

Strategies to genetically engineer T cells for cancer immunotherapy

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Abstract Immunotherapy is one of the most promising and innovative approaches to treat cancer, viral infections, and other immune-modulated diseases. Adoptive immunotherapy using gene-modified T cells is an exciting and rapidly evolving field. Exploiting knowledge of basic T cell biology and immune cell receptor function has fostered innovative approaches to modify immune cell function. Highly translatable clinical technologies have been developed to redirect T cell specificity by introducing designed receptors. The ability to engineer T cells to manifest desired phenotypes and functions is now a thrilling reality. In this review, we focus on outlining different varieties of genetically engineered T cells, their respective advantages and disadvantages as tools for immunotherapy, and their promise and drawbacks in the clinic.

Keywords T cell · T cell receptor (TCR) · Chimeric antigen receptor (CAR) · Gene-modified T cells · Adoptive cell therapy · Tumor immunity

Abbreviations

ACT	Adoptive cell transfer
AICD	Activation-induced cell death
B-ALL	Acute B lymphoblastic leukemia
CAIX	Carbonic anhydrase IX
CEA	Carcinoembryonic antigen

CLL	Chronic lymphocytic leukemia
CTA	Cancer–testis antigen
FBP	Folate-binding protein
HSV-tk	Herpes simplex virus thymidine kinase
iC9	Inducible caspase 9
iCAR	Inhibitory chimeric antigen receptor
NHL	Non-Hodgkin lymphoma
pMHC	Peptide–major histocompatibility complex
RCC	Renal cell carcinoma
siRNA	Small interfering ribonucleic acid

Introduction

T cells play a central role in mediating cellular immunity. These multi-functional adaptive immune cells protect us from disease throughout our entire lives with their ability to recognize bacterial, viral, and cancer-associated antigens. T cells harbor a T cell receptor (TCR), which dictates their antigen specificity and exists as a membrane heterodimer, associated with the CD3 signaling complex. The TCR binds a ligand consisting of a peptide processed from pathogens, malignant, and normal cells presented to the T cell by the major histocompatibility complex (MHC). In this way, T cells can recognize the presence of pathogens or transformed cells, leading to target cell killing, or the secretion of pro-inflammatory cytokines that recruit and support other immune effectors.

Unfortunately, most viruses and tumors have developed various mechanisms to evade the host immune system, leading to weak or ineffective immune responses, resulting in chronic infections or malignancies. To address the issue of immune responses ineffective at eliminating cancer, many therapeutic approaches are aimed at harnessing and optimizing the anti-tumor potential of T cells.

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One such strategy known as adoptive cell transfer (ACT) involves the transfer of ex vivo activated and expanded antigen-reactive T cells or genetic modification to redirect their specificity prior to transfer. ACT gained its first success using lymphokine-activated killer (LAK) cells [1] and later tumor-infiltrating lymphocytes (TILs) [2, 3] or antigen-stimulated autologous peripheral blood lymphocytes (PBL) [4–7].

The most promising of these early approaches utilized TIL following harvest from tumor, and short-term ex vivo expansion was pioneered by the Rosenberg group at the Surgery Branch of the National Cancer Institute. Animal models and studies in patients with melanoma demonstrated that lymphocytes isolated from TIL maintained tumor reactivity in vitro, having the capability to lyse tumor cells and secrete cytokines, such as IL-2, IFN γ , and TNF α [8, 9]. These cells were also able to mediate objective clinical responses when grown ex vivo and infused back into patients [10].

Despite these promising but preliminary clinical results, for a variety of reasons TIL has not been a universal approach for ACT. Aside from TIL production being a logistically and technically demanding method, oftentimes primary tumors harboring TIL are previously resected as part of cancer treatment or inaccessible depending on the tumor type and location. Additionally, the time it takes or the inability to expand available TIL to therapeutic numbers calls for an alternative cell-based approach for treating cancer or chronic viral infections.

Recent technological advances have facilitated efficient expression of transgenes in T cells allowing for normal circulating peripheral lymphocytes to be redirected targeting antigens of choice. Genetic engineering of T cells with chimeric antigen receptors (CARs), TCRs, and other receptors has been shown to successfully redirect the specificity of T cells. Such strategies are the focus of this review.

However, the ability to redirect a T cell to recognize a specific antigen is not enough to ensure an effective immunotherapy. Antigen recognition needs to be coupled with efforts to ensure a T cell's functionality specific to the target while limiting off-target or off-tumor recognition. T cells should also be able to functionally persist long term and be able to traffic to and accumulate at the target site. Additionally, optimal modified T cells should exhibit robust, multi-functional immune responses, resist mechanisms of anergy, exhaustion and immunosuppression, and be amenable to deletion on demand to diminish potential toxicity issues. While these strategies show promise at the bench and have had some clinical success at the bedside, many aspects of this type of therapy must be resolved before these effectors are good enough to become a standard treatment for patients.

