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# Single-cell RNA sequencing reveals the landscape of the cellular ecosystem of primary hepatocellular carcinoma

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**Abstract**

Hepatocellular carcinoma (HCC) cells, along with multiple nonmalignant stromal cells, such as fibroblasts, endothelial cells and immune cells, comprise an intricate cellular ecosystem, undergo dynamic phenotypic changes and present complicated cellular interactions, thus synergistically facilitating HCC initiation and progression and leading to treatment resistance. Clarifying the heterogeneity, cell plasticity and complexity of the cellular ecosystem of HCC will be highly beneficial for understanding HCC development and identifying novel therapeutic targets. Single-cell RNA sequencing (scRNA-seq) refers to profiling the transcriptome at single-cell resolution, and the development of scRNA-seq technology and analysis algorithms has greatly promoted the analysis of cell composition, cell subpopulation heterogeneity, development trajectory and cell-to-cell interactions in cell populations. In this review, we systematically summarized and discussed scRNA-seq in treatment-naive primary HCC and revealed the global cell composition of HCC; the widespread molecular heterogeneity of HCC cells; the molecular subtypes of fibroblasts; the cell composition, functional states and development trajectory of immune cells; and the frequent interactions between different cell types to systematically draw the landscape of the cellular ecosystem of primary HCC.

**Keywords** Hepatocellular carcinoma, Cellular ecosystem, Tumor microenvironment, Single-cell RNA sequencing

## Background

Hepatocellular carcinoma (HCC) is a lethal tumor with high morbidity and mortality rates and a poor prognosis worldwide; there is a lack of highly effective treatment methods in the advanced stage [1, 2]. Heterogeneity of HCC cells and surrounding nonmalignant stromal cells (e.g., endothelial cells, fibroblasts, immune cells) and their complicated interactions facilitate HCC initiation and progression and lead to treatment resistance [3–5]. Clarifying the heterogeneity and complexity of the cellular ecosystem is helpful for understanding the development of HCC and developing interventions.

Cell plasticity refers to the ability of cells to dynamically alter their phenotypic states in response to various internal and external stimuli. Cell plasticity in the cellular

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ecosystem of tumors, including HCC, is not restricted to only cancer cells; stromal cells, such as fibroblasts, endothelial cells and immune cells, also have the capacity to reprogram toward a different fate in response to intrinsic or extrinsic factors, which largely fuels the heterogeneity and complexity of the cellular ecosystem, further facilitates HCC initiation and progression and leads to treatment resistance [6, 7]. Clarifying the cell plasticity is helpful for understanding the heterogeneity and complexity of the cellular ecosystem of HCC and developing interventions.

Single-cell RNA sequencing (scRNA-seq) refers to profiling the transcriptome at single-cell resolution, and the development of scRNA-seq technology in recent years has greatly promoted the analysis of cell composition, cell subpopulation heterogeneity, development trajectory and cell-to-cell interactions in cell populations [8, 9]. ScRNA-seq in HCC has allowed a detailed understanding of the heterogeneity of HCC cells, the highly complex stromal cells constituting the tumor microenvironment (TME), the cell plasticity in dynamic phenotypic changes and the complicated cellular interactions [10, 11]. This review summarizes and discusses scRNA-seq in treatment-naive primary HCC to systematically describe the landscape of the cellular ecosystem of primary HCC.

#### ScRNA-seq delineates the global cellular landscape of HCC

HCC cells, along with multiple nonmalignant stromal cells, compose an intricate cellular ecosystem and synergistically affect HCC progression [3–5]. Systematically identifying cell types, proportions and spatial distribution among population cells that constitute HCC tissues and delineating the global cellular landscape is essential for further analysis of cell subpopulations and cell–cell interactions.

ScRNA-seq of single-cell suspensions enzymatically dissociated from HCC tissues has revealed the global cell composition of HCC [12–22]. In addition to malignant HCC cells, endothelial cells, fibroblasts, T cells, B cells, NK cells, and myeloid-derived cells, such as macrophages and neutrophils, were identified and constituted the main cell types of the TME of HCC, although there were significant individual and regional differences in the proportions of these cell types (Table 1).

Considering the significant intertumor heterogeneity, clearly defining clinicopathological features of HCC patients, such as the AFP level, HBV status and microvascular invasion, which could fundamentally change the landscape of the cellular ecosystem, and further comparing their effects may help more accurately reveal the landscape of the cellular ecosystem of HCC [21, 23–25]. Furthermore, considering the significant intratumor heterogeneity, clearly defining the spatial distribution of regional sampling may help more accurately reveal the landscape of the cellular ecosystem of HCC.

Considering the potential distortion of cell composition resulting from different dissociation efficiencies of different cell types during single-cell suspension preparation and loss of information about cell spatial distribution, especially spatial distribution information which is closely related to the spatial intratumoral architecture and microenvironmental niches that affect tumor progression and treatment, integrating scRNA-seq and spatial transcriptomics to capture the entire gene expression profile and assess cell spatial distribution simultaneously at single-cell resolution will be highly beneficial to delineate the global cellular landscape of HCC [26–28].

#### ScRNA-seq uncovers intertumor and intratumor heterogeneity and intertumor homogeneity of HCC cells

HCC is mostly induced by chronic inflammation and transformation from liver cirrhosis [29]. The chronic inflammatory and fibrogenic microenvironment drives oncogenic mutations, thus initiating tumorigenesis and forcing the dynamic clonal evolution of malignant HCC cells, which generally leads to the molecular heterogeneity of HCC cells [30]. Comprehensively uncovering the molecular heterogeneity of HCC cells greatly benefits the precise treatment of HCC.

ScRNA-seq of single-cell suspensions enzymatically dissociated from HCC tissues revealed that malignant HCC cells from different cases clustered into separate cell clusters, whereas nonmalignant stromal cells composing the HCC microenvironment tended to overlap and admixed together in cell cluster analysis, which suggested that HCC cells from different cases presented various global transcriptomic profiles and a relatively high level of intertumor heterogeneity [13–15], specifically manifested in the differential gene expression pattern of aggressive molecular subclasses [12], liver cancer stem cell (LCSC) markers and RTK gene families [14], and differentiation-related molecular subclasses [15]. Furthermore, differential gene expression patterns of aggressive molecular subclasses [12], LCSC markers [31], differentiation-related molecular subclasses [15] and proliferation-associated genes [17] were identified in HCC cells from the same cases, indicating a nonnegligible level of intratumor heterogeneity [32].

