CAR-T CELL THERAPIES FOR NON-HEMATOPOIETIC MALIGNANCIES: TAKING OFF THE TRAINING WHEELS

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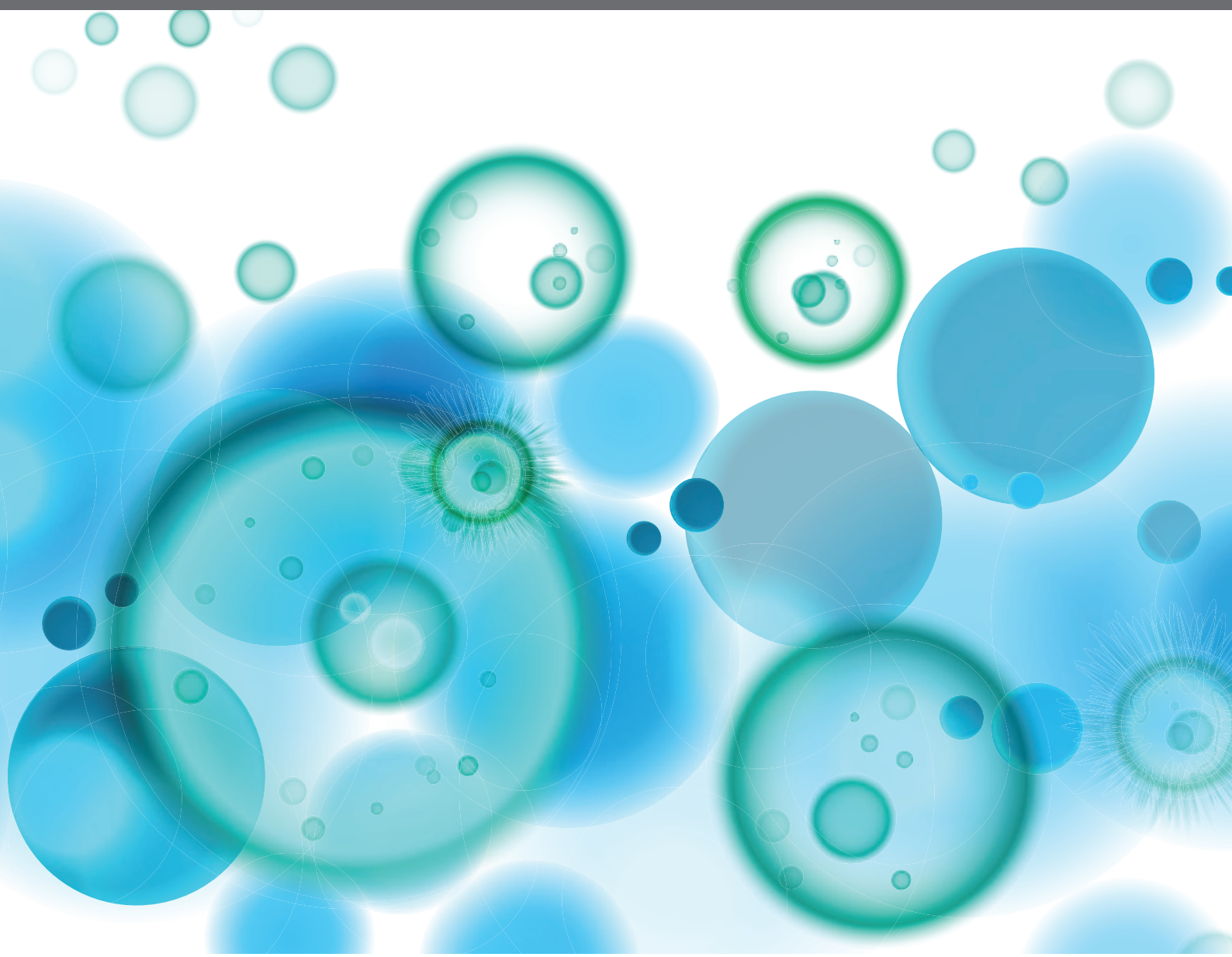

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CAR-T CELL THERAPIES FOR NON-HEMATOPOIETIC MALIGNANCIES: TAKING OFF THE TRAINING WHEELS

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Chimeric antigen receptor (CAR) T cell therapies for leukemia (e.g. tisagenlecleucel) and lymphoma (e.g. axicabtagene ciloleucel) have recently received regulatory approval in the United States. Phase I/II trials have demonstrated complete remission of refractory or relapsed tumors in 50% - 94% patients. However, the clinical successes of engineered T cells for the treatment of solid malignancies have thus far been few and far between. Furthermore, several instances of severe and lethal toxicities have arisen due to on-target, off-tumor recognition of antigen by T cell products.

Recent advances in phase I trials for solid tumors, as well as in pre-clinical models, have revealed several variables that will be important to consider for the successful use of CAR-T cells in treating solid tumors. These variables include (i) regional versus systemic delivery; (ii) scFv versus ligand interactions; (iii) antigen loss versus escape; (iv) epitope spreading and (v) checkpoint expression on immune cells or tumor cells. Also, there remains outstanding mechanistic questions related to why differences exist in the persistence and tonic signaling of second-generation CD28 versus 4-1BB co-stimulated CAR-T cells. In addition, we are now learning the roles of lympho-depleting regimens (and associated toxicities) in modifying the persistence of engineered T cell therapies.

A more comprehensive view of CAR-T cell strategies and important advances, both of pre-clinical and clinical evaluations, in solid tumors is necessary to drive these therapies forward.

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REVIEW
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CAR T Cells in Solid Tumors: Blueprints for Building Effective Therapies

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Genetic redirection of T lymphocytes with chimeric antigen receptors (CARs) has soared from treating cancers preclinically to FDA approval for hematologic malignancies and commercial-grade production scale in under 30 years. To date, solid tumors are less susceptible to CAR therapies and instead have been treated more successfully with immune checkpoint blockade or tumor-infiltrating lymphocyte therapy. Here, we discuss the current challenges in treating solid tumors with CAR T cells, and the obstacles within the host and tumor microenvironment hindering their efficacy. We present a novel three-pronged approach for enhancing the efficacy of CAR T cells whereby a single infusion product can synergize the power of an optimal CAR construct, a highly potent T cell subset, and rejuvenate the endogenous immune response to conquer therapeutically-resistant solid tumors.

Keywords: chimeric antigen receptor, T cell, solid tumor, adoptive cell transfer, checkpoint

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INTRODUCTION

Healing is a matter of time, but it is sometimes also a matter of opportunity—Hippocrates

The interactions between antigen-presenting cells and T cells enable high fidelity host protection against foreign pathogens and malignant cells. T cells have unparalleled ability to not only respond to these antigens but also to formulate memory, permitting a rapid and robust response upon future challenge against the same antigen. In terms of cancer, this potentially means long-term protection against recurrence of tumor cells expressing those antigens. Tumors can express antigens that are rapidly recognized by T cells, where mutations of self-antigens or germline cancer antigens differ sufficiently from normal antigens, or those that are less robustly detected, such as overexpressed self-antigens or differentiation antigens expressed by tissue from which the tumor originates (1). As a result, tumors that are more similar to normal cells, or those with highly immune-suppressive qualities, escape surveillance, permitting their outgrowth and potential to cause great harm. Many technological advances have created opportunities for cancer immunotherapists to bolster the power of T cells against cancer through reeducation and intelligent design to overcome the evasive barriers established by solid tumors. Perhaps immunotherapy represents one such opportunity posited by Hippocrates—a chance for intervention that could heal cancer patients much more effectively than time itself.

