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Engineered T cells for cancer treatment

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Abstract

Adoptively transferred T cells have the capacity to traffic to distant tumor sites, infiltrate even fibrotic tissue and kill antigen-expressing tumor cells. A variety of groups have investigated different genetic engineering strategies designed to enhance tumor specificity, increase T cell potency, improve proliferation, persistence, or migratory capacity, and increase safety. In this review we focus on recent developments in the T cell engineering arena, discuss the application of these engineered cell products clinically, and outline future prospects for this therapeutic modality.

Keywords

CAR T cells; cancer treatment; immunotherapy; genetic modification of T cells

Introduction

To date, monoclonal antibodies have been the most widely used form of immunotherapy for cancer. However, their limited, biodistribution, range of effector mechanisms recruited, and in vivo persistence have all restricted their clinical potency. In contrast, adoptively transferred effector T cells have the capacity to effectively traffic through multiple tissue planes to distant tumor sites [(1)], recruit multiple cellular and humoral effector mechanisms, and persist for many years, thereby producing complete and sustained disease remissions. Genetic engineering is a means by which we can further increase the potency of these tumor-targeted cellular products. In this review, we evaluate recent improvements in T cell engineering, describe their current clinical impact, and discuss the future prospects of this novel approach.

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Adoptive transfer of T cells with native antigen specificity

T cells have the capacity to identify and eradicate malignant disease through native receptor recognition of tumor-associated antigens (TAAs), even without modification. Examples of such activity are well described in melanoma, where Rosenberg and others have reported that infusion of melanoma-specific tumor-infiltrating lymphocytes (TILs) and T cell clones targeting melanoma-associated antigens produces clinical responses in approx. 50% of patients[2–5]. Similarly, we have infused over 100 hematopoietic stem cell transplant (HSCT) recipients with donor-derived polyclonal T cell lines targeting Epstein-Barr virus (EBV) to prevent and treat the often lethal EBV-associated lymphoproliferative disorder (post-transplant lymphoproliferative disease; PTLD) that frequently occurs in these severely immunocompromised patients[6,7]. Small doses (circa $2x10^7$ CTL/m^2) of *in vitro* expanded EBV-specific cytotoxic T lymphocytes (CTLs) proved to be both safe and effective for the prophylaxis and treatment of EBV reactivation post-transplant[8]. Subsequently this strategy was extended to EBV-associated malignancies that occur in immunocompetent individuals including Hodgkin disease (HD), non-Hodgkin lymphoma (NHL), and nasopharyngeal carcinoma (NPC). Although the viral antigen expression pattern in these patients is restricted to weakly immunogenic EBV proteins such as LMP1 and LMP2, the adoptively transferred CTLs trafficked to tumor sites, and produced complete remission in over half the subjects with refractory or relapsed disease[9–14].

In principle the successes described above should be extendable to any other TAAs that can be targeted by T cells. Unfortunately, however, most TAAs are self antigens and self-reactive T cells are largely anergized or deleted. Moreover, even if TAA-specific T cells can be generated and are then infused, these cells may fail to persist due to tumor immune evasion strategies such as (i) down-regulation of T cell target antigens, major histocompatibility complex (MHC) and co-stimulatory molecules; (ii) production of inhibitory/Th2-polarizing factors such as transforming growth factor (TGF) β, interleukin (IL) 10, IL13, and IL4, (iii) expression of pro-apoptotic molecules on the cell surface; and (iv) recruitment of regulatory T cells (Tregs) that inhibit the effector T cell response to tumor[15].

Nevertheless, advances in cell engineering technology has now allowed us to modify T cells with genes that can; increase the range of antigens they can recognize and/or augment their affinity for their targets; improve their homing to tumor sites; increase their resistance to tumor immune evasion strategies; enhance their proliferation and survival; and ensure their safety (Figure 1). Although it remains unclear as to which of these modifications, or combination thereof, will be most relevant in the clinical setting, in this review we will discuss the current status of T cell engineering.

Genetic modification of T cells

Effective genetic modification of T cells requires the use of systems that produce adequate gene transfer and expression of the desired transgene. The choice of gene transfer vector is dictated by the desired level and duration of expression necessary for the hoped-for therapeutic benefit.

Viral vectors have long been used as vehicles to deliver therapeutic genes to target cells. To permit sustained expression in a highly proliferative cell, such as the T cell, the majority of studies to date have used vectors that integrate in the host T cell genome, usually gammaretrovirus or lentivirus-based vectors, thereby avoiding the dilutional effect that would follow cell division if a non-integrating, non-replicating vector is used. Though gene-modified T cells have a long in vivo safety profile[16, 17] viral vectors are expensive to produce and test, and there is often a requirement for onerous and prolonged follow-up of treated patients that further adds to both cost and complexity[18]. This has ensured continued interest in the development of efficient non-viral gene transfer.

RNA or DNA-based expression plasmids are much less expensive than viral vectors to produce and test, and can be used to alter T cell biology when efficient transgene integration (and hence long-term expression) is not required. More recently, transposon-based gene delivery systems have been developed that offer the practical advantages of plasmids coupled with the integrative capabilities of retroviruses. Most transposons are binary systems, incorporating two expression plasmids, one encoding the transposase and the other containing the gene of interest flanked by the transposon terminal repeat sequence required for transposition. After delivery to the target cell, the transposase binds to the terminal repeat sequences of the donor plasmid and the host genome, excises the gene of interest, and inserts it into the host genome. Transposons, unlike retroviral vectors, do not preferentially integrate close to transcription start sites in the host cell genome, potentially improving their safety profile. The Sleeping Beauty transposon is now being used to gene-modify T cells that are then adoptively transferred to patients with B cell malignancies, while the Piggybac system is being evaluated for similar application[19–22].

Modifications that enhance T cell targeting

The generation of tumor-reactive T cells from cancer patients is often difficult due to the low immunogenicity of TAAs, which are either “self” antigens or “naïve” targets for the immune system. Therefore, investigators have explored genetic engineering approaches whereby autologous T cells are modified to express tumor-specific receptors. Two basic gene transfer approaches have been pursued clinically – (i) the transfer of antigen-specific receptor α and β chains, and (ii) the transgenic expression of chimeric antigen receptors composed of antibody-binding domains fused to T cell signaling domains (Figure 2).

αβTCR gene transfer

In the above process, T cells are modified *ex vivo* to express TCR αβ heterodimers directed against a specific tumor target. These α and β chains may be isolated from T cell clones, from mice that are transgenic for the human TCR, or from phage/yeast/T cell displays[23,24]. Once selected, the affinity of these TCRs can be further enhanced by mutation/selection. To date, transgenic α and β TCR chains targeting TAAs including melanoma antigens, minor histocompatibility antigens, and common oncoproteins have been generated and used to modify non-specifically activated T cells, rapidly producing a tumor-directed clinical grade product[25].

