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Design and Development of Therapies using Chimeric Antigen Receptor-Expressing T cells

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Summary

Investigators developed chimeric antigen receptors (CARs) for expression on T cells more than 25 years ago. When the CAR is derived from an antibody, the resultant cell should combine the desirable targeting features of an antibody (e.g. lack of requirement for major histocompatibility complex recognition, ability to recognize non-protein antigens) with the persistence, trafficking and effector functions of a T-cell. This article describes how the past two decades have seen a crescendo of research which has now begun to translate these potential benefits into effective treatments for patients with cancer. We describe the basic design of CARs, describe how antigenic targets are selected, and the initial clinical experience with CART cells. Our review then describes our own and other investigators' work aimed at improving the function of CARs and reviews the clinical studies in hematological and solid malignancies that are beginning to exploit these approaches. Finally, we show the value of adding additional engineering features to CAR-T cells, irrespective of their target, to render them better suited to function in the tumor environment, and discuss how the safety of these heavily modified cells may be maintained.

Keywords

cancer; immunotherapy; T cells; CAR; gene therapy

Introduction

Chimeric antigen receptor-expressing T (CAR-T) cells are examples of adoptive cellular immunotherapies (ACIs) which are themselves a subset of complex biological therapies (CBTs) (1, 2). While such therapies have been available for more than 20 years, it has proved difficult to develop them to a stage at which they can be predictably successful and widely implemented as a standard of care. In this review, we outline some specific approaches to overcome these barriers for CAR-T cells, but it is important also to understand the more general barriers that ACIs face in becoming approved therapies in clinical practice.

Many ACIs have to be individually made for each patient, a challenge to the robust scalability required for late phase clinical studies. Moreover, the standard pharmaceutical business model is to recoup the costs of initial drug development by selling cheap-to-manufacture licensed drugs that ameliorate rather than cure and that are administered over a prolonged period of time with exceedingly high profit margins. Many ACIs will remain expensive to produce even after approval, an effect compounded by the stacked license fees for the many patents covering the multiplicity of intellectual property incorporated in a

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single product. Unlike many conventional drugs, ACIs, including CAR-T cells, are intended to be curative not ameliorative, so that they will need to be given once, or a few times, only. Finally, the very specificity of these ACIs means that only a small subset of patients with any given cancer may be suited to treatment, making every ACI an orphan drug. In combination, these market issues can lead to an unaffordable pricing structure with little appeal to pharmaceutical companies. In this review, we describe how we are developing a "plug and play" approach to adoptive immunotherapy, using CAR-T cells directed to cancer. With this approach, it will be possible to use a multiplicity of genetic engineering strategies that can enhance access to and killing of many different types of tumor cells. The tumor targeted is then altered simply by changing the specificity of the targeting receptor on the adoptively transferred effector T cells. In other words, broadly applicable strategies will be made specific for individual tumors by coupling the engineered T-cell to specific chimeric antigen receptors.

For this concept to work, we must first define appropriate target antigens within and around tumor cells to provide specificity of action, and devise receptors that can signal to the T cell that it has engaged the appropriate antigen within the tumor or its microenvironment. We have to then develop generic approaches to enhance the anti-tumor effector activity of the adoptively transferred cells, increase their resistance to tumor immune evasion strategies, and allow the immune response to be terminated should it prove toxic or damaging to the host immediately or in the longer term.

Design of the chimeric antigen receptor

CARs combine the antigen-binding property of monoclonal antibodies with the lytic capacity and self-renewal of T cells and have several advantages over conventional T cells (3–5). CAR-T cells recognize and kill tumor cells independently of the major histocompatibility complex (MHC), so that target cell recognition is unaffected by some of the major mechanisms by which tumors avoid MHC-restricted T-cell recognition, such as downregulation of human leukocyte antigen (HLA) class I molecules and defective antigen processing.

Chimeric immune receptors were first developed in the mid-1980s and initially consisted of the variable (antigen binding) regions of a monoclonal antibody and the constant regions of the T-cell receptor (TCR) α and β chains (6). In 1993, Eshhar *et al*. modified this design to use an ectodomain, from a single chain variable fragment (scFv), from the antigen binding regions of both heavy and light chains of a monoclonal antibody (Figs 1 and 2), a transmembrane domain, and an endodomain with a signaling domain derived from CD3-ζ. Most CARs subsequently designed and used have followed this same structural pattern, with incorporation of co-stimulatory signaling endodomains, which are described in Fig. 1 and below (Provision of Co-stimulation to enhance T-cell activity after antigen-specific receptor engagement). In this section, we describe how the composition of the *ectodomain, hinge* and *transmembrane domain* influences CAR function and the consequent behavior of the T cell that expresses it (Fig. 2).

Ectodomain of CARs

ScFvs are the most commonly used ectodomains for CARs, and the affinity of the scFv predicts CAR function (7, 8). For example, T cells expressing CARs containing high affinity ROR1-specific scFv have superior effector function than low affinity scFvs (7). There is, however, a plateau above which further affinity maturation does not increase T-cell activation for any given CAR. The likely explanation for the plateau effect is that the avidity of the CAR needed for maximal T-cell activation is a function of the number and density of the expressed receptors as well as their affinity (8). In addition to CAR affinity, function is

also affected by the location of the recognized epitope on the antigen (9, 10). For example, CAR-T cells expressing an scFv that recognized an epitope on CD22 (an antigen expressed by normal and malignant B cells) that was proximal to the B cells' plasma membrane had superior anti-leukemic activity to CAR-T cells that recognized a membrane-distal epitope (10). Antigen binding and subsequent activation can also be modulated by introducing a flexible linker sequence in the CAR, which will also allow expression of two distinct scFvs that can recognize 2 different antigens (11) (Figs 1 and 2). T cells expressing these so-called tandem CARs (TanCARs) may be better able to kill tumor targets expressing low levels of each antigen individually and may also reduce the risk of tumor immune escape due to the emergence of single antigen loss variants.

Because the scFvs used to date in the clinic have almost all consisted of both heavy and light chain-derived antigen binding domains and are often derived from murine monoclonal antibodies, there is considered to be a significant risk of anti-idiotype or anti-mouse antibodies, either of which can block function. Single domain scFvs have therefore also been used to prepare CARs (12). Their smaller ectodomain may render them less immunogenic, although this may come with the cost of lower affinity/specificity. Another strategy to reduce CAR immunogenicity is to humanize the scFvs, an approach taken for HER2-, EphA2-, and mesothelin-specific CARs (13–15). Unfortunately, this approach does not preclude the development of anti-idiotype antibodies that may be equally inhibitory.

The CAR concept is not confined to using scFvs as the targeting ectodomain, and other ligands and receptors have been substituted. For example, IL13Rα2-specific CARs have been prepared by modifying IL13 molecules to form ectodomains and used clinically (16–18), while NKG2D-ligand and CD70-specific CARs have been constructed by adding a ζ-signaling domain to the cytoplasmic tail of NKG2D or the CD70 receptor (CD27) respectively (19–21). Peptide ligands have also been used as CAR ectodomains. For example, Davies *et al.* (22) designed a CAR containing the promiscuous T1E peptide ligand that will recognize and bind to target cells expressing the ErbB family of receptors. Finally, multiple antigens can be recognized by so called 'universal ectodomains' such as CARs that incorporate an avidin ectodomain to recognize targets that have been incubated with biotinylated monoclonal antibodies (23), or that contain a FITC-specific scFv, which has potent antitumor activity in preclinical animal models when given in combination with FITC-labeled monoclonal antibodies (24). These alternative CAR ectodomains have performed well in preclinical studies, but only the IL13Rα2-specific CAR have been tested in humans (25, 26).

