Chimeric Antigen Receptor T-Cell Therapy
Current Perspective on T Cell–Intrinsic, T Cell–Extrinsic, and Therapeutic Limitations

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Abstract: Genetically engineered chimeric antigen receptor (CAR) T-cell therapy leverages the ability of the immune system to eliminate tumors and redirects cytotoxic functions toward cells expressing specified tumor-restricted antigens. Although 6 CAR T-cell therapies have received Food and Drug Administration (FDA) approval for the treatment of many hematological malignancies, limitations involving T cell–intrinsic, T cell–extrinsic, and therapeutic factors remain in the treatment of both liquid and solid tumors. Chimeric antigen receptor design, signals from the tumor microenvironment, tumor antigen escape mechanisms, and systemic inflammatory consequences of CAR T-cell infusion all influence the efficacy and feasibility of CAR T-cell therapy in different malignancies. Here, we review the core structure of the CAR, the evolution of different CAR generations, CAR T-cell therapy limitations, and current strategies being investigated to overcome the T cell–intrinsic, T cell–independent, and therapeutic barriers to successful CAR T-cell therapy.

Key Words: CAR T-cell therapy, chimeric antigen receptor, hematological malignancies, solid tumors

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CANCER IMMUNOSURVEILLANCE

Paul Ehrlich[1] first proposed the cancer immunosurveillance hypothesis in the early 1900s postulating that the immune system can recognize and protect against tumors. Since then, much research in immunotherapy has focused on understanding and developing tools to strengthen the immune system's ability to effectively surveil, recognize, and target tumor cells for clearance. Tumors often express neoantigens as a result of mutations or aberrant gene expression. Dendritic cells at the site of the tumor capture and present antigen in the context of major histocompatibility complex (MHC) to T cells with a cognate T-cell receptor (TCR). After antigen presentation, T cells clonally expand and differentiate into effector and memory T cells. Specifically, effector $CD8^+$ T cells acquire cytotoxic function via expression of perforin and granzyme B as well as chemokine receptors and adhesion molecules that allow the T cells to migrate back to the tumor to perform its cytotoxic functions. Effective T-cell responses lead to the development of memory T cells that provide protection against any secondary occurrence of tumor antigen. However, T-cell exhaustion, characterized by impaired effector function, is usually associated with chronic antigen stimulation.

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CHIMERIC ANTIGEN RECEPTOR

Chimeric antigen receptors (CARs) are recombinant cell surface receptors conventionally engineered on αβ $CD4^+$ and $CD8^+$ T cells that can redirect the T cells to recognize and target cells expressing a specific antigen in an MHC-independent manner. Chimeric antigen receptors consist structurally of 4 domains: (1) an extracellular antigen-binding domain, (2) a hinge region, (3) a transmembrane domain, and (4) 1 or more intracellular signaling domains. All 4 domains influence the efficacy and persistence of CAR T cells in eliciting antitumor responses.

Antigen-Binding Domain

One advantage of the CAR is that it leverages and couples the wide range of targets of the B-cell receptor (BCR) to the power of the effector functions of helper and cytotoxic T cells. Unlike conventional TCRs, which are restricted to recognizing small peptide antigens (8–20 residues) in the grooves of MHCI or MHCII of a specific HLA,[2] BCRs have the capacity to recognize an infinite number of peptides, lipids, nucleic acid, carbohydrates, and even synthetic epitopes directly and not limited by the polymorphic MHC.[3,4] The BCR is secreted by plasmablasts and plasma cells of the B-cell lineage in soluble form as antibodies, consisting of variable heavy (V_H) and light (V_L) chains as well as constant heavy (C_H) and light (C_L) domains. In conventional CAR molecules, the antigen-binding domain of CARs consists of 1 V_H and V_L chain tethered by a flexible linker to form a single-chain variable fragment (scFv). Similar to the antibody, the affinity of the CAR for the target antigen is dependent on several factors including the sequence of the complementarity-determining regions and the 3D conformation of the V_H and V_L chains. In addition, analogous to natural BCR binding,[5] the affinity of CAR for its target has consequences on downstream function.[6] For instance, although there is a threshold affinity for CARs to bind target antigens, relatively lower affinity receptors have advantages in achieving greater selectivity, T-cell expansion, and antitumor efficacy[7–11] (Fig. 1A). Emerging evidence indicates that higher affinity and repeated antigen stimulation of CARs also lead to activation-induced cell death.[12,13] Thus, a major challenge to achieve best efficacy is to optimize and finely tune CAR affinity rather than striving for constructing the highest-affinity receptors.