A number of factors have limited the broader introduction of this approach. Transferred α and β chains can cross-pair with endogenous TCR chains, forming hybrid TCRs with either loss of activity or gain of new and unwanted (autoimmune) specificity. The frequency of this problem can be reduced by incorporating a murine-derived transmembrane region in the transgenic TCR, though this is not ideal given the potential immunogenicity[26], or through the introduction of sequences encoding cysteine residues to form additional disulfide bonds that stabilize pairing of the transgenic TCR and minimize cross pairing with endogenous α and β chains[27–29]. Alternatively, selective disruption of the endogenous αβTCR using zinc-finger nucleases (ZFNs)[30,31], or the substitution of γδ T cells as the platform for αβ transgenic TCR transduction may prevent this problem[32]. At the moment we do not know how crucial these modifications will be or which will be most favorable for clinical use. Perhaps a more important general limitation of this strategy is that conventional TCRs recognize only single peptides presented in the context of individual HLA alleles, thus limiting their use to individuals with the appropriate HLA polymorphism. Hence, application to the broadest possible range of patients requires large panels of TCRs to be made and tested in large numbers of patients. Even then, loss of the single targeted peptide epitope may lead to tumor immune escape. Finally, as investigators select TCR clones with non-physiologically high levels of affinity, the risk of toxicity due to off-target binding to related epitopes present on normal tissue becomes an increasing concern.

Clinical studies with αβTCRs

Despite these concerns a number of clinical trials using engineered T cells expressing αβTCR have been initiated. These trials have focused on targeting well studied and extensively characterized self antigens including MART1 and gp100 (melanoma)[33,34], carcinoembryonic antigen (CEA) (colorectal cancer)[35], NY-ESO-1 (melanoma, synovial sarcoma and multiple myeloma)[36] and MAGE-A3 (melanoma, multiple myeloma, synovial sarcoma and esophageal cancer)[37]. Though promising clinical responses support the therapeutic potential of this approach, there have also been a number of reported toxicities related to “on target” but “off tissue” effects. For example, investigators at the National Cancer Institute (NCI) reported the development of skin rashes, uveitis and hearing loss in patients treated with high affinity transgenic αβTCRs specific for MART1 or gp100[33], while the infusion of CEA-targeted T cells was associated with the development of severe inflammatory colitis[35]. More recently, Morgan et al treated 9 patients with MAGE-A3 positive tumors with T cells modified with a high-avidity TCR directed against an HLA-A2-restricted MAGE-A3 epitope and though 5 patients experienced clinical regressions, 3 experienced mental status changes, two of whom lapsed into comas and subsequently died. Brain biopsies or postmortem brain autopsies revealed infiltration of CD8+ T-cells into the white matter and perivascular spaces. Furthermore, expanded CSF cells from an affected patient produced IFNγ in response to MAGE-A3+ tumor cell lines. It subsequently transpired that the TCR used in this study recognized not only MAGE-A3 but also MAGE-A9 and MAGE-A12, which was found to be expressed in human brain possibly explaining the neuronal cell destruction that precipitated post adoptive transfer[37].

Finally, “off target” toxicity has also been reported using MAGE-A3 TCR-modified T cells. In this case T cells were modified to express an affinity-enhanced αβTCRs targeting an

HLA-A1 epitope from MAGE-A3 that was subsequently used as treatment for melanoma or relapsed multiple myeloma. The first patient, who received the cells as treatment for metastatic melanoma, died 4 days post-infusion of cardiac failure. Following extensive studies the cause of death was attributed to hemorrhagic myocardial infarction precipitated by demand ischemia and subsequently the trial was re-opened. However, the second patient, treated for relapsed multiple myeloma, also developed cardiogenic shock and died 5 days after infusion. Again, after ruling out the expression of MAGE-A3 or related MAGE proteins in cardiomyocytes/heart tissue, the group undertook a systematic investigation of TCR binding and reactivity, which revealed that their affinity-enhanced TCR recognized an unrelated peptide derived from Titin, which is highly expressed in muscle tissue and a target of auto-antibodies in some forms of myasthenia gravis, particularly in patients with thymomas[38]. Subsequently, iPS cardiomyocytes were confirmed to express Titin. However, Maus et al also indicated that endogenous T cells with native specificity for the same epitope did not demonstrate any activity against Titin. Thus, in this case the cross-reactivity and subsequent toxicity appears to be a function of the engineering process that was designed to enhance affinity rather than the endogenous specificity[39,40].

Chimeric antigen receptors (CARs)

T cell specificity can also be altered by expressing chimeric antigen receptors (CAR), which are artificial receptors composed of an extracellular domain that is responsible for antigen recognition, a transmembrane domain and one or more intracellular signaling domains. The extracellular domain is most commonly derived from the variable regions (i.e. antigen binding portion) of the heavy and light chains (V_H and V_L,) of a monoclonal antibody joined by a flexible linker. The intracellular signaling domain (endodomain) is most usually derived from the T cell receptor (CD3) zeta chain. CAR expression allows tumors to be targeted in an HLA-unrestricted manner, increasing the number of eligible patients, and extends the types of antigens that can be recognized by T cells to include carbohydrates and glycolipids. Second and third generation CARs incorporate additional endodomains that provide the necessary accessory signals or co-stimulation to allow T cells to pass through the multiple checkpoints that under physiological conditions regulate T cell activation, proliferation, differentiation and survival following receptor engagement[41].

Table I describes some of the CARs that have been developed for clinical use in solid tumors and hematological malignancies. Initial trials using T cells modified to express CARs that contained exclusively the CD3ζ signaling domain (so called first generation constructs) proved sub-optimal. Indeed, CAR engagement failed to induce either cytokine production or T cell expansion *in vivo*. Subsequently, second generation CARs, which contained additional co-stimulatory endodomains including CD27, CD28, 41BB, DAP10, OX40 or ICOS proved to confer greater strength of signaling and persistence to the T cells, resulting in improved potency. For example, in a head to head comparison, Savoldo and colleagues demonstrated that CAR-CD19 T cells encoding the costimulatory CD28 endodomain had strikingly enhanced expansion and persistence compared with their counterparts lacking this endodomain[42]. Porter and colleagues used CAR-CD19-modified T cells expressing the 41BB endodomain to treat chronic lymphocytic leukemia (CLL) and saw significant *in vivo* expansion, and persistence for at least 6 months, which resulted in

complete clinical responses in 2 of 3 treated patients[43,44]. More recently the same group has applied this strategy for the treatment of patients with relapsed and refractory pre-B cell acute lymphoblastic leukemia (ALL)[45]. Initial results from two treated patients confirmed the *in vivo* proliferative capacity of the infused cells in vivo, with detection in blood, bone marrow and cerebrospinal fluid post-infusion. Again, the transferred T cells produced initial clinical responses in both patients. However, this was sustained in only one while the second patient relapsed with a CD19 negative tumor two months post-treatment, demonstrating the potential for immune escape using a mono-specific therapy that targets a molecule that is not of direct pathogenic relevance[45]. In addition, many of the B-CLL and ALL responders have developed acute toxicities during the expansion phase of the T cells, associated with fevers and the release of high levels of cytokines and cytokine receptors including soluble IL1Rα, IL2R, IL2, IL6, IL10, TNFα and IFNγ. Monoclonal antibodies to TNFα and the IL6R (etanercept and tocilizumab, respectively) were apparently able to rapidly reverse these toxicities. Other investigators using second and third generation CARs (incorporating 3 or more endodomains) have described similar toxicities[46–48].