Hinge region of CARs

While the ectodomain is critical for CAR specificity, the connecting sequence between the ectodomain to the transmembrane domain (the hinge region) (Fig. 1), can also profoundly affect CAR-T-cell function by producing differences in the length and flexibility of the resulting CAR. For example Guest *et al.* (27) compared the influence of adding a CH2CH3 hinge derived from IgG1 to hingeless CARs specific for carcinoembryonic antigen (CEA), neural small adhesion molecule (NCAM), 5T4, or CD19. While 5T4- and CD19-specific CAR-T cells with a CH2CH3 hinge had enhanced effector function, CEA- and NCAM-specific CAR-T cells had optimal activity without a hinge. More recently, Hudecek *et al.* (7) compared the influence of a CH2-CH3 hinge [229 amino acids (AA)], CH3 hinge (119 AA), and short hinge (12AA) on the effector function of T cells expressing 3rd generation ROR1-specific CARs. They demonstrated that T cells expressing 'short hinge' CARs had superior antitumor activity. Conversely, other investigators found that a CH2-CH3 hinge impaired epitope recognition of a 1st generation CD30-specific CAR (28). While these results are

somewhat conflicting, they all clearly indicate the potential importance of the hinge region in determining outcome of CAR engagement.

At present we do not know the mechanisms underlying the above observational differences, and our dataset is too small for any general predictive rules or algorithm to have emerged as to which hinge will likely best work with which CAR. For the moment, therefore, empiric testing of scFv/hinge domain combinations is required to determine optimal CAR design, although this will likely change as more experimental data and validation studies are available for analysis.

Transmembrane domain of CARs

Between the hinge and the signaling endodomains lies the transmembrane domain. This is usually derived from CD3-ζ, CD4, CD8, or CD28 molecules. Like hinges, the transmembrane domains were initially viewed as inert structural links between the ectodomain and endodomain of the CAR. It is now evident that the transmembrane domain can indeed influence CAR-T-cell effector function. For example, first generation CD19-specfic CAR which contain a CD3-ζ transmembrane domain are less stable over time on the cell surface of T cells in comparison to 2nd generation CD19-specific CAR-T cells with a CD28 transmembrane domain (29). Simply replacing the CD3-ζ transmembrane domain with a CD28 transmembrane domain renders the expression of 1st generation CARs more stable (Dotti *et al.*, unpublished data). Other investigators have shown that an intact CD3-ζ transmembrane domain is essential for measurable signaling by a 1st generation CEA-specific CAR expressed in a T-cell line (30). CARs incorporating a transmembrane domain from native CD3-ζ chain could dimerize and form complexes with endogenous TCRs resulting in enhanced T-cell activation, while CARs containing mutated CD3-ζ transmembrane lacked these interactions.

Endodomain of CARs

Upon antigen recognition, CAR endodomains transmit activation and costimulatory signals to T cells. T-cell activation relies on the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) present in the cytoplasmic domain to the cytoplasmic CD3-ζ domain of the TCR complex (31). While the majority of current CAR endomains contain an activation domain derived from CD3-ζ, other ITAM-containing domains have been explored including the Fc receptor for IgE-γ domain, which proved to be less effective (32). The selection of costimulatory signaling domains is described in detail in a later section (Provision of co-stimulation to enhance CAR-T-cell activity after antigen-specific receptor engagement).

In conclusion, there is an intricate interplay between scFVs, hinge, transmembrane domain and endodomain that determines CAR function and there is no single optimal configuration that is ‘one size fits all’. For the moment, therefore, CAR receptor optimization remains largely empirical, with testing required in a range of pre-clinical models.

Antigens suited to CAR-targeted adoptive cellular immunotherapy

Unlike the native TCR, the majority of scFv-based CARs only recognize target antigens expressed on the cell surface, rather than internal antigens that are processed and presented by the cells’ MHC. While this limits the detection of a range of tumor specific antigenic epitopes (for example from mutant oncogenes and translocations), CARs have the advantage over the classical TCR that they can recognize structures other than protein epitopes, including carbohydrates and glycolipids. This increases the pool of potential target antigens. Like other cancer immunotherapy approaches, CARs should ideally target antigens that are

only expressed on cancer cells or their surrounding stroma (33), such as the splice variant of EGFR (EGFRvIII), which is specific to glioma cells (34). Few human antigens, however, meet this criterion, and the majority of target antigens (Tables 1 and 2) are expressed either at low levels on normal cells (e.g. GD2, CAIX, HER2) and/or in a lineage restricted fashion (e.g. CD19, CD20).

Antigens overexpressed by tumor cells

Although the majority of target antigens on tumor cells are shared with normal tissues and are only overexpressed in comparison to normal tissues, many have been targeted by CAR-T cells in preclinical animal models. Unfortunately, these models often cannot accurately predict human toxicities since many of the scFV ectodomains do not recognize non-human counterparts of the targeted antigens, the tissue distribution of which is also frequently species specific. This same limitation, of course, applied to studies with monoclonal antibodies. So to avoid unexpected toxicities, many of the first clinical studies selected target antigens based on the availability of monoclonal antibodies and their safety profile in humans. Since almost no monoclonal antibody has been free of at least some adverse effects, the decision on whether to proceed with a study in which the CAR is derived from a monoclonal antibody has been determined by careful assessment of the likely risk:benefit ratio for any given target antigen. For example, the EGFR monoclonal antibody cetuximab is FDA approved for the treatment of EGFR-positive cancers, but most patients treated with the monoclonal antibody develop a skin rash due to baseline EGFR expression in epithelial cells (110). Since the overall avidity of EGFR-specific CARs arrayed on a T cell would be greater than the avidity of a bivalent soluble antibody, significant safety concerns were raised about the severity and persistence of skin toxicity that have so far precluded clinical trials of EGFR-specific CAR-T cells by systemic administration. Conversely, GD2- and HER2-specific monoclonal antibodies have a favorable safety profile, which led to testing of CAR-T cells in humans (73, 91, 92, 111).

Lineage-specific antigens

Lineage-specific targets have been mainly explored for hematological malignancies (29, 37, 40, 42–44, 112). For example, CAR-T-cell treatment of B-cell malignancies can be used to target a highly and consistently expressed lineage-specific antigen (e.g. CD19, CD20), even though it is also expressed by normal B cells, since replacement therapy using intravenous immune globulin (IVIG) is feasible. In general, however, it might be preferable to target more lineage-associated antigens. For example, in many B-cell malignancies it is possible to target either the κ- or λ-light chain, since normal B cells express one or other of these antigens, while all the cells of the (clonal) malignancy will express a single light chain (54). In addition, as discussed below (Increasing the safety of CAR-T cells), the increasing potency of later generation CARs and their combination with other strategies to improve their function will almost inevitably increase their potential for ‘on-target antigen but off target tissue’, thereby producing unexpected toxicities even when targeting lineage specific antigens, since these might be aberrantly expressed at low levels elsewhere.

Other considerations for antigen selection

Targeting single antigens carries the inherent risk of immune escape (113–115), which can be reduced by targeting multiple antigens. In preclinical studies, targeting HER2 and IL13Rα2 with CAR-T cells resulted in enhanced antitumor effects in comparison to CAR-T cells that targeted a single antigen (116). As discussed below (Increasing the safety of CAR-T cells), expressing multiple CARs in T cells also has the potential to increase safety by generating T cells that recognize a unique antigen pattern that is only present on tumor cells. Targeting antigens exclusively expressed by cancer cells does not, however, address the fundamental problem of selecting variants that lack expression of the target antigen. Such

escape variants are common because of the marked genetic instability of most cancer cells (117). One solution is to target antigens expressed on the tumor stroma, a component that is critical for tumor growth and is more genetically stable. As discussed below (Targeting the cellular components of tumor stroma) investigators have therefore targeted fibroblast activation protein (FAP) expressed on cancer associated fibroblasts (CAFs) or vascular endothelial growth factor receptor (VEGFR)-2 expressed on the endothelial cells of the tumor vasculature (89, 108).

Initial clinical experience with CAR-T cells

Initial studies in humans were conducted with T cells expressing the 1st generation CARs illustrated in Fig. 1. Investigators used CAR-T cells to target hematological malignancies as well as solid tumors. Even when these CAR-T cells were combined with lymphodepletion (to reduce Treg mediated inhibition and favor homeostatic expansion of the infused cells), the results were uniformly disappointing, with minimal expansion or persistence *in vivo*, and with no unequivocal evidence of antitumor activity. For example, 2 patients received CD20-specific CAR-T cells post autologous stem cell transplant, and 4 patients received CD19-specific CAR-T cells in combination with IL-2 outside the transplant setting (36, 45). While high doses of T cells were administered (up to 2×10^9 cells/m^2), T-cell persistence was less than one week. Despite the short persistence, 2 patients developed antibodies directed to the CAR. The clinical efficacy of these CAR-T cells was difficult to assess since patients either received T cells post transplant or received additional therapies post T-cell infusion.

