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Review

Cancer immunotherapy with lymphocytes genetically engineered with T cell receptors for solid cancers

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ABSTRACT

Adoptive transfer of T cells genetically engineered with chimeric antigen receptors (CAR-T cells) have proven to be highly effective for treating $CD19^+$ B cell-derived hematologic malignancies. However, due to the lack of ideal tumor surface antigens, CAR-T cell therapy has limited success in treating solid tumors. T cells genetically engineered with T cell receptors (TCR-T cells) recognize intracellular and cell-surface antigens in the context of major histocompatibility complex (MHC) presentation and thus have the potential to access much more target antigens than CAR-T cells, providing great promise in treating solid tumors. There is an increasing interest in the application of TCR-T cell therapy for solid tumors, and fifty-six clinical trials are undergoing worldwide to confirm its validity. In this review, we summarize the recent progress in clinical studies of TCR-T cell therapy, describe strategies in the preparation and characterization of TCR-T cells, focusing on antigen selection, TCR isolation and methods to further enhance the potency of adoptively transferred cells.

1. Introduction

T lymphocyte is an essential component of adaptive immunity, and plays a key role in the surveillance and clearance of malignant-transformed or pathogen-infected cells. Adoptive transfer of *ex vivo* expanded autologous tumor-infiltrating lymphocytes (TIL) following non-myeloablative chemotherapy has generated complete and durable responses in patients with metastatic melanoma [1–3]. Despite notable success of TIL treatment in metastatic melanoma, it is laborious and challenging to identify and isolate tumor-reactive TIL from tumor lesions of patients with other cancer types. Besides, as a highly personalized therapeutic modality, it is difficult and cost-ineffective to industrialize the manufacturing processes of tumor-reactive TILs, which usually involves the following steps: isolation of TIL from tumor biopsies, identification of tumor-reactive TIL and expansion of TIL to sufficient doses for clinical study using a rapid expansion protocol. By introducing the transgene encoding a tumor-specific receptor (CAR or TCR) into T cells, T cells are able to be redirected to eradicate tumors. CAR-T therapy and TCR-T therapy are the other two types of mainstream T-cell based cancer immunotherapy. These genetically modified T cells can be applied to any tumors with the expression of target antigen. Streamlined manufacturing processes also enable the reproducible generation of engineered T cells with sufficient numbers in a relatively short period of time (usually less than one month) [4,5].

CAR is an artificial receptor composed of an antibody-derived domain, a TCR signaling domain (CD3ζ) and one or two costimulatory domains (like CD28, 4-1BB and I-COS) (Fig. 1). CAR-T cells recognize tumor antigens expressed on cell surface in an MHC-independent manner, but most tumor antigens are intracellular proteins, thus CAR-T cells are not able to recognize them. Conversely, natural TCR recognizes surface and intracellular antigens presented on the cell surface as peptide-MHC complexes. MHC molecules bind peptide fragments derived from both self- and foreign-antigens of a cell and display these peptides on the cell surface for T cell screening. When T cell recognizes foreign- or mutated self-peptide-MHC molecules, its cytotoxic function will be activated to eliminate the abnormal cells (Fig. 1). Currently, one of the greatest challenges in treating solid tumors is the identification of safe tumor antigens. Most surface antigens for solid tumors are also expressed on normal tissues raising a major concern with potential on-target/off-tumor toxicities mediated by CAR-T cells. Therefore, TCR-T cell therapy could be a promising alternative or complementary modality for treating solid tumors.

In this review, we first summarized the recent progress in clinical trials of TCR-T cell therapy. Based on the records published in

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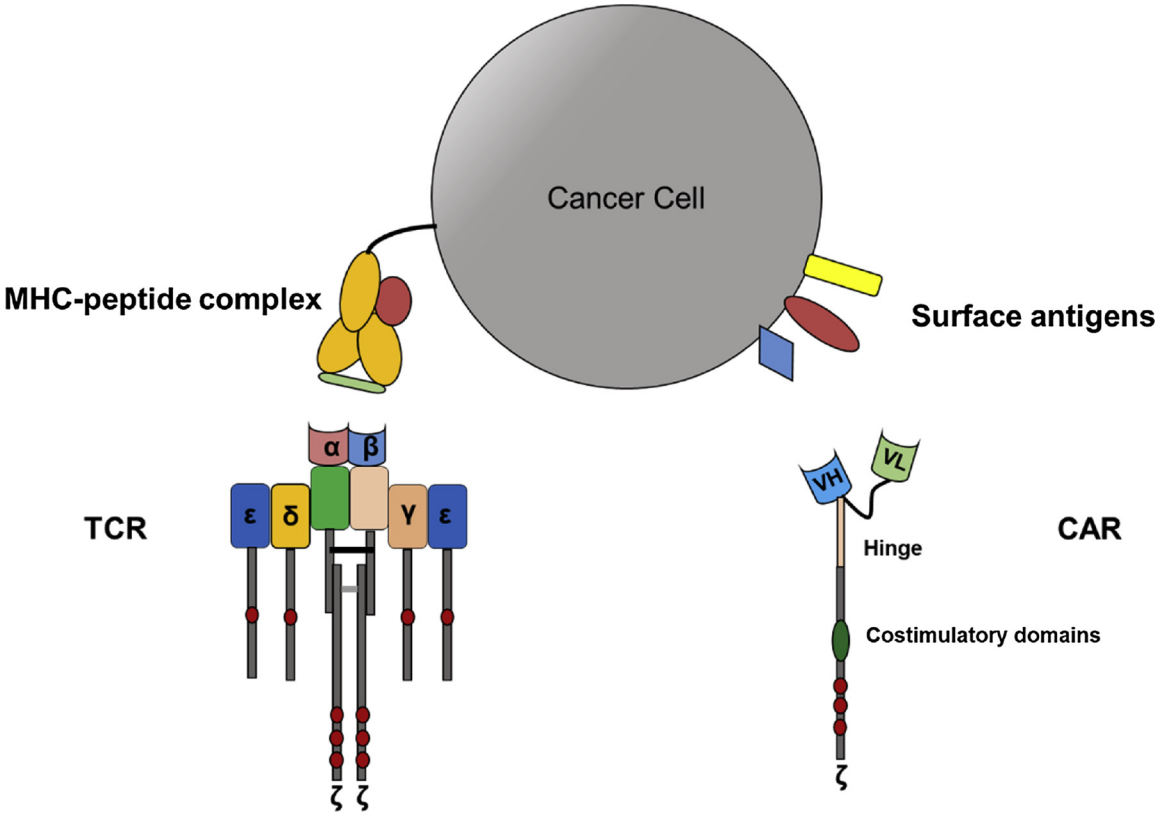

Fig. 1. Comparision between TCR-T and CAR-T therapy. TCR recognizes antigen-derived peptides in the context of MHC presentation which include surface antigens and intracellular antigens. CAR recognize surface antigens.

clinicaltrials.gov, we screened the TCR-T cell studies only for solid tumors and discussed the differences between clinical trials in China and United States (US), where the majority of trials are conducted. Then we described in detail each step involved in the development of TCR-T cell products, including antigen selection and characterization of TCR-T cells. Finally, we discussed the factors that limit the efficacy of TCR-T cells and possible solutions.

