

Engineering and Design of Chimeric Antigen Receptors

Sonia Guedan,¹ Hugo Calderon,¹ Avery D. Posey, Jr.,^{2,3,4} and Marcela V. Maus^{5,6}

¹Department of Hematology, Hospital Clinic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), 08036 Barcelona, Spain; ²Center for Cellular Immunotherapies, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA; ³Parker Institute for Cellular Immunotherapy at the University of Pennsylvania, Philadelphia, PA, USA; ⁴Corporal Michael J. Crescenz VA Medical Center, Philadelphia, PA, USA; ⁵Cellular Immunotherapy Program, Massachusetts General Hospital Cancer Center, Boston, MA, USA; ⁶Harvard Medical School, Boston, MA, USA

T cells engineered with chimeric antigen receptors (CARs) have emerged as a potent new class of therapeutics for cancer, based on their remarkable potency in blood cancers. Since the first clinical reports of their efficacy emerged 7 years ago, investigators have focused on the mechanisms and properties that make CARs effective or toxic, and their effects on T cell biology. Novel CAR designs coupled with improvements in gene transfer technology, incorporating advances in gene editing, have the potential to increase access to engineered cell therapies, as well as improve their potency in solid tumors.

Adoptive transfer of T cells collected from autologous peripheral blood and engineered to express chimeric antigen receptors (CARs) has produced impressive clinical responses in patients with hematologic malignancies.¹ Together with checkpoint blockade therapy,² CAR T cells are revolutionizing the field of cancer therapies, providing hope to patients with previously refractory cancers.^{3–9} CAR T cells targeting CD19 were recently approved by the US Food and Drug Administration (FDA) and the European Commission (EC) for the treatment of relapsed or refractory acute lymphoblastic leukemia (ALL) in pediatric and young adults (Kymriah), and for the treatment of relapsed or refractory diffuse large B cell lymphoma in adults (Kymriah and Yescarta). Emerging clinical data are proving the potential of CD22 and B cell maturation antigen (BCMA)-directed CAR T cells to eradicate leukemia and multiple myeloma, respectively, heralding a new era for the treatment of blood cancers.^{10,11}

Although the human T cell is a highly potent anti-tumor agent, several challenges remain for successfully applying CAR T cells to solid tumors. First, the potency of these “living drugs” can lead to lethal on-target toxicity if the target is expressed on a life-sustaining tissue.¹² Second, active proliferation of the CAR T cells in the body can itself lead to potentially serious side effects, such as cytokine release syndrome¹³ and neurologic toxicity,¹⁴ although these are typically transient and reversible. Third, although finding a single uniquely expressed tumor antigen is already a challenge,¹⁵ targeting only one antigen may be insufficient to obtain sustained responses, because antigen loss has been identified as a frequent cause of tumor resistance to CAR T cell therapies.^{3,10,16} Fourth, in order to be effective in solid tumors, CAR T cells will have to overcome multiple challenges, including an immunosuppressive tumor microenvironment. Recent

clinical reports suggest that CAR T cells can engraft in the peripheral blood, traffic to solid tumors, and respond to antigen.^{17–20} We define engraftment as the ability for CAR T cells to be detectable in peripheral blood at any time after infusion. Moreover, evidence of transient antitumor activity has been observed in patients with difficult-to-treat tumors.^{21,22} However, with rare exceptions, clinical responses in patients with solid tumors have been minor and transient, highlighting the importance of optimizing T cell therapeutics to tackle the solid tumor challenge. Finally, simplified methods to culture and engineer T cells are required to lower the costs, accelerate development, and improve access to this novel treatment.

In this review, we discuss the importance of fine-tuning each of the modules that form a CAR as an initial step to improve T cell specificity, antigen recognition, and T cell function. Second, we will summarize the current and novel tools that are being used to engineer CAR T cells, including recent developments in gene-editing technology that allow researchers to edit specific genes within the human genome, and even knock-in transgenes in specific integration sites. Finally, we will provide a comprehensive overview of exciting strategies that are being developed to generate new synthetic receptors with enhanced function and reduced toxicities.

For simplicity, this review focuses on modification of $\alpha\beta$ -T cells with CARs. Substantial effort has been invested in identifying the best T cell subsets for adoptive cell transfer. Moreover, strategies to introduce CARs into other immune cell types, including $\gamma\delta$ -T cells, natural killer cells, natural killer T cells, and macrophages have garnered growing attention. CAR design will need to be tailored to each cell type. The pursuit of the ideal cell type for redirection with CARs is beyond the scope of this review and has been covered elsewhere recently.^{23–25}

Design and Optimization of CARs

CARs are designed in a modular fashion that typically consists of an extracellular target-binding domain, a hinge region, a transmembrane

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Correspondence: Marcela V. Maus, Cellular Immunotherapy Program, Massachusetts General Hospital Cancer Center, 149 13th Street, Room 3.216, Charlestown, MA 02129, USA.

