

Special Issue: Cancer and the Organism

## Feature Review

## CARs: Synthetic Immunoreceptors for Cancer Therapy and Beyond

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Chimeric antigen receptors (CARs) are versatile synthetic receptors that provide T cells with engineered specificity. Clinical success in treating B-cell malignancies has demonstrated the therapeutic potential of CAR-T cells against cancer, and efforts are underway to expand the use of engineered T cells to the treatment of diverse medical conditions, including infections and autoimmune diseases. Here, we review current understanding of the molecular properties of CARs, how this knowledge informs the rational design and characterization of novel receptors, the successes and shortcomings of CAR-T cells in the clinic, and emerging solutions for the continued improvement of CAR-T cell therapy.

Adoptive transfer of T cells expressing chimeric antigen receptors (CARs) has shown remarkable clinical efficacy against advanced B-cell malignancies [1–5]. This clinical success has sparked urgent interest in the development of new CARs and the extension of CAR-T cell therapy to solid tumors as well as applications beyond cancer. As the field accumulates both research and clinical experience, the potential as well as limitations of this exciting new therapeutic paradigm are coming into clearer focus. In this review we begin with a discussion of current understanding of the biology behind CAR expression and signaling, which informs the rational design of new CAR molecules. We next survey the clinical outcomes of CAR-T cell therapy trials to date, and discuss challenges highlighted by these bedside experiences. Finally, we discuss emerging solutions that seek to improve upon current CAR designs and describe potential applications of CAR-T cell therapy beyond cancer.

## The Biology of CAR Expression and Mechanism of Signaling

CARs are fusion proteins with well-defined functional domains, including an extracellular antigen-binding domain, extracellular spacer, transmembrane domain, and intracellular signaling domain responsible for T-cell activation (Figure 1A) [6]. **Second- and third-generation CARs** (see Glossary) incorporate one or two additional intracellular costimulatory signaling domains, respectively, to enhance T-cell activation and proliferation (Figure 1B) [6]. A major strength of the CAR platform is its modularity, with interchangeable and structurally distinct options being available for each functional domain (Box 1). This modularity has enabled the construction of a variety of functional receptor molecules with diverse antigen specificities and structural properties. Although a full mechanistic understanding of CAR signaling and its effects on T-cell biology remains elusive, recent studies on T-cell receptor (TCR) signaling as well as detailed observations of CAR-T cell behavior *in vitro* have yielded valuable insights into the mechanism of CAR function and provide guidance for the design and construction of new CARs. Unless otherwise noted, all results discussed in this article refer to human **conventional T cells** (as opposed to **regulatory T cells**, Tregs), and all preclinical *in vivo* results refer to

## Trends

CAR-T-cell therapy has shown remarkable clinical efficacy against B-cell leukemias, but there is significant room for improvement in treatments for non-hematologic malignancies.

CAR design has relied heavily on historical experience and trial-and-error. Accumulating knowledge of T-cell biology, CAR signaling, and rational protein engineering is facilitating increasingly sophisticated CAR designs.

Novel *in vitro* and *in vivo* characterization methods for CAR function enable detection of subtle differences across CAR designs and may better predict CAR performance in the clinic.

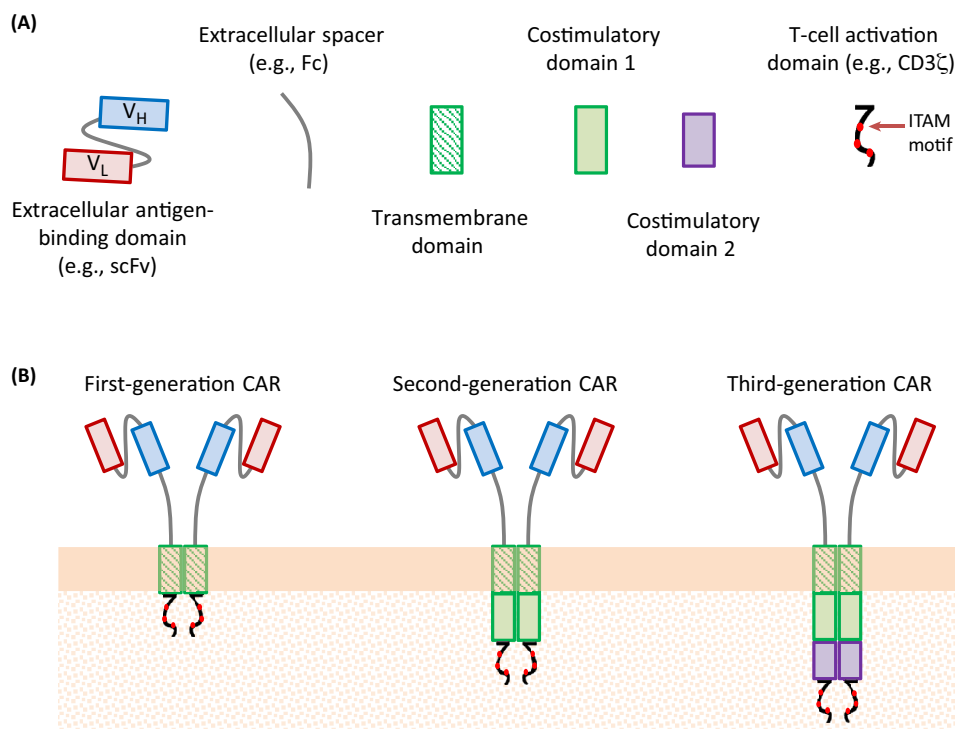
Various strategies have been employed to generate 'armored' T cells against immunosuppression and exhaustion, and to improve T-cell persistence.

Promising preclinical data support the extension of CAR-T cell therapy to the treatment of infections and autoimmune diseases.

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**Figure 1.** Chimeric Antigen Receptor (CAR) Structure and Designs. (A) CARs are modularly constructed fusion receptors comprising the following protein domains (from N terminus to C terminus): extracellular antigen-binding domain, extracellular spacer, transmembrane domain, costimulatory domain(s), and T-cell activation domain. (B) First-generation CARs contain a single intracellular signaling domain, most commonly CD3 $\zeta$ , that is capable of triggering T-cell activation. Second- and third-generation CARs incorporate one or two costimulatory domains, respectively, and enhance productive T-cell stimulation compared to first-generation CARs. Abbreviations: Fc, crystallizable fragment of an antibody; ITAM, immunoreceptor tyrosine-based activation motif; scFv, single-chain variable fragment; V<sub>H</sub>, heavy-chain variable fragment; V<sub>L</sub>, light-chain variable fragment.

findings obtained from the adoptive transfer of human T cells into immunocompromised mouse models.

#### Effect of CAR Expression on T-cell Biology.

CAR-encoding transgenes are most commonly introduced into CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells via viral transduction, resulting in strong constitutive CAR expression [2,7–9]. The gross over-expression of potent signaling domains that constitute the CAR, such as **CD3 $\zeta$**  and **CD28** or **4-1BB**, suggests that CARs have the potential to influence T-cell biology even in the absence of antigen stimulation. Indeed, cases of dramatic **tonic signaling** have been reported for multiple CAR constructs, with higher basal CAR expression levels correlating with increased tonic signaling and CAR- **T cell exhaustion** in the absence of antigen exposure (irresponsive cytotoxic T cells) [10–12]. It is worth noting that the specific effects of CAR expression on T-cell biology appear to correlate more strongly with the type of CAR expressed (e.g., CARs containing CD28 vs 4-1BB) than with the genetic background of the T cells, as illustrated by transcriptional profiling of CD28 and 4-1BB CAR-T cells generated from multiple donors [10]. Furthermore, the number of costimulatory domains incorporated into CAR molecules has been shown to affect the basal phosphorylation levels of signaling proteins important in human T-cell activation (LAT, ZAP-70, SYK, ERK, and LCK) [13], suggesting that the specific composition of CAR molecules can profoundly influence T-cell biology independently of antigen stimulation. A recent study compared the function of T cells expressing a CD19 CAR that was either randomly

#### Glossary

**4-1BB (CD137):** a member of the tumor necrosis factor (TNF) receptor family that is typically upregulated in activated T cells and provides costimulatory signals upon binding to 4-1BB ligand (4-1BBL); its cytoplasmic domain can be incorporated into CAR molecules.

**Affinity:** the strength of a given non-covalent binding interaction between two molecules, such as the binding strength of an antibody binding site to the epitope on its cognate antigen.

**Allogeneic:** adjective describing cells or tissues from a genetically dissimilar individual of the same species.

**Anaphylaxis:** an acute and severe allergic reaction that can be life-threatening in the absence of immediate medical treatment.

**Anti-Dsg3 antibody:** antibody targeting desmoglein 3 (Dsg3), a protein that is essential to cell–cell adhesion in the epidermis.

**Antigen escape:** escape of tumor cells from immune surveillance because of the loss of surface presentation of the targeted antigen on tumor cells.

**Avidity:** the overall, accumulated binding strength of multiple non-covalent binding interactions between two molecules or macromolecules; influenced by binding affinity, the number of binding interactions between the two molecules, and additional factors such as the proximity of the separate binding sites.

**B-cell aplasia:** depletion of B cells.

**Cas9:** a family of prokaryotic nucleases that, when complexed with the appropriate guide RNA sequences, can be directed to specific DNA sequences and generate double-stranded breaks in the DNA, leading to DNA repair processes that may or may not introduce insertions, deletions, and/or mutations in the targeted DNA sequence.

**CD28:** a surface receptor that naturally binds to CD80 (B7.1) and CD86 (B7.2) to provide costimulatory signals required for proper T-cell activation. Its cytoplasmic domain can be incorporated into CAR molecules.

**CD3 $\zeta$  (CD247):** a subunit of the T-cell receptor (TCR) complex that is phosphorylated to initiate T-cell activation signaling networks.

## Box 1. CAR Parts

## Antigen-Binding Moiety

The antigen-binding domain in a CAR can consist of any target-binding protein provided that the molecule remains functional when fused to an N-terminal signal peptide and C-terminal components that constitute the remainder of the receptor. Antibody-derived single chain variable fragments (scFvs) are the most commonly used antigen-binding domains, but CARs have also been constructed with other antibody-derived binding components such as **nanobodies** [151] or natural binding partners of the target antigen [65].

