Published in final edited form as:

Immunol Rev. 2023 November ; 320(1): 58–82. doi:10.1111/imr.13233.

Challenges and solutions for Therapeutic TCR-based agents

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Summary

Recent development of methods to discover and engineer therapeutic TCRs or antibody mimics of TCRs, and to understand their immunology and pharmacology, lag two decades behind therapeutic antibodies. Yet we have every expectation that TCR-based agents will be similarly important contributors to the treatment of a variety of medical conditions, especially cancers. TCR engineered cells, soluble TCRs and their derivatives, TCR mimic antibodies and TCR-based CAR T-cells promise the possibility of highly specific drugs that can expand the scope of immunologic agents to recognize intracellular targets, including mutated proteins and undruggable transcription factors, not accessible by traditional antibodies. Hurdles exist regarding discovery, specificity, pharmacokinetics and best modality of use that will need to be overcome before the full potential of TCR-based agents is achieved. HLA restriction may limit each agent to patient subpopulations and off-target reactivities remain important barriers to widespread development and use of these new agents. In this review we discuss the unique opportunities for these new classes of drugs, describe their unique antigenic targets, compare them to traditional antibody therapeutics and CAR T-cells, and review the various obstacles that must be overcome before full application of these drugs can be realized.

Keywords

TCR gene therapy; Soluble TCR; TCR mimic mAb; HLA restriction; Off-target toxicity; CAR-T-cells

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1. Introduction

Methods to produce monoclonal antibodies (mAbs) were described first in the late 1970's, but the first mAbs for the treatment of cancer were not approved until two decades later. Currently, after 20 more years, mAbs represent an increasingly dominant part of the drug armamentarium for cancers, autoimmune disease, inflammatory processes, infections, and neurologic disorders, among others. Although antibodies and T-cell receptors (TCR) represent the two dominant arms of the adaptive immune response in vertebrates, development of methods to discover and engineer therapeutic TCRs, and to understand their functions and pharmacology, lag two decades behind mAbs. In spite of this, we have every expectation that TCR-based agents will be similarly important future contributors to the treatment of a variety of medical conditions, especially cancers. As with antibodies, there exist now hurdles regarding discovery, specificity, pharmacokinetics and best modality of use that will need to be overcome before the full potential of TCR-based agents is realized.

In addition, the recent success of adoptive cellular immunotherapy with chimeric antigen receptor (CAR)-directed T-cells directed to hematologic malignancies has prompted interest in finding similar approaches for treating solid tumors. CAR molecules, which typically are restricted to cell surface protein targets, have largely been based on the IgG, but TCR-based agents, directed to peptide-MHC targets, have seen increased interest as a strategy to more specifically target solid tumors, in which there is a paucity of tumor-selective cell surface proteins available. Regardless of receptor format, upon binding to cognate tumor antigens, intracellular domains of CAR and TCR can be designed to recruit similar molecules for activating host effector cells for killing.

Why focus on TCR-based agents?

Currently, there are no FDA-approved mAbs that bind to surface antigens exclusive to cancer cells; however, conventional α TCRs can recognize numerous peptide-MHC (pMHC) antigens with exquisite sensitivity and variable specificity, including pMHC on cancer cells in the form of tumor-associated antigens (TAA) and tumor-exclusive neoantigens[1]. Among the first TAA found to be recognized by TCRs were those derived from MART1[2], gp100[3] MAGE-A[4], and Tyrosinase[5] all of which were first found to be recognized by either peripheral T-cells or tumor-infiltrating lymphocytes (TIL) from resected melanoma lesions. Similar to TAA, neoantigens produced by somatic mutations exclusive to cancer cells are becoming increasingly appreciated as tumor rejection antigens that can be targeted by TCR therapy. TIL present in several resected solid tumors recognize patient-specific neoantigens[6,7]. When such TIL are expanded ex vivo and reinfused, they can induce durable regressions in metastatic solid tumors,[8,9] thus demonstrating the therapeutic potential of neoantigen targeting. Moreover, various neoantigen qualities, such as clonality, MHC binding properties, and immunogenicity, have been shown to predict response to immune checkpoint blockade[10]. Because T-cells generated in vivo in patients are endowed with specificity for tumor antigens, there has been significant interest in clinical development of this class of TCR-based agents for cancer immunotherapy.

In this review, we will discuss the principles and uses of TCR and TCR mimic agents, illustrate some of the critical issues that are limiting the development of these agents,

provide possible solutions to the problems, and contrast and compare TCRs to monoclonal antibodies (mAbs) and to TCR mimic agents. Although TCR shares structural similarities to mAb, specific features differ markedly between mAb and TCR, rendering TCR more difficult to design as soluble drugs (Table 1). As a consequence, while mAbs have been used in various platforms successfully, ranging from fragments to conjugates to CAR T-cells; TCR's have had a more limited repertoire of platforms to date. In contrast, when engineered into cells, TCR more easily co-opt T-cell functionality that mAb cannot, requiring the latter to be more radically engineered to be effective drugs. Finally, mAbs are now being discovered and described that share some functions and specificity of the TCR (known as TCR mimic mAbs). Such agents may solve some of the pharmacologic obstacles encountered with TCRs themselves and add considerable scope to mAbs, but may also create unexpected new problems. These issues also will be discussed below.

2. Structural Issues

Traditional antibodies, TCRm antibodies, and TCRs bear structural similarities, belonging to the immunoglobulin superfamily of proteins, but have distinct features that influence their pharmacology and potential applications and platforms. Although TCRm and IgG are largely identical in structure and pharmacologic characteristics, they differ vastly in potential applications and specificity in that TCRm recognize a far larger universe of antigens, including intracellular targets, but the epitopes are HLA restricted. In contrast, TCR-based molecules are similar in recognition properties to TCRm, though of far lower affinity typically, but are much more limited currently in their platform applications because the native TCR structure is usually membrane associated (Table 2). An important difference between the traditional IgG and TCR-like agents is in their specificity. IgG recognize three-dimensional shapes of proteins, carbohydrates, and haptens, among other molecules, which can often confer near-perfect specificity for the target antigen. TCR-based agents recognize a linear peptide sequence buried in the groove of MHC molecules, as well as parts of the MHC sequence adjacent to the peptide. Therefore, the surface area of the recognized epitope bound by the TCR agent is limited, and the possibility of cross-reactive epitopes, both from recognition of the MHC and from sequence similarities to other peptides in the proteome that may be presented, is significant[11,12]. This distinction makes the discovery and development of specific TCR-based agents more complicated. In contrast, by selecting TCR directly from humans, such as from TILs, many cross-reactivities may be avoided because the thymus filters out most cross-reactive TCR during T-cell development [13].

3. Immunologic hurdles in selecting appropriate antigenic targets for TCR based agents

HLA restriction.

CD8+ T-cells detect and eliminate abnormal cells by recognizing peptide fragments of processed proteins that are presented by human leukocyte antigen class I (HLA I). HLA is highly polymorphic, with each variant (allotype) characterized by a different peptide binding groove, resulting in allotype-restricted peptide binding motifs. In humans, three classical HLA class I genes (HLA-A, HLA-B, and HLA-C) are expressed in nucleated cells with up

to six different allotypes per individual. The classical antigen presentation pathway for HLA class I presented peptides involves the proteolytic cleavage of proteins in the proteasome followed by the peptide fragment translocation to the ER by TAP; after further trimming, individual peptides get loaded onto HLA-class I molecules and transported to the cell surface for presentation to CD8 T-cells. However, alternate peptide presenting mechanisms exist, as can be seen in humans lacking TAP, that are still able to present peptides on cell surface, though with much lower abundance.

Characteristics of peptide antigens.

The peptides presented on HLA class I can be foreign (e.g virus, bacteria) or self. A recent study found that all proteins can potentially give rise to presented peptides [14]. However, presented peptides are often skewed towards proteins with a high abundance and high turnover rates [15].

Cancer-associated aberrant protein expression includes products of mutated oncogenes, passenger mutated genes, tumor suppressor genes, oncofetal genes, aberrantly or overexpressed genes, abnormal glycoproteins, and post-translationally modified proteins. In theory, these aberrant proteins or protein fragments can produce peptide fragments that can be presented on HLA class I where they can be detected by CD8+ T-cells. Hereby, a distinction is made between self-antigens and neo-antigens. Self-antigens derive from proteins that can also be found on other tissues, but are overexpressed or re-expressed in cancerous cells. Prominent examples are lineage-specific tumor-associated antigens (TAA) such as MART-1 and CEA, cancer germline antigens (CGA), including NY-ESO-1, which is usually exclusively expressed in testicular germ cells, but is re-expressed in various cancer cells due to genomic instability (for example, in 40% of epithelial ovarian cancer, 75% of synovial cell sarcoma, 25% of melanoma), MAGE, or PRAME. Neo-antigens are peptides that are exclusively found on cancer cells (tumor-specific antigens) and result from nonsynonymous somatic mutations, frameshift mutations, and sometimes from post-translational modifications such as phosphorylation or glycosylation. Due to the enormous heterogeneity between individuals in their allotypes and the resulting heterogeneity of the immunopeptidome between individuals, most neoantigens identified are patient-specific (that is, “private”). Targeting private neoantigens requires individual customization of TCR posing significant logistical and financial challenges. However, gain of function mutations in a cancer driver gene critical for tumor survival that is shared among patients with particular HLA allotype are called “public” neoantigens. Such targets might be used in broader populations of patients[16]. Recent studies have shown the successful identification of a public neoantigen derived from a PIK3CA mutation as well as the identification of four different TCRs that are able to detect this neoantigen in an HLA-A03 context which is one of the most prevalent HLA allotypes [17].

Using TCR T-cells for target identification.

T-cell-based immunotherapy is partly based on the assumption that T-cells found endogenously in the host can specifically detect and eliminate cancer cells. While the endogenous cytotoxic T-cell response is often insufficient to protect against tumor development due to the immunosuppressive tumor microenvironment, such TCR may be

used to identify the target epitope or create new more potent specific therapeutic agents. Isolated and sequenced TCRs used to produce genetically engineered T-cells infused into the same patient from which they were isolated have shown promising tumor control in clinical trials[7,18,19]. Rapidly identifying the target peptide as well as the TCR sequence able to react with the target remains one of the major challenges in TCR immunotherapy.

