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Review

Fifteen Years of Gene Therapy Based on Chimeric Antigen Receptors: "Are We Nearly There Yet?"

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Abstract

"T-body" or chimeric antigen receptor (CAR) technology, which combines the specificity of an antibody with the homing, tissue penetration, and target cell destruction of T cells, was first described in 1993. After many years of unmet promise, significant improvements in gene transfer, including the development of efficient retroviral vectors for transduction of human T cells, and better understanding of immunological pathways and immune cell interactions, are allowing this technology to reach a critical phase of evaluation, in which we will learn whether the approach can truly meet expectations. In this review we summarize the concept of CAR-based immunotherapy, describe the steps accomplished, and outline the future progress we need to make if this approach is truly to improve cancer immunotherapy.

Introduction

Adoptive transfer of tumor-infiltrating T lymphocytes (TILs) or antigen-specific cytotoxic T lymphocytes (CTLs) induces objective clinical responses in patients with melanoma (Rosenberg *et al.*, 2008) and Epstein-Barr virus (EBV)-related malignancies (Rooney *et al.*, 1998; Bollard *et al.*, 2004; Comoli *et al.*, 2005; Straathof *et al.*, 2005a). Gene transfer of peptide-specific native $\alpha\beta$T cell receptor (TCR) and genetic transfer of chimeric antigen receptors (CARs) are two means by which we can redirect the specificity of polyclonal T lymphocytes and thus more broadly extend these beneficial effects of T cell therapies to other malignancies. Both strategies are appealing because they provide a means of generating large numbers of antigen-specific CTLs directed to otherwise weakly immunogenic tumor-associated antigens. Although compelling results using gene transfer of native $\alpha\beta$TCRs have been obtained in patients with melanoma (Morgan *et al.*, 2006), and the approach will likely be extendable to other malignancies, in this review we focus on the use of CARs for T cell-based cancer therapeutics.

The term "T-body" was coined in 1993 to define chimeric molecules composed of a specific antigen-binding domain encoding the variable regions of a monoclonal antibody, linked together as a single chain antibody (scFv), and a signaling moiety derived from either the ζ chain of the TCR/CD3 complex or the γ chain of the FcεRI receptor (Eshhar *et al.*, 1993). When expressed by T cells, the chimeric molecules bind the specific antigen expressed on the cell surface of the target cells through their antibody-binding moiety, and activate the lytic pathway of the T cells on cross-linking of the chimeric ζ or γ chains that form the receptor endodomains (Fig. 1). Many researchers now term these T-bodies "chimeric antigen receptors (CARs)" or "chimeric immune receptors (CIRs)," because these hybrid receptors can also include ligands that are not antibody derived (Stastny *et al.*, 2007). Many different CARs have now been cloned, which target antigens expressed either by hematologic malignancies (Hombach *et al.*, 1999; Cooper *et al.*, 2003; Jensen *et al.*, 2003; Vera *et al.*, 2006) or solid tumors (Kershaw *et al.*, 2006; Lamers *et al.*, 2006; Ahmed *et al.*, 2007), and a comprehensive list of these is provided by Sadelain and colleagues (2009). Many of the target antigens for these CARs have been validated in preclinical and clinical studies of the native monoclonal antibody or other ligand used, which facilitates early adoption of CARs in human subjects.

Advantages of CAR-Based T Cell Immunotherapy

The advantages of CARs over the native antibodies or ligands from which they derive are a consequence of their physical association with effector T cells. Thus, CAR-modified T cells can have an active biodistribution, with migration through multiple tissue planes along chemokine

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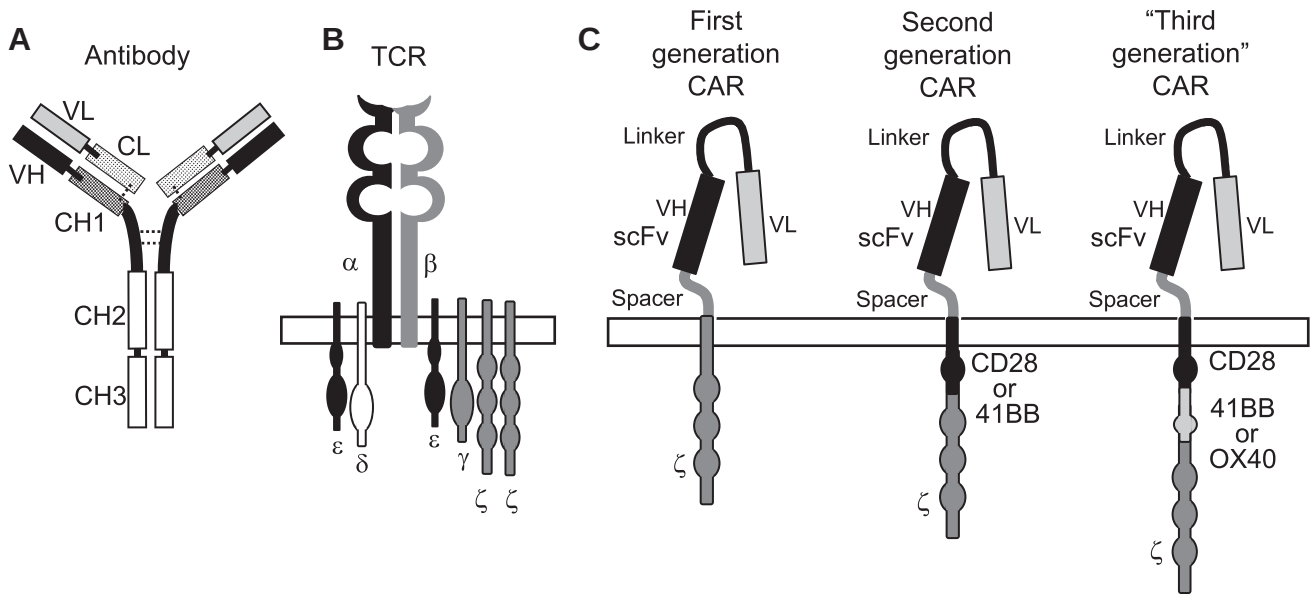