LCSCs are a small subpopulation of undifferentiated HCC cells with distinct cell surface protein markers, such as CD133, EpCAM, CD90, CD44, OV-6, CD13, CD24, DLK1, α2δ1, ICAM-1 and CD47, and exhibit self-renewal, differentiation, tumor formation and metastatic capacities. LCSCs have been proposed to be HCC-initiating cells and have been shown to be responsible for tumor metastasis, relapse and drug resistance. Targeting LCSCs is a promising therapeutic strategy for patients

**Table 1** Single-cell RNA sequencing (scRNA-seq) revealed the global cell composition of HCC

| Tissue sample | ScRNA-seq technology | Cell type (Marker genes) | Cell proportion (%) | | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fresh HCC tissue from one HCC case | 10 X Genomics | HCC cells (ALB, APOA1) | 5.8 | | | | | | | | Wen et al. [18] |
| | | Endothelial cells (PECAM1, PLVAP) | 2.3 | | | | | | | | |
| | | Fibroblasts (SOD3, ACTA2) | 0.8 | | | | | | | | |
| | | T cells (CD3D, CD3E) | 75.5 | | | | | | | | |
| | | B cells (CD79A, IGHG1) | 4.4 | | | | | | | | |
| | | NK cells (NCAM1, FCGR3A) | 5.7 | | | | | | | | |
| | | Macrophages (C1QA, C1QB) | 5.5 | | | | | | | | |
| Fresh HCC tissue from one HCC case | 10 X Genomics | HCC cells (ALB, ARG1, GPC3, KRT8) | 40.6 | | | | | | | | Liang et al. [17] |
| | | Endothelial cells (PECAM1, CDH5) | 3.9 | | | | | | | | |
| | | Fibroblasts (COL1A2, ACTA2) | 2.4 | | | | | | | | |
| | | T cells (CD3D, CD3E, CD3G) | 20.2 | | | | | | | | |
| | | B cells (CD79A) | 3.3 | | | | | | | | |
| | | NK cells (FGFBP2, KLRF1) | 3.2 | | | | | | | | |
| | | Macrophages (CD68, AIF1) | 25.8 | | | | | | | | |
| | | Neutrophils (FCGR3B, CXCR2) | 0.7 | | | | | | | | |
| Fresh HCC tissues from two HCC cases | 10 X Genomics | | P1 | | | | P2 | | | | Losic et al. [12] |
| | | HCC cells (ALB, FGG) | 75.7 | | | | 47.5 | | | | |
| | | Endothelial cells (KDR, VWF) | 8.8 | | | | 11.8 | | | | |
| | | Fibroblasts (ACTA2, TAGL) | 4.1 | | | | 15.8 | | | | |
| | | Cytotoxic T/NK cells (GNLY, NKG7, CCL5) | 0 | | | | 3.5 | | | | |
| | | B cells (IGJ, CD79A) | 0.1 | | | | 0.7 | | | | |
| | | Myeloid-derived cells (HLA-DQB1, CD68) | 11.3 | | | | 20.7 | | | | |
| Fresh HCC tissues from two HBV-associated HCC cases | mCEL-Seq2 | | P1 | | | | P2 | | | | Jühling et al. [15] |
| | | HCC cells (AQP1, KRT7, CD24, ALB, HBV-RNA, GLUL, CRP) | 29.2 | | | | 61.5 | | | | |
| | | Endothelial cells | 0 | | | | 0.2 | | | | |
| | | Fibroblasts (ACTA2, CD36, NOTCH3, COL4A2) | 53.9 | | | | 8.4 | | | | |
| | | Lymphocytes (LTB, CD3E, IL2RG) | 0.4 | | | | 20.8 | | | | |
| | | Macrophages (HLA-DQB1, C1QC) | 16.5 | | | | 9.1 | | | | |
| Fresh HCC tissues from eight HBV-associated HCC cases | 10 X Genomics | | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | Ho et al. [14] |
| | | HCC cells (AFP, GPC3) | 40.2 | 70.3 | 66.6 | 57.1 | 82 | 66.9 | 41.4 | 13.1 | |
| | | Endothelial cells (PECAM1, CD34) | 0 | 0 | 0.2 | 0.3 | 3.2 | 0 | 0.1 | 0.1 | |
| | | T cells (CD3E) | 0.6 | 2.8 | 18.4 | 22 | 6.4 | 20.4 | 43.8 | 72.2 | |
| | | B cells (CD19, CD79A) | 17.3 | 5.6 | 0.3 | 1 | 0 | 4.1 | 1.1 | 3.2 | |
| | | Macrophages (CD68) | 41.9 | 21.3 | 14.6 | 19.6 | 8.4 | 8.6 | 13.6 | 11.4 | |
| Fresh HCC tissues from two regions of three HCC cases | Singleron | | P1 | | P2 | | | P3 | | | Zhao et al. [20] |
| | | | A | B | A | | B | A | B | | |
| | | HCC cells (ALB, TTR, APOA2, APOA1) | 79.7 | 69.3 | 21 | | 37.6 | 77.6 | 47.4 | | |
| | | Hepatic stellate cells (ACTA2, RGS5, SERPINA1) | | | | | | | | | |
| | | Endothelial cells (CDH5, CLDN5, PECAM1, vWF) | 0.4 | 1.7 | 1.4 | | 0.8 | 4.7 | 6.1 | | |
| | | T cells (CD3D/E, CD2) | 1.8 | 2 | 1.4 | | 0.7 | 0.3 | 1.8 | | |
| | | B cells (MZB1, JCHAIN, MS4A1, CD79A, IGHG1) | 1 | 2 | 46.5 | | 18.3 | 0.1 | 4.6 | | |
| | | Myeloid-derived cells (CD68) | 17.1 | 25 | 29.7 | | 42.6 | 17.3 | 40.1 | | |

with HCC [33]. Notably, despite the intertumor and intratumor heterogeneity, there were LCSC subpopulations in each HCC case, these cells exhibited relatively high proliferation activity and stemness features, and TOP2A may be responsible for the maintenance/promotion of the stemness of HCC cells (Fig. 1) [20].

Recognizing the widespread intertumor and intratumor heterogeneity of HCC cells, identifying potential intertumor homogeneity and determining cell subclones highly responsible for treatment resistance, relapse and metastasis of HCC in each HCC case will be conducive to the individualized precision treatment of HCC.

### ScRNA-seq revealed molecular subtypes of fibroblasts in HCC

Fibroblasts are one of the major stromal cell types in the TME of HCC with a heterogeneous phenotype and function, which not only secrete an extracellular matrix rich in collagen and other macromolecules to provide mechanical support but also influence the growth, metastasis and therapeutic resistance of HCC. Furthermore, fibroblast levels were negatively correlated with HCC prognosis, and these cells are considered a potential target of HCC treatment [4]. Revealing the molecular landscape of fibroblasts at the single-cell level to systematically identify phenotypic and functional heterogeneity could accelerate the translation of fibroblast-targeted treatments for HCC.

