Insight into next-generation CAR therapeutics: designing CAR T cells to improve clinical outcomes

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Chimeric antigen receptor (CAR) T cell therapy has shown considerable promise for hematologic malignancies, leading to the US Food and Drug Administration approval of two CAR T cell–based therapies for the treatment of B cell acute lymphoblastic leukemia and large B cell lymphoma. Despite success in hematologic malignancies, the treatment landscape of CAR T cell therapy for solid tumors has been limited. There are unique challenges in the development of novel CAR T cell therapies to improve both safety and efficacy. Improved understanding of the immunosuppressive tumor microenvironment and resistance mechanisms has led to encouraging approaches to mitigating these obstacles. This Review will characterize challenges with current CAR T designs for hematologic malignancies and solid tumors and emphasize preclinical and clinical strategies to overcome them with novel CAR T cell therapies.

Introduction

In the last decade, the concept of harnessing the intrinsic and complex ability of the immune system to fight cancer has multiplied, with a pronounced expansion in the field of immuno-oncology demonstrating durable clinical responses in patients with solid tumors and hematologic malignancies (1). Adoptive T cell therapy (ACT) is an example of an immunotherapy employed in the clinic, a form of passive immunotherapy based on the infusion of lymphocytes to generate an antitumor response (2). Chimeric antigen receptors (CARs) are synthetic molecules that harness the antigen-recognition ability of antibodies and the effector functions of immune cells, typically T cells. T cells are genetically engineered to express CARs, which are composed of an extracellular antigen-recognition domain derived from an antibody, a hinge and transmembrane domain, and intracellular motifs that activate and augment cell function upon antigen engagement (Figure 1 and ref. 3).

Within this field, CD19-targeted CAR T cells for the treatment of B cell malignancies were the first cell-based therapeutic approved by the US FDA in 2017 (4, 5). Despite the successes of CAR T cell therapy for B cell malignancies, immune-mediated toxicities can lead to morbidity and mortality, limiting the widespread use of this therapy. Moreover, durable responses are observed in less than half of CAR T cell–treated patients, underscoring the importance of developing next-generation CARs that are safer and more effective (6–8). CAR T cell therapy for solid tumors has not yet translated into clinical practice due to challenges such as tumor antigen heterogeneity, difficulty trafficking and infiltrating into the tumor sites, and an immunosuppressive tumor microenvironment (TME).

Evolution in CAR design

CAR design evolved into different generations defined by modifications of the endodomain of the receptor. First-generation CAR T cells were composed of an antigen-recognition extracellular domain joined to a single intracellular motif encoding for the cytoplasmic T cell glycoprotein CD3ζ, mimicking normal activation through the T cell receptor (TCR) (Figure 1). This initial design was able to trigger a cellular response against its target antigen (9, 10), but the modified T cells displayed an inability to persist in patients, with limited clinical benefits (11). Second-generation CAR T cells added an additional intracellular motif composed of the signaling domain of costimulatory receptors such as CD28 (12) and 4-1BB/CD137 (13) to the CD3ζ-activating domain; these modifications rendered CAR T cells with enhanced effector functions and increased persistence in vivo compared with their first-generation counterparts (14). Third-generation CARs were defined by the inclusion of a second costimulatory signaling domain intended to improve even further the antitumor ability of the cells, most commonly combining CD28 with 4-1BB (15–17). However, the clinical advantages of using third- compared with second-generation CAR T cells are unclear.

In a recent clinical trial, second- and third-generation CD19-specific CAR T cells were coadministered to patients with B cell non-Hodgkin's lymphoma, demonstrating that third-generation cells had improved in vivo expansion and persistence (18). The study and selection of an optimal costimulatory domain continues to be an expanding field in CAR T cell biology and includes OX40 (19), ICOS (20), CD27 (21), and others (22). The evolution into different generations of CAR T cells is based on harnessing

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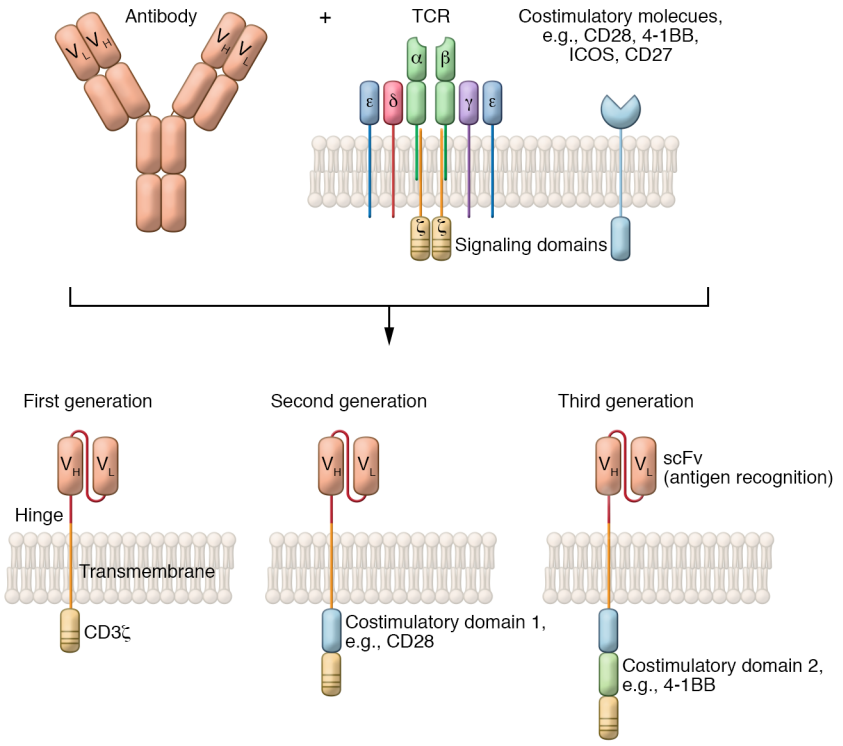

