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Review

The human application of gene therapy to re-program T-cell specificity using chimeric antigen receptors

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Abstract

The adoptive transfer of T cells is a promising approach to treat cancers. Primary human T cells can be modified using viral and non-viral vectors to promote the specific targeting of cancer cells via the introduction of exogenous T-cell receptors (TCRs) or chimeric antigen receptors (CARs). This gene transfer displays the potential to increase the specificity and potency of the anticancer response while decreasing the systemic adverse effects that arise from conventional treatments that target both cancerous and healthy cells. This review highlights the generation of clinical-grade T cells expressing CARs for immunotherapy, the use of these cells to target B-cell malignancies and, particularly, the first clinical trials deploying the Sleeping Beauty gene transfer system, which engineers T cells to target $CD19^+$ leukemia and non-Hodgkin's lymphoma.

Key words Chimeric antigen receptors, Sleeping Beauty, gene therapy, T cells, immunotherapy, CD19, receptor tyrosine kinase orphan-like receptor 1 (ROR1)

Allogeneic hematopoietic stem cell transplantation (HSCT) was initially performed to treat hematologic malignancies in order to restore hematopoiesis after myeloablative radiotherapy and/or chemotherapy. However, the attack of residual tumor cells by donor-derived lymphocytes exerts a beneficial effect, defined as the graft-versus-tumor (GVT) effect[1]. The T cells that participate in the GVT effect also cause toxicity due to the inadvertent recognition of major and minor histocompatibility antigens, causing graft-versus-host disease (GVHD). A central role of engrafted T cells in both the beneficial and damaging effects is supported by the finding that recipients exhibiting GVHD were less likely to relapse than patients who did not exhibit GVHD[2,3]. Even in the absence of GVHD, the targeting of leukemia by the T-cell-mediated alloresponse exerts a beneficial effect following HSCT. Patients with leukemia who received T-cell-depleted grafts were more likely to relapse than patients who did not exhibit GVHD after allogeneic HSCT[4-6]. Furthermore, patients who did not exhibit GVHD after receiving human leukocyte antigen (HLA)-matched bone marrow from a sibling donor were less likely to relapse than patients who received bone marrow from their genetically identical twins[5].

Donor leukocyte infusions (DLI) have been administered after transplantation to promote the regression of relapsed disease by exploiting these T-cell-mediated GVT effects. This strategy has been successfully used to treat chronic myelogenous leukemia (CML), resulting in prolonged and stable remission[7]. DLI has also been employed to treat other hematologic malignancies and solid tumors, with mixed results[8-10]. The major limitation of DLI can be ascribed to the lack of specificity for tumor-associated antigens (TAAs); thus, recipients are exposed to the potential of inducing or exacerbating GVHD, which outweighs the beneficial effects of DLI for most tumor types.

The engrafted T cells cultured from donor-derived hematopoietic stem cells (HSCs) or infused T cells from DLI are stimulated by antigen-presenting cells (APCs). In the microenvironment in which T cells engage APCs, the αβ T-cell receptor (TCR) recognizes an antigen presented in the context of the major histocompatibility complex (MHC). Mismatched HLA and the related minor histocompatibility antigens between allogeneic individuals stimulate T cells in an antigen-independent manner. The results from animal models suggest that recipient-derived APCs[11], rather than epithelial cells[12], are crucial for inducing GVHD. Similarly, patient-derived APCs also induce a GVT effect[13-15]. Thus, interactions between donor-derived T cells and recipient-derived APCs lead to divergent outcomes: a beneficial graft-versus-leukemia (GVL) effect and detrimental GVHD[16]. In addition to GVHD, other potential limitations of allogeneic HSCT include the high cost of the transplantation

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procedure and subsequent supportive care as well as the risk of infection during immunosuppressive treatment.

The task of generating an effective antitumor response is inherently difficult, as the genetic instability of tumors promotes adaptations to escape immune surveillance. This genetic instability is apparent, as patients with ostensibly healthy immune systems develop cancers. Tumors can generate an immunosuppressive microenvironment by increasing the expression of immunomodulatory molecules, such as programmed cell death ligand-1 (PD-L1)[17], or secreting immunosuppressive cytokines, such as transforming growth factor-β (TGF-β)[18-20]. In addition, tumors can render themselves functionally invisible to T cells by down-regulating the expression of MHC molecules and the antigen-processing machinery, which are necessary for antigen recognition by an αβ TCR[21,22]. Indeed, a study of primary breast cancer samples detected total loss of MHC class I molecules in greater than 50% of the samples[23]. Further increasing the difficulty of tumor recognition by T cells is that malignant cells present TAAs which are perceived by the immune system as "self." During the process of thymic education, the T cells that display an αβ TCR which is strongly reactive to a self-antigen are deleted from the repertoire to avoid the subsequent generation of an autoimmune response. Thus, the destruction of cancer is dependent on T cells overcoming the immunomodulatory adaptations of tumors and the propensity to ignore self-antigens while maintaining the ability to recognize and respond to TAAs.