## Extracellular Spacer

Commonly used extracellular spacers are taken from the CD4, CD8, and CD28 extracellular domains as well as the IgG **Fc region**. Amino acid substitutions are often made in the Fc domain to prevent unwanted interactions with Fc  $\gamma$  receptors (FcR $\gamma$ ) expressed by cells such as monocytes and natural killer cells [28,152–154].

## Transmembrane Domain

CAR transmembrane domains typically consist of the membrane-spanning domain of CD4, CD8, CD28, or CD3 $\zeta$ . Transmembrane domain choice is dictated by whether a molecule remains functional when fused to particular C-terminal signaling domains, and the decision is often based on historical experience. Investigations into CAR signaling mechanisms may shed light on whether the CAR transmembrane domain functions merely as a structural anchor or plays additional functional roles.

## Costimulatory Domain

Costimulation augments T-cell activation, leading to increased cytokine production, proliferation, differentiation, and persistence. Costimulatory domains in CARs borrow from a variety of native receptors that shape T-cell activation, and CD28 and 4-1BB intracellular domains are the most common [6]. The relative contributions of CD28 and 4-1BB to CAR-T cell function have been reviewed extensively elsewhere [32,155]. Efforts to combine the strengths of multiple costimulatory domains in third-generation CARs have yielded varying results thus far [32,156–162]. The ability to quantitatively predict the effects of costimulatory signal combinations will likely require a more in-depth mechanistic understanding of CAR signaling than is currently available.

## Activation Domain

CD3 $\zeta$ , CD3 $\epsilon$ , and FcR $\gamma$  intracellular domains were regularly used as the activation domain in first-generation CARs, but CD3 $\zeta$  has emerged as the activation domain of choice in recent years [6]. It remains unclear how the use of different activation domains may alter CAR behavior, but the CD3 $\zeta$  activation domain in second-generation CARs has been sufficient to mediate clinical efficacy in multiple clinical trials [1–5].

integrated via retroviral transduction or site-specifically integrated into the **T-cell receptor  $\alpha$  constant (TRAC) locus** or the  $\beta_2$ -microglobulin locus, with or without additional promoter/enhancer elements [12]. The study found that the dynamic pattern of CAR expression regulated by the native TRAC locus contributed to the most sustained antitumor efficacy *in vivo* [12], demonstrating the need to stringently control basal and dynamic CAR expression levels to achieve optimal therapeutic efficacy. Further investigation is necessary to determine whether CAR expression from the TRAC locus will serve as a superior strategy across multiple CAR designs and tumor targets.

## CAR Signal Initiation and Transduction

Few studies have directly assessed the mechanism by which CARs convert extracellular binding events into intracellular signaling. Nevertheless, research on immunoreceptor triggering and the specific signaling domains utilized in CARs provide a framework for inferring the biophysical and biochemical events in CAR signaling.

As discussed in Box 1, the most common signaling domains in CARs are derived from the cytoplasmic segments of CD3 $\zeta$ , CD28, and 4-1BB. Both CD3 $\zeta$  and CD28 contain stretches of

**CD45:** a receptor-type protein phosphatase with a long rigid extracellular domain; has been shown to play a role in T-cell activation by calibrating the phosphorylation state of kinases and phosphoproteins associated with TCR signaling.

**CD148:** a receptor-type protein phosphatase with a long rigid extracellular domain; has been shown to negatively regulate TCR signaling.

**Central memory T ( $T_{CM}$ ) cells:** a subset of memory T cells characterized by the expression of CD45RO, CD62L, and CCR7;  $T_{CM}$  cells can achieve long-term persistence and readily proliferate and differentiate into effector T cells in response to antigen stimulation.

**Checkpoint blockade:** a family of therapeutic strategies that aim to promote immune responses against diseases (primarily cancers) by blocking checkpoints, such as PD-1 and CTLA-4 signaling, that would otherwise inhibit immune-cell function.

**Conventional T cells:** T cells that are not of the regulatory phenotype; may be naive, stem-cell memory, memory, or effector T cells.

**CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9:** a prokaryotic system that enables the introduction of insertions, deletions, and/or mutations at specific genomic locations.

**Dectin-1:** a pattern-recognition receptor that specifically binds to  $\beta$ -glucans, which are glucose polymers expressed on fungal cell walls.

**Epstein-Barr virus (EBV):** a common virus of the herpes family; most humans have coexisting latent infection and anti-EBV adaptive immunity.

**Fc (fragment crystallizable) region:** the base region of an antibody that interacts with Fc receptors and the complement system.

**First-generation CARs:** CARs that do not contain any costimulatory domains.

**Graft-versus-host disease (GVHD):** a medical condition in which transplanted tissue from a genetically dissimilar person attacks host cells, leading to tissue damage.

**Immunological synapse:** the interface formed between a

positively charged, basic amino-acid residues in their cytoplasmic tails, which are known to closely interact with the negatively charged inner leaflet of the plasma membrane when the receptors are in their resting, 'off' state [14–16]. Similarly, 4-1BB contains a basic stretch of amino acids in its cytoplasmic tail, even though its membrane-interacting capabilities have not been investigated in detail. For both CD3 $\zeta$ , and CD28, receptor triggering leads to dissociation of the intracellular chains from the plasma membrane, suggesting membrane dissociation as a crucial step in signal transduction [15,16]. The exact functional role of receptor–membrane contact remains unclear, but the sequestration of receptor chains inside lipid bilayers could serve as a means to regulate the availability of binding sites that are crucial for downstream signaling (Figure 2A) [16,17]. Specifically, the masking and unmasking of **immunoreceptor tyrosine-based activation motif (ITAM)** phosphorylation sites on CD3 $\zeta$  chains, or the various signaling-molecule binding sites on CD28, could play a crucial role in signal transduction [14,16,17]. Furthermore, the configuration of the receptor chains could influence the clustering of receptors and their relative location to other membrane-tethered signaling molecules, thereby altering the initiation and/or maintenance of signal transduction [15,18].

If the association and dissociation of the intracellular domains from the plasma membrane played a role in CAR signaling, how might extracellular ligand-binding trigger the intracellular dissociation event? It has been shown that T cell/target cell conjugations can generate mechanical forces through spontaneous membrane motions and receptor engagement [19–21]. This observation supports the **receptor deformation model of TCR triggering** which posits that the tugging and pulling between a conjugated T cell/target cell pair would ultimately deform a ligated TCR to a signaling-competent conformation [22]. Target-cell ligation may similarly provide a mechanical force to dislodge the CAR intracellular domain from the T-cell plasma membrane and trigger receptor signaling (Figure 2B). Such a model, which relies on general mechanical coupling rather than on specific binding-induced conformational changes, is consistent with the fact that functional CARs can be constructed from a variety of domains with different physical shapes and biochemical properties. If true, this model would suggest that the extracellular spacer and transmembrane domain of a functional CAR must fall within a given range of conformational flexibility, and the mechanical properties of these linker domains would likely impact the sensitivity of a CAR.

The receptor deformation model is only one of several leading hypotheses for TCR signaling. Another model, termed the **kinetic segregation model**, posits that TCR signaling is triggered by the exclusion of bulky phosphatases such as **CD45** and **CD148** from the compact **immunological synapse** formed between a conjugated pair of T-cell and target-cell membranes [23,24]. It has been shown that cell contacts formed by CAR/cognate ligand conjugation exclude CD45 [25,26]. Furthermore, head-to-head comparisons of CARs with different extracellular spacer lengths revealed that a short-spacer CAR is most effective for targeting membrane-distal epitopes in CD19 and ROR1 [27,28], consistent with the hypothesis that a compact immunological synapse is essential for CAR signaling by excluding bulky phosphatases (Figure 2C). However, there is a limit to how close T-cell and target-cell membranes can favorably come together because of the thick glycocalyx surrounding each cell and the energy cost of membrane bending [29]. As such, shorter spacer lengths are not always optimal, particularly when targeting membrane-proximal epitopes [7,28,30], and both the location of target epitopes and the receptor's extracellular spacer length are important in CAR design. In addition to adjusting cell–cell distance in an immunological synapse, spacer length also affects the mechanical properties of the CAR molecule itself. Therefore, the ability of the CAR to access target epitopes, achieve phosphatase segregation, and transduce mechanical forces should be considered in concert when selecting target epitopes and designing corresponding CAR structures.

lymphocyte (e.g., a T cell) and an antigen-presenting cell or target cell.

**Immunoreceptor tyrosine-based activation motif (ITAM):** tyrosine-containing motifs that are found in stimulatory receptors in immune cells and are phosphorylated to initiate signal transduction.

**Kinetic segregation model of TCR triggering:** a proposed model for TCR signaling which posits that TCR signaling is triggered by the exclusion of bulky phosphatases such as CD45 and CD148 from the compact immunological synapse formed between a T cell and its target cell or an antigen-presenting cell.

**Lymphodepletion conditioning:** chemotherapeutic treatment administered to cancer patients to eliminate existing lymphocytes before the adoptive transfer of therapeutic cells.

**Naïve T (T<sub>N</sub>) cells:** a subset of T cells that have never encountered antigen stimulation and are characterized by the expression of CD45RA, CD62L, and CCR7.

**Nanobodies:** antibodies that contain a single, monomeric variable domain; initially derived from camelid and fish heavy-chain antibodies.

**Pemphigus vulgaris (PV):** an autoimmune disorder of the skin caused by antibodies that target desmoglein (Dsg) proteins present in epithelial cells, ultimately leading to the loss of cell–cell adhesion in the epidermis and to blisters in the skin.

**Peripheral blood mononuclear cells (PBMCs):** all blood cells found within circulating blood that possesses a round nucleus, including lymphocytes and monocytes.

**Receptor deformation model of TCR triggering:** a proposed model for TCR signaling which posits that the tugging and pulling between a conjugated T cell/target cell pair deforms the ligated TCR to a conformation that can initiate signaling.

