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Review Article

Target selection of CAR T cell therapy in accordance with the TME for solid tumors

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Abstract: Chimeric antigen receptor-engineered T (CAR T) cell therapy has made great progress in hematological malignancies and resulted in two newly FDA-approved drugs specific for CD19, *Kymriah* and *Yescarta*. To some extent, this success is attributable to the appropriately selected antigen, CD19, a cell surface protein that is uniformly and strongly expressed on malignant B cells. This result indicates that a proper CAR target is of great importance to the success of this technique. Another key factor contributing to the success of hematological malignancies can be ascribed to the nonphysical tumor microenvironment (TME). The TME in solid tumors is complicated and has a specific niche favorable for tumor progression with physical barriers, multiple mechanisms of immunosuppression, and a variety of biochemical factors, thus resulting in limited efficacy of CAR T cell therapy in clinical trials with cancer patients. Therefore, the inhospitable solid TME becomes a major hurdle in translating the success of CAR T cell therapy in hematological malignancies to solid tumors. Here, we provide our perspective on how to improve the success of CAR T therapy in solid tumors by focusing on the aspects of target selection and the related TME in CAR T cell design, especially stressing the interplay between them. With four kinds of antigenic CAR targets as examples in this review, we anticipate that the overall consideration of both factors will further expand CAR T cell therapy in clinical trials.

Keywords: CAR T cell therapy, target selection, tumor antigen, solid tumor, tumor microenvironment, anti-tumor immunity

Introduction

In recent years, genetically engineered T cells that recognize tumor antigens by either chimeric antigen receptors (CARs) or T cell receptors (TCRs) have developed rapidly as a very promising treatment for cancer patients, with CAR-engineered T cells (CAR T) at the forefront. The application of CAR T cells has achieved excellent clinical results in cancer patients, especially those with CD19-positive hematologic malignancies [1]. This directly prompted the US Food and Drug Administration (FDA) to approve two drugs, *Kymriah* of Novartis and *Yescarta* of KITE Pharma, the first CAR T therapy products [2, 3], which have induced intense interest in developing CAR T therapies for cancers. A typical CAR consists of an ectodomain, a transmembrane domain and an endodomain [4]. The ectodomain in this case contains a signal peptide, an antigen recognition region usually derived from a single-chain variable fragment (scFv) of a monoclonal antibody, and a spacer that connects the antigen recognition region to the transmembrane domain [4]. The transmembrane structure in a CAR is most commonly from CD28-, and less commonly from CD3ζ, CD4, CD8, and OX40. The main function of this structure is to provide stability to the CAR, with the transmembrane region from CD28 being more reliable than those of other proteins in most cases [5, 6]. The endodomain of a CAR is engineered with various intracellular signaling molecules. According to the characteristics of signaling molecules in a CAR, CARs have been categorized into four 'generations', which have been reviewed in detail by other researchers [7]. Along with the evolution of CARs from the first to the fourth generation, problems frequently occurred in practice, but were gradually overcome at different stages. The first-generation CARs contain a single signaling structure

from CD3ζ or FcεRIγ, accompanied by poor outcomes in most studies because of inadequate proliferation, a short life span *in vivo* and insufficient cytokine products [8]. The second-generation CARs added intracellular signaling domains from various costimulatory molecules such as CD28, 4-1BB and OX40 to the first-generation CARs, which improved the proliferation, cytotoxicity, sustained response, and life span of CAR T cells *in vivo* [4, 9]. In the third-generation CARs, two costimulatory molecules were fused to the CD3ζ signaling moiety, with the most common combinations being of p56-lck+CD28+CD3ζ, OX40+CD28+CD3ζ, or 4-1BB+CD28+CD3ζ [6]. The third-generation CARs can reduce the undesired anti-inflammatory effect of IL-10 [10], but takes the risk of signal leakage and cytokine cascade [11]. To optimize the anti-tumor effects of CAR T cells, the fourth-generation CARs have been developed recently by engineering the second-generation CARs with a cytokine expression cassette, which is known as T-cells redirected for universal cytokine-mediated killing (TRUCK). TRUCKs can strengthen T-cell activation and attract innate immune cells to the targeted lesion to eradicate antigen-negative tumor cells by releasing anti-tumor cytokines, thus producing better tumoricidal effects, especially on solid tumors [12]. One of the characteristics of all CAR structures is the ability to recognize tumor surface antigens independent of the expression of major histocompatibility complex (MHC) molecules [13], which endows genetically-modified T cells with the ability to target a broader spectrum of antigens than unmodified T cells, ranging from any proteins to carbohydrates, or lipid structures [14]. Therefore, the clinical application of CAR T cells is widely expanded.