Immune responses to transgenic receptors

Most CARs and many transgenic TCRs contain novel sequences or sequences derived from other species. As a consequence, the recipient may generate an immune response that eliminates the modified T cells. For example, Lamers and colleagues reported the development of anti-scFv antibodies in three patients treated with T cells expressing a carbonic anhydrase IX (CAIX)-specific CAR[49–51], while Kershaw and colleagues observed a CAR-specific antibody response in a patient treated with CAR T cells modified to recognize the ovarian-associated α-folate receptor (αFR)[52]. More recently, Jensen and colleagues reported lack of *in vivo* persistence due to the induction of endogenous cellular immune responses directed against a selection gene (neomycin phosphotransferase) incorporated in their CD20 CAR-containing plasmid[53]. Although cellular and humoral responses have not been observed in all treated patients, the substitution of humanized single chain CARs may reduce the risk of premature deletion of T cells due to immune responses.

Safety concerns

As for αβTCR-modified T cells, a major concern with CAR T cell transfer relates to the potential for “on target antigen” but “off target tissue” toxicity – an effect associated with targeting TAAs that are not exclusively tumor-restricted in their expression profile. Lamers et al reported the development of cholestasis following the infusion of T cells modified with a CAR targeting carbonic anhydrase as treatment for renal cell carcinoma, which correlated with expression of carbonic anhydrase on biliary epithelial cells[49–51]. Brentjens and colleagues reported renal and respiratory failure in a patient with CLL after a single infusion ($3x10^7$/kg) of T cells modified with a second generation CAR targeting CD19 that was given following high dose cyclophosphamide, administered to induce host lymphodepletion[54]. The authors hypothesized the combination therapy may have led to a cytokine storm *in vivo* or to rapid tumor lysis. Finally, Morgan et al infused $>10^{10}$ T cells modified with a third generation CAR targeting HER2 to a patient with widely metastatic colon cancer after intensive lymphodepletion. The subject rapidly developed pulmonary toxicity and died 4 days after infusion. After extensive analysis the investigators concluded

that the toxicity might have been due to targeting of low levels of HER2 on pulmonary endothelium - a known site at which intravenously infused human CAR T cells accumulate[55,56].

Selective targeting

While it is now clear that genetically modified T cells can be targeted to tumors, it is equally apparent that targeting a single epitope or antigen alone has a high risk of leading to tumor immune escape or to toxicity due to “on target, off organ” effects if the targeted antigen is not uniquely expressed on target tumor cells. To improve safety Wilkie et al modified activated T cells with two CARs targeting the breast cancer-expressed TAAs HER2 (coupled with the CD3ζ endodomain) and MUC1 (coupled to CD28) and demonstrated that these dual-targeted T cells were able to deliver complementary signals, leading to potent cytotoxicity and synergistically-enhanced proliferation in the presence of tumor cells expressing both target antigens[57]. Similar results have more recently been reported by Sadelain and colleagues[58]. In addition, investigators are developing hybrid receptors which will induce T cell activation at the tumor site by inverting the inhibitory effects of cytokines, such as IL4, produced in the local environment[59,60]. Finally, an alternate approach to promote potent anti-tumor effects while minimizing toxicity is to combine CAR and conventional therapies. The feasibility of such a strategy has recently been demonstrated by Sanchez and colleagues, who combined CAR T cells engineered to recognize MUC1 with anti-androgen therapy to provide additive anti-tumor effects in a prostate cancer model[61].

Genetic modification of T cells to improve *in vivo* migration, proliferation and survival

T cell migration

Once tumor-specific T cells are infused, they must migrate to distal tumor sites before exerting their cytotoxic effects. T cell migration occurs along a chemokine gradient so efficient trafficking requires that the chemokine receptors expressed by the infused T cells must match the chemokines produced by the tumor. In practice, however, tumor and surrounding stromal cells can produce a chemokine milieu that recruits T cell subsets such as Tregs that support rather than perturb the tumor microenvironment. In HD, for example, malignant Reed-Sternberg cells secrete chemokines (e.g. TARC) that attract immunoinhibitory/suppressive Th2 cells and Tregs, both of which contribute to the hostile immune microenvironment and directly impair the antitumor activity of effector T cells.

Gene transfer can alter the migration profile of infused, tumor-targeted (and pro-inflammatory) T cells through the forced expression of chemokine receptors that are matched with the chemokines produced by the target tumor, allowing the transferred cells to exploit the tumor’s own inhibitory mechanisms. Our group has expressed transgenic CCR4 receptors on T cells expressing CAR-CD30, an antigen that is highly expressed by many HD cells, allowing the tumor targeted effector cells to migrate toward the HD-generated TARC gradient, the cognate chemokine for CCR4[62]. Similarly, Moon et al increased the migration of mesothelin-directed CAR T cells towards malignant pleural mesotheliomas, by modifying them to express CCR2b, the cognate receptor for the chemokine CCL2 that the tumors

produce[63]. CCL2 is secreted by many tumor types, and modifying CAR-GD2 T cells with the same chemokine receptor (CCR2b) produces a >10-fold increase in the homing capacity of the transgenic cells towards CCL2-secreting neuroblastoma with increased anti-tumor activity[64]. A similar approach could be applied to other human malignancies in which a signature chemokine expression profile can be identified.

T cell proliferation and in vivo persistence

T cell proliferation requires continued antigenic stimulation, either via direct interaction with tumor cells or through professional APCs that present tumor antigens, as well as the presence of appropriate cytokines. Moreover, a proportion of the cells should enter the memory T cell compartment after infusion, so that protection can be assured long-term. Tumors have developed an array of strategies to prevent these events from occurring, necessitating countermeasures that will ensure T cell proliferation and survival.

Transgenic expression of growth factors/growth factor receptors

Recombinant IL2 has been systemically administered to support the expansion and persistence of adoptively-transferred T cells, but is associated with significant toxicity and the expansion of T regs, potentially offsetting the immunological benefits[65]. Investigators are now exploring alternative methods to expand T cells *in vivo*, by genetically modifying them to express the growth factors IL2 or IL15, thereby producing effector T cells that are self-sustaining. Both IL2 and IL15-modified cells have been shown to retain their antigen specificity, phenotype and function. Importantly, they also retain their dependence on antigenic stimulation for continued expansion. For example, Quintarelli and colleagues genetically modified EBV-specific T cells with retroviral vectors encoding either IL15 or IL2 and showed that both promoted *ex vivo* and *in vivo* expansion and antitumor activity, confirming that this was achieved without induction of Tregs[66,67].

