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Considerations for the Clinical Application of Chimeric Antigen Receptor T Cells: Observations from a *Recombinant DNA Advisory Committee Symposium Held June 15, 2010*

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Abstract

T cells that are genetically modified to express single-chain chimeric antigen receptors (CAR) have shown promise in early cancer immunotherapy clinical trials. Unfortunately, 2 recent deaths in cancer patients treated with CAR T cells have created some uncertainty on how to best mitigate patient risk, while continuing to advance this very promising therapeutic avenue. In order to address these concerns, the Recombinant DNA Advisory Committee (RAC) held a symposium, the objectives of which were to first review the reported treatment-associated toxicities and, second, to discuss methods for improving safety and efficacy. This report highlights the issues raised as part of this discussion, with a specific focus on protocols infusing CAR T cells. Because this was not a consensus conference, the opinions described should not be construed to represent those of any individual RAC member, the RAC as a body, conference participants, the National Institutes of Health, or the U.S. Food and Drug Administration. *Cancer Res*; 71(9); 3175–81. ©2011 AACR.

Evolution of Chimeric Antigen Receptors

Following appropriate preconditioning regimens that deplete circulating lymphocytes, the adoptive transfer of tumor-infiltrating lymphocytes (TIL) into patients with metastatic melanoma leads to objective clinical responses in select individuals (Supplementary references S1 and S2). Building on this approach, recent studies suggest that autologous T cells, genetically engineered to express tumor-associated antigen (TAA)-specific T-cell receptors (TCR), can replace TILs in certain clinical settings (1). Importantly, tumor regressions are observed even under conditions of widely metastatic and bulky disease and in patients who have failed both prior surgical extirpation and

medical therapy. Unfortunately, the potential for widespread use of adoptive transfer strategies using TILs or T cells with recombinant TCRs is limited (Supplementary reference S3).

In order to overcome the historic problems associated with the cellular targeting of human leukocyte antigen (HLA)-restricted TAA, Eshhar and colleagues developed a strategy to redirect T-cell specificity using chimeric antigen receptors (CAR) or T bodies (Fig. 1; ref. 2). First-generation vectors for the production of CAR T cells contain the heavy and light chain immunoglobulin (Ig) variable regions, fused as a single chain to the ϵ , γ , or ζ signaling sequences of the TCR or the signaling region of the Fc γ domain (3–8). T cells expressing such first-generation CARs recognize surface TAAs, independent of HLA restriction, but cannot recognize intracellular TAAs (Table 1). Early clinical experiences with first-generation CAR T cells showed that (i) the survival of adoptively transferred CAR T cells was limited in cancer patients and (ii) few objective antitumor responses were observed (8–10).

To enhance survival and/or increase proliferation of transferred CAR T cells, investigators have incorporated signaling moieties from costimulatory molecules including CD28 (B7–1), CD134 (OX40), and CD137 (4–1BB), alone (second generation) or in sequence (third generation; refs. 11–15). A recent study comparing simultaneously infused CD19-specific CAR T cells with or without the CD28 signaling sequence indeed shows improved survival of CD28-modified cells (16). Additionally, combining CD28 with 4–1BB signaling sequences further promotes engraftment of CAR T cells (17,

