Front. Med. 2024, 18(3): 430−445
https://doi.org/10.1007/s11684-023-1049-z

REVIEW

Cancer stem cell-immune cell crosstalk in the tumor microenvironment for liver cancer progression

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Abstract **Crosstalk between cancer cells and the immune microenvironment is determinant for liver cancer progression. A tumor subpopulation called liver cancer stem cells (CSCs) significantly accounts for the initiation, metastasis, therapeutic resistance, and recurrence of liver cancer. Emerging evidence demonstrates that the interaction between liver CSCs and immune cells plays a crucial role in shaping an immunosuppressive microenvironment and determining immunotherapy responses. This review sheds light on the bidirectional crosstalk between liver CSCs and immune cells for liver cancer progression, as well as the underlying molecular mechanisms after presenting an overview of liver CSCs characteristic and their microenvironment. Finally, we discuss the potential application of liver CSCs-targeted immunotherapy for liver cancer treatment.**

Keywords liver cancer; cancer stem cell; immune cell; immunotherapy

Introduction

Liver cancer, primarily hepatocellular carcinoma (HCC), is the sixth most common aggressive cancer [1]. Currently, several kinds of molecular targeted drugs have been approved for HCC treatment, including kinase inhibitors, angiogenesis inhibitors and immune checkpoint inhibitors, which only provide modest benefits [2]. Previous studies indicated that the main manifestations of malignant tumors related to the existence of cancer stem cells (CSCs) [3]. Emerging evidences show that a subset of stem cells or precursor cells in HCC, namely liver CSCs [4–6]. Heterogeneous HCC is largely explained by liver CSCs expressing different surface markers. Alterations in the tumor microenvironment (TME) are also known to promote tumor cell heterogeneity [7]. The liver CSC TME is essential for maintaining the properties of CSCs, protecting them from being attacked by the immune system and maintaining their metastatic potential [8]. Another confounding variable is the interaction of the immune cell lineage with CSCs, as both immune evasion and CSCs are considered integral parts of tumor growth and metastatic spread [9]. Harnessing the immune system to recognize and efficiently eliminate tumor cells has been successfully used in HCC treatment.

This review summarizes current knowledge of liver CSCs, and highlights the impact of the microenvironment niche on HCC stemness. Importantly, we focus on the significance of crosstalk between CSCs and the immune system, and discuss the potential application of liver CSCs-targeted immunotherapy in liver cancer therapy.

Liver CSCs characteristic

The origin of liver CSCs

The origins of liver CSCs are controversial. One potential origin of CSCs is from liver stem/progenitor cells, which is concluded from the fact that liver CSCs share a

Received June 9, 2023; accepted November 15, 2023

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massive number of similar characteristics with normal stem cells [10-13]. Hepatic progenitors expressing H-Ras and SV40LT proteins tend to transform into liver CSCs [14]. A leading cause of tumor cells acquiring CSC-like features is also thought to be the reprogramming and dedifferentiation of non-CSCs [14,15]. Another view is that liver CSCs may derive from the dedifferentiation of mature hepatocytes [16–18]. Chronic liver disease causes recurrent inflammatory response and regeneration of hepatocytes, resulting in genetic and epigenetic alterations in hepatocytes and hepatic progenitor cells, which also contribute to liver CSCs formation [14,19]. Additionally, the origin of liver CSCs is also investigated by lineage-tracing studies and ablation of CSC markers (namely, epithelial cell adhesion molecule (EpCAM) and LGR5) in intact mouse HCC tumors. $EpCAM^+$ ductal cells are the main cell source of liver cancer due to inflammation-associated tumorigenesis in a mouse model of HCC [20]. Furthermore, $LGR5^+$ hepatocytes are found to be responsible for diethylnitrosamine (DEN)-induced HCC and highly sensitive to neoplastic transformation triggered by ERBB pathway activation [21]. Collectively, these studies suggest that liver CSCs originate from stem/progenitor cells, proliferating ductal cells and hepatocytes.

Cell surface markers of liver CSCs

Although the pathogenesis of HCC remains unclear, liver CSCs are vital for HCC formation and exhibit heterogeneity, which due to the presence of tumor cell subsets expressing different markers in HCC [22–24]. Several surface markers for liver CSC subpopulations (CD133 [25], CD90 [26], CD44 [27], EpCAM [28], CD13 [29], oval cell marker (OV-6), CD47 [30], delta like 1 homolog (DLK1) [31], CD24, intercellular adhesion molecule 1 (ICAM1) [32], α2δ1 [33], and keratin 19 (CK19) [34]) in HCC have been identified. Recent studies indicate that CD133 regulates neurotensin, interleukin (IL)-8, CXCL1, and MAPK signaling to maintain the characteristics of CSCs [25,35,36]. Additionally, $CD90^+$ and $CD44^+$ HCC cells also display tumorigenic and metastatic potential [26,37]. Recently, CD44 has been found to regulate TGF-β-induced epithelial-mesenchymal transition (EMT) [27] and maintain CSCs properties via regulating redox status [38]. In addition, $EpCAM^+$ HCC cells show CSC traits that are highly invasive and tumorigenic [28,39]. Moreover, CD13 regulates the reactive oxygen species (ROS) scavenger pathway to prevent cells from apoptosis [29]. CD47 modulates the cathepsin S/protease-activated receptor 2 paracrine pathway to regulate HCC stemness [30]. $DLK1^+$ cells possess robust chemoresistance and self-renewal potential *in vitro* and tumor-initiating capacity *in vivo* [31]. Both CD24 and ICAM1 are functional liver CSC markers that activate stemness-associated factor Nanog to initiate HCC [32]. Furthermore, α2δ1 has been demonstrated to potentiate liver CSCs characteristics by activating pro-survival pathways in a calcium-dependent mechanism [33]. CK19 is also a functional CSC marker that contributes to tumor progression [34,40]. Overall, these results propose that liver CSC markers provide a promising therapeutic target for overcoming HCC treatment resistance.