First generation CAR-T cells targeting carbonic anhydrase IX (CAIX), CD171, folate receptor α (FR-α), and GD2 were evaluated in patients with advanced stage solid tumors (61, 87, 91, 92, 118). Lamers *et al.* (61) infused renal cell carcinoma RCC (RCC) patients with polyclonal T cells expressing a 1st generation CAIX-specific CAR. Two of the first 3 patients developed hepatitis due to CAIX expression on bile ducts. Both patients also developed potent anti-CAR immune responses resulting in limited T-cell persistence (61). Subsequently, pretreatment with CAIX monoclonal antibodies of 4 patients prior to CAR-T-cell transfer prevented hepatitis and abrogated the induction of anti-CAR immune responses (118). While this resulted in prolonged T-cell persistence, no clinical benefit was observed. Six neuroblastoma patients received up to $10^9/m^2$ of $CD8^+$ T-cell clones expressing 1st generation CARs specific for CD171 (65). Infusions were well tolerated, but T cells persisted only for 6 weeks, and only 1 out of 6 patients had a partial response 8 weeks post T-cell infusion. Kershaw *et al.* (87) evaluated the safety and efficacy of 1st generation FR-α CAR-T cells in patients with ovarian cancer. Eight patients received up to 5×10^{10} CAR-T cells in combination with IL-2, where as 6 patients received CAR-T cells in combination with an allogeneic PBMC vaccine. T cells persisted less than 3 weeks in all but one patient and did not specifically home to tumor sites as judged by ^{111}Indium scintigraphy. No antitumor activity was observed. Because of these disappointing outcomes, extensive efforts have been made to enhance the effector function of CAR-T cells *in vivo*, which are discussed in detail in the following sections.

Optimal T-cell subset in which to express CARs

The ideal T-cell target in which to graft CAR molecules should be the subset that can traffic to tumor sites, receive appropriate co-stimulation and retain a profile predictive of prolonged in vivo survival. Moreover, the T-cell chosen should preferably not be able to produce toxicity *in vivo* due to the inappropriate activation of signaling pathways in otherwise quiescent T-cell subtypes, or because of disruption to the otherwise tightly coordinated activation and subsequent contraction process of T cells that are dependent on both antigenic

stimulation and physiological costimulation and inhibition (see section “Provision of costimulation to enhance CAR-T-cell activity after antigen-specific receptor engagement”).

Identification of optimal T-cell surface phenotype

A number of investigators have tried to identify a phenotypic profile for the optimal subset to allow this component of the T-cell system to be separated and selectively transduced, to produce maximum benefit with minimum adverse effects (119, 120) (Fig. 3). These efforts are based on evidence that signals for memory T-cell development are received during the initial expansion of T cells and that the pattern of development and cell fate are influenced by the stimuli received during initial exposure to antigen (priming). The affinity of the TCR engagement, the balance of costimulatory versus inhibitory cellular and soluble signals, as well as other environmental cues (121, 122) all dictate the diversity of T-cell subsets which generate memory T cells and effector T cells (Teff) either linearly, progressively, or through asymmetric division (123).

The obvious population in which to express CARs is the Teff, since this subset are potent anti-tumor effector cells (124). Teff cells are, however, less appealing for adoptive immunotherapy because of their limited proliferative capacity and persistence in vivo. Investigators instead are now concentrating on memory T-cell subsets, which are traditionally divided into central and effector memory cells (Tcm and Tem) based upon the expression of CD62L and CCR7 (maintained on Tcm and lost on Tem) (125, 126). These cells are ‘antigen-experienced’, can expand substantially *in vivo* and are long-lived. Many studies have now shown that a balanced composition of both Tcm and Tem subsets can effectively and rapidly control repeated exposure to pathogens over a prolonged period (127). Because of these characteristics, Tcm and Tem cells have been considered ideal vehicles for grafting CARs. Since CD62L expression can be used to identify T cells with memory characteristics, investigators have used positively selected CD62L cells to express a CAR and shown superior activity in a mouse model (128). A clinical study based on this approach has been opened at the Fred Hutchinson Cancer Research Center.

More recently, a phenotypically defined T-cell subset with true stemness properties (the Tscm) has been identified (129). These cells have enhanced proliferative potential and survival *in vivo* and can differentiate into memory and effector populations. If they are indeed a truly distinct subset in humans, they may allow investigators to identify a T-cell subset suited to genetic manipulation that will also be best able to mediate complete and durable remission in cancer patients.

Functional T-cell selection

An alternative approach to using surface-phenotype based selection of T-cell subsets prior to CAR expression is to employ T cells already selected *in vivo* for their established capacity to act as Teff, to enter the memory pool, and to re-expand on re-exposure to antigens. Virus-specific cytotoxic T lymphocytes (CTLs) are an example of such cells, and in addition to their potential for life-long persistence, virus-specific CTLs contain both $CD8^+$ and $CD4^+$ subsets, with the latter compartment critical for long-term persistence of the former (130, 131). Virus-specific CTLs are also well characterized for expression of homing/chemokines receptors commensurate with their capacity for trafficking to and residing in the designated lymphoid or non-lymphoid tissue (132). Their potential value as CAR-expressing effector cells is considered below.

Provision of costimulation to enhance CAR-T-cell activity after antigen-specific receptor engagement

T-cell costimulation is mediated by a multiplicity of receptor-ligand interactions, and plays a fundamental role in preventing the induction of anergy in T lymphocytes upon engagement with the target antigen (121). Tumor cells and the tumor microenvironment are deficient in co-stimulation and favor the induction of T-cell anergy due to their lack of expression of co-stimulatory molecules such as CD80 and CD86 that are ligands for the CD28 receptor expressed by activated effector T cells (133). Adoptively transferred T lymphocytes engineered to express CARs are not immune from tumor-induced anergy. In the absence of CD80/CD86 co-stimulation, engagement of antigen through the CAR produces T-cell hyporesponsiveness (134). In addition, when tumor-specific T cells are expanded *ex vivo* they may lose expression of CD28, particularly if culture is prolonged, and thus become unresponsive to tumor cells or bystander cells expressing costimulatory molecules.

CAR molecules can be engineered to overcome the lack of co-stimulation by tumor cells. Endodomains derived from well characterized co-stimulatory molecules such as CD28 (54, 104), CD134/OX40 (134), CD137/4-1BB (135, 136), and CD27 (137) can be incorporated within CARs to provide direct T-cell co-stimulation after CAR antigen binding (Fig. 1). Since each of these co-stimulatory molecules activates different signaling pathways (e.g. phosphatidylinositol 3-kinase (PI3K) for CD28 versus tumor necrosis family (TNF)-receptor-associated factor (TRAF) adapter proteins for 4-1BB and OX40), multiple endodomains can be included in a single CAR and thereby recruit multiple signaling pathways, potentially maximizing the co-stimulatory benefits (134, 136). Although the mechanism of the effector cell immunological synapse formation by CAR molecules has not been elucidated, it is likely that CAR cross-linking upon antigen-binding leads to the physical recruitment and dimer formation of costimulatory endodomains incorporated within the CAR and consequent activation of the downstream signaling pathways. An alternative means of providing costimulation, that may occur independently of CAR cross linking relies on the trans- and auto-co-stimulation achieved when CAR-T cells are further engineered to express either CD80 or 4-1BBL molecules that are separated from CAR molecules, but this approach has not yet been clinically tested (138).