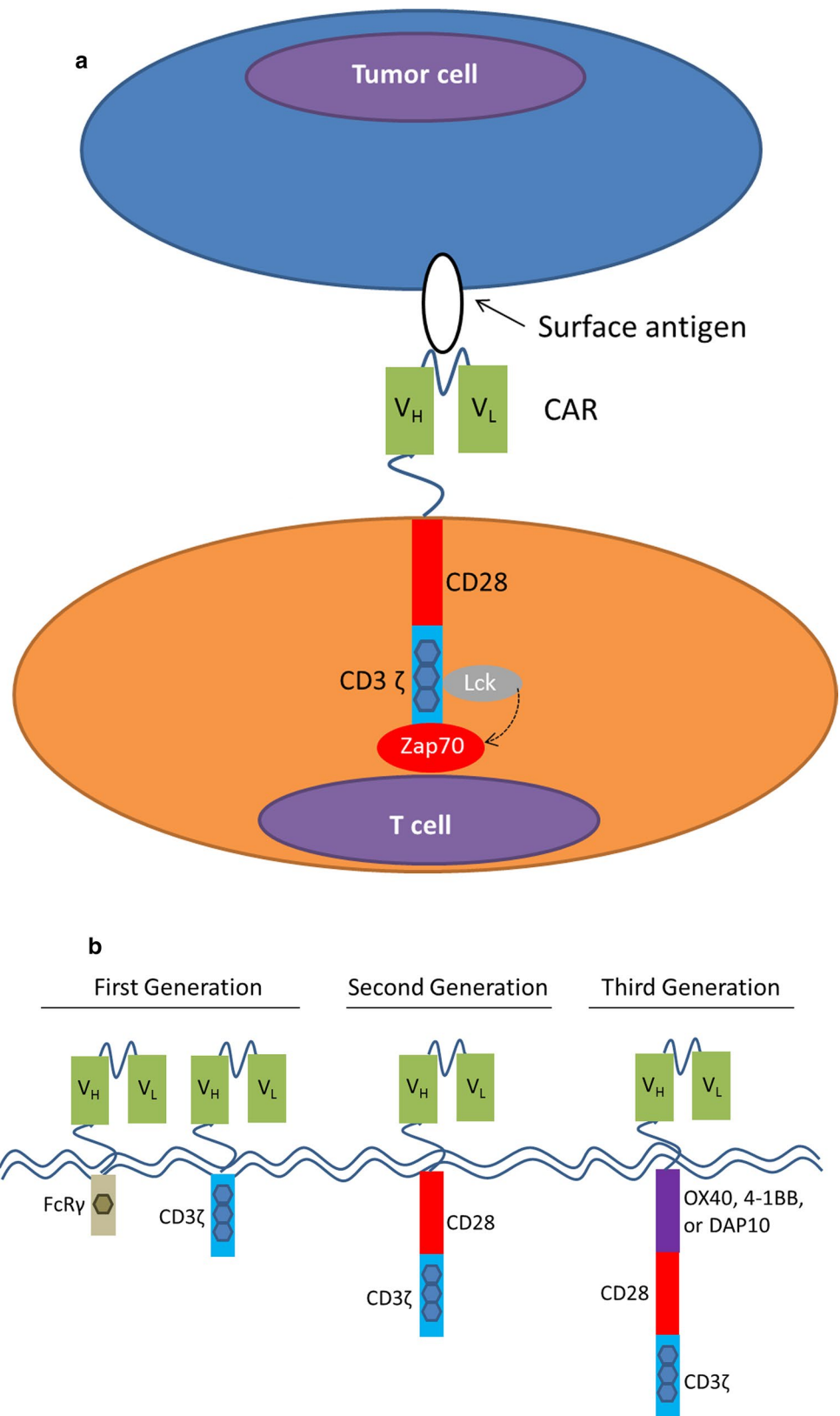
Chimeric antigen receptors

CAR design

One class of antigen receptors that have been widely examined for the use of redirecting T cell specificity is CARs (Fig. 1a). A CAR is simply described as combining the antigen-binding capability of an antibody with the intracellular signaling-associated component of a TCR. This unique juxtaposition allows for high affinity, three-dimensional epitope recognition by an immunoglobulin to be linked to the helper or effector responses of a T cell. Specifically, the extracellular antigen-recognizing domain is derived from the antigen-binding fragment (Fab) of mouse monoclonal antibodies (mAbs) that have high affinity for specific antigens. Unlike the normal structure of the Fab fragment of a mAb, the Fab fragment in a CAR exists as a single-chain variable fragment (scFv). The scFv is linked via hinge and transmembrane domains to an intracellular signaling domain. This allows for antigen recognition to be coupled to immune cell signal transduction by phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs). While the first CAR, described by Eshhar and colleagues, linked the scFv to the signaling components of the FcR γ chain [11], subsequent CARs used that of the ζ chain of the TCR/CD3 complex [12] as the CD3 ζ signaling domain contains three ITAMs as opposed to FcR γ 's single ITAM. This structural configuration allows for essentially any mAb to be engineered to make a CAR. But for targets where generating conventional antibodies in mice or other species is difficult, other techniques, including phage display, have been useful in generating the antigen-binding portion of the CAR [13, 14].

The use of a mAb or ligand receptor facilitates recognition of intact proteins, carbohydrates, and lipids and can alleviate the need for target antigens to be processed and presented by MHC molecules. However, CARs and TCR-like mAbs have also been designed to recognize conformational epitopes of pMHC [15–18]. Yet, avoiding MHC restriction would allow for CAR-mediated target recognition in spite of HLA downregulation or aberrant proteasomal antigen-processing mechanisms. This non-MHC-restricted antigen recognition also allows CAR use in patients of all HLA types, which is a distinct advantage from the use of engineered TCRs, as will be discussed. CARs are also independent of many of the signaling molecules or coreceptors required for TCR signaling and do not require association with the CD3 complex for T cell activation and function. As such, CARs contain all the minimal elements necessary to bind antigen and activate the T cell. Additionally, as a single-chain construct, CAR constructs are compact with relatively small vectors, allowing it to

Fig. 1 CAR-mediated target cell recognition. **a** Structure of a second-generation CAR interacting with tumor cell. A CAR consists of a single-chain variable fragment (scFv) composed of variable light (V_L) and heavy (V_H) chains linked via hinge, transmembrane domains, and intracellular signaling domains containing at least the γ chain of the FcR or the ζ chain of the TCR/CD3 complex. Identity of the scFv region dictates MHC-independent recognition of a surface antigen. ITAMs of CD3 ζ are denoted in blue octagons; **b** comparison of costimulatory domains included in first, second, or third generation of CARs. ITAMs are denoted as colored octagons



easily make high titer virus for transduction. Furthermore, single-chain CARs are not subject to chain pairing competition or mispairing, unlike when introducing exogenous TCRs as discussed in a later section.

However, there are some limitations to the use of CAR-engineered T cells [19]. CAR recognition only targets antigens expressed on the cell surface. Thus, they would not be effective against non-surface viral proteins that exist intracellularly and are processed and presented by MHC. Although CARs' lack of MHC restriction avoids immune escape mechanisms including HLA downregulation, antigen loss can still limit the effectiveness of antigen-specific CARs [20, 21]. Additionally, myeloid-derived suppressor cells (MDSCs) have been shown to inhibit the efficacy of CAR-engineered T cells through engagement of PD-1 in a murine model for metastatic colorectal cancer [22]. Also, the mAb–antigen interaction is much stronger than a TCR–antigen interaction, which may impact T cell function [23], and the identity of the scFv region is thought to impact the stability and activity of CAR T cells [24]. Moreover, use of murine-derived scFv causes concern for potential immunogenicity of these chimeric receptors [25, 26], although efforts to reduce immunogenicity have been used by humanizing murine-derived scFv or generating scFv from human scFv phage display libraries [27].

