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CAR-T design: Elements and their synergistic function

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ABSTRACT

Chimeric antigen receptor (CAR) T cells use re-engineered cell surface receptors to specifically bind to and lyse oncogenic cells. Two clinically approved CAR-T–cell therapies have significant clinical efficacy in treating CD19-positive B cell cancers. With widespread interest to deploy this immunotherapy to other cancers, there has been great research activity to design new CAR structures to increase the range of targeted cancers and anti-tumor efficacy. However, several obstacles must be addressed before CAR-T–cell therapies can be more widely deployed. These include limiting the frequency of lethal cytokine storms, enhancing T-cell persistence and signaling, and improving target antigen specificity. We provide a comprehensive review of recent research on CAR design and systematically evaluate design aspects of the four major modules of CAR structure: the ligand-binding, spacer, transmembrane, and cytoplasmic domains, elucidating design strategies and principles to guide future immunotherapeutic discovery.

1. Introduction

T cells expressing chimeric antigen receptors (CAR) have displayed remarkable efficacy at treating malignant cancers, particularly liquid tumors. The ability to custom design CARs for specific oncological applications has made them an attractive alternative to conventional cancer treatments such as radiation and chemotherapy. CARs consist of an extracellular ligand-binding domain, most commonly a single chain variable fragment (scFv), a spacer domain, a transmembrane domain, and one or more cytoplasmic domains [1]. First-generation CARs contain a single activatory domain, which in most cases is the CD3ζ cytoplasmic domain, while a few studies used the γ chain of Fc receptors. Second-generation CARs commonly contain an activatory domain (CD3ζ/γ chain of Fc receptors) connected to co-stimulatory domains obtained from native co-stimulatory molecules such as CD28 and 4–1BB [1]. More recent optimization has led to the development of third-generation constructs incorporating CD3ζ with two co-stimulatory cytoplasmic domains. The design of each module of the CAR structure can contribute to CAR-T–cell signaling mechanisms, effector functions, and its eventual efficacy and toxicity. It is evident that modules such as the scFv and intracellular cytoplasmic domains play a key role in ligand recognition and signaling. It has recently become clear that non-signaling domains such as the spacer and transmembrane domains can also influence CAR functions.

Highly encouraging preclinical and clinical results have spurred massive research efforts into CAR-T cell therapy, yet challenges still lie ahead of full clinical adoption due to their toxicity and challenges associated with non-hematological cancers. So far only two CAR-T–cell therapies, Kymriah® and Yescarta®, are clinically approved yet they have exhibited serious, sometimes lethal, side-effects. Cytokine release syndrome, the rapid release of cytokines into the bloodstream following administration of immunotherapies, as well as neurotoxicity have been frequently reported [2,3]. In addition to these side effects, responses to CAR-T therapy have been variable due to both heterogeneity in target-antigen expression in malignant cells as well as high rates of antigen escape and downregulation of target cancer

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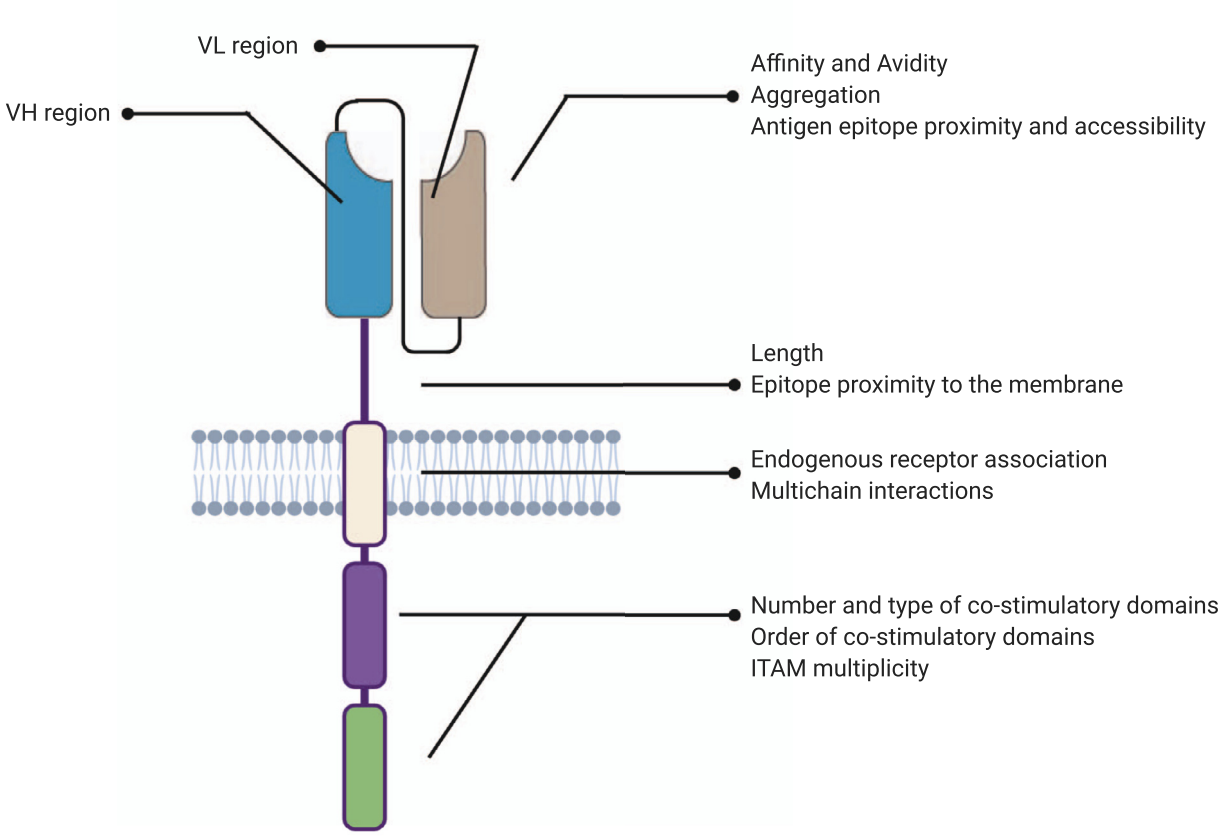

Fig. 1. Design parameters of each module of the CAR tested in literature.

markers [4,5]. CAR-T cells continue to be minimally effective against solid tumors, in part due to their inability to persist and maintain their effector functions in the neoplastic microenvironment [6]. Recent literature suggests that appropriate compositional and structural design of CARs can reduce off-target effects and enhance tumor eradication and persistence. While it is recognized that small variations in these CAR modules and characteristics can be critical functionality determinants, relationships among these factors are complicated, and currently no general design rules can predict *in vivo* functions. In this review, we will discuss recent advances in engineering the extracellular, spacer, transmembrane, and cytoplasmic domains of CARs and how they affect CAR-T function. We summarize a list of design parameters tested in literature for each module and describe their effects on the functionality of CAR-T cells (Fig. 1). This systematic analysis can help uncover design principles, which can be broadly applied toward future designer immunotherapies.

2. Ligand-binding domain

scFvs are the most commonly used ligand-binding domains in CAR structures, although other domains such as nanobodies, ligands to cognate receptors, native receptors against targets—including those such as NKG2D and T1E that target multiple ligands—and small peptides have been used [7–16]. Fig. 1 and Fig. 2 highlight critical design parameters of ligand-binding domain including affinity, avidity, antigen epitope location, and accessibility, as well as how they affect CAR-T–cell functionality. Interested readers can also refer to Supplementary Table 1 for a detailed list of representative publications that highlight the importance of these parameters.

