Cancer Therapy With TCR-Engineered T Cells: Current Strategies, Challenges, and Prospects

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To redirect T cells against tumor cells, T cells can be engineered *ex vivo* to express cancer-antigen specific T cell receptors (TCRs), generating products known as TCR-engineered T cells (TCR T). Unlike chimeric antigen receptors (CARs), TCRs recognize HLA-presented peptides derived from proteins of all cellular compartments. The use of TCR T cells for adoptive cellular therapies (ACT) has gained increased attention, especially as efforts to treat solid cancers with ACTs have intensified. In this review, we describe the differing mechanisms of T cell antigen recognition and signal transduction mediated through CARs and TCRs. We describe the classes of cancer antigens recognized by current TCR T therapies and discuss both classical and emerging pre-clinical strategies for antigen-specific TCR discovery, enhancement, and validation. Finally, we review the current landscape of clinical trials for TCR T therapy and discuss what these current results indicate for the development of future engineered TCR approaches.

Keywords: T cell receptor, TCR, chimeric antigen receptor, CAR, TCR-engineered T cells, TCR T, adoptive cell therapy

INTRODUCTION

The past decades have seen rapid advancements in our understanding of the mechanisms underlying the antitumor function of immune cells, and as such adoptive cell therapy (ACT) strategies have emerged as a major platform of cancer therapeutics. A milestone in ACT was the success of tumor infiltrating lymphocyte (TIL) therapy for metastatic melanoma beginning in the 1980s (1). While TIL therapy remains an important ACT modality, the manufacture of TIL products is logistically challenging. ACT efforts have thus largely transitioned towards strategies to engineer peripheral blood T cells with receptors that confer desired antigen specificity. These predominantly include chimeric antigen receptor T cell (CAR T) and T cell receptor engineered T cell (TCR T) therapies. Due to the remarkable efficacy of CAR T therapies in treating B cell malignancies (2), interest in CAR T therapy has eclipsed that of TCR T therapy. However, TCR T therapy is gaining interest as CAR T trials have so far failed to elicit satisfactory responses in the treatment of solid cancers (2), and many believe TCRs may be better suited for the treatment of solid

cancers (3). Indeed, exciting clinical results are now emerging that demonstrate safety and efficacy of TCR T therapies in both hematological and solid cancers. In this review we describe the biology of TCRs and tumor antigen targets and discuss state of the art techniques for TCR discovery and preclinical assessment. Finally, we describe the current landscape of TCR T trials and the challenges that remain.

BIOLOGY OF TCRs AND TUMOR ANTIGEN TARGETS

Redirecting T Cell Specificity Through Genetic Engineering

Conventional T cells recognize MHC-presented antigens through their T cell receptor (TCR), a disulfide-linked heterodimer comprised of an α and β chain. To form a functional receptor, TCR α/β heterodimers further complex with CD3ε/γ/δ/ζ subunits (4–7). TCRs recognize enzymatically

cleaved peptides that are presented at the cell surface by MHC molecules (pMHC). In humans, antigen-presenting MHC alleles are broadly classified as HLA class I (A, B, or C) or HLA class II (DR, DP, or DQ), which predominantly present cytosolic or extracellular derived peptides, respectively (4). The coreceptors CD8 and CD4 enhance TCR antigen sensitivity through interaction with MHC class I or II molecules, respectively (8). TCR binding to cognate pMHC leads to the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in intracellular regions of the CD3 subunits (5, 6), which results in T cell activation and initiation of effector functions including proliferation, cytokine secretion, and cytolysis *via* secretion of perforin and granzyme (**Figure 1**). In TCR T therapy, T cells are edited to express TCR α and β chains that confer a desired specificity. Here, introduced TCR α and β chains dimerize and complex with endogenous CD3 components to form a functional TCR that redirects T cell specificity towards an antigen of interest.

Another common method for redirecting T cell specificity is through the genetic transfer of chimeric antigen receptors
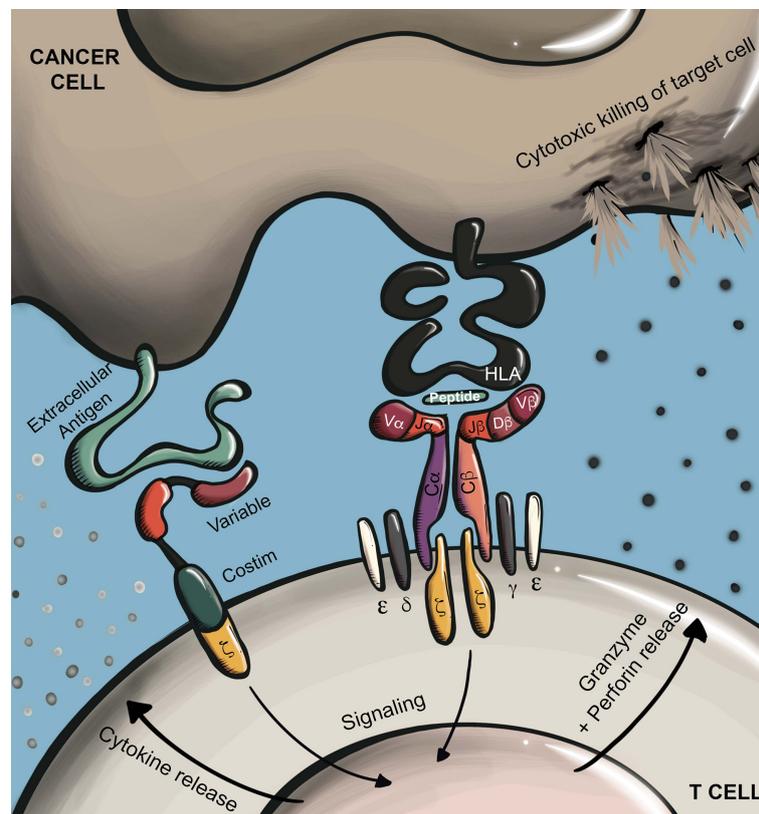

FIGURE 1 | Antigen recognition by CARs and TCRs. CARs recognize surface proteins typically through an antibody-derived scFv recognition domain. Antigen recognition leads to T cell activation *via* phosphorylation of ITAMs in a conjugated intracellular CD3ζ domain. In the case of later generation CARs, ligand binding also leads to additional stimulation of conjugated costimulatory receptors (e.g. CD28, 4-1BB). TCRs recognize HLA-presented peptides which may be derived from any cellular compartment. Antigen recognition by TCRs leads to T cell activation through phosphorylation of ITAMs in the associated CD3ε/γ/δ/ζ subunits. Depending on T cell subtype, T cell activation through either receptor type will trigger effector functions including proliferation, cytokine secretion, and target cell killing through directed secretion of perforin and granzyme.

(CARs), which are broadly comprised of an extracellular-facing antigen-binding domain linked to an intracellular immune cell activation signaling domain. Most often, CARs recognize antigen through the single-chain variable fragment (scFv) of an antibody. In a typical CAR design, the antigen-binding scFv is linked *via* a hinge, or spacer, region to a transmembrane domain that is further conjugated to an intracellular CD3ζ signaling domain. In this manner, antigen binding by the scFv drives CD3ζ phosphorylation and downstream T cell activation. Later generation CARs include the addition of intracellular costimulatory domains such as CD28 and 4-1BB, which further improve CAR T function and persistence (**Figure 1**) (9).

TCRs vs CARs

While TCRs recognize antigens in the context of HLA presentation, CARs recognize natively folded proteins at the cell surface. Therefore, CARs overcome clinical limitations imposed by the HLA-restriction of TCRs. HLA encoding genes are the most polymorphic in the human genome, with over 20,000 HLA-class I alleles identified to date (10). Therefore, unlike CAR T therapy, patients selected for TCR T therapy must express not only the targeted antigen, but also the corresponding antigen-restricting HLA allele. For this reason, TCR T therapies typically utilize TCRs that are restricted to relatively common HLA alleles, such as HLA-A*02:01, which is present in about 47.8% and 16.8% of Caucasian and African American populations in the United States, respectively (11).

As CARs and TCRs utilize differing signaling mechanisms, they exhibit several important differences in their functional response to antigen stimulation. While TCRs can elicit a cytotoxic response to as few as a single pMHC molecule, CARs typically require thousands of target surface molecules to mediate an effective response (12–14). A consequence of the reduced antigen sensitivity of CARs is seen in patients with B cell malignancies who initially respond to CAR T therapy but subsequently relapse with progression of antigen-low cancer cells (15). Upon stimulation, CARs mediate supraphysiologic T cell activation, leading to enhanced cytokine secretion. For this reason, CAR T cells are more likely to cause cytokine release syndrome (CRS) in patients as compared to TCR T cells; however, recent advancements in treatment have made CRS generally clinically manageable (16, 17).

Tumor antigens recognized by CARs must be located at the surface of cancer cells. Conversely, TCRs recognize HLA presented peptides that may be derived from any cellular compartment. As transmembrane proteins constitute only an estimated 14-26% of the proteome (18–20), CAR-targetable antigens are considerably more limited. However, the repertoire of CAR-targetable antigens is extended to some degree by the ability of CARs to recognize not only protein antigens, but also other molecules like glycoproteins and glycolipids (21). This difference in repertoire of potential targetable antigens has significant implications for CAR T and TCR T therapies, which must aggressively target tumor cells while avoiding toxicity directed towards healthy tissue. Indeed, expression of target antigen on normal cells can lead to T cell-mediated destruction of healthy tissue, known as 'on-target off-tumor' toxicity (22). Therefore, the degree to which a target

antigen is exclusively expressed by cancer cells is an important factor. So far, the primary success of CAR T therapy has been in the treatment of B cell malignancies targeting CD19, an antigen expressed ubiquitously on malignant and healthy B cells. While CD19-directed CAR T therapy leads to ablation of both malignant and healthy B cells, such on-target off-tumor toxicity is clinically manageable through replacement antibody therapy (22). However, in the case of many other types of cancer, including almost all solid cancers, such T cell-mediated ablation of healthy organ tissue is not clinically manageable, and thus target antigens with exclusivity of expression in cancer cells are best. Currently described tumor antigens with the greatest specificity of expression in cancer cells are predominantly intracellular derived antigens, accessible to TCRs but not CARs (23). Therefore, TCR T therapies may have an advantage over CAR T therapies in the ability to aggressively target cancer cells while minimizing toxicity.

TCR Targeted Tumor Antigens

Significant progress has been made in identifying the precise cancer antigens that mediate immune rejection. While the nomenclature describing tumor antigens varies, widely studied classes of tumor antigens include tumor-associated antigens (TAAs), cancer-germline antigens (CGAs), and tumor-specific antigens (TSAs) (24–26) (**Figure 2**).

TAAs

TAAs are expressed by tumor cells but are also expressed in at least some healthy tissue. As a result, therapies targeting TAAs must contend with potential T cell mediated on-target off-tumor toxicity. TAAs are further classified as differentiation antigens or overexpressed antigens.