ScRNA-seq of single-cell suspensions enzymatically dissociated from HCC tissues identified fibroblasts expressing high levels of canonical fibroblast markers, such as ACTA2, COL1A2, and COL1A1, and allowed the analysis of the molecular subtypes of fibroblasts in HCC [19]. Fibroblast subtypes expressed high levels of extracellular matrix (ECM) signature genes, ECM signature genes and lipid processing-related genes concurrently, lipid-processing genes, major histocompatibility complex II genes as well as chemokine-related genes and signature microvasculature genes were associated with ECM organization, both ECM and lipid processing, lipid metabolism, antigen presentation and vascular smooth muscle contraction, respectively, and presented dynamic transition (Fig. 2). Among fibroblast subtypes, the CD36-positive fibroblast subtype including the lipid-processing matrix fibroblast subtype expressed high levels of ECM signature genes and concurrently expressed lipid processing-related genes and the lipid-processing fibroblast subtype expressed high levels of lipid-processing genes potentiated the capacity of MDSCs to promote an immunosuppressive TME and tumor stemness and was

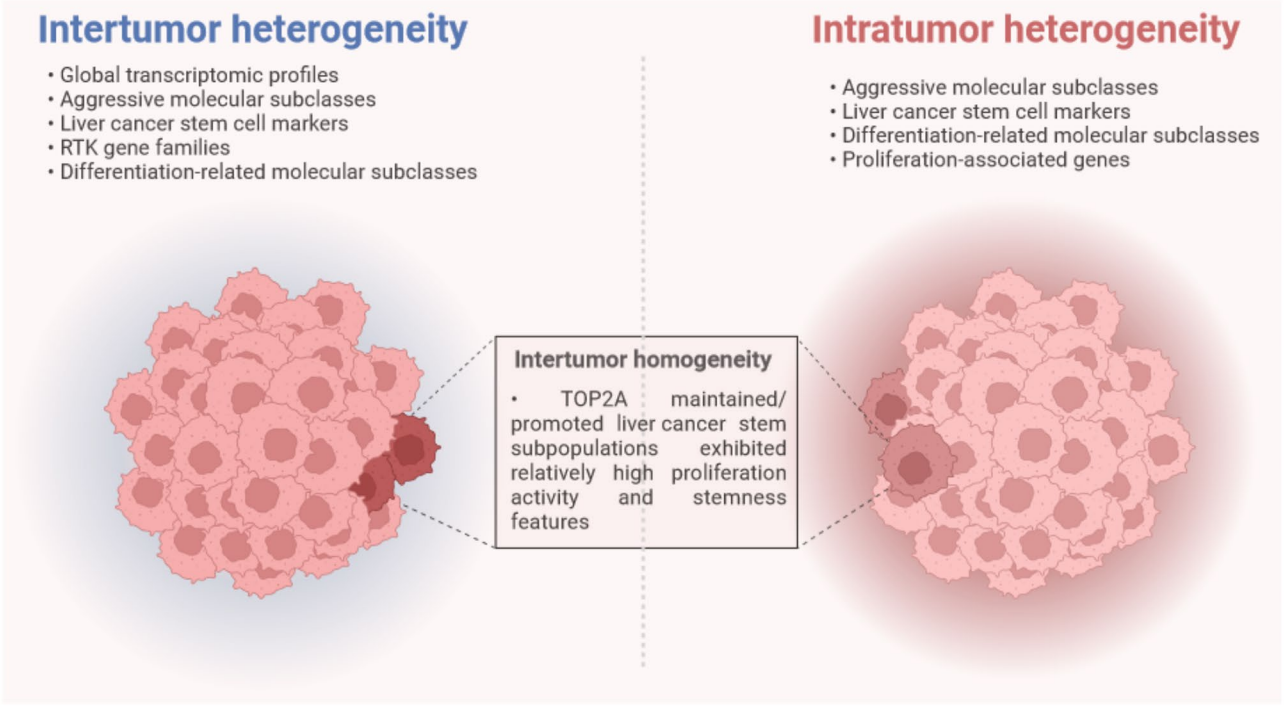


**Fig. 1** Intertumor and intratumor heterogeneity and intertumor homogeneity of HCC cells. HCC cells from different cases presented various global transcriptomic profiles and differential gene expression patterns of aggressive molecular subclasses, liver cancer stem cell (LCSC) markers, RTK gene families, and differentiation-related molecular subclasses. Differential gene expression patterns of aggressive molecular subclasses, LCSC markers, differentiation-related molecular subclasses and proliferation-associated genes were identified in HCC cells from the same cases. There were LCSC subpopulations in each HCC case, and TOP2A may be responsible for the maintenance/promotion of the stemness of HCC cells. Adapted from "Cold vs. Hot Tumors", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

