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Expanding the CAR toolbox with high throughput screening strategies for CAR domain exploration: a comprehensive review

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ABSTRACT

Chimeric antigen receptor (CAR)-T-cell therapy has been highly successful in the treatment of B-cell hematological malignancies. CARs are modular synthetic molecules that can redirect immune cells towards target cells with antibody-like specificity. Despite their modularity, CARs used in the clinic are currently composed of a limited set of domains, mostly derived from IgG, CD8α, 4-1BB, CD28 and CD3ζ. The current low throughput CAR screening workflows are labor-intensive and time-consuming, and lie at the basis of the limited toolbox of CAR building blocks available. High throughput screening methods facilitate simultaneous investigation of hundreds of thousands of CAR domain combinations, allowing discovery of novel domains and increasing our understanding of how they behave in the context of a CAR. Here we review the growing body of reports that employ these high throughput screening and computational methods to advance CAR design. We summarize and highlight the important differences between the different studies and discuss their limitations and future considerations for further improvements. In conclusion, while still in its infancy, high throughput screening of CARs has the capacity to vastly expand the CAR domain toolbox and improve our understanding of CAR design. This knowledge could be foundational for translating CAR therapy beyond hematological malignancies and push the frontiers in personalized medicine.

BACKGROUND

Treatment of B-cell leukemia and lymphoma has seen a tremendous improvement in clinical outcomes with the advent of chimeric antigen receptor (CAR-)T-cell therapies. This wave of novel therapeutics was led by the CD19-targeted CAR-T-cell products Kymriah[1] and Yescarta[2] in the context of relapsed/refractory diffuse large B-cell lymphoma and acute B-lymphocytic leukemia, which were approved in late 2017. Since then, we have seen the regulatory approval of two additional CD19-specific CAR-T-cell products for B-cell malignancies,[3 4] as well as two products targeted against B-cell maturation antigen for the treatment of myeloma.[5 6] Although revolutionary, current CAR therapies are not flawless. In addition to concerns of on-target off-tumor toxicities,[7 8] simultaneous activation of massive numbers of cancer-directed immune cells causes side effects, such as cytokine release syndrome and neurotoxicity, often requiring additional medical interventions.[9 10] Perhaps more importantly, a considerable portion of CAR-treated patients eventually relapses because of either antigen escape or lack of persistence.[11 12] Furthermore, tumor-specific target selection, migration towards and infiltration into the tumor site, and expansion and function in a hostile tumor microenvironment provide additional challenges for CAR-T-cell therapy in solid tumors.[13] These drawbacks led researchers to scrutinize the design of CARs currently used in the clinic.

CARs are synthetic molecules that generally consist of five major sequential components (figure 1A): (1) an antigen-binding domain (ABD), often derived from a conventional monoclonal antibody in single-chain variable fragment (scFv) format, (2) a flexible hinge domain (HD) or spacer, (3) a transmembrane domain (TMD), (4) one or more co-stimulatory domains (CSDs) and (5) an intracellular signaling domain (ISD). The specificity, sensitivity, therapeutic potency and persistence of the CAR-T-cell product are influenced by the choice of each of these components. The ABD determines the tumor antigen specificity and carries the largest variation across literature with over 64 target antigens spanning liquid and solid tumors.[14] Optimization efforts to improve tumor cell discrimination, safety or T-cell exhaustion include affinity modulation[15] and reconfiguration of the ABD to a different format.[16–21] The other CAR components are less diverse

