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The basic principles of chimeric antigen receptor (CAR) design

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Abstract

CARs are recombinant receptors that provide both antigen-binding and T cell activating functions. A multitude of CARs has been reported over the past decade, targeting an array of cell surface tumor antigens. Their biological functions have dramatically changed following the introduction of tri-partite receptors comprising a costimulatory domain, termed second generation CARs. These have recently demonstrated clinical benefit in patients treated with CD19-targeted autologous T cells. CARs may be combined with costimulatory ligands, chimeric costimulatory receptors or cytokines to further enhance T cell potency, specificity and safety. CARs represent a new class of drugs with exciting potential for cancer immunotherapy.

Introduction

CARs are recombinant receptors for antigen, which, in a single molecule, redirect the specificity and function of T lymphocytes and other immune cells. The general premise for their use in cancer immunotherapy is to rapidly generate tumor-targeted T cells, bypassing the barriers and incremental kinetics of active immunization.(1, 2) Once expressed in T cells, the CAR-modified T cells acquire supra-physiological properties and act as “living drugs” that may exert both immediate and long-term effects. The engineering of CARs into T cells requires that T cells be cultured to allow for transduction and expansion. The transduction may utilize a variety of methods, but stable gene transfer is required to enable sustained CAR expression in clonally expanding and persisting T cells. In principle, any cell surface molecule can be targeted through a CAR, thus over-riding tolerance to self-antigens and the antigen recognition gaps in the physiological T cell repertoire that limit the scope of T cell reactivity. Various T cell subsets, as well as T cell progenitors and other immune cells such as natural killer (NK) cells, can be targeted with a CAR. Redirecting immune reactivity towards a chosen antigen is not however the only purpose of smarter CARs, which are designed to accomplish much more than to target and initiate T cell activation. CARs with different strengths and quality of signaling have the potential to modulate T cell expansion and persistence, as well as the strength of T cell activation within the tumor microenvironment, features that dramatically alter the efficacy and safety of tumor-targeted T cells. In this regards, CARs provide a broader range of functional effects than transduced T cell receptors (TCRs), wherein strength of signaling, which is for the most part determined by the TCR’s affinity for antigen, is the principal determinant of T cell fate. CARs and TCRs have their respective advantages and disadvantages.(1-4) While the flexibility and “dynamic range” of CARs is attractive, current CARs are limited to recognizing cell surface antigens, whereas TCRs recognize both cell surface and intracellular proteins. CARs however do not require antigen processing and presentation by HLA, and are therefore more

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broadly applicable to HLA-diverse patient populations. We discuss here the targeting and signaling properties of CARs, focusing on their effects on T cell specificity, potency and safety. Other general aspects of adoptive T cell therapy that apply not only to the use of CARs but other T cell therapies as well, including T cell expansion methodologies, T cell subset selection and host conditioning, are beyond the scope of this review. Owing to the extraordinary potential of T cell engineering and the modular nature of their structure, CARs are rapidly evolving and show great promise for their successful utilization in a wide range of immunotherapies.

CAR targeting

CARs are recombinant receptors that typically target native cell surface antigens.(4) Unlike the physiological TCR, which engages HLA-peptide complexes, CARs engage molecules that do not require peptide processing or HLA expression to be recognized. CARs therefore recognize antigen on any HLA background, in contrast to TCRs, which need to be matched to the patient's haplotype. Furthermore, CARs can target tumor cells that have down-regulated HLA expression or proteasomal antigen processing, two mechanisms that contribute to tumor escape from TCR-mediated immunity.(5) Another feature of the broad applicability of CARs is their ability to bind not only to proteins but also to carbohydrate and glycolipid structures, again expanding the range of potential targets. A survey of antigens targeted to date by CARs is shown in Table 1.

The moieties used to bind to antigen fall in three general categories, either scFv's derived from antibodies, Fab's selected from libraries, or nature ligands that engage their cognate receptor (see Fig. 1, first generation CARs). Successful examples in each of these categories–too many to cite–have been reported (Table 1). scFv's derived from murine immunoglobulins are commonly used, as they are easily derived from well-characterized monoclonal antibodies. They however may prove to be more immunogenic than Fab's derived from human libraries or invariant human ligands.

The rules for selecting optimal epitopes for CAR targeting are still little known. The position of the epitope and its distance to the cell surface are expected to affect the binding to the antigen and the optimal formation of T cell-target conjugates and synapses,(6) but there is still little knowledge of the overall rules governing optimal epitope selection. Empirical observations indicate that the structure of the "spacer region" between an scFv and the transmembrane region (Fig. 1) can affect CAR specificity, but no definitive principles have yet emerged.(7) CAR length and protrusion from the T cell membrane are likely to affect synapse formation. The optimal affinity of CARs is also little defined. Few studies have attempted to address this question, which is of major importance in the case of TCRs(8, 9) and likely to impact CAR function as well. Informative studies comparing multiple CARs recognizing the same epitope with different affinities are still lacking. Finally, the effect of antigen density is not yet well defined. CARs typically target highly expressed antigens, but little is known about minimum thresholds. It is uncertain whether CARs are as exquisitely sensitive as TCRs.(9) If not, lesser sensitivity could represent a limitation in their activity against tumors expressing low antigen levels but may also turn into an advantage where avoidance of low-level antigen expression on normal cells is desirable. Thus, the antigen-binding moiety of the CAR is not just a targeting device but is integral to CAR function, which is largely but not solely defined by the signaling components incorporated into the CAR's cytoplasmic domain.