Identification of optimal endodomains

That incorporation of co-stimulatory endodomains in CARs enhances the proliferation and activation of CAR-T cells in humans *in vivo* has been clearly demonstrated by direct comparison of the fate of CAR-T cells with and without an included co-stimulatory endodomain (29) (see below ‘Direct comparison of co-stimulatory endodomain in humans’). Efforts to predict in advance which endodomains or combination of endodomains will prove to be optimal in humans has been much more problematic. *In vitro* experiments and xenogeneic mouse models have been used to dissect and compare the effects of incorporating each co-stimulatory endodomain into CARs, and it has become evident that the results are often contradictory and may not predict events in clinical studies. For example, when CD28, 4-1BB or combinations of multiple co-stimulatory endodomains were compared, the relative potency of each was dependent on the target antigen, the biology of the tumor cells and the mouse strains used (139, 140). Ultimately, identification of the optimum choice of costimulatory endodomains(s) for a given CAR and tumor cell target may have to be resolved in clinical trials.

Direct comparison of costimulatory endodomain activity in humans

One means of directly determining the value of each specific CAR costimulatory moiety in patients is to simultaneously infuse each patient with two or more T-cell products, each

expressing CARs with identical specificity and sequence, but with different costimulatory components. Using this approach, we infused six lymphoma patients simultaneously with two T-cell products expressing the same CD19-specific CAR but encoding either the CD3-ζ chain of the TCR alone or both the CD3-ζ chain and the CD28 endodomain. We demonstrated that CD28 costimulation within the CAR indeed promotes superior *in vivo* expansion of CD19-specific CAR-T cells (29). In a similar current study at Memorial Sloan-Kettering and University of Pennsylvania, investigators are comparing the simultaneous infusion of T cells expressing CD19-specific CARs incorporating either the CD28 or 4-1BB endodomains. We will initiate a new study to compare CD19-specific CARs incorporating either CD28 alone or the combination of CD28/4-1BB endodomains. Although impressive results have already been reported in B-cell malignancies incorporating the 4-1BB endodomain within a CD19-specific CAR (41, 42, 44), these direct comparator studies retain fundamental importance if we are to know definitively how CAR-mediated T-cell costimulation affects T-cell fate and anti-tumor activity, since this study design avoids the inevitable and significant confounding variables associated with small phase I studies enrolling heterogeneous patients with heterogeneous disease.

Role of lymphodepletion

The expansion, persistence and anti-tumor activity of CAR-T cells may be enhanced if the cells are given after administration of lymphodepleting drugs (141). These benefits may result from reduction of Treg in the lymphoid tissues and in tumor and from the production of cytokines such as IL-7 and IL-15 that may favor expansion of infused cells. At present, however, there is no unequivocal evidence that lymphodepletion benefits the outcome, and the potential toxicities may ultimately negate the putative benefits (38, 73).

Clinical experience with 2nd and 3rd generation CAR-T cells: Hematological malignancies

The most impressive clinical results with CAR-T cells so far has been achieved with polyclonal T cells expressing CD19-specific CARs either with CD28.ζ or 41BB.ζ signaling domains (37, 40–44). These studies have been recently reviewed in detail elsewhere (142). Complete responses were observed post infusion of 2nd generation CAR-T cells in patients with CD19^{+} hematological malignancies including NHL, chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL). Antitumor activity was dependent on significant T-cell expansion *in vivo*, which was associated in several patients with a life-threatening cytokine storm (42, 44). The clinical picture was reminiscent of hemophagocytic syndrome and patients responded either to a combination of steroids, TNF-α antibody (infliximab), and IL-6 receptor antibody (tocilizumab) or to monotherapy with tocilizumab (42, 44). While addition of co-stimulatory domains dramatically increased the expansion and persistence of polyclonal T cells, this benefit was not observed with T-cell clones. Three patients received T-cell clones expressing 3rd generation CD20-specific CARs after lymphodepletion with cytoxan (143). T-cell persistence was limited; 2 patients with no evaluable disease remained disease free for 12 and 24 months, and a 3rd patient had a partial response that lasted for 12 months. This study indicates that T-cell clones, regardless of the endodomain used, can have limited T-cell function *in vivo.*

Besides targeting hematological malignancies with CD19 and CD20 with CAR T cells, we are currently conducting clinical studies with polyclonal T cells expressing 2nd generation CARs specific for the κ-light chain of human immunoglobulin (see ‘Control of toxicities’) or for CD30. We have generated CARs specific to selectively target κ^{+} lymphoma/leukemia cells, while sparing the normal B cells expressing the non-targeted λ-light chain, thus minimizing the impairment of humoral immunity associated with the depletion of all normal B lymphocytes (54). This approach is now in clinical trial using a 2nd generation CAR encoding the CD28 endodomain and clinical responses including CRs have been observed

(144). We have also implemented clinical trials using a 2nd generation CAR specific for the CD30 antigen (50), which is present on most Hodgkin's lymphoma (HL) and some non Hodgkin's lymphoma (NHL) cells. The generation of functional CAR-T cells has been successful in the patients with refractory/relapsed diseases, of whom 4 have been treated so far without toxicities.

In contrast to B-cell malignancies, limited clinical experience is available for CARs redirected to T-cell or myeloid-derived malignancies. While preclinical studies have demonstrated the potent anti-acute myeloid leukemia (AML) effects of CD123-specific or Lewis-Y antigen-specific CAR T cells (53, 55), only one clinical study with Lewis-Y antigen-specific CAR T cells is in progress for AML patients (56).

Solid tumors

Limited clinical experience is currently available with targeting solid tumor antigens using 2nd or 3rd generation CARs. One patient who received 10^{10} T cells expressing a 3rd generation HER2-specific CAR and a lymphodepleting chemotherapy regimen consisting of cytoxan and fludarabine, in combination with IL-2 rapidly developed acute respiratory distress syndrome and died (73). We have infused up to $10^8/m^2$ T cells expressing a 2nd generation HER2-specific CAR to patients with osteosarcoma. While no overt toxicities were observed, the antitumor activity has so far been limited (111). Clinical studies with T cells expressing a 3rd generation EGFRvIII-specific CAR for glioma patients are in progress, and another study has treated patients with pancreatic cancer with a 2nd generation mesothelin-specific CAR, one of whom developed an anaphylactic reaction (145).

Using physiological costimulation through the native T-cell receptor

The costimulation provided by second or later generation CARs is non-physiological, since it does not occur in the same regulated tempero-spatial sequence that follows a T-cell encounter with antigen on professional antigen-presenting cells. Indeed, the overabundance of stimulation from a 2nd or 3rd generation CAR may be toxic to the T-cell itself (for example by activation induced cell death) or to the recipient of the T cells (for example by the rapid production of pro-inflammatory cytokines). An alternative approach outlined in the section 'Optimal T-cell subset in which to express CARs' relies instead on restricting expression of CARs to a specific T-cell subset such as virus-specific CTLs for which physiologic CD80/CD86 costimulation is provided by professional antigen-presenting cells when viral latent antigens processed by these APCs are encountered by CTLs through their native TCRs (35, 146, 147). While attractive in principle, the choice of suitable viral antigen specificity of a CTL is limited, as the virus associated antigens need to be encountered relatively frequently and in the presence of a broad array of costimulatory molecules. Epstein Barr virus (EBV)-infected cells may be a valuable resource for this purpose. B lymphocytes expressing EBV-antigens persist lifelong in seropositive individuals and boost both MHC class-I and class-II EBV-specific CTLs targeting latent antigens (132). Importantly, *ex vivo* expanded EBV-specific CTLs retain the same property of longevity as circulating cells and persist long term after adoptive transfer (130, 131). Based on this evidence, we demonstrated preclinically (146, 147) and then clinically that EBV-specific CTLs engrafted with CARs produce antitumor effects against tumors targeted by the CAR, while retaining their physiological costimulation through their native antigen-specific αβTCRs (91, 92).

Clinical experience with CAR-engrafted virus-specific CTLs

Our group expressed a 1st generation CAR directed to GD2 on EBV-specific CTLs and gave them to 11 children with advanced neuroblastoma (91, 92). By comparing EBV-specific

CTLs and activated T cells expressing the same but distinguishable 1st generation CAR, we found that CAR-expressing EBV-specific CTLs initially persisted in the circulation at a higher level and longer than activated T cells and that 5 of the 11 patients with active disease showed tumor responses or necrosis. Three of them had complete responses (sustained in 2), while an additional 2 with bulky tumors showed substantial tumor necrosis. Nevertheless, neither of the CAR-T cell populations expanded *in vivo*, and patients with massive tumor burdens were helped little. Subsequent GD2-CAR studies are using lymphodepletion in combination with second and third generation vectors and are described in the next sections.