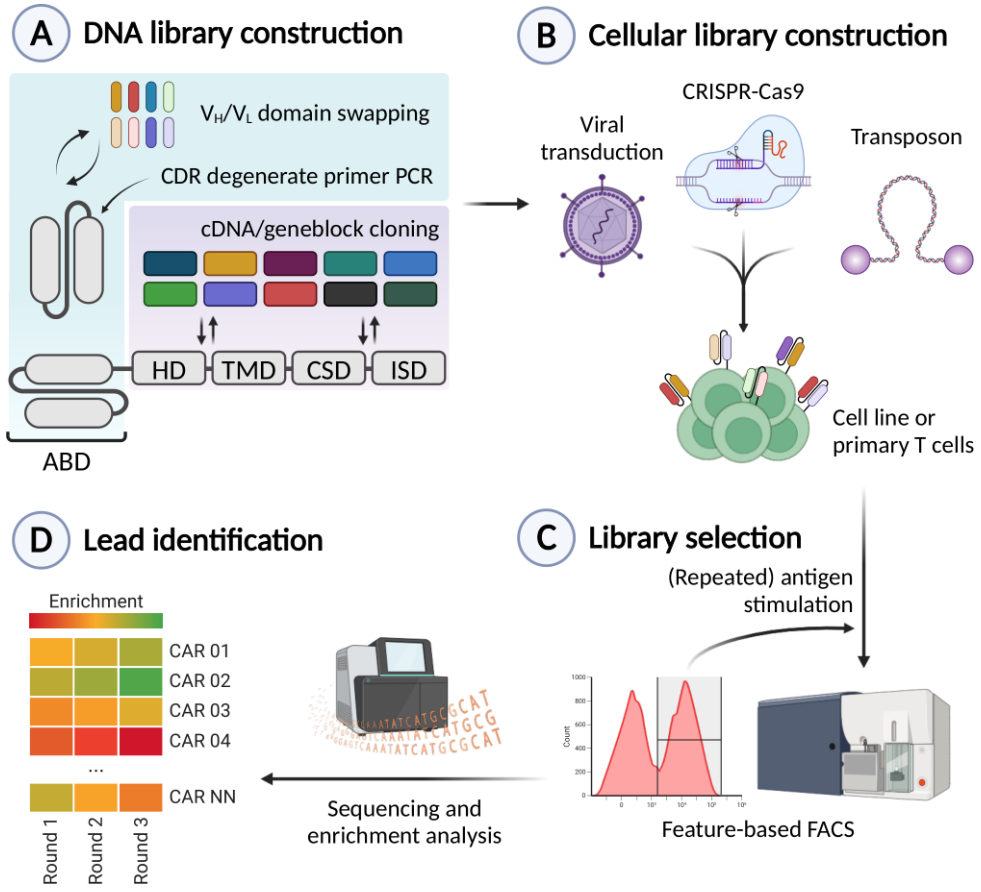

Figure 1 Generalized workflow for high throughput screening of CAR variants. (A) Diversity is created in the CAR library by means of PCR amplification with degenerate primers or permutation of domain modules. (B) Pooled library elements are integrated into cells using permanent modification methods. (C) Subsequent cycles of antigen stimulation and feature-based sorting enrich high-performing library elements. (D) Enrichment over rounds of stimulation is tracked via sequencing of the pools and high-performing lead candidates can be identified for further characterization. ABD, antigen-binding domain; CDR, complementary determining region; CSD, co-stimulatory domain; FACS, fluorescence-activated cell sorting; HD, hinge domain; ISD, intracellular signaling domain; TMD, transmembrane domain; VH, variable heavy chain; VL, variable light chain. Created with BioRender.

in the clinical landscape. Current CAR-T-cell products in the clinic are mostly limited to combinations of a CD8a-derived, CD28-derived or IgG-derived HD, CD8-derived or CD28-derived TMD, 4-1BB or CD28 CSD and a CD3ζ ISD.[22]

To fill this gap, preclinical studies have started exploring alternative domains, such as ABDs taken from receptors,[23 24] ligands,[20] autoantigens,[19] peptides[21] and nanobodies,[25] HDs derived from proteins including 4-1BB,[26] CD34,[27] low-affinity nerve growth factor receptor[28] and members of the Siglec-family,[29] TMDs isolated from proteins such as CD16 and natural killer (NK) cell-associated receptors,[30] and ICDs among which inducible costimulator (ICOS)[31] and OX40 have been most extensively studied.[32 33] However, these CAR domains are often studied in isolation with only one or, at most, a few candidates evaluated in parallel. Meanwhile, there are indications that certain domain combinations lead to favorable outcomes.[31 34 35] Current low throughput workflows are labor-intensive and time-consuming and do not allow for exhaustive evaluation of large CAR combinatorial libraries. Higher throughput screening would not only increase the number of domains that can be tested, but would also enable basic research on how the inclusion of a specific domain affects the overall outcome, which could eventually lead to the definition of basic CAR design rules. Recently, there have been a number of studies using such high throughput CAR screening approaches. Here we review the growing body of high throughput CAR screening campaigns and compare the methodologies used therein.

OVERVIEW OF CURRENT CAR LIBRARY SCREENING APPROACHES

Most CAR library screening approaches use similar sequential workflows with (1) (combinatorial) CAR library generation in DNA-space, (2) transfer of the library into cells, (3) assaying and selection of responding library members, and (4) identification and validation of lead candidates (figure 1). However, the actual implemented

methods to achieve each of these steps can differ between studies depending on the intended goal.

The cloning techniques used for library assembly are often dictated by the CAR domain that is being screened (table 1). For the ABD, scFv libraries are obtained by creating diversity in complementary determining regions (CDRs),[36–38] in entire variable chains[39 40] or in full scFvs.[41–44] CDR diversity is generated by amplifying the variable chain of interest with degenerate primers,[38] computational modeling[37] or by DNA synthesis.[36] Meanwhile, variable chain and scFv repertoires are created in vitro from human variable region banks[41] or by PCR amplification of immunoglobulin genes from healthy human peripheral blood mononuclear cells or B cells.[40 42–44] With regard to ISD libraries, researchers mostly opt for the synthesis of gene blocks encoding the library members,[12 45–47] presumably due to declining costs of DNA synthesis and the relatively low number of unique library components, though they can also be amplified from complementary DNA.[48] Subsequently, ISDs are PCR amplified and cloned into the acceptor vector using type IIs restriction enzymes[45 46 48] and/or overlap-extension PCR, Gibson Assembly, In-Fusion cloning or blunt-end ligation.[47 49–51]

High throughput CAR-screening efforts also differ in the cell type used (table 1). Most studies rely on cell lines because they offer a convenient unlimited supply of homogeneous cells. The Jurkat T-cell lymphoma line is often the cell line of choice due to its largely conserved T-cell signaling machinery.[38 41 42 44 47 48 51] While functional markers such as CD69 upregulation and interleukin (IL)-2 secretion are present in Jurkat cells, they show little cytotoxic capacity, do not secrete the full repertoire of primary T-cell cytokines and have higher basal signaling due to their nature as a continually dividing cell line.[48 52] Therefore, while they can be valuable as a first step in a screening campaign, any screening effort must be validated in primary T cells. Other cell lines used for screening include the NK-cell lymphoma line NK-92,[43] HEK293F[39] and the murine hybridoma line B3Z.[36] Cell lines also have the added advantage that they can be easily engineered to express a reporter gene in response to activation signals. They offer an attractive alternative to simple activation markers like CD69, which may not be sensitive enough to capture subtle differences in signaling. In contrast, multiple reporter genes driven by different transcription factors can capture a broader image of CAR-induced T-cell signaling pathways.[38 52–55] To date, only a few groups have used primary murine or human T cells for screening purposes,[45 46 49–51] directly assessing CAR functionality in the final cellular product.

A suitable method to genetically introduce the CAR gene is critical for proper evaluation of CAR libraries (table 1). Candidate selection is based on the enrichment of library members that show phenotypical and/or functional superiority (see below). Incorporation of multiple CAR constructs per cell inevitably leads to co-selection of poorly functional CARs with lead candidates,[38 44 48] causing the loss of valuable resources on unnecessary validation experiments. Consequently, transfection, electroporation, or nucleofection of transient CAR-encoding nucleic acids is not suitable for this application because of the need for high cytoplasmic concentrations of unique library elements to achieve sufficient CAR expression for functional evaluation.[44] Permanent modification methods, such as viral transduction,[37 39–44 46–48 50 51] transposon systems[38] and CRISPR-Cas9,[36 45] can be carefully titrated to ensure singular or site-specific integration.

