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REVIEW

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Novel insights into TCR-T cell therapy in solid neoplasms: optimizing adoptive immunotherapy

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Abstract

Adoptive immunotherapy in the T cell landscape exhibits efficacy in cancer treatment. Over the past few decades, genetically modified T cells, particularly chimeric antigen receptor T cells, have enabled remarkable strides in the treatment of hematological malignancies. Besides, extensive exploration of multiple antigens for the treatment of solid tumors has led to clinical interest in the potential of T cells expressing the engineered T cell receptor (TCR). TCR-T cells possess the capacity to recognize intracellular antigen families and maintain the intrinsic properties of TCRs in terms of affinity to target epitopes and signal transduction. Recent research has provided critical insight into their capability and therapeutic targets for multiple refractory solid tumors, but also exposes some challenges for durable efficacy. In this review, we describe the screening and identification of available tumor antigens, and the acquisition and optimization of TCRs for TCR-T cell therapy. Furthermore, we summarize the complete flow from laboratory to clinical applications of TCR-T cells. Last, we emerge future prospects for improving therapeutic efficacy in cancer world with combination therapies or TCR-T derived products. In conclusion, this review depicts our current understanding of TCR-T cell therapy in solid neoplasms, and provides new perspectives for expanding its clinical applications and improving therapeutic efficacy.

Keywords T cell receptor, TCR-T cell, Immunotherapy, Solid tumor, Tumor antigen, Clinical application

Introduction

Significant advances have been made in cancer immunotherapy, and the innate immune system has a vital role against tumor progression in the tumor microenvironment (TME), particularly in solid tumors [1]. Adoptive cell transfer (ACT) therapy, in combination with immune checkpoint inhibitors (ICIs), can induce tumor regression [2–5]. ACT represents a pioneering immunotherapy distinguished by its wide-ranging applicability, which has contributed to its rapid progress and therapeutic breakthroughs. ACT has evolved over several generations from autologous tumor-infiltrating lymphocyte (TIL) therapy [6, 7] to antigen-specific endogenous T cell therapy, culminating in chimeric antigen receptor (CAR) and TCR-T cell therapies. And

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the manufacturing process for ACT has developed from simple targeting of T cell populations for expansion from the TME, into now the use of genetic engineering in both CAR-T and TCR-T cells. This involves both modification of autologous or donor T cells and expansion [8] to obtain functionally engineered T cell populations aimed at specific target peptides.

CAR-T cells directly recognize extracelluar membrane antigens, eliminating restrictions related to major histocompatibility complex (MHC) extraction and delivery [9]. Mature applications of CAR-T cell therapy have been developed for a variety of hematologic malignancies, leading to remarkable progress in the treatment of B cell leukemia and B cell lymphoma [10, 11]. As of 2023, eight applications had been approved by the Food and Drug Administration, starting with Tisagenlecleucel, the first anti-CD19 CAR-T product to be approved. However, CAR-T cells can only recognize cell surface antigens, and there are difficulties in the treatment of solid tumors owing to the heterogeneity of cellular antigens, limitations in tumor-associated antigen (TAA) library to target [12, 13], and challenges of infiltration and T-cell depletion in TME [14–16]. It is hoped that with fourth- and next-generation CAR-T cell therapies, improved safety and a controlled therapeutic window will be achieved by co-expression of cytokines or other co-receptors [17, 18]. Also, in some clinical trials, CAR-T cells targeting the oncofetal antigen claudin-6 [19] and claudin 18 [20] for the treatment of solid tumors have been shown to be feasible.

TCR-T cells function via exogenously specific TCR to achieve CD8 cytotoxic T lymphocyte (CTL) lytic activity. TCR-T cells strictly recognize peptide epitopes presented by MHC class I molecules. Cytoplasmic proteasome breakdown and delivery of MHC molecules by means of biofilm fluidity make it possible for neoplasm cells to display diverse antigens. The innate cytosolic pathway creates more antigen targets for TCRs in whole-cell fractions to recognize and affinite. Sharable antigens for solid tumors remain to be found, and the therapeutic efficacy of TCR-T has been clinically validated. More critically, a range of tumor neoantigens has been identified, and some clinical trials have demonstrated the efficacy of TCR-T cell therapy against metastatic solid tumors [21–23]. In this review, first, we introduce the tumor-antigen libraries and the process of TCR acquisition and optimization. Second, we summarize the construction and application of TCR-T cells, including computer simulations and the complete flow from laboratory to clinic. We also discuss the methods for enhancing TCR-T affinity instead of cross-reactivity. Third, the constraints and future prospects for improving the efficacy of TCR-T cell therapy in cancer treatment are considered. In conclusion, this review elucidates current clinical applications of TCR-T cell therapy on solid neoplasms.

TCR-T design and manufacturing

Principles

Aβ TCR multimers activate the TCR signaling pathway by recognizing and binding extracellularly to peptide-MHC (p-MHC) complexes. These consist of a predominant heterodimeric duplex, usually paired by one α chain and one β chain, both of which have a transmembrane region and an antigen-binding region. The TCR chain is anchored to the T cell membrane by the constant region, and each chain can be reconfigured by rearrangement of variable regions [24]. Such rearrangements, which involve random and exponential differences, represent a good implementation of the inherent polymorphism and reserve of TCRs and can theoretically handle any epitope sequence. Rearranged TCRs then undergo physiological maturation of affinity through thymic selection [25]. The TCR recognizes and binds the p-MHC complex through interactions of its two variable regions [26]. TCRs do not function independently; activation of T cells depends on the cooperation of the TCR heterodimer and CD3 six-chain multimers, which comprise three dimers: one CD3 γε heterodimer, one CD3 δε heterodimer, and one CD3 ζζ homodimer [27, 28].

Artificially engineered TCRs use similar mechanisms to those of native TCRs to recognize specific MHC complexes with the HLA isotype (Fig. 1). The killing activity of T cells is directly related to the affinity of TCRs. T cell editing can be used to achieve recognition and effects on tumor cells beyond those possible in the physiological state. This requires the expression of specific relatively high-affinity TCRs; the natural CD complexes oscillating on the cell membrane are used to build functional receptors, which are provided in part by the T cells themselves. This means that the process can be physiologically activated by the T cells; however, co-stimulatory signals are essential, including CD28 and CD137 (4-1BB) on the surface of T cells. The production of equipped TCR-T cells, the search for and exploitation of novel specific TCR-T cells, manufacturing of personalized tumor neoantigens, screening of TCR genes, and localization and isolation can be progressively simplified and made more rapid using genomics techniques. Moreover, next-generation sequencing (NGS) and single-cell multi-omics sequencing technologies for screening and identification of target proteins can be used to comprehensively determine the tumor specificity of TCR sequences and structures and predict recognizable epitopes.

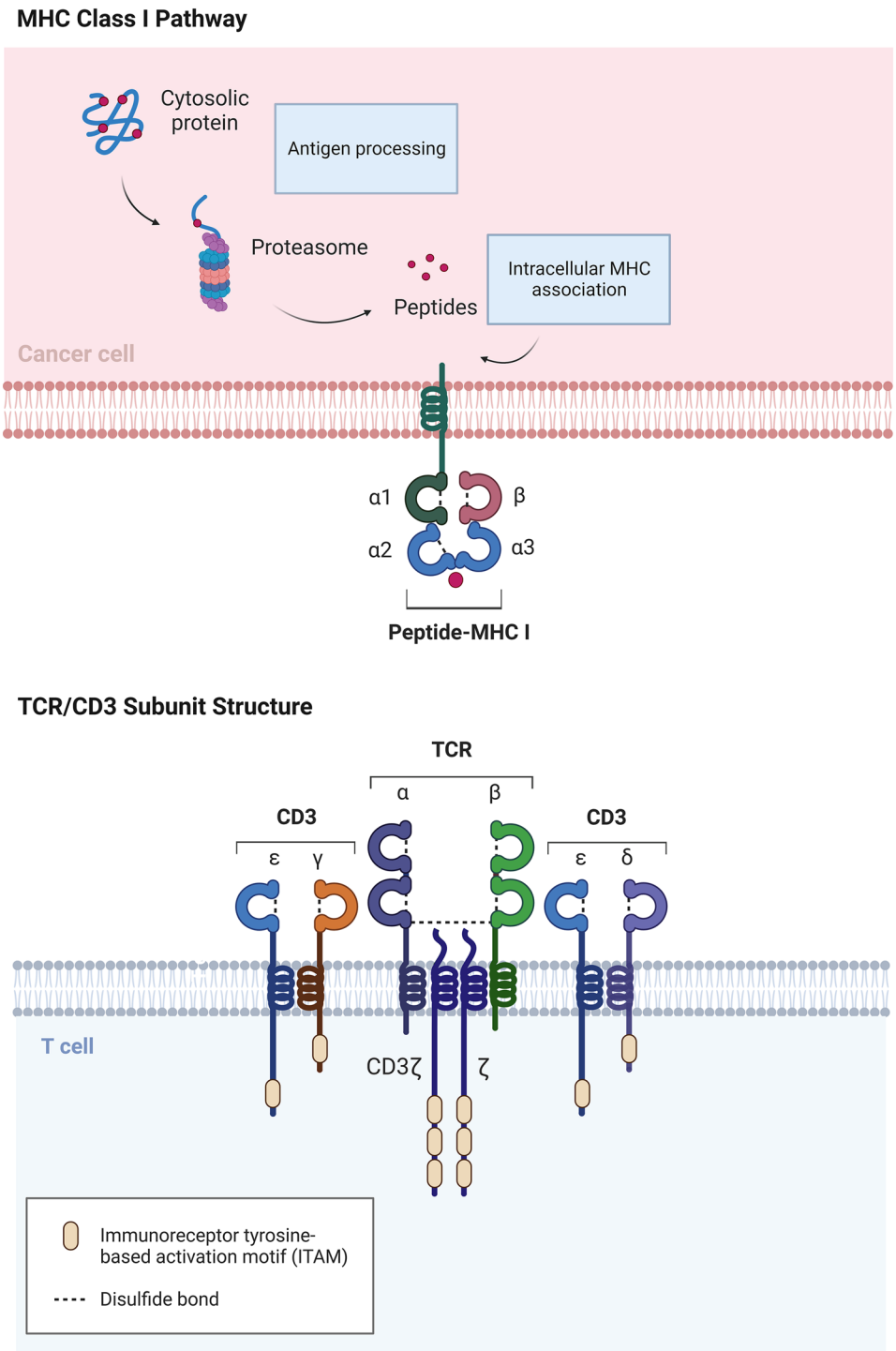

Fig. 1 Schematic diagram of TCR/CD3 and peptide-MHC structure. Cancer cells process and deliver intracellular antigenic peptides via MHC class I molecules on the cell membrane. The TCR complex is essentially an α, β-double-stranded heterodimer, recognizing the peptide-MHC complex with the CD3 γε heterodimer, CD3 δε heterodimer and CD3 ζζ homodimer cooperatively

Recognition, characterization, and acquisition of TCR-T cells and p-MHC

Screening and identification of antigens

In order for TCR-T cells to achieve specific tumor cell killing, the antigen used as a target for TCR recognition should possess the following qualities:

(1) expression solely or predominantly in tumor cell populations;
(2) correlation with key tumor events;
(3) known targets and coding sequence;
(4) immunogenicity that can trigger a T cell response capacity;
(5) no cross-reactivity with autoantigens;
(6) non-rapid induction of T cell depletion.

Existing immunotherapies for solid tumors can be divided into two broad categories based on the antigens involved: TAAs and tumor-specific antigens (TSAs), also known as neoantigens [29, 30]. Clinical trials of TCR-T cell therapy that are currently underway or have been initially completed for patients with solid tumors also focus on these two antigen categories. The main known clinical trials and their outcomes are listed in Table 1. In addition, HA-1-specific targeted T cells have demonstrated favorable safety profiles in some clinical tests of ACT [31, 32]. However, the requirement for donors and ligands to have compatible single nucleotide polymorphisms (SNPs) makes it challenging to find suitable matches for recipients [33].

Our current knowledge on target antigens for TCR-T cell therapy is summarized in Table 2.

(1) TAAs TAAs exhibit limited expression levels in a restricted number of cell types in normal tissues but are upregulated in tumor tissues owing to abnormal gene expression. TAAs arise from epitopes of endogenous wild-type proteins from specific populations and can be shared between different patients and tumor types, making them potentially suitable for universal immunotherapies [85, 86]. However, the recognition of these antigens by existing specific TCRs is often of low affinity owing to negative selection and tolerance processes against self-antigens in early development, leading to suboptimal clinical trial outcomes [87]. Furthermore, the engineering of high-affinity TCRs targeting TAAs may increase the risk of self-cross-reactivity to normal cells with low antigen levels. Reported severe autoimmune reactions include colitis, kidney damage, severe hepatitis, respiratory failure, and treatment-related fatalities [88].