Differentiation antigens are expressed by cancer cells as well as normal cells of the same tissue origin. Melanoma differentiation antigens were among the first discovered tumor antigens and include the widely studied melanoma-associated antigen recognized by T cells (MART-1) (27) and glycoprotein 100 (gp100) (28). Among tumor antigens, differentiation antigens typically pose the greatest risk for on-target off-tumor toxicity. As discussed in further detail in a later section, clinical experience has demonstrated that targeting differentiation antigens is likely only clinically appropriate when antigen expression is restricted to dispensable healthy tissue, such as CD19-expressing B cells. Likely for this reason, only one TCR T clinical trial targeting a solid cancer differentiation antigen has been initiated since 2012 (**Supplementary Table 1**).

Overexpressed antigens are expressed at high levels in cancer cells but are minimally expressed in healthy cells. While targeting such antigens continues to pose a risk for on-target off-tumor toxicity, the differential expression between cancer and normal cells allows for the possibility of achieving a therapeutic window by which adoptively transferred T cells may destroy high-antigen expressing cancer cells with minimal destruction of low-antigen expressing healthy tissue. An example of a widely studied overexpressed antigen is Wilms' Tumor Antigen 1 (WT1), a transcription factor with 10- to 1000- fold higher expression in leukemic cells as compared to normal cells (29, 30).
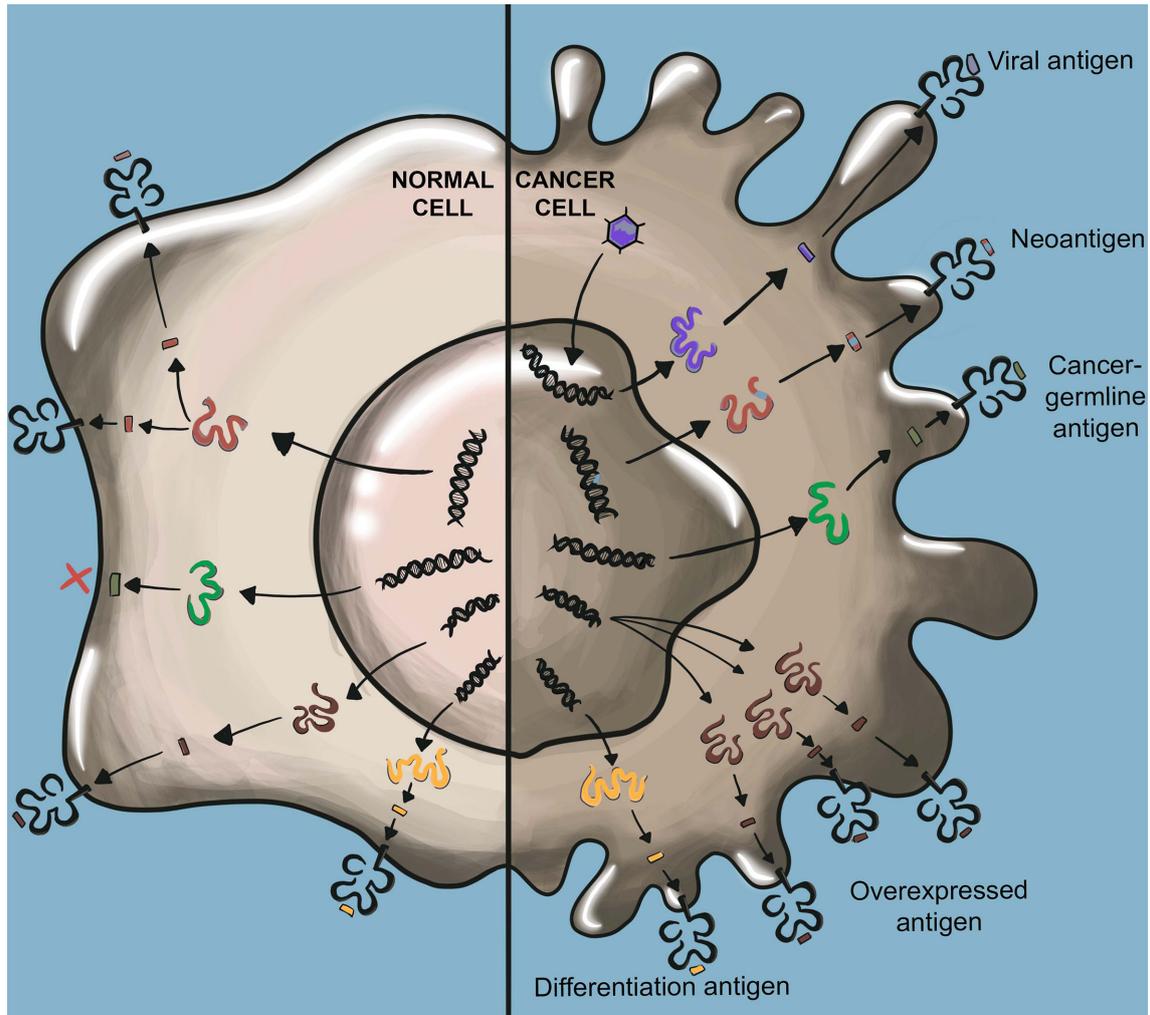
FIGURE 2 | TCR-recognized tumor antigens. Viral antigens result from viral oncogenes which are not present in normal cells. Neoantigens arise from somatic mutations not found in normal cells. Viral antigens and neoantigens are collectively referred to as tumor-specific antigens (TSAs). Cancer germline antigens (CGAs) are derived from proteins that are normally only expressed in germ cells such as testis which lack HLA class I expression. Overexpressed antigens arise from proteins highly overexpressed in cancer tissue as compared to normal tissue. Cancer differentiation antigens are expressed by cancer cells and their expression is otherwise limited to only the normal cells of the same tissue origin as the cancer. Overexpressed antigens and cancer differentiation antigens are collectively referred to as tumor-associated antigens (TAAs).

CGAs

CGAs are aberrantly expressed in cancer cells while their expression in normal tissue is restricted to germline cells, such as those of testis, which lack HLA-class I expression, thus greatly reducing the risk for on-target off-tumor toxicity. As such, CGAs are currently among the most aggressively pursued targets for TCR-based immunotherapies. Examples of CGAs with high clinical significance include NY-ESO-1 and MAGE-A4, which are detected with high levels of expression in various solid and hematological cancers (31–35). However, several studies have reported that CGAs are heterogeneously expressed within tumors, which could limit potential therapeutic efficacy when targeting a single CGA (36).

TSAs

TSAs are genetically encoded in cancer cells but are not present in the genome of any normal cells. TSAs are further classified as viral antigens or neoantigens.

Many human cancers are caused by viral infections, such as human papillomavirus (HPV) (37), hepatitis B virus (HBV) (38), and Epstein-Barr virus (EBV) (39, 40). In many cases, virus-driven cancers are mediated by the expression of viral oncogenes that drive cellular transformation and cancer progression (41–43). As viral oncogenes are often homogenously expressed in virus-driven cancers, and their expression is nearly absent in normal cells, they represent highly attractive tumor antigen targets. Specific examples of clinically relevant viral antigens

include the HPV viral oncogenes E6 and E7, which are expressed in several types of epithelial carcinoma (37, 44, 45), and the EBV viral oncogenes LMP1 and LMP2, which are expressed in several solid and hematological cancers (40, 46–49).

Genomic instability is a cardinal feature of cancer (50), which results in the accumulation of many tumor-specific mutations. Some of these mutations will give rise to new proteins, or neoantigens. As neoantigens are expressed exclusively by cancer cells, these serve as attractive targets for ACT that would pose essentially no risk for on-target off-tumor toxicity. However, a challenge is that the vast majority of cancer mutations are so-called bystander mutations, which do not enhance the fitness of the cancer cell. Such random, non-selected mutations are typically heterogeneously expressed and are unlikely to be shared across patients, rendering them ineffective antigen targets. Conversely, a small fraction of cancer mutations improve cellular fitness and directly promote cancer progression, which are known as driver mutations. These mutations may be expressed homogenously by cancer cells and shared among patients within particular cancer types (51–54). If immunogenic and restricted to a common HLA, such driver mutations give rise to so called 'public neoantigens' (55–57). While few public neoantigens have been discovered so far, these highly selective targets are of significant clinical interest. Examples of currently described public neoantigens include KRAS G12D/G12V, collectively found in 60-70% of pancreatic adenocarcinomas and 20-30% of colorectal cancers (58), and PIK3CA H1047L, detected in about 5% of metastatic breast cancers (59, 60).

STRATEGIES FOR THE ISOLATION AND TRANSGENIC EXPRESSION OF ANTIGEN-SPECIFIC TCRs

Enrichment of Antigen-Specific T Cells

V(D)J recombination of TCRs during thymic development results in a tremendous diversity of TCR sequences within the human T cell repertoire. It is estimated that in an average adult human, there are approximately 4×10^{11} total circulating T cells and an estimated 10^{10} unique T cell clonotypes (61). Thus, for the vast majority of T cell clones with specificity towards non-viral antigens, the clonal frequency in peripheral blood is far below what is needed to perform the various manipulations required to isolate antigen-specific TCRs given current technologies. Therefore, TCR isolation efforts generally begin with a method that allows for enrichment of T cells with the desired antigen specificity. The following section describes several common T cell enrichment methods employed for TCR discovery.

Expansion of Tumor Infiltrating Lymphocytes

In certain types of solid cancers there is often a large presence of tumor infiltrating lymphocytes (TILs) (62). Compared to peripheral blood T cells, T cells within the tumor tissue are often enriched in clones with tumor-antigen specificity. Several groups have used expanded TILs as sources for discovery of tumor specific TCRs (63–67). Of note, for decades *ex vivo* expanded TILs have in

themselves served as an effective ATC for several types of solid cancer (66–70). The TIL therapy lifileucel from Iovance has demonstrated strong efficacy in clinical trials and the company plans to submit for FDA approval (71).

Vaccination

T cells with specificity towards antigens of interest can be selectively expanded *in vivo* through vaccination strategies. A common approach is to vaccinate human HLA transgenic mice with an antigen of interest, which can result in robust enrichment of antigen-specific T cells harvested from the lymph nodes and spleen (46, 58, 72). In select cases, the peripheral blood of patients participating in cancer vaccine trials has been used as a source of antigen-enriched T cells for TCR discovery (73, 74).

Selective *In Vitro* Expansion of Peripheral Blood T Cells

Several strategies have been developed to stimulate peripheral blood T cells *in vitro* in an antigen-specific manner, driving the selective expansion of T cells with a desired specificity. Early pioneering work in this regard performed *in vitro* stimulations of peripheral blood T cells to preferentially expand virus-specific T cells (75–78). These stimulation methods have since been used to expand T cells enriched in specificity for TAAs (79–81) and neoantigens (60, 82, 83). These approaches typically stimulate T cells *via* autologous antigen presenting cells (APCs), usually dendritic cells (DCs), pulsed with the antigens of interest in the form of exogenous peptide or through cDNA/RNA delivery (60, 80, 82–84). In the case of patient-derived peripheral blood, several studies have shown that initial selection of PD-1+ and/or antigen-experienced (CD45RO+CD62L+, CD45RO+CD62L-, or CD45RO-CD62L-) T cells can further enhance *in vitro* enrichment of tumor-specific T cells (82, 83, 85–87).