E-mail: mvm Maus@mg h.harvard.edu





domain that anchors the CAR to the cell membrane, and one or more intracellular domains that transmit activation signals. Depending on the number of costimulatory domains, CARs can be classified into first (CD3z only), second (one costimulatory domain + CD3z), or third generation CARs (more than one costimulatory domain + CD3z). Introduction of CAR molecules into a T cell successfully redirects the T cell with additional antigen specificity and provides the necessary signals to drive full T cell activation. Because antigen recognition by CAR T cells is based on the binding of the target-binding single-chain variable fragment (scFv) to intact surface antigens, targeting of tumor cells is not MHC restricted, co-receptor dependent, or dependent on processing and effective presentation of target epitopes. In this section, we describe the properties and function of each of the modules that compose CARs. Despite intensive research efforts to define optimal CAR design, a universally improved CAR structure has not yet been discovered. As of now, each CAR construct needs empirical testing for evaluation, and several studies indicate that small modifications can have major consequences on the therapeutic outcome.

scFv

The antigen-binding capability of the CAR is defined by the extracellular scFv, not the targeted antigen. For instance, the scFv of both of the approved anti-CD19-targeted CARs, Kymriah and Yescarta, is derived from the murine anti-human CD19 antibody FMC63, which targets a specific epitope of CD19 found in exon 4 of the CD19 gene.²⁶ Other anti-CD19 CARs may recognize different epitopes of CD19, or the same immunodominant epitope but with differential affinities, such as the scFv derived from SJ25C1,¹⁴ which is in the JCAR015 product. Investigators targeting MUC1 with CARs have used scFvs derived from antibody clones HMFG2, SM3, and 5E5, among others. HMFG2 and SM3 both recognize the PDTR epitope of the MUC1 variable tandem repeat; however, MUC1 is a highly glycosylated protein, especially within these tandem repeats, and SM3 only recognizes the deglycosylated protein, whereas HMFG2 can recognize deglycosylated or fully glycosylated protein.²⁷ CAR T cells targeting MUC1, either utilizing SM3 scFv and co-expressing interleukin-12 (IL-12) or utilizing an HMFG2-based scFv, were employed in a phase I clinical trial to treat metastatic seminal vesicle cancer. HMFG2-based CAR T cells caused tumor necrosis, whereas SM3-based CAR T cells did not seem to have efficacy.²⁸ In contrast, CARs using the 5E5 scFv recognize the GSTA epitope of the MUC1 repeat only when glycosylated with Tn antigen, an abnormal glycoform found in many epithelial cancers.²⁹ Because glycosylation of tumor-associated antigens can be modulated by cell type or oncogenic transformation, this difference in epitope binding can define the target of CAR T cells and potentially discriminate between normal tissue expressing a TAA and a tumor cell expressing the same TAA. In fact, changes to the tumor cell surface glycome can affect cell signaling, affect cell-matrix and cell-cell interactions, and influence immune modulation and the cellular metastatic potential.³⁰ Investigating the changes of the tumor membrane glycolipids and glycoproteins, which are encompassed in a broader view of the tumor “surfaceome,” can provide additional targetable avenues for cancer immunotherapy.

The format of an scFv is generally two variable domains linked by a flexible peptide sequence, either in the orientation VH-linker-VL or VL-linker-VH. The orientation of the variable domains within the scFv, depending on the structure of the scFv, may contribute to whether a CAR will be expressed on the T cell surface or whether the CAR T cells target the antigen and signal.³¹ In addition, the length and/or composition of the variable domain linker may be an important contribution to the stability of the scFv; in studies generating scFvs from a TAG72 antibody (clone B72.3), linkers up to 6xGGGGS demonstrated higher molecular weight dimers and multimers, with clustering decreasing with increasing linker length.³² If these scFvs are utilized in CAR molecules, this clustering may lead to antigen-independent signaling, or “tonic signaling.” Another linker of a TAG72-based scFv (clone CC49) designed to enhance proteolytic stability (Whitlow “218” linker: GSTGSGSKPGSGEGSTKG) had the effect of enhancing scFv affinity,³³ which may alter CAR T cell behavior as well.

Two studies have demonstrated that variance of CAR scFv affinities, either through mutagenesis of complementary-determining regions while holding the epitope constant, or through CAR development with scFvs derived from therapeutic antibodies against the same target, but not the same epitope, can change the strength of the T cell signal and allow CAR T cells to differentiate overexpressed antigens from normally expressed antigens.^{34,35} In fact, researchers demonstrated that trastuzumab-based CAR T cells with reduced scFv affinity could degranulate in response to HER2⁺ breast cancer cells without reactivity against normal primary human cells that express HER2.³⁴ These results suggest that the scFv, a critical component of a CAR molecule, can be carefully designed and manipulated to influence specificity and differential targeting of tumors versus normal tissues. Given that these differences may only be measurable with CAR T cells (as opposed to soluble antibodies), pre-clinical testing of normal tissues for expression of the target, and susceptibility to on-target toxicities, requires live-cell assays rather than immunohistochemistry on fixed tissues.

Hinge

The hinge, also referred to as a spacer, is the extracellular structural region of the CAR that separates the binding units from the transmembrane domain (Figure 1). With the exception of a few CARs based on the entire extracellular moiety of a receptor, such as NKG2D,³⁶ the majority of CAR T cells are designed with immunoglobulin (Ig)-like domain hinges. These spacers generally supply stability for efficient CAR expression and activity.