FIG. 1. Chimeric antigen receptors (CARs). CARs are most commonly created by joining the heavy- and light-chain variable regions of a monoclonal antibody (**A**) that binds to a specific antigen to the intracellular portion of a T cell signaling molecule, such as components of the TCR-associated CD3 complex (ζ chain) (**B**). First-generation CARs only contain a T cell signaling domain that transmits the activation signal (**C**, *left*). Second-generation CARs incorporate in addition a single costimulatory molecule endodomain, such as the endodomain of CD28 or 41BB (**C**, *middle*). Third-generation CARs incorporate at least two costimulatory molecule endodomains, such as the endodomains of CD28 and 41BB (**C**, *right*).

gradients, and can recruit the multiple cytotoxic effector mechanisms available to a T cell, rather than the more restricted cytotoxic machinery associated with, for example, the Fc component of an antibody. CARs also offer advantages over transfer of native αβTCRs. Target cell recognition by αβTCRs is MHC restricted, precluding the design of a "universal" receptor for the treatment of patients with different HLA haplotypes. CARs, by contrast, like monoclonal antibodies, are essentially universal. Moreover, many tumors downmodulate MHC molecules and/or have dysfunctional antigen-processing machinery so that the target antigenic epitopes for αβTCR are simply not present. Because CAR-modified T cells bind directly to native proteins expressed on the surface of target cells without the need of antigen processing or MHC-restricted presentation, they are unaffected by this immune evasion strategy. Moreover, CARs can recognize nonprotein antigens, unlike conventional αβTCRs (Rossig *et al.*, 2001). The major limitation of CARs versus αβTCRs is that they are generally unable to recognize antigens that are internal, even when these are processed to peptides presented by HLA molecules.

Expressing CARs in T Lymphocytes: Do We Need New Tools?

The major goal of adoptive T cell therapy in patients with cancer is to establish effective antitumor activity that can recognize and destroy malignant cells irrespective of their site in the body. Moreover, this activity should be persistent, so that there will be destruction of resurgent malignant cells, even if these arise from phenotypically distinct tumor progenitor cells that are not themselves effectively targeted. For these benefits to be produced, CAR-modified T cells need to have adequate trafficking to the tumor site, be resistant to tumor-related immunosuppressive factors, and have robust and stable expression of their transgenic CAR.

Several methods are currently used to express chimeric molecules in T lymphocytes, each with a different profile of efficiency, cost, and complexity. Because T lymphocytes are highly proliferative, most studies of CAR gene transfer have used vectors that integrate into the host cell DNA. Such vectors were typically based on gammaretroviruses or lentiviruses, but more recent advances in the development of integrating nonviral vectors are beginning to challenge this monopoly.

Viral vectors

Gammaretrovirus-mediated gene transfer to T cells has been tested in several clinical trials (Rosenberg *et al.*, 1990; Bordignon *et al.*, 1995; Heslop *et al.*, 1996; Bonini *et al.*, 1997). Such retroviruses integrate into the host genome (Miller *et al.*, 1990; Hu and Pathak, 2000) and produce consistent gene expression in human T cells and their progeny. These vectors, however, have several limitations (Hu and Pathak, 2000). They can transduce only dividing cells (Miller *et al.*, 1990), have limited cargo capacity (Hu and Pathak, 2000), can cause insertional mutagenesis (Hacein-Bey-Abina *et al.*, 2008), and are expensive to produce and use clinically. Moreover, transgene expression tends to decline over time, although it can be increased after T cell activation through their CAR or native αβTCR (Pule *et al.*, 2008). These limitations notwithstanding, retroviral gene transfer has shown an acceptable profile of efficacy and safety for expressing CAR and other transgenes in T lymphocytes (Bonini *et al.*, 2003; Brenner and Heslop, 2003). Optimization of the vector cassette and advances in bicistronic and tricistronic vector design allow three or potentially more distinct cDNAs to be