Liver CSCs-associated signaling pathway

Liver CSCs mediate therapeutic resistance and relate with the poor prognosis of HCC patients [41,42]. Intricate liver CSC-associated signaling pathways are generally related to HCC development [43]. Evidence is emerging to support that Wnt/β-catenin signaling is activated in different liver CSC subsets [44–47], including $EpCAM^+$ [48], $CD133^+$ [49], $OV6^+$ [50], $Sox9^+$ [51], and $Lgr5^+$ [30] liver CSCs. Shp2 [52] and EPHB2 [53] favor liver CSC extension through β-catenin signaling. Of note, EpCAM is identified as a direct target of β-catenin, and Wnt/β-catenin signaling activates the liver CSC marker EpCAM [54]. AKT and AKT/GSK3β signaling is often altered in liver CSCs, which are related to the Wnt/β-catenin signaling. Accordingly, EPHB2 regulates cancer stemness and drug resistance through AKT/GSK3β/β-catenin axis [55]. The NOTCH signaling is hyperactive and plays physiologic roles in HCC. It is also involved in stem cell self-renewal and differentiation [56,57]. It is reported that C8orf4 decreases the proportion of $CD13^+CD133^+$ liver CSCs by inhibiting NOTCH2 signaling [58]. Consistently, $CD133^+$ liver CSCs have a higher NOTCH expression [49,56]. Moreover, iNOS/nitric oxide (NO) activates NOTCH1 via regulating TACE/ADAM17 signaling in liver CSCs to promote hepatocarcinogenesis [59]. IL-6/STAT3 activation in liver CSCs is also positively correlated with HCC development [60]. Hepatitis B virus X (HBx) activates IL-6/STAT3 signaling to facilitate the carcinogenicity of hepatic progenitor cells [61]. Additionally, STAT3 upregulates Nanog expression to promote the extension of $CD24^+$ liver CSCs [62]. Long-term tobacco smoke exposure enhances HCC cell stemness properties through IL-33/p38 axis [63]. Furthermore, the JAK/STAT pathway inhibits stem cell homeostasis by suppressing $SP/CD44^+$ CSCs properties in HCC [64]. Consequently, distinct signaling pathways are involved in regulating liver CSC properties in HCC, which provides various insights for studying the pathogenesis of HCC.

Tumor microenvironment of liver CSCs

HCC is a highly heterogeneous disease, which forms an immune tolerance microenvironment. CSCs exist in the

TME, maintaining an undifferentiated state and self-renewal potential [7]. Emerging evidence indicates the potential role and influence of the liver CSC microenvironment on modulating HCC tumorigenesis, EMT and tumor progression [65]. The liver CSC microenvironment encompasses a series of tumor-related components, such as tumor parenchymal cells (containing non-CSC cancer cells and CSCs), stromal cells [66], immune cells, cytokines, extracellular vesicles (EVs) [67], extracellular matrix (ECM), oxygen, nutrients [68], and hepatitis virus infection [69,70], which in turn promote tumor progression (Fig. 1).

In addition to the tumor parenchymal cells, the stromal cells and their secreted cytokines are the main components that constitute the TME. It is acknowledged that cancer-associated fibroblasts (CAFs) regulate the stem cell properties and tumorigenicity of CSCs in several malignant tumors [71]. CAFs drive NOTCH3 expression to regulate the self-renewal of liver CSCs through LSD1 activation [72]. Subsequently, CAFs are able to trigger HCC stem cell-like characteristics via IL-6-STAT3-NOTCH signaling [73]. Our previous study found that tumor-derived exosomal miR-1247-3p stimulates the CAF to product IL-6 and IL-8 and exert the cancer cells to an EMT phenotype [66]. The findings suggest that CAFs interact with HCC to maintain and progress liver disease. Additionally, endothelial cells are essential components of the TME and contribute to tumor

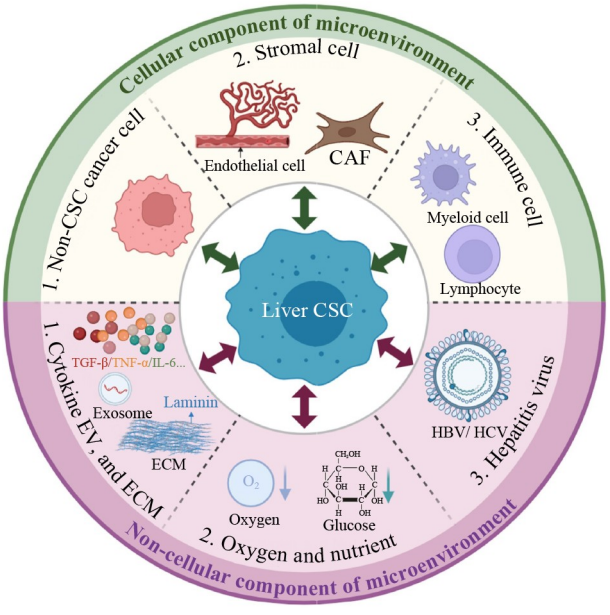

Fig. 1 Tumor microenvironment of liver CSCs. The tumor-associated stroma is vital for regulating cancer stemness in HCC. Abbreviations: ECM, extracellular matrix; HBV, hepatitis B virus; HCV, hepatitis C virus; CAF, cancer-associated fibroblasts; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6. This figure was created with BioRender.com.

metastasis and angiogenesis [74]. $CD133^+$ liver CSCs secrete IL-8 to promote angiogenesis through its effects on endothelial cells in the TME [35]. Moreover, $CD90^+$ liver CSCs modulate the phenotype of endothelial cells by releasing exosomes containing H19 long non-coding (lncRNA) [75], implying the interaction between angiogenesis and liver CSCs in TME contributes to HCC development [76]. Taken together, these results suggest the critical roles of the stromal cells in regulating liver CSCs properties.

