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Chimeric antigen receptor signaling: Functional consequences and design implications

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Chimeric antigen receptor (CAR) T cell therapy has transformed the care of refractory B cell malignancies and holds tremendous promise for many aggressive tumors. Despite overwhelming scientific, clinical, and public interest in this rapidly expanding field, fundamental inquiries into CAR T cell mechanistic functioning are still in their infancy. Because CAR T cells are manufactured from donor T lymphocytes, and because CARs incorporate well-characterized T cell signaling components, it has largely been assumed that CARs signal analogously to canonical T cell receptors (TCRs). However, recent studies demonstrate that many aspects of CAR signaling are unique, distinct from endogenous TCR signaling, and potentially even distinct among various CAR constructs. Thus, rigorous and comprehensive proteomic investigations are required for rational engineering of improved CARs. Here, we review what is known about proximal CAR signaling in T cells, compare it to conventional TCR signaling, and outline unmet challenges to improving CAR T cell therapy.

INTRODUCTION

Chimeric antigen receptor (CAR) T cell therapy, named "Advance of the Year" in 2018 by the American Society of Clinical Oncology, has revolutionized cancer treatment. Kymriah (tisagenlecleucel, Novartis) and Yescarta (axicabtagene ciloleucel, Gilead) were rapidly approved by the U.S. Food and Drug Administration, and the number of active clinical trials testing CAR T cells in patients has exploded. As CAR T cell therapies mature, the focus of the field is shifting from showing efficacy to making them work better. In particular, there is substantial interest in (i) minimizing the toxic side effects of hematologic malignancy–targeted CAR T cells and (ii) improving the efficacy of solid tumor–targeted CAR T cells. Although there are many approaches to addressing these major challenges, it is becoming clear that understanding how CARs signal, particularly as compared with canonical T cell receptors (TCRs), may be critically important for designing more effective therapies.

CARs, unlike the TCRs they mimic, consist of molecules in which tumor antigen recognition and intracellular activation are combined. Their structure and design have been extensively reviewed elsewhere (*1*, *2*) but minimally comprises an extracellular antigen recognition domain linked through a transmembrane domain to an intracellular activation domain or domains (*3*, *4*). Early CARs consisted of antibody single-chain variable fragments (scFvs) fused through a transmembrane domain to the cytoplasmic tail of the TCR signaling component CD3ζ; however, the addition of costimulatory signaling domains is required to achieve optimal clinical efficacy. CARs that incorporate a costimulatory domain membrane-proximal to the CD3ζ signaling domain, including both Kymriah and Yescarta (as well as most of the clinically used CARs), are referred to as second-generation CARs (*5*, *6*) (Fig. 1). Third- and fourth-generation CAR constructs are being developed, with each successive generation adding additional signaling capacity (*7*, *8*). Third-generation CARs contain two in-line costimulatory domains, whereas fourth-generation CAR T cells typically incorporate separate cytokine signals. Second-generation CARs differ in their choice of costimulatory domain, which affects the efficacy, response phenotype, and metabolic properties of the resulting CAR T cells (*9*). The most frequently used costimulatory domains derive from the CD28 family (CD28 and ICOS, Inducible T Cell Costimulator) and the tumor necrosis factor receptor (TNFR) family (4-1BB, CD27, and OX40). Kymriah and Yescarta use the same scFv, which recognizes the B cell antigen CD19, but Yescarta incorporates a CD28-derived costimulatory domain, whereas Kymriah incorporates a 4-1BB domain (the two constructs also have different hinge and transmembrane regions). T cells expressing these two different second-generation CARs have substantial and important functional differences, although the reasons for this are not entirely clear. CD28-based CARs seem to elicit stronger T cell activation as compared with 4-1BB–expressing CARs, tending toward an effector-like phenotype, with high interleukin-2 (IL-2) secretion and cytolytic capacity; they are sensitive to low antigen levels and highly proliferative and glycolytic (*2*, *10*, *11*). However, in vivo persistence of CD28-based CARs is limited, and they are more prone to activation-induced cell death (*12*–*14*). In contrast, 4-1BB–based CAR T cells tend toward a central memory phenotype with slower effector response and elevated oxidative metabolism (*2*, *10*, *11*). 4-1BB CAR T cells are also more persistent, due to decreased exhaustion and up-regulation of BCL-2 family members (*15*), and have been found in vivo even years after treatment (*16*, *17*). It is likely that these strikingly distinct phenotypes arise from their activation of different downstream pathways. In normal T cells, 4-1BB typically initiates downstream signaling through the recruitment of TNFR-associated factors (TRAFs) (*18*); CD28, in contrast, signals through the phosphatidylinositol 3-kinase (PI3K)–AKT pathway (*19*). It is presumed that these pathways are preserved in CAR T cells, but it is possible that when these domains are placed in the context of a CAR construct, they activate other pathways as well. It is abundantly clear that CAR design has significant functional implications, but the precise mechanisms responsible for this are unknown.

