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Review

# Spatial heterogeneity of the hepatocellular carcinoma microenvironment determines the efficacy of immunotherapy

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Received: 1 September 2024 / Accepted: 2 January 2025
Published online: 07 January 2025


**Abstract**
Hepatocellular carcinoma (HCC) remains a global health challenge owing to its widespread incidence and high mortality. HCC has a specific immune tolerance function because of its unique physiological structure, which limits the efficacy of chemotherapy, radiotherapy, and molecular targeting. In recent years, new immune approaches, including adoptive cell therapy, tumor vaccines, and oncolytic virus therapy, have shown great potential. As the efficacy of immunotherapy mainly depends on the spatial heterogeneity of the tumor immune microenvironment, it is necessary to elucidate the crosstalk between the composition of the liver cancer immune environment, from which potential therapeutic targets can be selected to provide more appropriate individualized treatment programs. The role of spatial heterogeneity of immune cells in the microenvironment of HCC in the progression and influence of immunotherapy on improving the treatment and prognosis of HCC were comprehensively analyzed, providing new inspiration for the subsequent clinical treatment of liver cancer.

**Keywords** Hepatocellular carcinoma · Spatial heterogeneity · Tumor microenvironment · Immunotherapy

**Abbreviations**

| | |
|---|---|
| HCC | Hepatocellular carcinoma |
| TACE | Transhepatic arterial chemoembolization |
| ICIs | Immune checkpoint inhibitors |
| TME | Tumor microenvironment |
| DC | Dendritic cell |
| NK | Natural killer cells |
| NKT | Natural killer T cells |
| Treg | Regulatory T cell |
| MDSC | Bone marrow derived suppressor cells |
| TANs | Tumor-associated neutrophils |

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|---|---|
| ROS | Reactive oxygen species |
| MMPs | Matrix metalloproteinases |
| IL | Interleukin |
| PD-1 | Programmed death-1 |
| PD-L1 | Pogrammed death ligand-1 |
| CTLA-4 | Cytotoxic T lymphocyte-associated protein-4 |
| MDSCs | Myeloid-derived suppressor cells |
| VEGF | Vascular endothelial growth factor |
| IFN-γ | Interferon-γ |
| TNF | Tumor necrosis factor |
| CSF1 | Macrophage colony stimulating factor 1 |
| MCP-1 | Monocyte chemoattractant protein-1 |
| BMP2 | Bone morphogenetic protein 2 |
| HGF | Liver cancer growth factor |
| CAR-T | Chimeric antigen receptor T cells |
| TILs | Tumor-infiltrating lymphocytes |
| CIK | Cytokine induced killer cell |
| GPC3 | Glypican 3 |
| ORR | Objective response rate |
| AFP | Alpha-fetoprotein |
| TCR-T | T cell receptor engineered T |
| CIK | Cytokine induced killer |
| TAA | Tumor-associated antigen |
| OVs | Oncolytic virus |

## 1 Introduction

The prevalence of primary liver cancer is increasing annually, and it is the third most common tumor-related cause of death worldwide, posing a severe challenge to global healthcare [1]. Primary liver cancers include hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma, and mixed liver cancer, among which HCC is the most common subtype, accounting for 75–85% [2]. Multiple risk factors are associated with the occurrence of liver cancer, including chronic hepatitis B virus(HBV), chronic hepatitis C virus (HCV), alcoholism, non-alcoholic steatosis, autoimmune liver disease, and aflatoxin exposure [3, 4]. As a special organ rich in immunoactive cells, the liver has a variety of immune cells and antigen-presenting cells (APCs), which play a key role in maintaining immune tolerance and immune escape. Although great progress has been made in surgical tumor resection, liver transplantation, transhepatic arterial chemoembolization (TACE), radiofrequency ablation (RFA), and systemic drug therapy, the overall effect is still unsatisfactory due to frequent tumor recurrence and metastasis and the emergence of drug resistance. In the past, systemic immunotherapy based on immune checkpoint inhibitors (ICIs) has shown encouraging results and has changed the treatment of cancer. However, in the clinical treatment of HCC, the remission rate is less than 20%, and immune-related adverse reactions (irAEs) caused by ICI activation are major challenges in clinical practice [5]. At present, with an in-depth understanding of the spatial heterogeneity and immune microenvironment of HCC tissues, emerging immunotherapies with stronger anti-tumor immunity have gradually become a research hotspot, which is expected to improve the survival time and quality of patients [6].

## 2 Cells spatial heterogeneity in the tumor microenvironment

The efficacy of immunotherapy depends on the tumor microenvironment(TME) formed by a variety of infiltrating immune cells, tumor-associated fibroblasts, vascular endothelial cells, extracellular matrix, chemokines, cytokines, etc., but in fact, immunotherapy is only effective in 20 to 30% of patients (Fig. 1). The dynamic interactions between infiltrating immune cells in tumor tissue and other cellular and non-cellular composition of the TME that affect HCC progression (Fig. 2), thus further elucidating the spatial heterogeneity of immune cells in the TME and seeking new or combined

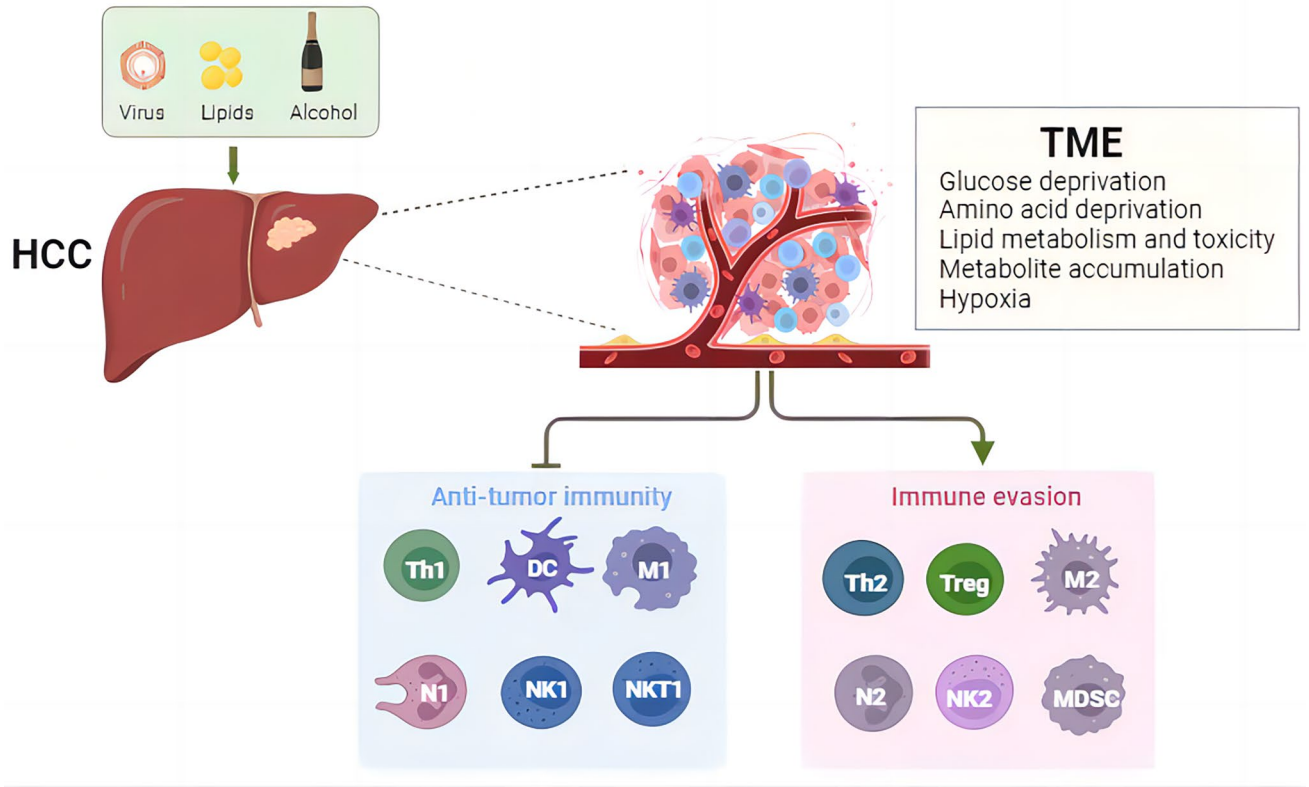


**Fig. 1** Spatial heterogeneity of immune cells in the HCC microenvironment. Complex interactions between immune cells, cancer-associated fibroblasts, endothelial cells, extracellular matrix, and cell-secreted cytokines in the TME play a critical role in HCC progression. Immune cells can be divided into types that recognize tumor cells and initiate cytotoxic responses to promote anti-tumor and types that exert immunosuppressive responses that lead to immune evasion. HCC: hepatocellular carcinoma; TME: Tumor microenvironment; Th: Helper T cells; DC: Dendritic cell; M1: M1 macrophage; M2: M2 macrophage; N1: N1 neutrophils; N2: N2 neutrophils; NK: Natural killer cells; NKT: Natural killer T cells; Treg: Regulatory T cell; MDSC: Bone marrow derived suppressor cells

immunotherapeutic targets to inhibit tumor development. Improving patients' sensitivity to immunotherapy is an urgent problem in all fields of liver cancer treatment [7, 8].