Generations of CARs

Over time, the design of CARs has been refined to provide better antigen recognition and a more efficient transfer of cellular signaling for T cell function and persistence [28]. As mentioned previously, the signaling domain of FcR γ was swapped with that of CD3 ζ because it included a greater number of ITAMs (Fig. 1b). Additionally, the single-chain antibody can be substituted by other receptors or a ligand of a receptor expressed on tumor cells. Such approaches include substituting the scFv region of a CAR for heregulin (a ligand for Her3 or Her4 receptors) [29], VEGF (anti-VEGFR2) [30], NKp30 (targeting B7-H6) [31], or the NKG2D receptor [32–34]. Moreover, multiple signaling domains have been added to the CD3 ζ or FcR γ domains to augment activation and costimulation mimicking immunologic signal 2 during physiologic T cell activation [35].

“Second-generation” CARs (Fig. 1b) utilize an additional cytoplasmic domain of a costimulatory receptor, such as CD28, 4-1BB, DAP10, OX40, or ICOS, providing greater strength of signaling and persistence to the T cells [36–42].

A third generation of CARs (Fig. 1b) was also developed using two costimulatory domains with an activating domain, conferring an even greater potency to redirected T cells [36, 43–48]. But these more complex structures

warrant further investigation as it is unclear whether the strong costimulation would always be advantageous [49]. Optimization of how many and which type of signaling domains included is necessary to determine which combination is best for augmenting activation, sustained function, and survival while minimizing anergy, premature death, and rapid exhaustion. Additionally, further efforts to examine how antigen location and density, and CAR binding moiety, affinity, and sensitivity affect its function may also help influence development of optimally designed CARs.

CAR targets

The first clinical trials using CAR targeted folate-binding protein (FBP) for patients with ovarian cancer [50] and carbonic anhydrase IX (CAIX) for patients with renal cell carcinoma (RCC) [51]. Both CARs were first generation containing the FcR γ signaling domain. In both of these trials, no objective clinical responses were seen, nor were the genetically altered T cells able to persist long term, but the potential for adverse events using CAR therapy was first evident. In the trial targeting CAIX⁺ RCC, the phenomenon described as “on-target, off-tumor” was clearly evident. Patients treated with the CAIX CAR gene-modified T cells experienced grade 2–4 liver toxicity because the transduced cells recognized CAIX antigen expressed on the epithelial cells of the bile duct [51].

As subsequent generations of CARs were developed and more efficient CAR targets were identified, we began to see greater clinical success with CARs. The efforts targeting pan-B cell antigen CD19 are the best examples of the proof of concept for CAR therapy of this. Initial trials targeting CD19 associated with relapsed indolent non-Hodgkin's lymphomas (NHL) and chronic lymphocytic leukemia (CLL) [52–58] demonstrated the safety of CAR T cells and modest clinical benefit. Later, patients with B cell acute lymphoblastic leukemia (B-ALL) receiving CD19-targeted CAR T cell treatment resulted in positive clinical outcomes with robust anti-tumor efficacy in two independent trials [21, 59]. One trial utilized a second-generation CD28/CD3 ζ anti-CD19 CAR and achieved rapid tumor eradication and minimal residual disease-negative complete remissions in all five patients treated [59]. Therapy was well tolerated, and steroid therapy ameliorated cytokine-mediated toxicities. The other trial used second-generation anti-CD19 CAR T cells including costimulatory cassette 4-1BB (in contrast to CD28 in the clinical study described above) to treat two children with B-ALL, achieving expansion of anti-CD19 CAR T cells and complete remission in both patients [21]. Cytokine-release syndrome developed in both patients, but was effectively reversed with etanercept and tocilizumab treatment without reducing antileukemic efficacy. However, one patient relapsed with CD19⁻ blast

Table 1 Active clinical trials using CAR-engineered T cells

Target antigen	Associated malignancy	CAR generation	Clinical trial ID#
CD19	Acute lymphoblastic leukemia	3rd (CD28:4-1BB)	NCT02186860
CD133	Various malignancies	1st	NCT02541370
CD171	Neuroblastoma	2nd (4-1BB) and 3rd (CD28:4-1BB); transgene includes truncated EGFR	NCT02311621
CEA	CEA ⁺ adenocarcinomas	2nd (CD28)	NCT01723306
EGFR	EGFR ⁺ solid tumors	1st and 2nd (4-1BB)	NCT01869166
GD2	Neuroblastoma	3rd (CD28:OX40); transgene includes iC9	NCT02439788
GD2	GD2 ⁺ sarcomas	3rd (CD28-OX40); transgene includes iC9	NCT01953900
Her-2	Breast cancer	2nd (CD28)	NCT02547961
Her-2	Glioblastoma multiforme	2nd (CD28)	NCT01109095
Mesothelin	Pancreatic, ovarian, and mesothelioma	2nd (4-1BB)	NCT02159716

cells 2 months after treatment, indicating a need to target other molecules in some patients with ALL.

The success of anti-CD19 CARs is facilitated by the near-universal expression of CD19 on B cell malignancies but limited and absent expression on B cells and bone marrow stem cells, respectively. These properties also limit the off-target potential of anti-CD19 CARs. Outcomes from this CD19 model also allowed for the observation of practical limitations of initial CAR designs and allowed for development of subsequent generations of CARs. Most recently, a clinical trial used a second-generation CD19-reactive CAR to treat both children and young adults with chemotherapy-resistant B-precursor acute lymphoblastic leukemia inducing a complete response in 70 % of patients with B-ALL and an minimal residual disease-negative complete in response in 60 % [60]. Additionally, all toxicities associated with the therapy were reversible.