Immune cells are essential in driving CSC extension and CSC-specific avoidance of immunosurveillance. Molecules secreted by immune cells regulate cancer cell properties in the TME [77]. These inflammatory factors generate a CSC-inducing niche. It has been found that tumor-associated macrophages (TAMs) induce EMT and CSC-like properties through IL-6, TNF-α, and TGF-β1 in HCC [78–80]. Conversely, CSCs could also regulate immune cell antitumorigenic properties. $CD47^+$ liver CSCs can escape phagocytosis by M1 macrophages in the TME [81,82]. Moreover, chemokine ligand 20 (CCL20) derived from hypoxia-induced liver CSCs lead to the recruitment of IDO^+ TAMs and the inhibitory effect of T cell responses [83]. In addition, tumor-associated neutrophils (TANs) are essential in driving liver CSC expansion by secreting bone morphogenetic protein 2 (BMP2) and TGF-β2 [84]. TAN-induced HCC stem cell like cells generate more CXCL5 to recruit more TANs infiltration, which forms a positive feedback loop [84]. Moreover, immune suppressive cytokines secreted by myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs) mainly mediate the formation of an immunotolerant niche [85]. Various tumors including HCC is infiltrated by Tregs [86]. Tregs inhibit the activities of effector T cells and avoid self-tolerance by secreting cytokines or direct contact. It has been investigated that Tregs are capable of promoting the invasion of HCC cells through TGF-β1-mediated EMT [87]. Liver CSCs can promote VEGF production, and thus inducing the recruitment of MDSCs in the TME [88]. Additionally, dendritic cells (DCs) could increase the apoptosis ratio of liver CSCs and exert the T cell-mediated antitumor immune response [89,90]. The ablation of Arf1 in CSCs could activate DCs and enhance T cell infiltration to potentiate anti-tumor immune response [90]. Accumulating evidence has demonstrated that liver CSCs contribute to the evasion of immunosurveillance by altering the phenotype of DCs and impairing their recruitment [91,92]. These investigations indicate the critical roles of immune cells in favoring the CSC niche.

T cells and natural killer (NK) cells are vital components of immune detection and play an essential role in long-term antigen-specific immune recognition [9]. Notably, they are able to directly identify and kill

cancer cells. Interactions between CSCs and $CD8^+$ T cells could be divided into two aspects: CSCs evade T cell-mediated cytotoxicity and inhibit the anti-tumor properties of T cells [9]. Programmed cell death 1 ligand 1 (PD-L1) expression on HCC cells interact with programmed cell death 1 (PD-1) on T cell to escape T cell-mediated immune surveillance. Interferon γ (IFN-γ) is a key factor secreted by the immune system to eliminate cancer cells [93]. IFN-γ derived from T cell could upregulate PD-L1 expression on HCC cells [94]. CSCs could impair cytotoxic T cell activity by altering their PD-L1 expression [95]. For example, the expression of PD-L1 is elevated in $CD44^+CD24^+ALDH^+$ CSCs [96]. Moreover, CSCs decrease the expression of MHC-I to limit the presentation of neoantigens to T cells [97]. Additionally, liver CSC-derived cytokines can impair NK cell activity, such as circular ubiquitin-like with PHD and ring finger domain 1 RNA (circUHRF1) and granulin-epithelin precursor (GEP) [98,99].

ECM remodeling is critical for tumor progression and metastasis. Notably, ECM stiffness is vital for regulating liver CSC differentiation [100]. Matrix stiffness is found to promote a higher proportion of $CD133^+$ or $EpCAM^+$ HCC cells through integrin β1-activated mTOR signaling [101]. It is known that HCC cells remodel the ECM by upregulating ECM components and matrix metalloproteinases (MMPs). Nevertheless, CD133 expression in liver CSCs induce the production of MMP2 and metalloproteinase 9 to degrade ECM and promote immune escape by reducing their sensitivity to NK cells [102]. Moreover, liver CSCs produce lysyl oxidase (LOX) to promote the formation of a stiff ECM, thus enhancing the stiffness-mediated cancer stemness [103].

Hypoxia and glucose deprivation are also the main factors that stimulate vascular proliferation and promote HCC development [104]. HCC is a solid tumor and the tumor body grows rapidly, which forms a hypoxic environment. Hypoxia promote HCC cells proliferation [105], cause angiogenesis, and accelerate invasion [106]. For instance, HIF1α upregulates *USP22* and *TP53* at the transcriptional level under hypoxic conditions, and then promotes the production of $CD24^+$ or $CD44^+$ liver CSCs in HCC [107]. Similarly, the positive feedback loop between HIF-1α and SENP1 contributes to $CD24^+$ subpopulation in HCC under hypoxia conditions [104]. Accordingly, it is necessary to clarify the changes in hypoxia-related response molecules in HCC cells. In addition, glucose deprivation generally appeared in large-growing tumors microenvironment such as HCC. Nutrient deprivation impacts the expansibility of liver CSCs [108]. Under low glucose conditions, hexosamine biosynthetic pathway (HBP) increases $CD133^+$ subpopulation and enhances CD133 expression in HCC cells, leading to a more aggressive HCC progression [109]. Moreover, FUT1-mediated fucosylation drives HCC stemness through CD147, ICAM-1, EGFR, and EPHA2 via AKT/mTOR/4EBP1 axis [68]. In summary, hypoxia and glucose deprivation significantly affects liver CSCs maintenance.