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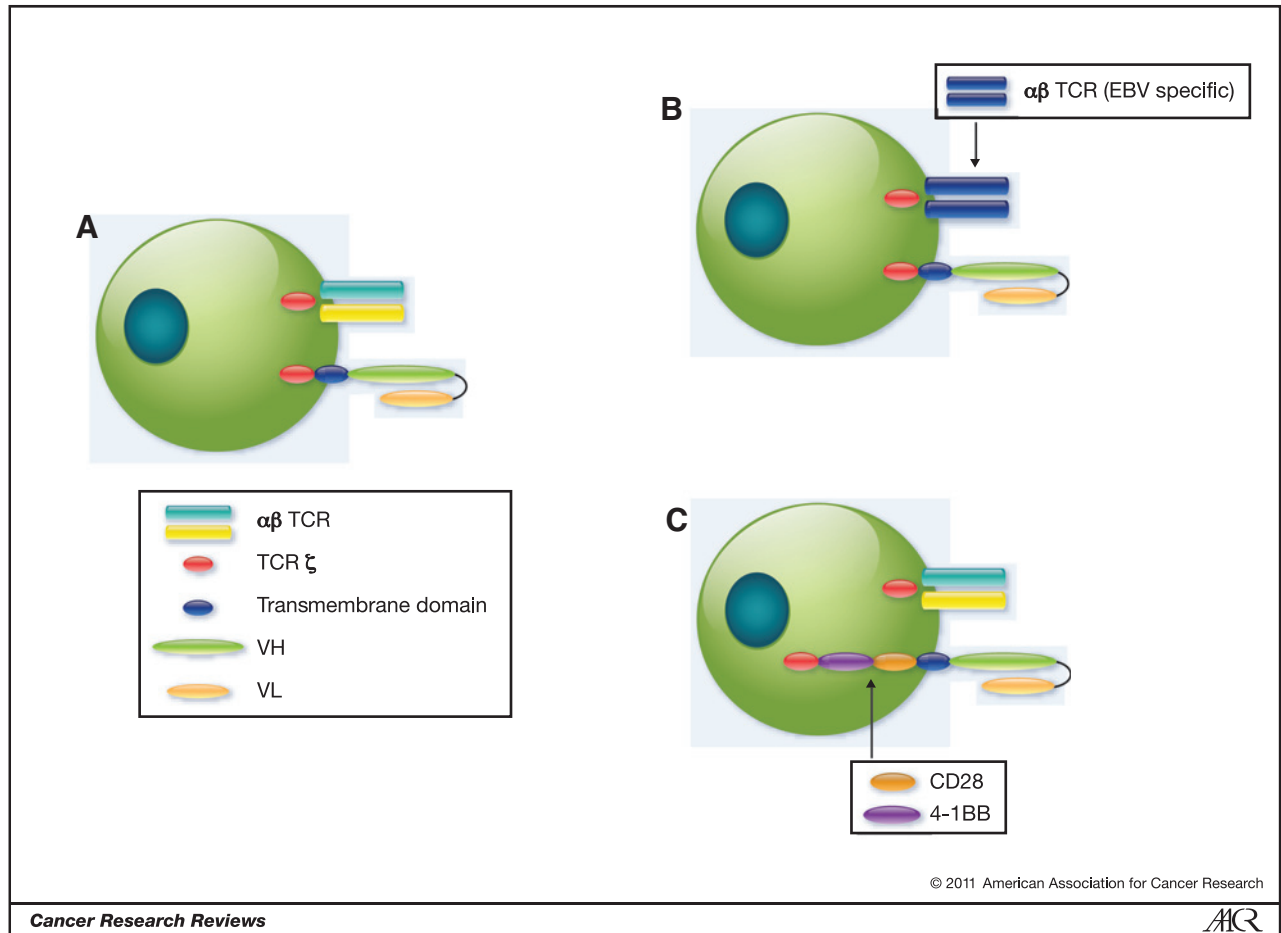


Figure 1. Evolution of CAR T-cell design. A, initial CAR T cells were composed of an antigen-specific scFv linked to the TCR ζ chain. These CARs were expressed in nonclonal T-cell populations with diverse specificities of their endogenous TCRs. B, in subsequent strategies, investigators have attempted to prolong gene-modified T-cell survival by expressing first-generation CARs in T cells whose endogenous TCRs recognize specific antigens of persisting viruses, such as Epstein Barr virus (EBV), allowing for the induction of the costimulatory cascade upon cognate TCR ligation. C, because of difficulty isolating TAA-specific T cells from most types of solid tumors, other investigators have used a modified CAR design in which the 4-1BB/CD28 intracellular signaling domains are linked to the transmembrane region. In these CARs, scFv antigen recognition results in T-cell activation through the TCR ζ chain, leading to prolonged survival secondary to appropriate costimulation. VH, heavy variable chain; VL, light variable chain.

18). As an alternative to incorporating costimulatory moieties into the vector, some investigators express first-generation CARs in T cells specific to endogenous viral antigens, for example, Epstein Barr virus (EBV). In this setting, antigen

recognition by the CAR provides TCR ζ signaling, whereas recognition of processed EBV peptides in the context of appropriate HLA molecules by the physiologic $\alpha\beta$ TCR allows for intrinsic costimulation (19).

