CAR models: next-generation CAR modifications for enhanced T-cell function

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T cells genetically targeted with a chimeric antigen receptor (CAR) to B-cell malignancies have demonstrated tremendous clinical outcomes. With the proof in principle for CAR T cells as a therapy for B-cell malignancies being established, current and future research is being focused on adapting CAR technology to other cancers, as well as enhancing its efficacy and/or safety. The modular nature of the CAR, extracellular antigen-binding domain fused to a transmembrane domain and intracellular T-cell signaling domains, allows for optimization by replacement of the various components. These modifications are creating a whole new class of therapeutic CARs. In this review, we discuss the recent major advances in CAR design and how these modifications will impact its clinical application.

Molecular Therapy — Oncolytics (2016) 3, 16014; doi:10.1038/mto.2016.14; published online 18 May 2016

T-cell-based immunotherapies have come of age as a feasible, safe, and efficacious approach to treating cancer. At the same time, the use of highly personalized, living therapeutics poses multiple challenges. Moreover, immunotherapies involving the genetic modification of T cells, such as those involving expression of chimeric antigen receptors (CAR) to modify T-cell specificity, require an additional level of optimization. In this article, we summarize our current understanding of the key aspects of CAR-T-cell design. This is not meant to be an extensive compilation of the full body of knowledge in the field, but rather an overview of the most relevant finding that have driven the field to its current stage, and those that will likely define its forthcoming directions.

Antigen-specificity for a T cell is encoded by the T-cell receptor (TCR).¹ T cells recognize and eradicate infected cells by TCR-mediated detection of microbial antigens, in the form of short amino acids presented by major histocompatibility complex proteins. TCR binding of a specific major histocompatibility complex and peptide combination initiates an intracellular signaling cascade that begins with phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) domains within TCR accessory proteins CD3ζ, CD3γ, CD3δ, and CD3ε.²,³ This signaling cascade terminates in T-cell activation and killing of the target cell. T cells can also target cancerous cells by detection of tumor antigens, which can be novel, or normally expressed only in germ cells, or mutated self-antigens (neo-epitopes).⁴ Investigators have confirmed the power of tumor-reactive T cells by isolating tumor-infiltrating lymphocytes (TILs) from patients with metastatic cancer, expanding them ex vivo, and infusing the TILs back into patients.⁵ Some of these patients have achieved durable complete remission (CRs), which is unheard of with standard cytotoxic chemotherapies.⁶ However, a major impediment to the adoption of TIL therapy to many cancer patients is the laborious and time-consuming (>2–3 months) process required to achieve a sufficient number of tumor-reactive T cells.⁷ CARs provided the requisite technological advance and allowed the creation of a large, bulk population of tumor-reactive T cells within as short as 1 week and mediated positive clinical outcomes in many patients with acute or chronic leukemia.⁸,⁹

The CAR is a hybrid antigen receptor, part antibody and part TCR, and is composed of an extracellular antigen-binding domain and intracellular signaling domain(s) (Figure 1a).¹⁰ Genetic retargeting of a T cell with a CAR endows a new antigen-specificity through the single-chain variable fragment (scFv), which is derived from a tumor-specific antibody.¹¹ The scFv allows the T cell to bind a tumor antigen and T-cell activation is initiated through the intracellular domains, which are derived from CD3ζ ITAM domains.¹,³ To complete the genetic construct for the CAR, a hinge and a transmembrane domain (TM), commonly from CD8α or immunoglobulin, bridges the extracellular scFv and intracellular CD3ζ ITAM domains. Early in vitro studies demonstrated that T cells gene-targeted with CARs that have an intracellular signaling domain composed of only CD3ζ supported T-cell activation and target killing, however, these first-generation CAR T cells had very limited persistence and antitumor efficacy in vivo.¹¹ Since one of the main advantages to the CAR technology is its modular nature it allows continual refinement and modification to optimize T-cell function, which is how first-generation CARs were replaced with second-generation CARs.