2. Clinical trials of TCR modified T cell therapy

Up to August 17, 2019, there are 78 clinical trials on the application of TCR-T cells to treat solid tumors (clinicaltrials.gov). Among these studies, 16 of them are either active but not yet recruiting or not yet recruiting, 40 trials are under the status of recruiting/enrolling by invitation, 15 studies are either suspended or terminated, and 7 studies are completed (Tables 1–4). Most active trials (active but not yet recruiting/not yet recruiting plus recruiting/enrolling by invitation) are conducted in US (66%, 37/56) or China (25%, 14/56), 3 trials are carried out in Japan and only one trial in Netherlands and Canada respectively (Fig. 2A). Fig. 2B shows the targets of these clinical trials. In US, 13 trials target cancer-testis antigen, NY-ESO-1, 7 trials target oncogenic viral antigens including HPV16-E7, Merkel cell polyomavirus (MCPyV) and human endogenous retrovirus E (HERV-E), 8 target tumor-associated antigens (MAGE protein family, MART-1, WT-1 and AFP) and 4 studies target neoantigens. In China, except for one study targeting tumor-associated antigen AFP (α-fetoprotein), which is a widely used biomarker in the diagnosis of hepatocellular carcinoma (HCC), most studies target NY-ESO-1 and oncogenic viral antigens.

NY-ESO-1 is a cancer-testis antigen with restricted expression to germ cells and placental cells, and aberrant re-expression in a wide range of tumor types [6]. Early studies have thoroughly examined the safety to target NY-ESO-1 with either wide-type or affinity-enhanced TCR-T cells as a monotherapy. Recent studies tend to use NY-ESO-1 reactive TCR-T cells as a part of combinatorial therapies to enhance efficacy. For example, to promote the persistence of infused T cells, the feasibility and safety of delivering NY-ESO-1 specific TCR-T cells along with NY-ESO-1(157-165) peptide pulsed dendritic cells are investigated in some studies (NCT01697527, NCT02775292). To circumvent heterogeneity of antigen expression in tumor and immune escape, decitabine (a demethylating agent), is used to induce re-expression of NY-ESO-1 specifically in tumor cells (NCT03017131, NCT02650986). The programmed death 1 (PD-1) receptor is a negative regulator of T cell-mediated immunity and its ligand (PD-L1) is expressed by multiple tumors to inhibit the function of cytotoxic T cells. Modulation of PD-1/PD-L1 axis by commercial monoclonal antibodies (mAbs) could unleash the suppressed function of T cells. Remarkable efficacy of PD-1 or PD-L1 mAbs has been proven in multiple malignancies, including head and neck squamous cell carcinoma, melanoma, non-small cell lung cancer, renal cell carcinoma, and urothelial cancer therefore, combination therapy with NY-ESO-1 specific TCR-T cells and PD-1/PD-L1 inhibitor may provide a synergetic effect (NCT03578406, NCT03709706). There is one trial using gene editing method (CRISPR) to eliminate endogenous TCR and PD-1 in NY-ESO-1 specific TCR-T cells (NCT03399448). Pre-clinical studies showed CRISPR/Cas9-mediated PD-1 disruption enhanced anti-tumor activity of CAR-T cells [7]. Besides, elimination of endogenous TCR may enhance the function of TCR-T cells by reducing the competitive combination of endogenous TCR and introduced tumor-reactive TCR with CD3 complexes and meanwhile may solve the safety concern about TCR mispairing. However, these studies are still in the early stage and the performance of gene-edited TCR-T cells in human is still unknown. We found that most NY-ESO-1 related clinical trials do not clearly indicate what kind of conditions the TCR-T cells will be used for, but for multiple solid tumors. It is worth to note that although theoretically, these TCR-T cells could target any tumors with the expression of NY-ESO-1, the expression frequency of the antigen differs greatly in different tumor types

Table 1
Clinical trials of TCR-T cells to treat solid tumors under the status of not yet recruiting/active, or not yet recruiting.

Antigen	HLA subtypes	Diseases	Phase	NCT number	Locations
LMP2	A*02, A*11 or A*24	NPC	1	NCT03925896	China
HPV16-E7	A*02:01	OPC (stage II and III)	2	NCT04015336	U.S.
HPV16-E7	A*02:01	OPC (stage I)	2	NCT04044950	U.S.
MART-1	A*02:01	MM	1/2	NCT02654821	Netherlands
AFP	A*02:01	HCC	1	NCT03971747	China
LMP1/LMP2/EBMA1	A*02:01, A*24:02 A*11:01	NPC	2	NCT03648697	China
MART-1	A*02:01	MM	2	NCT00910650	U.S.
NY-ESO-1	A*02:01	Solid tumors	1	NCT02366546	Japan
NY-ESO-1	A*02:01	Solid tumors	2	NCT01697527	U.S.
NY-ESO-1	A*02:01	SS	1/2	NCT03250325	Japan
Unknown	–	MM	1	NCT01586403	U.S.
HBsAg	–	HCC	1/2	NCT03634683	China
NY-ESO-1	A*02:01	NSCLC	1	NCT02588612	U.S.
WT1	A*02:01	NSCLC (stage III/IV)/ Mesothelioma	1/2	NCT02408016	U.S.
NY-ESO-1/LAGE-1a	A*02:01	SS and other solid tumors	2	NCT03967223	U.S.
NY-ESO-1	A*02:01	SS	1	NCT01343043	U.S.

with the highest and most homogenous expression in myxoid and round cell liposarcoma and synovial sarcomas [6]. Therefore, for other cancer types with antigen expression in the range of low to medium, like breast cancer, prostate cancer, head and neck cancer and non-small cell lung cancer, solving the problem of tumor heterogenicity with other agents, cells or creative methods is critical for reproducible success.

We found that the clinical trials carried out in China have apparently geographical features with 5 trials targeting HCC and nasopharyngeal carcinoma (NPC) respectively. It was estimated that about 80% of human HCC are attributable to chronic hepatitis B virus (HBV) infection [8]. The burden of HBV infection in China is the highest around the world. According to cancer statistics in China 2015, HCC is the third

Table 2
Clinical trials of TCR-T cells to treat solid tumors under the status of recruiting/enrolling by invitation.