Although less serious than the aforementioned “on-target, off-tumor” events, a potential drawback of the highly effective CD19 CAR campaign targeting B cell malignancies is the prolonged elimination of normal B lymphocytes and, thus, impairment of humoral immunity [52, 55, 57]. However, B cell aplasia and hypogammaglobulinemia can be easily mitigated by intravenous administration of gammaglobulin.

While CD19-targeted B cell malignancies have been the poster child advocating for CAR therapy, it is important to acknowledge another CAR target that has exhibited mixed clinical results. In 2010, it was reported that the use of a third-generation CAR was based on the widely used mAb trastuzumab targeting Her-2 induced respiratory distress resulting in death in one treated patient with metastatic colon cancer [49]. It was discovered that low-level Her-2 expression on normal lung epithelium caused this fatal side effect, characterized as a much more serious “on-target, off-tumor” adverse event. Additionally, the very high dose of CAR T cells (10^{10} T cells) may have precluded any

potential therapeutic window. With this in mind, a modified CAR-based approach targeting Her-2 led to a more successful recent trial [61]. In this report, a dose-escalation study using a second-generation Her-2-specific CAR with a different scFv was used in patients with recurrent/refractory Her-2⁺ sarcomas. This study also differed in that it administered lower doses of T cells (1×10^4 – 1×10^8 /m²), and it did not use coadministration of high-dose IL-2 or lymphodepleting chemotherapy prior to transfusion. Transduced cells persisted for 6 weeks and trafficked to tumor sites without any evident toxicities. Some patients even exhibited stable disease or partial response. The stark contrast between these two trials highlights the importance of mAb selection, generation of CAR, target distribution, and host preconditioning can have on the positive and negative outcomes in CAR clinical trials.

A wide variety of other CARs have been designed to target an array of antigens showing promise as potential therapies for cancer. Other targets evaluated in vitro and in vivo include but are not limited to EGFRvIII [62] for glioblastoma, GD2 [63, 64] for neuroblastoma, GD3 [65, 66], MAGE-1 [18], and HMW-MAA [67] for melanoma, CD20 [43, 45, 68–71], CD23 [72], CD30 [73–75] and others for hematologic malignancies, PSMA [39, 76] for prostate cancer, MUC-1 [46], Her-2 [77, 78], and CEA [79–81] for breast cancer, EGP-40 [82] for colorectal cancer, VEGFR2 [30, 83] and KDR [84] for tumor neovasculature, and MUC16 [85] for ovarian cancer. Table 1 lists a sampling of active clinical trials using CARs and their respective targets.

CAR summary

Overall, CARs provide MHC-independent recognition of a variety of extracellular target types with a compact, single-chain construct containing all the minimal elements necessary for T cell activation. For better or for worse, the

design of CARs prevents any chance of receptor mispairing but circumvents the natural process of T cell activation. While documentation of “on-target, off-tumor” is well noted, “off-target” effects are not as readily seen because the antigen-binding region of CARs is derived from monoclonal antibodies that have been well characterized. However, several groups conducting clinical trials with CARs have seen other serious adverse events including tumor lysis syndrome [57] and cytokine storm [55, 86, 87]. Additionally, anti-transgene immunogenicity has been described as a potential cause of low persistence of CAR T cells post-transfer [53] and has even caused severe anaphylactic reactions in patients treated with CAR T cells [26]. Overall, the majority of CAR studies have shown much promise for their clinical use, and continuing efforts are further elucidating how to limit improve clinical outcomes while minimizing adverse events.

T cell receptors

TCR design

TCRs compose an additional class of antigen receptors used to engineer T cells. Natively, CD8⁺ and CD4⁺ T cells express a TCR as a membrane-bound $\alpha\beta$ heterodimer, which recognizes antigenic peptide fragments bound to MHC class I or II, respectively. To date, TCRs investigated for ACT have been limited to mostly MHC-I-restricted candidates. These TCRs can recognize any intracellular antigen as it is processed and presented by MHC molecules and associate with the CD3 complex that initiates ITAM-mediated signal transduction in the T cell (Fig. 2).

Because TCRs recognize processed peptides presented on MHC, targeted antigens can be derived from the entire protein composition of the tumor cells, including intracellular proteins, whereas CARs can only be designed to recognize molecules expressed on the surface of target cells. This quality also allows TCRs to target a large number of non-surface antigens of virally infected cells and tumors associated with viral infection, such as hepatitis-associated hepatocellular carcinoma, papilloma virus-associated cervical cancer, and Epstein–Barr virus-related malignancies.

An important factor influencing the effectiveness of immunotherapeutic use of TCRs is the relationship between receptor affinity and cell function. Because antigen recognition depends on the interaction between the TCR and the peptide MHC (pMHC) complex, it might be predicted that T cells engineered with high-affinity TCRs are better effectors than T cells engineered with low-affinity TCRs. We and others have suggested that CD8 expression is only required for T cells with low-affinity TCRs [88–90]. The reliance on coreceptors, such as CD8, is one

of the hallmarks contrasting TCRs with CARs. Various CD8-independent TCRs have been characterized that can transfer antigen recognition to CD4⁺ cells in an MHC-I-restricted manner [89–93]. Together, these studies suggest high-affinity TCRs allow for lower TCR transgene expression to achieve functionality, create novel populations of MHC-I-restricted CD4⁺ cells, and offer enhanced tumor regression. Naturally occurring high-affinity, CD8-independent TCRs are relatively rare, however, and thus difficult to find. Rather than screening for such high-affinity TCRs, various methods have been developed to affinity-enhanced already characterized TCRs, which are discussed in a later section. It is important to recognize, however, that higher-affinity TCRs hold much greater potential to undergo activation induced cell death (AICD) upon antigen encounter [94, 95], or induce “off-tumor, on-target” and “off-tumor, off-target” reactivity [96–98], counterproductive to their therapeutic intention.

