CARs: Driving T-cell specificity to enhance anti-tumor immunity

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Abstract

Adoptive transfer of antigen-specific T cells is a compelling tool to treat cancer. To overcome issues of immune tolerance which limits the endogenous adaptive immune response to tumor-associated antigens, robust systems for the genetic modification and characterization of T cells expressing chimeric antigen receptors (CARs) to redirect specificity have been produced. Refinements with regards to persistence and trafficking of the genetically modified T cells are underway to help improve the potency of genetically modified T cells. Clinical trials utilizing this technology demonstrate feasibility, and increasingly, antitumor activity, paving the way for multi-center trials to establish the efficacy of this novel T-cell therapy.

2. Introduction

Allogeneic hematopoietic stem-cell transplantation (HSCT) cures a substantial portion of patients with hematological malignancies who are refractory to conventional chemotherapy, and underscores the powerful therapeutic effect of the T-cell immune response in controlling advanced disease. Polyclonal (non-targeted) T-cell therapy in the form of donor lymphocyte infusion (DLI) following HSCT has been used to effectively treat relapse of slow-growing malignancies in a subset of patients (1-7). However, disease relapse and graft-versus-host-disease (GVHD) following HSCT and DLI illustrate the two most significant limitations of non-directed cellular therapy, namely, immune evasion of the tumor leading to relapse, and on-target effects in which donor-derived T cells target major or minor histocompatibility antigens leading to GVHD.

To achieve remission, infused T cells must recognize and eliminate tumor cells that have arisen in the immunocompetent host and that have evolved a range of passive and active immune evasion strategies to avoid immunemediated destruction. Passive evasion strategies include the emergence of tumor escape variants that have lost the targeted tumor-associated antigen (TAA) such as described in a report by Vago and colleagues(8). In 5 of 17 patients who relapsed with acute myeloid leukemia following haploidentical HSCT, they identified antigen-loss variants of the original leukemic cells in which a region of chromosome 6 encoding the mismatched human leukocyte antigen (HLA) haplotype was deleted, with consequent loss of the tumor target for the donor T cells (8). Active evasion strategies are exemplified by the ability of tumors to adversely modulate the tumor microenvironment that impair T-cell effector functions, such as through secretion of TGFbeta (9).

The antigenic similarities of many tumors limit immune-mediated recognition and clearance by T cells. Many TAA expressed in the tumor microenvironment are self-antigens and endogenous T cells are tolerant due to the lack of their recognition of, or activation by, TAA. Investigators have used genetic tools to overcome the limitation of immune tolerance by genetically modifying T cells to express transgenic T-cell receptor (TCR) alpha and beta chains that recognize TAA in context of human leukocyte antigen (HLA), or by expressing a

single-chain chimeric antigen receptor (CAR) to redirect T-cell specificity to a TAA expressed on the cell surface independent of HLA(10, 11). In this review, we focus on the design and implementation of CARs.

3. The CAR structure

The prototypical CAR uses a mouse monoclonal antibody (mAb) that docks with a designated cell-surface TAA triggering desired T-cell activation and effector functions. The specificity of a CAR is achieved by its exodomain which is typically derived from the antigen-binding motif from a mAb that links V_H with V_L sequences to construct a single-chain fragment variable (scFv) region. In the event that the TAA is itself a receptor, exodomains of CARs have also been fashioned from ligands or peptides (e.g. cytokines) to redirect specificity to receptors (e.g. cytokine receptors), such as the IL-13Ralpha2–specific "zetakine" (12). The exodomain is completed by the inclusion of a flexible (hinge), such as from CD8α or immunoglobulin(13, 14) and is expressed on the T-cell surface via a transmembrane domain. Upon binding TAA, the CAR activates T cells via an endodomain which typically includes cytoplasmic domains from CD3 or high-affinity receptor FcεRI (15-17). The docking of CAR to TAA ideally provides the genetically modified T cell with a fully-competent activation signal, minimally defined as CAR-dependent killing, proliferation, and cytokine production. Specific effector functions can be engineered by the design of CARs, such as the inclusion of more than one chimeric activation domain. Thus, iterative modifications to the CAR have resulted in first-, second-, and third-generation CARs designed with one, two, or three signaling motifs within an endodomain (Figure 1) that include cytoplasmic signaling motifs derived from CD28, CD134, CD137, Lck, ICOS, and DAP10(14, 18-20).