Antigen	HLA subtypes	Diseases	Phase	NCT number	Locations
Unknown	–	NSCLC/ Other solid tumors	1	NCT03778814	China
NY-ESO-1	A*02:01	Sarcoma	1	NCT03462316	China
Unknown	–	Lung cancer/ melanoma	1	NCT03891706	China
NY-ESO-1	A*02:01	NSCLC	1	NCT03029273	China
HBsAg	–	HCC	1	NCT03899415	China
HBsAg	–	HCC	1	NCT02686372	China
HBsAg	–	HCC	1	NCT02719782	China
HPV16-E7	A*02:01	HSIL	2	NCT03937791	U.S.
HPV16-E6	–	HNSCC/CC	1	NCT03578406	China
MCPyV	A*02:01	MCC	1/2	NCT03747484	U.S.
HPV16-E7	A*02:01	HPV16-associated cancers	1/2	NCT02858310	U.S.
NY-ESO-1	A*02:01	Solid tumors	1	NCT03240861	U.S.
NY-ESO-1	A*02:01	OC/ fallopian tube cancer/ peritoneal cancer	1	NCT03691376	U.S.
NY-ESO-1	A*02:01	Solid tumors	1	NCT02457650	China
HERV-E	A*11:01	KC	1	NCT03354390	U.S.
NY-ESO-1	A*02:01	Solid tumors	1/2	NCT02650986	U.S.
MAGE-A3	DPB1*04:01	Solid tumors	1/2	NCT02111850	U.S.
NY-ESO-1	A*02:01	Solid tumors	2	NCT01967823	U.S.
Neoantigen	–	Solid tumors	2	NCT03412877	U.S.
MAGE-A4	A*24:02	Solid tumors	1	NCT02096614	Japan
NY-ESO-1	A*02:01	Solid tumors	1	NCT02869217	Canada
NY-ESO-1	A*02:01	Solid tumors	1	NCT03159585	China
Unknown	–	Solid tumors	1	NCT03686124	U.S.
NY-ESO-1	A*02:01	Solid tumors	1	NCT03399448	U.S.
Neoantigen	–	Solid tumors	1	NCT03970382	U.S.
NY-ESO-1	A*02:01	Solid tumors	1	NCT02774291	U.S.
Unknown	–	Solid tumors	1	NCT03441100	U.S.
Unknown	–	Solid tumors	1	NCT03247309	U.S.
KRAS G12D	A*11:01	Solid tumors	1/2	NCT03745326	U.S.
KRAS G12V	A*11:01	Solid tumors	1/2	NCT03190941	U.S.
NY-ESO-1	A*02:01	NSCLC	2	NCT03709706	U.S.
MAGE-A3/A6	DPB1*04:01	Solid tumors	1	NCT03139370	U.S.
NY-ESO-1	A*02:01	OC/FTC/PC	1	NCT03017131	U.S.
HPV16-E7	A*02:01	HPV + tumors	1	NCT03912831	U.S.
AFP	A*02:01	HCC	1	NCT03132792	U.S.
MAGE-A4	A*02:01	Solid tumors	1	NCT03132922	U.S.
NY-ESO-1	A*02:01	High Grade Myxoid Liposarcoma	2	NCT02992743	U.S.
MAGE-A10	A*02:01	Urothelial cancer/melanoma/ head and neck tumors	1	NCT02989064	U.S.
MAGE-A10	A*02:01	NSCLC	1	NCT02592577	U.S.
Unknown	–	Solid tumors	1	NCT02876510	U.S.

Table 3
Clinical trials of TCR-T cells to treat solid tumors under the status of suspended/terminated.

Antigen	HLA subtypes	Diseases	Phase	NCT number	Locations
TGFβRII frameshift antigen	A*02	CRC	1/2	NCT03431311	U.S.
gp100	A*02:01	Skin cancer/melanoma	2	NCT00509496	U.S.
MAGE-A3	A*01	Solid tumors	1/2	NCT02153905	U.S.
NY-ESO-1	A*02:01	Solid tumors	1	NCT02070406	U.S.
gp100	A*02:01	Advanced melanoma	2	NCT00610311	U.S.
MART-1	A*02:01	Advanced melanoma	2	NCT00612222	U.S.
TNF-related apoptosis inducing ligand (TRAIL)	–	KC	1	NCT00923390	U.S.
MAGE-A3/A12	A*02:01	Solid tumors	1/2	NCT01273181	U.S.
NY-ESO-1	A*02:01	Solid tumors	2	NCT00670748	U.S.
NY-ESO-1^{C259}	A*02:01	Melanoma	1/2	NCT01350401	U.S.
CEA	A*02:01	Solid tumors	1	NCT00923806	U.S.
NY-ESO-1	A*02:01	Advanced melanoma	2	NCT02062359	U.S.
p53	A*02:01	Solid tumors	2	NCT00704938	U.S.
NY-ESO-1	A*02:01	Solid tumors	1	NCT01457131	U.S.
MART-1	A*02:01	Melanoma	2	NCT00706992	U.S.

most common cancer in China, indicating a large market requirement for new therapy [9]. Tan et al. showed that HCC cells contain the integrated HBV-DNA fragments that encode peptide epitopes which are presented by MHC molecules and recognized by T cells [10]. Therefore, HbsAg, a surface antigen of HBV, was proposed as the possible target for T cell-based immunotherapy. Similarly, NPC has a high incidence in southern China and is related to the consistent infection of EBV. Antigens associated with latent virus infection, such as LMP1, LMP2 and EBNA1, have been demonstrated as targets of TCR-T cell therapy. In US, 4 trials target neoantigens with 2/4 trials against KRAS related epitope. Neoantigen specific TCR-T cell therapy may provide a treatment for any epithelium cancers, but as a highly personalized therapy, it still requires a lot of improvements on manufacture, quality controls and regulations to make the production process more efficient and economical.

3. Selection of target antigens for TCR engineered T cell therapy

Preclinical and clinical studies have illustrated the adoptively transferred T cells are able to traffic to virtually all anatomical sites [11,12] indicating that on the one hand, transferred T cells can attack antigen-expressing tumors spreading to any location, but on the other hand, any healthy cells or tissues expressing the same antigen would be the target as well. [13,14]. For example, melanoma-associated antigens (gp100 and MART1) were targeted by TCR-engineered T cells in early clinical trials to treat metastatic melanoma. These antigens are expressed by both melanoma cells and normal melanocytes. HLA-A2 restricted MART-1_{27-35} epitope was first targeted by a TCR (referred to as DMF4) cloned from TILs of a melanoma patient [15]. Fifteen patients were treated with this TCR modified autologous T cells and 2/15 patients experienced a sustained partial regression of metastatic lesions. None of the patients exhibited melanocyte-related toxicities. To enhance therapeutic efficacy, two high-avidity TCR were tested in subsequent clinical trials [16]. One TCR (DMF5) targeting the same epitope as DMF4 was identified by screening TIL clones from different melanoma patients and another TCR for gp100 was isolated from transgenic mice [17]. In this clinical trial, total nine patients exhibited partial regression (6/20 patients were treated with DMF5-transduced T cells and 3/16 patients were treated with gp100-specific TCR-T cells). However, transient toxicities in skin, eye and ears were found in most patients (16/20 for MART1 and 16/16 for gp100) and only one patient treated with gp100 specific TCR-T cells demonstrated a complete response (> 14 months). These results revealed that targeting tumor-associated antigens is challenging to achieve the balance between efficacy and safety at the same time.