Table 1. Limitations of gene-modified T cells by antigen recognition moiety

Limitations	Gene-modified T-cell type	
	$\alpha\beta$ TCR	CAR
Susceptible to loss and/or downregulation of HLA expression on tumor cells	+	–
Susceptible to dysfunction of the tumor cell antigen-processing machinery	+	–
Cell surface target antigen restriction	–	+
Potential for vector recombination with endogenous TCR	+	–

Table 2. Principles applicable to all trials in which CAR T cells are used

- A clear plan for monitoring should be in place and at a minimum include provision for collection of the following:
 - Physiologic data
 - Cytokines, e.g., IFN- γ , interleukin-6, TNF- α , and others
 - Plasma and lymphocytes for cryopreservation
 - Routine lab results from sera and urine
 - Target organ-specific lab results as indicated
- Subject screening should be provided for adequate pulmonary and cardiac function.
- Early reporting of SAEs and clinical outcomes is encouraged.
- Protocols that use retroviral or lentiviral vectors should monitor for the possibility of insertional mutagenesis.^a
- The informed consent should include the following:
 - Discussion of the risk of insertional mutagenesis
 - The term "gene transfer" as opposed to "gene therapy" to avoid the potential for therapeutic misconception
- Special considerations requiring extra care:
 - Effects of CAR expression on non-T-cell populations, e.g., natural killer cells.
 - Effects of CAR expression on specific T-cell subsets, e.g., CD8⁺ T cells vs. all T cells or subsets of T cells, e.g., memory vs. effector T cells.
 - Novel vectors used to improve transduction efficiency
 - New cytokine support regimens
 - New preconditioning regimens

^aThe potential for malignancy due to insertion mutagenesis is unknown. Although it has not been seen in the CAR trials to date, the long-term follow-up is small. An analysis by the NIH Office of Biotechnology Activities in 2007 of adverse events in trials using retroviral and lentiviral vectors in terminally differentiated cells identified 12 malignancies, none of which could be directly attributed to the vector. (Supplementary references S16 and S17).

Focus Points for Future Consideration

Reported serious adverse events following CAR T-cell transfer

At least 40 trials using CAR T cells for treatment of cancer have been registered with the NIH Office of Biotechnology Activities (OBA) and undergone NIH Recombinant DNA Advisory Committee (RAC) review, and at least 120 subjects have been dosed across all trials (Supplementary Table S1; ref. 20). Overall, most serious adverse events (SAE) that were viewed as possibly related to transfer of CAR T cells have been mild and self-limited and occurred shortly after infusion (Supplementary Table S2). Unfortunately, 2 patients died shortly after adoptive transfer of CAR T cells (21, 22).

Following RAC review of the death in the HER2-CAR T-cell trial at its December 2009 meeting (available at http://oba.od.nih.gov/rdna_rac/rac_past_meetings_2000.html), the Committee recommended holding a safety symposium to review clinical trials using CAR T cells and then to formulate a report both to facilitate subsequent RAC and Institutional Biosafety Committee reviews of these trials and to assist investigators in designing future studies. A planning committee, consisting of RAC members, leading investigators in this field, and OBA staff, identified key components of these trials on the basis of their potential impact on patient safety and their role in therapy. These issues were discussed in a panel format and are summarized in section 6 below. A paradigm for considering how to incorporate these considerations into clinical trial design is provided in Table 2 and Supplementary Tables S3–S5.

Points to Consider in the Design and Implementation of Clinical Trials Using CAR T Cells

1. Unwanted on-target effects of CAR T cells

Recognition of antigen expressed on nontumor cells by CAR T cells is emerging as the major risk factor of CAR T-cell transfer. Such recognition may become manifest as (i) immediate toxicity and (ii) late or sustained toxicity resulting from long-term depletion of cells with important homeostatic functions.