Adoptive cell transfer (ACT) comprises one of two arms of immunotherapy and involves *ex vivo* enrichment of tumor-specific cells, expansion to large numbers, and reinfusion into the patient

to specifically target and kill cancer cells. ACT is conducted *via* two approaches: (1) naturally arising T cells that infiltrate the tumor—called tumor-infiltrating lymphocytes (TILs)—can be expanded *ex vivo* from the malignant site or (2) non-therapeutic endogenous lymphocytes obtained from the peripheral blood can be rendered tumor specific *via* genetic redirection with a T-cell receptor (TCR) or chimeric antigen receptor (CAR). The second arm of immunotherapy includes immune checkpoint blockade (ICB), where enhancing priming or rejuvenating exhausted T cells can render a functional, albeit often transient, antitumor state. This review will focus on CAR T cell therapies and how future CARs may work synergistically with other immunotherapies to drive long-lasting cures in patients.

The CAR combines a single chain variable fragment (scFv) ectodomain that can target an antigen of choice with an endodomain comprised of the CD3ζ TCR signal and additional costimulatory domain. Its first use by Kuwana et al. and Gross et al. in the late 1980s revealed that redirection of a T cell with this receptor could induce antigen recognition without the major histocompatibility complex (2, 3). CAR-redirected T cell therapies have been successful in hematologic malignancies but are less effective in treating the majority of patients with solid tumors to date. For solid tumors, immunotherapy based in TIL generation or ICB has been more successful. Conceivably, harnessing a CAR therapy with mechanisms of success from TIL and ICB therapies is a logical approach to overcome the obstacles preventing their effective regression of solid tumors. This review will discuss the current status of CAR therapies for solid tumors and outline a three-pronged approach to enhance these therapies against treatment-resistant cancers based on lessons learned with adoptive immunotherapy.

DESTINATIONS OF CAR T CELL IMMUNOTHERAPY

The ability to harness an immune response against cancer through ACT or ICB has reinvigorated cancer therapies by improving outcomes in patient populations previously resistant to conventional treatment. Genetic redirection of T cells with specificity against a chosen antigen provides theoretical opportunity to invoke long-term immunity, but with varied results based on type of tumors targeted (4, 5). Herein, we will review recent triumphs of CAR T cells against B cell hematologic malignancies, and the challenges currently preventing similar efficacy in treatment of aggressive solid tumors.

Success in Hematologic Malignancies

Since 2010, numerous clinical trials have demonstrated the ability of CAR T cells directed against CD19 to promote clinical responses in acute lymphoblastic leukemia (ALL) (6–10), diffuse large B cell lymphoma (DLBCL) (11–13), chronic lymphocytic leukemia (CLL) (14, 15), and other B-cell non-Hodgkin lymphomas (16, 17) with remissions of up to 90% in some of these cases. Because CD19 is expressed ubiquitously in the B cell lineage, targeting CD19 ablates this cell compartment in patients, though sparing of some plasma cells with long-term humoral immunity is possible (18). Fortunately, B cell aplasia can be treated with immunoglobulins to prevent infections, making this a serious but manageable on-target/off-tumor toxicity (19). As a result of excellent responses in patients refractory to standard of care therapies, two constructs of CD19-CAR T cells have been granted FDA approval. Tisagenlecleucel (KYMRIAH, Novartis), with the 4-1BB/CD3ζ costimulatory domain, was approved in August 2017 for B-ALL (20) and in May 2018 for DLBCL, and axicabtagene ciloleucel (YESCARTA, Kite Pharmaceuticals), with the CD28/CD3ζ costimulatory domain, was approved for DLBCL in October 2017. Administration of these CAR T cell therapies requires specialized training under the FDA Risk Evaluation and Mitigation Strategies to manage adverse events such as cytokine release syndrome or neurotoxicity. These approvals render CAR T cells the first FDA approved personalized gene therapy and establish a major milestone in the field of cancer immunotherapy.

Unfortunately, the dramatic responses reported in patients with B cell malignancies have not yet been consistently reproduced with analogous therapies for individuals with solid tumors. However, it is important to appreciate that CAR T cell development for patients with solid tumors is still in early stages. The historical progress, current status, and major obstacles facing success of these therapies in conquering solid tumors are discussed below.

Clinical Challenges in Solid Tumors

While the results of CAR T cells in B cell malignancies are encouraging, treatment of solid tumors with similar approaches has yielded less favorable results. Similar to therapies for hematologic malignancies, the difficulty in initial design begins with constructing the CAR against an antigen expressed in the tumor—but not in normal tissue—to bolster efficacy while reducing off-tumor toxicity (21). Thus far, clinical trials with CAR T cells in solid tumors have demonstrated severe toxicities since the targeted antigens are often not completely foreign to the host, and even low expression in distant tissues can instigate devastating effects in the presence of a potent T cell therapy (22, 23). Several examples of off-tumor responses in clinical trials are as follows: in renal cell carcinoma, targeting carbonic anhydrase IX (CAIX) led to liver toxicity in 4/8 patients in 2/3 cohorts due to basal expression of CAIX on bile duct epithelium even with low doses of CAR T cells (24, 25). CAR T cells engineered against ERBB2 given in a high dose to a patient with metastatic colorectal cancer caused multi-organ failure with acute pulmonary toxicity due to antigen expression on lung epithelium (26). This resulted in death of the patient within 5 days post-transfer of the cellular product (26). Similarly, a trial for gastrointestinal tumors with CEACAM5-CAR T cells was closed due to poor efficacy and persistence of cells, in addition to toxicity from expression of the targeted antigen on lung epithelium (27). Careful consideration of target antigens is therefore warranted so that a balance between safety and efficacy can be maintained for patients.

Some antigens specific to tumors have been identified that result in more limited off-tumor effects, but many of these targets for CAR T cells have mediated poor clinical efficacy in patients. Several studies using HER2-based CAR in sarcoma (28), mesothelin-specific CAR in mesothelioma and pancreatic cancer (29–31), carcinoembryonic antigen for colorectal cancer (32),

EGFRvIII in glioblastoma (33), and α-folate receptor in ovarian carcinoma (34) have shown safer toxicity profiles but yield no better treatment outcomes than stable disease in most cases. Furthermore, similar to CD19$^+$ B cell malignancies (9, 35), solid tumors treated with therapeutic CAR T cells can undergo antigen escape due to selection pressure favoring tumor cells lacking the targeted antigen. High frequency of EGFRvIII loss in glioblastoma patients, though indicating the CAR T cells are potent against their target, highlights the importance of heterogeneity in antigen targeting for future solid tumor CAR treatments to be successful (36). Despite these challenges, there has been recent success with CAR T cell therapy in glioblastoma. Localized delivery of CAR T cells engineered against IL-13Rα for recurrent glioblastoma resulted in an objective response lasting 7.5 months in one patient with several intracranial and spinal tumors (37, 38). Obtaining responses in such aggressive, end-stage cancers emphasizes the vast potential for CAR T cell therapies and the importance of their future development.