Figure 1. Structure and evolution of CARs. CARs are composed of an antigen-recognition domain (mainly scFv of monoclonal antibodies) fused to a hinge/transmembrane domain and intracellular signaling domains able to transduce activation signals to CAR T cells. Modification in the intracellular domains gave rise to a different generation of CAR T cells from the first generation, which included a CD3ζ motif, to the second and third generations, which included one or two costimulatory domains, respectively. V_H, variable heavy chain; V_L, variable light chain.

immunological traits of T cells that are beneficial for an enhanced antitumor response. These include optimal cell activation received through signal 1 (TCR/CD3ζ) and signal 2 (costimulatory receptors) that will affect their effector function. For cell therapy, persistence after adoptive transfer is essential for durable responses, with a less differentiated phenotype being a key trait for enhanced engraftment and subsequent expansion after antigenic stimulation (23, 24). Optimizing designs of these different components of the CAR and/or T cells has the potential for improving clinical outcomes by addressing challenges identified from the early clinical application of CAR T cells.

Toxicities associated with CAR T cell administration

Cytokine release syndrome (CRS) and neurotoxicity are commonly observed toxicities after the administration of CAR T cells (25). While exact pathophysiology of these syndromes is not yet fully elucidated, one proposed mechanism is that systemic cytokines can be released when there is an interaction between the tumor and the CAR T cells (26). CRS manifests with fevers, hypoxia, and hypotension, which can lead to multiorgan dysfunction (25). Neurologic toxicity, termed immune effector cell–associate neurotoxicity syndrome (ICANS), can present with aphasia, altered level of consciousness, impaired cognitive function, motor weakness, seizures, and/or cerebral edema (25, 27). It is important to note that comparing toxicities across CAR T trials is challenging, as there is variability in the scoring systems used to grade the severity of toxicities (Table 1).

Although toxicities are usually reversible with supportive care and/or interventions such as corticosteroids or IL-6 receptor blockade, they can be fatal, highlighting the need for safer CAR T constructs that maintain antitumor ability (28). Current guidelines support the use of anti–IL-6 therapy in combination with corticosteroids for patients with grade 3 or higher CRS and/or neurotoxicity. In an analysis of the ZUMA-1 trial, prophylactic use of tocilizumab reduced the incidence of severe CRS from 13% to 5%, but did not reduce the incidence of neurotoxicity (29). Topp and colleagues reported that earlier intervention with tocilizumab and steroids in patients enrolled in cohort 4 of the ZUMA-1 trial improved the incidence of severe CRS and neurotoxicity without negatively affecting clinical outcomes (30). This study and others challenge the notion that early steroid intervention hinders CAR T cell expansion (28). Prospective studies are needed to address whether prophylactic cytokine blockade and/or corticosteroids reduce the incidence of severe toxicity without affecting CAR T cell expansion and clinical efficacy.

In order for infused CAR T cells to be therapeutically efficient, they need to reach an activation threshold that is influenced by antigen density on the target tissue, affinity of the antigen-binding site to said antigen (31), costimulatory domains present in the CAR, and other cell-intrinsic and -extrinsic factors (32). It was recently shown that low expression levels of CD22 on target cells modified CD22-specific CAR T cell function, with lower cytokine production and reduced persistence in mouse models of acute lymphoblastic leukemia (ALL) with low antigen expression (33). Another study demonstrated that the activity of CD19-specific CAR T cells depends on the antigen density on target cells, with constructs including CD28 costimulation showing better function against low-antigen tumor cells than 4-1BB constructs. This lower sensitivity is enhanced by including additional ITAM motifs to the 4-1BB–based CAR and shows increased signaling against low antigen density (34). The costimulatory domain was shown to modulate the activity of CAR T cells, with CD28 inducing more rapid responses that were higher