The hinge also provides flexibility to access the targeted antigen. Several studies have demonstrated that the optimal spacer length of a given CAR depends on the position of the targeted epitope.³⁷ Long spacers provide extra flexibility to the CAR and allow for better access to membrane-proximal epitopes^{37,38} or complex glycosylated antigens.³⁹ By contrast, CARs bearing short hinges are more effective at binding membrane-distal epitopes.^{40,41} It has been proposed that the length of the spacer is crucial to provide adequate intercellular

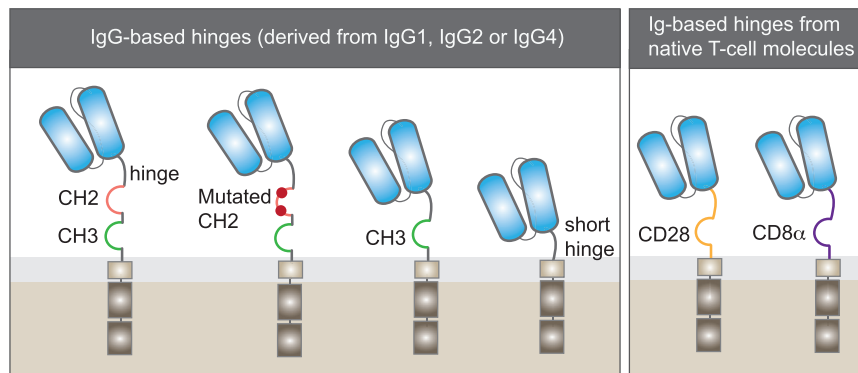


Figure 1. Two Families of Hinges

IgG-based hinges, derived from IgG1, IgG2, or IgG4, provide flexibility to the scFv to target membrane-proximal epitopes. However, binding of the CH2 domain to Fc receptors in myeloid cells can impair functionality. Modification or deletion of the CH2 domain can restore CAR T cell functionality. CARs with shorter hinges, including an IgG-derived hinge lacking the CH2-CH3 regions, or hinges derived from native CD28 and CD8 hinges, can be used to target membrane-distal epitopes.

distance for immunological synapse formation,⁴² highlighting the need to optimize spacers for individual epitopes accordingly.

Hinges can affect the overall performance of CAR T cells. Human IgG-derived spacers consisting of two Ig-like domains, CH2 and CH3, have been used for efficient antigen recognition and are especially useful in assessing the level of CAR expression at the surface of the T cell. However, several studies indicate that IgG-based spacers retain their capacity to bind to Fc gamma receptor (FcγR) through their CH2 domain even after they are incorporated into CARs. This feature leads to off-target activation by myeloid and lymphoid cells expressing FcγRs,⁴³ and correlates with off-tumor localization and poor engraftment in animal models.^{41,44,45} Point mutations to ablate the FcγR binding domain and spacers trimmed down to a single CH3 domain have improved CAR T cell engraftment and anti-tumor efficacy in *in vivo* experiments.^{41,45,46} Alternatively, extracellular domains naturally lacking FcγR binding activity, such as CD28 and CD8α, have been extensively used as spacers.^{47,48} In fact, these two spacers are found in Yescarta and Kymriah, respectively.

Transmembrane Domain

The transmembrane domain consists of a hydrophobic α helix that spans the cell membrane and is probably the least characterized region of the CAR. Although the main function of the transmembrane is to anchor the CAR in the T cell membrane, some evidence suggests that the transmembrane can be relevant for CAR T cell function.

The nature of the CAR signaling is not well understood; however, if CARs behave similar to TCRs, it is likely that CARs need to dimerize or associate with other accessory molecules for proper signaling initiation. In that regard, one study demonstrated that first generation CARs need specific regions within the CD3 transmembrane for CAR dimerization or incorporation into endogenous TCR clusters.⁴⁹

Second and third generation CARs have been designed with numerous single-span transmembranes, mainly derived from CD4, CD8α, or CD28.^{47,50,51} A recent study demonstrated that when using CARs containing the inducible T cell costimulator (ICOS) intracellular domain, incorporation of the ICOS transmembrane, instead of the extensively used CD8α transmembrane, was required for increased CAR T cell

persistence and overall anti-tumor efficacy.⁵² In another study, Morin et al.⁵³ analyzed the function of the extracellular and transmembrane domains of the endogenous CD28 molecule using a transgenic mouse that lacks the intracellular tail of CD28. Interestingly, the authors show that the extracellular and transmembrane domains of CD28 can partially induce T cell activation.⁵³

Altogether, these results suggest that the transmembrane domain of certain costimulatory molecules can be involved in synapse formation or T cell signaling. Linking the proximal intracellular domain to its corresponding transmembrane domain may enable proper CAR T cell signaling, while using the widely used CD8α or CD28 transmembrane domains may favor CAR expression or stability.

Costimulatory Domains: The Art of Signaling

Substantial effort at CAR T cell engineering has been invested in understanding the effects of CAR costimulation with the aim of identifying an optimal endodomain to include in CAR constructs. CAR endodomains, or intracellular domains, are usually derived from costimulatory molecules from the CD28 family (including CD28 and ICOS) or the tumor necrosis factor receptor (TNFR) family of genes (including 4-1BB, OX40, or CD27). The CD28 family signals through the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, with ICOS inducing a stronger PI3K activation than CD28.^{52,54} Unique to CD28 is the recruitment of Grb2 and Lck to its intracellular tail, which results in high levels of IL-2 production,⁵⁵ as well as the recruitment of Itk. The TNFR family members signal through recruitment of TRAF proteins⁵⁶ and are implicated in modulating T cell proliferation, differentiation, and survival.⁵⁷⁻⁵⁹