Tissue differentiation antigens (TDAs)

TDAs are present in specific stages of cell differentiation but may be shared with a small number of antigens in normal cells. Melanocyte differentiation antigens currently commonly used for human TCR target include MART-1/Melan-A [34–37], gp100 [35], and tyrosinase [89]. A number of clinical trials targeting MART-1 demonstrated some clinical efficacy, but some dose-dependent toxicities were documented, illustrating the potential risk of TDA expression of normal tissues. Besides, carcinoembryonic antigen (CEA) was targeted in an early clinical study of TCR-T to three patients with metastatic colorectal cancer. All patients presented with decreased serum CEA levels and one-third example of objective regression. However, it should be noted that all patients reported the occurrence of a severe transient inflammatory colitis, and the limitations of using CEA as a target for cancer immunotherapy were similarly described [90].

Cancer germline antigens (CGAs)

CGAs, also called "cancer testis antigens", are normally restricted to germ cells. CGAs have been targeted in the majority of the TCR-T cell clinical trials, with favorable objective remission outcomes. The New York Esophageal Squamous Cell Carcinoma Antigen 1 (NY-ESO-1) targeted product has achieved favorable results in clinical trials, particularly in cutaneous melanoma and synovial sarcoma [40–47]. However, it has been reported to have limited expression in metastatic cancers. Owing to the effects of tumor heterogeneity on NY-ESO-1, maintenance of long-term stability for individuals needs to be further explored [91]. Some studies have shown that preferentially expressed antigen of melanoma (PRAME), another germinal tissue-specific antigen, participates in the proliferation and survival of cancer cells in a variety of malignancies, including melanoma, sarcoma, lung cancer, head and neck cancer, and kidney cancer, as well as being expressed in healthy tissues such as gonads, adrenal glands, bone marrow, and brain [92, 93]. Recently, one clinical trial of TCR-T cell targeting PRAME has yielded a good clinical objective response rate in the trial cohort (NCT03686124) [61]. Melanoma antigens (MAGE), especially MAGE-A, have been widely used in the treatment of various solid tumors. However, clinical trials of MAGE-A10 have to date only yet reported results regarding usability in non-small-cell lung cancer [48], partly owing to the high degree of overlap between MAGE-A10 and MAGE-A4 expression. Clinical trials of MAGE-A4 have shown it to be relatively effective

Table 1 Current major target antigens and clinical trials of TCR-T cell therapy

Antigen type	Target antigen	HLA	Cancer type	Clinical trial	Phase	Objective response rate (ORR) (%)	Clinical response	References
TDA	MART-1	HLA-A*0201	Melanoma	n.s	n.s	2/17 (12)	2 PR	[34]
	MART-1	HLA-A*02:01	Melanoma	NCT00509288	2	6/20 (30)	6PR	[35]
	MART-1	HLA-A*02:01	Melanoma	NCT00910650	2	0/13 (0)	0	[36]
	MART-1	HLA-A*02:01	Melanoma	NCT02654821	1/2a	2/12 (16.7)	2PR	[37]
	gp100	HLA-A*02:01	Melanoma	NCT00509496	2	3/16 (16)	1CR, 2PR	[35]
	CEA	HLA-A*02:01	Colorectal cancer	NCT00923806	1	1/3 (33)	1PR	[90]
CGA	NY-ESO-1	HLA-A*02:01	Melanoma; synovial sarcoma	NCT00670748	1	5/11 (45) 4/6 (67)	2CR, 3PR; 4PR	[40]
	NY-ESO-1	HLA-A*02:01	Melanoma; synovial sarcoma	NCT00670748	2	11/20 (55); 11/18 (61)	4CR, 7PR; 1CR, 10PR	[41]
	NY-ESO-1	HLA-A*02:01	Melanoma; synovial sarcoma; liposarcoma; osteosarcoma; MPNST	NCT02070406; NCT01697527	1	2/10 (20)	2PR	[42]
	NY-ESO-1	HLA-A*02:01;HLA-A*02:06	Synovial sarcoma	NCT01343043	1/2	6/12 (50)	1CR, 5PR	[43]
	NY-ESO-1	HLA-A*02:01;HLA-A*02:06	Synovial sarcoma	NCT01343043	1/2	9/30 (30)	9PR	[44]
	NY-ESO-1	HLA-A*02:01;HLA-A*02:06	Synovial sarcoma	JMA-IIA00346	1	1/3 (33)	1CR	[45]
	NY-ESO-1 (CRISPR/Cas9)	HLA-A*02:01	Metastatic sarcoma; Myeloma	NCT03399448	1	0/3	0	[46]
	NY-ESO-1	HLA-A*02:01	Myeloma	NCT01352286	1/2	11/25 (44)	1SCR,1CR, 8VGPR, 1PR	[47]
	MAGE-A3	HLA-A*02:01	Melanoma; synovial sarcoma; esophageal cancer	NCT01273181	1/2	5/9 (56)	1CR,4PR	[52]
	MAGE-A3	HLA-A*01	Melanoma	NCT01350401	1	0/1	0	[51]
	MAGE-A3	HLA-DPB1*0401	Metastatic solid tumors	NCT02111850	1	4/17 (23.5)	1CR,3PR	[53]
	MAGE-A4	HLA-A*24:02	Esophageal cancer	UMNI000002395	1	0	/	[49]
	MAGE-A4	HLA-A*02	Relapsed/refractory metastatic solid tumors (9 types)	NCT03132922	1	9/38 (24)	9PR	[50]
	MAGE-10	HLA-A*02:01 OR HLA-A*02:06	Nsclc	NCT02592577	1	1/11 (9)	1PR	[48]

Table 1 (continued)

Antigen type	Target antigen	HLA	Cancer type	Clinical trial	Phase	Objective response rate (ORR) (%)	Clinical response	References
	PRAME	HLA-A*02:01	Cutaneous melanoma, uveal melanoma, endometrial carcinoma, synovial sarcoma, and ovarian cancer	NCT03686124	1	8/12 (64)	8PR	[61]
Viral antigen	HPV16-E6	HLA-A*02:01	Epithelial cancer	NCT02280811	1/2	2/12 (17)	2PR	[94]
	HPV16-E7	HLA-A*02:01	Epithelial cancer	NCT02858310	1	6/12 (50)	6PR	[95]
	HBV	HLA-A*02:01;HLA-Cw0801	HBV-	NCT03899415	1	1/8 (12.5)	1PR	[96]
	MCPyV	HLA-A*02:01	Merkel cell carcinoma	NCT03412877	1	1/5 (20)	n.s	[97]
Neoantigen	TP53	HLA-A*02:01	Metastatic breast cancer	NCT03412877	1	1/1 (100)	1PR	[21]
	KRAS G12D	HLA-A*08:02	Metastatic pancreatic cancer	IND 27501	1	1/1 (100)	1PR	[22]
	mutation-associated neoantigens (CRISPR/Cas9)	multiple HLA class I	Metastatic solid tumors	NCT03970382	1	0/16	0	[23]

MPNST malignant peripheral nerve sheath tumor, *NSCLC* non small cell lung cancer, *PR* partial response, *CR* complete response, *SCR* strictly complete response, *VGPR* very good partial response

against synovial sarcomas [49, 50]. However, the trial of engineered T cells on MAGE-A3 has reported fatalities [51], which have been confirmed to be caused by cross-reaction with the MAGE-A12 protein in the brain. Neurotoxicity and cardiac toxicity were reported in another clinical trial [52], with two treatment-related deaths, which preliminarily demonstrated cross-reaction with actin of cardiac myocytes. After that, one clinical trial of engineered MHC II-restricted MAGE-A3 TCR on autologous CD4 T cells provided preliminary evidence of safety and efficacy [53].

(2) TSAs TSAs are specific to neoplasms arising from oncogenic mutations, including genomic mutations. They are especially likely to occur as a result of key events in tumorigenesis (occurrence of single-nucleotide variations, indels, fusion genes, or chromosomal structural abnormalities), insertion and integration of foreign carcinogenic oncogenes (viral oncogenes) [94–97], or variants of events throughout transcription and expression of genes downstream (aberrant transcripts, aberrant post-translational modifications). TSAs are presented by MHC molecules and can be used to better characterize the heterogeneity of tumors and determine the therapeutic potential of TCR-T cells. In mutant cells, TSAs are also processed and transported by endosomal proteasomes to form p-MHC complexes with MHC-like molecules on the cell surface, whose epitopes are involved in receptor recognition. For immunodominant tumor TSAs, the sequential process of stimulating an immune response involves transcription, translation, and processing of the original peptide, presentation of the mutant peptide by the MHC molecule, the epitope of the pMHC complex, and the affinity of the TCR [98–100]. Thus, for the prediction and screening of TSAs, it is necessary to have full information about the types of MHC molecules possessed by the patient, and their genome and expression profile, as well as identification of the mutation and comprehensive follow-up analysis. Several shared neoantigens have achieved good results in clinical trials [21, 22, 101].

The major methods used to acquire TSAs are listed in Table 3. High-resolution mass spectrometry (MS) is commonly used to analyze samples and can be used in combination with a pan-HLA class I antibody for immunoprecipitation to capture tumor cell p-MHC complexes and determine polypeptide characteristics

[102–105]. Jaeger et al. achieved precise extraction of a mouse-specific H2-K1-presenting peptide from in situ tumor tissues by inserting an inducible affinity tag within this MHC allele, resulting in a neoantigen that could not be predicted by mRNA expression or translational efficiency. This approach provided the TME and tissue-specific stimuli that are lacking from in vitro cell cultures, as well avoiding and confounding heterogeneous interference [106]. Whole-exome sequencing (WES) and RNA sequencing (RNA-seq) can be used as complementary methods to compare abnormal expression of tumor genes with that of normal genes and identify the sequences of mutated genes. RNA-seq can also be used to detect alternative splicing events and to estimate the relative frequency of mutant allele expression [107, 108].

For TCRs for which concrete sequences have been determined by sequencing and manual design, undifferentiated screening of recognizable antigens can be performed using p-MHC homopolymer libraries constructed from baculovirus or yeast as vectors, with soluble TCRs as probes; this assists with the discovery of orphan TCRs, which have unknown antigen specificity in the natural state [109]. Mutation Associated NeoAntigen Functional Expansion of Specific T cells (MANAFEST) is a high-throughput platform that can screen known TCRs for recognizable neoantigens. T-SCAN is also a platform for homologous antigenic profiling of target T cells for large-scale genome-wide libraries [110, 111]. Signaling and antigen-presenting bifunctional receptors (SABR), a newly developed protein complex, recognizes a spectrum of homologous epitopes of specific TCRs [112]. Similarly, there are MHC-TCR double modified receptors that recognize antigenic targets of specific TCRs delivered by MHC-2 molecules on murine-derived CD4 T cells [113]. Notably, some new technologies have been used in antigen-searching biological toolboxes; for example, trogocytosis is a new T cell targeted ligand discovery principle that uses fluorescent labeling to trace membrane transfer of T cell membrane proteins to the p-MHC I of target cells, enabling isolation of the target cells and sequencing of the TCRs [114], as well as capture of orphan TCRs. Cattaneo et al. present HANSolo, a high-throughput system for unbiased p-MHC identification. In this method, the patient-matched Bcl-6/xL-immortalized B cell lines are modified for antigen-library expressing and specific T cell selection, which have all individual MHC genotypes [115].

Data from WES, RNA-seq, and proteomics in databases such as The Cancer Genome Atlas are used to initially screen for neoantigens across the cancer spectrum [116]. Databases have also been constructed with mature data accumulated from MS or from immunoprecipitation-MS or liquid chromatography-MS capture of antigens [117, 118], in conjunction with information obtained from NGS; these databases can be used for modeling and finding neoantigens, as well as for deep-learning-based prediction of peptide properties based on additional features such as liquid chromatography retention time, ion mobility, and MS/MS spectra [119].

Deep learning using databases and in silico prediction of possible sequences for obtaining and exhibiting neoantigens have become mainstream tools for neoantigen personalization. These require patient information related to tumorigenesis, infiltration, and metastasis, including genome, transcriptome, and proteome data, which can be compared with data from normal populations. The molecular properties of the MHC are polymorphic and diverse and influence binding to the TCR; the patient's HLA allele determines the value and size of his or her tumor-specific predictive neoantigen pool [120–123]. Personalized information on specific patient treatments from NGS, WES, and RNA-seq, together with p-MHC acquisition by MS, can be used to estimate possible neoantigens that may arise, construct TCR–pMHC binding-prediction models, and estimate the ability of patients to affinity and transmit epitopes for specific MHC molecules. Machine learning enables the prediction of peptides from mutant homology libraries via a series of key steps. Filters must be added to the initial prediction outcome to exclude antigenic peptides that are not valid in the process. The filtering procedure involves parameterization of the delivery process of the antigenic peptide, including the affinity of the peptide for the MHC molecule, the sequence consistency of the variant peptide, the frequency and expression of the variant allele, and the capacity for peptide cleavage at the proteasome and subsequent translocation.

