

Engineering CAR-T cells: Design concepts

Shivani Srivastava and Stanley R. Riddell

Program in Immunology, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N., Seattle, WA 98109, USA

Despite being empirically designed based on a simple understanding of TCR signaling, T cells engineered with chimeric antigen receptors (CARs) have been remarkably successful in treating patients with advanced refractory B cell malignancies. However, many challenges remain in improving the safety and efficacy of this therapy and extending it toward the treatment of epithelial cancers. Other aspects of TCR signaling beyond those directly provided by CD3 ζ and CD28 phosphorylation strongly influence a T cell's ability to differentiate and acquire full effector functions. Here, we discuss how the principles of TCR recognition, including spatial constraints, Kon/Koff rates, and synapse formation, along with in-depth analysis of CAR signaling might be applied to develop safer and more effective synthetic tumor targeting receptors.

Introduction

Advances in genetic engineering combined with an improved understanding of T cell recognition have led to the design of synthetic tumor targeting receptors, termed CARs, that can be introduced into human T cells to redirect antigen specificity and enhance function in adoptive immunotherapy. The basic concept underlying the design of CARs is to link an extracellular ligand recognition domain, typically a single-chain variable fragment (scFv), to an intracellular signaling module that includes CD3 ζ to induce T cell activation upon antigen binding. The modular structure has been extended from first-generation CARs with only a CD3 ζ signaling domain to second- and third-generation CARs that link the signaling endodomains of CD28, 4-1BB, or OX40 to CD3 ζ in an attempt to mimic costimulation (signal 2) that is provided during T cell receptor (TCR) recognition by antigen-presenting cells (APCs) and required for full physiological T cell activation (Figure 1) [1,2]. The approach of providing one or more costimulatory signals in *cis* in second- and third-generation CARs augments cytokine production and proliferation of CAR-T cells *in vitro* and second-generation CD19-specific CARs carrying CD28 or 4-1BB signaling moieties have demonstrated potent *in vivo* antitumor activity in preclinical models and clinical trials for B cell malignancies [3–14].

Despite being empirically designed based on the fundamentals of TCR signaling, T cells engineered with

CARs specific for the lineage restricted CD19 molecule have been remarkably successful in treating patients with advanced refractory B cell malignancies. Complete tumor regression is achieved in a substantial fraction of patients in multiple studies (Table 1); however, serious cytokine release syndromes and organ toxicities related to activation of T cell effector functions through the CAR are observed in patients with a high tumor burden [10,12–14]. Because CARs confer HLA-independent recognition, all patients with tumors that express the target antigen are potentially eligible for therapy and numerous laboratories in academia and the biotechnology industry are now working on extending this novel therapy to common epithelial cancers. Success in this endeavor may require improving the tumor specificity, sensitivity, and safety of CARs and understanding how synthetically constructed receptors direct T cell effector function and fate.

The design and function of synthetic CARs might be improved by applying recent insights from studies of TCR recognition. Downstream signaling, T cell function, and cell fate determination are significantly impacted by TCR affinity for MHC/peptide complexes determined by K_{on}/K_{off} rates, evolutionarily determined spatial constraints between the T cell and APC, immunological synapse formation, TCR clustering, and interaction of CD4 and CD8 coreceptors with MHC [15–17]. Below we discuss how specificity, structural constraints imposed by T cell:target cell interactions, and receptor affinity impact CAR design and function, within a framework of the current understanding of how these features of TCR signaling impact T cell recognition.

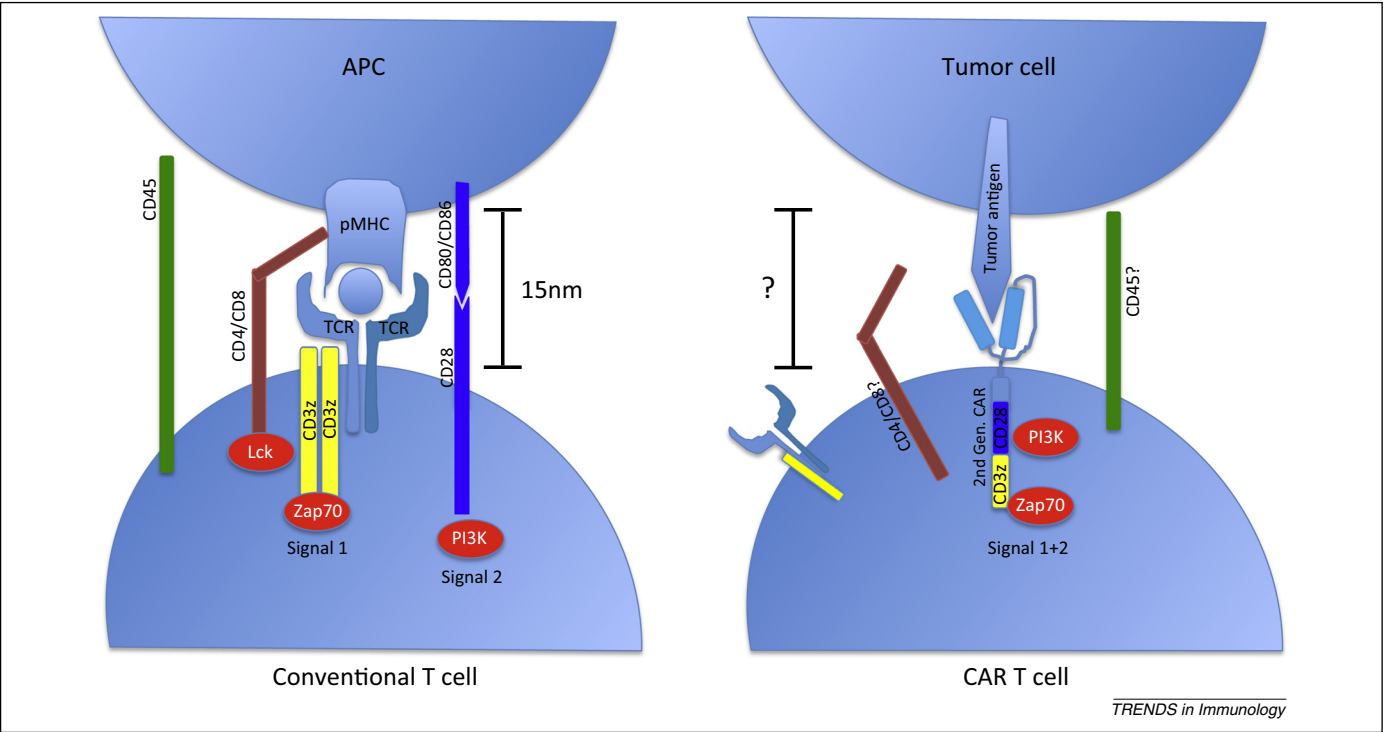
Specificity: discriminating tumor cells from normal cells

