Acta Pharmaceutica Sinica B 2021;11(7):1721–1739

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Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

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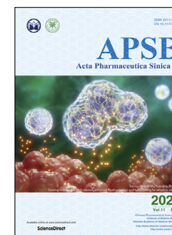

REVIEW

Cancer stem cell-targeted chimeric antigen receptor (CAR)-T cell therapy: Challenges and prospects

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Received 13 May 2020; received in revised form 3 August 2020; accepted 14 September 2020

KEY WORDS

Chimeric antigen receptor T cell;
Cancer stem cell;
Immunotherapy;
Tumor associated antigens;
Combination therapy;
Off-tumor toxicity;

Abstract Cancer stem cells (CSCs) with their self-renewal ability are accepted as cells which initiate tumors. CSCs are regarded as interesting targets for novel anticancer therapeutic agents because of their association with tumor recurrence and resistance to conventional therapies, including radiotherapy and chemotherapy. Chimeric antigen receptor (CAR)-T cells are engineered T cells which express an artificial receptor specific for tumor associated antigens (TAAs) by which they accurately target and kill cancer cells. In recent years, CAR-T cell therapy has shown more efficiency in cancer treatment, particularly regarding blood cancers. The expression of specific markers such as TAAs on CSCs in varied cancer types makes them as potent tools for CAR-T cell therapy. Here we review the CSC markers that have been previously targeted with CAR-T cells, as well as the CSC markers that may be used as possible targets for

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Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2020.12.015
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Clinical trial;
Tumor immunosuppressive microenvironment

CAR-T cell therapy in the future. Furthermore, we will detail the most important obstacles against CAR-T cell therapy and suggest solutions.

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1. Introduction

Cancer stem cells (CSCs) can be categorized as self-renewal cells with tumor initiation ability and high proliferative potential that firstly were identified in acute myeloid leukemia (AML). They have the property of being divided into other CSCs and heterogeneous differentiated cancer cells with limited proliferative potential[1]. It has been believed that CSCs are derived from normal stem cells with mutational transformation. Another theory says that mutation in normal stem cells may cause restricted progenitor or differentiated cells that can acquire characteristics of the CSCs[2]. Involving CSCs in stimulating the tumor growth, metastasis and angiogenesis have been revealed in various cancers. Hermann and colleagues in their study recognized a population of pancreatic CSCs with CD133 and CXCR4 markers which were essential for tumor metastasis[3]. Bao et al.[4] have found that glioma CSCs in comparison to non-CSCs produce elevated amounts of the vascular endothelial growth factor (VEGF) which promote angiogenesis *in vitro* and *in vivo*. Recent studies have suggested the footprint of the CSCs in treatment failure in different types of cancer. CSCs relate with tumor recurrence and patients' short survival after therapies like radiation and chemotherapy. There are evidences that CSCs are resistant to many cancer drugs and therapies, and this phenomenon is because of their stemness determinants[5]. Quiescence, expression of several ATP binding cassette (ABC) transporters, high expression of anti-apoptotic proteins, ability for DNA damage repair, and signaling pathways dealing with CSCs self-renewal like NOTCH, Hedgehog and WNT/β-catenin are the most important factors in the resistance of CSCs to cancer drugs[1,6]. The most common ABC transporters responsible for drug resistance in CSCs are ABCG2, ABCB1, and ABCC1[7]. In recent years, ABC transporter inhibitors, despite their side effects, have been used in various cancers that most of them had shown low clinical benefits[8]. In breast, hepatocellular carcinoma (HCC) and glioblastoma CSCs, an elevated expression of the anti-apoptotic proteins like BCL-xL, BCL-2 and AKT has been reported which are responsible for resistance against anti-cancer therapies[9,10]. Bao et al.[11] have shown that CD133^{+} glioma CSCs separated from both primary patient glioblastoma samples and human glioma xenografts, in response to radiotherapy, activated the DNA damage checkpoints prominently, and repaired the DNA damage related to radiation more efficiently than CD133^{-} tumor cells. Given that, CSCs share the most similarity with normal stem cells, therefore, most of the conventional therapeutic strategies can also interrupt the function of normal stem cells as well as those of the CSCs. Accordingly, new therapeutic strategies are needed for the specific elimination of CSCs in a variety of cancers.

Chimeric antigen receptor (CAR)-T cells are engineered T cells dedicated to eradicate tumors by recognizing tumor associated antigens (TAAs) on cancer cells and, subsequently inducing the release of cytotoxic cytokines, perforin and granzyme[12]. CARs, unlike conventional T cell receptors (TCRs), are not limited to major histocompatibility complex (MHC), so they can recognize the tumor antigens independently[13]. CARs are composed of a single-chain variable fragment (scFv) specific for a tumor antigen as an extracellular binding domain, an extracellular domain as a spacer, a transmembrane domain, and intracellular signaling domains. First produced CARs only had CD3ζ domain for signaling. In the second generation of CARs, another signaling domain has been used as a co-stimulatory domain which commonly is 4-1BB (CD137) or CD28, added to the CD3ζ. In third generation of CARs, two co-stimulatory signaling domains were used together along with the CD3ζ domain. Finally, the fourth generation of CARs which are called TRUCKs or armored CARs, are the second generation CARs which are able to secrete some inflammatory cytokines like IL-12 and IL-15 (Fig. 1)[14]. In recent years, CAR-T cell therapies have shown considerable results in solid tumor and hematologic malignancy treatment in preclinical and clinical studies. Until now, two CD19-specific CAR-T cells are approved for malignancies of B cells[15]. CAR-T cells are strong candidates for targeting CSCs because of their potential ability in killing cancer cells through recognition and binding to the TAAs. Chen et al.[16] have reported that GD2 CAR-T cells are able to significantly eliminate side population (SP) cells and eradicate established tumor in a neuroblastoma mouse model. In several studies using CSC markers-redirected CAR-T cells, it was shown that CAR-T cells effectively eliminate CSCs but ignore the normal stem cells or display low cytotoxicity on them[17–22]. There are various markers on CSCs possible to be targeted by CAR-T cells (Fig. 2), some of which having been targeted in recent years and showing hopeful results. In this review, we highlight the CSC markers that have been targeted by CAR-T cells previously. As well, we will describe the most important CSC markers which are powerful nominees for CAR-T cell therapy in future. Also, we issue barriers against CAR-T cell therapy and review some solutions. Finally, we offer some strategies for elevating CAR-T cell functions against CSCs.

2. How to find novel cancer-associated antigens?

A main priority in cancer research area has been the exploration of new tumor antigens, with the goal of targeting them by various immunotherapies. The approaches employed to identify such antigens have evolved over several years. Overall, earlier cell-based and biochemical-based (low throughput) methods such as serological analysis of recombinant cDNA expression libraries (SEREX), phage display, protein arrays and surface receptor screening have now led to higher throughput methods such as gene expression profiling by microarray and RNA-seq[23–26]. These approaches might be very helpful for identification of various intracellular and extracellular (cell surface) antigens which will be

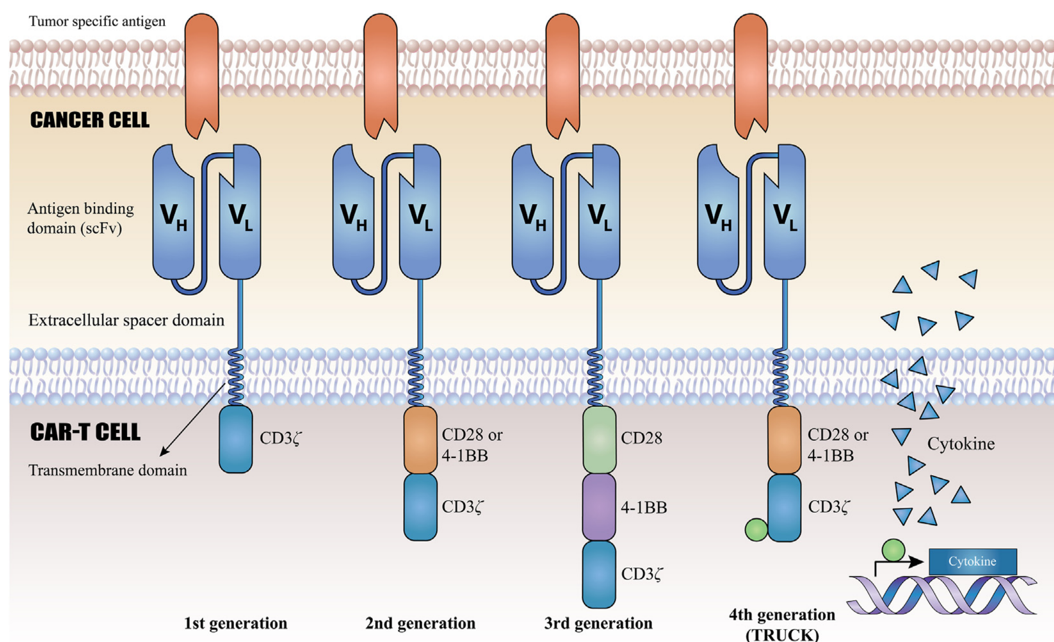

Figure 1 The structures of various generations of CARs which are different in their intracellular domains.

targeted by different immune-based therapies. However, the story of identification of cancer stem cells antigens might be a little different with conventional cancer antigens targeted by various immunotherapies such TCR-based immunotherapy. It seems that identification of CSC-specific antigens targeted by CAR-T cells is a big hurdle as (i) target antigens should exclusively express on the surface of CSCs but not in their cytosol as CAR-T cells only recognize cell surface antigens in a HLA-independent manner; (ii) target antigens should exclusively express by cancer cells but not normal cells aiming to prevent on-tumor off-target toxicity as this adverse effect is more severe in CAR-T cell therapy compared with other immunotherapeutic approach such monoclonal antibody-based therapies[27]; (iii) in many studies, CSCs are characterized by possessing some functional qualities such self-renewal activity rather phenotypical characterization of CSC subpopulations; (iv) some of CSC populations are characterized by the lack of cell surface expression of antigen. For example, leukemic cancer stem cells are characterized by $CD34^{+}CD38^{-}$[28]. So, lacks of expression of CD38 by leukemic cancer stem cells make it difficult to specifically target these cells by CAR-T cells. As identification of CSC-specific antigens is tricky at least by now, it seems that deploying new generations of CAR-T cells (*e.g.*, iCAR-expressing CAR-T cells) and/or combination therapy (*e.g.*, differentiation therapy + CAR-T cell therapy) might fairly alleviate and/or overcome some of these limitations.