We next designed a study in which adult patients with B-cell malignancies were infused simultaneously with EBV-specific CTLs engrafted with a 1st generation CD19-specific CAR and activated T cells expressing a 2nd generation CD19-specific CAR encoding the CD28 endodomains. Major limits, however, quickly emerged for this study. EBV-specific CTLs could be manufactured only in a minority of potential referrals, since the majority of the lymphoma patients had received anti-CD20 antibody (rituximab) as part of their standard of care, so eliminating B cells and precluding generation of the EBV+ B-lymphoblastoid cell lines required the *ex vivo* expansion of CTLs. Both products were available for 5 patients, but CD19-specific CAR-expressing EBV-specific CTLs did not persist longer than activated T cells expressing the 2nd generation CAR, likely because EBV-infected B cells in these patients were lacking (authors, manuscript in preparation). Recognizing that EBV-expressing target cells will not be available to use as stimulators in lymphoma patients, we broadened the virus specificity of our cells to include cytomegalovirus (CMV) that also has chronic persistence. This clinical trial has been opened for allogeneic stem cell transplant recipients (148). Similarly, a trial using CTLs specific for three viruses, adenovirus, EBV and CMV, engrafted with a GD2-specific CAR has been recently initiated in the post allogeneic transplant setting in patients with refractory/relapsed neuroblastoma (149). We are also exploring the use of CMV-specific T cells that are genetically modified with a 2nd generation HER2-specific CAR for the adoptive immunotherapy of glioblastoma (GBM). Since CMV antigens are present in latently infected leukocytes and are also detected in the tumor itself (150–153), this approach may enhance *in vivo* persistence and expansion of adoptively transferred T cells and also directly increase the anti-tumor activity of transferred T cells.

Beyond costimulation and other approaches to improving expansion, persistence and function of CAR-T cells

The clinical studies described above confirm the pre-clinical data showing that introducing costimulation for CARs is necessary but insufficient *per se* to reverse all the immune inhibitory mechanisms of cancer. This is particularly apparent when solid tumors rather than hematologic malignancies are being targeted. Solid tumors and their microenvironment lack the conventional costimulatory molecules that may be present on (for example) malignant and normal B lymphocyte targets and have developed intricate systems to suppress the immune system (133, 154, 155) (Fig. 4). Thus, malignant cells and their supporting stroma secrete immunosuppressive cytokines such as transforming growth factor-β (TGFβ) or IL-10, attract immunosuppressive cells such Tregs or MSDCs, inhibit dendritic cell maturation, express molecules on the cell surface that suppress immune cells including FAS-L and PD-L1, and create a metabolic environment (e.g. high lactate, low tryptophan) that is immunosuppressive.

While T-cell costimulation mediated by CD28 and 4-1BB endodomains in CAR molecules may overcome some of the above inhibitory effects, other causes of T-cell anergy are more resistant. Additional engineering of CAR molecules has therefore been exploited as

countermeasures to the inhibitory mechanisms developed by tumor cells and their microenvironment and to further enhance CAR-T-cell activity.

Three broad approaches have been adopted as countermeasures to overcome tumor immunosuppression: (i) increasing the level of CAR-T cell activation or decreasing physiological downregulation, (ii) engineering the CAR-T cells to be resistant to the immune evasion strategies used by the tumor, or (iii) targeting the cellular components of tumor stroma (Fig. 5). Any one countermeasure may affect more than one mechanism of tumor immunosuppression.

Addition of immunostimulatory cytokines/cytokine receptor genes

IL-2 is released by 2nd generation CAR-T cells following receptor engagement, but is not by itself sufficient to reverse T-cell anergy (156, 157). Moreover, this cytokine may increase the number and activity of local Tregs (158). Cytokines that are not directly produced by T cells upon antigen stimulation but are biologically active in T cells may be better placed to accomplish the goal of selective CAR-T-cell activation and reversal of anergy without Treg recruitment.

Of these cytokines, IL-15 is perhaps the most promising. IL-15, is mainly produced by monocytes/macrophages and dendritic cells. It shares a common γ-chain with IL-2 and IL-7 but also requires a private chain in the receptor, IL-15Rα, that is expressed on antigen-presenting cells, monocytes, and macrophages (159). IL-15 selectively stimulates these target cells through a cross presentation mechanism (160). IL-15 promotes the proliferation of T lymphocytes and also prevents apoptosis and exhaustion (156, 161), reverses anergy (156), stimulates long-lasting antigen-experienced memory cells (162), and overcomes Treg-mediated inhibition (157, 163–165). IL-15 can be used either as a growth factor for the *ex vivo* expansion of CAR-T cells, where it may 'imprint' long-lasting resistance to Tregs (39, 166), or as a recombinant protein *in vivo* to support T-cell expansion after adoptive transfer (166), thereby enhancing the antitumor activity of adoptively transferred T cells in animal models. Preliminary clinical studies showed that systemic administration of recombinant IL-15 may be highly toxic, and the cytokine may be better tolerated if production is confined to the tumor location. We have described how CAR-T cells can be genetically modified to produce their own IL-15 and achieve the hoped-for benefits at the tumor site while avoiding systemic toxicity (157, 164). Locally produced IL-15 improves CAR-T-cell expansion and persistence *in vivo* and renders CAR-T cells resistant to the inhibitory effects of Tregs by activation of the phosphoinositide 3-kinase (PI3K) pathway resulting in increased expression of anti-apoptotic molecules such BCL-2 (*CM2*). We plan to test this approach in a clinical trial.

Local production of other cytokines such as IL-7 and IL-12 may also be beneficial. IL-7 shares the γ-chain of its receptor with IL-2 and IL-15 and plays a crucial role in maintaining the homeostasis of mature T cells and the maintenance of memory T cells (167). Clinical studies have shown a good safety profile compared to the other γ-chain cytokines. Since systemic administration of recombinant IL-7 is well tolerated (168), we and other investigators are manipulating the IL-7/IL-7Rα signaling axis in antigen-specific T cells to selectively promote a robust and selective expansion of these cells following IL-7 exposure (169). Although this approach has not yet reached clinical trial, studies of a third cytokine, IL-12, are more advanced. IL-12 is a pro-inflammatory cytokine that promotes Th1 differentiation and links innate and adaptive immunity (170). Like IL-15, the transgenic expression of IL-12 in tumor-specific T cells significantly increases their anti-tumor activity (171, 172) by directly enhancing T-cell activity and by increasing their production of Th1 cytokines (173). In addition, IL-12 may help to reverse the immunosuppressive tumor environment by triggering the apoptosis of inhibitory tumor-infiltrating macrophages,

dendritic cells, and myeloid derived dendritic cells (MSDCs) through a FAS-dependent pathway (174). While there are safety concerns in regards to constitutive IL-12 expression, there are several mechanisms available to restrict IL-12 production to activated T cells at tumor site by using inducible expression systems (172). Transgenic IL-12 expression by tumor-infiltrating T lymphocytes is currently being tested in a clinical trial at the National Cancer Institute.

Ultimately, it may be possible to bypass the use of cytokines/cytokine receptors and to directly manipulate T-cell pathways that influence cell growth and survival. For example, expression of a constitutively active form of serine/threonine AKT (caAKT), which is a major component of the PI3K pathway, improves T-cell function and survival, since caAKT-expressing T cells have sustained higher levels of NF-κB and of anti-apoptotic genes such as Bcl-2, resulting in resistance to tumor inhibitory mechanisms (175). This approach is at an early stage and may lack the selectivity necessary for safe use.