The specificity of a TCR is for only a short peptide (8–12 amino acids) from a foreign (or nonself) antigen; so, there is potential for
cross-reactivity to similar sequences of amino acids. TCR ligation of host antigen could lead to T-cell activation, autoimmunity, and even death. To minimize this potential, T cells require at least two signals to fully activate. The first signal is through the TCR, but the second signal, or costimulation, is mediated through ligation of CD28 by CD80 or CD86, which are normally expressed on antigen-presenting cells (APC). Therefore, when a T cell encounters a cross-reactive peptide expressed on a normal (non-APC) cell, it is unable to provide costimulation and T-cell activation is unsuccessful. However, when APCs are activated, as during inflammation or infection, they upregulate CD80 and CD86 and can induce both signals 1 and 2, thereby supporting full T-cell activation, target killing, and long-term persistence.

Second-generation CAR T cells have been confirmed to mediate potent antileukemia responses in phase 1 clinical trials. CR rates up to 90% have been obtained when patients with relapsed and/or refractory B-cell acute lymphoblastic leukemia (B-ALL) were infused with second-generation CD19-targeted T cells that included a CAR with a 4-1BB or CD28 costimulatory domain. While great success has been noted with targeting CD19, there are significant safety and efficacy concerns with adapting this technology to other cancers. Anticipating these concerns, researchers have again looked to the modular nature of the CAR to further refine and optimize this novel antigen-receptor. For the remainder of this review, we will discuss how modifications to the scFv, hinge/spacer, and intracellular signaling domains can help to overcome these challenges.
domains may render this engineered cell therapy safer and/or more efficacious.

THE SINGLE CHAIN VARIABLE FRAGMENT

The scFv retargets a bulk population of autologous T cells with a new tumor-reactivity. However, recent studies have demonstrated that the scFv can impact CAR function beyond just tumor-specificity. In fact, investigators have demonstrated aspects of scFv design can modulate the safety and/or efficacy of CAR T cells. For example, the nonhuman origin of the antibodies used to create the scFv has resulted in anti-CAR immune responses, which may limit the persistence of the adoptively transferred CAR T cells. Furthermore, this anti-CAR immune response may have more profound implications considering that investigators postulate it resulted in fatal anaphylaxis in patients infused with multiple doses of CAR T cells. Therefore, while current lead CARs targeting B-cell malignancies include scFv with mouse origins, CARs targeting other cancers in the developmental pipeline will likely be “humanized” to minimize these immune responses.

The affinity of scFv for its target is another CAR feature being modified to optimize gene-targeted T-cell function. CD19 has been an optimal target to establish the proof-of-principle of adoptive CAR T-cell therapy. It has also demonstrated that on-target/off-tumor toxicity is a concern since patients have been induced into long-term periods of B-cell aplasia. No major complications have been described secondary to B-cell aplasia, presumably due to intervention with gamma globulin and/or antibiotics. However, expression of the target on critical tissues, such as ERBB2 on respiratory epithelium, has resulted in death in at least one patient. As CARs are developed against solid tumor malignancies the tumor targets will likely be shared on normal epithelial tissue, thereby increasing the possibility of dangerous complications. However, investigators have recently demonstrated that differential levels of antigen expression and scFv affinity can be used to differentiate between tumor targets and normal tissue. Investigators have demonstrated that low-affinity scFv’s support CAR T-cell-mediated killing of tumor cells that express high level of antigen, but does not support killing of normal cells that express low or normal levels of the same antigen. This discrimination between levels of antigen expression has been demonstrated both in vitro and in vivo and suggests that scFv affinity can be optimized to increase safety when a target is expressed on normal, healthy tissue.

A breakthrough in CAR design occurred when it was determined that inclusion of CD3ζ (signal 1) in tandem with CD28 costimulation (signal 2) on a single CAR genetic construct could replicate normal TCR activation and costimulation. However, research has demonstrated that requiring combinatorial ligation of separate, distinct tumor antigens by bispecific CARs can increase safety, while also supporting efficacious cancer killing. The underlying technology to this CAR advance relies on disassociating the activation and costimulatory domains onto separate complementary CARs. Therefore, CAR1 has only the CD3ζ activation domain, while CAR 2 has only costimulatory domains. Ligation of CAR1 by its antigenic target or ligation of CAR2 by its antigenic target is insufficient by itself to mediate full T-cell activation since they deliver only 1 signal. However, when both CAR1 and CAR2 bind their respective antigens, CD3ζ (signal 1) and costimulation (signal 2) occur in tandem and support T-cell activation and long-term in vivo function.