Hinge Region

The hinge region is the extracellular spacer that connects the antigen-binding and transmembrane domains of the CAR. The amino acid sequence and composition of the hinge region influence its length and flexibility and have consequences on CAR T-cell trafficking, survival, signaling, and cytokine secretion.[14–16] Longer hinges are necessary for reaching antigens proximal to the target cell surface or when embedded within heavily glycosylated structures, whereas short hinges are more appropriate when the

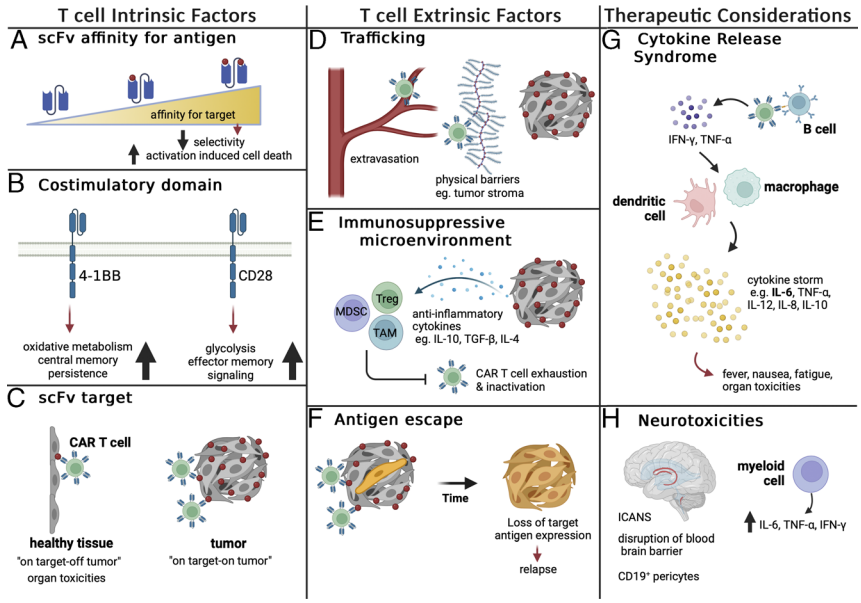

FIGURE 1. T cell–intrinsic and –extrinsic factors and therapeutic considerations that influence CAR T-cell therapy efficacy. A–C, T-cell intrinsic factors that impact CAR T-cell efficacy. A, The affinity of the scFv antigen–binding domain for the target antigen influences CAR T-cell function, with higher affinity receptors more likely to be less selective and induce activation-induced cell death. B, The selected costimulatory domain(s) influences downstream signaling in the CAR T cell, which has consequences on dominant metabolic pathways, differentiation, signaling strength, and longevity. C, Chimeric antigen receptor T cells are designed to target tumor antigens, but these epitopes are often also expressed (albeit at a lower density) on normal cells, potentially leading to CAR T-cell cytotoxic functions directed at healthy tissues. This phenomenon, coined "on-target, off-tumor effects," includes organ toxicities. D–F, T-cell extrinsic factors that impact CAR T-cell efficacy. D, The ability of infused CAR T cells to traffic toward the tumor is dependent on the tumor vasculature. Physical barriers such as extracellular matrix components often observed in the stroma of solid tumors also impede CAR T-cell infiltration. E, The immunosuppressive microenvironment in solid tumors induces CAR T-cell exhaustion through a variety of mechanisms, including production of anti-inflammatory cytokines and recruitment of immune cells, which elicit inhibitory functions toward CAR T cells. F, Tumor antigens targeted by CAR T cells may be expressed at varying levels on tumor cells because of tumor heterogeneity or downregulation mechanisms. Therefore, tumor cells with no/low expression of targeted antigen can evade CAR T-cell therapy eventually leading to relapsed disease. G–H, Therapeutic challenges to consider in CAR T-cell therapy. G, Chimeric antigen receptor T-cell engagement with their target results in production of proinflammatory cytokines released by both CAR T cells and other immune cells leading to a systemic inflammatory response called CRS in hematological cancers. H, Neurotoxicity, also referred to as immune effector cell–associated neurotoxicity syndrome, is commonly observed in patients receiving CAR T-cell therapy for hematological cancers and is often characterized by myeloid cell–driven inflammation. On target-off tumor effects due to CD19-expressing pericytes may lead to disruption of the blood-brain barrier in CD19-directed CAR T-cell therapies. Figure generated using Biorender.