Instead of evaluating each candidate independently, most high-throughput screening campaigns rely on enrichment of superior CAR constructs from a pool of constructs. To quickly eliminate oversensitive, non-specifically activating or tonic signaling constructs, some approaches incorporate an initial round of negative selection of their CAR library on antigen-negative cell lines or healthy peripheral blood mononuclear cells (PBMC).[39 42] Then, cellular CAR libraries are stimulated by plate-bound or soluble antigen, or antigen presenting cells (table 1). Expression of CD69,[41 44 46–48 51] cytokines[46] or activation-induced reporter genes[36 38 39 42] and proliferation[46 51] are functional markers that have been used to sort for the best responders. Incorporating multiple stimulation-and-sorting rounds and/or more stringent sorting gates will increase the relative frequency of the best performers, even if extremely rare in the initial population.[44 53 56] Alternatively, long-term stimulation assays rely on the outgrowth of top candidates over time because of their competitive advantage, followed by a sort via antigen or tetramer staining.[40 43] Finally, computational approaches can assist in the selection of lead candidates by modeling for orientation, conformation, antigen-receptor interactions and changes in stability.[37 50] Artificial intelligence has also been used for CAR selections, using sparse data to make predictions of functional outcomes of CAR designs.[49] Others have opted for the rational selection of lead candidates based on single-cell transcriptional profiling of pooled populations.[45]

As a final step, the enriched library is sequenced and the relative frequency of lead candidates is determined. Those lead candidates are subsequently validated and benchmarked against the wildtype receptor or a state-of-the-art CAR construct. Although not all novel CARs identified through high throughput screening outperform their benchmark, these screening efforts can still offer valuable information on the effects induced by specific changes in the CAR building blocks and aid in future rational CAR design. Below we provide an overview of current literature related to high throughput and computational methods for the discovery of improved CAR architectures.

SCREENING STUDIES OF DIFFERENT CAR DOMAINS

Antigen-binding domain

The ABD of a CAR is typically an scFv derived from a monoclonal antibody that was acquired via hybridoma

Table 1 Overview of published methods in high throughput screening of novel CAR domains

Ref	Library variable	Library generation	Cell type	Modification method	Stimulation method	Enrichment method
36	scFv (CDR)	Degenerate HDR template synthesis	B3Z cell line (IL2-EGFP reporter)	CRISPR-Cas9	Soluble rhHER2 antigen, or SKBR3 cell line	3× rhHER2 binding sort, or 3× GFP expression sort
38	scFv (CDR)	Degenerate primer PCR and restriction-ligation cloning	Jurkat cell line (NFkB-ECFP & NFAT-EGFP reporter)	Transposon	JeKo cell line	rhROR1 binding sort and ECFP$^+$EGFP$^+$ expression sort
39	scFv (VL/VH)	Not specified	HEK293F cell line (Gal4-VP64-BFP reporter)	Lentiviral	Healthy PBMC and CD38$^+$ K562 cell line	BFP expression sort
40	scFv (VL/VH)	Human B cell cDNA nested PCR and overlap extension PCR	Primary T cells	Retroviral	NY-ESO-1$_{157}$/HLA-A2-modified K562 or T2 cell line Raji cell line (CD19)	Repeated antigen stimulation followed by tetramer binding sort (NY-ESO-1$_{157}$/HLA-A2), or Repeated antigen stimulation followed by rhCD19 binding sort
41	scFv (full)	Degenerate primer PCR and restriction-ligation cloning	Jurkat cell line	Lentiviral	CEA-modified HeLa cell line	3× EGFP$^+$CD69$^+$ expression sort
42	scFv (full)	Human B cell cDNA PCR and restriction-ligation cloning	Jurkat cell line (NFAT-mCherry reporter)	Lentiviral	HEK293-6E cell line, or H-226 cell line, or AsPC-1 cell line	mCherry$^-$ expression sort followed by 2× GFP$^+$mCherry$^+$ expression sort
43	scFv (full)	Human PBMC cDNA, murine spleen or BM cDNA nested PCR and restriction-ligation cloning	Primary T cells; NK-92 cell line	Lentiviral	Subcutaneous mouse models with an EGFR$^+$-modified MCF-7, HER2$^+$-modified MCF-7, SW480, A549, SK-OV-3, SK-BR-3, N3 or BN16 cell line	In vivo enrichment for tumor-reactive CARs of unknown specificity
44	scFv (full)	Phage display library PCR and restriction-ligation cloning	Jurkat cell line	Retroviral	Plate-bound CEA	3× CD69$^+$ expression sort with subsequent gDNA PCR and recloning
45	ISD	Gene synthesis, PCR and restriction-ligation cloning	Primary T cells	CRISPR-Cas9	SKBR3 cell line	CAR$^+$ expression sort
46	ISD	Gene synthesis, PCR and Golden Gate assembly	Primary T cells	Lentiviral	CD19$^+$-modified K562 cell line	IFN-γ$^+$, IL-2$^+$ or CD69$^+$ expression or proliferation sort
47	ISD	Gene synthesis, PCR, overlap extension PCR and restriction-ligation cloning	Jurkat cell line	Lentiviral	rhCD19 antigen	3× CD69$^+$ and/or CD69$^+$PD-1$^-$ expression sort
48	ISD	cDNA PCR and restriction-ligation cloning	Jurkat cell line	Retroviral	Plate-bound c-Myc antibody	CD69$^+$ expression sort
49	ISD	Gene synthesis and In-Fusion cloning	Primary T cells	Lentiviral	Nalm-6 cell line	NA

Continued

Table 1 Continued

Ref	Library variable	Library generation	Cell type	Modification method	Stimulation method	Enrichment method
51	ABD, HD, ISD	Gene synthesis and restriction-ligation cloning	Jurkat cell line; Primary T cells	Retroviral	NA	$CD69^+$ expression sort proliferation sort
53	ISD	Site-directed mutagenesis	Jurkat cell line (NFAT-GFP and NFkB-CFP reporter)	Retroviral	Soluble rhB7H6	GFP^+CFP^+ expression sort