Some machine learning models identify mutations and predict neoantigens based on nucleic acid sequences; models with a focus on the identification of specific MHC molecules include NetMHC [124], NetMHCpan [125], and MHC flurry [126, 127], which consider the binding steps of specific MHC molecules but not the complex process of subsequent presentation. Some learning and prediction models use an HLA-ligand peptide dataset to improve prediction fidelity based on affinity; these include NetCTL [128] and NetCTLpan [129]. Experimentation is continuing with the introduction of more complex steps to train prediction tools and predict target TCRs that can bind to p-MHC and be recognized efficiently, for instance, McPAS-TCR [130] and VDJdb [131]. Although the feasibility of this approach has been demonstrated, the anticipated antigen pool may be exponentially larger than the actual antigen pool [132, 133], and most of the new antigens delivered by the predicted

Table 2 Classification and description of target antigens in TCR-T cell therapy

Categories		Shared antigens and genes in solid tumors	Advantages	Disadvantages
TAAs -Endogenous wild-type proteins	Tissue differentiation antigens	• MART-1/Melan-A [34–37] • gp100 [35] TYRP1[38] • mesothelin [39]	Antigen ubiquity: widely shared between patients; widely shared under tumor heterogeneity	Prone to immune evasion under affinity limitation Relatively poor effect, easily tolerated
	Cancer germline antigens	• NY-ESO-1 [40–47] • MAGE-A [48-53] • BAGE [54] • SAGE [55] • HAGE [56] [57] • SSX [58] • LAGE [59] • SCP1 [60] • PRAME[61]	Easier to recognize, isolate and validate Relatively mature, have undergone clinical tests	Autosomal cross-reactions have been widely reported, causing multiple injuries and even death
TSAs (neoantigens) -Somatic mutant proteins	Neoantigens from genomic mutations: SNVs; Indels; Fusion genes; Chromosomal structural abnormalities	SNVs • *TP53* [21, 62, 63] • *KRAS* [22, 64–67] • *IDH1* [68] • *JAK2* [69] • *BRAF* [70] • *CDK4* [71] • *CDK12* [72] • *NRAS* [65] • *CTNNB1* [73] • *GAS7* [74] Indels • *NPM1* [75] • *CALR* [76] • *TGFBR2* [77] Fusion genes • *BRD4-NUT* [78] • *NTRK1/2/3* [79–81] • *NRG1* [82]	No expression in normal somatic cells No T cell thymocyte selection and central immune tolerance Individuation, more in line with the heterogeneity of patients with tumors, new therapeutic potential	Need to predict and characterize, more cost of time and resources Need to find commonality Immature and difficult to validate Unknown risk of cross-reactivity
	Viral neoantigens (viral open reading frames)	• HPV-16 E7 • HPV-16 E6		
	Neoantigens of transcriptomic variants	• COL6A3-FLNV [83]		
	Neoantigens of proteomic variation/ abnormal antigenic peptide presentation	• LUAD [84]		
MiHAs	A large antigen library distinct from MHC presentation		Available applications in hematologic malignancies	Strict requirement of individual matching

TAA tumor-associated antigen, *TSA* tumor-specific antigen, *MiHA* minor histocompatibility antigen, *SNV* single nucleotide variation

MHC molecules do not trigger an effective immune response [134].

Obtaining TCRs

Sources of T cell clones include autologous T cells, derived from TILs within patients' tumor or circulating T cells from their peripheral blood; and peripheral blood lymphocytes from healthy donors (Fig. 2A). The two differ in terms of T cell use and processing; autologous T cells are used for development of personalized TCR-T cell therapies, and some studies have also induced differentiation from hematopoietic stem and progenitor cells in vitro, progressing to production of mature single-TCR-specific T cells [136]. One p-MHC complex can bind to different TCR clones, and the specificity of the low affinity of TCRs for p-MHC allows one TCR clone to bind to a variety of different epitopes with low sequence similarity and different structures, as confirmed in recent studies. Owing to the simplicity and diversity of TCRs and the epitopes they recognize, identification of optimally specific TCRs in screening is complex; it may be necessary to take into account the immunogenicity of the corresponding antigens. The best TCRs with validated encoding genes can be sequenced by TCR sequencing, RNA-seq, and NGS to obtain coding sequences of

Table 3 Acquisition strategies and main methods for neoantigens

Category	Introduction	Main method	Feasibility	Application scenarios
Direct acquisition of neoantigens	Sampling and screening of an existing population of tumor tissue, usually from biopsy or surgical resection Aim: to find available targets in their naturally occurring antigen pools	Immunoprecipitation-MS [102–105]	Patient tumor tissues	To capture primary tumor p-MHC
		Affinity-tag extraction [106]	Animal tumor tissues with specific MHC type tagged	To precisely extract known and neoantigens in situ
		RNA-seq and WGS [107, 108]	Patient tumor tissues	To obtain complete serial sequence information of one patient
		peptide-MHC libraries [109]	Specific TCR or acquired T cells, and constructed vector libraries	To undifferentiately screen one TCR-recognizable known epitopes
		MANAFEST, T-SCAN [110, 111]	Specific TCR	To high-throughput screen recognizable epitopes
		SABR [112]	Specific TCR	To screen homologous epitopes
		Trogocytosis [114]	T cells fluorescently labeled with membrane proteins	To trace target cells binding and then sequence involved TCRs
		Hansolo system [115]	Patient T cells and immortalized B cell lines	To construct unbiased mutanome minigene recognizable library of the patient
Predictive modeling of neoantigens	Acquisition of patient's MHC molecular profile (individual-specific MHC typing) In silico analysis and prediction of deliverable epitopes combined with simulation of realistic multi-step parameter optimization, with attention to distortion or overestimation of the predicted epitope library Aim: capture of possible key antigens for usable TCR design	TCR and antigen prediction 1. Personalized information and MHC typing 2. Computerized prediction models: i. HLA typing ii. mutation typing and calling iii. HLA binding prediction iv. TCR prediction v. TCR priority vi. TCR-recognizing HLA screen [135] 3. Design of the corresponding TCR at the optimal epitope-MHC	Databases for in silico pre-analysis: • whole-genome sequencing and WES • RNA-seq • proteomics • MS	To predict epitopes and also exclude self-reactive antigens on a large-scale, use sequence information and select models

both α and β strands and introduced into T cells via constructed vectors.

The strategy starts with T cell culture expansion using specific cytokines (interleukin-2, etc.) and T cell priming by co-culturing with antigen-presenting cells (APCs) loaded on antigen-homologous MHC molecules (incubation or gene introduction) to obtain specific T cell polyclonal populations. Assays of T cell proliferation, killing lysis activity by chromium release, enzyme-linked immunospot (ELISpot or FluoroSpot), intracellular cytokine staining (ICS) [137, 138], cytokine capture (IFN-γ), multiple intracellular staining, and activation or degranulation markers (41BB, CD107a) may be performed to detect the frequency, phenotype, and functional status of T cells [139, 140]. Alternatively, a fluorescent-protein reporter gene, coupled to the activation of the response element nuclear factors of T cells (NFAT), can be used to visualize characteristics of TCR-mediated activation. Fluorescence-activated cell sorting with fluorescent-labeled p-MHC tetramers, or MS sorting with heavy metal chelate-labeled p-MHC tetramers, can be used to screen antigen-specific T cells [141, 142]. Screening isolation of T cells can then be improved by various methods; tandem microgene (TMG) transduction or single-chain long peptide pulses on APCs is used to improve the screening efficiency of TILs at a higher order of antigen presentation [143, 144], although the expression levels of TMGs for the whole antigenic libraries may not be artificially controlled consistently [145]. DNA barcode-labeled p-MHC multimer- and tetramer-associated TCR sequencing has emerged as a method for high-throughput screening and precise TCR recognition of antigen-specific T cells [146–149]. Microfluidic technology has also enabled the dynamic detection and recognition of TCR–pMHC interactions at higher throughput [150, 151]; for instance, microfluidic antigen–TCR engagement sequencing technology allows high-throughput isolation and single-cell TCR sequencing of neoantigen-specific T cells [152]. In addition, a mouse model carrying a human-specific TCR has been constructed for the development and applications of high-affinity TCR [153]. The establishment of a heterologous immune system through mutagenesis of one of the TCR double-strands in the mouse thymus, or transfer of the entire human TCR αβ locus into mice to develop T cells targeting human self-antigens [154, 155], increases the probability of spontaneous occurrence of high affinity, without producing clonal deletion or tolerance of T cells as in humans [155].

Single-cell RNA-seq and single-cell TCR sequencing can be used to obtain phenotypic information of T cells carrying a single TCR clonotype, and targeted TCRs are obtained by sequencing TCR α and β single strands [156, 157]. High-throughput single-cell RNA-seq can be used to identify TCR transcripts from primed T cells [147], facilitating screening of early highly transcribed TCRs. Effective specific isolation of TIL in the unamplified state could avoid the problems of interference that occurs with high concentrations of IL-2 and deviation of the amplified state from the TIL polyclonal population [158]. Single-cell cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) and TCR-coupled TCR-CITE-seq can be used to target cell surface proteins of early TILs [159]. The new neoscreen platform was constructed to screen TILs early after specific antigen presentation and has achieved highly sensitive antigen-specific TCR isolation and identification [160]. Spindler et al. captured millions of natural TCRα/β clonotype libraries from primary T cells by massively parallel microfluidics processing and successfully constructed Jurkat cell lines for preservation, demonstrating that antigen-responsive TCRs can be screened with high throughput and specificity using a large-scale library [161]. The development of spatial transcriptomics has also enabled access to different phenotypes of tumor-infiltrating cells, as well as weighted localization, with applications in the definition of heterogeneity of metastatic tumors or tumors at multiple sites and in the search for shared immune cellular signatures. The newly developed Slide-TCR-seq method ensures fidelity and completeness of sequencing at scale of whole transcriptomes and TCR immunome libraries in the tissue environment, which has facilitated access to immunome libraries and enabled them to be compared with more sensitive and validated TCRs, including in different spatial contexts, even in a state of TME suppression [162, 163].

Progressively established decoy-RNA libraries targeting V and J regions of TRAC and TRBC pinpoint specific functional regions of specific segments of the TCR; they are used in pairwise deep sequencing of TCRs against oligoclonal populations of T cells [164, 165] and enable the identification of the full peptides of antigen-specific TCRs in human or human-derived mouse T cells. With single-cell sequencing information from T cells, Omer et al. applied a TRB prediction series pipeline based on IgDiscover, IgBlast, and TIgGER simulation software to multiple adaptive immune receptor repertoire sequencing (AIRR-seq) databases to retrace variants in the genetics upstream of the TCR haplotypes; this approach included tracing of unobserved coding genes, haplotypes, and loci, as well as mono-chromosomal or di-chromosomal deletions, and provided error-correction methods. This experiment confirmed the feasibility of using AIRR-seq information to resolve rearrangements and structural variants in the TCR V(D)J gene and to explain the germline variability caused by SNPs and changes in the expression profile of a specific TCR locus [166].