Blocking the immune check points

Instead of adding stimulatory signals (costimulation, cytokines/cytokine receptors), the function of CAR-T cells may be enhanced by blocking downregulatory signals. Antibodies that block the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) or the programmed death-1 (PD-1) receptor or the PD-L1 ligand have produced encouraging clinical results as single agents (176, 177). CTLA-4 is expressed by activated T cells and acts as a co-inhibitory molecule of T cells after engaging CD80 and CD86 expressed by antigen-presenting cells (178). PD-1 also acts as an inhibitory receptor for T cells by engaging the PD-L1 counter-receptor, expressed by antigen-presenting cells in a regulated manner and constitutively by many tumors (179). Antibodies that block the CTLA-4/CD80/CD86 or PD-1/PD-L1 axes therefore prevent the physiological contraction of the T-cell immune system. The CTLA-4 antibody ipilimumab has shown remarkable effects in a randomized clinical study in a subset of melanoma patients (176) and promising results have also been reported for antibodies blocking PD-1 or PD-L1 in patients with renal carcinoma (177). The combination of these antibodies with adoptive transfer of CART cells is a logical evolution of the current clinical protocols to prolong the effector function of CAR-T cells at sites of solid tumor. This combination does, however, have the potential for uncontrolled proliferation and activation of the CAR-T cells (180) and will need to be examined using careful dose escalation studies and - ideally - with other safety interventions in place (see ‘Increasing the safety of CAR-T cells’).

Instead of influencing receptors for inhibitory signals, it is also possible to directly silence genes that render T cells susceptible to inhibitory signals in the tumor microenvironment. For example, many tumor cells express FAS ligand (Fas-L), and silencing FAS expression in T cells prevents FAS-induced apoptosis (181).

Dominant negative and chimeric cytokine receptors

CAR-T cells can be engineered to be resistant to cytokines such as IL-4 and TGFβ that are widely used by tumors as an immune evasion strategy (Figs 4 and 5). TGFβ promotes tumor growth and limits effector T-cell function through SMAD-mediated pathways resulting in decreased expression of cytolytic gene products such as perforin, decreased cell proliferation, and increased apoptosis (182, 183). These detrimental effects can be negated by modifying T cells to express a dominant-negative TGFβ receptor type II (TGFβ-DNR), which lacks most of the cytoplasmic component including the kinase domain (184, 185). DNR expression interferes with TGFβ-signaling, thereby blocking TGFβ-induced SMAD2 phosphorylation so that T-cell effector function is sustained even in the presence of TGFβ. We have used the TGFβ-DNR to modify T cells directed to tumors through their native T-

cell receptors. We then gave these TGFβ-resistant T cells specific for the EBV antigens LMP1/LMP2 into patients with EBV-positive Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL) and have obtained clinical benefit including complete responses (186) even in patients who failed with LMP1/LMP2-specific T cells expressing only wildtype TGFβ receptor type II. Other clinical studies are now in progress in which the TGFβ-DNR is expressed in CAR-T cells.

It is also possible to engineer T cells to actively benefit from the inhibitory signals generated by the tumor environment, by converting inhibitory into stimulatory signals. For example, linking the extracellular domain of the TGFβ RII to the endodomain of toll-like receptor (TLR) 4 results in a chimeric receptor that not only renders T cells resistant to TGFβ, but also induces T-cell activation and expansion (187). Chimeric IL-4 receptors are another example of these 'signal converters'. Many tumors secrete IL-4 to create a Th2-polarized environment, and two groups of investigators have shown that expression of chimeric IL-4 receptors consisting of the ectodomain of the IL-4 receptor and the endodomain of the IL-7Rα (188) or the IL-2Rβ chain (189) enable T cells to proliferate in the presence of IL-4 and retain their effector function including Th1-polarization.

Targeting the cellular components of tumor stroma

Most solid tumors have a stromal compartment that supports tumor growth directly through paracrine secretion of cytokines, growth factors, and provision of nutrients and that also contributes to tumor-induced immunosuppression (190–193). This compartment may be a suitable target for CAR-T cell therapy. For example, we have shown in preclinical studies that T cells expressing CARs specific for fibroblast activation protein (FAP) expressed on cancer-associated fibroblasts (CAFs) have potent antitumor effects, which is enhanced when they were combined with tumor-specific CAR-T cells (89). Hence, targeting CAFs has the potential to improve the antitumor activity of adoptively transferred CAR T cells. Other investigators are targeting the tumor vasculature with CARs (108, 109). These studies initially targeted VEGFR-2 using a VEGF-based CAR, but more recent pre-clinical studies have used a VEGFR-2-specific scFv (108). The combination of CAR-T cells targeting vasculature and tumor cells was again more effective than CAR-T cells targeting either component alone. In addition combining VEGFR-2-specific CARs and IL-12 in T cells was sufficient to eradicate tumors, indicating another means of potentiating effects (194). Any approach that targets antigens present on normal tissue has to consider the inevitable safety concerns, but while these require consideration, clinical studies evaluating the safety and efficacy of the approach, for example with VEGFR-2-specific CAR-T cells, are in progress.

Increasing the safety of CAR-T cells

The efficacy of CAR-modified T cells has not been devoid of toxicities. These fall into three categories.

Toxicity from the gene delivery system

Up to now the most effective approaches to express CARs in human T lymphocytes have been based on γ-retroviral vectors and lentiviral vectors (41, 42, 44, 195). Both vectors allow robust and stable expression of CARs in T cells without requiring complex and long procedures of *ex vivo* selection. Genomic integration of viral vectors may, however, cause toxicities due to insertional mutagenesis. Unlike past experience with γ-retroviral vector-mediated gene transfer to $CD34^+$ hematopoietic progenitor cells (196), insertional mutagenesis leading to lymphoproliferation of T lymphocytes (including CAR-T cells) has not yet occurred, perhaps because integration is occurring into more differentiated cells with fewer developmental pathways open to disruption by integration events.

On target toxicities

This second type of toxicity is directly attributable to T cells engaging the targeted antigen. For example, the infusion of long-term persisting CD19-specific CAR-T cells is followed by long-term elimination of all cells bearing the CD19 antigen, irrespective of whether they are malignant or normal and leads to profound and prolonged B-cell aplasia and ultimately hypogammaglobulinemia (37, 41–44). This particular toxicity may be ameliorated by infusing immunoglobulin preparations.

On biological target but off organ toxicities

Other on-target toxicities may be more severe and less amenable to correction, particularly if they are on target but ‘out of organ’. For example, targeting the carbonic anhydrase IX that is highly expressed by renal cell carcinoma also causes significant liver toxicity, since the targeted antigen is also expressed by cells of the biliary tree (61, 118). Similarly, a fatal adverse event occurred in a patient infused with HER2-specific CAR-T cells which may have been due to low level HER2 expression in the pulmonary parenchyma or vasculature (73).

Systemic inflammatory response syndrome (SIRS) or cytokine storm

This toxicity is attributable to rapid and extensive activation of infused CAR-T cells upon antigen engagement, with general perturbation of the immune system, and the associated release of high levels of proinflammatory cytokines, such as TNF-α and IL-6 (42, 44). This toxicity has now been observed after administration of CAR-T cells with several different specificities and, while often associated with a tumor response and potentially reversible, it remains a major concern for broader introduction of the approach.

Controlling toxicity from the gene delivery system

Although oncogenicity from retroviruses is currently only a hypothetical concern for CAR-T cells, there is considerable interest in developing alternative vector systems that retain significant genomic integration capacity, but are based on DNA plasmids such as the transposon/transposes system which may be less likely to selectively integrate in critical sites in the genome (197, 198). A phase I study in which T cells are engineered to express a CD19-specific CAR encoded in a Sleeping Beauty transposon system has been recently approved by the FDA and the clinical trial is ongoing at MD Anderson Cancer Center.

Controlling on target toxicity

To prevent on target toxicity requires accurate antigen selection, so that careful dose escalation of CAR-T cells currently remains a fundamental aspects of CAR-T-cell therapies and T-cell therapies in general. It may also be possible to reduce this type of toxicity by targeting antigens that are more restricted in their expression. In B-cell malignancies, for example, we generated CARs specific to κ-light chain of immunoglobulin, since unlike a CD19 CAR-T-cell which will target both normal and malignant B cells equally well, a κ+CAR-T cell will target all the cells of a κ+ malignancy (since the tumor is clonal) while sparing the subset of normal B cells that express λ (54). Other groups are targeting ROR1 in clinical trials (7). Nevertheless, to extend CAR-T-cell therapies beyond hematological malignancies into the arena of solid tumors will likely continue to require targeting of antigens that are inevitably expressed by normal tissues. Preclinical models may be insufficient or inadequate to predict the organ toxicity and in these circumstances alternative safety strategies are required. One approach is to infuse T cells with only transient expression of the CAR, for example after electroporation of mRNA encoding the receptor (199–201). Unlike T cells transduced with a (genome-integrating) vector, in which each daughter cell contains the same transgene, translated to the same level, mRNA transduced T

cells express the transgene for a finite period of time (depending on the stability of the mRNA and the translated protein); moreover, levels of expression diminish as the cells divide, and the transcripts become progressively diluted. Since CAR-T cells may expand 1000–10,000-fold over 7–10 days, this dilutional effect may be rapid. The approach is being used at the University of Pennsylvania to test a CAR specific for mesothelin in patients with pancreatic cancer (145).