A. Mitigating the potential for early toxicity. A feared complication after infusion of CAR T cells is their massive activation leading to the research participant's death. This complication is especially of concern with CARs directed against untested and/or endogenously prevalent TAAs, because T cells with high-avidity receptors can respond to cells that express their targets at levels that are currently too low for detection by conventional means. One approach to limit such toxicity is an interpatient (and sometimes intrapatient) dose-escalation scheme. Alternatively, one could conduct the initial dose escalation with first-generation CAR T cells without conditioning or cytokine supplementation, under the expectation that stimulation of these adoptively transferred T cells may be suboptimal, although this is not yet proven. Once the first generation CAR T cells are

shown to be safe, second-, or perhaps third-generation CAR T cells, with a potential for more pronounced responses and prolonged persistence, could be explored in combination with supplementary treatments (23). An important caveat to this statement is that it is currently unclear whether lack of toxicity using first generation CAR T cells will translate into a similar safety profile for second or third generation CAR T cells combined with lymphodepletion and/or cytokine support. As an alternative approach, if second or third generation CARs targeting a new antigen are evaluated without previous experience with a first generation CAR, a very conservative dose-escalation strategy should be adopted (see section 6 below).

The question of whether the coexpression of conditional suicide genes might safeguard against some of the potential side effects of nontumor cell recognition by CAR T cells was discussed (24). As exemplified by the fatal SAE using HER2-CAR T cells, the CAR T cells may act within minutes after engagement of their target antigen, and symptoms are not expected until significant damage has occurred. Therefore, inclusion of a suicide gene, whose benefit will necessarily take time, was deemed to have limited potential for preventing acute toxicity, although it may have utility in modulating late toxicities.

A third approach to reduce risks of immediate toxicity is splitting the T-cell dose over 2 or more days, as is being tested in recipients of CD19-specific T cells (21). Using this scheme, it would be desirable to monitor cytokines or chemokines in the serum as potential indicators for toxicity after the first of the split doses. A fourth approach using a very conservative dose-escalation strategy is discussed in section 6 below. Importantly, in all trials, preclinical studies should carefully test for expression of the TCR's target antigen in healthy tissues. Furthermore, the use of CAR T cells against antigens that are widely expressed on nontumor cells essential for important physiologic functions should, to the extent feasible, not be chosen as targets, unless preclinical data show convincingly that their expression is at low enough levels that on-target toxicity is unlikely to occur.

B. Mitigating the potential for late toxicity. Another risk of CAR T cells is the long-term depletion of cells that are important for normal human function. For example, CAR T cells for treatment of B-cell malignancies have targeted CD19 and CD20, markers expressed on normal B cells (25). Treatment with CAR T cells directed against either of these antigens has the potential to deplete the patients' B cells (26). Furthermore, unlike the α -CD20 monoclonal antibody, rituximab, which has a defined half-life, these CAR T cells may potentially survive and function for the life of the patient. The lack of CD19 or CD20 expression on most plasma cells allows the maintenance of physiologic antibody levels in the majority of patients, following depletion of CD19⁺ or CD20⁺ cells (Supplementary reference S4). In addition, it is expected that B cells would rapidly be replaced from lymphoid progenitors once the CAR T cells die or become functionally impaired. Finally, even under conditions that would allow for long-term persistence of functioning CD19- or CD20-specific CAR T cells,

resulting in continuous depletion of B cells, this could be managed by Ig transfer and would thus be preferable to a fatal cancer.

A related concern is the potential for CAR T cells to negatively impact organ function due to low levels of target antigen expression in a nontumor site, for example, VEGF receptor 2 (VEGFR2)-specific CARs targeting the tumor vasculature (Supplementary references S5 and S6). VEGFR2 is expressed on endothelial cells during physiologic angiogenesis, vasculogenesis, arteriogenesis, and lymphangiogenesis and is needed for processes such as wound healing and embryogenesis (Supplementary reference S7). As such, VEGFR2-specific persisting CAR T cells could interfere with formation of indispensable vasculature. Although there is no universal strategy for mitigating these late CAR T-cell-associated toxicities, the resultant conditions could potentially be managed medically. Alternatively, risks of long-term toxicity could be mitigated by the insertion of suicide genes into the CAR T cells.