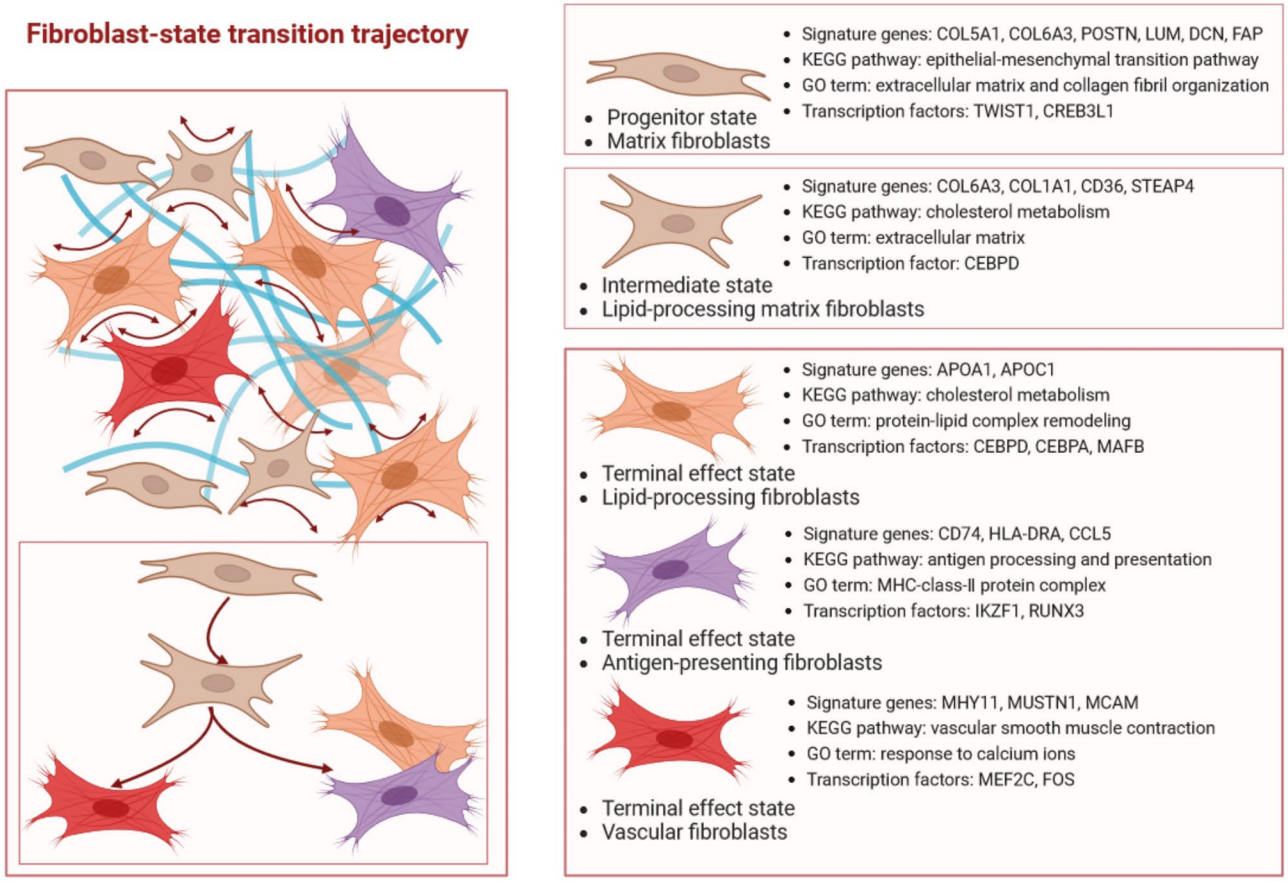


**Fig. 2** Molecular subtypes and transition trajectory of fibroblasts in HCC. Molecular subtypes of fibroblasts in HCC were divided into matrix fibroblasts, lipid-processing matrix fibroblasts, lipid-processing fibroblasts, antigen-presenting fibroblasts and vascular fibroblasts based on the expression of signature genes and presented dynamic transitions. The cells began as progenitor state matrix fibroblasts, transitioned to intermediate state lipid-processing matrix fibroblasts and finally diverged into a terminal effect state, including lipid-processing fibroblasts, antigen-presenting fibroblasts and vascular fibroblasts. Adapted from "Fibroblast and Fibrin Activity in Wound Healing", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

predictive of a poor prognosis and a better immunotherapy response in HCC patients. Targeting the CD36-positive fibroblast subtype with a CD36 inhibitor can be used to synergistically enhance the efficacy of immunotherapy.

Further elucidation of the molecular mechanisms involved in the transition of molecular subtypes of fibroblasts and the biological roles of various molecular subtypes of fibroblasts in the development of HCC may be beneficial to the translational application of fibroblast-targeted treatments in HCC.

### ScRNA-seq elucidates the cell composition, functional states and developmental trajectory of immune cells in HCC

The cellular components of the HCC immune microenvironment are highly complex, with diverse populations of lymphocytes and myeloid-derived cells playing critical roles in HCC immune evasion and responses to immunotherapy [34]. ScRNA-seq revealed that HCC-infiltrating lymphocytes mainly included T cells, B cells, and natural killer (NK) cells, myeloid-derived cells mainly consisted of macrophages and dendritic cells (DCs), and the cell subpopulations, functional states and developmental trajectory of each immune cell type were elucidated.

#### T cells

T cells are one of the major cell types in the HCC immune microenvironment and are greatly responsible for the immunosuppression/immune tolerance of HCC [3]. Although immune checkpoint inhibitors aimed at improving T-cell cytotoxic activity by checkpoint blockade improved clinical outcomes, there was a low disease remission rate in HCC [35, 36]. Elucidating the functional states and underlying immune state evolution of T cells in HCC will help with the development of novel immunotherapies.

ScRNA-seq elucidated that the majority of T cells in HCC were conventional $CD4^+$ T cells and $CD8^+$ T cells, and other cell types included unconventional MAIT, NKT and γδT cells. According to activated states and different functions, $CD4^+$ T cells included naïve $CD4^+$ T cells enriched for CCR7 and TCF7, regulatory T cells

(Tregs) enriched for FOXP3, helper T cells (Th), including Th1 cells enriched for STAT4 and IL2, Th2 cells enriched for GATA3 and CD40LG and Tfh cells enriched for CXCL13, and cytotoxic $CD4^+$ T cells enriched for cytotoxic molecules such as NKG7, GNLY and GZMB. $CD8^+$ T cells mainly included naïve CD8+T cells enriched for LEF1, CCR7, CCR6 and S100A8, liver-tissue-resident memory-like T cells enriched for NR4A1, cytotoxic $CD8^+$ T cells enriched for cytotoxic molecules such as GZMH, GZMK, INFG and GNLY, and gradually transitioned to exhausted $CD8^+$ T cells expressing high levels of the exhaustion markers CTLA4, PDCD1, TIGHT, LAG3, HAVGR2 and EOMES, and some NKT cells enriched for CD16, MAIT cells enriched for TRAV1-2 and Tregs expressing FOXP3 (Fig. 3) [13, 14, 20–22, 37–40].

Overall, T cells in HCC presented a dynamic immune state transition toward a more exhausted or immunosuppressive status with distinctive gene expression signatures. Further revealing the molecular mechanisms responsible for immune state evolution and identifying potential exhausted or immunosuppressive markers to reverse immune status will help with the development of novel immunotherapies.

### B cells

B cells can not only mediate the humoral immune response by activation, proliferation and differentiation into plasma cells to secrete antibodies but also play important roles in antigen presentation and immune regulation [41]. Recognizing the functional states of B cells in HCC helps to further clarify their biological functions in HCC progression and facilitate the development of novel immunotherapeutic strategies.

ScRNA-seq recognized that B cells in HCC presented naïve B cells, memory B cells and plasma cells and were divided into different molecular subtypes based on high expression of the corresponding genes, including three subsets of naïve B cells enriched in TCL1A, KLF4 and CLEC2B, four subsets of memory B cells enriched in LMNA, AIM2, heat shock proteins and activated