CAR signaling

The first fusion receptors shown to have T cell-activating potential on their own were chimeric molecules between CD3-ζ or Fc receptor γ and CD8, CD4, CD25 or CD16 (Fig. 1,

first generation CARs), which were shown to initiate phosphatidylinositol and tyrosine kinase pathways together with calcium influx in human T cell leukemias.(10-13) The addition of a hapten-specific scFv derived from amurine antibody to the extracellular portion of such fusions, termed a T-body (Fig. 1, first generation CARs), effectively redirected cytolysis by murine T cell hybridomas.(14) While CD3-ζ chain aggregation is sufficient to enable lytic activity in CTL lines, it is important to bear in mind that the strength of signal required for cytotoxicity is lower than that needed for other T cell functions. This likely underscores the limited therapeutic responses reported with activating receptors, the anti-tumoral effects of which are often confined to local administration models (15, 16) or short-term systemic models.(17) In transgenic mice, T cells expressing CARs that only comprise an activation domain in their cytoplasmic domain are prone to undergoing anergy.(18)

Once we could efficiently transduce human primary T cells, we found that CD3-ζ CARs failed to elicit a robust cytokine response, including interleukin-2, and support T cell expansion upon repeated exposure to antigen.(19) It would take the design of a tri-partite fusion receptor, possessing both activating and costimulatory properties (Fig. 1, second generation CARs), to obtain absolute T cell expansion of human peripheral blood T cells upon repeated exposure to antigen.(20) Significantly, these essential functions cannot be investigated in leukemic or immortalized T cell lines,(21) but only in primary T cells, which CAR investigators have now solidly embraced as the gold standard for evaluating CAR function in vitro or in vivo. Eventually dubbed second-generation CARs, receptors encompassing the CD3-ζ chain and the cytoplasmic domain of a costimulatory receptor such as CD28, 4-1BB, DAP10, OX40 or ICOS, were eventually reported (Table 1). The superior activity of dual-signaling receptors over the activating-only receptors was observed in several models utilizing mouse or human T cells.(22-24) The key attribute of dual-signaling receptors is to confer greater strength of signaling and persistence to the T cells, resulting in their overall greater potency. The enhanced persistence imparted by dual-signaling CARs has been confirmed in patients treated with a mixture of T cells transduced with either a CD28/CD3ζ or CD3ζ-only CAR.(25) Second generation CARs come in varied configurations, but exhaustive comparisons are still lacking. Some CD28 and 4-1BB–based second generation CARs were compared in animal models, but either one proved to be superior to the other in different contexts. In one study, Carpenito et al found that two CD28 and 4-1BB-based CD19-specific CARs had the same therapeutic activity, but noted that the T cells expressing the 19-BB CAR accumulated to greater levels over time, possibly in antigen-independent fashion.(26) This difference was not observed in another model.(27) More comparative studies are needed, noting that such studies must take into account the variability between CARs within any one given category. For example, different CD28/CD3ζ CARs differ in their ability to elicit interleukin (IL)-2 secretion.(20, 28) Furthermore, the location of the targeted epitope, its density, the affinity of the CAR and other topological effects of CAR structure affect CAR signaling, as discussed above. Comparisons will thus need to include multiple representatives of the evaluated categories to reach generalizable conclusions.

A third generation of CARs, encompassing two costimulatory domains combined with an activation domain in their cytoplasmic domain (Fig. 1, third generation CARs), has been described, which appears to confer yet greater potency to tumor targeted T cells in some mouse models.(26, 27, 29, 30) These more complex structures warrant further study as well. A first clinical study utilizing a CD20-specific CD28/4-1BB/CD3ζ did not shown dramatic responses,(31) but this early result should not in anyway detract from the potential value of these “triple-decker” CARs. Overall, more investigation is needed to attain a better understanding of optimal CAR signaling to promote sustained T cell function and survival, preventing premature death, rapid exhaustion or undue proliferation.

Potentiation and complementation of CAR function: costimulatory ligands, chimeric costimulatory receptors and cytokines

Costimulatory support can be engineered into T cells otherwise than through a CAR. (Fig. 2) The coexpression of chimeric costimulatory receptors (CCRs), costimulatory receptor ligands and cytokines, have all been utilized to modulate the function and/or survival of CAR-transduced T cells.

Costimulatory ligands

The constitutive expression of costimulatory ligands on the T cell surface (Fig 2) provides a powerful means to potentiate CAR-targeted T cells. Several ligands for Ig super-family and TNF receptor family costimulatory receptor, including CD80, CD86, 4-1BBL, OX40L and CD70, have been shown to enhance T cell proliferation and cytokine secretion upon antigen engagement.(32) The combination of two ligands, in particular CD80 and 4-1BBL, results in sustained in vivo T cell expansion and persistence, associated with the rejection of massive, established tumor burdens.(32) Both auto- and trans-costimulation have been shown to contribute to enhanced T cell activity in this context, which may be useful to enhance adoptive cell therapies utilizing CAR- or TCR-transduced T cells. The occurrence of costimulatory ligands found on tumor cells is also likely to influence the activity of CAR-modified T cells, whether they are activating (e.g., CD80, CD40L, 4-1BBL) or inhibitory (e.g., PD-L1).