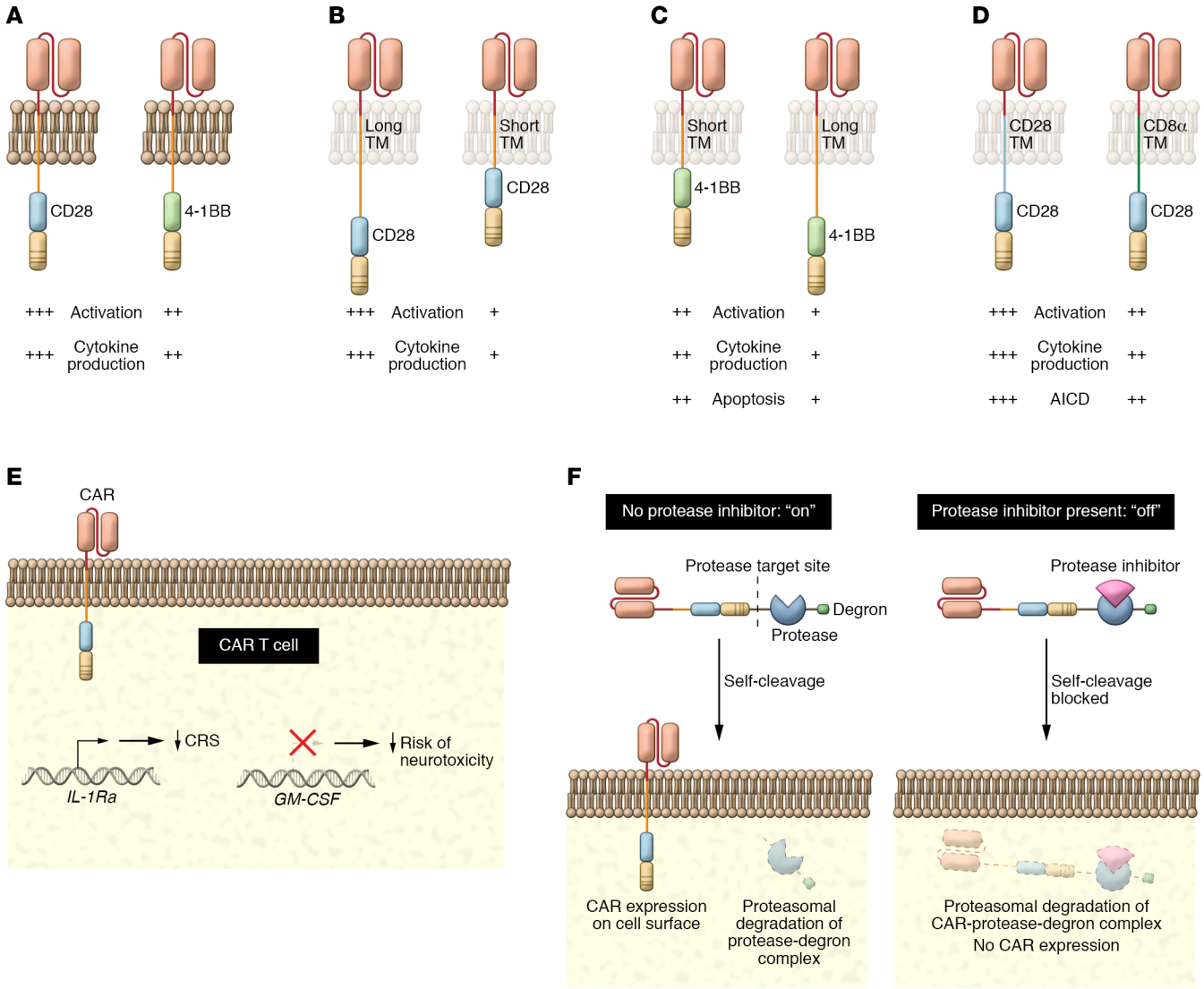

Figure 2. Overcoming toxicities through CAR-engineering strategies. CAR T cells show different levels of activation and cytokine production depending on (**A**) costimulatory domain, (**B** and **C**) length of the transmembrane domain, and (**D**) nature and composition of the hinge/transmembrane domain (**E**). CAR T cells have been engineered to secrete soluble IL-1 receptor antagonists or induce a targeted deletion on the gene encoding for GM-CSF associated with toxicities (**F**). Administration of a protease inhibitor controls CAR surface expression, acting as an "on/off switch" based on a complex composed of a self-cleaving protease and a degron moiety. TM, transmembrane; AICD, activation-induced cell death.

in magnitude compared with 4-1BB–containing CARs, with lower risks of cytokine-associated toxicities (Figure 2A and ref. 35). The choice of costimulatory domain affects not only CAR T cell fate; in addition, the length of the endodomain can also modulate activation. It was demonstrated that a shorter intracellular domain containing CD28 as costimulation reduced the ability of the CAR to interact with CD3ζ, with consequently less cell activation and cytokine production (Figure 2B and ref. 36). In another study, an anti-CD19 CAR with a longer hinge/transmembrane and intracellular fragments produced lower levels of cytokines with higher expression levels of antiapoptotic proteins with cytolytic ability comparable to that of the conventional 4-1BB–based CAR with shorter domains. A phase I clinical trial using this construct showed no neurotoxicity or CRS in any of the 25 treated patients (Figure 2C and ref. 37).

The transmembrane domain also modifies the ability of the CAR to induce cell activation. It was shown that an anti-CD19 CAR with a transmembrane moiety containing CD8α produced fewer cytokines and lower levels of activation-induced cell death compared with a CD28-based transmembrane with comparable ability to eliminate tumors in preclinical models (Figure 2D and ref. 38). Based on these findings, a phase I clinical trial utilizing a fully humanized anti-CD19 antibody plus hinge and transmembrane domains from CD8α (Hu19-CD828Z) was designed to test the hypothesis that single-chain variable fragments (scFvs) derived from a human antibody may be less immunogenic than those from a murine-derived antibody. Twenty patients with large B cell lymphoma (LBCL) were enrolled in this study with ongoing complete remission (CR) rates of 40%, comparable to that in the pivotal ZUMA-1 study. The incidence of severe grade 3 or higher

