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Engineering CAR-T cells for next-generation cancer therapy

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Summary

T cells engineered to express chimeric antigen receptors (CARs) with tumor specificity have shown remarkable success in treating patients with hematologic malignancies and revitalized the field of adoptive cell therapy. However, realizing broader therapeutic applications of CAR-T cells necessitates engineering approaches on multiple levels to enhance efficacy and safety. Particularly, solid tumors present unique challenges due to the biological complexity of the solid-tumor microenvironment (TME). In this Review, we highlight recent strategies to improve CAR-T cell therapy by engineering (1) the CAR protein, (2) T cells, and (3) the interaction between T cells and other components in the TME.

Keywords: cancer immunotherapy, chimeric antigen receptor (CAR), CAR-T cells, T-cell engineering, solid tumors, tumor microenvironment (TME), mammalian synthetic biology

Introduction

Chimeric antigen receptors (CARs) are synthetic receptors that enable T cells to recognize tumor-associated antigens (TAAs) in a major histocompatibility complex (MHC)-independent manner. CAR-T cells targeting the pan–B-cell marker CD19 have shown unprecedented response rates in treating refractory B-cell malignancies (Maude et al., 2014; Neelapu et al., 2017) and became the first genetically modified cell-based therapy to receive FDA approval (Bouchkouj et al., 2019; O'Leary et al., 2019). However, the development of effective CAR-T cell therapy for non–B-cell malignancies has required more sophisticated engineering approaches to overcome tumor-defense mechanisms such as immunosuppression, antigen escape, and physical barriers to entry into solid tumors. In this review, we examine current and prospective strategies to engineer CARs, T cells that express CARs or tumor-specific T-cell receptors (TCRs), and the interaction between engineered T cells and the tumor microenvironment (TME), with particular focus on improving the efficacy and safety of adoptive T-cell therapy for the treatment of solid tumors (Figure 1).

Engineering the CAR protein

Evolution of CAR Designs

Kuwana et al. reported the first proof of principle of combining antibody-type antigen specificity with T-cell signaling by fusing the TCR constant region to the variable regions of a bacterial antigen-recognizing antibody (Kuwana et al., 1987). Single-chain variable fragments (scFvs), comprised of the variable heavy (V_H) and light (V_L) chains of a monoclonal antibody (mAb) separated by a flexible linker, are still commonly used as the extracellular antigen-sensing domain of CARs. The first reports of tumor-targeting CARs demonstrated that an scFv recognizing antigens like human epidermal growth factor receptor 2 (HER2) fused to the CD3ζ signaling domain can elicit tumor-specific cytotoxicity (Eshhar et al., 1993; Moritz et al., 1994; Stancovski et al., 1993), but T cells expressing these “first-generation” CARs that included only the CD3ζ chain for T-cell signaling generally failed to elicit potent antitumor effects.

In the following years, second- and third-generation CARs emerged that included one or two costimulatory domains, respectively, drawing from the biological understanding that the endogenous TCR requires association with other costimulatory or accessory molecules for robust signaling (Chen and Flies, 2013). Most commonly derived from CD28 or 4-1BB, these costimulatory domains conferred more potent antitumor cytotoxicity, increased cytokine production, and improved proliferation and persistence of CAR-T cells (Haynes et al., 2002; Imai et al., 2004). The choice of costimulatory domain impacts a wide range of properties including metabolic pathways (Kawalekar et al., 2016), T-cell memory development (Kalos et al., 2011; Kawalekar et al., 2016), and antigen-independent tonic signaling (Long et al., 2015), prompting further research into other costimulatory domains. For example, a third-generation CAR with OX40 and CD28 costimulatory domains repressed CD28-induced secretion of interleukin (IL)-10, an anti-inflammatory cytokine that compromises T-cell activity (Hombach et al., 2012). Additionally, the inducible T-cell costimulator (ICOS) costimulatory domain in combination with either CD28 or 4-1BB costimulation increased *in vivo* persistence, and MyD88/CD40 costimulation improved *in vivo* proliferation of CAR-T cells (Collinson-Pautz et al., 2019; Guedan et al., 2018).

More recently, fourth-generation CARs that incorporate additional stimulatory domains, commonly referred to as “armored" CARs, have been reported. In one example, Chmielewski et al. engineered armored CAR-T cells termed “T cells redirected for universal cytokine-mediated killing” (TRUCK) to secrete the proinflammatory cytokine IL-12 to stimulate innate immune cells against the tumor and resist inhibitory elements of the TME, including regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs) (Chmielewski et al., 2014; Pegram et al., 2012). The secretion of other soluble factors have been studied, including IL-15 or IL-18 to enhance T-cell proliferation, as well as the combination of CCL19 and IL-7 to recruit endogenous immune cells and establish a memory response against tumors (Adachi et al., 2018; Hoyos et al., 2010; Hu et al., 2017).

In addition to the evolution of CAR designs outlined above, the modularity of the four major components of a CAR—extracellular antigen-sensing domain, extracellular hinge or spacer domain, transmembrane domain, and intracellular signaling domain—has enabled further optimization of each of these components to improve the efficacy of CAR-T cell therapy. These engineering efforts are well-summarized in other reviews (Labanieh et al., 2018; Rafiq et al., 2020). Here, we focus our attention on strategies that enable T cells to expand beyond the hard-wired, single-input, single-output signaling capability programmed by conventional CAR designs (Figure 2).

Combinatorial Antigen Sensing for Logic-Gated T-Cell Activation

Boolean logic gates have been utilized for the combinatorial detection of multiple antigens by CAR-T cells to improve their safety and antitumor efficacy (Figure 2A). AND-gate logic requires the co-presence of two different antigens to activate the CAR-T cell, and this increased specification reduces the risk of either off-target recognition or “on-target, off-tumor” toxicities, in which healthy tissues that express the same antigen as tumor cells suffer collateral damage. The synthetic Notch (synNotch) receptor—which triggers inducible target-gene expression upon recognition of a cell surface-bound ligand—was engineered to recognize a TAA and induce the expression of a CAR, which can subsequently trigger T-cell activation upon recognizing a second TAA (Roybal et al., 2016). This strategy has been shown to reduce systemic toxicity compared to constitutive CAR expression, provided that the off-tumor target is not spatially proximal to the tumor cells (Srivastava et al., 2019). Since there is a temporal delay between the recognition of TAA #1 by synNotch and the recognition of TAA #2 by CAR, a given T cell could have its synNotch receptor triggered by TAA #1 from a tumor cell but subsequently attack a healthy cell expressing TAA #2. An alternative AND-gate approach separates the CD3ζ chain and costimulatory domain into two constitutively expressed receptors each recognizing different antigens, such that the CAR-T cell is optimally activated only in the simultaneous presence of both antigens (Kloss et al., 2013; Wilkie et al., 2012). However, this approach often suffers from “leakiness” due to the fact that first-generation CARs containing only the CD3ζ chain are already signaling-competent. Yet another strategy programs T cells to deliver a conditionally active cytotoxic protein upon CAR- or TCR-mediated detection of TAA #1 on the cell surface; the engineered protein becomes cytotoxic if and only if it detects TAA #2 inside the target cell, thus requiring both antigens to be expressed by the same target cell to trigger robust killing (Ho et al., 2017).

CAR-T cells programmed to execute AND-NOT logic can also help prevent toxicities against healthy cells. This strategy utilizes an inhibitory CAR (iCAR) that targets an antigen found on

healthy tissue, and pairs it with an activating CAR that targets a TAA. In a proof-of-principle study, a prostate-specific membrane antigen (PSMA)-targeting iCAR that incorporates the programmed cell death protein 1 (PD-1) inhibitory signaling domain was co-expressed with a second-generation CD19 CAR, and the iCAR inhibited CAR-T cell activation in the presence of PSMA (Fedorov et al., 2013).

While AND and AND-NOT logic can improve the safety of CAR-T cells by increasing specificity, OR-gate logic has been utilized to increase antitumor efficacy by circumventing antigen escape, or loss of the targeted epitope by tumor cells. An OR-gate CAR can recognize two different TAAs, and the binding of *either* antigen induces T-cell activation. One OR-gate strategy utilizes the pooled mixture of two populations of CAR-T cells (CARpool), each expressing a monospecific CAR. A variation on this theme is to sequentially administer two different CAR-T cell products (Shah et al., 2020; Shalabi et al., 2018). Another strategy is the co-expression of two separate CARs in each T cell (dual CAR) (Ruella et al., 2016). Yet another approach uses tandem bispecific CARs (TanCAR) that comprise two scFv domains separated by a linker on one receptor chain, and this strategy was shown to be functionally superior to both the CARpool and dual-CAR approaches (Hegde et al., 2016; Zah et al., 2020). In particular, CD19/CD20 and CD19/CD22 bispecific CARs have been characterized for the treatment of B-cell malignancies (Fry et al., 2018; Qin et al., 2018; Zah et al., 2016), and are in clinical trials for lymphoma and ALL, respectively (NCT04007029, NCT04215016, NCT03919526, NCT04303520).

ON/OFF Switches for Controllability and Safety

CAR modifications to improve safety and controllability have also taken the form of externally inducible or self-regulating ON/OFF switches. Conventional CAR-T cells are “always on,” meaning their CARs are constitutively expressed and are always capable of signaling upon antigen stimulation. However, this is not always desirable when CAR-derived toxicity is an anticipated risk. In addition to the off-target and “on-target, off-tumor” toxicities discussed previously, various systemic toxicities have also been observed in patients treated with CAR-T cells. Common examples include cytokine release syndrome (CRS) (Fitzgerald et al., 2017; Grupp et al., 2013; Schuster et al., 2017), neurotoxicity or “CAR-related encephalopathy syndrome” (CRES) (Grupp et al., 2013; Lee et al., 2019a; Schuster et al., 2017), tumor lysis syndrome (TLS), and anaphylaxis (Maus et al., 2013). It has been hypothesized that modulation of CAR-T cell activity in space and time can prevent or significantly lessen the severity of toxicities associated with CAR-T cell therapy while maintaining its antitumor efficacy.

One approach to modulate CAR-T cell activity is to regulate the presence of functional CARs on the surface of engineered T cells by adjusting either the stability or the conformation of the CAR protein itself (Figure 2B). As examples of the former strategy, CARs have been fused to degradation tags that can be inactivated either by small-molecule binding (Weber et al., 2020) or under hypoxic conditions (Juillerat et al., 2017). The default state in such systems is “off,” and the removal or inactivation of the degradation tag is required to stabilize the CAR protein, thus enabling CAR-mediated T-cell activation upon antigen stimulation. An alternative design in which the CAR protein is present by default but turned off through the administration of a small-molecule drug has also be demonstrated (Juillerat et al., 2019).

Instead of modulating protein half-life, controlling the conformation of the CAR protein can also regulate the availability of functional CARs, such that the receptor is signaling-competent only under specified conditions. For example, the antigen-binding domain of CARs can be "masked" by a built-in inhibitory peptide, such that the CAR's functional conformation is acquired only after the inhibitory peptide has been cleaved by proteases commonly active in the TME (Han et al., 2017).

Adapter-Dependent CARs

Alternatively, T cells can be engineered to express a receptor that must be complemented by an additional protein component before the resulting complex is capable of translating antigen recognition to T-cell activation (Figure 2C). Urbanska et al. reported a "universal" receptor comprising a biotin-binding domain fused to an intracellular T-cell signaling domain. T cells expressing this biotin-specific receptor can, in principle, be directed against any target cell that has been labeled with a biotinylated antibody (Urbanska et al., 2012). Two advantages of this strategy include the abilities to (1) control the ON/OFF state of the T cell through administering or withholding the biotinylated antibody and (2) use the same biotin-specific receptor to target a wide variety of TAAs by changing the specificity of the biotinylated antibody. The concept of using a universal CAR coupled with one or more antigen-targeting adaptor proteins to overcome tumor heterogeneity and mitigate toxicity soon opened the floodgates for other adapter-dependent CAR designs (Bachmann, 2019; Cartellieri et al., 2016; Herzig et al., 2019; Lee et al., 2019c; Qi et al., 2020; Raj et al., 2019; Rodgers et al., 2016), including a small molecule adapter, which binds to both fluorescein isothiocyanate (FITC) and folate, that alleviated CRS-like toxicity in an NSG mouse model (Lee et al., 2019b).

Expanding upon the concept of constitutively expressing a universal receptor on the T-cell surface combined with an externally administered adaptor protein to dictate antigen specificity, Cho et al. reported a SUPRA CAR system that can program a variety of Boolean logic gates in engineered T cells by expressing multiple base receptors, each with multiple potential adaptor protein partners reconstituted by leucine-zipper dimerization (Cho et al., 2018). Such a system supports the possibility of simultaneously increasing specificity through the use of AND or AND-NOT gates while addressing tumor heterogeneity by targeting multiple antigens with different adaptor proteins. However, the versatility of multi-component systems comes at the cost of an increased number of parameters that must be optimized, including the half-life, biodistribution, and interaction dynamics among the adaptor protein, base receptor, and the engineered T cell itself. Therefore, it remains to be seen whether the complex signal processing achievable through adaptor-dependent CAR designs will translate to robust therapeutic candidates.

Engineering the CAR-Expressing Cell

Safety Controls on CAR-T Cell Activity

In addition to engineering the CAR protein itself, engineering approaches applied at a cellular level to modulate T-cell activity can also significantly impact therapeutic efficacy and safety. For example, transient CAR expression resulting from mRNA electroporation instead of viral integration can mitigate toxicities induced by CARs that cross-recognize healthy tissue (Beatty et

al., 2014; Tchou et al., 2017; Wiesinger et al., 2019). Another safeguard against severe toxicity is the implementation of suicide genes that enable the depletion of engineered T cells by the administration of small-molecule drugs (Figure 3A) (Marin et al., 2012). For example, CAR-T cells targeting the CD44v6 antigen for the treatment of leukemia and myeloma have been engineered to express herpes simplex virus thymidine kinase (HSV-TK), which enabled effective elimination of the CAR-T cells upon exposure to ganciclovir *in vitro* (Casucci et al., 2018). However, expression of viral HSV-TK raises concerns of immunogenicity and requires three days of ganciclovir exposure to achieve effective T-cell elimination (Marin et al., 2012). An alternative approach using the inducible caspase 9 (iCasp9) suicide gene has been shown to eliminate >90% of engineered cells within 30 minutes of drug administration in human patients (Di Stasi et al., 2011). iCasp9 contains the intracellular domain of the pro-apoptotic protein caspase 9 fused to FK506-binding protein (FKBP). The small molecule AP1930 facilitates the dimerization of FKBP and activation of the fused caspase 9, inducing apoptosis in cells expressing the iCasp9 protein (Straathof et al., 2005). Additionally, the expression of transgenes for the surface proteins CD20 or truncated epidermal growth factor receptor (tEGFR) can also facilitate suicide mechanisms. The FDA-approved mAbs rituximab and cetuximab bind to CD20 and tEGFR, respectively, and the resulting antibody-dependent cellular cytotoxicity (ADCC) can be used to eliminate T cells engineered to express these antigens (Griffioen et al., 2009; Serafini et al., 2004; Wang et al., 2011).

While suicide genes can efficiently deplete CAR-T cells to counter toxicities, the activation of a suicide gene also results in the irreversible termination of the therapy. An alternative reversible strategy utilizes dasatinib, a tyrosine kinase inhibitor that interferes with the lymphocyte-specific protein kinase (LCK), thus inhibiting CD3ζ phosphorylation and CAR activation. Dasatinib has been shown to function as a reversible ON/OFF switch of CAR-T cell activity, where the cessation of dasatinib administration rapidly reversed its inhibitory effects, highlighting its potential as an emergency drug for potentially lethal toxicities such as CRS and CRES (Mestermann et al., 2019; Weber et al., 2019).

Regulated CAR Expression to Improve CAR-T Cell Safety

As an alternative to post-translational regulation of CAR stability and function, transcriptional regulation can provide another tunable handle to improve CAR-T cell safety (Figure 3B). For example, in the Tet-ON system, the small molecule doxycycline (Dox) acts as an “ON switch,” where CAR expression is induced by the reverse tetracycline transactivator (rtTA) protein only in the presence of Dox (Sakemura et al., 2016). Conversely, in the Tet-OFF system, Dox acts as an “OFF switch” by abolishing the ability of tetracycline transactivator (tTA) to activate CAR transcription; this system was used to reversibly inhibit deleterious CAR signaling and T-cell fratricide in CD5 CAR-T cells (Mamonkin et al., 2018). Additionally, response to a tumor-associated environmental cue could be achieved through inducible promoters responsive to hypoxia-inducible factor (HIF)-1α, activating CAR expression only in hypoxic environments (Ede et al., 2016). Finally, as previously described, the synNotch receptor enables inducible CAR transcription upon binding to a membrane-bound ligand (Roybal et al., 2016; Srivastava et al., 2019).