In addition to this novel CAR design, other groups have created similar dual CAR systems and demonstrated the potential for enhancing safety and/or efficacy of tumor targeting. For example, a second CAR that is specific for a normal tissue antigen can be conjugated to a suppression domain to prevent killing of normal tissue. Investigators propose to use this inhibitory bispecific CAR design to prevent normal tissue destruction. The demonstration that combinatorial antigen ligation and/or signal domain dissociation can be used to regulate T-cell activation and function suggest a robust platform for future CAR design manipulations to further enhance safety and/or function.

While the scFv can be optimized to increase safety, others have replaced the scFv to create a ligand-based CAR or universal CAR. The ligand-based CAR replaces the scFv with a ligand for a tumor marker, for example, a CAR that expresses a ligand for the IL13 receptor (IL13R) allows redirection of T cells to IL13R expressed on glioblastoma. While ligands sometimes may have multiple binding partners, this IL13-zetakine is mutated to be highly-specific for the IL13R for glioblastoma. Universal CAR systems have also been developed to broaden the clinical applicability of CAR T-cell therapy (Figure 1b). These universal CARs retarget patients’ T cells and mediate an anti-tumor response, regardless of cancer diagnosis. This technology relies on replacing the scFv with a binding domain that is specific for a tagged protein or molecule, such as biotin or fluorescein isothiocyanate. The extracellular portion of the universal CAR is juxtaposed to a transmembrane domain, which is followed by intracellular sequences comprised of T-cell costimulatory and activation domains (Figure 1b). Therefore, after a tumor-specific antibody, labeled with fluorescein isothiocyanate or biotin, binds its target on a tumor cell it can then be bound by the universal CAR, which will support ligation, activation of the T cell, and killing of the tumor cell. Research has validated the function of both universal CAR T-cell systems in animal models. While the universal CAR T-cell technology has not been evaluated in patients as of yet, it has the major advantage that it can be applied to the many tumor-specific antibodies that have already been approved for clinical use or are currently in development.

HINGE AND Spacer DOMAINS

The hinge, spacer, and transmembrane domains connect the scFv to the activation domains and anchor the CAR in the T-cell membrane. Recent studies have demonstrated that these domains can have significant impact on CAR T-cell function and can be optimized to enhance antigen binding and T-cell signaling. This was revealed by demonstrating spacers are required to bind membrane-proximal epitopes and support efficacious target killing. In contrast, spacers reduce function when the epitope is expressed near the amino terminal portion of the cell surface protein. However, spacer length is not the only important consideration. Investigators have also determined that inclusion of Fc domains can support binding of antibodies that activate immune cells. Due to the abundance of antibodies present in vivo, these CARs can mediate such strong and persistent T-cell activation that it leads to activation-induced T-cell death or activate other immune cells that limit CAR T-cell persistence. Removal of these Fc binding sites enhances in vivo CAR T-cell function and persistence. These reports suggest that CAR hinge and spacer length and sequence should be optimized based on epitope position. It is important to note that most of this research is based on using an Immunoglobulin-derived hinge and spacer and they have not been evaluated for CD8-derived spacers and hinges.