2.1. Affinity and avidity of ligand-binding domain

scFv affinity is a key parameter that has been modulated to improve specificity of the CAR and reduce “on-target, off-tumor” side effects, which is of particular importance when the target antigen is ubiquitously expressed on healthy tissue. For instance, CARs constructed from an anti-ErbB2 scFv with a K_D (dissociation constant) of 0•3 μM showed selective cytotoxicity towards cells highly expressing ErbB2 while CARs bearing high-affinity scFv sequences (K_D <0•01 μM) ErbB2 did not [17]. Similarly, in another study anti-ErbB2 CARs were constructed from affinity-modulated scFv sequences derived from monoclonal antibody mAb 4D5. CAR-T cells using a lower-affinity 4D5 variant (K_D ~ 1 μM) showed an increased therapeutic index in mice compared to CAR-T cells bearing a high-affinity 4D5 variant (K_D ~ 0•6 nM) [18]. This was attributed to the ability of low-affinity scFv CARs to selectively discriminate between tumors which typically express ErbB2 at higher densities compared to normal tissues. Caruso et al. compared the specificity of anti-EGFR CARs constructed from Cetuximab and Nimotuzumab, which has a 10-fold lower affinity than Cetuximab [19]. Nimotuzumab-based CARs showed EGFR-density dependent activation *in vitro* and did not show potent recognition of low-density EGFR cells *in vivo*, unlike the Cetuximab-based CAR. In another study, an anti-CD38 CAR with a low-affinity scFv (K_D in the micromolar range), obtained from an affinity-tuned antibody library, was specifically cytotoxic to cells highly expressing CD38 both *in vitro* and *in vivo*, while having minimal effect on healthy $CD38^+$ hematopoietic cells [20]. Similarly, LFA-1 I domains modulated for micromolar affinity to ICAM-1 were more selective to cells expressing high levels of the target antigen (ICAM-1). CAR-T cells incorporating micromolar-affinity I domains

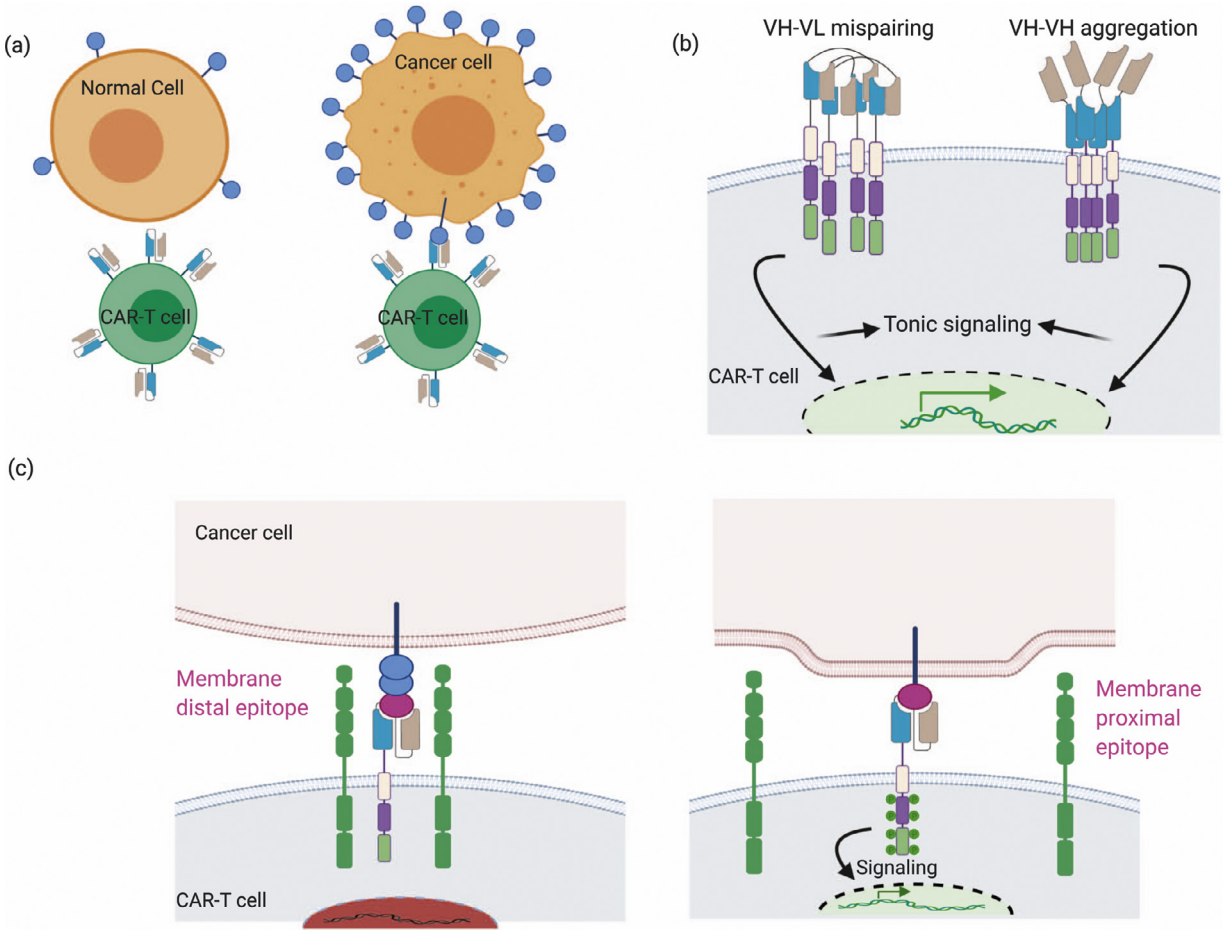

Fig. 2. scFv properties such as affinity, avidity, aggregation propensity, and its antigen epitope location are critical parameters that can affect CAR function. (a) scFv affinity and avidity can be modulated to improve selective recognition of target cells bearing higher ligand density, thus reducing on-target off-tumor effects.

(b) CAR surface aggregation can cause VH-VL mispairing, which can occur at high expression levels or with sub-optimal linker design that limits stabilizing inter-domain interactions. (c) Location of epitope targeted by scFv dictates synaptic cleft distances, which are important for kinetic segregation of phosphatases like CD45.

specifically cleared thyroid carcinoma xenografts in mice without systemic toxicity [9].

While reducing affinity has been shown to improve CAR-T–cell specificity, it can reduce anti-tumor potency in certain cases. Anti-ROR1 CARs constructed from a higher-affinity scFv (R12) showed greater anti-tumor potency compared to CARs constructed from a 2A2 scFv which has a 50-fold lower affinity [21]. Similarly, higher-affinity anti-FRβ CARs (K_D ~54•3 nM) showed specific and complete abrogation of tumors in mouse models of acute myeloid leukemia compared to lower-affinity anti-FRβ CARs (K_D ~2•4 nM) which were ineffective against the disease [22]. In another study, anti-GD2 CARs constructed from a low-affinity 14G2a scFv were ineffective against rapidly proliferating tumors, while a high-affinity scFv obtained by rationally engineering the 14G2a scFv conferred significant anti-tumor potency. However, the increased sensitivity and potency also resulted in severe neurotoxicity due to non-specific off-tumor effects [23]. Ligand-binding affinities should therefore be optimized by finely balancing the desired strength of anti-tumor response with the potential risk of on-target, off-tumor toxicity. Lastly, affinity thresholds are likely not universal and depend on interconnected factors such as antigen densities on target cells, CAR expression levels, and binding epitope location [24,25].