T cell growth and survival can also be increased by engineering cells to respond to cytokines, which do not normally induce proliferation of *in vitro* expanded T cells. Vera and colleagues have shown that transgenic expression of the IL7 receptor by antigen-specific T cells restores their responsiveness to the IL7 cytokine, and sustains their expansion *in vitro* and *in vivo* without affecting their antigen specificity or cytokine dependence[68]. Since this cytokine has been safely administered to human subjects without apparent enhancement of Treg cell number and function, the infusion of a tumor-targeted T cell product engineered to express the IL7R could be followed by exogenous administration of clinical grade IL7 cytokine to promote transgenic cell proliferation and survival[69–72].

Selected T cell populations for gene transfer

The persistence of gene-modified T cells may be favored by infusing T cell subsets with stem cell-like properties since these should have superior *in vivo* longevity[73]. One way to achieve this goal is to culture the cells ex vivo in cytokines, including IL15, IL7 and IL21, that have been shown to promote the expansion of T cells with a central memory phenotype[74,75]. In non-human primate proof of concept studies Berger et al infused effector (CD62L−CD28−CD8+Fas^{hi}) and central (CD62L+CD28+CD8+ Fas^{hi}) memory-

derived CMV-specific T cell clones and demonstrated that the central memory-derived T cells survived longer *in vivo*, suggesting that T cells isolated from different compartments have divergent fate[76]. More recently Wang and colleagues isolated human melanoma-specific T cells from the central memory compartment, grew them in culture, and then showed that these highly differentiated and expanded effector T cell clones nonetheless effectively targeted skin melanocytes and persisted long-term *in vivo*[77].

Another means of ensuring in vivo persistence of tumor directed T cells may be to retarget T cells that have native receptor specificity for latent viruses and are known to be long-lived memory cells. For example, adoptively transferred EBV-specific CTLs have been shown to persist long-term (>10 years) *in vivo*, likely due to the fact that the infused cells derived both from central and effector memory subsets and were able to receive physiological co-stimulation *in vivo* via exposure to EBV-infected APCs[8]. To assess whether the same was true if EBV-CTLs were used as a CAR platform, Pule et al compared the longevity of mitogen-activated T cells with that of polyclonal EBV-specific CTLs modified with a CAR targeting GD2 in patients with advanced neuroblastoma[78]. Early after infusion, CAR-GD2-modified EBV-CTLs circulated at higher levels than activated T cells, but in extended follow-up studies, cells derived from both activated T cells and EBV-CTL populations were detected long-term (192 and 96 weeks, respectively), and the duration of persistence correlated with the percentage of CD4+ helper T cells within the infused product as well as with their expression of the central memory markers ($CD45RO^{+}CD62L^{+}$)[79]. Importantly, *in vivo* persistence was associated with superior clinical outcome. Thus, future clinical studies using T cells that have been selected based on a central memory phenotype may extend the life span of adoptively-transferred cells and improve clinical efficacy, though the complexity, cost, and large blood volumes required for the up-front clinical grade selection of such cells must also be taken into consideration[80].

Co-stimulation

T cell proliferation and survival requires both antigenic stimulation and the sequential engagement of co-stimulatory molecules. Unlike “professional” APCs, which express both antigen and co-stimulatory molecules, tumor cells may express only the target antigen. Exposure to antigen in the absence of co-stimulation can lead to T cell apoptosis or anergy. One means of providing T cell co-stimulation is to force the expression of co-stimulatory ligands, such as CD80 and 41BBL by the engineered T cell that will engage their native co-stimulatory receptors in an autocrine or paracrine manner[81]. Alternatively, the signaling portions of co-stimulatory molecules including CD27, CD28, OX40 and 41BB, have been incorporated into the intracellular portion of second and third generation CARs so that CAR engagement with the target antigen delivers both the antigen activation and co-stimulation signals simultaneously, which may substitute for the lack of co-stimulation from the tumor cells themselves[41]. The effects of modifying CAR T cells with additional co-stimulatory endodomains have been summarized in Table I.

Increasing T cell resistance to the tumor

T cell survival can be increased by overexpressing pro-survival/anti-apoptotic genes. For example, T cells transduced with the human telomerase reverse transcriptase (hTERT) gene

have increased longevity due to the prevention of telomere erosion. Unfortunately, this modification may cause genomic instability, limiting both safety and clinical value[82–84]. An alternative means of increasing T cell persistence is to modify cells with anti-apoptotic genes, such as Bcl-2 and Bcl-xL[85,86], or to downregulate pro-apoptotic genes such as Fas, thus making the cells resistant to Fas/FasL-mediated apoptosis[87].

Counteracting the hostile tumor microenvironment

Genetic modification of T cells can also be used to counteract the immune-inhibitory tumor microenvironment that can neutralize adoptively transferred antigen-specific CTLs. One of the most widely used tumor evasion strategies is local secretion of TGFβ by the tumor or its stromal elements. TGFβ is a multifunctional cytokine that promotes tumor growth, limits effector T cell proliferation and function, activates Tregs, and induces tolerance. The detrimental effects of TGFβ can be negated by modifying cells to express a dominant-negative TGFβ receptor type II (dnTGFβ-RII), prolonging their persistence and enhancing tumor elimination in mice bearing TGFβ-expressing tumors[88–90], and we are now assessing the safety and efficacy of dnTGFβ-RII-modified tumor-specific CTLs in patients with relapsed/refractory HD or NHL.

Wilkie and colleagues took this approach one step further – they proposed not just negating the inhibitory effects of a tumor-produced immunosuppressive cytokine, but instead switching the signal into one that was activating for the transferred T cells. To accomplish this goal they modified CAR T cells to express a custom chimeric cytokine receptor, consisting of the exodomain of the IL4 receptor fused to the endodomain of the shared IL2/15 βc endodomain. They hypothesized that transgenic expression of this molecule on T cells would protect them from the inhibitory effects of IL4, a prototypic Th2-polarizing/inhibitory cytokine produced by a variety of tumors, whilst providing a pro-proliferative signal to T cells via the IL2/15 βc endodomain directly at the tumor site[60]. This approach has yet to be clinically translated.