Chimeric costimulatory receptors

CCRs mimic costimulatory signals but, unlike CARs, do not provide a T cell activation signal. Their purpose is to provide costimulation, e.g. a CD28-like signal,(33) in the absence of the natural costimulatory ligand on the antigen-presenting cell (Fig.2). They thus provide a means for the tumor to direct counterfeit costimulation specifically within the tumor microenvironment. CCRs targeting the glycolipid G_{D2}, MUC16, PSMA and the α-folate receptor have been described, utilized in conjunction with a TCR or a CAR to augment T cell reactivity against dual-antigen expressing T cells, reinforcing T cell activation in the absence of natural costimulatory ligands and in antigen-dependent fashion.(20, 33-35) Under particular conditions, CCRs may also be utilized to improve selective tumor targeting, as further discussed below.

Cytokines

Another approach to enhance the potency of CAR-targeted T cells is to further genetically modify the T cells to secrete pro-inflammatory or pro-proliferative cytokines. Its purpose is not only to provide autocrine support to enhance the function, proliferation and/or persistence of CAR-expressing T cells, but also to favorably alter the tumor microenvironment and recruit endogenous innate and cognate immune effectors. The expression of T cell-encoded cytokines additionally aims to limit the systemic toxicity of many cytokines. Preclinical reports investigating γ_c cytokines or IL-12 show great promise for this approach.

T cell-encoded IL-15 increases the viability and proliferation of human peptide-specific T cells despite withdrawal of exogenous IL-2. (36) Improved in vitro and in vivo expansion of human Epstein Barr Virus specific cytotoxic T cells (EBV-CTLs) following retroviral gene transfer of the IL-2 or IL-15 cDNA has also been reported.(37) The report of an isolated IL-15 modified $CD8^+$ T cell clone exhibiting logarithmic proliferation for over 1 year in the absence of exogenous cytokine support cautions against this approach, (38) although this concern may be mitigated by using a suicide gene to potentially remove T cells via drug-

induced apoptosis(37). Cytokine modified T cells used as antigen presenting cells to expand tumor targeted T cells, expressing either IL-7 and IL-12 (39) or IL-21, (40) successfully expanded tumor-targeted T cells, with a more favorable central memory phenotype in the latter case. Comparisons between cytokines expressed at different levels in different assays or tumor models are complex to interpret. Nonetheless, we compared CD19 CAR-targeted human 19z1$^+$ T cells that constitutively expressed either IL-2, IL-7, IL-15 or IL-21 under standardized conditions and found that all four γ_c cytokines enhanced tumor rejection in a xenotransplant model of human CD19$^+$ tumor, more so, in this context, with IL-7 and IL-21 than IL-2 and IL-15 (41).

In an immune competent syngeneic tumor model, CD19-targeted, CAR-modified T cells expressing IL-12 showed greater efficacy than CAR-modified T cells alone.(42) Significantly, IL-12 modified T cells eradicated CD19$^+$ tumors in the absence of any prior conditioning and, additionally, exhibited resistance to regulatory T cell (Treg) inhibition. In a murine melanoma model, transgenic Pmel-1 CD8$^+$ T cells, as well as Pmel-1 TCR-transduced murine T cells that were modified to express IL-12, eradicated established tumors with significantly greater potency than T cells expressing the Pmel-1 TCR alone(43). Similarly improved outcomes were obtained in tumor bearing mice treated with IL-12 secreting T cells targeted to tumor by an anti-VEGF receptor-2 CAR.(44) In both latter models, the effect of IL-12 appears to act at least in part by altering myeloid cells in the tumor microenvironment.(44, 45)

The titration of cytokine secretion by T cells is important because of the potential toxicity of elevated systemic levels. One may address this concern by appropriately calibrating promoter strength or through conditional cytokine release following T cell activation utilizing nuclear factor of activated T cells (NFAT)-inducible promoters.(46) Using this approach to control cytokine secretion, two trials treating metastatic NY-ESO-1$^+$ tumors with autologous TCR-targeted T cells or tumor-infiltrating T cells secreting IL-12, are under way at the NCI (NCT01457131, NCT01236573).

CARs in the clinic

The CD19 paradigm

The most investigated target to date is CD19, an attractive target for CAR-based therapy as it is present in most B cell leukemias and lymphomas but not in any normal tissue other than the B cell lineage.(47, 48) CD19+ malignancies were the first cancers to be eliminated by CAR-engineered human T cells administered intravenously to systemic tumor-bearing mice.(49) Successful B cell tumor eradication was eventually obtained with different CD19 CARs(15, 22-24), paving the way for several on-going clinical trials. The targeting of CD19 has thus become a paradigm for evaluating CAR technology.(50) We estimate that at least 50 patients with leukemia or lymphoma have been treated at the time this review is written, 28 of which were reported from 5 centers in the past year.(25, 51-56) The reported clinical outcomes were recently reviewed elsewhere (57, 58) and are briefly summarized here.