expressed by retrovirally transduced T cells (Quintarelli *et al.*, 2007; Di Stasi *et al.*, 2009). Most importantly perhaps, no adverse effects related to insertional mutagenesis of T lymphocytes by integrated provirus have been reported in any patient infused with retroviral gene-modified T cells during the past 20 years (Bonini *et al.*, 2003; Brenner and Heslop, 2003). This observation markedly contrasts with experience in patients receiving hematopoietic stem cells (HSCs) that have been similarly modified with gammaretroviruses (Hacein-Bey-Abina *et al.*, 2003, 2008). Such clinical observations parallel experimental data showing that retrovirally transduced T cells do not undergo malignant transformation in RAG1-deficient mice, unlike gene-modified HSCs (Montini *et al.*, 2006). Overall, it seems likely that highly differentiated cells, such as mature T lymphocytes, are less likely to undergo malignant change associated with the genotoxicity of integrating gammaretroviruses.

Lentiviral vectors

Lentiviral vectors may offer certain advantages over gammaretroviral vectors. They can transduce nondividing or minimally proliferating T lymphocytes (Naldini *et al.*, 1996; Hu and Pathak, 2000), and the reduced requirement for *ex vivo* activation before transduction may maximize long-term *in vivo* persistence of the transduced cells, by reducing activation-induced cell death or clonal exhaustion (Cavalieri *et al.*, 2003). Compared with gammaretroviruses, lentiviruses also have enhanced cargo capacity, and reduced susceptibility to gene silencing. Although genotoxicity due to insertional mutagenesis may still occur, the frequency is apparently lower as there is a reduced probability of integration into transcriptionally sensitive sites (Montini *et al.*, 2006). Modifications to lentiviruses to make them self-inactivating after integration may further lower this risk.

Nonviral vector gene transfer

Although DNA plasmid-based gene delivery has greatly reduced the cost of manufacturing and testing, the use of this approach for CAR gene transfer has been limited by the inefficiency of the process and the transience of expression due to lack of transgene integration—a lethal flaw for transduction of a rapidly dividing population such as T cells. Improvements in electroporation of T lymphocytes have significantly enhanced the efficiency of gene delivery, and reduced the toxicity of the procedure. Without a high rate of plasmid integration, however, investigators are compelled to extensively culture the transduced cells to expand the rare clones in which stable expression is achieved. Unfortunately, this often exhausts the T cells and renders them unfit to expand further and persist *in vivo* (Park *et al.*, 2007; Till *et al.*, 2008). Transposon-based gene delivery systems may overcome this limitation, by combining transposons and transposases with the gene of interest in the plasmid. Systems such as Sleeping Beauty (Huang *et al.*, 2008; Singh *et al.*, 2008) and PiggyBac (Wilson *et al.*, 2007) are currently being considered to express CARs in T lymphocytes. It remains to be seen whether genomic integration mediated by transposon-based gene delivery is safe and efficient *in vivo* or whether insertional mutagenesis and transgene silencing will occur.

Other technologies have also been explored for gene transfer to T lymphocytes. In principle, for example, messenger RNA (Mitchell *et al.*, 2008) or protein transfer can be used to force CAR expression in T lymphocytes. These approaches may be safe, but the transience of their effects means they are likely of limited clinical value for CAR transfer.

Initial Clinical Trials of CAR-Modified T Cells

Despite consistent and robust expression of CAR molecules in T cells, early clinical studies of the approach were disappointing. The first trials were in patients infected with HIV, who received T lymphocytes expressing a CAR (CD4ζ) binding to the HIV gp120 envelope expressed on the surface of infected cells. Infusions of syngeneic or autologous CD4ζ-modified $CD4^+$ and $CD8^+$ T cells were well tolerated but produced minimal antiviral activity. Although gene-modified T cells were detectable in the bloodstream for up to 1 year, and trafficked to the gut-associated HIV reservoir, the percentage of gene-modified cells was low and substantially declined with time (Mitsuyasu *et al.*, 2000; Walker *et al.*, 2000).