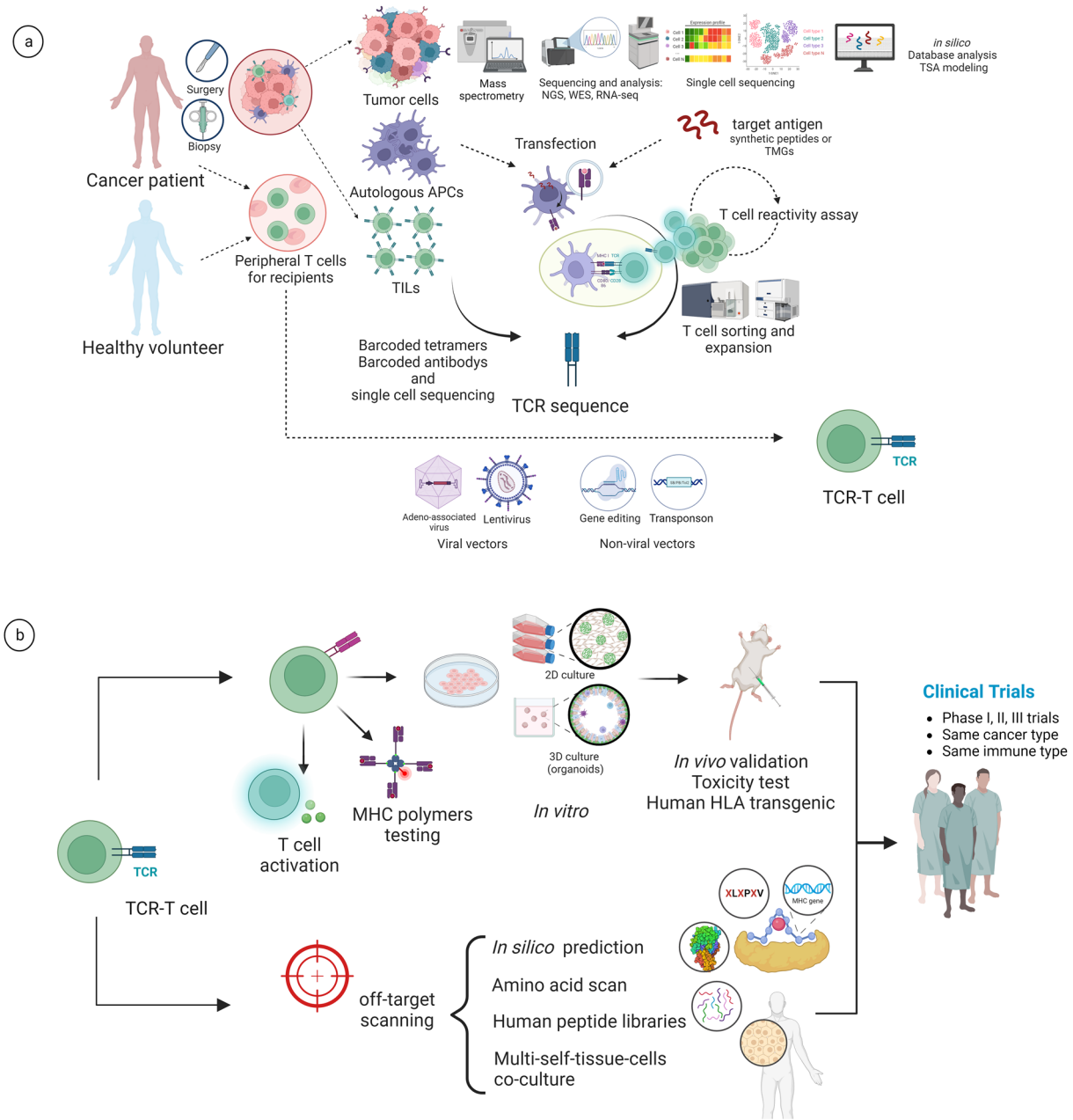

Fig. 2 Overview of the necessary steps to develop, integrate, and test one TCR-T product. **A** Workflow for reforming recipient T cells to express TCRs that aim target antigens. First, target antigens are derived from tumor cells of individual patients. A series of protein acquisitions and multi-omics identifications are performed to identify tumor antigens, and new TSAs are computationally predicted. Acquired natural or synthetic antigenic peptides, or TMG transduction, enable autologous APCs to stimulate T cells with antigens to produce reactive TCRs or to detect T cell reactivity. Reactive productive T cells or in situ tumor-infiltrating cells are sorted and expanded, or screened using barcode tetramers or barcode antibodies, followed by single-cell RNA-seq or TCR sequencing, ultimately yielding the target TCR and gene sequence. Recipient T-cells can be obtained from peripheral T cells from the patient or from an HLA-matched healthy donor. A single tumor-responsive TCR-T is obtained by expressing TCR in recipient T cells via viral or non-viral vectors. **B** The effectiveness of a TCR-T can be validated by T-cell activity assays and MHC-polymer soluble ligands or a constructed libraries test. T cell activity is determined by antigen titration, HLA-matched cell line culture, and two- or three-dimensional (2D or 3D) tissue cultures. In vivo mouse models are established with human HLA for detection of tumor killing activity and toxicity documentation. Off-target activities of TCR-Ts on self-tissues can also be recorded simultaneously, via computer prediction, screening of reactive peptides for alanine or whole-amino-acid substitutions, screening of a full library of human self-peptides, and co-culturing of a variety of self-HLA-matched cell and tissue lines. Finally, TCR-Ts are subjected to clinical trials in various phases and several results have been reported

TCR editorial optimization

The original structures of TCRs can be modified artificially. Genetic engineering artificially creates affinity maturation of the complementation determining region (CDR); the highly variable CDR3 is important in peptide interactions and sequence diversity. Research efforts have been directed toward detection of this region and identification of its core motifs and basic features [167–169]. Several studies have been conducted on point mutation of CD3 loops for TCR affinity enhancement, which was subsequently shown to be effective and safe in clinical trials [42, 43].

For protein primary structure, the prediction of TCR recognition peptides can be optimized to induce targeted point mutations to alter their affinity for target antigens [170]. However, this involves artificial interference with the original negative selection result of the thymus, and attention to the emergence of non-target peptides for further identification is necessary. The sequence specificity of the predicted identified peptides can be determined using computerized deep learning methods. These include ERGO, which is based on a combination of McPAS-TCR and VDJdb, the two currently available large TCR–pMHC (I or II) datasets for training [130, 131, 171]. There are also post-training TCR and peptide binding prediction methods using natural language processing, in which candidate peptides are screened for specific identifiable TCRs [172].

Analysis of the shared motifs of TCR recognition core sequences has highlighted key conserved residues that drive TCR recognition, which can be used for further screening of TCR variants with core conservation [170]. This can also be achieved by peptide complex crystal analysis [173]. After identification of TCR conservation and specific target-antigen-MHCs, diverse mutant libraries, mainly on CD3α or CD3β, which have higher variability, can be constructed independently to obtain a larger population of TCR mutations. This involves mutation and enrichment of the remaining peptide recognition structural residues and flanks after retaining key residues [174]. A superior TCR with high affinity was obtained by screening for allogeneic p-MHC-guided T cell activity, and many mature TCR affinity engineering platforms have recently been constructed using mammalian cell lines [161, 175] or screening after precursor T cell differentiation [153].

Methods for protein structure training and model prediction are also being developed, with emerging strategies using modeling analysis or structure-guided analysis in three dimensions, and introduction of micro-interactions between interacting peptides for model optimization [176, 177], enabling more accurate prediction of recognizable protein responses. It has also been demonstrated that the reactivities of TCRs and MHC presenting peptides are equally affected by the structural diversity of p-MHC molecules [178]. Deep learning algorithms have been used for three-dimensional (3D) prediction analysis to train TCRs to recognize the binding effects of p-MHC [179]. Using AlphaFold, deep neural networks can predict TCR and p-MHC interactions, accurately distinguishing the correct available peptide epitopes, with applications in the development of generalizable predictive models for TCRs and further p-MHC-specific binding [180].

TCR-T cell construction

(1) Viral vectors Once optimal TCR sequences and encoded genes have been obtained, they can be preserved or transferred by viral vectors into T cells suitable for tumor therapy. Adenoviral vectors were the first to be used for this purpose, although they are gradually being replaced owing to drawbacks such as their inability to integrate into the genome and encoding of heterologous proteins [181]. Replication-defective retroviruses, such as γ-retroviruses or lentiviruses, are now commonly used for the delivery of TCR target genes, and their stable delivery and safety have been demonstrated in human experiments [182]; however, the non-specific semi-random integration of their mechanism creates a risk of insertion of a random number of copies into the whole genome of the host cell [183]. This could interfere with the functioning of the transgene or even silence it [184], as well as posing unanticipated risks of other alterations in cytogenetic material. Adeno-associated viruses, which are widely used as vectors for gene therapy, can also be used for TCR-T construction, and good results have been achieved by combining them with CRISPR/CRISPR-associated protein 9 (Cas9) to achieve endogenous in situ knock-in (KI) of TCRs [185]. Recently, in an immunodeficient mouse model, Nyberg optimized an AAV synthetic subspecies, Ark313, for efficient transfection of murine-derived T cells; this could perform targeted transfer of large transgene expression cassettes with high efficiency, enabling nucleotide-free DNA delivery for CRISPR/Cas9-mediated gene knockdowns, with non-specific integration reported only as a rare event [186].

(2) Non-viral vectors Retrotransposon system

mRNA electroporation allows for transient gene introduction and its use has also been reported for direct transient TCR and CAR expression. Clinical data to date indicate its efficacy and safety; however, this technique has limited durability for a single safe transfer of a certain amount of TCR, and its efficacy is restricted. It is currently used to introduce other transgenic systems. These use transposons that are flanked by terminal inverted repeat (TIR) sequences and contain DNA double strands of transposase-coding sequences; there are

also semi-autonomous transposon systems that can be supplemented with the expression of transposases in the form of synthetic plasmids and mRNAs, enabling artificial control of transposon DNA expression [187] Such transposases are highly specific [188], ensuring to some extent stability and safety after transfer.

The Sleeping Beauty (SB) transposon was originally derived from an inactive copy of a DNA transposon of the Tc1/mariner superfamily [189] and has TIRs of approximately 230 bp at both ends, with internal substructural domains including a nuclear localization sequence, DNA-binding structural domain, and catalytic structural domain [190]. SB is now widely used in ACT, and its powerful transduction ability enables the transduction of long sequences of up to 6000 bp into mammalian cells for double-stranded cleavage and insertion. The integration profile of SB in mammalian genomes has been shown to be near-random in clinical trials; this reduces the risk of insertion mutagenesis [191–193]. SB has been shown to be an effective and safe approach for introduction of specific receptor genes into human T cells [194–196]. In CAR-T cell therapies, in particular, its applications are relatively mature, and it can be used to introduce transgenic CAR particles. A recent study combined CRISPR/Cas9 technology with knockdown disruption of alloreactive TCRs and subsequently reduced homozygous reactivity, thereby providing an alternative means of inactivating donor TCRs as a universal source of T cells. CD19 CARs were imported using SB and maintained stable and potent expression [197]. There have also been initial attempts to conduct a quantitative production process for CAR-T based on SB, illustrating that the efficiency of transposon systems for widespread production can be increased [198]. These valuable applications also contain lessons for further applications of SB in TCR-T cells.

The PiggyBac (PB) transposon system uses a transposon originally from insects, which is 2475 bp in length and contains one transposase-encoding open reading frame, encoding dimerization, DNA-binding domains, a catalytic domain, an insertion domain, one N-terminal domain, and one cysteine-rich C-terminal domain [199–204]. PB has been demonstrated to be active in in vitro assays and in yeast, mouse, and humans [202, 205]. The integrated PB tends to be inserted into a TTAA sequence, which is subsequently replicated and inserted into the transposon flanking sequence [206]. High efficiency has been achieved for gene transfer of PB as a vector in CD19-CAR-T cells [207]. PB is also available as an alternative to the non-viral vector approach for TCR-T gene introduction [208]. A recent study demonstrated the presence in the human genome of a homologous protein, PGBD5, derived from domesticated PB transposons [209], suggesting possible cross-reactivity between the PB endogenous human transposon and a risk of cross-reactivity of exogenous sequences. The applications of PB and the related clinical progress in CAR-T cell therapies for solid malignancies in recent years are worthy of note [210].

Tol2, the only transposon identified from vertebrates with autonomous transposition activity, contains incomplete TIRs of 17 bp and 19 bp and three subterminal repeats of ~ 30 bp near the right TIR and is capable of sustained transgene expression after gene delivery. In contrast to that of other transposon systems, the transgene efficiency of Tol2 is well stabilized in mouse strains and human systems and is not affected by endogenous factors such as gene silencing mechanisms in mammalian hosts [211–215].

The transposon types of listed above possess relatively low immunogenicity and a small genomic footprint; however, the gene transfer efficiency of various transposons varies from target cells, and one of their distinctive features is that their efficiency is negatively correlated with the size of the transposon expression cassette. In the context of proven efficacy and safety in ACT, large-scale transposon transfection with guaranteed efficiency is key to dissemination; however, the negative aspects of the randomized nature of the integrated genome should not be overlooked [195, 216].

Gene editing technologies represented by CRISPR/Cas9

Gene editing with CRISPR and the Cas9 endonuclease is emerging as a precise means of introducing exogenous TCR genes, enabling flexible reprogramming of TCR-T cell signaling.

Early milestones in gene editing were achieved using short interfering RNAs, ZEN, and TALENs to silence PD-1 or endogenous TCRs, or to create antigen-specific artificial T cells [217–220]. The superiority of CRISPR in precisely targeting multiple genes at different locations using guide RNA sequences allows for simpler preparation and target library expansion [221]; however, there is a relatively high risk of genetic modification toxicity and off-targeting according to clinical reports of ACT therapies [222]. Such gene-editing systems can be introduced into target cells using lentiviral or AAV vectors. Then, single-guide RNAs are used to fine-tune the DNA sequence target, and Cas9 proteins cleave the DNA to produce double-strand breaks (DSBs), followed by classical autonomous repair of host DNA by non-homologous end joining (NHEJ) and homology-directed repair (HDR) guided by the HDR template (HDRT) [223]. NHEJ, which usually involves a direct joining of DNA break ends, can be used for T cell knockout (KO); however, this simple repair process can lead to the introduction of new

insertion mutations and interference with gene function [224]. Instead, targeted T cell KI by HDR can be achieved through design and addition of exogenous HDRTs. The current preference is for the transfer of the HDRT into cells to be accomplished using single-stranded DNA donors and virus-independent gene electroporation, as this method has the advantages of ease of production, low cytotoxicity, and safety [225, 226]. However, the effective acquisition rate of HDR CRISPR individual gene KIs reported by Roth et al. was not high, and the harvesting of KI homozygotes in KI double strands was challenging [226]. Several protocols have been developed to improve HDR KI efficiency, including tuning of the parameters of the T cell introduction program and optimization of the HDRT design to improve nuclear translocation efficiency and KI efficiency [225]. Another study proposed a new way to improve the efficiency of HDR KI by intervening in the choice of repair mode after targeted editing and inhibiting the bypass of NHEJ repair by adding small-molecule interfering agents [227]. However, the T cell status and corresponding editing procedures need to be closely monitored and adjusted during the process of T cell editing using CRISPR/Cas9, so that the relative efficiency can be improved and optimal transformation and survival can be achieved [228, 229]. Finally, the composition, functional integrity, and genetic information of the product should be confirmed and verified.