A similar phenomenon was observed when targeting human carcinoembryonic antigen (CEA). CEA is a protein overexpressed in several cancer types, including gastrointestinal cancer, breast cancer and non-small cell lung cancer [18]. It is also a widely used serum tumor marker in diagnosis. In a clinical trial, three patients with refractory metastatic colorectal cancer were treated with autologous T cells genetically engineered to express a murine high avidity TCR against human CEA: HLA-A2/$CEA_{691-699}$ [19]. TCR engineered T cells induced profound decreases of serum CEA levels (74–99%) in all patients and 1/3 patient exhibited objective regression. However, since CEA was also expressed in normal epithelial cells throughout digestive tract, all patients experienced a severe transient inflammatory colitis. Even though the expression of cancer-associated antigens is much higher in malignant tissues than normal tissues, given the heterogenicity of tumors, it is still difficult to find a proper threshold of antigen expression to ensure serious killing and tolerable autoimmunity.

Table 4
Clinical trials of TCR-T cells to treat solid tumors under the status of completed.

Antigen	HLA subtypes	Diseases	Phase	NCT number	Locations
HPV16-E6	A*02:01	High Grade Squamous Intraepithelial Lesion	1	NCT03197025	U.S.
NY-ESO-1	A*02:01	Solid tumors	1	NCT02775292	U.S.
p53	A*02:01	Solid tumors	2	NCT00393029	U.S.
MART-1	A*02:01	Metastatic Melanoma	2	NCT00509288	U.S.
HPV16-E6	A*02:01	HPV-associated cancers	1/2	NCT02280811	U.S.
NY-ESO-1^{C259}	A*02:01	OC	1/2	NCT01567891	U.S.
MART-1/gp100	A*02:01	Metastatic Melanoma	2	NCT00923195	U.S.

NPC: Nasopharyngeal carcinoma; OPC: Oropharyngeal cancer; HCC: Hepatocellular carcinoma;
MM: Metastatic melanoma; SS: Synovial sarcoma; NSCLC: Non-small-cell lung carcinoma; HBsAg: hepatitis B virus surface antigen; HSIL: vulvar high-grade squamous intraepithelial lesions;
HNSCC: Head and neck squamous cell carcinoma; CC: Cervical cancer; MCC: Merkel cell cancer;
MCPyV: Merkel cell polyomavirus; OC: Ovarian carcinoma; HSC: hematopoietic stem cells; KC: Kidney cancer; FTC: Fallopian tube carcinoma; PC: Peritoneal Carcinoma; CRC: Colorectal Cancer;

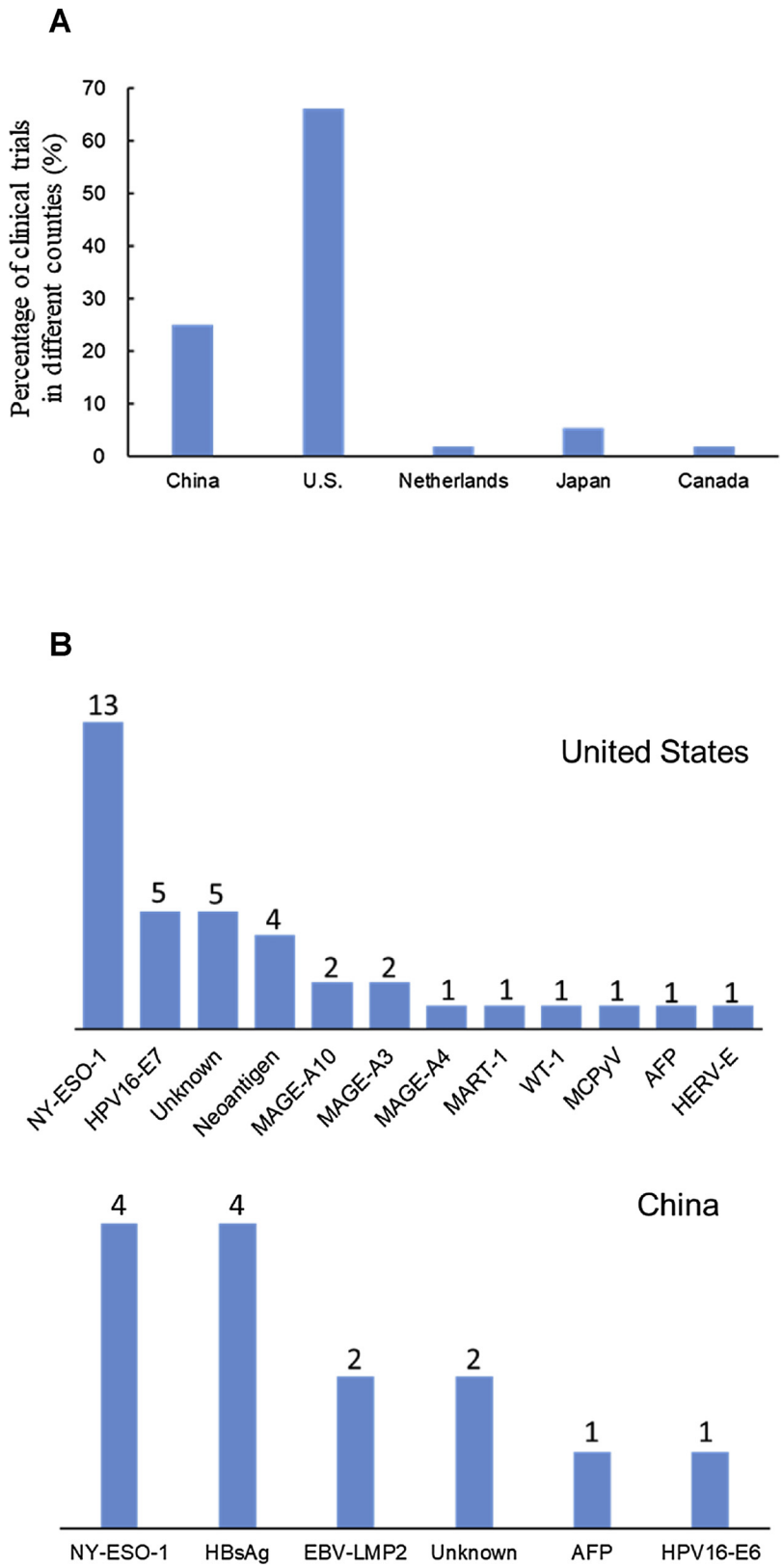

Fig. 2. The analysis of 56 active clinical trials on the application of TCR-T cells to treat solid tumors. (A) The percentage of clinical trials conducted in different countries. (B) Proportion of each type of antigen targeted in the clinical trials conducted in China and United States.