2. Effects of costimulatory signaling

Results to date show that, whereas target cell lysis by first generation CAR T cells is independent of costimulatory signaling, their proliferation, production of cytokines, and upregulation of other effector cell molecules depend on and/or are improved by costimulation (27). Furthermore, on the basis of what is understood about natural functions of CD28 and 4-1BB *in vivo*, it is anticipated that CAR T cells bearing these domains might be more resistant to activation-induced cell death (Supplementary reference S8). Therefore, the majority of participants concluded that the incorporation of CD28 and/or 4-1BB into CAR constructs offers an important potential for therapeutic benefit. Evaluations of other cosignaling moieties, which may enhance the proliferation and/or survival of CAR T cells, are appropriate. In addition to modifying the CAR to improve T-cell engraftment, it may also be possible to *a priori* identify T-cell subpopulations with the capacity for sustained proliferation. In fact, there is evidence that the starting population of T cells used for CAR modification may affect their ability to survive and expand (Supplementary reference S9). For example, in nonhuman primates, effector CD8⁺ T cells generated *ex vivo* from central rather than effector memory populations enjoy enhanced survival following adoptive transfer (Supplementary reference S10). Because the ratios of different T-cell subsets vary between patients, selective use of T-cell subsets prior to genetic manipulation may provide a more discriminating path to predict the long-term outcome of CAR T-cell transfer. Finally, clinical experience with the expression of CARs in non-T-cell populations, for example, natural killer cells, is limited (28). Therefore, special care must be taken with CAR use in T-cell subsets and non-T-cell lymphocyte populations.

3. Systemic conditioning

Adoptive transfer of CAR T cells can be further modified by auxiliary therapies, such as partial or complete myeloablation, to improve preservation of the transferred T-cell populations. The rationale behind lymphodepletion prior to T-cell infusion

is multifactorial and includes, but is not limited to, the elimination of "cytokine sinks," creation of space for the expansion of adoptively transferred cells, and removal of suppressor cell populations. In fact, depletion of lymphocytes in melanoma patients infused with *ex vivo* expanded TILs is requisite for treatment efficacy, and recent studies suggest a correlation between the degree of lymphocyte depletion and objective response rates (Supplementary reference S11). Despite the recognized risks of myelosuppression, the majority of the RAC meeting participants felt that administration of CAR T cells that do not carry an endogenous TCR to a persisting virus is unlikely to result in significant clinical benefit without prior conditioning.

4. Cytokine supplements

Recombinant soluble cytokines such as interleukin-2 (IL-2) are administered upon CAR T-cell transfer, with the expectation that they will promote the expansion and survival of transferred cells. If, and to what degree, cytokines may exacerbate the potential toxicity of second- or third-generation CAR T cells is uncertain. It is also unclear if second- and third-generation CAR T cells need to be supplemented with IL-2 either at all or at the high and potentially toxic doses used for TIL transfer (Supplementary reference S12). For the initial evaluation of CAR T cells to novel targets, dosing without addition of cytokines or with injection of low to moderate amounts of cytokines may be prudent and would facilitate differentiation of SAEs induced by CAR T cells alone from those caused by the potential synergy between these drugs. Furthermore, the design of later-phase studies using second- and third-generation CAR T cells should consider incorporating treatment arms with and without cytokine support. Ongoing studies exploring the use of CAR T cells genetically modified to secrete cytokines such as IL-15 or IL-12 may eliminate the need for systemic cytokine administration (29). If, and to what degree, this approach will improve the potential benefit of CAR T cells or instead pose additional risks is currently unknown.

5. Chimeric antigen receptors expressed in T cells targeting persisting viruses

Some groups have combined the advantages of CAR T cells with those of traditional antigen-specific T cells (Supplementary reference S13). Specifically, T cells to persisting viruses such as EBV have been enriched *in vitro* and then genetically modified by insertion of a transgene encoding a TAA-specific CAR. In a neuroblastoma trial, 11 individuals were treated simultaneously with EBV-cytotoxic T lymphocytes (CTL) expressing a GD2-specific first-generation CAR and activated T lymphocytes expressing the same CAR. Only CAR EBV-CTLs showed significant *in vivo* persistence, and 4 of 8 patients with evaluable tumors had evidence of tumor necrosis or regressions, including a sustained complete remission. Lymphodepletion did not improve CAR T-cell persistence or clinical outcome (19). Potential deleterious effects due to virus-specific CAR T cells without a costimulatory endodomain

are anticipated to be similar to those of other T cells expressing second- or third-generation CARs.

