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Targeting the interactions between lymphocytes and liver cancer stem cells in combination with immunotherapy is a promising therapeutic strategy

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, with a poor prognosis and high recurrence rate. Liver cancer stem cells (LCSCs), a small subset of HCC cells, have the capacity for self-renewal and the property of treatment resistance, suggesting that LCSCs are key factors in causing poor prognosis for HCC patients. In addition, LCSCs interact with immune cells to participate in the formation of an immunosuppressive microenvironment and escape the immune surveillance in HCC, especially lymphocytes. At present, immunotherapies for HCC are mainly based on reactivating the lymphocyte system, including immune checkpoint inhibitors, multifunctional antibodies, and adoptive cell therapy. Therefore, blocking the interactions between lymphocytes and LCSCs in combination with immunotherapy may be a promising therapeutic strategy. This review summarizes the interaction mechanisms of lymphocytes and LCSCs and the current exploration of combination therapy in HCC.

Keywords: Hepatocellular carcinoma, liver cancer stem cells, lymphocytes, immunotherapy, therapeutic resistance

INTRODUCTION

HCC is the most common type of primary liver cancer and a leading cause of cancer-related death worldwide[1]. HCC has a poor prognosis, and its 5-year overall survival (OS) rate is less than 20%, making it the second most lethal tumor after pancreatic cancer[2]. Early-stage HCC patients can largely improve 5-year OS rates through liver transplantation or radical surgery[3]. However, only 15% of HCC patients benefit from it, and most patients are diagnosed at an advanced stage[4]. Acquired drug resistance and tumor recurrence after treatment are the key factors leading to the poor prognosis of advanced HCC patients[5,6].

Cancer stem cells (CSCs) are a small subset of cancer cells with stem cell properties that play a major role in tumor growth, metastasis, recurrence, and resistance to therapy[7]. Liver cancer stem cells (LCSCs) often express biomarkers such as CD44, CD47, CD24, EpCAM, and CD133[8]. Targeting LCSCs therapy based on their specific biomarkers may be an effective strategy to eliminate tumor recurrence at the source. LCSCs are involved in the formation of an immunosuppressive microenvironment, altering or impairing the natural function of immune cells, thereby evading immune surveillance[9-13]. Mobilizing or reactivating the immune system can effectively eliminate cancer. Immunotherapy, especially the strategy for activating the immune activity of lymphocytes, is currently a promising tumor treatment[14]. Immune checkpoint inhibitors (ICIs), multifunctional antibodies and adoptive cell therapy are increasingly used for immunotherapy in the lymphocyte system, but the high drug resistance and relapse rate are still unsolved problems[15]. Combining immunotherapy and LCSCs targeted therapy may better solve the current problem. This review briefly summarizes the research on the mutual regulatory mechanism of lymphocytes and LCSCs and some explorations of combination therapy in HCC.

THE INTERACTIONS BETWEEN LYMPHOCYTES AND LCSCS

Lymphocytes, the main population targeted by immunotherapy, play an important role in the development and progression of HCC. LCSCs, although only a small fraction of HCC cells, possess the ability of self-renewal and tumor formation and are a key factor leading to poor prognosis[16]. This section describes the progress of research on the interactions of lymphocytes with LCSCs and non-LCSCs [Table 1 and Figure 1].

$CD8^+$ cytotoxic T lymphocytes

$CD8^+$ T cells are important lymphocytes in the tumor-adaptive immune response. $CD8^+$ T cells are modified into antigen-specific cytotoxic T lymphocytes (CTLs) via the T-cell receptor (TCR), which recognizes the major histocompatibility complex I (MHCI) on the antigen-presenting cells[17]. On the one hand, CTLs directly cause cell destruction by releasing cytotoxic substances such as granzyme, perforin and interferon γ (IFN-γ). On the other hand, $FasL^+$ CTLs bind to Fas receptors on tumor cells and trigger the caspase signaling pathway, which induces tumor cell apoptosis[18]. Recently, researchers have found that cytotoxic substances such as granzyme and perforin are not directly released around target cells. They are assembled with various proteins, including thrombospondin-1 (TSP-1), into supramolecular attack particles (SMAPs) that attach to the target cell membrane surface. SMAPs can prolong the cytocidal activity of CTLs. The glycoprotein coat TSP-1 of SMAPs is crucial to their ability to destroy cells. By interacting with the N-terminus of macrophage signal regulatory protein-α (SIRPα) on immune cells, CD47 on tumor cells prevents macrophages from phagocytosing them. TPS-1 can bind to the CD47 on tumor cells, which weakens the inhibitory effect of like macrophages and increases the long-lasting lethal power of SMAPs[19,20]. Generally, CTLs are the most important members for killing HCC cells. However, not all HCC cells are sensitive to the cytotoxicity of $CD8^+$ T cells. By upregulating PVRL1, HCC stabilizes the cancer cell surface poliovirus receptor, which interacts with T-cell immunoreceptor with Ig and ITIM domains (TIGIT) and simultaneously suppresses the antitumor immune response. The combination of PVRL1/TIGIT inhibitors with anti-PD1 effectively inhibits the development of HCC[21]. Moreover, in the immune microenvironment