Implicit in the design of CARs is the desire by investigators to improve the survival of adoptively transferred T cells, as their persistence correlates with their therapeutic potential. While the optimal CAR design remains to be determined, results from early clinical trials appear to indicate that 1st generation technology, in which a CAR signals solely through immunoreceptor tyrosine-based activation motif (ITAM) domains on CD3-zeta, is unlikely to sustain the *in vivo* persistence of T cells in most patients (21-25). Second-generation CARs, which have signaling domains in addition to CD3-zeta coupled to other co-stimulatory molecules, have improved T-cell effector functioning(21, 26, 27). For this reason, most clinical trials infusing CAR^{+} T cells at this point are using the 2nd generation CAR design. Indeed, Kochenderfer and colleagues treated a patient with advanced follicular lymphoma with fludarabine and cyclophosphamide preconditioning followed by infusion of autologous T cells modified via gamma retrovirus transduction to express a second generation CAR that recognized CD19 (28). Significant regression of the lymphoma was noted, which could be attributed to the chemotherapy and/or T cell infusion. Importantly, B-cell precursors were selectively eliminated from the patient's bone marrow and absent in the peripheral blood for at least 39 weeks following infusion of the modified T cell suggesting prolonged activity and efficacy of the modified T cell, rather than chemotherapy. Third-generation CARs that include a combination of co-stimulatory endodomains (e.g. CD28 and CD137) (14) are being tested in a limited number of clinical trials. Newer strategies to enhance T cell signaling following ligation of the CAR with the TAA (beyond the current 2nd generation design) will likely need rigorous safeguards to prevent excessive toxicity as these T cells may be capable of synchronous and supra-physiologic signaling which could lead to adverse events (29).

To improve the targeting of TAA, the avidity of the genetically modified T cell for TAA may be altered by changes to the scFv to improve functional affinity based on selecting high-affinity binding variants from phage arrays(30, 31). The approach to developing CAR[+]

T cells with a calibrated increase in functional affinity may be necessary to enable genetically modified T cells to target tumors with low levels of TAA expression or perhaps to target a cell-surface molecule in the presence of soluble antigen(32, 33). Other modifications to the scFv include reducing potential immunogenicity by using humanized scFv regions, for example to target carcinoembryonic antigen (CEA) (34) and ERBB2 (14). It is anticipated that these humanized CARs may avoid immune-mediated recognition leading to elimination of the genetically modified T cells. However, a benefit for using humanized scFv regions is yet to be established in the clinical setting.

4. Approaches to genetic modification of T cells to express CAR

Approaches to the genetic manipulation of T cells for the introduction of CAR transgene have mostly relied on transduction using recombinant retrovirus. As an alternative, we and others are investigating the clinical potential of non-viral approaches to gene transfer. The different approaches to the expression of transgenes are summarized in two recent reviews by June and Jena(35, 36). Recombinant retroviral systems can efficiently and stably genetically modify populations of T cells with the inherent goal to shorten their *in vitro* time to production and release, since prolonged time in culture can lead to terminal differentiation and replication senescence (37-39) (40-42). Despite the theoretical risk for insertional mutagenesis there has been no documented genotoxicity or tumor induction attributed to T cells genetically modified with retrovirus. However, for many investigators recombinant clinical grade retroviruses are often cumbersome and expensive to manufacture requiring specialized facilities and personnel skilled in current good manufacturing practice (cGMP). Nevertheless, the retroviral transduction systems have been most extensively studied and validated in the clinical setting.