epitope is either membrane distal or more easily accessible. Therefore, hinge length must be determined for each antigen-binding domain-target pair. Hinges are commonly derived from CD8α, CD28, or immunoglobulin G; however, immunoglobulin G–derived hinges often have off-target toxicities due to natural binding to Fcγ receptors expressed on immune cells. A CD34-derived hinge has also recently been described in preclinical studies.[17]

Transmembrane Domain

The transmembrane domain is a hydrophobic alpha helix that anchors the CAR in the cell membrane of the T cell and has been shown to impact CAR stability and function.[16] Most transmembrane domains are derived from CD3ζ, CD8α, or CD28. CD3ζ- and CD28-derived transmembrane domains mediate CAR dimerization with endogenous TCR complexes or the CD28 receptor, respectively, which may have consequences on downstream signaling.[18,19]

Intracellular Signaling Domain

The intracellular signaling domain has garnered most of the attention in CAR design for achieving effective antitumor immunity. Following antigen binding, the intracellular domains undergo conformational changes that enable phosphorylation and recruitment of downstream signaling proteins. The progression of CAR design over the past 30 years can be dissected into 5 generations of CAR based on the structure of the intracellular signaling domain. First-generation CARs, generated in the 1990s, contained a single CD3ζ or FcεRIγ domain but this was not sufficient to elicit effective T-cell activation, cytokine production, or persistence due to limited signaling capacity and likely skewed T cells toward anergy.[20]

Coupling of CD3ζ signaling domain with additional costimulatory signaling domains such as CD28 or 4-1BB introduced the second-generation CAR, which had improved activation, expansion, and longevity of the CAR T cells.[21,22] Food and Drug Administration–approved CAR T-cell therapies are derived from second-generation CAR design using either the CD28 or 4-1BB as costimulatory signaling molecules. Chimeric antigen receptor T cells costimulated by CD28 or 4-1BB differ in their metabolic profiles: T cells expressing CARs with CD28 primarily use glycolysis and differentiate into memory effector T cells, whereas T cells expressing CARs with 4-1BB rely heavily on mitochondrial respiration and differentiate into longer-lasting central memory T cells[23] (Fig. 1B). Many groups have provided evidence for the efficacy of other costimulatory domains CD27, OX40, GITR, ICOS, and a combined MyD88/CD40.[24–28] Third-generation CARs harbor 2 costimulatory domains—most commonly 4-1BB and CD28—and

often exhibit more robust antitumor immunity than second-generation CARs as in non-Hodgkin lymphoma.[29,30] However, other groups found that a third-generation CAR was less effective at eliciting antitumor effects than a second-generation CAR in a pancreatic cancer model.[31] Therefore, the advantage that third-generation CARs have over second-generation CARs is unclear and may depend on the setting.