Endogenous TCRs recognize peptide:major histocompatibility complex (MHC) antigen through a highly complex and interconnected process involving receptor components as well as intracellular kinases, substrates, and coreceptors. Highly coordinated and tightly regulated

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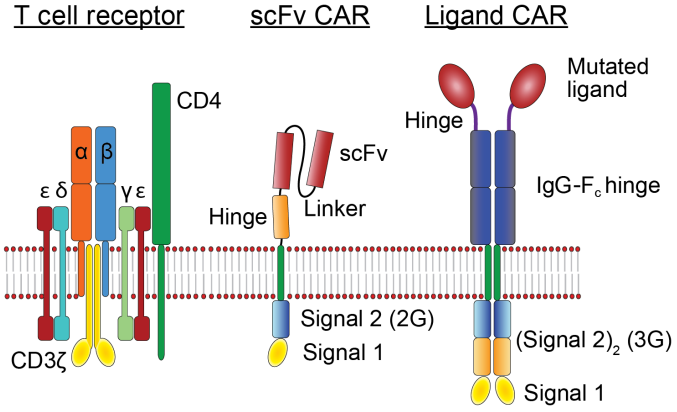

Fig. 1. CAR versus TCR structure. TCRs (left) are a multisubunit antigen recognition complex in which the TCRα and TCRβ chains recognize peptide in the context of major histocompatibility complex (MHC) molecules and associate with signaling molecules CD3δ, CD3ε, CD3γ, and CD3ζ (shown in gold). TCRs also associate with a coreceptor, either CD4 (shown in green) or CD8. Minimally, CARs (center and right) are built around an antigen-binding extracellular domain, either an antibody-derived scFv (center) or a receptor-binding ligand or peptide (right). These antigen recognition domains are linked through a flexible immunoglobulin domain-containing hinge region (for scFvs; center) or a hinge and immunoglobulin-based scaffold (for receptor-binding constructs; right) to a transmembrane domain (green) and then to signaling domains. First-generation CAR constructs (not shown) have only the cytoplasmic tail of CD3ζ, whereas subsequent generations contain one (second generation; center) or more (third generation example at right) costimulatory domains membrane-proximal to a CD3ζ tail (gold). IgG, immunoglobulin G; F_c, fragment crystallizable.