Currently, there is great enthusiasm in the exploration of new innovations in CAR design, manufacture development and toxicity management, which has been stimulated by the successes of *Kymriah* and *Yescarta* for treating CD19-positive B cell malignancies. In the meantime, attention to the research of CAR T therapy on solid tumors has also been intensified, with a rapidly growing number of clinical trials on solid tumors underway [15-17]. Considering that solid tumors have complicated mechanisms for tumor growth and progression compared with those of hematological malignancies, it is more challenging to conquer this type of cancer with CAR T cells. To achieve the same level of success as in B cell lymphoma for solid tumors, a reasonably designed CAR is crucial. In the first step, selecting a proper antigenic target should be extensively considered. In regard to this, we might learn from the experience of the successes of *Kymriah* and *Yescarta*, which were largely due to the choice of CD19, a B cell marker with high levels of expression on malignant B cells. CD19 is indispensable for the growth of B cell malignancies since it is required for the signal transduction of the B cell receptor (BCR) [18]. Although B cell aplasia in patients was found after CAR T therapy because CD19 is also expressed on healthy B cells, the aplasia was well tolerated by an intravenous immunoglobulin replacement [19]. In another aspect, the indiscriminate loss of B cells can contribute to the removal of both cancerous and precancerous B cells, thus decreasing the chance of relapse [20]. From this, we can see that target selection is of decisive importance for a CAR study with two factors being considered comprehensively. One is to maximally match the common standards for normal antigens in targeted immunotherapy, and another is to take the therapeutic effects into account together with the specific tumor microenvironment (TME). In this review, four kinds of CAR targets are exemplified by the advancements obtained in recent CAR T investigations, and they show our perspective on how to choose a suitable target for a CAR in accordance with the complicated TME in solid tumors. In **Figure 1**, we summarize the distribution of CAR targets within the TME and the CAR structures, with an emphasis on the CAR design according to the basic elements in a CAR. We anticipate that the notion will expedite the clinical application of CAR T therapy for solid tumors.

Antigenic targets for CAR T cells in solid tumors

As mentioned above, antigenic targets for CAR T cells can be a range of any proteins, carbohydrates or glycolipids, thus widely expanding the spectrum of the target selection. A properly selected target plays a central role in determining the success of a CAR T cell therapy. To the best of our knowledge, ideally, the molecule for CAR targeting should be overexpressed on tumor tissues, with zero or low expression on normal cells. In the meantime, the antigenic target

