



Involving stemness factors to improve CAR T-cell-based cancer immunotherapy

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Abstract

Treatment with chimeric antigen receptor (CAR) T cells indicated remarkable clinical responses with liquid cancers such as hematological malignancies; however, their therapeutic efficacy faced with many challenges in solid tumors due to severe toxicities, antigen evasion, restricted and limited tumor tissue trafficking and infiltration, and, more importantly, immunosuppressive tumor microenvironment (TME) factors that impair the CAR T-cell function adds support survival of cancer stem cells (CSCs), responsible for tumor recurrence and resistance to current cancer therapies. Therefore, in-depth identification of TME and development of more potent CAR platform targeting CSCs may overcome the raised challenges, as presented in this review. We also discuss recent stemness-based innovations in CAR T-cell production and engineering to improve their efficacy in vivo, and finally, we propose solutions and strategies such as oncolytic virus-based therapy and combination therapy to revive the function of CAR T-cell therapy, especially in TME of solid tumors in future.

Keywords Adoptive cell therapy · Cancer stem cell · iPSC · Oncolytic virus · Stemness · Tumor microenvironment

Abbreviations

ACT	Adoptive cell therapy	ICB	Immune checkpoint blockade
CAR	Chimeric antigen receptor	iCARs	Inhibitory CARs
CRS	Cytokine-release syndrome	ICIs	Immune checkpoint inhibitors
CSCs	Cancer stem cells	iPSCs	Induced pluripotent stem cells
DAMPs	Damage-associated molecular patterns	GvHD	Graft-versus-host disease
		PAMPs	Pathogen-associated molecular patterns

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PBMC	Peripheral blood mononuclear cell
MDSCs	Myeloid-derived suppressor cells
OV	Oncolytic virus
TAAAs	Tumor-associated antigens
TAMs	Tumor-associated macrophages
TILs	Tumor-infiltrating lymphocytes
TME	Tumor microenvironment

Introduction

Immunosuppressive tumor microenvironment (TME) due to molecular and cellular effectors, upregulation of inhibitory signaling pathways versus activating responses, and poor antigenicity of tumor cells all limited the antitumor responses delivered by both host normal immune T cells and transferred TILs (tumor infiltrated lymphocytes) during adoptive cell therapy (ACT) [1, 2]. These limitations have motivated immunologists and oncologists to introduce now approved immune checkpoint inhibitors (ICIs) and also to develop alternative ACT by engineering chimeric antigen receptors (CARs) expressed on T cells *ex vivo* to target tumor-associated antigens (TAAs) on the surface of tumor cells *in vivo* [2]. Over time and depending on the number and type of signaling domains, fourth-generation CAR T cells are developed that are enhanced to induce more enhanced cytolytic activity while producing inflammatory interleukins (ILs) (e.g., IL-12 and IL-15) and reprogrammed to have long-term survival of CAR T cells in the TME [3].

Until now, the CAR T-cell therapy was approved by FDA and limited only for hematologic tumors and targeting CD19 antigen on B cell (i.e., KYMRIAH and YESCARTA). KYMRIAH is a CD19-directed engineered autologous T-cell immunotherapy indicated for the treatment of adult patients with relapsed or refractory follicular lymphoma after two or more lines of therapy. YESCARTA is also a CD19-targeted autologous T-cell immunotherapy for adults with large B-cell lymphoma who are refractory to first-line chemo-immunotherapy or have relapsed within 12 months of first-line chemo-immunotherapy. However, preclinical studies indicated that the efficacy of CAR T-cell therapy of solid tumors is limited by several factors, including the selection of specific targetable antigens or epitopes, production of potentiated CAR, effective lymphodepletion, and infiltration, tumor homing, and survival of CAR T cells in the TME [4]. Therefore, profound solutions are needed to increase the chances of treating solid tumors with CAR T-cell immunotherapy.

Considering the survival of administered CAR T cells *in vivo*, selection of sources with stemness properties and reprogramming of mature cells into stem cells are examples of promising strategies under investigation, as discussed in this review. Moreover, the development of CAR

T cells targeting the population of cancer stem cells (CSCs) involved in tumorigenesis, metastasis, tumor recurrence, and resistance to conventional therapies is an alternative way to eradicate established solid tumors, as investigated in recent years and summarized here. Finally, combination of CAR T-cell therapy with powerful therapies such as ICIs, oncolytic virotherapy, and chemo/radiotherapy is perspective strategy for enhancing CAR T-cell functions against resistant solid tumors that we presented this review.