Hepatitis B virus (HBV) [110] and hepatitis C virus (HCV) infection [111] are the largest risk factors for HCC occurrence and development, indicating that hepatitis virus-induced chronic liver inflammatory environment promotes hepatocarcinogenesis [112,113]. HBV PreS1 upregulates CSCs-related markers (CD133 and CD90) expression and facilitates the capacity of CSCs in HCC [69]. Additionally, HBx upregulates miR-5188 expression to promote β-catenin nuclear translocation and HCC stemness [114]. Similarly, HCV and HCFD mediate TLR4-NANOG and the leptin receptor (OBR)-STAT3 activation to accelerate HCC progression [115]. Moreover, Sal-like protein4 (*SALL4*) gene re-expression is induced by DNA demethylation in HCC cells, which is associated with HBV or HCV infection [116]. In brief, hepatitis virus infection contributes to the self-renewal of liver CSCs and facilitates HCC progression.

Liver CSCs-immune cell crosstalk in tumor progression

Immune cells are prominent components in modulating the CSC subpopulation. Previous studies have shown the relationship between liver CSCs and immune cells. CSCs can promote tumor initiation and progression, revealing that they have the advantage of evading immune surveillance. Emerging evidence support that some primary immune cells are necessary for CSCs expansion and enable them to escape immune surveillance via distinct mechanisms [9].

Protumorigenic immune cells

Protumorigenic immune cells mainly refer to tumor-associated myeloid cells in the innate immune system, including macrophages, granulocytes, and monocytes [117,118]. Extensive studies support that myeloid cells interact with HCC cells and affect the TME to regulate cancer progression and recurrence [119]. Therefore, the interplay between myeloid cells and adaptive immunity is also an essential modulator of tumor progression, with tumor-associated myeloid cells playing vital roles in tumor immune evasion (Fig. 2).

Tumor-associated macrophages

Macrophages is known to be classified into two subtypes: M1 and M2 macrophages. M1 macrophages prompt both inflammatory and anti-tumor responses. Activated M2 macrophages secrete a series of inflammatory response factors that participate in regulating the inflammatory

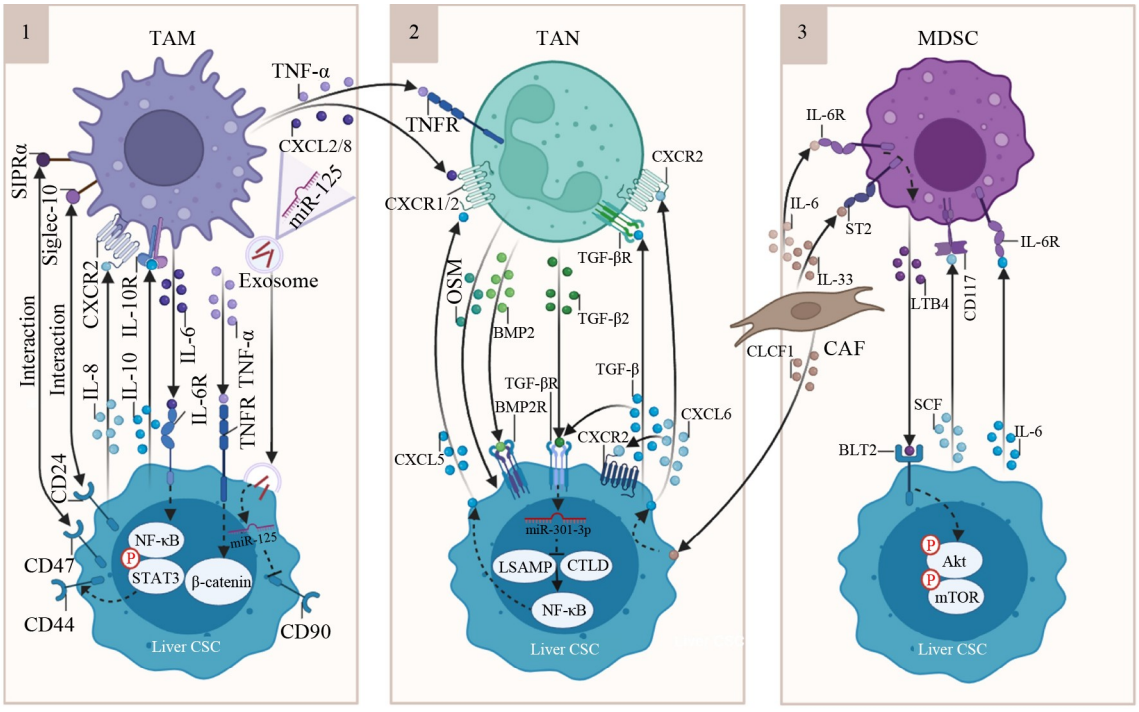

Fig. 2 The interactions between liver CSCs and protumorigenic immune cells. Mutual communication between liver CSCs and protumorigenic immune cells through soluble mediators or signaling regulates HCC stemness. Abbreviations: TAM, tumor-associated macrophage; SIPRα, signal regulatory protein alpha; TNF-α, tumor necrosis factor-α; Siglec-10, sialic-acid binding Ig-like lectin 10; IL-6/8/10/33, interleukin 6/8/10/33; NF-κB, nuclear factor-κB; TAN, tumor-associated neutrophil; miR-125/301-3p, micro RNA-125/301-3p; LSAMP, limbic system-associated membrane protein; CYLD, CYLD lysine 63 deubiquitinase; CXCL, C-X-C motif ligand; CXCR: C-X-C chemokine receptor type; OSM, oncostatin M; TGF-β, transforming growth factor-β; BMP2, bone morphogenetic protein 2; CLCF1, cardiotrophin-like cytokine factor 1; CAF, cancer-associated fibroblast; MDSC, myeloid-derived suppressor cell; LTB4, leukotriene B4; BLT2, leukotriene B4 receptor type 2; SCF, stem cell factor. This figure was created with BioRender.com.

microenvironment [120]. TAMs usually express an M2 phenotype and are crucial components of the tumor inflammatory microenvironment [121]. Furthermore, TAMs involve in tumor occurrence, progression and migration, which are pivotal immune cells and associated with liver CSCs formation [122]. This section will discuss the relationship between TAMs and liver CSCs to provide a novel insight for cancer treatment from the perspective of targeting TAMs.