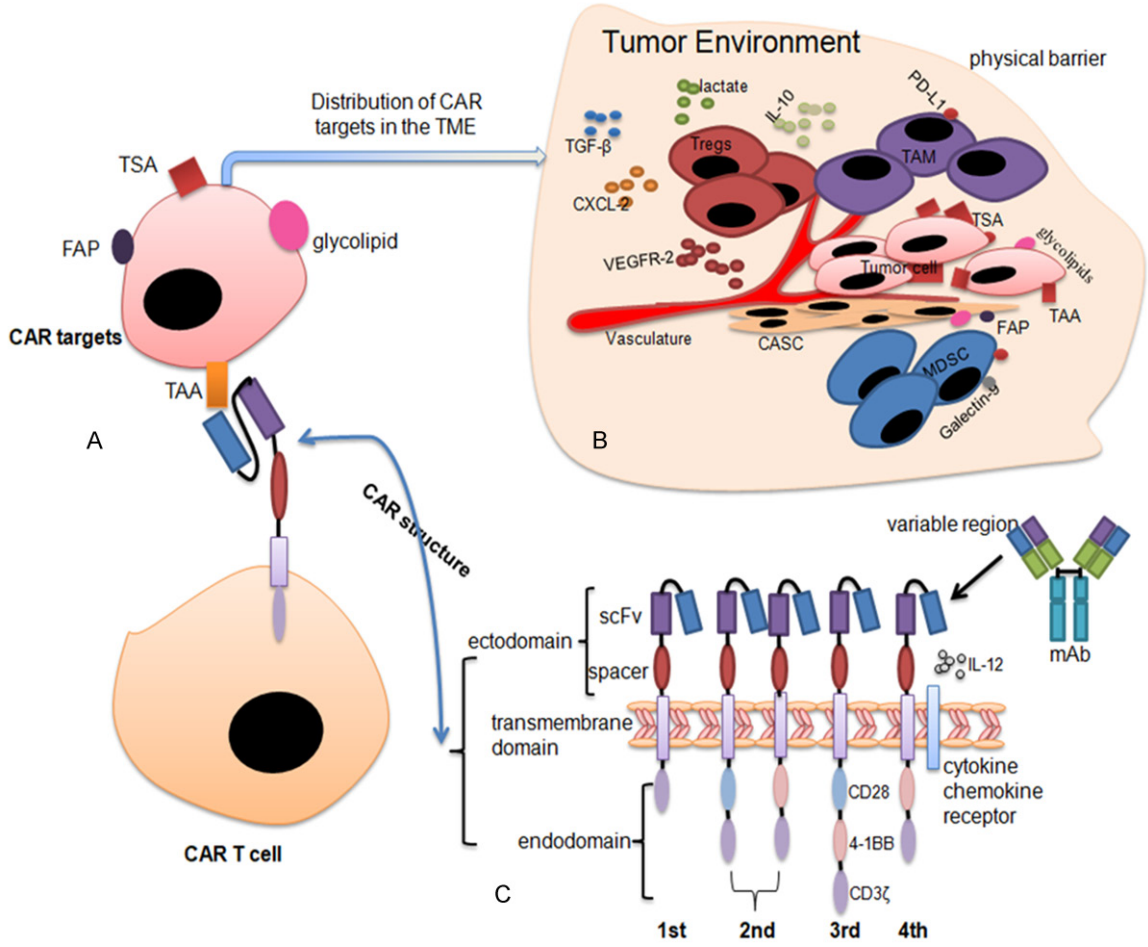

Figure 1. Schematic design of CARs according to the basic elements in a CAR. A. Specific binding between a CAR T cell and its corresponding target cell represented with the four kinds of potential antigens on the surface. B. Distribution of CAR targets in the complicated solid TME. The TME is the major hindrance of CAR T therapy due to many factors such as physical barriers and immunosuppressive components secreted by a variety of inhibitory cells in the TME. Overcoming the immunosuppressive functions of the TME in CAR T therapy is the key point to be considered for the right target selection. Numerous targetable molecules exist that can be categorized as TSA, TAA, CASC-expressed antigen, and glycolipid antigen. C. CAR structure. CAR T research has advanced for several decades and experienced four generations of evolution. A classical CAR is composed of an ectodomain, a transmembrane domain, and an endodomain. A CAR generation can be identified by the selected signal molecules in the endodomain.

should also have plasma membrane localization. In terms of the criteria, the tumor-specific antigen (TSA), a type of tumor antigen, is the most suited, partly due to its unique and abundant expression on tumor cells [21]. Since this type of antigen is not expressed by healthy tissues, great efforts have been made to expedite its potential usage in clinics. However, these antigens are highly heterogeneous among patients suffering from the same type of tumors, which in turn creates challenges for CAR T cell therapy against them. For instance, because of the antigenic heterogeneity, it is a prerequisite to identify a proper TSA for each patient and then be able to generate specific CAR T cells [22]. This is a very complicated and costly process that it is not affordable to most patients. Therefore, although TSAs can serve as ideal targets for CAR T cells, it is rare to see successful cases in CAR T therapy using them [23]. More efforts are needed to lower the cost and simplify the procedure for accelerating their applications. Accordingly, another type of tumor antigen called tumor-associated antigen (TAA) has been extensively explored at the same time. Nevertheless, targeting TAAs can often cause on-target/off-tumor toxicities because they are concurrently expressed on normal tis-

Table 1. Overview of the four types of CAR targets

Category	Expression pattern	Advantages	Disadvantages	Example
TSA protein form	Uniquely expressed on malignant cells	Increases anti-tumor efficacy and safety because of the unequivocal restriction to tumor tissues	Antigenic heterogeneity; difficult to find a specific target for each individual; immunoescape	EGFRvIII
TAA protein form	Overexpressed by cancer cells, with low expression by healthy tissues	Provides wide range of options for target selection; targetable in different tumor types	Extensive preclinical testing of expression in healthy tissues is required; on-target/off-tumor toxicities; immunoescape	Mesothelin
Glycolipid or ganglioside antigen	Expressed on both cancerous and normal tissues	Expands the range of target candidates; reduces immunoescape; high density on cell surface	Extensive preclinical testing of expression in healthy tissues is required; on-target/off-tumor toxicities	GD2
CASC surface antigen protein form	Mainly expressed by CASCs, with low or no expression on cancer cells	Avoids immunoescape; broadly applicable in various cancers	Severe side-effect toxicities	FAP

Note: TSA, tumor-specific antigen; TAA, tumor-associated antigen; CASC, cancer-associated stromal cell; EGFRvIII, type III of variant epidermal growth factor receptor; FAP, fibroblast activation protein; GD2, Disialoganglioside.