Site-Specific CAR Transgene Insertion and Allogeneic Compatibility Engineering

The examples of regulated gene expression provided above utilize synthetic inducible promoters, and the entire gene-expression cassette is typically integrated into the T-cell genome using retroviral or lentiviral vectors, leading to variable integration sites and copy numbers. An alternative method to achieve dynamic CAR expression profiles is to integrate the transgene into specific genetic loci regulated by endogenous transcriptional machinery. A variety of gene-editing technologies including clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9), transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs) have made this a feasible approach in T-cell engineering (Chen, 2015). For example, Eyquem et al. demonstrated that CRISPR/Cas9-mediated insertion of the CD19 CAR transgene into the TCRα constant (*TRAC*) locus results in CAR-T cells with superior *in vivo* function compared to retrovirus-mediated random CAR-transgene insertion (Eyquem et al., 2017), although more recent evidence suggests whether site-specific CAR integration into the *TRAC* locus improves T-cell function may depend on the specific CAR construct used (Zah et al., 2020). A compelling example of the transgene insertion site influencing CAR-T cell function comes from the case of a chronic lymphocytic leukemia (CLL) patient, whose disease regression was observed to coincide with the clonal expansion of T cells carrying the CD19 CAR transgene inserted into the methylcytosine dioxygenase ten-eleven translocation 2 (*TET2*) locus (Fraietta et al., 2018). Combined with a pre-existing hypomorphic mutation in the patient's other *TET2* allele, this CAR insertion resulted in the loss of wildtype *TET2*, a gene that encodes for a regulator of DNA methylation and blood cell formation. This fortuitous CAR insertion/*TET2* ablation event led to an altered epigenetic landscape that conferred profound proliferative capability and a central-memory phenotype to the engineered T cells (Carty et al., 2018; Fraietta et al., 2018).

Gene-editing technologies have also enabled the elimination of endogenous genes in support of allogeneic T-cell therapy (Table 1). T-cell products derived from healthy donors can overcome manufacturing challenges associated with autologous cell therapy, such as the difficulty to obtain enough high-quality T cells from heavily pre-treated patients with advanced disease. However, allogeneic T-cell transfer requires the elimination of the endogenous TCR to prevent graft-versus-host disease (GvHD), and the removal of class-I major histocompatibility complex (MHC-I) has been proposed to minimize allograft rejection (Liu et al., 2017). To this end, Torikai et al. demonstrated ZFN-mediated abrogation of TCRαβ or human leukocyte antigen (HLA)-A expression in CD19 CAR-T cells (Torikai et al., 2012; Torikai et al., 2013). In addition to preventing GvHD, gene editing has been utilized to protect CAR-T cells from lymphodepletion, which is a common preconditioning treatment administered prior to CAR-T cell infusion to improve the efficacy of the transferred cells. For example, TALEN-mediated simultaneous disruptions of TCRαβ/CD52 or TCRαβ/deoxycytidine kinase (dCK) have been shown to confer CD19 CAR-T cells with resistance to anti-CD52 or dCK phosphorylation-dependent lymphodepleting regimens, respectively (Qasim et al., 2017; Valton et al., 2015).

In the studies referenced above, endogenous gene disruption and CAR transgene integration were independently executed, yielding heterogeneous CAR-T cell populations. Georgiadis et al. coupled gene-editing to CAR integration by incorporating a single-guide RNA (sgRNA) element into the U3 region of the 3' long terminal repeat (LTR) sequence of the CAR-encoding lentiviral vector. After electroporation of Cas9 mRNA, magnetic bead-based selection for edited cells resulted in highly enriched CAR^+ TCR^- populations (Georgiadis et al., 2018). In another strategy, Ren et al. used a "one-shot" CRISPR system with multiple sgRNA expression cassettes in one CAR-encoding lentiviral vector to simultaneously knock out the endogenous TCR and Beta-2

microglobulin (β2M), an essential subunit of the HLA-I molecule, to generate CD19 CAR-T cells suitable for allogeneic therapy (Ren et al., 2017b).

Knockout of Negative Regulators

Gene-editing technologies can also be used to abrogate the expression of negative regulators of T-cell activity (Table 1). Tumor cells frequently upregulate ligands to immune checkpoint receptors, such as cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and PD-1 expressed on T cells, leading to inhibition of T-cell activity in the TME (Buchbinder and Desai, 2016). Immune-checkpoint blockade using antibodies targeting CTLA-4, PD-1, or PD-1 ligand (PD-L1) has revolutionized the field of immuno-oncology in recent years (Wei et al., 2018). An alternative approach to checkpoint blockade is the ablation of checkpoint-receptor expression on engineered T cells. Menger et al. demonstrated that PD-1 knockout through TALEN-mediated editing in melanoma-reactive $CD8^+$ T cells and fibrosarcoma-reactive polyclonal T cells enhanced the persistence of the modified T cells, augmenting their antitumor activity against syngeneic tumor models and establishing long-term antitumor memory (Menger et al., 2016). Additionally, CRISPR/Cas9-mediated disruption of PD-1 in CD19 CAR-T cells enhanced CAR-T cell cytotoxicity toward PD-$L1^+$ tumor xenografts *in vivo* (Rupp et al., 2017). The first CRISPR/Cas9-edited cell therapy trial conducted in the U.S. evaluated the adoptive transfer of autologous T cells genetically modified to express a New York esophageal squamous cell carcinoma 1 (NY-ESO-1)–targeting TCR while eliminating endogenous TCRαβ and PD-1 expression; recent results from the trial confirmed the safety and feasibility of CRISPR-edited cell therapy for cancer (Stadtmauer et al., 2020).

In addition to PD-1 knockout, elimination of the metabolic regulator REGNASE-1 and CD3-signaling regulator diacylglycerol kinase (DGK) has also been shown to enhance T-cell function *in vitro* and *in vivo* (Jung et al., 2018; Riese et al., 2013; Wei et al., 2019). Similar to PD-1, lymphocyte-activation gene 3 (LAG3) is known as a T-cell exhaustion marker, and it has been found to inhibit the effector function of T cells and enhance the suppressive function of Tregs (Zhang et al., 2017). However, CRISPR/Cas9-mediated deletion of *LAG3* in CD19 CAR-T cells did not result in a discernable phenotype, perhaps due to compensatory effects from PD-1 (Woo et al., 2012; Zhang et al., 2017).

Several studies have reported the feasibility of multiple knockouts using CRISPR/Cas9 in CAR-T cells to achieve the dual goals of reducing alloreactivity and improving T-cell function. Triple-targeting of the *TRAC*, *B2M*, and *PD-1* loci has been demonstrated by electroporation of Cas9:sgRNA ribonucleoprotein (RNP) complex (Liu et al., 2017) or by electroporation of mRNA encoding Cas9 and sgRNAs into CAR-T cells (Ren et al., 2017a). Furthermore, triple targeting of the *TRAC*, *B2M*, and *FAS* loci using a lentiviral vector encoding both the sgRNAs and a CD19-targeting CAR and electroporation of Cas9 mRNA resulted in reduced alloreactivity and prolonged T-cell survival by abrogating pro-apoptotic Fas/FasL signaling (Ren et al., 2017b). Finally, CRISPR/Cas9-mediated deletion has been used to improve the safety of CAR-T cells by disrupting the expression of CRS-associated cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sterner et al., 2019).

Receptors that Rewire an Inhibitory Input to a Stimulatory Output

While CAR-T cells have shown promising therapeutic efficacy against hematologic malignancies, their targeting of solid tumors has been stymied by a number of challenges. In addition to antigen heterogeneity, tumor cells produce tumorigenic and immunosuppressive factors in the TME, and this inhibitory environment is further compounded by immunosuppressive cell types such as MDSCs and Tregs (Labanieh et al., 2018). Among the immunosuppressive soluble factors commonly found in the TME, transforming growth factor beta (TGF-β) is a particularly potent cytokine that drives T-cell differentiation into Tregs, as well as macrophage polarization to the immunosuppressive M2 phenotype (Tormoen et al., 2018). The engineering of a TGF-β–responsive CAR demonstrated that CARs can be used to (1) sense a soluble factor and (2) rewire T-cells to convert an inhibitory signal to a trigger of antitumor activity (Figure 4) (Chang et al., 2018; Hou et al., 2018). TGF-β–responsive CAR-T cells were demonstrated to proliferate and produce T helper type 1 (Th1)-associated cytokines in the presence of soluble TGF-β, as well as protect nearby cells from the inhibitory effects of TGF-β, likely through the combined effects of TGF-β sequestration and paracrine Th1 cytokine signaling (Chang et al., 2018; Hou et al., 2018).

Signal rewiring can also be achieved with "switch receptors," which are chimeras comprising an ectodomain that binds a suppressive molecule fused to an endodomain that drives a stimulatory pathway (Figure 4). IL-4 is a cytokine with complex roles in the TME, such as inducing M2 polarization, promoting tumor growth, and suppressing tumor-specific effector T cells (Tormoen et al., 2018; Wilkie et al., 2010). IL-4 switch receptors consisting of the IL-4 receptor α (IL-4Rα) ectodomain fused to either the IL-7Rα endodomain or the β_c receptor subunit common to IL-2 and IL-15 signaling have been shown to paradoxically enhance T-cell proliferation in the presence of IL-4 (Leen et al., 2014; Wilkie et al., 2010). Consequently, the co-expression of the IL-4Rα:β_C switch receptor in CAR-T cells augmented their antitumor capacity (Wilkie et al., 2010). More recently, Roth et al. used a pooled knock-in screen using CRISPR/Cas9 coupled with single-cell RNA sequencing (scRNA-seq) to evaluate a panel of transgenes integrated into the *TRAC* locus. Through this analysis, a novel TGFβR2:4-1BB switch receptor was identified as the lead candidate for improved T-cell fitness and solid tumor clearance (Roth et al., 2020).

Switch receptors can also mitigate the suppressive effects of immune checkpoints. For example, CTLA-4:CD28 and PD-1:CD28 switch receptors were shown to enhance the activity of tumor-specific T cells (Liu et al., 2016; Shin et al., 2012). As another checkpoint receptor, T-cell immunoreceptor with Ig and ITIM domains (TIGIT) binds to CD155 or CD112 on tumor cells, and these interactions hinder cytokine production and effector function of T cells. To blunt these effects, Hoogi et al. designed a TIGIT:CD28 switch receptor that enhanced cytokine production and activation of T cells, as well as delayed tumor growth *in vivo* when combined with a melanoma-specific TCR (Hoogi et al., 2019).

Yet another set of engineering strategies focuses on addressing tumorigenic factors that are not directly expressed on T cells or tumor cells (Figure 4). For example, vascular endothelial growth factor-A (VEGF-A) is a tumor-derived soluble factor that promotes tumor growth and metastasis by facilitating angiogenesis. Chinnasamy et al. co-transduced T cells with a CAR targeting VEGF receptor 2 (VEGFR2) and an inducible transgene encoding IL-12. These CAR-T cells demonstrated trafficking to the tumor vasculature, elimination of $VEGFR^+$ MDSC subtypes that participate in tumor angiogenesis, and IL-12–mediated solid tumor regression (Chinnasamy et al., 2012). Additionally, T cells expressing a CAR that targets fibroblast activation protein (FAP) eliminated FAP^+ tumor stromal fibroblasts that support tumor growth, augmenting T-cell

immunotherapy (Wang et al., 2014). However, it bears noting that strategies targeting non-tumor tissues can cause significant toxicity. For example, Tran et al. reported that CAR-T cells cross-reactive to human and mouse FAP cause off-target bone marrow toxicities and cachexia (Tran et al., 2013).

Transgene Expression to Promote T-Cell Function

In addition to abolishing or rewiring inhibitory signals, the overexpression of stimulatory signals can also improve the activity of tumor-specific T cells. The constitutive expression of costimulatory ligands CD80 and 4-1BBL in PSMA-targeting CAR-T cells showed superior activity against prostate tumor cells by engaging costimulatory receptors *in cis* (autocostimulation) and *in trans* (transcostimulation of bystander cells) (Stephan et al., 2007). Furthermore, Zhao et al. co-expressed the 4-1BBL costimulatory ligand with a second-generation CD19-targeting CAR with CD28 costimulation, and this combined costimulation led to improved antitumor functions driven by the continuous activation of the interferon regulatory factor 7 (IRF7)/interferon beta (IFNβ) pathway (Zhao et al., 2015). Yet another strategy harnesses the endogenous IL-7 signaling mechanism that confers improved persistence in tumor-specific T cells. Shum et al. engineered a constitutively active IL-7 receptor and co-expressed it with a GD2-targeting CAR, which resulted in enhanced survival under repeated tumor challenges in neuroblastoma and glioblastoma xenograft models (Shum et al., 2017).

The aberrant expression of the colony-stimulating factor 1 (CSF-1) in the TME drives macrophages to the M2 phenotype and promotes tumor growth. Several clinical trials of small molecules and mAbs targeting the CSF-1/CSF-1R axis in combination with other mAbs and/or chemotherapy are under way for the treatment of solid tumors (NCT01525602, NCT02777710, NCT02323191, NCT02760797, NCT02923739). In the context of CAR-T cells, the co-expression of CSF-1R, which T cells do not naturally express, was shown to confer CSF-1 responsiveness and activated the RAS/MEK/Erk kinase pathway to enhance T-cell proliferation, cytokine production, and CSF-1–driven chemotaxis (Lo et al., 2008).

Metabolic Reprogramming of T Cells

T-cell metabolism, or the manner in which T cells utilize nutrient sources, has consequential effects on their differentiation state and effector function. The architecture of the CAR protein can impact the metabolic profiles of CAR-T cells. For example, T cells expressing CARs with 4-1BB costimulation favor the oxidative breakdown of fatty acids characteristic of the central-memory phenotype, accompanied by enhanced proliferation and persistence, while T cells expressing CARs with CD28 costimulation favor aerobic glycolysis characteristic of the effector-memory phenotype (Kawalekar et al., 2016).

Due to the intimate relationship between T-cell metabolism and function, reprogramming the metabolic profile of CAR-T cells can potentially increase their clinical efficacy. The TME of solid tumors has an overabundance of potassium (K^+) released by necrotic tumor cells, which increases the intracellular K^+ concentration in infiltrating T cells, downregulating Protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling and impairing T-cell activation after TCR ligation. As a counterstrategy, the overexpression of K^+ channels was shown to increase Akt/mTOR activity and rescue T-cell effector functions by facilitating K^+ efflux and lowering

intracellular K^+ levels (Eil et al., 2016). Additionally, elevated K^+ in the TME and the resulting perturbation of the transmembrane electrochemical gradient limit nutrient uptake by T cells. Interestingly, *ex vivo* conditioning and activation of tumor-specific $CD8^+$ T cells in elevated K^+ to mimic such functional starvation in the TME led to epigenetic and metabolic reprogramming that maintained T-cell stemness—evidenced by improved persistence, engraftment, self-renewal, and multipotency—thereby enhancing their antitumor function *in vivo* (Vodnala et al., 2019). Further, Geiger et al. showed supplementing L-arginine to balance increased arginine metabolism in activated T cells promotes the central-memory phenotype and improves antitumor activity (Geiger et al., 2016).

Altering the expression levels of metabolic genes can also promote an advantageous metabolic profile. Phosphoenolpyruvate carboxykinase 1 (PKC1) increases the production of phosphoenolpyruvate (PEP), which sustains TCR-mediated, Ca^{2+}-induced nuclear factor of activated T-cells (NFAT) signaling and effector functions, and the overexpression of PKC1 in T cells has been shown to restrict tumor growth in melanoma-bearing mice (Ho et al., 2015). Leukemic cells drive T-cell dysfunction by causing suppressed Akt/mTORC1 signaling, decreased expression of the glucose transporter Glut1, and reduced glucose uptake. Accordingly, the overexpression of Akt or Glut1 was demonstrated to partially rescue T-cell activity (Siska et al., 2016). Additionally, Yang et al. knocked out Acetyl-CoA acetyltransferase (ACAT1), a cholesterol esterification enzyme, in $CD8^+$ T cells, and the resulting increase in the plasma membrane cholesterol concentration enhanced TCR clustering and signaling (Yang et al., 2016). Lastly, PPAR-gamma coactivator 1α (PGC1α) is a metabolic regulator downregulated in tumor-infiltrating T cells. It facilitates mitochondrial biogenesis by transcriptional coactivation, and its overexpression in $CD8^+$ T cells rescued their mitochondrial function and protected their metabolic and effector activities in the TME (Scharping et al., 2016).