The main advantage of using CRISPR/Cas9 in ACT is that it enables precise editing of multiple loci simultaneously [230]. This allows for the precise introduction of engineered receptor genes and simultaneous KO of endogenous-related genes, such as endogenous TCR genes and T cell suppressor genes [23], resulting in streamlined production. Knocking tumor-specific receptors into the endogenous TCR constant motifs TRAC and TRBC by CRISPR/Cas9 has been shown to effectively improve the killing activity of gene-edited T cells [231]. The effectiveness of the obtained CAR-T [232] and TCR-T cells for the treatment of hematologic malignancies, some melanomas, and solid tumors such as synovial sarcoma has also been preliminarily demonstrated in mouse models [233, 234] and human clinical trials [235–237].

Stadtmauer et al. reported the first human phase I clinical trial of CRISPR-engineered TCR-T cells. Using CRISPR/Cas9 for multiple gene editing of T cells intended for therapeutic applications in three patients with refractory solid tumors, they introduced a specific artificial TCR NY-ESO-1, which simultaneously knocked out both the endogenous TRAC and TRBC genes to reduce mismatches, as well as supplementally knocking out the third gene, PD-1, to comprehensively enhance the anti-tumor activity of the T cells. Their study proved the in vivo feasibility of CRISPR gene editing for TCR modification in the clinic and the lasting nature of the modifications, and it has already been approved by the regulatory authorities as the first human safety study [238]. Subsequent phase I clinical trials have likewise confirmed the efficacy and promising long-term functionality of NY-ESO-1-specific TCR-T cells for refractory synovial sarcoma [45]. Recently, Foy et al., using a non-viral CRISPR/Cas9 editing approach, knocked out both the TRAC and TRBC genes in one single step and inserted two strands of a neo-TCR derived from a patient PBL into TRAC loci. In a phase I trial (NCT03970382) in 16 patients with refractory tumors, treatment was successful: five patients remained stable in terms of disease progression, whereas the remaining 11 showed a good response to the therapy [23]. Parallel knockdown of in situ TCRs is now routine practice, and the benefits of this are discussed in detail later. Notably, Stenger et al. in a summary study of various ACT editing methods, found that retention of endogenous TCRs resulted in significant improvement in T cell persistence compared with endogenous TCR retention when using TCR-KO-anti-CD19 CAR-T cells for the treatment of human patients with CD19 + leukemia [239, 240].

To reduce the risk of uncontrolled proliferation and toxicity of ACT cells, a number of strategies have been developed to set a start switch for small-molecule drugs such as rituximab or sirolimus [241, 242]. CRISPR/Cas9-mediated introduction of suicide genes has likewise been used to achieve switch-off of the functions of engineered T cells [243], ensuring their controllability and subsequent use in clinical applications.

More recently, studies have aimed to increase engineered T cell yields and enable scaled-up T cell editing by combining the newer homology-independent targeted insertion approach to DNA repair with the CRISPR/Cas9 system [244]. High-fidelity Cas9 proteins showing superior efficacy, precision and a shorter period of editing activity, in many Cas9 enzyme systems could create more favorable conditions for optimizing Cas9 in the clinical design of future engineered T cell products [245, 246]. In addition to NHEJ- and HDR-mediated CRISPR/Cas9 modifications, DSB and HDRT-free base editing are rapidly evolving. Base editing has been shown in preclinical studies to reduce the off-target and chromosomal translocation risks associated with earlier methods without inducing DSBs [247]. Webber et al. also achieved multiple base editing of the T cell genome using mRNA electroporation, with high efficiency in simultaneous editing of multiple T cell loci [248]. However, there is a risk of complex genomic alterations or rearrangements potentially resulting from lentiviral transduction or DNA-based delivery of CRISPR/Cas9 systems [249]. By

contrast, Cas9 ribonucleoprotein delivery systems for T cells have advantages including greater editing efficiency and less toxicity [250, 251].

Applications and constraints of TCR-T cells

Identification and applications of TCR-T cells

Evaluation of TCR affinities and recognizable epitopes

Molecular bioinformatics pre-assessment is used to elucidate the affinity process of target TCRs and clarify kinetic and cytological parameters, followed by a combination of computational scanning and experimental techniques to perform ultra-high-scale screening and save time in preclinical studies. The use of a computer-based in silico approach allows for the pre-modeling of large-scale libraries of TCR-recognizable epitopes, although specific p-MHC binding remains difficult to predict. The computational approach can also incorporate sequencing information for the construction of core sequence libraries [252].

Affinity prediction can be based on a deep-learning approach that predicts the immunogenicity of peptides and determines key residues for T cell recognition, as well as simulating the physicochemical properties and immunogenicity that define the corresponding real-world conditions [253]. Enhancing the recognition of tumor antigens by increasing TCR affinity has been of clinical interest [254]. High-affinity TCR-modified T cells can detect lower levels of tumor antigens, do not rely on the adjuvant role of CD8 co-receptors, and can produce MHC-1-restricted CD4 T cells to secrete positive cytokines and promote immune initiation [255–257], contributing to the tumor-suppressive milieu and the diversification of TSAs. However, there is a concomitant risk of high-affinity TCRs recognizing and attacking normal tissues.

Targeted TCR affinity sorting and affinity maturation based on the binding of a given antigen are usually performed using yeast display and phage display technologies; these, combined with the soluble tetrameric, dimeric, and monomeric p-MHC ligands, can be used in high throughput to screen natural high-affinity TCRs, identify libraries of TCR mutants with modified affinity, or validate the affinity of TCRs for specific antigens [167, 258–261]. A library of stably expressed TCR sentinel mutations can be obtained, and soluble p-MHC ligands can be prepared using high-throughput sorting techniques. However, these methods are unable to regulate and predict the complex specific binding or cross-reactivity of antigenic peptides [262]. In addition, the protein expression and modification capabilities of the cells used to display the libraries remain limited [258], potentially causing distortion of antigenic peptides.

The effective activation of p-MHC cannot be characterized based on TCR binding alone. It has been consistently found that high-affinity TCRs strongly bound to p-MHC do not elicit agonist-stimulated interactions [263]. Using a p-MHC yeast library and soluble TCRs to identify collected inactivating TCRs, the "catch bonds" at the TCR–pMHC binding interface have been defined using molecular dynamics simulations of the TCR–pMHC binding interface, and the formation and persistence time (lifetime) of this force have been shown to be was positively correlated with the functional potency of TCR–pMHC-associated interactions [264–266]. Follow-up studies demonstrated that this positive effect results from mechanical-force-induced conformational changes in p-MHC that enhance pre-existing contacts and activate new interactions at the TCR–pMHC binding interface to resist force-induced bond dissociation, leading to formation of TCR–pMHC catch bonds and activation of T cells; moreover, the balance of such conformational changes is correlated with the isoforms of HLA molecules [267]. Such force bonds have been used to assist in the identification of TCRs with strong activity and high specificity for specific HIV-Pol and MAGE-A3 antigens, enabling refinement of the design and functional screening of TCR libraries for structural characterization [268]. The opposite "slip bonds" are thought to reverse the force action of TCR and p-MHC interaction and down-regulate the bond lifetimes; their rupture under external forces leads to inactivation of homologous TCR recognition of specific peptides [264]. Further, in biophysics, the cell surface TCR is understood as a multi-module mechanosensor that is force-sensitive to the recognition module of a moving p-MHC, where dynamic recognition of the bond is instantly transmitted to the non-directly covalently associated TCR signaling module. Physical alterations, such as molecular deformation, enhance or attenuate this non-covalent binding and prolong the bond lifespan, and the recognition of an antigenic peptide by a TCR is understood to be the result of the bond's immunogenicity. Immunogenicity of TCR-recognized antigenic peptides is understood as an alteration of the bond [269]. However, these two bond-based explanations for the diverse affinity differences in the ability of TCRs to turn on, hold, and turn off the force of p-MHC recognition remain complex and open to refinement. Recent findings suggest that in non-cellular molecular experiments, low-affinity TCR–p-MHC pairs with faster solution off-rates have external force insensitivities that are more resistant to mechanical forces (weak sliding or capture bonds); i.e., low-affinity TCRs improve their retention of recognition for antigens [270]. In addition, covalent TCR-pMHC interactions such as disulfide bond formation can occur secondarily, enhancing the

interaction and activating TCR ligand-signaling T cells with limited affinity [271]. Therefore, current methods for screening TCRs may lead to unanticipated deviations between predicted functions and the natural state, and the real impact of their affinity on T cell actions needs to be further verified and corrected.

TCR affinity and activity are not always correlated; TCRs with high antigenic affinity (1–5 μM) tend to exhibit high activity in vitro, and TCRs with low to medium affinity (5–100 μM) usually show poor correlation between affinity and activity [264]. Therefore, the actual responsiveness of TCRs to the target p-MHC still needs to be assessed. A recent paper proposed using DNA origami technology for exploration of the complex problem of T cell sensitivity to p-MHC, for instance, in the case of TCRs with medium/low affinity, those present in small amounts, or even individual p-MHC agonists. This platform would enable precise intermolecular nanoscale microscopic distances to be determined on simulated APC membranes to achieve quantitative control and localization of TCR populations on the surface of individual T cells, as well as bio-interfacial mimicry for recognition and binding of p-MHCs [272].

Adverse event reporting

In clinical trials, artificial TCR-T cells have shown unanticipated post-administration cross-reactivity in humans, with fatal effects in some cases. Objective responses were observed in one-third of cases in a clinical trial of TCR-engineered T cells targeting CEA, with all three patients who received the drug experiencing severe post-treatment transient colitis as a side-effect [90]. Two early concurrent independent clinical trials of engineered high-affinity TCR-T cells targeting MAGE-A3 showed considerable neurological and cardiac off-target toxicity. One of these, using T cells with CD3 regionally directed mutagenesis of mouse-derived TCRs, showed neurotoxicity along with complete remission of clinical outcome in five of nine patients, and two cases of post-treatment brain death, the cause of which was later shown to be recognition of normally expressed MAGE A12 in the brain by the MAGE A3 high-affinity TCR [52]. Another experiment was terminated prematurely after participants suffered cardiogenic shock and there were two post-treatment deaths [51]. Subsequently, the affinity-modified TCR-T with an artificially engineered mutation in the CDR2 region produced cross-reactivity against titin in normal cardiomyocytes, resulting in cardiotoxicity [273]. Notably, these proteins exhibit a high degree of homology with the MAGE family. These two reports provide warnings, as well as ideas for subsequent target prediction development and cross-reactivity assays. Detection of TCR and peptide binding alone is not sufficient; detection of T cell activation can provide a more accurate (and more intuitive) assessment of TCR specificity and cross-reactivity. Negative selection of TCRs for specific peptides does not exclude their effects with homologous mutant peptides.

Rehearsing and ruling out off-target effects

When TAA-targeting TCRs are used to target tumors, TCR affinity above a certain threshold will recognize target cells and initiate cross-talk signaling owing to low levels of expression of these antigens or cognate antigens in normal tissues. Therefore, the actual TCR affinity must be controlled to remain below that threshold and tested for its strength, to avoid undermining the required tumor selectivity. When TCRs are evaluated, the sequence identifying the target antigen or specific epitope is known, and epitopes are initially mutated and evaluated using alanine scanning or full amino acid scanning for amino acid point mutations at each position outside the anchored position, which usually remains a 9-mer or 10-mer conserved core motif [273, 274].

Furthermore, human endogenous peptide databases can be scanned using epitope-wide all-amino-acid point mutations to screen for existing self-TCR-recognizing epitopes and potential cross-reactivities [275]. However, the relatively conservative alternative constructs result in antigenic display libraries of limited practical size, which may not be sufficient for the required exclusion. Further, there are artificial methods of screening for possible TCR reactions using yeast libraries [109, 276] or combinatorial peptide libraries [277], and extensive computerized search rules through combinatorial peptide libraries have been established and evaluated [278]. TCR interactions can be tested against self-tissue libraries to exclude possible off-target side-reactive antigens, and TCR fingerprints are confirmed by TCR reactivity to antigenic libraries corresponding to TCR sequence specificity [279]. TCR fingerprinting can be used to identify potential target epitopes for the presentation of specific MHC-1-like molecules, or in reverse to screen for the best-reacting TCRs for specific antigens. Research has confirmed the uniqueness of TCR fingerprints, which use the characteristic pMHC motifs of each individual TCR as intrinsic features of TCRs [279, 280]; these fingerprints can be used for fine differentiation and prioritization in the search for TCR clusters that recognize the same pMHC clusters. The universal T-SCAN platform for positive screening for tumor epitopes can likewise be used for cellular presentation and exclusion of self-reactive antigens at higher throughputs, reducing the risk of cross-reactivity in the context of large self-antigen

libraries [110]. Recently, bioinformatics analysis of protein sequence spatial landscapes has allowed for position-specific amino acid preferences to be complemented by conformation in the TCR–pMHC-bound state, accelerating the detection of potential homologous protein cross-reactivity trends [252]. Experimental identification and estimation in silico of off-target reactivity of TCRs against specific tumor antigens is usually performed on a large scale using MS-based immunopeptidomics [83, 281].