### 2.1 Tumor-associated neutrophils

Neutrophils, as innate immune cells, are one of the fastest responding cells in the processes of injury, infection, and tumors. When a large number of neutrophils infiltrate the liver to produce an inflammatory response, they are recruited into the TME under the action of chemokines and transformed into tumor-associated neutrophils (TANs) with plasticity. There are two types of TANs: N1 (anti-tumor) can produce type I interferon to inhibit tumor growth; N2 (tumor promoting) can secrete reactive oxygen species (ROS), matrix metalloproteinases (MMPs), arginine, and other molecules, inhibit the function of T cells and NK cells, and promote the occurrence and metastasis of tumors [9, 10]. Studies have shown that inhibition of TGF-β signaling can promote the transformation of TANs into the N1 type [11]. The synergistic effect of TGF-β and Axl can mediate the secretion of chemokine CXCL5 in HCC tissues, promote the proliferation and invasion of HCC cells, increase the number of infiltrating TANs, and promote the tolerance of tumor cells to chemotherapeutic drugs [12]. In the experiment of tumorous mice carrying liver cancer, the use of anti-GR1 antibody to remove TANs can further inhibit the growth and invasion of tumor cells and the formation of new blood vessels [13]. These results suggest that the spatial heterogeneity of TANs in the TME may affect the occurrence and development of tumors.

TANs increase the migration activity of regulatory T cells (Tregs) and macrophages by releasing CC chemokine ligand 2 (CCL2) and CCL17, allowing them to enter HCC tissues to promote tumor progression and drug resistance to sorafenib [14]. HCC patients with low cellular levels of CCL2 or CCL17 survived longer. Bone morphogenetic protein 2(BMP-2) and TGF-β2 secreted by TANs induce the expression of miR-301b-3p in HCC cells. The upregulation of miR-301b-3p, as a positive feedback loop regulating the stemness of tumor cells, can activate the NF-κB signaling

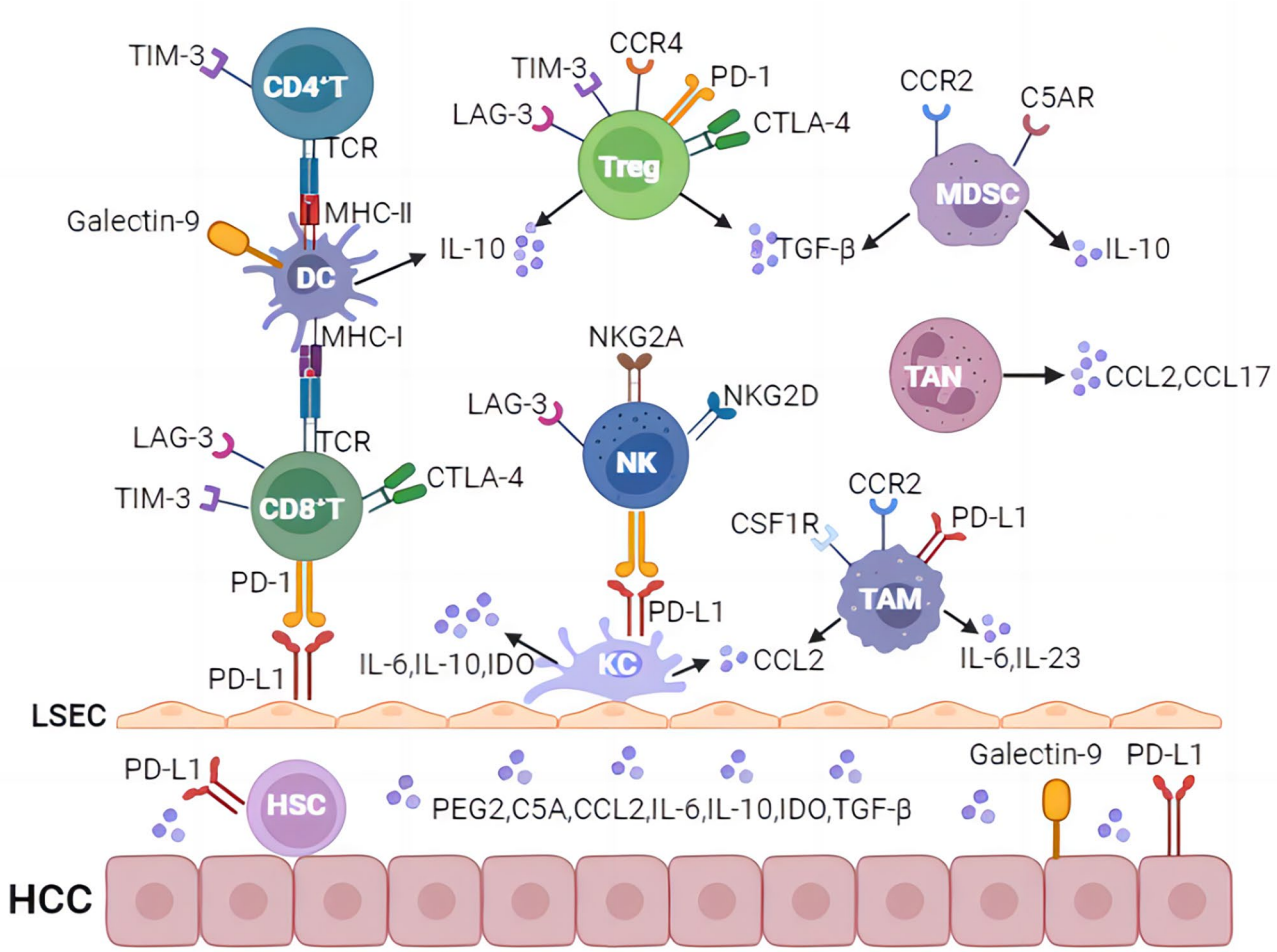


**Fig. 2** Schematic diagram of the interaction between cells and cytokines in the TME of HCC. The induction mechanism of immune tolerance inhibits the anti-tumor response of effector cells and promotes the formation of immunosuppressive environment and tumor progression. HCC and non-parenchymal cells (LSEC, KC, HSC) promote an immunosuppressive environment by secreting cytokines/chemokines to recruit ligands of immune tolerance cells (Tregs, MDSCs, TAMs) and expression suppressive effector cells (T cells and NK cells). LSEC: Hepatic sinus endothelial cells; KC: Kupffer cells; HSC: Hepatic stellate cell; IL: Interleukin; TGF-β: Transforming growth factor-β; CCL: CC chemokine ligand; TIM-3: T cell immunoglobulin mucin-3; PGE2: Prostaglandin E2; PD-1: Programmed death-1; PD-L1: Pogrammed death ligand-1; CTLA-4: Cytotoxic T lymphocyte-associated protein-4; LAG3: Lymphocyte activating gene 3; IDO: Indoleamine 2, 3-dioxidase; CSF1: Macrophage colony stimulating factor 1 receptor

pathway, leading to high secretion of CXCL5 and recruitment of more TANs for infiltration [15, 16]. In HCC, CXCL5 is an important chemokine that promotes TANs infiltration in tumor areas and produces poor prognosis through the protein kinase B(Akt)/extracellular signal-regulated kinase 1/2(ERK1/2) signaling pathway. Inhibition of CXCL5 secretion may become an important target for combination with tumor-targeted therapy [17]. Many studies have emphasized that crosstalk between HCC cells, TANs, and tumor-associated fibroblasts (CAFs) is an important factor affecting the progression of HCC. CAFs can inhibit the function of neutrophils by activating the SDF1α/CXCR4/IL-6 signaling pathway in the HCC TME, upregulating the expression of PD-L1, CXCL8/IL-8, CD66b, CCL2, and TNF, and inhibiting the proliferation and anti-tumor function of related T cells [18]. CAFs can also activate the IL-6/STAT3/PD-L1 signaling pathway and affect the survival and function of TANs in HCC [18]. Tumor-derived CXCL6 and TGF-β are responsible for the recruitment of neutrophils and polarization of M2 type, respectively, and enhance the function of CAFs to secrete cardiac trophic factor-like cytokine 1(CLCF1) [19]. In general, blocking the chemokine signaling pathway and reducing the infiltration of TANs may provide new ideas for the treatment of HCC.