Table 1. Interaction between lymphocytes and liver cancer stem cells

LCSCs	Molecule(s)	Activity of Lymphocytes	References
$SOX2^+$ LCSCs	PD-L1↑	TILs↓	[35,36]
$CK19^+$ LCSCs $SALL4^+$ LCSCs	PD-L1↑	TILs↓	[109]
$CD133^+$ LCSCs	Galectin-3↑	$CD8^+$ T cells↓	[134]
$CD44^+$ LCSCs	Histone macroH2A1↓	Tregs↑	[10]
$CD44^+$ LCSCs	TGF-β-miR-34a-CCL22↑	Tregs↑	[60,61]
GEP^+ LCSCs	MICA↑	NK↓	[12]
$EpCAM^+$ LCSC	CEACAM1↑	NK↓	[11]
$CD133^+$ LCSCs $SOX2^+$ LCSCs	HMBOX1↑	NK↑	[69]
$CD44^+$ LCSC	ceRNA CD44 3' UTR↑	NK↑	[70]

LCSCs: Liver cancer stem cells; PD-L1: programmed cell death ligand-1; TILs: tumor-infiltrating lymphocytes; CK19: cytokeratin 19; SALL4: Sal-like protein 4; Tregs: regulatory T cells; GEP: granulin-epithelin precursor; MICA: MHC class I-related chain A; NK: Natural killer; CEACAM1: carcinoembryonic antigen-associated cell adhesion molecule 1.

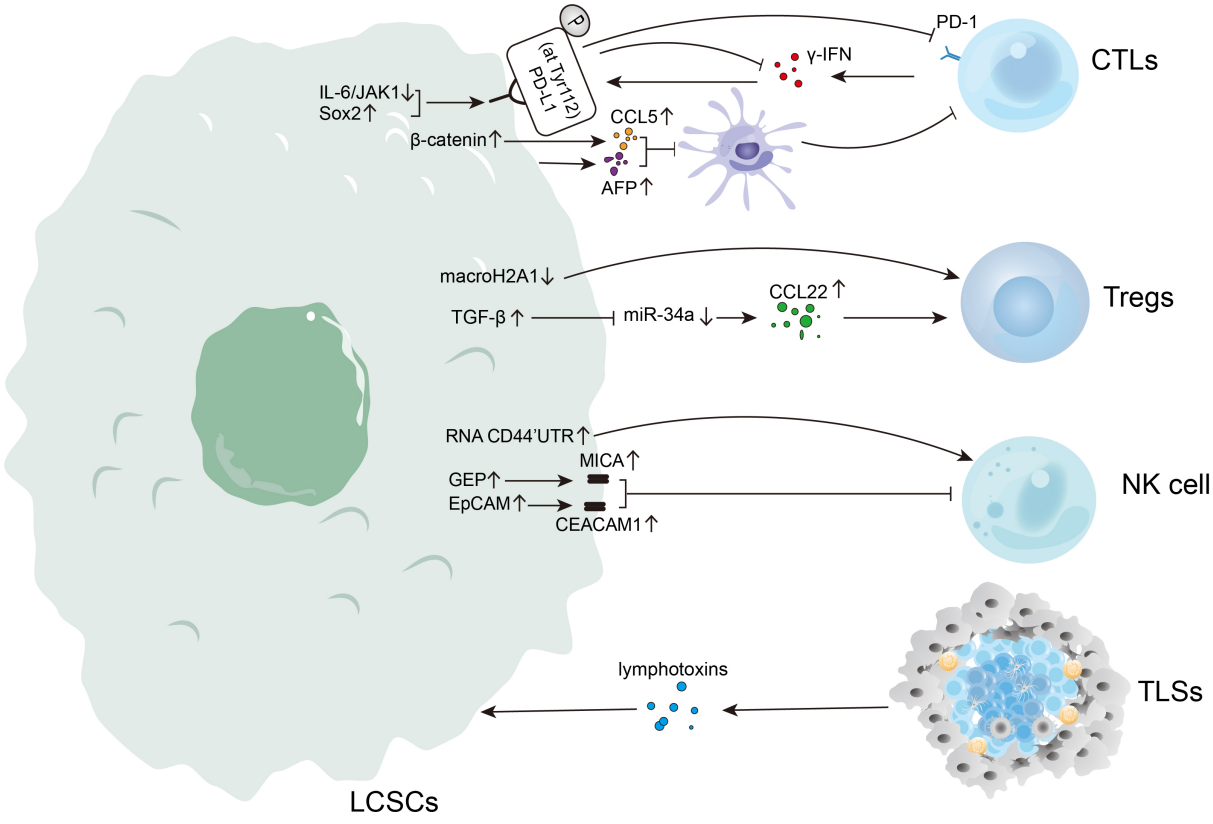

Figure 1. Liver cancer stem cells interact with lymphocytes.

of HCC, $CD8^+$ T cells express high levels of immune checkpoint proteins, such as programmed cell death-1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), which implies that these T cells enter a state of exhaustion with decreased antitumor activity[22].

Single-cell RNA-sequencing studies have revealed that the infiltrating T cells are characterized by an enrichment of regulatory T cells (Tregs) and exhausted $CD8^+$ T-cell clones. This landscape exemplifies an immunosuppressive microenvironment. 37% of exhausted $CD8^+$ T-cell clones shared TCR β chain with other $CD8^+$ T-cell clusters, suggesting that exhausted $CD8^+$ T cells were likely derived from other $CD8^+$ T cells in HCC microenvironment[23,24]. Subsequently, Wang *et al*. identified a T-cell exhaustion mechanism in which thymocyte selection-associated high mobility group box protein (TOX) in T cells promotes its own entry into a state of functional failure by stimulating the PD-1 endocytic cycle[25]. The latest study revealed that HCC patients with enrichment of severely exhausted $CD8^+$ T cells (TEX) had a lower survival rate, while those with a predominance of $CD103^+$ tissue-resident memory cells (TRM) had a higher survival rate. Dynamic changes in TEX and TRM affect the prognosis of HCC patients[26]. Therefore, reactivation of exhausted T-cell activity is important for treatment of HCC. Using antibodies to block immune checkpoints, including PD-1, CTLA-4, Lag-3, TIGIT and Tim-3, can reverse the function of exhausted T cells and restore the antitumor activity of tumor-infiltrating T cells[27,28]. Removing the inhibitory effect of immune microenvironment suppressor cells may also be a strategy to reshape the antitumor activity of T cells. Hypoxia-inducible factor-1α (HIF-1α) induces the expression of triggering receptor-1 expressed on myeloid cells-1 (TREM-1) in tumor-associated macrophages (TAMs), which reverses the activity of dysfunctional $CD8^+$ T cells and reduces resistance to programmed cell death ligand-1 (PD-L1) blockade[29]. Icaritin, an adjuvant, also inhibits myeloid-derived suppressor cells (MDSCs) to improve HCC therapeutic efficacy[30].