3. CSC markers that have already been targeted by CAR-T cells

It has been shown that different cell surface antigens are expressed on CSCs; however, the expression pattern is much varied in different type of tumors. In spite of current knowledge of CSC surface markers, more studies are essential to achieve the big picture understanding. The markers of CSCs on the cell surface seem to be different from other cell surface markers. Besides, the normal cells also express these CSC markers on their surfaces[29]. Thus, antigen selection is a key component for therapeutic application of CSC-targeted CAR-T cell therapy. In this regard, CSC antigen selection should follow these principles: (1) selecting a high-level antigen expression to target CSCs and blunt tumor relapse; (2) selecting highly specific CSC antigen to prevent on-target, off-tumor toxicities; and (3) selecting the common but tumor specific antigens that are expressed on various tumor types, because this results in the development of universal CSC- targeted CAR-T cell therapies. Furthermore, it is likely to identify new CSC surface antigens in the future. To this, we need more extensive sample analyses in different tumor types to detect the new surface antigens that show CSCs characteristics as well[30].

There are some CSCs markers that virtually expressed as private cell surface proteins including, GD2, LGR5, IGF-IR, CD44, CD47, EpCAM, Dll4, FZD and CD123. These cell surface proteins can be specifically targeted by CAR-T cells and or/novel monoclonal antibodies without prompting a substantial tissue toxicity and even an enrichment of CSCs observed following treatment with conventional anti-cancer therapies. Some of the CSC markers which use in CAR-T cell therapies has been reported in pre-clinical studies are including, CD133, CD166, CD20, CD38, CLL-1, EpCAM, CD123, CD171, ROR1, CD44, CD47, CD117, and c-Met. Because of their remarkable results in cancer regression, a noticeable number of them have recently undergone clinical trials (Table 1).

3.1. CD133

CD133, or prominin-1, a member of pentaspan transmembrane glycoproteins in human, is encoded by the *PROM1* gene. Plasma membrane organization is suggested as a physiologic function of CD133. CD133 is known as a remarkable marker for hematopoietic and neural cells as well as CSCs[31]. CD133 is expressed in various CSCs substantially in leukemia stem cells, brain, liver, breast, pancreatic, and ovarian CSCs[3,10,32,33]. It has been shown that glioblastoma patient-derived $CD133^{+}$ cells express great levels of anti-apoptotic proteins and are resistant to chemotherapeutic agents including temozolomide, carboplatin, paclitaxel and

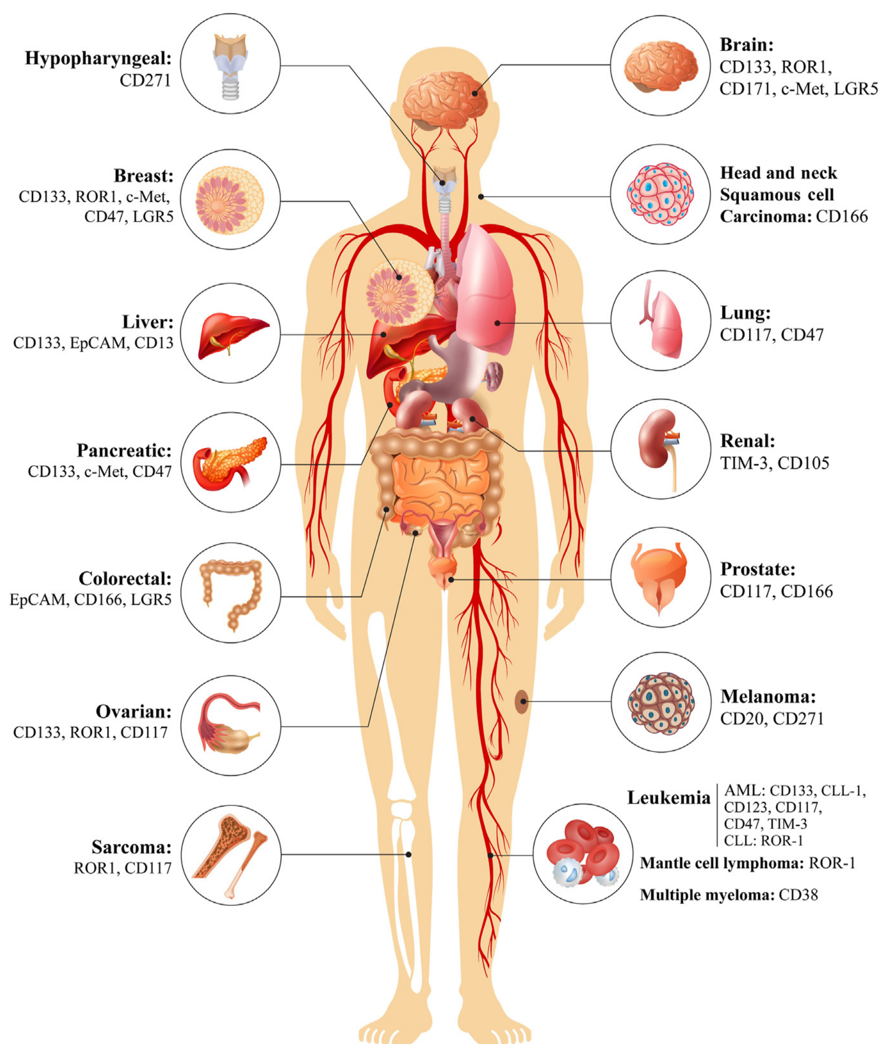

Figure 2 Distribution of potential CSC markers as targets for CAR-T cells in various types of cancers.

etoposide compared to CD133^{-} cells. Its elevated expression is also related with tumor recurrence in glioblastoma patients[32]. AKT and BCL-2 pathways are important in CSC survival, and it has been shown that CD133^{+} HCC cells display resistance to doxorubicin and fluorouracil *via* activating these pathways[10]. CD133 is a potential target in cancer therapy, so that in recent years, various agents such as monoclonal antibodies (mAbs) were used to target it[34]. The antitumor effects of the CD133 CAR-T cells have been surveyed in several pre-clinical studies. Hu et al.[35] in their study on a glioma orthotopic mouse model have reported that CD133 CAR-T cells can significantly suppress tumor growth, and increase survival rate in mice. In another study by Zhu et al.[36], it was indicated that CD133 CAR-T cells can remarkably eradicate glioblastoma CSCs both *in vitro* and *in vivo*. Also, one study has used CD133 CAR-NK cells instead of CAR-T cells which significantly could target and eradicate CD133^{+} primary ovarian cancer cells and ovarian cancer cell lines[37]. Considering these anti-CD133 CSC therapies, CAR-T cells targeting CD133 can likely efficiently eradicate CSCs. However, hitherto, there is only one investigation that has used CD133-targeted CAR-T cells to treat patient-derived glioblastoma stem cells[36]. As CD133 is expressed on malignant neural stem cells and normal neural stem cells, a risk of off-tumor toxicity should be considered. There are a couple of tactics such as intratumoral delivery of CD133-targeted CAR-T cells that can be employed to reduce off-tumor toxicity (Table 2[38]).

3.2. CD20

The CD20 antigen is a glycosylated transmembrane phosphoprotein which is encoded in human by the *MS4A1* gene and is effective in B cell development and differentiation into plasma cells[39]. CD20 is expressed in B cell chronic lymphocytic leukemia, hairy cell leukemia, B cell lymphomas, but has notably been proposed as a melanoma CSC marker[39,40]. The CD20 antigen designed for a therapeutic objective in case of certain hematological malignant tumors and which is a key marker for targeting the cells initiating melanoma, has shown promising results. In several studies, it has been reported that treatment with the anti-CD20 antibody rituximab, with or without combination with chemotherapy, results in striking outcomes like recurrence inhibition, survival rate elevation, and metastasis prevention in melanoma patients[41–43]. CD20 CAR-T cells mostly were used to target hematologic malignancies and have shown a robust function

Table 1 Clinical trials using CSC markers-specific CAR-T cells.