Identifying the TCR alpha and beta chain.

Upon target recognition, T-cells with a TCR able to recognize their target undergo clonal expansion. This expansion can be used to identify clonally expanded TCR sequences that are likely to be specific for antigens presented in a given disease state using single-cell or bulk RNA sequencing. Other high throughput methods for TCR identification include phage, yeast, and T-cell display libraries. A stimulation-induced functional TCR sequencing platform has been described in which naive T-cells from healthy donors are subjected to stimulation with autologous DC electroporated with a mutant or the respective wild-type driver oncogene[17]. Using qPCR to detect INFgamma, wells that are preferentially reactive towards the mutant antigen are further stimulated and subjected to sequencing in order to identify the TCR *alpha* and *beta chain* sequences.

To optimize the activity of genetically engineered T-cells, TCRs are often affinity enhanced by introducing mutations into the CDRs, which make direct contact with the pMHC complex. Since the immune system preferentially deletes high-affinity TCRs (K_d< 6uM) [20] in favor of low-affinity TCRs to prevent autoimmune reactions and to maintain highly promiscuous T-cells that are reactive against a wide range of antigens, affinity enhancement may lead to T-cells with increased off-target reactivity to structurally similar self-peptides, which can lead to severe or lethal toxicity in patients[21].

Why do TCRs have off-targets?

The affinity of T-cell receptor (TCR) for its target is determined by its complementarity-determining regions (CDRs) on each TCR alpha and beta chain. This highly variable sequence results from genetic rearrangement and diversification. There are six CDRs per TCR, and they typically recognize a peptide presented in the context of the major histocompatibility complex (MHC), which, in humans, is the human leukocyte antigen (HLA) [22].

There are two classes of canonical MHCs: class I MHC molecules are expressed in almost every nucleated cell in the body and present processed intracellular protein products. In contrast, class II MHCs are restricted to immune cells and present peptides derived typically from phagocytosis. Humans have six HLA class I alleles and six HLA class II alleles. High polymorphism results in the human population having more than 25,000 different HLA class I and 10,000 HLA class II alleles [23]. The diversity of these genes is primarily due to variations in the amino acid sequence within the peptide-binding cleft, increasing the variety of peptides displayed. The potential combination of peptide:MHC is predicted to be over 10E15 [24] and becomes even larger once all possible post-translational modifications are taken into consideration, such as phosphorylation, oxidation, glycosylation, and citrullination, among others [25].

However, it is estimated that there are only ~10E12 T-cells in the human body collectively representing ~10E8 TCRs [26], millions of times less than needed to recognize every epitope individually. If a TCR were to bind only one cognate peptide:MHC pair, it would fail to mount a protective immune response against the actively evolving microbiome, viruses, and oncogenic mutations. Therefore it is necessary that TCRs have to be cross-reactive, with each TCR capable of recognizing thousands of, and possibly up to a million, different peptide:MHC complexes [27]. This hypothesis has been validated while elucidating the mechanism of T-cell development and selection, as well as activation. A single peptide expressed in the thymus may lead to the elimination of polyclonal T-cells, and a monoclonal T-cell may be activated by multiple different foreign peptides. Such binding degeneracy provides the advantage of a single TCR being able to recognize similar pathogenic peptide groups and confer a wider protective effect [28].

However, this raises a concern regarding cross-reactive and auto-reactive therapeutic TCRs. Fortunately, all developing thymocytes undergo positive and negative selection in the thymus. TCRs that can recognize self-MHC molecules expressed by the cortical thymic epithelial cells (cTECs) are positively selected and migrate to the medulla, where they encounter multiple self-peptides presented by medullary thymic epithelial cells (mTECs) and resident dendritic cells (DCs). TCRs that bind too strongly to self-peptides are eventually eliminated by inducing apoptosis (central tolerance), leading to a final pool of T-cells unlikely to be autoreactive[24].

Autoreactive T-cells that escape selection and encounter their ligand in the periphery may remain inactive, given that TCR engagement without co-stimulatory signaling leads to T-cell anergy (peripheral tolerance) or the induction of regulatory T-cell differentiation (iTregs)[29,30]. Another mechanism to keep self-reactive T-cells quiescent is by anatomical exclusion. The brain, central nervous system, eyes, and testes [31] avoid auto-reactivity by actively maintaining an immunosuppressive microenvironment either by secretion of immunosuppressive cytokines, selective homing of tolerogenic immune cells, limited lymphatic drainage [32] or formation of a physical barrier [31].These layers of protection in the periphery against self-reactive T-cells are evidenced by their prevalence during steady state. Prior work has speculated that the total number of potential auto-reactive T-cells is in the range of 1–10% [33,34], and more recent claims have suggested that this number may be even as high as 30% of the total immature effector T-cell population. Therefore, it would not be uncommon to identify TCRs with self-reactivity potential. These cells, if taken out of their quiescent steady-state environment and introduced in the context of TCR-based cell therapies, could cause significant damage to the host, as was lethally evident in certain clinical trials[35], further emphasizing the importance of rigorous testing for potential off-targets before the application of a specific TCR in patients.

Consequences of off-targets.

Off-target toxicities may be due to (1) cognate-targeted antigens also being expressed in healthy tissue and (2) cross-reactivity to structurally similar peptides. In patients successfully treated with TCRs targeting MART1 and gp100, some patients developed severe side effects due to the target antigen also being expressed in melanocytes in the

skin, ear, and eyes [36,37]. Another clinical trial targeting CEA showed severe transient inflammatory colitis in three patients due to its expression on normal intestinal cells[38]. These studies show the limitation of using *tumor-associated antigens* as targets in cancer immunotherapy.

To optimize genetically engineered T-cells, TCRs are often affinity enhanced by introducing mutations into the CDRs, that bind to the MHC complex. Affinity enhancement, however, often increases the risk of T-cells' off-target reactivity because these engineered T-cells bypass the natural negative selection process to self-peptides[39]. While the threshold for negative selection in the thymus was proposed to be < 6uM, affinity-enhanced TCRs often have logs higher affinity reaching the nanomolar range or even the picomolar range [40,41]. Natural T-cell function has been proposed to plateau at an affinity of 5 uM [42] to 10 uM [43], and further increases in affinity may not lead to an increase in function.

In different trials, affinity-enhanced TCRs targeting MAGE A3 cross-reacted with peptides derived from self-proteins, leading to lethal toxicity in four patients. In one clinical trial (NCT01273181), the murine-derived TCR was affinity-enhanced through site directed mutagenesis in the CDR2 region, inducing tumor regression in 5/9 patients; however, also leading to lethal toxicities in two patients due to cross reactivity to a MAGE-A12 peptide expressed in the brain [44]. In two separate trials (NCT01350401 and NCT01352286), an affinity-enhanced TCR against MAGE A3 was cross-reactive to a peptide derived from the cardiomyocyte protein Titin, leading to cardiogenic shock and death of two patients [21,35].

Methods of identifying off-targets.

The identification of peptides presented in healthy tissue is crucial for excluding those peptides as targets for immunotherapy. Bioinformatic tools help by analyzing sequencing data from healthy tissue. Tools such as NetMHCpan[45] can assess how well peptides from the human proteome bind to different HLA alleles. Other computational methods can identify off-target peptides by considering factors such as charge, hydrophobicity, and structural information like predicted accessible surface area. The BLOSUM algorithm[46 47,48] is commonly used, as it allows for peptides of different lengths and can find biologically relevant off-targets by using evolutionary and functional similarities between amino acids. This is achieved by blasting potential sequences to the human reference proteome.

However, these approaches have a high false discovery rate, do not reliably represent what is actually presented by the cell, and do not reliably predict T-cell reactivity. Recent optimizations in mass spectrometry and bioinformatic tools have advanced the field of immuno-peptidomics of healthy tissue[49,50]. Projects such as the Human HLA Ligand Atlas and the Immune Epitope Database (IEDB) will improve to assess whether the target is also expressed in healthy tissue [51]. However, the sensitivity limit of detection for mass spectrometry is currently low, making the detection of infrequently-presented peptides difficult.

An empiric approach is anticipate potential TCR off-targets is to use alanine scans [52] by replacing each amino acid residue in a peptide sequence with an alanine and testing T-cell responses. This approach has the advantage of measuring the actual human T-cell

response to an epitope. However, this method may not be effective in identifying significant interactions if the substituted amino acid is structurally similar to alanine and typically relies on single alanine substitutions that does not reflect the diversity of structural modifications. Thus, alanine scans tend to favor identification of TCR interactions with larger and charged amino acids.

The X-scan method is similar to the alanine scan, but instead of substituting with alanine, it individually substitutes one position in the peptide sequence with each of the 19 other amino acids while keeping all other positions unchanged [53]. This results in 162 possible substitutions in a 9mer. Another method for screening peptides to identify TCR off-targets is to use combinatorial peptide libraries (CPLs) [54], where one position in the peptide sequence is held constant while the remaining positions are changed to any other amino acid. The peptides resulting from CPL scans are screened in subpools to determine TCR reactivity. Compared to alanine scans, X-scans and CPLs offer a more comprehensive understanding and ranking of potentially cross-reactive peptides by allowing for a wider range of peptides to be screened.

In vitro methods have limitations as they rely on predicted peptides that are based on the known target ligand sequence, and subsequently, cannot evaluate cross-reactivity of highly divergent sequences. Therefore, more empiric methods utilizing large libraries, where the peptide target is genetically encoded into expression systems, have been developed. Yeast-, baculovirus-, and phage-based display libraries of peptides, [55,56], [41,57] have been employed. In these methods, human MHC is expressed with the peptide attached by a linker. However, for these systems to work, the MHC must fold, and the peptide must bind the MHC properly, which may not successfully occur due to species-specific differences.