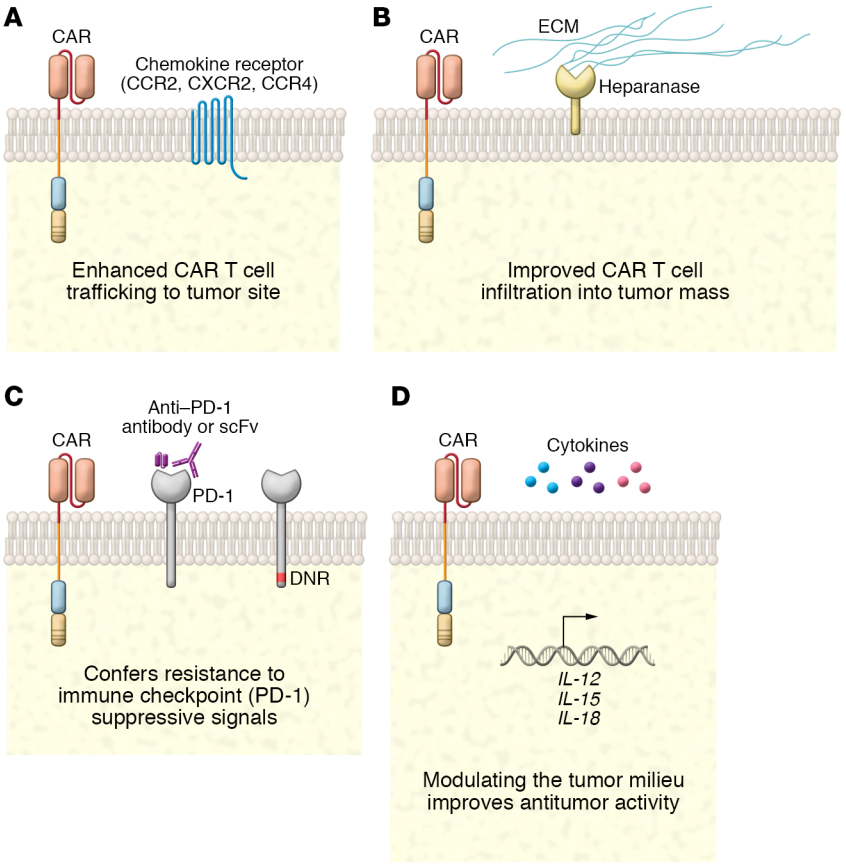

Figure 3. Strategies to enhance efficacy of CAR T cells against solid tumors. Several engineering strategies have been developed to improve CAR T cell function (**A**). Chemokine receptors coexpressed on CAR T cells enhance traffic to the tumor site (**B**). Expression of heparanase improves the ability of CAR T cells to penetrate the tumor stoma by degrading the ECM (**C**). Engineered CAR T cells can overcome inhibitory immune checkpoints by secreting anti–PD-1 antibodies or scFvs; another strategy includes the expression of dominant-negative forms of the receptors (DN–PD-1) (**D**). CAR T cells modified to secrete cytokines have improved function by modulating the tumor milieu. CCR4, CC-chemokine receptor 4; DNR, dominant-negative receptor.

neurologic toxicity was only 5% compared with 32% in the ZUMA-1 trial, with lower serum levels of cytokines compared with those of patients treated with an earlier construct, FMC63-28Z (4). This work demonstrated that newer generation CARs associated with lower levels of in vitro cytokine production are effective yet safer than prior CAR constructs (39).

One approach to mitigating CRS is to genetically delete cytokines associated with toxicities or to induce the secretion of molecules that inhibit their biological action, such as soluble cytokine receptors. The cytokine GM-CSF has recently been associated with the development of CRS. In a lymphoblastic leukemia xenograft model, the neutralization of GM-CSF by lenzilumab reduced neurotoxicity. The deletion of GM-CSF specifically on CAR T cells improved the antitumor activity and survival of mice (40). Based on these results, a clinical trial evaluating the combination of lenzilumab with axicabtagene ciloleucel (axi-cel) in patients with relapsed/refractory (R/R) LBCL is planned (NCT04314843). Similarly, IL-1 secreted by macrophages/monocytes has been shown to play a key role in CRS, and this effect can be countered by engineering CAR T cells able to secrete IL-1 receptor antagonist (IL-1Ra) (Figure 2E and ref. 41) or by administering anakinra, a soluble form of IL-1Ra (42). A phase II clinical trial is accruing patients to evaluate the efficacy of anakinra in reducing severe CRS and neurotoxicity in patients with LBCL treated with axi-cel (NCT04150913).

On/off switches by administration of exogenous small molecules. A novel strategy for controlling adverse events arising from CAR T cell therapy focuses on depleting or arresting infused cells by including safety mechanisms such as suicide genes or safety switches. One approach engineers cells able to engage full antitumor potential upon administration of bispecific adaptors that act as bridges between tumor cells and CAR T cells. These low molecular weight adaptors contain residues able to recognize molecules expressed by cancer cells, such as folate receptors, to engage the CAR-inducing cell activation. Consequently, CRS can then be controlled and even terminated by regulating the administration of the bispecific adaptor (43).

Another strategy for reducing severe toxicities is to selectively trigger apoptosis by introducing an inducible form of caspase 9 (iCasp9) that dimerizes upon exposure to an inert small molecule (AP1903). This approach was initially reported in pediatric patients with acute leukemia who underwent stem cell transplantation, and it led to a rapid deletion of infused T cells (44). This was further evaluated in a humanized preclinical model of CD19-specific CAR T cells with depletion of transferred cells and subsequent B cell reconstitution. The effect was dependent on the dose of dimerizing agent administered (45). A caveat of using suicide genes is the corresponding decrease in antitumor activity, and therefore these strategies may be best utilized in cases of life-threatening toxicity (46). Robust clinical data regarding suicide genes is lacking. Several phase I clinical trials are planned to determine the safety and tolerability of iCasp9 CAR T cells in patients with CD19-expressing B cell malignancies (NCT03016377, NCT03696784).