CSC marker	NCT#	Cancer type	Center	Status
CD133	NCT02541370	Liver, pancreatic, brain, breast, ovarian, colorectal carcinoma, and acute leukemia	Chinese PLA General Hospital	Unknown
	NCT03423992	Malignant gliomas	Xuanwu Hospital	Recruiting
	NCT03473457	AML	Zhujiang Hospital	Recruiting
	NCT03356782	Sarcoma	Shenzhen Geno-Immune Medical Institute	Recruiting
CD20	NCT03893019	Melanoma	University Hospital of Köln	Recruiting
CD38	NCT03464916	MM	University of Pennsylvania, Abramson Cancer Center	Recruiting
	NCT03767751	MM	Chinese PLA General Hospital	Recruiting
	NCT03473496	MM	Zhujiang Hospital	Recruiting
	NCT03271632	MM	Shenzhen Geno-Immune Medical Institute	Recruiting
CLL-1	NCT04010877	AML	Shenzhen Geno-Immune Medical Institute	Recruiting
	NCT03222674	AML	Shenzhen Geno-Immune Medical Institute	Recruiting
	NCT03795779	AML	The General Hospital of Western Theater Command, Peking University Shenzhen Hospital	Recruiting
EpCAM	NCT02915445	Malignant neoplasm of nasopharynx, breast	West China Hospital	Recruiting
	NCT03563326	Stomach	West China Hospital	Recruiting
	NCT03013712	Colon, pancreatic, prostate, gastric, hepatic, and esophageal carcinoma	IEC of Chengdu Medical College	Recruiting
	NCT02729493	Liver	Anhui No. two Province People's Hospital	Recruiting
	NCT02725125	Stomach	Anhui Provincial Cancer Hospital	Recruiting
CD123	NCT03672851	Acute leukemia	Second Affiliated Hospital of Xi'an Jiaotong University	Enrolling by invitation
	NCT02937103	Leukemia	Southwest Hospital of Third Millitary Medical University	Recruiting
	NCT03114670	AML	Affiliated Hospital to Academy of Military Medical Sciences	Recruiting
	NCT03796390	AML	Hebei Yanda Ludaopei Hospital	Recruiting
	NCT04014881	AML	Union Hospital, Tongji Medical College	Recruiting
	NCT02159495	AML	City of Hope Medical Center	Recruiting
ROR1	NCT02194374	CLL	M.D. Anderson Cancer Center	Withdrawn
	NCT02706392	ALL, CLL, MCL, breast, NSCLC	Fred Hutchinson Cancer Research Center	Recruiting
CD117	NCT03473457	AML	Southern Medical University Zhujiang Hospital	Recruiting
	NCT03356782	Sarcoma	Shenzhen Geno-Immune Medical Institute	Recruiting
CD171	NCT02311621	Neuroblastoma	Seattle Children's Hospital	Recruiting
c-Met	NCT01837602	Breast	Abramson Cancer Center	Completed
	NCT03060356	Melanoma, breast	University of Pennsylvania	Recruiting
	NCT03672305	HCC	The Second Hospital of Nanjing Medical University	Not yet recruiting

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; HCC, hepatocellular carcinoma; MCL, mantle cell lymphoma; MM, multiple myeloma; NSCLC, non-small cell lung cancer.

in the eradication of cancerous cells and cancer regression[44−46]. Regarding melanoma, Schmidt et al.[47] have reported that targeting melanoma stem cells by CD20 CAR-T cells can significantly eradicate tumors and elevate the surviving rate of NIH-III mice having melanoma. In aggregate, although anti-CD20 has shown remarkable results in the treatment of B cell malignancies, B cell aplasia can be one of the adverse treatment effects, making the patients prone to various infectious agents. However, this challenge can be resolved by administration of IVIG. In case of targeting CD20 on melanoma stem cells, it seems that a couple of approaches such as using CAR-T cells expressing combinatorial target-antigen recognition can be employed to somewhat overcome off-tumor toxicity (Table 2)[38].

3.3. *CD38*

CD38, a type II transmembrane glycoprotein, plays the role of an ectoenzyme in catalyzing the nicotinamide adenine dinucleotide (NAD^+) conversion into adenosine diphosphate−ribose (ADPR), and cyclic ADPR[48]. CD38 has been considered as a potent multiple myeloma (MM) stem cell marker. $CD38^+$ MM cells can initiate MM disease in SCID mice and cause drug resistance and recurrence in patients[49,50]. Several studies in the pre-clinical stage have investigated the potential of CD38 CAR-T cells in MM. Drent et al.[18] have shown that CD38 CAR-T cells produce inflammatory cytokines, and greatly lyse the primary malignant cells from patients displaying multi-drug resistant MM. In another

Table 2 Safety strategies for overcoming off-tumor on-target toxicity following CSCs targeting by CAR-T cells[38].

Overcoming strategy	Example
Using CAR-T cells expressing an anti-CAR	Anti-CAR19-CART cells
Using CAR-T cells expressing a suicide gene switch	a. HSV.TK- expressing CAR-T cells b. iCasp9-expressing CAR-T cells c. CD20 or EGFRt-expressing CAR-T cells
Using CAR-T cells expressing a combinatorial target-antigen recognition	a. Chimeric costimulatory receptor (CCR)-expressing CAR-T cells b. Tandem-CAR-T cells c. SynNotch receptor-expressing CAR-T cells
Using CAR-T cells expressing an inhibitory chimeric antigen receptor	Inhibitory chimeric antigen receptor (iCAR)-expressing CAR-T cells
Using universal CAR-T cells specific against bispecific small molecule switch	Universal anti-FITC-directed CAR-T cells
Using CAR-T cells expressing an on-switch CAR	CAR-T cells expressing an extracellular specific antigen-binding domain (scFv) with costimulatory domains and a key downstream signaling element
Using CAR-T cells expressing a transient CAR expression	Mesothelin-specific CAR mRNA-engineered T cells
Using CAR-T cells expressing a fully human CAR	Fully human CAR-T cells targeting human αFR
Using CAR-T cells expressing a CAR with decreased affinity to target	a. Low-affinity CAR-T cells targeting EGFR b. Low-affinity CAR-T cells targeting CD123 c. Low-affinity CAR-T cells targeting ErbB2
Regional delivery or intratumoral injection of CAR-T cells	a. Intratumoral delivery of ErbB-targeted chimeric antigen receptor T cells b. Regional delivery of mesothelin-targeted CAR-T cells
Symptomatic treatments following CAR-T cell therapy	Using cytokine-blocking agents and high-dose corticosteroids
Replacement therapy	Administration of intravenous immunoglobulin (IVIG) following infusion of anti-CD19 CAR-T cells

study, the ability of CD38 CAR-T cells with low affinity for CD38 markers and having 4-1BB and CD28 costimulatory domains together has been evaluated in a MM mouse model. These CAR-T cells have shown a good proliferative response being able to inhibit the tumor growth significantly, as well as rapidly de-bulk it[22]. An et al.[51] in their study have used nanobody-based CD38 CAR-T cells which resulted in significant lyse of MM cell lines and inhibiting the growth of tumor in NOD/SCID mice. As CD38 is also expressed on normal hematopoietic cells at intermediate levels, the caution should be taken for possible adverse off-tumor toxicity following CD38-targeted CAR-T cell therapy. For example, the one of the solutions can be relied on utilizing low affinity anti-CD38 CAR-T cells (Table 3). Drent et al.[52] have shown that affinity-optimized CD38-CAR T cells can very selectively target MM cells. However, if the affinity of the CAR is not cautiously optimized, high affinity CD38-CARs, similar to many other studies, can cause off-tumor adverse side effects[27,53].

3.4. *CLL-1*

C-type lectin-like molecule 1 (CLL-1) or CLEC12A is a class II transmembrane glycoprotein belonging to the group V of C-type lectin-like receptor family[54]. CLL-1 is considered as a leukemic stem cell (LSC) marker and is related with drug-resistance and recurrence in AML patients. So that, various strategies including CAR-T cells, antibody−drug conjugates, unconjugated mono-specific antibodies and bispecific antibodies have been used for targeting it in recent years[54,55]. Tashiro et al.[56] have shown that CLL-1 CAR-T cells outstandingly decreased the formation of leukemic colonies in primary AML patient peripheral blood mononuclear cells, and led to tumor growth limitation and survival rate elevation in a human AML xenograft mouse model. In another study, it was reported that CLL-1 CAR-T cells entirely eliminated tumor in an AML mouse model established with the CLL1^{+} HL60 cell line[19]. Wang et al.[57] have used third generation CLL-1 CAR-T cells and indicated they can lyse CLL-1^{+} cell lines, as well as eradicate human AML in xenograft models. To concomitantly target LSCs and AML blasts, Liu et al.[58] reported that CLL1-CD33 compound CAR (cCAR)-T cell therapy could encouragingly eradicate leukemia blasts and LSCs in the patients with relapsed/refractory AML. They also showed that antitumor activity of cCAR is superior to single target CAR-T cell therapies. cCAR therapy also could induce total myeloid ablation in bone marrow, signifying that it may perform as a safer alternative to circumvent the severe toxicities associated with standard bone marrow ablation regimens without mitigating the anti-AML efficacy[58]. Considering these data, it seems that targeting CLL-1 by CAR-T cells either as single target antigen or in combination with other target antigens (*e.g.*, CD33) may provide an additional therapeutic option for AML treatment (Table 2)[38].

3.5. *EpCAM*

The epithelial cell adhesion molecule (EpCAM) or CD326, a type I transmembrane glycoprotein, is involved in cell signaling, differentiation, proliferation and migration. EpCAM is regarded as a prominent CSC marker in HCC and colorectal cancer[59,60]. EpCAM^{+} HCC cells (HuH1 and HuH7) are acting as tumor initiating cells which can form large tumors in SCID mice[59]. In recent years, EpCAM CAR-T cells have shown powerful anti-tumor potentials. Zhang et al.[61] have reported that EpCAM CAR-T cells release cytotoxic cytokines like TNF-α and interferon

(IFN)-γ, and thus effectively eliminate EpCAM$^+$ cancer cells *in vitro*. Further, they have been found to significantly inhibit tumor growth in colorectal cancer xenograft models. Wu et al.[62] in their study have demonstrated that EpCAM CAR-T cells can eliminate PC3M prostate cancer cells *in vitro*, and significantly decrease the growth of tumor in NOD/SCID mice. In another report in which CAR-NK cells were used instead of CAR-T cells, EpCAM CAR-NK cells targeted colorectal cancer cells and secreted granzyme B, perforin, and IFN-γ cytokines. It has also been revealed that multimodality therapy of EpCAM CAR-NK cells in combination with regorafenib, a multi-kinase inhibitor, impressively results in tumor growth suppression in EpCAM$^+$ tumor xenografts[63]. Deng et al.[64] have also shown that EpCAM-specific CAR-T cells not only kill PC3M prostate cells (overexpressing EpCAM) but also of prolongs the survival of PC3 prostate cells (underexpressing EpCAM). In aggregate, it can be inferred that application of CAR-T cells with optimized (probably low affinity) CAR-T cells would further overcome off-tumor toxicity associated with EpCAM expression (Table 2)[38].