Controlling SIRS

To reduce the onset or severity of SIRS, investigators are modifying T-cell dose escalation and have introduced the prompt use of antibodies blocking the effects of TNF-α and IL-6.

General reduction in toxicity

While the specific measures outlined above may all be beneficial, the inherent potential of T cells to persist and expand means that the associated toxicities may show corresponding persistence and worsen with time. Thus, there is a strong incentive to use engineered T cells that also express a suicide or safety switch along with the CAR. These cells would then retain their long-term capacity for engraftment, expansion and expression but could be eliminated on demand by the activation of the suicide gene in the event of toxicity. We have selected this approach for our forthcoming clinical trial in which a 3rd CAR targeting the GD2 antigen has been coupled with the inducible caspase9 suicide gene (202, 203). This particular suicide gene can be selectively activated by an otherwise bioinert small molecule (chemical inducer of dimerization (CID). Compared to the more widely used herpes thymidine kinase/ganciclovir approach this system may act more rapidly by induction of apoptosis, and to have reduced immunogenicity since the sequences are all human derived (203, 204).

Although the possibility of rapidly eliminating CAR-T cells in the event of toxicity represents an important consideration for safety, the elimination of these cells also abrogates their anti-tumor effects. This may be problematic if, as seems likely, long term immune surveillance is necessary to prevent tumor relapse. Thus, the ultimate goal is to retain the beneficial antitumor effects of CAR-T cells even against antigens that are shared, with normal tissues. One means of accomplishing this is to preferentially express CARs on the T cells only at the tumor site, by exploiting metabolic conditions that are commonly developed within the tumor environment such as hypoxia (205). Targeting multiple antigens on a single cell may also reduce toxicity, by providing the engineered T cells with true pattern-recognition ability. For example, engineering T cells to express two CARs with complementary signaling domains restricts full T-cell activation only to tumor sites at which both antigens are expressed (206–208). These elegant models seem very promising in preclinical experiments, but it remains to be demonstrated whether the benefits can be recapitulated within heterogeneous human malignancies in which the levels and patterns of antigen expression may vary between one cell and another.

Conclusions

CAR-T cells are making the transition from merely ‘promising’ to being ‘effective’ treatments for hematological malignancies. As we continue to improve the functionality of the T cells that express chimeric tumor-targeting receptors and enable them to function in the tumor micro-environment, we can anticipate broader application beyond hematological cancers and into solid tumors. With increasing interest in the field from commercial entities we are hopeful that development and clinical implementation of this exciting approach will now accelerate.

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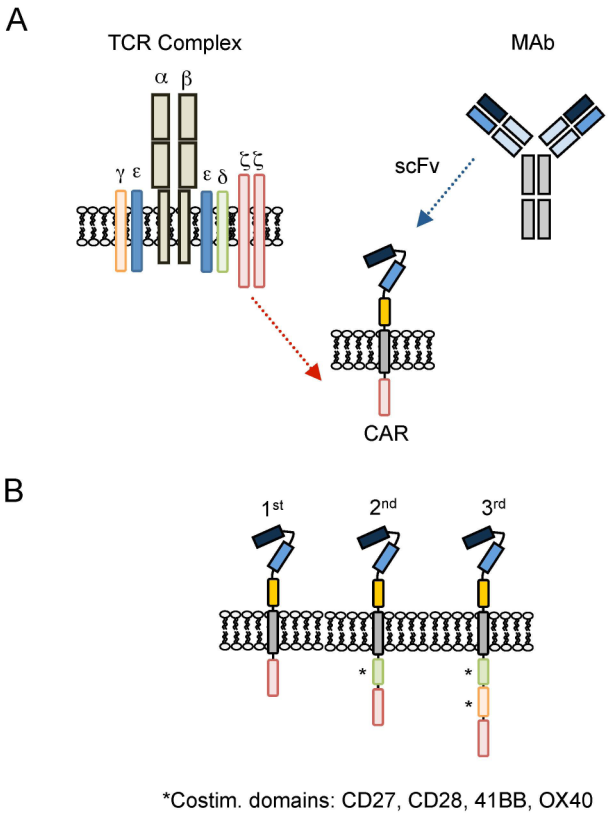

Fig. 1. CAR Design

(A) CARs consist of an ectodomain, commonly derived from a single chain variable fragment (scFv), a transmembrane domain, and an endodomain. (B) Depending on the number of signaling domains, CARs are classified into 1st generation (one), 2nd generation (two), or 3rd generation (three) CARs.

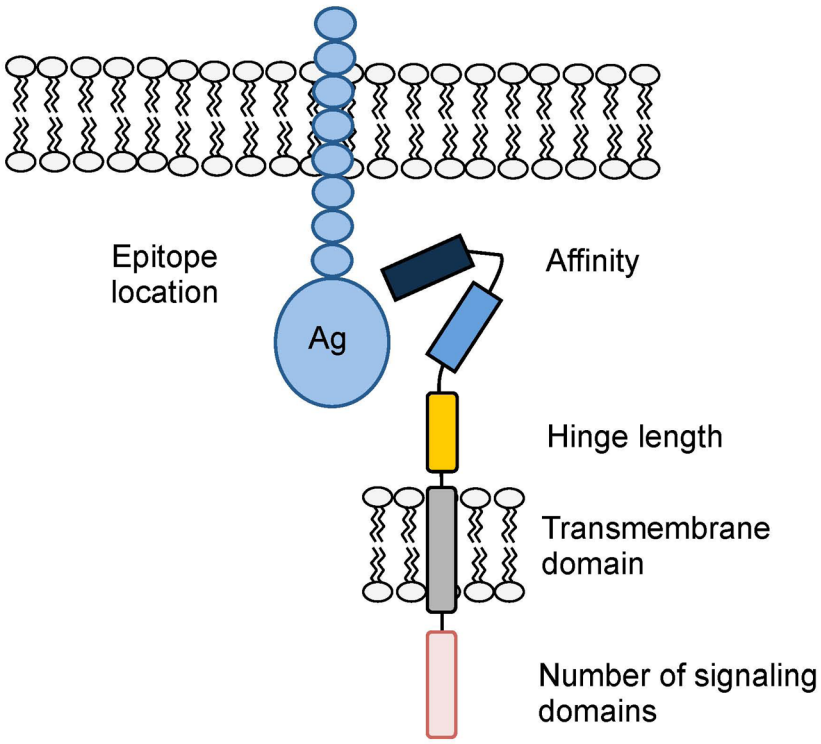

Fig. 2. Critical CAR components

Optimal CAR activity is determined by epitope location, scFv affinity, hinge and transmembrane domains, and number of signaling domains.