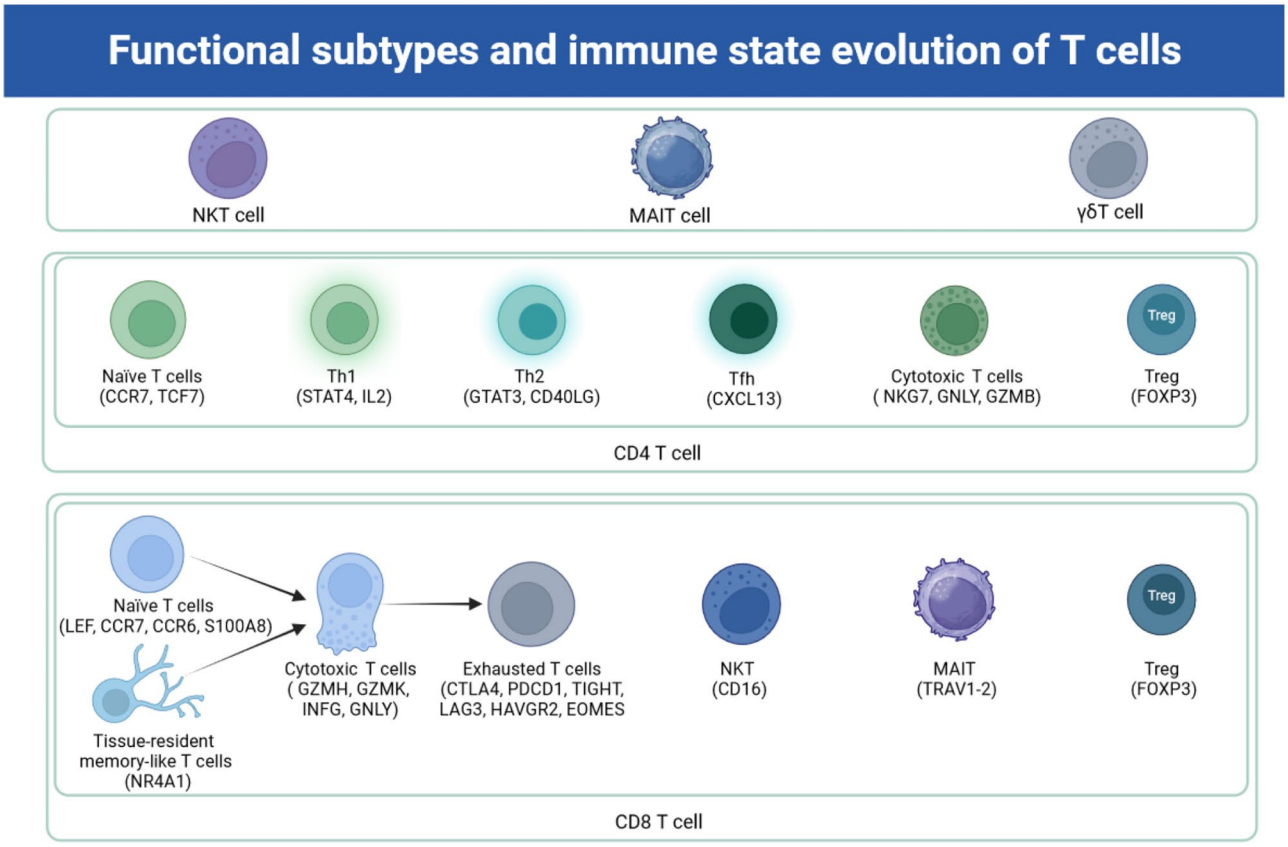


**Fig. 3** Functional subtypes and dynamic immune state evolution of T cells in HCC. T cells in HCC included $CD4^+$ T cells, $CD8^+$ T cells, MAIT cells, NKT cells and γδT cells. $CD4^+$ T cells included naïve T cells enriched in CCR7 and TCF7, helper T cells (Th), including Th1 cells enriched in STAT4 and IL2, Th2 cells enriched in GATA3 and CD40LG and Tfh cells enriched in CXCL13, cytotoxic T cells enriched in NKG7, GNLY and GZMB and regulatory T cells (Treg) enriched in FOXP3. $CD8^+$ T cells mainly included naïve T cells enriched in LEF1, CCR7, CCR6 and S100A8, liver-tissue-resident memory-like T cells enriched in NR4A1, cytotoxic T cells enriched in GZMH, GZMK, INFG and GNLY, gradually transitioned to exhausted T cells expressing CTLA4, PDCD1, TIGHT, LAG3, HAVGR2 and EOMES, and partially NKT cells enriched in CD16, MAIT cells enriched in TRAV1-2 and Tregs enriched in FOXP3. Adapted from "Icon Pack-T cell", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

memory B cells characterized by high expression of EGR1, JUNB, NR4A2 and GPR183, and three subsets of plasma cells characterized by high expression of IGHG1, IGHA1 and MKI67. Significantly, inferred developmental trajectories of B cells in HCC indicated that naïve B cells and memory B cells mainly differentiated into IgG plasma cells (Fig. 4). Further, IgG plasma cells preferentially fostered immunosuppression in the TME by promoting protumorigenic macrophage formation in HCC and were correlated with a worse survival of HCC patients [20, 42].

Further investigation of the differentiation induction mechanisms of naïve B cells and memory B cells and the biological functions of different subtypes of plasma cells in HCC progression will contribute to the development of novel immunotherapeutic strategies for HCC.

### NK cells

NK cells present cytotoxic activity to directly kill tumor cells and are involved in immune regulation by cytokine secretion [43]. Deeply characterizing NK cells in HCC is critical for understanding their roles in HCC immunological responses and NK cell-based immunotherapy.

ScRNA-seq revealed that NK cells in HCC were mainly composed of tissue-resident memory NK cells characterized by high expression of CD69, NR4A1, CD160, CXCR6 and EOMES, and circulating NK cells recruited from peripheral blood highly expressed FCGR3A, CX3CR1 and transcription factor T-bet (TBX21), were activated into conventional $CD56^{bright}$ NK cells overexpressing XCL1 and $CD56^{dim}$ NK cells highly expressing cytotoxic genes GZMB, GZMH, PRF1 and CCL5, and gradually transitioned toward the exhausted state with downregulation of activation marker FCGR3A and upregulation of inhibitory receptor KLRC1 (Fig. 5) [13, 14, 37, 44, 45].

Further investigation of the molecular mechanisms responsible for the activation of tissue-resident memory NK cells, the recruitment of circulating NK cells and the evolution toward the exhausted state may facilitate the development of NK cell-targeted immunotherapeutic strategies for HCC.

### Dendritic cells (DCs)

DCs present tumor antigens and induce the activation and proliferation of T cells, thus killing tumor cells and playing important roles in the tumor immune response [46]. ScRNA-seq identified that DCs in HCC consisted of plasmacytoid DCs that highly expressed