CSCs are one of the important factors contributing to immunosuppression. Reduction of MHC-I expression on CSCs suppresses CTL-mediated tumor killing[31]. Meanwhile, LCSCs recruit M2-type macrophages by activating Yes-associated protein to evade immune clearance[32]. Compared to non-LCSCs, LCSCs express more PD-L1 and show stronger immunosuppressive effects. In addition to generating negative regulatory signals to $PD\text{-}1^+$ effector T cells, PD-L1 derived from tumor cells also releases signals into themselves that enhance the expression of stemness-associated genes like cytokeratin 19 (CK19) and regulate LCSCs number and function[33,34]. Several studies have revealed that microenvironmental regulatory factors can upregulate PD-L1 expression on LCSCs to support their growth. On the one hand, the transcription factor Sox2 enhances PD-L1 transcriptional activity by binding to the PD-L1 promoter region to promote LCSCs survival[35,36]. On the other hand, the IL-6/JAK1 signaling pathway drives PD-L1 Y112 phosphorylation, which recruits the endoplasmic reticulum-associated N-glycosyltransferase STT3A to catalyze PD-L1 glycosylation and maintain PD-L1 stability[37]. Upregulation of PD-L1 expression impairs IFN-γ secretion from CTLs by a negative feedback regulatory mechanism, enhancing the ability of LCSCs to protect against T-cell killing and immune evasion[38]. Therefore, targeting the above sites, such as Sox2 combined with anti-PD-L1, may contribute to antitumor therapy.

Dendritic cells (DCs) pulsed with total HCC RNA induce effector T lymphocyte activation. Activated effector T lymphocytes enhance the ability to kill tumors and secrete more IFN-γ[39,40]. LCSCs can inhibit cytotoxic T-cell activity by inhibiting the recruitment of DCs and promoting tumor cell growth. $EpCAM^+/AFP^+$ LCSCs have the ability to self-renew and differentiate, regulated by Wnt/β-catenin signaling, and are capable of triggering highly aggressive HCC in NOD/SCID mice[41]. β-catenin activation in HCC cells impairs the recruitment of $CD103^+$ DCs by downregulating the expression of the chemokine CCL5. This defective DC recruitment impairs the function of liver antigen-specific $CD8^+$ T cells, promoting immune evasion and suppressing anti-PD-L1 efficacy[42]. In addition, the $EpCAM^+/AFP^+$ LCSCs secreted AFP into the tumor microenvironment, which significantly inhibited DCs differentiation. These immature DCs reduce inflammatory mediator levels and fail to induce a robust T-cell proliferative response[43]. Researchers indirectly enhanced the killing effect of CTLs on LCSCs by modifying DCs. Annexin A3

(ANXA3) may regulate the activity of LCSCs through the HIF-1α/Notch pathway. More functionally active T cells were induced by ANXA3-transfected DCs, and these effector T cells can specifically recognize and kill $CD133^+$ LCSCs both *in vitro* and *in vivo*[44]. These studies suggested the multiple interactions between the LCSCs and the tumor immune microenvironment.

Regulatory T Cells (Tregs)

Tregs are a subpopulation of $CD4^+$ T cells that are characterized by the expression of CD25 and Foxp3[45,46]. Tregs are the main subpopulations that maintain the body's immune tolerance to tumors. Tregs derived from peripheral blood and tumor-infiltrating lymphocytes (TILs) of patients with HCC are increasing and are more inhibited than those derived from normal subjects[47,48]. An elevated level of Tregs in the tumor microenvironment is associated with poor prognosis[49]. Mechanistically, intratumor Tregs upregulate the expression of glucocorticoid-induced tumor necrosis factor receptors (GITRs) and inhibit tumor-specific T-cell activity[50,51]. In addition, Tregs stimulate their own differentiation and promote HCC immune escape via the upregulation of lnc-EGFR[52].

Tregs interact with other immune cells via cell contact and cytokine secretion to inhibit their activity and thus participate in the formation of the immunosuppressive tumor microenvironment. For example, Tregs mediate the loss of the human leukocyte antigen-DR isotype (HLA-DR) on type 2 conventional dendritic cells (cDC2s), which impairs DC antigen presentation and thus suppresses antitumor immunity in HCC[53]. Indeed, HCC cells can also secrete cytokines or nutrients to maintain and enhance the immunosuppression of Tregs. Mechanistically, HCC-derived growth differentiation factor 15 (GDF15), by recognizing the receptor CD48, promotes the production of induced Tregs and enhances the suppressive function of natural Tregs, which induces tumor immunosuppression[54]. In addition, tumors phagocytose nutrients that are necessary for effector T cells to exert cytotoxicity while flushing out lactate to provide nutrients to Tregs. Tregs use the lactate transporter protein monocarboxylate transporter 1 (MCT1) to convert lactate into energy and maintain a state of tumor immune tolerance[55].