Autoimmunity mediated by T cells could be very severe and even result in patient death. The specificity of TCR towards antigen epitopes is the crucial consideration to evade potential toxicities. Targeting different HLA-restricted epitopes derived from the same antigen could lead to entirely different results. For example, the MAGE-A gene family are widely expressed in multiple epithelial malignancies including melanoma [20], esophageal cancer [21], breast cancer, head and neck cancer, and lung cancer [22]. As a member of MAGE-A family, MAGE-

A3 was reported to have restricted expression in normal tissues but in a wide range of tumors, therefore MAGE-A3 was initially proposed as a good target for more patients with common epithelial malignancies. In a clinical trial, the HLA-A2 restricted MAGE-A3$_{112–120}$ epitope was targeted by T cells engineered with an avidity-modified TCR to treat patients with advanced cancers. Although five out of nine patients experienced objective tumor responses, three patients in the treatment showed severe neurologic toxicities within two days post-cell infusion and two of them died. Careful investigation revealed the infiltration of CD8$^+$ T cells into two patients' brains and low-level expression of MAGE-A12 in brain tissues was involved in the cross-reaction of transferred T cells against a homologous peptide epitope [23]. In another clinical trial, two patients (one with melanoma and another with multiple myeloma) were treated with autologous T cells genetically modified with an affinity-enhanced TCR against HLA-A1 restricted MAGE-A3$_{168-176}$ epitope. A few days post T cell-infusion, the patients developed cardiogenic shock and died. Myocyte necrosis mediated by infused T cells was later observed in the autopsy. Although no detectable expression of MAGE-A3 was found in heart tissues, muscle-specific protein titin which was expressed in normal myocardium produced a similar HLA-A1 restricted peptide epitope resulting in the unexpected severe cross-reaction [24]. By contrast, another epitope from MAGE-A3 (HLA-DPB1*0401 restricted) was demonstrated to be safe and effective. Seventeen patients with various metastatic cancers were treated with TCR transduced autologous CD4$^+$ T cells. One patient (1/17) with metastatic cervical cancer experienced a complete response (ongoing at $\geq$ 29 months), 3/9 patients treated with the highest dose level (up to 1.23×10^{11} cells) experienced partial responses and no treatment-related deaths occurred [25]. These clinical results indicated some unique features of TCR-gene modified T cell therapy. First, a specific target antigen epitope is an essential requirement for successful TCR-T cell therapy. Secondly, MHC-restriction confines the application of TCR-T cells to individuals with matched MHC alleles, which seems to be inferior to MHC-independent treatment (like CAR-T cells), but for most solid tumors with a lack of genuinely tumor-specific antigens, TCR-T cells can target multiple epitopes in the context of different HLA of the same protein and thus are accessible to much broader spectrum of antigen pools.

There are two criteria that determine the feasibility of a tumor antigen for TCR-T cell therapy. First, the antigen should be expressed exclusively in tumors. Second, there should be a sufficient number of patients who may potentially benefit from the treatment. Therefore, the frequency of antigen expression in tumor types and the frequency of defined HLA in population (The allele frequency net database, www.allelefrequencies.net) need to be carefully considered. Currently, three categories of tumor antigens are proposed as suitable targets for TCR transduced T cell therapy, including cancer-testis antigens, oncogenic viral antigens and neoantigens (Fig. 3) [26].

3.1. Targeting cancer-testis antigens

Till now, it is still challenging to identify and validate that the selected cancer-testis epitopes are genuinely absent in normal tissues. Bioinformatics methods, like the CGA database (www.cta.lncc.br), the protein atlas (www.proteinatlas.org), GTExPortal (gtexportal.org) and genevestigator (genevestigator.com) can provide initial information about the expression profile of target antigens. Commercially available cDNA libraries of a variety of healthy and malignant tissues can be further used to validate the specificity of target antigens by qPCR [27]. One defect is that most commercial cDNA libraries are constructed by using tissues of Caucasians and may do not apply to other ethnic groups. IHC and tissue microarrays can test protein expression of target antigens. Although these methods provide valuable information for antigen selection, rare antigen-positive normal cells may exist, thus potential toxicities cannot be entirely excluded.

As mentioned above, NY-ESO-1 is an archetypical example of cancer-testis antigens. Previous clinical trials targeting HLA-A2 restricted NY-ESO-1$_{157-165}$ epitope revealed good tolerance and efficacy [28–30]. The first study conducted by Robbins et al. has shown the feasibility of NY-ESO-1 specific TCR engineered T cell therapy for patients with synovial and melanoma. [31]. In this study, all patients (six with synovial sarcoma and eleven with melanoma) showed moderate to high expression of NY-ESO-1 antigen in tumors (> 50%, evaluated by IHC staining). After receiving a non-myeloablative lymphocyte-depleting preparative regimen of cyclophosphamide and fludarabine, TCR-T cells (medium cell number: 5×10^{10}, range: $1.6–130 \times 10^9$ TCR-T cells) were infused into patients. Four out of six patients with synovial sarcoma and 5/11 patients with melanoma experienced objective clinical responses. Moreover, two melanoma patients had complete responses lasting for one year. One responsive synovial sarcoma patient experienced stable disease for 18 months. No severe toxicities occurred in this study. Four years later, a subsequent study of additional 12 synovial cell sarcoma patients and 9 melanoma patients in the same clinical trial was published [29]. The updated data showed that the ORR of patients with synovial cell sarcoma was 61% (11/18), and the ORR of patients with melanoma were 55% (11/20). Another clinical trial conducted by D'Angelo et al. showed that 6/12 (50%) patients who received an affinity-enhanced TCR transduced autologous T cells against HLA-A2/NY-ESO-1$_{157-165}$ experienced clinical responses with the persistence of infused T cells in all responders for at least six months. [32].

3.2. Targeting oncogenic viral antigens

It was estimated that around 12% of all cancers are associated with viral infection [33]. Viral antigens, especially viral oncogenic proteins, are attractive targets for adoptively transferred T-cell therapy. These antigens are constitutively and exclusively expressed on cancer cells, playing a pivotal role in cancer survival and proliferation. Moreover, viral antigens are usually heterogeneous enough to produce a pool of immunogenic peptide epitopes. For example, Epstein-Barr virus (EBV) is associated with nasopharyngeal carcinoma, Burkitt lymphoma, Hodgkin disease, and some gastrointestinal carcinoma [34]. More than twenty-five years ago, the adoptive transfer of donor-derived EBV-specific T cells had been successfully used to treat virus-related lymphoma of immunocompromised patients who had experienced stem cell transplantation. Bollard et al. reported that infusion of enriched EBV-LMP1- or EBV-LMP2-specific T cells into twenty-one high-risk or multiple-relapsed lymphoma patients resulted in clinical responses in thirteen patients [35]. Eleven out of twenty-one patients achieved complete responses, and no severe toxicities occurred. Notably, epitope-spreading of peripheral T cells occurred only in patients with clinical responses indicating polyclonal T cell responses triggered by the transferred T cells might be necessary for effective treatment.

Infection of the high-risk human papillomavirus (HPV-16 and HPV-18) is associated with cancers of cervix, oropharynx, anus and vagina [36]. Oncoproteins, E6 and E7, are constitutively expressed in cancer cells, serving as ideal targets [37]. Several clinical trials were conducted to evaluate the safety and efficacy of TCR-gene engineered T cells against E6 or E7 epitopes. A TCR specific to HLA-A2 restricted HPV16-E6$_{29-38}$ epitope was isolated from the metastatic lesion of a rectal cancer patient. In a clinical trial, twelve late-stage, HPV-positive cancer patients were treated with the TCR-T cells, and 2/12 patients experienced clinical responses and achieved sustained tumor regression for 3-month and 6-month, respectively [38]. Another TCR targeting HPV16-E7$_{11-19}$ was isolated from TIL cultures of a woman with cervical cancer and was demonstrated to have a higher functional avidity than above mentioned E6-specific TCR. Currently, the clinical trial using this E7-specific TCR transduced T cells is undergoing (NCT02858310).