A major challenge in CAR design is ensuring elimination of tumor cells while sparing healthy tissue and minimizing toxicity. Conventional T cells have an extraordinary ability to distinguish foreign peptide MHC (pMHC) from self-pMHC through their TCRs. As few as one to ten agonist pMHC in a sea of thousands of self-pMHC can trigger T cell activation [18–20]. This specificity is achieved in part by selection in the thymus of a TCR repertoire enriched for low avidity to self-pMHC but containing sufficient diversity to include TCRs with high avidity for foreign pMHC the host may encounter. Specificity is further dictated by the requirement for two simultaneous signals delivered through TCR/CD3 ζ (signal 1) and costimulatory receptors (signal 2) on T cells, both of which are provided only by activated APCs.

Corresponding author: Riddell, S.R. (sriddell@fredhutch.org).

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Figure 1. Signaling of conventional and chimeric antigen receptor (CAR)-T cells. Left: Delivery of signals 1 and 2 to conventional T cells is initiated by the T cell receptor (TCR) interacting with foreign-peptide MHC (pMHC) on an antigen-presenting cell (APC). The spatial distance between the T cell and the APC is ~15 nm, which physically excludes from the synapse the inhibitory receptor CD45 because of its large ectodomain. Interaction of CD4/CD8 coreceptors with MHC recruits Lck to the TCR complex, where it phosphorylates and activates Zap70, which provides signal 1. Ligation of the costimulatory receptor CD28 by CD80/CD86 results in PI3K activation and delivers signal 2 for full T cell activation. Right: Single-receptor design showing a second-generation CAR containing CD3 ζ and CD28 endodomains *in cis*. Activation of these CARs by a single tumor antigen is sufficient to deliver both signals 1 and 2 *in cis*, resulting in T cell activation. The spatial distance between CAR-T cells and target tumor cells is not known, nor is it known whether this distance is small enough to physically exclude the phosphatase CD45 from the synapse. It is also unknown whether CARs interact with endogenous TCR/CD3 ζ or CD4/CD8 coreceptors.

In the case of CARs, specificity and safety are determined by the choice of target molecule, and most targets identified thus far are not entirely tumor restricted in their expression. For example, CD19 and CD20 are expressed on both malignant and healthy B cells and thus T cells

expressing second-generation CARs specific for these antigens eradicate tumor cells but also destroy healthy B cells, resulting in B cell depletion that is sustained as long as functional CAR-T cells persist *in vivo* [10,12–14]. The coexpression of a conditional suicide gene in CAR-T cells that

Table 1. Clinical trials of CD19 CAR-T cell therapy.

Study	CAR design	Trial design	Malignancy	Outcome ^a	Refs
Kochenderfer <i>et al.</i> 2010	Murine CD19scFv–CD28/CD3 ζ	Case report	Follicular lymphoma	PR	[3]
Brentjens <i>et al.</i> 2011	Murine CD19 scFv–CD28/CD3 ζ	Pilot (nine patients)	CLL (<i>n</i> = 8), acute lymphoblastic leukemia (<i>n</i> = 1)	One PR	[4]
Kalos <i>et al.</i> 2011 Porter <i>et al.</i> 2011	Murine CD19 scFv–4-1BB/CD3 ζ	Pilot (three patients)	CLL (<i>n</i> = 3)	Two CR, one PR	[5,6]
Savoldo <i>et al.</i> 2011	Murine CD19 scFv–CD3 ζ and CD28/CD3 ζ	Pilot (six patients)	Non-Hodgkin lymphoma (<i>n</i> = 6)	Two SD	[7]
Kochenderfer <i>et al.</i> 2012	Murine CD19scFv–CD28/CD3 ζ	Pilot (eight patients)	Non-Hodgkin lymphoma (<i>n</i> = 4) CLL (<i>n</i> = 4)	One CR, five PR	[8]
Brentjens <i>et al.</i> 2013 Davila <i>et al.</i> 2014	Murine CD19 scFv–CD28/CD3 ζ	Phase I (16 patients)	Acute lymphoblastic leukemia	CR 88%	[9,10]
Grupp <i>et al.</i> 2013	Murine CD19 scFv–4-1BB/CD3 ζ	Pilot (two patients)	Acute lymphoblastic leukemia	Two CR	[11]
Maude <i>et al.</i> 2014	Murine CD19 scFv–4-1BB/CD3 ζ	Phase I/II (30 patients)	Acute lymphoblastic leukemia	CR 90%	[12]
Lee <i>et al.</i> 2015	Murine CD19scFv–CD28/CD3 ζ	Phase I (21 patients)	Acute lymphoblastic leukemia	CR 70%	[13]
Kochenderfer <i>et al.</i> 2015	Murine CD19scFv–CD28/CD3 ζ	Phase I (15 patients)	Non-Hodgkin lymphoma (<i>n</i> = 11) CLL (<i>n</i> = 4)	CR 53% PR 26%	[14]