Interplay of T-Cell Phenotypes, Function, and Versatility

The differentiation state of T cells influences their longevity and efficacy, motivating the isolation or enrichment of specific T-cell subtypes in CAR-T cell manufacturing. Generally, the selection of less differentiated phenotypes—naïve (T_N), memory stem (T_{SCM}), and central memory (T_{CM})—imparts greater engraftment and efficacy than the more differentiated counterparts—effector (T_E) and effector memory (T_{EM}) (Sadelain et al., 2017). Different costimulatory domains in the CAR protein can affect T-cell subtype distribution: CD28 costimulation tends to promote the short-lived, potent T_{EM} phenotype, while 4-1BB results in enrichment of the longer-lived, self-renewing T_{CM} phenotype (Kawalekar et al., 2016). It has also been shown that T_N, T_{CM}, and T_{EM} $CD4^+$ and $CD8^+$ T cells can all be transduced and expanded as CD19 CAR-T cells, but the combination of $CD8^+$ T_{CM} and $CD4^+$ T_N subsets yields synergistic antitumor activity *in vivo* (Sommermeyer et al., 2016). T_{SCM} cells comprise only 2–3% of peripheral blood mononuclear cells (PBMCs), but this memory subset possesses the highest self-renewal capacity and superior persistence, and they are suggested to be the primary precursors of T-cell memory establishment (Hurton et al., 2016). IL-15 is a pro-survival cytokine fundamental to T-cell memory, and it can preserve a T_{SCM}-like phenotype by inhibiting mTORC1 activity, reducing glycolysis, and improving mitochondrial fitness (Alizadeh et al., 2019). Hurton et al. incorporated IL-15 costimulation in CAR-T cells by co-expressing a membrane-bound chimeric IL-15, which led to a T_{SCM}-like molecular profile with improved T-cell persistence regardless of CAR stimulation (Hurton et al., 2016). In addition, the miR-17-92 microRNA cluster was found to be upregulated in IFNγ-producing Th1 cells compared

to T helper type 2 (Th2) cells, and it was found to be downregulated in T cells derived from glioblastoma patients (Sasaki et al., 2010). To induce Th1-like phenotype in glioblastoma-targeting CAR-T cells, Ohno et al. co-transduced miR-17-92 with a third-generation EGFRvIII-specific CAR; in combination with the chemotherapeutic agent temozolomide, this strategy led to improved cytolytic activity and protection against tumor re-challenge *in vivo* (Ohno et al., 2013).

Stem cells are a versatile starting material for adoptively transferred cellular products due to their abilities to self-renew and differentiate into various cell types. Schmitt et al. showed that OP9-DL1, a bone marrow stromal cell line that ectopically expresses the Notch ligand Delta-like-1, can induce the differentiation of hematopoietic progenitor cells (HPCs) into T lymphocytes (Schmitt and Zúñiga-Pflücker, 2002). In a subsequent study, functional $CD8^+$ T cells were generated from human umbilical cord blood hematopoietic stem cells (HSCs), which possess greater self-renewal and potency than HPCs, in OP9-DL1 cocultures (Awong et al., 2011). More recently, an artificial thymic organoid (ATO) system has been shown to facilitate *in vitro* differentiation of human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) into mature T_N-like cells with potent antitumor efficacy and a similar transcriptional profile as primary $CD8^+$ T_N cells (Montel-Hagen et al., 2019).

Expansion of CAR Effectors beyond T cells

Exosomes derived from CAR-T cells, rather than the T cells themselves, have been studied as an alternative effector for CAR-mediated antigen specificity and cytotoxicity. Exosomes released by CAR-T cells can carry the CAR and a high level of cytotoxic molecules; these tumor-specific cytotoxic packages can traffic into solid tumors due to their nanoscale size, while incurring a lower risk of CRS-related toxicities and conferring protection against the immune-checkpoint molecule PD-L1 due to their lack of PD-1 expression (Fu et al., 2019).

Programming CARs into cell types other than T cells can further expand the versatility of the therapy by realizing new functions unachievable by CAR-T cells. Klinchinsky et al. recently demonstrated the feasibility of adenoviral transduction of a CAR into primary macrophages. The resulting CAR-M cells exhibited tumor-specific phagocytosis, inflammatory cytokine production, polarization of bystander macrophages to the immunostimulatory M1 phenotype, and cross-presentation of the TAA to bystander T cells. Although a comparison between CAR-T and CAR-M cells was not evaluated, the established role of macrophages as professional antigen presenting cells (APCs) warrants the potential of CAR-M cells to more effectively stimulate an adaptive antitumor immune response (Klinchinsky et al., 2020).

CAR-natural killer (NK) cells can be generated from cord blood or iPSCs (Li et al., 2018; Liu et al., 2020), making them an attractive candidate for allogeneic, off-the-shelf products. Moreover, CD19-targeting CAR-NK cells have achieved robust clinical efficacy without inducing CRS, neurotoxicity, or GvHD in patients with B-cell lymphoid tumors, highlighting their relative safety compared to their T-cell counterparts (Liu et al., 2020). CAR-NK cells have been shown to exert potent and specific cytotoxicity toward a variety of tumor models, including leukemia, multiple myeloma, ovarian cancer, and glioblastoma (Chu et al., 2014; Genßler et al., 2016; Li et al., 2018; Quintarelli et al., 2020); as well as toward immunosuppressive cell types such as MDSCs and follicular helper T cells (T_{FH}) (Parihar et al., 2019; Reighard et al., 2020). Lastly, natural killer T (NKT) cells possess antitumor and tumor-homing capabilities, and GD2-targeting CAR-NKT cells

that harness these inherent advantages exhibited effective localization to and lysis of neuroblastoma cells without significant toxicity (Xu et al., 2019). Taken together, these developments highlight both the potential for cell-based immunotherapy to expand beyond T cells and the applicability of the CAR technology across a variety of immune cell types.

Engineering CAR-T Cell Interactions with the Tumor and TME

The immune-evasive and immunosuppressive nature of the TME contributes to the poor therapeutic efficacy of CAR-T cells observed in solid tumors. Hallmarks of the TME, which have been extensively reviewed elsewhere (Gajewski et al., 2013; Whiteside, 2008), include (1) physical barriers to tumor penetration by immune cells, (2) upregulated checkpoint ligands, (3) a pro-tumor stromal niche, (4) abundant immunosuppressive and pro-metastatic soluble factors, and (5) modulated expression of chemokines to preferentially recruit leukocytes with an immunosuppressive phenotype. These factors have in turn driven the design of CAR-T cells that respond to TME elements to enhance CAR-T cell efficacy (Figure 5).

Tumor Homing and Penetration

The efficacy of CAR-T cell therapy in solid tumors is significantly hindered by poor immune-cell infiltration (Newick et al., 2017). T-cell migration is regulated through chemokine axes. Tumor cells can upregulate or downregulate chemokines, as well as modulate chemokine expression by tumor-associated cells, contributing to the poor recruitment of CAR-T cells (Oelkrug and Ramage, 2014). Engineering CAR-T cells to overexpress receptors for chemokines that are overexpressed in the TME can turn a tumor's defense mechanism against itself. For example, GD2- and mesothelin-targeting CAR-T cells have been engineered to co-express CCR2b, the dominant isoform of the chemokine receptor of CCL2, resulting in enhanced T-cell homing to CCL2-expressing neuroblastoma and malignant pleural mesothelioma xenografts, respectively (Craddock et al., 2010; Moon et al., 2011). Similarly, CAR-T cells that co-express CCR4 showed improved migration towards tumors expressing CCL17 and CCL22 *in vivo*, while those expressing CXCR1 or CXCR2 exhibited enhanced homing towards tumor-derived IL-8 (Di Stasi et al., 2009; Jin et al., 2019). Once CAR-T cells reach the tumor site, their infiltration is hindered by the high-density structural extracellular matrix (ECM) associated with solid tumor nodules. Accordingly, CAR-T cells engineered to express heparinase, an enzyme that degrades ECM, have been shown to improve tumor infiltration and overall survival in multiple xenograft models (Caruana et al., 2015).

CAR-T cells that successfully reach solid tumors are next faced with a multitude of suppressive and evasive features that induce CAR-T cell dysfunction (Newick et al., 2017). To improve therapeutic efficacy in this immunosuppressive environment, CAR-T cells have been engineered to produce proteins that (1) improve CAR-T cell function in an autocrine fashion; (2) disrupt immunosuppressive elements; and/or (3) induce TME remodeling to enhance the endogenous antitumor immune response (Figure 5). Each of these strategies is discussed in detail below.

Autocrine Stimulation of CAR-T Cells in the TME

Cytokines are signaling proteins with the ability to drastically augment or abrogate CAR-T cell function. Co-expressing the CAR with immunostimulatory cytokines could significantly enhance CAR-T cell proliferation, survival, and effector function in the immunosuppressive TME. For example, T cells that constitutively co-express a CD19-targeting CAR plus IL-2, IL-7, IL-15, or IL-21 have been shown to achieve greater *in vivo* tumor control compared to T cells expressing the CAR alone (Markley and Sadelain, 2010). Interestingly, although the receptor complexes of these four cytokines contain the common gamma chain (γ_c), each cytokine differentially impacted the proliferation, subtype differentiation, and function of the engineered T cells, underscoring the complexity of T-cell biology and variety of potential outcomes achievable through different engineering strategies (Markley and Sadelain, 2010). T cells co-expressing IL-12, IL-15, IL-18, and/or IL-21 plus a CAR targeting a variety of antigens have also been described, resulting in improved efficacy, proliferation, and/or persistence *in vivo* (Batra et al., 2020; Hoyos et al., 2010; Hu et al., 2017; Koneru et al., 2015; Krenciute et al., 2017; Pegram et al., 2012). However, constitutive overexpression of immunostimulatory cytokines can also increase toxicity (Zhang et al., 2011). Regulatory strategies previously discussed in this review, such as inducible promoters, can be implemented to modulate cytokine production and associated toxicity (Liu et al., 2019).

Disruption of Immunosuppressive Axes

The expression of immune-checkpoint receptors and ligands such as PD-1 and PD-L1 are prevalent in the TME, and they can potently inhibit CAR-T cell cytotoxicity and induce anergy (Drake et al., 2006). Thus, immune-checkpoint blockade has strong synergistic potential with CAR-T cell therapy, and several ongoing clinical trials are evaluating combination therapy with CAR-T cells and exogenously administered checkpoint inhibitors (Grosser et al., 2019). Furthermore, CAR-T cells have been engineered to secrete immune-checkpoint inhibitors, including anti–PD-1 scFvs and anti–PD-L1 antibodies, or to express PD-1 dominant-negative receptors (DNRs) (Chen et al., 2017; Cherkassky et al., 2016; Li et al., 2017; Rafiq et al., 2018; Suarez et al., 2016). In addition to enhancing efficacy, this approach may also avoid toxicities associated with systemic immune-checkpoint blockade by restricting checkpoint inhibitor distribution to the immediate environment of the producer T cells. For example, it has been shown that anti–PD-1 scFvs secreted by intraperitoneally (IP) injected CAR-T cells remained localized at the injection site. However, when an equal number of conventional CAR-T cells were administered IP with exogenous anti–PD-1 antibody, the antibody was detected systemically within three hours (Rafiq et al., 2018).

The solid-tumor milieu also houses a diverse collection of soluble factors that promote tumorigenesis and inhibit CAR-T cell function. For example, prostaglandin E2 (PGE_2) is a bioactive lipid often upregulated in tumors, where it contributes to tumor survival through regulation of cell proliferation, migration, apoptosis, and angiogenesis (Ricciotti and FitzGerald, 2011; Wang and Dubois, 2006). In the context of CAR-T cell therapy, PGE_2, along with adenosine, inhibits T-cell signaling and activation though the activation of protein kinase A (PKA), thereby reducing T-cell proliferation and effector function (Newick et al., 2016). In two solid-tumor models that highly express PGE_2, CAR-T cells engineered to express a peptide inhibitor of ezrin-mediated PKA translocation to the immune synapse exhibited improved tumor infiltration and killing (Newick et al., 2016). Similarly, elevated concentrations of bio-reactive chemicals such as reactive oxygen species (ROS) in the TME play an important role in tumorigenesis (Weinberg et al., 2019). Catalase is an enzyme that facilitates the decomposition of hydrogen peroxide (H_2O_2), an ROS

that impairs T-cell activity in the TME. Increasing intracellular levels of catalase by co-expressing the *catalase* gene in HER2- and carcinoembryonic antigen (CEA)-specific CAR-T cells has been shown to enable CAR-T cells to metabolize the suppressive H_2O_2, improving their tumor cytolytic capacity (Ligtenberg et al., 2016).

The aberrant expression of cytokines in the TME plays a critical role in tumor progression and resistance to CAR-T cell therapy. In particular, TGF-β plays a multiplexed role in cancer progression through interactions with tumor cells, stroma, and both innate and adaptive immune cells to induce (1) the secretion of immunosuppressive chemokines, cytokines, and growth factors; (2) ECM remodeling and matrix deposition; (3) immunosuppressive reprogramming of macrophages, neutrophils, and T cells; and (4) inhibited maturation or proliferation of T cells and NK cells (Pickup et al., 2013). To ablate these powerful effects, CAR-T cells have been engineered to express a TGF-β DNR that potently inhibits endogenous TGF-β signaling, resulting in T cells with enhanced proliferation and antitumor efficacy in a prostate cancer xenograft model (Kloss et al., 2018). Based on these results, a phase-I clinical trial has been initiated to assess T cells co-expressing a PSMA CAR and the DNR for the treatment of relapsed and refractory metastatic prostate cancer (NCT03089203). The DNR is distinct from the TGF-β–targeting CAR and TGF-β switch receptor discussed in a previous section in that the DNR does not transduce any signals that can stimulate the engineered T cell. It remains to be seen whether the stimulatory effects of the CAR and switch receptors will confer additional clinical benefits compared to the DNR.

In the tumor microenvironment, IL-6 is often overexpressed by tumor cells, tumor associated macrophages (TAMs), and other resident cells (Kumari et al., 2016). IL-6 supports tumorigenesis through a number of mechanisms, and plays a central role in the induction of CRS after CAR-T cell infusion (Lee et al., 2014). Systemic administration of tocilizumab, an mAb targeting IL-6 receptor alpha (IL-6Rα), has become standard treatment for CRS after CAR-T cell therapy (Kotch et al., 2019). More recently, CD19-targeting CAR-T cells that co-express a non-signaling, membrane-bound IL-6 receptor (mbaIL6) were shown to sequester IL-6 while retaining *in vivo* antitumor efficacy (Tan et al., 2020). However, it remains to be seen if CAR-T cells engineered in this fashion can prevent CRS.

TME Remodeling to Promote the Endogenous Immune Response

Tumors are adept at selectively attracting or evading subsets of leukocytes, including CAR-T cells, to promote immune regulation or suppression (Rabinovich et al., 2007). In addition, tumors are often capable of inducing an immunosuppressive or pro-metastatic phenotype on the local stroma, as well as an anti-inflammatory or dysfunctional phenotype on resident leukocytes (Morgan and Schambach, 2018). Another approach to enhancing the efficacy of CAR-T cell therapy is to reverse this immunosuppressive-cell niche through remodeling the tumor-cellular composition and phenotype. To realize this, CAR-T cells have been be engineered to secrete cytokines or other soluble factors that induce TME remodeling in a paracrine or endocrine fashion.

In germinal-center lymphomas, loss of herpesvirus entry mediator (HVEM) expression induces the secretion of non-redundant stroma-activating factors, resulting in acute lymphoid-stroma activation. The hyperactivated stroma recruits T_{FH} cells, which support malignant B cells through CD40/CD40L interactions and cytokine stimulation. As a counterstrategy, CD19 CAR-T cells

engineered to secrete a soluble form of HVEM were shown to enhance tumor control *in vivo* (Boice et al., 2016).

CAR-T cells engineered to secrete IL-12 have been shown to remodel the TME by reprogramming TAMs to an M1 phenotype and decreasing the presence of MDSCs and Tregs in syngeneic mouse models (Chinnasamy et al., 2012; Liu et al., 2019; Yeku et al., 2017). Similarly, CAR-T cells that constitutively secrete IL-18 can alter the TME makeup by increasing intratumoral M1 macrophage, activated dendritic cell (DC), and activated NK cell numbers, while decreasing M2 macrophage and Treg levels. A direct comparison of IL-12– to IL-18–expressing CAR-T cells indicated that IL-18 is more effective at remodeling the immunosuppressive TME in a syngeneic murine pancreatic-cancer model (Chmielewski and Abken, 2017). Furthermore, CD19 CAR-T cells expressing IL-18 induced the expansion of endogenous $CD8^+$ T cells, NK cells, NKT cells, and DCs in the bone marrow, potentially contributing to the control of tumors with heterogenous CD19 expression in a syngeneic mouse model (Avanzi et al., 2018).