Other prevalent cancer-associated viruses include Hepatitis B virus (HBV), Hepatitis C virus (HCV) and human Cytomegalovirus (CMV). Chronic infection of HBV or HCV is associated with hepatocellular

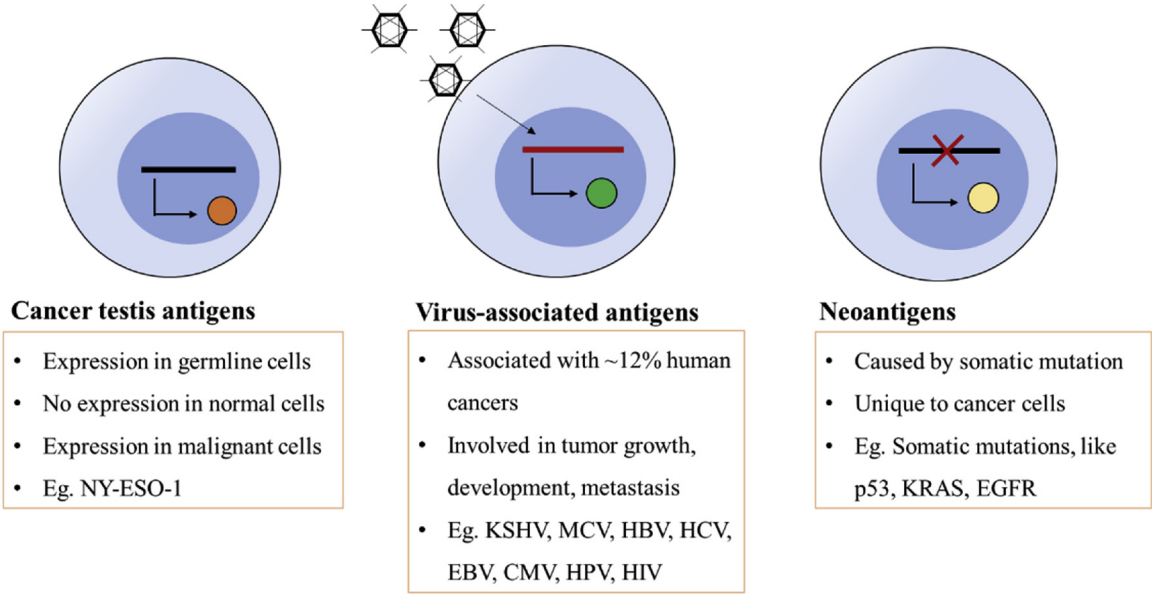

Fig. 3. Proposed ideal targets for TCR-T cell immunotherapy. Cancer-testis antigens are exclusively expressed on tumors and non-MHC-bearing testis. Virus-associated antigens are expressed in virus-associated cancer cells. Neoantigens are derived from somatic mutations. These tumor antigens are considered as ideal targets for TCR-T cell immunotherapy.

carcinoma (HCC) [39]. The CMV antigens, like 1E1 and pp65, were observed to be present in glioblastoma tissues and may be used as targets for TCR transduced T cells [40,41]. Besides, endogenous viruses that reactivated during oncogenesis can also serve as target antigens. For example, an HLA-A11-restricted epitope derived from human endogenous retrovirus type E (HERV-E) selectively expressed in the clear cell variant of renal cell carcinoma [42]. Clinical trial targeting this epitope with TCR transduced T cells is undergoing (NCT03354390).

3.3. Targeting neoantigens

Somatic mutations accumulated during oncogenesis produce mutated peptides. Some antigenic peptides can bind to MHC molecules to form neoantigens on the surface of malignantly transformed cells. Neoantigens are usually exclusively expressed in cancer cells and are unique to individual patients, serving as ideal targets for T-cell based immunotherapy. Robbins et al. showed that neoantigen-reactive T cells identified by exome sequencing could mediate objective tumor regression of patients with metastatic melanoma demonstrating the feasibility of isolation and application of these mutation-specific T cells in the treatment of solid cancer patients [43]. Gros et al. subsequently found that neoantigen-specific T cells cannot only be isolated from tumor-infiltrating lymphocytes but also peripheral blood of patients with melanoma, providing a more accessible cell source for isolation of neoantigen-reactive T cells [44]. Among neoantigens, driver mutation is one of the most attractive targets due to their essential role in tumor progression and possible more homogenous expression in cancer cells. For example, mutations in the KRAS oncogene contribute to the formation and progression of a wide variety of human cancers. Tran et al. identified $CD8^+$ T cells recognizing mutated KRAS epitope ($KRAS^{G12D}$) in the context of HLA-C*08:02 from patients with metastatic colorectal cancer, and one patient treated with $KRAS^{G12D}$ specific T cells experienced objective regression of all lung metastases [45]. Except for site mutations of peptide epitopes, T cell epitopes are generated from alternative open reading frames (frameshift mutations) [46,47], fusion proteins [48,49] and non-coding RNA as well [50]. For example, transforming growth factor β receptor II (TGFβRII) frameshift mutation is found in Lynch syndrome, colorectal cancer and gastric cancer. A TCR specific to the $TGF\beta RII^{mut}$ peptide epitope was isolated from a colon cancer patient who had experienced a response after $TGF\beta RII^{mut}$ peptide-vaccination [51]. In a xenograft mouse model, the TCR transduced T cells significantly delayed the growth of colorectal cells, and one clinical trial (NCT03431311) was performed using this TCR-T cells. To identify epitopes recognized by $CD4^+$ T cells, Wang et al. developed a genetic targeting expression system by introducing cDNA encoding DRα, DRβ, DMα, DMβ, and Ii into 293 cells. The resulting 293ECIIDR cells can be used as "professional" APC and efficiently present MHC class II-restricted T cell epitopes [52,53]. As mentioned before, there are four clinical trials using TCR-T cells against neoantigens in the US, and two trials in China using TCR-T cells against uncovered targets, which is possibly targeting patient-derived neoantigens (NCT03778814, NCT03891706). We found an increasing interest in the application of neoantigen-reactive TCR-T cells recently. With the development of methods to identify and manufacture these highly personalized cells, neoantigens might serve as universal targets for patients with different cancer types.

4. Construction and characterization of TCR-T cells

After identification of the tumor target, isolation of antigen-specific T cell clones and genes encoding desired TCRs are the second step to construct TCR-T cells. Generally, strategies to clone tumor-reactive T cells rely on the proliferative characteristics of T cells after antigen encounter. Following TCR identification, TCR-T cells are constructed using viral or non-viral vector-based transduction methods. In preclinical studies, a series of *in vitro* and *in vivo* functional tests will be conducted to assess the transduction efficiency, specificity, avidity, and efficacy of TCR-gene modified T cells.