sues at a low level, even though the most TAAs are overexpressed by tumors [24]. Fortunately, the problem is being resolved gradually, as methods in the field of gene engineering are progressing, resulting in several clinical trials underway with TAAs for solid tumors [25-27]. Although TSAs and TAAs are the first choice for solid CAR T therapies in most cases, challenges frequently exist as just described. When these therapies cannot satisfy the demand in applications, finding other types of targets becomes essential. One category of alternative antigens that includes fibroblast activation protein (FAP) [28] and vascular endothelial growth factor receptor-2 (VEGFR-2) [29], has been well studied. This type of target is not expressed on the tumor cells themselves but is highly expressed in tumor-associated fibroblasts for FAP [28] or tumor vasculature for VEGFR-2 [29], forming a supportive niche for tumor cells. Therefore, targeting them can destroy tumorigenesis by hindering stromal formation or angiogenesis. Reported documents have confirmed that it is practical to choose FAP or VEGFR-2 as targets for CAR immunotherapy [30, 31]. In addition to the above mentioned targets, there is a distinctive type of antigen, glycolipid antigens, that is also suitable to be targeted by a CAR, which is ascribed to the characteristics of CARs, i.e., MHC-independence. The most investigated glycolipid antigen target is ganglioside GD2, which is highly overexpressed in neuroblastoma and many other types of solid tumors [32], and has been applied as a CAR target in several clinical trials: NCT02992210, NCT02761915, NCT03373097, NCT02765243 (clinicaltrials.gov). Here, we classified the above antigens into four types of CAR targets for solid tumors, i.e., TSA, TAA, cancer-associated stromal cell (CASC) surface antigen and glycolipid antigen, which include almost any target currently being studied. As described in **Table 1**, we can see that potential targets for CAR T therapy can be able to be chosen in a broad range with great flexibility.

Tumor microenvironment to be considered

The tumor microenvironment (TME) represents a complex ecosystem for a solid tumor that involves innumerable interactions between immune cells, cancer cells, stromal cells, cytokines, chemokines, and the extracellular matrix. TME supports tumor proliferation, survival, and metastasis by forming a highly immunosuppressive niche [33]. Immunosuppression in the niche can be achieved through different mechanisms. Tumor-associated macrophages, cancer-associated fibroblasts, and tumor cells can all secrete suppressive cytokines and chemokines, and there can be metabolic competition over the consumption of nutrients by tumor cells or a shortage of oxygen. The production of inhibitory metabolites, migration failure because of the rigid extracellular matrix, poor antigen presentation, chronic T cell receptor (TCR) signaling, and inhibitory receptor expression by tumor cells and stromal cells can all pose formidable barriers for the effective anti-tumor function of the immune system [34, 35]. To establish a favorable TME for developing therapeutic efficacy in cancer immunotherapy, inhibiting tumor-induced immunosuppressive mechanisms is key. Current strategies mainly consider

the following aspects: (1) the use of immune modulators aimed at immune checkpoints to enhance endogenous anti-tumor immunity in the TME [36]; (2) targeting regulatory cells within the TME, such as Tregs (regulatory T cells), TAMs (tumor-associated macrophages), and MDSCs (myeloid-derived suppressor cells) [37, 38]; and (3) modifying the cytokine and chemokine profile of the TME [39]. Similarly, in CAR T cell therapy for solid tumors, the same strategies can be adopted so that the inhospitable TME can be converted to be hospitable for the infused CAR T cells. It is crucial to take the global TME into account during target selection for programming a CAR T therapy. By targeting components within the TME, researchers have reported positive results. In 2015, Lo et al. demonstrated that CAR T cells specific to the FAP expressed on stromal cells, inhibited stromagenesis, reduced vascular density, and disrupted the spatial orientation of tumor cells [40]. Another protein highly expressed on endothelial cells of the tumor vasculature, integrin $\alpha_v\beta_3$, has also been targeted, leading to significant tumor shrinkage [41]. Furthermore, since the components in the TME are the main cause of immunosuppression, the combination of blocking these components with a regularly selected CAR target has been a well-studied approach that has produced promising results. Chimeric switch receptors that can convert a negative signal of IL-4 (a cytokine frequently present in the TME) to a positive signal of IL-7 have been developed for designing prostate stem cell antigen (PSCA)-specific CAR T cells [42]. The combination of blocking checkpoint molecules with a regularly selected CAR target is another example since checkpoint inhibition promotes the function of effector-T cells. Several studies have demonstrated increased activity against solid tumors using this modality [43, 44]. The above data suggest that the hostile immunosuppressive TME is modifiable to become beneficial to CAR T cells, thus leading to improved tumor eradication. As a key step in CAR T therapy, selecting appropriate antigenic targets according to the specific TME exerts far-reaching effects on the results.