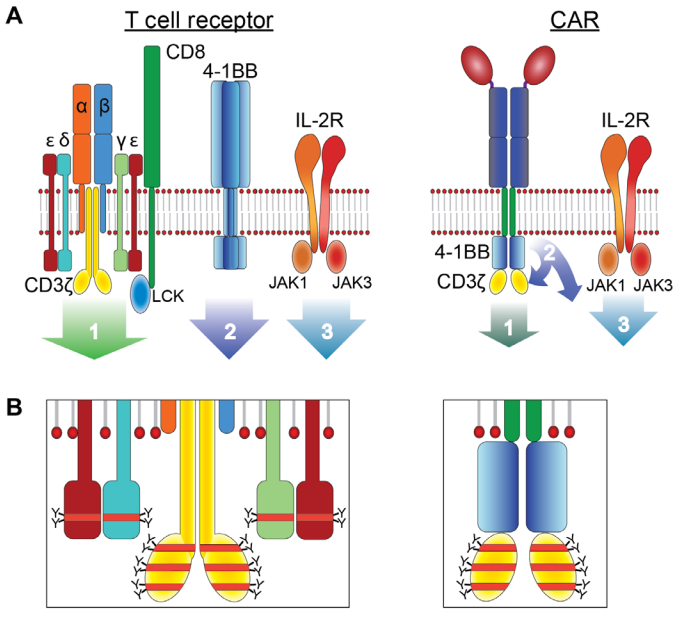

Fig. 2. Canonical TCR-mediated activation requires three distinct signals, whereas CAR signaling is less discrete. (**A**) Antigen-dependent ligation of the TCR complex (left), termed signal 1, initiates T cell activation. The TCR:peptide-MHC complex stabilizes coreceptor-MHC interactions (CD8 shown in green), which results in more recruitment and activation of the Src family tyrosine kinase LCK. Signal 2 is mediated by costimulatory molecules such as CD28, 4-1BB, and OX40; in T cells, signal 2 is not completely contemporaneous with signal 1 and may not occur in exactly the same place. In CAR T cells (right), in contrast, signal 1 and signal 2 are mediated by the same physical event of antigen recognition. Signal 3 for both canonical T cells and CARs is provided through cytokine signaling and usually occurs later than both signal 1 and signal 2. (**B**) The TCR complex (left) comprises 10 immunoreceptor tyrosine-based activation motifs (ITAMs), depicted in red. Each ITAM incorporates two tyrosines (Y), each of which is phosphorylated by LCK and other Src family tyrosine kinases. Phosphorylated ITAMs serve as docking sites for ZAP-70 and other Src homology 2 (SH2) domain–containing proteins, which nucleate signaling cascades, leading to full activation. CAR constructs (right) only comprise six ITAMs if the CAR dimerizes, and three if it does not. Although it is clear that ITAM multiplicity has strong effects on CAR signaling, how to incorporate ITAM number and position into CAR design has not yet been optimized.

mechanisms including kinetic proofreading (*20*, *21*), kinetic phase separation (*22*), mechanotransduction (*23*), conformational change (*24*, *25*), and coreceptor complex stabilization converge to permit exquisite control and sensitivity of TCR stimulation (*26*). It is not clear whether CAR ligation activates CAR T cells using entirely conserved endogenous TCR signal transduction mechanisms, but it is clear that CARs are effective at recognizing antigen and triggering T cell activation. This suggests that CARs are capable, optimally, of recapitulating the effects of canonical T cell activation events, typically referred to as signal 1, signal 2, and signal 3 (Fig. 2A).

In this review, we discuss what is known about how CARs signal in T cells, using TCR signaling as an initial conceptual framework. We review recent studies that shed light on the downstream signaling cascades initiated by CAR ligation, discuss the signaling and functional implications of design differences between CARs and TCRs, and propose a paradigm for further efforts to improve CAR function.

Signal 1

Signal 1 refers to the immediate downstream consequences of antigen recognition by the TCR, largely initiated by lymphocyte-specific protein tyrosine kinase (LCK), an Src family tyrosine kinase (SFTK). LCK phosphorylates the tyrosines in immunoreceptor tyrosine-based activation motifs (ITAMs), canonical immunoreceptor activation domains consisting of two Yxx(I/L) motifs separated by six to eight amino acids (*27*, *28*). LCK also facilitates CD4/CD8 coreceptor recruitment (*29*, *30*), which strengthens the TCR-peptide:MHC interaction and creates a positive feedback loop to recruit more LCK. This results in phosphorylation of the 10 ITAMs found in the TCR complex on CD3ε, CD3δ, CD3γ, and CD3ζ chains (*31*, *32*) (Fig. 2B), which then serve as recruitment and activation sites for Src homology 2 (SH2)– and SH3-containing kinases such as ZAP-70, which further activate signaling proteins such as linker of activated T cells (LAT), SLP-76, and phospholipase C–γ (PLC-γ) (*27*, *33*).

Almost all CAR constructs contain the intracellular CD3ζ domain, which contains three ITAM motifs (*3*) (Fig. 2B); these are typically the only ITAMs present in CAR constructs. Thus, whereas the endogenous TCR complex contains 10 ITAMs, CARs contain 3 (or 6, if they dimerize). Coreceptor-independent phosphorylation of CD3ζ ITAMs may be critical for the initiation of CAR activity, as CARs do not engage coreceptors. In addition, both the number and the position of ITAM motifs have been shown to be important for CAR T cell function. For instance, studies in computational and reductive model systems demonstrated that increasing ITAM number from 3 to 6 or 10 seemed to improve CAR activation (*34*). Similarly, ablation of the two N-terminal ITAMs (XX3) in a second-generation CD19-targeting CAR construct led to decreased efficacy relative to the

wild-type construct. However, this study also created a construct in which the C-terminal ITAMs were ablated (1XX); this construct outperformed the wild-type construct, as did a construct containing only the third ITAM (although this construct deleted the first two ITAMs, such that the third ITAM was located where the first ITAM would have been) (*35*). Thus, although it is clear that both ITAM number and position have a considerable effect on CAR function, this effect is not always conserved across systems and is likely to be influenced by numerous other factors.