**Regulatory T cells (Tregs):** a CD4<sup>+</sup>/CD25<sup>hi</sup>/FOXP3<sup>+</sup> T-cell subtype whose primary function is to maintain tolerance toward self-antigens and prevent autoimmunity by suppressing the proliferation and activity of immune cells, particularly effector T cells.

**Second-generation CARs:** CARs that contain one costimulatory domain.



CAR stimulation yields many of the same signal-transduction events that occur upon TCR stimulation, such as the phosphorylation of CD3 $\zeta$  ITAMs, LCK, ZAP70, and LAT [13]. CARs also appear to mimic TCRs at the immunological synapse, forming initial microclusters that exclude CD45 and organizing, together with native receptors, into the characteristic bull's-eye structure of the **supramolecular activation cluster** (SMAC) (Figure 2D) [25,26]. The importance of clustering in CAR signaling was further highlighted by the observation of a tonically signaling CAR that led to antigen-independent CAR-T cell exhaustion [10]. The propensity of the CAR's extracellular **single-chain variable fragment** (scFv) domain to spontaneously aggregate was proposed as the cause of antigen-independent receptor clustering and signaling [10]. These results suggest that care should be taken to avoid domains with innate oligomerization tendencies when designing CARs, so as to prevent spontaneous receptor clustering that leads to premature T-cell exhaustion.

The intensity of the costimulatory signal required for proper T-cell activation has been observed to vary inversely with the extent of TCR stimulation [31]. However, CARs provide hard-coded stoichiometry between CD3 $\zeta$  and costimulatory domains. This fixed dosing of CD3 $\zeta$  and costimulatory domains potentially underlies the observation that a nonfunctional subpopulation can emerge upon antigen stimulation of second-generation CD19 CARs in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells [9]. While native costimulatory receptors can still be involved in CAR-T cell activation, exactly how these native receptors interface with CAR-mediated signaling remains to be explored. Nevertheless, overexpression of costimulatory receptor ligands in conjunction with CARs was shown to increase the ability of human CD19 CAR-T cells to reject NALM6 leukemia xenografts in mice [32], suggesting that CAR signaling might benefit from additional costimulation in productively activated T cells.

### CARs, TCRs, and BCRs

Both CARs and TCRs activate T cells and likely share many of the biophysical and biochemical signaling events discussed above. However, CARs differ from TCRs in several prominent ways: CARs harbor fewer subunits and fewer ITAMs, do not need coreceptors to support ligand binding, have several-order higher affinities for their targets, typically interface with much more abundant cognate antigens, and can be activated by a molecularly diverse set of ligands (Figure 3) [33]. In these aspects, CARs are actually more similar to B-cell receptors (BCRs) (Figure 3). The BCR extracellular domain consists of an antibody. Therefore, like CARs, BCRs exhibit high ligand affinity and recognize targets in a major histocompatibility complex (MHC)-unrestricted manner.

BCRs also share many of the signaling characteristics of TCRs, such as the phosphorylation of ITAM motifs and the formation of receptor microclusters that mature into an immunological synapse [34,35]. Notably, BCRs undergo tonic signaling, which is an emerging challenge in CAR engineering efforts [10,11]. These shared themes in natural immunoreceptor signaling may underlie the success of CARs in synthetically combining BCR and TCR properties to activate T cells and trigger productive effector functions.

### Predicting CAR Efficacy in the Wet Lab

CAR-T cells are typically assessed for their therapeutic potential first *in vitro*, then in mouse models, and finally in Phase I clinical trials, with financial and time commitments rising exponentially at each transition. As such, there is a need for *in vitro* assays that can reliably identify promising CARs at early stages of the bench-to-bedside pipeline. *In vitro* quantifications of cytokine production, T-cell proliferation, and target-cell lysis are the standard assays by which CARs are evaluated for basic function. However, these assays often fail to predict relative *in vivo* performance when comparing multiple CARs that demonstrate basic *in vitro* function [36].

#### Single-chain variable fragment

**(scFv):** a fusion protein comprising the variable regions of the heavy and light chains of an antibody connected via a linker peptide sequence.

#### Stem-cell memory T (T<sub>SCM</sub>) cells:

a subset of T cells that exhibit properties associated with both naïve and memory T cells; T<sub>SCM</sub> cells have been suggested to have superior self-renewal capability compared to memory T cells, and to possess the ability to differentiate into both memory and effector T cells.

#### Stress tests: *in vitro* or *in vivo*

assays designed to test the limits of T-cell effector functions, typically by repeatedly challenging T cells with antigen stimulation or by reducing the T-cell dosage relative to tumor burden.

#### Supramolecular activation cluster

**(SMAC):** an assembly of receptors arranged in concentric circles at the interface between a lymphocyte (e.g., a T cell) and an antigen-presenting cell or target cell.

**T-cell exhaustion:** a state of dysfunction in which T cells exhibit reduced functional activities, including proliferation, cytokine production, and cytotoxicity.

#### Type 1 T helper (Th1) response: a

response characterized by the production of interferon- $\gamma$ , interleukin (IL)-2, and TNF- $\alpha$ , among other cytokines, that is particularly effective against intracellular pathogens.

#### Type 2 T helper (Th2) response: a

response characterized by the production of IL-4, IL-5, IL-6, IL-10, among other cytokines, that is particularly effective against helminthes and other extracellular pathogens.

**Third-generation CARs:** CARs that contain two costimulatory domains.

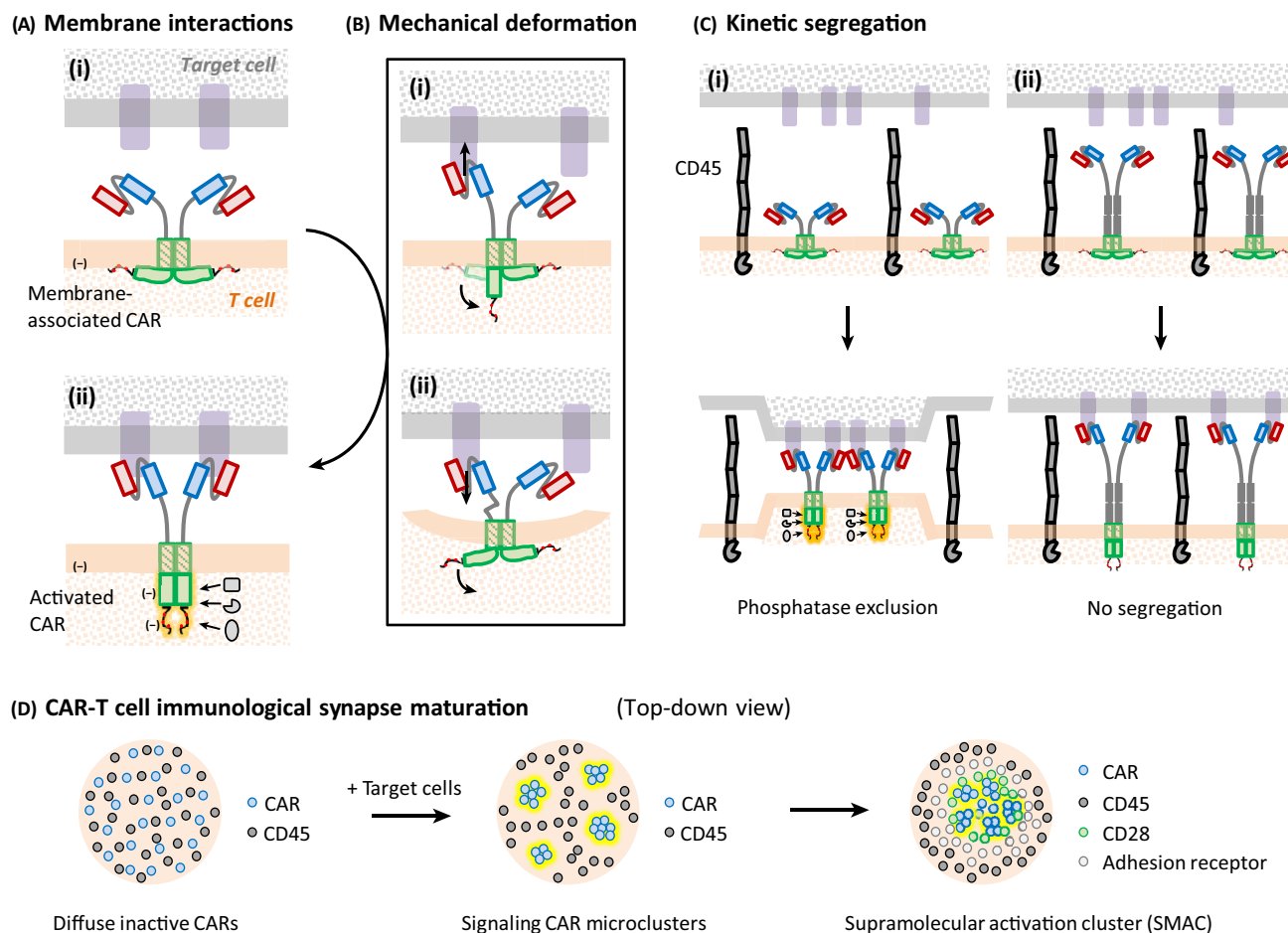
**Tonic signaling:** receptor signaling in the absence of ligand engagement.

#### T-cell receptor $\alpha$ constant (TRAC)

**locus:** genetic location within the T-cell receptor  $\alpha$  locus that encodes the constant region of the TCR $\alpha$  subunit of the TCR complex.