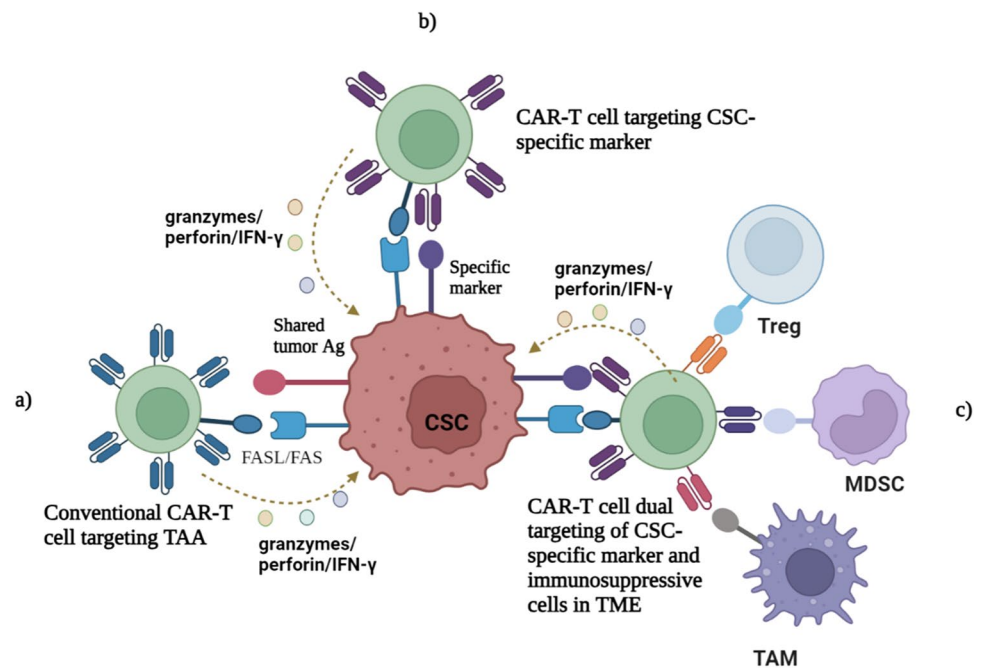
CAR T cells targeting resistant CSCs and TME

Similar to non-CSCs, CSCs express the cell surface antigens that could be targeted by CAR T cells in an HLA-independent manner, and preclinical and clinical studies have investigated to achieve an effective outcome [5, 6]. It should be noted that CSCs are heterogeneous cells for the expression or even non-expression of targeted antigens, different types of CSCs in many tumors express different pattern of TAAs, and moreover, CSCs may express the antigens shared with normal stem cell, which resulted in off-target toxicity [7] (Fig. 1a). Overcoming these challenges requires the development of new generations of CAR T cells, such as the selection of antigens with high expression levels compared to normal or CSC-specific antigens and the discovery of new CSC surface antigens [8, 9]. There are some CSCs markers that can be specifically targeted by monoclonal antibodies (mAb) for CSCs enrichment and identification and also immunotherapies by CAR T cells in clinical and preclinical studies (Table 1).

CD133, or prominin-1, is a transmembrane glycoprotein found in CSCs of human tumors and drive resistance to chemotherapeutic agents that treatment with CD133-targeted CAR T cells resulted eradication of glioblastoma CSCs as well as off-tumor toxicity, as CD133 is expressed on normal neural stem cells [10]. Epithelial cell adhesion molecule (EpCAM) or CD326 is frequently overexpressed in tumor-initiating cells or CSCs and its overexpression is correlated with activation of Wnt/ β -catenin signaling in CSCs [11, 12]. Therefore, EpCAM may represent a promising target for immunotherapy of EpCAM-expressing tumorigenic CSCs. For examples, CSC of liver and colorectal cancer express another transmembrane glycoprotein called EpCAM that its targeting by EpCAM-specific CAR T cells demonstrated the potential to inhibit tumor growth in EpCAM+ tumor xenografts while inducing secretion of cytotoxic cytokines such as TNF- α and IFN- γ [13]. In addition, CD123-directed CAR T cells have the potential to be highly effective in the elimination of CD123+ leukemic CSCs in a primary AML model [14].