Initial studies in patients with cancer were equally discouraging. T cells with a CAR targeting the α-folate receptor (FR) were infused into patients with metastatic ovarian cancer. Fourteen patients received 3×10^9 to 1.7×10^{12} cells without toxicity. However, antitumor responses were not measurable and transgenic T cells were barely detectable in the circulation beyond 3 weeks after infusion, with only one exception of persistence 1 year after treatment, albeit at low levels (Kershaw *et al.*, 2006). A second study was conducted in patients with neuroblastoma, using T cells electroporated with a plasmid encoding a CAR targeting the L1-cell adhesion molecule (L1-CAM) expressed by neuroblasts. A total of 6 patients with refractory neuroblastoma received up to 3 infusions of gene-modified T cells (10^8 and/or 10^9 cells). The toxicity was acceptable, although bone pain requiring narcotics was reported shortly after the infusion of T cells in a patient with extensive osseous metastases. Tumor responses were modest and incomplete, and again persistence of the infused cells was limited (Park *et al.*, 2007). Finally, subjects with relapsed or refractory B cell lymphomas received T cells transfected by electroporation with a DNA plasmid encoding a CAR targeting the CD20 antigen. Seven patients were treated with multiple infusions and some also received subcutaneous injections of recombinant interleukin (IL)-2. No adverse events attributable to the T cell infusions were observed. T cells were documented to traffic to the bone marrow, although the number of circulating $CD20^+$ B cells remained stable or slightly increased after treatment in all patients, with marginal antitumor effects. The administration of low-dose IL-2 prolonged the persistence of modified T cells but not beyond 9 weeks (Till *et al.*, 2008).

CAR-Modified T Cells and Lack of Costimulation

Although the results of these studies were clinically disappointing, they proved instrumental in showing that a major problem of CAR-modified T cells is their lack of expansion and persistence *in vivo*, which is in sharp contrast with the behavior of adoptively transferred antigen-specific CTLs (Heslop *et al.*, 1996). A number of factors likely contribute to these differences. Elimination of the cells by an immune response directed to the CAR can certainly occur

(Lamers *et al.*, 2006), and it is also true that the culture conditions used to generate T cells were suboptimal. Thus activation and expansion of naive T cells before gene transfer require optimal costimulation provided by CD28 and tumor necrosis factor (TNF) family members (Paulos *et al.*, 2008; Boesteanu and Katsikis, 2009). Because the clinical-grade reagents for this costimulation are not readily available, investigators have cross-linked CD3 with monoclonal antibodies, with suboptimal consequences for T cell survival. More recently, activation with beads coexpressing OKT3 and CD28 has been used and may produce a more active and persistent product (Paulos *et al.*, 2008). The substitution of cytokine cocktails such as IL-7, IL-15, and IL-21 for IL-2 alone also serves to better preserve the subset of T cells with a central-memory phenotype (T_{CM}), and thereby favor long-term persistence (Ma *et al.*, 2006; Andorsky and Timmerman, 2008).

While the previously described mechanisms may help to explain the poor *in vivo* persistence and activity of CAR-modified T cells, it is increasingly apparent that a major factor is the inability of CAR engagement alone to recapitulate the costimulatory events that follow the physiologic engagement of the native $\alpha\beta$TCR. Full activation and proliferation of T cells require not only TCR engagement (first signal) but also costimulation provided by antigen-presenting cells (APCs, second signal) and cytokines (third signal). A multiplicity of these costimulatory receptor–ligand and cytokine signals is required, in an optimal temporal and spatial sequence. CAR-redirected T cells lack any such costimulation when they engage tumor cells, because these target cells are deficient in costimulatory molecule expression (e.g., CD80 and CD86) and do not release helper cytokines. Moreover, CAR-modified T cells cannot receive activation through stimulation provided by professional APCs in secondary lymphoid organs because the native receptors on CAR-modified T cells are not specifically directed toward antigens on the hosts' APCs.

Two approaches have been adopted to compensate for the lack of costimulation after CAR engagement

Costimulatory endodomains

The first approach is to incorporate costimulatory signaling domains as part of the CAR itself. Thus, domains derived from CD28 (Finney *et al.*, 1998; Maher *et al.*, 2002; Kowolik *et al.*, 2006; Vera *et al.*, 2006), 4-1BB (Imai *et al.*, 2004), or OX40 (Pule *et al.*, 2005) molecules have been incorporated as single or multiple (Pule *et al.*, 2005; Milone *et al.*, 2009) endodomains in tandem in the CAR molecule to generate "second- and third-generation" CARs (Fig. 1). Antigen engagement of these CARs is followed by T cell activation, proliferation, and IL-2 secretion, even without cross-presentation by APCs (Maher *et al.*, 2002; Vera *et al.*, 2006). Experiments in severe combined immunodeficient (SCID) mice are being conducted to define the best combinations of costimulation required for CAR-modified T cells. So far the data seem to suggest that the optimal combination may depend on the affinity of the single-chain antibody, the level of CAR expression and its intrinsic structure (hinge region, spacer region, and transmembrane domain), as well as on the density of antigen expression by tumor cells and the tumor environment. It is difficult for a murine SCID system to accurately model all these variables, particularly because CAR-modified T cells also interact with other immune system cells in an immunocompetent individual. Because of these limitations, it is essential to validate any conclusions from animal models in small early-phase clinical trials. Such studies using polyclonal activated T lymphocytes expressing these novel "second-generation" CARs are currently open at several institutions. It will likely remain difficult to predict which costimulatory combination will be superior in any given setting, and a side-by-side comparison of CARs containing different costimulatory endodomains infused simultaneously in the same patient may be the most efficient way of optimizing the approach.