Affinity modulation can impact CAR signaling and other effector functions such as cytokine secretion, proliferation, and persistence. Low-affinity anti-CD19 CAR (CAT-CAR) (K_D = 14•3 nM) showed increased antigen-specific proliferation and increased persistence *in vivo* compared to the conventional FMC63-based CARs (K_D = 0•32 nM), even though both were found to target similar epitopes on the CD19 antigen. IL-2 and IFNγ secretion levels were comparable for the two CARs, while TNFα showed a small increase in the case of the low-affinity CAT-CAR (both *in vitro* and *in vivo*) [26]. The authors also note that the faster dissociation constant (K_{off}) of the scFv used in the CAT-CAR ($3 \times 10^{-3}\ s^{-1}$) compared to the FMC63 scFv ($6•8 \times 10^{-5}\ s^{-1}$) contributes to its low affinity and could result in reduced duration of receptor-ligand interactions. Faster K_{off} values could also result in increased serial killing and hence improved therapeutic performance. On the contrary, another work using anti-CD123 CAR-T cells indicated that effector functions such as cytokine secretion levels and proliferation were more dependent on CAR expression levels than on affinity [25]. It is likely that once the affinity is sufficiently high, further enhancements to affinity do not translate to further enhancements in CAR performance [17,25]. Indeed, how ligand-binding domain affinity mechanistically affects CAR-T–cell effector functions is as of yet unclear. In physiological TCRs, the strength and duration of TCR-pMHC interactions influence early signaling events as well as later effector responses [27–29]. Similar relationships between signal strength and affinity parameters such as K_{on} and K_{off} may also impact how ligand-binding domain affinities impact CAR function.

The affinity of the ligand-binding domain has proved to be a crucial parameter in CAR design. Yet, it is still a measure of monovalent receptor-ligand interactions. In CAR-T cells as well as in native T cells, the overall strength of interactions is additionally determined by multiple receptor-ligand interactions occurring both due to multiple

synapses at the T cell–target cell interface, as well as clustering of receptors at the immune synapse [30–32]. Avidity is a parameter that accounts for multiple receptor-ligand interactions and is influenced by CAR expression levels, ligand densities on target cells, and affinity of individual ligand-binding domains. In one study, CARs were constructed from scFv sequences targeting HLA-A2 displaying WT1 (Wilms tumor suppressor gene 1) peptide. High affinity as well as high avidity of CARs (concomitant with high CAR expression levels) were implicated in non-specific cross-reactivity with pMHCs displaying irrelevant peptides [32]. In another study, a high-affinity anti-CD123 CAR (K_D = 2 nM) expressed at relatively low levels showed significantly lower proliferation and cytokine production despite similar cytotoxicity against target cells compared to a similarly high-affinity anti-CD123 CAR (K_D = 1 nM) expressed at a much higher level, indicating avidity-related effects on effector functions [25]. Similarly, Weitjens et al. noted that cytokine secretion (TNFα, IL2) correlated with expression levels of anti-G250 CARs when the CAR-T cells were exposed to high antigen densities [33]. Considerations of avidity necessitate evaluation of ligand-binding domains in the context of CAR-T cells. For instance, in a recent report a library of ~120 affinity-modulated scFv sequences was constructed against CD38. Some of these candidates did not show detectable binding using standard affinity measurement techniques such as bilayer interferometry, but did display binding when tested with ligand-coated beads. Ligand-binding was further confirmed by CAR-T cell killing, indicating avidity-reinforced activation of CAR-T cells [20].

Lastly, multiple molecular engineering methods exist for controlling CAR expression. For example, self-inactivating lentiviral vectors with EF1α promoter have been shown to result in reduced CAR expression levels compared to LTR (long terminal repeat) promoter-based gammaretroviral vectors [34]. Furthermore, integrating CARs into the *TRAC* locus of T cells resulted in lower but dynamically regulated CAR surface expression compared to retrovirally integrated CARs, and T cells expressing CARs from the *TRAC* locus exhibited reduced tonic signaling and improved *in vivo* anti-tumor efficacy [35].

2.2. scFv aggregation

scFv aggregation also plays a role in regulating CAR-T–cell activity, where it has been implicated in tonic signaling. Excessive tonic signaling—signaling in an antigen-independent manner—can eventually cause early exhaustion of T cells [34,36–38]. In one study, framework regions of anti-GD2 14G2a scFv were proposed to be responsible for CAR surface aggregation resulting in tonic signaling and exhaustion [36]. In the same study, tonic signaling was observed in several other CARs such as an anti-ErbB2 CAR (4D5 scFv) and anti-CD22 (H22 and m971 scFv) CARs but not an anti-CD19 CAR (FMC63 scFv). In this study, the authors found that replacing the framework regions of anti-CD19 FMC63 CAR-scFv with the framework regions of anti-GD2 14G2a scFv resulted in increased exhaustion. However, anti-GD2 14G2a CAR modified with framework regions from FMC63 scFv did not express on the cell surface, making it difficult to ascertain whether removal of scFv aggregative sequences would prevent tonic signaling. Another study on tonic signaling also identified that antigen-independent proliferation without exogenous IL-2 was observed in CD28-CD3ζ second-generation anti–c-Met-and anti-Mesothelin CAR-T cells but not in CD28-CD3ζ FMC63-based anti-CD19 CAR-T cells [24]. In addition, the authors also noted a correlation between higher CAR expression and increased continuous antigen independent proliferation. Although the authors did not specifically implicate scFv aggregation, the combinatory effect of the scFv and CD28 costimulatory domain is likely the cause of the continuous proliferation phenotype observed. scFv aggregation or misfolding could be caused by low folding stabilities of the VH or VL domain or exposure of hydrophobic residues at the VH-VL interface; scFv linkers can sterically constrain VH-VL domain interaction and result in oligomerization [37,39]. Elevated CAR expression levels can facilitate dynamic swapping of VH-VL domains between different CAR units and enhance aggregation potential on the cell surface [37]. Particularly, in cases where antigen densities on target cells require higher CAR expression, one should carefully balance the trade-off between high expression and aggregation propensities.

2.3. Antigen epitope location and accessibility

The flexibility of the CAR's modular structure allows for targeting difficult epitopes including larger, bulky cell surface receptors, especially heterogeneously glycosylated tumor-associated molecules like MUC1 or mesothelin (MSLN). Suboptimal performance of an anti-MUC1 SM3-scFv–based CARs was attributed to glycosylation independent steric hindrance [40]. CARs based on an scFv targeting the membrane-proximal region (Region III) of the MSLN molecule showed increased functional response (both cytotoxicity and cytokine secretion) *in vitro* and *in vivo* compared to a membrane-distal epitope targeting CAR. The authors attributed this to the rigid structure of the membrane-proximal region that enabled better signal transduction. Additionally, the membrane-distal region of MSLN functionally interacts with proteins such as CA125 (MUC16), which might impede CAR binding [41]. This suggests that apart from steric availability, structural as well as functional aspects of the target epitope need to be included in design considerations for CARs. Novel CAR designs such as bispecific CARs utilizing tandemly connected scFv sequences targeting two antigens may require additional design efforts to identify appropriate CAR structures that allow accessibility to both targets [42]. Epitope location is also important to modulate immune synapse distance, which determines effective cytotoxic granule delivery and kinetic segregation of phosphatases [8,43,44]. A CAR targeting the membrane-distal epitope of CD22 was found to have weaker signaling, lower lytic efficiency, and defective degranulation compared to CARs binding to a membrane-proximal epitope [45]. While the immune synapse distance can be more easily tuned by the spacer domain, as we will articulate below, it is still important to consider epitope location along with functional and structural constraints imposed by the nature of the target.