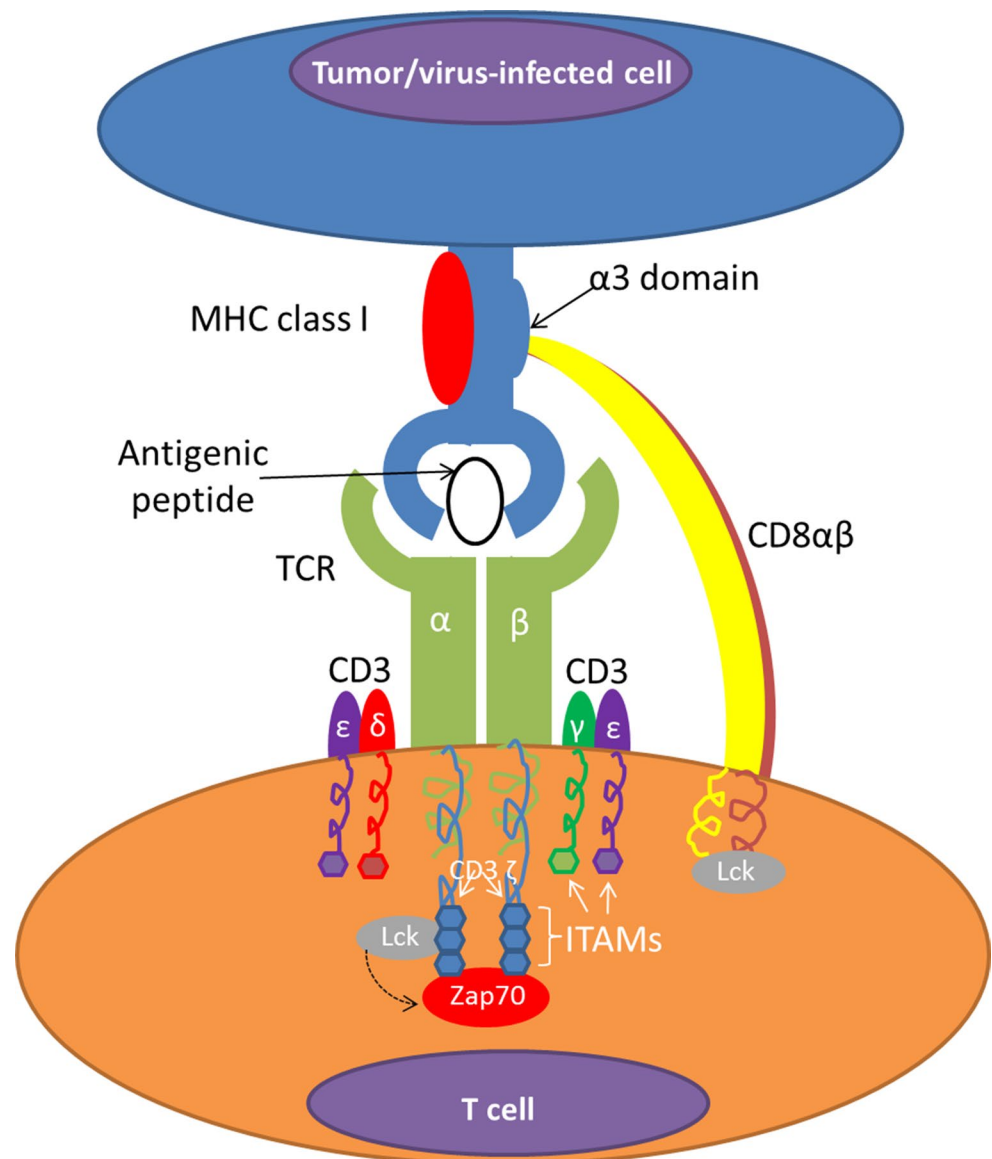
It is also important to acknowledge that MHC restriction of TCRs limits the number of patients that can be treated using a single TCR, and engineered T cells would need to be HLA matched to patients. Because HLA class I expression has a tendency to be downregulated on tumor cells [99, 100], this may also serve as a barrier to effective therapy. Additionally, because TCRs require interaction with the CD3 complex, its expression and functionality are limited by available CD3 complex components. Competition for the CD3 complex with endogenous TCR limits functional transgene expression, although strategies discussed below may allow for improved surface competition for CD3 by the transgene receptor.

TCR pairing

As a heterodimer, TCRs also differ from CARs in that two chains need to be expressed rather than just one to redirect specificity, resulting in larger construct formation and potentially non-uniform chain expression. The addition of viral 2A self-cleavage peptide sequences between the α and β chains within the construct [101–103] and codon optimization of the construct [104, 105] are two examples that allow increased and more uniform TCR expression without altering the TCR sequence itself. The presence of endogenous TCR also allows for the opportunity of chain mispairing between endogenous and introduced α and β chains. These interactions reduce the level of expression of the introduced TCR [89, 106] and could lead to novel and unpredictable self-reactive TCRs with potential for serious adverse events.

Such “off-target, off-tumor” adverse events have been reported as a result of high-affinity TCR recognizing unanticipated epitopes or as a result of the phenomenon of TCR chain mispairing, generating novel self-reactive

Fig. 2 TCR-mediated target cell recognition. Depicted is the structure of an MHC class I-restricted TCR interacting with tumor or virus-infected cell. TCR α and β chains are specific to both MHC and presented antigenic peptide. The TCR complexes with various CD3 components on the cell surface, and the CD8 coreceptor stabilizes the TCR–pMHC interaction while recruiting Lck to facilitate TCR signaling. ITAMs are denoted as colored octagons. Lck = lymphocyte-specific protein tyrosine kinase; Zap70 = zeta-chain-associated protein kinase 70



TCRs. Two recent studies demonstrate undesirable reactivity propagated from TCR mispairing between endogenous and introduced TCR α and β chains. One study observed graft-versus-host disease (GVHD) in a mouse model in five different TCR systems [107], and the other demonstrated the ability of engineered human T cells to develop MHC class I- and II-restricted allo- and auto-reactivity through mispairing [108]. However, to date, no evidence of GVHD has been seen in over 100 patients treated with this gene therapy approach [109].