Investigators evaluated selective expression of the CAR in the cell surface by engineering CAR constructs with an "off switch" encoded as part of the receptor. In this design, a self-cleaving site is controlled by a protease paired to a "degron" moiety (a degrada-

Table 1. Summary of select clinical trials highlighting various mechanisms to reduce CAR T cell toxicity and improve efficacy

Mechanism of intervention	Trial design (NCT) (reference)
Costimulatory/transmembrane domains	
Anti-CD19 CAR derived from the CD19-BBz prototype with costimulatory 4-1BB and CD3ζ domains.	Phase I trial in adults with R/R LBCL. No grade ≥2 neurologic toxicities or CRS in 25 patients treated (NCT02842138) (37)
Anti-CD19 antibody plus hinge and transmembrane domains from CD8α (Hu19-CD828Z)	Phase I trial in 20 adults with R/R B LBCL with reported 5% grade ≥3 neurotoxicity (NCT02659943) (39)
Cytokine depletion	
Neutralization of GM-CSF by lenzilumab	Phase I/II trial in adults with R/R B cell lymphoma combining lenzilumab with axi-cel (ZUMA-19) (NCT04314843) (40)
Blockade of IL receptor by anakinra, an IL-1 receptor antagonist	Phase II trial in adults with R/R B cell lymphoma combining anakinra with axi-cel (NCT04150913) (41)
On/off switches	
CD19 CAR with iCasp9 safety switch	Phase I/I trial in R/R B-ALL or LBCL using i-C9 CD19 CAR T cells. Patients who experience severe CRS or neurotoxicity are given rimiducid to activate the safety switch (NCT03016377, NCT03696784) (44)
NK cells derived from cold blood and modified to express CD19 CAR. NK cells were transduced with iCasp9 as a safety switch	Phase 1/2 trial of 11 patients with R/R CD19 B cell malignancies. There was no severe CRS, neurotoxicity, or GVHD reported; 73% of patients had a clinical response (NCT03056339) (56)
Small molecule inhibitors	
Utilizing a BTK inhibitor, ibrutinib, to improve CAR T cell function and efficacy	Pilot study of 19 patients with R/R CLL treated with CD19 CAR T in combination with ibrutinib. One year PFS of 38% in patients treated with CAR T alone vs. 50% in patients treated with ibrutinib combination (P = 0.91) with lower levels of severe CRS in the ibrutinib cohort (50)
Antigen escape	
Sequential use of CD19 followed by CD22 CAR T cells to prevent antigen escape	Phase I trial of 20 pediatric patients with R/R B-ALL treated with sequential CAR T cell therapy. LFS and OS not reached. Two patients relapsed with CD19-negative disease (66)
Checkpoint inhibitors	
Combination of anti–PD-L1 antibody with CD19-directed CAR T cells	Phase I/II trial combining atezolizumab with axi-cel in patients with R/R LBCL (ZUMA-9). Incidence of CRS, neurotoxicity, and efficacy comparable to the ZUMA-1 trial (NCT02926833) (105)
Combination of PD1 inhibitor with mesothelin-targeted CAR T cells	Phase I/II trial combining pembrolizumab with autologous mesothelin-targeted CAR T cells in patients with malignant pleural disease (NCT02414426) (107)
PD-1 knockout autologous CAR T cells for solid tumors	Esophageal cancer (NCT03706326) Lung cancer (NCT03525782) Mesothelin-expressing tumors (NCT03545815)

LFS, leukemia-free survival; OS, overall survival; PFS progression-free survival.

tion signal) that is able to induce the proteolysis of the CAR protein. In the "on" state, the self-cleavage will generate a CAR able to be expressed on the cell surface, while the degron will undergo proteolysis. The administration of a protease inhibitor (asunaprevir) switches to the "off" state in which the CAR is not cleaved and retains the degron moiety that induces proteasomal degradation of the CAR-degron protein, thus preventing cell-surface expression (Figure 2F and ref. 47).

Dasatinib, a tyrosine kinase inhibitor, disrupts signaling downstream of the activation domain CD3ζ by inhibiting phosphorylation of the lymphocyte-specific protein tyrosine kinase (LCK). Therefore, dasatinib temporarily inactivates CAR T cells in vitro and in vivo, with full antitumor effect restored upon removal of the drug. In a preclinical model of lymphoma, the administration of dasatinib rapidly mitigated CRS (48). Hence, as dasatinib targets the activation motif CD3ζ, it could be used to rapidly reduce CRS of CAR T cells independently of their costimulatory domains.

Small molecular inhibitors can be used to increase the efficacy of CAR T cells. Bruton's tyrosine kinase (BTK) is highly expressed in B cell malignancies, and its inhibitor ibrutinib has been used to treat these malignancies. In a combinatorial approach with CAR T cell therapy, it was shown that ibrutinib does not alter the gene transfer of CD19-CAR T cells, their proliferation, or their in vitro cytotoxic ability. Combining anti-CD19 CAR T and ibrutinib increased engraftment and antitumor efficacy on in vivo models of ALL and chronic lymphocytic leukemia (CLL) (49). It was recently reported that the concomitant administration of CD19-CAR T cells and ibrutinib in patients with CLL showed lower severity of CRS with expansion similar to that of CAR T cells alone (50).