To overcome the requirements of generating autologous mature DCs for antigen stimulation, several groups have developed so called artificial antigen presenting cells (aAPCs). One common aAPC system uses the myelogenous leukemia cell line K562, which is negative for HLA-A, B, and DR. This cell line serves as a modular aAPC through the stable transduction of various HLA alleles and costimulatory molecules. Other cell-free aAPC systems have been developed that conjugate HLA and costimulatory molecules onto beads and nanoparticles (88–90).

Isolation of Antigen-Specific T Cells

After obtaining polyclonal T cell products that are enriched for T cells with specificities of interest, it is necessary to isolate the antigen-specific T cells from the bulk T cell population.

Approaches in this regard typically involve stimulating T cells with the cognate antigen of interest, and then isolating antigen-responsive T cells based on increased expression of known T cell activation-associated molecules. This includes antibody staining of transmembrane proteins that are transiently upregulated following T cell stimulation (e.g., 4-1BB and OX40 in CD8+ and CD4+ T cells, respectively), allowing for isolation of these cells by FAC sorting or magnetic bead separation (82, 83). Another approach is IFN-γ-capture, whereby antigen stimulated T cells are identified and captured based on

production of IFN-γ, which is rapidly secreted by antigen-stimulated CD8+ and Th1 CD4+ T cells. In certain cases, staining with peptide-HLA multimers followed by FAC sorting or magnetic bead separation is an efficient method to identify and isolate antigen specific T cells (63). However, this requires upfront knowledge of an antigen restricting HLA and minimal epitope. Although the repertoire of HLA multimer reagents is expanding, these reagents remain limited to relatively common HLA alleles.

TCR Sequencing

TCR α and β chains of T cells of interest are then cloned from cDNA through PCR amplification. However, a unique challenge is that their 5' regions are highly variable. To overcome this, one of two PCR variations are typically employed, 5' RACE or multiplex PCR (66, 67, 73, 79, 82, 91). The fact that TCR specificity is encoded by regions of two separate genes imposes a unique challenge for determining functional TCR sequences from a population of T cells; that is, once T cells are lysed for RNA extraction, the TCR α and β transcripts from each T cell clone intermix, making it ambiguous as to which TCR α sequence pairs with which TCR β sequence. Therefore, prior to sequencing the functional TCR α and β chain transcripts of antigen-specific T cells, it is typically required to first separate individual T cell clones. While by no means exhaustive, we outline here several classical and emerging strategies to isolate T cell clones for TCR sequencing.

Limiting Dilution

A classical method for obtaining T cell clones is the outgrowth of T cell clones in individual wells. In the limiting dilution method, T cells are diluted to obtain a cell concentration allowing for approximately one cell to be deposited into each well of a 96-well plate. An alternative method is to FACs sort the T cell population to deliver a single cell into each well. The goal is to obtain expanded clonal populations of the T cells of interest, which can then be additionally screened for antigen-specificity and sequenced *via* Sanger sequencing (27, 46, 72, 73, 84, 92–97).

Single Cell RT-PCR

Several studies have obviated the need to expand T cell clones following antigen-specific T cell separation by instead performing single cell RT-PCR to amplify TCR α and β chains. In such methods, single T cells are FAC sorted into wells containing RT-PCR reaction buffer, and from a single cell RT-PCR is performed and the TCR α and β chains are PCR amplified (66, 79, 82, 83, 91, 98). This method reduces the time and labor required for expansion of individual T cell clones; however, a downside to this approach is that confirmatory assays to assess antigen specificity cannot be performed on the T cell clones prior to sequencing.

Single-Cell RNA Sequencing

Single-cell RNA sequencing (scRNAseq) is a rapidly advancing technology that has emerged as a uniquely effective platform for TCR discovery, as it allows for single-cell assessment of cellular gene expression as well as the sequence of gene transcripts. As such, several recent studies have successfully used this platform for TCR discovery by stimulating T cells with antigens of interest and then performing scRNAseq. This allowed the researchers to identify antigen specific T cells through their increased expression of effector cytokines such as IFN-γ, TNF-α, and/or IL-2, and from this same data set the researchers then obtained the sequences of transcripts for the TCR α and β chains from the activated cells (60, 65, 99).

PRECLINICAL ASSESSMENT OF CANDIDATE TCRs

TCR discovery efforts often yield sequences of several TCRs with a desired antigen specificity. How does one select an optimal TCR from this list of candidates? And once a lead TCR is selected, what preclinical evaluations can be performed that may predict the likelihood of clinical success? The following section describes several TCR features that are commonly evaluated in preclinical TCR studies.

HLA Restriction

Often the first characteristic of an isolated TCR that must be determined is its HLA restriction (**Figure 3**). Not only is understanding the HLA restriction of a TCR necessary to identify patients that may respond to a TCR T therapy, it is also needed to perform many of the experiments for preclinical assessment. In the case of several TCR discovery approaches, knowledge of HLA restriction is already incorporated into the pipeline, such as in the case of vaccination of HLA-transgenic mice or the use of HLA tetramers to select T cells, in which case the HLA restriction of resulting TCRs will be near certain. However, in TCR discovery approaches that do not incorporate *a priori* knowledge of HLA restriction, such as through stimulating with autologous DCs, HLA restriction must be determined experimentally. A commonly used approach is to deliver individual cloned HLA alleles into the non-human primate COS-7 cell line, which possesses antigen processing and presentation capabilities but does not express potentially confounding endogenous human HLA alleles. The COS-7 cells are then induced to express one of the HLA candidates and the antigen of interest through delivery of cDNA/RNA or peptide loading, and cocultured with TCR T cells. Here, the antigen restricting HLA is evident as the HLA that elicits a TCR T cell response, observed through functional responses such as cytokine secretion and/or 4-1BB/OX40 upregulation (58, 66, 82, 83, 87, 93, 100–104).

TCR Affinity/Avidity

The successful interaction between a TCR and the appropriate pMHC complex is a critical component of effective antitumor immune responses. TCR affinity and avidity describe the binding and kinetic interactions between the TCR and the pMHC (105). Affinity plays a central role in TCR sensitivity and specificity, and refers to the physical strength of the interaction (105–107). Affinity is quantified *via* surface plasmon resonance (SPR), a 3D interaction that measures binding in terms of an association rate (K_{on}) and a dissociation rate (K_{off}) (105, 107, 108). Together, K_{off} and K_{on} make up the binding constant (K_D), where $K_D = k_{off}/$
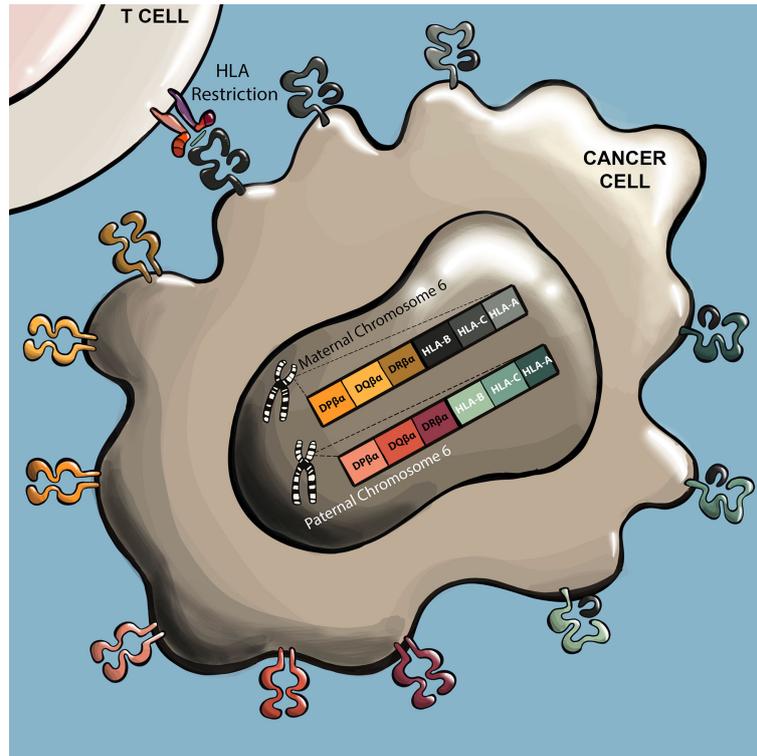
FIGURE 3 | TCRs recognize antigens presented by specific HLA alleles. TCR antigens are predominantly presented by six HLA genes. These include genes for HLA class I (A, B, and C), and class II (DR, DP, and DQ). These HLA genes are highly polymorphic, with many allele variants in the human population. Humans inherit one set of each gene from each parent, and human cells can therefore express up to twelve different HLA presenting alleles. For a given TCR, the specific HLA allele that presents the cognate peptide is referred to as the 'restricting HLA' of the TCR.

k_{on} (105, 107). High affinity TCRs recognize lower levels of antigen, do not require the CD8 coreceptor, and can enable CD4+ T cells to recognize and lyse tumor cells in an MHC class I-dependent manner (106, 109).

TCR avidity usually correlates with affinity, and refers to the combined effect of multiple TCR-pMHC interactions, coreceptors (CD8), TCR density, and T cell functional status (105, 110). Different aspects of avidity (i.e. structural or TCR avidity) can be measured *via* staining with pMHC monomers or multimers with defined valency (105). TCR affinity, avidity, and the various kinetic constants all contribute directly/indirectly to functional avidity, which describes how well T cells expressing a specific TCR respond to decreasing abundance of peptide, and is sometimes referred to as antigen sensitivity (105, 108, 111). Assessments of TCR functional avidity typically include measurement of TCR T cell cytokine secretion or cytolytic function in response to target cells that are pulsed with titering concentrations of peptide.

Antigen Processing by the Standard or Immunoproteasome

HLA class I epitopes are peptide fragments, typically 8-12 amino acids in length (112, 113), generated through processing of ubiquitinated proteins by the proteasome. The proteasome is a large protein complex responsible for the degradation of endogenous proteins that have been damaged or are not needed by the cell and have been tagged by ubiquitin conjugation. The subunits β1, β2, and β5 of the proteasome's 20S catalytic core are associated with the three major catalytic activities of the proteasome. While proteasomes that incorporate subunits β1, β2, and β5 are referred to as the 'standard proteasome', hematopoietic cells and cells stimulated with certain inflammatory cytokines (e.g., INF-γ, IFN-α, IFN-β, and TNF-α) alternatively express β1i, β2i, and β5i subunits that displace β1, β2, and β5 subunits in the proteasome, forming an isoform termed the 'immunoproteasome'. The immunoproteasome displays several biochemical differences that influence peptide cleavage activity. This results in the immunoproteasome producing peptide products with enhanced immunogenicity compared to the standard proteasome, as these immunoproteasome-generated peptides are more likely to contain C-terminal hydrophobic residues, which are associated with more efficient HLA-class I binding (24, 114). In addition, there are 'intermediate proteasomes' that contain a mixture of standard and immunoproteasome subunits, specifically substituting only β5i or β1i plus β5i and result in a peptide repertoire similar to that produced by the immunoproteasome, but includes additional unique peptide products (24, 115).