The PresentER system was developed to enable the upscaled testing of tens of thousands of candidate peptides for their presentation using endogenous human MHC [11]. This system involves transducing TAP1- and TAP2-deficient T2 cells with a library of peptides along with an endoplasmic reticulum signaling sequence. Cross-reactive peptides are identified through DNA sequencing of the transduced minigene encoding potential off-target peptide sequences. Another library screening technique, called signaling and antigen-presenting bifunctional receptors (SABR) [58], involves expressing peptides linked to MHC receptors fused to intracellular CD3ζ and CD28 domains. The target cells are identified through fluorescence, and the presented target peptides are subsequently identified through sequencing as well. In contrast to the genetic encoding of short antigenic peptides used in PresentER and other libraries, SABR libraries encode larger numbers of amino acid sequences including all known A2 binding epitopes from IEDB database. However, both methods rely on HLA-binding or peptide cleavage algorithms. Therefore, these screens must be combined with mass spectrometry data or use of T-cells as surrogates for further validation.

4. Soluble TCR-based Therapies

Non-cellular TCR-based therapies bypass many of the limitations of an adoptive T-cell transfer approach. Two main approaches are via a TCR or an antibody that mimics a TCR's reactivity.

ImmTacs:

Examples of the most clinically advanced soluble TCR therapies are the Immune Mobilizing Monoclonal TCRs Against Cancer (ImmTac) molecules, which comprise a soluble disulfide stabilized, affinity enhanced TCR fused to an anti-CD3 single chain variable fragment (ScFv). One arm of the ImmTac molecule engages pMHC, while the anti-CD3 ScFv arms engage CD3 on T-cells, redirecting powerful polyclonal T-cells to kill the targets. ImmTACs thus overcome the challenges of natural TCRs as soluble drugs (weak affinity towards tumor antigens, difficulties in manufacturing, lack of solubility). An ImmTAC molecule, Tebentafusp, (reactive with a gp100 epitope presented by HLA-A2) was the first approved soluble TCR therapy for the treatment of adult patients with unresectable or metastatic uveal melanoma in the United States and the European Union in 2022.

For TCRs, a relatively small number of mutations is sufficient to improve their affinity to the 100 picomolar range, while still maintaining specificity. In addition, the removal of the transmembrane domain and the addition of a non-native disulphide bond creates a soluble protein with exceptional stability [59]. Each of the four described ImmTAC molecules (reactive with gp100/HLA-A*02:01, MAGE-A3/HLA-A*01:01, Melan-A/MART-1/HLA-A*02:0, and NY-ESO-1/ HLA-A*02:01) generated were able to redirect T-cells to tumor cell lines presenting the respective tumor-associated peptide antigens. The affinity of the TCR receptor component correlates closely with the degree of T-cell activation and, importantly, provides greater sensitivity to the expected low numbers of cell surface target antigens. ImmTACs are the first soluble bispecific agents to combine high-affinity recognition of MHC-presented tumor antigens with the simultaneous redirection and activation of bulk T-cells [60,61]. Therapeutic ImmTac molecules targeting other tumor antigens PRAME, PIWIL1, or MAGE-A4 in the complexes of HLA-A2 or A24 have been recently developed, and some of these agents have entered clinical trials. Others target viral epitopes such as hepatitis B virus (HVB) and human immunodeficiency virus (HIV) [62,63]. As a therapeutic class, ImmTACs offer a tailored, off-the-shelf solution possessing high specificity, in turn mediating efficacious cancer cell cytotoxicity.

TCR mimic monoclonal antibodies (TCRm).

The application of mAb or CAR T-cells in cancer therapy remains limited by the lack of cancer-specific cell surface targets not found on normal cells. Most targets in clinical development are tissue lineage antigens that are shared with normal tissues; therefore, targeting these conventional surface proteins with a high potency of modalities such as CAR-T, bispecific mAbs (bisAbs) or antibody-drug conjugates (ADCs) often causes on-target, off-tumor toxicities. In contrast to hematologic cells, in which deletion of a lineage (for example, B cells) may be tolerated by the patient for moderate time periods, the lack of specific antigens particularly limits the therapeutic applications of these agents among

patients with AML or most solid tumors. To target the larger universe of intracellular tumor antigens, a new class of mAbs, TCRm, has been developed. TCRm mAbs are designed to recognize peptide/MHC complexes, similar to TCRs. However, the traditional antibody structure also allows the advantages and versatility of a mAb: easy protein engineering, high affinity and specificity, long half-lives in plasma, solubility, and off-the-shelf dosing flexibility [64]. Most importantly, a mAb can be engineered to various formats to improve its therapeutic potency [65]. While TCRm can access intracellular peptide/HLAs, the antibody structure offers possible advantages of intrinsic effector functions of mAbs and advanced therapeutic antibody formats. These include antibody-dependent cellular cytotoxicity (ADCC) either as an Ig or as a bispecific format, complement-dependent cytotoxicity (CDC), CAR T-cells, and antibody-dependent cellular phagocytosis (ADCP). In addition, mAbs can serve as antigen-specific vehicles that specifically deliver potent cytotoxic agents such as toxins, drugs, or radionuclides to cancer cells.

Several murine TCRms were developed to monitor antigen processing and presentation in mouse models as experimental tools [66]. In the last decade, the use of TCRm mAbs for cancer therapy was greatly advanced. Traditionally, TCRm antibodies have been difficult to generate by conventional hybridoma technology. Advances in antibody display library methodology provided a breakthrough leading to the isolation of many mouse and human TCRm antibody fragments such as Fabs or scFvs, as well as several full-length human TCRm, thus allowing the investigation of these TCRms as potential therapeutic agents. Following the first two therapeutic TCRm mAbs, a murine hybridoma-generated TCRm (8F4) reactive with the myeloid leukemia antigen proteinase 3-derived epitope PR-1 (VLQELNVTV) presented by HLA-A*02:01[67] and the first fully human TCRm, ESK1, specific for a Wilm's tumor protein 1 (WT1)-derived epitope/HLA-A*02:01 complex [68], a growing number of TCRm targeting various tumor or viral antigens have been reported (Table 3). TCRm 8F4 has been humanized and engineered to bispecific antibody (BisAb) and was in clinical trials. ESK1, has been converted to bispecific T-cell engager (BiTE) and CAR T formats, radioconjugates, and also engineered to enhance Fc functions, demonstrating versatile usage of a TCRm mAb in various therapeutic settings as a typical mAb [69,70]. A TCRm specific for an epitope derived from alpha fetal protein (AFP) in the context of HLA-A2 has entered clinical trial in a CAR T-cell format for hepatocellular carcinoma [71].

TCRm-CAR T-cells.

In comparison to antigen targets of traditional antibodies, which may exist in the tens to hundreds of thousands on the cell surface, peptide/HLA complexes are typically low-density antigens on the cell surface, ranging from less than 10 to hundreds per cell [72]. While antibody maturation has often been used to increase the antigen antibody interactions, using CAR T-cells to increase avidity has been shown to be an efficient way to overcome this hurdle. The first TCRm-CAR T, derived from ESK1, showed potent activity against leukemias *in vivo*[70]. Recently, more than a dozen more TCRm have been engineered into CAR T-cell formats recognizing NY-ESO-1, gp100, MAGE-A1, minor antigens, among other antigen, in the context of HLA molecules (Table 3).

Bispecific mAbs (BisAb).

Similar to the ImmTACs above, BisAbs are designed to recognize both a cancer antigens and an effector cell antigen and they comprise a large family of molecules, with a wide variety of formats. Such bispecific molecules function by recruiting and activating polyclonal T-cells, NK cells, or other effector cells. The successive conceptual and technical innovations in generating bisAbs have led to the extensive collection of over 100 BisAbs known today [73].

Bispecific T-cell engager molecules (BiTE) are a subtype of BisAb, composed of a scFv specific for tumor antigen on one arm, linked to a scFv for CD3 on the other arm. BiTEs are completely devoid of constant regions of the antibodies, with a small size (55KDa) and are highly flexible, thus enabling close interactions between CD3T-cells and cancer cells, and consequently facilitating potent polyclonal cytotoxicity of CD3 T-cells against cancer cells. Such a BiTE molecule functions by recruiting and activating polyclonal T-cells at tumor sites, thereby bypassing MHC restriction and co-stimulation, while retaining epitope specificity needed for traditional TCRs. Upon crosslinking, T-cells are activated to form an immunologic synapse, which induces apoptosis in tumor cells via the perforin/granzyme B pathways [74]. Blinatumomab, an anti-CD19 and anti-CD3 BiTE, is the first BisAb approved by FDA in 2016[75]. Bispecific molecules directed against targets in low abundance like MHC presenting specific epitopes, require an extremely high potency to be effective. ESK1-BiTE was the first TCRm-based BiTE, which showed superior cytotoxicity than an Ig form against a wide range of tumor cells expressing WT1 *in vitro* and *in vivo* in mice. Interestingly, The ESK1-BiTE also induced robust secondary CD8 T-cell responses against other epitopes via epitope spreading [69]. Such a mechanism may be important for long-lasting anti-tumor immunity by controlling the outgrowth of tumor cells that have lost the target protein or that have downregulated the primary target during tumor evolution. This biological function is possibly analogous to that of the check-point blockade antibodies, which unleashes tumor-specific T-cell responses that had been suppressed or dormant in the tumor microenvironment. In addition, as a small molecule, BiTEs may penetrate more easily than CAR T-cells into the tumor microenvironment (TME) of solid tumors, where it can bridge tumor targets with tumor-infiltrating lymphocytes (TILs). Moreover, BiTE can be delivered by CAR T-cells, achieving dual targeting strategy [76].

Full length BisAbs.

The omission of antibody Fc domains from BiTEs, also has pharmacokinetic implications; BiTEs have a short plasma half-life (4–5 hours), which requires continues infusion and are therefore not ideal as convenient drugs. To overcome this problem, various bisAbs with full length antibody architecture have been developed to engage targets with CD3 T-cells, while silencing the Fc domains of the antibody. For the low-density antigens such as peptide/HLA complexes, bivalent mAb structures would provide more stable binding. Recently, a TCRm 11D06, specific for WT1 RMF epitope presented by HLA-A2, was engineered to a bivalent mAb (in a 2+1 format) IgG with a prolonged half-life. We engineered five different BisAbs derived from a TCRm specific for the phosphopeptide derived from insulin receptor substrate 2 (pIRS2) in the context of HLA-A2 molecule. Among which, we found that mAbs 1+1 and 2+2 format structures, effectively redirected T-cell cytotoxicity against the tumor

cells [77]. These studies demonstrated that a variety of currently advanced bisAb formats can be applied to TCRm as well.

Challenges for TCR mimics and solutions.