3.6. *CD123*

CD123, IL-3 receptor subunit α, pairs with common β subunit (CD131) and in binding with IL-3 which results in promotion of cell survival and proliferation[65]. CD123 was confirmed as a unique marker for AML stem cells which are resistant to conventional therapies such as allogeneic hematopoietic cell transplantation and chemotherapy, in AML patients[66]. In xenograft models of human AML, CD123 CAR-T cell administration was found to eliminate leukemia burden and lead to long-term survival. Also, the same authors have established a CD123 memory CAR-T cell subset[67]. Pizzitola et al.[20] in their study have reported that CD123 CAR-cytokine-induced killer (CIK) cells significantly inhibit leukemic growth in immunodeficient NOD-SCID IL2Rg null (NSG) mice and prevent AML from dissemination to peripheral organs like spleen. They have also observed that residual AML cells do not expand any resistant mechanism to this CAR-mediated immunotherapy approach[20]. Tettamanti et al.[21] have shown that CD123 CAR-CIK cells strongly killed both primary AML blasts and CD123$^+$ cell lines *in vitro*, while spare normal hematopoietic stem/progenitor cells (HSPCs) and normal low-expressing antigen cells[21]. These findings might be attributed to suboptimal affinity of ScFv which constituted anti-CD123 CAR. Optimized affinity-CAR might help to discriminate between cells with low- and high-density surface antigen expression, the latter being characterized by the AML tumor cells. Considering above data, it seems that manipulation of CAR affinity might overcome issues of off-tumor toxicity (Table 2)[38].

3.7. *ROR1*

Receptor tyrosine kinase-like orphan receptor 1 (ROR1), a type I transmembrane protein, is related to CSC phenotype in a number of cancers including mantle cell lymphoma, chronic lymphoblastic leukemia (CLL), breast, ovarian, sarcoma, and brain cancers[68–70]. Zhang et al.[68] have confirmed that ROR1 expression was elevated in breast cancer cells following treatment with chemotherapy and was associated with invasion and drug resistance. They have indicated that the treatment with cirmtuzumab, anti-ROR1 mAb, significantly suppressed metastasis and the growth of tumors in mice bearing breast cancer[68]. It has been shown that ROR1 CAR-T cells in exposure to ROR1$^+$ CLL cells released great levels of TNF-α, IFN-γ, and IL-2 cytokines. As well, they strongly lyse the CLL SP cells[71]. Deniger et al.[69] have revealed that ROR1 CAR-T cells eliminated ROR1$^+$ tumor cells in both autologous and allogeneic cases *in vitro*, and on the other hand, can diminish the bulk of tumor and increase survival rate in NSG mice engrafted with Kasumi-2 cancer cells[70]. In another study, Huang et al.[72] have confirmed that ROR1 antigen had high expression in sarcoma cell lines including osteosarcoma, fibrosarcoma, alveolar or embryonal rhabdomyosarcoma as well as Ewing sarcoma. They indicated that ROR1 CAR-T cells could alleviate tumor growth and increase the surviving rate in osteosarcoma and sarcoma mouse models, respectively[72]. To avoid off-tumor toxicity related to elimination of mature myeloid lineage, Lynn et al.[73] have transiently overexpressed mRNA *CAR* by electroporation. They found that mRNA-electroporated HA-FRβ CAR-T cells preserved effective antitumor activity *in vitro* and *in vivo*. Furthermore, to rescue CAR-T cell-mediated toxicity of normal tissues, Srivastava et al.[74] have designed a logic-gated ROR1 CAR. Their data showed that SynNotch EpCAM-inducible ROR1 CAR-T cells can selectively detect EpCAM$^+$ROR1$^+$ tumor cells while spared EpCAM$^-$ROR1$^+$ normal tissues. In aggregate, these data not only highlighted the significance of CAR affinity and synthetic biology in CAR development and target detection, but also suggested that transient delivery of antigen-specific CARs as well as logic-gated control of CAR recognition might mitigate the risk of off-tumor toxicity (Table 2).

3.8. *CD117*

CD117 or c-Kit, a type III receptor tyrosine kinase, binds to its ligand, a stem cell factor, and plays a valuable role in maintaining cell performances including cell survival, metabolism, cell growth, proliferation, apoptosis, cell migration, and cell differentiation[75]. CD117 is acting as a CSC marker in some cancers like sarcoma, ovarian, prostate, lung and AML[76,77]. CD117$^+$ ovarian and lung cancer cells, which were self-renewal, have displayed resistance to chemotherapy[75]. Harris et al.[77] have shown that CD117 activation results in prostate cancer progression, invasion, and resistance to tyrosine kinase inhibitors, including sunitinib, imatinib, and ISCK03 *in vivo*. Arai et al.[78] have reported that CXCR4$^+$ CD117 CAR-T cells can successfully traffic in bone marrow of infused mice and eliminate CD117$^+$ cells. Another study has affirmed that CD117 CAR-T cells can eradicate CD117$^+$ cells in bone marrow of humanized mice which were transplanted with bone marrow of AML patients[79]. As CD117 is widely expressed in hematopoietic and non-hematopoietic cell lineages, it seems modulation of anti-CD117 CAR-T cells through a couple of strategies such as optimization of CAR antigen recognition module and transient expression of CAR (Table 2)[38] could significantly reduce the risk for off-tumor toxicity while retaining an effective antitumor activity.

3.9. *CD171*

CD171 or L1 cell adhesion molecule (L1CAM), a transmembrane glycoprotein being a part of L1 protein family, regulates cell adhesion, survival, growth, migration, and invasion. CD171 has been suggested as a prominent CSC marker in glioblastoma; however, it is also overexpressed in some cancers such as colon,

ovarian, and pancreatic cancer[80]. Hong et al.[81] have shown that the CE7 epitope of CD171 had an expression in several primary solid tumors including glioblastoma, neuroblastoma, renal carcinoma, lung cancer, and ovarian adenocarcinoma, while having a restricted expression in normal tissues. In another study, Hong et al.[82] have indicated that CE7 CAR-T cells could eradicate tumor burden, inhibit ascite formation, and significantly increase survival rate in an experimental model of ovarian cancer. Intratumoral injection of CD171 CAR-T cells was reported to cause tumor elimination and regression, and extended survival in NSG mice bearing human neuroblastoma xenografts[83]. Altogether, these findings revealed that intratumoral delivery of CAR-T cells may be a way to increase efficacy, gain complete tumor regression, and avoid off-tumor toxicities. Besides intratumoral delivery of CAR-anti-CD171 CAR-T cells, various strategies can be also employed to reduce the possibility of off-tumor toxicity (Table 2)[38].

3.10. c-Met

c-Met or hepatocyte growth factor receptor (HGFR) is a receptor tyrosine kinase which is activated by its ligand HGF and leads to cell motility, invasion, and metastasis in various cancers. It is considered as a CSC marker in glioblastoma, breast, and pancreatic cancer[84−86]. It has been found that c-Met induces the expression of stem cell reprogramming factors including Nanog, SOX2, and CD133 in human glioblastoma neurosphere lines[86]. Targeting c-Met by XL184, a kinase inhibitor, or small hairpin RNAs has diminished tumor growth and the population of CSCs in pancreatic tumors established in NOD SCID mice[84]. Frigault et al.[87] have shown that c-Met CAR-T cells strongly cause the tumor bulk shrink and prolong survival in NOD-SCID mice bearing tumors established by intraperitoneal injection of SKOV3 ovarian cancer cells or human lung adenocarcinoma L55 cells subcutaneous injection. Thayaparan et al.[88] in their study have reported that Met CAR-T cells released IFN-γ and IL-2 cytokines and potently killed MET^{+} H28 mesothelioma cells *in vitro*. The same authors have also revealed that these CAR-T cells significantly decreased tumor bulk in mice with an established mesothelioma xenograft. As c-Met is commonly expressed in stem cells, progenitor cells and CSCs, MET-directed CAR-T cells could potentially cause off-tumor toxicity in various tissues. It seems that the strategies such as local delivery of CAR-T cells, utilizing CAR-T cells expressing a suicide gene switch and transient expression of CAR could reduce the off-tumor toxicity. However, this remains to be assessed.

3.11. CD166

CD166 or activated leukocyte cell adhesion molecule (ALCAM) is a part of the immunoglobulin superfamily which has a role in cell migration, neurogenesis, hematopoiesis, and immune responses. Its high expression is related to different cancers such as, prostate, breast, osteosarcoma, pancreatic, melanoma, and colorectal carcinomas[89]. CD166 is regarded as a prostate, colorectal and head and neck squamous cell carcinoma CSC marker which leads to chemoresistance and maintenance of cancer cells[60,90,91]. Wang et al.[92] have confirmed that CD166 is highly expressed in the lines of the human osteosarcoma including MG-63, Saos-2, MNNG/HOS and U2OS, which are eliminated after being co-cultured with anti-CD166 CAR-T cells. CAR-T cells could efficiently hamper tumor growth without any cytotoxic activity against healthy tissues (such as lung, heart, liver, spleen, intestine and kidney) in an orthotopic osteosarcoma model[92]. Their data suggested that CD166-redirectd CAR-T cell therapy might be safe in humans. However, it should be noted that Wang et al. have not evaluated off-tumor toxicity in an immunocompetent mice model. Therefore, safety issues should be further addressed probably through development of approaches that reduce possible adverse events by anti-CD166 CAR-T cells. These approaches might be including generation of multi-specific CAR-T cells, modulation of ScFv affinity and optimization of therapeutic dose of CAR-T cells. As other potential targets (such as HER2) exist for anti-osteosarcoma therapy, construction of bi-specific CAR-T cells against CD166 and HER-2 might be beneficial in mitigating possible off-tumor toxicity[93,94].