The largest series and most dramatic early results were reported from the National Cancer Institute (NCI), Memorial Sloan-Kettering Cancer (MSKCC) and the Abramson Family Cancer Research Institute at the University of Pennsylvania (UPenn). These clinical trials followed the same overall procedures, including patient T cell apheresis, retroviral or lentiviral CAR transduction, T cell expansion and host conditioning prior to T cell infusion. They however differ in several regards, including not only CAR design (same CD28/CD3z dual-signaling domain utilized at the NCI and MSKCC, 4-1BB/CD3z utilized at UPenn), but also T cell manufacturing, conditioning chemotherapy, tumor burden, tumor chemo-sensitivity, and T cell dosage, which are reviewed in detail in ref. (57) Kochendorfer and the

NCI group reported on 8 patients (4 with CLL, 3 with follicular lymphoma, and one with marginal zone lymphoma) conditioned with fludarabine and cyclophosphamide, and further given IL-2 after T cell infusion. Amongst the 4 CLL patients, one achieved a complete response (CR) and another stable disease (SD). Four of the 8 treated patients exhibited B cell aplasias.(53) Brentjens and colleagues reported on 8 patients with CLL and 1 patient with B cell acute lymphoblastic leukemia (B-ALL), whose diseases were resistant to the milder cyclophosphamide conditioning regimen used in 5 of the 8 CLL patients (the first 3 were given T cells without any prior conditioning; no significant response was obtained). In the CLL cohort, 2 patients had stable disease and one patient demonstrated a substantial lymph node reduction. None of the CLL patients developed B cell aplasia, in contrast to the one patient with relapsed B-ALL. (47) June and colleagues (54, 55) treated 3 patients with bulky CLL that were conditioned with bendamustine, a highly active agent in these in these patients. Two of them achieved dramatic, long-lasting CR's. The reasons for the different outcomes in these 15 CLL patients treated at 3 different centers, which include significant differences in CAR design, conditioning intensity and the selection of chemosensitive patients amongst many variables,(57) still remain to be elucidated. Altogether, better responses were observed following more active conditioning, resulting in a 25% CR rate in 12 CLL patients treated with T cells following chemotherapy conditioning. Much will undoubtedly be learned about the role of the CAR and other parameters by comparing biological and clinical outcomes using similarly manufactured T cells in similarly selected patients.

Solid tumors

One next frontier for CAR-based therapies is to take on solid tumors. Early attempts with first generation CARs did not yield very encouraging data,(59) although one recent study targeting the G_{D2} ganglioside in children with neuroblastoma showed 2 CRs in 13 patients. (60) Solid tumors present a different set of challenges compared to B cell malignancies: overall lesser sensitivity to T cell mediated cytotoxicity, a microenvironment that presents with an array of immunosuppressive mechanisms differing between tumor types, and a paucity of target antigens with an expression profile as favorable as CD19. Despite an impressive number of investigated targets (Table 1), few target candidates are tumor-specific, or restricted to the tumor and a "dispensable" normal cell type or a tissue that is sheltered from an immune attack. In this perspective, identifying valid targets to achieve efficacious tumor rejection while ensuring patient safety is an essential goal that requires further investigation. Nonetheless, several trials utilizing first and second generation CARs are under way and listed on the US clinicaltrials.gov web site.

CAR safety

The two main safety concerns associated with the use of CARs are the targeted destruction of normal tissues and cytokine storms associated with large-scale immune responses. The toxicity of the different conditioning regimens used in conjunction with adoptive T cell therapies is also a significant issue to consider but is beyond the scope of this review.

On-target, off-tumor responses

The immune-mediated rejection of normal tissues that express the targeted antigen is referred to as an "on-target, off-tumor" response. This occurrence is best illustrated in the B cell aplasias induced by CD19-targeted CARs.(52-54) Whereas B cell aplasia can be effectively managed by administering intravenous immunoglobulin, such collateral damage may not be tolerable in many other instances. This may for example be the case for her2, (61) which is expressed at low level in several normal tissues, including heart and pulmonary vasculature. Other examples, for which no toxicities have been reported to date,

include PSMA, which is highly expressed in castrate-resistant, metastatic prostate cancer, (62) but is detected in astrocytes type II, the renal proximal tubule and the jejunum brush border; ROR1, which is expressed in a subset of leukemias and lymphomas, but is also detected in adipocytes.(63) T cells are very effective at destroying normal tissues that express the targeted antigen, as exemplified by the ocular and vestibular effects of MART-1-specific T cells(64) and the cholestatic effect of T cells targeted to carbonic anhydrase IX. (65) It is presently unknown whether the very low level expression of antigens such as PSMA and ROR1 on normal tissues will expose these to immune destruction. This problem would be easily resolved if there were truly tumor-specific cell surface molecules to target, but such molecules are so far very rare. The identification of restricted CAR targets is therefore a high priority.