Similar to TCRs, TCRm also recognize a linear peptide sequence bound to HLAs; therefore, cross-reactivity to other similar complexes poses a potentially significant toxicity challenge. One argument against TCRm usage vs TCR, is that TCRm are not naturally selected structures filtered by thymic selection to preferentially recognize foreign, and not self, peptide-HLA complexes. In addition, most selection methods using sequence libraries that may introduce unnatural unstable structures. Therefore, TCRm may never completely mimic natural TCR recognition [72]. TCRs generally dock onto peptide-HLA complexes using a conserved canonical binding mode, forming a large binding interface between the TCR and peptide-HLA, enabling broader contacts across both peptide backbones and HLA heavy chain. In earlier studies of TCRm, X-ray crystallography studies have shown that the binding of the TCR-mimic antibody to MAGE-1(161–169)–HLA-A*01:01 was focused on the HLA-α1 helix with no contact between the antibody and N-terminal MAGE-A1 peptide residues [78]. A similar phenomenon was reported for ESK1, that the ESK1 Fab primarily interacts with the N-terminal residue of the peptide and HLA-A*02:01 [12]. However, other binding motifs of TCRms have also been reported. One TCR-mimic antibody engineered to bind to the NY-ESO-1(aa 157–165)/HLA-A*02:01 epitope adopts a TCR-like canonical binding geometry. In this study, crystal structures of 2 Fab antibodies to NY-ESO-1 peptide (SLLMWITQV) presented by HLA-A*0201 were compared to a TCR recognizing the same pMHC,1G4. Binding to the central methionine-tryptophan peptide motif and orientation of binding were almost identical for Fabs and TCR [79].

Alanine substitution assays have shown that various peptide residues could be recognized by TCRm, depending on the individual TCRm mAbs. For example, a TCRm mAb specific for the PRAME peptide/HLA-A*02:01 mainly recognized C-terminal residues of the peptide [80]. A recent TCRm mAb to WT1 RMF/HLA-A2 recognized peptide residues 1, 3, 5 and 6 [74]. A TCRm (6B1) generated for the phosphopeptide pIRS2/HLA-A2 complex had an alanine scan that showed that the mAb primarily recognized the phosphate on the serine of the residue 4, which closely resembled the TCR recognition of the phosphopeptides/HLA-A2 complexes [77]. Although a growing number of TCRms have been reported, most lack detailed analyses of recognition mode and specificity data. As a result, the factors that contribute to the recognition modes of TCRms remain complex and unclear. Even the well-established model of TCR-peptide/MHC interactions has also been constantly updated with exceptions, as a recent study revealed a reverse docking topology relative to the established TCR/p/MHC docking paradigm [81]. Future work will focus on discovering TCR mimic mAbs that better recognize peptide/MHC complexes with fine specificity and with TCR-like conformations. This may be achieved by more rigorous screening algorithms, better filtering of hits, and structure-based analyses.

Another way to improve the selection process of finding better TCR-likeTCRm, could be the design of improved the phage libraries and protein re-engineering to create molecules that engage peptide/MHC in a manner structurally similar to that of conventional αβ-TCRs

[82]. Crystallographic analysis of one selected pMHC-restricted Ab revealed highly peptide-specific recognition, validating this engineering strategy.

Improved screening strategies to select TCRms that interact with the amino acids of the peptide/HLA complex that are broader and more central may be preferred as well. Specificity to desired middle amino acids should reduce binding to many potential human proteomic off-target peptides. With this strategy, we were able to select more specific TCRm clones for the WT1 RMF/HLA-A2 complex than we had identified before (unpublished).

Furthermore, the availability of more crystallographic studies would provide direct structural information to improve our current understanding of the interactions between TCRm and the peptide/HLA complexes. Recent studies of TCRs have demonstrated that off-target peptides do not need to share sequence, physiochemical, or backbone geometry with the cognate peptide and that peptides, HLAs, and TCRs all have flexibility and adaptability during the TCR recognition of the peptide/HLAs [83,84]. This leads to a question if such conformational plasticity also exists in the TCRm recognition that are not always captured by crystallographic analysis alone. Although conventional mAb binding to protein targets is fundamentally different from TCR recognition, TCRm, which recognize peptide/HLA, may share certain similarity with TCRs. Thus, it is vital to understand the dynamic characteristics of peptide/HLA interactions with TCRm. To better understand the contribution of allostery, protein dynamics, and protein flexibility, during peptide/HLA interactions with TCRm mAbs, dynamic studies using isotope-edited infrared spectroscopy, nuclear magnetic resonance (NMR), Förster resonance Energy transfer (FRET), and molecular dynamics (MD) stimulation, may offer new insights into the recognition of TCRm to peptide/HLA complex. Such methods have shed light on both TCR-p/MHC interactions, antibody orientation and function [85–88].

5. Cancer vaccines

As cancer vaccines are not a direct use of a TCR-based drug, but rather a means to induce a host TCR-based response in which the host provides the cytolytic agent, we will only briefly discuss their uses and issues for comparison here. Cancer vaccines consist of synthetic peptides, mRNAs, DNAs or proteins derived from tumor antigens that are used for active vaccinations to induce or boost naturally occurring tumor-reactive T-cells' TCR that recognize peptides presented by MHCs. Cell-based vaccines have also been tried using dendritic cells loaded with tumor antigens or modified tumor cells [89]. Cancer vaccines have been the subject of intense preclinical and clinical investigation for a variety of malignancies over the past 40 years, however, the successful clinical translation from bench to marketing approval has been elusive. Many clinical trials of cancer vaccines, including our studies [90,91] have shown to be able to induce vaccine-specific immune responses. However, responses alone do not always translate, lacked into immediate clinical benefits especially in the setting of active, bulky cancers or leukemia. Because most cancer vaccines were targeting TAAs, a major obstacle is the induction of potent adaptive immune responses against self-antigens that is limited by the inherent self-tolerance of the host.

The recent success of checkpoint blockade therapy and recent advances in neoantigen identification revived the enthusiasm for current cancer vaccine development [92,93]. The adaptive immune system's ability to discriminate between "non-self" and "self," coupled with the vast diversity of T-cell repertoire, yields neoantigen-specific T-cells that are present in the blood or TILs of cancer patients. The key role of neoantigens in antitumor immune responses has been demonstrated in patients with solid tumors, whose tumors showed substantial regression after treatment with adoptively transferred neoantigen-specific T-cells [6,8,94]. However, neoantigens are generally patient tumor-specific, requiring a patient-specific vaccine to be prepared, making this approach logistically complex and expensive.

Clinical experience suggests that cancer vaccines are safe and can elicit long-term immune memory responses important for durable disease control [89,95,96]. This suggests that vaccines may be particularly well-suited in the minimal residual disease setting. In addition, neoantigens are key targets of checkpoint blockade immunotherapy-driven responses; therefore, priming tumor-specific T-cells and mobilizing them to the tumor, vaccine therapies could help checkpoint blockade to unleash T-cell-mediated tumor-specific responses. While several neoantigen vaccines have been tested in human trials, from historical experience, combinations of neoantigen vaccines with checkpoint blockade and other therapies may achieve better therapeutic efficacy [97]. While most neoantigen-targeting vaccines are patient-specific, new searches for public neoantigens such as p53 mutations and RAS mutations, could offer a broader application of vaccines [98,99].

6. Cellular TCR-based therapeutic approaches: Choosing the right cell vehicle

Engineering cells with a tumor antigen-specific TCR requires a sufficient quantity of healthy cells for expansion ex-vivo before infusion and an appropriate effector capable of achieving the desired response. If the cell source is the autologous patient, this precondition may limit the types of cells that may be used, especially if the patient has a comorbidity or received prior therapy that reduces cell numbers. The necessity for patient-specific cells and the difficulties of controlling doses, proliferation and persistence of cells once infused, may limit optimal clinical applications at this time. Allogeneic off-the-shelf sources would overcome some of these limitations, but are less well-described and clinically developed. Here we discuss the different types of immune cells that can be engineered with tumor antigen-specific TCR-based agents for adoptive T-cell therapies against cancer.

CD8 T-cells:

Cytotoxic CD8 T-cells, as the most efficient cancer-killing cells that inherently recognize MHC-class I-associated antigens via their TCR [100] have been a top choice of cells to express an exogenous TCR. However, the presence of native TCR within these cells poses challenge. For example, exogenous TCR chains can mis-pair with endogenous TCR αβ chains, which could lead to less specific activity, cross-reactivity towards self-antigens, autoimmunity, and reduced potency. Solutions to this issue include introduction of cysteines into the constant regions or the use of murine constant regions, framework region engineering, domain-swapping, single chain exogenous TCRs, and knocking-out endogenous TCR

αβ chains (including knocking in the new TCR into the TCR alpha site)[101–106]. TCR-engineered CD8 T-cells generally need to be infused together with helper CD4 T-cells for optimal function[107]. Early TCR-engineered T-cell therapies [108]used allogeneic T-cells with exogenous TCR targeting MART-1AFP, CEA, GD2, gp100, MAGE-A3, MAGE-A4, Mesothelin, and NY-ESO-1 among others [60,108–111]

CD4 T-cells:

Because CD4+ T-cells make up two-thirds of the total blood T-cell population, CD4 T-cells are being investigated for their cancer-killing efficiency, after engineering them to express tumor pMHC-class I restricted exogenous TCR [112]. Challenges to this approach include a reduced potency due to a lack of CD8 co-receptors on CD4 T-cells and, as described above, mispairing of exogenous TCR with endogenous TCR of CD4 T-cells. Strategies to overcome these issues include the transfer of CD8αß co-receptor genes and improving the pairing of exogenous TCR using techniques discussed above [113]. For example, one clever and robust approach was to make therapeutic CD4+ T-cells capable of providing MHC Class I-restricted immunity against MHC Class II-negative tumors by use of MHC Class I-restricted CD4+ T-cells specific for Epstein-Barr virus (EBV) and cytomegalovirus (CMV) that recognized HLA-A2/peptide multimers [114]. In a xenogeneic mouse model, this work demonstrated that human TCR and CD8 genes engineered into CD4+ T-cells conferred efficient protection against the growth of tumors expressing the EBV or CMV antigens recognized by the TCR.