INTRACELLULAR DOMAINS

All the CARs currently tested in the clinic contain either CD28, 4-1BB (CD137) or a combination thereof. However, other alternatives have
been explored preclinically. A clear clinical comparison of first- versus second-generation CARs was performed by Savoldo and collaborators. In this work, they administered, to the same patient, T cells expressing a first-generation CAR, along with T cells expressing a second-generation CAR (containing a CD28 costimulation domain). Both CARs had the exact same antigen domain, and their persistence postadministration was analyzed by polymerase chain reaction, showing that the CD28-containing CAR-T cells outperformed the first-generation counterparts. This study provided the ultimate confirmation that CD28 costimulation enhances survival of CAR-T cells in patients.46

While CD28 costimulation is responsible for clonal expansion of activated T cells and secretion of IL-2 in early phases of activation, another costimulatory receptor, 4-1BB (CD137), is associated with long-term survival of T cells. Costimulatory domains derived from 4-1BB have been used in second- and third-generation CARs. Incorporation of this 4-1BB costimulation has been shown to prevent exhaustion due to tonic CD3-signaling present in CD28 CARs.48

Beyond CD28 and 4-1BB, a CD4-associated costimulatory receptor, OX40 (CD134) has been tested preclinically as part of CAR signaling. A third-generation CAR containing CD28 and OX40 costimulation induced superior survival of CCR7+ T cells than a CD28-only counterpart.49 In addition, a CD28-OX40 CAR induced less secretion of IL-10 than a CD28-based second-generation CAR, without altering the secretion of IFN-γ.50

ICOS, a costimulatory receptor involved in TH17 polarization was also integrated in the intracellular domain of a CAR. Its presence was associated with enhanced in vivo persistence of TH17-polarized CAR-T cells, compared to CD28 or 4-1BB costimulation.51 In turn, CD27 costimulation was shown to provide better survival than CD28.52 In elegant work by Duong and colleagues, a combinatorial approach was used for random generation and selection of intracellular signaling domains. They found that, within a library of CAR constructs containing variable numbers for costimulatory domains, the CAR displaying the greatest antitumor effect was a third-generation design containing DAP10 and CD27 in addition to CD3ζ.53

More recently, a CAR containing a DAP12-derived signaling domain was developed for adoptive transfer of NK cells, showing a more robust performance than CD3ζ-based CARs.54 In T cells, a “KIR-CAR” consisting of a scFv fused to the transmembrane domain of KIR2DS2, along with DAP12 as a separate molecule, displayed more robust antitumor effect in vivo than a second generation, CD3ζ-based CAR. This novel design displayed also superior surface expression than its CD3ζ-counterpart, probably related to different membrane dynamics, which may impact the function of CAR-T cells.55

A thorough understanding of the molecular pathways triggered by CARs, in presence and absence of antigen ligation, will be required to rationally design the most efficient receptors. This will be achieved by the implementation of systems biology approaches to preclinical experimentation, and by integration of the molecular, biological and pharmacodynamics data obtained from the analysis of gene-modified T cells administered in the clinic.55

**JOINT EXPRESSION OF CAR AND ACCESSORY GENES**

CARs have also been combined with accessory proteins to improve the function of T cells, and/or to use lymphocytes as a carrier to deliver a payload that will alter the tumor microenvironment. T cells modified to express a tumor-specific CAR in tandem with a chemokine receptor that binds chemokines present in the tumor microenvironment enhances tumor targeting, while also modulating CAR T-cell homing and trafficking.56 This dual-protein system allows dual targeting as well as functional modification. In another direction with accessory proteins, Zhao et al. demonstrated that a second-generation CAR containing a CD28 costimulatory domain with CD3ζ, expressed together with 4-1BB ligand in trans (CD28ζ/41BBL), induced a more potent antitumor effect than a third-generation CAR containing both CD28 and 4-1BB in cis (Figure 1c). In addition, T cells expressing a CAR with the CD28ζ/41BBL domains, expressed less markers of exhaustion and persisted longer in vivo in a mouse model of B-cell leukemia.57 On the other hand, Condomines et al. reported that the second-generation 28Z design was more efficacious than a first-generation CAR complemented in trans by CD80 costimulation. This observation was associated with a decreased sensitivity to CTLA4-mediated inhibition in T cells expressing the second-generation CAR,58 and suggests that different costimulatory ligands may lead to different outcomes. Finally, CAR-T cells coexpressing CD40L also outperformed second-generation CAR-T cells. This particular design, applied to the treatment of B-cell malignancies, enhanced CAR-T cells by multiple mechanisms including the stimulation of IL-12 secretion by dendritic cells and the induction of costimulatory molecules by CD40-expressing target cells.59