Several tumor antigens have now been characterized as being produced by the standard, intermediate, and/or immunoproteasomes (101, 115–118). Because the dendritic cells used to enrich tumor specific T cells express predominantly intermediate and immunoproteasomes (115), it is likely that most preclinical TCRs will recognize antigens produced by immunoproteasome and/or intermediate proteasome. These cognate peptides may or may not be additionally produced by the standard proteasome. The proteasomal requirements of a cognate peptide can be determined in several ways. Many cell lines predominantly express the standard proteasome, but will upregulate immunoproteasome subunits in response to IFN-γ. Therefore, the response of TCR T cells can be compared against antigen expressing cell lines with or without pretreatment with IFN-γ (73, 101, 116, 117). Peptide proteasomal requirements have also been determined with further resolution by testing T cell responses against antigen expressing 293 cells with or without overexpression of specific inducible proteasomal β subunits (101, 115–117, 119). There is a growing appreciation of the importance of proteasomal processing dynamics in immunotherapies such as immune checkpoint blockade (120) and TCR T therapy (121). In cases in which a therapeutic TCR recognizes a peptide processed exclusively by the immunoproteasome, it may be useful to select patients whose tumors have confirmed expression of immunoproteasome subunits. As downregulation of immunoproteasome subunits has now been observed in some cancer types (122, 123), it is likely ideal for a therapeutic TCR to recognize an antigen that is generated by both the standard and immunoproteasome.

Safety Assessment of Lead TCRs

Of major importance is the identification of potential safety concerns of lead TCRs, as previous clinical trials have observed severe cases of both on-target off-tumor toxicities (124–126) and off-target toxicities (127, 128). This section describes state-of-the-art techniques to assess the safety of preclinical TCRs and their cognate-antigen targets.

In cases in which a candidate TCR targets a novel or putative TAA or CGA epitope, it is imperative to preclinically assess its pattern of expression and HLA-presentation in healthy tissue. The importance of such validation was made clear by the fatal neurotoxicity that occurred in two patients following administration of T cells expressing an affinity enhanced TCR recognizing an epitope shared by MAGE-A3 and MAGE-A12. Autopsy performed on these patients revealed infiltration of CD3+CD8+ T cells in the brain. Further investigation identified unexpected expression of MAGE-A12 in a subset of neurons in the human brain (129). Several strategies are now available for preclinical assessment of expression profiles of putative CGA or TAA targets that should be employed. Kunert et al. provide suggested strategies based on their experience of assessing the expression profile of a MAGE-C2 derived epitope that is now being targeted clinically (NCT04729543). As an early step in the assessment of putative TAAs or CGAs, the authors suggest consulting online databases such as The Human Protein Atlas (proteinatlas.org) and the CTdatabase (cta.lncc.br) (130), which compile extensive data throughout literature concerning RNA and protein expression of many genes in both healthy and cancerous tissue (131). An additional tool that has recently emerged for the validation and/or discovery of CGAs and TAAs is the HLA Ligand Atlas (hla-ligand-atlas.org), an open-source, community resource of comprehensive human HLA ligandome data collected originally from 29 distinct non-malignant tissues derived from 21 individuals (113). To experimentally and independently evaluate antigen expression in healthy tissue, Kunert et al. suggest performing qPCR on commercially available cDNA libraries derived from a wide array of healthy tissues types, and if possible, further evaluating protein expression by performing IHC on a panel of healthy tissue types (131).

In addition to establishing the safety of the cognate-antigen target, it is of critical importance to investigate potential off-target reactivities of candidate TCRs. The clinical importance of such investigation was highlighted by the deaths of two patients resulting from off-target reactivity of an affinity-enhanced MAGE-A3-specific TCR towards a Titin-derived epitope expressed in cardiomyocytes (127, 128). Several preclinical strategies are now commonly employed by investigators to identify possible off-target reactivities of candidate TCRs. Several groups have identified specific amino acids within the cognate peptide that are necessary for TCR recognition. This is accomplished by mutating each residue within the cognate peptide and identifying the mutant versions unable to elicit a T cell response. The investigators then searched for all other human peptides containing an identical or similar amino acid motif through the use of webtools such as BLAST and ScanProsite, and then assessed whether these structurally similar peptides elicited a response by the candidate TCR (60, 63, 131–134). In cases in which one or several off-target peptides were identified, the researchers further investigated the immunogenicity of these peptides by determining TCR T cell response at titering concentrations (63, 131, 134) or determining if the off-target peptide is actually capable of being naturally processed (132, 133). While this approach is highly valuable for identifying cross-reactive peptides that are structurally similar to the cognate peptide, it would not identify structurally dissimilar peptides that mediate cross-reactivity (135).

Several groups have also assessed potential alloreactivity of therapeutic TCRs by performing functional assays in which TCR T cells are cultured with many different lymphoblastoid cell lines (LCLs) expressing various HLA alleles (131–133). The utility of this approach is highlighted by Sanderson et al., who identified that a lead HLA-A*02:01 restricted MAGE-A4 specific TCR mediated an alloresponse to HLA-A*02:05, indicating that patients that express HLA-A*02:05 should be excluded from treatments using this TCR (133).

ENGINEERING STRATEGIES TO IMPROVE TCR SAFETY AND EFFICACY

Promoting Proper Pairing of TCR α/β Chains

TCR α/β chains form heterodimers largely through interactions within TCR constant regions. A challenge facing

TCR T applications is that the endogenous TCR α/β chains expressed by conventional T cells can pair with introduced TCR α/β chains. Several consequences arise from such TCR mispairing. Firstly, TCR mispairing reduces the surface expression of introduced TCRs, as a significant fraction of the introduced TCR α/β chains will participate in non-productive mispairings with endogenous TCR α/β chains. Furthermore, mispaired TCRs compete with the engineered TCR heterodimer for association with limiting CD3 components (136). A second consequence of TCR mispairing is the production of brand new TCRs that have not undergone thymic selection, and which may have unexpected specificity for autoantigens. Indeed, TCR mispairing was shown to cause lethal graft-versus-host-disease (GVHD) in mice (137), and led to the formation of alloreactive and autoreactive human T cells *in vitro* (138). However, incidence of GVHD has not been observed in human TCR T clinical trials to date, including early TCR T trials utilizing unmodified human TCRs (139). To avoid issues associated with TCR mispairing, the vast majority of TCR T applications now use at least one strategy to reduce TCR mispairing (**Figure 4**).

Murinization

Extending on the serendipitous observation that human T cells exhibited greater biological activity when engineered with a murine-derived TCR as compared to human-derived TCRs, Cohen et al. demonstrated that murine TCR α/β chains preferentially dimerize with each other in the presence of endogenous human TCR α/β chains. The investigators further demonstrated that preferential pairing of introduced TCRs is also achieved when the constant regions of human TCRs are replaced with murine constant regions (140). A concern of using engineered TCRs with murine constant regions is that the foreign murine sequences may elicit an immune response in patients, as has been observed in other cell therapy trials utilizing foreign proteins such as green fluorescent protein (141, 142) and the HyTK suicide gene (143). One study identified anti-murine TCR antibodies in the post-treatment sera of 6/26 patients treated with TCR T cells expressing fully murine TCRs. However, epitope mapping revealed that the antibodies were specific for the variable regions of the TCRs, not the constant regions (144). In a separate TCR T trail utilizing a human TCR with murinized constant regions, anti-TCR serum antibodies were not detected in any of the 11 patients screened (44). Together, these clinical findings suggest that murine TCR constant regions have low or negligible immunogenicity. Nonetheless, strategies have also been developed to partially murinize TCRs by substituting specific murine amino acid sequences (145, 146).

Additional Disulfide Bond

Endogenous TCR α/β chains form a disulfide bond between TCR α constant region (Cα) residue 94 and TCR β constant region (Cβ) residue 130 (147). The proper pairing of introduced TCRs can be improved by introducing a second stabilizing disulfide bond through cysteine substitutions at Cα residue 48 and Cβ residue 57, which increases interchain binding affinity of

introduced TCR α/β chains while decreasing binding affinity with endogenous TCR α/β chains (148, 149).

Transmembrane Hydrophobic Substitutions

The endogenous TCR α chain has a relatively low stability, which can be increased by substituting leucine and valine residues within the Cα transmembrane region. TCR α chains containing these stabilizing mutations, termed α-LVL, demonstrate increased TCR surface expression and biological activity. While this strategy promotes pairing of an introduced TCR by stabilizing the TCR α chain, the TCR β chain remains unmodified and thus susceptible to mispairing. However, this can be addressed by incorporating the α-LVL substitutions into murinized TCRs, the combination of which can synergistically enhance TCR expression and biological activity (150).

Domain Swapping/Conjugation

Through TCR crystal structure analysis, Voss et al. identified several amino acids mediating TCR α/β dimerization. By swapping two such interacting residues, a Cα glycine and Cβ arginine, the authors generated mutant TCR α/β chains with a similar propensity for dimerizing with each other, but with a significantly reduced propensity to bind with unmodified endogenous TCR α/β chains (151). Bethune et al. employed a similar strategy when they designed TCRs with large regions of Cα and Cβ segments exchanged, referred to as dsTCR$_c$. Interestingly, mispairing of introduced dsTCR$_c$ α/β chains with endogenous α/β chains was completely undetectable (152). Other examples of TCR domain swapping/conjugation strategies include swapping with γδ TCR constant regions (152), replacing regions with CD3ζ (153, 154) or CD28/CD3ε (155), or conjugation to leucine zipper dimerization motifs (156, 157).