Theoretically, even if the perfect antigen for a solid tumor could be identified and targeted, CAR T cell therapies for solid tumors face further obstacles including poor trafficking to the tumor site (39), as well as limited persistence and proliferation within the host (27, 34, 40–42). Moreover, CAR T cells can be functionally suppressed within the hostile tumor microenvironment (43). These collective hurdles set solid tumor CAR-based therapies apart from liquid tumors (21, 44). The question puzzling the medical community today is how—or if—we can overcome these significant barriers and cure solid tumors with a CAR T cell therapy approach. Reflecting upon these challenges, we hypothesize that the ultimate CAR therapy for solid tumors may be established *via* a three-pronged approach, as illustrated in **Figure 1**. The most therapeutic strategy should (1) encompass specificity through the CAR construct, (2) select for a T cell subset with enhanced persistence, trafficking, and long-lived memory responses, and (3) synergize with the endogenous host response to neoantigens. We will review our field's progress on encompassing these three axes thus far and present our blueprint for what may be necessary to combat solid tumors with next-generation CAR-based approaches.

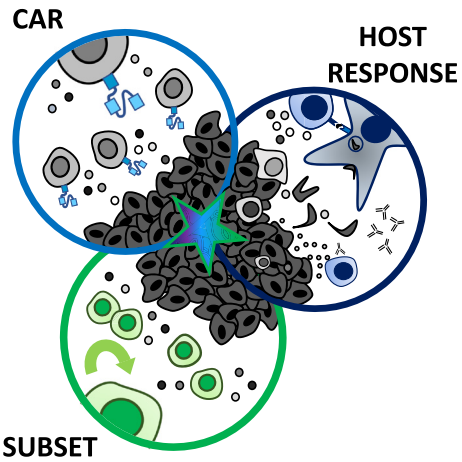

FIGURE 1 | Three-pronged approach to improve chimeric antigen receptor (CAR) T cell therapies in solid tumors. A multi-faceted attack on solid tumors resistant to standard CAR T cell therapies may best augment their efficacy in clinical trials. The ultimate CAR T cell therapy should encompass three axes: (1) a CAR with high fidelity targeting of more than one tumor antigen and trafficking capacity, (2) selection of a T cell subset with potent self-renewal and migratory capacity for long-term persistence and immunity, and (3) ability to harness and rejuvenate the host response to tumor neoantigens. A single arm (CAR, subset, or host response) has not been sufficient for long-term responses against aggressive solid tumors to date.

EVOLUTION OF THE CAR DESIGN

The first three generations of CAR construct design have evolved to incorporate two activating signals (TCR-signaling domains and costimulation) to enhance functionality of therapies *in vivo* and have been reviewed previously (45–47). Briefly, the first-generation CAR, pioneered by several groups in the late 1980s (2, 3, 48–50), consisted of only the scFv region and CD3ζ intracellular domain. These cells demonstrated poor efficacy and expansion in response to antigen, especially if given without exogenous IL-2 (51). The second-generation CAR includes an additional costimulatory domain while the third generation (**Figure 2A**) uses two costimulatory domains in tandem (52); both have greatly enhanced efficacy over the first generation. CD28 was incorporated first (53), followed by ICOS (54), OX40 (CD134) (54, 55), and 4-1BB (CD137) (54, 56, 57). While the optimal costimulatory signals are under debate and may depend on the T cell subset itself, 4-1BB signaling has been shown to improve persistence (15, 58) and enhance metabolic fitness and memory potential of CAR T cells over CD28 (59), and the combination of 4-1BB and ICOS appears promising preclinically (60).

Due to a lack of clinically successful CAR therapies in patients with solid tumors, numerous groups have been inspired to design "fourth-generation" CAR constructs incorporating novel mechanisms to improve antitumor activity. These approaches include enhancing migration and efficacy of the engineered cell, as well as the ability to resist immunosuppression and off-tumor toxicity, illustrated in **Figure 2** and discussed directly below.

Enhancing Migration

Tumors that express fewer chemokines often evade host surveillance *via* impairing effector T cell recruitment and infiltration into the tumor (61). Several different chemotherapeutics have been shown to induce CXCR3-ligand and CCL5, which enhance CD8$^+$ T cell recruitment and reduce tumor growth (62). One chemokine in particular, MCP-1/CCL2, has been correlated with enhanced CCR2-expressing T cell trafficking when secreted by tumors such as gliomas, neuroblastoma, renal cell carcinoma, and mesothelioma (63). For CAR T cells, *ex vivo* activation protocols can alter expression of chemokine receptors, where those such as CCR2 are frequently downregulated (64). Two groups have shown that forced expression of CCR2 on CAR T cells (**Figure 2B**) targeting either GD2 in neuroblastoma (65) or mesothelin for malignant pleural mesothelioma (64) enhances T cell infiltration and augments antitumor activity of the transferred cells. In melanoma, poor T cell infiltration within tumor has been correlated with high tumor IL-8/CXCL8 expression (61); therefore, future CAR