As an alternative to transduction, electroporation has been adapted as an approach to the nonviral gene transfer of DNA plasmids to generate CAR$^+$ T cells (24, 43, 44). Early clinical data demonstrates the feasibility and safety of infusing autologous CAR$^+$ T cells in patients with lymphoma and such T cells have been attributed to have an antitumor effect (22, 23). The electrotransfer and integration of naked plasmid DNA into T cells is considered inefficient because it depends on illegitimate recombination for stable genomic insertion of nonviral sequences. As a result, lengthy *in vitro* culturing times were required to select for stably transfected T cells, leading to senescence of some of the T cells and decreased efficacy (45, 46). The efficiency of integration can be greatly improved leading to shortened time in tissue culture using transposon and transposase systems such as derived from *Sleeping Beauty* (SB) (43, 47, 48) and *piggyBac* (49, 50) to stably introduce CAR from electrotransferred DNA plasmids (44, 49, 51-55). The electroporation of T cells in compliance with cGMP with clinical-grade DNA plasmids is less costly compared with retrovirus systems. We have shown that the SB system can be used to introduce CAR and other transgenes into primary human T cells with approximately 60-fold improved integration efficiency, compared with electrotransfer of DNA transposon plasmid without transposase (47). After electroporation, T cells can be rapidly expanded in a CAR-dependent manner by recursive culture on γ-irradiated artificial antigen-presenting cells (aAPC) achieving clinically sufficient numbers of cells for infusion within 3 to 4 weeks after electroporation.

5. Improved persistence of CAR$^+$ T cells

Limited survival of infused genetically modified T cells has been noted in many of the initial Phase I trials described in Table 1. While these trials were not powered for efficacy, the lack of long-term persistence of the infused T cells likely contributes to the lack of major clinical responses reported from these studies. The reasons for poor persistence are (i) incomplete T-

cell activation through the CAR, (ii) diminished proliferative capacity of the T-cell sub-population into which CAR was inserted, (iii) unfavorable environment into which T cells are infused and/or home to, and (iv) immune response by the recipient leading to clearance of infused T cells. As previously mentioned, the CAR design has been modified to improve the ability of T cells to undergo a fully competent activation signal to ultimately improve efficacy (Figure 1).

Additionally, there is increasing awareness that the type of T cell into which the CAR is expressed impacts the ability of the T cell to proliferate and survive after adoptive transfer. Initially, pools of T cells directly obtained from peripheral blood were genetically modified to express CAR (Figure 1, Paradigm 1). However, sub-sets of T cells may be selected for improved persistence and thus improved therapeutic effect (Figure 2, Paradigm 2). These T cells signal through an additional receptor, for example, via an endogenous alpha-beta TCR with specificity for known antigen (e.g. viral antigen or allo-antigen)(25, 56, 57) or via enforced expression of a co-stimulatory molecule such as CD80 and CD137L (58). Triggering such TCRs *in vivo* can lead to enhanced T-cell proliferation thereby improving antitumor effect delivered by the introduced CAR. This was recently demonstrated in a report by Pule and colleagues describing patients infused with autologous bispecific T cells that recognized EBV-specific antigens via the endogenous alpha-betaTCR and the disialoganglioside antigen GD_2 on neuroblastoma cells via introduced CAR. The EBV-specific CAR^{+} T cells had prolonged persistence *in vivo* compared with CAR^{+} T cells for which the specificity of the TCR was not known (25). Moreover, the CAR can be expressed on naive and memory T cells that were pre-selected prior to genetic modification in order to generate a desired population of T cells with an enhanced ability to persist after adoptive transfer (Figure 1, Paradigm 3)(59, 60). A debate remains regarding the preferred T-cell phenotype. For example, infusion of central memory T cells persisted to a greater extent than more differentiated T cells in the non-human primate model (61), whereas infusion of naive T cells were more long lived in a murine model (59).

Another strategy to improve the survival of T cells is to deplete lymphocytes in the recipient prior to the infusion of the genetically modified T cells. The efficacy of this strategy is demonstrated by the adoptive transfer of *ex vivo* expanded tumor-infiltrating lymphocytes (TIL) after iatrogenic lymphodepletion in patients with advanced melanoma (62). Sustained persistence of genetically modified T cells has also been observed when patients received lymphodepleting chemotherapy prior to T-cell infusion (28) (23). Infused T cells may proliferate more efficiently in the lymphopenic host through homeostatic mechanisms mediated by the removal of regulatory and suppressor cells, and increased availability of cytokines, while immune responses that develop against the CAR may be attenuated.