Antigen-specific CTLs

The second approach for providing costimulation is to express the CAR in antigen-specific CTLs, whose native $\alpha\beta$TCR is targeting for an antigen known to be present on host professional APCs. In this way, native receptor engagement ensures all costimulation is received physiologically, and chimeric receptor engagement serves exclusively as a means of retargeting an already costimulated cell. This approach has been validated preclinically *in vitro* (Rossig *et al.*, 2002; Landmeier *et al.*, 2007) and *in vivo* (Duraiswamy *et al.*, 2003; Savoldo *et al.*, 2007), and we extended it to EBV antigen-specific CTLs redirected to the G_{D2} antigen present on many human tumors including neuroblastoma (Pule *et al.*, 2008) (Fig. 2). Adoptive transfer of EBV-specific CTLs effectively treats EBV-driven lymphomas after allogeneic hematopoietic stem cell (HSC) transplantation (Rooney *et al.*, 1995, 1998). Studies using a "gene-marking" approach demonstrated the persistence *in vivo* of EBV-specific CTLs for more than 10 years after their adoptive transfer (Heslop *et al.*, 1996). These clinical observations indicate that the infused CTLs, although phenotypically predominantly effector T cells (T_E) and effector-memory T cells (T_{EM}) (Pule *et al.*, 2008), contain a fraction of cells that have functionally long-term memory properties and persist when receiving an efficient costimulation from latently EBV-infected APCs (Heslop *et al.*, 1996). We therefore engrafted EBV-specific CTLs and primary T cells with distinguishable G_{D2}-specific CARs and infused both sets of autologous cells into patients with neuroblastoma. We found that CAR-modified EBV-specific CTLs had enhanced and prolonged survival compared with polyclonal activated T cells expressing the same CAR, and produced objective tumor responses including complete remission (Pule *et al.*, 2008). We are now using this approach for patients with lymphoma, comparing the expansion and persistence of EBV-specific CTLs expressing a "first-generation" CAR targeting the CD19 molecule with that of polyclonal T lymphocytes expressing a "second-generation" CAR targeting the same CD19 molecule but also encoding the CD28 endodomain. This study will allow us to formally evaluate whether physiologic or synthetic costimulation is superior for CAR-modified human T cells *in vivo*. Finally, in addition to appropriate *in vivo* costimulation, expressing CARs on antigen-specific CTLs of defined specificity may allow the use of vaccination to boost CAR cellular immune responses.

Does CAR Expression in Specific T Cell Subsets Matter?

In the previous section we discussed the potential advantages of expressing a CAR in a defined CTL population.