Among DCs, conventional type 1 DCs (cDC1s) in particular excel at inducing immunity against tumors via their ability to cross-present cellular antigens and prime Th1 cells. Recently, it has been shown that T cells engineered to secrete Fms-like tyrosine kinase 3 ligand (Flt3L), a hematopoietic cell growth factor, promote intratumoral cDC1 and DC-precursor proliferation (Lai et al., 2020). Furthermore, when T cells were co-transduced to express Flt3L and an anti-HER2 CAR, a combined treatment with these CAR-T cells and adjuvants induced an enhanced antitumor response and endogenous T-cell epitope spreading *in vivo* (Lai et al., 2020).

CAR-T cells have also been engineered to co-express multiple immune-modulatory proteins. In one example, CAR-T cells were programmed to co-express CCL19 and IL-7 to induce endogenous immune-cell recruitment and stimulate the recruited cells, respectively. In a syngeneic hCD20-expressing mastocytoma mouse model, these CAR-T cells induced robust recruitment of endogenous T cells and DCs, resulting in enhanced and durable tumor clearance (Adachi et al., 2018).

CAR-T cells have also been designed to modulate the TME through the expression of surface-bound proinflammatory ligands. For example, CD40L is normally transiently expressed on T cells after TCR stimulation, and its interaction with the CD40 receptor on different immune cell types can lead to activation of APCs, licensing of DCs, as well as apoptosis of $CD40^+$ tumor cells (Cella et al., 1996; Eliopoulos et al., 2000; Ridge et al., 1998). Constitutive CD40L expression on CD19 CAR-T cells resulted in elevated surface expression of costimulatory molecules, adhesion molecules, HLA molecules, and the Fas death receptor on $CD40^+$ tumor cells, thus increasing their immunogenicity (Curran et al., 2015). These T cells also induced the secretion of proinflammatory IL-12 by monocyte-derived DCs *in vitro*, and showed enhanced antitumor efficacy *in vitro* and *in vivo* (Curran et al., 2015). It was subsequently demonstrated that CD40L-expressing CAR-T cells can license APCs in lymphatic tissues in a syngeneic immunocompetent mouse model, and this licensing was found to be dependent on the CD40L/CD40 interaction (Kuhn et al., 2019). Furthermore, increased recruitment of macrophages, DCs, and endogenous $CD4^+$ and $CD8^+$ T cells to lymphatic tissues was observed, along with the recruitment of DCs, $CD4^+$ and $CD8^+$ T cells to the tumor. Treg levels were also observed to slightly increase in the tumor, but the ratio of $CD8^+$ T cells to Tregs was unchanged. Thus, CD40L-expressing CAR-T cells capable of remodeling the TME and lymphatic tissue activated endogenous T cells to

suppress antigen-negative tumor re-challenge, strongly suggesting induced epitope spreading (Kuhn et al., 2019). Similarly, the surface expression of 4-1BBL on CAR-T cells is proposed to remodel the TME through autocrine-induced secretion of type I IFNs, which may improve DC cross-priming, Treg inhibition, and angiogenesis suppression (Zhao et al., 2015).

Finally, CAR-T cells can be engineered to facilitate the engagement of tumor cells by endogenous, non-engineered T cells through the secretion of bispecific T-cell engagers (BiTEs), which are composed of two fused scFvs. Choi et al. engineered BiTEs with one scFv targeting EGFR, which are overexpressed in glioblastoma cells, and the other targeting CD3 on T cells. EGFRvIII-targeting CAR-T cells engineered to secrete EGFR/CD3 BiTEs have been shown to eliminate orthotopic tumor xenografts with heterogenous EGFRvIII expression (Choi et al., 2019).

Conclusion

CAR-T cell therapy has shown great promise in treating hematologic malignancies. However, solid tumors pose unique challenges that require further engineering and tuning of the technology to successfully treat these intractable malignancies. Recent protein- and cell-engineering strategies have made great strides in boosting the intrinsic fitness and anti-tumor function of T cells, increasing tumor-targeting specificity, preventing tumor escape and relapse, as well as modifying the TME to enhance immunotherapeutic outcomes. Although most engineering strategies reported to date have focused on delivering individual desirable features, advancements in genome-editing methodologies and genetic circuitry design offer the possibility of multi-layered approaches that can simultaneously address multiple critical needs in T-cell therapeutics development.

At the same time, the biological complexity of and potential crosstalk among different engineered features within the T cell, as well as among engineered and endogenous immune cells, tumor cells, and other tumor-associated factors, must be carefully balanced when advancing the clinical translation of CAR-T cells for the treatment of solid tumors. The decreasing cost and increasing capacity of next-generation and single-cell sequencing methods, as well as proteomic and metabolomic analyses, could significantly enhance our ability to understand and rationally manipulate these complex interactions while engineering the next generation of CAR-T cell therapy for solid malignancies. The growing toolbox of T-cell engineering strategies that can be synergistically implemented and modularly calibrated for maximum safety and efficacy will continue to enable innovations that aim to generate new treatment options for currently intractable diseases.

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Figure Legends

Figure 1. CAR-T Cell Engineering Approaches
Strategies to engineer CAR-T cells for improved function in solid tumors include a focus on CAR engineering, T-cell engineering, and TME interaction optimization.

Figure 2. Protein Engineering Strategies to Improve the Programmability, Safety, and Efficacy of CAR-T Cells
(A) Combinatorial antigen recognition by AND- and AND-NOT logic using a synNotch receptor and iCAR, respectively, can increase antigen specificity and safety. Tandem bispecific OR-gate CARs can circumvent antigen escape and increase efficacy.
(B) Engineered ON and OFF switches can easily and efficiently alter CAR-T cell activity.
(C) Programming CARs to activate only in the presence of an adaptor or by leucine-zipper–mediated reconstitution can increase controllability over CAR-T cell activity.

Figure 3. Engineering strategies to improve CAR-T cell safety
(A) Co-expression of suicide genes such as HSK-TV, iCasp9, CD20, and tEGFR enables induction of T-cell death to abort the therapy in the case of adverse events.
(B) Tet-ON and -OFF systems allow the control of the CAR expression on the transcriptional level.

Figure 4. Rewiring T-cell signaling with synthetic receptors
Switch receptors rewire T-cell responses by triggering co-stimulatory signaling in the presence of normally inhibitory ligands. CARs responsive to environmental cues such as soluble TGF-β or surface antigens present on tumor-supportive tissues can enhance anti-tumor function by removing and converting immunosuppressive factors.

Figure 5. Strategies in Optimizing CAR-T Cell and Tumor Interactions
CAR-T cells have been engineered to utilize, reverse, or circumvent tumor-driven immunosuppressive factors and axes through a variety of mechanisms.

Tables

Table 1. Targeted genome-editing strategies to enhance T-cell function

Target locus[a]	Motivation[b]	Technology[c]	Reference
TRAC	Ablate TCRαβ expression to reduce alloreactivity	ZFN	(Torikai et al., 2012)
		TALEN	(Qasim et al., 2017; Valton et al., 2015)
		CRISPR/Cas9	(Georgiadis et al., 2018)
		CRISPR/Cas9 CAR knock-in	(Eyquem et al., 2017; MacLeod et al., 2017)
TRAC, TRBC	Enhance transgenic TCR expression	CRISPR/Cas9	(Stadtmauer et al., 2020)
B2M	Ablate HLA expression to reduce alloreactivity	CRISPR/Cas9	(Ren et al., 2017b)
HLA-A	Ablate HLA expression to reduce alloreactivity	ZFN	(Torikai et al., 2013)
CD52	Confer resistance to lymphodepletion	TALEN	(Qasim et al., 2017)
dCK	Confer resistance to lymphodepletion	TALEN	(Valton et al., 2015)
PD-1	Inhibit immune-checkpoint signaling	TALEN	(Menger et al., 2016)
		CRISPR/Cas9	(Liu et al., 2017; Ren et al., 2017a; Rupp et al., 2017; Stadtmauer et al., 2020)
REGNASE-1	Disrupt a negative regulator of T-cell activity	CRISPR/Cas9	(Wei et al., 2019)
DGKα, DGKζ	Disrupt a negative regulator of T-cell activity	CRISPR/Cas9	(Jung et al., 2018)
LAG3	Disrupt a negative regulator of T-cell activity	CRISPR/Cas9	(Zhang et al., 2017)
FAS	Abolish pro-apoptotic signaling	CRISPR/Cas9	(Ren et al., 2017b)
GM-CSF	Inhibit CRS-related toxicities	CRISPR/Cas9	(Sterner et al., 2019)

[a]*TRAC*, T-cell receptor alpha constant; *TRBC*, T-cell receptor beta constant; *B2M*, Beta-2 microglobulin; *HLA*, human leukocyte antigen; *dCK*, deoxycytidine kinase; *PD-1*, programmed cell death protein 1; *DGK*, diacylglycerol kinase; *LAG3*, lymphocyte-activation gene 3; *GM-CSF*, granulocyte-macrophage colony-stimulating factor.
[b]TCR, T-cell receptor; CRS, cytokine release syndrome.
[c]ZFN, zinc-finger nuclease; TALEN, transcription activator-like effector nuclease; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9.

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Engineering CAR-T cells for next-generation cancer therapy

Deleted: Multilayered CAR-T Cell Engineering Strategies for Next-Generation Cancer Therapy¶

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Summary

T cells engineered to express chimeric antigen receptors (CARs) with tumor specificity have shown remarkable success in treating patients with hematologic malignancies and revitalized the field of adoptive cell therapy. However, realizing broader therapeutic applications of CAR-T cells necessitates engineering approaches on multiple levels to enhance efficacy and safety. Particularly, solid tumors present unique challenges due to the biological complexity of the solid-tumor microenvironment (TME). In this Review, we highlight recent strategies to improve CAR-T cell therapy by engineering (1) the CAR protein, (2) T cells, and (3) the interaction between T cells and other components in the TME.

Keywords: cancer immunotherapy, chimeric antigen receptor (CAR), CAR-T cells, T-cell engineering, solid tumors, tumor microenvironment (TME), mammalian synthetic biology

Introduction

Chimeric antigen receptors (CARs) are synthetic receptors that enable T cells to recognize tumor-associated antigens (TAAs) in a major histocompatibility complex (MHC)-independent manner. CAR-T cells targeting the pan–B-cell marker CD19 have shown unprecedented response rates in treating refractory B-cell malignancies (Maude et al., 2014; Neelapu et al., 2017) and became the first genetically modified cell-based therapy to receive FDA approval (Bouchkouj et al., 2019; O'Leary et al., 2019). However, the development of effective CAR-T cell therapy for non–B-cell malignancies has required more sophisticated engineering approaches to overcome tumor-defense mechanisms such as immunosuppression, antigen escape, and physical barriers to entry into solid tumors. In this review, we examine current and prospective strategies to engineer CARs, T cells that express CARs or tumor-specific T-cell receptors (TCRs), and the interaction between engineered T cells and the tumor microenvironment (TME), with particular focus on improving the efficacy and safety of adoptive T-cell therapy for the treatment of solid tumors (Figure 1).

Engineering the CAR protein

Evolution of CAR Designs

Kuwana et al. reported the first proof of principle of combining antibody-type antigen specificity with T-cell signaling by fusing the TCR constant region to the variable regions of a bacterial antigen-recognizing antibody (Kuwana et al., 1987). Single-chain variable fragments (scFvs), comprised of the variable heavy (V_H) and light (V_L) chains of a monoclonal antibody (mAb) separated by a flexible linker, are still commonly used as the extracellular antigen-sensing domain of CARs. The first reports of tumor-targeting CARs demonstrated that an scFv recognizing antigens like human epidermal growth factor receptor 2 (HER2) fused to the CD3ζ signaling domain can elicit tumor-specific cytotoxicity (Eshhar et al., 1993; Moritz et al., 1994; Stancovski et al., 1993), but T cells expressing these “first-generation” CARs that included only the CD3ζ chain for T-cell signaling generally failed to elicit potent antitumor effects.

In the following years, second- and third-generation CARs emerged that included one or two costimulatory domains, respectively, drawing from the biological understanding that the endogenous TCR requires association with other costimulatory or accessory molecules for robust signaling (Chen and Flies, 2013). Most commonly derived from CD28 or 4-1BB, these costimulatory domains conferred more potent antitumor cytotoxicity, increased cytokine production, and improved proliferation and persistence of CAR-T cells (Haynes et al., 2002; Imai et al., 2004). The choice of costimulatory domain impacts a wide range of properties including metabolic pathways (Kawalekar et al., 2016), T-cell memory development (Kalos et al., 2011; Kawalekar et al., 2016), and antigen-independent tonic signaling (Long et al., 2015), prompting further research into other costimulatory domains. For example, a third-generation CAR with OX40 and CD28 costimulatory domains repressed CD28-induced secretion of interleukin (IL)-10, an anti-inflammatory cytokine that compromises T-cell activity (Hombach et al., 2012). Additionally, the inducible T-cell costimulator (ICOS) costimulatory domain in combination with either CD28 or 4-1BB costimulation increased *in vivo* persistence, and MyD88/CD40 costimulation improved *in vivo* proliferation of CAR-T cells (Collinson-Pautz et al., 2019; Guedan et al., 2018).

More recently, fourth-generation CARs that incorporate additional stimulatory domains, commonly referred to as "armored" CARs, have been reported. In one example, Chmielewski et al. engineered armored CAR-T cells termed "T cells redirected for universal cytokine-mediated killing" (TRUCK) to secrete the proinflammatory cytokine IL-12 to stimulate innate immune cells against the tumor and resist inhibitory elements of the TME, including regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs) (Chmielewski et al., 2014; Pegram et al., 2012). The secretion of other soluble factors have been studied, including IL-15 or IL-18 to enhance T-cell proliferation, as well as the combination of CCL19 and IL-7 to recruit endogenous immune cells and establish a memory response against tumors (Adachi et al., 2018; Hoyos et al., 2010; Hu et al., 2017).

In addition to the evolution of CAR designs outlined above, the modularity of the four major components of a CAR—extracellular antigen-sensing domain, extracellular hinge or spacer domain, transmembrane domain, and intracellular signaling domain—has enabled further optimization of each of these components to improve the efficacy of CAR-T cell therapy. These engineering efforts are well-summarized in other reviews (Labanieh et al., 2018; Rafiq et al., 2020). Here, we focus our attention on strategies that enable T cells to expand beyond the hard-wired, single-input, single-output signaling capability programmed by conventional CAR designs (Figure 2).

Combinatorial Antigen Sensing for Logic-Gated T-Cell Activation

Boolean logic gates have been utilized for the combinatorial detection of multiple antigens by CAR-T cells to improve their safety and antitumor efficacy (Figure 2A). AND-gate logic requires the co-presence of two different antigens to activate the CAR-T cell, and this increased specification reduces the risk of either off-target recognition or "on-target, off-tumor" toxicities, in which healthy tissues that express the same antigen as tumor cells suffer collateral damage. The synthetic Notch (synNotch) receptor—which triggers inducible target-gene expression upon recognition of a cell surface-bound ligand—was engineered to recognize a TAA and induce the expression of a CAR, which can subsequently trigger T-cell activation upon recognizing a second TAA (Roybal et al., 2016). This strategy has been shown to reduce systemic toxicity compared to constitutive CAR expression, provided that the off-tumor target is not spatially proximal to the tumor cells (Srivastava et al., 2019). Since there is a temporal delay between the recognition of TAA #1 by synNotch and the recognition of TAA #2 by CAR, a given T cell could have its synNotch receptor triggered by TAA #1 from a tumor cell but subsequently attack a healthy cell expressing TAA #2. An alternative AND-gate approach separates the CD3ζ chain and costimulatory domain into two constitutively expressed receptors each recognizing different antigens, such that the CAR-T cell is optimally activated only in the simultaneous presence of both antigens (Kloss et al., 2013; Wilkie et al., 2012). However, this approach often suffers from "leakiness" due to the fact that first-generation CARs containing only the CD3ζ chain are already signaling-competent. Yet another strategy programs T cells to deliver a conditionally active cytotoxic protein upon CAR- or TCR-mediated detection of TAA #1 on the cell surface; the engineered protein becomes cytotoxic if and only if it detects TAA #2 inside the target cell, thus requiring both antigens to be expressed by the same target cell to trigger robust killing (Ho et al., 2017).