In addition, the choice of costimulatory domain has been shown to be important for CD3ζ ITAM phosphorylation. It has been shown that CD3ζ ITAM phosphorylation occurs in the same places upon ligation of CD28- or 4-1BB–containing second-generation CAR T cells, with greater intensity at earlier time points in cells expressing CD28-containing CARs (*36*, *37*). This is likely due to the fact that CD28 has a proline-rich region with which LCK associates; LCK can be coimmunoprecipitated with CD28-containing constructs, but only minimally associates with 4-1BB–bearing constructs (*36*). Mutating the LCK association domain in CD28 greatly reduces phosphorylation of CD3ζ upon CAR ligation (*36*), suggesting that CD28 in CARs acts similarly to coreceptors in the TCR complex, recruiting LCK to potentiate a positive-feedback loop.

Furthermore, in silico and reductive molecular reconstitution models demonstrate that the kinetics of phosphorylation by LCK differ between ITAMs within one CAR (*34*, *38*). For example, expression of liposome-bound LCK and CD3ζ in isolation leads to phosphorylation at each ITAM tyrosine with distinct kinetics, with fastest phosphorylation of the first and fourth tyrosines (ITAMs 1 and 2) and slowest phosphorylation of the sixth and fifth tyrosines (ITAM 3). In addition, although the order of tyrosine phosphorylation appears to be random within a particular CD3ζ molecule, there seems to be competitive inhibition of subsequent phosphorylation events once initial phosphorylation has occurred (*38*). Further evidence to support the differential kinetics of ITAM phosphorylation in CARs comes from peptide screen experiments and in vitro phosphorylation studies of second-generation CARs (*36*, *39*), although these population-based studies were unable to assess the effects of ITAM phosphorylation on the kinetics of further ITAM phosphorylation.

After the CD3ζ ITAMs have been phosphorylated, ZAP-70 docks via its dual SH2 domains and phosphorylates a number of known substrates including LAT, SLP-76, and PLC-γ. It has been shown that ZAP-70, SLP-76, and PLC-γ are also phosphorylated upon CAR ligation (*36*, *37*) and that this phosphorylation, as with CD3ζ, is more robust at early time points (within minutes) in CAR T cells expressing CD28-containing CAR constructs than in those expressing 4-1BB–containing constructs. There are limited data on later time points, but ZAP-70 phosphorylation appears to be roughly equal across all of these cell types after 24 hours of stimulation (*40*). It should be noted that these findings likely depend on many other factors (such as CAR density and affinity, T cell population, and method of activation), as antigen recognition does not universally lead to phosphorylation of these early signaling proteins (*41*). How ZAP-70, LAT, SLP-76, and other proximal signaling molecules are recruited to CARs and subsequently activated is an area of active investigation. It seems probable that SFTK activity is required for ZAP-70 phosphorylation, as ZAP-70 phosphorylation decreases upon treatment with the tyrosine kinase inhibitor dasatinib (*42*), but specific mechanistic data are lacking.

Circumstantially then, it seems that the signaling events most immediately downstream of CD28-containing CARs are likely to be similar to those downstream of TCRs. 4-1BB–containing CARs initiate similar receptor-proximal phosphorylation events upon ligation, but less robustly. In addition, LCK does not appear to associate with CD28-lacking CARs; whether LCK is recruited in some other way or whether other SFTKs compensate for LCK in these cases is unknown but important to investigate. It has also been shown that specific residues in the cytoplasmic tail of CD3ζ affect ITAM accessibility and LCK binding (*43, 44*); it is possible that similar factors affect the kinetics of CAR activation.

Signal 2

After initiation of signal 1, T cells require costimulation (signal 2) to achieve optimal activation and to prevent anergy (*45*). Endogenous T cell costimulation is typically provided by CD28, which is recruited to the site of T cell antigen-presenting cell (APC) contact by its ligands B7.1 and B7.2. Ligation of CD28 results in phosphorylation of a membrane-proximal YMNM motif by SFTKs, enabling the p85 subunit of PI3K to bind and activating AKT, which leads to the initiation of distal signaling cascades including the mTOR (*46, 47*), glycogen synthase kinase 3 (GSK3) (*48*), and GRB2-SOS (*19*) pathways. CD28 also contains two proline-rich motifs (PRRP and PYAP), which bind LCK and ITK as well as other SH3-containing proteins such as GRB2 and filamin A (*19, 49*). In addition, the YMNM and PYAP motifs have been shown to be essential for the formation of the immunological synapse (IS), a bull's eye–like superstructure required for optimal T cell activation (*50*).