^aCR, complete remission; PR, partial remission; SD, stable disease.

could be activated to eliminate them or regulating expression of the CAR could overcome this side effect since the B cell pool is constantly repopulated from hematopoietic stem cells. CAR-T cells can be transduced with an inducible form of caspase-9 (iCasp9), which dimerizes and is activated on administration of the drug AP1903, leading to rapid apoptosis of the T cell. The iCasp9 gene has been incorporated into vectors for preclinical studies and demonstrates effective inducible suicide activity in Phase I clinical trials [21]. Coexpressed cell surface markers such as a truncated epidermal growth factor receptor and CD20 have also been incorporated into CAR vectors and could serve as targets for antibody-mediated elimination of CAR-T cells [22,23]. Another approach is to regulate expression of the CAR itself by placing it under the control of regulatory elements that can be turned on or off by the delivery of small molecules, such as the Tet-on or doxycycline-inducible systems that have been used *in vitro* [24]. In other cancers, complete tumor specificity might be achieved by targeting neo-antigens resulting from oncogenic mutations such as EGFRvIII, which is deleted in exons 2–7 and exhibits constitutive signaling in the absence of ligand binding, or molecules expressed in development and on tumor cells but not normally expressed in adult tissue such as ROR1 and GD2 [25–27]. Other candidate molecules for CAR-T cell therapy such as Her2/Neu, mesothelin, and MUC16 are overexpressed in tumors relative to normal tissue [28–30], but it remains to be determined whether

CARs can be designed with avidity thresholds that are sufficiently tuned to distinguish target cells based on high and low levels of antigen expression. It seems likely that, with such targets, toxicity will be a potentially serious issue, as will the escape of tumor variants that express low levels of antigen.

The requirement for costimulation (signal 2) provided by activated APCs ensures that TCR signaling is effective only when antigen is encountered in the appropriate context. Several groups are attempting to apply this two-signal concept to CAR design to improve tumor specificity and limit off-tumor recognition of normal cells by coexpressing two CARs with different binding domains (Figure 2A). This dual-receptor strategy has proved to be challenging and its success may depend on the choice of CARs to use and antigens to target. For example, Wilkie *et al.* used a split-receptor system to target human epidermal growth factor 2 (Her2) and mucin 1 (Muc1), both of which are commonly overexpressed in many tumors. The Her2-binding CAR was designed with only a CD3 ζ signaling domain while a second CAR specific for Muc1 provided the costimulatory signaling domain of CD28, essentially reproducing the signal 1 and 2 checkpoints of T cell activation [31]. Such dual-specificity CAR-T cells proliferated when they received both signals 1 and 2 in response to Her2/Muc1 double-positive target cells, but not in response to Her2 or Muc1 single-positive cells. However, these dual-specificity CAR-T cells were capable of lysing Her2⁺Muc1[−] and Her2⁺Muc1⁺ cells alike