Cytokines have also been explored as combination partners that may enhance the activity and/or persistence of CAR-T cells, resulting in enhanced anti-tumor effects. Hoyos et al. tested the combination of a second-generation CAR targeting CD19, along with interleukin-15 (IL-15) and an inducible suicide gene (iCasp9). The CAR/cytokine/suicide gene triad effectively enhanced proliferation, reduced PD-1 expression and provided superior anticancer killing in a humanized mouse model of B-cell lymphoma.59 In another study, Markley and Sadelain showed that anti-CD19 CAR-T cells that constitutively expressed IL-2, IL-7, IL-15, or IL-21 displayed greater antilymphoma activity in vivo than CAR-T cells with no cytokine; IL-7 and IL-21 being the most efficacious.60

Interleukin-12 (IL-12) is a pleiotropic cytokine that may induce potent antitumor responses by stimulating the immune system at multiple levels. Systemic administration of IL-12, however, causes severe toxicity, precluding its clinical application.61 To circumvent this limitation, systemically administered CAR-T cells can be used as “Trojan horses” to deliver IL-12 to the desired tissues (Figure 1d). Using this approach, Pegram and collaborators62 showed that expression of IL-12 by CAR-T cells eliminated the need for lymphodepleting preconditioning, and rendered CAR-T cells resistant to inhibition by regulatory T cells, in a syngeneic mouse model of CD19+ malignancies. Furthermore, their results showed that the mechanism of action of IL-12 was dependent on the presence of both CD8+ and CD4+ T-cell subsets, and of functional IFN-γ secretion.63 IL-12 secretion can be further restricted to sites of T-cell activation by coupling its expression to signaling pathways activated by engagement of CAR. For instance, Zhang and collaborators generated γ-retroviral vectors where the expression of IL-12 by transduced T cells is controlled by a nuclear factor of activated T cells (NFAT)-responsive promoter.64 Using this type of transcriptional control, Chmielewski et al. showed that IL-12 secretion by anti-carcinoembryonic antigen CAR-T cells resulted in elimination of carcinoembryonic antigen-expressing tumor cells, but also of carcinoembryonic antigen-negative cells. This indirect effect was mediated by the activation of a potent macrophage-driven, TNF-α-dependent, cytotoxic effect.65 Chinnasamy et al. similarly demonstrated that T cells targeted to the tumor vasculature, which also expressed NFAT-driven IL-12, modified the tumor microenvironment by eliminating myeloid-derived suppressor cells.66
To this date, the expression of IL-12 by CAR-T cells has been extensively explored in preclinical models, allowing for a better understanding of the biology behind IL-12-driven immunotherapy. However, the clinical efficacy of CAR-T cells armed with IL-12 remains to be demonstrated. A clinical trial of adoptive transfer of TIL expressing NFAT-driven IL-12 in melanoma patients, showed the induction of objective responses in patients treated with T-cell infusions that contained a remarkably lower number of cells than conventionally used in TIL trials. In addition, treatments were administered in absence of preparative chemotherapy and, most importantly, a patient who had failed TIL treatment experienced an objective response upon treatment with TIL+NFAT-IL-12 (ref. 67). These findings suggest that IL-12 expression by adoptively transferred T cells may result in clinical benefit, but the ultimate answer will be provided by randomized clinical trials testing combination treatments versus CAR-T cells alone.