TAMs form a "niche" to maintain the self-renewal of CSCs. Cytokines secreted by macrophages regulate CSCs properties via multiple pathways [123]. For example, TAMs secret IL-6 to activate IL-6/STAT3 axis to promote $CD44^+$ HCC cells expansion and liver tumorigenesis [78]. Similarly, CCL17 produced by activated M2 macrophages triggers the activation of TGF-β1 and Wnt/β-catenin signaling in HCC cells, which promotes the stemness and EMT process of HCC cells [124]. TAMs produce TNF-α to promote HCC cell stemness via Wnt/β-catenin axis [79]. Consistently, TAMs also produce TGF-β1 to induce EMT and stemness properties in liver cancer cells, which gain higher metastasis and invasive capability [80]. Moreover, HCC cell-derived TGF-β drives T cell immunoglobulin and mucin domain 3 (TIM-3) expression on TAMs, then TIM-3 stimulates STAT6 and nuclear factor-κB (NF-κB) pathway, which foster TAM to produce IL-10 and stimulate HCC cell migration [125]. Furthermore, IL-8 secreted by macrophages regulates downstream JAK2/STAT3/Snail signaling to trigger the invasion properties of HCC cells [126]. Moreover, TAMs secret CXCL2, CXCL8, and TNF-α to recruit neutrophils into HCC milieus and increase the production of pro-stemness factor oncostatin M (OSM) in neutrophils, finally leading to HCC metastasis and malignant progression [127]. In addition, HCC cells treated with TAM extracellular vesicles or miR-125a/b targeting liver CSC marker CD90 could suppress cell proliferation and stem cell properties. This finding suggests that targeting CD90 has an inhibitory effect on liver CSCs [128]. Consequently, TAM-CSC communication is related with tumor development.

Liver CSCs promote migration and recruit macrophages to tumor sites by secreting chemokines. CSCs possess an immunosuppressive program involved in macrophages recruitment and trigger them toward M2 polarization at the tumor locus, this ability of CSCs is generally found in HCC through activating NF-κB

signaling pathways and secreting cytokines such as IL-8 and IL-10 [129]. For instance, $CD133^+$ cells secrete IL-8 to induce TAMs M2 polarization in HCC [123]. Similarly, HCC-derived IL-8 attracts more M2-type TAMs to promote a pro-oncogenic inflammatory microenvironment, and aberrant activation of the neurotensin (NTS)/IL-8 pathway stimulates the invasion potential of HCC cells [123]. Notably, CSCs escape immune surveillance by exerting TAMs immunosuppressive functions. CD24 expressed on tumor cells interacts with sialic-acid binding Ig-like lectin 10 (Siglec-10) on TAMs to promote HCC progression [130]. Similarly, CD47 expressed on tumor cells interacts with signal regulatory protein alpha (SIPRα) on macrophages to protect liver CSCs from being phagocytosed [30]. These observations demonstrate that both immunosuppression and stemness phenotypes can be targeted simultaneously between the crosstalk of CSCs and macrophages.

Tumor-associated neutrophils

TANs are classical inflammatory cells associated with tumorigenesis and development in the TME [84]. However, the direct interplay between TANs and HCC cells remains unclear [131]. Recently, it has been demonstrated that BMP2 and TGF-β2 upregulate miR-301b-3p expression in HCC cells, and subsequently block limbic system-associated membrane protein (LSAMP) and CYLD lysine 63 deubiquitinase (CYLD) expression, thus contributing to HCC stemness [84]. In addition, NF-κB signaling is activated in TAN-induced HCC stem cell like cells, higher CXCL5 is generated, and more TANs infiltration is recruited, which forms a positive feedback network. It is also validated in HCC clinical patients [84]. Of note, the interaction of CAFs, HCC cells, and TANs are connected with a network of cytokines. For instance, cardiotrophin-like cytokine factor 1 (CLCF1) derived from CAFs upregulates the expression of CXCL6 and TGF-β in tumor cells, driving TANs N2 polarization and subsequently promoting HCC cells stem-cell like characteristics [132]. In conclusion, all of above observations suggest that TANs modulate liver CSCs and are effective targets for HCC treatment.

Myeloid-derived suppressor cells

MDSCs represent a heterogeneous population of immature myeloid cells. MDSCs could disrupt the effect of DCs and inhibit T cell infiltration [133–135]. Emerging evidence implicates that MDSCs inhibit antitumor immune response in HCC patients [135]. Drug-resistant HCC cells are generally considered to have CSC properties [136]. Drug-resistant HCC cells-derived IL-6 activates MDSCs and facilitates the immunosuppressive function of MDSCs with a higher production of NO and ROS [136]. Furthermore, tumor cell-derived stem cell factor (SCF) drives MDSCs expansion and promotes the proliferation of liver cancer cells [137]. Moreover, HSC-monocytic MDSCs (M-MDSC) crosstalk promotes HCC progression. Activated HSC triggers monocyte-intrinsic p38 MAPK signaling to induce M-MDSCs accumulation and promote HCC development [138]. In addition, IL-1β triggers solute carrier family 7 member 11 (SLC7A11) expression and subsequently upregulates PD-L1 and colony stimulating factor-1 (CSF1) expression via α-ketoglutarate (α-KG)-HIF1α signaling pathway, which promotes TAMs and MDSCs infiltration, and thus contributing to HCC invasion [139]. Interestingly, CAFs secret IL-6 and IL-33 to mediate the activation of 5-lipoxygenase (5-LO) metabolism in MDSCs, 5-LO mediates the function of $CD33^+$ MDSCs to stimulate intrahepatic cholangiocarcinoma (ICC) stemness through its downstream leukotriene B4 (LTB4)-leukotriene B4 receptor type 2 (BLT2) axis via the activation of AKT/mTORC1 signaling [140]. Therefore, targeting MDSCs probably provides a novel strategy for improving HCC clinical treatment.