Gene Therapy

Allogeneic T cells mediate GVT effects in the specialized environment of allogeneic HSCT. It is unclear how to extrapolate these clinical data to infuse autologous T cells to avoid GVHD and induce an antitumor response. In gene therapy, a promising approach for cancer treatment, cells are genetically modified *ex vivo* for *in vivo* administration to patients. Due to their natural effector functions and established role in mediating anti-leukemia responses, T cells are an attractive vehicle to be engineered for targeted cancer immunotherapy. One goal of combining T-cell therapy with gene therapy is to abolish tolerance of the body's immune system to the tumor, thereby leading to recognition and eradication of cancer cells. In addition, such modifications must be compliant with current Good Manufacturing Practices (GMP) to achieve human application of the genetically modified T-cell product. GMP-complaint manufacture and release of T cells can be accomplished using viral and non-viral methods.

Viral vectors have been effectively used to promote the integration of exogenous DNA into T cells. Both recombinant lentivirus and γ-retrovirus stably introduce transgenes into primary human T cells and have been successfully used in clinical trials. However, there are drawbacks to this approach. First, the construction of GMP-compliant viral vectors requires extensive validation and involves considerable cost in terms of expense, specialized reagents and labor. There is also a significant turnaround time for viral production, partially due to a bottleneck in GMP-compliant viral production facilities. In addition, the size of the viral cargo can be restricted due to the necessary inclusion of viral packaging components and limited size of the viral capsid. Moreover, considerable safety concerns remain due to the nature of the viral vector, which may be assuaged by assessing each T-cell product for its replication competency, but this release test is expensive and time consuming.

Studies have also described a potential for mutagenesis associated with the integration of genetic material delivered by a recombinant viral particle. For example, γ-retroviruses based on the murine leukemia virus are prone to integration near transcriptional start sites of actively transcribed genes[24]. Likewise, lentiviral vectors prefer integration into certain genetic loci, with 57%[24] and 69%[25] of integration events occurring within genes, which is higher than what is expected due to random integration. These risks were illustrated by the development of T-cell leukemia in 25% of patients infused with HSCs that were transduced with γ-retrovirus to treat X-linked severe combined immunodeficiency disease[26]. These cases of induced leukemia were traced to viral integration near the *LMO2* proto-oncogene[27,28]. However, it should be emphasized that the cell type transduced impacts the potential for insertional mutagenesis. In contrast to HSCs, T cells appear to be significantly more resistant to oncogenic transformation after infection with retrovirus[29,30] and have been successfully and safely transduced hundreds of times for use in clinical trials[31].

In contrast to the production of clinical-grade virus, naked DNA plasmids are manufactured in a much faster turnaround time due to a greater number of GMP-approved vendors and the relative simplicity of their production. In addition, the production of plasmids occurs in the absence of eukaryotic cells, reducing the manufacturing burden and post-production validation, all of which contributes to the reduced cost of producing DNA compared to virus for human application. In addition, naked DNA plasmids do not exhibit the same size constraints as plasmids that must be packaged into capsid particles. The major limitation of naked DNA is its low efficiency of stable transfection into primary T cells. This limitation can now be overcome using transposon/transposase systems. Multiple Class II DNA transposons display activity in human cells, including *Tol2*, *piggyBac*, and Sleeping Beauty (SB). Class II DNA transposons are advantageous, as they typically insert a transposon using a copy/paste mechanism, minimizing disruption to the surrounding DNA.

Tol2 is a fish-derived transposon containing an autonomous transposase that retains activity in human cells[32]. *Tol2* is advantageous due to its ability to catalyze the integration of large DNA sequences (greater than 10 kb) without a substantial loss in transposition efficiency[33,34]. However, *Tol2* displays preference to integrate near transcriptional start sites[35]. In addition, *Tol2* does not display the enzymatic activity of *piggyBac*, a transposable element derived from the cabbage looper moth[36]. Similar to *Tol2*, *piggyBac* is capable of catalyzing the transposition of large elements (greater than 14 kb) of DNA without a detrimental loss of efficiency[37]. Integration by *piggyBac* targets TTAA sites, rarely resulting in mutations to the surrounding sequences[37-39]. Moreover, overexpression of the *piggyBac* transposase does not greatly inhibit its transposase activity[39], reducing the need for the optimization of transposase expression based on the transposase activity. This characteristic contrasts that of other transposases, including SB (discussed below). However, the use of *piggyBac* is potentially compromised by its propensity to integrate transposons in and around actively transcribed genes[37,40], which increases the probability of deleterious effects.

The transposase SB (**Figure 1**) was reconstructed from an extinct transposase found in salmonid fish[41]. Since the "awakening" of SB, molecular phylogenetics coupled with mutagenesis has been used to increase the activity of the SB system. The activity