Multiple characterizations of TCR-T cells

Immunological studies titrate homologous peptide antigens to estimate the affinity of TCR-T cells and assess their relationship with peptide concentration. Cytological studies are conducted to measure the response of "target immune cells" to a set of HLA-typing-matched progenitor cells in co-culture and to detect the activity of T cells (such as proliferation, cytotoxicity, and release of cytokines) in response, as described in the previous section. A new method uses peptide library APCs to express anti-cytokine antibodies and identify defined HLA molecules and peptide epitopes recognized by orphan TCRs by detecting the secretion of specific cytokines IL-2 and IFN-γ from T cells, as well as converting binding interactions into universal signals for different types of HLA molecules (not limited to CD4 and CD8 T cells) to identify peptide libraries to be detected on a scale of thousands of peptide oligonucleotide libraries; this scaling up has a lower cost compared with ELISPot and ICS [149]. Cells and cell lines to be tested should include tumor cells, autologous cells, and HLA-diverse lymphocytes for in vitro validation of tumor killing activity, autologous reactivity, and allogeneic reactivity of engineered TCR-T cells [282] (Fig. 2B). Histologically, cells are cultured in standard cultures or organoid cultures using two-dimensional (2D) or three-dimensional (3D) materials, and tissue cell phenotypes are characterized by immunohistochemistry to detect T cell reactivity. The use of 2D and 3D materials in vitro to mimic organoid environments can contribute to a better understanding of the cell-to-cell actions of TCR-T cells. Joseph et al. cultured cardiomyocytes, astrocytes, and endothelial cells, as well as terminally differentiated human cells derived from induced pluripotent stem cells, as normal cells to observe T cell reactivity, and an immortalized B-cell line, B-LCLs, to characterize the alloantibody response. They also used 2D and 3D materials to observe the killing effect of TCR-T cells on microtissues [274]. To ensure autoantigen coverage, human cells can be subjected to specific events to expose certain potential epitopes, for instance, treatment with IFN-γ to induce immunoproteasomes and replication of immune peptides exposed during inflammatory events [274].

Detection of antitumor activity in vivo begins with a mouse xenograft model of tumor cells, which should be constructed and used according to the Scientific Procedural Approach to Animal Testing, with the regulatory authorities FDA and EMA supporting the 3R principle [283]. Construction of an immunodeficient xenograft mouse model can be used to confirm TCR-engineered human T cell efficacy; however, toxicity assessment in this model is limited by the lack of HLA molecules in the host and the poor persistence of human T cells in mice [284]. Reliable in vivo models to assess TCR cross-reactivity and allogeneic reactivity are still being developed. The T2EVOLVE Consortium, a public–private partnership, describes currently available preclinical models, tools, and specifications for TCR-T cells and their clinical safety and efficacy [285]. Innovative animal models for TCR-T cells have been created that are more in line with desired characteristics, such as the humanized SGM3 model used to more sensitively discriminate subtle/nuanced differences between ACT cells produced from different starting cell sources [9]. Studies of antigen-specific TCR-T in patients with solid tumors have been carried out in multiple phases of clinical trials, and lymphocyte clearance of drugs such as cyclophosphamide and fludarabine is usually applied earlier in clinical trials to achieve better T cell implantation, proliferation, and persistence [286, 287]. However, lymphocyte depletion predictably increases the incidence of several hematologic toxicities (neutropenia, anemia, and thrombocytopenia) and infectious complications, with consequent increases in the incidence and severity of cytokine release syndrome (CRS) and, in some cases, tumor lysis syndrome [288]. The solid tumor response of patients can be documented according to the Response Evaluation Criteria in Solid Tumors (RECIST ver. 1.1) [289]. Some modified and proposed versions such as immune-related RECIST (irRECIST) [290] and immune RECIST (iRECIST) [291], have been not standardized in evaluation of TCR-T clinical trials.

Existing constraints and approvals

TCR α/β chain mispairing

Endogenous TCR-encoded TCR α/β chains continue to be expressed intact [292]. As endogenous and transgenic TCRs are simultaneously expressed, they may heterodimerize to generate four distinct TCRs and persist in the circulatory system. Simply introducing exogenous TCR genes upstream of the host expression set and additionally increasing TRAC and TRBC expression will result in the coexistence of endogenous TCRs, exogenous TCRs, and heterodimerization with endogenous and transgenic

TCRs [292] (Fig. 3). TCR heterodimers, owing to their unselected and unscreened thymus, may be of unknown immunogenicity and auto-antigenic reactivity, and the infusion may trigger an auto-crossing immune reaction. This reaction may even be fatal, for instance, graft-versus-host disease (GVHD) demonstrated in mouse models [293, 294], although GVHD-like toxicity has not been documented in patients receiving TCR-T products to date [295, 296]. In addition, coexistence of multiple TCRs can lead to competition for limited CD3 downstream molecules and co-stimulatory signals, reducing the efficiency of the target TCR.

Treatment for such mismatches has been systematically established to reduce the structural similarity between exogenous and endogenous TCR single chains and increase the structural specificity of the engineered α/β chains to match. The first method for modification of exogenous TCRs involved modification of the extracellular constant region. For example, the residues in the constant region of human TCR were partially or completely replaced with the constant region of mouse TCR. A human immune response against the TCR region from mice does not impair the efficacy of T cell therapy or increase the body's additional response. Mouse TCR constant region replacement may enhance the safety and functionality of TCRs [86]; however, further observation of its use in TCR-T cell therapy is required, as the murine modification has been found to affect the anti-CD19 CAR effect in clinical tests [297]. Modification of TCR residues has also been achieved by the introduction of two complementary cysteine residues, adding additional pairing of the engineered TCR α and β chain disulfide bonds [298], by replacing the TCR α chain TM region with hydrophobic residues to stabilize its expression [299], by co-expressing a TCR single chain fused to CD3ε [300], or by using structural domain inversion or swapping of the constant domain of TCR double strands to minimize mismatches [301–303].

KO or silencing of endogenous TCRs can also be considered as a means of reducing the incidence of mismatches. This can be assisted by additional modules such as RNA interference [304] or KO of endogenous TCR loci with CRISPR/Cas9, which is a more precise method and an easier one to use. KI of the target TCRs at the location of the original locus saves processing and effectively achieves the engineered TCR dimer pairings; this approach has already been tested in clinical trials, as described in the previous section.

Adverse reactions

Owing to low levels of TAA expression in normal tissues, introduction of exogenous TCR-T cells may cause cross-reactivity elsewhere in the body. For example, severe events were reported in the two MAGE A3 TCR-T cell clinical trials, where the homologous antigen MAGE A1 was expressed in ocular or cutaneous melanocytes. In addition, in clinical trials of TCR-T cell therapies targeting MART 1, patients treated with the therapy developed unanticipated ocular uveitis and hearing loss [35–37]. Significant adverse events on MAGE-A homologous antigens emphasize the exclusion of potentially reactive antigens of candidate TCRs within the whole space-wide proteome and the elimination of autoimmune cross-reactivity. TCRs should recognize peptide with an identical epitope sequence, furthermore, may recognize structurally similar peptide-HLA class I complexes despite differences in peptide sequences. Some means of removing excess T-cell toxicity, such as inducible suicide gene introduction [243], are also worthy of consideration.

CRS is the most common and serious immune-related adverse event encountered in recent clinical trials of engineered T cells and mostly occurs within 14 days of ACT infusion [36]. Symptoms of CRS range from mild fever to life-threatening symptoms and multiple organ system failure, and include headache, encephalopathy, tremors, and seizures. CRS is currently thought to be associated with T cells or to involve immune cell activation and secretion and tumor cell lysis, which is related to the variability in levels of antigen expression among patients. Moreover, immune effector cell-associated neurotoxicity syndrome, often referred to as neurotoxicity, is very common, especially in patients receiving CD19 CAR-T cells [305].

Although CRS in ACT is strongly associated with elevated levels of ILs such as IL-2, IL-6, IL-5, IL-8, IL-10, and of TNF-α, and some of these cytokines have synergistic effects on T cell activity and lifespan, the IL-6 receptor antibody tocilizumab is currently considered to be a suitable option for drug control [306]. Attempts to improve transgenic T cells are also underway, and it has been found that spatial site-blocking effects can be attenuated over time; in situ polyethylene glycol affixed to the surface of CAR-T cells slows monocyte activation and effectively attenuates CRS symptoms and neurotoxicity [307].

Limited therapeutic effect

One of the main features of refractoriness in solid tumors is the easily acquired T cell resistance of the tumor tissue and mesenchyme, the causes of which may be primary or secondary. However, the impairment of T cells is regarded as the result of a complex network of negative signaling interactions.

Primary resistance arises from tumor antigenic characteristics, including antigenic expression drift,

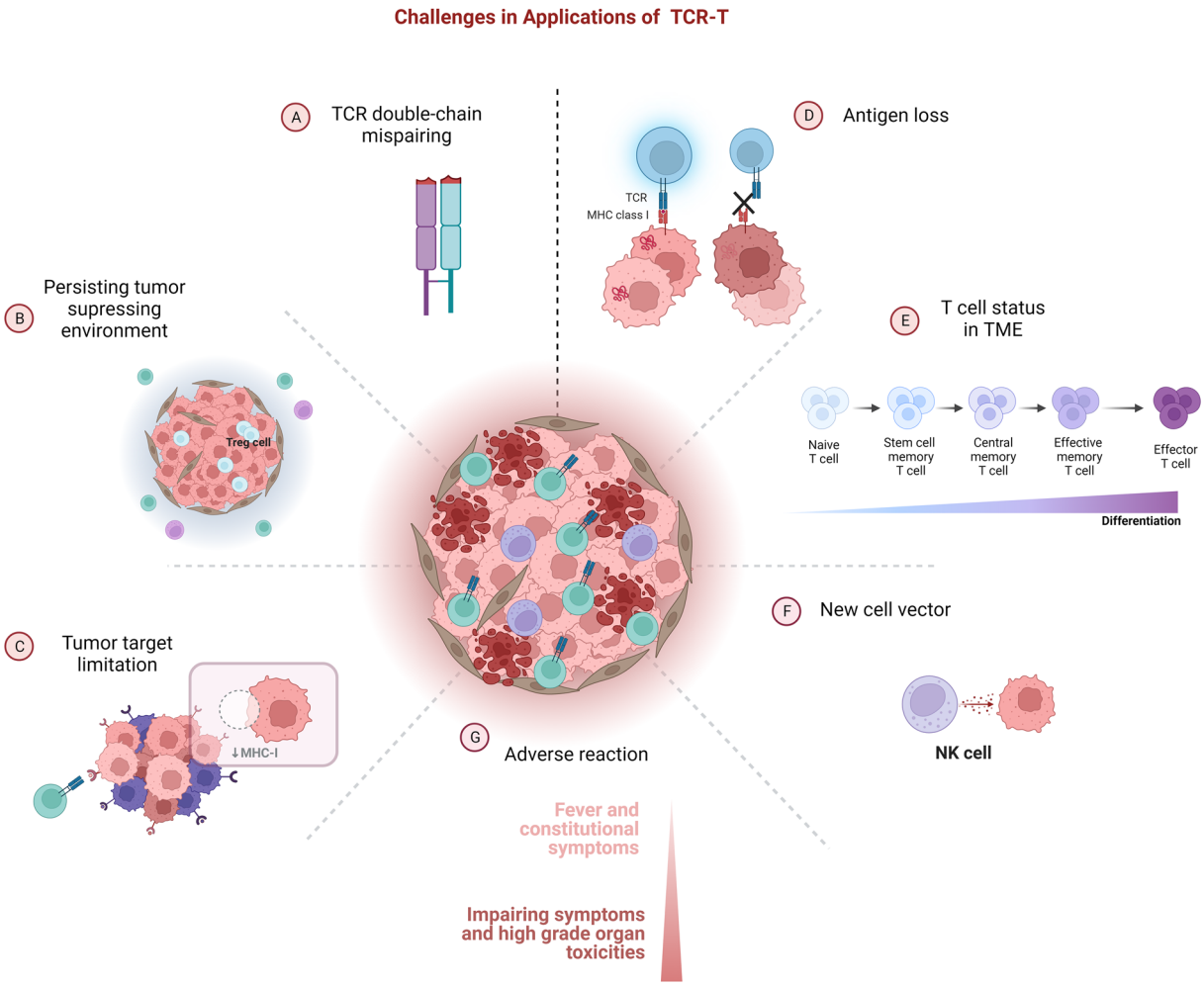

Fig. 3 Challenges in applications of TCR-T immunotherapy for tumors. Applications of TCR-Ts in tumor immunotherapy still have some limitations and potential improvements: **A** Mispairing of introduced TCRs and endogenous TCRs may occur on TCR-Ts. **B** Multiple factors contribute to an immunosuppressive TME. **C** Antigenic heterogeneity of solid tumor tissues and decreased MHC-I molecules pose obstacles to TCR-T recognition. **D** Antigenic drift and loss in tumor cells occur during solid tumor development. **E** T cells are heterogeneous in different states of differentiation, with respect to characteristics such as activation capacity and lifespan. **F** Applications of natural killer (NK) cells or natural killer T cells as vector bring new advantages and possibilities. **G** TCR-Ts have demonstrated numerous adverse effects in clinical trials, most commonly cytokine release syndrome

heterogeneity, or low antigenic expression [308], especially in cancers carrying high mutational loads. The genetic heterogeneity in the targeting of TCRs to some limited neoantigen also leads to functional limitations of TCR-T cells that accompany this heterogeneity, analogous to the inactivation of ICIs [309], and the tumor epigenetic heterogeneity of patients also generates antigenic variants and generates new TCR tolerance [310–312]. Thus, the need for shared antigenicity has been emphasized during TCR development, in order to provide deep coverage of tumor tissues in both spatial and temporal dimensions, better selection of early key antigens with less susceptibility to antigenic drift is needed, as well as an emphasis on achieving broadness in the individualization of the antigenic screening process, in a single dose or in phases, by administering therapies targeting multiple target antigens.