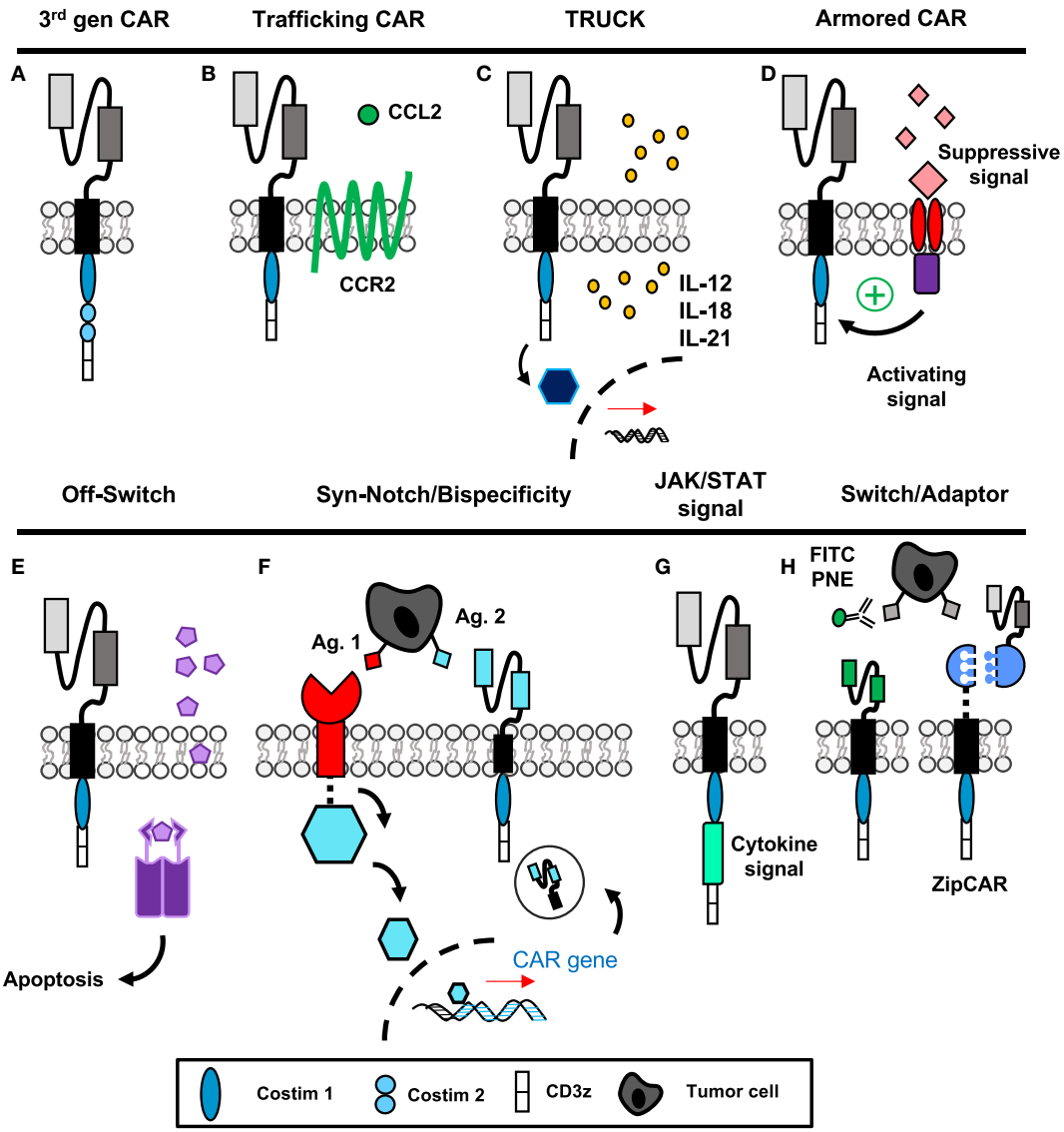

FIGURE 2 | "Fourth-generation" chimeric antigen receptor (CAR) constructs incorporate novel mechanisms to enhance targeted antitumor efficacy. **(A)** The third-generation CAR incorporates the extracellular scFv with intracellular CD3ζ signaling and two tandem costimulatory domains. **(B)** CAR T cells with additional chemokine receptors have improved trafficking to tumors. **(C,G)** T cells secreting additional cytokines or engineered with cytokine signaling domains have enhanced activation and can modulate surrounding microenvironment. **(D)** Armored CARs redirect suppressive signals from the tumor to activating signals to resist exhaustion. **(E)** Suicide genes and **(F)** bispecificity mitigate off-tumor toxicity through the ability to deplete transferred cells or enhance specific targeting to tumors, respectively. **(H)** Switchable CAR targeting *via* adaptor molecules provides versatile opportunity to control CAR activation, specificity, and longevity after transfer of cells. Abbreviations: Ag, antigen; PNE, peptide neo-epitopes.

T cells engineered to express CXCR1 or CXCR2 may also be more efficient at targeting melanoma. As various solid tumors express unique combinations of chemokines, further understanding of these chemokine profiles could aid in the design of novel CAR T cells that can traffic more robustly to the particular cancer they are intended to destroy.

Augmenting Efficacy

As solid tumors have proven to be formidable foes, CAR T cells fortified with enhanced properties of cytokine secretion or cytokine signaling domains have several unique advantages to overcome limitations of the tumor microenvironment, as depicted in **Figures 2C,G**. If the T cell produces a cytokine related to cytotoxic effector programming upon ligation of the CAR, autocrine signaling can activate and support the antitumor activity, persistence, and survival of the transferred cells. In addition, tumor-targeting CAR T cells can deliver cytokines to modulate the cancer microenvironment in an advantageous manner to either activate host effectors or hinder host suppressors to bolster memory T cells in the patient long term.

These cytokine-producing "TRUCKs" (T cells Redirected for Universal Cytokine Killing) have shown efficacy when

delivering IL-12, IL-15, IL-18, or IL-21 to the tumor microenvironment (**Figure 2C**) (66). Of particular clinical importance, IL-12-producing CARs were reported to be therapeutic against lymphoma even without preparative lymphodepletion (67), and significantly enhanced efficacy of MUC-16^{ecto} CAR against a preclinical model of ovarian carcinoma (68). IL-12-producing CD8$^+$ T cells modulated suppressive host myeloid cells within the tumor microenvironment, and as a result improved therapeutic efficacy (69). In a clinical trial for metastatic melanoma patients, autologous TIL engineered to secrete IL-12 yielded objective responses in lower doses compared with unmodified TILs and without systemic administration of IL-2; however, many of these responding patients developed severe liver toxicities and hemodynamic instability (70). Moving forward, it will be critical to deliver localized and inducible IL-12 production within the tumor microenvironment *via* TILs or CAR T cells to more specifically direct its potency while minimizing risk of unacceptable toxicity. IL-15 production similarly improved survival and proliferation of CAR T cells specific for CD19 in leukemia/lymphoma (71) and IL-13Rα2 in glioblastoma (72), as did membrane-bound IL-15 for CD19$^+$ leukemia without significant toxicity (73). Recently, IL-18-producing CAR T cells have been developed. Administration of IL-18 has been shown to augment immunity in solid tumors *via* activating natural killer (NK) cells (74) and is known to induce IFN-γ production from Th1 cells in the presence of IL-12 (75, 76). In CD19$^+$ tumors, IL-18-producing TRUCKS improved engraftment and long-term survival of hosts bearing established tumors (77). Importantly, in mouse models of pancreatic carcinoma and metastatic lung adenocarcinoma—classically highly resistant to treatment—Chmielewski and Abken established that IL-18 secretion and autocrine signaling can induce a T-Bet^{High} FoxO1^{low} signature in the CAR T cells and augment tumor infiltration of NKG2D$^+$ NK cells, while reducing the frequency of regulatory T cells (Tregs) and suppressive macrophages in the tumor microenvironment (78). While improved proliferation and cytokine production within the host are important to antitumor efficacy, the possibility of cytokine-induced dysregulation of CAR expansion or toxicity highlights the need for a form of safety switch or suicide gene within the CAR (71).