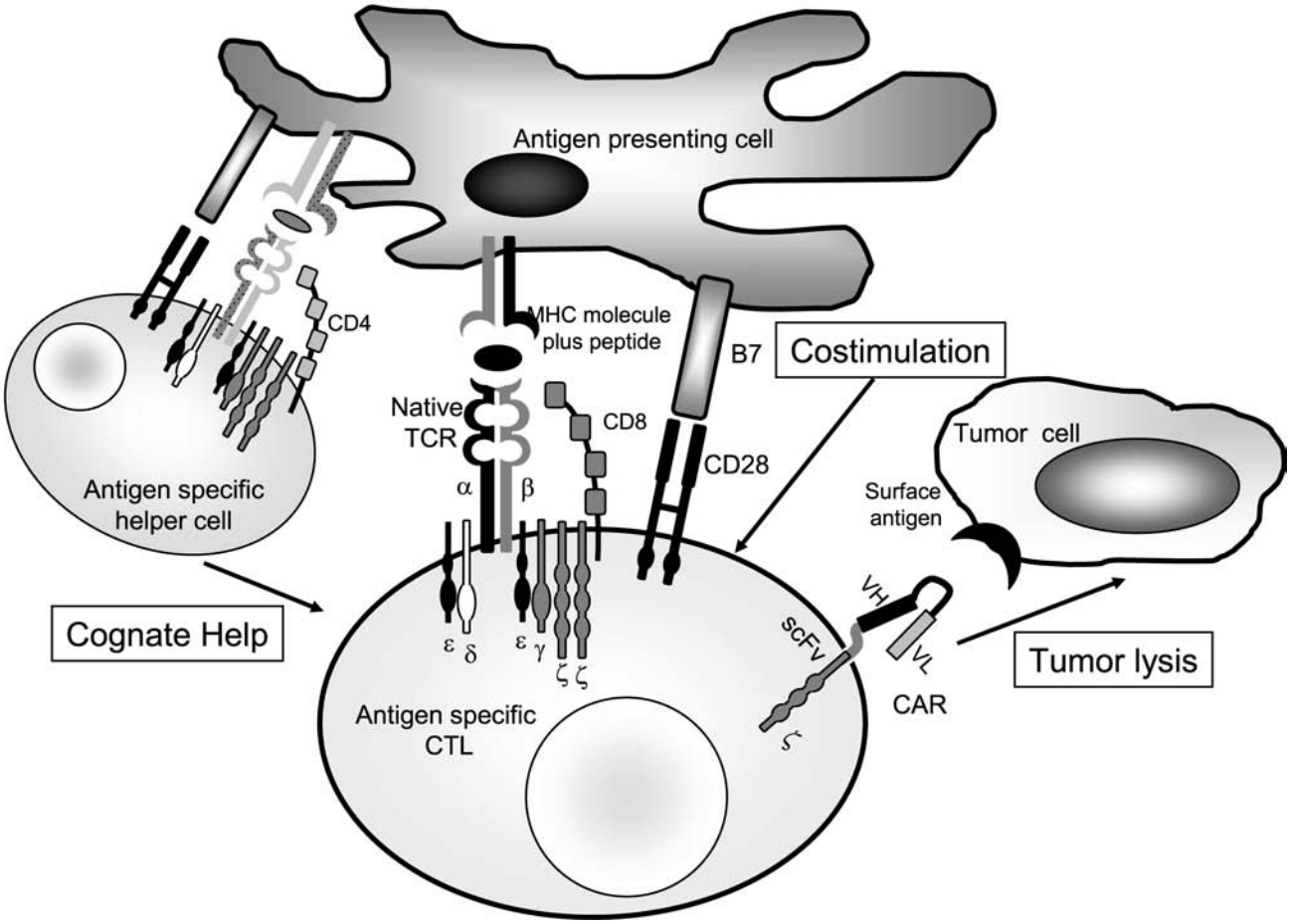

FIG. 2. CARs and costimulation. Several studies have demonstrated that first-generation CARs that transmit just a CD3-ζ signal fail to induce sustained T cell activation or division likely because CAR activation is not accompanied by the required sequence of costimulatory stimuli. To circumvent this limitation, CARs can be expressed in antigen-specific T cells, which will receive costimulation when their native receptor is engaged by the appropriate antigen on a professional antigen-presenting cell. In addition, cognate help can be provided by antigen-specific helper cells.

There are, of course many other subsets of T cells in which CAR can be expressed, and some of these may have superior overall functionality to others. In addition to polyclonal activated T lymphocytes, other T cell subsets including γδT cells and natural killer (NK) cells have been used as platforms for CAR modification.

γδT lymphocytes

γδT lymphocytes represent less then 5% of human T cells, and are preferentially localized in organs containing epithelial cells (skin, lung, intestine, and genitourinary tract) (Nanno *et al.*, 2007). They have MHC-unrestricted cytotoxic activity. *Ex vivo*-expanded human $V\gamma9V\delta2^{+}$ T cells have antitumor activity against several human malignancies and, when engineered to express CAR molecules, they acquire specific cytotoxic activity against other tumor cells (Rischer *et al.*, 2004). Despite the scarcity of γδT cells in the peripheral blood, these cells can be expanded *ex vivo* and *in vivo* using a combination of IL-2 and zoledronate, a pharmacologic agent that promotes the accumulation of metabolites, such as isopentenyl pyrophosphate, which act as endogenous ligands to stimulate $V\gamma9V\delta2^{+}$ T cells (Dieli *et al.*, 2007). Clinical trials using CAR-modified γδT cells are planned, although we know little about the likely persistence of these cells *in vivo* after adoptive transfer. However, their high tropism for epithelia may be useful for treating several types of carcinomas.

Natural killer cells

NK cells represent less than 15% of lymphocytes and are characterized by the expression of CD56 (bright or dim) and of Fcγ receptor III (FcγRIII, CD16) and by their lack of CD3 (Cooper *et al.*, 2001). Although the mechanisms by which NK cells recognize target cells are complex and incompletely understood, NK receptors for MHC class I molecules are crucial for discriminating normal from pathogenic cells (Cooper *et al.*, 2001). NK cells have long been considered as important for tumor immune surveillance (Cooper *et al.*, 2001) and after HLA-haploidentical HSC transplantation for patients with myeloid malignancies recognition of killer-immunoglobulin receptors (KIRs) and MHC disparities may contribute greatly to the graft-versus-leukemia effect (Ruggeri *et al.*, 2008). NK cells have been used as an alternative lymphocyte platform for the expression of CARs, and in

preclinical studies they have potent antitumor activity (Imai *et al.*, 2005). As for $\gamma\delta$T cells, little is known of the survival and persistence of NK cells after adoptive transfer.

Central memory cells

Studies in nonhuman primates have shown that virus-specific CTLs reexpanded from the $CD62L^+$ fraction have the highest capacity to persist *in vivo* after adoptive transfer (Berger *et al.*, 2008). Preclinical evaluations of CAR-modified T cells obtained from the $CD62L^+$ subpopulation will determined whether they are superior to unfractionated T lymphocytes in establishing a long-term memory pool.