### 2.2 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a group of heterogeneous cells with immunosuppressive activity in response to innate and adaptive immune responses, and can be divided into monocytes(M-MDSCs) displaying monocyte-like morphological characteristics. Polymorphonuclear cells(PMN-MDSCs) have the morphological characteristics of neutrophils. MDSCs are pathologically activated in the context of chronic inflammation or cancer, accumulate in local tissues and systemic blood, and have been shown to induce nitrification of lymphocyt-specific protein tyrosine excitation(LCK) through the production of highly active molecule "active nitrogen", thereby inhibiting the ability of T cells to kill tumor cells and participate in anti-tumor immunity [20].Compared to healthy individuals, the proportion of specific M-MDSCs in the peripheral blood of HCC patients is higher, and a high MDSCs population is associated with poorer overall survival(OS) and relapse-free survival (RFS) [21]. Cytokine-induced killer(CIK) adoptive immunotherapy can induce the increase of inflammatory mediators such as CX3CL1 and IL-13 in the TME, as well as the increase of MDSCs, which can suppress the cytotoxic activity of CIK and reduce the efficacy [22]. It has long been shown that HCC specific cell cycle related kinase(CCRK) up-regulates IL-6 expression and activates NF-κB by enhancing the enzyme enhancer of zeste homolog 2(EZH2). PMN-MDSC accumulation and T-cell activation in the TME are inhibited, and high expression of CCRK/IL-6/CD11b/CD33 is associated with the worst prognosis [23].

The hepatic stellate cell (HSC) -peripheral blood monocyte co-culture system and MDSCs derived from patients with fibrotic HCC were studied. This release factor activates the innate p38 MAPK signal of monocytes, which can lead to M-MDSC-specific gene expression and immunosuppressive enhancer reprogramming. The use of p38 MAPK inhibitors can eliminate the interference of HSC-M-MDSCs, thereby inhibiting the growth of HCC cells [24]. Accumulation of M-MDSCs can lead to a decrease in tumor-infiltrating lymphocytes (TILs) and increase tumorigenicity. In addition, the BET domain inhibitor i-BET762 can reduce M-MDSCs in peripheral blood mononuclear cells (PBMCs) of HCC patients, and the combination of anti-PD-L1 and i-BET762 in CCL4-induced fibrotic HCC mouse models can synergistically enhance TILs therapy, eliminate tumors, and prolong survival [24]. MER proto-oncogene tyrosine kinase (MerTK) is highly expressed in numerous types of cancer, thereby leading to the activation of carcinogenic signaling pathways and play a crucial role in the occurrence, development, and drug resistance of tumors. It has been discovered that targeting MerTK could not only eliminate the iron death resistance of HCC cells caused by immune checkpoint inhibitor treatment but also reduce the immunosuppressive effect of MDSC in the drug-resistant HCC microenvironment, activate $CD8^+$T cells, and synergistically enhance the anti-tumor activity of immune checkpoint inhibitors. This provides a novel idea for the inadequate immunotherapy response and immunoresistance of patients with liver cancer and it is anticipated to become an important strategy for individualized immunotherapy and management of patients with liver cancer [25].

### 2.3 Tumor-associated macrophages

Tumor-associated macrophages (TAMs) are immune cells closely associated with the immune response in the TME of HCC. Due to their heterogeneity and plasticity, they can be polarized into tumor-inhibiting M1 and tumor-promoting M2 macrophages in different microenvironments. M1 macrophages are induced by Th1-related cytokines interferon-γ (IFN-γ) and liposolysaccharide (LPS) and can express ROS, nitric oxide synthase (iNOS), IL-12, IL-6, IL-1β, and CXCL8/10 to exert pro-inflammatory functions, activate T cell activity,and induces an anti-tumor immune response [26, 27]. M2 macrophages are induced by Th2-related cytokines, such as IL-4, IL-13, and IL-10, with high expression of scavenger receptor (CD163) and mannose receptor (CD206), and can secrete anti-inflammatory cytokines that play an important role in tissue repair and immunosuppression [28]. M2 macrophages are the main composition of the HCC tumor immune microenvironment that promote the immune escape of HCC cells, inhibit immune response, and promote neovascularization and tumor cell invasion. The high expression of CCL2 in patients with HCC is closely related to macrophage migration and poor prognosis. Blocking the CCL2/CCR2 signaling pathway can inhibit the polarization of monocytes to TAMs, activate $CD8^+$T cells to participate in anti-tumor responses, and inhibit tumor growth [29, 30].

OPN/CSF1/CSF1R signalling plays a key role in immunosuppression and is an important cause of TAMs infiltration and immune checkpoint inhibitor treatment failure [31]. OPN can promote chemotactic migration of TAMs in HCC and polarization towards the M2 type, promote the expression of PD-L1 in HCC cells by activating the CSF1 receptor in macrophages, and stimulate the production of immunosuppressive factors. OPN can also induce macrophages

to secrete CSF1 through the PI3K/Akt/p65 signaling pathway. The combination of the CSF1R blocker PLX3397 and anti-PD-L1 can promote the polarization of M1 macrophages and change the TME of HCC by affecting the number of MDSCs and activation of T cells, thereby improving the efficacy of immune checkpoint inhibitors [31]. In the context of continuous hypoxia, necrotic fragments of HCC cells regulate the interferon (TRIF)/NF-κB signaling pathway through the adaptor of the TLR4/TIR domain, induce TAMs to release IL-1β, and promote epithelial-mesenchymal transition (EMT) and tumor cell migration [32]. IL-6 is an important proinflammatory factor that mediates the immune responses of various cells. Elevated IL-6 expression was found in the serum of patients with HCC and was associated with the expression of PD-L1 in macrophages and HCC progression. In fact, IL-6/STAT3 signaling promotes the proliferation of HCC cells and accelerates the malignant progression of tumors by regulating the kinase pathway [33]. Studies have reported that PD-L1 induces the generation of M2 macrophages through Erk/Akt/mTOR [34]. In addition, tumor-derived Wnt ligands can induce M2 polarization of macrophages through the Wnt/β-catenin signaling pathway, and inhibition of the Wnt/β-catenin signaling pathway in TAMs or Wnt signaling in HCC cells plays a key role in delaying HCC progression [35]. The ratio of CD86(+)/CD206(+) in HCC tissues was significantly correlated with advanced clinical stage, time to recurrence (TTR) and OS [36]. TAMs are an important part of the HCC immune microenvironment and can promote angiogenesis, cancer cell metastasis, immunosuppression, and maintain cancer cell stemness and drug resistance [37]. Inhibiting the recruitment of monocytes and the accumulation of TAMs in HCC, consuming TAMs in tumor tissues, reshaping the phenotype of TAMs, and improving the phagocytic ability of macrophages have become potential strategies for the treatment of HCC.

### 2.4 Natural killer cells

Natural killer(NK) cells are large granular lymphocytes that have cytotoxic effects on tumor cells and are considered the first line of defense against tumors, accounting for 30%-50% of the total number of lymphocytes in the liver [38, 39]. NK cells are critical in preventing fibrosis and warding cancer by producing various cytokines and chemokines, particularly IFN-γ. NK cells express a variety of activating receptors that transmit activation signals, and inhibitory receptors that transmit inhibitory signals. Abnormal cells regulate the secretion of cytokines, proliferation, killing, and other effector functions of NK cells by balancing these two types of receptors [40]. For example, killer cell immunoglobin-like receptor (KIR) and C-type lectin-like receptor NKG2A inhibitory receptor expressed by NK cells are bound to the major histocompatibility complex MHC-I(MHC-I) and the non-classical MHC-I complex HLA-E on normal hepatocytes to avoid damage to normal hepatocytes. However, tumor cells can escape the killing effect of $CD8^+$T lymphocytes by down-regulating MHC-class I molecules [41, 42]. Compared to NK cells from healthy individuals, NK cells from the peripheral blood of HCC patients express high levels of T-cell immunoglobulin mucin-3(TIM-3). TIM-3 promotes NK cell maturation by binding to its ligand galectin 9(GAL-9), which helps tumor cells evade immune monitoring [43, 44]. When TIM-3 continues to be highly expressed, PI3K/mTORC1/p-S6 signaling can be blocked to inhibit NK cell secretion of IFN-γ and TNF-α, resulting in dysfunction [45].

Studies have found that high expression of circUHRF1 in HCC tissues and HCC-derived exosomes is significantly correlated with poor prognosis, and circUHRF1 can mediate miR-449c-5p to stimulate TIM-3 upregulation, thereby inducing NK cell exhaustion [46]. The CD48 protein expressed by monocytes and macrophages in the HCC microenvironment interacts with 2B4 receptors on circulating NK cells to trigger their depletion and death of NK cells [47]. CD96 and TIGIT are immune checkpoints of the same immunoglobulin superfamily, which are mainly responsible for transducing inhibitory signals and compete with the activator receptor DNAM-1 to bind CD155 and inhibit the function of NK cells [48]. Blocking the interaction between CD96, TIGIT, and CD155 can restore the cytotoxicity of NK cells and reverse their depletion of NK cells in vitro [49, 50]. CAR-NK cells have been used to improve the targeting and efficacy of NK cells, making them a new choice for adoptive therapy. GPC3-CAR-NK-92 and CD147-CAR-NK cells have been shown to be effective in killing HCC cells [51, 52].