3. Spacer domain

Spacer domains that connect the scFv to the transmembrane domain lend flexibility to the scFv and help improve efficacy. Functional effects of spacer design modulations are illustrated in Fig. 3. A comprehensive list of selected publications investigating various spacer designs is available in Supplementary Table 2. Appropriate spacer domain engineering can enable recognition of target epitopes that are otherwise sterically inaccessible. The use of a highly flexible IgD hinge instead of a CD28 hinge resulted in better recognition of the sterically hindered MUC1 epitope [40]. Spacer domain modulation can also be used to regulate synaptic cleft distances and hence signaling phenomena such as kinetic segregation. To maintain optimal synapse distance, membrane-distal epitopes usually require shorter spacers whereas membrane-proximal epitopes require longer spacers [21]. Apart from limiting exclusion of inhibitory phosphatases, increasing epitope-paratope distance can also result in impaired delivery of granzymes and perforins to the target cell, thus reducing lytic efficiency. In a physiological T-cell setting, the highly dense immune synapse hinders diffusion of lytic granules, which enhances pore formation by perforins and granzyme delivery [46]. Despite the non-classical nature of CAR-T immune synapses, kinetic segregation and lytic granule delivery are still considered key to CAR-T–cell signaling and killing mechanisms [43,47]. Thus, altering spacer lengths can have a profound effect on cytolytic activity and signaling of CAR-T cells. In an earlier report, first-generation anti-CEA CARs, which bound to a membrane-distal epitope of CEA, were tested with

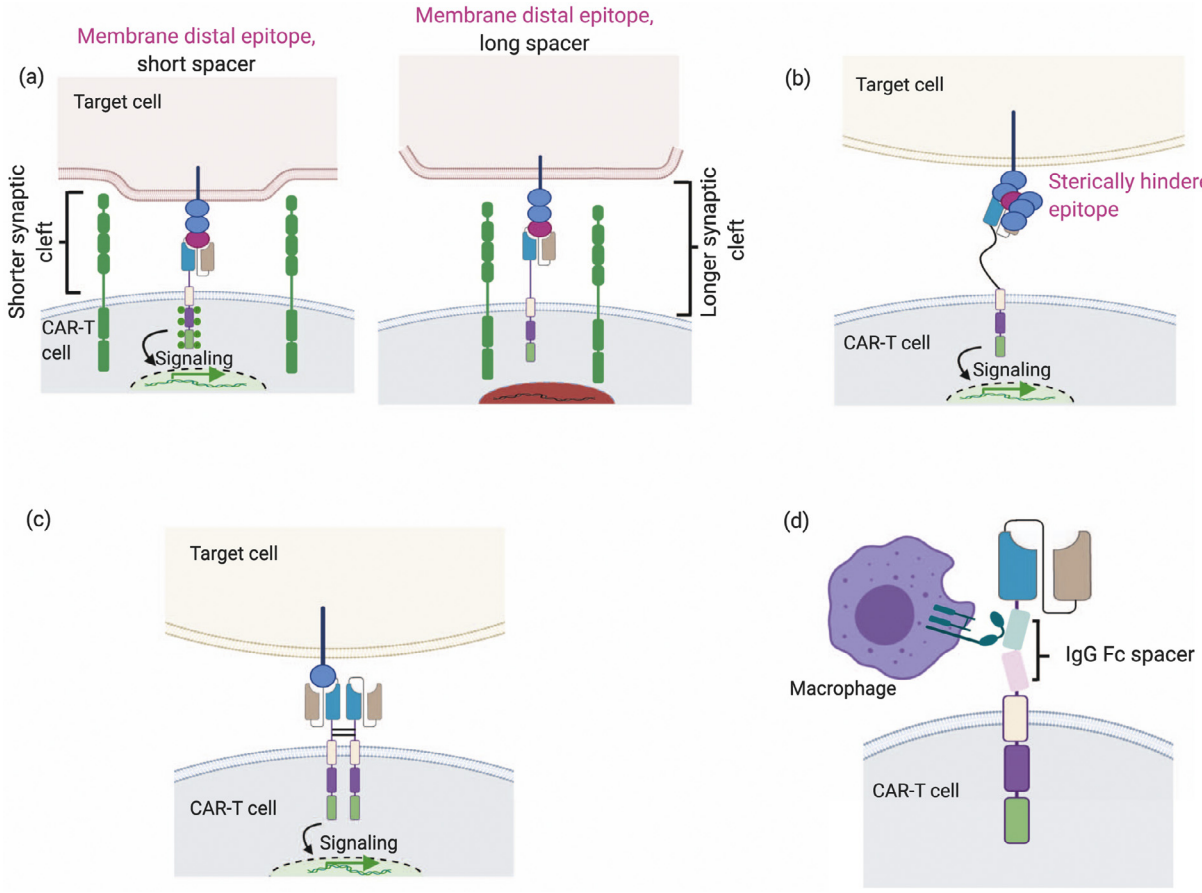

Fig. 3. Spacer design can be used to modulate synaptic cleft distances, allow flexibility and dimerization and reduce non-specific innate immune responses. (a) Spacer length can be modulated to control synaptic cleft distances, which can possibly regulate signaling. When targeting membrane distal epitopes, short spacers (left) shorten the synaptic cleft, enabling exclusion of phosphatases such as CD45 and hence enhancing phosphorylation of cytoplasmic ITAMs while long spacers (right) lengthen the synaptic cleft and possibly do not exclude phosphatases. (b) Flexible spacers can enable access to sterically hindered epitopes. (c) Dimerization of spacer domains results in increased signal strength and activation stimulus. (d) FcγR interactions arising from IgG based spacers results in activation of innate immune system and decreased efficiency.

either an IgG1-Fc spacer or no spacer [48]. It was found that addition of the IgG1-Fc spacer reduced secretion of IFNγ without a concomitant loss in lytic efficiency. In an effort to evaluate whether this effect was due to epitope location, the authors tested the same CARs in cell lines that expressed a truncated form of the antigen in a membrane-proximal position. However, this did not affect the trend in IFNγ or lytic efficiency that was noted earlier, which the authors attributed to possible steric hindrances. This study underscores the fact that spacer-domain design considerations should take into account factors such as steric accessibility and ligand density. Spacer length has also been purported to affect mechano-transduction of ligand recognition. CARs with longer spacers (IgG4-Fc) that were generated against soluble homo-dimeric TGF-β showed decreased activation profiles compared to shorter (IgG4 hinge only) spacers [49]. Given that these novel CARs respond to soluble antigens and a synaptic cleft does not exist in this case, it clearly exemplifies the role of the spacer region in mechanically transducing ligand-recognition to T cells.

Interestingly, although use of a long IgG-Fc spacer can produce the strongest *in vitro* response for some CARs, overtly strong CAR signaling can also result in impaired *in vivo* function due to activation-induced cell death (AICD) [50]. In addition to fratricidal AICD, non-specific FcγR interactions can trigger AICD and also elicit an innate immune response, limiting CAR-T–cell persistence *in vivo* [51–54]. For CARs where a long spacer is required to achieve optimal spacing between T cells and target cells, IgG1-Fc and IgG4-Fc–based spacers can be mutated to minimize FcγR interactions by substituting the CH2 domain with an IgG2 CH2 domain and/or introducing mutations in other regions that minimize interactions with FcγR. A list of these mutational modifications in IgG1 and IgG4 spacers tested in CAR-Ts is available in Supplementary Table 2. Despite its extremely low binding affinity to FcγR, IgG2-based spacers have only been sparsely used in CARs [55].