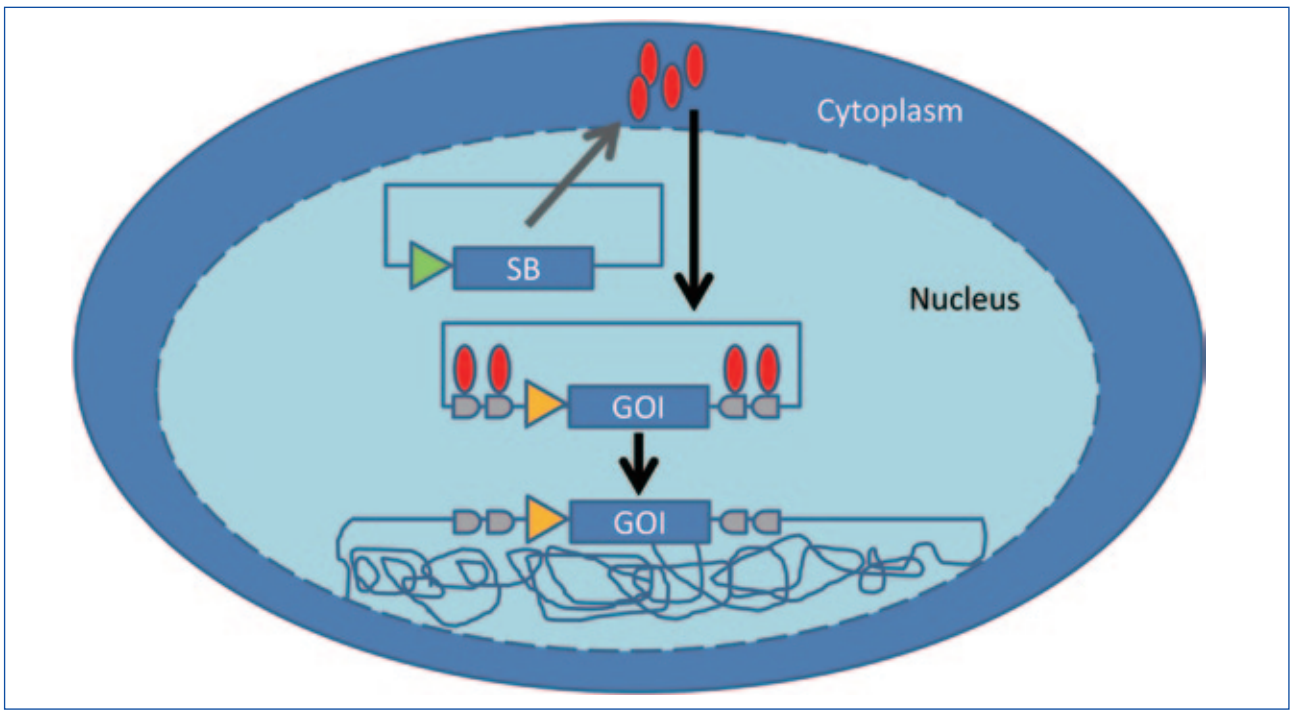

Figure 1. **Sleeping Beauty (SB)-mediated DNA transposition. The SB transposase (red ovals) expressed by a transiently transfected plasmid binds to the inverted repeat sequences (in gray) flanking the gene of interest (GOI) and catalyzes the cut-and-paste transposition of the GOI into the genome of the target cell (tangled lines) at thymine and adenine dinucleotide base pairs. Transposon integration and loss of the plasmid expressing SB transposase results in stable gene expression. The triangles denote promoter sequences, which often vary between the transposase and the GOI.**

of the original transposase was increased[42] and rendered 100-fold more potent in the *SB100X* mutant[43]. Approaches have also been taken to optimize the inverted terminal repeat sequence of the transposon[44]. Compared to genetic engineering using a virus or other transposons, SB is appealing because it can efficiently integrate a gene of interest (flanked by the appropriately inverted and direct repeat sequences) into primary T cells at one (or more) of the 2×10^8 TA dinucleotide sites in the human genome[45-47]. Based on analysis of the resulting integration sites, SB-mediated transposition appears to be less likely to alter gene expression than other vectors that preferentially integrate near transcriptional sites. However, there are some disadvantages of the SB system. Chief among these is an apparent decrease in the efficiency of transposition with increasing cargo load, particularly for transposons greater than 6 kB[42,48,49]. Decreased transposition of large vectors by SB can be ameliorated by increasing the number of transposase-binding sites in the transposon vector[44]. Further complicating the SB system is that the ratio of SB transposase to SB transposon must be optimized, as excess transposase inhibits the transposition reaction[42].

Transposon-mediated gene delivery presents a risk of remobilization. In contrast to infection with replication-deficient viral particles, active introduced transposase could theoretically promote the continuous excision and reintegration of transposons. The likelihood that each integration event induces a deleterious effect is the same as that of the original transposition. However, excision of a SB transposon typically results in a 5-bp insertion, although other small insertions and deletions are possible[50]. Insertions and deletions resulting from excision of the SB plasmid vary depending on the cell type and may reflect the preferred DNA repair mechanisms in each cell type[50]. This "footprint" can lead to a frameshift mutation in the unlikely event that SB integrates into and is mobilized from an exon. Nonetheless, excision of a transposon is estimated to be rare (probability less than 1×10^{-4} transpositions), and the probability of an adverse event is even lower[51,52]. Risks from continual transposition are limited by transient expression of the transposase by a non-integrating vector. This risk is further diminished by expressing the SB transposase using electro-transferred, *in vitro* transcribed mRNA[53].

Strategies to Target TAAs

The goal of virus- or transposon-mediated gene therapy for cancer is to abolish tolerance of the immune system to cancer cells and promote the specific recognition and destruction of tumor cells. This therapeutic strategy involves targeting T cells to TAAs. Ideally, TAAs are presented on tumor cells but not healthy cells, ensuring that TAA-specific T cells achieve antitumor effects without causing on-target off-tissue toxicity. TAA-specific T cells can be numerically expanded *ex vivo* by culturing tumor-infiltrating T cells, or those from the peripheral blood, in the presence of autologous tumors and stimulatory cytokines[54,55] to promote propagation in the absence of the immunoinhibitory environment that may exist *in vivo*. This approach has demonstrated clinical success, particularly for expanding melanoma-specific T cells[56]. Gene therapy can be used to re-direct T cells to specific TAAs by genetically manipulating the T cells *ex vivo* via the introduction of an exogenous TCR or a novel chimeric antigen receptor (CAR).