Single-Chain TCRs

To combine the antigen recognition properties of a TCR α/β heterodimer into a single chain, several groups have developed so-called three-domain single-chain TCRs (scTCR), which are composed of Vα/Vβ regions fused by a short peptide linker and conjugated to a Cβ domain (153, 158, 159). To mediate signal transduction, three-domain scTCRs are typically further conjugated to CD3ζ (153, 158, 160). Zhang et al. compared the function of nine three-domain scTCR constructs conjugated to CD3ζ with or without conjugation to the additional stimulatory domains CD28 and Lck. Although the addition of both CD28 and Lck improved scTCR function, none of the scTCR constructs performed as well as native TCRs in terms of functional avidity (160). scTCR constructs utilizing CD3ζ transmembrane and signaling domains function independently of the CD3 complex (160), which theoretically allows for higher surface expression to be achieved with scTCRs than with native TCRs, as scTCRs are not limited by the abundance of CD3 components. The CD3-independence of scTCRs may also be beneficial in applications where it is desirable to maintain levels of endogenous TCR expression. However, scTCRs utilize signaling mechanisms distinct from those of CD3-dependent native TCRs, which may partially explain the reduced functional avidity of scTCRs (160). To generate scTCRs that preserve CD3-dependence, Voss
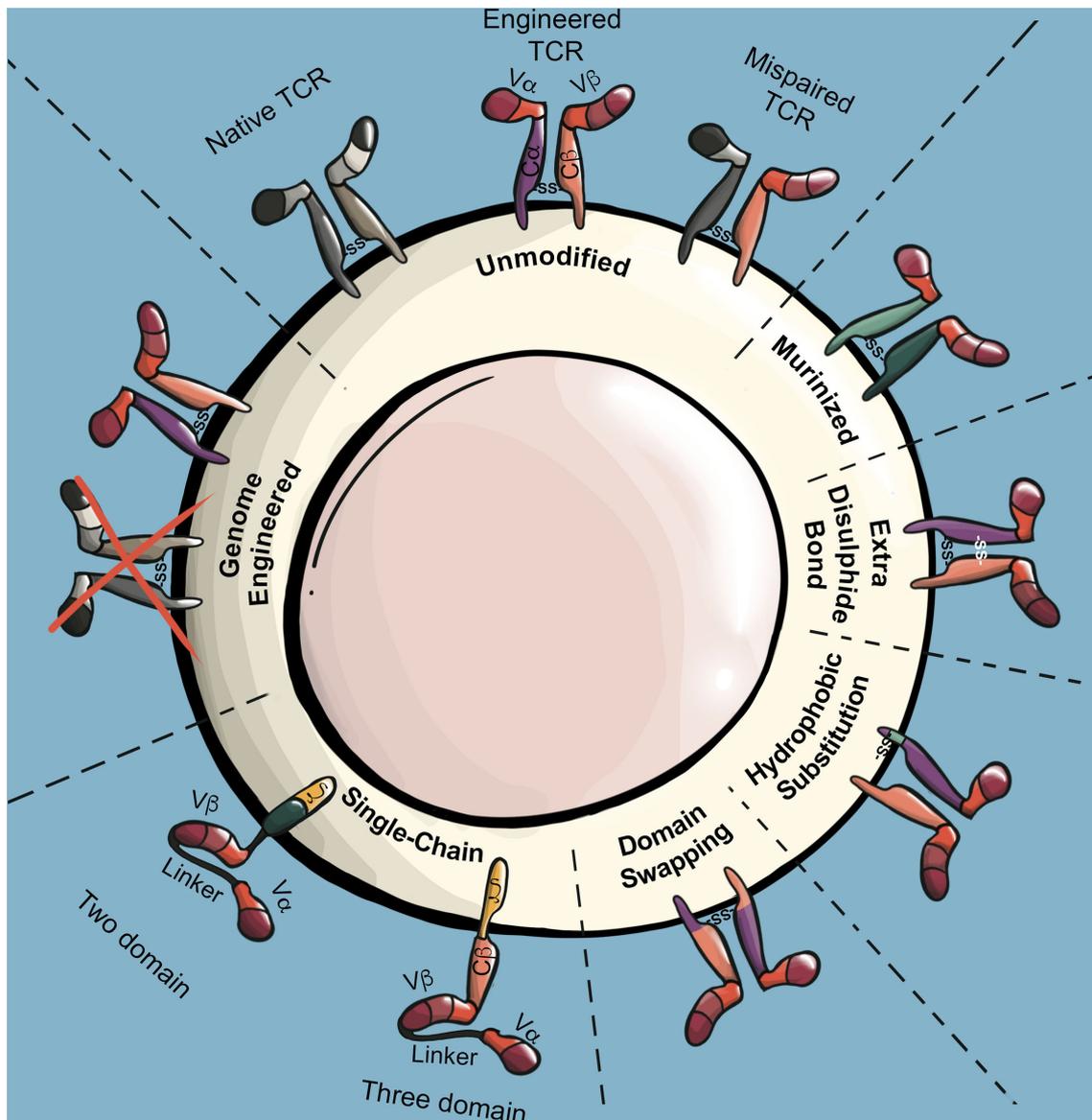
FIGURE 4 | TCR modifications to prevent mispairing and maximize surface expression. Illustration of mispairing between endogenous TCR and engineered TCR. Murinized TCRs replace the human TCR constant regions with those of a mouse TCR constant region. The addition of an extra disulfide bond in the TCR constant region through cysteine substitutions stabilizes interchain binding affinity of engineered TCR α/β chains while reducing their binding affinity with endogenous TCR α/β chains. Stability of the engineered TCR α chain can be increased through select hydrophobic substitutions in its transmembrane region. Domain swapped TCRs invert large or specific segments of the engineered TCR α/β constant regions, which reduces propensity of engineered TCRs to mispair. Single-chain TCRs (scTCR) encode TCR antigen recognition and signaling domains into a single chain. Three-domain and two-domain scTCRs differ by the inclusion or absence of the TCR β constant region, respectively. Genome engineering strategies utilize RNA interference or endonuclease technologies to reduce or ablate endogenous TCR expression.

et al. designed a system whereby three-domain TCRs without CD3ζ conjugation were coexpressed with a Cα domain. In this manner, the three domain scTCR dimerizes with the coexpressed Cα domain, presenting at the cell surface in a four-domain structure similar to that of a native TCR heterodimer. Intriguingly, these scTCR/Cα constructs have similar functional avidities as native TCRs (161). However, Aggen et al. demonstrated that three-domain scTCRs continue to

mispair to some extent with endogenous TCR α chains due to the presence of the Cβ domain. To generate a scTCR system that completely eliminates mispairing, the group generated two-domain scTCRs, which utilize stabilizing Vα/Vβ mutations to obviate the need for a Cβ domain (162). To mediate signaling, two-domain scTCRs are conjugated to intracellular signaling domains such as CD3ζ and CD28. Intriguingly, CD3ζ/CD28 containing two-domain scTCRs are essentially CARs that utilize

a Vα/Vβ antigen recognition domain. As such, two-domain scTCRs display features typical of CARs including CD3-independent signaling and decreased sensitivity to low antigen density. However, unlike typical CARs, two-domain scTCRs are still dependent on HLA presentation (14).

Genome Engineering Strategies

Rather than modifying the introduced TCR, other strategies address mispairing through knock-down or knock-out of the endogenous TCR. Provasi et al. combined the use of zinc-finger nucleases (ZFN) to knock-out endogenous *TRAC* and *TRBC* genes with lenti-viral delivery of a WT-1 specific TCR. Here, the authors described an elegant, although relatively extensive manufacture system utilizing sequential rounds of *TRAC/TRBC* disruption, magnetic bead separation, and TCR α/β chain delivery. This resulted in a TCR T product with enhanced expression of the introduced TCR and a complete absence of endogenous TCR α/β chains (163). In a recent first-in-human trial, Stadtmauer et al. employed multiplexed CRISPR/Cas9 editing to disrupt T cell *TRAC*, *TRBC*, and *PDCD1* genes in combination with lenti-viral delivery of a NY-ESO-1 specific TCR. In the four patient-derived products described in this report, disruption of *TRAC* and *TRBC* was achieved in an average of 45% and 15% of cells, respectively. However, as *TRAC/TRBC* edited T cells were not selected prior to lenti-viral transduction with the NY-ESO-1 TCR, a significant fraction of the TCR-engineered T cells likely continued to express endogenous TCR α/β chains (164). Several groups have also developed virus-free systems to deliver TCRs and/or disrupt endogenous *TRAC/TRBC* genes, which may also aid in improving TCR T clinical cost and feasibility. Davo et al. electroporated T cells with Dicer-substrate small interfering RNAs (DsiRNA) targeting the endogenous *TRAC* and *TRBC* loci, achieving an approximately 6-fold and 3-fold reduction in expression of *TRAC* and *TRBC*, respectively. The authors then electroporated the T cells with a codon optimized WT1-specific TCR that isn't recognized by the DsiRNA. This resulted in T cell products with relatively high engineered TCR expression (60.2% tetramer+) with no observable TCR mispairing. However, as transgene expression in this system is transient, this would likely necessitate multiple infusions in a clinical setting (165). Roth et al. used CRISPR/Cas9 editing to mediate targeted insertion of TCR α and β variable regions into the first exon of the *TRAC* and *TRBC* loci, respectively. This mediated the combined effect of disrupting the endogenous TCR α/β chains, while placing expression of the introduced TCR under physiologic control. A potential challenge of this approach is the relatively low editing efficiency, with about 3% of cells expressing the introduced TCR, which could therefore necessitate sorting and/or extended selective expansion (166).

Affinity/Avidity Enhancement

Given that affinity plays a central role in TCR function, the manufacturing of high-affinity TCRs is an attractive method to improve the efficacy of TCR T therapies. Naturally occurring TCRs, including those that recognize self/tumor antigens, have relatively low affinities as a result of negative selection (105, 167).

Methods to improve TCR affinity focus on the introduction of amino acid sequence (AAS) variations into the TCR complementarity-determining regions (CDRs). For example, single and dual AAS substitutions that enhanced the functions of TCRs specific for NY-ESO-1 (1G4) and MART-1 (DMF4, DMF5) were generated through overlapping PCR (168). These TCRs had affinities in the low μM and even nM range, which surpass the affinity of most naturally occurring TCRs, which range from 1-100 μM (106, 110). Yeast and bacteriophage display are additional powerful, high-throughput tools that can generate TCRs with affinities in the pM range (109, 167, 169). While these techniques are effective at identifying high affinity TCR variants, higher affinity has been associated with increased cross-reactivity (106, 109, 168). TCRs with affinities greater than the normal range (1-100 μM) are more likely to demonstrate cross-reactivity to similar or completely different peptides (109, 135, 170). In various studies of high affinity TCRs, increasing the affinity within the nM and pM range resulted in recognition of control antigen and antigen negative target cells (109, 168). Efforts to improve the affinity of TCRs should thus proceed with caution and thorough evaluation as these high affinity TCRs could have detrimental effects when used as patient therapies.

Recent work in TCR affinity maturation has focused on incorporating a more thorough assessment of the structure of the TCR and how it interacts with the target pMHC. Hellman et al. utilized a structure-guided design that incorporated both positive and negative designs (106, 170). In other words, they utilized mutations that either enhanced or weakened the interaction of the TCR with the MHC protein. These mutations redistributed the binding free energy in a way that forces the TCR-pMHC interaction to rely more on the presence of the correct target peptide, leaving less flexibility for off-target peptides. In the MART-1 specific DMF5 TCR, these structure-guided modifications decreased cross-reactivity to MART-1 homologs and eliminated cross-recognition of a selection of divergent peptides. Structure-guided approaches, therefore, have the potential to improve ACT while minimizing the risk of off-target toxicities.