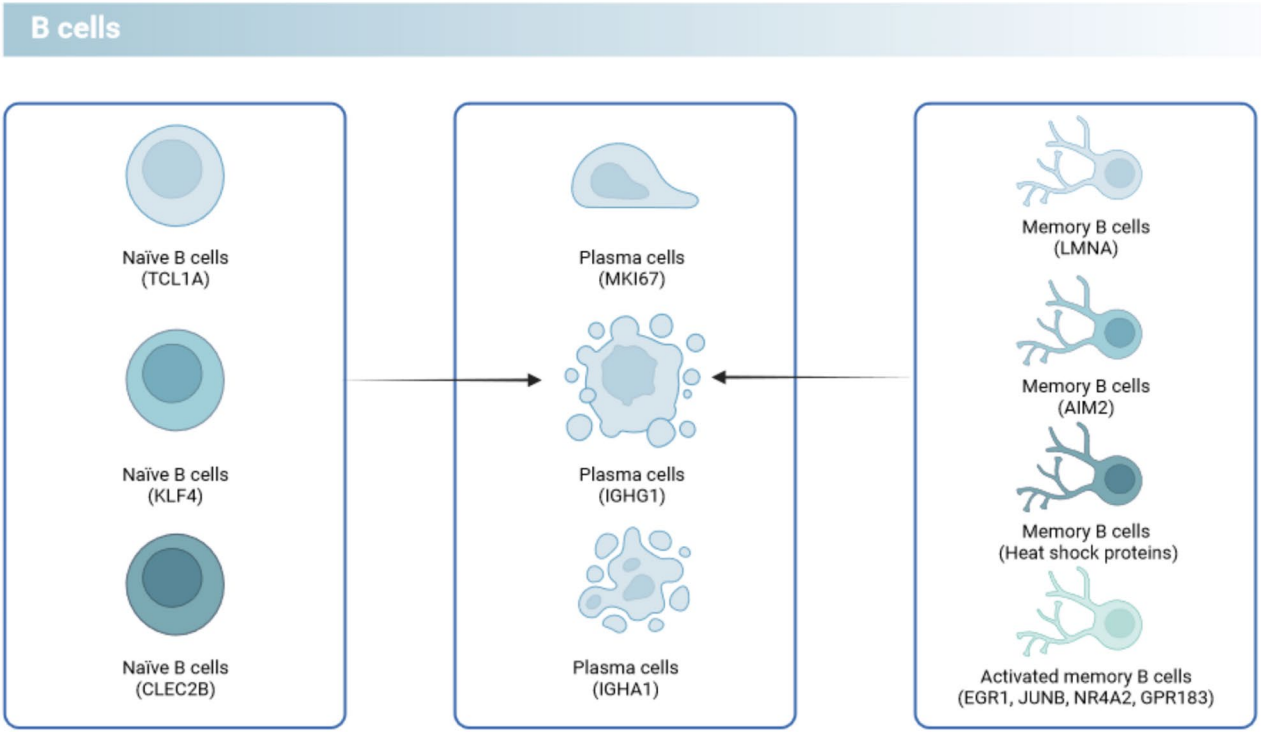


**Fig. 4** Functional states and molecular subtypes of B cells in HCC. B cells in HCC included naïve B cells, memory B cells and plasma cells. Naïve B cells were divided into three molecular subtypes enriched in TCL1A, KLF4 and CLEC2B; memory B cells included four molecular subtypes enriched in LMNA, AIM2, heat shock proteins and activated memory B cells characterized by high expression of EGR1, JUNB, NR4A2 and GPR183; plasma cells presented three molecular subsets characterized by high expression of IGHG1, IGHA1 and MKI67. Inferred developmental trajectories indicated that naïve B cells and memory B cells mainly differentiated into IgG plasma cells. Adapted from "Try Our New Cells!", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

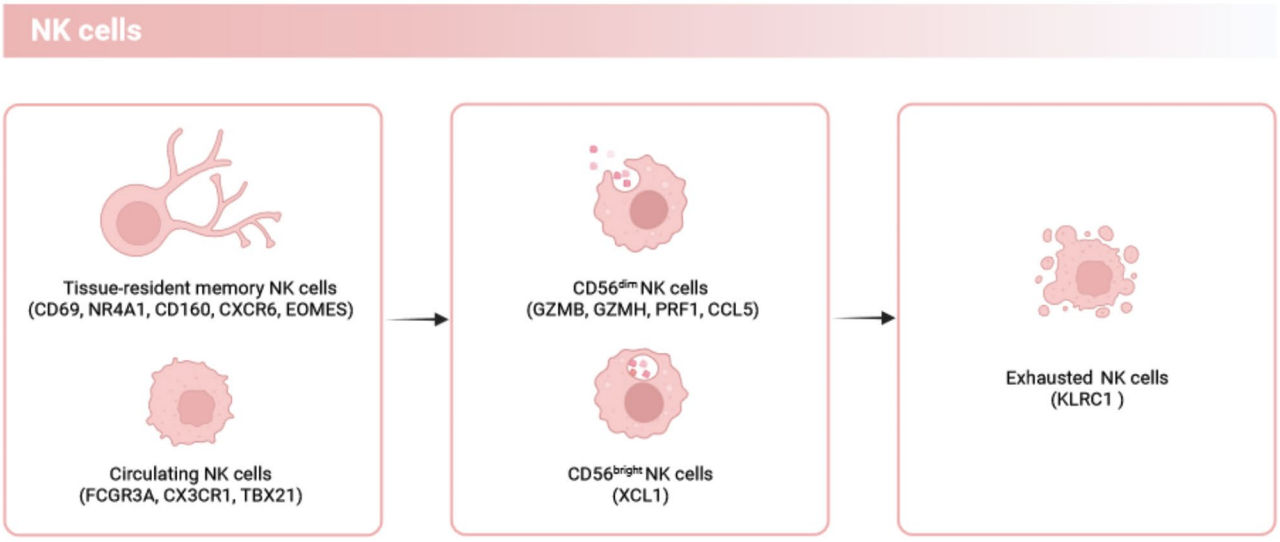


**Fig. 5** Dynamic immune state evolution of NK cells in HCC. Tissue-resident memory NK cells characterized by high expression of CD69, NR4A1, CD160, CXCR6 and EOMES and circulating NK cells highly expressing FCGR3A, CX3CR1 and TBX21 were activated into conventional $CD56^{bright}$ NK cells overexpressing XCL1 and $CD56^{dim}$ NK cells highly expressing the cytotoxic genes GZMB, GZMH, PRF1 and CCL5 and gradually transitioned toward the exhausted state with upregulation of the inhibitory receptor KLRC1. Adapted from "Try Our New Cells!", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

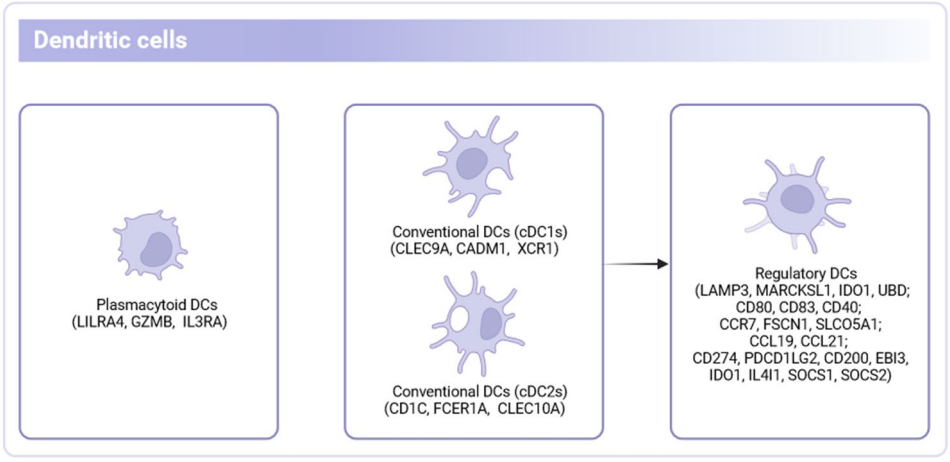


**Fig. 6** Functional subtypes and immune state evolution of dendritic cells (DCs) in HCC. DCs in HCC consisted of plasmacytoid DCs that highly expressed LILRA4, GZMB, and IL3RA; conventional DCs (cDCs) that highly expressed CLEC9A, CADM1, and XCR1 (cDC1s); cDCs that highly expressed CD1C, FCER1A, and CLEC10A (cDC2s); and regulatory DCs with higher expression levels of the maturation markers LAMP3, MARCKSL1, IDO1, and UBD; activation markers CD80, CD83, and CD40; migration markers CCR7, FSCN1, and SLCO5A1; lymphocyte recirculation chemokines CCL19 and CCL21; and immune-suppressive markers CD274, PDCD1LG2, CD200, EBI3, IDO1, IL4I1, SOCS1, and SOCS2. The inferred developmental trajectory suggested that both cDC1s and cDC2s might undergo a transition to regulatory DCs. Adapted from "Try Our New Cells!", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