Both CSCs and Tregs are responsible for tumor immune tolerance and tumor recurrence. The preoperative peripheral blood quantity of $EpCAM^+$ circulating tumor cells and Tregs is positively correlated with the risk of postoperative recurrence and metastasis in HCC patients[56]. The interactions between CSCs and Tregs further lead to tumor immunosuppression. Enriched $LGR5^+$ LCSCs are associated with poor prognosis in HCC, whereas Tregs increased LGR5 of CSCs expression in gastric cancer through TGF-β1[57-59]. Tregs and $LGR5^+$ CSCs may also be associated in HCC. For instance, $CD44^+$ LCSCs regulate TGF-β1 expression, and TGF-β1 has been shown to recruit Tregs through the miR-34a-CCL22 axis, which promotes immune escape[60,61]. In addition, downregulation of histone macroH2A1 in HCC cells increases LCSCs and $CD4^+/CD25^+/FoxP3^+$ Tregs proliferation[10].

Natural killer cells

Natural killer (NK) cells, a subpopulation of normal human peripheral blood lymphocytes, can nonspecifically kill tumor cells. This natural killing activity requires neither specific antigen nor MHC restriction. NK cells express a large number of immune recognition receptors, including kill-activated receptors (KARs) and inhibitory receptors, to recognize ligands on normal cells and tumor cells, thus maintaining a balance between the immune response and immune tolerance of NK cells. Correspondingly, abnormal NK cell receptors could promote HCC development[62].

Natural killer group 2D (NKG2D) is a typical KAR on the surface of NK cells, and its expression plays a significant role in the innate immunity of NK cells against the malignant transformation of HCC[63]. Increased expression of the soluble form of MHC class I-related chain A (MICA) in patients with advanced

HCC correlates with downregulation of NKG2D expression and impairs NK-cell function[64]. However, recent studies have found that activation of the NKG2D system perhaps contributes to a strong inflammatory response that exacerbates liver tissue damage. Cadoux *et al.* discovered for the first time that NKG2D ligands MICA/B and ULBP1/2 are linked with poor prognosis and early tumor recurrence in HCC[65]. In addition, β-catenin signaling downregulates the expression of murine NKG2D ligands Rae-1 through binding to TCF4, which attenuates the invasive capacity of HCC[65]. The above findings suggest that targeting NK cells to treat HCC is a double-edged sword. We should pay attention to indicators of liver injury and inflammatory response in the treatment.

Cytokine-induced killer (CIK) cells recognize and kill LCSCs via the NKG2D system. Intravenous infusion of CIK cells can significantly retard tumor growth[66]. However, LCSCs can attenuate the toxicity of NK cells by synthesizing inhibitory proteins, such as granulin-epithelin precursor (GEP). GEP is a hallmark of the LCSCs and functionally increases the stemness of CSCs by regulating the expression of stemness-associated signaling molecules, including β-catenin, Oct4, Nanog and Sox2[67]. In addition, GEP confers the ability of HCC cells to evade NK cytotoxicity by regulating MICA expression[12]. Another study found that $EpCAM^{High}$ LCSCs also resisted NK-cell-mediated cytotoxicity by upregulating carcinoembryonic antigen-associated cell adhesion molecule 1 (CEACAM1)[11]. Restoring the sensitivity of LCSCs to NK-cell-mediated cytotoxicity can enhance innate immune suppression in HCC. Blocking the expression of GEP and CEACAM1 restores the natural killing activity of NK cells[11,12,68]. In addition, LCSCs overexpressing HMBOX1 suppress their self-renewal and improve NK-cell-mediated antitumor immune responses[69]. Recently, researchers also found that LCSCs enhance their own sensitivity to NK cells by upregulating the competitive endogenous RNA (ceRNA) CD44 3' UTR[70].

Tertiary lymphoid structures

Tertiary lymphoid structures (TLSs) are classically defined as lymphoid aggregates formed in nonhematopoietic organs. TLSs do not exist under physiological conditions but are formed during chronic and non-resolving inflammatory processes such as infection, transplant rejection, autoimmune diseases and cancer[71]. TLSs are characterized by $CD20^+$ B surrounded by $CD3^+$ T-cell structures, similar to the lymphoid follicles in secondary lymphoid organs. In addition, TLSs contain components such as distinct DC populations, dense stromal networks, and specialized vascular systems provided by peripheral node addressin (PNAd)-positive high endothelial venules (HEVs) that are thought to mediate lymphocyte recruitment[72].

TLSs are involved in antigen-specific antitumor immune responses mainly by promoting the induction of effector T cells, central memory T cells and plasma cells. In almost all solid tumors, TLSs are associated with a reduction in recurrence risk and an improvement in survival rate[73]. However, the role of TLSs in the pathogenesis of HCC is controversial, as they may promote the growth of tumor progenitor cells. Finkin *et al.* found that patients with abundant TLSs after tumor excision were more likely to have late recurrence and death. In the $IKK\beta(EE)^{Hep}$ model, NF-κB activation induces TLSs formation[74]. This model provided the first functional information for TLSs, showing that they support LCSCs growth by activating and secreting protumor cytokines such as lymphotoxins[74]. This is also the first study to reveal the relationship between TLSs and LCSCs. On the other hand, Calderaro *et al.* analyzed data from patients with HCC undergoing surgical resection from the pathological and public database Liver Cancer Institute (LCI) and found that intratumor TLSs were associated with a lower risk of early recurrence[75]. Intratumor TLSs may reflect the presence of sustained and effective antitumor immunity. Wu *et al.* first revealed the heterogeneity of TLS function across spatial locations[76]. The cellular scoring and indicator gene expression levels of $CD8^+$ central memory T cells and $CD8^+$ effector memory T cells correlate with the distance from TLSs to tumor cells, indicating that TLSs function is influenced by tumor cells to some extent. These comparative data show that

TLSs and LCSCs interact and constantly reach a new balance, thus promoting the occurrence and development of HCC to reach a new balance. PD-L1/PD-1 checkpoint inhibitors can enhance the tumor-killing effect of T cells. Further understanding of the mechanism of action of TLSs on LCSCs may improve the effectiveness of immunotherapy in HCC.