CD28 and 4-1BB are the most widely used costimulatory endodomains in CARs. Clinical trials with CARs incorporating CD28 or 4-1BB intracellular domains showed similar response rates in patients with hematologic malignancies. However, the persistence of T cells engineered with these two CAR designs is strikingly different. Pre-clinical studies identified these T cell persistence differences in head-to-head comparisons of CD28- and 4-1BB-based CAR T cells in animal models.^{48,60} Clinical trials for B cell malignancies have shown that CD28-based CAR T cells are typically undetectable beyond 3 months,^{6,61} whereas 4-1BB-based CAR T cells can persist



in patients for several years after treatment.⁶² Exhaustive studies indicate that signaling through CD28-based CARs results in more rapid T cell activation, proliferation, cytolysis, and increased glycolysis, but shorter T cell persistence. By contrast, 4-1BB signaling induces a slower T cell effector response and promotes mitochondrial biogenesis, greater oxidative metabolism, and sustained T cell persistence.^{52,63-65} The high effector function and self-limited expansion of CD28-based CARs may be ideal to transiently treat diseases with a rapid tumor elimination and short-term persistence of the CAR (i.e., as a bridge therapy for allogeneic hematopoietic stem cell transplantation). By contrast, 4-1BB-based CARs may be used to treat diseases in which complete responses require sustained T cell persistence.⁶²

Similar to 4-1BB, CD27 signaling has been shown to enhance CAR T cell survival when compared with CD28.⁶⁶ Incorporation of ICOS into a CAR drives CD4⁺ T cells toward a Th1/Th17 phenotype, enhancing T helper functions and increasing *in vivo* T cell persistence when compared with the widely used CD28 and 4-1BB intracellular domains.⁶⁷ Recent data suggest that various lymphocyte subsets require distinct costimulation signals for optimal function and persistence. For instance, whereas the ICOS intracellular domain enhances the persistence of CD4⁺ CAR T cells, the 4-1BB intracellular domain provides optimal persistence in CD8⁺ T cells.⁵² One option to join the properties of different intracellular domains in one single T cell is to combine two intracellular domains in a third generation CAR. Typically, such combinations include one intracellular domain from the CD28 family and one intracellular domain from the TNFR family, resulting in the simultaneous activation of different signaling pathways.^{52,60,68,69} Third generation CARs are consistently expressed at lower levels when compared with second generation CARs, but they usually show improved effector functions. Optimization of these more complex receptors, including the position of each intracellular domain in relation to the cell membrane, is required to attain complete synergy of the combination.⁵²

Altogether, these findings indicate that the nature of costimulation during CAR T cell activation critically regulates the metabolism, survival, and effector function of T cells. Each costimulatory domain has unique properties, and it is unlikely that an optimal costimulatory domain will serve all purposes. Differences in the affinity of the scFv, the intensity of antigen expression, the probability of off-tumor toxicity, or the disease to be treated may influence the selection of the intracellular domain. Further understanding of the molecular signaling effects of CARs may help in choosing the right intracellular domain or combination of intracellular domains for each condition. Ultimately, carefully designed clinical trials may be required to understand the effects of costimulation on the success of CAR T cell therapies.

Avoiding Tonic Signaling

Although the main purpose of engineering T cells with CARs is to drive activation upon antigen-specific recognition in tumors, some CAR constructs can also induce antigen-independent activation, usu-

ally referred to as tonic signaling. High density of CARs at the T cell surface, usually driven by strong constitutive promoters, can result in clustering of CAR molecules that leads to chronic T cell activation. Tonic signaling can be detected during *in vitro* primary T cell expansions and is characterized by differences in the growth patterns, phenotype, and function of CAR T cells when compared with control T cells. The level and consequences of tonic signaling are highly dependent on the selection and configuration of CAR modules, but they usually include chronic T cell activation with accelerated differentiation and exhaustion, and impaired anti-tumor effects.^{52,70} Moreover, tonic signaling in cMET-specific CARs containing the CD28 signaling domain can result in continuous *in vitro* proliferation, with constitutive secretion of diverse cytokines, including IL-2.⁷¹ In a GD2-specific CAR, substitution of the CD28 intracellular domain for the 4-1BB intracellular domain ameliorated the T cell exhaustion caused by tonic signaling. Although low levels of tonic signaling in 4-1BB-based CARs may be beneficial because of enhanced T cell persistence,⁴⁸ recent reports indicate that in some cases, 4-1BB-driven tonic signaling may result in augmented T cell apoptosis, impaired *in vitro* expansion, and progressive downregulation of CAR expression.^{52,72} The level of tonic signaling can also depend on the length of the hinge,⁴⁵ the expression system,^{52,71} and the vector used.⁷² In third generation CARs, tonic signaling may be mitigated by placing the 4-1BB intracellular domain distal to the cell membrane.⁵²

Tonic signaling is not usually assessed systematically in most CAR reports. However, emerging evidence underscores the importance of avoiding the use of CARs with configurations that may induce this chronic activation. Mitigating tonic signaling may require testing different combinations of scFvs, hinges, and intracellular domains. CAR aggregation can be attenuated by using alternative promoters to reduce the levels of CAR expression; however, this can also result in impaired efficacy.⁵² A recent report suggests that knocking in the CAR into the TRAC locus through homology-directed recombination averts tonic CAR signaling by optimizing CAR internalization and re-expression kinetics.⁷³

Genetic Engineering Strategies

Viral- and non-viral-based genetic engineering tools have been used to generate CAR T cells, resulting in permanent or transient expression of therapeutic genes (Table 1). However, most of the current clinical trials developing modified T cells employ the latest generations of retroviral (gamma retroviral and lentiviral) vectors.