CLINICAL LANDSCAPE OF TCR T THERAPIES

Trends in TCR T Trials

As of October 3rd, 2021, the search term "TCR" (and synonyms "T Cell Receptor" and "T Cell Antigen Receptor") in clinicaltrials.gov yielded 538 interventional trials. Through manual inspection of these trials, 119 were identified to include the adoptive transfer of TCR T cells. One TCR T trial that did not include these search terms (NCT04044768) was also identified and included in this analysis (**Supplementary Table 1**). The first TCR T trial was initiated in 2004 by Steven Rosenberg at the National Institutes of Health (NIH) targeting the melanoma differentiation antigen gp100 (NCT00085462). Since then, the number of new TCR T trials initiated has steadily increased, with a particular acceleration between the years of 2017 – 2019, in

which 51 new TCR T trials were initiated (**Figure 5A**). Of the TCR T trials to-date, 53% have been in phase I, 24% in phase I/II, and 22% in phase II. To date, no phase III TCR T trials have been initiated (**Supplementary Table 1**). The status of these TCR T trials was assessed as of November 3rd, 2021 (**Figure 5B**). There were 118 antigens targeted in the 116 TCR T trials with specified targets. Of the targeted antigens, the majority are CGAs (47%), followed by viral antigens (24%), tumor-associated antigens (21%), neoantigens (7%), and fetal oncogenes (3%) (**Figure 5C**). The CGA NY-ESO-1 is by far the most targeted

antigen in TCR T trials to-date (36 trials). Although not first targeted until 2014, HPV now constitutes the second most common TCR T target (10 trials). While the melanoma differentiation antigen MART-1 is the third most targeted antigen in TCR T trials (7 trials), these largely constitute early TCR T trials, as MART-1 targeted-trials have not been initiated since 2012 (**Figure 5D**). The majority of the TCR T trials to date have been for the treatment of solid cancers (85%), followed by hematological malignancies (9%), and trials targeting both solid and hematological cancers (2%). A small subset of TCR T trials
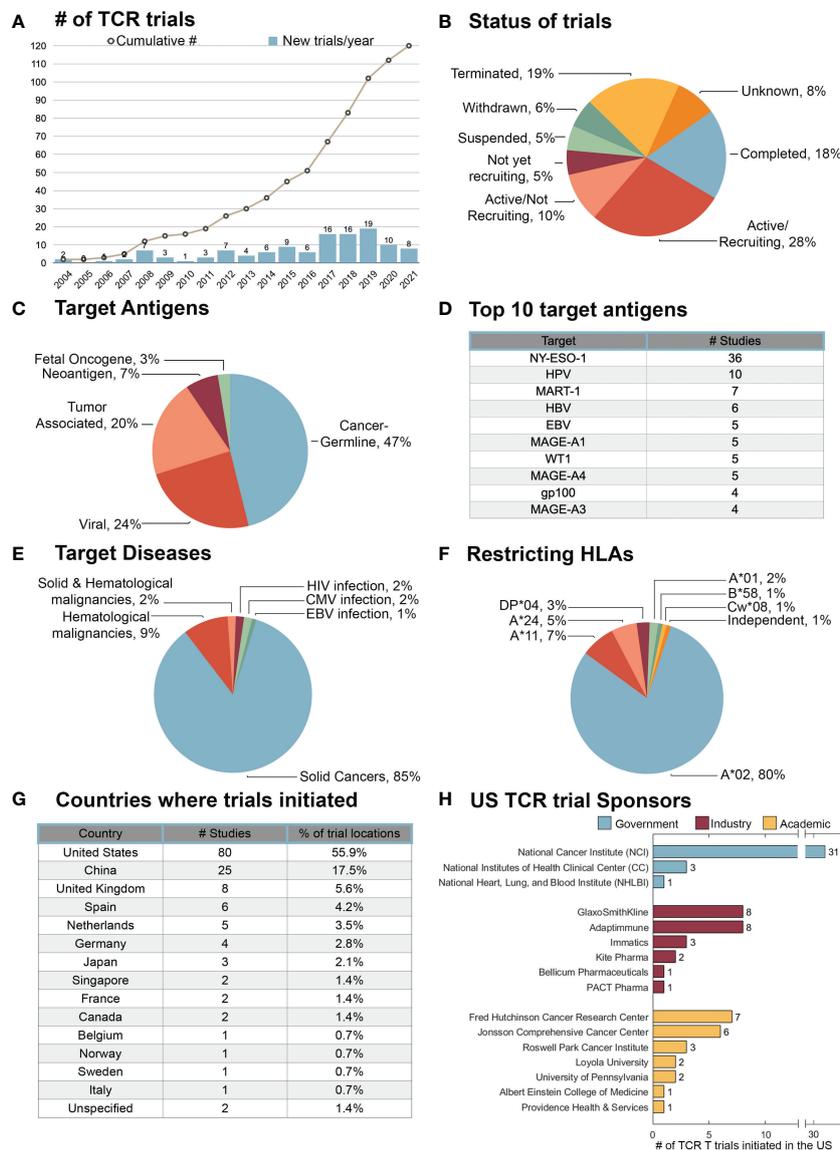

FIGURE 5 | Trends in TCR T trials initiated thus far. TCR T trials registered in clicaltrials.gov were assessed as of October 3rd 2021. **(A)** The number of new TCR T trials initiated each year and the cumulative number of registered TCR T trials by year. **(B)** Clinical status of the 120 TCR T trials. **(C)** Classifications of 118 tumor antigen targets in 116 TCR T trials with specified target antigens. **(D)** Ten most common targets in TCR T trials. **(E)** Diseases targeted in TCR T trials. **(F)** Frequency of 111 target antigen-restricting HLAs in 100 TCR T trials that specified HLA restriction. **(G)** Locations where TCR T trials have been conducted by country. **(H)** Primary sponsors of the 80 TCR T trials conducted in the United States.

have been for the treatment of HIV, CMV, or EBV infections (4%) (**Figure 5E**). More information about the precise disease targets of these TCR T trials can be found in **Supplementary Table 1**. Of the 100 trials with specified HLA-restrictions, 111 restricting-HLA alleles were listed, as some trials included multiple antigen targets and HLA restrictions. HLA-A*02 was by far the most common restricting HLA allele (80%), followed by HLA-A*11 (7%) and HLA-A*24 (5%). HLA class II restricted antigens were targeted in 3 trials, all of which were restricted by HLA-DP*04 (3%) (**Figure 5F**). The majority of TCR T trial locations were in the United States (56%), followed by China (18%), and the United Kingdom (6%) (**Figure 5G**). Among the 80 TCR T trials occurring in the United States, 44% were sponsored by the NIH, 28% by academic institutions, and 29% by industry. The support of industry in TCR T, which accelerated around 2017 (**Supplementary Table 1**), will likely aid in the development of later phase TCR T trials. The NCI has been by far the most active individual institution in United States TCR T trials, sponsoring 31 trials to date. Among academic institutions, the Fred Hutchingson Cancer Research Center has sponsored the most trials (7 trials), followed by the Johnson Comprehensive Cancer Center (6 trials). Among TCR T trials sponsored by pharmaceutical companies, Adaptimmune and GlaxoSmithKline have sponsored the most trials (8 trials each) (**Figure 5H**).

Assessment of Safety and Efficacy of TCR T Trials to Date

There is a quickly expanding body of literature detailing the results of TCR T trials. Clinical results encompassing over twenty-five TCR T trials are detailed in **Table 1**. This section discusses broad findings that have emerged from these early phase trials, particularly relating to TCR T safety and efficacy. Of note, many TCR T clinical protocols include lymphodepleting regimens prior to TCR T infusion, which has been demonstrated in early ACT trials to improve T cell engraftment and persistence. Many studies also include systemic administration of IL-2 following TCR T infusion to support T cell activity and persistence. Both interventions consistently induce various toxicities that, while undesirable, are generally clinically manageable. Detailed description of the impact of these interventions is beyond the scope of this review, but is extensively reviewed elsewhere (189–191). As such, discussion of toxicities observed in TCR T trials will focus on those mediated directly by the infused T cells. Finally, it is worth noting that to date all TCR T trials have been early phase and almost exclusively treating patients with highly advanced, treatment refractory disease.

Trials Targeting Cancer Differentiation Antigens: Evidence of Efficacy and On-Target Off-Tumor Toxicity

In one of the earliest TCR T trials, patients with metastatic melanoma were treated with autologous T cells transduced with a TCR (DMF4) recognizing the melanoma differentiation antigen MART-1. The objective response rate in these patients was relatively modest, with 2/17 (12%) patients achieving durable partial responses. No TCR T induced toxicity was observed (171). In a later related study, patients were treated with TCR T cells that expressed a higher affinity MART-1 specific TCR (DMF5). Compared to the prior trial using the lower affinity DMF4 receptor, this trial observed enhanced efficacy, with objective responses observed in 6/20 (30%) patients. However, the increased biological activity mediated by the DMF5 TCR was also associated with the emergence of on-target off-tumor destruction of melanocytes, leading to widespread erythematous skin rash (14 pts), uveitis (12 pts), and hearing loss (10 pts). Similar results were observed in patients treated with T cells expressing a high affinity mouse-derived TCR targeting the melanoma differentiation antigen gp100 (126). In a later study, three patients with metastatic colorectal cancer were treated with an affinity-enhanced TCR recognizing the cancer differentiation antigen carcinoembryonic antigen (CEA). While a partial response was observed in 1/3 (33%) patients, all three patients presented with severe transient colitis as a result of on-target off-tumor destruction of CEA-expressing colonic mucosa (124). Ultimately, these studies demonstrated that while TCR T therapies targeting cancer differentiation antigens can mediate objective clinical responses, they are often associated with potentially dangerous on-target off-tumor toxicities. Likely owing to this, few TCR T trials targeting cancer differentiation antigens have been initiated in the past decade (**Supplementary Table 1**).

Trials Targeting NY-ESO-1: A Safe and Effective Target

The CGA NY-ESO-1 has been the most widely targeted antigen in TCR T trials, and several groups have now published promising results from trials targeting this antigen. An early landmark trial treating patients with refractory melanoma or synovial sarcoma with TCR T cells expressing an affinity-enhanced NY-ESO-1 specific TCR reported objective responses in 11/20 (55%) melanoma patients, including four durable complete responses, and 11/18 (61%) synovial sarcoma patients, including one durable complete response. No TCR T associated toxicities were observed (174). Later, a large study of synovial sarcoma patients treated with TCR T cells expressing an affinity enhanced NY-ESO-1 specific TCR (SPEAR T cells) observed clinical responses in 15/42 patients (36%), including one complete response, and 24/42 patients presenting with stable disease. These patients were also divided into treatment cohorts based on magnitude of NY-ESO-1 tumor expression and the lymphodepleting regimen they received. The greatest clinical responses were observed in a cohort of twelve patients (cohort 1) whose tumor expressed +2 or +3 NY-ESO-1 staining by immunohistochemistry in ≥50% of cells and who received a relatively intensive lymphodepleting regimen. Here, clinical responses were observed in 6/12 (50%) patients, including one complete response, and a median duration of response of 30.9 weeks (179). Of these twelve patients, five experienced cytokine release syndrome of grades 1 (2 pts), 2 (1 pt), or 3 (2 pts) (192). Several other studies reporting TCR T trials targeting NY-ESO-1 to treat various cancer types have also observed clinical responses

TABLE 1 | Available TCR T trial results.