Introduction of an exogenous cloned αβ TCR is advantageous in that it should signal as a conventional TCR and mimic an endogenous immune response. The two chains can be co-expressed by a single vector using an internal ribosome entry site (IRES) or a pircornavirus 2A peptide sequence. Equimolar α/β chain partners are required for efficient expression of the exogenous TCR, rendering the 2A method more desirable[57], as genes expressed downstream of an IRES are typically not expressed as efficiently as the upstream gene[58]. Although vectors have been developed that stably express TCR genes, this field has been limited by obtaining immunoreceptors that are specific for TAAs.

The TCRs of T cells which are responsive to tumor cells can be isolated and cloned for transfection/infection into peripheral blood T cells to enhance the tumor-specific immune response. Such techniques have been used to produce T cells specific to melanoma antigens, such as melanoma antigen recognized by T cells (MART-1)[59,60] and testis antigen NY-ESO-1, which are found in synovial cell sarcoma and metastatic melanoma[61,62], and to general TAAs, such as p53[63,64] and survivin[65]. T cells expressing TAA-specific TCRs have successfully promoted clinical responses in patients, including complete responses (CRs) in 2 of 11 patients with metastatic melanoma treated with T cells expressing a NY-ESO-1-recognizing TCR[66]. However, there are drawbacks to this approach. First, the introduced TCR requires a specific TAA-derived peptide to be presented in the context of a particular HLA molecule. In addition, the affinity of the cloned TCR appears to correlate with the therapeutic potential of the genetically modified T cells. There are multiple approaches to increase the association and dissociation rate constants of a TCR[67,68]. However, *ex vivo* alterations of TCR affinity may lead to inadvertent recognition of alternative processed antigens, as was the case for patients receiving melanoma antigen family A, 3 (MAGE-A3)-specific T cells who experienced cardiac toxicity due to the recognition of titin by the infused genetically modified T cells[69]. In addition, the exogenous TCR α/β pairs could partner with the endogenous TCR components, reducing the expression of the desired TCR complex. Moreover, these altered TCR pairings could result in the recognition of self-antigen and the development of autoimmune disease[70]. The latter effect could be diminished by silencing the endogenous TCR using short hairpin RNA (shRNA)[71] or by permanently removing the endogenous TCR using artificial nucleases, such as transcription activator-like effector nucleases (TALENs)[72] or clustered regularly interspaced short palindromic repeat (CRISPR)-associated proteins (CRISPR/Cas9)[72-75]. However, T cells expressing an exogenous TCR specific for a TAA would continue to be restricted by HLA expression on tumors, which may be down-regulated[21-23].

In contrast, CARs recognize TAAs independent of HLA. The typical CAR consists of four separate domains that are assembled from individual units: 1) the single chain variable fragment (scFv) domain, 2) the extracellular scaffold/linker, 3) the transmembrane domain, and 4) the signaling endodomain[76]. The specificity of the CAR is imparted by the scFv domain, which is directed against a TAA. Other molecules can be substituted for the scFv domain, including cytokines[77], pattern-recognition receptors[78], and cell surface molecules[79], to dock the CAR to the respective ligands of these molecules. The scFv domain is attached to the transmembrane region via an extracellular scaffold. Optimal recognition of the TAA is affected by the extracellular length of the CAR. TAAs presented proximal to the plasma membrane may be more efficiently recognized by a CAR containing a longer scaffold, and conversely, TAAs presented further from the cell membrane may be more efficiently recognized by a CAR containing a shorter scaffold[80,81]. Moreover, the source of the scaffold may greatly affect the effectiveness and *in vivo* function of the CAR. CARs targeting the B-cell antigen CD19 that have been used for clinical trials contain scaffolds derived from CD28, CD8, IgG1, or IgG4. The IgG motifs found in CARs may bind to endogenous Fc receptors, leading to clearance of CAR^+ T cells expressing the IgG motifs and the non-specific activation of NK cells[82]. *In vivo* binding between CAR^+ T cells containing the IgG scaffolds and endogenous cells expressing Fc receptors can apparently be reduced by mutagenesis of the IgG scaffolds[82]. The transmembrane domain links the extracellular scFv domain and scaffold to the signaling endodomain. Oligomerization of the transmembrane domain may favor CAR signaling, but few studies have systematically analyzed different transmembrane domains[83]. Signaling via the CAR molecule occurs via the addition of various endodomains to the cytoplasmic portion of the chimeric molecule. First-generation CARs signaled via immunoreceptor tyrosine-based activation motifs (ITAMs) from the CD3-ζ chain or FcR-γ[84]. A second co-stimulatory signal is included in second-generation CARs: the signaling endodomains of proteins such as CD28, 4-1BB, OX-40, and ICOS[85]. CARs containing two or more co-stimulatory molecules in addition to CD3-ζ are commonly referred to third-generation CARs. A competitive repopulation experiment demonstrated a survival advantage for second-generation CAR^+ T cells infused into lymphoma patients compared to first-generation designs[86]. The ability to infuse more than one population of CAR^+ T cells into a given recipient is appealing to help understand the immunobiology which favors the sustained persistence and, thus, the enhanced therapeutic activity of a given CAR species. However, the expense associated with generating two infusion products from two recombinant viral vectors diminishes the enthusiasm for this otherwise appealing experimental approach.