In CARs, costimulation is usually provided in series through the in-line addition of one (in second-generation) or two (in third-generation) membrane-proximal costimulatory domains (Fig. 1). As mentioned above, the most frequently used domains derive from the CD28 family (CD28 and ICOS) or the TNFR family (4-1BB, CD27, OX40). Given the notable differences between how the CD28 family and TNFR family molecules function in their endogenous contexts, it is perhaps not unexpected that engineered CAR T cells comprising CD28 and 4-1BB costimulatory moieties function differently. Data show that distinct signals are initiated from these CAR constructs. Most studies of CAR signaling show that CD28-containing CARs signal more rapidly and intensely than 4-1BB–containing CARs at early time points, using a variety of readouts (*15, 36, 37, 41, 51*). Specific evaluation of proximal signaling pathways also indicates that signal transducer and activator of transcription 3 (STAT3) and PI3K appear to be more robustly activated (*36*). However, comprehensive analyses of the signaling cascades initiated by CAR ligation are still in their infancy, and the consequences of concatenating costimulatory domains with signal 1 domains have not yet been elucidated.

4-1BB is typically up-regulated on T cells 24 hours after activation (*52*), at which point, ligand binding inhibits apoptosis and stimulates proliferation and effector function (*53*). 4-1BB comprises binding sites for TRAF1, TRAF2, and TRAF3 (*54*), and these adaptor molecules can modulate the activation of the canonical and noncanonical nuclear factor κB (NF-κB) pathway as well as mitogen-activated protein kinases (MAPKs) (*55, 56*). Signaling downstream of these TRAF family members up-regulates the transcription of prosurvival proteins such as BCL-2 and BCL-XL and of cell cycle regulators such as MYC, cyclin D1 (CCND1), and $p21^{Cip1}$ (CDKN1A), as well as the production of proinflammatory cytokines such as IL-4, IL-2, IL-5, and interferon-γ (IFN-γ) (*57*). In TNFR-containing CARs, however, pathway activation occurs immediately upon CAR ligation,

which potentially leads to distinct biochemical and functional consequences. For instance, 4-1BB–bearing CARs have been shown to associate with TRAF1 and TRAF3 to activate NF-κB, but not TRAF2 (*15*). In addition, studies have demonstrated that ligation of 4-1BB–containing CARs leads to higher levels of BCL-2 and BCL-XL expression than ligation of CD28-containing CARs (*15*), in keeping with the idea that TRAFs stimulate transcription of these antiapoptotic proteins. However, 4-1BB CAR T cells do not typically produce as much IFN-γ or IL-2 as CD28 CAR T cells upon stimulation (*15, 58*), suggesting that the pathways triggered by TRAF activation in T cells are not entirely conserved in CAR T cells.

There is some evidence that 4-1BB can have a dominant moderating effect on CAR signaling; two groups have shown that third-generation constructs expressing both CD28 and 4-1BB initiate early phosphorylation events with kinetics more similar to those of 4-1BB second-generation constructs than to those of more potent CD28 second-generation constructs (*37*, *40*). In contrast, two other recent publications demonstrated that third-generation CAR T cells expressing 4-1BB and CD28 outperformed second-generation CARs in terms of cytokine release, CAR T cell survival, and tumor elimination (*41, 51*). There is therefore some controversy about whether third-generation constructs are consistently more or less potent than second-generation constructs or whether they are directly comparable from a signaling standpoint. Available evidence suggests that many third-generation CAR constructs function very potently in vitro but are subject to early exhaustion in vivo; however, very little signaling analysis has been done of third-generation CAR designs that incorporate OX40 or CD27. Thus, it is probable that the addition of signaling modules to CAR constructs has complex and unpredictable consequences, and it is also probable that canonical signaling events do not predict functional outcomes in CARs as directly as they would in TCRs. Much that is assumed about CAR signaling relies on knowledge gained through studies of endogenous TCR signaling, but it is likely that colocating signaling domains from different pathways in CAR constructs create new and emergent signaling cascades.

Signal 3

In T cells, signal 3 is mediated by soluble factors such as cytokines and is often initiated after stimulation of signals 1 and 2 (*59*). Signal 3 is required for optimal T cell activation (*60*) and is widely viewed as essential for full CAR T cell function (*17*). Cytokines such as IL-2, IL-7, and IL-15 are often used to stimulate CAR T cells during the manufacturing process (*61, 62*); proliferation is required for optimal transduction as well as CAR T cell therapeutic activity (*63*). Cytokine signaling is also an essential part of optimal CAR T cell activity in vivo; IL-6/STAT3 signaling has been shown to improve anti–chronic lymphocytic leukemia (CLL) CAR T cell activity (*17*), and exogenous provision of cytokine signals such as IL-2, IL7, CCL19, and IL-15 has been shown to improve function in solid tumor–targeted CAR T cells (*64–66*). Many groups are working to incorporate signal 3 directly into CAR design to further improve survival, persistence, and antitumor properties of CAR T cells. For instance, a recent study incorporated STAT3 and STAT5 signal-initiating motifs in its CAR construct and demonstrated that this improved proliferation while delaying differentiation (*67*).