Non-IgG–based spacers such as CD8 and CD28 hinge regions have proved equally effective and have been used in clinically approved CAR-T–cell therapies. Alabanza et al. reported that the CD28α hinge region incorporated into an anti-CD19 CAR was shown to increase AICD compared to CD8α hinge CARs [56]. Cytokine production levels (IFNγ and TNFα) were also elevated in CD28 hinge-incorporating CAR-T cells, although there was no significant difference in cytotoxicity or *in vivo* tumor control. The authors attributed this to structural aspects of the CD28 hinge, which is more prone to dimerization than the CD8α hinge. The authors hypothesized that increased dimerization of CD28 hinge-CARs on the cell surface results in increased activation signals and consequently, greater AICD. Recent work from Majzner et al. demonstrated that CD28 hinge and transmembrane domains decrease the antigen-density threshold for T-cell activation in CD19 CARs compared to their CD8 counterparts [57]. We note in the two examples above the hinge and transmembrane domains are varied together as a single variable, so it is difficult to determine whether the observed differences in CAR function are due to the hinge or transmembrane domain alone or their combination. Additionally, size differences in the CD28 hinge region (45 amino acids) and the CD8 hinge region (39 amino acids) may also contribute to their differing functionalities [57]. Furthermore, another study showed that enhancing antigen-independent dimerization of CARs with S228P mutation in IgG4 hinge improved both *in vitro*

cytotoxicity and *in vivo* tumor regression [58]. While this study did not delve into AICD, it is evident that spacer-mediated CAR dimerization caused an anti-tumor response different from that reported by Alabanza et al. [56]. Thus, while structural aspects of the hinge region can be exploited to modulate CAR avidity and valency, it is clear from these studies that the functional effects of such changes are not generalizable.

In addition, it is possible that spacer domains can alter the CAR's structural conformation. For instance, in one study the length of CD8α hinge was modulated by truncating or adding a few residues [59]. These modifications produced CARs with altered extracellular conformations, and resulted in dramatically different *in vivo* and *in vitro* responses. In this study, the authors were able to identify a novel modified CD8α hinge CAR that elicited no severe cytokine release or neurotoxicity *in vivo*. While the mechanistic effect of these modifications to CD8α is not yet understood, this study underscores that fact that spacer design critically contributes to CAR-T efficacy.

Apart from playing a key role in CAR signaling, spacers are also commonly used to quantify and purify CAR-positive subsets of T cells after engineering. Anti-Fc antibodies are commonly used for quantifying cell surface expression of IgG-Fc spacer-based CARs. A novel design incorporating Strep-TagII sequence in the CAR spacer region has also been used for detection and purification of CAR-positive cells [60].

4. Transmembrane domain

Transmembrane domains in CAR structures serve as a fulcrum for transducing ligand recognition signals to the intracellular cytoplasmic domain. In a physiological T cell, the transmembrane domains of the TCR-CD3 complex play an imperative role in the assembly of the complex. Of relevance to the CAR structure is the CD3ζ transmembrane domain incorporated in *cis* with the CD3ζ cytoplasmic domain in first-generation CARs. Dimerization of CD3ζ is mediated by a cysteine residue at position two in the transmembrane domain of CD3ζ. First-generation anti-CEA CARs constructed with a C2G mutation in the transmembrane domain that abrogates dimerization showed a significant impairment in CD69 upregulation when incubated with antigen compared to CARs using the native CD3ζ transmembrane domain [61]. Interestingly, exogenous expression of the CD3ζ domain in a first-generation CAR format led to increased expression of CD3ε as well. In the same study it was noted that first-generation CD3ζ CAR interacts with TCRα and TCRβ chains via ionic interactions between transmembrane domains. These interactions result in both *cis* and *trans* signaling mechanisms where cytoplasmic signaling units of endogenous TCR-CD3 complex are involved [61,62]. In another study, Guedan et al. noted that third-generation CARs incorporating ICOS, 4−1BB, and CD3ζ cytoplasmic domains had significantly improved anti-tumor potency when the ICOS cytoplasmic domain was connected to an ICOS transmembrane domain (ICOSTM-ICOS-41BB-CD3ζ) as opposed to a CD8α transmembrane (CD8TM-ICOS-41BB-CD3ζ) [63]. While Wan et al. reported that the transmembrane domain of ICOS constitutively associates with Lck and promotes proximal signaling, it remains to be conclusively tested if this is responsible for increased anti-tumor potency and persistence of the third-generation ICOSTM-ICOS-41BB-CD3ζ observed by Guedan et al. [63,64].

Transmembrane-mediated interactions can also be used to generate novel CAR designs such as split CAR systems that can deliver *trans*-cytoplasmic domain signaling. Anti-Mesothelin and anti-CD19 CAR systems were constructed by using an scFv-linked KIR domain and a DAP12 domain. This design exploited the natural transmembrane interactions between the KIR2DS2 domain and DAP12 [65]. T cells maintained more stable surface expression of KIR2DS2/DAP12 CARs as compared to CD3ζ CARs, and the authors attributed this to the stability of the KIR2DS2/DAP12 domain in the plasma membrane. Similarly, NKG2D CARs constructed with full-length NKG2D receptors containing the native NKG2D extracellular, transmembrane and cytoplasmic domains allow for transmembrane-mediated interactions with DAP10 and offer the potential for additional "in-trans" co-stimulation with DAP10 [13,14]. Another CAR transmembrane design was based on the FcεRI domain, which consists of three subdomains: α, β, and γ, which are associated through transmembrane interactions. A second-generation "*trans*" signaling CAR was constructed by replacing the native cytoplasmic domains of γ and β with CD3ζ and 4−1BB respectively. The extracellular domain of the α domain was replaced with an anti-CD19 scFv [66]. Such novel designs have the potential to offer different functionality and modularity to CARs. While the actual signaling mechanisms of "trans" configurations (as opposed to linear CARs) have not been fully elucidated, they may mimic multi-chain TCR architecture and can potentially result in a more effective physiological signaling profile. Fig. 4 and Supplementary Table 3 elucidate the novel engineering strategies afforded by the transmembrane domain.

5. Cytoplasmic domains

In T cells, the intracellular signaling domain of the TCR-CD3 complex transduces the necessary "signal 1″ to kick-start the signaling cascade. Co-stimulatory receptors, especially CD28, convey "signal 2″

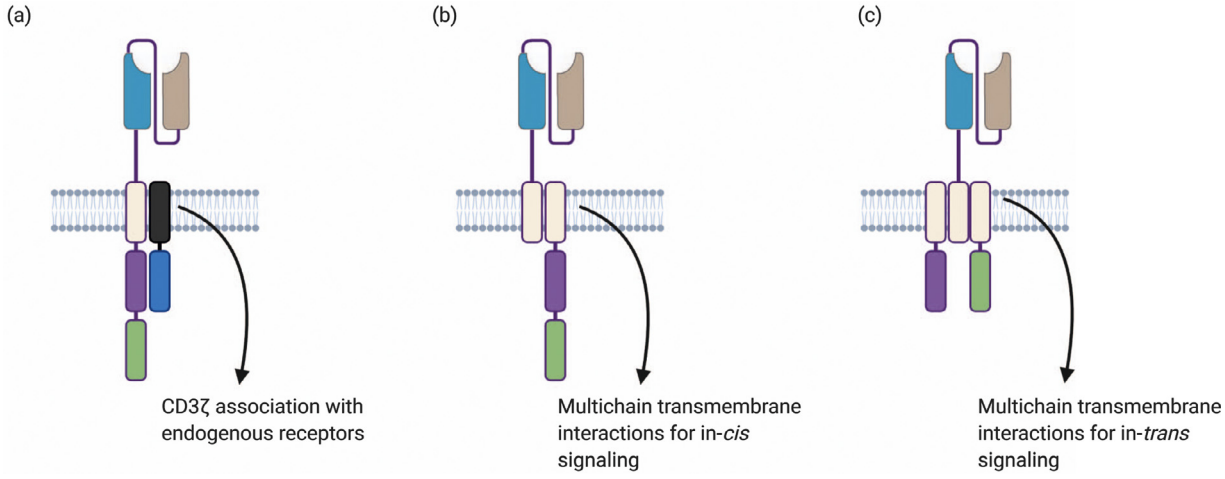

Fig. 4. Transmembrane domain interactions can afford novel CAR designs. (a) Association of CAR transmembrane domain with endogenous receptors/endogenous transmembrane domains. (b) Multichain transmembrane association for split CAR systems. (c) Multichain transmembrane associations to create in trans co-stimulatory domain signaling.