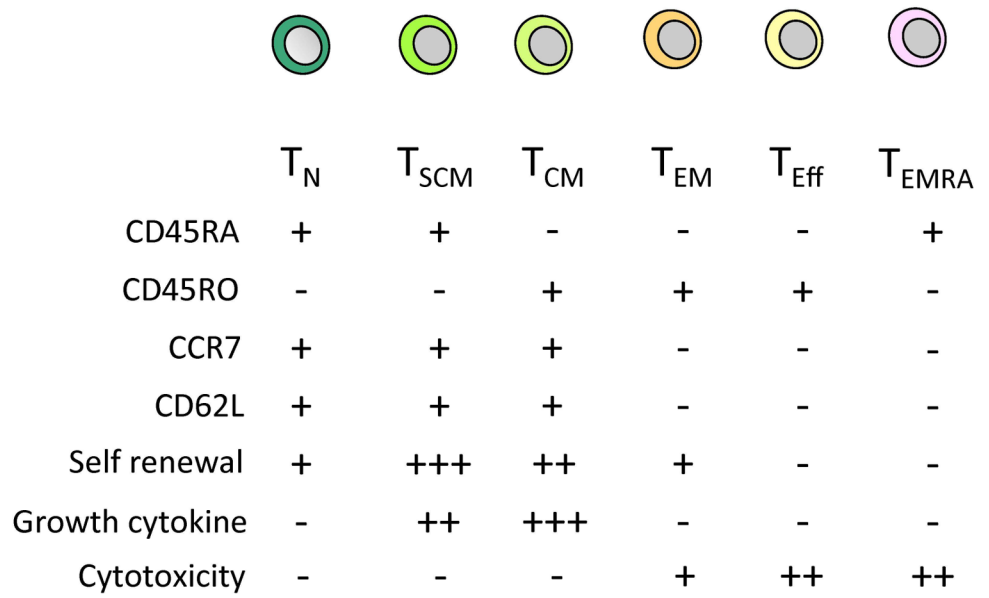

Fig. 3. T-cell subsets

The phenotypic properties of T-cell subsets. − indicates absence and + presence of the specific cell surface marker. Functional properties for each subset are also shown. − indicates lack of the functional property; +, ++, +++ indicates the degree (low, intermediate, high) of the specific property.

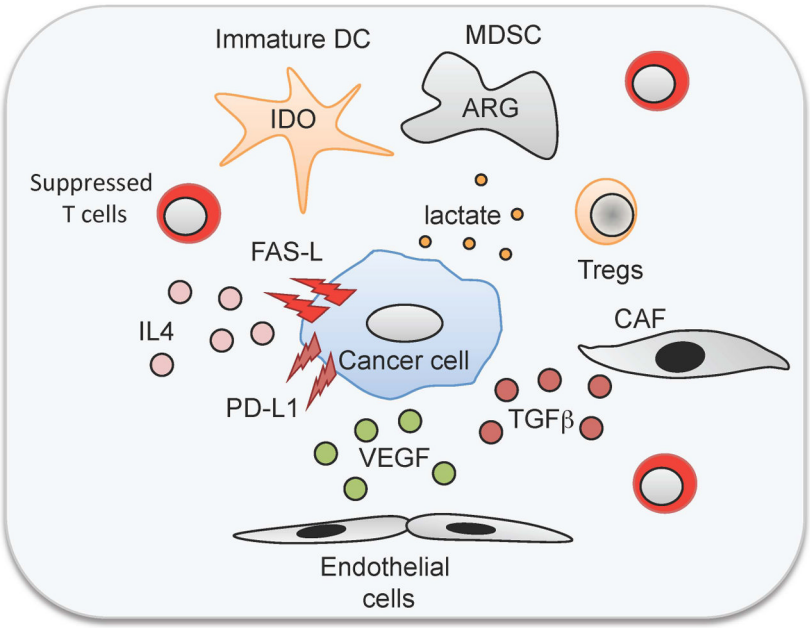

Fig. 4. Immune evasion strategies of tumors

Malignant cells and their supporting stroma 1) secrete immunosuppressive cytokines such as transforming growth factor-β (TGFβ) or interleukin-10 (IL-10); 2) attract immunosuppressive cells such Tregs or MSDCs; 3) inhibit dendritic cell maturation; 4) express molecules on the cell surface that suppress immune cells including FAS-L and PD-L1, and; 5) create a metabolic environment (e.g. high lactate, low tryptophan) that is immunosuppressive.

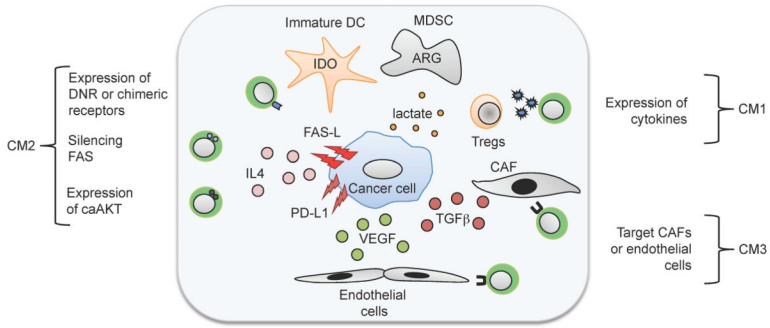

Fig. 5. Overcoming tumor-mediated immunosuppression

Countermeasures can be divided into the following strategies: 1) increasing the level of CAR-T-cell activation or decreasing physiological downregulation; 2) engineering the CAR-T cells to be resistant to the immune evasion strategies used by the tumor; and 3) targeting the cellular components of tumor stroma.

Table 1

CAR targets for hematological malignancies

Antigen	Malignancy	CAR ectodomain, antigen class	Clinical Trial
CD19 (29, 35–44)	B-cell	scFv, protein	published
CD20 (36, 45–48)	B-cell	scFv, protein	published
CD22 (10)	B-cell	scFv, protein	
CD30 (49–51)	B-cell	scFv, protein	ongoing
CD33 (52)	Myeloid	scFv, protein	
CD70 (21)	B-cell/T-cell	ligand, protein	
CD123 (53)	Myeloid	scFv, protein	
Kappa (54)	B-cell	scFv, protein	ongoing
Lewis Y (55, 56)	Myeloid	scFv, carbohydrate	ongoing
NKG2D ligands (20, 57–59)	Myeloid	ligand, protein	
ROR1 (7)	B-cell	scFv, protein	

Table 2

CAR targets for solid tumors

Antigen	Malignancy*	CAR ectodomain, antigen class	Clinical Trial
B7H3 (60)	sarcoma, glioma	scFv, protein	
CAIX (61, 62)	kidney	scFv, protein	published
CD44 v6/v7 (63, 64)	cervical	scFv, protein	
CD171 (65)	neuroblastoma	scFv, protein	published
CEA (66)	colon	scFv, protein	ongoing
EGFRvIII (67, 68)	glioma	scFv, protein	ongoing
EGP2 (69, 70)	carcinomas	scFv, protein	
EGP40 (71)	colon	scFv, protein	
EphA2 (14)	glioma, lung	scFv, protein	
ErbB2(HER2) (72–79)	breast, lung, prostate, glioma	scFv, protein	published
ErbB receptor family (22)	breast, lung, prostate, glioma	ligand, protein	
ErbB3/4 (80, 81)	breast, ovarian	scFv, protein	
HLA-A1/MAGE1 (82, 83)	melanoma	scFV, peptide/protein complex	
HLA-A2/NY-ESO-1 (84)	sarcoma, melanoma	scFV, peptide/protein complex	
FR-α (85–88)	ovarian	scFv, protein	published
FAP** (89)	cancer associated fibroblasts	scFv, protein	
FAR (90)	rhabdomyosarcoma	scFv, protein	
GD2 (91–93)	neuroblastoma, sarcoma, melanoma	scFv, ganglioside	published
GD3 (94)	melanoma, lung cancer	scFv, ganglioside	
HMW-MAA (95)	melanoma	scFv, proteoglycan	
IL11Rα (96)	osteosarcoma	ligand, protein	
IL13Rα2 (16–18, 25)	glioma	ligand, protein	ongoing
Lewis Y (55, 97, 98)	breast/ovarian/pancreatic	scFv, carbohydrate	published
Mesothelin (15, 99)	mesothelioma, breast, pancreas	scFv, protein	ongoing
Muc1 (100)	ovarian, breast, prostate	scFv, glycosylated protein	
NCAM (101)	neuroblastoma, colorectal	scFv, protein	
NKG2D ligands (20, 57–59)	ovarian, sacoma	native receptor, protein	
PSCA (102, 103)	prostate, pancreatic	scFv, protein	
PSMA (104, 105)	prostate	scFv, protein	
TAG72 (106, 107)	colon	scFv, carbohydrate	
VEGFR-2** (108, 109)	tumor vasculature	ligand/scFv, protein	ongoing

* many antigens are expressed on several malignancies; due to space limitations only examples are listed

** expressed on the tumor stroma