To help reduce the barriers to combining gene therapy with T-cell therapy, our laboratory at MD Anderson Cancer Center in Houston, Texas has employed electroporation using a commercially available Nucleofector device (Lonza Inc, Allendale, NJ, USA) to stably express CARs in primary human T cells for clinical trials[52]. Circulating T cells are electro-transferred with both the CAR-containing transposon and a hyperactive SB transposase, which catalyzes the cut-and-paste insertion of the transposon into the host genome. The apparent transient expression of the SB transposase in manufactured T cells helps to minimize the genotoxicity that arises via the continuous reintegration of the transposon. Transient expression of this fish-derived transposase also reduces the risk of the recipient developing an immune response to the transposase after the cells are infused. This improvement is significant, as an anti-transgene immune response could prompt the clearance and rejection of the genetically modified cells[87]. After gene transfer, the T cells are selectively propagated on activating and propagating cells (AaPCs) in the presence of stimulatory soluble cytokines for 4 weeks. The clinical-grade AaPCs are genetically modified K-562 cells that are available as a master cell bank. The AaPCs support the selective numeric expansion of CAR^+ T cells by expressing a TAA and the co-stimulatory molecules CD86, 4-1BB, and membrane-bound interleukin (IL)-15[88].

Currently, to maintain patient safety, the manufacture of clinical-grade SB-modified CAR$^+$ T cells occurs over a period of 3 to 5 weeks to enable the loss of the DNA plasmid containing the SB transposase[88-90]. Indeed, polymerase chain reaction (PCR) is used to confirm the absence of the SB transposase prior to infusion. Redirected T-cell specificity mediated by the CAR is evaluated via a chromium release assay. Sterility is verified via visual inspection, a negative result for mycoplasma based on a PCR assay, and a negative result for endotoxin based on the limulus amoebocyte lysate (LAL) test. The absence of genotoxicity is determined by the lack of growth of the genetically modified T-cell population upon removal of the AaPCs and cytokines, as well as by demonstrating a normal karyotype. The electroporated and propagated T cells are evaluated via flow cytometry for 1) viability, 2) the expression of the CAR, 3) the memory phenotype, 4) the absence of AaPCs, 5) exhaustion markers, and 6) telomere length. The presence of a polyclonal TCR repertoire is validated by flow cytometry or using bar-coded probes to confirm the lack of skewing of the numerically expanded T cells[91].

CAR$^+$ T Cells in Clinical Trials

CAR$^+$ T cells typically engineered by introducing DNA via a virus or a SB system have been successfully infused into patients with either solid or hematologic malignancy (**Table 1**). Transfer of autologous T cells transduced with retrovirus to enforce the expression of a CD19-specific CAR containing the CD28 and CD3-ζ endodomains after a preparative chemotherapy regimen led to the prolonged eradication of CD19$^+$ cells in a follicular lymphoma patient[92]. Later studies conducted using CD19-specific CAR$^+$ T cells in 8 lymphoma and chronic lymphocytic leukemia (CLL) patients found favorable responses in 6 of these patients, including 1 CR lasting more than 15 months and 5 partial responses (PRs) lasting at least 7 months[93]. The expected adverse events associated with targeting CD19 include long-term B-cell aplasia in about half of the patients. In addition to these long-term toxicities, acute adverse events manifested as symptoms of inflammation, such as fever, fatigue, and hypotension. The loss of humoral immunity can be life-threatening, as 1 patient has died of culture-verified influenza 18 days after receiving an infusion of CAR$^+$ T cells[93].

A separate group has also demonstrated success in treating patients with CLL and B-lineage acute lymphocytic leukemia (ALL) with CAR$^+$ T cells that target CD19 and signal via the CD28 and CD3-ζ endodomains. Out of 8 patients with CLL who were infused with CAR$^+$ T cells, 1 was partially responsive to therapy, and 2