Higher-order CAR interactions

The TCR-CD3 complex is stabilized by polar contacts between the extracellular and transmembrane domains of each subunit (*68, 69*). CAR constructs incorporate immunoreceptor extracellular and transmembrane domains and, therefore, have the capacity to associate with endogenous receptor components both at baseline and upon stimulation. In first-generation constructs containing the CD3ζ transmembrane domain, CAR ligation effected signaling through both the CAR-CD3ζ and endogenous CD3ζ to improve overall antigen response (*70, 71*). It has also been shown that second-generation constructs containing the CD28 and CD8 transmembrane domains associate with endogenous CD3ζ (*37*). How this affects CAR T cell response is unknown, but CD28-containing second-generation constructs associate with higher levels of endogenous CD3ζ than 4-1BB–containing constructs (when both express the CD8 transmembrane domain), and these constructs have been demonstrated to be more responsive to antigen stimulation at early time points (*36*, *37*). Third-generation CAR constructs, in contrast, appear to have minimal, if any association with endogenous CD3ζ at baseline (*37*); as mentioned above, these constructs have also been shown to function less well than second-generation constructs. Failure to associate with endogenous CD3 subunits may play a role in this phenotype, although why third-generation constructs should fail to associate with endogenous CD3ζ is unclear.

Baseline CAR oligomerization

CAR constructs also associate with each other in the absence of stimulation (*70, 72*). However, direct assessment of CAR homodimers indicates that unligated dimerization does not cause CAR T cell activation. Ligand-induced CAR dimerization, in contrast, does cause activation, suggesting that this is a qualitatively different mechanism. However, unstimulated CAR dimerization occurs at much lower levels than ligand-induced dimerization (*72*), raising the possibility that observed differences are quantitative in nature. In keeping with this hypothesis, it has been proposed that ligand-independent CAR oligomerization is a mechanism of low-level tonic signaling that predisposes CAR T cells to exhaustion and poor function (*73*).

Extracellular spacer length

The spatial configuration of the CAR itself has significant functional consequences, and many groups have experimented with varying the distance between the antigen-binding portion of the CAR and the cell membrane. The effects of these manipulations are highly context dependent and are influenced by myriad factors including, but not limited to, the affinity of the CAR, its inherent signaling capacity, whether it signals tonically, and where the antigen epitope is physically located. Longer spacers seem to be more effective in CARs that bind to antigen eptiopes located close to the cell membrane, whereas shorter spacers benefit CARs targeted to membrane-distal epitopes (*74, 75*). Independent of epitope location, spacer length variation has pleiotropic and contradictory effects (*76, 77*). For instance, it has been shown that lengthening the spacer can increase Erk/MAPK signaling, although this paradoxically leads to increased activation-induced cell death and decreased in vivo function through Fas-mediated mechanisms (*78*). In a different system, shortening the spacer abrogates tonic signaling; however, in this case, spacer shortening also impairs antigen recognition and cellular activity (*79*). These considerations are clearly extremely important in CAR design, although their effects on CAR function are idiosyncratic, and their influences on CAR signaling are unlikely to be consistent across contexts.

The immunological synapse

In T cells, engagement of signals 1 and 2 results in the formation of an IS, a large and well-organized supramolecular activation complex (SMAC) centered on the interface between the T cell and the target cell (Fig. 3). This bull's eye–like structure comprises three regions to concentrate TCR-coreceptor complexes, spatially segregate kinases, and phosphatases, and facilitate delivery of lytic granules to the target (*80*). While it has been shown that cytotoxic T cells can kill before formation of a mature IS (*81*), the IS is required for optimal T cell killing (*82*, *83*).