Reasonable target selection alleviates toxicities in CAR T therapy

In CAR T therapies, safety and effectiveness are the major concerns for cancer patients. Unfortunately, these concerns remain a great challenge because of the frequently reported toxicities elicited by the treatment, with even death incidences reported in some trials [24, 45]. Reported toxicities following CAR T therapy include cytokine release syndrome (CRS), neurologic toxicity, and on-target/off-tumor toxicity. The most significant and life-threatening mechanism is CRS induced by the rapid and extensive activation of infused CAR T cells after antigen engagement [46]. This syndrome happens at a frequency of 18%-100% of patients [47]. Fortunately, this type of toxicity can be well-controlled with the IL-6R inhibitor tocilizumab [48] and other cytokine-directed methods [49]. For the neurologic toxicity described in 13%-52% of patients across institutions, the syndrome is frequently self-limiting, and the etiology remains unclear, although it often accompanies CRS [1]. More studies are needed to fully understand the biology of the syndrome and to subsequently prevent or abrogate this toxicity. As far as it is concerned, the on-target/off-tumor toxicity directly relates to the specific recognition of a target by CAR T cells [50], thus the antigenic specificity of the modified T cells decisively affects the outcomes. Given that most antigens that are currently being targeted in CAR T cell studies are also expressed on normal tissues, this type of toxicity will remain a safety problem for CAR T therapy. Emerging strategies aiming to minimize the on-target/off-tumor toxicity such as affinity-tuned CARs [51] and combinational-targeting CARs [52, 53] have been developed. Notably, tumor recognition and bystander discrimination as well as the control of CAR T cell activity can be intervened by selecting more suitable antigens [14, 54], thus alleviating the syndromes caused by toxicities. In finding such suitable target antigens, completely understanding the TME in which the antigens reside is of great importance.

Target selection in accordance with the TME

Appointing an appropriate antigenic target to a CAR has the most profound effect because failure in this step will restrain subsequent efforts owing to the adverse events in the first place. The TME adds another critical factor that can exert a similar impact on this approach. The TME not only is essential to tumor growth but also creates a powerful immunosuppressive niche in the tumor so that tumor cells can

escape from the host's immuno-surveillance. With the increasing number of clinical trials in CAR T therapy for solid tumors, it is appropriate to recapitulate these trials to explore ways to select proper targets based on the specific TME. The following section takes four antigenic targets as examples to illustrate the interplay between target selection and the TME in CAR T therapy, with each target as a representative of the above-mentioned antigen classes.

EGFRvIII, a type of TSA, is ideal for GBM-CAR T targeting

Glioblastoma (GBM) is the most common malignant primary brain tumor that is incurable thus far. To discover novel therapies for GBM, various immunotherapeutic strategies such as CAR T therapy, immune checkpoint blockade, and anti-tumor vaccination are currently evaluated. Fueled by the success of treating B cell acute lymphoblastic leukemia and chronic lymphoblastic leukemia with CARs targeting CD19, CAR T therapy targeting GBM-relevant antigens such as EGFRvIII, IL-13Rα2, HER2, and EphA2 [55] is drawing much attention. Among those, EGFRvIII-specific CAR T therapy has made the most progress in preclinical models, which have expedited the translation of this novel therapy into clinical application.

Epidermal growth factor receptor variant III (EGFRvIII) is the most common variant of the epidermal growth factor receptor (EGFR) observed in human tumors and results from the in-frame deletion of a portion of the extracellular domain [56]. The resultant mutant with a novel sequence at the fusion junction, thus creates a tumor-specific and immunogenic epitope that is not expressed in normal tissue. The mutant is not only expressed in a large majority of GBM patients, but also with other malignancies. Notably, EGFRvIII was found to be common on $CD133^+$ glioblastoma cancer stem cells and confers a high degree of self-renewal and tumor-initiating ability to $EGFRvIII^+/CD133^+$ cells [57]. Meanwhile, EGFRvIII plays important roles in tumorigenesis and invasiveness as a constitutively active tyrosine kinase [58]. These properties, including surface neoantigens specifically expressed on malignant cells, high expression in GBM and cancer stem cells, and the ability to induce phenotypic transformation to malignancy make EGFRvIII an ideal target for GBM treatment with CAR-modified T cells [59]. Based on the above nature of EGFRvIII, a second-generation CAR derived from a murine 3C10 single-chain variable fragment (scFv) fused with 4-1BB and CD3 was generated and tested in subcutaneous and orthotopic xenograft models of human EGFRvIII positive GBM [60]. The results demonstrated that targeting EGFRvIII specifically and lacking reactivity to wild-type EGFR significantly delayed tumor progression. A clinical trial at the University of Pennsylvania treating 10 patients with EGFRvIII-positive GBM with anti-EGFRvIII CAR T cells was reported to be safe and without evidence of off-tumor toxicity, CRS and cross-reactivity to wild-type EGFR [61]. Other forms of EGFRvIII-oriented CARs have also revealed satisfactory efficacy in preclinical models plus a first-in-man exploratory study for patients with newly diagnosed GBM [62]. However, it is also important to note that although the above data demonstrated successful CAR T cell trafficking to the tumor and effective antigen targeting, the evaluation of tumor tissue resected at the time of recurrence showed that EGFRvIII expression was lost over time [63]. The characteristics of antigen loss and the intratumoral as well as interindividual heterogeneity in GBM increase difficulties in treatments with CAR T cells. In this situation, alternative targets should be found to reduce the chance of immune escape and augment the anti-tumor immune response. Clinical studies on the overall safety and efficacy of CAR T cells targeting IL-13Rα2 and HER-2 in GBM are ongoing [55].