Table 1. Chimeric antigen receptor (CAR) T cells in clinical trials

Reference	Cancer	CAR target	CAR endodomains	Number of patients	Clinical outcomes
Kochenderfer *et al.*, 2010[92]	Lymphoma	CD19	CD28 and CD3-ζ	1	PR
Kochenderfer *et al.*, 2012[93]	Lymphoma	CD19	CD28 and CD3-ζ	4	3 PR, 1 died of influenza
	CLL	CD19	CD28 and CD3-ζ	4	1 CR, 2 PR, 1 SD
Kochenderfer *et al.*, 2013[101]	CLL	CD19	CD28 and CD3-ζ	4	1 CR, 1 PR, 2 PD
	Lymphoma	CD19	CD28 and CD3-ζ	6	1 PR, 5 SD
Brentjens *et al.*, 2011[94]	CLL	CD19	CD28 and CD3-ζ	8	1 PR, 2 SD, 3 NR, 1 PD, 1 died of sepsis-like disease (symptoms preceded T-cell transfer)
	ALL	CD19	CD28 and CD3-ζ	1	CR
Brentjens *et al.*, 2013[96]	ALL	CD19	CD28 and CD3-ζ	5	5 CR
Davila *et al.*, 2014[97]	ALL	CD19	CD28 and CD3-ζ	16	10 CR, 4 CRi, 2 NR
Porter *et al.*, 2011[98]	CLL	CD19	4-1BB and CD3-ζ	1	CR
Kalos *et al.*, 2011[99]	CLL	CD19	4-1BB and CD3-ζ	3	2 CR, 1 PR
Grupp *et al.*, 2013[100]	ALL	CD19	4-1BB and CD3-ζ	2	2 CR
Jensen *et al.*, 2010[87]	Lymphoma	CD19	CD3-ζ	2	2 NR
	Lymphoma	CD20	CD3-ζ	2	2 NR
Till *et al.*, 2008[102]	Lymphoma	CD20	CD3-ζ	7	1 PR, 4 SD, 2 NED maintained
Till *et al.*, 2012[103]	Lymphoma	CD20	CD28,4-1BB,CD3-ζ	3	1 PR, 2 NED maintained
Kershaw *et al.*, 2006[104]	Ovarian cancer	αFR	CD3-ζ	14	14 NR
Lamers *et al.*, 2012[106]	Renal cell carcinoma	CAIX	CD3-ζ	12	12 NR
Park *et al.*, 2007[107]	Neuroblastoma	CD171	CD3-ζ	6	1 PR, 5 PD
Louis *et al.*, 2011[108]	Neuroblastoma	GD2	CD3-ζ	11	3 CR, 3 PR, 1 SD, 4 PD
Morgan *et al.*, 2010[109]	Colorectal cancer	HER2	CD28,4-1BB,CD3-ζ	1	Died of cytokine release syndrome

CLL, chronic lymphocytic leukemia; ALL, acute lymphocytic leukemia; PR, partial response; CR, complete response; SD, stable disease; PD, progressive disease; NR, no objective response; CRi, complete response with incomplete count recovery; NED, no evidence of disease.

maintained stable disease[94]. Early in the study, 1 patient with CLL died 48 h post T-cell infusion; however, serum cytokine abnormalities observed prior to this administration suggested that T-cell therapy was not the primary cause of this patient's death[95]. Treatment of ALL with CAR$^+$ T cells was more successful and has resulted in clinical responses in 20 of 22 treated patients reported in three studies[94,96,97]. In the earliest study, the sole ALL patient demonstrated protracted B-cell aplasia following infusion of CD19-specific CAR$^+$ T cells. To consolidate the antitumor effect, this recipient underwent allogeneic HSCT[94]. A subsequent study demonstrated that 4 of 5 patients exhibited CRs following infusion of CAR$^+$ T cells, and these patients also went on to receive allogeneic HSCT[96]. The fifth recipient with ALL in that study initially responded to CAR$^+$ T cells but was ineligible for HSCT and relapsed 13 weeks later before succumbing to the disease[96]. The most recent study found responses in 14 of 16 patients, including clinical CRs in 10 patients[97].

Similarly, patients with CD19$^+$ CLL or ALL treated with autologous CD19-specific CAR$^+$ T cells that signal via the 4-1BB and CD3-ζ endodomains after lymphodepleting chemotherapy experienced prolonged remissions[98-100]. Porter *et al*.[98] administered CAR$^+$ T cells to a patient with CLL, resulting in CR lasting longer than 10 months. A related study transferred CAR$^+$ T cells to 3 CLL patients, leading to 2 CRs and 1 PR lasting at least 7 months[99]. CRs were also observed in 2 patients with ALL; however, CD19$^-$ leukemia cells were detected in 1 patient 2 months after treatment[100]. Toxicities associated with this treatment include tumor lysis syndrome, elevated serum cytokine levels, fever, and hypotension. One of the patients with ALL displayed acute vascular leak syndrome, requiring pressor support, and acute respiratory distress syndrome, requiring intubation[100].

CD19-specific CAR$^+$ T cells have also demonstrated success in reducing the tumor burden after allogeneic HSCT in patients with tumors resistant to DLI. A single dose of donor-derived CAR$^+$ T cells mediated tumor regression in 3 of 10 patients, including 1 who exhibited CR[101]. Importantly, none of these recipients exhibited GVHD.

CD20 represents another target for the treatment of B-cell malignancies with infused CAR$^+$ T cells. PRs were observed in patients who received first- or third-generation CAR$^+$ T cells targeting this B-lineage antigen[102,103]. Only 3 patients were treated with the third-generation CAR signaling via the CD28, 4-1BB, and CD3-ζ endodomains: 2 maintained no evidence of disease for at least 1 year, and 1 exhibited PR prior to relapse 12 months after the infusion. Significantly, PCR revealed that these third-generation CAR+ T cells persisted for at least 9 months in all 3 patients[103]. A lack of persistence of first-generation CAR$^+$ T cells, which contain the signaling endodomain of only one molecule (CD3-ζ), has been attributed to a failure to control the cancer in some clinical studies[86,87].

Non-hematopoietic cell surface antigens have also been targeted using CAR$^+$ T cells in humans. In aggregate, these trials targeting solid tumors have not displayed the same level of success as those targeting hematologic malignancies. These results may be partially related to the deployment of first-generation CAR$^+$ T cells in some of these trials. No patients exhibited a clinical response after receiving first-generation CAR$^+$ T cells targeting the α-folate receptor (αFR) (0 of 14)[104] or carbonic anhydrase IX (CAIX) (0 of 12)[105,106] for the treatment of ovarian or renal cell carcinoma, respectively. Some positive results were obtained for the treatment of neuroblastoma using first-generation CAR$^+$ T cells targeting CD171 or GD2. One of 6 patients treated with CAR$^+$ T cells targeting CD171 exhibited PR[107], and 3 of 11 patients with active disease exhibited CRs following the infusion of CAR$^+$ T cells targeting the ganglioside GD2[108]. Of these 3 responders, 1 remained disease-free for 6 weeks prior to relapse, whereas the responses of the other 2 lasted longer than 21 and 60 months[108].