The issue of TCR mispairing has been addressed in a variety of ways. One main strategic effort involves a host of modifications to the TCR construct summarized in Fig. 3. As mentioned before, codon optimization of the TCR α and β genes can help promote efficient translation and surface assembly of the introduced receptor without altering the

TCR sequence itself [104, 105]. Introduction of cysteine residues in the α and β constant regions has been shown to promote inter-chain disulfide bridge formation to limit mispairing [110]. Other approaches to help improve pairing, expression, and function of the introduced TCRs include the addition of leucine zippers at the end of the intracellular tails [111] and altering the glycosylation of the TCR [112]. Substituting human constant regions with portions of or the entire murine constant regions in the transgene also promotes proper pairing and enhanced surface expression [113–118]. It was observed that elements of the murine constant regions do not efficiently interact with the human constant regions [115]. Additionally, the murine constant region has a higher affinity for human CD3, which can favor the murinized receptor in competing for limited surface CD3 [113]. Receptors with murine constant regions

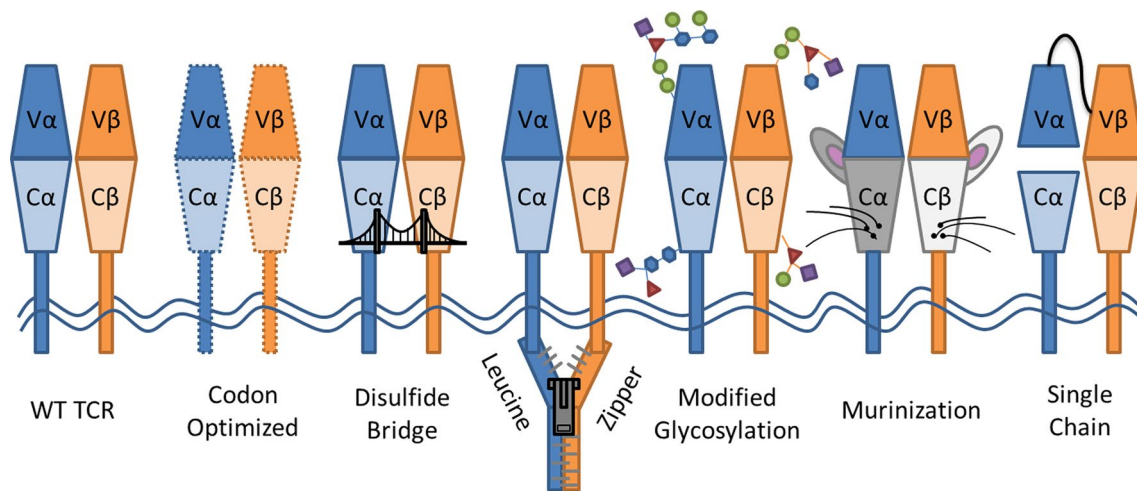


Fig. 3 TCR modifications to limit mispairing and improve cell surface expression and function. Modifications of wild-type TCR structure (far left) include codon optimization, introduction of a disulfide

bridge, addition of leucine zipper, modification of glycosylated residues, substitution with murine constant regions, or use of single-chain $V\alpha$ - $V\beta$ - $C\beta$ with a $C\alpha$ chain

were also found to mediate higher levels of cytokine secretion in vitro [113, 114], but this technique poses additional problems including evoking an immune response and possible clearance of the engineered T cells as they express potentially antigenic domains in the transgene [114].

Another novel approach to alleviate mispairing was demonstrated by recent reports using single-chain TCRs. One group engineered a recombinant TCR consisting of a single-chain $V\alpha$ - $V\beta$ - $C\beta$ and a $C\alpha$ chain, which only paired with each other and not the endogenous TCR [115]. Other groups have used a stabilized $V\alpha$ - $V\beta$ single-chain TCR linked to intracellular signaling domains to elicit functional activation of T cells in the absence of coreceptors and to circumvent mispairing with endogenous TCRs [119, 120]. Efforts to limit mispairing also focus on altering the endogenous TCR by downregulation via small interfering RNA (siRNA) [121] or using designer zinc-finger nucleases [122]. All these alternate strategies share the same goal to reduce competition for the CD3 complex and allow for stable pairing of the introduced TCR.

TCR targets

More than 20 years after TCR gene-modified T cells were first used to redirect T cell specificity in mice [123], this technology was first evaluated in humans. TCR-engineered T cells' first use in the clinic targeted HLA-A2⁺/MART-1⁺ melanoma [124, 125]. These initial clinical studies demonstrated that TCR gene-modified T cells were generally safe, well tolerated, and have the potential to be effective therapeutically in cancer patients. A later study targeting melanoma antigens MART-1 and gp100 demonstrated further clinical benefit using

TCR-transduced T cells, while highlighting potential for adverse events [96]. Of note, the gp100-reactive TCR in this study was high affinity and of mouse origin, and many patients treated with this TCR exhibited toxicities in the eye and inner ear and displayed destruction of normal melanocytes. This well-characterized example of “on-target, off-tumor” effects first cautioned the use of high-affinity TCRs. Fortunately in this case such toxicities resolved naturally or with administration of topical steroids in almost all patients.

Since these early studies, identification of TCR genes encoded to recognize epitopes expressed by human tumors and improvements in TCR gene transfer technology has allowed for normal T cell antigen specificity redirection and targeting of a variety of antigens and malignancies, including carcinoembryonic antigen (CEA), cancer–testis antigen (CTA) family members, and viral protein family members.

CEA is overexpressed in many epithelial cancers, particularly in colorectal cancer. A recent clinical trial noted objective clinical response in a patient treated with high-affinity CEA-reactive T cells [98]. However, “on-target, off-tumor” adverse events, including inflammatory colitis, were documented in these patients.

The CTA family includes NY-ESO-1 and MAGE and is expressed by the normal testis and a panel of human cancers including that of breast, bladder, colon, lung, melanoma, head and neck, gastric, ovarian, thyroid, neuroblastoma, synovial cell sarcoma, and prostate. Clinical studies have shown T cells redirected to recognize NY-ESO-1 provided objective clinical response in patients with synovial cell sarcoma and melanoma without any evidence of previously reported adverse events [126].