Ref	Year	Trial	Phase	Sponsor	Target	HLA	Construct Details	Disease	# Pts	Pre. Cond.	Combination	Responses	TCR T Induced Toxicity
(124)	2011	NCT00923806	I/II	NCI	CEA	A*02:01	affinity enhanced TCR	colorectal cancer	3 pts	Cy + Flu	IL-2	**1/3, 33.3% (ORR/PR)**	Severe colitis (3 pts)
(126)	2009	NCT00509496	II	NCI	gp100	A*02:01		melanoma	16 pts	Cy + Flu	IL-2	**3/16, 18.8% (ORR)** 1/16 (CR), 2/16 (PR)	Skin toxicity (15 pts), Uveitis (4 pts), Hearing loss (5 pts)
(171)	2006	Not Specified (NS)	NS	NS	MART-1	A*02:01	DMF4 TCR	melanoma	17 pts	Cy + Flu	IL-2 Peptide vaccine	**2/17, 12% (ORR/PR)** 1/17 (MR)	None
(126)	2009	NCT00509288	II	NCI	MART-1	A*02:01	DMF5 TCR	melanoma	20 pts	Cy + Flu	IL-2	**6/20, 30% (ORR/PR)**	Skin toxicity (14 pts), Uveitis (12 pts), Hearing loss (10 pts)
(172)	2014	NCT00910650	II	JCCC	MART-1	A*02:01	DMF5 TCR	melanoma	10 pts	Cy + Flu	IL-2 Peptide-pulsed DC vaccine	**0/14, 0% (ORR)** 7/14 (SD) *ORR assessed at Day 90*	Erythematous skin rash (3 pts), Acute respiratory distress (2 pts)
(173)	2018	NCT01586403	I	Loyola University	tyrosinase	A*02	TIL1383I TCR	melanoma	3 pts	Cy + Flu	IL-2	**1/3, 33% (ORR/PR)**	Vitiligo (2 pts)
(174)	2015	NCT00670748	II	NCI	NY-ESO-1	A*02:01	1G4-a95:LY TCR (affinity enhanced)	melanoma synovial sarcoma	38 pts	Cy + Flu	IL-2 ± AVIPOX- ESO vaccine	**22/38, 58% (ORR)** 5/38 CR, 17/38 PR	None
(175)	2019	NCT02366546	I	Mie University	NY-ESO-1	A*02:01 A*02:06	affinity enhanced TCR siRNA *TRAC/TRBC*	various solid tumors	9 pts	Cy	None	**3/9, 33.3% (ORR/PR)**	CRS (3 pts)
(176)	2019	NCT02070406 NCT01697527	I	JCCC	NY-ESO-1	A*02:01	1G4-a95:LY TCR (affinity enhanced)	various solid tumors	10 pts	Cy + Flu	IL-2, DC-peptide vaccine, ± ipilimumab	**2/10, 20% (ORR)** 1/10 (CR), 1/10 (PR), *4/10 (tR)*	None
(177)	2019	NCT02869217	Ib	University Health Network	NY-ESO-1	A*02:01 A*02:06	TBII-1301 (MS3II-NY-ESO1-SiTCR)	various solid tumors	9 pts	Cy	None	**2/9, 22.2% (ORR/PR)** 5/9 (SD), *1 pending*	CRS grade 1-2 (5 pts)
(178)	2019	NCT01352286	I/IIa	GlaxoSmithKline	NY-ESO-1/LAGE-1	A*02:01	NY-ESO-1 SPEAR T cells (NY-ESOc259 TCR)	multiple myeloma (Post-HSCT)	25 pts	melphalan	lenalidomide	**21/25, 84% (ORR)** 2/25 (sCR), 1/25 (CR), 13/25 (VGPR), 5/25 (PR), 4/25 (SD)	GVHD (6 pts). *Likely not related to the engineered TCR.*
(179)	2019	NCT01343043	I/II	GlaxoSmithKline	NY-ESO-1	A*02	NY-ESO-1 SPEAR T cells	synovial sarcoma	42 pts	Cy ± Flu as per cohort	None	**15/42, 35.7% (ORR)** 1/42 (CR), 14/42 (PR), 24/42 (SD)	CRS grades 1 (2 pts), 2 (1 pts), and 3 (2 pts)
(164)	2020	NCT03399448	I	University of Pennsylvania	NY-ESO-1	A*02:01	8F TCR, CRISPR KO *TRAC/TRBC/PDCD1*	multiple myeloma, MRCLS	3 pts	Cy + Flu	None	**0/3, 0% (ORR)** 2/3 (SD)	None
(128)	2013	NCT01350401 NCT01352286	I/II	Adaptimmune GlaxoSmithKline	MAGE-A3	A*01	MAGE-A3a3a TCR (affinity enhanced)	melanoma, myeloma (Post-ASCT)	2 pts	Cy (melanoma pts)	None	NA	Off-target toxicity in cardiac tissue leading to 2 pt deaths
(129)	2013	NCT01273181	I/II	NCI	MAGE-A3	A*02:01	affinity enhanced TCR	various solid tumors	9 pts	Cy + Flu	IL-2	**5/9, 55.6% (ORR)** 1/9 (CR), 4/9 (PR)	Neurological toxicity (3 pts) 2 pt deaths.
(194)	2017	NCT02111850	I/II	NCI	MAGE-A3	DPB1*04:01		various solid tumors	17 pts	Cy + Flu	IL-2	**4/17, 23.5% (ORR)** 1/17 (CR), 3/17 (PR)	Fever (10 pts), Elevated ALT, AST, and creatinine (2 pts)
(181)	2015	UMIN000002395	I	Mie University	MAGE-A4	A*24:02		esophageal cancer	10 pts	None	Peptide vaccine	**0/10, 0% (ORR/PR)** 3/10 SD	None
(182)	2020	NCT03132922	I	Adaptimmune	MAGE-A4	A*02	ADP-A2M4 SPEAR T cells	various solid tumors	34 pts	Cy + Flu	None	**7/28, 25% (ORR/PR)** 11/28 (SD)	2 pt related deaths (aplastic anemia and CVA), not likely off target toxicity
(183)	2020	NCT04044859	I	Adaptimmune	MAGE-A4	A*02	ADP-A2M4CD8 SPEAR T cells	various solid tumors	5 pts	Cy + Flu	None	**2/5, 40% (ORR/PR)** 3/5 (SD)	No DLTs or SAEs
(184)	2021	NCT04044768	II	Adaptimmune	MAGE-A4	A*02	ADP-A2M4 SPEAR T cells	synovial sarcoma, MRCLS	37 pts	Cy + Flu	None	**13/33, 39% (ORR)** 11/33 (PR), 2/33 (CR), 15/33 (SD)	CRS grades 1-2 (21 pts), 3 (1 pt)
(185)	2018	NCT02989064 NCT02592577	I	Adaptimmune	MAGE-A10	A*02	MAGE-A10c796 TCR (affinity enhanced)	various solid tumors	8 pts	Cy ± Flu	None	**0/8, 0% (ORR/PR)**	CRS (1 pt), Increase in serum amylase (1 pt)
(186)	2019	NCT02280811	I/II	NCI	E6	A*02:01	E6 TCR	HPV-associated solid cancers	12 pts	Cy + Flu	IL-2	**2/12, 16.7% (ORR/PR)** 4/12 (SD)	None
(44)	2021	NCT02858310	I	NCI	E7	A*02:01	E7 TCR	HPV-associated carcinomas	12 pts	Cy + Flu	IL-2	**6/12, 50% (ORR/PR)** 4/12 (SD)	1 DLT, not likely off target toxicity
(187)	2017	UMIN000011519	I	Several sponsors	WT1	A*24:02	TAK-1 TCR, siTCR	AML, MDS	8 pts	None	Peptide vaccine	Transient decrease of blasts in BM in 2 pts. SD in 1 pt.	No adverse events greater than grade 3
(188)	2019	NCT01640301	I/II	Fred Hutchinson CRC	WT1	A*02:01	TCR$_{C4}$ TCR, Allo EBV-specific T cells	AML (Post-HCT)	12 pts	None	IL-2	100% RFS vs 54% in comparative group	GVHD in several patients, not likely caused by TCR T cells, but rather HCT.

NA, Not Applicable. Bolded values highlight the overall response rates of the trials.

without instances of on- or off- target toxicity attributed to TCR T cells (164, 176–178, 193). Together, these studies demonstrate that NY-ESO-1 targeting TCR T therapies are safe and capable of eliciting potent antitumor responses.

Trials Targeting MAGE-A Family Antigens: Early Toxicity and Recent Success

The MAGE-A family of proteins have also served as highly attractive CGA targets, and results from several TCR T trials targeting members of this family are now available. Unfortunately, two early reports of trials targeting MAGE-A3 described fatal TCR T mediated toxicity. In one study, two patients treated with TCR T cells expressing an affinity enhanced MAGE-A3 specific TCR died of cardiac toxicity following TCR T infusion. Post hoc investigation into the cause of these fatalities revealed cross-reactivity of the affinity enhanced TCR towards Titin expressed in cardiomyocytes (127, 128). This study emphasized the need for extensive preclinical investigation into potential off-target reactivities of lead TCRs. In a study published the same year, nine patients with metastatic cancer were treated with TCR T cells expressing an affinity enhanced TCR recognizing a similar MAGE-A3 and MAGE-A12 epitope. Clinical responses were achieved in 5/9 (56%) of patients, including a durable complete response in one patient. However, three patients experienced severe neurotoxicity following TCR T cell infusion, with two patients dying as a result. Post hoc analysis identified unexpected expression of MAGE-A12 in a subset of neurons in the brain, and the observed toxicity was thus presumed to be due to on-target off-tumor recognition of MAGE-A12 the brain (129). This study demonstrated the need for extensive preclinical characterization of cognate antigen targets in healthy tissue. A later study targeted MAGE-A3 with a high affinity HLA-DPB1*04:01 restricted TCR derived from a T regulatory cell. Objective responses were achieved in 4/17 (24%) of patients, with one patient achieving a durable complete response. Following TCR T treatment, one patient experienced grade 4 toxicities including increased ALT, AST, and creatinine, and eventually developed respiratory failure requiring hospitalization. A second patient experienced grade 3 toxicities of increased ALT, AST, and creatinine lasting two days. The cause of these toxicities was not described (194). Two recent reports of phase I trials of TCR T cells expressing an affinity-enhanced TCR specific for MAGE-A4 (ADP-A2M4 SPEAR T cells) to treat various solid cancers observed antitumor responses without evidence of serious TCR T mediated toxicity (182, 183). This year, Adaptimmune reported results of a phase II trial of ADP-A2M4 SPEAR T cells (now afamitresgene autoleucel) to treat patients with synovial sarcoma or myxoid/round cell liposarcoma (MRCLS). Here, objective responses were observed in 13/33 (39.4%) patients, including two durable complete responses, and disease control was achieved in 28/33 (84.8%) patients. Grades 1-2 CRS were observed in 21 patients and grade 3 CRS was observed in one patient. Based on this data, the company plans to file for afamitresgene autoleucel approval next year (184). In summary, results of early TCR T trials targeting MAGE-A3 demonstrated the need for more extensive preclinical testing of

both on-target and off-target effects of lead TCRs and enhanced preclinical safety assessment strategies have emerged as a result of these studies. However, recent trials of Adaptimmune's MAGE-A4-targeting afamitresgene autoleucel have demonstrated strong efficacy treating solid cancers in the absence of major TCR T cell mediated toxicity and may be nearing FDA approval.