Genetic modification to improve safety

Suicide Genes

Along with enhancing potency, increasing longevity or conferring resistance to inhibitory signals genetic engineering approaches can also be employed to incorporate a “safety switch’ so that the infused cells can be eliminated should adverse effects occur. Transgenic expression of the B cell antigen CD20 by T cells has been proposed as a suicide gene strategy, and this is being currently being evaluated preclinically[91,92]. In the clinical setting one of the most well-studied suicide systems utilizes the herpes simplex viral thymidine kinase (*TK*) gene, which converts the pro-drug ganciclovir (GCV) to a purine analog, inhibiting DNA polymerase. Thus, GCV can be administered as a means of eliminating actively proliferating cells and the activity of this approach is currently being tested in late phase clinical studies[93–96]. There are, however, several shortcomings to using *TK* as a suicide gene[97]. One is the inherent immunogenicity of this virus-derived gene, which might lead to premature clearance of infused cells. Second is the removal of a therapeutically valuable drug as an option for the treatment of viral infections post-

transplant. Another concern is the time required to ablate infused cells - usually days to weeks. Even a recently developed codon-optimized HSV-*TK* required 3 days ganciclovir exposure to produce transgenic cell death[98] – a time frame that would be inadequate in cases where infused cells cause acute on- or off-target toxicity. An attractive alternative suicide strategy is the inducible *Caspase9* transgene (*iCaspase9*)[99–100], which is non-immunogenic and rapidly (within 24 hours) produces apoptosis, even in non-dividing cells[101]. iCaspase9 is trigged upon administration of a small molecule dimerizer, AP20187, and produces apoptosis in >95% of transgenic cells. Thus, incorporation of this safety switch in combination with other modifications may be prudent as T cell potency is increased.

Commercialization Strategies

Broader clinical use of complex biologics such as engineered T cells for human disease will require strategies that address limitations due to the personalized nature of the therapy and the lack of scalability of the complex manufacturing processes associated with the genetic modification and cell expansion process. Investigators have begun to explore strategies to generate “universal T cells,” which can be use in the allogeneic setting as an “off the shelf” product, as well as to develop simplified methodologies to generate modified T cells that use new, scalable and cost effective manufacturing processes.

Universal T cells

To develop a “one size fits all” CAR T cell therapy, Tamada and colleagues demonstrated that a variety of tumors could be targeted using fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies in combination with T cells engineered to express a FITC-directed CAR[102]. Powell and colleagues proposed a similar approach whereby T cells were modified with a universal immune receptor specific for biotinylated antigen-specific molecules (biotin binding immune receptor; BBIR). These BBIR T cells can specifically recognize and be activated by various biotinylated molecules, including ScFVs and antibodies. Although this is an interesting approach, the biodistribution and immunogenicity of the modified cells is unclear[103,104]. Alternatively, ZFN technology can be used to delete endogenous HLA molecule expression facilitating the generation of a less allostimulatory T cell product[30], but it is not clear how such cells would avoid being killed by NK effector cells, which may be activated when they engage an HLA negative target[31].

Scalability of Process

Independent of advances in developing universal T cells, commercialization will require manufacturing platforms for engineered T cells to become more scalable and robust. Conventional processes rely on *ex vivo* cell propagation in plates, flasks or bags, all of which have limitations with respect to the availability of nutrients and oxygen (O_2), and the accumulation of metabolic waste products including lactic acid and carbon dioxide (CO_2). This occurs because conventional cultureware is restricted to the use of a shallow media volume to allow sufficient gas diffusion from above the cells, which restricts both available nutrients and the buffering capacity of the media. In addition, O_2 and nutrient requirements progressively increase with cell concentration and rate of growth, so that cultures must be fed and split regularly. These frequent medium changes and cell manipulations are time

consuming, expensive, reduce the reproducibility of the cell product manufacture and increase the risk of contamination.

Scale-up using hollow fiber or stirred tank bioreactors or plastic bags may overcome the above issues, but are not always easy to adapt for T cell culture[105–107]. Hollyman and colleagues used a culture system based around the WAVE Bioreactor for the expansion of CAR-CD19 T cells, obtaining between 0.8–2.4×10^{10} T cells in 13–18 days of culture[108]. Indeed the same platform was also shown to support the reliable expansion of tumor infiltrating lymphocytes, with no adverse effects on T cell phenotype or function[109,110]. The major advantage of the WAVE bioreactor is the potential for large scale T cell production (>10^{10} cells). However, the system is expensive, requiring the purchase of the bioreactors themselves as well as ancillary equipment. Supported in part by the NHLBI - Production Assistance for Cellular Therapies (PACT) mechanism, we have taken a different approach by using a gas-permeable culture device (G-Rex: Wilson Wolf Manufacturing). In this G-Rex platform, O_2 and CO_2 are exchanged across a silicone membrane at the base of the flask, which allows for an increased depth of medium above, providing more nutrients and diluting waste products. This system supports the expansion of a range of suspension cell types including genetically-modified T cells[111–115]. Importantly, the platform is highly scalable, GMP-compliant, and reduces the number of technician interventions approximately 4-fold while increasing the cell output by 3–20-fold compared with conventional methods. These and other manufacturing improvements will help gene-modified products have a broader clinical utility and become more attractive from a commercial perspective. Indeed, we are seeing the first evidence of such interest with the recently formed partnerships between Novartis and the University of Pennsylvania as well as between bluebirdbio, Celgene and Baylor College of Medicine to advance novel T cell therapies for the treatment of cancer.

Conclusions

T cell immunotherapy has the potential to cure patients with advanced cancer and has already had impressive successes. However, many obstacles remain before this approach can reach its full potential and become a standard of care. By using genetic modification to improve target recognition, enhance T cell persistence, improve migration, and increase safety, investigators are steadily increasing the range of cancers that can be treated and the potency of the benefits obtained. However, even if successful, broader implementation will also depend on the development of T cell manufacturing processes that are robust and scalable, which will enable T cell therapies to become more accessible, in part by attracting interest from commercial entities who will in turn ultimately transform adoptive T cell transfer from “boutique to chain store”.

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Abbreviations

ALL	acute lymphoblastic leukemia
APCs	antigen presenting cells
BBIR	biotin binding immune receptor
CARs	chimeric antigen receptors
CLL	chronic lynphocytic leukemia
CTLs	Cytotoxic T lymphocytes
dnTGFβ-RII	dominant-negative TGFβ receptor type II
EBV	Epstein-Barr virs
GCV	ganciclovir
G-Rex	Gas-permeable culture device
HD	Hodgkin disease
HSCT	hematopoietic stem cell transplant
hTERT	human telomerase reverse transcriptase gene
iCaspase9	inducible Caspase 9 transgene
IL	interleukin
MHC	major histocompatilibity complex
NHL	non-Hodgkin lymphoma
NPC	nasopharyngeal carcinoma
PACT	Production Assistance for Cellular Therapies
PTLD	post-transplant lymphoproliferative disease
TAAs	tumor-associated antigens
TGF	transforming growth factor
TILs	tumor-infiltrating lymphocytes
TK	thymidine kinase
Tregs	regulatory T cells
ZFNs	zinc-finger nucleases
αFR	α-folate recptor