Clinical data reveals that the recipient can recognize immunogenic transgenes which might lead to immune-mediated clearance of infused genetically modified T cells. As initially reported by Lamers and colleagues in 2006 (63), and recently updated (64), 11 patients with metastatic renal cell carcinoma (RCC) received multiple doses (without prior lymphodepletion) of autologous T cells modified via retrovirus transduction to express a 1st generation CAR specific for carbonic anhydrase IX (CAIX), that is over-expressed on RCC. Humoral and cellular anti-CAIX-CAR T-cell immune responses were noted that were associated with limited persistence of the transferred T cells (64). Similarly, development of anti-CAR humoral immunity was thought to curtail persistence of CAR^{+} T cells in the study reported by Kershaw and colleagues, in which 14 patients with ovarian cancer received autologous T cells modified via retroviral transduction to express a 1st generation CAR specific for the ovarian cancer-associated antigen α-folate receptor (65). Patients received a single dose of T cells with or without IL-2, without prior lymphodepletion (65). Immune responses to the CAR may be avoided using a humanized CAR (66) (31). However, the

vector itself may be a target for an immune response as two patients developed immunity directed against the retroviral vector epitopes in the study reported by Lamers and colleagues (64). Immune responses to epitopes encoded by the viral vector may be avoided by using non-viral, electroporation techniques to introduce DNA plasmids to express CAR.

The ability to genetically modify T cells with different extracellular receptors and intracellular signaling domains also offers an opportunity to engineer for improved persistence. Investigators have enforced expression of cytokines that signal through the common γ-cytokine receptor, to replace the dependence of T cells on exogenous cytokines for survival. For example, animal experiments (67) as well as clinical experience (68) have shown that long-lived T cells are associated with expression of the IL-7Rα chain (CD127), but genetically modified and propagated T cells tend to down-regulate this cytokine receptor. Therefore, to enhance the ability of T cells to respond to the pro-survival cytokine IL-7, investigators have enforced the expression of IL-7Rα to demonstrate the improved survival of genetically modified EBV-specific T cells in an animal model (69). A novel membrane-bound variant of IL-7 when co-expressed on the cell surface improved persistence of CAR+ T cells (Hurton ASH 2009). Furthermore, T-cell over-expression of receptors for IL-2 and IL-15, as well as enforced expression of cytokines for secretion, have improved persistence of T cells *in vitro* (70) (71) (72-74) (75). However, when this approach was tested in a clinical trial infusing TIL genetically modified to constitutively secrete recombinant IL-2, the persistence of the adoptively transferred T cells was not improved compared with genetically unmodified TIL (76). The reasons are likely multifactorial, but the authors concluded that extensive manipulation and prolonged culture of the T cells, resulting in shortened telomere length, likely contributed significantly to the truncated persistence of the modified T cells. Cytokine receptors have also been modified in T cells to improve their ability for effector functioning within a tumor-suppressive environment. For example, investigators have introduced a dominant-negative receptor for TGFβ receptor to enable genetically modified T cells to resist the suppressive effects of this cytokine (77). Recognizing that the tumor microenvironment contains regulatory T cells, the CAR signaling motif has been adapted to resist the suppressive effects of these cells by the expression of chimeric CD28 (78).

6. Improved trafficking of CAR+ T cells

To effectively penetrate the tumor, genetically modified T cells must home to the sites of malignancy. Migration may be compromised by the loss of desired chemokine receptors during genetic modification and passage *ex vivo*, or may result from the selection of T cells that are inherently unable to localize to certain tissues. Panels of tissue-specific homing receptors which are typically composed of integrins, chemokines, and chemokine receptors are associated with T-cell migration to anatomic sites of malignancy. Therefore, flow cytometry can be used to describe the potential migration of the T cells that will be infused (79, 80). Whether specific subsets of T cells expressing the desired endogenous homing receptors can be genetically modified to express CAR is unclear. As a result, investigators are manipulating the homing potential of T cells through the enforced expression of chemokine receptors such as CCR4 (81).