Cytokine storms

The second major concern is that of “cytokine storms” associated with intense anti-tumor responses mediated by large numbers of activated T cells.(53-55) These typically cause high fever and hypotension, potentially resulting in organ failure. Their management may require steroids, vasopressors and/or supportive therapy delivered in the intensive care unit. Grupp and colleagues have observed that IL-6 blockade utilizing Tocilizumab may be effective in steroid-refractory circumstances, without compromising T cell efficacy.(66) Unlike many conventional drug-induced side effects, this toxicity cannot be controlled by simply reducing drug dosage, as proliferating T cells will increase in numbers and eventually reach critical levels where a synchronous cytokine response exceeds tolerability. Split T cell dosing or short-lived T cells may partially reduce this effect, but more fundamental solutions are needed to reduce and ideally prevent the occurrence of overwhelming T cell activity..

Emerging solutions to improve CAR safety

Recognizing that CAR-modified T cells are in general well tolerated, their broader use requires having solid strategies to treat or, better, prevent on-target, off-tumor effects and cytokine storms. On therapeutic option is to utilize suicide genes to have a means to eliminate an excessive response. Herpes simplex virus thymidine kinase(67) and inducible caspase-9(68) are clinically tested systems that could be used to halt deleterious responses. (69) The downsides to this approach are that it is reactive, not preventative, and that active T cells will be eliminated, possibly curtailing the therapy. A better understanding of cytokine storms may offer novel prospects for reducing toxicity without compromising therapy and limiting the use of corticosteroids.(66) Significantly, we find, in ALL patients treated with a CD19-specific CD28/CD3z CAR, that stronger cytokine responses occur in patients with large tumor burdens but not in those with minimal residual disease at the time of T cell infusion, a finding that suggests that reducing tumor burden by alternative means prior to T cell infusion will reduce the risk of T cell-induced cytokine-mediated toxicity following a subsequent T cell infusion (unpublished observations). Ultimately, the design of T cells that are effective, highly tumor-specific, and regulated in their maximal accumulation and activation (so as to prclude toxic cytokine elevation), will represent a valuable advance for the use of CARs. One approach to improve tumor selectivity, based on combinatorial antigen recognition, is reviewed below.

New directions in the CAR industry

New technologies or concepts other than the design of better CARs and their combination with costimulatory ligands, chimeric costimulatory receptors or cytokines, are emerging to broaden or improve the use of CARs. These include improved CAR delivery systems, the design of CARs that recognize intracellular antigens, and combinatorial antigen recognition to increase T cell specificity and potency.

CAR delivery

The mechanics of T cell transduction are beyond the scope of this review but are briefly addressed here. CARs began to be investigated in meaningful ways when methods for the transduction of human primary T cells became available(70-72). For the past 15 years, virtually all CAR studies have relied on retroviral vectors, including gamma-retroviral and lentiviral vectors.(73) Most current clinical trials utilize retroviral vectors derived from murine leukemia virus or human immune deficiency virus-1. Although retroviral vectors can induce insertional oncogenesis in hematopoietic progenitors(74, 75), T cells appear to be far less susceptible to retroviral vector-induced transformation(76-79). Transposases, which also provide random vector integration(80), are starting to be evaluated in the context of CAR therapy.(81) The relative advantages/disadvantages of these different integrating systems have not yet been elucidated, but will hinge on CAR expression levels, silencing over time, safety features, ease of manufacturing and usage, and cost. Although T cell transformation secondary to insertional mutagenesis has not been reported to date, site-directed vector integration into genomic safe harbors(82) may eventually enable to achieve long-term CAR expression without any risk of insertional oncogenesis. Alternative approaches that do not rely on transgene integration, which utilize RNA electroporation,(83, 84) or cell surface conjugation,(85) result in transient CAR expression, precluding effective T cell persistence beyond a week or two. The usefulness of transiently CAR-expressing T cells, which would presumably require multiple infusions to provide meaningful tumor responses but may reduce destruction of normal tissues or prevent T cell accumulation to levels exposing to the risk of cytokine storms, remains to be established.

Another key aspect of CAR delivery is the addressee and identifying what T cells, expanded under what conditions, are better suited for optimal tumor eradication. As stated above, this topic is beyond the scope of this review, recognizing that different T cell subsets (CD4+ or CD8+ αβ T cells, γδ T cells, naïve, central memory, effector memory, virus-specific T cells and the recently described stem-like memory T cells) (86, 87) warrant further investigation to delineate whether different CAR designs are best suited for different T cell types. CARs are also functional in Tregs(88) and in the progeny of transplanted T cell progenitors(89).

CAR-like TCRs and TCR-like CARs

The transfer of TCRs into T cells poses two particular challenges that CARs elude: the risk of mispairing between endogenous and transduced TCR chains(90), and competition for rate-limiting CD3 complex(91), which is required for TCR signaling. Several approaches have been proposed to prevent TCR chain mispairing, including partial murinization of the constant regions, the addition of disulfide bonds and altering the knob-in-hole directional interaction between constant regions. Another approach is to add signaling domains to the intracellular portions of the transduced TCR (92), similarly to first generation CARs, which Willemsen and Debets showed could at once avert TCR mispairing and reduce association with CD3.(92, 93)

Conversely, HLA-peptide complexes can be targeted by antibody structures that mimic TCR recognition. CARs may be advantageous in this regards as they do not interfere nor compete with the native TCR and CD3, and can be further endowed with costimulatory capabilities. Human Fab fragments specific to peptide/MHC molecules have been derived from phage display libraries.(94). While many preferentially bind to MHC, (95) some high-affinity Fabs with greater binding affinity for the peptide have been generated and shown by crystallographic analysis to have a binding footprint to MHC/peptide complexes similar to that of TCRs(96). The therapeutic potential and toxicity of these TCR-like CARs remain to be established.