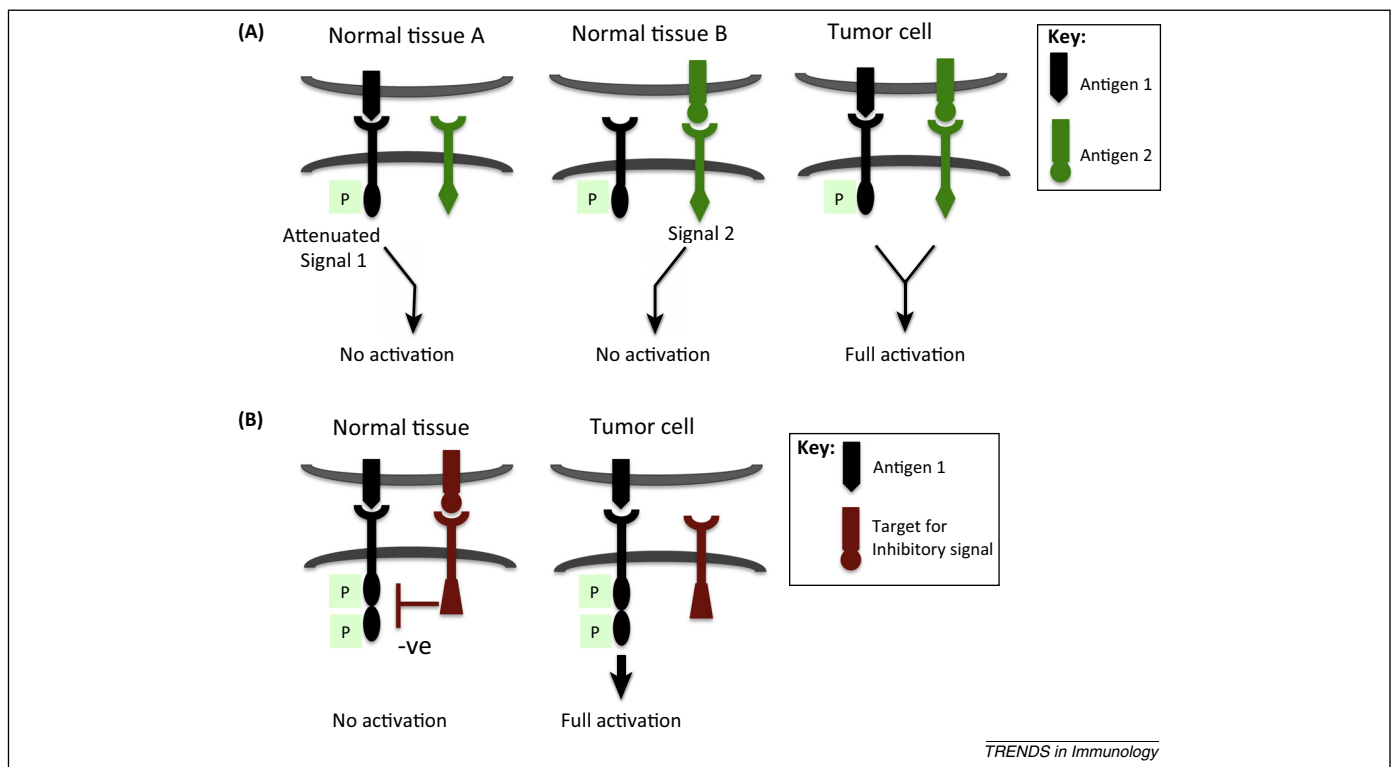


Figure 2. Split-receptor designs to enhance tumor selective recognition. (A) Enhancing tumor selectivity using chimeric costimulatory receptors. To reduce the potential for chimeric antigen receptor (CAR)-T cells to recognize normal tissues that may express the target antigen, dual CARs have been designed such that two molecules, both of which are expressed on tumor cells, must be engaged to deliver signals 1 and 2 and fully activate CAR-T cells. Normal cells that express only one of these antigens do not signal T cells sufficiently for full activation. The CAR that delivers signal 1 may need to be attenuated in signaling, such as by mutating one or more ITAMs in CD3 ζ or by reducing the affinity of the single-chain variable fragment (scFv). (B) Enhancing tumor selectivity using inhibitory chimeric receptors. In this scenario, a CAR that delivers a dominant inhibitory signal such as might be mediated via a PD-1 endodomain is coexpressed with a CAR capable of full T cell activation. Engagement of both target antigens on normal cells would suppress T cell activation, whereas recognition of only the activating ligand on tumor cells would result in T cell activation and effector function.

in vitro, demonstrating that the delivery of signal 1 only was sufficient for cytolysis. Grada *et al.* encountered a similar problem in their split-receptor system targeting CD19 and Her2, where dual-specificity CAR-T cells lysed both single CD19⁺ and Her2⁺ target cells [32]. The inability to discriminate between single- and double-positive target cells seriously limits the ability of these CARs to improve targeting specificity over that of single-input CARs. Kloss *et al.* demonstrated that it may be possible to titer down the signaling strength of the CAR delivering 'signal 1' so that T cell cytotoxicity requires signaling through the costimulatory CAR. They screened scFvs specific for the prostate stem cell antigen (PSCA) in a CAR format and selected a suboptimal CAR that when expressed in T cells alone was insufficient to induce killing of PSCA⁺ tumor cells *in vitro* or *in vivo*. When this suboptimal CAR was coexpressed with a second costimulatory CAR that delivered CD28 and 4-1BB signals on recognition of prostate-specific membrane antigen (PSMA), the T cells were capable of recognizing dual PSCA⁺PSMA⁺ tumor cells *in vivo* [33]. This strategy thus provides one way in which CAR specificity can be increased to minimize the toxicity of off-tumor effects.

Another way to increase the tumor specificity of CARs is to use a split-receptor system to provide negative signaling in the presence of normal, but not tumor, tissue. Such dominant inhibition of TCR signaling is yet another principle of conventional T cell biology. On activation, T cells upregulate inhibitory receptors like PD-1 and CTLA-4 that dampen TCR signaling, either through competition for costimulatory ligands (e.g., CTLA-4 for the CD28 ligands CD80 and CD86) or via engagement of ligands upregulated on APCs by proinflammatory cytokines (e.g., PD-1 for PD-L1 and PD-L2). These negative feedback mechanisms prevent excess T cell activity and minimize proinflammatory damage to the host. The importance of this regulation is demonstrated by the autoimmunity and lymphoproliferative disease that develop in PD-1 and CTLA-4 knockout mice [34,35]. Moreover, PD-L1 is constitutively expressed at sites of immune privilege such as the neurons of the eye and trophoblasts in the placenta, thereby protecting these tissues from immune attack [36]. Such inhibitory receptors are also commonly upregulated on 'exhausted' T cells during chronic infection and cancer after prolonged exposure to antigen, and immunotherapies aimed at blocking the PD-1 and CTLA-4 pathways have met with significant clinical success in boosting T cell activity toward viral and tumor antigens [37,38].