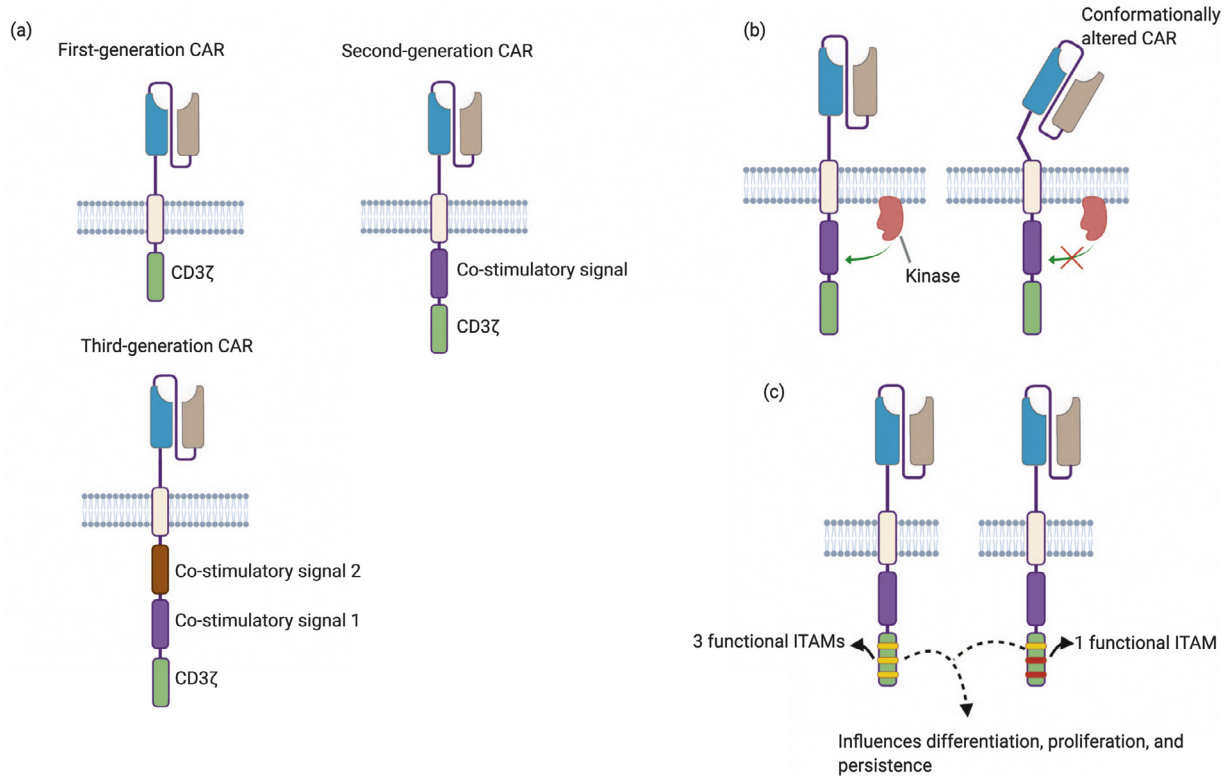

Fig. 5. Number, type and order of co-stimulatory domains as well as ITAM multiplicity can affect CAR-T functionality. (a) Based on the number of co-stimulatory domains used, CARs are classified as first-generation

(no co-stimulatory domain), second-generation (one co-stimulatory domain), or third-generation (two co-stimulatory domains). (b) The order of the co-stimulatory domains can possibly dictate structural compatibility with the transmembrane region and influence conformation of the CAR. It can also affect accessibility of membrane proximal kinases that are critical for signaling. (c) The number of ITAMs on CD3ζ can be modulated to alter effector functions. Mutations in signaling residues in co-stimulatory domains can also be used to regulate effector functions.

which is important for sustained signaling, prevention of anergy, and proliferation. 4−1BB, ICOS, and OX40 are other co-stimulatory receptors that affect T-cell differentiation pathways, metabolic cycles, as well as apoptosis and activation-induced cell death [67]. In CAR-T cells, co-stimulatory signals are usually included in-*cis* with the CD3ζ cytoplasmic domain. The number and type of co-stimulatory signals, as well as their order and proximity to the membrane, are critical parameters that have been addressed in literature (Fig. 5). Immunoreceptor Tyrosine Activation Motifs (ITAMs) present on cytoplasmic domains of TCR-CD3 complexes are the phosphorylation sites, which recruit ZAP70, critical for signaling cascades. In T cells, ITAM diversity and number of functional CD3ζ ITAMs are important for optimal signaling [68,69]. In CAR-T cells, the number of functional ITAMs is gaining attention as an important design strategy to ensure efficacy. We have organized a concise list of relevant publications investigating these parameters in Supplementary Table 4. We acknowledge that the breadth of literature in this field is large and encourage readers to also refer to other key review papers [67,70].

5.1. Number and type of co-stimulatory domains

First-generation CARs are typically engineered with primary signaling motifs that provide activating signal (signal 1) upon ligand engagement. Second- and third-generation CARs are engineered to provide one or more co-stimulatory signals (signal 2) along with activating signal (signal 1) upon ligand engagement. Most first-generation CAR-T cells used only the intracellular CD3ζ domain as the primary signaling motif, and showed lack of proliferation and persistence *in vivo* [71]. Early studies also used the γ-chain of Fc receptors as the primary activating domain, as reviewed by Sadelain et al. [72]. One particular study by Haynes et.al compared first-generation anti-CEA CARs containing FcεR1-γ chain *versus* CD3ζ cytoplasmic domain [73]. This study showed that CD3ζ-based CARs elicited greater cytotoxicity and IFNγ production compared to FcεR1-γ based CARs, potentially due to the presence of more ITAMs in the CD3ζ domain (3 ITAMs in monomeric CD3ζ *vs* 1 ITAM in FcεR1-γ). The effects of ITAM multiplicity are discussed in Section 5.3 below.

Second-generation CARs, especially CD28− and 4−1BB−based CARs, have become particularly attractive due to their ability to confer functionalities such as long-term persistence and increased efficacy and are currently utilized in clinically approved therapies, namely Kymriah® and Yescarta® [70,74−79]. Both 4−1BB and CD28 are intensely investigated in literature and some of the key functional modulations they affect are listed in Table 1. It is worth noting that despite poorer *in vitro* performance compared to its CD28 counterpart, 4−1BB−based CARs tend to result in greater long-term persistence, and the two co-stimulatory domains' relative clinical efficacy remains unclear due to a lack of head-to-head clinical comparisons [80−84]. Xiong et al. made the interesting observation that 4−1BB−based CARs form higher-quality immunological synapses than CD28-based CARs and argued that synapse quality can be used as a predictor of *in vivo* efficacy [85]. In another study, the signaling of CD28− and 4−1BB−based second-generation CARs were compared using a phosphoproteomic mass spectroscopic analysis [86]. Interestingly, it was shown that CARs with CD28 co-stimulatory domains showed faster and higher intensities of phosphorylation, indicating higher signal strengths compared to CARs with a 4−1BB domain. Interestingly, divergent phosphorylation pathways were not detected, suggesting that differences in signaling kinetics and intensity—rather than the types of signaling pathways activated—explain the diverse functional effects of the CD28 and 4−1BB second-generation CARs. In T cells, the signal strength received by the TCRs is

Table 1
Comparison of functional aspects of CD28 and 4−1BB co-stimulatory domains.