Fourth-generation CARs are engineered to express the CAR and secrete specific cytokines either constitutively or upon CAR engagement to aid in the antitumor responses. These fourth-generation CARs are coined "armored CARs" or "T cells redirected for universal cytokine-mediated killing."[32] T cells redirected for universal cytokine-mediated killing/armored CAR T cells promote a proinflammatory environment in solid tumors by secreting a variety of cytokines, including interleukin 7 (IL-7), IL-12, IL-15, IL-18, and IL-23, which act either on the CAR T cell itself or in a paracrine fashion, recruiting innate immune cells to the site of the tumor.[33] Alternatively, armored CARs can secrete other proteins like checkpoint inhibitors or nanobodies to bind secondary targets.[32] Lastly, fifth-generation CARs, like fourth-generation ones, are based off the second-generation, but also express truncated intracellular domains of cytokine receptors that promote JAK-STAT signaling. Thus, fifth-generation CARs have the capacity to send all 3 activation signals of natural T cells: (1) TCR engagement, (2) costimulation, and (3) cytokine engagement rendering better persistence *in vivo* and antitumor effects in liquid and solid tumors than second-generation CARs.[34]

CAR T-CELL THERAPY LIMITATIONS

Although there have been advancements with CAR T-cell therapy in hematological cancers, therapy in solid tumors has met limited success. After infusion, CAR T cells need to successfully migrate to malignant sites, interact optimally with their target, elicit cytotoxic functions, and persist to avoid relapse. Many intrinsic and extrinsic factors as well as therapeutic limitations contribute to undesirable outcomes of CAR T-cell therapy in liquid and solid tumors, which are detailed in the following section.

T Cell–Intrinsic Limitations

Antigen Selection—"On-Target, Off-Tumor" Effects

One of the major challenges with designing CAR T cells is identifying unique tumor-specific targets with which to create the antigen-binding domain. Current FDA-approved CAR T-cell therapies for B lineage–derived cancers target CD19 and BCMA, which are expressed on both malignant and nonmalignant B-lineage cells and therefore result in B-cell aplasia or hypogammaglobulinemia. Although patients receive intravenous immunoglobulin replacement to compensate for loss of normal B-cell or plasma cell function, some are prone to infection and exhibit long-term defects in humoral immunity.[35,36] In addition, neurotoxicity as an adverse effect of anti-CD19 therapy suggests "on-target, off-tumor" adverse effects likely due to CD19 expression on brain mural cells.[37]

Antigen selection in solid tumors is more challenging than in hematological cancers because the "on-target, off-tumor" effects of solid tumor antigens often have more severe consequences in normal tissue function compared with B-cell aplasia (Fig. 1C). Many solid tumor antigens, like HER2, have been identified because of their heightened expression on epithelial cancer cells; however, their lesser expression on normal tissues is often still sufficient to lead to toxicities and multiorgan failure.[38,39]

Poor CAR T-Cell Expansion and Persistence

Chimeric antigen receptor T-cell expansion and persistence *in vivo* are often critical for maintaining long-term remission in patients. Both intrinsic variables related to the CAR T-cell design and extrinsic factors related to environmental signals the CAR T cell receives may limit their durability. The scFv region of the CAR T is often derived from mice and therefore can elicit strong immune responses against the CAR that greatly hinder their persistence of CAR T cells.[40] In addition, scFvs that bind to antigen with relatively higher affinities may lead to T-cell exhaustion and poor expansion.[11]

The selected costimulatory domain in CAR design has been shown to have consequences on long-term persistence. Chimeric antigen receptor T cells harboring the 4-1BB intracellular signaling domain preferentially use oxidative and fatty acid metabolism, which closely resembles the natural memory T-cell metabolic phenotype and may delay T-cell exhaustion.[23,41,42] 4-1BB–based CAR T cells persisted for several weeks after CD28-based CAR T cells met their decline in murine models of B-cell acute lymphoblastic leukemia and in non-Hodgkin lymphoma patients.[43,44] By contrast, CD28 CAR T cells elicit stronger and faster signaling with an effector T-cell phenotype compared with 4-1BB CAR T cells.[41] Depending on the nature of pathogenesis, the preference between a fast-acting CD28 or more durable 4-1BB CAR T cell may play a role when treating cancers with different remission rates. Ideally, CAR T-cell therapy would treat fast-growing tumors immediately and persist for long-term control to prevent tumor relapse.