IL-21 is a homeostatic cytokine that has shown promise in preclinical TIL and CAR studies, and may be a desirable future candidate to bolster responses in adoptive transfer clinical trials. Programming CD8$^+$ tumor specific lymphocytes *ex vivo* with IL-21 was reported to reduce the activation/exhaustion phenotype of terminally differentiated cells observed after long-term expansion with IL-2 (79). While *ex vivo* cytolytic function of CD8$^+$ T cells upon antigenic stimulation was reduced with IL-21 priming, the *in vivo* melanoma regression was greatly enhanced long term compared with CD8$^+$ T cells primed with IL-2 or IL-15. Systemic administration of IL-21 also enhanced efficacy of tumor-specific CD8$^+$ T cells against melanoma in a preclinical model (80). IL-21 fosters generation of antitumor T cells expressing Tcf7, L-selectin, and Lef1 in the Wnt/β-catenin pathway, inducing a signature of stem-like properties that may support long-lived memory of transferred CAR T cells clinically (79). IL-21 programming of human CAR T cells *ex vivo* was also shown to improve efficacy against CD19$^+$ tumors *in vivo* (81). Furthermore, in a direct comparison, CAR T cells producing IL-21 were superior to IL-15- or IL-2-producing CARs against CD19$^+$ malignancies (82). Therefore, maintenance of memory characteristics *in vivo* through inducible IL-21 expression in CAR T cells, theoretically also supporting memory of endogenous tumor-specific T cells, may greatly improve the longevity of future CAR therapies for long-lasting curative responses.

Another related application of this concept has been described where the CAR construct encodes a costimulatory domain as well as a cytokine signaling domain for IL-2Rβ (**Figure 2G**) (83). Therefore, the CAR T cell does not produce the cytokine, but the pathway downstream of the desired signal is activated upon engagement of the scFv fragment with antigen. Unfortunately, this approach is restricted to augmenting the CAR T cell's efficacy and not the endogenous host response. However, with cytokines like IL-2, which signal to both effector and Tregs, this approach can restrict signal activation to the effector arm of the antitumor response. Collectively, manipulating cytokine production or cytokine signaling has opened new possibilities for generating CARs with desirable traits to bolster their efficacy against tumors and improve immunity of other infiltrating immune cells.

Evading Immunosuppression: Turning Lemons into Lemonade

Even when CAR T cells successfully invade the tumor, they face a microenvironment rich in suppressor cytokines, such as TGF-β and IL-4, and inhibitory molecules including PD-L1 that poise the cancer to escape immunity. To make these limitations advantageous, tumor immunologists are now redirecting TCR or CAR-specific T cells with additional domains that either (1) limit suppressive signaling or (2) convert suppressive signals into activating signals, thus "armoring" T cells against the suppressive elements of the tumor (**Figure 2D**). The earliest studies using this approach were with Epstein–Barr virus-specific T cells engineered with a dominant negative mutation of the TGF-β receptor, which allowed tumor-specific T cells to resist suppression by the tumor-derived TGF-β (84). Likewise, PSMA-specific CAR T cells for prostate cancer engineered with a dominant negative TGF-β receptor demonstrated enhanced proliferation post-transfer and are now being used in clinical trials (NCT03089203, **Table 1**) (85). New studies with CAR T cells have used a chimeric cytokine receptor that binds IL-4, a cytokine that suppresses immunity, *via* an ectodomain but transmits a therapeutic IL-7 signal *via* the endodomain. When IL-4 binds the receptor, instead of the anti-inflammatory STAT6 translocation, the IL-7 pathway phosphorylates STAT5 and polarizes the cell toward an inflammatory Th1 response (86). Similarly, a PD-1/CD28 chimeric switch receptor has been designed to convert an exhaustive stimulus into a costimulatory signal; this construct was shown to enhance cytokine production and *in vivo* efficacy in the presence of PD-L1$^+$ prostate cancer cells compared with CAR-only transduced cells (87). Two clinical trials are ongoing in China with the use of chimeric switch receptors and are described in **Table 1**. These advances in T cell engineering may now enable reversal of mechanisms driving CAR T cell failure in solid tumors.

TABLE 1 | Clinical trials of fourth-generation chimeric antigen receptor (CAR) T cells in solid tumors.

4th Generation CAR T cells in solid tumors		
ClinicalTrials.gov identification	**Trial description**	**Location(s)**
"Armored" CAR		
NCT03089203	CAR T cells targeting PSMA for castration-resistant prostate cancer with dominant negative TGF-β receptor	University of Pennsylvania
NCT02937844	Pilot study of autologous chimeric switch receptor modified T cells in recurrent glioblastoma multiforme	Sanbo Brain Hospital Capital Medical University, Beijing, China
NCT02930967	Chimeric switch receptor with PD-L1$^+$ recurrent or metastatic malignant tumors	China Meitan General Hospital
Suicide genes		
NCT00730613	CAR T against IL-13Ra2 in glioblastoma with Hy/TK suicide switch	City of Hope Medical Center
NCT02992210	4SCAR-GD2 targeting CAR with iCaspase9 domain in refractory solid tumors	Shenzhen Geno-Immune Medical Institute
NCT02414269	Malignant pleural disease treated with Meso-CAR T cells, modified with iCasp9/M28ζ	Memorial Sloan Kettering Cancer Center
NCT01822652	GD-2-CAR T (28-Ox40ζ) and iCaspase9 Suicide safety switch for Neuroblastoma	Baylor College of Medicine
NCT03185468	4SCAR-GS2 with iCaspase9 domain in advanced/metastatic urothelial carcinoma	Shenzhen Geno-Immune Medical Institute
Antibody-producing CAR T cells		
NCT03179007	CTLA-4/PD-1 antibody expressing MUC-1 CAR T for MUC1$^+$ advanced solid tumors	Shanghai Cell Therapy Research Institute
NCT03182803	CTLA-4/PD-1 antibody expressing mesothelin-CAR T for Meso$^+$ advanced solid tumors	Shanghai Cell Therapy Research Institute
NCT03182816	CTLA-4/PD-1 antibody expressing EGFR-CAR T for EGFR$^+$ advanced solid tumors	Shanghai Cell Therapy Research Institute
NCT02862028	PD-1 antibody expressing CAR T cells for EGFR family member positive advanced solid tumor (liver, lung, stomach)	Shanghai International Medical Center, Shanghai, China
NCT02873390	PD-1 antibody expressing CAR T cells for EGFR family member positive advanced solid tumor	Ningbo Cancer Hospital, Zhejiang, China
NCT03030001	PD-1 antibody expressing mesothelin-specific CAR T cells for meso$^+$ malignant tumors (recurrent or refractory)	Ningbo Cancer Hospital, Zhejiang, China
NCT03170141	4SCAR-IgT against EGFRvIII on glioblastoma multiforme, producing PD-1 and PD-L1 antibodies	Shenzhen Geno-Immune Medical Institute