Secondary resistance is triggered extrinsically by the tumor after T cells have been equipped with a specific TCR library. Arising from the evolved immune escape of tumor cells, it resembles the natural pathway of immunosuppression of T cells by the TME and features a variety of molecular mechanisms. Its most direct manifestation is the downregulation or loss of MHC-1-like molecules and altered expression of corresponding immune factors.

Downregulation of MHC-1-like molecules prevents the effective presentation of target antigens, or the suppression of co-activating signals and activation of co-suppressive signals. Impairment of natural T cell function also occurs via restriction of dendritic cell (DC) migration towards the draining lymph nodes, or through inhibition of the antigen cross-presentation process in paracrine cells [313, 314], which may also interfere with the sustained activation state of the engineered T cells. A recent study identified a new axis of T cell killing action in tumor cells escaping from MHC1 downregulation [315]—natural killer group 2D ligand (NKG2DL) and T cell NKG2D interaction. During classical tumor escape by downregulation of MHC molecules and antigens, CD8 T cells maintain their killing capacity [315], suggesting that it is beneficial to maintain or enhance the original natural killing pathways and new targets of T cells for the maintenance of TCR-T cell activity. Moreover, to enhance intracellular TCR signaling to improve T cell activity under existing conditions, simple general modifications in the variable region of the TCR have been found to increase levels of cell surface expression of the TCR. Three amino acid residue substitutions in the framework of the variable structural domain of the TCR effectively enhanced TCR recognition ability and TCR-mediated proliferation and secretion of killer cytokines from T cells [316].

T cell exhaustion

Clinical and infusion of TCR-T cells has demonstrated that these engineered T cells can generate a memory phenotype and maintain long-term survival in vivo [317, 318]. However, T cell depletion, defined by a progressive decline in T cell function due to the presence of the TME, continued exposure to tumor antigens, and TCR stimulation, may lead to transition to a state of terminal depletion [319], characterized by TCR signaling co-suppressor receptors such as classical PD-1, CTLA4, and LAG3 [320]; active immunosuppressive enzymes such as CD39 [321, 322]; or the expression of intracellular negative factors that downregulate the intracellular cascade of responses in which the TCR is involved [323, 324].

Even when an effective TCR is deployed, the short persistence of activated T cells will result in reduced therapeutic efficacy, especially in the face of compromised effector function and inhibitory receptor expression of T cells owing to the TME [325]. CD8 T cell dysfunction can be characterized by inability to secrete IL-2, loss of proliferative capacity, and inability to secrete TNF-a and interferon; these functional impairments are incremental and are accompanied by increased expression of inhibitory receptors [326] and decreased numbers of CD4 T cells, which have been shown to inhibit CTL depletion [327].

Regarding the natural exhaustion and escape that occurs in endogenous T cells in the TME, tissue PD-1 upregulation inhibits the function of T cells and induces them toward terminal differentiation. Moreover, modified T cells from TME tend to perform poorly in terms of efficacy [328].

There are a range of optimization options to extend the effective time of TCR-T cells, like removing dysfunctional cells from the circulation to provide opportunities for preferential and stable proliferation of effector and memory T cells. Optimization of the metabolic environment, where hypoxia and mitochondrial dysfunction have been found to be associated with T cell depletion. Glycolytic metabolism, mitochondrial respiration, and metabolites can decisively influence the development and function of T cell populations, including CD8 T cells and regulatory T (Treg) cells [329, 330]. The acidic, hypoxic, nutrient-uptake environment created by the TME in solid malignancies is detrimental to the metabolism of effector T cells. The TME affects key enzymes in glycolysis and respiration, decreasing the glycolytic capacity of infiltrating T cells and leading to their dysfunction. Improved metabolic reprogramming strategies for CD8 T cells could improve the effectiveness ACT [331, 332]. Upregulation of glucose transporters and amino acid transporters in engineered T cells improve the metabolic capacity and subsequent activity of ACT through elevated expression of certain key pro-metabolic factors, such as mTORC1, and cellular proliferation factors, such as c-Myc [333, 334]. Currently, researchers are attempting to improve the uptake rate and metabolic capacity of T cells competing for nutrients and thus their effects through the development of tumor-cell-selective inhibitors of glucose uptake and metabolism in the TME.

In addition, CD8 TILs in hypoglycemia and hypoxia can continue to be active through metabolic pathways that enhance peroxisome activation signaling and fatty acid catabolism, and promotion of fatty acid bypass metabolism has also been shown to enhance the tumor-suppressive ability of TILs [335]. Moreover, in highly glycolytic tumor subtypes, Treg cells were found to promote NFAT-1 translocation and upregulate surface PD-1 expression by actively transporting lactate in a low-glucose and high-lactate TME. Upon PD-1 inhibitor administration, the Treg cells strongly competed with CD8 T cells, enhancing TME inhibition and interfering with therapeutic efficacy [336]. Therefore, predicting glycolytic and lactate modulators of TME is likely to be instrumental in enhancing the adjuvant capacity of ACT activity. Some "preservative" drugs to reprogram the T-cell state are also being investigated, like the use of metformin to regulate CD8 T cell differentiation, which

elevated the conversion of T cells to a memory stem-like phenotype, and promoted cytotoxicity in vivo [337, 338].

Incorporation of cytokines has promotion of T cell growth factors, in addition to the benefits derived from lymphocyte pre-elimination, also has the effect of avoiding intrinsic immune cell competition for these cytokines [49]. Improvements have been achieved by antagonizing co-inhibitory signaling molecules such as PD-1 by co-infusion of signal blocking antibodies with engineered T cells [5, 339], or by modification of T cells expressing the PD-1 negative receptor; the anti-tumor efficacy of an IL-6 and PD-1 antibody blockade combination [340] and knockdown of the minus-regulatory protein site using precision editing with CRISPR/Cas9 also enhanced the anti-tumor function of transgenic T cells [341].

Carrier cell optimization

The expectation that a uniform allogeneic source of T cells will have advantages for production has led to calls for improved strategies to reduce the risk of GVHD; there has also been optimization of the primitive naïve state and differentiation capacity of the T cells to avoid early emergence of an end-stage T cell depletion state. Studies have also used patient-personalized solid tumor endogenous-derived T cells, such as tumor antigen-specific T cells, which have the potential to produce TCR-T cells [317]. The use of different types of T cell to construct TCR-T cell can maintain the anti-tumor effect and avoid adverse effects [342]. Natural killer (NK) T cells are especially interesting because they are non-allogeneic reactive. Current CAR-T strategies have included the use of NK T cells and other innate-like T lymphocytes "piggybacking" on engineered T cell receptors, an approach that is exempt from the limitations of HLA molecules and has the potential to directly target tumor cells with low-density antigens [343]. NK cells as recipient cells also naturally avoid mismatches triggered by endogenous TCR expression [344]. For surface modification of T cells, functional enhancements and on/off control of T cells have been achieved by introducing the concept of multiple antigen–antibody targeting axes, or multi-gated strategies. Co-stimulatory switch receptors prevent depletion of genetically engineered T cells and may increase their persistence [345–347]. Switch receptors consist of the extracellular portion of inhibitory receptors (e.g., PD-1, TIGIT, TIM-3) and the intracellular signaling domain of co-stimulatory receptors (e.g., CD28, 4-1BB). For example, targeting of low ACT homing in solid tumors, enhanced in vivo homing, and killing of antigen-specific CTLs by cell surface fucosylated CTLs have been demonstrated in a mouse model [348].

Prospects for combination therapies

Joint applications

The body's anti-tumor immune response is complex, multi-component, and not a simple series reaction. TCR-T cell therapies could be combined with other immunotherapies, including ICIs, cytokines, such as IFN-a, monoclonal antibodies targeting specific receptors, and tumor vaccines targeting modifications of tumor cells or APCs (Fig. 4A, B). Recent advances in the direction of anti-tumor nanomedicines, including targeting of ACT cells for prolongation of somatic circulation and inhibition of degradation, have created the possibility of advancing the widespread use of ACT therapy in cancer. Various options for improving the TME are available, and the TME improvements demonstrated in recent studies are applicable to enhancing the efficacy of engineered T cells.

ICIs

Although ineffective on their own for certain malignancy outcomes, the commonly used PD-1/PD-L1 inhibitors and anti-CTLA-4 antibodies [349], 350, in combination with ACT, show efficacy in the treatment of various solid malignancies, with each compensating for the deficiencies of the other. In addition, adenosinergic signaling has been found to be an important tumor immunometabolic checkpoint, and adenosine axis blockers have been shown to have promising anti-tumor activity in combination with ACT [351].

Anti-tumor cytokines

IL-2, IL-7, IL-15, and IL-21 have been shown to prolong the survival time of memory and naïve T cells in vitro and to favor T cell proliferation [334, 352, 353], but their differentiation-promoting effects on T cells may lead to depletion of subsequent products. IL-18, a member of the IL-1 family, is a pro-inflammatory cytokine capable of promoting a type I immune response and activating a variety of immune cell types, such as stimulating NK cells and promoting the transformation of Th1 cells [354]. The introduction of secreted biologically responsive mature IL18 to enhanced the anti-tumor capacity of Pmel-1-specific T cells infused into melanoma B16F10 hormonal mice to secrete IFN-γ and express CD25, and reduced the aggregation of immune-suppressor cells in the TME, effectively prolonging the survival rate of the mice. In a human melanoma xenograft model, additional transduction of NY-ESO-1-specific TCR-T cells expressing active IL18 resulted in a significant increase in the number of viable T cells in peripheral blood and inhibition of tumor progression [355]. The promotional effect of IL-18 on T

cell survival and tumor killing activity was again demonstrated in a recent CAR-T model of advanced refractory solid tumors and in anti-tumor-engineered T cells with increased transformation of the memory phenotype, reduced depletion, and maintenance of a more durable response [356]. Moreover, IL-18 did not cause relevant therapeutic toxicity in patients [357]. Thus, IL-18 should be available as an effective adjuvant anti-cancer factor in combination with TCR-T cell therapies, as it would have multiple benefits. In addition, fusion protein complexes combining IL-12, IL-15, and IL-18 signaling have been developed and validated in an in vivo model, which could promote memory-type differentiation of NK cells and improve their metabolism as well as enhancing anti-tumor cytotoxicity such as secretion of IFN-γ in the short term. Each of them shows a positive promotional effect on TCR-T cells [358], suggesting that such multi-cytokine complexes would be beneficial for TCR-T cell therapies. Given their respective positive promotional effects on TCR-T cells, these multi-cytokine complexes are also likely to have potential for the development and applications of effector enhancement and functional modulation of TCR-T cells.

Tumor vaccines

APC vaccines such as DC vaccines have been shown to enhance TCR-T expansion and tumor suppression following TCR-T cell vaccination; in a study that enrolled 14 HLA-A2.1 + patients with metastatic melanoma, signs of tumor regression were observed in 13 of 14 patients following co-vaccination using genetically modified MART-1 TCR-T cells made from autologous T cells and a MART-1 peptide-pulsed DC vaccine. A rapid expansion response of TCR-T cells was observed in vivo, suggesting that dual-cell therapy with concurrent vaccination with DC vaccine further enhances the in vivo expansion of TCR-T cells and exerts anti-tumor effects [36]. By contrast, in a multi-cohort study (n = 6, n = 4) that included 10 patients with advanced sarcoma or melanoma, autologous short-term preparations of NY-ESO-1 TCR-T cells were concomitantly over-transfected with a DC vaccine pulsed with NY-ESO-1 peptide, and signs of tumor regression were observed in two-thirds and one-half of the patients, respectively. However, this study also found that the addition of ipilimumab did not provide any greater clinical benefit [42].