Table 2 Active clinical trials using TCR-engineered T cells

Target antigen	Associated malignancy	MHC restriction	Clinical trial ID#
gag	HIV	HLA-A2	NCT00991224
gp100	Melanoma	HLA-A2	NCT00509288
HPV E6	HPV-associated cancers	HLA-A2	NCT02280811
MAGE-A3	Various malignancies	HLA-A1	NCT02153905
MAGE-A3	Various malignancies	HLA-DP04	NCT02111850
NY-ESO-1	Various malignancies	HLA-A2	NCT02457650
Thyroglobulin	Thyroid cancer	HLA-A2	NCT02390739
Tyrosinase	Melanoma	HLA-A2	NCT01586403
WT1	Myelodysplastic syndrome, AML	HLA-A2	NCT02550535
WT-1	Non-small cell lung cancer, mesothelioma	HLA-A2	NCT02408016

A pair of clinical trials targeting widely expressed CTA, MAGE-A3, recorded much more mixed results. The first clinical trial used a mouse-derived, high-avidity TCR that was further modified by site-directed mutagenesis in the CDR3 region [127]. While targeting MAGE-A3, it was also reactive against MAGE-A9/A12. Although five out of nine patients experienced clinical regression of their cancers, one-third of the patients experienced neurological toxicity, and two patients died because of these adverse events. Unknown expression of MAGE-A12 in the brain is believed to be the cause of this toxicity.

Another clinical trial using an affinity-enhanced TCR targeting MAGE-A3 resulted in cardiogenic shock and death of the first two patients treated [128, 129]. While no MAGE-A3 expression was detected in cardiac tissue at autopsy, it was determined the TCR designed with affinity-enhancing mutations in the CDR2 regions exhibited an unpredicted cross-reactivity to titin, a sarcomeric protein expressed in striated muscle. Interestingly, the parental TCR from which the engineered TCR was derived from was isolated from a patient without cardiac toxicity, attributing the adverse events in the clinical trial to the intentionally induced CDR2 mutations. These mixed clinical results yield concern for use of such high-affinity receptors.

Virus-induced tumors can express members of a third family of antigens targeted by engineered T cells. Studies have engineered T cells to target antigens, such as CMV [130, 131], EBV [132–134], HIV [135–137], HCV [91–93, 138, 139], HPV [140, 141], and others. No clinical reports have yet been published testing virus-reactive TCR-transduced T cells in humans. Table 2 lists some of the active clinical trials using TCRs and their respective targets.

TCR summary

Overall, TCR-transduced T cells offer the ability to target a wide variety of self and non-self-targets through the normal biology of a T cell. Although MHC-restricted, dependent on coreceptors, and introduction of a second TCR causes

potential for mispairing, great efforts are being made to better engineer high-affinity TCRs hope to enhance therapeutic efficacy while minimizing potential adverse events. Some success has been seen in clinical trials, and many are hopeful and greater objective clinical results will be observed in the future.

Other engineering approaches

Other receptor types

While CAR and TCR engineering have been well studied, ongoing efforts to design other receptor types for T cell engineering hold merit and clinical potential as well. For example, engineering T cells to express the NKG2D receptor allow T cells acquire the reactivity of natural killer (NK) cells [32–34]. Another NK cell receptor that has been investigated for T cell engineering is NKp30, which recognizes the tumor-prone antigen B7-H6. A recent study combined the specificity of NKp30 and the signaling capability of CARs, designing a novel NKp30-based CAR replacing the scFv domain with the ligand-binding domain of NKp30 [31]. Adoptive transfer of the NKp30-CAR allowed for non-MHC-restricted recognition of B7-H6-expressing RMA, a murine lymphoma, in vivo and interestingly provided protection against subsequent challenge with wild-type RMA tumor cells. Similar efforts are being made to examine potential for CAR-related therapy replacing the scFV domain with other ligands or ligand-binding receptors described earlier.

Another unique approach being investigated engineers dual-expressing T cells. In this way, T cells can coexpress complementary CARs, TCRs or other receptors, each specific to a distinct target. This approach is thought to optimize T cell homing and tumor specificity while reducing toxic potential. In this way, it would promote selective enhancement of T cell survival in the tumor microenvironment with synergistic signaling and provide a means to

combat downregulation of antigen in tumor escape mechanisms. One study validated this tactic by generating T cells coexpressing CARs reactive to MUC1 and ErbB2 targeting breast cancer *in vitro* [142].

Additionally, recent work has demonstrated the design of anti-specific inhibitory chimeric antigen receptors (iCARs) linked to a powerful acute inhibitory signaling domain that can recognize T cell inhibitory molecules, such as CTLA-4 and PD-1, limiting T cell responsiveness even in the presence of an engaged activating receptor [143]. This dual-receptor approach provides a means to curb unwanted off-target responses.

Cytokine production

Another approach to modify or enhance potency of receptor-engineered T cells is to further genetically modify the cells to secrete pro-inflammatory or pro-proliferative cytokines. This approach not only provides autocrine support to enhance T cell function, proliferation, and/or persistence, but also favorably alters the tumor microenvironment. This would allow for innate and cognate immune effector recruitment and limitation of the systemic toxicity of exogenously delivered cytokines in therapeutic approaches. For example, engineering melanoma-reactive T cells to express IL-2 resulted in continued cell growth in the absence of exogenous IL-2, which may be a viable approach to help T cell persistence post-adoptive transfer [144]. A similar tactic using T cells modified to express IL-12 have exhibited enhanced anti-tumor function and were better able to resist immunosuppression by regulatory T cells [145, 146]. Similarly, engineering T cells to secrete IL-12 allowed transformation of myeloid cell within tumors from immunosuppressive to immunosupportive [147]. This strategy was even used in a recent phase I clinical trial where IL-12 secreting MUC-16(ecto)-targeting CAR-transduced T cells were infused into patients with recurrent ovarian cancer [148].

Chemokine recognition

In addition to cytokine-engineered T cells, modifying them to express chemokine receptors can aid in migration patterns, increasing the efficiency of trafficking to and infiltration of tumors. Transferring of genes encoding CCR4, CCR2B, or CXCR2 enables T cells to home toward CCL17, CXCL1, and macrophage chemoattractant proteins [73, 149, 150]. Also, VEGFR-2-engineered T cells allowed T cells to find tumor-associated neovasculature in one study [151]. Additional efforts to improve T cell homing may include engineering T cells with certain integrins or their ligands [152] or by blocking inhibitors of migration like endothelin [153].