CAR-T cells programmed to execute AND-NOT logic can also help prevent toxicities against healthy cells. This strategy utilizes an inhibitory CAR (iCAR) that targets an antigen found on

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healthy tissue, and pairs it with an activating CAR that targets a TAA. In a proof-of-principle study, a prostate-specific membrane antigen (PSMA)-targeting iCAR that incorporates the programmed cell death protein 1 (PD-1) inhibitory signaling domain was co-expressed with a second-generation CD19 CAR, and the iCAR inhibited CAR-T cell activation in the presence of PSMA (Fedorov et al., 2013).

While AND and AND-NOT logic can improve the safety of CAR-T cells by increasing specificity, OR-gate logic has been utilized to increase antitumor efficacy by circumventing antigen escape, or loss of the targeted epitope by tumor cells. An OR-gate CAR can recognize two different TAAs, and the binding of *either* antigen induces T-cell activation. One OR-gate strategy utilizes the pooled mixture of two populations of CAR-T cells (CARpool), each expressing a monospecific CAR. A variation on this theme is to sequentially administer two different CAR-T cell products (Shah et al., 2020; Shalabi et al., 2018). Another strategy is the co-expression of two separate CARs in each T cell (dual CAR) (Ruella et al., 2016). Yet another approach uses tandem bispecific CARs (TanCAR) that comprise two scFv domains separated by a linker on one receptor chain, and this strategy was shown to be functionally superior to both the CARpool and dual-CAR approaches (Hegde et al., 2016; Zah et al., 2020). In particular, CD19/CD20 and CD19/CD22 bispecific CARs have been characterized for the treatment of B-cell malignancies (Fry et al., 2018; Qin et al., 2018; Zah et al., 2016), and are in clinical trials for lymphoma and ALL, respectively (NCT04007029, NCT04215016, NCT03919526, NCT04303520).

ON/OFF Switches for Controllability and Safety

CAR modifications to improve safety and controllability have also taken the form of externally inducible or self-regulating ON/OFF switches. Conventional CAR-T cells are “always on,” meaning their CARs are constitutively expressed and are always capable of signaling upon antigen stimulation. However, this is not always desirable when CAR-derived toxicity is an anticipated risk. In addition to the off-target and “on-target, off-tumor” toxicities discussed previously, various systemic toxicities have also been observed in patients treated with CAR-T cells. Common examples include cytokine release syndrome (CRS) (Fitzgerald et al., 2017; Grupp et al., 2013; Schuster et al., 2017), neurotoxicity or “CAR-related encephalopathy syndrome” (CRES) (Grupp et al., 2013; Lee et al., 2019a; Schuster et al., 2017), tumor lysis syndrome (TLS), and anaphylaxis (Maus et al., 2013). It has been hypothesized that modulation of CAR-T cell activity in space and time can prevent or significantly lessen the severity of toxicities associated with CAR-T cell therapy while maintaining its antitumor efficacy.

One approach to modulate CAR-T cell activity is to regulate the presence of functional CARs on the surface of engineered T cells by adjusting either the stability or the conformation of the CAR protein itself (Figure 2B). As examples of the former strategy, CARs have been fused to degradation tags that can be inactivated either by small-molecule binding (Weber et al., 2020) or under hypoxic conditions (Juillerat et al., 2017). The default state in such systems is “off,” and the removal or inactivation of the degradation tag is required to stabilize the CAR protein, thus enabling CAR-mediated T-cell activation upon antigen stimulation. An alternative design in which the CAR protein is present by default but turned off through the administration of a small-molecule drug has also be demonstrated (Juillerat et al., 2019).

Instead of modulating protein half-life, controlling the conformation of the CAR protein can also regulate the availability of functional CARs, such that the receptor is signaling-competent only under specified conditions. For example, the antigen-binding domain of CARs can be "masked" by a built-in inhibitory peptide, such that the CAR's functional conformation is acquired only after the inhibitory peptide has been cleaved by proteases commonly active in the TME (Han et al., 2017).

Adapter-Dependent CARs

Alternatively, T cells can be engineered to express a receptor that must be complemented by an additional protein component before the resulting complex is capable of translating antigen recognition to T-cell activation (Figure 2C). Urbanska et al. reported a "universal" receptor comprising a biotin-binding domain fused to an intracellular T-cell signaling domain. T cells expressing this biotin-specific receptor can, in principle, be directed against any target cell that has been labeled with a biotinylated antibody (Urbanska et al., 2012). Two advantages of this strategy include the abilities to (1) control the ON/OFF state of the T cell through administering or withholding the biotinylated antibody and (2) use the same biotin-specific receptor to target a wide variety of TAAs by changing the specificity of the biotinylated antibody. The concept of using a universal CAR coupled with one or more antigen-targeting adaptor proteins to overcome tumor heterogeneity and mitigate toxicity soon opened the floodgates for other adapter-dependent CAR designs (Bachmann, 2019; Cartellieri et al., 2016; Herzig et al., 2019; Lee et al., 2019c; Qi et al., 2020; Raj et al., 2019; Rodgers et al., 2016), including a small molecule adapter, which binds to both fluorescein isothiocyanate (FITC) and folate, that alleviated CRS-like toxicity in an NSG mouse model (Lee et al., 2019b).

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Expanding upon the concept of constitutively expressing a universal receptor on the T-cell surface combined with an externally administered adaptor protein to dictate antigen specificity, Cho et al. reported a SUPRA CAR system that can program a variety of Boolean logic gates in engineered T cells by expressing multiple base receptors, each with multiple potential adaptor protein partners reconstituted by leucine-zipper dimerization (Cho et al., 2018). Such a system supports the possibility of simultaneously increasing specificity through the use of AND or AND-NOT gates while addressing tumor heterogeneity by targeting multiple antigens with different adaptor proteins. However, the versatility of multi-component systems comes at the cost of an increased number of parameters that must be optimized, including the half-life, biodistribution, and interaction dynamics among the adaptor protein, base receptor, and the engineered T cell itself. Therefore, it remains to be seen whether the complex signal processing achievable through adaptor-dependent CAR designs will translate to robust therapeutic candidates.

Engineering the CAR-Expressing Cell

Safety Controls on CAR-T Cell Activity

In addition to engineering the CAR protein itself, engineering approaches applied at a cellular level to modulate T-cell activity can also significantly impact therapeutic efficacy and safety. For example, transient CAR expression resulting from mRNA electroporation instead of viral integration can mitigate toxicities induced by CARs that cross-recognize healthy tissue (Beatty et

al., 2014; Tchou et al., 2017; Wiesinger et al., 2019). Another safeguard against severe toxicity is the implementation of suicide genes that enable the depletion of engineered T cells by the administration of small-molecule drugs (Figure 3A) (Marin et al., 2012). For example, CAR-T cells targeting the CD44v6 antigen for the treatment of leukemia and myeloma have been engineered to express herpes simplex virus thymidine kinase (HSV-TK), which enabled effective elimination of the CAR-T cells upon exposure to ganciclovir *in vitro* (Casucci et al., 2018). However, expression of viral HSV-TK raises concerns of immunogenicity and requires three days of ganciclovir exposure to achieve effective T-cell elimination (Marin et al., 2012). An alternative approach using the inducible caspase 9 (iCasp9) suicide gene has been shown to eliminate >90% of engineered cells within 30 minutes of drug administration in human patients (Di Stasi et al., 2011). iCasp9 contains the intracellular domain of the pro-apoptotic protein caspase 9 fused to FK506-binding protein (FKBP). The small molecule AP1930 facilitates the dimerization of FKBP and activation of the fused caspase 9, inducing apoptosis in cells expressing the iCasp9 protein (Straathof et al., 2005). Additionally, the expression of transgenes for the surface proteins CD20 or truncated epidermal growth factor receptor (tEGFR) can also facilitate suicide mechanisms. The FDA-approved mAbs rituximab and cetuximab bind to CD20 and tEGFR, respectively, and the resulting antibody-dependent cellular cytotoxicity (ADCC) can be used to eliminate T cells engineered to express these antigens (Griffioen et al., 2009; Serafini et al., 2004; Wang et al., 2011).

While suicide genes can efficiently deplete CAR-T cells to counter toxicities, the activation of a suicide gene also results in the irreversible termination of the therapy. An alternative reversible strategy utilizes dasatinib, a tyrosine kinase inhibitor that interferes with the lymphocyte-specific protein kinase (LCK), thus inhibiting CD3ζ phosphorylation and CAR activation. Dasatinib has been shown to function as a reversible ON/OFF switch of CAR-T cell activity, where the cessation of dasatinib administration rapidly reversed its inhibitory effects, highlighting its potential as an emergency drug for potentially lethal toxicities such as CRS and CRES (Mestermann et al., 2019; Weber et al., 2019).

Regulated CAR Expression to Improve CAR-T Cell Safety

As an alternative to post-translational regulation of CAR stability and function, transcriptional regulation can provide another tunable handle to improve CAR-T cell safety (Figure 3B). For example, in the Tet-ON system, the small molecule doxycycline (Dox) acts as an “ON switch,” where CAR expression is induced by the reverse tetracycline transactivator (rtTA) protein only in the presence of Dox (Sakemura et al., 2016). Conversely, in the Tet-OFF system, Dox acts as an “OFF switch” by abolishing the ability of tetracycline transactivator (tTA) to activate CAR transcription; this system was used to reversibly inhibit deleterious CAR signaling and T-cell fratricide in CD5 CAR-T cells (Mamonkin et al., 2018). Additionally, response to a tumor-associated environmental cue could be achieved through inducible promoters responsive to hypoxia-inducible factor (HIF)-1α, activating CAR expression only in hypoxic environments (Ede et al., 2016). Finally, as previously described, the synNotch receptor enables inducible CAR transcription upon binding to a membrane-bound ligand (Roybal et al., 2016; Srivastava et al., 2019).

Site-Specific CAR Transgene Insertion and Allogeneic Compatibility Engineering

The examples of regulated gene expression provided above utilize synthetic inducible promoters, and the entire gene-expression cassette is typically integrated into the T-cell genome using retroviral or lentiviral vectors, leading to variable integration sites and copy numbers. An alternative method to achieve dynamic CAR expression profiles is to integrate the transgene into specific genetic loci regulated by endogenous transcriptional machinery. A variety of gene-editing technologies including clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9), transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs) have made this a feasible approach in T-cell engineering (Chen, 2015). For example, Eyquem et al. demonstrated that CRISPR/Cas9-mediated insertion of the CD19 CAR transgene into the TCRα constant (*TRAC*) locus results in CAR-T cells with superior *in vivo* function compared to retrovirus-mediated random CAR-transgene insertion (Eyquem et al., 2017), although more recent evidence suggests whether site-specific CAR integration into the *TRAC* locus improves T-cell function may depend on the specific CAR construct used (Zah et al., 2020). A compelling example of the transgene insertion site influencing CAR-T cell function comes from the case of a chronic lymphocytic leukemia (CLL) patient, whose disease regression was observed to coincide with the clonal expansion of T cells carrying the CD19 CAR transgene inserted into the methylcytosine dioxygenase ten-eleven translocation 2 (*TET2*) locus (Fraietta et al., 2018). Combined with a pre-existing hypomorphic mutation in the patient's other *TET2* allele, this CAR insertion resulted in the loss of wildtype *TET2*, a gene that encodes for a regulator of DNA methylation and blood cell formation. This fortuitous CAR insertion/*TET2* ablation event led to an altered epigenetic landscape that conferred profound proliferative capability and a central-memory phenotype to the engineered T cells (Carty et al., 2018; Fraietta et al., 2018).

Gene-editing technologies have also enabled the elimination of endogenous genes in support of allogeneic T-cell therapy (Table 1). T-cell products derived from healthy donors can overcome manufacturing challenges associated with autologous cell therapy, such as the difficulty to obtain enough high-quality T cells from heavily pre-treated patients with advanced disease. However, allogeneic T-cell transfer requires the elimination of the endogenous TCR to prevent graft-versus-host disease (GvHD), and the removal of class-I major histocompatibility complex (MHC-I) has been proposed to minimize allograft rejection (Liu et al., 2017). To this end, Torikai et al. demonstrated ZFN-mediated abrogation of TCRαβ or human leukocyte antigen (HLA)-A expression in CD19 CAR-T cells (Torikai et al., 2012; Torikai et al., 2013). In addition to preventing GvHD, gene editing has been utilized to protect CAR-T cells from lymphodepletion, which is a common preconditioning treatment administered prior to CAR-T cell infusion to improve the efficacy of the transferred cells. For example, TALEN-mediated simultaneous disruptions of TCRαβ/CD52 or TCRαβ/deoxycytidine kinase (dCK) have been shown to confer CD19 CAR-T cells with resistance to anti-CD52 or dCK phosphorylation-dependent lymphodepleting regimens, respectively (Qasim et al., 2017; Valton et al., 2015).

In the studies referenced above, endogenous gene disruption and CAR transgene integration were independently executed, yielding heterogeneous CAR-T cell populations. Georgiadis et al. coupled gene-editing to CAR integration by incorporating a single-guide RNA (sgRNA) element into the U3 region of the 3' long terminal repeat (LTR) sequence of the CAR-encoding lentiviral vector. After electroporation of Cas9 mRNA, magnetic bead-based selection for edited cells resulted in highly enriched CAR^+ TCR^- populations (Georgiadis et al., 2018). In another strategy, Ren et al. used a "one-shot" CRISPR system with multiple sgRNA expression cassettes in one CAR-encoding lentiviral vector to simultaneously knock out the endogenous TCR and Beta-2

microglobulin (β2M), an essential subunit of the HLA-I molecule, to generate CD19 CAR-T cells suitable for allogeneic therapy (Ren et al., 2017b).

Knockout of Negative Regulators

Gene-editing technologies can also be used to abrogate the expression of negative regulators of T-cell activity (Table 1). Tumor cells frequently upregulate ligands to immune checkpoint receptors, such as cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and PD-1 expressed on T cells, leading to inhibition of T-cell activity in the TME (Buchbinder and Desai, 2016). Immune-checkpoint blockade using antibodies targeting CTLA-4, PD-1, or PD-1 ligand (PD-L1) has revolutionized the field of immuno-oncology in recent years (Wei et al., 2018). An alternative approach to checkpoint blockade is the ablation of checkpoint-receptor expression on engineered T cells. Menger et al. demonstrated that PD-1 knockout through TALEN-mediated editing in melanoma-reactive $CD8^+$ T cells and fibrosarcoma-reactive polyclonal T cells enhanced the persistence of the modified T cells, augmenting their antitumor activity against syngeneic tumor models and establishing long-term antitumor memory (Menger et al., 2016). Additionally, CRISPR/Cas9-mediated disruption of PD-1 in CD19 CAR-T cells enhanced CAR-T cell cytotoxicity toward $PD\text{-}L1^+$ tumor xenografts *in vivo* (Rupp et al., 2017). The first CRISPR/Cas9-edited cell therapy trial conducted in the U.S. evaluated the adoptive transfer of autologous T cells genetically modified to express a New York esophageal squamous cell carcinoma 1 (NY-ESO-1)–targeting TCR while eliminating endogenous TCRαβ and PD-1 expression; recent results from the trial confirmed the safety and feasibility of CRISPR-edited cell therapy for cancer (Stadtmauer et al., 2020).

In addition to PD-1 knockout, elimination of the metabolic regulator REGNASE-1 and CD3-signaling regulator diacylglycerol kinase (DGK) has also been shown to enhance T-cell function *in vitro* and *in vivo* (Jung et al., 2018; Riese et al., 2013; Wei et al., 2019). Similar to PD-1, lymphocyte-activation gene 3 (LAG3) is known as a T-cell exhaustion marker, and it has been found to inhibit the effector function of T cells and enhance the suppressive function of Tregs (Zhang et al., 2017). However, CRISPR/Cas9-mediated deletion of *LAG3* in CD19 CAR-T cells did not result in a discernable phenotype, perhaps due to compensatory effects from PD-1 (Woo et al., 2012; Zhang et al., 2017).

Several studies have reported the feasibility of multiple knockouts using CRISPR/Cas9 in CAR-T cells to achieve the dual goals of reducing alloreactivity and improving T-cell function. Triple-targeting of the *TRAC*, *B2M*, and *PD-1* loci has been demonstrated by electroporation of Cas9:sgRNA ribonucleoprotein (RNP) complex (Liu et al., 2017) or by electroporation of mRNA encoding Cas9 and sgRNAs into CAR-T cells (Ren et al., 2017a). Furthermore, triple targeting of the *TRAC*, *B2M*, and *FAS* loci using a lentiviral vector encoding both the sgRNAs and a CD19-targeting CAR and electroporation of Cas9 mRNA resulted in reduced alloreactivity and prolonged T-cell survival by abrogating pro-apoptotic Fas/FasL signaling (Ren et al., 2017b). Finally, CRISPR/Cas9-mediated deletion has been used to improve the safety of CAR-T cells by disrupting the expression of CRS-associated cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sterner et al., 2019).