3.12. CD47

CD47, a transmembrane protein, a part of the immunoglobulin superfamily expressed in many cancerous cells, and also in its binding with the signal regulatory protein-α (SIRPα) in phagocytes including macrophages, sends a “don’t eat me” alarm thus inhibiting phagocytosis[94]. CD47 is a potent CSC marker in AML, pancreatic, lung, and breast cancer which stimulates self-renewing and causes chemoresistance[85,95−97]. Cioffi et al.[95] have shown that treatment with anti-CD47 antibodies ameliorated phagocytosis of primary human pancreatic CSCs. Also, targeting CD47 in combination with gemcitabine or abraxane resulted in sustained tumor regression in patient-derived xenograft models[95]. Golubovskaya et al.[98] have reported that CD47 CAR-T cells significantly killed pancreatic, ovarian and other cancerous cells and secreted large amounts of IL-2 cytokine *in vitro*. Furthermore, intratumoral delivery of CD47 CAR-T cells could significantly inhibit tumor growth in a BxPC3 pancreatic xenograft model[98]. By monitoring mouse body weight, the authors have proposed that anti-CD47 CAR-T cells have no toxic effect against normal tissues. However, more detailed toxicological studies will further validate the safety data of this therapy. Altogether, as CD47 is expressed in normal cells and normal tissues, it seems that, besides intratumoral delivery, tight regulation of CAR-T cells through different approaches such as regulation of CAR-T cell by switch-on and switch-off mechanisms as well as bi-specific CARs could significantly avoid adverse effects associated with off-tumor toxicity of administrated CAR-T cells (Table 2)[38].

4. *In vivo* studies on CSC-targeted CAR-T cells

It is clear that antitumor efficacy of the CSC-targeted CAR-T cells should be evaluated in a preclinical setting before their clinical application. Besides, appropriate expression of CAR molecules as well as proper function of CAR-engineered T cells, especially in terms of *in vitro* antigen-specific cytolytic and proliferative functions must be investigated as well. Importantly, appropriate xenograft models should be used to precisely evaluate the antitumor function of CSC-targeted CAR-T cells *in vivo*. New reports demonstrated that although patient-derived xenograft (PDX) and cell line-derived xenograft (CDX) models are more appropriate for evaluating preclinical efficacy, PDX studies are more predictive in terms of clinical outcome compared with CDX[99,100]. There are also some studies, in which fresh tumor tissues of the patient are transplanted into immunodeficient mice. These studies suggest the feasibility of preclinical assessment of

new cancer therapies[99]. It seems that for proof-of-concept experiments such as CSC-targeted CAR-T cell therapies, PDX might be a potential animal model to examine the efficacy of CAR-T cells especially eliminating CSCs. The resultant data may afford predictive data for setting up of CSC-targeted CAR-T therapies in a clinical setting. To date, few studies using CSC-targeted CAR-T cells have been described in animal models. For example, CAR-T cells specific for EpCAM antigens could eradicate CSCs in experimental models of prostate cancer[64]. In another study, CAR-T cells engineered with membrane-tethered IL-15 could induce anti-CSC memory CAR-T cells in an experimental model of $CD19^+$ leukemia[101]. CD123-targeted CAR-T cells could also strongly eliminate $CD123^+$ leukemic cancer stem cells in a primary acute myeloid leukemia model[21]. Zhu et al.[36] have also reported that anti-CD133 CAR-T cells can efficiently kill patient-derived glioblastoma stem cells both *in vitro* and *in vivo*. A further study have exhibited that intraperitoneal immunotherapy with anti-EpCAM CAR-T cells could efficiently eradicate established tumors and leads to significantly prolonged animal survival in xenograft models of peritoneal carcinomatosis[102]. These data indicated that of this approach might be an effective therapeutic clinical approach for the patients with gastrointestinal and gynecologic malignancies. A recent case report has reported that cocktail targeting of CSCs with EGFR and anti-CD133-CAR-T cells is a safe and feasible approach for clinical cancer treatment of a patient with advanced cholangiocarcinoma[103]. In aggregates, all above studies suggest that the adoptive transfer of CSC-specific CAR-T cells is a potential and encouraging therapeutic approach for various cancers. In addition to assessing the efficacy of CAR-T cells therapy, conducting *in vivo* studies are also crucial for assessment of safety of CAR-T cell therapy. For example, CAR-T cell therapy might be accompanied by some adverse effects such as on-target/off-tumor toxicity, cytokine release syndrome (CRS), and soluble tumor syndrome that have a tremendous risk to patient health. Thus, *in vivo* studies must be carried out prior any clinical application of CAR-T cell therapy.

5. Potential CSC markers as possible targets for CAR-T cell therapy in the future

Meticulous research in the area of CSC markers has led to marker delineation of CSCs in the cells of every tissue lineage. So far, each of the CSC antigen listed in the above section has different safety issues, and no doubt antigens other than the ones we have focused above and below will be brought forward for further evaluation in the future. We have here attempted to provide examples of potential CSC markers that may be beneficial for targeting by CAR-T cells. Targeting of CSC markers of CD271, LGR5, TIM-3, CD13, and CD105 have been evaluated in various cancers. The safety and efficacy of different therapeutic approaches that target some of these markers in various cancers have been and/or being examined in clinical trials (Table 3).

5.1. CD271

CD271 or low-affinity nerve growth factor receptor (LNGFR) is a part of the tumor necrosis factor receptor superfamily, which binds with low affinity to several ligands, including brain-derived neurotrophic factor (BDNF), neurotrophin-4, nerve growth factor (NGF)

Table 3 Clinical trials using CSC markers-specific agents in various cancers.

CSC marker	Agent	NCT#	Cancer type	Center	Status
LGR5	BNC101 mAb	NCT02726334	Colorectal	Flinders Medical Centre	Terminated
	Bispecific antibody MCLA-158	NCT03526835	Colorectal and other advanced solid tumors	Sarah Cannon Research Institute, Institut Jules Bordet, Institut Gustave Roussy, Vall d'Hebron	Recruiting
TIM-3	MBG453 mAb	NCT03066648	AML	Novartis Investigative Sites	Recruiting
CD13 or aminopeptidase N	CHR-2797	NCT00737555	Advanced or refractory solid tumors	UMC St. Radboud	Completed
	Tosedostat	NCT01636609	AML	M.D. Anderson Cancer Center	Completed
CD105	TRC105	NCT01806064	RCC	Multi-center	Active, not recruiting
	CD105/Yb-1/SOX2/CDH3/MDM2-polyepitope plasmid DNA vaccine	NCT02157051	Breast	Fred Hutch/University of Washington Cancer Consortium	Recruiting
	TRC105	NCT03418324	Prostate	Cedars Sinai Medical Center	Active, not recruiting
	TRC105	NCT01648348	Recurrent glioblastoma	Multi-center	Completed
	TRC105	NCT03181308	NSCLC	University of Alabama, Birmingham, Moffitt Cancer Center	Active, not recruiting
	TRC105	NCT01326481	Metastatic breast	University of Alabama, Birmingham, Roswell Park Cancer Institute	Completed

AML, acute myeloid leukemia; NSCLC, non-small-cell lung carcinoma; RCC, renal cell carcinoma.

and neurotrophin-3[104]. CD271 has been characterized as a CSC marker in melanoma and hypopharyngeal cancer[104,105]. Redmer et al.[106] have revealed that the expression of CD271 was increased in response to DNA-damaging chemotheraputic agents, including etoposide, fotemustine, and cisplatin, in drug-sensitive cancer cells, and subsequently led to elevating DNA repair components and resistance to these agents[106]. The authors also showed that knockdown of CD271 in fotemustine-resistant cells restores sensitivity to fotemustine. Furuta et al.[107] have shown that the overexpression of CD271 in melanoma cells and its binding to NGF in activated cytotoxic T lymphocytes (CTLs) downregulated melanoma antigens, and afterward hampered the *in vitro* activation of melanoma-specific CTLs. It has been confirmed that the combinatory treatment with anti-CD47 and anti-CD271 antibodies effectively blunted tumor metastasis in a metastatic melanoma mouse xenograft[108]. Using a humanized anti-CD271 mAb has resulted in tumor shrinkage and reducing the number of CD271$^+$ cells in melanoma and hypopharyngeal tumor bearing mice[104]. Although some studies reported that CD271 is not a perfect marker for identification of melanoma stem cells, but it seems that CD271 might be useful to identify and target melanoma cells with an augmented stemness and tumorigenic potential[109]. Altogether it seems that utilizing bi-specific CAR-T cells targeting CD271 and an additional melanoma CSC marker IFN inducible transmembrane family (IFITM1 and IFITM2)] could be a promising strategy for treatment of melanoma.