Mesothelin, a type of TAA, is suitable for CAR T targeting in a variety of solid tumors

Tumor-associated antigens (TAAs) are potential candidates for immunotherapy targets because they are overexpressed on tumor cells but have little to no expression on most normal tissues. As a type of TAA, mesothelin is highly expressed in a broad spectrum of solid tumors, such as epithelioid mesotheliomas, extrahepatic biliary cancers, pancreatic ductal adenocarcinomas (PDACs), ovarian carcinomas, and gastric carcinomas [64]. Moreover, reported data demonstrated that its increased expression is associated with poorer prognosis for patients with ovarian cancer [65], cholangiocarcinoma [66], pancreatic cancer [67], triple-negative breast cancer [68], and lung adenocarcinoma [69]. Re-

garding its expression in normal tissues, it is restricted to mesothelial cell layers and less so in epithelial cell layers [64]. Mesothelin is a cell-surface glycoprotein with an unclear physiologic function, but it may be a key element in malignancy since its aberrant expression plays important roles in both malignant transformation and tumor aggressiveness through boosting cancer cell proliferation, metastasis and invasion [70]. The above features of mesothelin have made it an attractive candidate as a therapeutic target. Over the past decades, strategies targeting mesothelin have been developed in preclinical studies, as well as in early phase clinical trials, including protein carrying immunotoxins, the use of monoclonal antibodies, antibody drug conjugates, specific vaccines, and CAR T cells [71]. Among these strategies, CAR T cell therapy draws the most attention for its great potential application in clinics with several preclinical studies underway. Jiang et al. showed that the anti-mesothelin CAR T cells were able to suppress tumor growth and penetrate the mesothelin-positive PDAC patient-derived xenograft models [72]. The engineered CAR T cells expressing an affinity-enhanced TCR against mesothelin were also assessed in PDAC and demonstrated efficient infiltration into the tumor site along with tumor cell death and prolonged survival of the treated mice [73]. In light of the anti-tumor effects of mesothelin-specific CAR T cells, researchers started a clinical trial in patients with epithelial ovarian cancer, malignant epithelial pleural mesothelioma and PDAC, which resulted in the direct anti-tumor efficacy in the clinical trial, NCT02159716 (clinicaltrials.gov). In another phase I trial, the activity of mesothelin-specific CAR T cells against pancreatic carcinoma metastases was evaluated, and the results showed the safety, feasibility and therapeutic potential with a CAR recognizing mesothelin [26]. Notably, a variety of mesothelin-specific CARs are being investigated in ongoing trials such as NCT02414269 and NCT02465983 (clinicaltrials.gov). All these results indicate that mesothelin is an effective target for CAR T therapy in mesothelin-expressing solid tumors.

Ganglioside GD2, a surface glycolipid antigen CAR target

Ganglioside GD2-specific CARs are a contrast to protein antigens for engineered CAR T cells since GD2 is a type of glycolipid found on the outer cell membrane. GD2 is highly overexpressed on a broad spectrum of tumor cells, including neuroblastoma, astrocytoma, retinoblastoma, Ewing's sarcoma, rhabdomyosarcoma, small cell lung cancer, melanoma and breast cancer, but is restrictedly expressed on normal tissues, including the central nervous system, predominantly in neuronal cell bodies and mesenchymal stem cells, and is expressed at low levels on peripheral nerves and skin melanocytes [32]. Reported data have shown that its expression density on neuroblastoma cell membranes can reach up to 5-10 million molecules/cell [74]. Moreover, levels of circulating GD2 are not high enough to interfere with binding to its specific monoclonal antibodies in circulation [75]. These properties make it an ideal target for CAR T cells. To date, anti-GD2-CARs have been well investigated in preclinical programs and clinical trials on several diseases such as neuroblastoma, osteosarcoma and melanoma [16, 76-78]. In a study testing the cytotoxicity of anti-GD2 CAR T cells in melanoma, the results revealed the specific lysis of GD2-positive melanoma cells *in vitro*. In two patient-derived xenograft (PDX) models, rapid tumor regression was observed in mice that received intravenous or local intratumor injections of anti-GD2 CAR T cells. Accordingly, researchers have concluded that anti-GD2 CAR T cells can both efficiently lyse melanoma in a GD2-specific manner and release Th1 cytokines *in vitro* and *in vivo*, representing a potential strategy for treating melanoma patients in the future [79]. The potential antitumor efficacy of anti-GD2 CAR T cells in H3-K27M$^+$ diffuse midline gliomas (DMG) was also reported recently. In this study, anti-GD2 CAR T cells demonstrated robust antigen-dependent cytokine release and the killing of DMG cells *in vitro*. In five PDX models, systemic administration of GD2-CAR T cells cleared engrafted tumors [80]. Based on the accumulated data, several clinical trials such as NCT02992210, NCT02761915, NCT03373097 and NCT02765243 (clinicaltrials.gov) are under way with GD2-targeted CARs in various solid tumors.