CAR T cells, in contrast, appear to form only a primitive and disorganized IS (*84*, *85*). The reasons for this are not clear, although many mechanisms appear to contribute. For instance, extracellular domain size is important for forming and maintaining the conventional IS; proteins with large extracellular domains and no ligand in the cell-cell contact zone, such as the phosphatase CD45, are excluded from the central SMAC (cSMAC) and pushed out into the distal SMAC (dSMAC; Fig. 3) (*86*). As the IS matures over a period of 10 to 15 min, CD45 is recruited back into the center (*87*). Artificial receptors in reconstituted cellular systems seem to exclude CD45 from the IS to a lesser extent, perhaps in part because the extracellular domain of most CARs is much larger than that of a conventional TCR (*86*). Confocal imaging of a CAR-mediated IS in a nonimmune cell model system demonstrated significant convolution of the CAR-bearing cell membrane, suggesting that the close physical approximation between T cells and APCs may not be entirely conserved in CAR T cells (*86*); this possibility is corroborated by images in CAR T cells as well (*84*).

In addition, the concatenation of signal 1 and signal 2 cytoplasmic domains in CARs seems to lead to faster signaling than in conventional TCRs, which may not provide sufficient time for full IS formation (*84*). Recent microscopy studies suggest that disorganized IS formation may, paradoxically, accelerate the speed at which CAR T cells degranulate as well as the rate at which they detach from target cells (*84*). It is important to note that these studies were done in CD28-containing second-generation CAR T cells, which are independently able to recruit the Vav guanine nucleotide exchange factor through their CD28 domains. Because Vav is critical for the cytoskeletal rearrangement necessary for degranulation, it is possible that this acceleration would not be seen in 4-1BB–containing second-generation CAR T cells. Nonetheless, these findings in the aggregate again suggest that processes that are canonically considered critical for optimal T cell function are not necessarily conserved in CAR T cells.

Downstream events

The consequences of the signaling differences described above are amplified over time; expression of downstream activation molecules