Sustaining *in Vivo* Expansion of Adoptively Transferred CAR-Modified T Cells

Several studies of adoptive T cell therapy suggest that robust and sustained clinical responses are best obtained by ensuring *in vivo* expansion of adoptively transferred antigen-specific CTLs (Dudley and Rosenberg, 2003; Robbins *et al.*, 2004). Although costimulation is undoubtedly one component of successful longevity, the contribution of exogenous cytokines is likely also to be considerable. These cytokines, including IL-2, IL-7 and IL-15, promote both T cell expansion and survival *in vivo* (Ma *et al.*, 2006).

At the simplest level, systemic administration of recombinant IL-2 has been used to support adoptive T cell therapies, including gene-modified T cells by CAR or transgenic $\alpha\beta$TCR expression (Yee *et al.*, 2002; Dudley and Rosenberg, 2003; Rosenberg *et al.*, 2008; Till *et al.*, 2008). Unfortunately, prolonged administration is associated with significant toxicity (Dudley *et al.*, 2005; Rosenberg *et al.*, 1994, 2008) and the concomitant expansion of IL-2 receptor-positive regulatory T cells (Treg) may progressively impair the function of effector T cells (Ahmadzadeh and Rosenberg, 2006). One means of capturing the benefits of the other cytokines important in T lymphocyte expansion and survival is to produce lymphodepletion before T cell infusion (Gattinoni *et al.*, 2005). This manipulation induces release of homeostatic cytokines such as IL-7 and IL-15, promoting expansion of adoptively transferred lymphocytes including CAR-modified T cells (Dudley *et al.*, 2005). However, the toxicities of agents required to produce such profound lymphodepletion may be unacceptable in patients who have already endured multiple rounds of intensive chemotherapy, and may promote adverse effects mediated by abrupt cytokine release by infused T cells (Brentjens *et al.*, 2009).

Administration of the relevant cytokines directly may obviate the requirement for lymphodepletion. Studies in nonhuman primates have shown the safety and clinical benefits of systemic administration of IL-15, and this cytokine will soon be tested in clinical trials (Berger *et al.*, 2009). Transgenic production of IL-15 by CAR-modified T cells may also be an option (Quintarelli *et al.*, 2007), with the advantage of providing the cytokine only where needed. Similarly, IL-7 is essential for homeostatic expansion of naive T cells (Schluns *et al.*, 2000; Ma *et al.*, 2006) and for maintaining the memory T cell population (Kaech *et al.*, 2003; Nanjappa *et al.*, 2008). Administration of IL-7 accelerates immune reconstitution in murine models (Alpdogan *et al.*, 2003) and the cytokine has been well tolerated in early-phase clinical trials, in which it produced polyclonal expansion of naive $CD4^+$ and $CD8^+$ T lymphocytes and no evident Treg expansion (Rosenberg *et al.*, 2006; Sportes *et al.*, 2008). IL-7 administration could enhance the expansion of adoptively transferred T cells if they physiologically reexpressed the IL-7 receptor α chain (Powell *et al.*, 2005) or if such expression were forced by genetic manipulation (Vera *et al.*, 2009).

Rendering CAR-Modified T Cells Resistant to Tumor Immune Evasion

Although CAR-modified T cells need to be present in sufficient numbers and for long enough to eradicate resurgent tumor cells, achieving these end points will likely not, per se, be sufficient to eradicate tumors. CAR-modified T cells must also efficiently traffic to the tumor sites and, once there, sustain their effectiveness in the presence of an array of immune evasion strategies used by the tumor cells (Zou, 2005).

Many tumor cells or their associated stroma produce cytokines that attract inhibitory rather than effector T cells. Further genetic modification of T lymphocytes to express the relevant chemokine receptor can overcome this difficulty, and ensure that the CAR-modified T cell can efficiently arrive at the tumor. The feasibility of this approach has already been shown for melanomas, which produce Groα and for Hodgkin's lymphoma (HL), which produces TARC (thymus and activation regulated chemokine) (Poppema *et al.*, 1998). T cells engineered to express CXCR2 will preferentially traffic to melanomas (Kershaw *et al.*, 2002), whereas T cells expressing CCR4 will traffic to Hodgkin's lymphoma (Di Stasi *et al.*, 2009). Thus, coexpression of a CAR targeting the CD30 antigen on HL and of CCR4 enhanced antitumor activity *in vivo* in an HL xenograft model (Di Stasi *et al.*, 2009) (Table 1).