γδ T -cells:

Gamma-delta (γδ) T-cells are an alternative cytotoxic effector population that can be engineered to express tumor-antigen specific αβ TCR [115,116]. Since γδ TCR chains do not pair with αβ TCR chains, γδ TCR are not subject to the problems associated with the use of exogenous αβ TCR chains, such as incorrect mispairing with endogenous TCR leading to alloreactivity and GvHD [117]. Many studies have successfully demonstrated engineering of cytotoxic γδ T-cells expressing HLA-class I restricted αβ TCR [118,119]. In a similar approach, γδ T-cells could also be equipped with TCR derived from iNKT to target CD1d-restricted tumor antigens [120].

The γδ T-cells have limited expression in the blood, with only 1–10% of total circulating T-cells making manufacturing difficult [121,122]. Therefore, in an alternate approach, αβ T-cells can be armed with tumor-specific TCR from γδ T-cells. Hence arming abundantly available αβ T-cells with γδ TCR will make them kill tumor cells in an HLA-independent manner [123]. In addition, expression of γδ TCR downregulates the endogenous αβ TCRs, thereby reducing the chance of off-target HLA-antigen recognition and alloreactivity by engineered T-cells.

NK-cells:

Natural killer (NK) cells are innate lymphoid cells with the inherent ability to identify and kill cancer and virus-infected cells [124]. They can identify the cancer cells in a TAA and pMHC-independent manner and kill them via several cytotoxic mechanisms such as inducing apoptosis by Fas-FasL interaction, secreting cytolytic molecules such as perforin

and granzyme, ADCC, and secreting cytokines that can recruit cells of other innate and adaptive immunity [125–128]. Blood-derived primary NK cells and the NK cell line "NK-92" have provided rapid killing of cancer cells in allogeneic settings without causing significant graft-versus-host disease [129–132]. CAR -NK cells have also reached human trials and appear to be safe and effective [133]. Hence, tumor antigen-directed TCR-engineered NK cells may be alternative off-the-shelf, ready-to-use allogenic cells with enhanced anti-tumor effector functions that combine the effect of TCR mediated tumor cells lysis as well as NK cells' intrinsic activation mechanisms. However, engineering NK cells with a functional exogenous TCR also requires the expression of exogenous CD3 molecules, as NK cells do not express CD3 components [134,135]. For example, enhanced HLA-B*07:02 restricted BOB1-specific TCR-engineered NK cell efficacy against B-cell leukemia compared with TCR-negative NK cells has been shown [105].

NK T-cells:

Natural Killer T-cells (NKT) share the properties of both conventional T-cells and NK cells. They express NK cell's specific markers and semi-invariant αβ TCR that recognizes lipids and glycolipids antigens presented by CD1d molecules [136,137]. There are two types of NKT-cells; Type-1 NKT-cells with limited TCR diversity, also called invariant NKT-cell (iNKT-cells), and other CD1d restricted T-cells called Type-2 NKT-cells. These NKT-cells are naturally potent cytotoxic against cancer cells and also confine immunosuppressive myeloid cells in the tumor microenvironment via CD1d-cognate detection, stimulating anti-tumor responses irrespective of the CD1d expression by cancer cells [138–141]. Since CD1d molecules are identical in all individuals, NKT-cells can be adoptively transferred across MHC barriers without the risk of allo-reaction and graft vs. host disease [142]. Hence, allogenic NKT-cells also can be exploited as readily available, off-the-shelf donor-unrestricted effector cells for adoptive cell therapies against cancer [143–147]. Adoptive cell therapy with tumor antigen-redirected exogenous TCR-engineered NKT-cells could provide combinatorial anti-tumor effects by utilizing both the exogenous tumor-specific TCR to recognize pMHC on tumor and CD1d restricted endogenous TCR against the cancer cells that could boost the overall therapeutic effect. TCR-engineered iNKT-cells demonstrated efficacy against various tumor models [148] in which bispecific effector functions for CD1d- and MHC-restricted antigens were seen.

CIK-cells:

The cytokine-induced killer (CIK) cells are heterogeneous populations of ex-vivo differentiated immune cells with high tumor-killing potency and characteristics of both NK cells and cytotoxic T-cells [149,150]. Among them, CD3+CD56+ cells are the most efficient cytotoxic CIK cells, which can kill tumor cells in both MHC-dependent [151] and independent manners by deploying effector molecules such as NKG2D, TRAIL, FasL, DNAM-1, NKp30, LFA-1, perforin and granzyme secretion [152–155]. Hence, strategies for engineering CIK cells with tumor antigen-redirected TCR could provide an adequate number of effector cells for adoptive cell therapy with the possibility to target surface and intracellular antigens. CIK cells genetically engineered to express HLA-A2+ restricted anti-Mart-1 and anti-NY-ESO-1 melanoma-antigens specific exogenous TCRs can kill tumor cells in a cognate pMHC specific manner and also maintain their MHC-independent anti-tumor activity [156].

Hematopoietic Stem Cells:

Adoptive cell therapy with tumor antigen-specific TCR-engineered hematopoietic stem cells (HSCs) could provide a continuous supply of effector T-cells against tumors by replacing the exhausted T-cells in the tumor microenvironment. In addition, the expression of exogenous TCR in HSCs will suppress the expression of endogenous TCR via allelic exclusion, which might solve the problem of TCR mismatch and off-target reactivity. However, an exogenous TCR c-terminal linked to CD3z or a co-expressed CD3 may be required to produce fully functional cells. Autologous or donor-matched CD34 positive HSCs can easily be isolated from peripheral blood stem cells, umbilical cord blood, or bone marrow for TCR engineering and transplantation [157]. For example, antigen-specific HLA-restricted cytolytic activity by modified T-cells differentiated from NY-ESO-1 and anti-p53-antigens-specific TCR-engineered UCB were demonstrated [158].

7. Protecting cells from host attack

Universal “off-the-shelf” allogeneic don,or cells engineered with tumor antigen-specific TCR are proposed to solve many logistical hurdles of autologous T-cell therapy. However, a mismatch in donor and recipient HLA haplotypes can lead to either host rejection or cell graft-versus-host-disease (GVHD) [159]. Ongoing strategies to evade the allo-rejection include HLA-matching to the donor or lymphodepletion of the recipient. However, these strategies are not completely effective and often toxic [160] [161]Gene editing of the donor cells may provide alternative approaches (Figure 1). For example, deleting endogenous genes of TCRα/β chains, HLA, β2-microglobulin (B2M), and MHC class II transactivator (CIITA) may shield donor cells; alternatively adding genes for HLA-E, alloimmune defense receptor (ADR) and immunoglobulin-degrading enzyme of Streptococcus pyogenes (IdeS) have been attempted to improve the persistence and functionality of the infused allogeneic cells [161–166]. Deleting the genes of endogenous TCRα/β chains also can significantly reduce the chances of mispairing with exogenous TCR, potential off-target reactivity, and rejection [105,164]. Similarly, deleting genes of B2M and CIITA blocks the expression of HLA class I and II on the cell surface, making these cells not detectable by the recipient T-cells. While deleting the B2M gene leads to the downregulation of all HLA-class I molecules on the cell surface, it also puts these cells at risk of host NK cell killing. Therefore, to escape NK cells’ attack, non-polymorphic exogenous HLA class E and G genes can be inserted in these cells [161–163]. Expression of ADR on the cell surface has increased evasion of host T-cell cytotoxicity [165]. Expressing IdeS can protect cells from any potential antibody attack on the injected allogeneic cells [166]. Similarly, overexpressing CD47 in the donor cells, a “don’t eat me” signal molecule could stop macrophage-mediated phagocytosis of the injected cells [167].

Unfortunately, with multiple genetic edits, there are risks of structural genomic abnormalities and lack of uniformity in every cell. One possible solution to this problem would be performing serial gene edits in iPSC or HSC to make a single clone-based uniform cell line that can be deposited for future use. These gene-edited stem cells could be differentiated into tumor antigen-specific TCR-engineered T-cells for adoptive cell therapy without the risk of batch-to-batch variability [162,163,168]

8. Comparing a TCR versus CAR as the receptor for an effector cell.

CAR T-cell therapy involves genetically combining high-affinity single-chain variable fragments (scFv) of mAb with enhanced intracellular T-cell activating domains and transducing them into T or NK effectors. It has resulted in remarkable clinical results in B cell neoplasms [133,169], but has demonstrated limited benefit in solid tumors. There is still a need for enhanced specificity and potency, as well as mitigation of common side effects, such as cytokine release syndrome (CRS)[170], which may be due to abnormally strong signal transduction CD3ζ.

CARs have the advantage of MHC-independent antigen recognition, making this therapy more easily adaptable across different patient populations[171]. (Figure 2). As a consequence, a major drawback for CAR T-cell therapies, unlike TCR T-cells, is their inability to target intracellular antigens. Cell surface tumor antigens are generally expressed on normal tissues as well[172]. A prominent example is CAR T-cell therapy targeting CAIX for renal cell carcinoma patients which resulted in off-tumor toxicity at the bile ducts[173]. TCR T-cell therapies circumvent this roadblock through targeting of intracellular antigens in the context of MHC, and therefore access the enormous immunopeptidome that may be cancer specific[174]. Additionally, CRS severity is known to correlate with high tumor burden and high T-cell therapy dosing, highlighting T-cell overactivity as a major contributor [170]. Because of the low target antigen density, as well as more natural control of T-cell activation and function via the TCR, TCR T-cells may also be less toxic with a decreased incidence of cytokine release syndrome[171,175]. Recent advances have allowed for endogenous TCR deletion with the incorporation of the transgenic TCR using CRISPR-Cas9 editing to knock out the TRAC and TRBC loci while simultaneously incorporating the new transgenic TCR[176,177]. This results in increased expression of the transgenic TCR with less mixed dimer formation between the transgenic TCR and endogenous TCR.

Though TCR T-cell therapy may be difficult to adapt across multiple patient populations due to its MHC restriction, rapidly expanding identification of epitopes for many of the common HLA types is broadening the scope of accessible targets. This will require advancements in computational and empiric screening strategies. Additionally, TCR T-cell therapies are able to have a cytotoxic effect on cancer cells even at low antigen densities (perhaps 10’s of epitopes) because of the high sensitivity of the TCR to effectively trigger controlled clonal T-cell expansion.