Retrovirus-based gene delivery is a mature, well-characterized technology, which permanently integrates the CAR into the host cell genome. Although serious adverse events were linked to retroviral gene transfer in CD34⁺ stem cells,^{74,75} mature T cells have been safely modified with this framework now for decades with hundreds of treated patients, resulting in stable engraftments and without cell transformation.^{76,77} The downsides of this approach are the cost associated with clinical-grade production and the limited amount of genetic material that can be included in the vector. A recent study using lentiviral vectors in patients with ALL reported that unintentional

**Table 1. A Summary of the Current Strategies Being Used to Genetically Modify T Cells *Ex Vivo* to Express Chimeric Antigen Receptors**

	Transgene Insertion	Transgene Expression	Transgene Lifespan	Transgene Delivery	Types	Features and Observations
Retroviral vectors	non-targeted integration	exogenous promoter	long	<i>ex vivo</i> transduction	gamma retrovirus	safe, optimized, and FDA-approved protocols; the production of the therapeutic cells is expressive
					lentivirus	transgene size limitation
Transposase enzymes	non-targeted integration	exogenous promoter	long	<i>ex vivo</i> electroporation	Sleeping Beauty	FDA-approved protocols, more economical than viral vectors, but less developed technology; transposase have to be electroporated along with the donor DNA (plasmid or minicircles)
					piggyBac	
mRNA	non-integrative	N/A	short	<i>ex vivo</i> electroporation	N/A	fast and economical method to produce CAR T cells; the transgene expression is rapidly diluted over the expansion of the T cells; ideal when first introducing a novel CAR into patients
Non-integrative lentivirus	non-integrative, episomal	exogenous promoter	mid-short	<i>ex vivo</i> transduction	NILV-S/MAR	the transient expression can be extended up to 30 days; the production of the non-integrative lentivirus is expensive and will still require constant re-dosing
Endonuclease enzymes	targeted integration	endogenous promoters	long	<i>ex vivo</i> electroporation	zinc-finger	directed transgene insertion into the host cell genome; ability to ablate specific host cell genes; endonucleases have to be electroporated along with donor DNA (AAV or linear dsDNA); further protocol optimization is required
					TALENs	
					CRISPR/Cas9	

NILV-S/MAR, non-integrating lentiviral vector containing a scaffold/matrix attachment region.

transduction of a single leukemic B cell during the CD19-CART manufacturing resulted in resistance to CAR T cell therapy through masking of the CD19 epitope. Although this is a rare event, this finding highlights the importance of properly isolating T cells from leukemic cells before redirection.⁷⁸

Non-viral DNA transfection transposition systems have also been used for permanent expression of therapeutic payload. Sleeping Beauty (SB) transposon is the most developed system of its kind to engineer CAR T cells,⁷⁹ complying with current good manufacturing practice (cGMP),⁸⁰ and is currently under investigation in the clinic (ClinicalTrials.gov: NCT00968760 and NCT01653717). Multiple SB enzymes^{81,82} have been used to deliver more than one transgene from a multi-cistronic single plasmid^{83,84} or multiple plasmids.⁸⁵ Others have developed CAR T cells using the *piggyBac* transposon system,^{86–88} which can integrate larger transgenes.^{89,90} In general, transposon technology might be easier and more economical to produce than viral vectors. However, the longer expansion protocols currently employed may result in T cell differentiation, as well as impaired activity and poor persistence of the infused cells.⁹¹ In this regard, recent developments using minicircle vectors might help address these issues because of higher efficiency integrations.⁹²

Non-integrative gene transfer, such as RNA transfection, enables transient expression of the CAR for up to 1 week. This strategy has

been used to evaluate potential toxicities or to limit the side effects of the therapy.^{20,93} However, the weaknesses of a transient CAR expression are that the transgene is rapidly lost, and patients will require multiple infusions for clinical effect. Alternatively, non-integrative CAR delivery, such as episomal gene transfer, may result in longer-term, but not permanent, expression.⁹⁴

Finally, in order to simplify and reduce the cost of CAR T cell manufacturing, alternative approaches using *in situ* T cell modification are being explored. *In situ* T cell programming using DNA nano-carriers⁹⁵ or lentiviral vectors specifically targeting human CD8⁺ T cells⁹⁶ have been proved safe and efficacious in pre-clinical models of B cell malignancies.

Gene Editing: The New Era

Improvements to site-directed nuclease gene-editing tools are unveiling a new era for CAR T cell therapy. Most efforts at T cell gene editing thus far have been focused on developing off-the-shelf universal CAR T cells from allogeneic healthy donors. The majority of current clinical trials use autologous CAR T cells, because use of allogeneic CAR T cells could induce graft-versus-host disease (GVHD) due to TCR-mediated recognition of the recipient alloantigens. The immune system of the recipient could also attack the infused human leukocyte antigen (HLA)-disparate CAR T cells, causing rapid T cell rejection. In order to prevent GVHD, different groups have focused on removing the TCR from allogeneic CAR T cells using genome editing.



Infusion of gene-edited universal CD19-CAR T cells in two pediatric patients with ALL induced complete responses in both patients, proving the feasibility of this approach.⁹⁷ Strategies to prevent CAR T cell rejection by the recipient are also being explored and include the elimination of HLA class I expression by editing the HLA-A or β_2 -microglobulin genes.