AA, amino acid; ABD, antigen-binding domain; BFP, blue fluorescent protein; BM, bone marrow; CD, cluster of differentiation; cDNA, complementary DNA; CDR, complementary determining region; CEA, carcinoembryonic antigen; ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein; HD, hinge domain; HDR, homology directed repair; HER2, human epidermal growth factor receptor 2; IFN, interferon; IL, interleukin; ISD, intracellular signaling domain; ML, machine learning; NA, not applicable; NFAT, nuclear factor for the activation of T cells; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; NGFR, nerve growth factor receptor; NK, natural killer; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death protein-1; rh, recombinant human; scFv, single-chain variable fragment.

technology, or phage, bacterial or yeast display methods.[44 57] However, these scFvs are not guaranteed to maintain desired binding properties in the context of CARs. Indeed, improper protein folding may diminish CAR expression, and antigen-independent crosslinking of CARs through the scFv can cause tonic signaling and compromise CAR functionality.[44 52 58 59] Soluble or plate-bound antigen used in these enrichment platforms may not maintain the same structure as membrane-bound antigen, leading to selection of scFvs specific for epitopes that are not available on antigen-presenting cells,[56] or loss of scFvs that recognize epitopes present in the membrane-bound conformation.[42] Similarly, ABD conformation and binding characteristics are also context-dependent and may not be properly translated from the antibody to the scFv typically found in a CAR, potentially changing CAR affinity and functionality.[37 57 58 60] High throughput screening of scFv variants in CAR format can overcome these challenges as both target antigen and scFv conformation are selected in their clinically relevant configurations.

The ability to enrich rare events from a highly diverse population is essential when screening scFv libraries. To our knowledge, Alonso-Camino and colleagues were the first to demonstrate the feasibility of rare event enrichment of CARs with their lymphocyte display platform.[56] Three rounds of stimulation and sorting for $CD69^+$ Jurkat cells resulted in a nearly 1,000-fold increase in frequency of carcinoembryonic antigen (CEA)-specific Jurkat cells spiked into Jurkat cells expressing no or an irrelevant CAR. The same group used this procedure to isolate HeLa cell-specific scFvs from a starting scFv library of 1.5×10^5 members.[41] Likewise, Lipowska-Bhalla *et al* transduced Jurkats with a mix of viral particles spiked with a CEA-targeting CAR at low frequency (0.0001%), which could be enriched 10^5-fold in just three rounds of fluorescence-activated cell sorting-based selection on the top 2% CD69 expressing cells.[44] Subsequent application of this method to an scFv library saw a 65-fold enrichment of CEA-binding Jurkat cells. While sequence analysis revealed three dominant scFv clones to be present in this population, only one clone was verified to be able to recognize CEA, indicating the enriched population was still contaminated by non-binders, presumably as a result of cross-contamination during library preparation or co-selection of non-binders with binders within the same cell.

The heavy chain CDR3 loop is a major determinant of binding specificity of scFvs and therefore often targeted for optimization. Rydzek *et al* obtained a 10^6 member CAR library via site-directed mutagenesis of the V_H CDR3 loop of an ROR1-specific CAR.[38] Single-cell sorting of $NFAT^+NF\kappa B^+$ Jurkat reporter cells on ROR1 stimulation led to the selection of 15 unique clones. Similar to other reports,[44] multiple genomic integrations had occurred in the best performers.[38] Expression of the wildtype receptor was probably driving the majority or entirety of the observed responses. While this study reaffirms the feasibility to pick up dominant clones present at very low frequency (0.0005%) in the initial library, it also highlights the importance of single integration events. Fluorescent reporters can also be coupled to an endogenous marker of activation, avoiding artificially enhanced signal amplification by synthetic promoters. For example, Di Roberto and colleagues linked green fluorescent protein (GFP) to the last exon of IL-2 separated by a P2A peptide in the B3Z murine T hybridoma cell line.[36] As such, CAR-modified B3Z cells recognizing their cognate antigen could be discriminated based on GFP expression while leaving IL-2 secretion intact. Deep mutational scanning of the V_H CDR3 region generated 190 variants of the human epidermal growth factor receptor 2 (HER2)-specific trastuzumab scFv.[36] After three rounds of soluble antigen stimulation and sorting on activation-induced GFP, two lead candidates were detected that had a similar sensitivity to $HER2^{high}$-target, but lower reactivity to $HER2^{low}$-target cells compared with the original scFv. By employing an affinity-based selection strategy against soluble HER2 in parallel to the activation-based screening, the authors were able to pinpoint residues

that can be altered to modulate affinity without affecting antigen-specific signaling.

Instead of focusing on the V_H CDR3, some have pursued swapping variable chains, generating diversity in entire CDR sets. Ochi *et al* interrogated sets of CDRs by generating scFv libraries by coupling V_H and $V_{L\lambda}$ or $V_{L\kappa}$ derived from healthy human B cells to a V_L or V_H of a validated functional scFv, respectively.[40] Two scFv libraries were created; one targeted to CD19 and one targeted to the New York esophageal squamous cell carcinoma 1 $(NY\text{-}ESO\text{-}1)_{157}$ peptide bound to HLA-A2. The libraries were stimulated three times and subsequently sorted for soluble CD19 or HLA-A2/NY-ESO-1_{157}-specific tetramer binders, respectively.