5.2. *LGR5*

The leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) or GPR49, a member of the G-protein-coupled receptors family, is involved in signal transduction from extracellular factors[110]. Next to a functional stem cell marker, LGR5 is considered as a potent CSC marker with a high expression in glioblastoma, colorectal, and breast cancers[110−112]. Yang et al.[110] have shown that LGR5 overexpression was strikingly correlated with breast cancer recurrence and poor outcome, and stimulated tumor formation, cell mobility and epithelial mesenchymal transition in breast cancer cells through WNT/β-catenin signaling activation. Junttila et al.[113] have tested an anti-LGR5 antibody-drug conjugate in two LGR5$^+$ xenograft models, including the LoVoX1.1 (colon cell line xenograft model) and D5124 (a primary human pancreatic xenograft model), confirming its significant role in the inhibition of tumor growth and tumor regression in both models. Gong et al.[114] have shown that using an anti-LGR5 antibody−drug conjugate effectively induced cytotoxicity in LGR5$^+$ gastrointestinal cancer cells *in vitro*, and eliminated tumors and prevented those from recurring in a human colon cancer xenograft. As LGR5$^-$ cancer cells can dynamically replenish LGR5$^+$ CSCs upon their elimination[112], it seems that CAR-T cell therapy combined with chemotherapeutic drugs not only can efficiently enrich and kill CSCs but also it can blunt the evasion of non-CSC plasticity.

5.3. *TIM-3*

T cell immunoglobulin mucin-3 (TIM-3), a cell-surface glycoprotein of type 1, first determined surface molecules expressed in IFN-γ-producing CD4$^+$ Th1 cells. It has been reported that galectin-9 connection with TIM-3 led to apoptosis in Th1 cells and negatively regulates Th1 cell functions[115]. Besides, TIM-3 is considered as a CSC marker in AML and renal cell carcinoma (RCC)[55,116]. Particularly, Komohara et al.[116] have found that the high expression of TIM-3 in tumor-associated macrophages (TAM) and tumor cells in RCC is positively in harmony with patients' shorter progression-free survival. Also, TIM-3$^+$ RCC cells were resistant to sunitinib and mTOR inhibitors. The authors also showed that a treatment with anti-TIM-3 mAb repressed its tumorigenic effects *in vitro* and *in vivo*. Kikushige et al.[117] tried out an anti-human TIM-3 mouse IgG2a antibody with antibody-dependent and complement-dependent cellular cytotoxic activities in mice grafted with human AML, and observed that it can alleviate leukemic burden and eradicate leukemic stem cells with the ability of reconstituting human AML. In another study, they also reported that the TIM-3−galectin-9 interaction leads to leukemic stem cell self-renewing and human AML development through activation of the NF-κB and β-catenin signaling pathways, which were inhibited by using anti-human galectin-9 antibodies[118]. As TIM-3 is overexpressed in leukemic stem cells of AML in the most FAB types (except for M3) but not in normal hematopoietic stem cells, it seems that TIM-3-targeted CAR-T cells therapy could be an empirical approach for the treatment of human AML. Moreover, targeting TIM-3 by CAR-T cells next to other surface antigens or in combination with other therapeutic modalities (Table 2) might be other alternative approaches for the treatment of cancers while avoiding off-tumor toxicity.

5.4. *CD13*

Haraguchi et al.[119] have shown that CD13 or aminopeptidase N, a membranous glycoprotein, can be considered as a potent HCC stem cell marker. They have further revealed that CD13 protects cells against ROS-induced DNA damage following chemo/radiotherapy. They confirmed that combining therapy with a CD13 inhibitor and fluorouracil (5-FU), a chemotherapy agent, strongly reduced tumor burden in mouse xenograft models. Yamashita et al.[120] have revealed that ubenimex, a CD13 inhibitor, sensitized HCC cell lines PLC/PRF/5 and HuH7 to anti-cancer drugs including 5-FU, cisplatin, doxorubicin, and sorafenib by increasing intracellular ROS levels. Another study, using poly(ethylene glycol)−poly(-lysine) block copolymer−ubenimex conjugates, resulted in tumor growth suppression and tumor cell apoptosis in a HuH7 subcutaneous xenograft model[121]. In another interesting study, He et al.[122] have reported that utilizing bispecific and split CAR (BissCAR)-T cells against CD13 and TIM-3, the antigens that are upregulated in AML leukemia stem cells, can eradicate patient-derived AML, with much reduced-off tumor toxicity to human HSCs and peripheral myeloid progenitors. This approach allows the researchers to the target new cell surface antigens, thereby potentially expanding the ability to develop functional CAR-T cells for the treatment of AML and other cancers. In aggregate, it seems that development of more tolerable and safer CAR-T cell through different approaches such as dual targeting of TAAs with affinity-optimized ScFvs may overcome off-tumor toxicity while preserving highly effective antitumor activity.

5.5. *CD105*

CD105 or endoglin, a disulfide-linked homodimer cell membrane glycoprotein, is overexpressed in tumor-associated vascular endothelium and is highly effective in vascular development and remodeling[123]. Bussolati et al.[124] have introduced CD105$^+$ human renal carcinoma cells as CSCs, which displayed some properties including Nanog, nestin, OCT4 stem cell marker expression, clonogenic ability, *in vivo* generation of serially transplantable carcinomas, and the ability to expand in non-adhesive spheroids. It

has been shown that CD105 knockdown with RNA interference conduces to N-cadherin downregulation and E-cadherin upregulation, and attenuates movement and invasion of $CD105^{+}$ RCC cells[125]. Ziebarth et al.[123] have demonstrated that CD105 inhibition declined cell viability, drove double stranded DNA damage, and increased apoptosis and cisplatin sensitivity in ovarian cancer cells. Madhav et al.[126] have used TRC105 (carotuximab), an endoglin neutralizing antibody, in combination with radiotherapy and observed that tumor burden significantly diminished in a 22Rv1 xenograft model.

Current immunotherapies might fail to eradicate most of cancer entirely, perhaps because all CSCs shall be also eliminated. Identification of CSCs by their cell-surface immunophenotype may permit patient-by-patient selection for more effective immunotherapies and/or drugs to be examined in clinical trials. In aggregate, it seems that based on currently available data on antigens expressed by CSCs (*e.g.*, CD133, ALDH and EpCAM), these antigens could act as targets for CAR-T cells and the strategy of CAR-T cells targeting CSCs can likely be viewed as a significant treatment in several cancers. However, at present, it remains to be assessed by robust preclinical and clinical studies to further reveal the safety and feasibility of this strategy. Moreover, in the future, it might be possible to develop novel and specific therapies that diminish tumorigenesis by eradication of CSCs or by targeting "stemness" in cancers. Hence, endeavors should be made to combine CAR-T cell therapy with drugs that endorse differentiation of CSCs, and/or selectively target these tumor cells, and/or specifically inhibit their self-renewal and expansion.

6. Hindrances regarding CAR-T cell therapy and possible solutions

Immune targeting of cancer stem cells using CAR-T cells carries some risks probably due to many factors including: (i) shared antigens with normal adult stem cells; (ii) antigen heterogeneity of CSCs; (iii) poor persistence and insufficient trafficking of CAR-T cells to CSCs niche; and (iv) presence of a wide range of cellular and acellular immunosuppressors.

6.1. On-target off-tumor toxicity

On-target off-tumor toxicities are caused by the low expression of some TAAs in normal tissues, and their subsequent attack by CAR-T cells is the major safety concern in CAR-T-cell therapy. Some strategies are evaluated in a number of studies to surmount this problem. One of them is the use of bispecific CAR-T cells which are able to target two separate TAAs and are just activated when both antigens are expressed in tumor cells. Thus, they correctly target tumor cells without paying attention to normal tissues[45]. Another way is the use of antigen-specific inhibitory CAR-T cells, which express an inhibitory receptor against normal antigens in addition to TAA-specific CAR. So, when they bind to a TAA expressing normal cell, these send inhibitory signals preventing from cytotoxicity[127]. The affinity of CAR is a key factor in binding and inducing cytotoxicity. It has been indicated that CARs with low affinity to antigens can effectively bind to high density TAAs in tumor cells and transduce activation signals, though ignoring low density TAAs in normal cells[22]. Using CAR-T cells transduced with a suicide gene including the inducible activation of caspase 9 (iCasp9), which can be induced by the administration of the small molecule dimerizer drug AP1903, is an executable strategy for elimination of CAR-T cells when they cause target-on cytotoxicity[44]. Table 2 summarized potential safety strategies which can be employed to overcome off-tumor toxicity following CSC-targeted CAR-T cell therapy.

6.2. Tumor heterogeneity: antigen loss and tumor escape

The clonal evolution and cancer stem cell (CSC) models have been proposed as drivers of tumor heterogeneity[128]. The concept of CSC plasticity and bidirectional conversion between CSCs and non-CSCs may help explain the tumor heterogeneity observed in tumors. The process of CSC plasticity may be modulated by specific microenvironmental signals and cellular interactions arising in the tumor niche[129]. Such CSC plasticity may limit the effect of CSC treatments. Therapies targeting CSCs would only be effective in eliminating this cell subset in a short period of time as new CSCs may arise from untargeted non-CSCs. Therefore, effective cancer therapy should not only target CSCs but also prevent generation of new CSCs from differentiated cancer cells (non-CSCs) as CSCs can be generated from bulk differentiated cancer cells at any stage of tumorigenesis. It is well-documented that CSC-targeted CAR-T cell monotherapy cannot completely and potently eliminate tumor cells highlighting the significance of combination therapy for CSCs. In this regard, it seems that combination therapeutic strategies including radio-/chemo-therapeutic drugs and CAR-T cells could be highly effective. Weiss et al.[130] have shown that the combination of a subtherapeutic dose of regional radiotherapy with NKG2D CAR-T cells generated synergistic activity in a glioma model of syngeneic mouse. Klapdor et al.[37] have confirmed that treatment with cisplatin followed by CD133 CAR-NK92 cells significantly eradicated ovarian CSCs. In another study, Zhang et al.[63] have indicated that a combination therapy including regorafenib and EpCAM CAR-NK92 cells suppressed tumor growth more significantly compared to monotherapy with CAR-NK92 cells or regorafenib. It has been also shown that ionophore antibiotic salinomycin, metformin and pharmacological inhibitors of S phase kinase associated protein 2 (SKP2) could effectively eliminate CSCs in different types of cancers in preclinical studies and are also being greatly effective while utilized in combinations with other conventional chemotherapeutics agents[131–133]. It seems that metronomic low doses of chemotherapeutic drugs can be used in combination of CAR-T cells.