Gene editing is also being explored to selectively remove the expression of T cell-suppressive genes.^{98–100} Checkpoint inhibitor PD-1 is probably the most prominent gene currently targeted, with PD-1 knockout anti-MUC1 CAR T cells entering clinical trials this year (ClinicalTrials.gov: NCT03525782). Recently, Ren and colleagues⁹⁹ set up a versatile system for editing multiple genes simultaneously in CAR T cells through a single-vector lentiviral system.

Target-specific endonucleases have also been exploited to insert transgenes inside loci relevant for T cell biology (Table 1). Unlike random insertion strategies, this method may result in finely regulated CAR expression independent of constitutive promoters.^{73,101} In a landmark study, Eyquem et al.⁷³ demonstrated that targeting the CAR insertion into the TCR locus results in more uniform expression and increased potency over conventionally generated CAR T cells.

Different endonucleases have been used to knock out genes in T cells, including zinc-finger nucleases (ZFNs),¹⁰² TALENs,^{97,103,104} and Cas9.^{98,99} For knock-in approaches, the transgene can be supplied from donor DNA using non-integrative viral vectors, such as adeno-associated viral vector type 6 (AAV6),^{73,101} or non-viral approaches, such as single-stranded DNA.¹⁰⁵ The enzymes are typically electroporated into T cells as mRNA or protein.^{98,99} More recently, a study using the CRISPR/Cas9 system has demonstrated that donor DNA can be electroporated as endonuclease-complexed and long linear double-stranded DNA (dsDNA), which is otherwise toxic, yielding relatively high T cell viability and transgene insertion efficiencies.¹⁰⁵ This system avoids the use of viral vectors, which is expensive and time-consuming to generate and test, and could therefore substantially reduce the cost and time for gene editing in T cells. Overall, this report underscores the potential to develop next generation CAR T cell therapies; however, further protocol optimization will be needed to achieve large-scale manufacturing of gene-edited CAR T cells.

Finally, a better understanding of the potential deleterious effects of genome editing is required to ensure that benefits derived from further modifications in CAR T cells are greater than risks. In this regard, whole-genome sequencing of CRISPR/Cas9 and TALEN-targeted cell lines revealed a low incidence of off-target mutations.¹⁰⁶ Recent studies suggest that Cas9 can induce a p53-mediated toxicity in certain cell types, which can lead to a selection of p53 mutant cells more tolerant to DNA damages.^{107,108} Careful monitoring of the p53 status in gene-edited cells together with efforts to enhance nuclease specificity may help reduce the potential risks of genome editing in CAR T cells.

The Next Generation of CARs

Several new approaches to enhance the therapeutic outcome of CAR T cells are being explored based on the lessons learned in recent clinical trials and fueled by rapid advances in synthetic biology. These approaches are mainly focused on improving T cell activation and persistence, overcoming tumor immunosuppression, addressing tumor escape, mitigating toxicities, and increasing access. CAR T cell therapies incorporating costimulatory molecules, inhibitory-to-costimulatory switch signals, cytokines, or safety switches have been developed to address these challenges and have been reviewed elsewhere.^{23,109–111} Here, we will highlight some of the innovative strategies based on new antigen receptors able to recognize a combination of antigens in an effort to avoid antigen escape or reduce toxicity (Figure 2).

Limiting Tumor Escape

A major challenge for CAR T cell therapies is tumor escape due to antigen loss in the setting of inherent tumor heterogeneity and plasticity. Loss or decreased expression of the targeted antigen after CAR T cell therapy and outgrowth of antigen-negative cells after treatment has been observed in several clinical studies,^{10,17,18,112,113} highlighting the importance of simultaneously targeting more than one antigen.

One approach to overcome this obstacle is to express two different CARs in one single cell in order to target two different antigens, ideally in one single bicistronic vector to ensure dual-antigen targeting in all cells, an approach known as “dual CARs.”¹¹⁴ Expression of two CAR molecules in one viral plasmid may require codon optimization of duplicated DNA sequences (i.e., CD3 ζ) to reduce the chances of DNA recombination. Another alternative is to design a receptor fusing two scFvs with different specificities with a single intracellular module in a tandem CAR.^{10,115,116} Tandem CARs have the advantage of a smaller transgene size when compared with dual CARs, which is especially important when other transgenes (i.e., safety switches or cytokines) are also incorporated into the CAR plasmid. The main disadvantage is that in order to achieve optimal antigen recognition and full T cell activation, tandem CARs require optimization of the spacer length and linker sequence between the two scFvs, as well as the orientation of scFvs, which is more complex than with second generation CARs.¹¹⁷ However, computational modeling methods can help predict the structure of tandem CARs, making design and development of these receptors more feasible.¹¹⁵

Dual and tandem CARs are known as OR-gate CARs, as binding of either CAR to its cognate antigen is enough to drive full T cell activation. Interestingly, enhanced T cell function is observed when both targets are present, which makes them more efficient at inducing anti-tumor responses than a pooled combination of CAR T cells.^{114,116,118} One of the main limitations of OR-gate CARs is that targeting two antigens may result in increased risk for toxicity, especially when treating solid tumors. Ongoing clinical trials are currently testing dual (ClinicalTrials.gov: NCT03330691) and tandem (ClinicalTrials.gov: NCT03185494, NCT03097770, and NCT03019055) CAR T cells