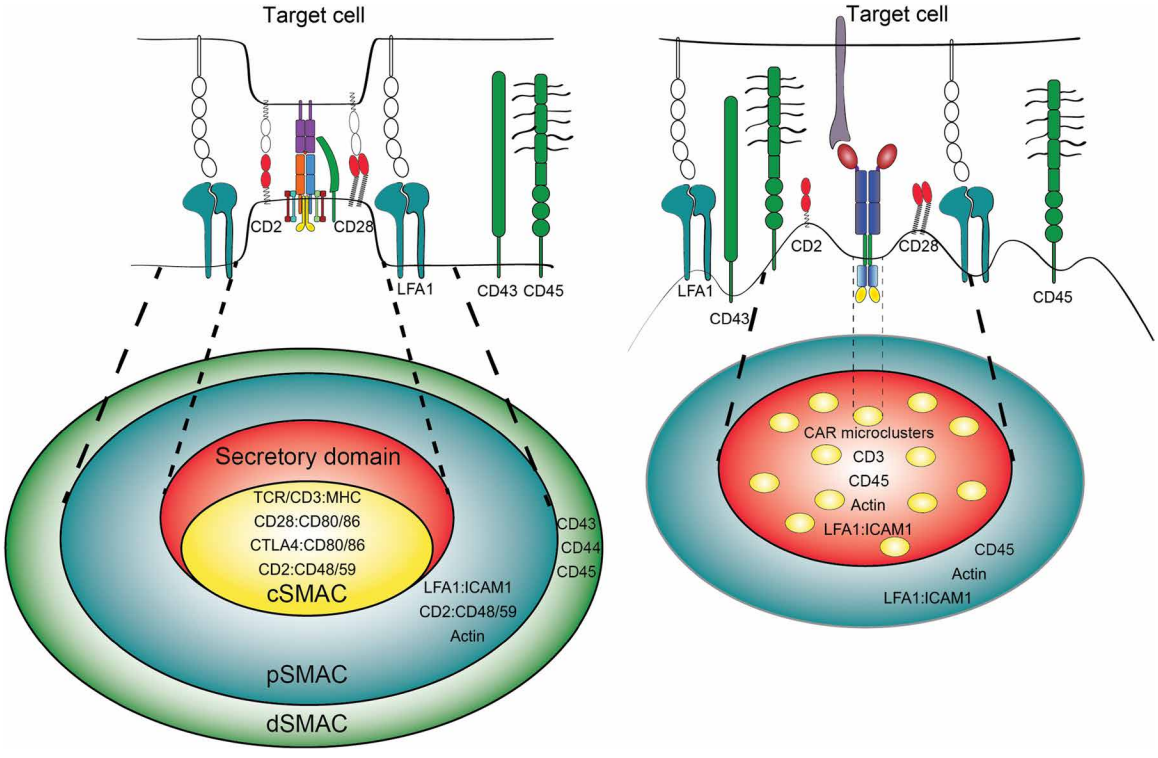

Fig. 3. TCRs and CARs form distinct ISs. Canonical TCR signaling (left) leads to the formation of an IS with clearly demarcated zones: the central supramolecular activation complex (cSMAC), the zone of closest physical proximity between the T cell and the target cell, incorporates TCR:peptide-MHC complexes as well as costimulatory molecules such as CD28 and CD2; the peripheral SMAC (pSMAC) includes larger molecules such as LFA1, in addition to molecules like CD2; and the distal SMAC (dSMAC) includes large, ligandless proteins such as CD45. This stereotypical bull's eye–like structure evolves over time, and CD45 is eventually recruited back to the cSMAC before degranulation occurs at the secretory domain. CAR synapses (right), on the other hand, are both spatially and temporally disorganized. They form more quickly and are less stable and comprise much less well-demarcated zones. Rather than a cSMAC, there are central CAR microclusters interspersed with and surrounded by adhesion molecules such as LFA-1 and signaling molecules such as CD45. Around this central area, there is an actin-rich pseudoring that is relatively CAR poor. Moreover, the area of cell-cell contact between CAR T cells and their targets appears to be quite convoluted, perhaps due to the varying extracellular sizes of the numerous proteins at the IS. Nonetheless, there is evidence that this disorganized IS leads to more rapid degranulation and disengagement from target cells, which ultimately may result in faster killing.

such as CD69, CD137, perforin, and granzyme B, as well as negative regulatory molecules such as PD-1, CTLA-4, LAG-1, and tissue inhibitor of matrix metalloproteinase 3 (TIMP3) are significantly different between CD28-based and 4-1BB–based CARs. Similarly, these cells differ drastically in functional indices such as proliferation and cell killing. The mechanisms underlying the differences in these downstream events are murky and lie outside of the scope of this review. However, at a very fundamental level, these notable functional differences likely result from disparities in signaling immediately downstream of CAR ligation, highlighting the importance of understanding the implications of proximal CAR signaling events.

CONCLUSIONS

CAR T cell therapy has transformed many aspects of clinical and translational oncology, and the stunning successes achieved to date have led to a rapid expansion of clinical and basic science research efforts in this area. However, this explosion has, in many ways, outpaced fundamental investigations into how these receptors work, which threatens to limit our ability to improve upon current designs in an optimal fashion. Evidence to date suggests that myriad factors contribute to CAR signaling, and many more remain still to be elucidated. Gaining a comprehensive mechanistic understanding of how CARs function will be an important step toward engineering more successful cellular therapies for difficult-to-treat diseases.

This review has focused primarily on the intracellular and supramolecular signaling events initiated by CAR ligation, envisioned through the lens of conventional TCR signaling. However, it is clear that there are substantive differences between how CARs and TCRs signal, raising the question of whether endogenous TCR signaling is an appropriate conceptual framework for CAR signaling inquiries. It is probable that agnostic, comprehensive signaling analyses will need to complement traditional candidate-based approaches in identifying key pathways in CAR signaling. These studies are ongoing in several institutions.

Furthermore, intracellular signaling is clearly a consequence of extracellular events in CAR receptors. Characteristics such as CAR avidity, scFv affinity, antigen-binding domain structure and size, hinge/spacer region length and design, and transmembrane domain choice all affect the kinetics and dynamics of signaling pathway activation, just as choice of cytoplasmic signaling moieties affects the specific pathways that are proximally activated. However, these design features are likely to be interdependent, and therefore, the effect of (for instance) altering scFv affinity probably differs in constructs with different (for instance) spacer lengths, transmembrane domains, or costimulatory domains. Most researchers focus primarily on a small subset of possible CAR designs; due, in part, to the tremendous success of CD19-targeted CAR T cells, much of the research discussed above has been done on CARs that target CD19. Moreover, the intellectual property ramifications are such that groups are highly incentivized to focus on designs they already own as well as to discourage others from working on these proprietary designs. This siloed approach makes studies that address generalizability exceedingly rare. Last, there is a vast array of signals that moderate CAR signaling—checkpoint molecules, immunosuppressive cytokines, oxygen tension, and other microenvironmental cues are all important for CAR T cell function. Nonetheless, the prime motivator of CAR T cell efficacy remains signaling initiated by the chimeric receptor itself, and as CAR T cell therapies are used to treat increasingly challenging diseases, it will become even more important to understand how generalized design features affect intracellular signaling. Ideally, this knowledge will be gained through rigorous, collaborative studies that compare signaling and function across a multitude of CAR constructs.

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