Thus, CAR$^+$ T cells can expand and remain persistent in recipients in a variety of clinical settings, resulting in encouraging clinical outcomes. Despite the relative safety of CAR$^+$ T-cell therapy in these studies, the risk remains that the hyper-physiologic activation of CAR$^+$ T cells when synchronously activated by a large bio-burden of antigen-expressing cells could lead to toxicities resulting from systemic cytokine release, referred to as cytokine release syndrome[93,96,97,100,109]. Indeed, rapid and systemic cytokine release following the infusion of third-generation CAR$^+$ T cells targeting HER2 into a colorectal cancer patient directly resulted in the patient's death[109]. Furthermore, not all patients respond to this therapy, and there remains considerable room for improvement in both the efficacy and specificity of infused CAR$^+$ T cells.

Improving the Specificity and Effectiveness of CAR$^+$ T Cells

A major obstacle is that the expression of some targeted TAAs is not restricted to tumor cells. Clinical trials targeting B-cell malignancies use an scFv domain specific for the kappa light chain[110], CD19[76], CD20[102,103,111], or CD22[112]. These antigens are expressed on both healthy and malignant B cells. Thus, targeting these TAAs may lead to partial or complete B-cell aplasia and compromised humoral immunity[93]. Although the clinical issues caused by loss of normal B cells can be circumvented by intravenous immunoglobulin infusion, TAAs targeted in association with non-hematopoietic malignancies are also often expressed on healthy tissues[113], potentially leading to severe adverse events, including death[109]. Much effort is being devoted to the discovery of TAAs that are indeed tumor-specific. One promising candidate TAA is the receptor tyrosine kinase orphan-like receptor 1 (ROR1). ROR1 is an appealing TAA because it is apparently absent from the cell surface of most adult tissues[114]. However, ROR1 is expressed on a variety of cancers, including B-cell malignancies, such as CLL and mantle cell lymphoma[115], pancreatic cancer, ovarian cancer, and lung cancer[116]. In contrast to targeting CD19, targeting ROR1 should lead to the specific destruction of cancerous B cells while avoiding damage to healthy B cells and the resulting hypogammaglobulinemia.

The functional specificity of a CAR can be increased by separating the activation and co-stimulatory endodomains into two CARs that are co-expressed on a single T cell. Thus, the activation of CAR$^+$ T cells occurs upon co-recognition of two TAAs by separate scFv domains: one provides the activation signal, and one provides co-stimulation[117]. This technique has demonstrated promise but has yet to be evaluated in a clinical setting. A related technique co-expresses a CAR and an inhibitory CAR (iCAR)[118]. The iCAR targets an antigen expressed on normal tissue and contains an inhibitory, as opposed to an activating, endodomain. Recognition of the target antigen by the iCAR inhibits signaling via the separate activating CAR, should that CAR recognize a TAA on a healthy cell[118].

A separate approach to limit off-target damage caused by CAR$^+$ T cells involves the co-expression of a death switch capable of rapidly shutting down the CAR$^+$ T-cell effector response. One such switch involves inducible caspase-9 (iCasp9). iCasp9 remains dormant until the administration of a biologically inert, cell-permeable molecule that dimerizes and subsequently activates iCasp9[119,120], inducing the genetically modified T cells to undergo apoptosis[121]. A single dose of the dimerizing drug administered to patients whose donor-derived T cells expressed iCasp9 resulted in the rapid and near complete removal of the infused T cells, causing GVHD[122]. The herpes simplex virus thymidine kinase (TK), which promotes death upon administration of the pro-drug ganciclovir, has also been used in human cells[123]. However, the expression of a viral antigen in CAR$^+$ T cells likely leads to an anti-transgene immune response and the rejection of the infused products[87]. Moreover, killing mediated by ganciclovir in TK$^+$ T cells apparently takes longer than killing via dimerized caspase-9[124], which may be detrimental in cases in which an induced immune response must be rapidly and efficiently halted. Alternatively, non-enzymatic death switches include the surface expression of CD20, which can be targeted using antibodies such as rituximab to induce cell destruction[125,126]. Similar to iCasp9, the targeting of CD20-expressing cells with an antibody promotes rapid cell death[124]; however, *in vivo* administration of this antibody would also deplete healthy B cells.

Perhaps the greatest opportunity to increase the therapeutic effectiveness of CAR$^+$ T cells is the modification of the CAR endodomain. Most current clinical trials targeting CD19 employ CAR designs that contain the CD3-ζ with either CD28 or CD137 signaling domains. There is apparent equipoise in the literature regarding the potential of these two second-generation CARs to eliminate malignant B cells. The endodomain used in a CAR may impact the nature of the induced immune response. Signaling via CD28 has been shown to favor the production of IFN-γ and inhibit the production of IL-17, whereas signaling via inducible costimulator (ICOS) favors the secretion of IL-17[127]. As both IFN-γ and IL-17 can alter the tumor microenvironment[128], modifying the CAR endodomain to favor production of different cytokines may change the *in vivo* antitumor activity of a CAR$^+$ T cell. It is likely that different combinations of endodomains (e.g., CD3-ζ with CD28, CD137, ICOS, 4-1BB, or OX-40) differentially affect the CAR$^+$ T-cell response to the corresponding TAA.