Monocytes from tumor patients with the ability to be APCs were used in a trial to restore APC function using ascites monocyte Toll4 receptor 4 (TLR4) lipopolysaccharide and TLR9 CpG oligodeoxynucleotides. An antibody blocking the IL-10 receptor (IL -10R Ab) restored the function of APCs and could carry and stably conserve a wide range of TAAs, including MUC1, ERBB2, mesothelin, MAGE, PRAME, GPC3, PMEL and TP53. The antibody exhibited potential activation of T cells in vitro and long-term T cell memory effects [359].

Peptide vaccines based on TCR recognition-specific epitopes can also carry information that the target TCR recognizes as homologous immune peptide antigens. Previous studies have demonstrated the ability of such vaccines to reside and remain active in lymph nodes in the region of administration and to enhance the effects of endogenous T cells throughout the body, as well as the ability of the carriers to carry other drugs, such as anti-inflammatory factors [360]. The amphiphile (AMP) vaccine can be conjugated with equipped homologous TCR-T antigen peptides, and results are expected to be published soon. In in vitro assays, pulses of AMP-loaded melanoma antigen-gp100 peptide were co-incubated with mouse DC cells and pre-given homologous TCR-T. This vaccine increased TCR-T cell expression of CD25 and CD69 co-activation markers and secretion of IFN-γ, as well as increasing specific lysis of co-cultured tumor cells [361]. In an in vivo model of homozygous tumor-bearing mice that underwent AMP pre-inoculation of lymph nodes, numbers of TCR-T cells and paracrine immune cells, such as DC cells, in lymph nodes and their ability to secrete IFN-γ increased after TCR-T infusion. Further transcriptomic studies revealed that the AMP-homologous peptide vaccine increased the transcription of genes associated with T cell anti-cancer and immune activation, including the co-stimulatory molecules CD40 and CD86, inflammatory IL12β, IFNγ, and GZMB, as well as TAP1 and TAPBP, which are associated with antigen presentation. The vaccine did not enhance the transcription of immunomodulatory factors associated with T cell depletion or incapacitation, such as FoxP3, CTLA4, and Ceacam1 [361]. In addition, enhanced infiltration of overlying T cells and enhanced expression of reactive substances were found at the tumor site [362]. AMP-matched homologous peptides are easy to fabricate after the design of a TCR-targeting peptide; this series of studies suggests that the combination of an AMP peptide vaccine and relay cell therapy could improve therapeutic efficacy in solid tumors. Furthermore, in a phase 1 clinical trial in a cohort of patients with advanced soft tissue sarcoma, a pullulan nanogel (long peptide antigen) vaccine was used in combination with NY-ESO-1-specific T cells, and one significant tumor shrinkage was observed when all three patients with tumor shrinkage lasting longer than 2 years, none of whom underwent lymphatic clearance. This nanogel vaccine contains a TCR-T cell-recognized NY-ESO-1 epitope, and preclinical studies in an immunosuppressant-resistant mouse model confirmed that the vaccine significantly increased levels of TCR-T cells in draining lymph nodes and tumor tissue [45].

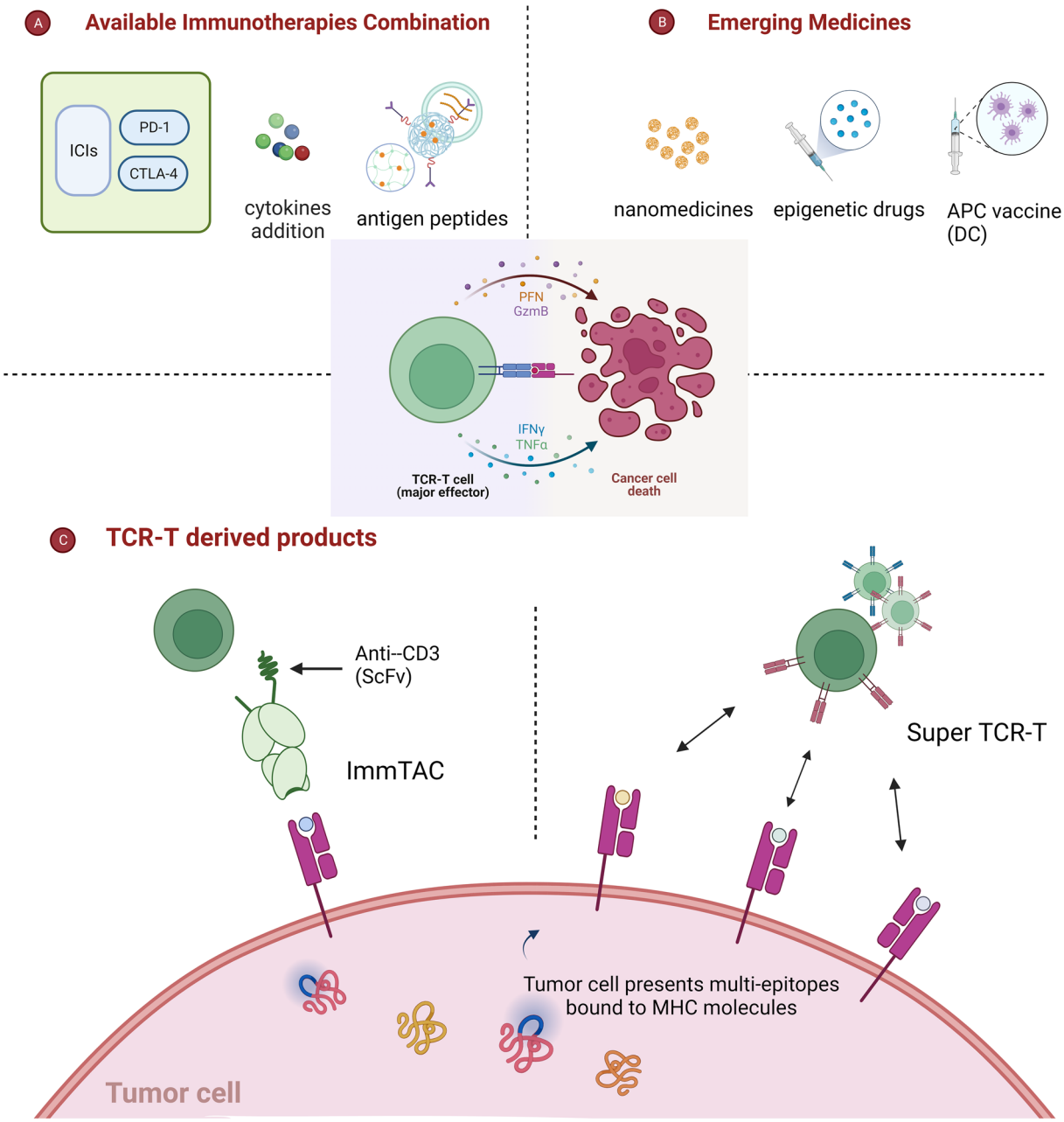

Fig. 4 The outlook for TCR-T development. **A** Amplifications of TCR-Ts in conjunction with the main existing immunotherapies to improve efficacy. **B** Amplifications of TCR-Ts in conjunction with some new drugs. **C** Some derivatives of TCR-T: immune-mobilizing monoclonal TCRs Against Cancer (ImmTACs) are not restricted by fixed HLA molecular typing; and Super TCR-T is able to recognize multiple antigenic epitopes of a tumor cell

mRNA vaccines can also cooperatively expand specific T cell clones and induce high-intensity T cell response. Besides, they can be sequenced from patient tumour tissues and present personal epitopes. One clinical trial showed the efficacy of the multi-neoantigen autogene cevumeran, individualized neoantigen encoding mRNA lipoplex in the immunotherapy on patients of pancreatic ductal adenocarcinoma. Personalized administration of this vaccine after surgical resection achieved vaccine-induced expansion of neoantigen-specific, long-lived polyfunctional effector T cells and the desired therapeutic effect was observed [363].

Epigenetic drugs

Significant upregulation of certain epigenetic factors, such as histone acetylase (HDAC), has been found in

various solid tumors, and HDAC inhibitors such as panobinostat show good anti-tumor effects [364]. Aesha et al. found that panobinostat, combined with human T cells transduced with an anti-Her2 CAR and a gp100-TCR, enhanced the transformation of gp100-directed T cells into a central memory phenotype while achieving effective clearance of human pancreatic cancer grafts in a mouse model [365].

Parental intermediaries

Addition of membrane amphiphilic markers targeting tumor cells effectively improves engineered T cell recognition and affinity, as a membrane-inserting ligand demonstrated in CAR-T cell trials on solid tumors in vivo [366]. This approach is independent of tumor antigen and tissue of origin and thus has broader applicability in TCR-T, it is based on adding additional common ligands to the heterogeneous population of tumor cells.

TCR-derived products

Based on engineered TCR-assisted T cell recognition of antigens, TCR or TCR-like therapeutics have been developed that do not require the production of follow-on T cells (Fig. 4C). This avoids the chimeric production and safety issues of genetically engineered modifications of T cells and facilitates production and specification uniformity for such therapies. TCR-mimetic monoclonal antibodies are monoclonal antibodies referencing the structure of the human TCR; they can trigger antibody-, cell-, and complement-mediated cytotoxicity and directly induce apoptosis [367–369]. The main mature products of TCR-derived drugs are immune-mobilizing monoclonal TCRs against cancer (ImmTACs), engineered reagents consisting of soluble specific monoclonal TCRs and anti-CD3 binding domains. ImmTACs are capable of homologous MHC complex recognition by high-affinity TCRs, relocalizing endogenous T cells to kill tumor cells and directing CD3 cross-linking to trigger activation of subsequent T cells. For example, in clinical trials against uveal melanoma, an ImmTAC, tebentafusp (also known as gp100), targeting shared antigens has shown promising results [370, 371].

In addition, the simultaneous application of multiple single-targeted TCR-T cells to counteract tumor antigen escape and infusion of patients with different single-antigen specific CAR-T or TCR-T cell mixtures are viable options. These approaches were recently used in patients with solid tumors, who received up to three new TCR-T cells of different specificities, with no evaluated effect [23]. Notably, multi-targeting of different antigens appears to be a viable strategy to overcome tumor antigen escape, and preliminary results suggest that it may enhance anti-tumor immunity against tumor cells co-expressing multiple antigens. However, this approach is limited by the number of known tumor antigens that can be applied, and the risks of multidrug combinations require consideration. A recent study found that a single TIL extracted from a patient whose advanced solid tumor had regressed after treatment with autologous TILs expressed one individual TCR capable of recognizing all three TAAs. This led to clinical cure for a prolonged period of time [372], indicating hope for the future with respect to the development of a monoclonal TCR-T cell capable of broadly recognizing multiple tumor epitopes. In addition, the ability of TCRs to recognize shared motifs such as x-x-x-A/G-I/L-G-I-x-x-x enables a wider range of effects of TCR-T cell therapies, with the expectation that super "multipronged" TCR-T cells with multiple epitopes targeted by individual engineered T cells will have superior ability to recognize and attack modalities and multiple refractory tumor types [372]. Current studies show promise of continual progress in this regard (e.g., NCT00937625).

Conclusions

The development of TCR-T cell therapies and the progress made in clinical trials have brought immunotherapy for refractory solid tumors to a new stage. Such therapies are constantly being updated and great advances have been made, including the prediction and screening of tumor neoantigens, assessment of off-target responses, and refinement and establishment of preclinical and clinical trial processes and protocols. Based on existing treatment protocols and clinical trial results, TCR-T cell therapy appears to have unique immunotherapeutic characteristics. Notably, combinations with other therapies, such as radiotherapy and chemotherapy, could improve T cell homing and proliferation, enhance T cell persistence, delay T cell depletion, improve the affinity of tumor peptides, and enhance the active effects. This could improve TCR-T cell oncology treatment and compensate for insufficiencies in previous immunotherapies. By providing more precise and powerful tools for use in the fight against malignant tumors, TCR-T cell therapies promise a brighter future for human tumor immunology.

Abbreviations

ACT	Adoptive cell transfer
APC	Antigen-presenting cell
CAR	Chimeric antigen receptor
CDR	Complementation determining region
CEA	Carcinoembryonic antigen

CGA	Cancer germline antigen
CRS	Cytokine release syndrome
DSB	Double-strand break
FDA	Food and Drug Administration
GVHD	Graft-versus-host disease
HDR	Homology-directed repair
HDRT	HDR template
ICI	Immune checkpoint inhibitor
IL	Interleukin
ImmTAC	Immune-mobilizing monoclonal TCR against cancer
KI	Knock-in
LC	Liquid chromatography
MS	Mass spectrometry
NGS	Next-generation sequencing
NHEJ	Non-homologous end joining
NK	Natural killer
PBL	Peripheral blood lymphocyte
PRAME	Preferentially expressed antigens of melanoma
RECIST	Response evaluation criteria in solid tumors
SB	Sleeping beauty
SNP	Single nucleotide polymorphism
TAA	Tumor-associated antigen
TCR	T cell receptor
TCGA	The Cancer Genome Atlas
TDA	Tissue differentiation antigen
TIL	Tumor-infiltrating lymphocyte
TIR	Terminal inverted repeat
TSA	Tumor-specific antigen
WES	Whole-exome sequencing

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