#### Tumor-associated antigens:

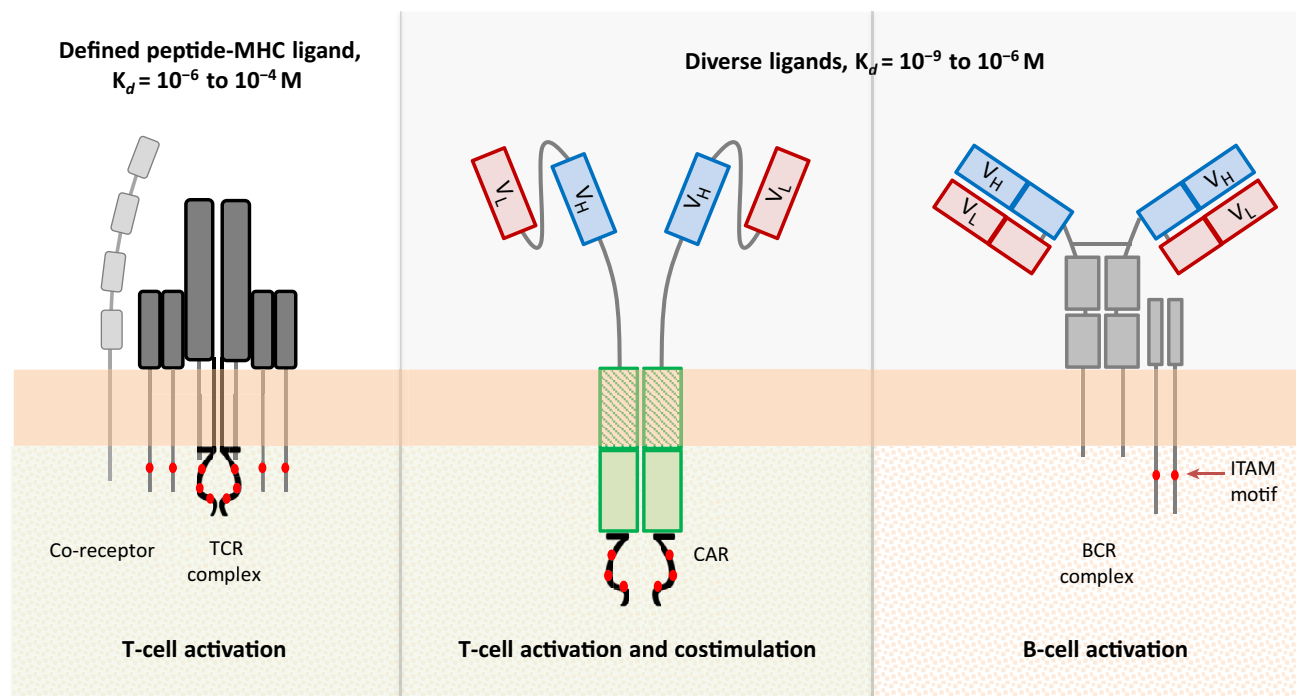
antigens that are expressed by tumor cells but can also be present on normal tissues.



Trends in Molecular Medicine

**Figure 2.** An Integrated Mechanistic Model of Chimeric Antigen Receptor (CAR) Signaling Initiation. Research on T-cell receptor (TCR) triggering and the specific signaling domains utilized in CARs suggests that the following potential mechanisms work in concert to initiate CAR signaling. (A) Ligand binding could generate mechanical forces that lead to the dissociation of CAR intracellular domains from the plasma membrane, thereby unmasking crucial binding sites for downstream signaling molecules. (i) At rest, CAR intracellular domains (e.g., CD28 and CD3 $\zeta$ ) may interact with the plasma membrane, as they do in their native receptor contexts, through basic residue motifs that bind to the negatively charged inner leaflet of the plasma membrane [14–16]. (ii) Upon antigen binding, CAR intracellular domains dissociate from the plasma membrane and adopt a signaling-competent conformation that allows interactions with downstream signaling molecules, including kinases such as ZAP-70 and LCK [15,16]. Phosphorylation of the intracellular domains is thought to lock the domains in the membrane-free state [15]. (B) Extending the receptor deformation model of TCR triggering to CARs suggests that the changes in CAR conformation from (A) to (Aii) may arise from mechanical pulling or pushing between the T cell and the target cell. (i) A pulling force can be transmitted via tension in the CAR extracellular and transmembrane domains to dislodge the intracellular domains from the plasma membrane. (ii) A pushing force may alter the local membrane curvature, thereby reducing the stability of the membrane-associated state of the CAR intracellular domains. (C) In the kinetic segregation model of TCR triggering, bulky phosphatases must be physically segregated from TCRs for T-cell activation domains to transduce signal. Thus, in addition to having accessible (i.e., membrane-free) intracellular signaling domains, CARs may also need to be segregated from phosphatases to initiate signal transduction. (i) Segregation of phosphatases and CARs can occur when CAR/ligand interactions force the T cell and target cell into close apposition and exclude bulky phosphatases from the immunological synapse (IS). (ii) By this logic, CARs with excessively long extracellular spacers that allow phosphatases to congregate with CARs at the IS would not be able to robustly activate T-cell signaling. (D) CARs in resting T cells are localized diffusely together with other surface receptors such as CD45. As the events in (C) take place in response to target-cell engagement, ligated CARs can coalesce into microclusters which have been confirmed to exclude CD45 and transduce T-cell activation signals [26]. With time, microclusters at the CAR-containing immunological synapse are hypothesized to coalesce and organize with other native surface receptors into the supramolecular activation cluster (SMAC) that is commonly observed at TCR synapses.

To increase the predictive power of *in vitro* assays, **stress tests** have become an increasingly common technique to mimic the repetitive stimulations and low effector-to-target ratios characteristic of *in vivo* environments. For example, it has been shown that repeated challenges with fresh tumor cells can reveal differences across CAR-T cell lines in terms of their long-term



Trends in Molecular Medicine

**Figure 3.** Comparison of Stimulatory Immunoreceptors. Standard CARs combine the antibody-like target-binding properties of B-cell receptors (BCRs) with the T-cell activation abilities of T-cell receptors (TCRs). T-cell costimulatory properties are further incorporated into CARs with the addition of costimulatory domains. Abbreviations: ITAM, immunoreceptor tyrosine-based activation motif;  $K_d$ , dissociation equilibrium constant (with typical range shown for each receptor type);  $V_H$ , heavy-chain variable fragment;  $V_L$ , light-chain variable fragment.

survival [36] and their propensities for PD-1-mediated exhaustion [37] in *ex vivo* cultures, thus providing a means to distinguish high-performing CARs from a pool of merely functional CARs. An *in vivo* version of this stress test calls for rechallenging a mouse with an additional tumor load once a previous tumor is cleared, and this method was used to demonstrate the superiority of 4-1BB over CD28 in the context of a mesothelin-specific CAR against mesothelioma xenografts [37]. In a similar vein, Sadelain and colleagues developed an *in vivo* stress test in which mice were treated with serially reduced CAR-T cell doses to determine the lowest dose required for a given CAR construct to achieve antitumor efficacy [32]. Using this test, the researchers compared six methods of providing CD28 and/or 4-1BB costimulation, and identified a rank order of varying therapeutic efficacies [32]. As these *in vitro* and *in vivo* approaches gain credence in unmasking subtle differences in CAR therapeutic efficacy, future work will be able to assess additional fine-tuned adjustments, such as variations in dosing schedule and mixing of various CAR-T cell products in treatment protocols.

Mouse models of CAR-T cell therapy typically employ immunocompromised mice to allow the adoptive transfer of human T cells. Such systems differ sharply from the immune environment in the eventual human therapeutic context, and they offer limited insight into how CAR-T cells may interface with the endogenous immune components of the patient. However, the alternative—evaluating CARs in murine T cells adoptively transferred into immunocompetent mice—comes with the drawback that optimized murine CAR designs do not necessarily translate well to human T-cell performance. As with many other therapeutic modalities, the efficacy of CAR-T cell therapy in mouse models has not always translated to clinical success (e.g., **first-generation CARs** performed well in mice but consistently underperformed in clinical trials [38–42]). Furthermore, the use of murine models prevents accurate evaluation of CAR

crossreactivity against healthy human tissues that might express the target antigen at low levels [43]. The emerging methods described above are improving our ability to detect subtle functional differences across various CAR-T cell treatments. Nevertheless, definitive validation of these methods will only come when future clinical trials are carried out to evaluate the resulting conclusions.

### Clinical Experiences and Challenges

The number of CAR-T cell therapy clinical trials has grown steadily since the turn of the century (Box 2), with 147 clinical trials recruiting as of March 2017 according to [www.clinicaltrials.gov](http://www.clinicaltrials.gov). Early clinical trials evaluated first-generation CARs targeting several solid-tumor antigens, including CD171 for neuroblastoma [39], folate receptor (FR) for ovarian cancer [40], and carbonic anhydrase IX (CAIX) for renal cell carcinoma [42]. These trials demonstrated the feasibility of generating patient-specific T-cell products, but they also highlighted important challenges. First, no antitumor efficacy was observed, and lack of CAR-T cell persistence after adoptive transfer was cited as a major factor [39,40]. Furthermore, although anti-CD171 and anti-FR CAR-T cells were well tolerated by patients, anti-CAIX CAR-T cells led to severe liver toxicities associated with on-target, off-tumor T-cell-mediated attacks on CAIX<sup>+</sup> bile-duct human epithelial cells [42]. Subsequent clinical trials incorporated changes in both antigen target and CAR components such as additional costimulatory domains to address the challenges observed in these early clinical experiences.

#### CD19 CAR-T Cell Therapy for B-cell Malignancies.

The most prominent CAR-T success story thus far has been the use of second-generation CARs targeting the pan-B-cell marker CD19 [1–5]. In particular, CD19 CAR-T cell therapy has achieved >85% complete response rate in the treatment of acute lymphocytic leukemia (ALL) across multiple trials [44–46]. The success of CD19 CAR-T cell therapy has been attributed to multiple factors. First, CD19 is highly expressed on the vast majority of malignant B cells, and off-tumor toxicity is primarily limited to **B-cell aplasia**, a condition that can be clinically managed with prophylactic infusions of  $\gamma$ -globulin [6]. Second, the incorporation of costimulatory domains such as CD28 and 4-1BB has significantly enhanced the potency of T-cell responses and survival upon adoptive transfer (Box 1) [47–51]. Third, liquid tumors such as malignant B cells present a uniquely accessible environment for CAR-T cells, without several

#### Box 2. Clinician's Corner

CARs are synthetic receptors that can be expressed in T cells to redirect T-cell responses toward specific targets of interest.