Allogeneic CARs. Despite the clinical efficacy observed with autologous CAR T cell therapy for hematologic malignancies, the complex manufacturing period and financial burden of treatment may limit the widespread use of these therapies. It was recently shown that patients who required bridging therapy between the time of apheresis and CAR T cell infusion had worse overall response. Furthermore, in the US CAR T Cell Lymphoma Consortium of patients with R/R LBCL, 7% of patients did not receive axi-cel after apheresis due to progression and/or death, highlighting the downside of waiting for the manufacturing of cell therapy products (51). One way to mit-

igate this challenge is by developing "off the shelf" allogeneic CAR T products. Nonetheless, the use of allogeneic T cells can give rise to graft-versus-host disease (GVHD). In order to mitigate this, cells have been modified to disrupt the TCR gene and/or the HLA I gene (52). Some groups have employed CRISPR-Cas9 to knock out genes such as TRAC, B2M, and HLA class I for CD19-specific allogeneic CAR T cells maintaining antitumor activity (53, 54). However, the safety and efficacy of this approach may be affected by a proportion of cells still expressing the intended knocked out genes.

Compared with T cells, allogeneic NK cells have the benefit of being relatively safe to infuse even without complete HLA matching, as they do not express a TCR. However, their low persistence after administration limits their therapeutic efficacy (55). In a phase I trial of allogeneic CD19-directed CAR NK cells derived from cord blood, 8 out of 11 patients showed response (56). Notably, none of the patients developed CRS, neurotoxicity, or GVHD, and the maximum tolerated dose was not reached (56). Although the numbers in this study are small, the capability of producing a safe off-the-shelf product can increase access to treatment. Ongoing clinical trials will inform the durability of response and toxicity profile of allogeneic CAR T cell therapies.

Antigen escape

Despite the high response rates of CD19 CAR T cell therapy, the loss or downregulation of CD19 has been reported as a common mechanism of tumor resistance (57, 58). Antigenic escape has been attributed to mutations and splice variants of CD19, leading to low or absent expression of the protein on the surface of malignant cells (59). A recently described mechanism mediating antigen escape consists of the transfer of the target antigen from the tumor cell to the CAR T by a mechanism of trogocytosis. This process of antigen transfer is dependent on CAR engagement to its target, leading to a reduction in the antigen density on the tumor cell and subsequent fratricide. This phenomenon was observed in CAR T cells specific for CD19, CD22, B cell maturation antigen (BCMA), and mesothelin (60).

Current strategies focused on overcoming antigen escape include the use of CAR T cells able to recognize more than one antigen present in the tumor cell. The use of multi-targeted CAR T cell products is an attractive approach that can be obtained by mixing single-targeted cells against different antigens prior to infusion (coadministration) or by simultaneously transducing T cells with different CAR constructs (cotransduction). Another multi-targeted strategy involves employing a single CAR molecule able to recognize two antigens. A bicistronic construct involves one single vector encoding for two independent CAR molecules, while bispecific constructs encode for a single bivalent CAR molecule able to recognize two different antigens (61).

The administration of bispecific CAR T cells able to recognize CD19 and CD20 demonstrated enhanced antitumor activity in preclinical models of B cell malignancies compared with a single CD19 construct. This effect was attributed to a reduced antigen escape by tumor cells (62). In another study, bispecific CAR T cells engineered against both CD19 and CD22 showed in vitro antitumor activity comparable to that of monospecific CD19 CAR T cells, but were able to eradicate CD19-negative patient-derived xenografts (63).

While several groups are evaluating dual-targeted CAR T cells in clinical trials as a strategy for overcoming antigen escape, data are not yet mature enough to determine whether these approaches affect clinical outcomes. Another unique strategy is the sequential infusion of two third-generation CARs targeting CD19 and CD22, respectively. In a single-center study of 89 patients with R/R B cell malignancies, Pan et al. demonstrated the feasibility of this approach and potential for clinical benefit (64). However, patients who relapsed did have evidence of antigen escape with loss of CD19. The use of two different cell populations raises concerns of additive toxicity. While this approach could work in hematologic malignancies, it would be challenging to translate to solid tumors in which there is much more heterogenous antigen expression.

A strategy focused on targeting multiple molecules includes engineering CAR T cells to produce bi-specific T cell engagers (BiTEs). BiTEs are soluble molecules that can be secreted and consist of two scFvs: one able to ligate CD3 and the other to bind a desired molecule expressed on the tumor cell. Therefore, BiTEs can act as physical links between T cells and tumor cells. A CD19-specific BiTE, blinatumomab, was granted approval in 2018 by the FDA for the treatment of B cell precursor ALL (B-ALL) (65). Engineering CAR T cells able to engage and enhance the endogenous immune system of the patient by secreting BiTEs is one approach that can enhance an antitumor T cell response, especially in patients with solid tumors where there is heterogenous antigen expression. A recent report found that CAR T cells able to secrete BiTEs can overcome antigen escape in preclinical models of B-ALL and circumvent antigen heterogeneity in a solid model of glioblastoma (66).

Solid tumors

Despite the impressive clinical results observed in hematological malignancies, progress for CAR T cell therapy in solid tumors has been met with many obstacles. One major factor is the lack of tumor-specific cell-surface antigens in solid tumors. The biologic heterogeneity in solid tumors differs from that in B cell hematologic malignancies and leads to high incidence of "on-target/off-tumor" toxicities due to the expression of target antigens on normal healthy tissues. Moreover, the complex TME in solid tumors negatively affects both endogenous T cells and transferred CAR T cells. This immune-suppressive microenvironment composed of soluble factors and suppressive cells such as myeloid-derived suppressor cells (MDSCs) and Tregs, in addition to the physical barriers that inhibit T cell infiltration into the tumor, add to the challenges for successful CAR T development for solid tumors.