4.1. T cell cloning

Tumor-specific T cells can be isolated from T cell repertoire of antigen-experienced or antigen-unexperienced individuals. A traditional method to identify tumor-specific T cells is using *in vitro* stimulation of PBMC and limiting dilution cloning. The success rate of this method depends on the initial frequency of tumor-reactive T cells in the bulk T cells and the antigenicity of tumor antigen. Many tumor-specific TCRs, especially for those against neoantigens, were isolated from TIL cultures of cancer patients [54,55]. Although the frequency of antigen-reactive T cells is usually lower in peripheral blood than in TILs, PBMC of

patients who have received vaccination [56,57], checkpoint inhibitors [58] or experienced complete responses after TIL therapy [59] were found to be good starting material to find desired T cell clones. To further enrich antigen-specific T cells, multiple rounds of *in vitro* antigen stimulation are required. Some studies use synthetic peptides to stimulate the bulk PBMC. In this case, cells other than T cells in the PBMC serve as antigen-presenting cells (APCs). Other studies use antigen-pulsing or antigen-expressing APCs to expand T cells. These APCs include autologous dendritic cells (DC) or artificial APCs, which are engineered to express several co-stimulators [60]. After several rounds of *in vitro* stimulation, antigen-reactive polyclonal T cells are routinely screened using ELISA or ELISpot assay. Then antigen-reactive polyclonal T cells are subjected to limiting dilution cloning to isolate monoclonal antigen-reactive T cells. This traditional method is labor-intensive and time-consuming especially for peptide-epitopes with low immunogenicity. Multimer-guided T-cell sorting permits quick enrichment of antigen-specific T cells or direct isolation of tumor-specific single cells for TCR sequencing, which dramatically facilitates the efficiency of T cell cloning [61,62]. Except for peptide-MHC multimers, cell surface markers, like 4-1BB and PD-1, are also frequently used to identify antigen-specific T cells [63]. It is challenging to clone TCRs against epitopes with low immunogenicity. Hence, optimized cytokine cocktail is also investigated to promote the growth advantage of desired T cells. [64]. Another method to isolate TCRs against epitopes with low immunogenicity is using transgenic mice. Transgenic mice with the entire human TCR α- and β-gene loci are potent tools to isolate TCR with high avidity [65]. For example, MAGE-A1 and NY-ESO-1 specific TCRs were isolated from the transgenic mice system successfully and demonstrated better *in vitro* and *in vivo* function than TCRs isolated from humans [66]. However, the usage of TCR from non-tolerant repertoires should be cautious to ensure no cross-reaction and other side-effects in humans. Following the establishment of antigen-specific T cell clones, genes encoding TCRs can be retrieved using PCR-based techniques with a set of degenerate primers binding to consensus sequences of TCR α- and β-gene or using rapid amplification of cDNA ends (RACE) [67]. Other methods to clone a TCR include emulsion PCR [68] and pairSEQ [69].

4.2. Construction of TCR-T cells using viral- or nonviral-vector based methods

Viral- or nonviral-vector based methods are used to transfer the gene encoding defined TCR into T cells of patients. Viral-vector for commercial production of TCR-T cells includes retroviral and lentiviral vectors [70]. Retroviral and lentiviral vectors can introduce exogenous genes into the genome of infected host cells, which leads to permanent transduction and inheritable expression of transgenes. The main difference between lentiviruses and retroviruses is that lentiviruses are capable of infecting non-dividing and actively dividing cells, whereas retroviruses can only infect dividing cells. Activation of primary T cells is usually required before viral-infection for both retroviruses and lentiviruses to reach a high transduction efficiency. Safety issues have been the primary concern about the use of viral vector-based transduction methods. Random integration of viral DNA into the host genome may result in dysfunction or dysregulation of coding sequences, activation of proto-oncogenes, and inactivation of cancer suppressor genes [71,72]. Despite a low chance of having replication-competent retrovirus (RCR) or lentivirus (RCL) for the latest generation of viral-vectors and packing systems, time- and cost-consuming quality control tests are compulsory for clinical application. Therefore, nonviral-vector based transduction methods may provide a rapid, safe, and cost-effective alternative. Recently, some studies investigated the application of the transposon system to integrate transgenes into primary T cells stably. The transposon system is composed of transposase and a transposon. Transposons are DNA sequences that can move around in a genome. Transposase is an enzyme that binds to the end of a transposon and catalyzes its movement. Through cloning the gene of interest into a transposon vector, the transgene can be inserted into the genome of host cells during translocation. The Sleeping Beauty transposon/transposase system has been exploited to produce genetically modified T cells and showed promising results [73,74].

4.3. Functional assessment of TCR-T cells

Following the construction of TCR-T cells, detailed characterization of these cells is essential to find suitable candidates for clinical application. First, peptide-MHC multimers or V-region specific antibodies will be routinely used to detect the expression level of transgenic TCR on T cells. One potential reason for the low expression level of transgenic TCR is mispairing between exogenous and endogenous TCR chains. Several strategies were exploited to optimize the pairing of transgenic TCR and to enhance their function. Cohen et al. showed that the hybrid TCR with human variable regions and murine constant regions had enhanced expression levels on transduced T cells and exhibited greater *in vitro* anti-tumor responses [75]. Bialer et al. showed that the replacement of several selected sites in the constant region of human TCR with mouse residues was enough to endow human TCR with improved expression and function [76]. Other approaches to improve the pairing and expression of exogenous TCR include the introduction of a second disulfide-bone between TCR α and β chains [77] or incorporation of hydrophobic mutations into the transmembrane regions of TCR chains [78]. The application potential of TCR-T cells is next to be evaluated using a series of *in vitro* functional assays. These assays detect the potency of T cells to release inflammatory cytokines and kill target cells after antigen encounter. Two important indexes here are specificity and efficacy. The specificity of a TCR is a prerequisite for a clinical application. Screening against normal tissues and self-peptides with a similar sequence as the antigen epitope with TCR-T cells is required to eliminate the potential off-target risk. Peptide titration can evaluate the avidity of TCRs and give an effective concentration (EC50) to compare the potency of different TCRs [27]. Cytotoxicity of TCR-T cells is usually evaluated using a co-culture method with T cells (effector cells) and target cells at different ratios. If available, multiple cancer cell lines or freshly isolated tumor cells should be tested as target cells to give a thorough evaluation of the function of TCR-T cells. Finally, *in vivo* functional assessment can be conducted in either HLA transgenic mice transplanted with a syngeneic tumor expressing the human target antigen [79] or immune-deficient mice transplanted with human cancer cell lines or tumors [80].

5. Design of better TCR-T cell immunotherapy

Currently, the adoptive transfer of gene-modified T cells faced several challenges in treating solid tumors. First, TCR-T cells have poor trafficking ability to tumor sites. Several preclinical and clinical studies showed that only a small fraction of infused T cells could infiltrate into tumors, and an even smaller number of T cells could access tumor cells. Second, unlike hematological malignancies that cancer cells circulate through the human body, solid tumors locate in specific anatomical sites and form compact masses consisting of a variety of cell types, extracellular matrix and vasculature. Even if T cells infiltrate through these masses, the harsh tumor microenvironment suppresses the cytotoxic function of these T cells. Besides, heterogenicity of tumors leads to the limited efficacy of T cell-based monospecific therapies, and results in the relapse of antigen-negative tumors. Strategies to overcome the hurdles mentioned above are essential to design better T-cell therapies.