Combinatorial antigen recognition

T cells may also be rendered more tumor-selective through combinatorial antigen recognition. We recently described a strategy that integrates combinatorial antigen recognition, split signaling and, critically, balanced strength of T cell activation and costimulation, to generate T cells that eliminate target cells that express a combination of antigens while sparing cells that express each antigen individually.(97) In this approach, T cell activation requires TCR or CAR-mediated recognition of one antigen, while costimulation is independently mediated by a CCR(33) specific for a second antigen. To achieve tumor selectivity, we diminished the efficiency of T cell activation to a level where it was ineffective without rescue provided by simultaneous CCR recognition of the second antigen.(98) Novel approaches to enforce tumor specificity in the face of truly unique target antigens are an important direction for future immunotherapies.

Perspectives

While there remains a vast number of important biological questions to address – optimizing CAR signaling, defining optimal targets, working out optimal combinatorial strategies, identifying the best and most practical processes for T cell subset selection and T cell manufacturing, reducing T cell-mediated toxicity and the toxicity of host conditioning – the first clinical successes of CAR therapy are being registered. The prospect of meeting the challenging premise of adoptive T cell therapy – to achieve specific tumor destruction with one or few T cell infusions and limited collateral damage to normal tissues– may be within reach. The targeting of B cell malignancies through CD19 has emerged as the paradigm for the CAR field. At the present, it also stands as an exception. The identification of safe targets in a broad range of tumor types, eventually in combinatorial fashion, and harnessing CAR technology for the treatment of solid tumors, are future challenges for all adoptive T cell therapies including those utilizing CARs. As this review aims to convey, there are many exciting strategies in the pipeline, and as many reasons to be optimistic about the prospects for CAR therapies. Providing access to targeted T cell therapies to all medical centers and their patients will pose additional biological, logistical and economic challenges, which are beyond the scope of this review. The fact that models for broad access to targeted T cell therapies are increasingly discussed is testimony to the therapeutic potential and rising credibility of CARs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

Chimeric antigen receptors (CARs) are a new class of drugs with great potential for cancer immunotherapy. Upon their expression in T lymphocytes, CARs direct potent, targeted immune responses that have recently shown encouraging clinical outcomes in a subset of patients with B cell malignancies. This review focuses on the design of CARs, including the requirements for optimal antigen recognition and different modalities to provide costimulatory support to targeted T cells, which includethe use of second and third generation CARs, costimulatory ligands, chimeric costimulatory receptors and cytokines.

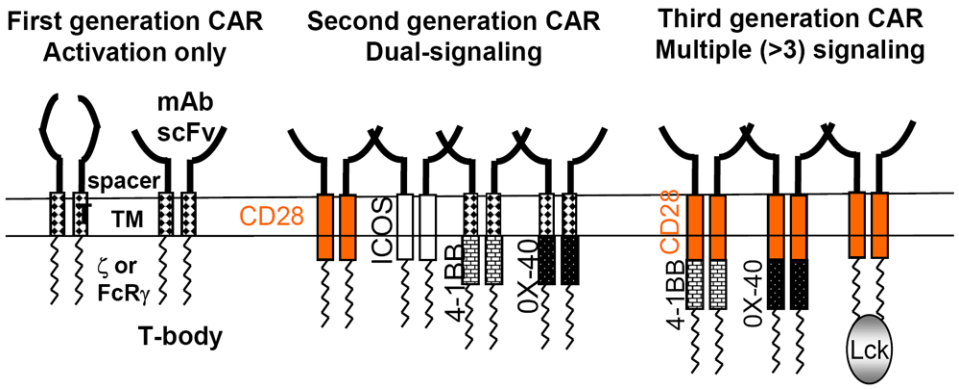

Figure 1. Three generation of CARs

Left: First generation CARs, including activating receptors such as CD8/CD3z fusion receptors and T-bodies; middle: Second generation CARs providing dual-signaling to direct combined activating and costimulatory signals; right: Third generation CARs comprising more complex structures with 3 or more signaling domains.