Newly discovered scFv variants were demonstrated to improve proliferation, reduce cytokine release, enrich naïve and memory T cells and have better in vivo tumor control.[40] Ma and coworkers took this approach even further and generated an scFv library with a diversity of 10^{11} through V_L/V_H randomization.[39] As it is not possible to reach sufficient coverage of such a large library in a cellular screen, phage display against recombinant CD38 was performed to reduce the library size to 10^6 members. This pre-enriched scFv library was subsequently screened in the form of a synNotch receptor driving a blue fluorescent protein (BFP) reporter gene. Following an additional negative selection round against healthy PBMC, removing any hypersensitive clones, the remaining library was sorted for BFP expression after antigen challenge. The discriminatory power between healthy and tumor cells of two lead candidates, R02 and R03, was confirmed in subsequent co-cultures. Although R02 had a 28-fold lower affinity than R03, its cytokine response against $CD38^{high}$ cells was consistently higher. The faster on-rate (k_{on}) and slower off-rate (k_{off}) of R02 compared with R03 allow it to quickly engage with and release target antigen, which is implicated in the potential for improved clinical performance.[61] In a similar fashion, Fierle and colleagues used a Jurkat NFAT reporter cell line to screen a naïve scFv library of 2×10^{10} members by first pre-enriching the library to only 10^5 members through affinity-based phage display against their target antigen mesothelin (MSLN).[42] When assessing scFv recognition characteristics against three isolated MSLN extracellular domains, it was discovered that the majority bound the membrane distal domain 1, whereas only one scFv bound the membrane proximal domain 3. Few scFvs also only bound to the full MSLN extracellular domain, pointing towards conformational or interdomain epitopes. Context-dependent recognition of MSLN was further demonstrated with some hits being able to bind to membrane-bound MSLN, but not soluble, bead-bound MSLN. Importantly, these screening methods inherently optimize the formation of the immune synapse while accounting for the effect of other extracellular domains of the CAR because the library is not restricted to the epitope of the wildtype scFv.

Thus far, most selection strategies were performed completely in vitro and were aimed at identifying a single lead scFv. However, immunosurveillance is achieved through immune cells with a repertoire of receptors. Fu *et al* attempted to create an artificial immune system of CAR-engineered immune cells that is capable of recognizing a variety of non-self-antigens.[43] A naïve scFv library generated from B cells of 200 healthy individuals and subjected to four rounds of negative selection through in vivo phage display in NSG mice to remove any autoreactive clones before subcloning the 5×10^5 member CAR library primary mouse T cells. Epidermal growth factor receptor $(EGFR)^+$ SW480-bearing or MCF7-bearing NSG mice treated with the CAR-T-cell library showed no tumor control for the first month, after which the majority of mice started to experience tumor growth reversal and ultimately total clearance, even in a challenging SW480 tumor model. Similar kinetics of tumor control were observed with $HER2^+$ mouse models. Formation of memory was illustrated by the absence of tumor growth in mice rechallenged with the same tumor. Most impressively, rechallenge with $EGFR^+$ SW480 of mice previously cured of $HER2^+$ tumors again showed initial tumor outgrowth with a rapid decline in tumor mass from the 1-month time point onwards, suggesting long-term maintenance of diversity. This work shows that a pooled therapeutic approach is feasible, though it remains unknown whether antitumor responses were driven by a few dominant clones or a broader scFv repertoire.

Screening of scFvs does not have to be entirely carried out experimentally. In some cases, computational modeling can be used to improve binding characteristics. As such, Krokhotin *et al* introduced single residue mutations in all six CDRs of an scFv derived from the $HER2_{369\text{-}377}$/HLA-A2-specific antibody SF2 in silico.[37] At least two hits had improved specificity and sensitivity for the $HER2_{369\text{-}377}$/HLA-A2 antigen facilitated by enhanced stability of the binding complex. Notably, using feedback loops of the experimental data, the models can be further refined. Another group used three-dimensional modeling of the scFv for in silico mutagenesis to evaluate the effect of amino acid (AA) substitutions in framework regions on the stability of scFv, which elicits tonic signaling through antigen-independent CAR clustering.[62] FMC63 CAR destabilization by modifying key framework region residues led to severe tonic signaling and reduced functionality. Conversely, the authors could rescue a natively unstable chondroitin sulfate proteoglycan 4 (CSPG4)-targeted CAR using this method without compromising specificity. Of interest, humanization of the murine CSPG4-specific scFv with the human stable framework rFW1.4 avoided CAR aggregation and signaling in the absence of antigen.

Hinge domain

An HD was initially incorporated into CARs with the aim of providing the ABD with the necessary reach and flexibility for effective binding to the target antigen and enhancing antitumor activity.[63 64] Despite the established benefits of integrating an HD, there remains a lack of

consensus regarding how specific properties, such as length, physical size, and binding sites precisely influence CAR function. For instance, shorter HDs tend to outperform longer ones by maintaining a tighter immune synapse on antigen binding,[65 66] while longer HDs may provide more effective access to membrane-proximal or heavily glycosylated targets.[33 35 67] This was elegantly demonstrated by McComb and colleagues, who showed that progressive truncation of a CD8α HD resulted in progressive attenuation of CAR functionality.[25] Similarly, CARs designed to target a membrane-proximal epitope often exhibit superior performance compared with those directed towards a membrane-distal epitope.[66 68–70] One possible mechanism behind this is the physical exclusion of phosphatases from the immune synapse on antigen binding, akin to mechanisms key for endogenous T-cell receptor (TCR) signaling.[65 66 71] If correct, the field would have to adjust its view on hinge length from the number of AA to the physical size as those are not always linearly correlated (eg, an IgG4 hinge is 240 AA and ~7 nm long while a CD8α hinge is 55 AA and ~5 nm long).[66] Recent work by Rios *et al* added valuable insights by creating a combinatorial library of different CAR domains.[51] This library included three different HDs of similar length (CD8α, CD28, and a short IgG4 hinge), two ABDs (CD19 and GD2), and five CSDs (wild-type CD28, mutant CD28, 4-1BB, OX40, and DAP12). Consistent with previous research, their findings indicated that the ABD-HD combination significantly influenced CAR expression and CAR-T cell proliferation, expansion, and tumor control. Moreover, similar to the dependency of ICOS TMD-CSD,[31] there were indications of a CD28 HD-TMD dependency for proper CAR functionality.[51] While larger HD library screening efforts are yet to be performed, the recent studies by McComb and Rios highlight the potential of higher throughput approaches in unraveling the complexities of hinge design and interdomain interactions.[25 51]