Antitumorigenic immune cells

Antitumorigenic immune cells in the TME predominantly include tumor-infiltrating lymphocytes (TILs) and DCs infiltrate the TME. The presence of a large number of TILs and DCs indicates that the body has initiated an immune response against the tumor. If the human immune system response to cancer is likened to a war, antitumorigenic immune cells are a “warrior” that rushes to the front line from all over the body to fight against cancer cells [141]. The amount of TILs and DCs in tumors significantly indicates the prognosis and response to immunotherapy in HCC patients (Fig. 3) [142].

T lymphocytes

T cells are vital components of immunosurveillance and play an essential role in long-term antigen-specific immune responses. The process of liver CSCs regulating T cell activity is involved in distinct pathways. Liver CSCs can negatively regulate T cell activity through transforming the activity of Treg cells [143]. TGF-β signaling could suppress miR-34a and increase the production of chemokine CCL22 in $CD44^+$ liver CSCs, which leads to Treg cells recruitment and thus favoring HCC immune escape [144]. Tumor cells and immune cells within immune-high HCC tumors linked to highly PD-L1 expression [145]. The presence of lymphocytes significantly correlates with HCC patient outcomes, PD-L1 expression is related to the HCC progenitor properties

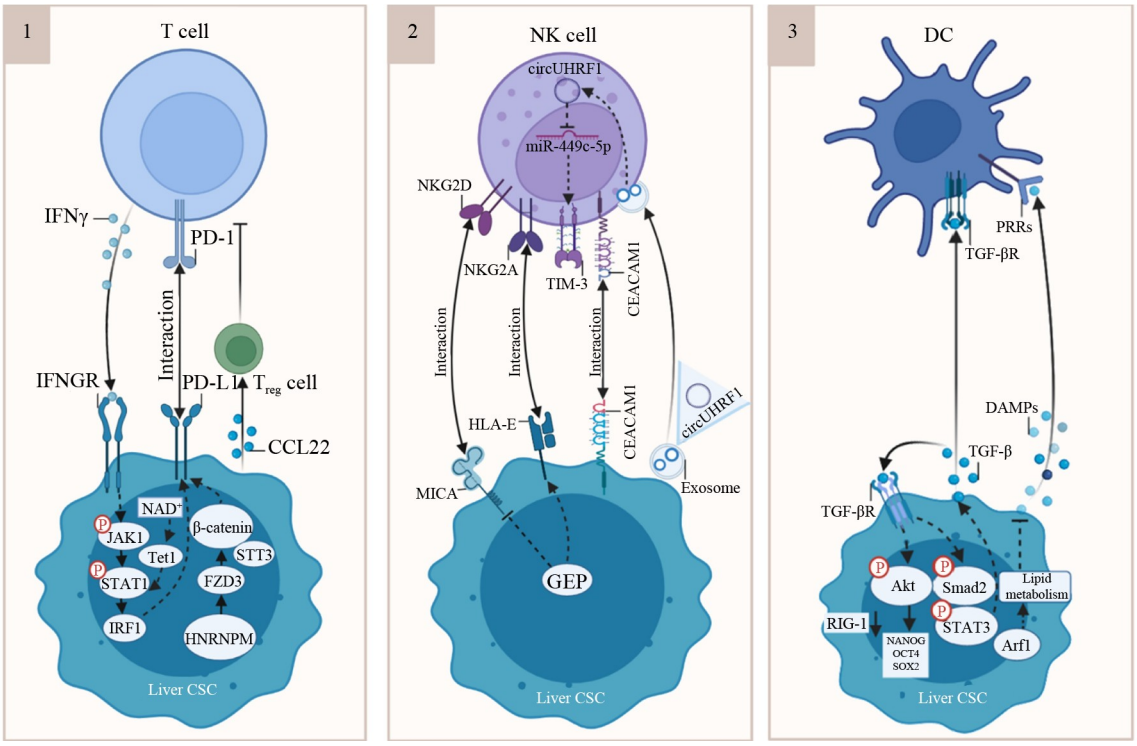

Fig. 3 The interactions between liver CSCs and antitumorigenic immune cells. The crosstalk between liver CSCs and antitumorigenic immune cells via soluble mediators or signaling mediates cancer stemness. Abbreviations: IFN-γ, interferon γ; PD-L1, programmed death-1-ligand-1; PD-1, programmed cell death 1; HNRNPM, heterogeneous nuclear ribonucleoprotein M; CCL22, C-C-motif chemokine ligand 22; T_{reg} cell, regulatory T cell; NK cell, natural killer cell; MICA, MHC class I chain-related molecule A; GEP, granulin-epithelin precursor; miR-449c-5p, microRNA-449c-5p; NKG2D, NK group 2 member D; HLA-E, human leukocyte antigen-E; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; circUHRF1, circular ubiquitin-like with PHD and ring finger domain 1 RNA; TIM-3, T cell immunoglobulin and mucin domain 3; PRRs, pattern recognition receptors; DC, dendritic cell; DAMPs, damage-associated molecular patterns. This figure was created with BioRender.com.