Ex vivo expansion of T cells is essential for transduction of CAR genes and to generate enough CAR T cells for infusion. Techniques used during CAR T-cell manufacturing such as cryopreservation, dosing of activation signals received in culture, and duration in culture influence T-cell persistence and efficacy *in vivo.*[45] In addition, several groups provide evidence that preselection of naive or stem memory T cells before expansion and CAR manufacturing lead to better persistence and antitumor immunity.[46,47]

T Cell–Extrinsic Limitations

CAR T-Cell Trafficking and Hostile Immunosuppressive Environment

After intravenous infusion, CAR T cells must successfully migrate to the site of the target to elicit appropriate effector function. In contrast to hematological cancers, which reside in niches readily available for intravascular T cells to successfully encounter their target, solid tumors often exhibit abnormal vasculature and a dense stroma, limiting the ability of CAR T cells to access the tumor (Fig. 1D). Furthermore, after extravasation, CAR T cells targeting solid tumors often must overcome a hostile tumor microenvironment, which deters effector function. Strong anti-inflammatory signals in the microenvironment of the tumor, such as cytokines IL-10 and transforming growth factor β, induce macrophage and dendritic cell polarization toward immunosuppressive activity, which in turn inhibit CAR T-cell function (Fig. 1E). T regulatory cells, myeloid-derived suppressor cells, and cancer-associated fibroblasts also contribute to antagonize CAR T cells from eliciting their function. Ultimately, the hostile microenvironment leads to CAR T-cell exhaustion and inactivation. External obstacles that hinder antitumor immune responses naturally (reviewed in Labani-Motlagh et al.[48]) also apply to the hurdles of CAR T-cell therapy.

Antigen Escape

One negative potential consequence of CAR T-cell therapy is the selection of tumor cells that express low or no detectable levels of antigen (Fig. 1F). Although most acute lymphoblastic leukemia patients (70%–90%) exhibited durable responses after CD19-directed CAR T-cell therapy in phase I trials, 11% of patients relapsed coinciding with undetectable expression of CD19.[49] Antigen loss after

CAR T-cell therapy has also been observed in solid tumors with both EGFRvIII- and IL13Rα2-directed CAR T cells in glioblastoma leading to decreased expression of the respective target.[50,51] One mechanism for antigen escape is trogocytosis where the target antigen is transferred to CAR T cells, thereby not only decreasing antigen density on the tumor but also promoting T-cell fracticide.[52]

Therapeutic Limitation Toxicities

Although CAR T-cell therapy is a promising avenue for cancer treatment, it is also associated with significant adverse effects in hematological cancers, including cytokine release syndrome (CRS), a systemic inflammatory response involving elevated cytokines (e.g., IL-6, interferon γ, tumor necrosis factor, IL-2, IL-8, and IL-10) in the sera of patients who experience fever and hypotension[53] (Fig. 1G). Cytokine release is due to extensively activated CAR T cells and other immune cells in response to the infused cells. In addition to CRS, some patients suffer from neurotoxicities after receiving CAR T-cell therapy[54] (Fig. 1H). Several options are being explored to ameliorate CRS and neurotoxicity including suicide genes and adjusting CAR T-cell dose.

CAR T-CELL ADVANCES

Overcoming T Cell–Intrinsic Limitations

One avenue to broaden the limited solid tumor associated antigens with which to design cognate antigen–binding domains is to identify and target tumor-specific posttranslational modifications such as overexpression of O-glycans Tn (GalNAcα1-O-Ser/Thr) and sialyl-Tn (NeuAcα2-6-GalNAcα1-O-Ser/Thr).[55,56] Other groups have proposed to identify tumor mutations unique to each patient creating the opportunity to develop fully personalized CAR T-cell therapy. However, the financial ability to support this strategy long-term in all patients is unclear.