COMBINATION OF IMMUNOTHERAPY AND TARGETING LCSCS

Multifunctional antibody against LCSCs

Several LCSCs-specific markers have been directly discovered, such as CD24, CD44, CD47, CD90, CD133, EpCAM and ANXA-3. Antibody-mediated targeted therapeutic strategies can effectively target CSC subpopulations and inhibit tumor growth or recurrence [Table 2]. CD44 is a surface feature of CSCs, and its expression level correlates with poor patient prognosis. Anti-CD44 antibody effectively eliminates CSCs from tumors and prevents pancreatic cancer tumor formation and tumor recurrence after radiotherapy[77]. Under low glucose conditions, CD133 antibody induces apoptosis of LCSCs by inhibiting autophagy and enhancing chemotherapeutic efficacy[78]. IgG1-iS18, an antibody targeting the 37 kDa/67 kDa laminin receptor (LRP/LR), impairs the adhesion and invasive ability of HCC cells and is a strategy for the treatment of metastatic HCC[79]. Antibody 1B50-1, which targets calcium channel α2δ1, reduces the self-renewal and tumorigenic capacity of LCSCs by affecting intracellular calcium signaling, thereby inhibiting HCC recurrence[80]. An anti-ANXA-3 antibody blocks the MKK4/JNK pathway in $CD133^+$ LCSCs, attenuating cell self-renewal and inhibiting tumor growth[81]. When combined with sorafenib, the antitumor effect of anti-ANXA-3 antibodies was better. In addition, ANXA-3 can stratify resistance to sorafenib, which is beneficial to help HCC patients develop better treatment plans on their own[82]. Anti-Dickkopf-1 (DKK-1) inhibits angiogenesis and cancer cell proliferation *in vitro* and suppresses LCSCs growth *in vivo*[83].

The interaction between CD47 and SIRPα inhibits the phagocytosis of CSCs by macrophages[84,85]. By blocking the binding of CD47 and SIRPα, an anti-CD47 antibody restores the phagocytic activity of macrophages and inhibits HCC tumor growth[86]. Lo *et al.* found that treatment with chemotherapeutic drugs or sorafenib in combination with an anti-CD47 antibody reduced the occurrence of drug resistance and enhanced the antitumor effect of the drugs[87,88]. Recently, Du *et al.* invented a bispecific antibody targeting the HCC-associated antigen Glypican-3 (GPC3) and CD47, which could inhibit HCC development by enhancing the innate immune response involving macrophages and neutrophils[89]. In addition, blockade of CD47 can increase microenvironmental $CD8^+$ cytotoxic T-cell infiltration as well as enhance tumor cell sensitivity to radiation therapy[90]. Anti-CD47 antibodies combined with T-cell immune checkpoint inhibitors may be an effective therapeutic strategy to achieve stronger antitumor benefits by exploiting the respective advantages.

Antibody-drug couples use specific monoclonal antibodies to transport cytotoxic agents, which can selectively kill tumor cells[91]. He *et al.* designed a CD24-targeting monoclonal antibody, G7 mAb, and used its combination drug to target LCSCs[92]. G7 mAb-conjugated doxorubicin (Dox) was shown to effectively inhibit tumor growth with less systemic toxicity in tumor-bearing nude mice[93]. G7 mAb can also form an immunoconjugate targeting $CD24^+$ HCC with NO donor HL-12. This immunoconjugate not only induces apoptosis of LCSCs via antibody-dependent cytotoxicity, but also inhibits tumor growth by upregulating intratumor NO levels, exerting effective antitumor effects *in vivo* and *in vitro*[94].

Antibodies can also be conjugated to biomaterials such as nanoparticles or thermosensitive liposomes to achieve local-specific imaging and elimination of tumors. Anti-CD44 antibody-conjugated liposomal nanoparticles can specifically deliver the chemotherapeutic drug Dox or killer genes to target cells and induce their apoptosis. Such nanoparticles can also be combined with bioluminescence imaging to quantify

Table 2. Combination therapy of immunotherapy and stem cells targeting

Therapeutic setting	Target cell	Molecule(s)	References
Anti-CD133 antibody	LCSCs	CD133	[78]
Antibody(1B50-1)	LCSCs	α2δ1	[80]
Anti-ANXA-3 antibody	LCSCs	ANXA-3	[81]
Anti-Dickkopf-1 antibody	LCSCs	Dickkopf-1	[83]
Antibody(G7mAb)	LCSCs	CD24	[92]
G7mAb-conjugated doxorubicin	LCSCs	CD24	[93]
G7mAb-conjugated HL-12	LCSCs	CD24	[94]
Anti-CD44 antibody-targeted liposomal nanoparticles	LCSCs	CD44	[95]
Anti-CD44mAb-conjugated $Nd3^+$-doped UCNPs	LCSCs	CD44	[96]
CD90@TMs	LCSCs	CD90	[97,98]
CD90@17-AAG	LCSCs	CD90	[98]
Anti-EpCAM-UPGs-MX	LCSCs	EpCAM	[99]
Anti-GEPmAb (A23)	LCSCs	GEP	[11]
Anti-CEACAM1 antibody	LCSCs	GEP	[68]
Bispecific antibody(cG7-MICA)	NK/LCSCs	NKG2D/CD24	[102]
CAR-T	LCSCs	CD44	[125]
CAR-T	LCSCs	CD133	[127]
EpCAM/CD3 bispecific antibody	T-cell/LCSCs	CD3/EpCAM	[136]