FAP, a CAR target on the surface of cancer-associated fibroblasts

Most CAR T cells are genetically engineered to target antigens on cancer cells, however, some

antigenic targets expressed on the surface of nonmalignant cancer-associated stromal cells (CASC) are also proper for CAR T cells. One attractive candidate of these targets is FAP, a transmembrane serine protease highly expressed on the CASCs in over 90% of epithelial cancers and with low expression on healthy adult tissue [81]. Selecting this type of target has several advantages. First, stromal cells are more genetically stable than cancer cells. Therefore, it is easier to target stromal cells in a stable way with an assigned antigenic target. Second, the tumor stroma has functions to support tumor cell growth, invasion, and angiogenesis to form a physical barrier against targeted tumor immunotherapy and to build an immunosuppressive niche by attracting immunosuppressive cells, regulating T cell functions, and expressing inhibitory molecules. Targeting stromal cells can damage these functions while retarding tumor growth. Third, the mechanisms by which the tumor stroma supports tumor growth are common; hence, targeted therapies against such mechanisms may have the potential to be used in a broad spectrum of tumors [82]. To date, several groups have reported their results from the use of anti-FAP CAR T cells. A study by Wang et al. developed an anti-mouse FAP-CAR construct comprising a sc-Fv from mAb 73.3 with a framework of CD8α-4-1BB-CD3ζ [82]. *In vitro*, the transduced FAP-CAR T cells secreted interferon-γ and specifically killed FAP-expressing 3T3 cells. In mice adoptively transferred 73.3-FAP-CAR T cells, FAP^{hi} stromal cells were reduced and the growth of subcutaneously transplanted tumors was inhibited. Furthermore, the results showed that the off-tumor toxicity in their models was minimal following the FAP-CAR T therapy. Therefore, researchers concluded that treatment with 73.3-FAP-CAR T cells directed to FAP-expressed tumor stroma can be safe and effective and suggested further clinical development of anti-human FAP-CAR. Based on the data obtained in this study, researchers performed a follow-up investigation on the impact of FAP-CAR T cells on tumor-induced desmoplasia. In highly desmoplastic tumor models, FAP-CAR T cells reduced tumor growth and was accompanied by a disruption of the desmoplastic stroma and reduced angiogenesis and cancer cell proliferation [40]. Although encouraging data were observed, the work by Tran et al. revealed severe side effects caused by the administration of FAP-CAR T cells generated from FAP-specific mAb FAP5 and sibrotuzumab. The results not only showed limited effects on tumor growth in a broad panel of murine models but also caused morbidity and mortality in most of the mice [83]. The differences among different groups may be related to the specificity and affinity of the scFvs that were used. Regardless of these contrasting results, FAP-targeting CAR T therapy has advanced into a phase I clinical trial, NCT01722149, for patients with malignant pleural mesothelioma, sponsored by the University of Zurich (clinicaltrials.gov).

Conclusion and perspective

In CAR T cell investigations, the first step is to choose an appropriate antigenic target. Ideally, CAR targets should be expressed on the cell surface of all tumor cells, but not on normal cells, and should be frequently shared among patients and contribute to the pathobiology of tumors such that downregulation would hamper tumor growth. TSA is the most satisfactory with these standards, largely due to its unique expression on tumor cells. A well-characterized TSA can provide a powerful tool to develop novel CARs for solid tumors. However, TSAs are genetically unstable and heterogeneous among patients and their expression can easily be manipulated by tumor cells to be downregulated or completely lost, hence escaping from the targeted immunotherapy. The study by targeting EGFRvIII confirmed the issue of antigen escape in TSA-specific CARs [63]. Another problem in targeting TSAs is the difficulty of finding appropriate TSAs because there are few TSAs in tumor tissues. Due to these limitations, the application of TSA-targeting CAR T therapies remains challenging and leaves a broad space to be filled with more efficient CARs. As another option, TAAs provide various candidates to be targeted since they are frequently overexpressed on tumor cell surfaces with a wide variety of types but are also simultaneously expressed on healthy tissues [84]. The expression profile of TAAs often causes unexpected on-target/off-tumor toxicities when this type of target is selected. To minimize this side effect, the highest priority should be given to antigens with fewer expression levels in vital normal tissues. Meanwhile, extensive preclinical studies should be performed to conduct extensive analyses of target expression by healthy tissue panels