Ways to improve

Although *in vivo* studies and clinical trials have shown great promise for engineered T cells as an effective immunotherapy for cancer, many efficacy and safety issues still prevent this treatment from becoming a standard of care. Efforts to pass these hurdles aim to allow the host to be most receptive to this kind of therapy, gain a better understanding of engineered receptors' impact on the biology of the T cell, and optimize transgene engineering and expression. Some of these strategies are discussed here.

Host conditioning

Host conditioning prior to ACT has been a point of interest to better allow long-term persistence of function of transferred cells. This evidence came from preliminary mouse studies that were followed up in the clinic by the Rosenberg group. They first noted that persistence and anti-tumor activity of transferred T cells *in vivo* was greatly increased with non-myeloablative lymphodepleting treatment using cyclophosphamide and fludarabine before adoptive transfer [154]. Other conditioning regimens, including fludarabine only [155], or dacarbazine [156], have also been used in studies with improved clinical responses. Lymphodepletion is thought to create space for the transferred T cells and eliminate competition for cytokines [157], remove competition at the surfaces of antigen-presenting cells [158, 159], and remove immunosuppressive regulatory T cells [160]. It is believed these changes in the host may lead to induction of a memory phenotype and enhance effector function. Systemic administration of cytokines, such as IL-2 and IFN α , post-ACT has also enhanced transferred T cell function and persistence [7, 156, 161, 162]. Post-conditioning with other cytokines including IL-15 or immunomodulatory therapies such as PD-1 and CTLA-4 blockade should also be considered. However, the type of and need for host conditioning remain controversial as numerous studies have observed clinical responses even when no host conditioning was administered [163, 164].

Suicide switch

Given the capability for “on- or off-target” toxicities and well-documented tumor lysis syndrome and cytokine storm seen with the use of engineered T cells, it would be beneficial to preserve the ability to eradicate the transferred T cells, if needed. Such strategies aim at turning off antigen receptor expression or eliminating engineered cells after transfer by incorporating certain “suicide genes” into the transgene. A long-studied approach in cell therapy investigations utilizes the herpes simplex virus thymidine kinase

(HSV-tk) gene. Its incorporation into the transgene vector makes engineered T cells susceptible to ganciclovir treatment [165, 166]. However, because HSV-tk is potentially immunogenic, its expression may create unwanted immune-mediated destruction of transferred T cells and decreased persistence [167, 168]. Another common approach and non-immunogenic technique incorporate caspase 9 under an inducible promoter (iC9) to initiate apoptosis of transduced cells [169–172]. Additionally, studies incorporating CD20 [173] or EGFR [174] in transgene vectors generated transduced cell susceptible to rituximab and cetuximab treatments, respectively.

Affinity modulation

As discussed earlier, TCR affinity is known to play a significant role in determining sensitivity of a T cell to antigen recognition. Thus, it is logical to think that higher-affinity TCRs may provide a better therapeutic candidate. Altering the affinity of the TCR may better equip engineered T cells to combat immune suppression mechanisms that alter T cell function including MDSC-mediated nitration of TCR tyrosine residues which can weaken TCR-pMHC binding [175]. Additionally, high-affinity TCRs oftentimes exhibit CD8 independence, meaning T cells equipped these receptors can recognize their tumor target without CD8's stabilization of the TCR-pMHC complex. This would allow for the generation of tumor-reactive MHC class I-restricted CD4⁺ T cells, allowing for production of helper cytokines upon antigen stimulation and a novel population of T cell help at the tumor site [90, 176, 177]. Moreover, a population of reactive CD4⁺ T cells may facilitate cross-priming or epitope spreading allowing for a broad systemic anti-tumor response.

One challenge in identifying tumor-reactive high-affinity TCRs is their inherent low frequency in patients. During T cell maturation and thymic selection, TCRs whose affinity for the host antigen/MHC is too low (death by neglect) or too high (negative selection) are eliminated from the pool of mature T cells [178]. This selection process often restricts the amount of high-affinity, self-reactive TCRs from existing in the periphery, thus limiting the discovery for high-affinity TCRs in tumor-bearing patients.

One way to identify and clone high-affinity TCRs is screening for CD8 independence against human MHC-restricted antigens in mice. It is documented that mouse CD8 does not bind to the $\alpha 3$ domain of human MHC class I molecules [113, 179]. Thus, in a vaccinated HLA-A2 transgenic mouse model, isolated T cell clones reactive against human tumors in vitro would likely express high-affinity TCRs. This approach has been used successfully to identify high-affinity TCRs targeting CEA [180] and p53 [181]. Additionally, TCRs have also been genetically modified to improve their biophysical properties using yeast and phage

display [182–184], a technique also used in CAR design [13, 14]. Interestingly, these modified receptors can have amino acid alterations in any of three CDR regions that can contribute to enhanced TCR-pMHC affinities, not only in the CDR3 region which is responsible for the majority of TCR diversity and specificity [185].

These methods in selecting for or enhancing receptor affinity, however, may not necessarily lead to a better therapeutic. As already pointed out, it is well documented that T cells equipped with extremely high-affinity TCRs can undergo AICD, which would selectively delete the effector cells intended for therapeutic benefit [94, 95]. Additionally, two clinical trials [96, 98] and a transgenic mouse model [97] have documented autoimmunity with high-affinity TCR-engineered cells. Taken together, these observations indicate that TCR affinity can make a difference in target recognition by TCR-transduced T cells, but the question of the need to use a high-affinity TCR for effective anti-tumor immunity remains unresolved.

Conclusions

The potential for engineering T cells for immunotherapy seems limitless. Specifically targeting tumor antigens with autologous T cells is a powerful and attractive strategy for cancer therapy. While clinical trials have had initial success, there still remain many issues in optimizing efficacy and safety of this type of immunotherapy. Additionally, the design and implementation of genetically engineered T cells are often constrained by regulatory authorities in both the USA and Europe, largely influencing the feasibility of generating effective and safe CAR T cells [186–188]. Ultimately, more extensive studies are needed to determine whether this type of therapy can deliver improvements in progression-free and overall survival when compared to the standard of care.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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