Identifying a tumor-associated antigen in solid tumors. In contrast to B cell malignancies, solid tumors have heterogeneous tumor antigen expression, and these antigens can be expressed in low levels on normal tissues. Even low-level expression in healthy tissues can lead to fatal events (67, 68). Preclinical approaches to improving safety in targeting antigens found in healthy tissue include development of CARs with lower affinity for target proteins, which demonstrated less exhaustion and enhanced proliferation in vivo (69). Several groups have used immunoproteomics, DNA or RNA sequencing, and whole gene exome sequencing to identify novel tumor-associated antigens and neoantigens, including prostate-specific membrane antigen (PSMA) and prostate stem cell antigen (PSCA). Kloss et al. reported a strategy for

enhancing tumor specificity by combinatorial recognition of two antigens, PSMA and PSCA, in a preclinical model. The novelty of this approach was that one CAR had a CD28-signaling domain while the other had a CD3ζ-signaling domain. This led to an improved toxicity profile by reducing reactivity against healthy tissues expressing either antigen alone (70).

Another strategy includes synthetic Notch receptors that were developed to conditionally express the CAR upon cognate ligand engagement on the tumor site. This dual antigenic trigger controls CAR expression in a transcriptional level that can mitigate systemic toxicities and expand the antigen repertoire (71, 72). Efforts are being made to broaden the antigen-binding domain of CARs beyond scFvs. Some approaches include molecules with a lower molecular size, such as camelid single-domain antibody fragments or "nanobodies" (73), ankyrin repeats (DARPins) (74, 75), and the recently described D-domain CARs. In a recent study, T cells armed with a nanobody targeting CD13 were shown to induce antitumor response against preclinical models of myeloid leukemia (76). Moreover, D-domains also have a smaller size than scFvs. These molecules are single-domain structures derived from α-helical bundle protein αD3 able to recognize antigens. In another recent study, T cells armed with a CD123-specific D-domain showed durable antitumor activity in xenograft models of acute myeloid leukemia (77).

Delivery and trafficking to the tumor site. In order for CAR T cells to target surface antigens in solid tumors, they must first traffic to the tumor site and subsequently infiltrate the tumor. To overcome this challenge, local administration of CAR T cells into the tumor site is an approach used to increase the number of available cells tested in brain (78), breast (79), peritoneal carcinomatosis (80), head and neck (81), liver metastasis (82), and lung (83). Although this approach showed promising results, it may be limited to certain types of tumors and could have limited efficacy against distal lesions from the inoculated primary site.

Different chemokines mediate trafficking of immune cells to the tumor bed (84). To exploit this feature, CAR T cells have been engineered to express chemokine receptors in order to increase trafficking to the tumor site (Figure 3A). The coexpression of CC-chemokine receptor 2 (CCR2), receptor for CCL2, together with a CAR specific for the target antigen GD2 was shown to improve trafficking and tumor infiltration in a xenograft model of neuroblastoma (85). Another study demonstrated that expressing CCR2 together with a mesothelin-specific CAR enhanced tumor infiltration compared with CAR T cells with no expression of the chemokine receptor (86). Using another chemokine receptor, CXC-chemokine receptor 2–expressing (CXCR2-expressing) CAR T cells directed against $\alpha_v\beta_6$ were able to control tumor growth with higher efficacy than the conventional counterpart in xenograft models of solid tumors (87).

In order to enhance the penetration ability of CAR T cells into the tumor, cells can be engineered to produce and secrete enzymes that degrade the extracellular matrix (ECM) and thus facilitate tumor infiltration. GD2-specific CAR T cells transduced to overexpress the enzyme heparanase improved the ability of transferred cells to degrade ECM, promoting tumor infiltration and antitumor activity in xenograft models (Figure 3B and ref. 88). In one study, the coexpression of IL-7 and CCL19 in CAR T cells improved the tumor-infiltrating ability of these cells, showing regression of preestablished solid tumors in mouse models (89). Another study reported that nanobody-based CAR T cells directed against molecules highly expressed in the TME, such as PD-L1 and the EIIIB splice variant of fibronectin, showed tumor growth inhibition in solid tumors engrafted in immunocompetent mice (90).

Overcoming the TME. The microenvironment within solid tumors is immunosuppressive due in part to the presence of cells with immune-suppressive activity and tumor-derived cytokines as well as checkpoint inhibitory ligands (91). CAR T cells can be engineered to be resistant to immune checkpoints or secrete scFvs mimicking systemic administration of these therapeutic antibodies (Figure 3C). In one study, CAR T cells modified to secrete anti–programmed cell death 1 (anti–PD-1) scFvs have been shown to act in a paracrine and autocrine manner, enhancing the antitumor activity of both the transferred CAR T cells and the endogenous T cells (92). In a similar approach, CAR T cells engineered to secrete an anti–PD-L1 antibody showed improved antitumor function in a preclinical model of renal cell carcinoma (93). These results were achieved in xenografts and immunocompetent mouse models by blocking the PD-1/PD-L1 axis in situ.

Another approach focused on this axis includes CAR T cells cotransduced to express a dominant-negative form of PD-1 lacking the intracellular signaling domain of the receptor. The inclusion of this truncated form of PD-1, unable to transduce inhibitory signals, improved the antitumor activity of mesothelin-specific CAR T cells in a xenograft model of mesothelioma (94). Other studies have reported that by disrupting the endogenous expression of PD-1 on CAR T cells by CRISPR/Cas9, the antitumoral activity of the transferred cells is enhanced in different mouse models (95–97). It was recently demonstrated that exosomes derived from CAR T cells express CAR molecules and carry cytotoxic molecules that showed antitumor activity upon injection into models of mice bearing solid tumors. These CAR-containing exosomes do not express PD-1, as opposed to CAR T cells, and their in vivo antitumor function is not affected by PD-L1 engagement in the TME (98). Clinical trials with PD-1 knockout autologous CAR T cells are underway for esophageal cancer (NCT03706326), lung cancer (NCT03525782), ALL (NCT03298828), and mesothelin-expressing tumors (NCT03545815).