Transmembrane domain

The TMD is a short peptide sequence of 21-27 AA forming a membrane-spanning alpha helix. Most clinical CAR designs make use of TMDs derived from CD3ζ, CD8α or CD28, often matching the source protein of the adjacent HD or CSD, but TMDs from OX40,[32] CD4 (64), CD7 (64), ICOS,[31] CD16,[30] CD27[23] and NK-cell receptors[30] have been explored as well. Based on the oligomeric state of the protein of origin, the TMD can determine CAR multimerization and may in some cases (eg, CD8α and CD28) facilitate interactions with endogenous proteins, which can increase antigen sensitivity, but also induce tonic signaling.[72–74] To gain better control over CAR interactions, oligomeric state and geometry through the TMD, Elazar and colleagues developed a computational design approach to generate TMDs de novo that can control the structure and function of CARs.[50] They generated artificial TMDs that were predicted to form CARs in a monomeric, dimeric, trimeric or tetrameric state, called proCARs. ProCAR-T cells showed in vitro and in vivo antitumor potency that scales linearly with the oligomeric state, and lower inflammatory cytokine release than CARs with a natural CD28 TMD. Notably, it was only the tetrameric proCAR that matched the in vivo tumor control of CD28 TMD CARs. This study demonstrates that controllable and more predictable CAR-T responses can be achieved with computer-assisted design of the transmembrane domain.

Intracellular signaling domain

The composition and spatial configuration of the intracellular signaling domains of the CAR, which recruit signaling intermediates to produce downstream effector functions in response to antigen detection, have been found to be integral to their clinical performance. This is well demonstrated by the paradigm shift from first-generation CARs, which comprise only FcRγ or CD3ζ signaling moieties and show no efficacy in a clinical setting due to poor persistence, to second-generation CARs bearing an additional signaling domain—most commonly derived from co-stimulatory receptors CD28 or 4-1BB. Indeed, leukemia cells lack expression of ligands for naturally expressed costimulatory receptors, and hence, these additional stimuli needed to be provided artificially.[75] As such, known costimulatory molecules, such as CD28, were initially incorporated in the form of chimeric costimulatory receptors, before being included into the CAR construct itself.[76 77] Because of its recent link to full T-cell activation, CD28, a member of the B7 family, was first considered as a potential CSD. Through a distinct PI3-kinase-mediated signaling cascade, it could induce strong cytokine production, proliferation and differentiation. Following the development of the first second-generation CAR, incorporation of members of the tumor necrosis factor receptor (TNFR) family, such as CD134 and 4-1BB, were further evaluated, with 4-1BB showing superior function. In contrast to CD28, 4-1BB does not associate with PI3-kinase, but its signal transduction occurs through the recruitment of TNFR-associated factor signaling molecules, enhancing T-cell proliferation and lytic capacity, and rescuing T cells from anergy and exhaustion.[78] These modifications boosted CAR persistence and enabled the first demonstration of efficacy in patients, which catalyzed Food and Drug Administration approval of a handful of second-generation CARs targeting CD19 or B-cell maturation antigen (BCMA).

Studies comparing CD28-based and 4-1BB-based second-generation CARs have yielded important insights into the functional consequences of choice of signaling domains. CD28 is known to induce a switch to aerobic glycolysis that enables rapid T-cell activation, proliferation and antitumor function.[79] 4-1BB CARs, on the other hand, signal through TRAF and have slower activation kinetics; they rely on fatty acid oxidation and exhibit enhanced mitochondrial biogenesis, which enhances T-cell longevity.[79] There is some evidence to suggest that incorporating both CD28 and 4-1BB in addition to

CD3ζ, commonly referred to as third-generation CARs, produces a synergistic combination of both properties. However, all three signaling components of the canonical CD19-28BBζ CAR may contribute to antigen-independent constitutive signaling, which likely causes premature T-cell exhaustion.[80]

Incorporating signaling domains from other members of the CD28 and TNF families of receptors has produced diverse results, such as distinct cytokine secretion profiles, enhanced proliferation and persistence, lower tonic signaling, and improved in vivo efficacy.[31 32 81] More broadly, adding functionality from other families of signaling receptors has produced promising results. For example, use of signaling domains derived from NK cells and macrophages has yielded unique advantages over the CD28 and 4-1BB-based CARs.[82 83] Addition of a cytokine signaling (signal 3) component could also warrant further investigation. Integrating IL-2Rβ and STAT3 signaling enhanced proliferation, cytokine polyfunctionality, and cytolytic activity on repeated antigen exposure in addition to preventing terminal differentiation.[84] Cytokine signaling is known to have a profound effect on the differentiation status of T cells, with IL-2 promoting an effector state and IL-7 and IL-15 supporting memory.[85] Signaling also influences downstream epigenetic changes that should be taken into consideration, since certain NFAT and AP-1-mediated T-cell signaling pathways reinforce epigenetic changes that stabilize an exhausted state.

Although most CARs use CD3ζ to induce T-cell activation, investigation of other signaling components from the TCR-CD3 complex may be warranted. A recent study reported that not all of the CD3ζ ITAMs are required for optimal signaling in a CD19-28ζ CAR and, in fact, a CAR that only contains the membrane proximal immunoreceptor tyrosine-based activation motif (ITAM) outperformed CARs with all three[86]; additionally, they found that the distance of the ITAM from the membrane was crucial for function. Moreover, fine tuning ITAM multiplicity has demonstrated enhanced control over potency and selectivity to ligand density, with higher multiplicity resulting in an increased proportion of T cells being activated at lower antigen concentrations.[87]

Duong and colleagues reported on high throughput screening of ISDs almost a decade ago.[48] 14 signaling domains were linked in random order and number into an HER2-specific CAR, generating a library of an estimated size of 10^8 elements. However, diversity was severely reduced in their Jurkat library (3×10^4), presumably due to a bias towards smaller inserts. Two rounds of stimulation through an myc-tag in the CAR and single-cell sorting for high CD69 expression identified 39 clones that showed response to antigen. Analyzing those hits for IL-2 production on stimulation with c-myc antibody or HER2-expressing target cells revealed that the DAP10-CD3ζ-CD27 ISD combination generated the highest responses. Although in vitro cytotoxicity was comparable to a CD28-CD3ζ control, in vivo tumor control of a subcutaneous tumor mouse model was significantly better.