CARs that respond to the pan-B-cell marker CD19 have shown robust efficacy in clinical trials for chemotherapy-refractory B-cell malignancies, including lymphocytic leukemia and non-Hodgkin's lymphoma. In particular, multiple trials have achieved >85% complete response rate in patients with acute lymphocytic leukemia.

Prominent side effects of CAR-T cell therapy observed in the clinic thus far include cytokine release syndrome (CRS, characterized by sudden and dramatic spikes in serum cytokine levels); neurological toxicities (e.g., cerebral edema); and on-target, off-tumor toxicities (e.g., T-cell infiltration into healthy heart or lung tissues). Severe side effects have contributed to cases of patient fatality in CAR-T cell trials targeting CD19 and HER2.

CAR-T cell therapy against non-hematologic malignancies has yielded limited success thus far. Potential obstacles include inefficient T-cell localization to the tumor site, physical barriers to tumor infiltration by T cells, and potent immunosuppressive factors that render T cells dysfunctional in the tumor microenvironment. Active preclinical research and clinical trials are attempting to overcome obstacles in the application of CAR-T cells to other cancer types by assessing different target antigens, treatment protocols, and methods to 'armor' the CAR-T cell.

In addition to cancer therapy, preclinical work has demonstrated the potential efficacy of CAR-engineered T cells in diverse therapeutic applications such as the control of viral infections and the treatment of autoimmune disorders.



immunosuppressive factors such as hypoxia and high local transforming growth factor (TGF)- $\beta$  activity that are typically associated with solid tumor microenvironments [52,53]. However, despite these important advantages, clinical experience with CD19 CAR-T cell therapy has also revealed ongoing challenges.

One important obstacle is the frequent occurrence of cytokine release syndrome (CRS, also known as ‘cytokine storm’)—the sudden and dramatic increase in serum levels of various cytokines, including interleukin (IL)-1, IL-2, IL-6, IL-8, IL-10, tumor necrosis factor (TNF)- $\alpha$ , and interferon (IFN)- $\gamma$  [44,46]. Cytokine production is an important T-cell effector function that appears to correlate with therapeutic efficacy [54,55]. However, severe CRS has also been implicated in the death of multiple patients in CAR-T cell therapy trials [43,56]. Although the administration of tocilizumab (an anti-IL-6 receptor  $\alpha$  antibody), etanercept (a TNF- $\alpha$  inhibitor), or corticosteroids can often provide effective clinical intervention, these agents are not uniformly effective across patients and, in the case of corticosteroids, can be lymphotoxic and directly counter CAR-T cell therapy [57,58]. This in turn can lead to relapses in patients that had previously achieved complete remission [46]. Another challenge is that severe neurotoxicities have been observed in multiple patients after CD19 CAR-T cell infusion, the cause of which remains unclear [45,46,59]. A Phase II clinical trial conducted by Juno Therapeutics was halted in July 2016 following the death of three patients from cerebral edema. The addition of the chemotherapy drug fludarabine to a **lymphodepletion conditioning** regimen that already included cyclophosphamide was cited as the source of toxicity, and the US FDA lifted the clinical hold after 3 days. However, the same trial was suspended again in November 2016 following the deaths of two more patients as a result of cerebral edema. The cause of this complication remains under investigation, and it is not yet certain whether this toxicity is unique to CD19 CAR-T cell therapy. Finally, a sizable fraction of ALL patients who achieved complete remission after CD19 CAR-T cell therapy eventually relapsed with the emergence of CD19<sup>+</sup> tumor cells [45,60]. It has been shown that **antigen escape**—escape of tumor cells from immune surveillance as a result of loss of the targeted antigen—can be achieved by malignant B cells via frameshift and missense mutations in CD19, as well as alternative splicing of the *CD19* mRNA, each leading to the loss of binding epitopes recognized by CD19 CARs [61].

Despite these challenges, the clinical efficacy of CD19 CAR-T cells has been remarkably robust, and multiple companies are aiming for FDA approval of their CD19 CAR-T cell products in 2017. As such, CD19 CAR-T cells will likely become the first commercially available adoptive T-cell therapy for cancer. Beyond CD19, additional B-cell malignancy antigens such as CD20, CD22, and BCMA are also under active clinical investigation, highlighting exciting advancements in the treatment of refractory B-cell leukemia and lymphoma.

#### CAR-T Cell Therapy for Non-Hematologic Malignancies

In contrast to the success observed in the treatment of B-cell malignancies, clinical evaluations of CAR-T cell therapy for solid tumors have yielded more-modest results. The majority of completed clinical trials on solid tumors have utilized first-generation CARs, and their limited therapeutic efficacies are perhaps unsurprising in retrospect [62]. However, trials with second- and third-generation CARs targeting the **tumor-associated antigens** mesothelin and HER2, respectively, have also been completed, each revealing distinct challenges [43,63].

Specifically, in a Phase I clinical trial, patients with malignant pleural mesothelioma or pancreatic cancer were treated with T cells that had been transfected with mRNA encoding a second-generation mesothelin CAR containing a 4-1BB costimulatory domain [63,64]. Transient, partial response was observed in one patient [63], but the same patient eventually developed **anaphylaxis** attributed to the generation of IgE antibodies specific to the CAR, which contained a murine scFv [64]. Human anti-mouse antibodies (HAMAs) were detected in a second

patient among the group of four patients reported, highlighting the risk of immunogenicity arising from CAR expression.

In a separate Phase I trial, a patient with metastatic colon cancer was treated with  $1 \times 10^{11}$  CD8<sup>+</sup> T cells expressing a third-generation HER2 CAR containing both CD28 and 4-1BB. The patient experienced severe respiratory distress within 15 minutes of T-cell infusion and died of cardiac arrest 5 days later [43]. Postmortem analysis confirmed massive T-cell infiltration into the lung, and it was speculated that T-cell activation by low levels of HER2 expression on lung epithelial cells triggered severe CRS and contributed to patient mortality [43].

Although the path to solid tumor treatment with CAR-T cells has been littered with obstacles, several ongoing clinical trials continue to evaluate multiple potential targets, including CEA for colorectal cancers, disialoganglioside GD2 for neuroblastoma and sarcoma, prostate-specific membrane antigen (PSMA) for prostate cancer and melanoma, epidermal growth factor receptor variant three (EGFRvIII) and IL-13 receptor  $\alpha 2$  (IL13R $\alpha 2$ ) for glioblastoma, as well as new trials targeting mesothelin and HER2 [62]. A recent report presented the case of a patient with recurring multifocal glioblastoma who attained complete tumor resolution after intraventricular infusion of IL13R $\alpha 2$  CAR-T cells [65]. Remarkably, the CAR-T cells were able to not only eliminate multiple intracranial tumors but also resolve spinal metastases. The patient eventually relapsed 228 days after the first CAR-T cell treatment, possibly due to decreased expression of IL13R $\alpha 2$  on tumor cells [65]. Nevertheless, such results provide evidence that new antigens coupled with novel delivery modalities may lead to improved therapeutic outcomes against solid tumors. Indeed, new methods for local delivery of T cells to the tumor site—such as direct injection into the pleural cavity of the lung for pleural malignancies, or implantation of a T-cell-laden biopolymer at the site of surgical tumor resection—are active areas of research [8,66].

### Current Pitfalls and Potential Remedies

Clinical experiences thus far highlight both the promise and the current limitations of CAR-T cell therapy. Several different strategies are under active investigation to address the various challenges that have been identified, with a general trend toward engineering multifunctional T cells that enable greater control after T-cell deployment into the patient.

#### Increasing Specificity

On-target, off-tumor toxicity has been a major challenge in CAR-T cell therapy, and its potential lethality lends the problem particular urgency. Given a general lack of tumor-exclusive surface antigens, the vast majority of CARs are directed against tumor-associated antigens that are also present on at least a subset of healthy cells. This shared antigen expression profile is responsible for off-tumor toxicities observed in CARs targeting CD19, HER2, and CEA, among others [67]. Several cancer germline antigens (CGAs, also known as cancer testes antigens) are being evaluated as potential targets, but it remains to be seen whether CGAs that are widely expressed on tumor cells but are absent from essential tissues can be identified [68]. An alternative approach has focused on targeting ‘neoantigens’—antigenic epitopes that arise from tumor-specific somatic mutations and are thus unique to diseased (cancerous) cells [68]. Most neoantigen-targeted therapies have focused on the isolation of neoantigen-reactive T cells and/or genes encoding the neoantigen-reactive TCR [69–71]. Nevertheless, mutations in surface-bound receptors (e.g., EGFRvIII) are readily compatible with CAR-mediated targeting [72], and it is also possible to engineer CARs to recognize MHC-presented antigens [73,74].

Given the dearth of ‘perfect’ antigens that are both unique to and uniformly expressed on tumor cells, researchers have actively explored alternative strategies to increase targeting specificity. One approach is to fine-tune the binding affinity and **avidity** of CARs to identify a ‘sweet spot’

where the CAR could bind tumor cells harboring high antigen expression while sparing healthy tissues with low antigen expression. By systematically examining mutations in EGFR- and HER2- binding scFv domains, researchers have generated CARs that could specifically target glioma and ovarian carcinoma xenografts, respectively, while avoiding normal cells expressing the same cognate antigen in mice [75,76]. The possibility of tuning the therapeutic window of CAR-T cells by adjusting their ligand-binding affinity and avidity has sustained clinical interest in shared antigens such as HER2 and CEA despite past observations of severe toxicity [43,77].

Since 2012 a series of studies have explored the idea of increasing targeting specificity by incorporating logical computation capabilities into CAR signaling [78–81]. By using Boolean logic, AND- and NOT-gate CAR-T cells require that the target cells present the correct ‘combination’ of antigens (e.g., ‘A AND B,’ or ‘A but NOT B’) instead of a single antigen before triggering T-cell activation (Box 3 and Figure 4). Such strategies have the potential to significantly increase tumor-targeting specificity, generally at the cost of increasing complexity in the transgenic constructs that must be introduced into the T-cell product. It remains to be seen whether these strategies would support robust therapeutic efficacy in the clinical setting.