7. Non-invasive imaging of CAR+ T cells

The ability to genetically modify T cells also provides investigators with a platform to express other transgenes such as those that permit noninvasive imaging by positron emission tomography (PET). The ability to visualize trafficking and localization of the modified T cells would provide significant biological information. One imaging transgene coexpressed with CAR is thymidine kinase (TK) (and associated TK mutants) from herpes simplex

virus-1 (HSV-1) (82) which can be used to enzymatically trap radioactive substrates within the cytoplasm to image the trafficking of T cells by PET (83, 84). Furthermore, expression of TK also renders CAR$^+$ T cells sensitive to conditional ablation using ganciclovir (85, 86). The potential immunogenicity of using viral-derived TK may be overcome by enforcing the expression of mitochondrial human TK in the cytoplasm (87). Recently, another naturally occurring human gene has been adapted for PET. Deoxycytidine kinase (dCK) has been imaged with designer PET probes and dCK itself has been redesigned for efficient uptake of PET probes which are currently in use (88, 89).

8. Safety of CAR$^+$ T cells

While the safety profile of adoptive transfer of non-modified *ex vivo* propagated autologous T cells is established (90), there are several potential concerns with the use of engineered lymphocytes. Firstly, toxicity attributable to undesired, on-target effects of the transgene has been observed (63, 64, 91). Significant hepatic toxicity necessitating discontinuation of treatment was seen in one patient, and dose reduction in 2 patients were required after infusion of T cells engineered to express CAR specific for CAIX. While CAIX is over-expressed on RCC cells it is also expressed, to a lesser extent, on epithelial cells lining the digestive tract, including liver bile ducts. Biopsy of the liver in one of these patients indicated infiltration by modified T cells around bile ducts expressing CAIX resulting in cholangitis (63, 64). Furthermore, evidence it appears that low-level expression of ERBB2 on normal lung cells may have been associated with the sudden death of a patient who received autologous HER2-specific T cells expressing a 3rd generation CAR (29). The elimination of normal B cells in the study reported by Kochenderfer and colleagues (28) is another example of an undesired, on-target toxicity. However, this toxicity was expected as CD19-specific T cells can not distinguish between CD19 expressed on malignant B cells versus normal B cells. In this situation, the risk of loss of normal B-cell function is offset by the potential benefit from an antitumor effect and that patients can tolerate B-cell lymphopenia. (We note that the report by Brentjens describing the death of a patient after infusing CD19-specific CAR$^+$ T cells was not attributed to the genetically modified T cells (92).)

Secondly, there is concern for genotoxicity attributable to the vector. This remains a theoretical concern for genetically modified T cells, in contrast to hematopoietic progenitor cells (93). The stable expression of CAR currently requires the introduction of a promoter and the transgene which raises the possibility of insertional mutagenesis (94). To date there have been no genotoxic events attributed to genetically modified T cells that have been transduced by recombinant virus or electroporation (54, 95). The risk for insertional mutagenesis may be alleviated by electrotransfer of *in vitro* transcribed mRNA coding for a CAR. RNA-based electroporation mediates transient expression (96), but as technologies improve to synchronously electroporate large numbers of cells, it may be possible to overcome the expected loss of CAR expression from transient transfection by repeatedly electroporating and administering CAR$^+$ T cells(97).

The third area of safety concern relates to the type of T cell being infused. For example, will the genetically modified T cells develop autonomous proliferation independent of binding TAA? Or, in the allogeneic setting, will the donor-derived T cells target major or minor histocompatibility antigens that could lead to GVHD? Co-expression of a conditional suicide gene, such as TK or dimerizable caspase, may prevent long-term toxicity (74), however, administration of the appropriate "suicide substrate" may not impact the pace of a potential CAR-mediated clinical toxicity arising in the acute setting. Currently, as infused CAR$^+$ T cells are not (yet) long-lived, there does not appear the need to co-express a conditional suicide gene for CAR$^+$ T cells. As T-cell subsets are isolated for genetic

modification and as CARs and other co-stimulatory molecules are refined to provide supra-physiologic signaling, the decision to include a conditional suicide gene may then be warranted.