(expressed CK19 and SALL4) in an analysis of 217 HCC clinical patients [145,146], implying the efficacy of anti-PD-1 immunotherapy directly acting on $CD8^+$ T cells when it is applied for HCC treatment. Additionally, β-catenin/STT3 signaling contributes to PD-L1 enrichment through modulating glycosylation and stabilizing PD-L1 in CSCs, which is crucial for CSC immune evasion [147]. Moreover, IL-6 activates JAK1 signaling and JAK1 phosphorylates PD-L1 to catalyze PD-L1 glycosylation and stabilization, which is also observed in HCC patient samples [148]. Of note, nicotinamide adenine dinucleotide (NAD^+) metabolism is necessary for the maintenance of liver CSCs. MRPS5 increases the proportion of NAD^+, which is essential for enhancing the function of mitochondria in liver CSCs [149]. Recently, we have demonstrated that NAD^+ metabolism maintains PD-L1 expression on HCC cells in response to IFN-γ, which is derived from T cells. In turn, PD-L1 expression on HCC cells interacts with PD-1 on T cells to escape T cell-mediated immune surveillance [94]. In addition, $Trp53^{-/-}$ HCC cells activate β-catenin signaling pathway to escape immune surveillance by impairing T cell cytotoxic activity, indicating that β-catenin reduces the DCs recruitment and subsequently downregulates T cell activity and thus contributing to tumor progression [150]. β-catenin activation could influence HCC immune microenvironment and promote HCC progression. Heterogeneous nuclear ribonucleoprotein M (HNRNPM) deficiency inhibits Wnt/β-catenin signaling and contributes to $CD8^+$ T cell infiltration in HCC, thus increasing the efficiency of anti-PD-1 immunotherapy. HNRNPM upregulates *FZD3* expression and activates Wnt/β-catenin signaling by regulating the alternative splicing (AS) of *MBD2*, *FZD3* and β-catenin further regulate stemness by targeting CSC marker OCT4 and SOX2 [151]. Separately, the hyaluronan synthesis inhibitor 4-methylumbelliferone (4mu) reduces the proportion of $CD47^+$ liver CSCs and elicits a T cell response induced by IL-12 [152]. In conclusion, the cytotoxic activity of tumor infiltrates contributes to immune surveillance by inhibiting liver CSC stemness properties. HCC forms a robust immune tolerance microenvironment because HCC patients have been in a state of low immune response for a long time and tended to develop resistance to tumor immunotherapy. Therefore, it has important clinical and therapeutic

significance to study how to improve HCC immune resistance and enhance T cell-mediated immune response.

Natural killer cells

The NK cell population is an important subpopulation of cytotoxic lymphocytes in HCC progression. It is known that GEP and ABCB5 expression in liver CSCs contribute to HCC poor outcomes [153]. GEP reduces MHC class I chain-related molecule A (MICA) expression, ligand for NK stimulatory receptor NK group 2 member D (NKG2D), and upregulates human leukocyte antigen-E (HLA-E), ligand for NK inhibitory receptor CD94/NKG2A on NK cells. Thus, GEP-expressing CSCs resistant NK cell-mediated cytotoxicity and promote HCC tumor immune escape [154]. Additionally, carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is upregulated on EpCAM-high liver CSCs, it contributes to resistance to NK cell cytotoxicity and promotes HCC development, which indicates that homophilic interaction of CEACAM1 is critical for alleviative NK cell cytotoxic effect on CEACAM1^{high} liver CSCs [155]. Moreover, HCC cell-derived exosomal circular ubiquitin-like with PHD and circUHRF1 inhibits NK cell-secreted IFN-γ and TNF-α. circUHRF1 suppresses NK cell-mediated effect by increasing TIM-3 expression and downregulating miR-449c-5p in HCC [156]. In summary, NK cell-mediated killing activities are regulated by liver CSCs through some critical mediators, which further suggest that NK cell-mediated immune response is vital for HCC progression.

Dendritic cells

DCs are antigen-presenting cells that are closely related to immune responses and have a vital role in exerting the T cell-mediated antitumor immune response [157]. A recently research has suggested that DCs and cytokine-induced killer cells (CIKs) upregulate caspase-3 expression and decrease proliferating cell nuclear antigen (*PCNA*) expression to increase the apoptosis ratio of liver CSCs [89]. Arf1-mediated lipid metabolism maintains CSCs. Arf1 ablation in CSCs results in metabolic stress including mitochondrial defects and endoplasmic reticulum (ER) stress, releasing damage-associated molecular patterns (DAMPs), which potentiate anti-tumor immune response through DCs activation and T cell infiltration enhancement [90]. Accumulating evidence has demonstrated that liver CSCs contribute to the evasion of immunosurveillance by altering the phenotype of DCs and impairing their recruitment [91,92]. *RIG-1* deficiency leads to accumulation of p-STAT3, and p-STAT3 could induce TGF-β1 expression. *RIG-I*-deficient HCC cells with stemness-associated characteristics alter the roles of DCs from immunostimulatory to immunosuppressive through upregulating TGF-β1 [91]. Moreover, *RIG-I* deficiency causes more TGF-β1 production that triggers Smad2 and Akt phosphorylation and facilitates the interaction of Smad2/p-Smad2 and Akt/p-Akt in HCC cells, thus promoting HCC stemness [91]. Additionally, β-catenin activation facilitates HCC against anti-PD-1 therapy, which is associated with defective recruitment of DCs [150]. Through the summary of the recent findings about CSCs regulate DCs recruitment, there still remains a need to figure out the association between DCs and liver CSCs.

Liver CSCs-targeted immunotherapy

Due to the lack of specific therapeutic targets, HCC has a high mortality rate [158]. Liver CSCs-targeted immunotherapy could be promising therapeutic strategies for HCC [95]. With the emergence of immunotherapy, exciting developments have been made that utilize CSC-specific antigens to harness the immune system's power and increase the efficacy of HCC treatment. Approaches with direct inhibitory effects include monoclonal antibodies targeting CSC-related cell surface markers, cytotoxic T lymphocyte (CTL)-based immunotherapy, and the use of DC vaccines.