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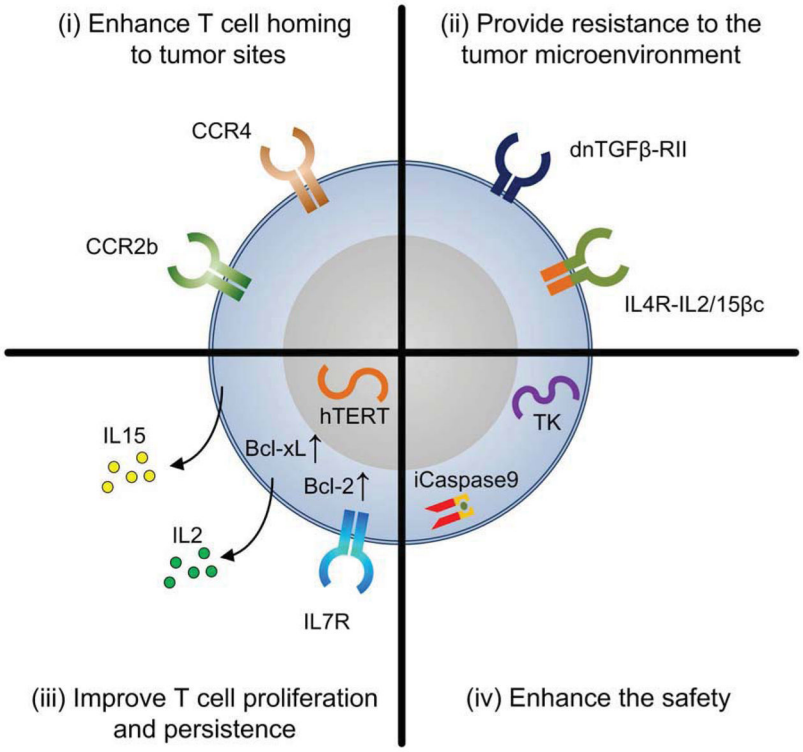

Figure 1.

Examples of Genetic modifications that have been explored individually or in combination with the purpose of improving the function and safety of T cells. These modifications include the transgenic expression of proteins that (i) enhance T cell homing to tumor sites, (ii) provide resistance to the tumor microenvironment, (iii) improve their proliferation and persistence and (iv) enhance their safety.

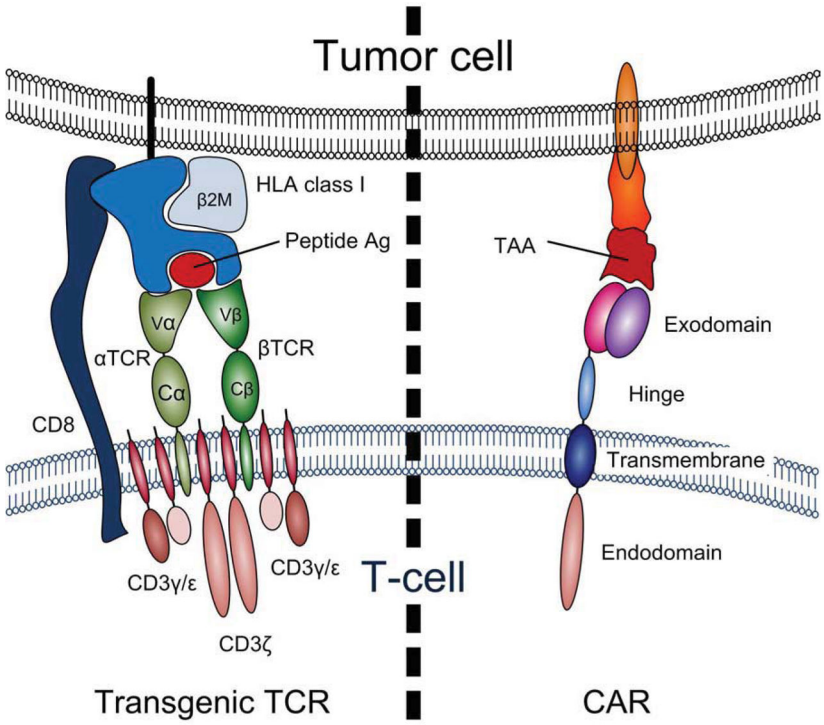

Figure 2.

a schematic of transgenic α and β T cell receptors (αβT CRs) and chimeric antigen receptor (CARs) and shows the differences and similarities between these two most common strategies used to redirect the immune T cell response.

Table I

shows the different types of CARs that have been developed for the treatment of hematological and solid malignancies and their stage of preclinical and clinical development.

Hematologic malignancies	CAR construct						Preclinical data	Clinical data	References
Target Antigen	Exodomain (clone)	Hinge	Trans membrane	Endo domain 1	Endo domain 2	Endo domain 3			
CD19	ScFV(FMC63)	CH2CH3	CD4	CD3ζ	–	–	+	+	53, 116–118
	ScFV(FMC63)	FcRγ	FcRγ	CD3ζ	–	–	+	–	119,120
	ScFV(FMC63)	CD8α	CD8	CD3ζ	–	–	+	–	121,122
	ScFV(FMC63)	CH2CH3	CD28	CD3ζ	–	–	+	+	42
	ScFV(SJ25C1)	CD8α	CD8	CD3ζ	–	–	+	–	123,124
	ScFV(FMC63)	CD28	CD28	CD28	CD3ζ	–	+	+	120,125–127
	ScFV(FMC63)	CH2CH3	CD28	CD28	CD3ζ	–	+	+	19,42, 117,128
	ScFV(FMC63)	CD8α	CD8	41BB	CD3ζ	–	+	+	43–45,121,122
	ScFV(FMC63)	CD8α	CD28	CD28	CD3ζ	–	+	–	121,122
	ScFV(SJ25C1)	CD28	CD28	CD28	CD3ζ	–	+	+	106,124,129–131
	ScFV(FMC63)	CD8	CD8	CD28	41BB	CD3ζ	+	–	125
CD19	ScFV(FMC63)	CD8α	CD28	CD28	41BB	CD3ζ	+	–	121,122
CD20	ScFV(Leu16)	CH2CH3	CD4	CD3ζ	–	–	+	+	53,132–137
	ScFV(Leu16)	CH2CH3	CD4	CD28	CD3ζ	–	+	–	137
	ScFV(Leu16)	CH2CH3	CD4	CD28	CD137	CD3ζ	+	+	137,138
CD22	ScFV(M791)	CH2CH3	CD28	CD28	CD3ζ	–	+	–	139
	ScFV(HA22)	CH2CH3	CD28	CD28	CD3ζ	–	+	–	139
	ScFV(BL22)	CH2CH3	CD28	CD28	CD3ζ	–	+	–	139
	ScFV(HA22SH)	CH2CH3	CD28	CD28	CD3ζ	–	+	–	139
	ScFV(HA22SH)	CH2CH3	CD8	41BB	CD3ζ	–	+	–	139
	ScFV(M791)	CH2CH3	CD8	CD28	41BB	CD3ζ	+	–	139
	ScFV(HA22)	CH2CH3	CD8	CD28	41BB	CD3ζ	+	–	139
	ScFV(BL22)	CH2CH3	CD8	CD28	41BB	CD3ζ	+	–	139
	ScFV(HA22SH)	CH2CH3	CD8	CD28	41BB	CD3ζ	+	–	139