Common pitfalls shared between both CAR T-cell therapies and TCR T-cell therapies include some degree of off-tumor toxicity, lack of rapid and cost-effective product manufacturing, slow identification of truly tumor-specific targets, and immune escape of tumor. Strategies to overcome some of these challenges include soluble mAb and TCR bispecific agents circumventing the need for cell production thus providing efficiency and affordability[61]. Hybrid approaches, such as TCRm gives CAR cell therapies the ability to access intracellular antigens[70]. AbTCR is another hybrid approach that gives TCRs typical antibody recognition[178,179] and are currently being studied further (Figure 3).

9. Cancer escape mechanisms: Downregulation of epitope presentation

Cancer induced downregulation:

One of many resistance mechanisms in solid tumors to TCR based immunotherapy is the downregulation or loss of cell surface HLA. [180–182]. Forty – ninety percent of human tumors are prone to HLA class I loss or downregulation, which is found to correlate with worse clinical responses, shorter overall and progression-free survival, an increase in metastasis[183–189] as well as the amount of tumor-infiltrating lymphocytes in the TME[190]. Thus, dysfunctional HLA antigen presentation may predict resistance to adoptive cell therapy and checkpoint inhibition in a clinical setting[190–192].

Genetic mechanisms for HLA loss or downregulation have been grouped into difficult-to-treat, DNA-encoded lesions, and epigenetic, transcriptional, as well as post-transcriptional alterations that are potential therapeutic targets [193]. Mutations in structural genes of the pMHC I complex, or the antigen presentation pathway have been shown to abrogate peptide antigen presentation[181,193]. The genetic HLA locus on chromosome 6p21 is frequently mutated or lost in several cancers, encoding several genes crucial for antigen presentation (i.e. HLA heavy chains, TAP1/2, tapasin)[194]. Loss of heterozygosity (LOH) associated with chromosome 6p21 is a major mechanism of reduced antigen presentation in several human tumors[183,195,196], represented in up to 17% of cancers[182]. The loss of single HLA class I molecules through somatic mutations in the HLA heavy chain genes have been reported [197,198]. Beta-2-microglobulin (B2M), which stabilizes the pMHC complex, is mutated in a variety of cancers, including melanoma, metastatic colon cancer and up to 25% of lymphomas[199–202],[201,203]. Complete loss or a functionally defective allele of TAP1/2 or loss of Tapasin and ERAP has been seen in several solid tumors including, renal cell carcinoma, colorectal-, cervical- and esophageal cancer[204–207], [208–210]. Because Interferon type I or type II signaling can induce HLA class I expression through Janus kinase and STAT signaling[211,212], LOH and mutations in JAK1/2 and STAT, as well as JAK1/2 upstream receptor APLNR also have been found to promote resistance to immune checkpoint blockade[199,213–215].

Changes in antigen presentation that are not the result of genetically encoded lesions may allow for therapeutic intervention with small-molecule drugs[193]. This includes, epigenetic silencing, mainly due to hypermethylation events on key promoters or histone modifications [216–218]. Several studies suggest that DNA methyltransferase inhibitors and histone deacetylase (HDACs) inhibitors effectively upregulate HLA class I expression in several cancer types[219–221], [222], [222–224]. MicroRNA-mediated degradation of mRNA encoding HLA class I heavy chains and transcripts of other crucial members of the peptide presentation pathway (TAP1/2, tapasin, calnexin, etc.)[193] may also be a target for intervention. [225],[226–228].

Cancers may utilize post-translational mechanisms to degrade HLA proteins, such as endoplasmic reticulum-associated degradation (ERAD)[229], autophagy-dependent mechanisms[230], and increased lysosomal degradation[231]. Interestingly, oncogenic signaling mechanisms such as MAPK activation and c-MYC and n-MYC overexpression have been found to reduce HLA class I, TAP, and B2M transcript levels and protein expression[232],

[233–235]. Finally, oxygen tension was found to reduce HLA class I expression in a HIF-1a dependent manner [236].

MHC I downregulation induced by viral infections:

Viruses also use mechanisms to evade immune recognition by downregulation of HLA class I expression when infecting host cells. Due to the focus of this review on TCR-based cancer immunotherapy, this work will not review viral mechanisms for HLA downregulation in detail. For an in-depth review, please refer to [237],[238]. Of relevance is that by specifically inhibiting steps of the antigen presentation pathway, viral immune-evasins may have the potential to be leveraged pharmacologically in gene therapy, transplantation, and auto-immunity.

10. Cellular micropharmacies

T-cell therapies alone still face many limitations in the treatment of solid tumors [239]. One very promising effort to overcome these limitations is the engineering of targeted cellular micropharmacies (TCM), a novel pharmacologic paradigm to genetically engineer or chemically modify immune cells to serve as vectors for drug delivery [240]. For example, our Synthetic Enzyme Armed Killer (SEAKER) cells secrete bacterial enzymes that accumulate in the TME. Systemic delivery of a non-toxic prodrug results in localized enzymatic unmasking in the TME, which vastly increases the therapeutic index and potential dose of the unmasked cytotoxic small molecule drug. T-cells are ideal pharmacologic vehicles to deliver payload specifically to tumors since they retain the advantages of adaptive immune cells to allow for a precise localized release of pharmacologic payload that reduces systemic toxicities of highly toxic cancer therapeutics or potent cytokines. There is also the promise of temporal control of payload release and regulation of cellular activity levels by choice of cell type and synthetic gating strategies [240–242], [240,243]. In the last 10 years, several TCM constructs have been published, carrying diverse therapeutic payloads, ranging from immune checkpoint- or TAA-targeting antibodies [244–246], scFvs [247–249] and BiTEs [250–253]; over proinflammatory cytokines[254–265], chemokines[266,267] and viral particles[268]; to ECM degrading[269] or pro-drug activating enzymes[270], immune modulatory soluble proteins[271,272] and small molecule drugs[273,274].

mAb blockade of regulatory immune checkpoints like PD-1 or CTLA-4 showed clinical efficacy in several tumors by combating T-cell exhaustion and prolonging tumor-specific immune responses[275], but are still limited by low TME penetrance in solid tumors and severe immune-related side effects.[275,276] Cellular delivery may solve these problems but have to date been restricted largely to CAR T-cells. Examples include secretion of full-length and scFv mAb to PD-1[244], [247–249], and CTLA-4 [246], and to CD47 [277–279]. Cells can also be engineered to secrete specific TAA blockers directly, as BiTEs against EGFR[250], CD3, EphA2+[251], CD19[252], and CD123[253].

Cells also are a promising approach to more safely deliver immunomodulatory cytokines (such as IL2, IL7, IL15, IL12, IL18, Flt3 ligand, GMCSF, CCL19, CCL21,) directly into the TME, initiating or potentiating tumor-specific immune responses, without life-threatening toxicity [241], [254], [255], [256,280],[281]. [266], [267].

11. Clinical Applications of TCR

TCR-based agents currently being studied in the clinic are predominantly in the forms of T-cells genetically modified to express an antitumor TCR and soluble TCR agents. Conventional αβ TCRs can recognize a massive number of peptide-MHC (pMHC) antigens with exquisite sensitivity and variable specificity, including pMHC on cancer cells in the form of tumor-associated antigens (TAA) and tumor-exclusive neoantigens. Among the first TAA found to be recognized by TCRs were those derived from MART1 [2], gp100 [3] MAGE-A1 [4,282], and Tyrosinase [5], all of which were first found to be recognized by either melanoma patient peripheral T-cells or tumor-infiltrating lymphocytes (TIL) from resected melanoma lesions. TIL present in several resected solid tumors recognizes patient-specific neoantigens [6,7,98]. When such TIL are expanded ex vivo and reinfused, they can induce durable regressions in solid metastatic tumors [8,283], thus demonstrating the therapeutic potential of neoantigen targeting. Similar to TAA, neoantigens produced by somatic mutations exclusive to cancer cells are becoming increasingly appreciated as tumor rejection antigens that can be targeted by TCR therapy. Moreover, various neoantigen qualities, such as clonality, MHC binding properties, and immunogenicity, have been shown to predict response to immune checkpoint blockade [284,285]. Because T-cells generated in vivo in patients are endowed with specificity for tumor antigens, there has been significant interest in the clinical development of a class of TCR-based agents for cancer immunotherapy.

Following the observation that melanoma patient TIL recognizes TAA and can induce cancer regression [286], early clinical studies utilized TAA-specific TCR-transduced T-cells to treat metastatic melanoma [36,287]. Though targeting TAA was initially thought to be safe due to their restricted expression, an affinity-enhanced MAGE-A3 TCR was found to exert off-target reactivity to cardiac tissue, causing fatal toxicity when expressed in T-cells adoptively transferred to melanoma and myeloma patients [21,35]. Similarly, a MAGE-A3/A12 TCR was found to cause fatal on-target/off-tumor reactivity to neuronal tissue [44]. The toxicities observed with affinity-enhanced TCRs targeting conserved TAA have shifted clinical interest towards using patient-derived TCRs to target neoantigens, of which entirely non-self-peptides can be targeted if sufficient somatic mutations are acquired in the tumor. Two allogeneic TCRs targeting the public KRAS G12D/C*0802 neoantigen were used to engineer autologous T-cells, which were reinfused to induce objective regression of metastatic pancreatic cancer [288]. In a similar approach, a library of 39 patient-derived TCRs to common TP53 mutations with various HLA restrictions were used to select an allogeneic TCR to redirect patient T-cells to the HLA-A*02-restricted p53 R715H public neoantigen [289]. The resulting TCR-engineered T-cells were reinfused and induced objective regression of breast cancer lasting six months. To demonstrate the feasibility of neoantigen calling and TCR identification at a scale to treat a large cohort of patients, a recent effort demonstrated the feasibility of identifying patient-specific neoantigens, their cognate TCRs, and manufacture of neoantigen TCR-engineered T-cells, dosing 16 patients with various solid tumors [290]. TCR-engineered T-cells are also being investigated for treating hematologic malignancies, particularly for AML/MDS by targeting the differentially expressed TAA WT1 [18,291–293]. Interestingly, relapse after WT1 TCR therapy was associated with antigen escape not by WT1 mutation or HLA downregulation

but by immunoproteasome regulation [294], a challenge that can be overcome by informed epitope selection.