However, targeting these markers has different safety issues and requires the identification and engineering of

Fig. 1 Development of CAR T cells targeting CSCs. **a** Convection CAR T cells developed to target common TAA on CSCs and induce apoptosis. **b** Modified CAR T cells developed to target specific stemness markers or antigens on CSCs and induce apoptosis. **c** Engineering CAR T cells to dual-target CSC-specific antigens and markers of any immunosuppressive cells in the TME such as Tregs, MDSCs, and TAMs. CAR chimeric antigen receptor, CSCs cancer stem cells, MDSCs myeloid-derived suppressor cells, TAAs tumor-associated antigens, TAMs tumor-associated macrophages, TME tumor microenvironment



CAR T cells targeting only potent CSC markers such as CD271, LGR5, TIM-3, CD13, and CD105 (Fig. 1b). Over-expression of LGR5 (leucine-rich repeat-containing, G protein-coupled receptor 5) was strikingly correlated with human cancer recurrence, and application of the anti-LGR5 mAb-drug conjugate demonstrated inhibition of tumor growth in both in vitro and in vivo models [22]. In addition, the low expression of critical CSC-specific antigens limits the efficacy of therapies, and thus targeting surface markers that drive stemness or reprogramming in cancer cells enhances the function of CAR T cells to selectively target and eliminate these CSCs. For example, c-Met is a receptor tyrosine kinase that induces the expression of stem cell reprogramming factors (i.e., Nanog, SOX2, and CD133) and their targeting by kinase inhibitor [23] and CAR T cells [24] potentially suppressed the growth of MET+ CSCs in xenograft tumors. Considering the tumor-supportive role of tumor-associated macrophages (TAMs) in the TME of solid tumors, targeting antigens such as TIM-3 (T-cell immunoglobulin mucin-3) expressed on the surface of both TAMs and CSCs may be a promising approach for the treatment of human tumors [25] (Fig. 1c). TAMs also support CSCs in breast cancer by upregulating stemness drivers such as SOX2, OCT4, and NANOG [26]. Human myeloid-derived suppressor cells (MDSCs) are myeloid-originated progenitor cells that are classified into two subsets known as monocytic MDSCs and polymorphonuclear MDSCs. CD11b+ CD14- CD33+ MDSCs secrete cytokines and chemokines in TME providing immunosuppressive niche and thus reducing the efficacy of immunotherapy [6]. CSCs activating mTOR [27] and TGFβ1 signaling pathways

promoted infiltration, accumulation, and expansion of MDSCs in tumor site [28], which followed by impairment of T-cell responses. Reciprocally, MDSCs contribute to the stemness and survival of CSCs through the production of tumor-supportive PEG-E₂ (prostaglandin E₂) [29]. Regulatory T cells (Treg) are a T-cell-derived subset that has been shown to have multiple mechanisms of crosstalk with CSCs to promote an immunosuppressive TME [6]. Similar to MDSCs, PEG-E₂ induces FOXP3+ CD4+ CD25+ Tregs to generate COX-2, suppressing the failure of effector T cells in cancer immunotherapy [30]. Tregs, mainly by secreting inhibitory cytokines such as TGF-β and IL-10, suppress CAR T cells, endogenous T cells and a variety of other immune cells [31]. Therefore, depleting (e.g., using cyclophosphamide or fludarabine) and targeting Tregs (e.g., CTLA-4 inhibition or CD25-targeting) may become an important aspect of CAR T-cell therapy for PDAC. These findings revealed that the TAM/Treg/MDSC-CSCs interaction reshapes the stemness in tumor cells resulting in tumor formation and progression and also opens the field for enhancing the antitumor potentials of CAR T cells targeting stemness markers in CSCs.