Hematologic malignancies	CAR construct						Preclinical data	Clinical data	References
Target Antigen	Exodomain (clone)	Hinge	Trans membrane	Endo domain 1	Endo domain 2	Endo domain 3			
CD30	ScFV(HRS3)	CH2CH3	FcεR1γ	CD3ζ	−	−	+	−	62,140, 141
	ScFV(HRS3)	CH2CH3	CD28	CD28	CD3ζ	−	+	−	62
Kappa	ScFV(CRL1785)	CH2CH3	CD28	CD3ζ	−	−	+	−	142
	ScFV(CRL1785)	CH2CH3	CD28	CD28	CD3ζ	−	+	−	142
CD70	CD27	CD27	CD27	CD3ζ	−	−	+	−	143
CD123	ScFV(7G3)	CH2CH3	CD28	CD3ζ	−	−	+	−	144
NKG2D	mNKG2D	mNKG2D	mNKG2D	CD3ζ	−	−	+	−	145–148

Solid tumors	CAR construct						Preclinical data	Clinical data	References
Target Antigen	Exodomain (clone)	Hinge	Trans membrane	Endo domain 1	Endo domain 2	Endo domain 3			
GD2	ScFV(14.G2a)	CH2CH3	FcεR1γ	CD3ζ	−	−	+	−	149, 150
GD2	ScFV(7A4)	CH2CH3	FcεR1γ	CD3ζ	−	−	+	−	149, 150
	ScFV(14.G2a)	CH2CH3	CD3	CD3ζ	−	−	+	+	78,79,149, 150
HER2	ScFV(NY29)	N/A	N/A	CD3ζ	−	−	+	−	151
	ScFV(NY29)	CD28	FcR1γ	CD3ζ	−	−	+	−	152, 153
	ScFV(FRP5)	CH2CH3	FcεR1γ	CD3ζ	−	−	+	−	154
	ScFV(4D5)	CD28	CD28	CD3ζ	−	−	+	−	155
	ScFV(4D5)	CD8	CD8	CD3ζ	−	−	+	−	155
	ScFV(ICR12)	CD8α	CD3	CD3ζ	−	−	+	−	57
	ScFV(FRP5)	N/A	CD28	CD28	CD3ζ	−	+	−	156,157
	ScFV(FRP5)	CH2CH3	CD28	CD28	CD3ζ	−	+	−	158
	ScFV(FRP5)	CD8α	CD28	CD28	CD3ζ	−	+	−	159
	ScFV(4D5)	CD28	CD28	CD28	CD3ζ	−	+	−	155
	ScFV(4D5)	CD8	CD8	CD28	CD3ζ	−	+	−	155
HER2	ScFV(4D5)	CD8	CD8	41BB	CD3ζ	−	+	−	155

Solid tumors	CAR construct						Preclinical data	Clinical data	References
Target Antigen	Exodomain (clone)	Hinge	Trans membrane	Endo domain 1	Endo domain 2	Endo domain 3			
	ScFV(ICR12)	CD28	CD28	CD28	CD3ζ	–	+	–	57
	ScFV(4D5)	CD28	CD28	CD28	41BB	CD3ζ	+	–	155
	ScFV(4D5)	CD28	CD28	CD3ζ	CD28	41BB	+	–	155
	ScFV(4D5)	CD8α	CD8	41BB	CD28	CD3ζ	+	–	155
	ScFV(4D5)	CD8α	CD8	CD28	41BB	CD3ζ	+	+	55,155
	ScFV(4D5)	CD8α	CD8	41BB	CD3ζ	CD28	+	–	155
FBP	ScFV(MOv18)	N/A	N/A	CD3ζ	–	–	+	–	160, 161
	ScFV(MOv18)	N/A	FcRγ	CD3ζ	–	–	+	+	52,162, 163
CD171	ScFV(CE7)	Fc	CD4	CD3ζ	–	–	+	+	164, 165
CAIX	ScFV(G250)	N/A	FceR1γ	CD3ζ	–	–	+	–	166, 167
	ScFV(G250)	CH2CH3	CD4	CD3ζ	–	–	–	+	50,51,168
PSMA	ScFV(J591)	CD8	CD8	CD3ζ	–	–	+	–	169, 170
PSMA	ScFV(J591)	CD28	CD28	CD28	CD3ζ	–	+	–	170
IL13-zetakine	ScFV(E13Y)	Fcγ4	CD4	CD3ζ	–	–	+	–	171–173
	ScFV(E13Y)	N/A	CD3	CD28	CD3ζ	–	+	–	174
	ScFV(E13Y)	CD8	CD3	CD28	CD3ζ	–	+	–	174
EphA2	ScFV(4H5)	CH2CH3	CD28	CD28	CD3ζ	–	+	–	175
FAP	ScFV(MO36)	Ch2CH3	CD28	CD28	CD3ζ	–	+	–	176
KDR	ScFV(P3S5)	CD28	CD28	CD3ζ	–	–	+	–	177
	ScFV(mVEGFR-164)	cMyc-CD8	CD8	CD3ζ	–	–	+	–	178
	ScFV(mDC101)	CD8	CD8	CD28	CD3ζ	–	+	–	179,180
	ScFV(KDR-1121)	CD28	CD28	CD28	CD3ζ	–	+	–	179, 180
	ScFV(mDC101)	CD8	CD8	CD28	41BB	CD3ζ	+	–	179, 180
	ScFV(mSP6)	CD8	CD8	CD28	41BB	CD3ζ	+	–	179, 180
	ScFV(KDR-1121)	CD28	CD28	CD28	41BB	CD3ζ	+	–	179, 180