LILRA4, GZMB, and IL3RA; conventional DCs (cDCs) that highly expressed CLEC9A, CADM1, and XCR1 (cDC1s); cDCs that highly expressed CD1C, FCER1A, and CLEC10A (cDC2s); and regulatory DCs with higher expression levels of the maturation markers LAMP3, MARCKSL1, IDO1, and UBD; activation markers CD80, CD83, and CD40; migration markers CCR7, FSCN1, and SLCO5A1; lymphocyte recirculation chemokines CCL19 and CCL21; and immune-suppressive markers CD274, PDCD1LG2, CD200, EBI3, IDO1, IL4I1, SOCS1, and SOCS2. The inferred developmental trajectory suggested that both cDC1s and cDC2s might undergo a transition to regulatory DCs with decreased antigen-presenting capacity and increased immune-suppressive ability (Fig. 6) [13, 20–22, 44].

### Macrophages

Macrophages are one of the major cell types in the tumor immune microenvironment and present heterogeneous functional phenotypes, such as proinflammatory M1 macrophages and anti-inflammatory M2 macrophages, with dynamic transitions [47]. Exploring the functional states and underlying evolution of macrophages in the HCC immune microenvironment helps to clarify their dual roles in HCC immunological responses and contributes to the development of novel immunotherapeutic strategies for HCC.

ScRNA-seq identified that some macrophages in the HCC microenvironment were liver-resident Kupffer cells with the expression of MARCO but were mainly circulating macrophages recruiting and originating from monocytes. According to signature genes, macrophages in the HCC immune microenvironment were mainly divided into myeloid-derived suppressor cell-like macrophages enriched in the S100A family genes FCN1 and VCAN and tumor-associated macrophages expressing APOE, C1QA, C1QB, C1QC and TREM2. Remarkably, although these cells could not be dichotomously distinguished as the proinflammatory M1 phenotype or the anti-inflammatory M2 phenotype using known marker genes such as FCGR3A (M1) and CD163 (M2) because of their coexistence in macrophages, there was a dynamic transition toward a more immunosuppressive M2 phenotype characterized by increased CD163 expression, upregulation of coinhibitors HAVCR2, LAIR1, LGALS9, and CD276, and downregulation of coactivators TNFSF14, CD48, and CD44 along the developmental trajectory (Fig. 7). Notably, a specific subset of tumor-associated macrophages with positive expression of SPP1, which presented an immunosuppressive M2 phenotype, appeared to promote T-cell exhaustion via the SPP1-CD44 axis. Targeting the SPP1-CD44 axis with an anti-SPP1 or anti-CD44 antibody can be used to synergistically enhance the efficacy of immunotherapy [13, 14, 20–22, 37, 44].

Overall, immune cells in the HCC microenvironment presented heterogeneous functional subtypes and dynamic immune state transitions toward a more exhausted or immunosuppressive status with distinctive gene expression signatures.

### ScRNA-seq-mediated exploration of cellular interactions in HCC

HCC cells, along with nonmalignant stromal cells (e.g., endothelial cells, fibroblasts, immune cells), comprise the intricate cellular ecosystem and present complicated cellular interactions, thus synergistically facilitating HCC initiation and progression and leading to treatment resistance [3–5]. Systematic exploration of cellular interactions in HCC is essential to understanding HCC development and discovering novel therapeutic targets for HCC.

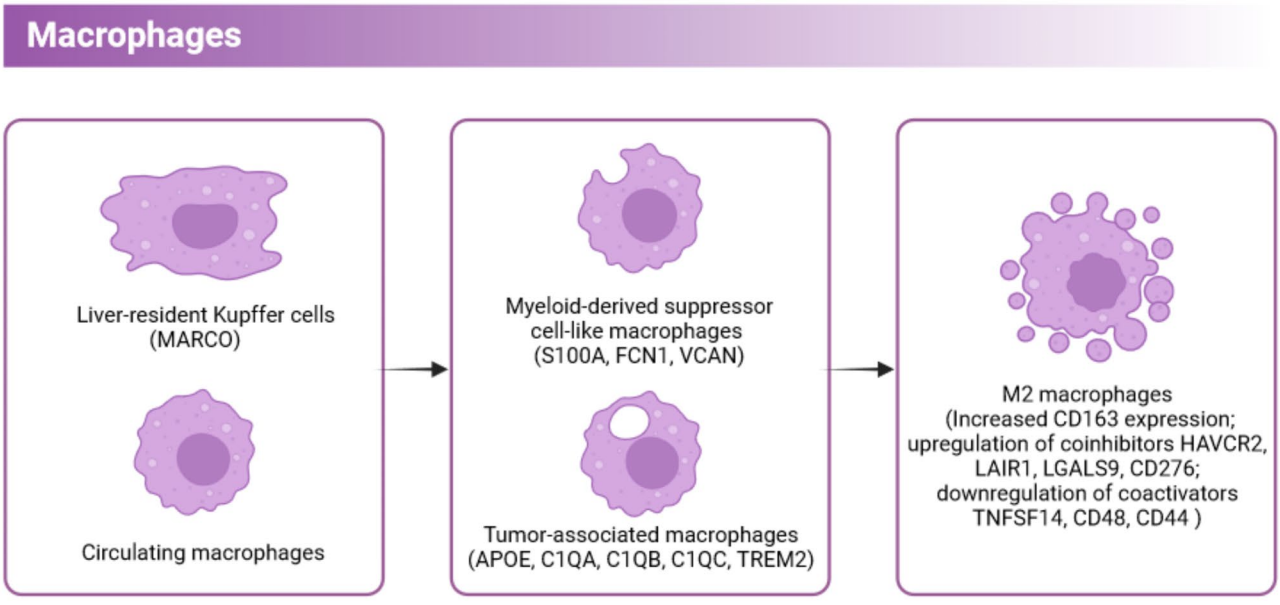


**Fig. 7** Cellular origins, functional states and immune state evolution of macrophages in HCC. Macrophages in the HCC microenvironment included liver-resident Kupffer cells with the expression of MARCO and circulating macrophages recruited and originating from monocytes and were mainly divided into myeloid-derived suppressor cell-like macrophages enriched in the S100A family genes FCN1 and VCAN and tumor-associated macrophages expressing APOE, C1QA, C1QB, C1QC and TREM2 with dynamic transition toward a more immunosuppressive M2 phenotype characterized by increased CD163 expression, upregulation of coinhibitors HAVCR2, LAIR1, LGALS9, and CD276, and downregulation of coactivators TNFSF14, CD48, and CD44. Adapted from "Try Our New Cells!", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