CD28	4−1BB
- Lower persistence and differentiation towards effector memory phenotype compared to 41BB second-generation CARs[120] -More prone to tonic signaling and causes early exhaustion[24] -Imparts resistance to Tregs *in-vitro*, in-vivo models however suggested that CD28 co-stimulation causes increased infiltration of Tregs and were less effective against tumors in presence of Tregs[121,122] -Resistant to CTLA4 inhibition[123] -Faster and higher signaling intensity[86] -Does not alter scFv "affinity ceiling" −affinity beyond which IFNγ, IL2 secretion and cytotoxicity do not increase[124]	-Greater persistence and differentiation towards central memory phenotype compared to CD28 second- generation CARs[120] - Can reduce tonic signaling at optimal expression levels and decrease exhaustion [24,34,36] -Slower and less intense signaling[86]

dependent on pMHC affinity and density [28,87]. In CAR-T cells, the signal strength is dependent on all of its structural components, as each of them affects signal transduction. In the case of T cells, TCR signal strength is critical in determining positive and negative selection of T cells, differentiation phenotypes, as well as cytokine secretion [27]. Signal strengths can similarly influence CAR function and hence their ultimate efficacy.

Third-generation CARs utilizing both CD28 and 4−1BB domains have been tested against various targets such as CD19, PSMA, GD2 and mesothelin [76,88−91]. In one particular study, differences in intracellular signaling were evaluated for anti-CD19 second-generation CARs with a CD28 co-stimulatory domain and compared with third-generation CARs containing 4−1BB and CD28 co-stimulatory domains. This study revealed that third-generation CAR-T cells showed an overall increase in the phosphorylation status of signaling proteins, indicating potentially higher signal strengths for the third-generation CARs compared to second-generation CARs [92]. Preclinical studies of third-generation anti-PSMA and anti-mesothelin CD28−4−1BB−CD3ζ CARs indicated superior tumor eradication and an increased persistence compared to their second-generation formats [89,91]. Similarly, third-generation ICOS−4−1BB−CD3ζ−based anti-mesothelin CARs had increased anti-tumor potency as well as increased persistence [63]. With regards to B-cell malignancies, a direct comparative clinical study of second-generation CD28-based CARs to third-generation 4−1BB−CD28−based CARs indicated higher persistence and expansion of the third-generation CARs compared to the second-generation formats, particularly in cases where disease burden was low [88]. However, the superiority of third-generation CARs over second-generation counterparts is still a subject of debate. For instance, a study by Abate-Daga et al. comparing the efficacy of second-generation, CD28-based anti-PSCA CARs with third-generation CD28−4−1BB−based CARs indicated that while *in vivo* persistence of third-generation CARs were generally improved in preclinical mouse xenograft models of pancreatic cancer, the second-generation CARs still outperformed the third-generation formats in terms of anti-tumor potency [93]. In another case, *in vitro* cytokine secretion (IL-2, TNFα) and proliferation were improved in third-generation anti-GD2 CARs containing CD28-OX40-CD3ζ domains as compared to second-generation (CD28-CD3ζ or OX40-CD3ζ) and first-generation (CD3ζ) formats [94]. However, a clinical study from the same group revealed that the clinical performance of the third-generation CAR was not significantly improved compared to previous studies on first-generation CARs reported by Pule et al. and Louis et al. [95−97]. Apart from the structural differences in co-stimulatory domains, patient heterogeneity and differing treatment regimens may also contribute to the lack of improvement from third-generation CARs. Another study by Hombach et al. reported that third-generation, anti-CEA CD28-CD3ζ-OX40 CARs performed suboptimally compared to second-generation CD28-CD3ζ CARs [98]. The fact that this study used cytokine-induced killer cells (CIKs) makes it difficult to generalize these results to conventional CAR-T cell engineering. Parallel clinical comparisons and detailed mechanistic studies on different co-stimulatory designs will be required to affirm which of these designs are clinically effective. Lastly, Zhao et al. analyzed seven chimeric antigen receptors structures and showed that a second-generation CD28-based CAR co-expressed with 4−1BB ligand (4−1BBL) performed better than a third-generation CAR with both 4−1BB and CD28. This study suggests that both the type and the spatial configuration of co-stimulatory modules matter in CAR function [76].

Other co-stimulatory domains such as OX40 and ICOS also have been utilized in various studies. ICOS signaling domains have been shown to promote Th17 polarization [99]. Anti-GD2 third-generation CARs constructed with CD28 and OX40 co-stimulatory domains showed improved proliferation and expansion compared to second-generation CARs using only the CD28 co-stimulatory domain [94]. Another interesting CAR construct is a third-generation CAR using CD28 and the cytoplasmic domain from TLR2 (Toll-like receptor 2), which not only increased *in vivo* tumor eradication but also upregulated genes related to migration, synaptic transmission, and cell adhesion [100]. Another novel design utilizes truncated IL2Rβ along with a STAT3-binding motif with a CD28 and CD3ζ domain to activate downstream JAK-STAT cytokine signaling pathways [101]. These studies indicate that guided modulation of the cytoplasmic domain can equip CAR-T cells with novel functionalities.

Multiple co-stimulatory domains and their combinations have been tested *in vitro* and *in vivo*, yet there is no consensus on the ideal design for CAR-T cells. The choice of co-stimulatory domain is likely dependent on clinical indications as well as on other CAR modules. Tonic signaling in anti-GD2 second-generation CARs caused by scFv-mediated aggregation was eliminated when a 4−1BB cytoplasmic domain was used instead of CD28 [36]. Anti-GD2 CARs based on the same scFv were tested in second-generation format with 4−1BB co-stimulation or third-generation format with both 4−1BB and CD28 [34,90]. Interestingly, these studies showed a uniform distribution of CAR molecules on the surface and minimal surface aggregation unlike the aggregated punctae of CARs observed in the CD28-based second-generation CAR in the study by Long et al. [36]. These studies indicate that co-stimulatory domains could influence CAR surface expression and distribution. Affinity thresholds, most commonly associated with the ligand-binding domain, can also be altered by the number and type of cytoplasmic domains used. The *in vitro* lytic efficiency of 4−1BB−based CARs was reduced with a lower-affinity scFv, while CD28-based CARs showed comparable lytic efficiencies with a wide range of scFv affinities [102]. Second-generation anti-PSCA CARs with a 4−1BB domain were less reactive to target cells presenting lower antigen densities compared to CD28-based CARs [103]. In light of these studies, it is evident that the optimal design for the cytoplasmic domain should be based on its synergistic effect with other components of the CAR.

5.2. Order of co-stimulatory domains

For second-generation and subsequent CAR designs, the order of the co-stimulatory domains has been found to influence their effector functions. An early study exploring functional differences between CD28-CD3ζ CARs and CD3ζ-CD28 CARs found that CD28-CD3ζ T cells secreted higher levels of IL-2 and importantly were able to undergo repeated cycles of stimulation and expansion, critical for sustained activity [74]. The authors posit that the superior performance of CD28- CD3ζ could be due to better structural integrity, which enhances signal transduction or the proximity of CD28 to membrane-proximal signaling kinases such as Lck.

Recent work has shown that CD28-CD3ζ, OX40-CD3ζ and CD28-OX40-CD3ζ produced comparable amounts of IL-10, an anti-inflammatory cytokine that is known to reduce anti-tumor efficacy [94]. Hombach et al. found that CD28-CD3ζ-OX40 T cells secreted significantly lower levels of IL-10 compared to a CD28-CD3ζ control [104]. It would be interesting to test whether these contradictory effects of OX40 are due to different positioning of the cytoplasmic domains or due to differences in the nature of the scFvs, target antigen, or other components of the CAR. Guedan and coworkers explored a variety of cytoplasmic domain combinations of 4−1BB, ICOS, and CD3ζ and found that T cells expressing a CAR with an ICOS-4−1BB-CD3ζ cytoplasmic domain and ICOS transmembrane domain resulted in enhanced expansion and persistence *in vivo* and 100% tumor regression within 35 days. Notably, a CAR with ICOS−4−1BB−CD3ζ but using a CD8 transmembrane domain displayed only modest tumor regression. Given that this CD8-modified CAR also expressed lower levels of IL-13 and IL-6, the authors posit that the membrane-proximal section of the cytoplasmic domain has a significant role in determining the CAR-T−cell's cytokine profile [63].