9. Future directions of CARs

The initial results of early phase trials using current CAR technology demonstrate the feasibility and therapeutic potential of genetically modified T cells. The technology will continue to improve, and future directions will likely include combination therapies. For example, CAR^{+} T cells may benefit from concomitant therapy with therapeutic monoclonal antibodies (98) or immunocytokine support (99). As the technology becomes more complex, involving multiple genes, the regulatory oversight for the production and release of the T cells and the safety of the recipient becomes more complex. Most pilot trials, including those accruing patients to receive cell and gene therapy, enroll research participants with advanced disease and it is not unexpected for a subset of these medically fragile patients to unfortunately expire during the trial. While it is incumbent on the clinical team to safeguard patient well-being, it is also important for regulatory bodies to monitor gene therapy trials in such a way as to maintain safety, but not hinder progress. The technology to manufacture CAR^{+} T cells has now reached a point of "mass-production" so that many investigators can readily participate in the design and implementation of clinical trials. Future clinical, multicenter trials will require streamlining the current regulatory processes governing T-cell manufacturing so that studies powered for efficacy can be efficiently conducted to definitively establish the therapeutic potential of CAR^{+} T cells.

Acknowledgments

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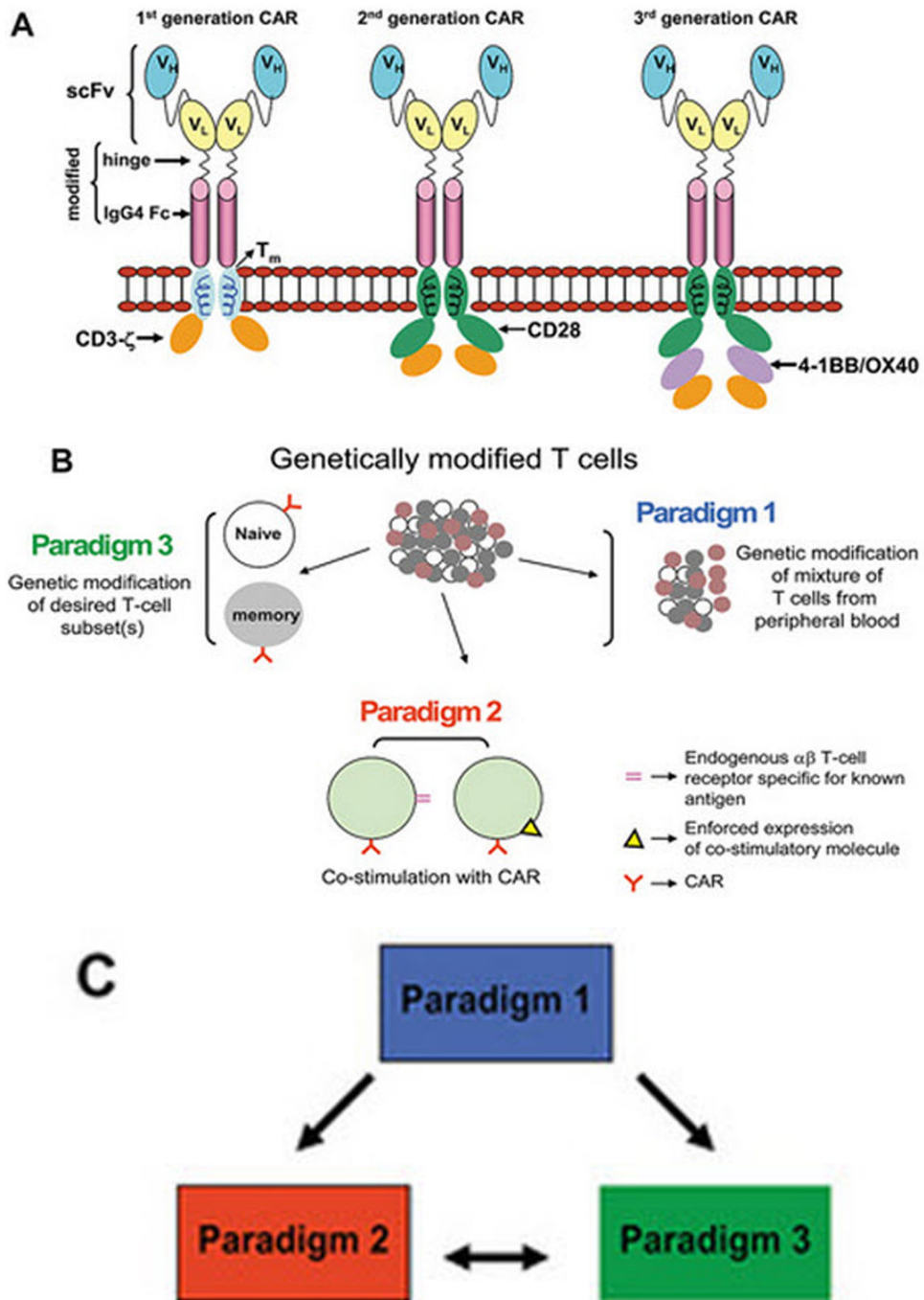
Figure 1. Descriptions of prototypical CARs and genetically modified T cells from which they are expressed