UCNPs: Upconversion nanoparticles; TMs: thermosensitive magnetoliposomes; CAR-T: chimeric antigen receptor-specific T.

the effect of *in vivo* killer gene therapy, thereby dynamically monitoring the tumor treatment process[95]. $Nd3^+$-doped core-shell upconversion nanoparticles (Nd-CSUCNPs) are a novel multimodal imaging reporter combining magnetic resonance (MR) and real-time upconversion luminescence (UCL). Anti-CD44 antibody conjugated with Nd-CSUCNP can deliver the imaging tool to HCC and improve the accuracy of preoperative multimodal imaging-guided HCC surgical resection[96]. Thermosensitive magnetoliposomes (TMs) loaded with anti-CD90 antibodies (CD90@TMs), which are transported to LCSCs in a targeted manner, effectively kill $CD90^+$ LCSCs by magnetic hyperthermia therapy[97]. Heat shock protein (HSP) inhibitors encapsulated in thermosensitive magnetic liposomes make LCSCs continuously sensitive to hyperthermia and then induce apoptosis[98]. Han *et al.* invented a nanoparticle micelle combining mitoxantrone (MX) and anti-EpCAM antibody, which can synergistically treat $EpCAM^+$ HCC cells with drugs and optical targeting. Nanoparticle micelles can be used for MR/UCL dual-mode imaging, which can help to diagnose HCC more accurately[99]. A variety of specific antibodies have been shown to have good antitumor effects in preclinical trials, but their application in the clinical setting needs to be further explored. On this basis, the application of first-line drugs for the clinical treatment of HCC, such as sorafenib combined with LCSCs-specific antibodies, may be a promising therapeutic strategy. The combination therapy now seems to be effective, at least in preclinical trials, greatly inhibiting tumor growth and recurrence.

Lymphocytes targeting LCSCs

NK cells

NK cells protect the organism that produces them from damage while recognizing and killing harmful foreign substances such as viruses and tumors by expressing a series of activated and inhibitory receptors[100]. A preclinical trial found that the NK-cell-activated receptor ligands MICA/MICB, Fas, and DR5 were upregulated in CSCs, suggesting that NK cells preferentially target cancer stem cells[101]. Enhancing the strength and targeting of NK cells to kill CSCs may be an effective therapeutic strategy. Several studies have shown that NK cells can effectively kill LCSCs through antibody-dependent cell-

mediated cytotoxicity (ADCC). As previously described, both GEP and CEACAM1 are upregulated in LCSCs[67]. Anti-GEP monoclonal antibody A23 or anti-CEACAM1 antibody can enhance the killing activity of NK cells and promote tumor regression[11,68]. cG7-MICA, a bispecific antibody against CD24 and NKG2D, recruits NK cells to the periphery of CSCs and promotes the release of IFN-γ and TNF-α from NK cells. In addition, cG7-MICA can enhance the inhibitory effect of sorafenib on tumors, suggesting that cG7-MICA can be used as an adjuvant therapy to alleviate the problem of sorafenib resistance[102].

The infusion of autologous or allogeneic NK cells after activation, culture and expansion *in vitro* can break the immune tolerance of the body and enhance the antitumor immune response[103]. The NKG2D-CD3ζ-DAP10 receptor, which is made up of the NK cell activation component NKG2D and two important signaling molecules, DAP10 and CD3ζ, can improve NK cell killing capabilities against cancer cells[104]. Kamiya *et al.* used the K562-mb15-41BBL cell line to stimulate NK-cell activation and expansion *in vitro* and then reinfused NK cells into mice. Activated NK cells significantly inhibited the growth of HCC, and the chimeric NKG2D-CD3ζ-DAP10 receptor could greatly enhance the antitumor activity of NK cells[105]. NK cells can be used as CAR carriers to form CSC-specific CAR-NK cells. CAR-NK cells retain the ability of NK cells to target abnormal cells through natural recognition receptors and the ability of CAR to target antigens, effectively killing cells and inhibiting cell escape[106]. In addition, NK cells do not need to be matched with patients. Therefore, they can be derived from a variety of sources, including pluripotent stem cells (PSCs) and peripheral blood mononuclear cells (PBMCs), which makes CAR-NK-cell therapy safer and more affordable[106,107]. Relying on CSCs antigen markers to modify CAR is beneficial to NK cells to inhibit the immune escape of CSCs.