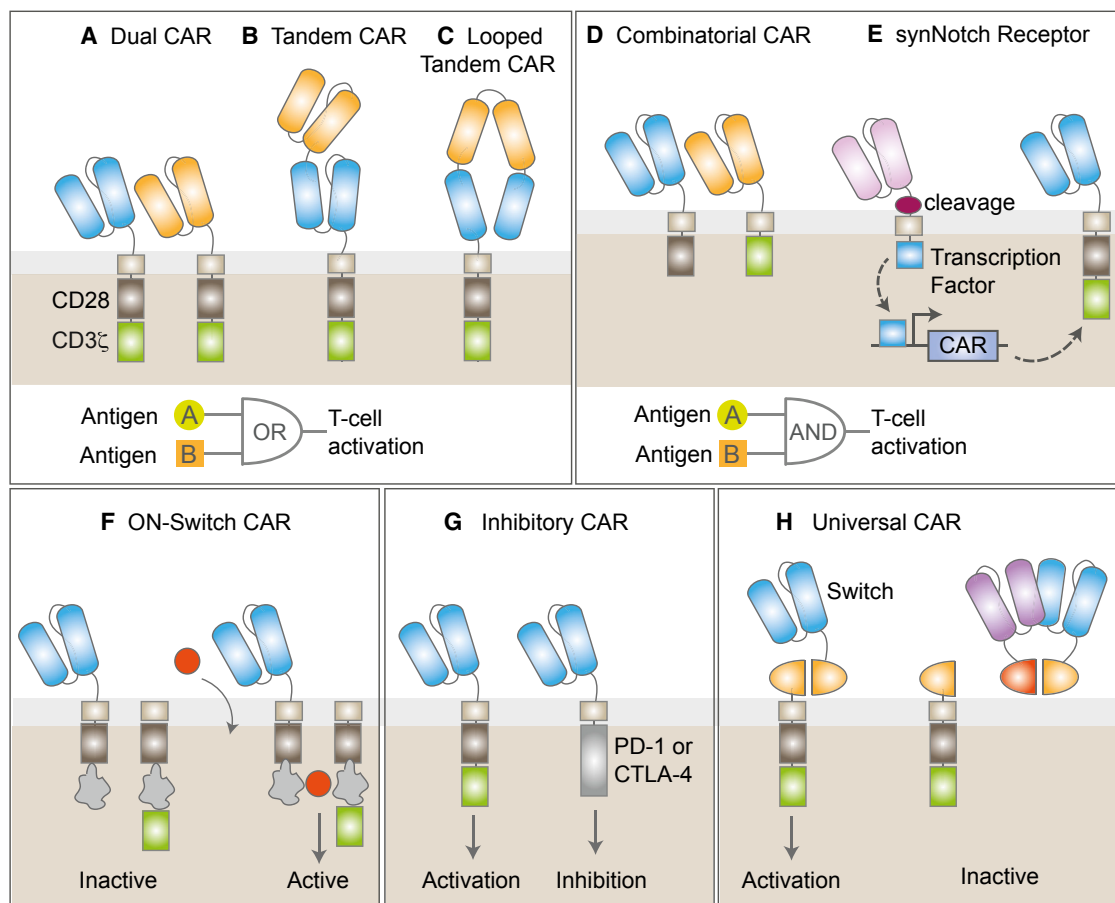


Figure 2. The Next Generation CARs: New Strategies to Avoid Tumor Escape and Mitigate Toxicities

Schematic representation of novel CAR configurations. (A–C) Multitargeted CAR configurations. Dual CARs co-express two different CARs in one cell (A). Tandem CARs contain two different scFvs in a single CAR molecule that can either be stacked (B) in series or as a looped structure (C). Dual and tandem CARs function as OR-gate circuits: CAR T cells are activated following recognition of antigen A OR antigen B. (D and E) Combinatorial CARs combine two constructs: one bears the CD3z signaling motif and the other bears the costimulatory signaling domain (D). Synthetic Notch (syn-Notch) receptors induce the transcription of a CAR after antigen recognition of their cognate antigen (E). Combinatorial and syn-Notch CARs function as AND-gate circuits: CAR T cells are fully activated only when antigen A AND antigen B are recognized. (F) On-switch CAR T cells are inactive until specific activating agents are added, assembling a fully functional receptor. (G) Inhibitory CARs (iCAR) inhibit T cell activation following antigen recognition in normal cells. (H) Universal or switchable CAR T cells remain inactive until antibody-based molecules targeting a tumor antigen are supplied to reconstitute a fully active CAR construct.

simultaneously targeting CD19 and CD22 or CD20 for the treatment of leukemia and lymphoma.