Receptors that Rewire an Inhibitory Input to a Stimulatory Output

While CAR-T cells have shown promising therapeutic efficacy against hematologic malignancies, their targeting of solid tumors has been stymied by a number of challenges. In addition to antigen heterogeneity, tumor cells produce tumorigenic and immunosuppressive factors in the TME, and this inhibitory environment is further compounded by immunosuppressive cell types such as MDSCs and Tregs (Labanieh et al., 2018). Among the immunosuppressive soluble factors commonly found in the TME, transforming growth factor beta (TGF-β) is a particularly potent cytokine that drives T-cell differentiation into Tregs, as well as macrophage polarization to the immunosuppressive M2 phenotype (Tormoen et al., 2018). The engineering of a TGF-β–responsive CAR demonstrated that CARs can be used to (1) sense a soluble factor and (2) rewire T-cells to convert an inhibitory signal to a trigger of antitumor activity (Figure 4) (Chang et al., 2018; Hou et al., 2018). TGF-β–responsive CAR-T cells were demonstrated to proliferate and produce T helper type 1 (Th1)-associated cytokines in the presence of soluble TGF-β, as well as protect nearby cells from the inhibitory effects of TGF-β, likely through the combined effects of TGF-β sequestration and paracrine Th1 cytokine signaling (Chang et al., 2018; Hou et al., 2018).

Signal rewiring can also be achieved with “switch receptors,” which are chimeras comprising an ectodomain that binds a suppressive molecule fused to an endodomain that drives a stimulatory pathway (Figure 4). IL-4 is a cytokine with complex roles in the TME, such as inducing M2 polarization, promoting tumor growth, and suppressing tumor-specific effector T cells (Tormoen et al., 2018; Wilkie et al., 2010). IL-4 switch receptors consisting of the IL-4 receptor α (IL-4Rα) ectodomain fused to either the IL-7Rα endodomain or the β_c receptor subunit common to IL-2 and IL-15 signaling have been shown to paradoxically enhance T-cell proliferation in the presence of IL-4 (Leen et al., 2014; Wilkie et al., 2010). Consequently, the co-expression of the IL-4Rα:β_C switch receptor in CAR-T cells augmented their antitumor capacity (Wilkie et al., 2010). More recently, Roth et al. used a pooled knock-in screen using CRISPR/Cas9 coupled with single-cell RNA sequencing (scRNA-seq) to evaluate a panel of transgenes integrated into the *TRAC* locus. Through this analysis, a novel TGFβR2:4-1BB switch receptor was identified as the lead candidate for improved T-cell fitness and solid tumor clearance (Roth et al., 2020).

Switch receptors can also mitigate the suppressive effects of immune checkpoints. For example, CTLA-4:CD28 and PD-1:CD28 switch receptors were shown to enhance the activity of tumor-specific T cells (Liu et al., 2016; Shin et al., 2012). As another checkpoint receptor, T-cell immunoreceptor with Ig and ITIM domains (TIGIT) binds to CD155 or CD112 on tumor cells, and these interactions hinder cytokine production and effector function of T cells. To blunt these effects, Hoogi et al. designed a TIGIT:CD28 switch receptor that enhanced cytokine production and activation of T cells, as well as delayed tumor growth *in vivo* when combined with a melanoma-specific TCR (Hoogi et al., 2019).

Yet another set of engineering strategies focuses on addressing tumorigenic factors that are not directly expressed on T cells or tumor cells (Figure 4). For example, vascular endothelial growth factor-A (VEGF-A) is a tumor-derived soluble factor that promotes tumor growth and metastasis by facilitating angiogenesis. Chinnasamy et al. co-transduced T cells with a CAR targeting VEGF receptor 2 (VEGFR2) and an inducible transgene encoding IL-12. These CAR-T cells demonstrated trafficking to the tumor vasculature, elimination of $VEGFR^+$ MDSC subtypes that participate in tumor angiogenesis, and IL-12–mediated solid tumor regression (Chinnasamy et al., 2012). Additionally, T cells expressing a CAR that targets fibroblast activation protein (FAP) eliminated FAP^+ tumor stromal fibroblasts that support tumor growth, augmenting T-cell

immunotherapy (Wang et al., 2014). However, it bears noting that strategies targeting non-tumor tissues can cause significant toxicity. For example, Tran et al. reported that CAR-T cells cross-reactive to human and mouse FAP cause off-target bone marrow toxicities and cachexia (Tran et al., 2013).

Transgene Expression to Promote T-Cell Function

In addition to abolishing or rewiring inhibitory signals, the overexpression of stimulatory signals can also improve the activity of tumor-specific T cells. The constitutive expression of costimulatory ligands CD80 and 4-1BBL in PSMA-targeting CAR-T cells showed superior activity against prostate tumor cells by engaging costimulatory receptors *in cis* (autocostimulation) and *in trans* (transcostimulation of bystander cells) (Stephan et al., 2007). Furthermore, Zhao et al. co-expressed the 4-1BBL costimulatory ligand with a second-generation CD19-targeting CAR with CD28 costimulation, and this combined costimulation led to improved antitumor functions driven by the continuous activation of the interferon regulatory factor 7 (IRF7)/interferon beta (IFNβ) pathway (Zhao et al., 2015). Yet another strategy harnesses the endogenous IL-7 signaling mechanism that confers improved persistence in tumor-specific T cells. Shum et al. engineered a constitutively active IL-7 receptor and co-expressed it with a GD2-targeting CAR, which resulted in enhanced survival under repeated tumor challenges in neuroblastoma and glioblastoma xenograft models (Shum et al., 2017).

The aberrant expression of the colony-stimulating factor 1 (CSF-1) in the TME drives macrophages to the M2 phenotype and promotes tumor growth. Several clinical trials of small molecules and mAbs targeting the CSF-1/CSF-1R axis in combination with other mAbs and/or chemotherapy are under way for the treatment of solid tumors (NCT01525602, NCT02777710, NCT02323191, NCT02760797, NCT02923739). In the context of CAR-T cells, the co-expression of CSF-1R, which T cells do not naturally express, was shown to confer CSF-1 responsiveness and activated the RAS/MEK/Erk kinase pathway to enhance T-cell proliferation, cytokine production, and CSF-1–driven chemotaxis (Lo et al., 2008).

Metabolic Reprogramming of T Cells

T-cell metabolism, or the manner in which T cells utilize nutrient sources, has consequential effects on their differentiation state and effector function. The architecture of the CAR protein can impact the metabolic profiles of CAR-T cells. For example, T cells expressing CARs with 4-1BB costimulation favor the oxidative breakdown of fatty acids characteristic of the central-memory phenotype, accompanied by enhanced proliferation and persistence, while T cells expressing CARs with CD28 costimulation favor aerobic glycolysis characteristic of the effector-memory phenotype (Kawalekar et al., 2016).

Due to the intimate relationship between T-cell metabolism and function, reprogramming the metabolic profile of CAR-T cells can potentially increase their clinical efficacy. The TME of solid tumors has an overabundance of potassium (K^+) released by necrotic tumor cells, which increases the intracellular K^+ concentration in infiltrating T cells, downregulating Protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling and impairing T-cell activation after TCR ligation. As a counterstrategy, the overexpression of K^+ channels was shown to increase Akt/mTOR activity and rescue T-cell effector functions by facilitating K^+ efflux and lowering

intracellular K^+ levels (Eil et al., 2016). Additionally, elevated K^+ in the TME and the resulting perturbation of the transmembrane electrochemical gradient limit nutrient uptake by T cells. Interestingly, *ex vivo* conditioning and activation of tumor-specific $CD8^+$ T cells in elevated K^+ to mimic such functional starvation in the TME led to epigenetic and metabolic reprogramming that maintained T-cell stemness—evidenced by improved persistence, engraftment, self-renewal, and multipotency—thereby enhancing their antitumor function *in vivo* (Vodnala et al., 2019). Further, Geiger et al. showed supplementing L-arginine to balance increased arginine metabolism in activated T cells promotes the central-memory phenotype and improves antitumor activity (Geiger et al., 2016).

Altering the expression levels of metabolic genes can also promote an advantageous metabolic profile. Phosphoenolpyruvate carboxykinase 1 (PKC1) increases the production of phosphoenolpyruvate (PEP), which sustains TCR-mediated, Ca^{2+}-induced nuclear factor of activated T-cells (NFAT) signaling and effector functions, and the overexpression of PKC1 in T cells has been shown to restrict tumor growth in melanoma-bearing mice (Ho et al., 2015). Leukemic cells drive T-cell dysfunction by causing suppressed Akt/mTORC1 signaling, decreased expression of the glucose transporter Glut1, and reduced glucose uptake. Accordingly, the overexpression of Akt or Glut1 was demonstrated to partially rescue T-cell activity (Siska et al., 2016). Additionally, Yang et al. knocked out Acetyl-CoA acetyltransferase (ACAT1), a cholesterol esterification enzyme, in $CD8^+$ T cells, and the resulting increase in the plasma membrane cholesterol concentration enhanced TCR clustering and signaling (Yang et al., 2016). Lastly, PPAR-gamma coactivator 1α (PGC1α) is a metabolic regulator downregulated in tumor-infiltrating T cells. It facilitates mitochondrial biogenesis by transcriptional coactivation, and its overexpression in $CD8^+$ T cells rescued their mitochondrial function and protected their metabolic and effector activities in the TME (Scharping et al., 2016).

Interplay of T-Cell Phenotypes, Function, and Versatility

The differentiation state of T cells influences their longevity and efficacy, motivating the isolation or enrichment of specific T-cell subtypes in CAR-T cell manufacturing. Generally, the selection of less differentiated phenotypes—naïve (T_N), memory stem (T_{SCM}), and central memory (T_{CM})—imparts greater engraftment and efficacy than the more differentiated counterparts—effector (T_E) and effector memory (T_{EM}) (Sadelain et al., 2017). Different costimulatory domains in the CAR protein can affect T-cell subtype distribution: CD28 costimulation tends to promote the short-lived, potent T_{EM} phenotype, while 4-1BB results in enrichment of the longer-lived, self-renewing T_{CM} phenotype (Kawalekar et al., 2016). It has also been shown that T_N, T_{CM}, and T_{EM} $CD4^+$ and $CD8^+$ T cells can all be transduced and expanded as CD19 CAR-T cells, but the combination of $CD8^+$ T_{CM} and $CD4^+$ T_N subsets yields synergistic antitumor activity *in vivo* (Sommermeyer et al., 2016). T_{SCM} cells comprise only 2–3% of peripheral blood mononuclear cells (PBMCs), but this memory subset possesses the highest self-renewal capacity and superior persistence, and they are suggested to be the primary precursors of T-cell memory establishment (Hurton et al., 2016). IL-15 is a pro-survival cytokine fundamental to T-cell memory, and it can preserve a T_{SCM}-like phenotype by inhibiting mTORC1 activity, reducing glycolysis, and improving mitochondrial fitness (Alizadeh et al., 2019). Hurton et al. incorporated IL-15 costimulation in CAR-T cells by co-expressing a membrane-bound chimeric IL-15, which led to a T_{SCM}-like molecular profile with improved T-cell persistence regardless of CAR stimulation (Hurton et al., 2016). In addition, the miR-17-92 microRNA cluster was found to be upregulated in IFNγ-producing Th1 cells compared

to T helper type 2 (Th2) cells, and it was found to be downregulated in T cells derived from glioblastoma patients (Sasaki et al., 2010). To induce Th1-like phenotype in glioblastoma-targeting CAR-T cells, Ohno et al. co-transduced miR-17-92 with a third-generation EGFRvIII-specific CAR; in combination with the chemotherapeutic agent temozolomide, this strategy led to improved cytolytic activity and protection against tumor re-challenge *in vivo* (Ohno et al., 2013).

Stem cells are a versatile starting material for adoptively transferred cellular products due to their abilities to self-renew and differentiate into various cell types. Schmitt et al. showed that OP9-DL1, a bone marrow stromal cell line that ectopically expresses the Notch ligand Delta-like-1, can induce the differentiation of hematopoietic progenitor cells (HPCs) into T lymphocytes (Schmitt and Zúñiga-Pflücker, 2002). In a subsequent study, functional $CD8^+$ T cells were generated from human umbilical cord blood hematopoietic stem cells (HSCs), which possess greater self-renewal and potency than HPCs, in OP9-DL1 cocultures (Awong et al., 2011). More recently, an artificial thymic organoid (ATO) system has been shown to facilitate *in vitro* differentiation of human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) into mature T_N-like cells with potent antitumor efficacy and a similar transcriptional profile as primary $CD8^+$ T_N cells (Montel-Hagen et al., 2019).

Expansion of CAR Effectors beyond T cells

Exosomes derived from CAR-T cells, rather than the T cells themselves, have been studied as an alternative effector for CAR-mediated antigen specificity and cytotoxicity. Exosomes released by CAR-T cells can carry the CAR and a high level of cytotoxic molecules; these tumor-specific cytotoxic packages can traffic into solid tumors due to their nanoscale size, while incurring a lower risk of CRS-related toxicities and conferring protection against the immune-checkpoint molecule PD-L1 due to their lack of PD-1 expression (Fu et al., 2019).

Programming CARs into cell types other than T cells can further expand the versatility of the therapy by realizing new functions unachievable by CAR-T cells. Klinchinsky et al. recently demonstrated the feasibility of adenoviral transduction of a CAR into primary macrophages. The resulting CAR-M cells exhibited tumor-specific phagocytosis, inflammatory cytokine production, polarization of bystander macrophages to the immunostimulatory M1 phenotype, and cross-presentation of the TAA to bystander T cells. Although a comparison between CAR-T and CAR-M cells was not evaluated, the established role of macrophages as professional antigen presenting cells (APCs) warrants the potential of CAR-M cells to more effectively stimulate an adaptive antitumor immune response (Klinchinsky et al., 2020).

CAR-natural killer (NK) cells can be generated from cord blood or iPSCs (Li et al., 2018; Liu et al., 2020), making them an attractive candidate for allogeneic, off-the-shelf products. Moreover, CD19-targeting CAR-NK cells have achieved robust clinical efficacy without inducing CRS, neurotoxicity, or GvHD in patients with B-cell lymphoid tumors, highlighting their relative safety compared to their T-cell counterparts (Liu et al., 2020). CAR-NK cells have been shown to exert potent and specific cytotoxicity toward a variety of tumor models, including leukemia, multiple myeloma, ovarian cancer, and glioblastoma (Chu et al., 2014; Genßler et al., 2016; Li et al., 2018; Quintarelli et al., 2020); as well as toward immunosuppressive cell types such as MDSCs and follicular helper T cells (T_{FH}) (Parihar et al., 2019; Reighard et al., 2020). Lastly, natural killer T (NKT) cells possess antitumor and tumor-homing capabilities, and GD2-targeting CAR-NKT cells

that harness these inherent advantages exhibited effective localization to and lysis of neuroblastoma cells without significant toxicity (Xu et al., 2019). Taken together, these developments highlight both the potential for cell-based immunotherapy to expand beyond T cells and the applicability of the CAR technology across a variety of immune cell types.

Engineering CAR-T Cell Interactions with the Tumor and TME

The immune-evasive and immunosuppressive nature of the TME contributes to the poor therapeutic efficacy of CAR-T cells observed in solid tumors. Hallmarks of the TME, which have been extensively reviewed elsewhere (Gajewski et al., 2013; Whiteside, 2008), include (1) physical barriers to tumor penetration by immune cells, (2) upregulated checkpoint ligands, (3) a pro-tumor stromal niche, (4) abundant immunosuppressive and pro-metastatic soluble factors, and (5) modulated expression of chemokines to preferentially recruit leukocytes with an immunosuppressive phenotype. These factors have in turn driven the design of CAR-T cells that respond to TME elements to enhance CAR-T cell efficacy (Figure 5).