### Preventing and Mitigating Toxicities

In addition to on-target, off-tumor toxicities, severe side effects such as CRS and neurotoxicity have prompted a search for methods to prevent and manage toxicities. Dose reduction has been considered as a general approach to reduce toxicity. For example, a high dose of T cells ( $1 \times 10^{11}$ ) was used in the original HER2 CAR-T cell trial that led to patient death [43]. A subsequent Phase I/II trial followed a dose-escalation protocol, starting with  $1 \times 10^4$  cells and ending at a maximum dose of  $1 \times 10^8$  CAR-T cells [82]. No dose-limiting toxicity was observed within the range tested, but efficacy was also limited to a transient partial response in 1 of 17 evaluable patients. T-cell persistence correlated with T-cell dosage, suggesting that T-cell dosing may need to operate within a limited window to support therapeutic efficacy while minimizing toxicity.

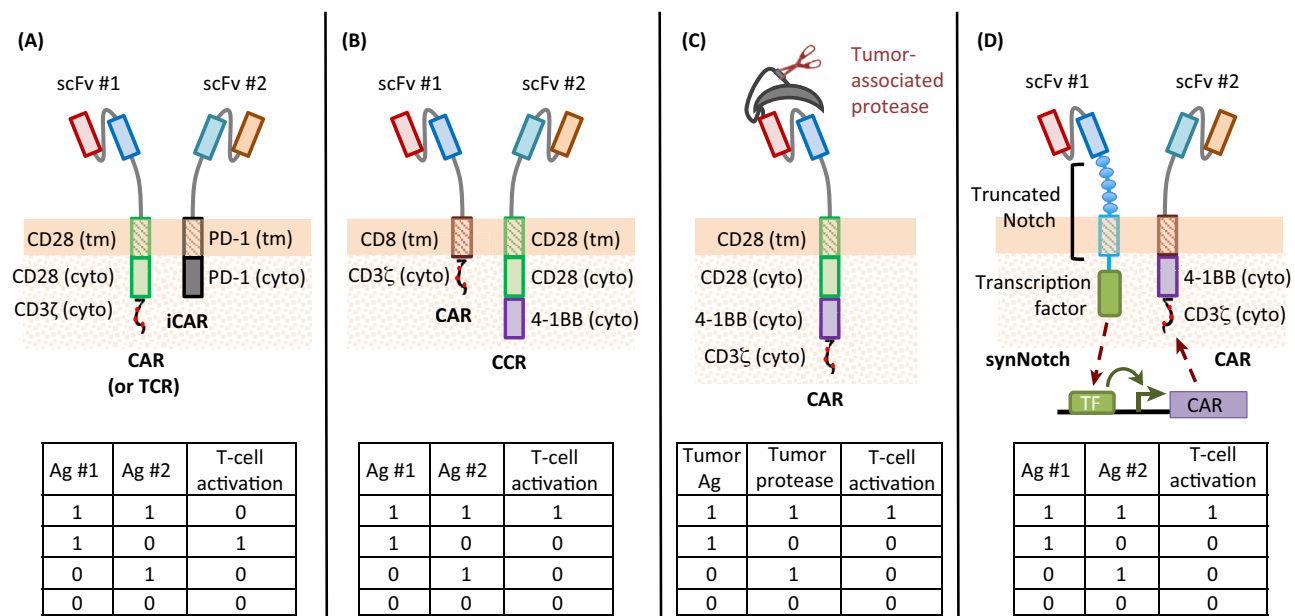
Another strategy for preventing or lessening toxicity is to reduce the longevity of CAR expression. Instead of virally integrating CAR-encoding genes into patient-derived T cells, transient

#### Box 3. Boolean Logic Computation to Increase CAR-T Cell Specificity

Tumor-targeting specificity of CAR-T cells can be increased by programming T cells to recognize combinations of biomarkers instead of single antigens. Sadelain and colleagues demonstrated the NOT-gate concept by pairing a conventional CAR or TCR with an inhibitory CAR (iCAR) that contains the signaling domains of CTLA-4 or PD-1. The conventional CAR or TCR can trigger T-cell activation upon binding to antigen A, but the presence of antigen B would trigger an inhibitory cascade via the iCAR that overrides any activation signal, yielding ‘A but NOT B’ signal integration (see Figure 4A in main text) [79].

Several AND-gate CAR designs have also been recently described. One design separates the CD3 $\zeta$  and CD28/4-1BB domains into two receptor chains targeting different antigens, such that full-intensity T-cell activation can only be achieved if both antigens are present to trigger both receptors (see Figure 4B in main text) [78]. In another approach, Wang and colleagues designed a ‘masked CAR’ whose antigen-binding domain is blocked by a masking peptide which can be removed by tumor-associated proteases in the tumor microenvironment (see Figure 4C in main text) [81]. As a further alternative, Lim and colleagues reported a novel ‘synNotch’ receptor [80] which releases a synthetic transcription factor (TF) upon ligand binding to upregulate CAR expression from a cognate synthetic promoter (see Figure 4D in main text). The net effect is an AND-gate response in which antigen 1 triggers the synNotch receptor and CAR expression, and a temporally delayed antigen 2 activates the T cell via CAR signaling. In principle, the synNotch platform can be adapted to a wide range of ligand inputs and genetic outputs, but the use of various murine and viral components in this system poses immunogenicity challenges.

It remains to be seen whether these highly engineered systems can achieve the robust T-cell effector function and fine-tuned temporal resolution required to yield clinical efficacy while preventing off-tumor toxicities. Answers to such questions are anticipated in the near future as the field of synthetic immunology moves beyond proof of concept into more physiologically relevant systems.



Trends in Molecular Medicine

**Figure 4.** Increasing Targeting Specificity of Chimeric Antigen Receptors (CARs) by Boolean Logic Calculations. (A) NOT-gate CAR developed by the Sadelain group paired a conventional CAR or T cell receptor (TCR) with an inhibitory CAR (iCAR) that contains either PD-1 or CTLA-4. Antigen binding to the iCAR triggers an inhibitory signal that overrides the activation signal from the conventional CAR or TCR [79]. (B–D) Three AND-gate CAR designs. (B) A chimeric costimulatory receptor (CCR) that is equivalent to a third-generation CAR lacking the CD3ζ chain was developed. This CCR is paired with a first-generation CAR, and both receptors must be triggered by their respective cognate antigens to achieve full T-cell activation [78]. (C) The Wang group engineered a ‘masked CAR’ whose antigen-binding domain is blocked by a masking peptide until the peptide is removed via cleavage by a tumor-associated protease [81]. (D) The Lim group developed a synNotch receptor that releases a synthetic transcription factor (TF) upon ligand binding (signal 1). The TF subsequently drives the expression of a CAR from a synthetic cognate promoter, and the CAR can then respond to its cognate antigen (signal 2) [80]. Abbreviations: Ag, antigen; cyto, cytoplasmic; tm, transmembrane; scFv, single-chain variable fragment; V<sub>H</sub>, heavy-chain variable fragment; V<sub>L</sub>, light-chain variable fragment.

transfection of CAR-encoding mRNA can enable temporary CAR expression [63,83]. A major trade-off associated with transient CAR expression is the need to infuse multiple doses of T cells into each patient; indeed, repeated infusion is accompanied by an increased risk of immune rejection of CAR-T cells, as illustrated by the previously discussed clinical trial targeting mesothelin [64].

Taking a different approach, Lim and colleagues proposed a system in which a second-generation CAR is split into two chains that can be reconstituted into a functional receptor by the dimerization-inducing molecule rapamycin or an analog [84]. Such a system enables temporal control over the availability of functional CAR molecules via the use of the trigger molecule, but the inability to spatially constrain the trigger molecule could limit its utility in preventing off-tumor toxicity [84].

In lieu of toxicity prevention, strategies to rapidly eliminate CAR-T cells if and when patients experience severe toxicity have also been proposed. Incorporation of herpes simplex virus thymidine kinase (HSV-TK) into engineered cells allows their specific removal by the administration of ganciclovir [85,86]. However, immunogenicity associated with HSV-TK can lead to rapid depletion of T cells expressing this transgene [87]. Another suicide strategy with clinical promise is the inducible caspase 9 (iCasp9) protein, which dimerizes into a functional, pro-apoptotic form upon the addition of AP1903, a chemical inducer of dimerization [88]. A third strategy is to express a truncated, non-ligand-binding, non-signaling copy of human EGFR

(huEGFRt) which allows selective depletion of huEGFRt<sup>+</sup> T cells with the anti-EGFR monoclonal antibody cetuximab [89]. Furthermore, the huEGFRt protein can simultaneously serve as a marker for engineered T cells in cell sorting as well as in immunochemistry [4,5], rendering it a multipurpose addition to therapeutic CAR-T cells.