Mitigating Off-Tumor Toxicity

Finally, CAR T cell depletion in patients experiencing uncontrolled toxicity and engineering approaches to enhance specificity to solid tumor antigens are two methods to reduce severe toxicities previously discussed. Suicide genes to deplete CAR T cells, incorporation of epitopes for antibody neutralization, and logic gate control of CAR T cell function have been described. The first examples of suicide genes involved use of HSV-thymidine kinase, which converts ganciclovir into a toxic metabolite (88). However, the problem with this approach is that the response is slow (89) (several days) and the viral proteins themselves may be immunogenic leading to rejection of the cells (90). In recent development, the inducible-caspase 9 system armors the CAR with a homodimer iCasp9 domain that dimerizes upon administration of a small molecule (**Figure 2E**) (89). Dimerization leads to cleavage of caspase 3 and apoptosis of the CAR T cells. Several clinical trials are now incorporating such safety switches into their CAR programs, which are outlined in **Table 1**. In addition, incorporating epitopes like RQR8/CD20 into the CAR construct provides a target for their depletion with antibodies such as rituximab (91). This approach depletes the majority of CAR T cells within a few hours (91). As rituximab is widely used clinically, this is a non-toxic and relatively inexpensive method for rapid deletion of CAR T cells in case of severe toxicity. Though protective against severe toxicities, the iCasp9 and antibody-directed depletion approaches do not differentiate cells causing off-tumor side effects from cells with therapeutic efficacy, which could result in loss of any clinical benefit against tumors.

To improve the discriminatory nature of strategies used to reduce toxicity, design of CAR T cells equipped with tetracycline-inducible systems or AND/NOT Boolean logic gates permit enhanced control over effector responses and improved sensing of tumor targets. Sakemura and colleagues established a Tet-on inducible system for CD19$^+$ malignancies, where administration of a tetracycline turns on CAR expression—useful for a period of heavy tumor burden—while withdrawal of the drug ceases CAR expression but permits survival of the cell—important for periods of off-tumor toxicity (92, 93). Boolean logic gates aim to prevent toxicity while maintaining efficacy, rather than irreversibly deleting CAR T cells that are toxic against both tumor and host. First, AND gates require a combination of antigens for full T cell activation. In prostate cancer, Kloss and colleagues demonstrated that high affinity CAR and chimeric costimulatory receptors targeting two antigens, such as PSMA and PSCA, leads to eradication of cells bearing either target (94). However, with low affinity receptors, activation of one receptor was not sufficient for full T cell activation, making the presence of both antigens necessary for activation (94). Wendell Lim and colleagues have pioneered the use of syn-Notch receptors in CAR T cells where engagement of a tissue-specific antigen by a surface receptor induces transcription of a CAR against a tumor-specific antigen (**Figure 2F**) (95–97). These approaches allow increased sensitization to tumor cells and reduced toxicity against healthy tissues bearing only one of the targeted antigens. Alternatively, NOT gates employ receptors that prevent T cell activation. For example, the iCAR developed by Fedorov et al. has two receptors with opposite functions: first, a receptor for an off-target antigen such as one found on healthy tissue signals the inhibitory cascade downstream of CTLA-4 or PD-1, while a second tumor-specific receptor signals CD3ζ and costimulation for T cell activation (98).

Therefore, CAR T cells can be designed to discriminate between on- and off-tumor targets without compromising survival of the transferred T cells. With these novel CAR T cell designs, toxicities can be managed without loss of antitumor function, though indication of each approach may vary depending upon the type of tumor and immunogenicity of the antigens targeted.

While combinatorial or logical sensing may enhance specificity of CAR T cells to tumor targets in the future, the search for antigens specific for tumors remains an important ongoing approach. Self-antigens are frequently modified through processes such as glycosylation as they undergo mutagenesis and cells experience malignant transformation (99). CAR T cells targeting glycosylated self-antigens in the tumor are potent against several solid tumor types and minimally toxic to the host due to the specificity of glycosylation sites for the tumor (100). Overall, a better understanding of how self-antigens are modified in tumors may represent a simpler approach to achieve high potency and low toxicity clinically.

Remote-Controlled CARs

Very recently, CAR T cells active only in the presence of a soluble, inert adaptor molecule have been brought to life in preclinical systems (**Figure 2H**). Early studies incorporated CAR T cells engineered for specificity against FITC (101) or PNEs (102), which are linked to antibodies specific for antigens on tumor. Recently, "SUPRA" (split, universal, and programmable) CAR T cells were developed where a "zipCAR" domain links an intracellular costimulatory domain and an extracellular leucine zipper (103). This zipper can be targeted with a complementary zipper fused to an scFv region to render the SUPRA CAR T cell tumor specific. These approaches would be particularly useful for generating universal CAR T cells for various tumors; adaptor molecules could be designed for tumor specificity and would provide options for altering specificity post-adoptive transfer, key for situations of selection pressure and antigen escape. The feasibility and speed of developing a new adaptor with specificity for tumors is likely to be much greater than generating a new, personalized CAR T cell product.

As collectively revealed in **Figure 2**, the scientific community's response to challenges in treating solid tumors has been robust and impressive. Indeed, many opportunities now exist for design of future clinical trials incorporating more specialized CAR constructs. However, since persistence of T cells and a long-lasting memory response are ideal for a successful therapy, it is likely that the quality of the lymphocyte itself is as important for building a better CAR to target the antigen. Consequently, we will next discuss what is known about the optimal properties of a T cell for adoptive transfer and future implications of their clinical use in patients with solid tumors.

BEYOND THE CAR: PURSUIT OF THE OPTIMAL T CELL

Ex vivo manipulation of T cells provides a unique opportunity to select the most highly therapeutic cells before transfer, including generation of $CD8^+$ lymphocytes with a distinct memory lineage or polarized $CD4^+$ helper T cell subsets. Despite the advantages of precisely defining the most effective infusion product composition through cell sorting, most clinical trials of CAR T cells to date infuse bulk products in efforts to transfer large numbers of cells (104). One recent clinical trial at Fred Hutchinson used this selective approach by infusing a 1:1 ratio of $CD4^+$ and $CD8^+$ cells with a central memory (CM) signature to treat patients with B-ALL; however, only 16 of the 30 patients had enough $CD8^+$ T_{CM} cells in the peripheral blood to meet their minimum criteria to enrich this population (105). As technologies advance to permit more efficient T cell purification, so will the feasibility of selecting the optimal T cells for achieving long-term cures in patients. By enriching cell subsets with greater potency, reliance on large doses of T cells may become obsolete. Highlighted below are current novel ways in which investigators are generating T cell subsets with enhanced properties for ACT.

$CD8^+$ Memory Subsets

Debate exists about which memory $CD8^+$ T cell subset is ideal for sustaining durable responses to cancer. Some investigators believe that effector $CD8^+$ T cells that secrete heightened IFN-γ are more effective against tumors while others argue that less-differentiated or even naïve $CD8^+$ T cells are the most ideal lymphocytes to foster long-lasting immunity (106, 107). Therefore, we review previous work defining the role of $CD8^+$ T cell differentiation and memory in the context of adoptive T cell transfer therapy.