Tumor Homing and Penetration

The efficacy of CAR-T cell therapy in solid tumors is significantly hindered by poor immune-cell infiltration (Newick et al., 2017). T-cell migration is regulated through chemokine axes. Tumor cells can upregulate or downregulate chemokines, as well as modulate chemokine expression by tumor-associated cells, contributing to the poor recruitment of CAR-T cells (Oelkrug and Ramage, 2014). Engineering CAR-T cells to overexpress receptors for chemokines that are overexpressed in the TME can turn a tumor's defense mechanism against itself. For example, GD2- and mesothelin-targeting CAR-T cells have been engineered to co-express CCR2b, the dominant isoform of the chemokine receptor of CCL2, resulting in enhanced T-cell homing to CCL2-expressing neuroblastoma and malignant pleural mesothelioma xenografts, respectively (Craddock et al., 2010; Moon et al., 2011). Similarly, CAR-T cells that co-express CCR4 showed improved migration towards tumors expressing CCL17 and CCL22 *in vivo*, while those expressing CXCR1 or CXCR2 exhibited enhanced homing towards tumor-derived IL-8 (Di Stasi et al., 2009; Jin et al., 2019). Once CAR-T cells reach the tumor site, their infiltration is hindered by the high-density structural extracellular matrix (ECM) associated with solid tumor nodules. Accordingly, CAR-T cells engineered to express heparinase, an enzyme that degrades ECM, have been shown to improve tumor infiltration and overall survival in multiple xenograft models (Caruana et al., 2015).

CAR-T cells that successfully reach solid tumors are next faced with a multitude of suppressive and evasive features that induce CAR-T cell dysfunction (Newick et al., 2017). To improve therapeutic efficacy in this immunosuppressive environment, CAR-T cells have been engineered to produce proteins that (1) improve CAR-T cell function in an autocrine fashion; (2) disrupt immunosuppressive elements; and/or (3) induce TME remodeling to enhance the endogenous antitumor immune response (Figure 5). Each of these strategies is discussed in detail below.

Autocrine Stimulation of CAR-T Cells in the TME

Cytokines are signaling proteins with the ability to drastically augment or abrogate CAR-T cell function. Co-expressing the CAR with immunostimulatory cytokines could significantly enhance CAR-T cell proliferation, survival, and effector function in the immunosuppressive TME. For example, T cells that constitutively co-express a CD19-targeting CAR plus IL-2, IL-7, IL-15, or IL-21 have been shown to achieve greater *in vivo* tumor control compared to T cells expressing the CAR alone (Markley and Sadelain, 2010). Interestingly, although the receptor complexes of these four cytokines contain the common gamma chain (γ_c), each cytokine differentially impacted the proliferation, subtype differentiation, and function of the engineered T cells, underscoring the complexity of T-cell biology and variety of potential outcomes achievable through different engineering strategies (Markley and Sadelain, 2010). T cells co-expressing IL-12, IL-15, IL-18, and/or IL-21 plus a CAR targeting a variety of antigens have also been described, resulting in improved efficacy, proliferation, and/or persistence *in vivo* (Batra et al., 2020; Hoyos et al., 2010; Hu et al., 2017; Koneru et al., 2015; Krenciute et al., 2017; Pegram et al., 2012). However, constitutive overexpression of immunostimulatory cytokines can also increase toxicity (Zhang et al., 2011). Regulatory strategies previously discussed in this review, such as inducible promoters, can be implemented to modulate cytokine production and associated toxicity (Liu et al., 2019).

Disruption of Immunosuppressive Axes

The expression of immune-checkpoint receptors and ligands such as PD-1 and PD-L1 are prevalent in the TME, and they can potently inhibit CAR-T cell cytotoxicity and induce anergy (Drake et al., 2006). Thus, immune-checkpoint blockade has strong synergistic potential with CAR-T cell therapy, and several ongoing clinical trials are evaluating combination therapy with CAR-T cells and exogenously administered checkpoint inhibitors (Grosser et al., 2019). Furthermore, CAR-T cells have been engineered to secrete immune-checkpoint inhibitors, including anti–PD-1 scFvs and anti–PD-L1 antibodies, or to express PD-1 dominant-negative receptors (DNRs) (Chen et al., 2017; Cherkassky et al., 2016; Li et al., 2017; Rafiq et al., 2018; Suarez et al., 2016). In addition to enhancing efficacy, this approach may also avoid toxicities associated with systemic immune-checkpoint blockade by restricting checkpoint inhibitor distribution to the immediate environment of the producer T cells. For example, it has been shown that anti–PD-1 scFvs secreted by intraperitoneally (IP) injected CAR-T cells remained localized at the injection site. However, when an equal number of conventional CAR-T cells were administered IP with exogenous anti–PD-1 antibody, the antibody was detected systemically within three hours (Rafiq et al., 2018).

The solid-tumor milieu also houses a diverse collection of soluble factors that promote tumorigenesis and inhibit CAR-T cell function. For example, prostaglandin E2 (PGE_2) is a bioactive lipid often upregulated in tumors, where it contributes to tumor survival through regulation of cell proliferation, migration, apoptosis, and angiogenesis (Ricciotti and FitzGerald, 2011; Wang and Dubois, 2006). In the context of CAR-T cell therapy, PGE_2, along with adenosine, inhibits T-cell signaling and activation though the activation of protein kinase A (PKA), thereby reducing T-cell proliferation and effector function (Newick et al., 2016). In two solid-tumor models that highly express PGE_2, CAR-T cells engineered to express a peptide inhibitor of ezrin-mediated PKA translocation to the immune synapse exhibited improved tumor infiltration and killing (Newick et al., 2016). Similarly, elevated concentrations of bio-reactive chemicals such as reactive oxygen species (ROS) in the TME play an important role in tumorigenesis (Weinberg et al., 2019). Catalase is an enzyme that facilitates the decomposition of hydrogen peroxide (H_2O_2), an ROS

that impairs T-cell activity in the TME. Increasing intracellular levels of catalase by co-expressing the *catalase* gene in HER2- and carcinoembryonic antigen (CEA)-specific CAR-T cells has been shown to enable CAR-T cells to metabolize the suppressive H_2O_2, improving their tumor cytolytic capacity (Ligtenberg et al., 2016).

The aberrant expression of cytokines in the TME plays a critical role in tumor progression and resistance to CAR-T cell therapy. In particular, TGF-β plays a multiplexed role in cancer progression through interactions with tumor cells, stroma, and both innate and adaptive immune cells to induce (1) the secretion of immunosuppressive chemokines, cytokines, and growth factors; (2) ECM remodeling and matrix deposition; (3) immunosuppressive reprogramming of macrophages, neutrophils, and T cells; and (4) inhibited maturation or proliferation of T cells and NK cells (Pickup et al., 2013). To ablate these powerful effects, CAR-T cells have been engineered to express a TGF-β DNR that potently inhibits endogenous TGF-β signaling, resulting in T cells with enhanced proliferation and antitumor efficacy in a prostate cancer xenograft model (Kloss et al., 2018). Based on these results, a phase-I clinical trial has been initiated to assess T cells co-expressing a PSMA CAR and the DNR for the treatment of relapsed and refractory metastatic prostate cancer (NCT03089203). The DNR is distinct from the TGF-β–targeting CAR and TGF-β switch receptor discussed in a previous section in that the DNR does not transduce any signals that can stimulate the engineered T cell. It remains to be seen whether the stimulatory effects of the CAR and switch receptors will confer additional clinical benefits compared to the DNR.

In the tumor microenvironment, IL-6 is often overexpressed by tumor cells, tumor associated macrophages (TAMs), and other resident cells (Kumari et al., 2016). IL-6 supports tumorigenesis through a number of mechanisms, and plays a central role in the induction of CRS after CAR-T cell infusion (Lee et al., 2014). Systemic administration of tocilizumab, an mAb targeting IL-6 receptor alpha (IL-6Rα), has become standard treatment for CRS after CAR-T cell therapy (Kotch et al., 2019). More recently, CD19-targeting CAR-T cells that co-express a non-signaling, membrane-bound IL-6 receptor (mbaIL6) were shown to sequester IL-6 while retaining *in vivo* antitumor efficacy (Tan et al., 2020). However, it remains to be seen if CAR-T cells engineered in this fashion can prevent CRS.

TME Remodeling to Promote the Endogenous Immune Response

Tumors are adept at selectively attracting or evading subsets of leukocytes, including CAR-T cells, to promote immune regulation or suppression (Rabinovich et al., 2007). In addition, tumors are often capable of inducing an immunosuppressive or pro-metastatic phenotype on the local stroma, as well as an anti-inflammatory or dysfunctional phenotype on resident leukocytes (Morgan and Schambach, 2018). Another approach to enhancing the efficacy of CAR-T cell therapy is to reverse this immunosuppressive-cell niche through remodeling the tumor-cellular composition and phenotype. To realize this, CAR-T cells have been be engineered to secrete cytokines or other soluble factors that induce TME remodeling in a paracrine or endocrine fashion.

In germinal-center lymphomas, loss of herpesvirus entry mediator (HVEM) expression induces the secretion of non-redundant stroma-activating factors, resulting in acute lymphoid-stroma activation. The hyperactivated stroma recruits T_{FH} cells, which support malignant B cells through CD40/CD40L interactions and cytokine stimulation. As a counterstrategy, CD19 CAR-T cells

engineered to secrete a soluble form of HVEM were shown to enhance tumor control *in vivo* (Boice et al., 2016).

CAR-T cells engineered to secrete IL-12 have been shown to remodel the TME by reprogramming TAMs to an M1 phenotype and decreasing the presence of MDSCs and Tregs in syngeneic mouse models (Chinnasamy et al., 2012; Liu et al., 2019; Yeku et al., 2017). Similarly, CAR-T cells that constitutively secrete IL-18 can alter the TME makeup by increasing intratumoral M1 macrophage, activated dendritic cell (DC), and activated NK cell numbers, while decreasing M2 macrophage and Treg levels. A direct comparison of IL-12– to IL-18–expressing CAR-T cells indicated that IL-18 is more effective at remodeling the immunosuppressive TME in a syngeneic murine pancreatic-cancer model (Chmielewski and Abken, 2017). Furthermore, CD19 CAR-T cells expressing IL-18 induced the expansion of endogenous $CD8^+$ T cells, NK cells, NKT cells, and DCs in the bone marrow, potentially contributing to the control of tumors with heterogenous CD19 expression in a syngeneic mouse model (Avanzi et al., 2018).

Among DCs, conventional type 1 DCs (cDC1s) in particular excel at inducing immunity against tumors via their ability to cross-present cellular antigens and prime Th1 cells. Recently, it has been shown that T cells engineered to secrete Fms-like tyrosine kinase 3 ligand (Flt3L), a hematopoietic cell growth factor, promote intratumoral cDC1 and DC-precursor proliferation (Lai et al., 2020). Furthermore, when T cells were co-transduced to express Flt3L and an anti-HER2 CAR, a combined treatment with these CAR-T cells and adjuvants induced an enhanced antitumor response and endogenous T-cell epitope spreading *in vivo* (Lai et al., 2020).

CAR-T cells have also been engineered to co-express multiple immune-modulatory proteins. In one example, CAR-T cells were programmed to co-express CCL19 and IL-7 to induce endogenous immune-cell recruitment and stimulate the recruited cells, respectively. In a syngeneic hCD20-expressing mastocytoma mouse model, these CAR-T cells induced robust recruitment of endogenous T cells and DCs, resulting in enhanced and durable tumor clearance (Adachi et al., 2018).

CAR-T cells have also been designed to modulate the TME through the expression of surface-bound proinflammatory ligands. For example, CD40L is normally transiently expressed on T cells after TCR stimulation, and its interaction with the CD40 receptor on different immune cell types can lead to activation of APCs, licensing of DCs, as well as apoptosis of $CD40^+$ tumor cells (Cella et al., 1996; Eliopoulos et al., 2000; Ridge et al., 1998). Constitutive CD40L expression on CD19 CAR-T cells resulted in elevated surface expression of costimulatory molecules, adhesion molecules, HLA molecules, and the Fas death receptor on $CD40^+$ tumor cells, thus increasing their immunogenicity (Curran et al., 2015). These T cells also induced the secretion of proinflammatory IL-12 by monocyte-derived DCs *in vitro*, and showed enhanced antitumor efficacy *in vitro* and *in vivo* (Curran et al., 2015). It was subsequently demonstrated that CD40L-expressing CAR-T cells can license APCs in lymphatic tissues in a syngeneic immunocompetent mouse model, and this licensing was found to be dependent on the CD40L/CD40 interaction (Kuhn et al., 2019). Furthermore, increased recruitment of macrophages, DCs, and endogenous $CD4^+$ and $CD8^+$ T cells to lymphatic tissues was observed, along with the recruitment of DCs, $CD4^+$ and $CD8^+$ T cells to the tumor. Treg levels were also observed to slightly increase in the tumor, but the ratio of $CD8^+$ T cells to Tregs was unchanged. Thus, CD40L-expressing CAR-T cells capable of remodeling the TME and lymphatic tissue activated endogenous T cells to

suppress antigen-negative tumor re-challenge, strongly suggesting induced epitope spreading (Kuhn et al., 2019). Similarly, the surface expression of 4-1BBL on CAR-T cells is proposed to remodel the TME through autocrine-induced secretion of type I IFNs, which may improve DC cross-priming, Treg inhibition, and angiogenesis suppression (Zhao et al., 2015).

Finally, CAR-T cells can be engineered to facilitate the engagement of tumor cells by endogenous, non-engineered T cells through the secretion of bispecific T-cell engagers (BiTEs), which are composed of two fused scFvs. Choi et al. engineered BiTEs with one scFv targeting EGFR, which are overexpressed in glioblastoma cells, and the other targeting CD3 on T cells. EGFRvIII-targeting CAR-T cells engineered to secrete EGFR/CD3 BiTEs have been shown to eliminate orthotopic tumor xenografts with heterogenous EGFRvIII expression (Choi et al., 2019).

Conclusion

CAR-T cell therapy has shown great promise in treating hematologic malignancies. However, solid tumors pose unique challenges that require further engineering and tuning of the technology to successfully treat these intractable malignancies. Recent protein- and cell-engineering strategies have made great strides in boosting the intrinsic fitness and anti-tumor function of T cells, increasing tumor-targeting specificity, preventing tumor escape and relapse, as well as modifying the TME to enhance immunotherapeutic outcomes. Although most engineering strategies reported to date have focused on delivering individual desirable features, advancements in genome-editing methodologies and genetic circuitry design offer the possibility of multi-layered approaches that can simultaneously address multiple critical needs in T-cell therapeutics development.

At the same time, the biological complexity of and potential crosstalk among different engineered features within the T cell, as well as among engineered and endogenous immune cells, tumor cells, and other tumor-associated factors, must be carefully balanced when advancing the clinical translation of CAR-T cells for the treatment of solid tumors. The decreasing cost and increasing capacity of next-generation and single-cell sequencing methods, as well as proteomic and metabolomic analyses, could significantly enhance our ability to understand and rationally manipulate these complex interactions while engineering the next generation of CAR-T cell therapy for solid malignancies. The growing toolbox of T-cell engineering strategies that can be synergistically implemented and modularly calibrated for maximum safety and efficacy will continue to enable innovations that aim to generate new treatment options for currently intractable diseases.

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Figure Legends

Figure 1. CAR-T Cell Engineering Approaches
Strategies to engineer CAR-T cells for improved function in solid tumors include a focus on CAR engineering, T-cell engineering, and TME interaction optimization.

Figure 2. Protein Engineering Strategies to Improve the Programmability, Safety, and Efficacy of CAR-T Cells
(A) Combinatorial antigen recognition by AND- and AND-NOT logic using a synNotch receptor and iCAR, respectively, can increase antigen specificity and safety. Tandem bispecific OR-gate CARs can circumvent antigen escape and increase efficacy.
(B) Engineered ON and OFF switches can easily and efficiently alter CAR-T cell activity.
(C) Programming CARs to activate only in the presence of an adaptor or by leucine-zipper–mediated reconstitution can increase controllability over CAR-T cell activity.

Figure 3. Engineering strategies to improve CAR-T cell safety
(A) Co-expression of suicide genes such as HSK-TV, iCasp9, CD20, and tEGFR enables induction of T-cell death to abort the therapy in the case of adverse events.
(B) Tet-ON and -OFF systems allow the control of the CAR expression on the transcriptional level.

Figure 4. Rewiring T-cell signaling with synthetic receptors
Switch receptors rewire T-cell responses by triggering co-stimulatory signaling in the presence of normally inhibitory ligands. CARs responsive to environmental cues such as soluble TGF-β or surface antigens present on tumor-supportive tissues can enhance anti-tumor function by removing and converting immunosuppressive factors.

Figure 5. Strategies in Optimizing CAR-T Cell and Tumor Interactions
CAR-T cells have been engineered to utilize, reverse, or circumvent tumor-driven immunosuppressive factors and axes through a variety of mechanisms.