ScRNA-seq systematically explored the interactions between different cell types by inferred ligand–receptor binding pairs and indicated frequent cellular interactions in HCC. HCC cells frequently interact with various immune cells, especially macrophages, via the MHCI-LILRB, MIF-CD74, CCL15/16-CCR1, CXCL12-CXCR4, C3-C3AR1/ITGB2 and TGFβ1-TGFβR2/3 axes. In addition, HCC cells and endothelial cells interact through the VEGFA-VEGFR and INHBB-ACVR1/2 axes. Moreover, the MIF-CD74/CXCR4 axis is the most common mediator of interactions between fibroblasts and other cells, including B cells, DCs, MDSCs, monocytes/macrophages, NK cells and T cells. Among the cellular interactions between immune cells, macrophages interact with T cells via SPP1-CD44, CCL18-CCR8, ICAM1-ITGAL, SELPLG-SELL, ICAM1-AREG and HAVCR2-LGALS9, and DCs present strong potential interactions with T cells via the costimulator CD28/B7 family (CD86-CD28, CD86-CTLA4, ICOSLG-CD28, and ICOLG-CTLA4) and interleukin-15 (IL-15- IL-2RB and IL-15-IL-2RG). Among the DC subtypes, regulatory DCs interact with T cells via the CD80/CD86-CTLA4/CD28, ADORA2A-ENTPD1, CD70-CD27, CCL19-CXCR3, CXCL10-CXCR3, TIGIT-PVR/NECTIN2, CCL19-CCR7, CCL22-CCR4, and CD274/PDCD1LG2-PDCD1 axes and interact with NK cells through the NECTIN2-CD226 and NECTIN2-TIGIT axes (Fig. 8). Notably, the MIF-CD74/CXCR4 axis-mediated interactions between CD36-positive fibroblasts and MDSCs and the SPP1-CD44 axis-mediated interactions between macrophages and T cells have been found to promote an immunosuppressive TME and are predictive of a poor prognosis and a better immunotherapy response in HCC patients. Targeting the CD36-positive fibroblast subtype with a CD36 inhibitor or targeting the SPP1-CD44 axis with an anti-SPP1 or anti-CD44 antibody can be used to synergistically enhance the efficacy of immunotherapy [14, 17–22, 44].

Although frequent interactions between different cell types in the cellular ecosystem of HCC have been widely inferred by scRNA-seq via ligand–receptor communication analysis, further addressing the spatial resolution and specific biological roles of these cell types in HCC development and their potential applications as novel therapeutic targets will aid in HCC treatment.

## Conclusions and prospective

ScRNA-seq revealed the global cell composition of HCC, the widespread molecular heterogeneity of HCC cells and fibroblasts, the cell composition, functional states and development trajectory of immune cells, and the frequent interactions between different cell types, which systematically illustrate the landscape of the cellular ecosystem of primary HCC. Further integrating scRNA-seq and spatial transcriptomics to capture deep gene expression information and cell spatial distribution information simultaneously at single-cell resolution, which enables the identification of transcriptionally characterized single cells within their native tissue context and the investigation of their specific biological functions to explore the heterogeneity, cell plasticity and complexity of the cellular ecosystem of HCC, will be highly beneficial for understanding HCC development and identifying novel therapeutic targets.

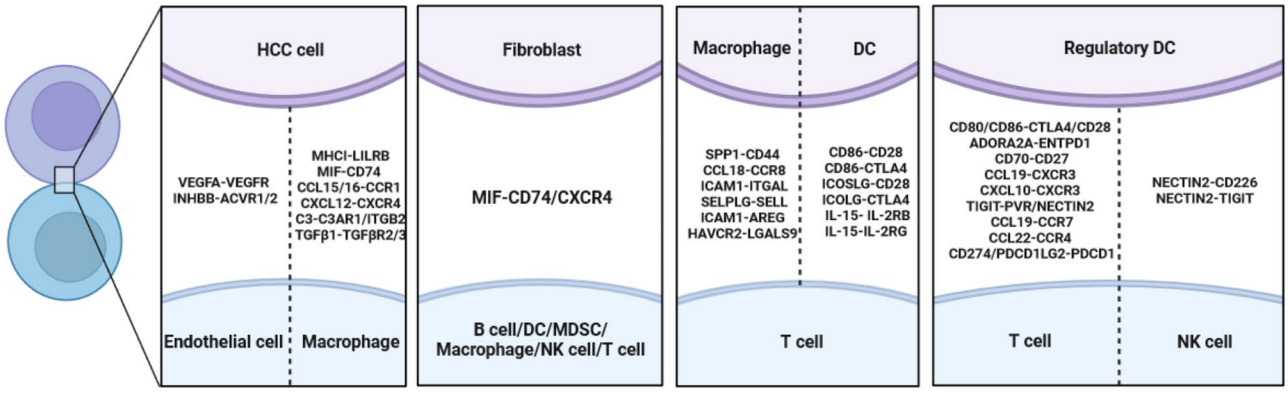


**Fig. 8** Frequent cellular interactions between different cell types in HCC inferred by ligand–receptor binding pairs. HCC cells frequently interact with macrophages via the MHCI-LILRB, MIF-CD74, CCL15/16-CCR1, CXCL12-CXCR4, C3-C3AR1/ITGB2 and TGFβ1-TGFβR2/3 axes. HCC cells and endothelial cells interact through the VEGFA-VEGFR and INHBB-ACVR1/2 axes. The MIF-CD74/CXCR4 axis is the most common mediator of interactions between fibroblasts and other cells, including B cells, DCs, MDSCs, monocytes/macrophages, NK cells and T cells. Macrophages interact with T cells via SPP1-CD44, CCL18-CCR8, ICAM1-ITGAL, SELPLG-SELL, ICAM1-AREG and HAVCR2-LGALS9. DCs present strong potential interactions with T cells via CD86-CD28, CD86-CTLA4, ICOSLG-CD28, ICOLG-CTLA4, IL-15- IL-2RB and IL-15-IL-2RG. Regulatory DCs interact with T cells via the CD80/CD86-CTLA4/CD28, ADORA2A-ENTPD1, CD70-CD27, CCL19-CXCR3, CXCL10-CXCR3, TIGIT-PVR/NECTIN2, CCL19-CCR7, CCL22-CCR4, and CD274/PDCD1LG2-PDCD1 axes and interact with NK cells through the NECTIN2-CD226 and NECTIN2-TIGIT axes Adapted from "T Cell & Dendritic Cell Interaction with Callouts (Layout)", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

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**Author contributions**
W.L. revised the manuscript. Y.Z. and S.Y. wrote the manuscript and prepared figures. All authors reviewed the manuscript.

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**Data availability**
No datasets were generated or analysed during the current study.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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