6. Starting dose of CAR T cells in phase I trials

Phase I trials are primarily designed to assess safety and feasibility rather than efficacy; biological activity and proof of concept are usually secondary aims. Phase I trials for CAR T cells typically start with reduced cell doses, which are then gradually escalated. The starting dose should be adjusted depending on the type of CAR; T cells with second- or third-generation CARs should start at a lower dose than those with a first-generation CAR. Similarly, transfer of CAR T cells into partially preconditioned patients should commence at a lower dose than transfer into nonmyeloablated patients. Although one would expect that immediate toxicity owing to transfer of CAR T cells may directly correlate with numbers of injected cells, late adverse events may be independent of the injected dose due to construct-dependent dynamic changes in cell numbers.

Providing reliable guidelines for starting doses of CAR T cells is currently not possible. To date, clinical trials have used a fairly wide range of starting doses; some investigators dosed according to weight, others according to body surface area, and still others used flat dosing schedules (Supplementary Table S1). The use of unadjusted dosing should be avoided, and a more uniform dosing scheme, such as that based on cells/kg, should be considered. An open question is whether cells/kg should be based on an ideal body weight or actual weight, given that weight increases due to obesity may not justify an increase in dose (Supplementary reference S14).

7. Ethical considerations

One of the ethical dilemmas facing investigators is the requirement in early-phase research to design studies that are safe, while simultaneously hoping to show biological activity or possibly even benefit to the individual subject, many of whom have few if any other therapeutic or even palliative options. In response to the unexpected death in the trial infusing HER2-specific CAR T cells, initial starting doses in a number of trials administering HER2-specific CAR T cells were lowered to 10^4 cells/m². RAC members were in general agreement that a lower starting dose of 10^4 cells/m² would likely be safe but would be unlikely to have potential benefit to the subject. Therefore, the risk-benefit calculus was such that any benefit realized from the research would most likely be a societal benefit, that is, an increase in generalizable knowledge with minimized risk, but little potential benefit for the patient subject. It will be a challenge to design early-phase studies that appropriately balance accurate disclosure of the risks and benefits posed by this research and appropriate informed consent among a seriously ill patient-subject population with few therapeutic options. Although the expectation of clinical benefit must be discouraged in clinical trials, especially those at the earliest stages of research, selecting a dose that would be relatively safe but have potential biologic activity is an appropriate goal. Whether the acceptable level of risk should be adjusted

in relation to the disease prognosis for a given patient cohort remains a subject worthy of future debate.

Summary

CAR T cells have shown some benefit in cancer patients (19, 26, 30). The major challenge for achieving therapeutic benefit by CAR T-cell transfer remains lack of sustained engraftment and loss of T-cell functions. These hurdles may be overcome, in part, by incorporation of costimulatory domains into CAR constructs or by modifying EBV-specific T cells. Additional studies are needed to assess the performance of EBV-specific CAR T cells in cancer patients of diverse ages. Results to date (excluding those based on EBV-specific T cells) suggest that partial myeloablation is required for survival of CAR T cells. Mechanisms that cause loss of transferred second- and third-generation CAR T cells remain poorly understood and may relate to the differentiation status of the transduced T-cell subsets. It is unclear if, and at what doses, cytokines are needed to improve the clinical outcome of second- and third-generation CAR T cells. Until this issue is clarified, the upfront use of high doses of systemic cytokines should be carefully justified.

The major immediate risk factor for CAR T cells remains their activity against nontumor cells, rather than genotoxic-

ity from the gene transfer event. Long-term risk factors, such as sustained depletion of normal cell populations or exacerbated expansion of CAR T cells, cannot yet be assessed. Although the risk of insertional mutagenesis remains a concern, this has neither been observed clinically nor in recent preclinical studies that have attempted to recreate insertional mutagenesis (Supplementary reference S15). However, as the field moves toward the use of less differentiated CAR T cells, the risk of insertional mutagenesis may increase.

Disclosure of Potential Conflicts of Interest

Dr. Strome is the cofounder and a major stockholder in Gliknik, Inc., a biotechnology company. He also receives royalties through the Mayo Clinic College of Medicine for the licensure of IP relating to 4-1BB (CD137) and B7-H1 (PD-L1).

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