Tables

Table 1. Targeted genome-editing strategies to enhance T-cell function

Target locus[a]	Motivation[b]	Technology[c]	Reference
TRAC	Ablate TCRαβ expression to reduce alloreactivity	ZFN	(Torikai et al., 2012)
		TALEN	(Qasim et al., 2017; Valton et al., 2015)
		CRISPR/Cas9	(Georgiadis et al., 2018)
		CRISPR/Cas9 CAR knock-in	(Eyquem et al., 2017; MacLeod et al., 2017)
TRAC, TRBC	Enhance transgenic TCR expression	CRISPR/Cas9	(Stadtmauer et al., 2020)
B2M	Ablate HLA expression to reduce alloreactivity	CRISPR/Cas9	(Ren et al., 2017b)
HLA-A	Ablate HLA expression to reduce alloreactivity	ZFN	(Torikai et al., 2013)
CD52	Confer resistance to lymphodepletion	TALEN	(Qasim et al., 2017)
dCK	Confer resistance to lymphodepletion	TALEN	(Valton et al., 2015)
PD-1	Inhibit immune-checkpoint signaling	TALEN	(Menger et al., 2016)
		CRISPR/Cas9	(Liu et al., 2017; Ren et al., 2017a; Rupp et al., 2017; Stadtmauer et al., 2020)
REGNASE-1	Disrupt a negative regulator of T-cell activity	CRISPR/Cas9	(Wei et al., 2019)
DGKα, DGKζ	Disrupt a negative regulator of T-cell activity	CRISPR/Cas9	(Jung et al., 2018)
LAG3	Disrupt a negative regulator of T-cell activity	CRISPR/Cas9	(Zhang et al., 2017)
FAS	Abolish pro-apoptotic signaling	CRISPR/Cas9	(Ren et al., 2017b)
GM-CSF	Inhibit CRS-related toxicities	CRISPR/Cas9	(Sterner et al., 2019)

[a]*TRAC*, T-cell receptor alpha constant; *TRBC*, T-cell receptor beta constant; *B2M*, Beta-2 microglobulin; *HLA*, human leukocyte antigen; *dCK*, deoxycytidine kinase; *PD-1*, programmed cell death protein 1; *DGK*, diacylglycerol kinase; *LAG3*, lymphocyte-activation gene 3; *GM-CSF*, granulocyte-macrophage colony-stimulating factor.
[b]TCR, T-cell receptor; CRS, cytokine release syndrome.
[c]ZFN, zinc-finger nuclease; TALEN, transcription activator-like effector nuclease; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9.

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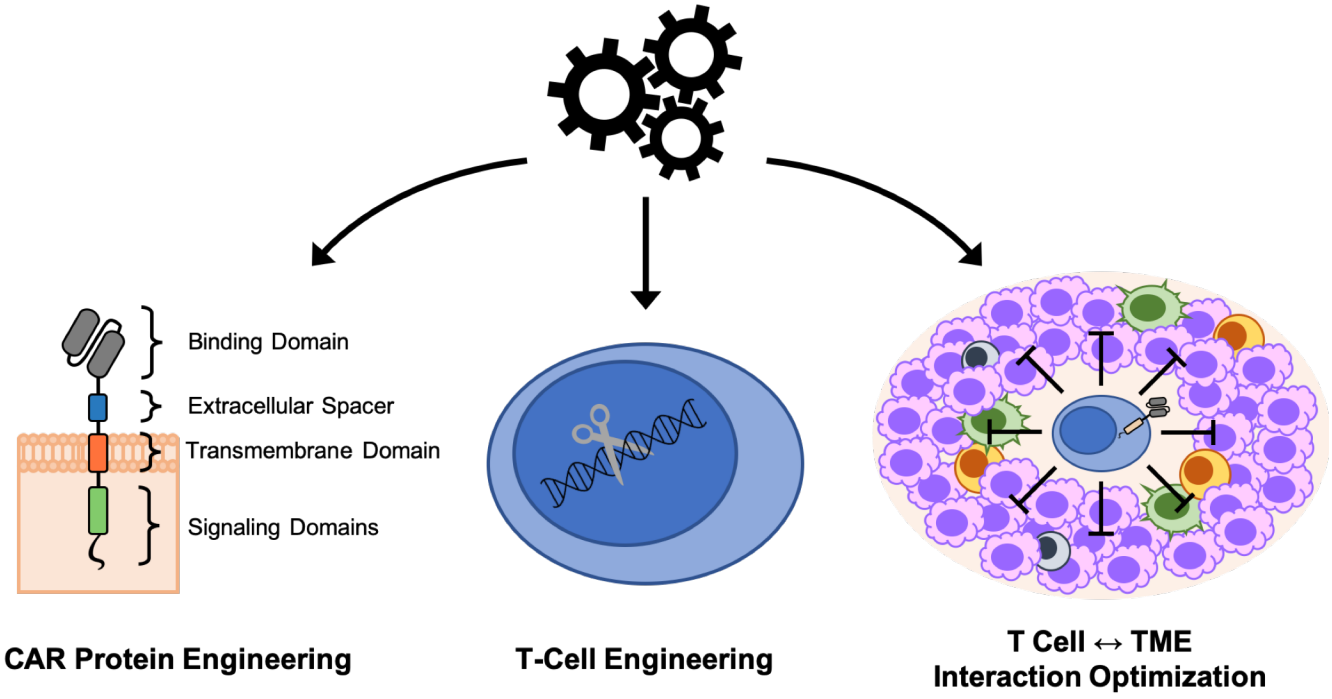
Figure 1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CAR Protein Engineering
T-Cell Engineering
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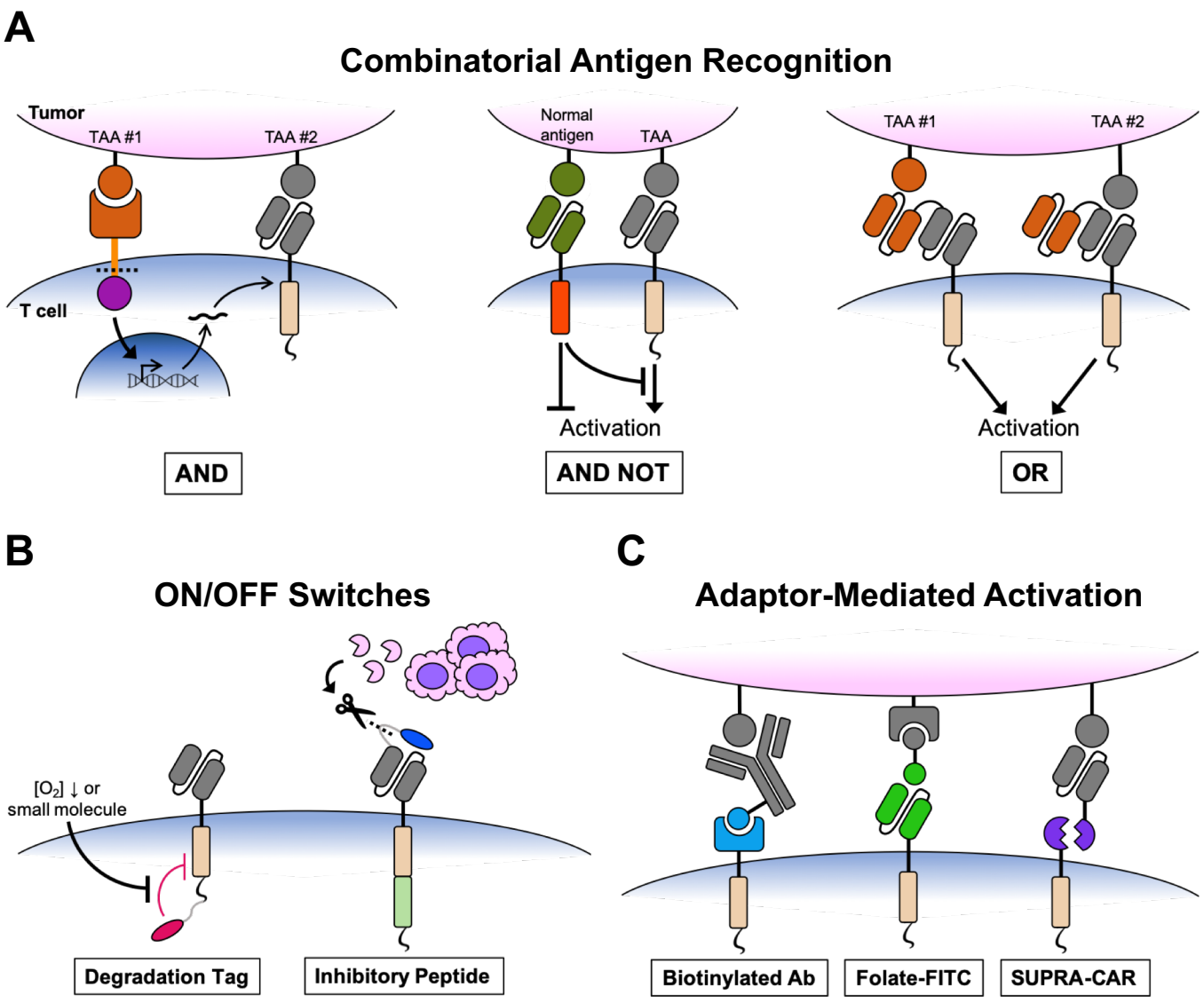
Interaction Optimization

Figure 2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AND
Normal antigen
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Activation
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TAA #2
Activation
OR
B
ON/OFF Switches
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Figure 3

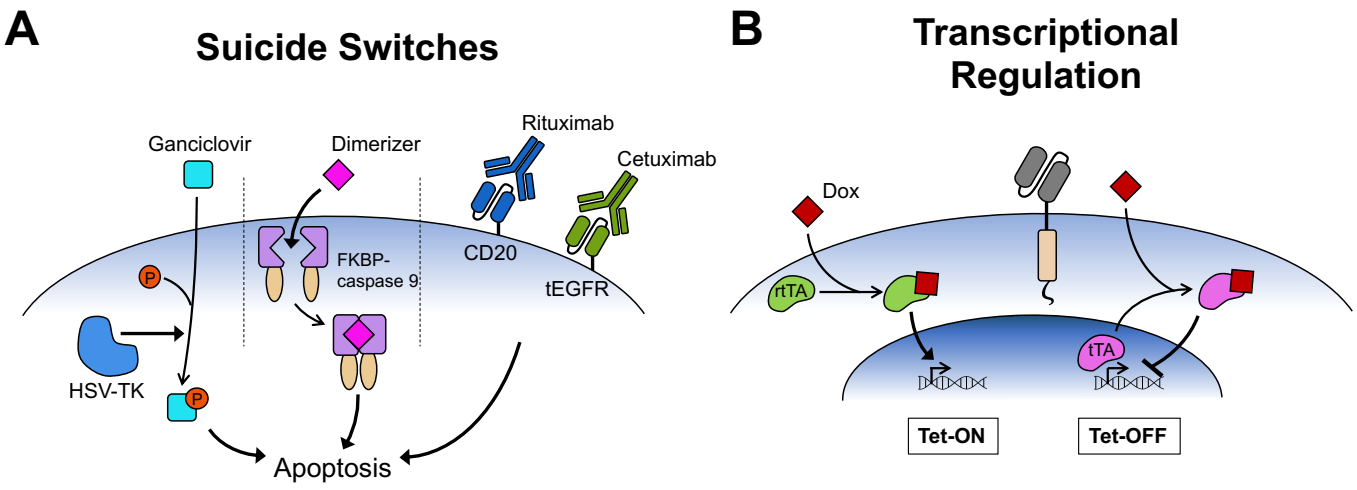
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Figure 4

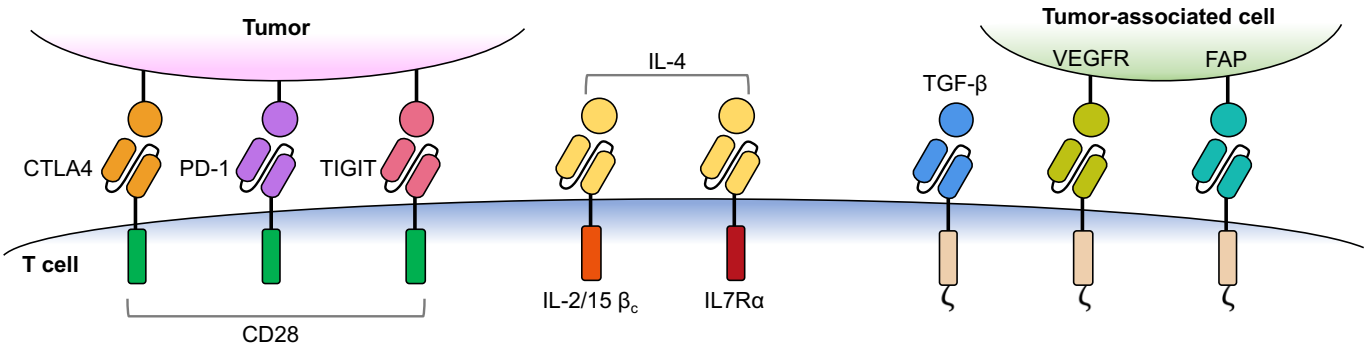
Tumor
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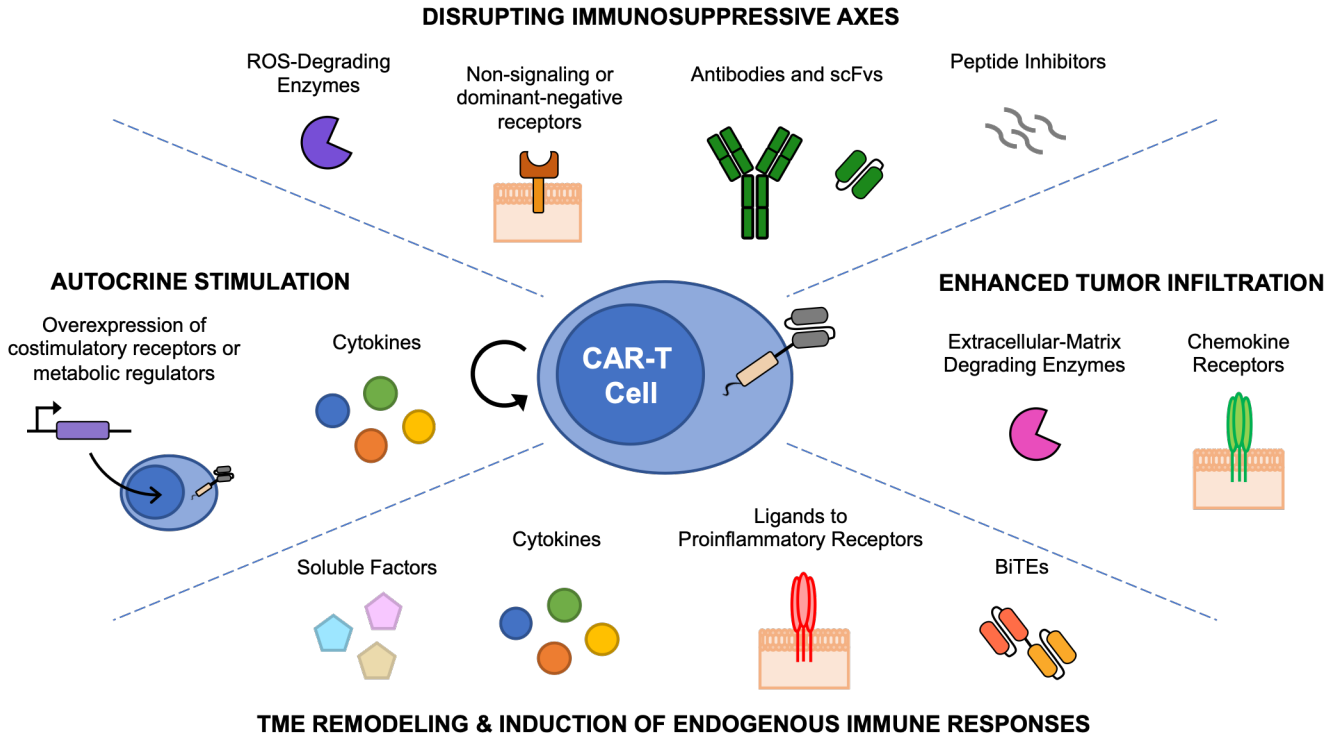
Figure 5


DISRUPTING IMMUNOSUPPRESSIVE AXES
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Antibodies and scFvs
Peptide Inhibitors
AUTOCRINE STIMULATION
Overexpression of costimulatory receptors or metabolic regulators
Cytokines
CAR-T Cell
ENHANCED TUMOR INFILTRATION
Extracellular-Matrix Degrading Enzymes
Chemokine Receptors
Soluble Factors
Cytokines
Ligands to Proinflammatory Receptors
BiTEs
TME REMODELING & INDUCTION OF ENDOGENOUS IMMUNE RESPONSES