Minimizing Toxicities

Lack of truly tumor-specific antigens is a major limitation for the development of CAR T cells. Most of the antigens currently targeted with CAR T cells are also expressed in normal cells, where even low levels of antigen expression can potentially result in severe on-target off-tumor toxicities.^{12,119,120} Novel strategies in which engineered T cells require a specific combination of two antigens for activation are being explored to prevent toxicities. In a first approach in this direction, T cells were engineered to co-express two different CARs, one containing signal 1 (CD3z) and the other containing signal 2 (costimulation).^{121,122} In this split-signal approach, recognition of a single

antigen, which may happen in normal cells, leads to suboptimal T cell activation and limited killing of normal cells. In tumors, where both antigens may be expressed, full T cell activation is achieved after engagement of both scFvs to their cognate antigens.¹²² In a different approach, the activation of CAR T cells can be controlled *in situ* by the addition of a small molecule. “ON-switch” CARs are designed as fragmented CAR receptors, where the extracellular antigen-binding module is dissociated from the intracellular signaling components.¹²³ Assembly of these two receptors in the presence of a heterodimerizing small molecule, such as tacrolimus-based analogs, leads to CAR T cell activation. The magnitude of responses depends on the dosage of the drug, which allows for titratable control. A more recent strategy involves an AND-gate recognition system in which activation of one receptor (synthetic Notch) induces the expression



of a second receptor (CAR) that induces T cell activation following antigen recognition.¹²⁴ Full T cell activation and tumor elimination occur only when both antigens are expressed. However, tumor escape of CAR T cells through loss of the first antigen targeted by the synthetic Notch receptor is a major limitation of this strategy.

A distinct class of strategies to limit toxicity includes the co-expression of a classic CAR with an antigen-specific inhibitory CAR (iCAR) bearing the signaling domain of an immunoinhibitory receptor (i.e., CTLA-4 or PD-1).¹²⁵ Engagement of iCARs to antigens in normal cells can constrain T cell functions, which can be resumed in the absence of the iCAR-targeted antigen and following antigen recognition by the activating CAR. One of the challenges of this approach is the identification of cell surface antigens that are expressed in normal cells but absent in tumor cells, a problem that is complementary to the obstacle of identifying tumor-specific antigens absent in normal tissues.

Universal CARs: Increasing Versatility

A different strategy that could simultaneously address antigen escape while mitigating toxicities is the utilization of so-called universal CARs. Instead of engineering T cells with fixed antigen specificities, these CAR therapies are composed of two parts: (1) an antibody-based molecule that recognizes a tumor antigen and is modified to express a “switch”; and (2) a universal CAR T cell without tumor specificity by itself, which contains a construct with an extracellular portion that binds to the switch and is linked to the intracellular signaling domains. In a first approach, universal CAR T cells were designed to bind to biotinylated antigen-specific molecules.¹²⁶ Other approaches incorporated the small molecule fluorescein isothiocyanate (FITC) or a short peptide in the antibody-based switch, allowing activation of the corresponding switchable CAR T cells after tumor recognition by the antibody-switch.^{127,128} In a more recent approach, a split-CAR system named SUPRA CAR combines zipCAR T cell containing an extracellular leucine zipper with an scFv domain fused to a second leucine zipper (zipFv).¹²⁹ These versatile systems allow researchers to easily combine universal CAR T cells with zipFvs targeting different antigens to avoid tumor escape. At the same time, modulating the dose or affinity of the antibody-based switches may control toxicities. In the case of SUPRA CAR T cells, T cell activation can be prevented when necessary by addition of a competitive zipFv that can bind with high affinity to the zipFv with tumor specificity.¹²⁹

Conclusions

The clinical development of CAR T cells has undergone tremendous growth over the past decade. However, this novel therapeutic modality is still in its infancy, and much remains to be learned and improved. Clinical trials evaluating CAR T cells in patients with both hematologic malignancies and solid tumors have revealed insights into the parameters that determine clinical outcome. Based on the lessons learned, a new arsenal of novel receptors and strategies has been developed and is poised to enter clinical trials.

Preclinical studies have unveiled the importance of optimizing CAR constructs. The affinity and flexibility of the extracellular binding domain is key for improved tumor recognition, whereas the nature of costimulation during CAR T cell activation critically regulates the metabolism, survival, and effector function of T cells. A major challenge to the field is that the optimal CAR structure for each given antigen to be targeted remains empirical and involves the generation and testing of several constructs. As novel, more complex receptors emerge, optimizing each module may lead to an unrealistic number of constructs to test, both in animal models and in humans. In this regard, bridging the scientific divide between chimeric molecular engineering and protein structure identification and modeling may help predict which molecules would function optimally and reduce the number of constructs to evaluate. Identifying general rules to guide CAR design would also simplify this process. Also, a better understanding of the interactions of CAR proteins with endogenous T cell molecules and detailed mechanisms of CAR signaling would facilitate the design of more effective CAR T cell treatments.

One major limitation, especially when treating solid tumors, is the absence of truly tumor-specific antigens that are expressed in tumor cells but absent in normal tumors. Expression of the targeted antigen in normal cells can result in severe toxicity, whereas absence or decreased antigen expression in tumor cells can lead to tumor escape. Thus, it is critical to explore the tumor surfaceome, including differential post-translational modifications of tumor-associated antigens, in order to identify new targets for antibody-based therapies, such as glycan-directed CAR T cells.¹³⁰ Strategies to optimize CAR specificity and reduce toxicity will be critical as we increase the number of tumor antigens to target. In this regard, recent technological advances in single-cell analysis tools and precision bioinformatics may help unveil additional differences between normal and tumor cells,¹³¹ albeit with increasing tumor heterogeneity.

Engineered T cells have shown their potential to dramatically change clinical outcomes in patients with B cell malignancies. Remarkable advances in the fields of molecular biology, immunology, gene editing, synthetic biology, and computational analysis provide new tools for CAR T cell development and are critical to achieving a greater therapeutic success when expanding this therapy to other cancer types.

CONFLICTS OF INTEREST

S.G., A.D.P., and M.V.M. are listed as inventors on various patents related to chimeric antigen receptors.

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