5.3. ITAM multiplicity

ITAMs are the sequence motifs (YXXL/I) in the cytoplasmic domains of receptors in hematopoietic cells [105]. In physiological T cells, ZAP70, a protein kinase, is recruited to doubly phosphorylated ITAMs resulting in activation of the signaling cascade. CAR-T cells, despite forming non-classical immune synapses, have also been shown to activate the ZAP70 kinase in an antigen-dependent manner [47,92]. Key differences exist between T cells and conventional CAR-T cells in terms of the number of ITAMs engaged in signaling (three for monomeric and six for homodimeric CARs *vs*. ten for T cells) [106]. Significant differences in activity of CARs and TCRs have been attributed to the difference in number of ITAMs [106,107]. Increasing the number of ITAMs from three to six by having two CD3ζ domains in *cis* resulted in an increased fraction of activated cells. Reducing the number of ITAMs from three to two enabled selective recognition of cells expressing high target densities [108]. Majzner et al. also demonstrated that doubling of CD3ζ ITAMs from three to six in 4−1BB−based second-generation CARs increased T-cell proliferation, IL-2 production, as well as *in vivo* anti-tumor response [57]. In another study each of the ITAMs of CD3ζ were mutated and it was found that both the number of functional ITAMs and their position influenced CAR-T−cell proliferation, *in vivo* tumor eradication, as well as differentiation phenotype [109]. In the same study, the authors showed that a single membrane-proximal functional ITAM in conjunction with a CD28 cytoplasmic domain was able to sustain proliferation, persistence, and cytotoxicity. In physiological T cells, ITAM multiplicity has been implicated in negative selection of T cells in the thymus, proliferation, and cytokine secretion [110]. In CAR-T cells, signaling mechanisms that are affected by ITAMs are not clear. Particularly, the availability of in-*trans* ITAMs potentiates distinct signaling mechanisms in T cells. The position of ITAMs themselves influences its affinity towards ZAP70 kinase in T cells [105]. These aspects of ITAMs have implications in CAR design and improved understanding of these aspects of signaling could immensely inform CAR-T design.

6. Conclusions and outstanding questions

Each module of the CAR structure influences CAR function both independently and synergistically. Reducing the affinity of scFv has been shown to improve selective recognition of tumor and thus reduce on-target, off-tumor toxicity. However, lowering the affinity can also result in reduced activation and anti-tumor cytotoxicity. Choosing the right cytoplasmic domains can augment these signals. For example, in one study, lytic efficiencies of CARs incorporating a CD28 cytoplasmic domain were not affected by the scFv affinities, while 4−1BB−incorporating CARs showed differential lytic efficiencies correlating with scFv affinities [102]. Multiple parameters such as CD28 co-stimulation, elevated levels of CAR expression, and aggregation-prone scFv sequences have been implicated in tonic signaling, a phenomenon that can result in early CAR-T−cell exhaustion and reduced tumor eradication. The length and nature of spacer domains impact immune synapse distances and are critical in determining cytolysis. In conjunction with scFv, the spacer domain is crucial in enabling target-epitope accessibility. Transmembrane domains are key structural components that have the potential to be exploited for novel CAR systems with *in trans* coupling of co-stimulatory domains and CD3ζ domains. In this review, we have focused on the design of the CAR molecule itself, but it is also important to acknowledge that parameters other than the CAR protein can significantly impact CAR-T−cell function. For example, choice of vector system for transduction and site of insertion of CARs have a deep impact on efficacy [34,35,111].

Lastly, a number of novel innovations in CAR designs has led to development of "universal" and switchable systems which can be used to conveniently tune CAR-modified T cells to target, in principle, any antigen of interest [93,112]. These novel designs usually consist of T cells engineered with an extracellular adapter domain that binds to soluble ligand-binding domains. Similar to conventional CARs, the adapter domain is connected to transmembrane and intracellular cytoplasmic signaling units. SUPRA CARs utilize a leucine-zipper extracellular domain connected to the transmembrane and cytoplasmic signaling domains (zipCAR); scFvs targeting the antigen of interest are fused to a cognate leucine-zipper domain that binds to the extracellular moiety of the zipCAR [113]. Other designs use specific affinity tags such as biotin or peptide neoepitopes (PNE) [58,114]. These novel "universal" designs offer a number of functional benefits, such as targeting multiple antigens simultaneously and allowing for regulation of effector functions by modulating the dose of the ligand-binding moiety. Despite these advantages, the design of novel CAR systems still suffers from similar challenges as conventional CARs, and their implementation requires additional soluble components, each with its own design and delivery considerations. Due to the functional similarity of conventional and novel CARs, design principles and strategies developed for conventional CARs can be translated to novel CAR design and development.

Many studies have employed rational protein design and/or library screening to optimize CAR sequences. CAR components can be modified in a combinatorial or sequential manner depending on their function *in vitro* and *in vivo*. For example, CARs targeting the orphan G protein-coupled receptor class C group 5 member D (GPRC5D) were constructed using select scFv sequences obtained from a phage-display library generated against the target epitope. scFv sequences were chosen based on binding capacity and incorporated into CARs in VH/VL or VL/VH formats. A combinatorial library was constructed with varied spacer domains and orientations of the select scFvs [55]. In another study, a bispecific CAR using two distinct scFv sequences targeting CD19 and CD20 sequentially identified spacer and scFv linker requirements to enable optimal targeting of both antigens

[42]. While these studies and many others have provided immense information about CAR function and design strategies, they are largely empirical, tedious, and are limited in the number of designs one can exploit in an experiment. The optimal CAR design for a given application will likely depend on interrogation of many variables of each module in an interdependent and combinatorial fashion, a process that can benefit from single-cell–based, high-throughput functional assays in the future [115].

A second major hurdle in CAR-T development is to identify *in vitro* quantitative parameters that can be correlated to *in vivo* efficacy. It has been shown that parameters such as *in vitro* activation and cytokine release do not always correlate with desired *in vivo* outcomes [53,116–118]. Ultimately, *in vitro* functional assays for CAR-T design need to be developed and validated in correlation with *in vivo* outcomes not only from animal models but also from clinical data. Along this line, polyfunctionality, namely the ability of T cells to produce more than one type of cytokine, has gained attention as an *in vitro* measurement that correlates with *in vivo* efficacy. Pre-infusion anti-CD19 CAR-T products were tested for 32 different cytokines and chemokines using a novel single-cell–based assay, and it was reported that increased polyfunctionality correlated with improved patient response [119]. Functional assay platforms that can quantitatively interrogate parameters that are predictive of *in vivo* CAR-T–cell function would greatly facilitate the elucidation of relevant CAR design principles, enable better understanding of CAR-T–cell biology, and facilitate the discovery of CAR formats with novel functionalities [115]. Ultimately, such efforts can streamline the immunotherapeutic discovery process, resulting in effective CAR-T candidates at lower costs and reduced development time.

7. Search strategy and selection criteria

Relevant papers on CAR-T design were chosen from searches on Google scholar and PUBMED. Search terms “CAR-T design”, “CAR-T cytoplasmic domain”, “CAR-T spacer”, “CAR-T scFv”, “CAR-T biology” were used to identify literature relevant to this article. In addition, we also used “TCR-CD3 complex”, “signal strengths” “polyfunctionality”, “TCR biology” to identify relevant articles from T-cell biology to corroborate and compare functional mechanisms between T cells and CAR-T cells (in some instances). We explored relevant and selected literature ranging from 1997 - March 2020.

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Supplementary materials

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