T cells

Immune checkpoint

ICI therapy is an important approach in current immunotherapy, especially targeting the PD-1/PD-L1 pathways. Calderaro *et al.* found that PD-L1 was highly expressed in a subset of LCSCs[108]. Nishida *et al.* also demonstrated that PD-L1 expression in HCC was positively correlated with the presence of tumor stem cell markers, including cytokeratin 19 (CK19) and Sal-like protein 4 (SALL4)[34]. In addition, the accumulation of PD-L1 on CSCs promotes the occurrence of immune evasion[109]. Considering that CSCs induce CTLs apoptosis by binding to PD-1, immune checkpoint inhibitors may indirectly inhibit the growth of CSCs. Atezolizumab selectively targets PD-L1, restoring T cells' ability to destroy tumor cells. The Food and Drug Administration (FAD) authorized the combination immunotherapy of atezolizumab and bevacizumab for the treatment of hepatocellular cancer in 2020. The combined drug group outperformed the sorafenib group in terms of OS rates, progression-free survival, and objective response rate in the Phase III clinical study (NCT03434379)[110]. Single ICI immunotherapy improved OS rates but did not reach prespecified statistical significance[111,112]. Combining ICIs with other treatments may be more effective and have more durable responses[113]. Antiangiogenic drugs can improve the microenvironment of solid tumors and enhance the sensitivity of ICI therapy to tumors, thereby improving their efficacy. Several clinical trials (NCT03434379, NCT03006926, NCT03794440, NCT03463876, *etc.*) have shown that anti-PD-1/PD-L1 inhibitors combined with antiangiogenic drugs have good tumor effects and prolong the survival of HCC patients[114-118].

An increased frequency of mutations in genes involved in the phosphatidyl inositol 3-kinase (PI3K)-Akt pathway was also observed in HCC with high PD-L1 expression[34]. In glioma, loss of phosphatase and tensin homolog (PTEN) function and activation of the PI3K pathway lead to upregulation of PD-L1 in tumor cells[119]. Correspondingly, downregulation of PTEN and upregulation of PI3KCA in HCC increased

the expression of LCSC markers, such as CK19 and CD133[120]. Therefore, activation of the PI3K-Akt pathway may lead to PD-L1 overexpression and increased tumor self-renewal. Activation of the PI3K-Akt pathway is one of the hallmarks of CSCs, and PI3K inhibitors preferentially reduce the levels of CSCs in tumors[121]. Therefore, the combined targeting of PI3K-Akt pathway inhibitors and anti-PD-1/PD-L1 inhibitors may help suppress HCC growth, especially the LCSCs. This treatment may be a promising strategy for relapse-prone HCC patients, which needs to be confirmed by further preclinical studies.

Chimeric antigen receptor-specific T (CAR-T) cells

CAR-T cells express specific antigen receptors and recognize tumor-associated antigens (TAAs), thereby targeting tumor areas to perform cytotoxic T-cell functions. CAR-T-cell therapies targeting CD19 are efficient against hematological tumors, so the FDA has approved two related CAR-T products[122,123]. However, for solid tumors, CAR-T-cell efficacy still faces many challenges, such as virus-related risks and appropriate targets[124]. CSCs have the ability of self-renewal and differentiation and play an important role in tumor initiation and recurrence. Therefore, an increasing number of studies use markers of CSCs as TAAs recognized by CAR-T cells. After CAR-T cells targeting $CD44^+$ LCSCs were infused into $CD44^+$ HCC xenograft mice, they accumulated in $CD44^+$ tumor regions and effectively inhibited tumor growth[125]. NKG2D-BBz CAR-T cells can effectively eliminate $NKG2DL^+$ HCC cells *in vitro* and *in vivo*[126]. A phase I clinical trial involving $CD133^+$ advanced metastatic malignancies (NCT02541370) revealed that CD133-specific CAR-T cells (CART-133) were a feasible and effective therapeutic strategy to stabilize the disease and prolong patient survival[127].

Tumor heterogeneity is a key factor affecting the efficacy of CAR-T therapy. Biopsy specimens from two patients revealed that CART-133 effectively eliminated $CD133^+$ tumor cells, but some $CD133^-$ tumor cells that were not eliminated may cause tumor progression and recurrence[127]. This suggests that it is essential to inhibit the rapid growth of antigen-negative cells while improving the efficacy of CAR-T targeting antigen-positive cells. A phase I clinical trial (NCT02414269) revealed that the efficacy of CAR-T cells in the treatment of malignant pleural disease can be enhanced with the help of anti-PD-1 drug[128]. PD-1 blockade works through endogenous tumor-specific T cells, potentially eliminating antigen-negative cells left behind after CAR-T-cell treatment. The combination of the two complementary advantages is a promising antitumor strategy[129].

The limited expansion of CAR-T cells *in vivo* is also one of the important factors affecting their antitumor efficacy[130,131]. Coexpression of cytokines enhances the ability of CAR-T cells to persist against tumors *in vivo*. Batra *et al.* found that coexpression of IL15 and IL21 enhanced the antitumor strength and persistence of GP3-CAR T cells. Two clinical trials (NCT02932956 and NCT02905188) are exploring the therapeutic efficacy of this modified GP3-CAR T biohybrid in HCC patients[131]. Another clinical trial (NCT03198546) showed that CAR-T cells with upregulated IL-7 and CCL19 expression increased patient survival[132,133]. Hence, modification of CSC-specific CAR-T cells by coexpressing cytokines may enhance their efficacies for patients with HCC.