Solid tumors	CAR construct						Preclinical data	Clinical data	References
Target Antigen	Exodomain (clone)	Hinge	Trans membrane	Endo domain 1	Endo domain 2	Endo domain 3			
VEGFR-1	ScFV(V-1)	CH2CH3	CD4	CD3ζ	−	−	+	−	181
EGFRvIII	ScFV(3C10)	CD8	CD8	CD3ζ	−	−	+	−	182
	ScFV(mab139)	CD8α	CD8	CD3ζ	−	−	+	−	183
	ScFV(mab108)	FcIgG2	CD28	CD28	CD3ζ	−	+	−	184
	ScFV(mab139)	CD8α	CD8	ICOS	CD3ζ	−	+	−	185
Mesothelin	ScFV(SS1)	CD8α	CD8	CD3ζ	−	−	+	−	186
	ScFV(P4)	CD8α	CD8	CD3ζ	−	−	+	−	187
	ScFV(SS1)	CD8α	CD8	41BB	CD3ζ	−	+	−	63,186,188
	ScFV(SS1)	CD8α	CD28	CD28	CD3ζ	−	+	−	186
	ScFV(P4)	CD8α	CD28	CD28	CD3ζ	−	+	−	187
	ScFV(SS1)	CD8α	CD28	CD28	41BB	CD3ζ	+	−	176
CD44 v6/v7	ScFV(1.1ASML)	CD8α	CD3	CD3ζ	−	−	+	−	189
	ScFV(VFF17)	cMyc-CD8α	CD3	CD3ζ	−	−	+	−	190
TAG72	ScFV(B72.3)	N/A	N/A	FCεR1γ	−	−	+	−	140,191
	ScFV(CC49)	CH2CH3	CH2CH3	CD3ζ	−	−	+	−	140,191
	ScFV(CC49)	Fc1γ-CH3	CD4	CD3ζ	−	−	+	−	192
Lewis-Y	ScFV(Mluc1)	N/A	N/A	FcRγ	−	−	+	−	193
	ScFV(Hu3S193)	CD8	CD28	CD28	CD3ζ	−	+	−	194–196
MUC1	ScFV(SM3)	CD28	CD28	CD3ζ	−	−	+	−	197
	ScFV(SM3/HMFG2)	CH2CH3	CD28	CD3ζ	−	−	+	−	61
	ScFV(SM3)	IgD-CD28	CD28	CD28	CD3ζ	−	+	−	197
	ScFV(SM3/HMFG2)	FcIgG1	CD28	CD28	CD3ζ	−	+	−	197
	ScFV(HMFG2)	FcIgG1	CD28	CD28	CD3ζ	−	+	−	197
	ScFV(HMFG2)	FcIgG1	CD28	CD28	CD3ζ	−	+	−	57
	ScFV(HMFG2)	IgD-FcIgG1	CD28	CD28	OX40	CD3ζ	+	−	60,197,198
	ScFV(HMFG2)	FcIgG1	CD28	CD28	41BB	CD3ζ	+	−	197
FAR	ScFV(Fab35)	FcIgG1	CD3	CD3ζ	−	−	+	−	199,200

Solid tumors	CAR construct						Preclinical data	Clinical data	References
Target Antigen	Exodomain (clone)	Hinge	Trans membrane	Endo domain 1	Endo domain 2	Endo domain 3			
	ScFV(Fab35)	FcIgG1	CD28	CD28	CD3ζ	−	+	−	200
NCAM	ScFV(D29)	CD3	CD3	CD3ζ	−	−	+	−	201,201
CEA	ScFV(MFE23,22)	CD3	CD3	CD3ζ	−	−	+	−	201–203
	ScFV(MD45)	CD8	FCγR	FCγR	−	−	+	−	204
	ScFV(pUC19)	CD8	CD3	CD3ζ	−	−	+	−	205
	ScFV(BW431/26)	FcIgG1	CD3	CD3ζ	−	−	+	−	206
	ScFV(MFE23)	CD8α	CD3	CD3ζ	−	−	+	−	207
	ScFV(A3B3)	CD8α	N/A	CD3ζ	−	−	+	−	208
	ScFV(pUC19)	CD8	CD3	CD3ζ	−	−	+	−	209
	ScFV(C2-45)	CD8α	CD3	CD3ζ	−	−	+	−	210
	ScFV(SCA431)	Fc	CD4	CD3ζ	−	−	+	−	211
	ScFV(MFE(23,22)	CD28	CD28	CD28	CD3ζ	−	+	−	203
CEA	ScFV(BW431/26)	FcIgG1	CD28	CD28	CD3ζ	−	+	−	159
	ScFV(MFE23)	CD8α	CD28	CD28	CD3ζ	−	+	−	207
	ScFV(F11-39)	CD8α	CD28	CD28	CD3ζ	−	+	−	212,213
	ScFV(C2-45)	CD8α	CD28	CD28	CD3ζ	−	+	−	210,214
	ScFV(pUC19)	CD8-CD28	CD28	CD28	CD3ζ	−	+	−	209
	ScFV(SCA431)	Fc	CD4	CD28	CD3ζ	−	+	−	215
EGP2	ScFV(GA733.2)	CD8α	FcRγ	FcRγ	CD3ζ	−	+	−	216,217
EGP40	ScFV(BR3E4/FG1)	N/A	N/A	FcRγ	−	−	+	−	218
ERBB3/4	ScFV(C11)	cMyc-CD8α	CD3	CD3ζ	−	−	+	−	219
	ScFV(FRP5)	cMyc-CD8α	CD3	CD3ζ	−	−	+	−	219
	ScFV(RAK)	cMyc-CD8α	CD3	CD3ζ	−	−	+	−	219
	ScFV(heregulinβ1)	cMyc-CD8α	CD3	CD3ζ	−	−	+	−	219
	ScFV(hrgα)	CH2CH3	CD34	CD3ζ	−	−	+	−	220
ERBB3/4	ScFV(hrgβ)	CH2CH3	CD34	CD3ζ	−	−	+	−	220

Solid tumors	CAR construct						Preclinical data	Clinical data	References
Target Antigen	Exodomain (clone)	Hinge	Trans membrane	Endo domain 1	Endo domain 2	Endo domain 3			
ErbB	ScFV(T1E)	CD28	CD28	CD28	CD3 ζ	−	+	−	221
GD-3	ScFV(MB3.6)	CD8α	CD3	CD3ζ	−	−	+	−	222,223
	ScFV(MB3.6)	CD8α	CD28	CD28	CD3ζ	−	+	−	222
PSCA	ScFV(7F5)	βC2	CD3	CD3ζ	−	−	+	−	224
	ScFV(hu1G8)	CH2CH3	CD28	CD3ζ	−	−	+	−	225
	ScFV(hu1G8)	CD8α	CD8	CD3ζ	−	−	+	−	226
	ScFV(7F5)	CD8α	CD8	CD3ζ	−	−	+	−	226
	ScFV(MB1)	CD8α	CD8	CD3ζ	−	−	+	−	226
HLA-A1+MAGE1	ScFV(MZ2-82/30)	FcIgG1-CD3	CD3	CD3ζ	−	−	+	−	227
	ScFV(G8)	N/A	CD4	FcεR1γ	−	−	+	−	228,229
	ScFV(G8)	CD28	CD28	FcεR1γ	−	−	+	−	229
	ScFV(Hyb3)	CD28	CD28	FcεR1γ	−	−	+	−	229
NKG2D	mNKG2D	mNKG2D	mNKG2D	CD3ζ	−	−	+	−	230–234
NKG2D	hNKG2D	hNKG2D	hNKG2D	CD3ζ	−	−	+	−	235,236
	hNKG2D	FcIgG1	CD28	CD28	CD3ζ	−	+	−	237