A similar logic can be applied to regulating CAR recognition, where coexpression of a second inhibitory CAR specific for an antigen expressed on normal, but not tumor, tissue would protect normal tissue from CAR-T cell-mediated attack (Figure 2B). Fedorov *et al.* demonstrated the feasibility of this approach by coexpressing a CD19-specific activating CAR carrying the CD3 ζ and CD28 endodomains and a PSMA-specific inhibitory CAR carrying the endodomain of PD-1 or CTLA-4 [39]. CAR-T cells were activated when exposed to target cells expressing CD19 in the absence of PSMA, but their ability to proliferate, lyse target cells, and secrete proinflammatory cytokines was inhibited by target cells coexpressing PSMA. This principle is particularly attractive for enhancing the specificity of activating

CARs that recognize ligands expressed on tumor cells and some normal cells. However, in addition to the issue of which ligands restricted to normal cells to select to deliver the negative signal, a significant challenge in designing inhibitory CARs is that the mechanisms by which these inhibitory receptors exert their function in TCR signaling are not fully understood. For example, PD-1 inhibits TCR signaling by colocalizing with TCR microclusters in the synapse, and this colocalization is required for its ability to recruit SHP2 and dephosphorylate proximal TCR signaling proteins like CD3 ζ , Zap70, and PKC θ [40]. Thus, the ability of the inhibitory CAR to colocalize with the activating CAR may be particularly important when using the PD-1 endodomain. By contrast, CTLA-4 is thought to inhibit TCR signaling by competing with CD28 for binding to CD80/CD86 and physically excluding CD28 from the synapse, thereby dampening costimulatory signaling [41]. Recent work even suggests that the primary purpose of the CTLA-4 endodomain is to regulate the surface expression of CTLA-4 rather than to induce downstream inhibitory signaling [42]. This mechanism of inhibition would be predicted to be less effective against second- or third-generation activating CARs, which already carry costimulatory endodomains. There is some evidence that the PD-1 endodomain is more effective than the CTLA-4 endodomain at inhibiting second-generation CAR activity [39].

Ultimately, we will need better assays to compare how the use of split receptors differs from conventional TCR signaling and might be engineered to better mimic the regulation of TCR signaling. Past studies on TCR signaling have taken a reductionist approach, using planar lipid bilayers as artificial APCs to monitor the spatial-temporal dynamics of specific proteins during formation of the immunological synapse [43]. Such a method could be particularly informative for estimating the density of ligands needed to deliver activating or inhibitory signals via coexpressed CARs and might provide a platform for testing whether specific antigens would make good targets for costimulatory or coinhibitory CARs based on their expression levels. In addition, several studies have demonstrated that the ability of costimulatory or coinhibitory receptors to colocalize with TCRs at the synapse is critical for their stimulatory and inhibitory function, respectively [40,41]. Thus, live-cell imaging techniques like confocal microscopy should prove useful in providing detailed insight into the spatial and temporal dynamics of CAR signaling and identifying how CAR split-receptor systems deviate from conventional T cell signaling.

Structural constraints in T cell:target cell interactions

Structural and spatial elements of TCR recognition have evolved for the precise regulation of the interaction between a T cell and its target cell and can be challenging to recapitulate with synthetic receptors. During formation of the immunological synapse, the ~15-nm distance between a T cell and an APC is dictated by the structures of the TCR and pMHC. This close proximity is important to exclude the phosphatases CD45 and CD148, which have large ectodomains longer than 15 nm, from the synapse to allow TCR-induced tyrosine phosphorylation to be initiated in the absence of these negative regulators. In support of the

importance of this molecular segregation at the site of TCR engagement, a mutated form of CD45 with a shortened ectodomain attenuates T cell signaling [44,45].

The spatial distance between CARs and their target antigens may be equally important for effective initiation of T cell signaling but depends on an entirely different set of structural elements related to the location of the epitope on the target molecule and the spacer domain between the scFv and the T cell membrane. Several studies have demonstrated that the same epitope can activate CAR-T cells with greater efficiency when expressed in a more membrane-proximal position than a membrane-distal position [46–50]. For example, Hombach *et al.* demonstrated that CAR-T cells recognizing the membrane-distal 'N' epitope of carcinoembryonic antigen (CEA) were only modestly activated; however, when they engineered recombinant CEA protein to express the N epitope in a membrane-proximal position, the same CAR-T cells were activated more efficiently [46]. This suggests that targeting some membrane-distal epitopes on tumor cells may permit large phosphatases like CD45 and CD148 to enter the synapse and inhibit phosphorylation events initiated by CAR engagement. Tailoring the extracellular spacer sequence between the T cell membrane and the ligand-binding scFv to promote synapse formation can assist in overcoming steric constraints imposed by the location of the target epitope. Work in our laboratory has demonstrated that, when targeting a membrane-distal epitope on the receptor tyrosine kinase ROR1, which is expressed on many solid tumors, CARs with a shortened extracellular spacer conferred superior recognition of ROR1⁺ tumor cells and induction of T cell effector function *in vitro* compared with the same scFv with a longer spacer [51]. The shortened spacer may decrease the distance between T cell and target cell and maintain exclusion of larger inhibitory molecules from the synapse. By contrast, when targeting a membrane-proximal epitope on ROR1, a longer extracellular spacer resulted in greater effector T cell function, indicating that the optimal spacer length of CARs is likely to vary based on the position of the target epitope [52]. Thus, the design of CARs for novel targets should factor in the location of the target epitope and customize the spacer length to optimize CAR-T cell signaling.