Other approaches

Therapeutic modalities such as small molecule inhibitors, bispecific antibodies, and cancer vaccines can also enhance the immune response of cytotoxic T cells to tumors. LDN193189, a small molecule compound, relieves the inhibition of Galectin-3 on the proliferation activity of $CD8^+$ T cells by downregulating the expression of Galectin-3 in CSCs, thereby inhibiting the immune evasion ability of LCSCs[134]. Bispecific

antibodies can simultaneously bind to two different specific antigens, such as TAAs of tumor cells and CD3 of T cells, which facilitates the accumulation of cytotoxic T cells at the tumor site. Anti-GPC3/CD3 bispecific antibodies rely on GPC3 to recruit cytotoxic T cells to the HCC xenograft region and effectively inhibit tumor growth[135]. Zhang *et al.* developed a bispecific antibody targeting EpCAM and CD3-specific antigens that induced strong cytotoxicity and inhibited the expression of stemness-associated genes in LCSCs. An EpCAM/CD3 bispecific antibody significantly inhibited the growth of HCC xenografts *in vivo*[136]. Catumaxomab, a trifunctional receptor targeting EpCAM and CD3, enhances antitumor activity by recruiting T cells and Fcγ receptor (FcγR)-positive helper cells to tumor sites. Several clinical studies have shown that catumaxomab can effectively remove tumor cells and prevent ascites accumulation in patients with ovarian cancer and gastric cancer[137,138]. Furthermore, compared with paracentesis alone, catumaxomab can prolong the survival time and maintain a better quality of life in malignant patients[139]. Catumaxomab has a good effect in the prevention and treatment of malignant patients, but its efficacy in solid tumors, including HCC, needs to be further explored in clinical trials.

Pulsed DCs carrying specific antigens can induce the activation of naive T lymphocytes to generate tumor cell antigen-specific CTLs, thereby inhibiting tumor growth in a targeted manner[140]. DCs loaded with $CD133^+$ LCSCs RNA stimulate specific CTL proliferation and IFN-γ secretion *in vivo* and effectively inhibit tumor growth[141]. Choi *et al.* demonstrated that using EpCAM peptides as loaders for DC vaccines can effectively promote specific CTL activation and kill $EpCAM^+$ LCSCs[142]. In addition to using proteins and nucleic acids as loaders for DC vaccines, fusion of LCSCs and DCs is also a potential strategy that may effectively activate multiple antigen-specific CILs for the immune response. Pang *et al.* revealed that $CD90^+$ HepG2/DC fusion cells upregulate T lymphocyte-mediated specific antitumor immune responses both *in vivo* and *ex vivo*[143]. Attenuated listeria monocytogenes (LM) is also a promising tumor vaccine vector that induces the activation and proliferation of antigen-specific T lymphocytes *in vivo* by upregulating the MHC-I and MHC-II pathways. Yang *et al.* prepared a novel cancer vaccine using LM replication-deficient LMΔdalΔdat strain-loaded CD24, which can reduce the number of Tregs in tumor TILs and enhance the activity of specific $CD8^+$ T cells[144]. This vaccine effectively inhibits tumor growth by altering the balance between cells in the immunosuppressive tumor microenvironment and is a promising therapeutic tool. Cancer vaccines targeting CSCs produce antigen-specific CTLs *in vivo* and potently kill LCSCs. This therapeutic strategy may be effective in killing tumors and inhibiting tumor recurrence, and its efficacy needs to be further explored in the clinic.

PROSPECTIVE AND CONCLUSION

In the process of antitumor immunity, lymphocytes play an important role in eliminating tumors. CSCs are currently recognized as a key factor in tumor heterogeneity and tumor recurrence. During HCC progression, LCSCs participate in the formation of an immunosuppressive microenvironment by damaging or altering lymphocyte phenotypes, thereby evading immune surveillance. Understanding the interaction mechanisms between lymphocytes and LCSCs and reversing the immunosuppressive microenvironment could help develop more effective treatment strategies.

With the development of technologies such as single-cell sequencing and lineage tracing, we have the opportunity to further explore the origin of lymphocytes and CSCs in the tumor immune microenvironment and their interaction mechanisms in tumorigenesis and development. The current strategy of immunotherapy is mainly to reshape the immune function of lymphocytes, including immune checkpoint inhibitor therapy, CAR-T therapy, multifunctional antibodies, *etc.* Although immunotherapy has a good effect, high drug resistance and recurrence rates are still problems that need to be solved. Combination immunotherapy and other treatments, such as antiangiogenic drugs, can effectively improve

the treatment effect and prolong the survival time of patients. Immunotherapy targeting LCSCs is also a promising treatment modality. This strategy not only kills tumors but also inhibits tumor recurrence in a targeted manner. At present, some preclinical experiments have shown that immunotherapy targeting LCSCs has good antitumor effects and low toxicity. For instance, anti-ANXA3 antibodies can block $CD133^+$ LCSCs' capacity for self-renewal and slow the growth of tumors, with no overtly harmful side effects reported[80,81]. Furthermore, anti-CD24 mAb-conjugated Dox can considerably increase the longevity of Huh7 tumor-bearing mice. Analysis of the in vitro activity of tumor cell proliferation revealed that anti-CD24 mAb-conjugated Dox specifically suppressed the proliferation of Huh7 and HT29 cells under comparable conditions, but had no appreciable inhibitory effect on HCT116 cells[93]. Of course, more research is needed to confirm its combined treatment effect and its safety. The combined treatment is predicted to benefit liver cancer patients when the mechanism is further explored. In addition, there is great heterogeneity between tumors in different patients, and even within the same patient, there is great heterogeneity within tumors. Therefore, which subgroup of LCSCs to target is also one of the issues to be considered. Moreover, antiangiogenic drugs, epigenetic modifiers, oncolytic virotherapy and phage therapy have been shown to have better tumor-killing effects in combination with immune checkpoint inhibitors[145-148]. Therefore, integrating the advantages of the above combination therapy into therapy targeting LCSCs may be a therapeutic direction worth exploring in HCC.

DECLARATIONS

Authors' contribution

Wrote the manuscript: Chen WM, Zhang XP
Assistance : Sun X, Yan YY
Supervised the project: Li L
All the authors have read and approved the final manuscript.

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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