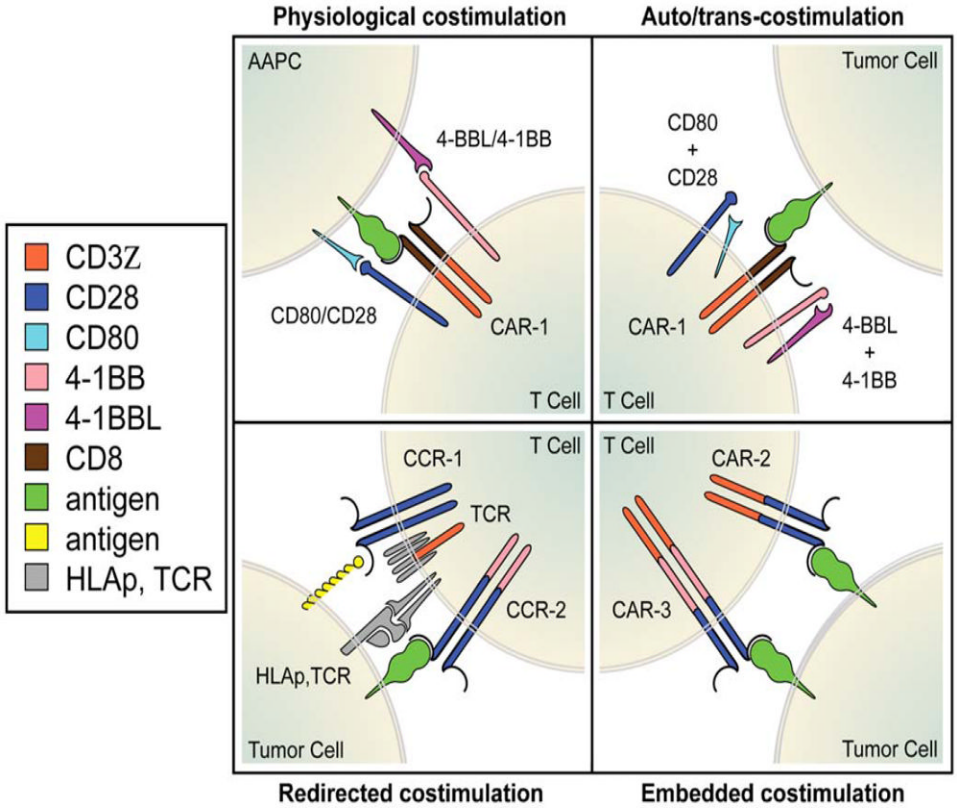

Figure 2. Strategies to provide costimulatory support to CAR-modified T cells

From upper left: UL, physiological costimulatory ligand display by professional or artificial antigen presenting cells; UR, auto- and trans-costimulation by T cells expressing costimulatory ligands; LR, embedded costimulation provided by second or third generation CARs; LL, redirected costimulation mediated by an antigen-specific chimeric costimulatory receptor (CCR).

Table 1

Antigens targeted by CARs

Target Antigen	Associated Malignancy	Receptor Type (Other specificity)	In vivo studies	Reference
α-Folate receptor	Ovarian cancer	ScFv-FcεRIγ	Phase I	(1)
	epithelial cancers	scFv-41BB-CD3ζ	+	(2)
CAIX	Renal-cell carcinoma	scFv-CD4- FcεRIγ	Phase I	(3-5)
	Renal cell carcinoma	G250-FcεRIγ	-	(6-8)
CD19	B cell malignancies	scFv-CD3ζ (EBV)	-	(9)
	B cell malignancies	scFv-CD3ζ	+	(10, 11)
	B cell malignancies	scFv-CD28-CD3ζ	+	(12-16)
	Refractory Follicular Lymphoma	scFv-CD3ζ	Phase I	(17, 18)
	B cell malignancies	scFv-CD28-CD3ζ	+	(19-22)
	ALL	scFv-41BB-CD3ζ	-	(23)
	ALL	scFv-41BB-CD3ζ	+	(24)
	B cell malignancies	scFv-CD3ζ (Influenza MP-1)	+	(25)
	B cell malignancies	scFv-CD3ζ (VZV)	-	(26)
	ALL	FMC63-CD28-41BB- CD3ζ	+, -	(27-29)
	B cell malignancies	FMC63-41BB-CD3ζ	+	(30)
	Follicular lymphoma	FMC63-CD28-CD3ζ	NCT00924326	(31)
	B cell malignancies	FMC63-CD28-CD3ζ	NCT00924326	(32)
	CLL & ALL	SJ25C1-CD28-CD3ζ	(NCT00466531 NCT01044069)	(33)
	CLL	FMC63-41BB-CD3ζ	NCT01029366	(34, 35)
	Lymphoma	scFv-CD3z + scFv-CD28-CD3ζ	Phase I	(36)
CD20	Lymphomas	scFv-CD28-CD3ζ	-	(37)
	B cell malignancies	scFv-CD4-CD3ζ	-	(38)
	B-cell lymphomas	scFv-CD3ζ	-	(39, 40)
	Mantle cell lymphoma, indolent B cell lymphomas	scFv-CD28-41BB-CD3ζ	NCT00621452	(41, 42)
CD22	B cell malignancies	scFV-CD4-CD3ζ	-	(38)
CD23	CLL	scFv-CD28-CD3ζ	+	(43)
CD24	Pancreatic adenocarcinoma	scFv- CD28-FcεRIγ	+	(44)
CD30	Lymphomas	scFv-FcεRIγ	-	(45)
	Hodgkin lymphoma	scFv-CD3ζ (EBV)	+	(46)
		scFv-CD28-CD3ζ (EBV)	+	(47)
CD33	AML	scFv-CD28-CD3ζ	-	(48)
		cFv-41BB-CD3ζ		
		scFv-CD28-CD3ζ (EBV)	+	(49)
CD38	Non Hodgkin lymphoma	scFv-41BB-CD3ζ	+	(50)
CD44v7/8	Cervical carcinoma	scFv-CD8-CD3ζ	+	(51)
CEA	Colorectal cancer	scFv-CD3ζ	+	(52-56),
		scFv-FcεRIγ	+	(55, 57)
		scFv-CD3ε	-	(58)