Challenges and perspectives approaches to improve CAR T cells targeting CSCs

CAR production and CAR T-cell expansion

Moreover, produced adenosine in hypoxic solid tumor mediated the reduction of IFN-supported CD8+ or CAR

Table 1 Examples of clinical trials CAR targeting cancer stem cells

CAR platform	Cancer type	Major finding(s)	Limitation	Status/NCT#	References
Anti-CD133-CAR vector-transduced T cells	Liver cancer, pancreatic cancer, brain tumor, breast cancer, ovarian tumor, CRC, AML and ALL	In patients with previously treated advanced HCC, CAR T-133 cell therapy demonstrates promising antitumor activity and a manageable safety profile	The most common high-grade adverse event was hyperbilirubinemia	Phase I/Phase 2 NCT02541370	[15]
MB-CART20.1	Melanoma	Ongoing	Ongoing	Early Phase 1 NCT03893019	[16]
CAR2 Anti-CD38 A2 CAR T cells	Refractory multiple myeloma	No results posted	No results posted	Phase I/completed NCT03464916	[17]
EpCAM CAR T cells	Stomach, malignant neoplasm of nasopharynx, recurrent breast cancer, gastric cancer	No results posted	No results posted	Phase I-II/recruiting NCT02915445 NCT03563326 NCT03013712 NCT02725125	[18]
Anti-CD123 CAR T treatment	AML	-	Terminated (adverse effect)	Phase 1 NCT03672851	[19]
c-Met RNA CAR T cells	Metastatic Breast Cancer, TNBC	mRNA c-Met-CAR T-cell injections were well tolerated and evoke an inflammatory response within tumors	Not reported	Phase 1 NCT01837602	[20]
CD171-specific CAR T cells expressing EGFRt	Neuroblastoma Ganglioneuroblastoma	No results posted	No results posted	Phase 1 NCT02311621	[21]

AML acute myeloid leukemia, ALL acute lymphoid leukemia, CRC colorectal cancer, EGFRt epidermal growth factor receptor that is expressed on the T cell, EpCAM epithelial cell adhesion molecule, HCC hepatocellular carcinoma, TNBC triple negative breast cancer

T-cell cytotoxicity. Therefore, the production of adenosine receptor-negative CAR T cells by applying CRISPR/Cas9 technology resulted in the generation of adenosine-resistant CAR T cells with enhanced antitumor function in vivo [32]. The antitumor responses of infused CAR T cells are also limited by their poor persistence in vivo, which could be improved by producing CAR T cells with memory or stem-like phenotypes that have long-term survival in vivo (Fig. 2a). These phenotypes could be achieved by stemness-related factor, metabolic, marker-based reprogramming of CAR T cells ex vivo. In clinical trials, CAR T cells that utilize oxidative phosphorylation (OXPHOS) have shown improved patient responses. Metabolically, strategies that redirect metabolism from glycolysis to OXPHOS/FAO or enhance mitochondrial fusion by blocking fission factors have shown promise. Restriction of glycolysis in favor of OXPHOS and FAO can be achieved by limiting glucose uptake, blocking glycolytic enzymes, or inhibiting positive regulators of glycolysis. Loss-of-function strategies, small molecule inhibitors, or upregulation of negative regulators are used in such approaches [33]. For example, glutamine promotes the differentiation of memory T cells into mature T cells and thus incubation of the source of CAR T cells with the inhibitor of glutamine uptake maintained these cells in memory-like phenotype with long persistence and better cytotoxic function in vivo [34].

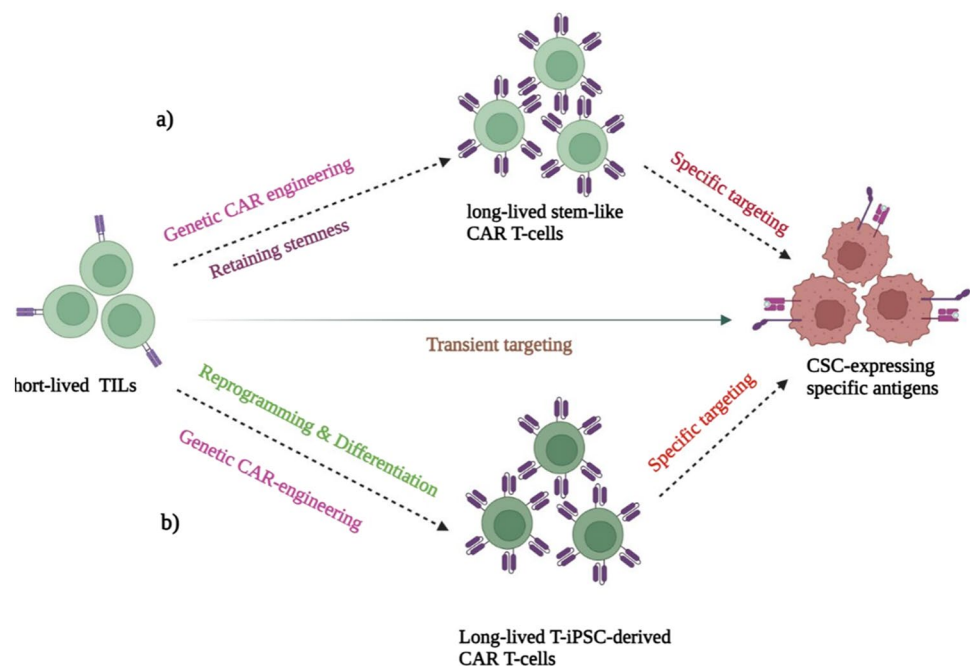
Interestingly, transduction of $\alpha\beta$ or $\gamma\delta$ TCR into induced pluripotent stem cells (iPSCs) resulted in the production of iPSC-derived T-cell products with low cytotoxic function but long-term persistence due to their reprogrammed stemness properties [35, 36] (Fig. 2b). Indeed, the low