Emerging Targets

Recent results have demonstrated efficacy of TCR T cells targeting HPV antigens in patients with various HPV-associated solid cancers. Relatively modest efficacy was achieved in a study treating twelve patients with TCR T cells expressing an E6-specific TCR, with objective responses observed in 2/12 (17%) patients, with no dose-limiting toxicities (186). In a related study using a higher affinity TCR recognizing E7, objective responses were achieved in 6/12 (50%) patients, with one dose-limiting toxicity that was presumably unrelated to TCR T mediated toxicity (44). However, the duration of clinical responses observed in both trials were relatively short (2 – 9 months). Recent studies have also demonstrated safety and efficacy of TCR T trials targeting the cancer overexpression antigen WT1. In one study, twelve AML patients at high risk for relapse following hematopoietic cell transplant (HCT) were treated with allogenic EBV-specific T cells engineered to express a WT1 specific TCR. Relapse free survival was achieved in 100% of the TCR T treated patients, as compared to 54% relapse free survival post-HCT in a comparative group of eighty-eight AML patients at similar risk of relapse. Nine patients exhibited grade 1-2 GVHD following TCR T infusion, with one patient developing grade 3 acute GVHD. However, the GVDH was determined to be most likely caused by the use of allogenic T cells rather than the introduced TCR (188).

Experiences of TCR T vs CAR T Trials to Treat Solid Tumors to Date

Recent years have seen the emergence of clinical trials reporting on the safety and efficacy of TCR T therapeutics in the context of both hematological malignancies and solid tumors (**Table 1**). Several TCR T trials focused on the latter have achieved improved therapeutic outcomes as compared to those achieved in CAR T trials (195), and it now appears that TCR T therapeutics are closer to receiving FDA approval for the treatment of solid cancers. A possible explanation for this disparity lies in the biological differences between TCRs and CARs (**Figure 1**), including 1) the ability of TCRs to recognize HLA-presented antigens derived from any cellular compartment including high specificity antigens such as CGAs and viral antigens, 2) TCRs are considerably more sensitive to low concentrations of a target antigen as compared to CARs, particularly in the case of affinity-enhanced TCRs (12–14), and 3) unlike CAR T cells, engineered TCRs do not drive ligand-independent tonic signaling (196, 197), making them better equipped to maintain function in vivo. Ultimately, the aforementioned factors are speculated to play a role in the comparatively improved performance of TCR T therapeutics

targeting solid cancers, however, both fields are evolving rapidly with new antigen discoveries and further genetic engineering of the T cells, which should lead to improved efficacy in the upcoming solid tumor CAR T trials.

Remaining Challenges

Intense efforts have been made to understand and address factors that limit the efficacy and clinical applicability of T cell-based ACTs, including TCR T therapy. This section describes select challenges currently facing TCR T therapy and the strategies available to meet them.

Manufacturing Cost and Complexity

TCR T products typically require 1-3 weeks of manufacture and involve several relatively complex processing steps that must be performed in highly regulated good manufacturing process (GMP) facilities. Despite the relative complexity, current TCR T manufacturing processes generally result in high rates of successful product manufacture. However, the cost of these T cell products is high. This is demonstrated by the high costs of FDA approved CAR T therapies, which utilize similar manufacturing processes. For example, single infusions of axicabtagene ciloleucel or tisagenlecleucel cost $373,000 and $475,000, respectively (198). In response to this, a strategy under development by many groups is the use allogeneic, or off-the-shelf, TCR T and CAR T products. However, a major challenge facing this approach is the likelihood of GVHD mediated by allogeneic T cells. Strategies to mediate GVHD of allogeneic T cell products include the use of oligoclonal virus-specific T cells with tightly restricted antigen specificities (188, 199), invariant T cell subsets such as γδ T cells (200) and iNKT cells (201), or *TRAC/TRBC* disrupted T cells (202). Another strategy to reduce the manufacturing cost of both TCR T and CAR T products is the use of non-viral gene delivery methods such as RNA electroporation (203, 204), transposons (205–207), and CRISPR/Cas9 (166).

T Cell Persistence

The ability of infused T cells to persist within the patient is an important factor mediating ACT antitumor efficacy (208). As such, intense efforts have been made to develop strategies that improve T cell persistence. Perhaps the most common strategy is to administer non-myeloablative lymphodepleting chemotherapy prior to T cell infusion, which alleviates competition of infused T cells with endogenous T cells for homeostatic cytokines such as IL-7 and IL-15, among other likely mechanisms (189). Several studies in mice have demonstrated that less differentiated T cell subsets (e.g., central memory and stem cell memory), have improved *in vivo* engraftment and persistence compared to highly differentiated T cells subsets (e.g., effector memory and terminally differentiated effectors) (209–211). As such, several strategies are now commonly employed to preserve less differentiated T cell subsets during ACT manufacture, including reduced expansion times and use of less differentiation-inducing cytokines (181, 212, 213). Some TCR T trials have further extended this approach by selecting specific T cell differentiation subsets prior to infusion (NCT02062359, NCT02408016). Another factor that limits T cell persistence is the paucity of costimulatory ligands within the tumor microenvironment. Several early TCR T trials performed coadministration of vaccines in efforts to provide TCR stimulation and costimulation *in vivo*. However, several of these studies failed to observe increased TCR T efficacy mediated by combination with vaccination (174, 176, 180). Genetic strategies to improve T cell costimulation include modified TCRs incorporating costimulatory domains (155) and coexpression of costim-only CARs (214) or domain swapped inhibitory receptors (215). Finally, several genetic strategies are also being explored to maintain T cell homeostatic cytokine signaling, including auto-secreting IL-15 or IL-12 elements (216–219) and a constitutively active IL-7 receptor (220).

Immunosuppressive Tumor Microenvironment

Another obstacle faced by adoptively transferred T cells is the immunosuppressive tumor microenvironment (TME). The TME supports tumor survival by recruiting immunosuppressive cell types, including myeloid derived suppressor cells (MDSCs), regulatory T cells (Tregs), and tumor-associated macrophages (TAMs) (221). Several pre-clinical studies of ACT are working to incorporate strategies to enable T cells to function in the hostile TME, including cotreatment with immune checkpoint blockade (222, 223), and genetic incorporation of inverted cytokine receptors (224) or MDSC targeting costimulatory receptors (214). A thorough understanding of the TME and its effect on T cells is necessary for the future success of ACT for solid cancers.

Tumor Intrinsic Escape Mechanisms

TCR T efficacy is influenced by the heterogeneity of cognate-antigen expression by tumor cells. This is especially the case when targeting CGAs, such as NY-ESO-1 and MAGE-A family proteins, which are often heterogeneously expressed by tumors (36). Nonetheless, complete responses have been observed in patients treated with TCR T cells targeting NY-ESO-1 and MAGE-A family proteins (**Table 1**). This may be partially explained by a phenomenon known as epitope spreading, whereby the immune response mounted by the infused TCR T cells leads to priming and activation of endogenous T cells to other non-cognate tumor antigens. Indeed, epitope spreading has been observed in humans following vaccination (225) and ACT (226, 227). Several genetic engineering strategies have now been developed to promote epitope spreading, including constitutively expressing inflammatory cytokines IL-12 (217, 218) and IL-18 (228), CD40L (229), or the DC growth factor FLT3 (230). Another approach to address antigen heterogeneity is to genetically encode specificity towards multiple antigens. While several multitargeting approaches have been developed in the CAR T realm (231), far less progress has been made in the development of multitargeting TCR T therapies to date.

TCR T therapies are also susceptible to tumor cell escape through perturbations in APM pathways. Downregulation of HLA-class I and other APM components such as TAP1 has been observed in many cancer types (232, 233). The impact of these escape mechanisms has been clearly demonstrated in TCR T and TIL trials where mutations in tumor APM components resulted

in tumor escape and cancer progression (44, 67). These studies highlight an urgent need for strategies to address such tumor intrinsic mechanisms of escape, especially as we see further improvements of the antitumor efficacy of TCR T therapeutics. In cases of tumor downregulation of APM pathways, expression of these components may be recovered through administration of interferon (234) or epigenetic modifying drugs (235). Addressing cases where APM components are lost through hardwired genetic mutations is considerably more challenging. One strategy that has been investigated in mice is *in situ* gene delivery of *β2M* with an adenoviral vector (236). However, this approach delivered adenovirus into relatively small tumors (7 – 10 mm in diameter) by intratumoral injection, therefore it is unclear if this approach will be effective in patients whose tumors are large, inaccessible, and/or dispersed. Therefore, patients with hardwired loss of APM components will likely need to be treated with HLA-independent therapies such as CAR T. Interestingly, HLA-independent TCRs are also currently under development. These TCRs bind natively folded surface proteins similar to CARs, but possess binding affinities within the range of pMHC-TCR interactions (237).

CONCLUSIONS

Immunotherapies have swiftly risen to become one of the major pillars in cancer treatment. Among them, TCR-engineered T cell therapies are a rapidly growing, active, and evolving field. Since the first report of redirected T cell specificity through TCR transfer in 1986 (238), tremendous progress has been made in TCR T therapies and their applications. Emerging technologies and enhanced strategies have made TCR discovery efforts considerably more time and cost effective. This will allow for more groups to become involved in TCR discovery and will ultimately lead to an increase in TCRs targeting new tumor antigens and restricted to a broader range of HLAs. Several clinical findings from early TCR T trials have shaped the past decade of TCR T development. Toxicities observed in early trials have led to improved preclinical safety assessments of TCRs and a transition towards antigen targets with increased tumor specificity. Since 2015 there has been an influx of results of TCR T trials treating various solid cancers and hematological malignancies. Several of these trials have demonstrated impressive clinical responses in the absence of serious toxicities, and it now seems that the first approval of TCR T therapies for solid cancer may be around the corner. These early results give reason for optimism in the continued development of TCR T therapies for cancer.

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PS and LK contributed to the development, writing, and review of the article. VH contributed to the figures and review of the article. All authors contributed to the article and approved the submitted version.

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