Since this initial report, there were no new high-throughput ISD screening studies published until 2022. In the span of a few months, multiple groups reported their findings with each their unique approach. Gordon *et al* employed a pooled screening method to assemble a 700,000 member library of third-generation CD19-targeted CARs comprising 87 different signaling domains and identify CARs that exhibited increased CD69 upregulation and/or decreased programmed cell death protein-1 expression in Jurkat T cells on three rounds of serial antigen challenge with recombinant human CD19.[47] The enriched pool showed substantial enrichment of ITAM-containing and co-stimulatory domains whereas inhibitory domains were relatively de-enriched. Two hits from this screen were selected for extensive characterization. In human primary T cells, the first—harboring CD40, CD3ε ITAM, and DAP12 signaling domains—showed enhanced cytotoxicity, increased polyfunctional cytokine secretion and stemness, lower exhaustion, and better long-term persistence, proliferation, and tumor control in vitro on rechallenge than BBζ control. It was also the most transcriptionally distinct on single-cell sequencing. However, it showed similar tumor control in an in vivo xenograft model of B-cell acute lymphoblastic leukemia (B-ALL). The other—composed of FceR1y, OX40 and CD3ζ ITAM3 signaling domains—performed similarly to BBζ in vitro by all metrics but showed better tumor control in an in vivo model of $CD19^+$ melanoma, as was predicted by single-cell sequencing gene sets.

Goodman *et al* subjected a library of second-generation CD19-targeted CARs using 1 of 40 different co-stimulatory domains expressed in human primary CD4+ or CD8+ T cells to 11 challenges and subsequently selected for CD69 upregulation, interferon (IFN)-γ or IL-2 secretion, or CellTrace dilution as a proxy for proliferation.[46] They identified BAFF-R as a highly cytotoxic hit with high IFN-γ secretion and an innate NK-like phenotype that showed lower proclivity to exhaustion and robust memory formation. Meanwhile, CD28 and transmembrane activator and CAML interactor (TACI) showed higher degrees of tonic signaling and basal proliferation, while CD40 exhibited the lowest degree of non-specific proliferation and outperformed 4-1BB in long-term expansion on rechallenge. Principle component analysis (PCA) divided the CAR domains into groups that showed (1) slow kinetics and robust long-term performance, (2) faster activation kinetics and poor persistence, and (3) better long-term killing, CD8 survival, and less terminal differentiation and long-term contraction. The study also showed that increasing the distance of CD3ζ from the transmembrane domain decreased early activation and tonic signaling. On the other hand, they identified KLRG1 as a robust inhibitory receptor that maintains a naive state. On testing their novel BAFF-R CARs in a TRAC locus targeted system, these CARs performed similarly to BBζ control in a $CD19^+$ mesothelioma model but showed more robust

tumor control in a BCMA$^+$ multiple myeloma model at the minimally effective dose for BBζ.

Daniels *et al* sought to predict CAR design rules for increased cytotoxicity and stemness by training a neural network on enrichment data following three antigen challenges of human primary T cells expressing an arrayed library of 2379 CARs in which 13 signaling motifs were shuffled into three positions between the transmembrane domain and a CD3ζ signaling domain.[49] They determined that the PLCy-mediated LAT signaling motif and TRAF-mediated CD40 and LMP1 signaling motifs elicited high levels of activation and cytotoxicity, with the latter showing increased stemness. Design principles were established by rank ordering the CARs by cytotoxicity or stemness phenotype and then analyzing the resulting distribution after filtering for a particular motif to determine its contribution to a particular phenotype, spatial preference, and synergistic function with other motifs. This revealed that a combination of TRAF and PLCy1 binding domains produced high cytotoxicity and stemness, and predicted that the PLCy1 domain would improve 4-1BB but not CD28-bearing CARs due to complementary versus redundant signaling pathways, respectively. The addition of two PLCy1 binding domains to the 4-1BB second-generation CAR was found to improve tumor control in a xenograft model of B-ALL.

To better probe functional states that capture many dimensions of effector response, Castellanos-Rueda *et al* conducted single-cell RNA sequencing (scRNA-seq) of primary T cells expressing a 180-member combinatorial library that incorporated two pools of ISD domains: (1) a membrane proximal pool of 15 co-stimulatory and inhibitory domains and (2) a set of single ITAM-containing domains, respectively, followed by the two membrane distal ITAMs of CD3ζ.[45] Following tumor cell co-cultures, unsupervised clustering of the transcriptional data revealed 13 unique clusters. CAR-induced clusters (CICs) were those that were relatively de-enriched in TCR-negative and unstimulated 28ζ controls, and pseudobulked PCR analysis revealed that low enrichment in CICs was correlated with memory and resting genes whereas high enrichment correlated to cytotoxic and effector genes. 10 CAR variants were selected from different phenotypic clusters to characterize relative to 28ζ and BBz. All showed activation with no significant differences in exhaustion and all showed tumor cell killing at low tumor burden, although with varying kinetics in vitro. Notably, four CAR ICD combinations (FCRL6-CD3G, CD28-FCGR2A, 4-1BB-FCER1G, and FCRL6-FCGR2A followed by truncated CD3ζ) showed potent and even enhanced tumor control at low E:T ratios. The selected CARs also showed distinct cytokine secretion profiles, though 28ζ showed the highest levels of proinflammatory cytokine secretion. In particular, CD79B was prominent in the transcriptional data—potentially owing to a proliferative advantage—while Fc or Fc-like receptors were enriched in CICs; meanwhile, CD28 and 4-1BB were enriched, but individual CARs harboring these domains generally showed poor effector function both in the transcriptional data and in functional assays. This paper demonstrates that scRNA-seq could provide a useful additional layer of information for the selection of novel CAR variants. In a case where multiple library members show a similar desired phenotype, researchers can decide to give preference to selecting a transcriptionally more diverse set of CAR variants to explore a larger functional space, or focus on more similar transcriptional profiles that are thought to be uniquely beneficial. scRNA-seq of pre-infusion and post-infusion of CAR-T-cell products could prove to be informative in this regard.