With the progression of HCC, the expression of the intrahepatic anti-inflammatory factors IL-10 and TGF-β is upregulated, which inhibits immune cell-mediated immunoanti-inflammatory functions. The enhanced expression of hypoxia-induced gene 2(HIG2) in the HCC microenvironment can stimulate the upregulation of IL-10 expression, activate the STAT3 pathway in NK cells, inhibit the killing function of NK cells, and promote HCC recurrence and metastasis [53]. Blocking IL-10 can specifically inhibit the expression of NKG2A in NK cells, indicating that the depletion of NK cells can be reversed by targeting IL-10 to restore immune monitoring function [54]. When cytokine-induced Sh2-containing protein (CISH) is removed from NK cells for metabolic reprogramming, the JAK-STAT signaling activity mediated by IL-15 can be upregulated, and the proliferation, survival, and cytotoxicity of NK cells can be stimulated [55]. IL-21 can

activate STAT1 and PI3K-Akt-Foxo1 signaling pathways to reverse TIM-3 and PD-1-mediated NK cell depletion and work with IL-15 to promote IFN-γ production and NK cell proliferation [56]. Unlike other immune cells, NK cells can recognize and spontaneously exercise the ability to kill abnormal cells without excessive sensitization or activation time and have no MHC restriction and other characteristics, showing advantages in terms of functionality and safety. Based on their combination with NK cells, they have great potential for HCC treatment.

### 2.5 Dendritic cells

Dendritic cells (DCs), which are highly specialized APCs, are an important bridge between innate and adaptive immunity. They can efficiently uptake, process, and present antigens. DCs in healthy livers are mostly in an immature state, with strong migration and antigen endocytic abilities. DCs can effectively activate unsensitized initial T-cells after maturation by capturing antigens, which is the central link in regulating and maintaining the immune response. DCs are usually divided into myeloid dendritic cells (cDCs), plasma cell-like dendritic cells (pDCs), and monocyte-derived dendritic cells (mo-DCs). cDCs can be further divided into cDC1 and cDC2 according to the pathway of differentiation and development. Specifically, cDC1 cells are associated with the cross-presentation of $CD8^+$T cells and mediate the polarization of $CD4^+$T cells to the Th1 phenotype, whereas cDC2 accumulates in tumors and can activate $CD4^+$T cells and $CD8^+$T cells to present specific antigens[57, 58]. DCs present antigens on MHC class II and MHC Class I molecules to $CD4^+$T and $CD8^+$T cells, respectively, and CD80/CD86 on DCs interacts with CD28 on the surface of T cells to transmit co-stimulatory signals, inducing T cells to present antigens[59]. DCs are important for activating the immune system of the body to resist cancer invasion. In most solid tumors, DCs absorb tumor antigens from infiltrated tissues and migrate to lymph nodes to stimulate $CD8^+$T cells. Therefore, the more DCs infiltrate, the better the prognosis of the patients.

DCs in tumor tissues express ligands of checkpoint pathways such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) or TIM-3, which induce Treg cell differentiation. The CTL response is inhibited by the immunosuppressive factor IL-10, thereby improving immune tolerance [59]. TGF-β, IL-10, and vascular endothelial growth factor secreted by HCC can induce the immaturity of DCs. When the expression of TGF-β and IL-10 receptors is downregulated, $CD8^+$T cells cannot be cross-activated, which reduces the cytotoxicity of T cells to tumor cells. MDSCs in the TME of HCC that affect DCs maturation, antigen uptake, and migration ability, and CAFs upregulate DCs ability of DCs to express PD-L1 in the microenvironment by activating the IL-6/STAT3 signaling pathway, thereby forming an inhibitory TME [59]. When exposed to appropriate antigenic stimulation, immature DCs differentiated into mature DCs, MHC class I and Class II molecules, co-stimulatory molecules were upregulated, and endocytosis activity decreased. Mature DCs exhibit high levels of antigen-MHC complexes, which transmit major stimulatory signals to T cells, and express high levels of the chemokine receptor CCR7 and co-stimulatory molecule CD40, which secrete cytokines necessary for T cell activation, thereby enhancing the activity of cytotoxic T cells [60, 61].

DCs, as key activators of adaptive immune responses, play a central role in initiating antigen-specific immunity and inducing anti-tumor immune responses and can help rebuild the immune surveillance function of tumor cells. Current DC-based vaccines are expected to reduce tumor growth and induce immune memory by inducing tumor-specific cellular and humoral immunity, restoring effective anti-tumor responses, and enhancing the dialogue between DCs and $CD8^+$T cells. DC vaccine preparation is mainly achieved through the following two methods: DCs derived from monocytes are isolated and cultured in vitro, loaded with tumor-related antigens, and then activated DCs are transfused back into the host to trigger the antitumor response of patients, and the other is to directly induce DCs to take up tumor antigens in vivo. Several clinical studies have demonstrated that DC-based vaccines are safe and tolerable in inducing the expansion of circulating tumor-specific $CD4^+$T cells and $CD8^+$T cells, and can induce a strong anti-tumor immune response in the body. In a HCC mouse model, compared with monotherapy, the combination of DC vaccine and PD-1 inhibitor showed longer OS, induced tumor cell apoptosis, prolonged recurrence time, and reduced tumor volume [62]. In addition, DC vaccines combined with CIK can change the immune balance, delay tumor progression, and improve patient prognosis [63].

### 2.6 Cytotoxic T lymphocytes

Cytotoxic T lymphocytes (CTL) is the "main force" that kills tumor cells in HCC, mainly $CD8^+$T cells. CTL can secrete the key factor of anti-tumor immunity, IFN-γ, to promote the antigen presentation of tumor cells, directly kill tumor cells through the release of perforin and granase, or mediate cell apoptosis through FASL-FAS. There are many immunomodulatory cells in the microenvironment of liver cancer tissues, and the heterogeneity of these cells regulates the fate of liver cancer