Live-imaging technologies could prove informative for studying the spatial and temporal dynamics of synapse formation between CAR-T cells and tumor cells. Using retroviral or lentiviral gene-transfer methods, signaling proteins fused to GFP can be imaged in primary T cells [53], and T cell:APC conjugates can even be imaged *in vivo* [54]. These methods can be used to determine whether altering the CAR spacer length affects synapse formation and/or the ability of CD45 or CD148 to localize to the synapse. Interestingly, a recent study demonstrated that co-culturing HEK cells expressing a CD19 CAR with CD19⁺ Raji cells resulted in clustering of receptors at the APC:T cell interface, recruitment of Zap70, and exclusion CD45 from the synapse [55]. A caveat of this study is the use of HEK cells rather than T cells; nevertheless, the results suggest that, at least in some contexts, CD45 is excluded from the synapse established between CAR-T cells and their targets. The CD19 CAR-HEK cell interface,

however, was highly convoluted and irregular, distinct from the more even and ordered TCR–pMHC interface, suggesting that some aspects of synapse formation may be distinct in CAR-expressing cells. A more comprehensive analysis of primary CAR-T cells and target tumor cells, both *in vitro* and *in vivo*, will be needed to understand how the formation, composition, and organization of the synapse compares with conventional T cells.

Affinity and sensitivity considerations

Tumor antigens are often expressed at low levels on the cell surface and this can present an obstacle to eliciting effective CAR-T cell signaling. In tumors in which CAR-T cells are effective in the clinic, such as chronic lymphocytic leukemia (CLL) and ALL, the number of CD19 molecules per tumor cell has been estimated to be ~10 000, which is two- to fivefold higher than alternative targets such as ROR1. The lower level of target antigen density that is required to elicit effective T cell activation is likely to vary for different target antigens and CAR constructs depending on CAR expression, scFv affinity, spatial constraints, and tumor cell expression of adhesion and costimulatory ligands that provide signals distinct from the CAR. A recent study demonstrated that for a CD20-specific CAR, ~200 CD20 molecules per target cell were required for lytic activity, while the requirement for cytokine production was approximately tenfold higher. Consequently, CD20-specific CAR-T cells could efficiently kill even CD20-downregulated lymphoma and leukemia targets *in vitro* [56]. If further refined, such studies may be useful in determining the antigen density threshold required for CAR activity and predicting whether tumor cells or healthy tissues will elicit CAR activation.

Some direction for designing CARs that elicit T cell activation toward very low-density antigens may be provided from the study of conventional TCRs, which display an exquisite sensitivity to low-density antigen. Imaging studies suggest that even a single-agonist pMHC can trigger cytokine production from some naïve T cells [18]. The mechanisms underlying TCR sensitivity and concomitant specificity are not fully understood and continue to be an important area of investigation. One way sensitivity is achieved is through the activation of costimulatory receptors, which can lower the activation threshold for TCRs. In the absence of CD28 ligation, T cells require very high numbers of TCR–pMHC interactions and prolonged stimulation, whereas CD28 costimulation allows T cells to respond to lower numbers of TCR–pMHC interactions [57,58]. Likewise, other receptors, like NKG2D, have been shown to function as costimulatory receptors by enhancing IL-2 production and T cell proliferation on TCR triggering [59]. Thus, signaling domains from such costimulatory receptors may be incorporated into the CAR itself or into split-receptor systems to enhance T cell sensitivity to target antigens.

Another explanation for the exquisite sensitivity of TCRs is provided by the 'serial triggering' model, which suggests that multiple TCRs should sample the pMHC for efficient T cell activation, a process which requires sufficiently low binding affinity and short K_{off} rates of the receptor–ligand interaction [60]. By contrast, the function

of some CARs, which typically have much higher affinity measured by surface plasmon resonance than that measured for TCR/pMHC interactions, has been shown to improve with increasing scFv affinity. For example, Hudecek *et al.* tested a panel of ROR1-specific CARs, and demonstrated that CARs derived from a higher-affinity scFv conferred greater effector function against ROR1⁺ mantle cell lymphoma and epithelial cancer cell lines *in vitro* and *in vivo* [51]. However, other studies indicate the existence of an avidity threshold for both CARs and TCRs, above which T cell function is not improved or is actually reduced [61–63]. In a study using CARs specific for pMHC, increased affinity and surface expression of the CAR paradoxically resulted in reduced cytolytic activity and sensitivity to low peptide concentrations relative to an $\alpha\beta$ TCR specific for the same epitope [63]. These studies suggest that a specific range in binding affinity is required for optimal CAR functionality.

The rational design of CARs will benefit from the development of tools for the precise study of K_{on}/K_{off} rates, preferably using live cells that express the CAR, to determine the target range for receptor affinity and optimize CAR sensitivity, especially to low-density antigens. One such tool was recently developed by Busch and colleagues, who used a real-time microscopy assay to study the K_{off} rates of TCRs on living T cells [64]. In this approach, monomeric pMHC conjugated to fluorescent dyes is multimerized on a *Streptactin* backbone via the *Streptag* sequences and used to label viable epitope-specific T cells. Addition of D-biotin displaces the pMHC by binding to *Streptactin* at *Streptag* sequences with higher affinity, leaving only monomeric pMHC bound to TCRs. Using fluorescence microscopy, the dissociation rate of pMHC from TCRs can then be quantified as the decay in fluorescence intensity over time. Such technology may similarly be used to rapidly measure K_{off} rates of CARs expressed in T cells that might correlate with enhanced CAR-T cell functionality in *in vitro* and *in vivo* assays against tumors that express low or high densities of target antigen.