cytotoxic function of CAR T-iPSCs is due to the rearrangement of TCR genes during iPSC manipulation, which subsequently produces the antigen specificity of effector T cells. This challenge could be overcome by using genome editing tools such as CRISPR to knock out *RAG2* (recombinase gene 2), which is responsible for additional TCR rearrangement in T-iPSCs [37]. Overall, stem cell-like T cells and CAR T-iPSCs appear to be attractive products for fractionated cell therapy given their potentials to differentiate cells presenting long-term persistence in vivo such as memory and effector T cells as well as introducing a powerful resource toward improving current CAR T-cell therapy in the clinic.

Combination therapy

The immunosuppressive TME impaired the activity of transfused CAR T cells in vivo, which could be addressed by applying combination therapies. Oncolytic viruses are another live platform that is being considered as an immunotherapy approach within the ability to infect and lyse cancer cells [38]. OV's induce antitumor immune responses by releasing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) in tumor niches, followed by interaction of AMPs/DAMPs and pattern recognition receptors (PRRs) in cancer or innate immune cells and expression of proinflammatory cytokines to activate immunosuppressive TME [39, 40]. OV's can promote the intratumoral infiltration and expansion of effector immune cells [41], which could be used in cancer therapy to enhance the efficacy of CAR T-cell therapy (Fig. 3a). In addition, OV's engineered to express chemokines such as

Fig. 2 Prospective methods to improve CAR T-cell persistence against CSC-specific antigens. **a** Short-lived T cells isolated from tumor mass, expanded ex vivo, and genetically CAR engineered plus induction of stemness factors to generate stem-like CAR T cells with long-term persistence in vivo while targeting CSC-specific markers. **b** T-iPSC-derived CAR T cells can be generated by reprogramming, differentiation, and genetic CAR engineering of TILs to present long and superior anti-CSC activity in vivo