Even in the case of efficient targeting and killing by CAR$^+$ T cells, tumors can escape clearance by the immune system via the down-regulation of the targeted TAA. Indeed, a recent clinical trial observed the emergence of CD19$^-$ ALL cells after CD19-specific CAR$^+$ T cells induced tumor regression[100]. Similar observations have been made in CD20-expressing tumors targeted using rituxamab[129,130]. To circumvent tumor escape via the down-regulation of the targeted TAA, multiple CARs targeting separate TAAs can be expressed in a single cell in two populations of T cells. In one study, a tandem CAR specific for CD19 and HER2 remained responsive to each individual antigen and displayed increased activity against targets expressing both TAAs[131].

Future Directions

Thus far, the CARs used for immunotherapy in clinical trials have been expressed in T cells expressing αβ TCR, which is partially due to the relative abundance of αβ T cells in the peripheral blood compared to other lymphocyte populations, such as γδ T cells. However, γδ T cells may have distinct advantages over αβ T cells for adoptive immunotherapy. For example, γδ T-cell populations exhibit endogenous antitumor activity mediated by the γδ TCR. The tumor specificity of some αβ TCRs is becoming apparent based on antigens that have already been described, such as the cellular stress indicator MHC Class I chain-related A (MICA), which is targeted by Vγ1Vδ1 TCR[132]. Both MICA and MHC Class I chain-related B (MICB) expressed on tumor cells activate γδ T cells[133]. γδ T cells are also reactive to other stress indicators, such as heat shock proteins[134] and members of the UL16-binding protein (ULBP) family[135], and expression of these markers by tumor cells triggers lysis via γδ T cells[135-138]. In addition, γδ T cells express invariant innate immune receptors with antitumor activity, such as NKG2D[139]. Another apparent advantage of γδ T cells over αβ T cells is that antigen recognition by γδ T cells is not restricted by HLA[140,141]. This advantage has implications for the development of "off-the-shelf" CAR$^+$ T cells for immunotherapy. Current T-cell immunotherapy relies on harvesting T cells either from the patient, who may produce few or defective T cells due to the malignancy itself and prior treatment of the malignancy, or from an HLA-matched allogeneic donor, which includes the risk of GVHD. Due to their inherent antitumor activity and lack of HLA restriction, γδ T cells are an intriguing candidate therapy that can be pre-prepared from unrelated third-party donors and infused on demand.

The practical difficulties in using γδ T cells for CAR therapy include their relative low frequency in the peripheral blood[142] and the lack of a clinically appealing approach to propagate functional polyclonal γδ T cells. Until recently, γδ T cells were expanded for human use via the exogenous addition of phosphoantigens, such as zoledronic acid, which is only recognized by the Vγ9Vδ2 TCR[143,144]. As a result, γδ T cells responsive to phosphoantigens have been infused in clinical settings[145-151]. We have successfully expanded polyclonal CAR$^+$[152] and CAR$^-$[153] γδ T cells on modified K-562-derived AaPCs. These CAR$^+$ γδ T cells retain the expression of receptors displaying inherent antitumor activity, such as the γδ TCR and natural killer (NK) receptors, potentially enabling the tumor to trigger γδ T-cell activation via both engineered and innate mechanisms. Future studies will compare the effectiveness of γδ CAR$^+$ T cells to that of αβ CAR$^+$ T cells.

Artificial nucleases are another means to improve T cells for adoptive immunotherapy. Zinc-finger nucleases (ZFNs)[154], transcription activator-like (TAL) effector nucleases (TALENs)[72], and CRISPR/Cas9[73-75,155] introduce double-stranded DNA breaks at specific sites, leading to repair by non-homologous end joining, which may result in gene inactivation. These technologies display potential for modulating the immune response to generate a favorable outcome in cancer immunotherapy. Artificial nucleases, such as CTLA-4 and programmed cell death-1 (PD-1), can be used to remove negative regulators of the anticancer response[156-158]. However, the removal of negative regulators of T-cell function must be approached with caution. Deaths have resulted following the infusion of CAR$^+$ T cells[95,109], one of which was attributed to the rapid and systemic release of inflammatory proteins[109]. Furthermore, artificial nucleases could be used to remove the TCR from the αβ T cells used for immunotherapy to eliminate the HLA alloreactivity of the infused T cells to reduce the risk of GVHD and to generate an off-the-shelf T-cell product[159].

Conclusions

The promise of T cells for the killing of cancer cells has been demonstrated for decades. Recent advances in gene therapy have facilitated the successful *ex vivo* re-programming of primary human T cells to express engineered CARs to achieve therapeutic effects *in vivo*. Both virus- and transposon-mediated technologies enable the re-specification of T cells to target TAAs. We believe that the SB system displays particular promise to help democratize T-cell therapy and personalize the genetically modified products to meet the needs of a patient with cancer. In the future, gene transfer will be augmented by genome editing to further increase the effectiveness of adoptive T-cell immunotherapy.

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