5.1. Methods to improve T-cell trafficking to solid tumors

Trafficking of T cells to inflammatory sites involves a series of tightly controlled processes. Firstly, activated T cells downregulate the expression of receptors responsible for homing to second lymph nodes,

such as CD62 L and CXCR7. Then, the expression of integrins and selectins are upregulated to mediate the adhesion and rolling of T cells on vascular endothelium. Simultaneously, chemokine receptors expressed on T cells mediate the sense of corresponding chemokines released by inflammatory tissues and promote chemotaxis and extravasation [81]. Bellone et al. showed that pericytes, a cell population wrapping around endothelial cells and being responsive for vessel maintenance and remodeling, have an aberrant function in tumors. Dysfunction of pericytes leads to leakiness of the vessel, irregular blood flow, and inefficient trafficking of T cells [82]. Buckanovich et al. demonstrated in a mouse model of ovarian cancer, overexpression of the endothelin B receptor on the tumor endothelium inhibited the adhesion of T cells on endothelium and infiltration to tumor sites [83]. Angiogenic factors, like VEGF, released by tumor cells can cause the downregulation of adhesion molecules such as VCAM-1, ICAM-1, ICAM-2 and CD34 on surrounding endothelium and block the extravasation of cytotoxic T cells to tumor sites. In order to overcome the tumor endothelial barrier, antibodies targeting angiogenic factors to normalize tumor vasculature were used, and data showed that antiangiogenic agents could improve the infiltration of infused T cells [84,85].

5.2. Methods to enhance T cell persistence

Several studies showed that the long-term persistence of tumor-specific T cells was associated with durable tumor regression [86–88]. Strategies to enhance T cell persistence *in vivo* include using unique cytokine cocktails or activation system to induce less-differentiated phenotype of T cells in ex vivo culture or using T cells with intrinsic longevity. For example, Yang et al. reported that a defined cytokine combination (IL-12 plus IL-7 or IL-21 for three days followed by withdrawal of IL-12) was able to expand genetically engineered T cells with the less-differentiated phenotype and expanded T cells showed upregulated expression of genes associated with stemness. In a mouse model, $CD8^+$ T cells expanded with this cytokine cocktail had increased persistence. Besides, this cytokine combination can even turn the phenotype of TIL with a highly differentiated status into a less-differentiated status [89]. Butler et al. developed an artificial antigen-presenting cell system to generate CTLs with a memory phenotype that showed long-term survival both in culture and in patients [90]. Some virus-specific T cells were engineered to co-express tumor-specific receptors and showed enhanced persistence and antitumor activities in patients [91]. The virus-specific native receptors are proposed to confer T cells with optimal co-stimulation and better survival. Also, for virus-associated tumors, these dual-specific T cells can simultaneously target two cancer antigens and may have more potent antitumor activities.

5.3. Overcoming of immune escape of cancer cells

Given the complex immunosuppressive microenvironment and intrinsic heterogenicity of solid tumors, it is still challenging to induce a durable response by current TCR modified T cell therapies. To further enhance the antitumor activity, one strategy is to modify T cells with cytokine-releasing capacity, which are known as cytokine armed T cells or armored T cells. Immunostimulatory cytokines are incorporated into engineered T cells not only to enhance the intrinsic survival, proliferation, and function of T cells but also to counteract immunosuppressive factors in the tumor microenvironment. Cytokines released by T cells can also recruit additional effector cells, such as NK cells, to eliminate tumors. For instance, Interleukin-12 (IL-12) was recognized as an immunostimulatory cytokine to enhance the cytotoxic function of NK cells and T cells, and promote the differentiation of naive CD4 + T cells to Th1 type [92–95]. Zhang et al. investigated the efficacy of IL-12 armored T cells in a xenograft mouse model of melanoma, and the release of IL-12 was under the control of an NFAT-responsive promoter. The result showed that IL-12 armed TCR-T cells significantly enhanced antitumor activity and prolonged animal survival in comparison with standard treatment (TCR modified T cells plus IL2 and vaccination) [96]. However, in the first-in-man trial of TIL armed with inducible IL-12 (NCT01457131), the administrated T cells exhibited short-term survival in patients and mediated clinical toxicities [97]. Other cytokines, such as IL-15 and IL-18, were also investigated in the design of armored T cells. IL-15 was recognized as an essential cytokine for the development of CD8+ memory T cells, proliferation, and cytotoxic activity of NK cells and T cells [98,99]. IL-18 was discovered to be a stimulator to activate T cells and monocytes without severe side effects in clinical studies [100]. These cytokine-armed T cells showed enhanced proliferation and antitumor activities in both *in vitro* experiments and several animal models [101,102], but the efficacy and safety of these cells in humans are still waiting for evaluation. Another strategy to enhance anti-tumor responses is to explore novel combination therapies, including genetically modified T cells with small molecule inhibitors [103], checkpoint modulators [104,105] or oncolytic viruses [106]. Currently, checkpoint inhibitors are commonly used in combination with T cell therapy to enhance anti-tumor activity. After TCR engagement, the expression of a set of inhibitory receptors (CTLA4, PD1, Tim3 and Lag3) is naturally upregulated on T cells to block continued activation and thus prevent autoimmunity. However, the protection mechanism could be hijacked by tumor cells to inhibit the function of adoptively transferred T cells. Moon et al. showed that the adoptively transferred TCR-T cells became hypofunctional with upregulation of PD-1, Tim-3 and Lag3 in a mouse model of lung cancer, and anti-PD1 antibody could augment the efficacy of transferred TCR-T cells [107]. Several preclinical studies showed that CTLA4 blockade could also enhance the effectiveness of adoptively transferred T cells [108,109]. However, in a clinical trial (NCT01697527), Nowicki et al. found no apparent benefit associated with the combination of Ipilimumab (anti-CTLA4 antibody) with NY-ESO-1-specific TCR-T cells and peptide-pulsed DC vaccine [110]. Given the small number of patients in this cohort, further clinical studies would be needed to evaluate the efficacy of this combination.

6. Conclusion

TCR-T cells are considered as a promising modality to treat solid cancers. However, only a few antigen epitopes are available as ideal targets, thus identification of novel epitopes with restricted expression in tumors is essential for the development of TCR-T cell therapy. Although cancer testis antigens, virus-associated antigens, and neoantigens are proposed as ideal tumor targets, comprehensive studies on the specificity of target antigen expression are still needed. In order to enhance the potency of TCR-T cells, several engineering methods can be considered for the transduction of T cells and various cytokine cocktails may be used to improve the function or enhance the longevity of T cells. In the future direction, TCR-T cell-based combination therapy might be an optimal strategy for the treatment of solid cancers.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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