Given the prominent role of T-cells in clearing viral infections, viral malignancies are expected to be amenable to TCR therapy. To this end, TCRs targeting HPV antigens are under investigation for cell therapy of various HPV+ epithelial malignancies [19]. Unlike TAA, HPV targeting can induce objective responses without significant toxicities, a safety feature most likely attributable to the non-self character of viral antigens. HBV antigens are also under clinical investigation for TCR therapy of hepatocellular carcinoma [295–297]. In a small cohort, HBV TCR-engineered T-cells could cause stabilization of HBV antigen or DNA levels in most patients and tumor lesion reduction in some patients. The conclusion of future trials will elucidate the potential of TCR-engineered T-cells for treating advanced viral and non-viral cancers. Clinical studies of adoptive cellular therapies utilizing antitumor T-cells from allogeneic sources without genetic modification are reviewed elsewhere [298].

Soluble agents with TCR-like recognition have also generated significant clinical interest. If shown to be efficacious, it may significantly advance cancer immunotherapy by redirecting T-cells to tumor antigens without lengthy and complex ex vivo cell engineering protocols. ImmTACs are the first soluble TCR-based agents to be approved by the FDA [299]. Tebentafusp utilizes a TCR domain specific for an HLA-A*02-presented gp100 epitope to redirect T-cell killing to melanoma cells, which manifests in a clinical benefit of a 14% higher 1-year overall survival in uveal melanoma patients. Other ImmTAC molecules currently in clinical trials include IMC-F106C specific for HLA-A*02/PRAME, and IMC-C103C specific for HLA-A*02/MAGE-A4 for the treatment of advanced solid tumors (NCT04262466, NCT03973333). For AML treatment, RO7283420, a T-cell bispecific in IgG format targeting HLA-A*02/WT1, is currently in phase I trials (NCT04580121).

12. Conclusions

Although the TCR-based therapeutic agents are nearly two decades behind mAb-based agents in their scientific and clinical development, TCR-based agents, whether incorporated into cells or as soluble drugs, are poised to be increasingly important therapies for cancer. Recent advances in understanding TCR structure and recognition features has accelerated their transition into both soluble agents, with platforms similar to mAb such as bispecific agents and engineered cells. In principle, the ability of TCRs to recognize truly cancer-specific epitopes, and intracellular targets, unlike traditional antibodies and most small molecules, opens the door to a new class of potentially non-toxic and effective drugs not previously envisioned. The number of potentially useful targets for TCRs will ultimately dwarf that available to traditional mAb. Already, TCR-based tools are available for transcription factors, cancer-germline antigens, oncofetal antigens, neoantigens, post-translationally modified proteins, tumor-associated antigens, and oncogenically mutated proteins. There is every expectation that drugs for each of these classes of targets will become available for use within the decade. Because TCRs are the natural receptor for T-cells, their use may also provide both better potency and control than CAR-engineered cells. However, a number of open areas of study remain, including: 1. Better understanding of the targets and off-targets of the agents, 2. New ways to render the molecules more

stable and with longer plasma half-lives when soluble, 3. Controlling TCR protein signaling and protein associations within engineered cells, 4. Improving approaches to affinity enhancement without loss of specificity, 5. Methods of creating drug- or radio-conjugates that may be clinically useful. 6. Automating and expediting the retrieval of patient's TCRs. Notably, the pace of discovery of tools and prototypes to address these issues has accelerated, and many academic and industrial laboratories are currently tackling these problems. Therefore, the future appears promising.

Funding Information:

NIH RO1 CA55349, PO1 CA23766, NCI P30 008748, R35 CA241894 and NCI 1R50CA265328-01A1.

Conflict of Interests

DAS is on a board of, or has equity in, or income from: Lantheus, Sellas, Iovance, Pfizer, Actinium Pharmaceuticals, OncoPep, Repertoire, Atengen, Sapience, Coimmune, and Eureka Therapeutics. TD is a consultant for Eureka Therapeutics.

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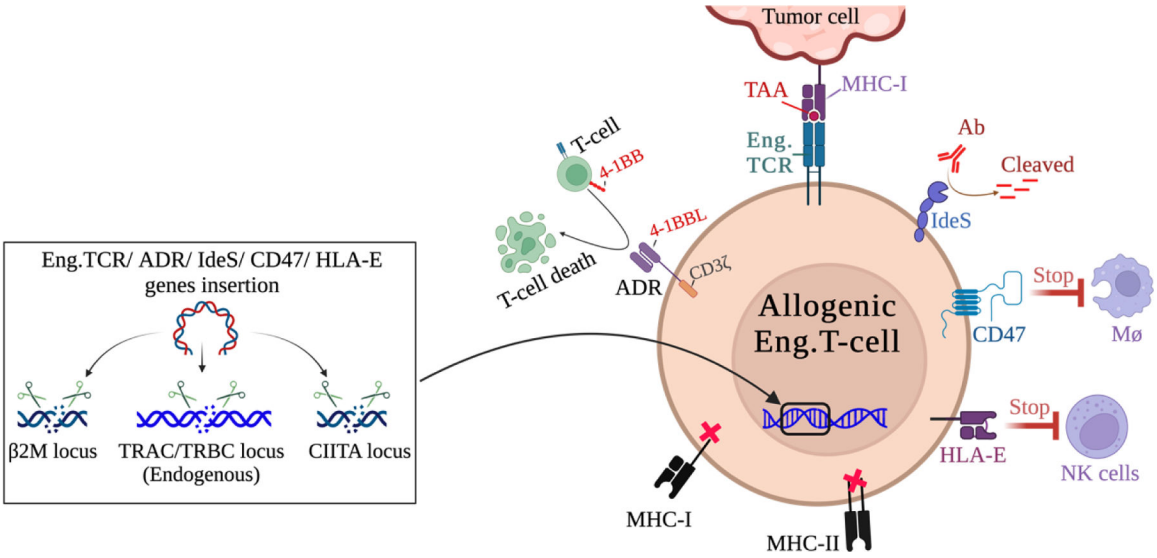

Figure 1. Protecting the Engineered cell.

There are two general strategies to generate stealthy, off-the-shelf allogenic donor cells for adoptive cell therapy. Methods include editing endogenous genes of TCRα/β chains, β2-microglobulin (B2M), and MHC class II transactivator (CIITA); by CRISPR mediated gene deletion or disruption of their expression by insertion of new genes such as engineered TCR can reduce recognition by the host. Alternatively, alloimmune defense receptor (ADR), "Do not eat me" CD47, Streptococcus pyogenes (IdeS), and HAL-E proteins can protect allogeneic cells from rejection by the host immune cells. See section 7 for more information. All figures were created by using BioRender.

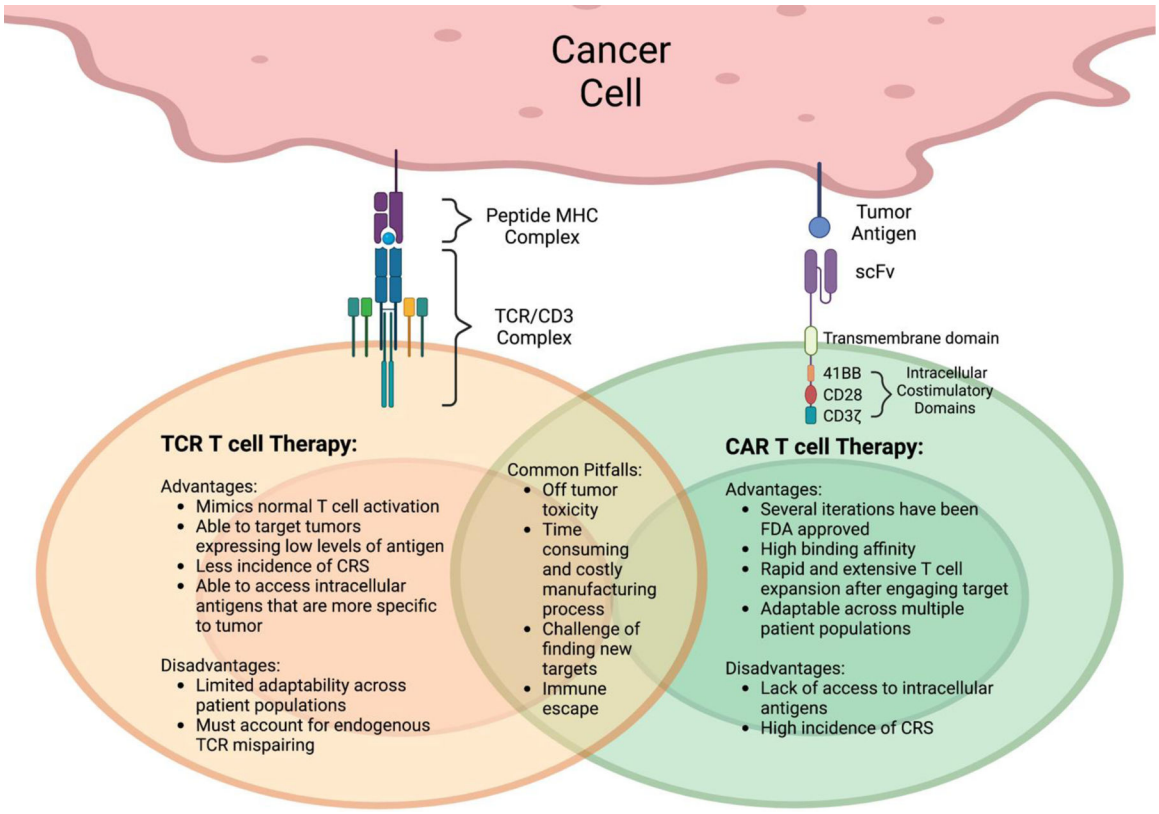

Figure 2. Comparison of Characteristics of TCR T-cell and CAR-T-cell formats.

(left) TCR therapy targets a large universe of intracellular tumor antigens that are presented on the cell surface as peptide fragments by MHC molecules. The recognition of the antigen occurs via alpha beta TCR/CD3 complex. **(right)** Most current CAR-T-cell therapies target cell surface and lineage proteins that are shared between tumor and normal cells. The recognition of the target is through the scFv of an mAb directed to the surface target protein, which was linked to the T-cell activation molecules CD28 or 41BB and CD3 zeta chain. All figures were created by using BioRender.