When activated with a cognate tumor antigen, $CD8^+$ T cells differentiate into a short-lived effector phase poised with cytotoxicity against their target. The exact mechanism of this differentiation pathway remains under dispute and two differentiation models have been proposed. One model suggests that naïve cells differentiate directly into the effector phase, followed by de-differentiation into long-lived memory cells. New evidence supporting this model is highlighted by the ability of an individual cell to lose and regain expression of L-selectin without cell division (108). A second model, known as the linear differentiation model, suggests that naïve cells are programmed into T_{SCM} [stem-cell memory (SCM)], T_{CM} [central memory (CM)], and T_{EM} [effector memory (EM)] subsets with varied capability of responding to antigenic rechallenge, terminating with differentiation into effector cells (**Figure 3A**) (109, 110). Recent epigenetic findings add to this body of literature supporting the linear model of T cell differentiation by showing that after priming, the histone methyltransferase Suv39h1 silences memory genes to direct $CD8^+$ T cells into the effector phenotype (111). Without Suv39h1, the memory subsets are preserved after activation while generation of effector subsets is impaired, suggesting that memory phenotypes are enriched before effector phenotypes (111). For a patient in complete remission from CLL after CD19-CAR T therapy, CAR integration into the tumor suppressor gene, *TET2*, resulted in robust clonal proliferation of CAR T cells with a predominantly CM phenotype (112). While this particular integration site was not by design, this clinical example highlights the intricacy of memory differentiation and the implications of driving the T cell toward a particular memory phenotype on patient outcomes. This suggests that epigenetic or genetic manipulation of T cells *ex vivo* could be a novel approach to control memory differentiation of

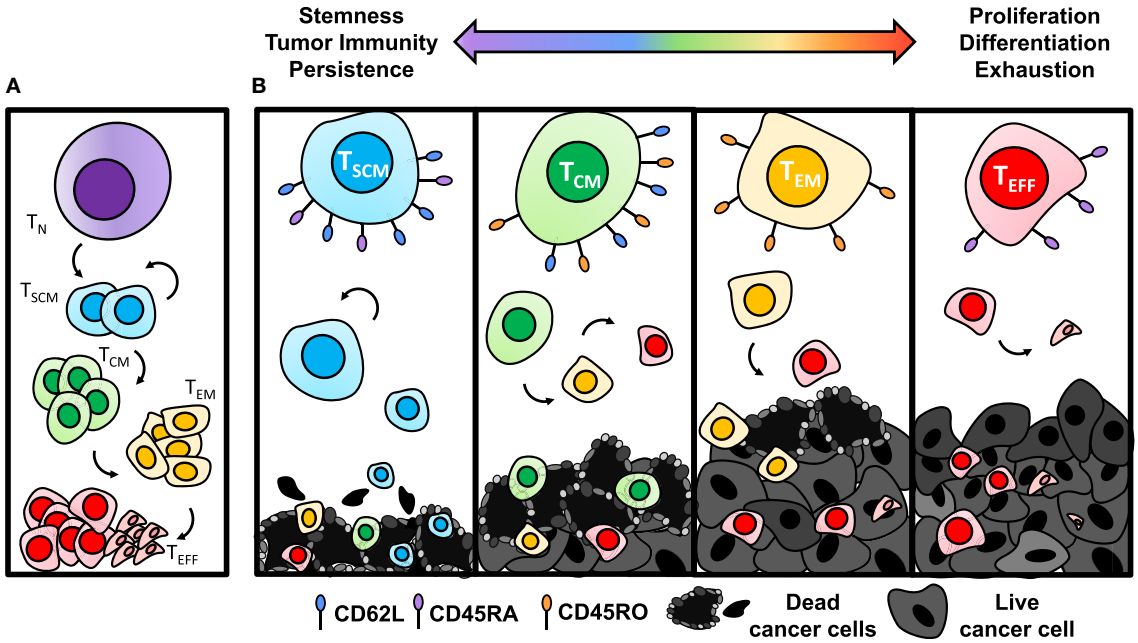

FIGURE 3 | Antitumor efficacy of memory CD8⁺ T cell subsets diminishes with differentiation. **(A)** Once activated with cognate antigen, CD8⁺ T cells progressively differentiate from stem-cell memory (SCM), with the highest capacity of self-renewing properties, to central memory (CM), effector memory (EM), and finally to terminal effector (EFF) phenotypes. **(B)** Antitumor immunity of T_{SCM} cells is enhanced due to establishing long-term memory responses to tumor antigens and heightened ability to persist. As cells become more differentiated through the T_{CM}, T_{EM}, and T_{EFF} stages, they lose capacity for self-renewal and become exhausted, resulting in poor antitumor immunity.

cells and generate a more therapeutic product before transferring cells into patients.

The antitumor efficacy of adoptively transferred memory subsets has been shown to progressively worsen as cells expand logarithmically and often approach the T_{EFF} phase (**Figure 3B**) (106, 113, 114). By contrast, T_{SCM} cells, characterized by $CD45RO^{-}CD45RA^{+}CD95^{+}CD62L^{+}$ expression (107), were most potent in a direct comparison of human meso-CAR-engineered memory subsets due to their enhanced proliferative capacity and survival (110). In addition, T_{SCM} cells have the ability to self-renew across several cell divisions when reactivated (110). Following the path of differentiation, tumor specific T_{CM} cells are traditionally more effective for long-term regression of established solid tumors than T_{EM} (114), while all memory subsets are superior to T_{EFFs} (113).

As a result of finding that less-differentiated memory cells are superior in regressing tumors in ACT models, there is now a growing clinical interest in the ability to expand T cells to large numbers for ACT, while concomitantly inhibiting phenotypic differentiation to foster more stem-like features and enhanced potency against tumors. One approach to accomplish this objective includes targeting downstream of the IL-2 pathway during *ex vivo* expansion through inhibiting subunits of GSK-3β (115), AKT (116), and PI3K (**Figure 4**) (117). GSK-3β inhibition was shown to bolster Wnt/β-catenin signaling and maintain the T_{CM} phenotype with *ex vivo* expansion, thus improving efficacy of infused antitumor CD8⁺ T cells (115). Inhibition of AKT (AKT inhibitor VIII) (116) or the p110δ subunit of PI3K (Idelalisib/CAL-101) (117) *ex vivo* were also two strong approaches to enrich the frequency of T_{CM} cells in infusion products and improve ACT with CAR-engineered cells for leukemia and mesothelioma models, respectively. However, when directly compared in a transgenic model of melanoma, CAL-101 improved persistence of $CD8^{+}KLRG1^{lo}CD62L^{hi}$ cells in the peripheral blood and significantly enhanced tumor regression compared with AKTi (117). CAL-101 has also been shown to improve antitumor efficacy of Th17 cells by enhancing the proportion of T_{CM} cells and reducing Tregs in culture (118). These reports reveal that adding small molecules to cultures can propagate T cells with a stem-like memory signature. This approach presents a simple and translatable way to improve both the quality and longevity of antitumor responses.