Loss of the target antigen is one of the mechanisms responsible for treatment failure and relapse after CAR-T cell therapy (Table 4[134–151]). Using bispecific CAR-T cells may be efficient by targeting other TAAs instead of lost antigens[45]. Another reliable mechanism is the use of factors that can enhance TAAs expression in tumor cells. It has been reported that all-*trans* retinoic acid can upregulate CD38 expression and folate receptor-β antigens in AML cell lines, and afterward increase the killing capability of CD38 and folate receptor-β CAR-T cells[152,153]. Biberacher et al.[154] have shown that bryostatin 1, a macrocyclic lactone, increased CD22 expression *via* activation of the protein kinase C, and so sensitized CLL cells for the cytotoxic effects of an anti-CD22 immunotoxin. Altogether these data demonstrated that combination therapy can enhance antitumor efficacy of CAR-T cells and overcome the shortcomings of CAR-T cell monotherapy. Moreover, it seems that combination of CSC-targeted CAR-T cell therapies which target multiple CSC-specific antigens (*e.g.*, utilization of bispecific CAR-T cells) either with

Table 4 Challenges for CSC-targeted CAR-T cell therapy.

Challenge		Overcoming strategy
Heterogeneity of CSC antigens		Use of CARs targeting multiple CSC antigens Use of dual-specific CAR-T cells Monitoring of patients for expression of CSCs antigen Combination therapy[134–136]
Trafficking		Use of CAR-T cells overexpressing chemokine receptors or combined application of CAR-T cells with an oncolytic virus armed with the chemokines that match the chemokines produced either by tumor or tumor associated cells Genetic addition of molecules which improve CAR-T localization Local delivery of CAR-T cells[137–140]
Persistence		Optimized *ex vivo* CAR-T cell culture conditions Pre-treatment with specific conditioning regimens and pharmacological inhibitors Manipulations of genes involved in CAR-T cell survival (*e.g.*, anti-apoptotic and proapoptotic genes and cytokines) Modification of different parts of CAR construct Modulation of redox regulation system Reversing CAR-T cell exhaustion Blunting host immune responses against CAR-T cells T cell selection procedures Ectopic expression of genes regulating T cell survival (*e.g.*, *TERT*)[141]
Tumor microenvironment	Soluble molecules	The using of single/multiplex genome-edited CAR-T cells resistant to various inhibitory signals such as adenosine, PGE2 and kynurenine[142–146]
	Immunosuppressive immune cells	The simultaneous use of CAR-T cells with blockage and depletion of various immunosuppressive molecules and cells such as Tregs and MDSCs Use of armored CAR-T cells[143,147–149]
	Physical barriers	Generation of CAR-T cells which penetrate the extracellular matrix and tumor-associated stroma to assist infiltration of CAR-T cells into tumor masses Local and/or intratumoral delivery of CAR-T cells[134,150,151]

appropriate chemotherapy, radiation therapy and/or other immunotherapeutic modalities (such as immune checkpoint inhibitors) can significantly improve antitumor response.

6.3. Persistence and trafficking

In vivo persistence and trafficking of CAR-T cells are two crucial factors predicting treatment responsiveness in patients. Persistence is related to several circumstances including CAR structure and preventing T cells from exhaustion and senescence (Table 4). Pre-clinical studies have clarified that 4-1BB and ICOS co-stimulatory domains lead to good persistence of CAR-T cells *in vivo* in comparison to the CD28 domain[155]. Other studies showed that the combination of CAR-T cells with the inhibitors of various immune checkpoints, like anti-PD-1, anti-CTLA-4, anti-TIM-3, anti-LAG-3, and anti-adenosine 2A receptor (A2AR), can upgrade the durability of CAR-T cells and prevent from exhaustion and senescence[156]. One study has pointed out that CAR-T cells which were engineered for expressing high levels of anti-apoptotic factors including AKT, were resistant to tumor-induced apoptosis, and therefore can persist and fight strongly against cancer cells[62]. It has also been shown that engineering CAR-T cells to produce IL-7 and IL-15 cytokines, elevated their persistence *in vivo*[16,157]. In solid tumors, unlike hematologic malignancies, infused CAR-T cells cannot easily reach tumor sites to kill cancer cells. Some strategies have been proposed to solve this problem. CAR-T cell local delivery has been suggested in which CAR-T cells can concentrate around tumors and effectively eliminate them in comparison to systemic infusion procedures[158]. Another way is engineering CAR-T cells expressing specific chemokines and chemokine receptors, allowing them to specifically go forward and infiltrate into tumors. Chemokine receptors CCL19, CCR4, CXCR2, and CCR2b were used in pre-clinical studies which caused CAR-T cells homing improvement in tumor sites[157,159–161]. It is possible that the intratumoral delivery of CSCs-specific CAR-T cells will directly target the CSC niches which are inaccessible with conventional therapies.

6.4. Suppressive tumor microenvironment

CAR-T cells even after a successful transport, find themselves in contact with an immunosuppressive tumor microenvironment which displays different inhibitory properties against their anti-

tumor functions. Various solutions have been investigated to fix this problem (Table 4).

6.5. *Physical and metabolic barriers*

Physical barriers prevent from CAR-T cell migration inside tumors, and a possible solution is destructing these hurdles. Targeting tumor stromal cells with fibroblast activation protein (FAP)-directed CAR-T cells can break up tumor structure and uncover cancer cells and CSCs for other CAR-T cells or therapeutic agents[150]. Caruana et al.[162] have indicated that engineering CAR-T cells to express an extracellular matrix degrading enzyme, including heparanase, improved infiltration of T cells inside tumors. Tumor hypoxia and nutrient starvation are leading factors in effector T cell inactivation. Hatfield et al.[163] have reported that oxygen supplementation in the tumor microenvironment enhanced the intratumoral infiltration of CTLs and decreased immunosuppressive factors, like the transforming growth factor (TGF)-β. Lack of glucose and amino acids, such as tryptophan, inhibited the activation of T cells and their cytokine production inside tumors. Hence, providing these factors for CAR-T cells probably increases their functions against tumors[164].

6.6. *Presence of immunosuppressive cells and molecules*

Immune system suppressor cells including T regulatory cells, TAMs, tumor associated neutrophils (TANs) and myeloid-derived suppressor cells (MDSCs) as well as inhibitory cytokines and factors like IL-10, TGF-β, prostaglandin E2 (PGE2), arginase, cyclooxygenase 1 (COX1), galectin-9, and indoleamine 2,3-dioxygenase (IDO) are involved in reducing the effector functions of cytotoxic T cells inside tumors. Therefore, targeting these factors along with CAR-T cell therapy will improve treatment efficiency. Burga et al.[165] have shown that MDSC depletion can restore the reduced antitumor functions of carcinoembryonic antigen (CEA) CAR-T cells in mice bearing CEA^{+} liver cancer. It has been signified that CAR-T cells which release effector cytokines, including IFN-γ and GM-CSF, can re-educate and activate TAMs. Then TAMs decrease the expression of cancer cell inhibitory factors, lyse cancer cells, increase IL-12 production, and process and present antigens to T cells[166]. Yazdanifar et al.[167] have shown that CAR-T cell combination with biological inhibitors of IDO, COX1, and galectin-9, remarkably increases CAR-T cell cytotoxicity versus resistant pancreatic ductal adenocarcinoma cells. Prostate specific membrane antigen