For instance, patients who responded to CAR-T-cell therapy show enrichment of clones expressing high levels of genes associated with cytotoxicity, effector molecules, chemokines, activation and proliferation, whereas non-responders had higher scores on genes associated with dysfunction and exhaustion.[88–91] Additionally, in vivo CRISPR knock-out screens in adoptively transferred T cells in mice have identified negative functional regulators (eg, *FLI1, DHX37, PPP2R2D*) of T-cell responses that could further inform candidate selection based on scRNA-seq data sets.[92–94]

Future prospects and limitations

CARs have shown incredible efficacy in treating B-cell acute lymphoblastic leukemia, large B-cell lymphoma and multiple myeloma, but have yet to show meaningful translation to other cancer indications. Hypothesis-driven approaches to improve these therapies often rely on rational design of a handful of novel CARs followed by characterization, which can be costly in terms of time, effort, and resources. Furthermore, the rules of CAR design are often not intuitive. In an effort to expedite the process of discovering next-generation CAR designs while also establishing CAR design principles, many groups have begun taking a more unbiased approach in which many potential CARs varying in some aspects of their architecture are screened simultaneously for function. This has produced several exciting new CAR compositions that show efficacy in preclinical studies, as well as identified new groups of CAR signaling domains and uncovered their impact on T-cell function. Selection data has shown the capacity to power machine learning algorithms that could predict CAR function, with the potential to expand such principles to consider cancer genotype and patient-specific factors such as gender, age and medical history.

Despite significant progress within this approach, some limitations should be highlighted. Present high-throughput screening methodologies necessitate either the direct sequencing of domain pools or the establishment of a barcode-domain look-up table through long-read sequencing. Unfortunately, this approach can result in the loss of low-frequency library members after bottlenecking.[51] An alternative method involves serial barcoded DNA assembly, which streamlines the identification of unique library elements using only short-read sequencing techniques.[51] Nevertheless, the challenge persists in

maintaining consistent barcode identities throughout the processes of cloning and viral transduction as viral vectors exhibit a susceptibility to recombination events during replication, giving rise to erroneous barcode-element combinations.[95] The utilization of viral vectors in CAR-T-cell therapy has sparked an ongoing debate, particularly in the wake of a rising number of reports on secondary malignancies post-treatment, potentially attributed to random integration events.[96] This indiscriminate integration into the genome also exerts an impact on gene expression profiles, resulting in more heterogeneous therapeutic products.[97] Non-viral, integrating cell engineering methods, exemplified by CRISPR-Cas9, present a highly specific and efficient means of generating safer and more uniform therapeutic products. For high throughput screening campaigns, these modification strategies ensure singular integration events within a predefined genomic locus, thereby mitigating confounding factors that might otherwise affect CAR performance.[98 99] Additionally, CRISPR-based knock-in protocols have become highly efficient, obtaining around 40% CAR-expressing T cells, though cell yield and viability remain a point of attention due to the harsher transfection methods used.[99] Current high-throughput CAR screening strategies employ a relatively simple selection scheme assaying a narrow part of T-cell responses to CAR signaling, often based on target binding or T-cell activation. However, selecting for the best-performing domains for one property does not necessarily guarantee the best performance overall. Integrating a broader spectrum of readouts, such as cytokine release, proliferation, differentiation, degranulation or synapse formation, would provide a more complete view on CAR domain characteristics in vitro. An additional layer of information on CAR-T-cell migration, tumor infiltration, expansion and persistence could be extracted from in vivo pooled screens. These multiparametric strategies would be particularly useful when evaluating combinatorial libraries of different CAR domains. While the focus is currently on unraveling the intricacies of each CAR domain in isolation, it is likely that co-dependencies between CAR domains exist. For example, antigen-ABD interactions can be influenced by the choice of the HD,[25 66] CAR functionality can change based on the HD-TMD combination,[51] and T-cell activation may be affected by TMD-CSD pairs.[31] The exponential increase in library size for combinatorial libraries introduces another hurdle. Currently, library size is often limited to 10^5–10^6 elements in in vitro mammalian cell screens to remain practical and maintain decent library coverage. In vivo CAR libraries would be smaller—approximately 10^4 members—to accommodate higher library coverage and minimize stochastic dropout throughout the screening process. Hence, one can design a stepwise screening approach, wherein initial library enrichment is performed using functional or phenotypical markers to achieve a library size that can be comfortably transferred to in vivo screens for further selection. However, we would caution that the more enriched the library is for functional clones, the more paracrine activity might be observed in a pooled setting. Therefore, validation and profiling of lead candidates in an arrayed screen would be necessary to provide isolated and more in-depth information, including safety profiles through subsequent in vivo assays. We recognize that as data sets and computational methods continue to develop, it is possible that machine learning-aided sublibrary design could prove sufficient to train models that can predict outcomes of larger CAR domain libraries and expand the scope of the physical assays.

The application of the discussed high throughput screening and computational methods can certainly be expanded. For example, library construction can be designed to enhance any aspect of the CAR structure or similar proteins of interest as it relates to cell function, including length and rigidity of structural elements such as the HD and linkers, or mutation of individual residues within a binding or signaling domain. In addition, multicell per well screens could identify synergistic combinations of CAR therapies. CARs can also be screened in vehicles beyond T cells such as macrophages and NK cells, which may have advantages in solid tumor infiltration and allogeneic cell transfer, respectively.[100 101] Similarly, most publications to date rely on healthy donor PBMCs rather than those derived from often heavily pretreated patients. Finally, AAs, sequence motifs and protein domains could be viewed as a natural language, opening the door for using deep-learning large language models for the de novo design of protein sequences with any given characteristics or functions.[102] For a comprehensive discussion on how artificial intelligence can advance cellular therapies, including CAR-T-cell therapy, we refer to a review by Caponni and Daniels.[103]

CONCLUSION

It is clear that the field has only just begun to unlock the potential of high throughput screening of CARs, with major implications in translating CAR-T cells to diseases beyond hematological malignancies such as solid tumors, autoimmunity and infectious diseases. We envision that the influx of novel domains in the CAR toolbox and the improved understanding of CAR design could fuel major advancements in personalized medicine as CAR therapies can be adjusted to challenges faced in different indications.

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