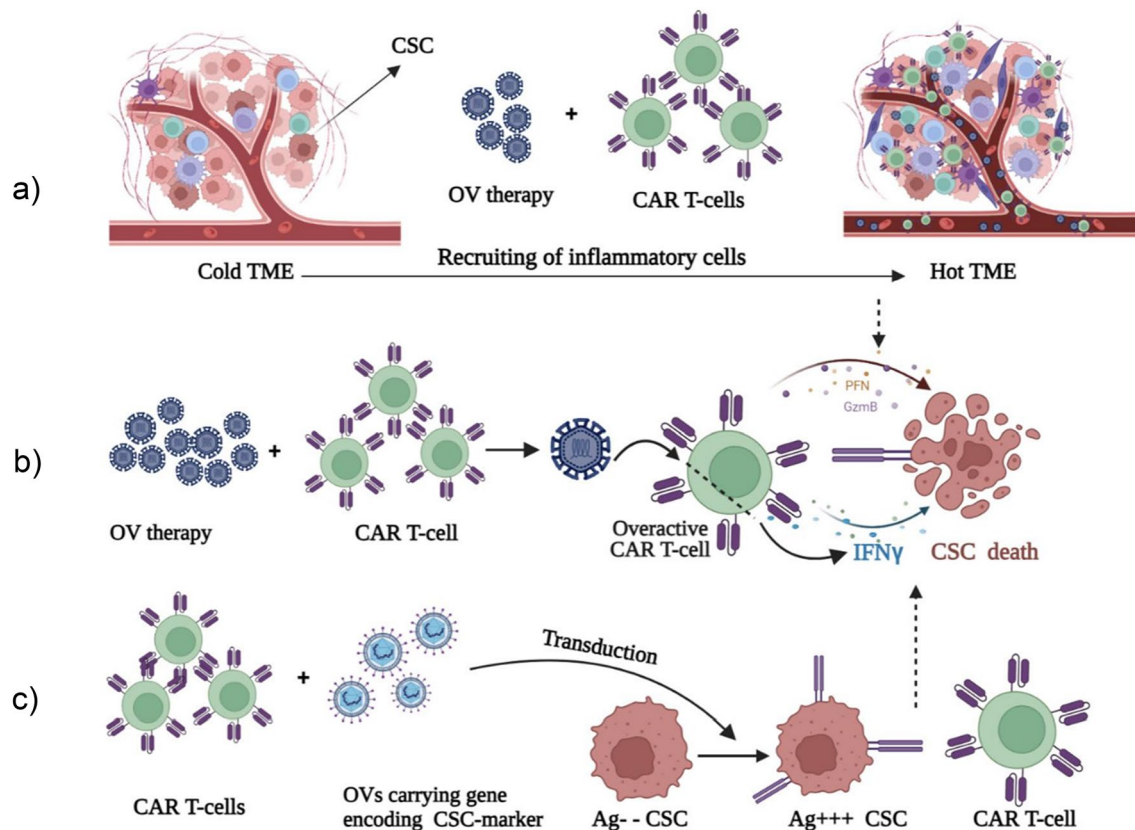


Fig. 3 Prospective pathways that Oncolytic virus (OV) and CAR T cells combination therapy can target and kill the CSCs in solid tumors. **a** OVs converted immunosuppressive and cold TME to the immunosuppressive and hot once by recruiting inflammatory immune cells and thus may promoted the infiltration of CAR T cells into tumor site for targeting and elimination of CSCs. **b** Co-treatment

of cancer cells with oncolytic viruses lead to overproduction of cytokines (ILs and IFNs) that augmented the antitumor activity of infused CAR T cells. **c** Genetic engineering of oncolytic viruses with DNA encoding the CSC-specific marker can promote effector functions of CAR T cells to bind and kill CSCs

CCL5, CXCL11, and CXCR3 ligand recruited CAR T cells to the tumor site and facilitated their antitumor activity [42, 43].

OVs directly lyse tumor cells, and with respect to CSCs, the Zika virus (ZIKV) and herpesvirus (HSV) have demonstrated the potential to target and kill glioblastoma CSCs [44]. OVs also induce the secretion of proinflammatory IFNs and ILs in the immunosuppressive TME or with cytokine-armed OVs, both of which contribute to the survival and expansion of CAR T cells and enhance their cytotoxic effects [38, 45, 46] (Fig. 3b). In fact, OVs activate apoptosis or lysis of tumor cells, resulting in the release of PAMPs and DAMPs, which are recognized by PRRs such as Toll-like receptors (TLRs) on immune cells, as well as the production of proinflammatory cytokines (such as type I IFNs, IL-1 β /6/12, TNF- α and GM-CSF) and chemokines (such as CCL2/3/5 and CXCL10), all leading to the formation of a proinflammatory TME [47]. In addition, some CSCs are negative for markers that are selected for CAR, and OVs as carriers can provide expression of that specific marker

on the surface of CSCs or tumor cells to enhance the anti-tumor activity of CAR T-cell therapy [48] (Fig. 3c). OVs also induce the expression of PD1/PD-L1, which sensitizes tumor cells to ICI therapy [44] and provides the combination therapies of OV, ICI, and CAR T cells. Taken together, OVs induce a switch from “cold” TME to “hot” TME by facilitating the recruitment of effector immune cells and activating systemic antitumor adaptive immunity to suppress tumor growth [49, 50]. Promising results of oncolytic virotherapy in combination with CAR T cells in preclinical studies for solid tumors lead to initiation of related ongoing clinical trials (Trials ID: NCT03740256 and NCT01953900).