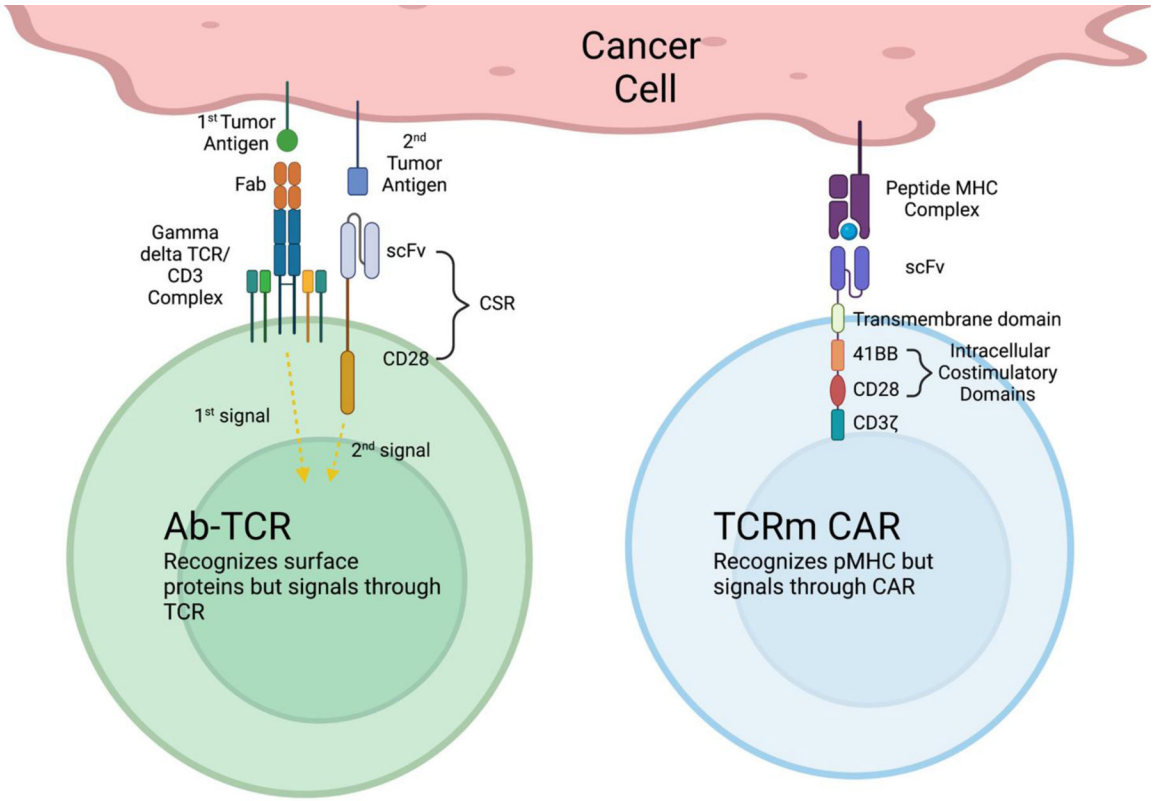

Figure 3. Characteristics of new hybrid T-cell formats.

(left). Ab-TCR is a new TCR/CAR-T format, which consists of two separate activation domains: the first domain uses a Fab specific for a tumor antigen, linked to gamma/delta TCR to facilitate a natural T-cell activation. The second domain uses a scFv of an mAb targeting a second tumor antigen, linked to a costimulatory molecule, CD28, down-stream of a signaling molecule needed to for fully activate T-cells and duel targeting tumor antigens This new format of CAR-T-cells could avoid excessive synthetic activation and toxicity caused by traditional CAR-T-cells that assemble T-cell activation molecules in one construct. (right). TCRm CAR-T-cells use traditional CAR-T construct, however, they are able to target intracellular tumor antigen-derived peptide/MHC complexes, by using scFv derived from TCR mimic mAbs. CSR: Costimulatory signaling receptor. All figures were created by using BioRender.

Table 1.

Hurdles to TCR-based Therapeutics.

Issues for each class of agent	Possible Solutions Available	Citations
TCR engineered cells.		
Patient specific cells	Use off the shelf allogenic cells	
TCR mispairing	Use cells with CRISPR deleted native TCR; knob and hole paired chains; mouse chains; framework engineering, domain swapped TCRs, single chain TCRs.	101–106
Immunosuppressive TME	Use of cytokine or chemokine armored cells, deletion of checkpoint molecules, ex vivo selection of optimal subsets or conditioning with cytokines	241, 254, 255, 256,280,281. 266, 267,300
Poor Penetration into tumor	Use of cytokine or chemokine armored cells, ex vivo selection of optimal subsets or conditioning with cytokines	241, 254, 255, 256,280,281. 266, 267,300
Lack of Persistence	Use of cytokine or chemokine armored cells, overexpression of transcription factors that promote persistence or protect against exhaustion, ex vivo selection of optimal subsets or conditioning with cytokines	241, 254, 255, 256,280,281. 266, 267,300,301
Manufacturing logistics	Automated techniques; off the shelf cells including HLA-matching banked cells and differentiation from iPSC	162,163,168 161 161 165
GVHD if allogeneic cells	Delete native TCR from cells, cell subset selection such as EBV/CMV sensitization, CD137- or CD8-depletion	176,177. 114.302
Graft rejection, if allogeneic cells	Delete HLA, B2M, & other presentation machinery. Introduce HLA-E or IdeS into cells.	161–166.
Soluble TCR-based agents.		
Low affinity	Affinity maturation	59.
Difficult protein engineering	New technology is advancing	
Both soluble and cell-based agents.		
Lack of broad “public” neoantigens	Extensive in silico searches. Empiric MS-based searches.	52 53. 54, 55,56 41,57
Antigenic heterogeneity	Use of multiple agents; Use of essential (driver oncogene) targets	
Escape by HLA loss or downregulation	Pharmacologic interventions	219–221, 222, 222–224.
Escape by antigen presentation loss	Pharmacologic interventions	219–221, 222, 222–224.
Escape by epitope mutation	Use of driver mutations or essential targets	
Off-target toxicities (cross-reactivities)	Better in silico screening and empiric screening of TCR	52 53. 54, 55,56 41,57
On-target, off-tumor toxicities	Better proteomic screening via healthy tissue HLA ligand databases and empiric MS search	11. 58,

Table 2.

Typical Features of Immunoglobulin Super Family Therapeutic Agents

Feature	IgG Antibody	TCR mimic	TCR
Isoforms	Multiple	Multiple	Alpha/beta or gamma/delta
Structure	Homodimer	Homodimer	Heterodimer
Mass (Daltons)	150,000	150,000	40,000/ 80,000
Affinity (typical; native)	High: 0.1–10nM	High: 0.1–10nM	Low: 0.1–10uM
Target antigens	All accessible molecules	Peptide/MHC	Peptide/MHC; Lipid, peptide, metabolite/CD1, MR1, HLA-E; Non peptidic-phospho-antigen/BNT3A1
HLA restriction	No	Yes	Yes and No
Soluble forms (native)	Yes	Yes	No
Membrane bound (native)	No	No	Yes
Typical Platforms:			
● Native	Yes	Yes	Yes
● Fc modified	Yes	Yes	N/A
● Truncated forms	Various	Various	Yes
● Bispecific forms	Yes	Yes	Yes
● ADC *	Yes	Yes	No
● RIC *	Yes	Yes	No
● CAR * or T-cell	Yes	Yes	Yes
Half-life (soluble forms)	Long (weeks)	Long (weeks)	Short (hours)
Specificity	High	Variable	Variable
Marketed	Multiple	No	One (as of 2022)
Current clinical Indications	Diverse & many	Cancer	Cancer
Discovery/development	Simple	Complex	Complex

* ADC =Antibody Drug Conjugate; RIC = RadioImmunoConjugate; CAR =Chimeric Antigen Receptor.

Citations for this are [303–307], [306–308], [308,309]

Table 3.

Human TCRm and Their Formats

Antigen target	HLA restriction	Diseases	Formats	Citations
Proteinase 3	A*02:01	Myeloid Leukemias	IgG, BisAb, CAR T-cell	67,309
WT1	A*02:01	Leukemias and various solid tumors	IgG, BiTE, full length BisAb, CAR T-cell	68–70,74
PRAME	A*02:01	Leukemias and various solid tumors	IgG, BiTE, CAR T-cell	310
FOXP3	A*02:01	Tregs, FOXP3+ T-cell malignancies and other types of cancers	IgG, BiTE	311
HPV-E7	A*02:01	Cervical cancer, many other HPV-associated tumors, head and neck cancers	IgG, BiTE	312
pIRS2	A*02:01	Ovarian, breast, colon, pancreatic, hepatocellular carcinoma, neuroblastoma, glioblastoma, melanoma, prostate, bladder, NSLC, CLL, MCL	IgG, BisAb	77
p53 mutation (R175H)	A*02:01	Multiple myeloma, ovarian cancer and many solid tumors	Fab, scDb	313
Ras G12V	A*03 A*01	Wide range of solid tumors: pancreatic, colon, ovarian and more	scDb	314
Epstein Barr Virus	A*02:01	B cell lymphoma and carcinoma	IgG	315
WT1	A*24:02	Leukemias and various solid tumors	CAR T-cell	316
Minor HA-H1	A*02:01	Leukemias	CAR T-cell	317
AFP	A*02:01	Hepatic carcinoma	CAR T-cell	71
hCG-beta	A*02:01	Ovarian, colon, and breast cancer	hIgG1, mIgG2a	318
NY-ESO-1	A*02:01	Melanoma and solid tumors	Fab, CAR T-cell	79,319
MAGE-A1	A*01:01	Melanoma	CAR T-cell	78
GP100	A*02:01	Melanoma	CAR T-cell	320
MUC-1	A*02:01	Breast cancer	Fab	321
hTERT	A*02:01	Melanoma and prostate cancer;	Fab	322
HIV	A*02:01	HIV	scDb	323
NDC80	A*02:01	Leukemias and various solid tumors	CART	323,324

Only TCRm mAbs against human targets are listed.