Once at the tumor site, countermeasures to more active immune evasion strategies can be implemented. Both the hypoxic environment, which accompanies large tumors, and the antiinflammatory cytokines they release normally impede T cell function. Inhibitory molecules such as transforming growth factor (TGF)-β, Fas ligand, IL-10, and indoleamine 2,3-dioxygenase (IDO) also attract cells with inhibitory function, such as Th2 cells, suppressor dendritic cells, or regulatory T cells (Zou, 2005). Genetic modification of the CAR itself (Loskog *et al.*, 2006), or of CAR-redirected T cells can overcome all of these (Bollard *et al.*, 2002; Dotti *et al.*, 2005) (Table 1).

"On" and "Off" Target Toxicities

One potential concern about the clinical effects of CAR is toxicity related to the normal tissue distribution of the targeted antigen. Ideally, the targeted antigen should be chosen on the basis of its selective expression by malignant cells, but for many tumor antigens the target is expressed only at differential levels on the malignant cell. For example, carbonic anhydrase IX (CAIX) is frequently overexpressed by clear cell renal carcinoma (RCC) but is also expressed by bile duct epithelial cells. Cholangitis with periductal T cell infiltration was observed when patients with metastatic renal cell carcinoma were treated with autologous T lymphocytes expressing a CAR targeting CAIX (Lamers *et al.*, 2006). One way to obviate this type of problem may involve selection of single-chain antibodies that have low target affinity, so that

Table 1. Methods to Overcome Tumor Immune Evasion

Immune evasion mechanism	*Example*	*Overcoming strategy*
Release of inhibitory molecules	TGF-β	Dominant negative TGF-β receptor (Bollard *et al.*, 2002)
	FasL	Downregulation of the receptor (Dotti *et al.*, 2005)
Impaired tumor trafficking	TARC/Groα	Forced expression of the specific chemokine receptor (CCR4 [Di Stasi *et al.*, 2009], CXCR2 [Kershaw *et al.*, 2002])
Tumor infiltration by inhibitory cells	Th2 and Treg cells	Second-generation CAR (Loskog *et al.*, 2006)
Limited *in vivo* T cell expansion	Lack of local cytokines	Infusion of exogenous cytokines (IL-2 [Rosenberg *et al.*, 1994], IL-7 [Rosenberg *et al.*, 2006; Sportes *et al.*, 2008], IL-15 [Berger *et al.*, 2009])
		Transgenic production of cytokines (IL-2 and IL-15) (Quintarelli *et al.*, 2007; Liu and Rosenberg, 2001)
		Lymphodepletion (Dudley *et al.*, 2008)
		Forced expression of IL-7 receptors (Vera *et al.*, 2009)
Limited *in vivo* T cell persistence	Lack of local costimulation	Second- or third-generation CARs (Sadelain *et al.*, 2009)
		Expression of CAR on antigen-specific CTLs (Pule *et al.*, 2008; Savoldo *et al.*, 2007)

Abbreviations: CAR, chimeric antigen receptor; CTLs, cytotoxic T lymphocytes; FasL, Fas ligand; IL, interleukin; TARC, thymus and activation regulated chemokine; TGF, transforming growth factor; Th2, helper T cell type 2; Treg, regulatory T cells.

(normal) low-expressing cells are spared. Alternatively, an inducible system may be used to favor the expression of the transgene at the specific tumor site (Kim *et al.*, 2008). For other tumors, the CAR is deliberately directed toward a lineage-restricted antigen present on both normal and malignant cells. For example, CARs targeting B lymphocyte self-antigens, such as CD19 and CD20, will inevitably target both leukemic cells derived from the B cell compartment and normal lymphocytes, with potential effects on humoral immunity. In this example, infusion of human gamma globulins may compensate for the deficit, which would be deemed acceptable if the approach treats an immediately life-threatening condition. As we increase the potency of CAR-modified T cells and enhance their ability to survive, function, and expand *in vivo*, it is likely that the adverse consequences of these "on and off" target effects will become more evident. For this reason, there is increasing interest in the possibility of including a suicide gene in the CAR-modified T cells so that progressive or unwanted "on and off" target effects could be more readily controlled (Bonini *et al.*, 1997; Straathof *et al.*, 2005b; Quintarelli *et al.*, 2007).

Conclusion and Future Perspectives

Although a long stretch of CAR-modified T cell development lies ahead, the first glimmers of effectiveness are with us, and the signposts to future success are clearly illuminated. Although we are unfortunately not "nearly there" yet, the second half of the journey should be more clinically productive than the first. Imminent clinical trials will use T cells that have been more extensively engineered, to contain countermeasures to tumor immune evasion strategies such as production of inhibitory cytokines, and to incorporate more effective killing mechanisms such as immunotoxins. A broader range of solid tumors will also be targeted. But beyond these scientific developments, there will also be concomitant technical improvements in cell preparation and testing that will simplify and accelerate administration of these cells to patients with disease, and that will facilitate application outside the current limited number of highly specialized centers. These technological and distributive advances will themselves be driven by demonstrations of the incontrovertible effectiveness of CAR-modified T cells and by confirmation of their superior pharmaco-economics due to fewer adverse effects than conventional agents.

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Author Disclosure Statement

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