from multiple donors. Recently, some research groups have proposed another solution to this side effect by tuning the sensitivity of CARs. In 2015, two different groups developed low-affinity CARs against HER2 and EGFR for glioblastoma and demonstrated excellent cytotoxicity in cells with high-density expression levels of targets while sparing cells with low-density expression levels that represented similar levels in normal tissues [85, 86]. As advances in CAR T therapy are increasingly being made, there are more options for proper target selection. In most cases, protein antigens are the first explored. However, it is feasible to choose a totally different type of antigen, such as carbohydrates or lipids [32], since the antigen-binding domain in a CAR is non-MHC restricted. In this way, the choices of CAR targets are expanding widely. As described above, ganglioside GD2-specific CARs have been extensively investigated, showing promising application in clinics [79, 80]. Furthermore, vital components expressed by tumor stromal cells can also be targeted by CARs, given the obvious drawback that target antigens on tumor cells are able to launch the adaptive mechanism of downregulating or completely losing their expression while being targeted [87]. In solid tumors, the TME is essential for tumor survival and CAR T cells can adjust the TME properties to facilitate T cell infiltration and promote antitumor activity after the relative tumor stromal cells are targeted, as exemplified by targeting FAP above [82]. To date, the application of CAR immunotherapy for solid tumors has been limited, partly due to the lack of antigenic targets that are constantly expressed in tumors yet entirely absent in healthy tissues. Combining stroma-targeting CARs with either TAA- or TSA-specific CARs may greatly enhance antitumor efficacy, providing various options for highly effective target selection.

In addition to the first-line role of CAR targets in the application of CAR T therapy, the TME also exerts significant effects on whether the approach is viable in solid tumors. On one hand, the solid TME is immunosuppressive and composed of malignant and nonmalignant cells [88], in which the infiltrated CAR T cells can be inhibited by a variety of factors. On the other hand, well-documented pathways inhibiting T cell immunity within tumors, including immune checkpoints, regulatory T cells, myeloid-derived suppressor cells, and metabolic alterations, can change after CAR T cell infusion, which means that the TME can either dampen or enhance immune responses in an adaptive manner [61]. This suggests that the hostile immunosuppressive TME can be converted to be beneficial to the infused CAR T cells if an appropriate approach is used. A recent study conducted by Chen et al. confirmed the idea by constructing CARs to target a soluble ligand, TGF-β, an otherwise immunosuppressive factor in a variety of solid tumors [89]. Another study by Batchu et al. also reversed the negative effect of the TME on mesothelin-CAR T cells through suppressing interleukin-10 in pancreatic cancer [90]. Data are accumulating to convince researchers of the tunable TME barriers and tumor eradication by appropriately targeted CAR T cells. Through optimizing the CAR structure and surmounting tumor-induced immunosuppression, the application of CAR T therapy to solid tumors will become closer to success as observed in hematologic malignancies. As the characteristics of the TME are depicted in more detail, more compelling evidence will be obtained for precise CAR target selection. The complicated interplay between the TME and antigenic targets can significantly determine if the efficacy of tumor killing is positive. It is promising to find novel strategies for changing the TME from an immunosuppressive mode towards one that supports the improvement of antitumor immunity by elucidating the molecular mechanism of interactions between the TME and CAR targets.

For the application of CAR T therapy against solid tumors, the main challenge is to inhibit tumor-induced immunosuppressive mechanisms and remodel a TME to be favorable for developing efficacious antitumor immunity. Both the property of a CAR target and the TME can contribute to producing new strategies to resolving this challenge. In one aspect, the antigenic specificity of a CAR directly defines the precise activity and safety of the genetically engineered T cells. Meanwhile, the heterogeneity of tumor antigens in solid tumors usually leads to invalid immune surveillance and thereby a refractory and relapsed tumor [91]. Given that it is too difficult to find entirely ideal antigenic targets that have a high frequency in common cancers, are constitutively expressed exclusively by malignant cells, are functionally

important for tumor growth and progression, and are targetable with MHC-independent systems, appointing relatively consistent targets to a CAR T therapy program continues to be the pursuit of researchers. In another aspect, the TME is extremely complicated and provides a favorable niche for tumor progression. A large number of immune cells together with stromal cells and extracellular matrix create an inflammatory milieu responsible for tumor expansion and dissemination and for tumor evasion [92]. The functional subversion of these TME components to a favorable antitumor state is also a practical goal for CAR T therapy. Recently, TME component-specific CARs have been developed and extensively investigated [89, 90], showing good prospects in promoting the antitumor efficacy.

In the aggregates, judicious target-antigen selection in accordance with the specific TME is a key element to be considered to effectively direct CAR T cells against diverse cancers. A well-selected antigenic target not only helps to reduce side effects and partly conquer the TME barriers of solid tumors but also plays decisive roles in the final anti-tumor efficacy. To obtain expected therapeutic effects, extensive preclinical investigation on the selected antigen should be performed according to the tumor microenvironment. At the same time, the immunosuppressive components in the TME are also good candidate targets because their hostile functions can be subverted to be favorable for tumor eradication under the appropriate treatment conditions. The selected target and TME are interrelated factors to be considered circumspectly when designing a CAR structure with the expectation of effectiveness. The tumor type determines its particular TME, accordingly requiring a specific target. Elucidating the interactive mechanisms between the target and the TME will help develop novel treatment modalities with CAR-modified T cells.

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Disclosure of conflict of interest

None.

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