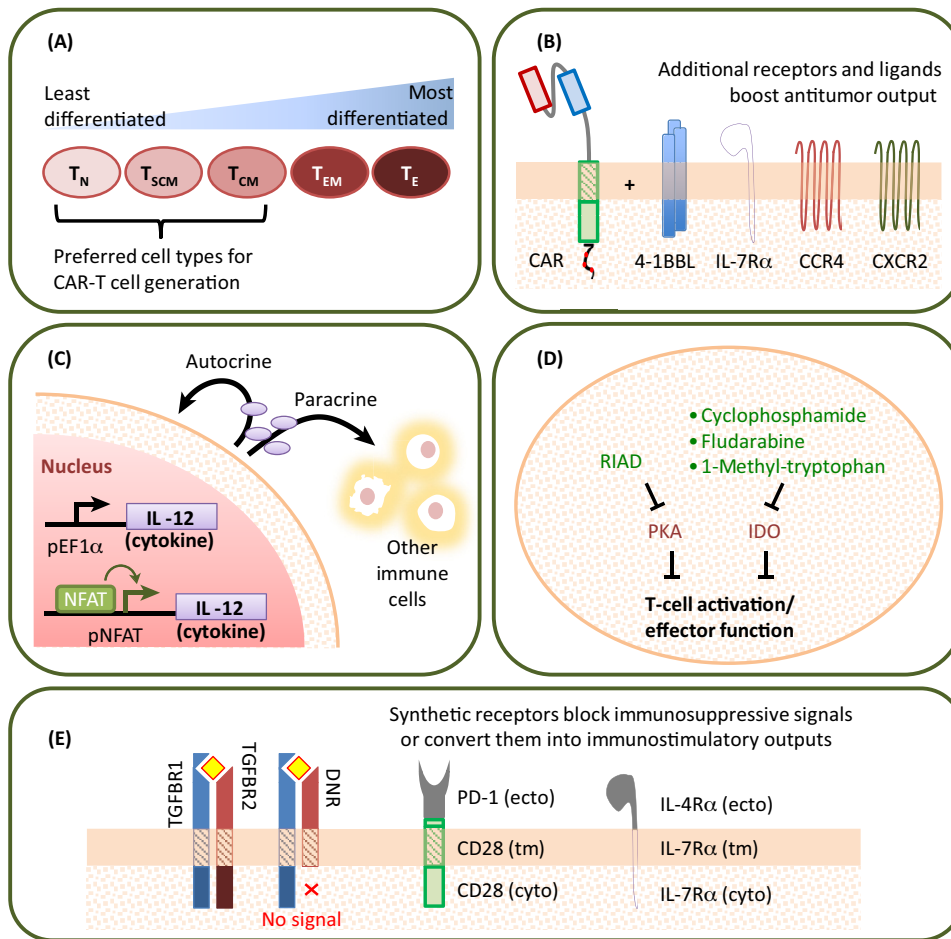
#### Increasing T-Cell Persistence and Effector Function

Although toxicity management has been a major focus in CAR-T cell development, an equally important challenge is generating T cells with sufficient antitumor activities to achieve therapeutic efficacy. Which starting T-cell population is optimal for the generation of therapeutic CAR-T cells remains an unresolved question [90]. Empirical evidence generated in non-CAR adoptive T-cell therapy models has variously supported the use of **naïve** [91], **central memory** [92], and **stem-cell memory T cells** [93]. Despite lack of definitive proof that any particular T-cell subtype is the optimal therapeutic candidate, an emerging consensus is that less-differentiated phenotypes are more likely to provide the proliferative potential required for long-term engraftment, which is strongly correlated with superior therapeutic efficacy (Figure 5A) [68]. Recent work by Riddell and colleagues in a CD19 CAR mouse model identified CD8<sup>+</sup> central memory and CD4<sup>+</sup> naïve T cell populations as being superior among naïve, central memory, and effector memory compartments in eradicating lymphoma xenografts in mice [94]. Moreover, the CD4<sup>+</sup> and CD8<sup>+</sup> T cells subsets presented synergistic antitumor efficacy when combined [94]. These results informed the design of an ongoing clinical trial that uses CD19 CAR-T cells derived from defined starting T-cell subsets (NCT01865617). The initial report of the trial described 30 patients who received bulk CD4<sup>+</sup> CAR-T cells mixed with CD8<sup>+</sup> CAR-T cells that were derived from either central-memory or bulk CD8<sup>+</sup> T cells [95]. No statistically significant inferences could be made when contrasting the two CD8<sup>+</sup> CAR-T cell groups, in part due to large patient-to-patient variability in cell doses and lymphodepletion regimens, as well as to the high overall remission rate regardless of the type of CD8<sup>+</sup> CAR-T cells used (27 out of 29 evaluable patients achieved bone-marrow remission) [95]. However, the trial is ongoing and may eventually provide sufficient statistical power to identify superior T-cell phenotypes in CAR-T cell therapy.

Beyond choosing an appropriate starting population, the addition of transgenic features may also enhance T-cell persistence and effector function. The incorporation of costimulatory molecules such as the 4-1BB ligand (4-1BBL) [32], cytokine receptors such as IL-7 receptor  $\alpha$  [96], and chemokine receptors such as CCR4 and CXCR2 [97,98] has been shown to enhance the persistence, reduce the exhaustion, and increase the antitumor efficacy of human CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 5B). Another way to generate 'armored' CAR-T cells with enhanced function involves the transgenic overexpression of cytokines such as IL-2, IL-12, and IL-15, which promote T-cell proliferation and effector functions (Figure 5C) [99]. For example, it has been reported that human CD4<sup>+</sup> and CD8<sup>+</sup> T cells programmed to inducibly express IL-12 can enhance tumor rejection by resisting Treg-mediated inhibition [100]. However, the pleiotropic effects of potent cytokines makes their transgenic overexpression a delicate balancing act between therapeutic immune activation and pathologic overstimulation. For example, the administration of T cells engineered to inducibly express IL-12 resulted in transient clinical responses in multiple patients with metastatic melanoma, but at the cost of severe liver toxicities [101].

The maintenance of T-cell activation and effector function is regulated by an intricate balance between stimulatory and inhibitory signals. Therefore, the negation of inhibitory signals can be as effective as the promotion of activating signals in optimizing T-cell function. For example, both genetic and chemical approaches have been used to introduce inhibitors into human CAR-T cells to counter the activity of tumor-associated immunosuppressive factors such as adenosine, prostaglandin E2, and indoleamine 2,3 dioxygenase (IDO) metabolic products





Trends in Molecular Medicine

**Figure 5.** Strategies to Enhance T-Cell Persistence and Effector Function. Several engineering approaches have been shown to increase T-cell proliferation, persistence, and anti-tumor effector functions such as cytotoxicity and cytokine production. These strategies include: (A) Using less differentiated cell types as starting material for chimeric antigen receptor (CAR)-T cell manufacturing. (B) ‘Armoring’ CAR-T cells with additional transgenic receptors or receptor ligands provide costimulation, enhance cytokine signaling, and/or promote migration [32,96–98]. (C) Equipping T cells with stimulatory cytokines expressed from constitutive or inducible promoters (pNFAT, pEF1α) [99,163]. (D) Blocking inhibitory signaling pathways through either the expression of transgenic peptides or the administration of pharmaceutical drugs [102,103]. (E) Generating chimeric receptors that either abolish endogenous signaling pathways or convert inhibitory ligand inputs into stimulatory signal outputs [104,105,107–109]. Abbreviations: cyto, cytoplasmic; CCR4, C-C motif chemokine receptor 4; CXCR2, C-X-C chemokine receptor 2; DNR, dominant negative TGF-β receptor; ecto, ectodomain; IDO, indoleamine 2,3-dioxygenase; IL-12, interleukin 12; IL-4Rα and IL-7Rα, interleukin receptors 4α and 7α; PD-1, programmed-death 1; PKA, protein kinase A; RIAD, regulatory subunit I anchoring disruptor; T<sub>CM</sub>, central memory T cell; T<sub>E</sub>, effector T cell; T<sub>EM</sub>, effector memory T cell; TGFBR, TGF-β receptor; tm, transmembrane; T<sub>N</sub>, naïve T cell; T<sub>SCM</sub>, stem cell memory T cell.

(Figure 5D) [102,103]. In addition, studies on the adoptive transfer of both murine and human tumor-targeting T cells have demonstrated that expression of a dominant negative TGF-β receptor (DNR)—a truncated TGF-β receptor 2 chain that lacks the cytoplasmic signaling domain—can enhance the eradication of established tumors in both immunocompetent and immunodeficient mice (Figure 5E) [104,105,164]. The combination of the DNR with a HER2 CAR is currently under clinical evaluation (NCT00889954). Furthermore, inhibitory signals can also be removed by genetic knockout of inhibitory receptors such as PD-1 [106], and this strategy is now being evaluated in the clinic (NCT02793856).

Going one step further, fusion receptors that actively convert inhibitory ligand inputs to stimulatory functional outputs have also been reported. Specifically, fusion proteins that combine the extracellular ligand-binding domain of an inhibitory receptor (e.g., PD-1 or IL-4 receptor) with the cytoplasmic signaling domain of an immunostimulatory receptor (e.g., CD28, IL-7, or IL-2/15 $\beta_c$  receptor) have been shown to effectively rewire T-cell responses to otherwise inhibitory input signals (Figure 5E) [107–109]. The ability to maintain T-cell persistence, sustain antitumor effector functions, and prevent premature T-cell exhaustion will play a crucial role in the development of robust therapeutic T cells for cancer immunotherapy.

### Preventing Tumor Escape

Loss of antigen expression has been cited as the cause or potential cause of tumor relapse in CAR-T cell therapy targeting CD19 and IL13R $\alpha$  [45,60,65]. Furthermore, tumor populations are known to be highly heterogeneous and are characterized by considerable intratumor variations in mutational profiles and gene-expression signatures [110]; thus, a single CAR may not be sufficient to recognize all tumor clones in a given patient. To address the challenge of antigen escape, we and others have developed bispecific CAR-T cells with a broadened range of antigen recognition [30,111–114]. Three general strategies have been explored for the generation of bispecific CAR-T cells: dual CAR (expressing two full-length CARs in each T cell), pooled CAR (combining two T-cell products, each expressing one CAR), and single-chain bispecific CAR (expressing a single CAR molecule that can recognize two different antigens).

The dual-CAR and pooled-CAR strategies can readily make use of conventional, single-input CAR constructs. However, the dual-CAR strategy must contend with either (i) the packaging limit of viral vectors used to transduce T cells, with increasing payload size being strongly correlated with decreasing transduction efficiency [115,116], or (ii) the toxicities and inefficiencies associated with multiple transductions steps required to introduce each CAR separately. Although the pooled-CAR strategy avoids these drawbacks, it requires the generation of two cell products for each patient, and only a portion of the infused cells will be able to recognize a particular antigen. In a head-to-head comparison, the dual-CAR strategy has been shown to be superior to the pooled-CAR approach [114].