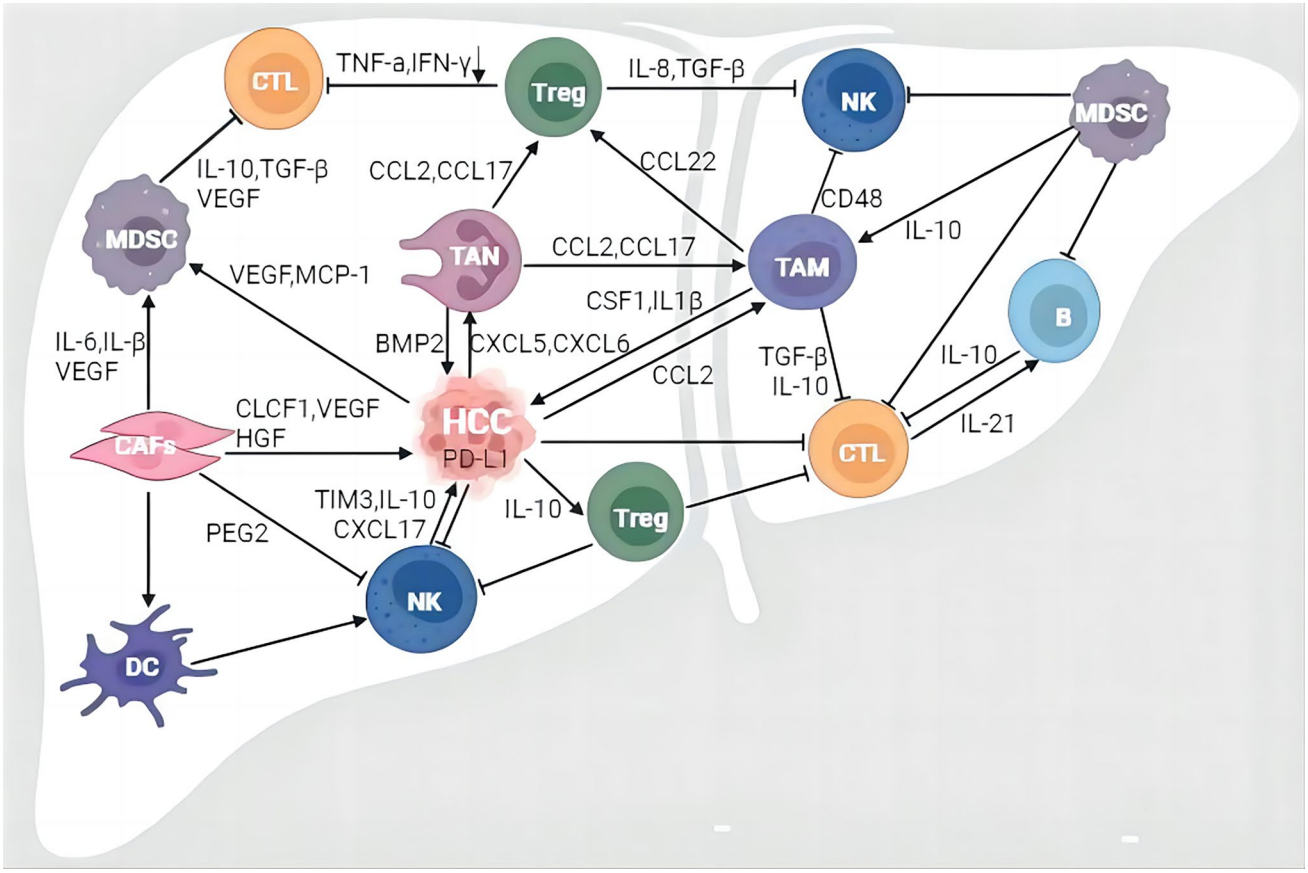


**Fig. 3** Diagram of immune cells and tumor cells forming an immunosuppressive environment in HCC. HCC cells secrete CXCL5, CXCL6 and CXCL8 to recruit TANs into tumor stroma, and produce IL-10 and VEGF to inhibit CTL and NK cells to kill tumor cells. CAFs secrete IL-6, TGF-β, VEGF, HGF and CLCF1, responsible for neutrophil recruitment and N2 polarization, and play a pro-inflammatory and pro-tumor role. The crosstalk between MDSCs and TAMs leads to increased secretion of IL-10 and decreased secretion of IL-6 and IL-12, which weakens the function of CTL and the toxicity of NK cells. Treg reduces the secretion of TNF-α and IFN-γ, thus inhibiting the activation of CTL, and inhibits the anti-tumor of NK cells by producing IL-2, IL-8 and TGF-β. DC reduced the secretion of IL-10 and IL-12 and inhibited the anti-tumor response of CTL. The immunosuppressive environment can lead to the differentiation of Treg and induce the polarization of TAMs into M2 macrophages, and the expression of CSF1 by TAMs leads to the expression of PD-L1 by HCC cells. In addition, the release of IL-10 and TGF-β by TAMs led to the inhibition of T cells and NK cells, and promoted the differentiation and drug resistance of TREGs. VEGF: Vascular endothelial growth factor; IFN-γ: Interferon-γ; TNF: Tumor necrosis factor; CSF1: Macrophage colony stimulating factor 1; CLCF1: Cardiac trophic factor-like cytokine 1; MCP-1: Monocyte chemoattractant protein-1; BMP2: Bone morphogenetic protein 2; HGF: Liver cancer growth factor

occurrence and development (Fig. 3). Co-suppressor molecules, such as PD-1, TIM-3, CTLA-4, Lymphocyte-activation gene 3(LAG-3), and their ligands have been shown to be key regulators of CTL depletion [64, 65]. The presence of hypoxia, amino acid deficiency, and lactic acid accumulation in the HCC microenvironment, as well as the high expression of immunoregulatory molecules such as IL-10, CXCL17, vascular endothelial growth factor (VEGF), and indoleamine 2, 3-diadoxidase (IDO), leads to limited tumor-associated antigen-specific response and CTL dysfunction [65–68]. One study highlighted that upregulation of PD-1 and LAG-3 can make tumor antigens tolerant and suppress the immune system, leading to progressive depletion of CTL [69]. Significant infiltration of $CD8^+CXCR5^+$ T cells in local HCC tissues implies a good prognosis, and T cells with $CD8^+CXCR5^+$ receptors can produce IL-21 and promote B cell differentiation to promote humoral immunity in HCC [70]. These results suggest that CTL depletion plays an important role in the survival of liver cancer and that the spatial heterogeneity of CTL in the liver cancer microenvironment affects the immunotherapy effect of HCC.

### 2.7 Regulatory T cells

Regulatory T cells (Tregs) belong to the immunosuppressive $CD4^+$T cell subgroup and express high levels of CD25, mainly via the Foxp3 transcription factor. Tregs can inhibit the production of perforin, degranulation, and granase in $CD8^+$T cells,

thereby damaging the effector function of $CD8^+$T cells and mediating the killing function of tumor cells through various contact-dependent and non-contact mechanisms. Granulocytes have been reported to mediate the differentiation of $CD4^+$T cells into Tregs by activating Toll receptor signaling pathways, and macrophage-derived CCL22 can promote Treg recruitment [71]. When the expression of CCL20 and its receptor CCR6, which recruits Tregs, is increased in HCC, the activity of Tregs can be increased through TCR contact with IL-10 and TGF-β signals, thereby promoting the escape and metastasis of tumor cells [72]. The high secretion of IL-10 in HCC tissues induces an increase in STAT5 phosphorylation levels and upregulation of JAK1 expression, thus enhancing the stability of Tregs [73]. The number of Tregs in peripheral blood and tumor tissues of HCC patients is higher than that in healthy individuals, and sorafenib can reduce the degree of Treg invasion by inhibiting TGF-β signaling [14, 74]. Overexpression of LCC-epidermal growth factor receptor (LCC-EGFR) in Tregs can induce the activation of activator protein 1(AP-1)/ nuclear factor of activated T cells 1 (NFAT1) in Tregs. AP-1/ NFAT1 axis of nuclear factors to block their ubiquitination, further promoting immunosuppression.

### 2.8 Infiltrating B cells

B cells dominate humoral immunity, promoting their activation by presenting tumor-associated antigens to T cells, producing antibodies to promote the uptake of tumor antigens by TAMs and DCs, and secreting IFN-γ and other key factors of anti-tumor immunity to promote anti-tumor immunity or directly kill tumor cells. There are a large number of B cells with low/activity phenotype of FcγRII in HCC tissues, which are mainly activated by the CD95L pathway stimulated by monocytes and inhibit the immunity of autologous tumor-specific cytotoxic T cells through IL-10 signaling, leading to early recurrence after tumor resection [75]. In the tumor margin area, the number of $CD20^+$B cells is closely related to tumor size, degree of vascular invasion, number of $CD8^+$T cells, and prognosis [76]. In the HCC mouse (Hepal-6) model, removal of B cells with a CD20 antibody resulted in reduced activation of $CD4^+$T cells and enhanced expression of PD-1 on $CD8^+$T cells, thus promoting tumor growth [77]. However, in another inflammation-associated HCC mouse model (Mdr2−/−), the removal of B cells with a CD20 antibody inhibited the TNF-α/NF-κB pro-cancer pathway and inhibited the occurrence and progression of HCC [78]. These studies suggest that B cells are involved in HCC progression, including immunosuppressive regulatory B cells (Bregs). It has been found in co-culture studies of Bregs and HCC cells that inhibition of the CD40/CD40L axis leads to decreased secretion of TGF-β1 and IL-10 and increased secretion of TNF-α, indicating that Bregs promote the production of an anti-inflammatory environment[79]. The major effect of immune cells and the cellular molecules is indicated in Table 1.

### 2.9 Non-immune cell

Hepatic stellate cell (HSC), cancer-associated fibroblast (CAF), and liver sinusoidal endothelial cell (LSEC) of non-bone marrow origin are significant non-immune cells in the tumor microenvironment (TME). Besides providing a scaffold structure in normal liver tissue, they are also essential components in the construction of the HCC immune environment. As an important constituent of stromal cells in the HCC microenvironment, HSCs can secrete extracellular matrix proteins such as type I collagen and type III collagen, and their activation contributes to liver fibrosis and HCC development [93]. HSCs can secrete various signaling molecules, such as cytokines (IL-6, IL-1B, CCL5, CCL21) and growth factors (TGF-α and TGF-β), which lead to HCC cells escape immune surveillance by recruiting Treg cells and activating endothelial cells[94]. Studies have indicated that the up-regulation of the Stathmin 1 (STMN1) gene can promote the activation of HSCs and acquire the CAFs phenotype, and blocking the complex signal transduction between HSCs and HCC mediated by STMN1 might be a potential target for HCC treatment [95].

CAFs as an important component of the tumor matrix, can inhibit the cytotoxicity and cytokine production of NK cells by releasing immunosuppressive prostaglandin E2(PGE2) and dioxygenase, thus forming an immune-tolerant environment for HCC development [96]. CAFs recruit circulating MDSCs by secreting CCL2, enabling them to recognize and bind to CCL2 receptors, thereby suppressing the immune response [97]. Additionally, CAFs induce the formation of an inhibitory immune microenvironment by secreting IL-6 to recruit neutrophils expressing PD-L1, and IL-6 can also promote the accumulation of DCs by activating signal transduction and transcriptional activator 3 and inhibit the anti-tumor role of related T cells [98].

LSECs of non-bone marrow origin play a pivotal role in maintaining liver function. As APCs, they have the ability to cross-activate effector T cells and can regulate the immune response by releasing cytokines. LSECs is involved in angiogenesis, coagulation promotion, and fibrinolysis during tumor development. Its fenestrum structure, hyperendocytosis clearance, and paracrine functions play an indispensable role in liver pathology [99].