Antibody-based immunotherapy

Several monoclonal antibodies with direct inhibitory effects have been developed to target CSC-related cell surface markers. The basis of antibody-mediated therapy for targeting CSCs are mainly separated into two segments: antibody-dependent cellular cytotoxicity (ADCC) and the direct inhibitory effect of mAbs [159]. CSC-targeted immunotherapy has been attracted more attention. For instance, EpCAM is recognized as a liver CSC marker and a promising therapeutic target in HCC [160]. An EpCAM/CD3 bispecific antibody (anti-EpCAM bispecific T cell engager (BiTE) 1H8/CD3) recognizing EpCAM can effectively target and eliminate liver CSCs *in vitro* and *in vivo* [161]. Additionally, cytokine-induced killer (CIK) cells bound with anti-CD3/anti-CD133 bispecific antibodies induced strong elimination of HCC cells *in vitro* and *in vivo* [162]. Other liver CSC marker-specific therapies, such as CD47, CD44, and CD24, related mAbs significantly inhibit HCC tumor progression. CD47 blockade inhibits tumor progression in HCC xenograft models, indicating that targeting CD47 may provide a better prognosis for HCC treatment [163]. CD44 antibody-targeted liposomal nanoparticles effectively target CD44$^+$ liver CSCs in preclinical models [164]. Meanwhile, a high-affinity humanized anti-CD24 antibody (hG7-BM3-VcMMAE conjugate) is designed for HCC treatment *in vivo* [165]. However, it is necessary to validate the clinical efficacy

of these liver CSC marker-specific, antibody-based therapies.

Chimeric antigen receptor-T cells

CTL-based immunotherapy has broad application prospects [166–168]. Alternative methods of generating CSC-specific T cells include genetically engineered chimeric antigen receptor (CAR)-T cells. Indeed, CAR-T cells could recognize specific antigens on CSCs to achieve their targeted effects [169]. Based on the liver CSC markers, several CAR-T-related clinical trials are ongoing. Various $CD133^+$ late-stage metastatic malignancies, including HCC, have been treated with CD133-directed CAR-T cells in phase I clinical trials published in 2018 [170]. Moreover, a phase I clinical study (NCT02541370) using CAR-CD133 T cells to treat patients with advanced and $CD133^+$ tumors, including 14 HCC patients, results show that 1 HCC patient achieve partial remission, 9 HCC patients acquire stable disease, implying that CD133-directed CAR-T cell infusion has antitumor activity in advanced HCC [170]. Similarly, a phase II clinical trial (NCT02541370) verified that CAR-CD133 T cells is feasible and have controllable toxicities in HCC [171]. Moreover, DLK1-directed CAR-T cells effectively eliminate DLK1-positive HCC cells *in vitro* and *in vivo*, indicating that DLK1 also could act as a therapeutic target for HCC treatment [172]. Additionally, one CAR-EpCAM T cell clinical trial (NCT03013712) has been organized and is recruiting EpCAM-positive cancer (including HCC), the efficacy is yet to be determined. Collectively, using CAR-T cells to target CSCs is a promising approach for HCC treatment [173]. Although these results are promising, one hindrance in the effective use of CAR-T therapy is toxicity-related challenges (the reduced levels of hemoglobin and platelets) [174]. The other challenge is antigen selection for CAR designing, as tumor antigens are also expressed on the surface of non-cancer cells, leading to target miss-effect and off-target effects, thus hindering therapeutic efficacy [175].

Dendritic cell vaccines

DC vaccines facilitate the immune system's recognition and eradication of tumor cells. Immune response mediated by DCs and triggered by tumor-specific antigens is generated using CSC-derived materials. For instance, pulsing DCs with CD44 and EpCAM peptides facilitate the production of mature DCs, which stimulate T cell activity and produce effective CTLs [176]. In addition, ICT-121, a dendritic cell vaccine targeted at CD133, is shown to promote cytolytic T cell responses against $CD133^+$ CSCs in a phase I trial (NCT02049489) [177]. Moreover, ANXA3-transfected DCs could also potently stimulate the killing activity of liver CSCs, indicating that ANXA3 maybe a novel target for CSC-targeted immunotherapy [178]. Additionally, a new work focus on increasing the efficacy of DC-based HCC neoantigen nano-vaccine by remodeling TANs [179].

Conclusions

Liver CSCs are a fraction of HCC cells with stem cell characteristics account for HCC initiation and therapeutic resistance. Much effort has been made to identify liver CSC markers and altered signaling pathways during recent decades. In this perspective, these discoveries are essential for improving HCC clinical treatment. To achieve stable remission and even cure aggressive malignancies, it is imperative to eliminate CSCs and block their signaling pathways. Remarkably, we illustrate the interplay of CSCs with individual immune cell lineages, and the mechanisms of crosstalk between them. These findings will aid in the development of novel strategies for effectively targeting CSCs. Liver CSCs-specific therapies require to be further investigated for clinical application. Nevertheless, some important issues remain unresolved. For instance, some of the crucial markers of liver CSCs are also shared by normal stem cells. Molecular differences between CSCs and normal stem cells need to be identified in order to target them specifically without implicating normal stem cells. Ultimately, targeting CSCs specifically increases the efficacy of existing therapies and provides a framework for the development of new therapeutic regimens with long-term clinical benefits. It is expected to identify more effective liver CSC markers and develop more specific anti-CSC marker therapies in the future.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Nos. 82273176, 81902894, 81972779, 81903036, 81622039, 81830054, 91859205, and 81988101), Chinese National Key Project (No. 2018ZX10723204-006-003), Shanghai Municipal Commission of Education Project (No. 201901070007E00065), and Program of Shanghai Academic Research Leader (No. 23XD1404800).

Compliance with ethics guidelines

Yue Ma, Hongwei Lv, Fuxue Xing, Wei Xiang, Zixin Wu, Qiyu Feng, Hongyang Wang, and Wen Yang declare no potential conflicts of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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