that aid in the advancement of multiomics single-cell techniques that will allow the characterisation of genetic, epigenetic, transcriptional, proteomic, and metabolic changes in the same cell are being developed. Single-cell multiomics will allow an even more comprehensive understanding of liver biology and disease at a single-cell resolution. Future research should focus on reducing the expense of high-throughput single-cell technologies and identifying molecular, cellular, histological, or radiological deputy biomarkers that facilitate the characterisation and stratification of liver diseases, which will help predict drug efficacy or patient prognosis instead of conducting full single-cell analysis of samples per patient. As high-throughput single-cell techniques are further developed, they will result in a more comprehensive analysis of single cells in healthy and diseased livers, potentially producing novel therapeutic targets. These approaches, which generate more high-dimensional, complicated, and informative resources, necessitate the advancement of cutting-edge

bioinformatic algorithms to extract the optimal amount of related information from these integrated datasets. We need to continuously evolve computational analysis pipelines synchronously with methodological advancement to offer new strategies for visualising, analysing, and interpreting single-cell omics datasets (Additional file 1: Fig. S2).

Conclusion

In summary, high-throughput single-cell and spatial omics are revolutionising our understanding of liver physiology and pathology in healthy and diseased states. These ever-advancing techniques have rapidly and profoundly accelerated the identification of rare- and disease-driving cellular subpopulations within the liver microenvironment. As we continue to become proficient in this technology, expand applications to map initial stages of infection in human tissue, and sample liver tissue longitudinally alongside disease progression, we will unveil novel and effective therapeutic targets and treatment options for patients suffering from various infections.

Abbreviations

ACE2	Angiotensin-converting enzyme 2
COVID-19	Corona virus disease 2019
CdnA	Complementary deoxyribonucleic acid
cccDNA	Covalently closed circular DNA
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPR-Cas9	CRISPR-CRISPR-associated nuclease 9
CHB	Chronic hepatitis B
CAVIN2	Caveolae-associated protein 2
CITE-seq	Cellular indexing of transcriptomes and epitopes by sequencing
DCs	Dendritic cells
DLL4	Delta-like ligand 4
DNAse	Deoxyribonuclease
DAA	Direct-acting antiviral
EGTA	Ethylene glycol tetraacetic acid
FACS	Fluorescence-activated cell sorting
FVH	Fulminant viral hepatitis
GO	Gene ontology
GSVA	Gene set variation analysis
GZMB	Granzyme B
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HSCs	Hepatic stellate cells
HVGs	Highly variable genes
HCC	Hepatocellular carcinoma
HLJ1	Human liver DnaJ-like protein
HSP40	Heat shock protein 40
IFN	Interferon
ICIs	Inhibitor
JAG1	Jagged 1
JAG2	Jagged 2
KCs	Kupffer cells
KEGG	Kyoto Encyclopedia of Genes and Genomes
LSECs	Liver sinusoidal endothelial cells
LPS	Lipopolysaccharides
MmRNA	Messenger ribonucleic acid
miRNAs	MicroRNAs

MHV-3	Hepatitis virus strain-3
NETs	Neutrophil extracellular traps
NK	Natural killer cells
NAFLD	Non-alcoholic fatty liver disease
NC	Normal control
PCR	Polymerase chain reaction
PBMCs	Peripheral blood mononuclear cells
PD-L1	Programmed cell death-ligand 1
<i>P. vivax</i>	<i>Plasmodium vivax</i>
QFPDD	Qing-Fei-Pai-Du decoction
RAS	Rat sarcoma protein
RCA	Rolling circle amplification
RYBP	Ring1 and YY1 binding protein
scATAC-seq	Single-cell assay for transposase-accessible chromatin using sequencing
scRNA-seq	Single-cell RNA sequencing
scATAC	Single-cell assay for transposase-accessible chromatin using sequencing
scMT	Single-cell methylome and transcriptome sequencing
snRNA-seq	Single nuclear sequencing
ST	Spatial transcriptomics
STING	Stimulator of interferon genes
SJ	<i>S. japonicum</i>
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
Tex cells	Exhausted CD8+ T cells
TRM	Tissue-resident memory
TGFB1	Transforming growth factor B1
TMPRSS2	Type II transmembrane serine protease
TROP2	Trophoblast cell surface antigen 2
TCM	Traditional Chinese medicine
UMI	Unique molecular identifier

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12929-023-00945-z>.

Additional file 1: Figure S1. Single cell perspective of liver in HBV infection. **A** HBV maintenance in hepatocytes. The structure diagram of NTCP is quoted from REF [160]. **B** An unbiased and comprehensive landscape of the intrahepatic immunological characteristic in HBV-infected patients. **C** The roles of Kuffer cells in HBV-infection. **D** A systematic and distinguishing gene regulatory network of CHB-related PBMCs. **E** The immune microenvironment of infected liver in FVH. **Figure S2.** Clinical implication of high throughput single cell technology.

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