(A) Dimerized CARs demonstrating the extracellular scFv (V_H linked to V_L) via a linker) region, linked to a flexible hinge and Fc region (for example, from IgG4) fused to intracellular signaling motifs via a transmembrane domain. The 1st generation CAR is shown as activating T cells through an endodomain composed of only CD3-ζ. The 2nd generation CAR activates T cells through chimeric CD3-ζ and CD28. The 3rd generation CAR activates T cells through three signaling motifs, e.g., CD3-ζ with CD28 and CD134 or CD137. The modular structure of the extracellular and intracellular domains can be readily

altered to achieve fully competent CAR-dependent signaling. (B) Paralleling the design changes to CAR is an understanding that the type of T cell into which the CAR is expressed can impact the therapeutic potential of adoptive immunotherapy. Paradigm 1 refers to the collection of (naïve, memory, effector) T cells in peripheral blood which can be genetically modified to express just the CAR without further manipulation. Paradigm 2 generates CAR$^+$ T cells that can signal with other desired receptor(s) such as endogenous αβ TCR or introduced co-stimulatory molecule(s). Paradigm 3 expresses the CAR in desired T-cell subsets such as naïve or central memory. (C) The three proposed paradigms are not mutually exclusive of each other as T cells from peripheral blood can be used as cellular templates for Paradigms 2 and 3. Clinical trials are needed to determine whether the CAR design or the type of T cell from which the CAR is expressed, or both, will result in superior persistence and anti-tumor response.

Table 1

Completed clinical trials with CAR+ T cells

1st Author, Year	Type of T cell	CAR construct	Cell Dose (T cells/ m2)	Cancer/No. Pt.	Serious Adverse Events	Persistence	Anti-tumor response
Park, 2007 (24)	OKT3 activated T cells	CE7R-1st gen. CAR plasmid with HyTK, nonviral transduction	$10^8 - 10^9$	Neuro-blastoma/ 6	None	1-42 days	1/6 PR
Kershaw, 2008 (65)	OKT3/allo-antigen activated T cells	α-folate receptor, 1st gen. CAR, retroviral vector	$3 \times 10^9 - 5 \times 10^{10}$ (OKT3) 4×10^9-1.69 $\times 10^{11}$ (alloantigen)	Ovarian/ 14	None	Up to 3 wks (OKT3)12 mo. in one patient (alloantigen)	None reported
Till, 2008 (23)	OKT3 activated T cells	CD20, 1st gen, CAR, nonviral transduction	$10^8 - 3.3 \times 10^9$	CD20+ NHL/7	None	1-3 wks (clones) 5-9 wks (lines+ IL2)	5 eval: 4 SD, 1 PR
Pule, 2008 (25)	OKT3 activated T cells and EBV-specific CTLs	GD2-CAR retroviral vector	$2 \times 10^7 - 2 \times 10^8$ of each product	Neuro-blastoma/ 11	None	Up to 3 wks for activated T cells; up to 6 mo. CTLs	8 eval: 4 response with 1 CR
Lamers, 2010 (64)		G250-1st gen. CAR, retroviral vector	$0.38 - 2.13 \times 10^9$	Renal/ 11	Liver	Up to 53 days	None reported
Kochen-derfer, 2010 (28)		CD19, 2nd gen. CAR, retroviral vector	4×10^8 in 2 divided doses	Follicular/1	Absent Ig	27 wks	PR