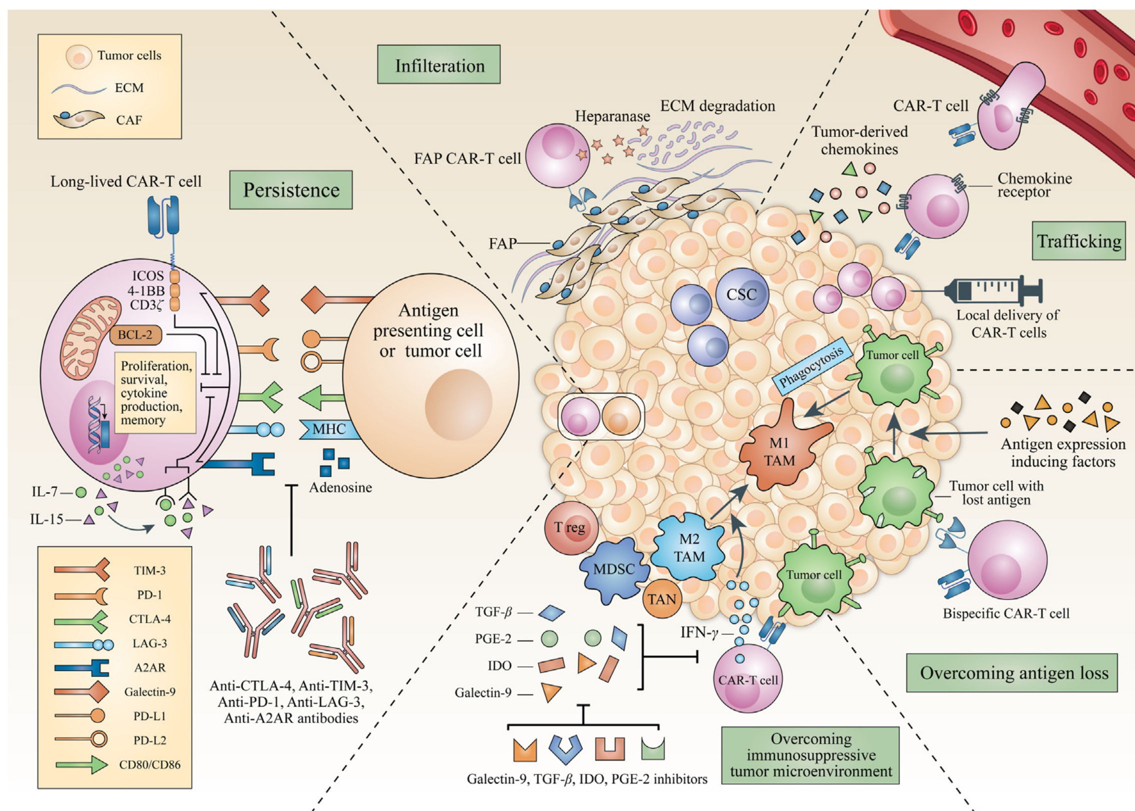

Figure 3 The most common obstacles in CAR-T cell therapy and proposed solutions. Regional installation and engineering CAR-T cells to express tumor-derived chemokines-specific receptors for trafficking improvement, CAFs-specific CAR-T cells and ECM degrading enzymes (such as heparanase) producing CAR-T cells for effective penetration, bispecific CAR-T cells and using antigen expression inducing factors to overcome antigen loss, inflammatory cytokines producing CAR-T cells plus immunosuppressive factors-specific biological inhibitors for overcoming hostile tumor microenvironment, and CAR-T cells engineered to produce IL-7, IL-15, and anti-apoptotic proteins, as well as CARs with ICOS and 4-1BB co-stimulatory domains instead of CD28 domain plus T cells' inhibitory receptors blockade for increasing persistence, have been evaluated and suggested as persuasive solutions. A2AR, adenosine 2A receptor; CAF, cancer associated fibroblast; CAR, chimeric antigen receptor; ECM, extracellular matrix; FAP, fibroblast activation protein; IDO, indoleamine 2,3-dioxygenase; MDSC, myeloid derived suppressor cell; PGE, prostaglandin E; TAM, tumor associated macrophage; TAN, tumor associated neutrophil; TGF, transforming growth factor.

(PSMA) CAR-T cells have been engineered to express a prevailing negative TGFβRII, a decoy receptor binding TGF-β without downstream signaling. This engineered decoy receptor resulted in elevated proliferation, augmented cytokine secretion, resistance to exhaustion, and long-term *in vivo* persistence of CAR-T cells, and afterward, tumor elimination induction in metastatic human prostate cancer mouse models[168] (Fig. 3).

7. Combination therapy

The combination of therapeutic treatments to specifically target cancer cells has been a cornerstone of cancer therapy for many years. Recent data revealed that CSC-targeted CAR-T cell monotherapy cannot completely and potently eliminate tumor cells highlighting the significance of combination therapy for CSCs. Weiss et al.[130] have shown that the combination of a subtherapeutic dose of regional radiotherapy with NKG2D CAR-T cells generated synergistic activity in a glioma model of syngeneic mouse. Klapdor et al.[37] have confirmed that treatment with cisplatin followed by CD133 CAR-NK92 cells significantly eradicates ovarian CSCs. In another study, Zhang et al.[63] have indicated that a combination therapy including regorafenib and EpCAM CAR-NK92 cells suppressed tumor growth more significantly compared to monotherapy with CAR-NK92 cells or regorafenib. Altogether these data demonstrated that combination therapy can enhance antitumor efficacy of CAR-T cells and overcome the shortcomings of CAR-T cell monotherapy. Moreover, it seems that combination of CSC-targeted CAR-T cell therapies which target multiple CSC-specific antigens (*e.g.*, utilization of bispecific CAR-T cells) either with appropriate chemotherapy, radiation therapy and/or other immunotherapeutic modalities (such as immune checkpoint inhibitors) can significantly improve antitumor response (Fig. 4).

8. Conclusions and future perspectives

Preconditioning with lymphodepleting chemotherapy prior to CAR-T cell therapy has been proved as a functional strategy to achieve effective cure in hematologic malignancies. It has been confirmed that some toxicities in relation with CAR-T cell therapy such as cytokine release syndrome are because of high tumor burden, and probably preconditioning makes CAR-T cell therapy safer by reducing tumor bulk[169]. The chemotherapy drugs, dacarbazine, temozolomide, and cisplatin, have the ability to induce T cell-attracting chemokine expression like CCL5, CXCL10 and CXCL9 in melanoma which takes part in recruitment of immune cells with anti-tumor activities. Hence, using these drugs as well as other agents for preconditioning, which can induce the production of such chemokines in the tumor microenvironment, maybe reinforce CAR-T cell therapy[170]. As discussed, antigen density is an important factor in CAR affinity determining, therefore antigen density specification in patients' cancer cells is a possible way for efficient CAR-T cells designing.

Immune targeting of stem cells carries risk of autoimmunity because of the existence of shared antigens between normal stem cells and CSCs. Therefore, it is key to discover private markers to CSCs[74]. There are still some questions that remain to be addressed: to what extent will an appropriate number of makers be recognized to define the actual population of CSCs? Would these biomarkers alter during cancer development and progression? To answer these questions, future studies not only should identify and/or redefine markers exclusive to CSCs but also should include

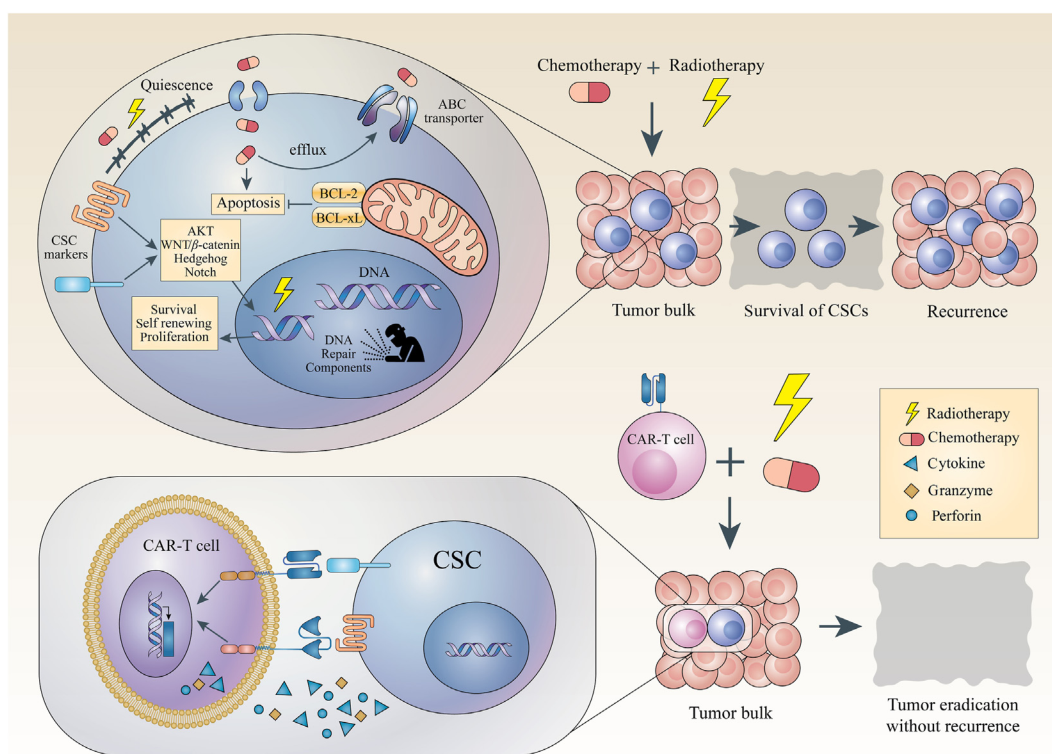

Figure 4 Use of CSCs-specific CAR-T cells in combination with chemo/radiotherapy and other therapeutics may completely eradicate tumors without risk of recurrence, which is mostly because of CSCs existing in tumors.

the increasing of specificity and efficiency in targeting CSCs. To overcome these existing challenges, development of strategies that exploit the distinctive characteristics of CSCs and the cooperation of multidisciplinary areas are greatly required.

Current evidences also suggest that CSCs are key targets for clinical cancer therapy to overcome resistance and recurrence. The specific surface CSC markers are well known in various cancers and are potent candidates for being targeted by novel anti-cancer therapeutics including CAR-T cell therapy. CAR-T cells can effectively target CSCs and disable the reconstitution ability of tumors. CAR-T cell therapy combination with chemo/radiotherapy is expected to guarantee tumor eradication without recurrence. Moreover, cure of cancer will ultimately need the eradication of all malignant cells (*i.e*., CSCs and their progeny) within a patient's cancer. Hence, it will be important and promising to concomitantly use CSC-targeting CAR-T cells with other anti-cancer therapeutic modalities. Such combinatorial regimens may perform in concert to eradicate CSCs, differentiated progenitors and bulk tumor cells in cancer patients. These data foresee the use of CAR-T cell-based combination therapies for the future treatment of cancer.

Acknowledgments

This work was supported by Dr. Kazemi Ashtiani Award of Iran's National Elites Foundation (INEF, Iran) awarded to Hamid Reza Mirzaei.

Author contributions

Javad Masoumi, Hamed Mirzaei and Hamid Reza Mirzaei contributed in conception, design, and drafting of the manuscript. Abdollah Jafarzadeh, Jalal Abdolalizadeh, Javad Masoumi, Haroon Khan and Jeandet Philippe contributed in data collection and manuscript drafting. All authors approved the final version for submission.

Conflicts of interest

Authors declare that there are no potential conflicts of interest.

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