The generation of single-chain bispecific CARs requires structural optimization, and therefore a potential increase in up-front cost at the receptor development stage compared to the other two strategies [30]. However, once a bispecific CAR has been developed, the generation of CAR-T cell products would benefit from increased transduction efficiency or a lower number of distinct cell products compared to the dual-CAR or pooled-CAR strategy, respectively. Accumulating knowledge on the structure–function correlation in CAR designs will continue to facilitate the development of novel CARs with increased functional capabilities.

### Moving Beyond Artisanal T-cell Production

To date, CAR-T cell therapy has been driven largely by academic centers that have developed in-house expertise on the design, production, and administration of CAR-T cell products. Although several companies have emerged in the wake of promising clinical reports, the prospect of widespread application of CAR-T cell therapy hinges on the ability to move T-cell manufacturing toward robust and standardizable production processes. In its current form, CAR-T cells are primarily a personalized medicine that must be tailor-made for each patient. This individualization increases treatment costs and imposes a treatment-time delay while new cell products are generated. Furthermore, standardization of product quality is extremely difficult because the starting material necessarily varies with individual patients.

Several ongoing clinical trials are evaluating the use of donor-derived T cells, with **graft-versus-host disease** (GVHD) as the primary safety consideration [117]. Early clinical reports

indicate promising outcomes for the use of **allogeneic** CD19 CAR-T cells in the treatment of patients who have received prior allogeneic hematopoietic stem-cell transplants [118]. Efforts toward the generation of ‘universal’ CAR-T cells have focused on eliminating the endogenous TCR using gene-editing techniques such as **CRISPR/Cas9**, transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs) [106,119–122]. Notably, two recent studies introduced CAR transgenes into the *TRAC* locus to simultaneously yield CAR expression and eliminate native TCR activity [12,123]. Interestingly, expressing a CAR in the *TRAC* locus yielded particularly potent CAR-T cells with reduced tonic signaling, terminal differentiation, and exhaustion [12]. In a first-in-human clinical application, TALEN-edited CD19 CAR-T cells lacking the endogenous TCR  $\alpha$  chain and CD52 (whose absence renders engineered T cells insensitive to the lymphodepleting agent alemtuzumab) demonstrated clinical efficacy in the treatment of two infants with progressing ALL [124]. However, both patients also exhibited symptoms of skin GVHD that were attributed to the presence of residual TCR<sup>+</sup> donor T cells, highlighting room for further improvement [124]. Instead of eliminating the endogenous TCR, an alternative strategy is to integrate the CAR into a starting T-cell population with known TCR specificity, particularly toward **Epstein–Barr virus** (EBV) – which can be found in the majority of the human population in the form of latent infection [125–128]. The use of T cells with a known TCR specificity reduces the risk of unexpected off-tumor toxicities. Furthermore, it has been proposed that generating CAR-T cells from EBV-specific T cells can enhance productive T-cell activation through costimulatory signals provided by latently infected antigen-presenting cells *in vivo* [125–127]. GD2 CARs integrated into EBV-specific T cells are currently being evaluated in the clinic for the treatment of GD2<sup>+</sup> sarcomas (NCT01953900).

### Better Together: Prospects of Combination Therapy

CAR-T cell therapy is one of several new advances in immunotherapy in recent years, and the prospect of combining different treatment modalities has been under active evaluation. Current protocols for adoptive T-cell transfer typically include conditioning chemotherapy, which enhances antitumor efficacy by depleting endogenous lymphocytes (including Tregs), and maximizing homeostatic proliferative support for transferred T cells [1,90,129]. In the case of patients with B-cell malignancies, conditioning chemotherapy may also reduce disease burden, which can further enhance T-cell proliferation and persistence [1]. More recently, synergistic possibilities between CAR-T cell therapy and **checkpoint blockade** have become a topic of great interest [37,130,131]. Because T-cell exhaustion has been cited as a primary cause of failures in T-cell-mediated anti-tumor immunity [132–135], combination therapies that support sustained T-cell effector function have the potential to significantly improve treatment outcomes. Indeed, a recent clinical report suggests that PD-1 blockade can enhance the antitumor efficacy of CD19 CAR-T cells in patients who fail to respond to CD19 CAR-T cells alone, although additional studies will be necessary to elucidate detailed mechanisms behind the observed therapeutic synergy [136].

### Diverse Therapeutic Applications

Beyond cancer therapy, efforts have also been underway to use CARs to combat infectious and autoimmune diseases in preclinical models. Antiviral approaches have targeted hepatitis B [137], hepatitis C [138], and HIV [139,140] infections, where virus-infected cells can be distinguished by surface presentation of specific viral proteins. An antifungal CAR uses the extracellular domain of the pattern-recognition receptor **dectin-1** as its antigen-binding domain and effectively targets carbohydrate epitopes on the opportunistic fungus *Apergillus*, disrupting its germination [141]. The dectin-1 CAR demonstrates that pattern-recognition receptors from the innate immune system may serve as a source of antigen-binding domains for CARs that target pathogens.

A recent study demonstrated the novel use of CAR-T cells against the antibody-mediated autoimmune disease **pemphigus vulgaris** (PV) [26]. This strategy takes advantage of the fact that PV pathology is largely attributed to a single cellular source—pathogenic, **anti-Dsg3 antibody**—expressing memory B cells—whose surface marker can be recognized by CARs [26]. CAR-T cells have also been engineered to attack autoreactive T cells, including both CD4<sup>+</sup> and CD8<sup>+</sup> targets. Specifically, these CARs utilize an MHC-I or MHC-II/autoantigenic peptide complex as their extracellular domain, which enables ligation with autoreactive TCRs on target T cells [142,143]. Intriguingly, such redirected T cells were reported to ameliorate experimental autoimmune encephalomyelitis (EAE, a murine model of multiple sclerosis) by inhibiting the activity of multiple autoreactive T cell clones, including those whose cognate epitopes were different from that targeted by the CAR-T cells [142]. Furthermore, the pathologic type 1 T helper (Th1)-dominant responses to autoantigens were replaced by protective type 2 T helper responses after CAR-T cell treatment, suggesting a true reprogramming of the immune response mediated by adoptively transferred T cells [142].

The potent immunosuppressive capabilities of Tregs have also been harnessed with CAR engineering to address therapeutic approaches for autoimmune diseases. For instance, Tregs engineered with the aforementioned CARs can recognize autoreactive TCRs and both prevent and treat EAE in mice [144]. In cases where autoreactive immune cells cannot be identified or do not exist, CARs can direct Treg activity to the site of inflammation—such as the brain (in the case of EAE) [145] or the colon (in the case of murine colitis) [146]—to attenuate local inflammatory activity. Furthermore, human CAR-Tregs with engineered specificity to the human major histocompatibility complex HLA-A2 have been shown to both attenuate GVHD caused by adoptively transferred HLA-A2<sup>+</sup> **peripheral blood mononuclear cells** (PBMCs) and minimize rejection of HLA-A2<sup>+</sup> human PBMCs and skin tissues in mice [147–149]. In addition, Tregs engineered with CARs that bind to factor VIII (FVIII, a blood-clotting protein used as replacement therapy in hemophilia A patients) were found to suppress the production of anti-FVIII antibodies and reduce the proliferation of FVIII-reactive T cells *in vitro* [150], thus returning tolerance for the FVIII clotting factor. Taken together, these results suggest that CAR Tregs might be used to promote tolerance in the host to autoimmunogenic stimuli, allogeneic tissues, and allergens.

## Concluding Remarks

The clinical success of CD19 CAR-T cells has inspired tremendous interest in adoptive T-cell therapy in recent years. Accumulating knowledge on T-cell biology, CAR signaling, and rational protein engineering promises to support the continuing improvement of CAR-T cell therapy for applications in cancer and beyond. Novel engineering strategies that produce T cells with increasingly complex functionalities have been developed to address issues such as tumor-targeting specificity and longevity of T-cell response *in vivo*, and a major task facing the field is the transition from proof-of-concept studies to the development of clinically implementable technologies. Although imperfect, preclinical mouse models have been successfully used to identify an arsenal of strategies to improve CAR-T cell efficacy against immunosuppressed solid tumors; future work must identify which strategies or combinations of strategies will hold true in the human clinical context. As CAR-T therapy advances through clinical trials toward widespread application, a significant number of important questions remain to be answered (see Outstanding Questions). Nevertheless, as a highly programmable, living immunotherapeutic strategy, CAR-T cells are poised to provide an alternative treatment paradigm for a variety of diseases still awaiting effective treatment options.

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## Outstanding Questions

Which antigens can serve as effective and non-toxic targets for non-B-cell malignancies? Antigens that facilitate effective tumor elimination while avoiding off-tumor toxicities remain elusive. The ability to identify better antigens for safe and efficacious tumor targeting will widen the applicability of adoptive T-cell therapy for cancer.

How can we overcome challenges associated with solid tumors—including antigen choice, immune suppression, access to ‘cold’ tumors that lack inflammatory signatures, and infiltration into solid tumor masses? Multiple factors surrounding tumor access and maintenance of T-cell functionality within tumor microenvironments will need to be addressed to increase therapeutic efficacy against solid tumors.

How can we achieve high-throughput optimization of new CAR constructs with predictable *in vivo* functionality? CAR engineering has relied on labor-intensive and time-consuming *in vitro* characterizations that often fail to predict *in vivo* functionality. The identification of *in vitro* assays with higher predictive value, and the development of higher-throughput T-cell isolation, expansion, and characterization methods, will facilitate the generation of novel CARs for new disease targets.

How can we develop widely accessible T-cell manufacturing processes that can generate high-quality T-cell products? Current knowledge on T-cell manufacturing is concentrated in a few academic centers and companies affiliated with such centers. Widespread application of T-cell therapy will necessitate the development of robust T-cell manufacturing processes that can be consistently replicated in non-specialist medical centers.

Could we generate donor-derived ‘universal CAR-T cells’ that can be pre-manufactured for off-the-shelf use? The personalized nature of current CAR-T cell therapy increases treatment cost and limits its availability. Off-the-shelf CAR-T cells would significantly broaden the accessibility of this treatment paradigm.

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