**Table 1** Summary of molecular targets of HCC in the tumor immune microenvironment

| Immune cell | Molecule(s) | Major effects | Reference(s) |
|---|---|---|---|
| TANs | TGF-β/Axl/CXCL5 | The synergistic effect of TGF-β and Axl induces CXCL5 secretion and increases the number of TANs infiltrated in HCC tissues | [12] |
| TANs | CCL2,CCL17 | Increased migration activity of Tregs and macrophages was associated with tumor prognosis | [14] |
| TANs | CXCR2/CXCL1 | The CXCR2-CXCL1 axis can regulate neutrophil infiltration into HCC tumor tissues | [80] |
| TANs | miR-301b-3p | High secretion of CXCL5 and recruitment of TANs | [15, 16] |
| TANs | HIF-1α | TANs infiltration associated with HCC progression | [81] |
| CAFs | miRNA-21 | High level of serum exosomal miRNA-21 was correlated with greater activation of CAFs and higher vessel density in HCC patients | [82] |
| CAFs | IL-6/STAT3/PD-L1 | The survival and function of TANs in HCC are affected by the IL-6/STAT3/PD-L1 pathway | [18] |
| CAFs | IL-6/STAT3 | Promote stem-like properties of HCC cells by enhancing STAT3/Notch signaling pathway | [83] |
| MDSCs | CCL26 | Mediates MDSC recruitment in HCC hypoxic region | [66] |
| MDSCs | ENTPD2 | Converting extracellular ATP to 5′-AMP blocks the differentiation of MDSCs in HCC | [84] |
| MDSCs | CCL9/CCR1 | MDSCs were recruited to the spleen | [85] |
| MDSCs | CCRK | Enhanced Zeste congener 2 enzyme activates NF-κB, leading to PMN-MDSC accumulation and inhibiting T cell activation | [23] |
| MDSCs | C5AR | Recruit MDSCs to TIME | [86] |
| TAMs | IL-6,IL-23,TNF-α | Expansion of IL-17-producing $CD4^+$ Th17 cells | [26, 27] |
| TAMs | TGF-β | Promote the expression of TIM-3 in TAMs | [87] |
| Immune cell | Molecule(s) | Major effects | Reference(s) |
| TAMs | IL-1β | Promote immune evasion of EMT and HCC | [32] |
| TAMs | CCR2 | Inhibit the recruitment of inflammatory monocytes and promote the EMT transition of TAMs | [30] |
| TAMs | CSF-1 | Reprogram the polarization of TAMs | [66] |
| TAMs | IL-6/STAT3 | Promote the proliferation of HCC cells and accelerate the malignant progression of tumor | [33] |
| TAMs | CCL2/CCR2 | Blocking CCL2/CCR2 signaling pathway inhibits TAMs polarization and activates $CD8^+$T cells to participate in anti-tumor response | [29, 30] |
| CTL | PD-1,TIM-3, CTLA-4,LAG-3 | Co-suppressor molecules and their ligands are key regulators of CTL depletion | [64, 65] |
| CTL | VEGF,CXCL17, IL-10,IDO | The tumor associated antigen specific response is limited and CTL function is dysfunctional | [65–68] |
| Tregs | CTLA-4 | Tumor-induced regulatory DC subsets suppress anti-tumor immune responses through CTLA-4 dependent IL-10 and IDO production | [88] |
| Tregs | TIM-3 | Antibodies against TIM3 can restore the immune response of HCC-derived T cells to tumor-specific antigens | [89] |
| Tregs | GITR | Enhanced TIL function in HCC | [90] |
| Tregs | OX40 | OX40 expression in HCC is associated with a unique immune microenvironment, specific mutational characteristics, and poor prognosis | [91] |
| Tregs | LAG3 | Antibodies against LAG3 can restore the immune response of HCC-derived T cells to tumor-specific antigens | [89] |
| Tregs | AP-1,NFAT1 | Promotes immunosuppression in HCC | [92] |

The spatial heterogeneity of a tumor is composed of a variety of cells, each of which plays a different role in the development of the tumor. Studies have shown that spatial heterogeneity of tumor affects immunotherapy [100–103], and tumor spatial heterogeneity of liver cancer leads to tolerance of immunotherapy [104]. These results suggest that tumor spatial heterogeneity plays an important role in inhibiting immunotherapy, so exploring the cell subtypes of tumor spatial heterogeneity has important scientific significance for understanding tumor immunotherapy tolerance. The treatment of immune checkpoints combined with targeting certain specific cell subtypes of tumor spatial heterogeneity is a novel strategy to enhance immunotherapy of cancer.

## 3 Adoptive cell therapy(cellular immunotherapy) for liver cancer

Adoptive cell therapy (ACT) activates and expands the host lymphocytes in vitro through gene modification and then injects them back into the host body to directly kill tumor cells or stimulate the immune response to suppress the tumor. Advanced acts include chimeric antigen receptor T cells (CAR-T), T-cell receptor-engineered T cells(TCR-T), tumor-infiltrating lymphocytes(TILs), and cytokine-induced killer cells(CIK) (Fig. 4).

### 3.1 Chimeric antigen receptor T cell therapy

The key to CAR-T therapy is to modify T cells using genetic engineering technology, introduce CARs into T cells through viruses or plasmids, and induce T cells to express tumor-associated antigens (TAAs) and chimeric antigen receptors that can activate T cells. In the absence of MHC restriction, T cells specifically kill the recognized tumor cells, and a long-lasting anti-tumor immune response is generated [105, 106]. CAR-T therapy has achieved good efficacy in hematological tumors, but clinical trials in solid tumors, especially HCC, face many challenges due to the lack of ideal specific surface antigens, cytokine release syndrome (CRS), tumor load, CAR-T cell transport, and infiltration efficiency [107–109]. According to previous studies, the secretion of IL-7 and CCL19 improved the survival and infiltration of CAR-T cells in mice and enhanced cell proliferation and migration [110]. Phosphatidyl inositide proteoglycan 3(GPC3) is a carcinomatoglycan expressed on HCC cell membranes, which is usually very low or not expressed in normal adult tissues, but the expression

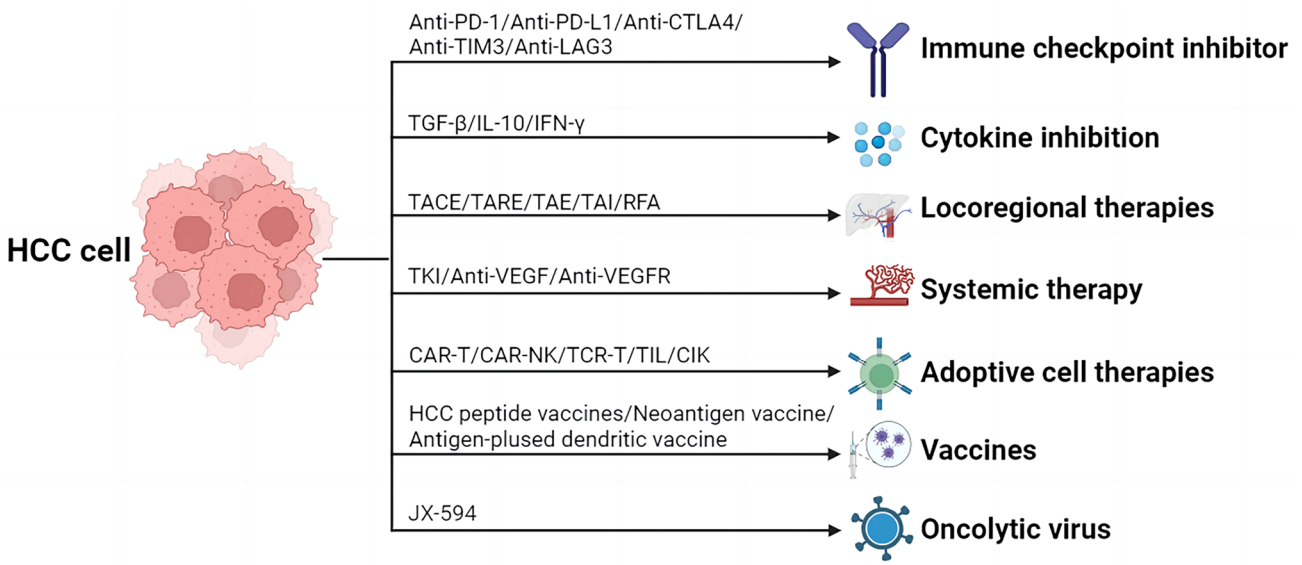


**Fig. 4** Schematic diagram of treatment options for HCC. Surgical resection, local ablation and liver transplantation are the most effective treatments for liver cancer, suitable for patients with early stage liver cancer, while local, systemic therapy and immunotherapy have become the most important treatments for unresectable liver cancer. ICIs in combination with local therapy, targeted anti-angiogenic drugs, tyrosine kinase inhibitors, adoptive cell therapy, tumor vaccines, oncolytic viruses, and dual immunotherapy as first-line treatment can provide patients with appropriate individualized options. TACE: Transarterial chemoembolization; TARE: Transarterial radiation embolization; TAE: Transcatheter arterial embolization; TAI: Transcatheter arterial perfusion chemotherapy; RFA: Radiofrequency ablation; TKI: Tyrosine kinase inhibitor; CAR-T: Chimeric antigen receptor T cells; CAR-NK: Chimeric antigen receptor NK cells; TCR-T: T cell receptor engineered T cells; TIL: Tumor infiltrating lymphocytes; CIK: Cytokine induced killer cell