Chimeric antigen receptor T cells are historically generated using retroviral vectors, which may integrate in multiple places in the T-cell genome, potentially posing a risk for oncogenic transformation and other undesirable consequences.[57] Advances in gene delivery using CRISPR/Cas9 genome editing allow for precise targeting of the CAR to the locus of the TCR-α constant (*TRAC*) gene.[58] Targeting the CAR to the *TRAC* locus resulted in uniform CAR expression, enhanced *in vivo* killing of tumor cells, and reduced exhaustion compared with conventionally generated CAR T cells.

Chimeric antigen receptor T-cell exhaustion, characterized by loss of antitumor function, is characterized by expression of inhibitory receptors PD-1, TIM-3, LAG-3, CTLA-4, among other modifications. Simultaneous knockdown of 3 inhibitory receptors using short hairpin RNA improved CAR T-cell tumor infiltration and persistence.[59] These "exhaustion-resistant" CAR T cells, which have also been generated by modulating expression of transcription factors,[60,61] retain their effector function despite continuous contact with their cognate antigen.[62]

Overcoming T Cell–Extrinsic Limitations

To mitigate antigen escape, much attention is now directed toward the development of tandem CAR T cells, which express at least 2 scFvs to target at least 2 antigens. Tandem CARs have successfully decreased antigen escape mechanisms in both liquid and solid tumors.[63–65] Furthermore, tandem CAR technology is one strategy to address antigen heterogeneity within a tumor.

The use of CAR T cells and oncolytic viruses (OVs) are potentially complementary strategies to combat the limited number of tumor-restricted antigens and antigen escape. Oncolytic viruses have been used to overcome the immunosuppressive environment as they promote a proinflammatory environment in response to viral infection.[66] Recent studies have further exploited OVs to deliver ectopic antigens to tumor cells, which allows for targeting of antigen-negative tumor cells.[67,68] This strategy may also be applied to settings with on-target off-tumor effects by delivering novel antigens followed by cognate CAR T cells. Many groups have also investigated the efficacy of cytokine-producing CAR T cells to modulate the milieu of the tumor microenvironment. For example, IL-12–producing CAR T cells can promote a proinflammatory environment through inducible or constitutive secretion of IL-12.[69]

One strategy to improve CAR T-cell trafficking into solid tumors involves the expression of chemokine receptors that correspond to chemokines found in the tumor microenvironment.[70] For example, mesothelin-directed CAR T cells, designed to also express chemokine receptors CCR2b and CCR4, were better able to infiltrate into MCP-1–producing tumors in a non–small cell lung carcinoma model.[71] Direct administration of CAR T cells at the site of the tumor[72] and arming CAR T cells with enzymes to disrupt tumor stroma[73] are other strategies developing to improve CAR T-cell infiltration.

Toxicity Management

One avenue for limiting the systemic and neurological toxicities that often follow CAR T-cell therapy is the introduction of inducible suicide or inhibitory genes, which establish the ability of CAR T-cell activity to be idled. In addition, pharmaceuticals have been used to target and deplete CAR T cells to decrease adverse effects of treatment.[74] Interleukin 6 receptor blockade by humanized monoclonal antibody tocilizumab received FDA approval for treatment of CRS when the first CAR T-cell therapy was approved in 2017.[75]

CONCLUSIONS

Chimeric antigen receptor T-cell therapy has seen success in hematological malignancies with 6 FDA-approved treatments so far and advancements continuously being made in solid tumors. Limited tumor antigens, poor trafficking, immunosuppressive microenvironment, and exhaustion all contribute to the delay success in CAR T-cell therapy in solid tumors. All 4 engineered components of the CAR: the antigen-binding domain, hinge region, transmembrane domain, and intracellular signaling domain(s), contribute to their persistence and antitumor efficacy. However, new strategies involving tandem CARs, T cells redirected for universal cytokine-mediated killing/armored CARs, and OVs, among others, continue to evolve and show promise in future therapies.

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