Few early phase clinical trials have evaluated the efficacy of combining CAR T cells with checkpoint inhibitors. The phase 1/2 ZUMA-6 trial investigated the combination of axi-cel with the anti–PD-L1 antibody atezolizumab in patients with R/R LBCL (NCT0926833). Although there were a small number of patients (n = 28), the best objective response rate of 75% (46% CR) is encouraging. The incidence of CRS and neurotoxicity was comparable to that in the ZUMA-1 trial (99). In patients with R/R neuroblastoma, the addition of pembrolizumab to CAR T cell therapy did not enhance persistence. However, no conclusions can be drawn, as only three patients were treated in this cohort (100). In another report, patients with malignant pleural disease were treated with regionally delivered mesothelin-targeted CAR T cells. Fourteen of the patients received checkpoint blockade agents off protocol and did not experience any toxicity (101). Therefore, the second phase of this trial is evaluating the efficacy of adding pembrolizumab after CAR T infusion (NCT02414269).

Modifying the intratumoral cytokine pool. In order to modulate the suppressive soluble milieu present in solid tumors, CAR T cells can be engineered to secrete immunostimulatory cytokines that enhance activation, proliferation, and antitumor activity (Figure 3D). Some studies showed that CAR T cells designed to secrete IL-12 have enhanced antitumor function in preclinical models of solid tumors, with an additional stimulation of an endogenous antitumor T cell response (102–105). This same beneficial effect of IL-12–secreting CAR T cells was observed for B cell malignancies (106). Another cytokine involved in T cell homeostasis and survival is IL-15. It was demonstrated that CAR T cells armored to secrete IL-15 were able to eradicate more efficiently a xenogeneic metastatic model of neuroblastoma (107). In a similar report, IL-13Rα2–specific CAR T cells engineered to produce IL-15 showed increased in vivo persistence and antitumor activity against glioblastoma models compared with the conventional counterpart (108). Furthermore, CAR T cells designed to inducibly secrete IL-18 showed superior antitumor activity against preclinical pancreatic and lung tumors. Importantly, an increase in numbers of intratumoral M1 macrophages and NK cells, which are associated with antitumor response, was reported for IL-18–armored CAR T cells, while protumoral populations such as Tregs and M2-like macrophages were reduced (109). In a similar study, both human and murine IL-18–secreting CAR T cells showed enhanced proliferative capacity and antitumor immunity in mouse models of solid tumors (110).

Resisting immunosuppressive cells. The presence of immunosuppressive cells in the TME, such as Tregs, MDSCs, and M2-like macrophages, has been shown to inhibit the antitumor ability of tumor-infiltrating T cells (111, 112). It has been demonstrated that these cells also inhibit the antitumor function of transferred CAR T cells in solid tumor models (113). An approach to limiting this suppression is the administration of CAR T cells in combination with agents that reduce the number of these immunosuppressive cells. A study showed that pediatric sarcoma xenografts induced the accumulation of murine MDSCs that inhibited GD2-specific CAR T cell function. The coadministration of GD2-CAR T cells with all-trans retinoic acid reduced the number of MDSCs and improved the antitumor ability of the transferred cells in this model of sarcoma (114). In another report, the transfer of Her2-specific CAR T cells in combination with an anti–4-1BB antibody enhanced the antitumor function of CAR T cells and showed reduced numbers of Tregs and MDSCs in a preclinical orthotopic model of breast cancer (115). Harnessing the capacity of macrophages to penetrate tumors, investigators demonstrated that genetically engineered human macrophages with CARs (CAR-Ms) decreased tumor burden and prolonged survival in xenograft mouse models. Furthermore, CAR-Ms remodeled the TME and were capable of antigen presentation in humanized mouse models (116).

Although chemotherapy-based lymphodepletion reduces the number of immunosuppressive cells to favor the engraftment of transferred cells (100, 117), the idea of engineering CAR T cells that are able to resist the action of these immunosuppressive cells is promising. It was recently demonstrated that introducing mutations at the LCK-binding motif in the CD28 intracellular domain of EGFRvIII-targeting CAR T cells improved antitumor activity against a melanoma tumor model. These mutations rendered CAR T cells resistant to suppression by Tregs without the need for lymphodepleting schemes (118). In another study, VEGFR-2–targeted CAR T cells reduced the number of a subset of intratumoral MDSCs that expressed VEGFR-2 in different models of vascularized subcutaneous tumors (119). These approaches focused on reducing the number of immunosuppressive cells in the TME or rendering CAR T cells resistant to this suppression, which could improve the therapeutic efficacy of this treatment for solid malignancies.

Concluding remarks

CAR T cell therapy has shown remarkable clinical results against B cell malignancies, with early indications that its use for solid tumors could pose a new therapeutic option. Promising bioengineering strategies are being developed to reduce CAR T cell–associated toxicities and improve therapeutic efficacy. Here, we reviewed preclinical and clinical approaches employed to mitigate toxicities and overcome common mechanisms of tumor evasion. Furthermore, we discussed major barriers imposed by solid tumors together with strategies that can enhance and broaden the therapeutic application of CAR T cells in these scenarios. Data obtained from both preclinical research on CAR design optimization and clinical trials are key to shaping next-generation CAR therapeutics with superior outcomes for cancer patients.

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