level of GPC3 in HCC tissues increases with an increase in HCC malignancy and is associated with poor prognosis of patients. Clinical data from GPC3-targeting CAR-T cell therapy presented at the American Society of Clinical Oncology showed that Ori-CAR-001 demonstrated good safety and efficacy in GPC3-positive relapsed/refractory patients, with an objective response rate (ORR) of 44% and disease control rate (DCR) of 78%. In the world's first clinical trial of CAR-T cell therapy targeting GPC3 protein for advanced HCC, two patients showed significant efficacy with local combined with CAR-T cells targeting GPC3, achieving long-term tumor-free survival and normal tumor marker levels, providing new hope for the therapeutics of patients with advanced HCC [111]. Inhibitory receptors on the surface of T cells, such as PD-1, CTLA-4, and LAG-3, are key obstacles to treatment, and CRISPR-Cas9 knocks out the genes that code for these surface receptors; for example, blocking the expression of PD-1 in GPC3-CAR-T cells can improve the anti-tumor activity, invasiveness, and persistence of CAR-T cells [112, 113]. GPC3-CAR-T cell therapy combined with sorafenib enhanced its anti-tumor effect in a mouse model, with encouraging results [114]. GPC3-CAR-T cells co-expressing IL-15 and IL-21 have good proliferative ability and anti-tumor activity and increase the number of T memory stem cells [115]. In addition, alpha-fetoprotein(AFP) can be used as an ideal target for CAR-T therapy, which has advantages over conventional CARs and AFP-CAR binding to peptide MHC complexes and intracellular antigens. In a HepG2 NOD/SCID mouse model, intravenous injection of AFP-CAR-T cells rapidly inhibited tumor cell proliferation [116]. At the same time, AFP-CAR-T cells showed strong anti-tumor activity in an established intra-abdominal liver cancer xenotransplantation model [117]. However, at present, there are few clinical application studies on AFP, so how to make AFP-CAR-T cells more accurate and widely used is a problem worth considering.

### 3.2 T cell receptor engineered T(TCR-T) cell therapy

TCR-T cell therapy extracts the TCR α chain and TCR β chain, which determine the antigen specificity of T cells, from effector T cells induced by tumor antigen, and introduces them into mature T cells, so that the modified T cells have tumor targeting and specificity, and can effectively recognize and kill tumor cells [118, 119]. TCR combines with CD3 to form a complex with non-covalent bonds, carries out antigen recognition in an MHC-dependent manner, and activates T cells to perform tumor monitoring by triggering a signaling cascade [119, 120]. In a Phase I clinical trial, eight patients with advanced HBV-HCC who were not eligible for liver transplantation had reduced or stable circulating HBsAg and HBV DNA levels in most patients after treatment with TCR-T cells, which were safe and well tolerated without immunosuppressive intervention [121]. HBV was highly expressed in both normal HBV-infected hepatocytes and HBV-DNA-integrated HCC cells. Affinity-modified TCR-engineered T cells (Ai-TCR-T) retain homologous HBV antigen specificity and can recognize multiple HBV genotypic variants with higher sensitivity and cytotoxicity in xenografted mouse models. Thus, HCC can be targeted more effectively [122]. The infusion dose can be gradually increased owing to the transient nature of the mRNA. In 8 patients with chronic HBV infection and diffuse inoperable HBV-HCC, weekly incremental doses of HBV-TCR-encoded mRNA modified T cell therapy were well tolerated, with no severe systemic inflammation or neurotoxicity. Long-term clinical benefits associated with transient immune changes have also been observed in patients with HBV-HCC [123]. AFP158-specific TCR-T cells can kill HLA-$A2^{+}AFP^{+}$ HepG2 tumor cells but have no significant toxicity to normal primary hepatocytes in vitro. Human T cells can be redirected to specifically recognize and kill HCC cells [124]. Other studies have reported that the construction of AFP158-166 specific TCR-T cells can promote the secretion of IFN-γ in tumor-bearing NOD/SCID mice, significantly increasing the specific cytotoxicity of HpeG2 [124]. In patients with solid cancer, autologous T cells using CRISPR non-viral knockout and knock-in editing techniques can generate individualized T cells with good applicability and specificity, avoid T cell exhaustion, and improve the feasibility and safety of TCR-T cell therapy [125].

### 3.3 Tumor infiltrating lymphocyte therapy

Tumor infiltrating lymphocyte**(**TIL) therapy involves obtaining infiltrating lymphocytes from tumor samples, adding the cell stimulating factor IL-2 in vitro for activation and expansion, and then injecting them back into the patient for anti-tumor therapy [126–128]. TILs are a heterogeneous group of lymphocytes composed of $CD4^{+}$ T lymphocytes, $CD8^{+}$T lymphocytes, B cells, and NK cells, among which $CD8^{+}$T lymphocytes are the most important CTL in the liver, which can promote the production of pro-inflammatory cytokines and plasma cells. The Fas/FasL, TNF-TNFR, and perforin/granase pathways induce tumor cell apoptosis and play a role in tumor killing [129–131]. The degree of TILs infiltration in HCC is significantly correlated with tumor progression and survival. Patients with HCC with significant lymphocyte infiltration have a reduced recurrence rate and an increased survival rate after resection [132, 133]. TIL therapy has unique advantages in the treatment of solid tumors owing to its diverse TCR cloning, superior tumor-homing ability, and low

off-target toxicity [134]. However, the successful application of TIL therapy is limited to a few tumors. Compared to other adoptive cell therapies, TIL may be superior in targeting tumor heterogeneity and exhibit better clinical efficacy than CAR-T in high mutation-loaded solid tumors, including melanoma [135, 136]. In a Phase I clinical trial, 15 HCC patients were treated with autologous tumor-infiltrating lymphocytes after tumor resection and adoptive immunotherapy, and after a median follow-up of 14 months, 15 patients (100%) were alive, 12 patients (80%) had no signs of disease, and the time to diagnosis of tumor recurrence after treatment ranged from 105 to 261 days [137]. These results preliminarily demonstrated the safety and efficacy of immunotherapy with activated and amplified autoTILs. However, further optimization of TIL isolation, purification, and in vitro amplification methods, and selection of more reasonable target genes are still problems that need to be solved.

### 3.4 Cytokine induced killer(CIK) cell therapy

CIK therapy involves isolation of human peripheral blood mononuclear cells (PBMC) after in vitro stimulation with antiCD3 antibodies, IFN-γ, IL-2, and other cytokines to obtain T cells ($CD3^+CD56^-$), NK cells ($CD3^-CD56^+$), and NKT cells ($CD3^+CD56^+$). The population is composed of heterogeneous lymphocyte populations and is injected back into the body. CIK cells have non-MHC-restricted cytotoxicity and antitumor activity, proliferate rapidly in vitro, and have little effect on normal cells. CIK therapy mainly exerts its anti-tumor effect by directly killing tumor cells or by releasing inflammatory cytokines and inducing apoptosis of tumor cells and has been widely used in clinical practice as a new generation of anti-tumor adoptive cellular immunotherapy. The interaction between the activated receptor NKG2D on CIK cells and the corresponding NKG2D ligand on HCC cells can effectively kill tumor stem cells and improve the survival time of patients with HCC. In a randomized Phase II study, patients with HCC ineligible for surgery experienced significant benefits after CIK treatment, with an overall extension of progression-free survival [138]. After adjuvant immunotherapy in 230 patients with HCC treated with surgical resection, radiofrequency ablation, or percutaneous ethanol injection, the median RFS was 44 months in the activated CIK immunotherapy group and 30 months in the control group. The risk of all-cause and cancer-related deaths was lower in the treatment group than in the control group, and the proportion of patients with serious adverse events did not differ significantly between the two groups [133]. A total of 162 patients (89 in the immunotherapy group and 73 in the control group) were followed up for 60 months, with recurrence-free survival of 44.8% in the immunotherapy group and 33.1% in the control group, and the risk of recurrence and death was significantly reduced in the immunotherapy group. In patients receiving radical treatment for HCC, adjuvant CIK cell immunotherapy resulted in significant improvements in RFS and OS, lasting more than 5 years [139]. In a clinical study, 58 HCC patients received RFA + TACE therapy (RFA + TACE group) and 58 patients received RFA + TACE + CIK cell immunotherapy (RFA + TACE + CIK group), and AFP levels in both groups were significantly lower after treatment than before treatment. The overall 5 year survival rates are 13.8 and 29.3%, respectively [140]. These results indicate that RFA and TACE combined with CIK cell reinfusion can reduce the recurrence rate of tumors, improve the postoperative quality of life and immune status of patients, and extend the progression-free survival. $^{125}$I radioactive seed implantation upregulates the expression of MHCI chain-related gene A in HCC cells and enhances cytokine-induced killer cell-mediated apoptosis by activating caspase-3. $^{125}$I radioactive seed implantation combined with CIK treatment significantly inhibited the growth of BALB/c nude mouse tumor cells. Moreover, the survival time of BALB/c nude mice was improved by the mutual promotion of antitumor immunity [141]. Silencing the expression level of PD-1 in CIK cells with synthetic small interfering RNA (siRNA) can target HCC cells and enhance the effect of immunotherapy, reducing tumor cell activity and tumor volume in mice with HCC [142]. Complete response (CR) can be achieved after CIK immunotherapy by chemically coupling antibodies targeting cancer-associated antigens mucin 1(muc1) or glypican 3(GPC3) with T cell-recognizing CD3 antibodies and anti-PD-1 antibody therapy [143]. CIK combination therapy may be a viable strategy for the targeted treatment of HCC, but more clinical trials are needed to demonstrate the true effectiveness of relevant CIK treatments.