Accessory molecules can also be included in CAR-T cells to serve as safety switches, which allow for the elimination of CAR-T cells from circulation, in the event of T-cell-mediated toxicity. This approach was originally tested in the setting of hematopoietic stem cell transplants, in order to mitigate the symptoms of graft-versus-host disease. Early designs relied on the expression of a protein that metabolized an inactive prodrug, into a cytotoxic metabolite, only in gene-modified cells (Figure 1e). A different approach involves the coexpression of a membrane-bound protein (usually, a truncated receptor lacking the intracellular signaling domain) that can be efficiently targeted by an antibody causing the depletion of cells that express such protein. For instance, Wang et al. 68 described the use of a truncated form of the human epidermal growth factor receptor (huEGFRT) in adoptively transferred cells, which can later be ablated by systemic treatment with a clinical grade antibody against epidermal growth factor receptor. A thorough review of the different suicide genes developed to date has recently been published, 69 and therefore we will focus on a more recent technology that is currently being evaluated in a clinical setting: the inducible caspase-9 (iCas9). This system involves the expression of a synthetic construct consisting of an incomplete proapoptotic caspase-9 (lacking its caspase recruitment domain) fused to a mutated peptide derived from the FKBP12 protein. Interaction of the mutated FKBP12 domain with a small molecule induces dimerization of the fusion protein, resulting in activation of caspase-9-induced apoptosis (Figure 1e). 70 Clinical testing proved the efficacy and safety of the iCas9 system in the context of allogeneic stem-cell transplant, where the suicide gene was expressed in donor T cells adoptively transferred to the patients. Those patients who developed graft-versus-host disease after transfer were treated with an intravenous administration of a clinical grade CID (AP1903), and subsequently experience a rapid and nearly complete elimination of circulating donor T cells. 71 Synthetic control switches such as the iCas9 system will likely be used extensively in clinical trials of adoptive immunotherapy, either for the removal of toxic CAR-T cells, or for a more sophisticated fine tuning of CAR-T-cell activity, as recently described by Wu and collaborators. 72 In this elegant work, the authors generated a dissociated CAR that expressed the antigen binding and transmembrane domains in one protein, and the intracellular signaling domains in a separate protein. Both subunits contain a heterodimerization module that, in presence of a small molecule, assembled the CAR into a fully functional unit. Thus, CAR-T-cell activation depends on two input signals: (i) antigen binding, and (ii) presence of dimerizing agent. This design allows for real-time and dose-dependent control of CAR-T-cell activity.

**FUTURE DIRECTIONS**

Roughly two decades after its inception, the use of synthetic immune receptors expressed in T cells via genetic manipulation has proven efficacious in treating oncologic patients. Despite reaching this important milestone, the field is still in its infancy and we anticipate that the next few years will see a large increase in the number of scientific papers reporting clinical successes with CAR-T cells. In retrospect, preclinical testing appears as a key component of the clinical success achieved in recent years, providing valuable information such as the need for integrated costimulation for optimal performance of CAR-T cells. Preclinical testing will likely again play a key role in the advancement of this field, now complementing the information collected from clinical samples of patients treated with CAR-T cells. Efforts will be focused on the translation of the clinical success of CD19-targeted therapies to other antigens and, most importantly, to solid tumors. This challenge will require a multidisciplinary approach involving clinicians, clinical laboratories, basic and translational researchers. Capitalizing on the legacy of the genomic era, the functional and structural design of CARs is likely to evolve from mere T-cell activation switches, in response to antigen binding, to more sophisticated pieces of biological engineering. Gene modified T cells will likely become therapeutic agents with complex sensing and effector functions, integrating information from the tumor cells and the tumor microenvironment for increased potency and specificity. The combination of CAR-T cells with other treatment modalities such as oncolytic virotherapy remains largely unexplored, and preclinical modeling will aid in the identification of the best combination strategies to achieve synergism among multiple modalities. At the cellular level, a detailed analysis of the molecular events that govern CAR-T-cell function and persistence will provide the building blocks for the development of the next generation of T-cell therapies.

**CONFLICT OF INTEREST**

Marco I Davila receives research funding from the H. Lee Moffitt Cancer Center and Research Institute, American Society of Hematology, and Damon Runyon Cancer Research Foundation. He receives compensation from Celldar and Geneious Biotechnologies as a consultant. He receives compensation from Precision Biosciences and Adaptive Biotechnologies as an Advisory Board Member. Daniel Abate-Daga receives research funding from the H. Lee Moffitt Cancer Center and Research Institute, Moffit’s Lung Cancer Center of Excellence, and a Career Enhancement Award from NIH SPORE grant P50 CA168536.

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