Another strategy is to directly screen ‘CAR bodies’ using scFv libraries formatted as CARs and expressed through lentiviral transduction in T cells, which are then stimulated with the tumor cells of interest [65]. Current methods for screening potential scFvs involve testing against cell surface-expressed antigens. Although scFvs in solution may recognize the antigen in this context, they may not be as effective when expressed as a CAR on the T cell surface, where adhesion and other cell surface molecules affect T cell:tumor cell interactions. By expressing a library of scFvs as CARs on T cells and screening the T cells for activation following antigen exposure, this strategy allows the selection of scFvs based on their ability to induce T cell activation and proliferation rather than just selecting for the highest-affinity binders. Because current data suggest that the optimal CAR affinity cannot be determined *a priori* for individual target molecules, such an unbiased approach that relies on CAR expression in a T cell may be particularly important to identify the ideal affinity range for a given scFv and target antigen.

Missing signal transduction modules

Another mechanism of signal amplification in TCR recognition that is lacking with CARs involves the interaction of CD4 and CD8 coreceptors with pMHC. The coreceptors CD4 and CD8 play a critical role in the initiation of TCR signaling by bringing Lck, which is bound to their cytoplasmic tails, into proximity with the TCR/CD3 ζ complex, where it phosphorylates ITAMs in the CD3 ζ chain, facilitating the binding and phosphorylation of Zap70 to promote its catalytic activity. However, once Lck is activated coreceptors can associate with other TCRs weakly bound to self-pMHC and mediate activation of those TCRs [66]. As a result of positive selection, TCRs are weakly self-reactive and these interactions with self-pMHC are important for the amplification of signals from foreign pMHC/TCR interactions [67,68]. Although CARs are clearly able to phosphorylate Zap70 and initiate downstream TCR signaling [69], whether they interact structurally with CD8 coreceptors or endogenous TCR/CD3 ζ complexes is unclear. One study demonstrated that use of the CD3 transmembrane domain in the construction of a CAR enabled it to associate with endogenous CD3 ζ and be incorporated into TCR clusters [70], as CD3 ζ is known to associate with $\alpha\beta$ TCRs via its transmembrane region [71,72]. This study, however, was performed in Jurkat cells, which have varied expression of proximal TCR signaling components relative to primary T cells, and other studies found that use of the CD3 transmembrane domain resulted in reduced protein stability and surface expression of the CAR compared with the CD28 transmembrane domain [7]. Thus, whether the CAR forms a synapse and clusters in a manner similar to TCRs or interacts with endogenous TCR/CD3 complexes and coreceptors remains unresolved. Strategies in CAR design that could enhance interaction with coreceptors may allow the activation of a single CAR to be amplified by serial triggering of endogenous TCRs.

Alternative ligand-binding domains

ScFv-based extracellular recognition domains have dominated the synthetic antigen receptor field. Thus, development of new CARs relies heavily on constructing scFvs from murine monoclonal antibody V_H and V_L sequences specific for tumor-associated molecules from already available hybridomas. In addition to being limited by the small repertoire of tumor-selective monoclonal antibodies, scFvs differ significantly from TCRs in at least two respects that can limit their utility: (i) their affinity for antigen is significantly higher, which can interfere with optimal signaling as discussed above; and (ii) their specificity is limited to antigens expressed on the tumor cell surface, not intracellular antigens. Developing CAR recognition domains that more closely mimic TCRs is an intriguing strategy for enhancing T cell functionality. Several groups have employed phage display and hybridoma strategies to isolate ‘TCR-like’ antibodies, which bind tumor-derived peptides in an HLA-restricted manner [63,73]. These antibodies imitate the specificity of TCRs in their ability to recognize intracellular tumor antigens expressed on MHC; however, they still exhibit the kinetics and high-affinity binding properties of antibodies rather than TCRs [63].

Efforts to design recognition domains *de novo* that mimic TCR binding from proteins other than scFvs are being made. One platform uses designed ankyrin repeat proteins (DARPin), which are derived from normal ankyrin proteins that mediate high-affinity protein–protein interactions and can be extensively tuned for affinity, on and off rates, and multiplexed specificities [74]. DARPins have been selected from phage or ribosomal display libraries to bind to Her2 and EGFR and our group is currently investigating the functionality of CARs constructed using a DARPin-binding domain. Computational methods have also been demonstrated to be effective at designing synthetic proteins *de novo* to target various antigens with highly defined properties [75–77]. This approach may be particularly powerful in designing more TCR-like CARs that are selected not just for their specificity for a particular tumor antigen but also for many other physical criteria such as K_{on}/K_{off} rates, binding distance from the target epitope, and the ability to interact with endogenous TCR/CD3 and/or coreceptors.

A concern when considering these strategies, however, is the potential for enhanced immunogenicity of synthetic polypeptide binding domains and/or novel sequences at fusion sites between components of CARs. Immunogenicity of scFv based CARs may be overcome in part by selecting scFvs from human libraries. In addition, fusion sites can be designed with the help of bioinformatics tools to limit the binding of peptide sequences to MHC, although antibody recognition of these sequences may remain a limitation. Efforts to ‘humanize’ such proteins and limit their immunogenicity will be critical for their effectiveness in the clinic.