Considering the plasticity of CSCs and the insufficiency of CAR T-cell monotherapy to completely and potently eliminate resistant tumor cells or CSCs, combination therapy with other standard radio/chemotherapy regimens may also be highly effective for CSC eradication. For example, treatment with local radiotherapy followed by infusion of NKG2D CAR T cells produced synergistic antitumor activity in a glioma model, indicating that radiotherapy promoted

the infiltration of CAR T cells to the tumor site to exert their effector functions [51]. Similar to OV, the combination of CSC-targeted CAR T-cell therapies with agents that induce the expression of CSC-specific antigens may overcome the obstacle posed by CSCs' loss of the target antigen [52]. Finally, PD-1/PD-L1 overexpression resulted in CAR T-cell exhaustion in the TME [53], and the combination of CD19 CAR T-cell therapy and PD-1 blockade improved the survival and function of immunosuppressive-resistant CAR T cells in vivo [54]. In addition, M2 macrophages expressing PD-L1 limited the CAR T-cell activity that PD-L1 blockade in combination with CAR T-cell therapy resulted in a phenotypic shift toward more M1-like subsets and a loss of M2 macrophages through IFN- γ signaling, leading to enhanced antitumor activity of CAR T cells [55]. The combination of CAR T cells and ICI has proven to be safe and effective in hematological malignancies. However, the efficacy and safety of combining ICI and CAR T-cell therapies in advanced solid tumors has not been well established. With regard to the aforementioned advantages, several trials (NCT04003649, NCT03726515) are underway to evaluate the safety and efficacy of CAR T cell and ICI combinations in solid tumors, including glioblastoma. Overall, combination therapies provide durable expansion and function of CAR T cells in tumor-supportive TME, while limiting resistance mechanisms and toxicities at low desired doses of CAR T-cell infusion.

Safety issues

Besides the side effects such as cytokine release syndrome (CRS), the VH and VL domains of CAR, the scFv of a mAb or, can induce the production of anti-scFv antibodies and thus reduce their antitumor effect. Instead of the complete scFv, the construction of an extracellular domain consisting of a single variable domain on a heavy chain (VHH) resulted in nanobody as the targeting domain of CARs with the required functions for recognition and binding of cell surface-expressed target antigens while overcoming the obstacle of neutralizing antibodies [56]. CAR T cells are indicated to target and eradicate CSCs, but CSCs express antigens that are shared with normal stem cells and other normal cells, leading to off-tumor toxicity. One strategy to overcome this challenge is the direct or intratumoral administration of CAR T cells [57]. However, this method is not possible for non-surface available in tumor tissues. Other strategies involve the development of multi-CAR T cells that target more than one marker in heterogeneous CSCs [58] or modified CAR T cells expressing inhibitory CARs (iCARs) such as PD-1 and CTLA-4, which are switched off when bound to normal cells in vivo [59, 60], or the production of suicide-inducible CAR T cells to overcome off-tumor toxicity after infusion of CAR T cells [61]. Given the low density

of TAA in normal cells, engineering CAR T cells with low affinity binding to TAA resulted in unbinding to normal cells and reduced cytotoxicity [62].

With regard to OV theory, patient safety is of paramount importance in cancer treatment, and treatment with oncolytic viruses appears to be the most promising in this regard. Completed trials have shown no dose-limiting toxicity. The major disadvantages of oncolytic viruses include fevers and flu-like symptoms, pre-existing antibodies, and OV replication in normal tissues, particularly the brain, especially in immunosuppressed cancer patients, which may hinder systemic delivery of the virus [63]. To overcome this problem, it is necessary to deliver the OVs to their target using a cellular vehicle, to suppress intracellular pathways associated with innate immunity by encoding one or two immunosuppressive genes of the wild-type OV in the OV vaccine, and to combine oncolytic therapy with immunosuppressive drugs [64].

Conclusion

Collectively, the suppressive TME impairs the cytotoxicity of CAR T cells, whose therapeutic potential could be enhanced by designing CAR T cells to target multiple antigens, re-expressing T engagers on the surface of memory T cells or iPSCs, reactivating the stemness or memory-induced pathway or genes in mature T cells, and combining them with other therapeutic modalities such as ICIs and OVs to enhance CAR T-cell trafficking and expansion at the tumor site, and also reprogramming the immunosuppressive TME at the molecular and cellular level into immunosuppressive once. Of note, the introduction of controllable keys to manage CAR T cells in case of adverse events is a critical safety recommendation for clinical establishment.

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