Target Antigen	Associated Malignancy	Receptor Type (Other specificity)	In vivo studies	Reference
		scFv-CD28-CD3ζ	-	(59)
		scFv-CD28-CD3ζ	+	(60, 61)
EGFRvIII	Glioblastoma	scFv-CD28-41BB- CD3ζ	NCT01454596	(62)
EGP-2	Multiple malignancies	scFv-CD3ζ	-	(63)
		scFv-FcεRIγ	-	(63),(64)
EGP-40	Colorectal cancer	scFv-FcεRIγ	-	(65)
EphA2	Glioblastoma	scFv-CD28-CD3ζ	+	(66)
erb-B2	Breast and others	scFv-CD28-CD3ζ	+	(67, 68)
		scFv-CD28-CD3ζ (Influenza)	+	(69)
		scFv-CD28mut.-CD3ζ	+	(70)
	Prostate cancer	scFv-FcεRIγ	+	(71)
	Colon cancer			(72)
	Various tumors	scFv-CD28-41BB- CD3ζ	+	(73, 74)
erb-B 2,3,4	Breast and others	Heregulin-CD3ζ	-	(75),(76)
		scFv-CD3ζ	+	(77)
FBP	Ovarian cancer	scFv-FcεRIγ	+	(78-80)
	Ovarian cancer	scFv-FcεRIγ (alloantigen)	+	(81)
Fetal acethylcholine e receptor	Rhabdomyosarcoma	scFv-CD3ζ	-	(82)
G_{D2}	Neuroblastoma, Melanoma	scFv-CD3ζ	-	(9, 10)
		scFv-CD3ζ	NCT00085930	(83, 84)
		scFv-CD28-OX40-CD3ζ	-, +	(74, 85, 86)
		scFv-CD3ζ (VZV)	-	(26)
	Ewing sarcoma	scFv-CD28-CD3ζ	+	(87)
G_{D3}	Melanoma	scFv-CD3ζ, ScFv-CD3ε	-	(88)
		scFv-CD28-CD3ζ	+	(89)
Her-2	Medulloblastoma	scFv-CD3ζ	+	(90)
		scFv-CD28-CD3ζ	+	(91)
	Pancreatic adenocarcinoma	scFv-CD28-41BB- CD3ζ	+	(44)
	Glioblastoma		Phase I	(92)
	Osteosarcoma	scFv-CD28-CD3ζ	+	(93)
	Ovarian	scFv-CD28-CD3ζ	+	(94)
HMW-MAA	Melanoma	scFv-CD3ζ, ScFv-CD28-CD3ζ	-	(95)
IL-11Rα	Osteosarcoma	scFv-CD28-CD3ζ	+	(96)
IL-13R-α2	Glioma	IL-13-CD28-4-1BB- CD3ζ	+	(97)
	Glioblastoma	IL-13- CD3ζ	+	(98, 99)
	Medulloblastoma	IL-13- CD3ζ	+	(100)
KDR	Tumor neovasculature	scFv-FcεRIγ	-	(101)
κ-light chain	B-cell malignancies (B-NHL, CLL)	scFv-CD3ζ	+	(102)
		scFv-CD28-CD3ζ	+	(102)
Lewis Y	Various carcinomas	scFv-FcεRIγ	-	(103)

Target Antigen	Associated Malignancy	Receptor Type (Other specificity)	In vivo studies	Reference
	Epithelial derived tumors	scFv-CD28-CD3ζ	+	(104-106)
L1-cell adhesion molecule	Neuroblastoma	scFv- CD3ζ	Phase I	(107, 108)
MAGE-A1	Melanoma	scFV-CD4-FcεRIγ	-	(109)
		scFV-CD28-FcεRIγ		
Mesothelin	Mesothelioma	scFv-41BB-CD3ζ	+	(73, 110, 111)
Murine CMV infected cells	Murine CMV	Ly49H- CD3ζ	+	(112)
MUC1	Breast, Ovary	scFV-CD28-OX40- CD3ζ	+	(113, 114)
MUC16	Ovary	scFV-CD28-CD3ζ		(115)
NKG2D Ligands	Myeloma, ovarian & other tumors	NKG2D-CD3ζ	+	(116-121)
NY-ESO-1 (157-165)	Multiple myeloma	scFv-CD28-CD3ζ	+	(122)
Oncofetal antigen (h5T4)	Various tumors	scFV-CD3ζ (vaccination)	+	(123)
PSCA	Prostate carcinoma	7F5-β2-CD3ζ	-	(124)
		scFv-CD3ζ		(125)
PSMA	Prostate cancer/ tumor vasculature	scFv-CD3ζ	+	(126, 127)
	Prostate/tumor vasculature	scFv-CD28-CD3ζ	-	(128)
		scFv-CD3ζ	+	(129)
ROR1	B-CLL and mantle cell lymphoma	scFv-CD28-CD3ζ	+	(130)
Targeting via mAb IgE	Various tumors	FcεRI-CD28-CD3ζ	+	(131)
TAG-72	Adenocarcinomas	scFv-CD3ζ	+	(132),(133)
VEGF-R2	Tumor neovasculature	scFv-CD3ζ	-	(134)
Biotinylated molecules	Various tumors-ovarian	BBIR-z/CD28z	+	(135)