Concluding remarks

Despite the initial and remarkable successes of CAR-T cells in the treatment of leukemia and lymphoma, the application of CAR-T cells to the treatment of common epithelial tumors, which have few truly tumor-specific target molecules and an immunosuppressive microenvironment hostile to T cells, is likely to prove challenging.

Deeper analysis of CAR signaling using new tools is necessary to provide insights into how CARs differ from TCRs and might be engineered to perform more effectively in clinical settings. Future areas for research will need to incorporate more sophisticated, global approaches to define how CAR signaling pathways differ from TCRs and identify what missing elements of TCR signaling might best be incorporated into CAR design (Table 2). Mass spectrometry-based techniques enable the quantification of changes in intracellular phosphorylation events that are initiated and propagated by signals through TCRs and CARs. This technology has been employed by several groups to gain system-wide insights into the role of phosphorylation during TCR signaling in human Jurkat and murine transgenic T cells [78,79]. Unbiased analysis of the phosphoproteome in CAR-T cells versus conventional T cells, both before and after receptor triggering, would help identify which signaling pathways are engaged and how these may differ between the two cell types. Mass spectrometry can even be extended to quantify other post-translational modifications, such as ubiquitylation and

Table 2. Unresolved issues in CAR-T cell biology.

Unresolved issues in CAR-T cell biology	Experimental approaches to answering these questions
Intracellular signaling pathways	<ul style="list-style-type: none"> • Mass spectrometry • Phosphoproteome analysis • Genome-wide transcriptional pathway analysis • Single-cell RNA-seq
Synapse formation/organization	<ul style="list-style-type: none"> • Confocal microscopy • <i>In vivo</i> live imaging
Receptor characteristics <ul style="list-style-type: none"> • Binding affinity • K_{on}/K_{off} rates 	<ul style="list-style-type: none"> • Real-time microscopy (Busch <i>et al.</i>)
Cell fate decisions <ul style="list-style-type: none"> • Effector differentiation • Memory formation 	<ul style="list-style-type: none"> • Epigenetic analysis (ChIP-seq) • Histone modifications • DNA methylation

acetylation, which would provide a more comprehensive overview of the protein modifications that result from CAR or TCR triggering. Likewise, genome-wide transcriptional analysis would provide another global, unbiased insight into how CAR-T cell signaling and activation dictates effector function and cell fate. Single-cell RNA sequencing may be even more informative, revealing differences in gene transcription that may be obscured by heterogeneity at the cell population level.

An aspect of CAR-T cell biology that has not been well studied is how signaling through CARs affects the cell's ability to become a long-lived memory cell, which may be essential for complete eradication of malignant cells. Epigenetic modifications, in the form of changes to DNA and/or histones, are known to play an important role in CD4⁺ helper T cell differentiation and CD8⁺ memory formation [80,81]. Using ChIP-seq, genome-wide changes in transcriptionally ‘permissive’ and ‘repressive’ histone modifications can be quantified and mapped in various cell subsets. For example, O’Shea’s group used this technology to study the epigenetic modifications that occur during the differentiation of naïve T cells into fully polarized Th1, Th2, and Th17 T helper cells and to provide insights into the plasticity of differentiated subsets [82]. Next-generation sequencing can also be used to generate comprehensive genome-wide mapping of DNA methylation sites, which have been shown to play a role in the lineage-specific differentiation of hematopoietic progenitors [83]. Global analysis of the ‘epigenome’ in CAR-T cells may provide a deeper understanding of how signaling through these synthetic receptors affects the cytokine profiles, differentiation, and memory potential of CAR-T cells.

The principle that T cells that are genetically modified to express novel synthetic CARs can effectively treat advanced refractory cancers has been established but many questions remain to realize the full potential of this new therapeutic modality (Box 1). Engineering safer, more effective CARs for cancer therapy will require moving beyond the traditional empirical approach to receptor design and cell engineering, ideally guided by our knowledge of TCR signaling, T cell biology, and manipulation of the tumor microenvironment. It is now apparent that binding affinity, K_{on}/K_{off} rates, and spatial constraints between CARs and target cells can influence the ability of CARs to optimally activate T cells for tumor recognition.

Box 1. Outstanding questions

- Can common epithelial cancers be successfully treated with CAR-modified T cells?
- What are the optimal signaling and costimulatory domains for CARs and will these differ depending on the tumor being targeted?
- Can measurements of the affinity of scFvs and target ligand density on tumor cells be used to inform more effective CAR design?
- What additional modifications to CAR-modified T cells will be necessary to overcome immunosuppressive tumor microenvironments?
- Can split-receptor systems using 'and' and 'not' logic gates be effective in the clinic for specifying selective recognition of tumor and not normal cells?
- How does CAR signaling differ from TCR signaling in directing cell fate decisions including formation of central and effector memory subsets?
- What are the alternatives to scFvs as ligand-binding domains in CAR design?
- How great a barrier will the immunogenicity of CAR-modified T cells be to their utility as cancer therapeutics?

The precise study of these parameters should provide insights for the design of new receptors that may improve antitumor function, particularly against solid tumors. Other genetic modifications to make T cells resistant to tumor-induced immunosuppression and combining CAR-T cells with other modalities, such as checkpoint inhibitors, can be envisioned as future approaches in this rapidly evolving field.

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