## 4 Immunotherapy of liver cancer of vaccine and oncolytic virus

Cancer vaccines exert a specific antitumor response by recognizing tumor-associated antigen(TAA) and tumor-specific antigen(TSA)-presenting cells. With the identification of an increasing number of tumor-associated antigens, some HCC peptide vaccines targeting TAAs have been identified as immunotherapeutic targets for HCC vaccines, including AFP, human telomerase reverse transcriptase(hTERT), and GPC3 [144]. In a prospective study of 15 patients with HCC, the AFP polypeptide vaccine produced T cells whose receptors reacted to the peptide, resulting in a complete response in one patient and a

slowdown in tumor growth in eight patients with no adverse events [145]. The DEX vaccine, a bionanobasic vaccine targeting HCC-targeted peptide(P47-P) and AFP epitope(AFP212-A2), was found to participate in host innate and adaptive immunity and induce an effective antitumor immune response, eradicating tumors in tumor-carrying in situ HCC mice. It can also provide long-term protective immune memory to resist re-attack by the tumor [146]. In a randomized Phase I clinical trial, 33 patients with advanced HCC received GPC3 peptide vaccination, of which 1 patient experienced a partial response, 19 patients were stable 2 months after initiation of treatment, and 30 patients had induced GPC3-specific CTL responses [147]. In addition, patients with a high GPC3-specific CTL frequency had significantly longer OS than those with a low frequency. In a phase II study of 41 patients with primary HCC who underwent surgical resection or radiofrequency ablation, patients treated with surgery plus the GPC3 peptide vaccine had a lower recurrence rate (24% vs. 48% at one year, respectively) [148]. XCL1 chemokines were linked to GPC3 to construct XCL1-GPC3 fusion molecules as liver cancer vaccines. In a mouse in situ liver cancer model, XCL1-GPC3 targeting DC enhances the proliferation of antigen-specific $CD8^+$T cells, induced the production of GPC3-specific $CD8^+$T cells, and increased the infiltration of activated NK and NKT cells. In addition, tumor formation and growth in immunized mice were significantly inhibited, which could further enhance the anti-tumor effect of anti-PD-1 [149]. HepaVac-101, the first human clinical vaccine trial against HCC, employs multiple novel HLA and Class II-restricted TAAs that bind a polypeptide antigen (IMA970A) to a TLR7/8/RIG I agonist, CV8102 [150]. These results showed that the vaccine was safe and induced a TAA-specific immune response. Tumor-targeting lipid-dendritic calcium phosphate (TT-LDCP) nanoparticles(NPs) designed with thymine-functionalized dendritic macromolecules can deliver siRNA targeting the immune checkpoint ligand PD-L1 and immunostimulating IL-2 encoding plasmid DNA to HCC, increasing tumor invasion and $CD8^+$T cell activation. Enhancing the efficacy of cancer vaccine immunotherapy [151]. This approach selectively targets and reprograms the immunosuppressive TME to improve cancer immunotherapy, selectively replicates tumor cells, kills tumor cells in a targeted manner without damaging normal cells, leading to the release of soluble antigens, danger signals, and type I interferon, and promotes the invasion of antigen-specific CTL cells into the tumor. It can also induce long-term effective antitumor immunity [152]. These results show that tumor vaccines can change the spatial heterogeneity of T cells in the microenvironment of liver cancer, inhibit the TME of liver cancer, and enhance the aggressiveness of T cells toward liver cancer cells.

JX-594 is an engineered oncolytic virus (OVs) that disrupts the viral thymidine kinase (TK) gene for cancer selectivity and inserts human granulocyte–macrophage colony-stimulating factor (hGM-CSF) and beta-galactosidase transgenes for immune stimulation and replication evaluation. The results of a phase II clinical trial showed that overall survival (OS) was significantly correlated with dose (14.1 and 6.7 months in the high-dose and low-dose groups, respectively), and that the median OS was longer in the high-dose group than in the low-dose group [153]. Ovs for cancer are a good idea and show promising therapeutic effects [154–156]. OVs have the potential to influence the heterogeneity of liver cancer cells and can be used to treat liver cancer [157]. Although OVs in HCC show promising clinical applications, further clinical trials are needed to confirm these therapeutic effects.

Single cells have unique functional characteristics in the microenvironment of liver cancer. In the cellular composition of spatial heterogeneity of liver cancer, the dynamic changes of single cells affect immune escape and immunotherapy of liver cancer [158]. It has been found that the depletion characteristics of T cells in liver cancer tissues and the immune landscape could predict the prognosis of HCC [159], suggesting that the immune landscape plays an important role in the treatment of liver cancer. Single-cell RNA sequencing can accurately peer into the immunosuppressive landscape of liver cancer [58, 160], which can promote the exploration of immunotherapy for liver cancer.ICIs have revolutionized cancer therapy and are gaining increasing interest in the treatment of HCC. Atezolizumab and bevacizumab combined with sorafenib improved overall survival, leading to U.S. Food and Drug Administration (FDA) approval as a first-line treatment for patients with advanced HCC. Despite these significant advances, there is a need to better understand the cellular and molecular characteristics of the tumor microenvironment and its role in the development and progression of HCC [161–163]. However, other immune-targeting strategies, such as adoptive T cell transfer, vaccination, and viral therapy, are currently being developed. The immune microenvironment of liver cancer, different cellular composition, currently available immunotherapy approaches and potential immunotherapy modalities are the directions of immunotherapy for HCC.

## 5 Conclusions and prospects

The TME plays an important role in patient prognosis and immunotherapy responses. An in-depth and comprehensive analysis of the cellular spatial heterogeneity of the HCC immune microenvironment and its dynamic changes during treatment can better guide clinical treatment and achieve precision medicine. Although the expression of

AFP, a specific marker for liver cancer, is closely related to the spatial heterogeneity of HCC tissue and also affects the immunotherapy of HCC [164, 165], AFP as a new target for liver cancer immunotherapy has not been thoroughly studied. ICIs therapy is currently the most widely used immunotherapy regimen, but the response rate in HCC clinical treatment is limited, and personalized combination therapy of multiple ICIs or ICIs in combination with ACT, tumor vaccines, and OVs is a better option to improve objective response rate (ORR).

Heterogeneity constitutes one of the traits of solid tumors, and not all tumors at one site in the same patient express the same tumor-associated antigen. Consequently, if merely a single-target CAR-T cell is employed to treat solid tumors, the highly heterogeneous nature of solid tumors theoretically dictates that they will inevitably relapse or cannot be eradicated. Despite these challenges, it is still worthy of anticipation that the future application of CAR-T and TCR-T therapy for precise and multi-targeted substitution of the tumor attack might transform the treatment strategy of HCC and offer patients more curative options [166].

Currently, most research focuses on the preclinical stage. Translating basic research results into the clinic and selecting appropriate immunotherapy targets to improve the therapeutic effect without affecting normal immune function are key problems that must be solved. Finding new biological targets that affect the spatial heterogeneity of HCC tissue and applying these targets as liver cancer immunotherapy is an effective strategy to improve liver cancer immunotherapy. Spatial heterogeneity of the TME and spatial transcriptional reprogramming are determinants of the efficacy of liver cancer immunotherapy. These research fields have revolutionary significance for the treatment and prognosis of liver cancer. With further exploration of HCC immunotherapy, targeting the spatial heterogeneity of the TME will be a great target for immunotherapy in patients with HCC.

**Author contributions** M. Zhu and M. Li designed the concepts and revised the manuscript; M. Zhang, K. Huang, and Q. Yin wrote the main manuscript text. M. Zhang prepared Figs. 1 and Table 1; K. Huang prepared Figs. 2–3; Q. Yin and X. Wu prepared Figs. 4.

**Funding** This work was supported by The National Natural Science Foundation of China (Nos. 82060514, 82460602 and 81960519), The Natural Science Foundation of Hainan Province (No. 824RC517 and 822QN311), the Hainan Medical University Talent Launch Fund (No. XRC220017), and Research Project of Take off the Proclamation and Leadership of the Hainan Medical University Natural Science Foundation (No. JBGS202106), and Hainan Provincial Science and Technology Special Fund (No. ZDYF2021SHF222).

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

## Declarations

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

**Code availability** Not applicble.



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