An alternate approach to generating more naïve-like T cells for ACT involves genetic reprogramming of induced pluripotent stem cells (**Figure 4**) (iPSCs). Theoretically, reprogramming T cells in this manner poses the opportunity to de-differentiate terminally exhausted tumor- or neoantigen-specific T cells, such as found in a TIL culture, into "younger" more memory-like cells, while retaining their rearranged TCR (119). Early reports on this concept showed the feasibility of generating iPSCs from peripheral blood mononuclear cells *via* induction of Oct4, Sox2, Klf4, c-Myc, and Lin28 (120–122). To move this approach into CAR T cell therapies, Sadelain and colleagues engineered peripheral T lymphocyte-derived iPSC cells to express a CD19-CAR, and subsequently differentiated them back into the lymphoid lineage (123). However, upon phenotypic analysis, they were genetically more closely related to innate γδ T cells and functionally demonstrated weaker antitumor efficacy compared with the desired αβ

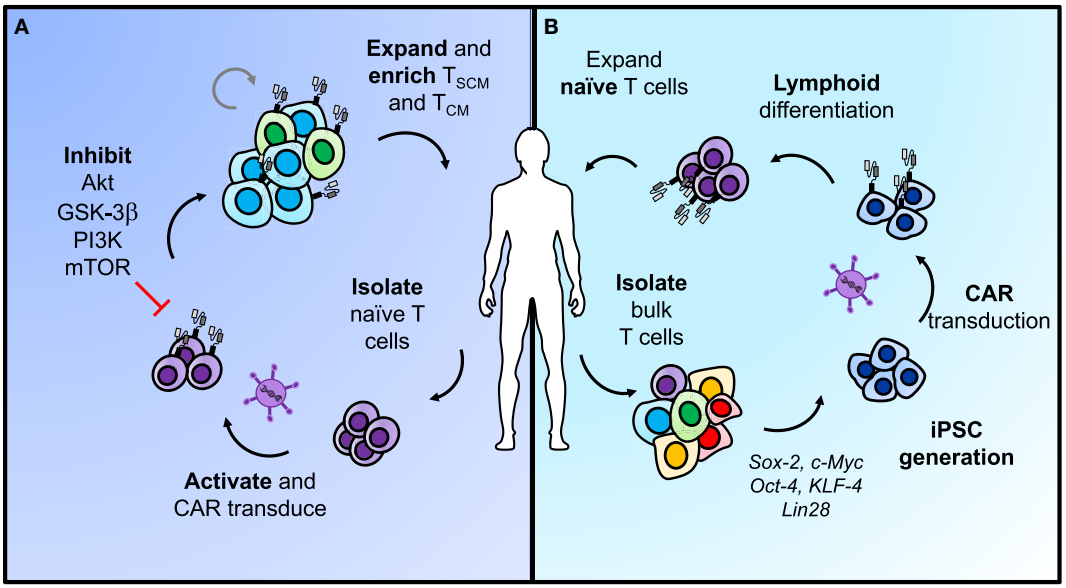

FIGURE 4 | Two approaches for generating less-differentiated T cells after *ex vivo* expansion for adoptive cell transfer. **(A)** Naïve T cells sorted from peripheral blood can be activated and transduced with chimeric antigen receptors (CARs) for antigen specificity. Adding pharmacologic inhibitors of AKT, GSK-3β, PI3K, or mTOR to the T cell culture helps retain cells in a less-differentiated state as they expand. This approach can enrich T_{SCM} and T_{CM} phenotypes in CAR T cells from naïve populations before adoptive transfer to enhance long-term immunity. **(B)** Differentiated T cells can be reprogrammed with stem-like qualities using iPSC technology. In brief, bulk T cells are isolated from the blood, programmed into iPSCs, and transduced with a CAR before lymphoid differentiation into naïve T cells. The most efficient approaches for lymphoid differentiation into naïve phenotypes are still under development.

T cells normally used in ACT (123). This process can also take up to 2 months to generate these CAR T cells, making the time investment on par with or even greater than expanding TIL *ex vivo* (123). Other attempts to program iPSCs down the lymphoid pathway *in vitro* have resulted in abnormal T cell development due to the absence of thymic selection or have generated T cells with effector-like phenotypes (123–125). To generate a potent response against tumors, the αβ$^+$ TCR indicative of a more natural T cell is required.

In response to this need, the Restifo lab devised a new approach for generating tumor-specific T cells from iPSCs *in vitro* with a phenotype closer to endogenous, thymic-derived T cells (126). Their 3D thymic culture system generated tumor specific CD8αβ$^+$ naïve-like T cells that regressed melanoma and prolonged survival comparably with *bona-fide* naïve T cells obtained from the pmel-1 transgenic mouse spleen (126). This new approach is exciting as it may permit generation of a more robust supply of CAR-engineered naïve-like T cells to mediate long-term cures in patients whose peripheral T cells were previously dysfunctional. Moving forward, inhibition of memory differentiation pathways in *ex vivo* culture and further developments in the feasibility of genetically reprogramming iPSCs will support generation of memory-like CD8$^+$ subsets with enhanced antitumor properties, thereby improving patient outcomes.

CD4$^+$ T Cell Subsets

While ACT with CD8$^+$ T cells has been more thoroughly studied, the impact of CD4$^+$ T helper cells on tumor immunity has recently emerged both preclinically and clinically (127, 128). This body of work indicates that CD4$^+$ T lymphocytes may play a key role in enhancing cancer immunotherapy. Since CD4$^+$ T cells classically support CD8$^+$ T cell activation and proliferation through cytokine secretion, an infusion product containing only CD8$^+$ cytotoxic T cells, as is used frequently in the clinic, may show poor persistence simply due to flawed design. Recently, adoptive transfer of a CD4$^+$ dominant T cell product resulted in tumor regression in a patient with metastatic cholangiocarcinoma (127) and a complete durable remission in a patient with metastatic breast cancer (128). These cases hint that CD4$^+$ lymphocytes may be a powerful subset that should not be selected against. Could it be possible that contrary to accepted dogmas, CD4$^+$ T cells may be able to lyse tumor cells themselves without reliance on CD8$^+$ T cells? The quality of tumor immunity may ultimately depend upon the CD4$^+$ subset transferred, and whether these subsets require CD8$^+$ T cells to exert antitumor effects is unclear and will be discussed further below. Herein, we will examine the role of CD4$^+$ T cells in tumor immunity (**Figure 5**), new discoveries of potent subsets within the CD4$^+$ lineage, and clinical implications of engineering human CD4$^+$ T cell subsets with CAR-specificity to extend treatment outcomes.

Cytokine and costimulatory cues can polarize naïve CD4$^+$ T cells into distinct subsets, such as Th1, Th2, Th17, Th9, Th22, T follicular helper, and Treg. The presence of various cytokines needed during activation by antigen-presenting cells to generate these various subsets is reviewed elsewhere (129–131). In the context of tumor immunity, CD4$^+$ T helper cells aid activation of CD8$^+$ cytotoxic lymphocytes (132, 133), but can also eradicate tumors in the absence of CD8$^+$ T cells (134, 135). The relative

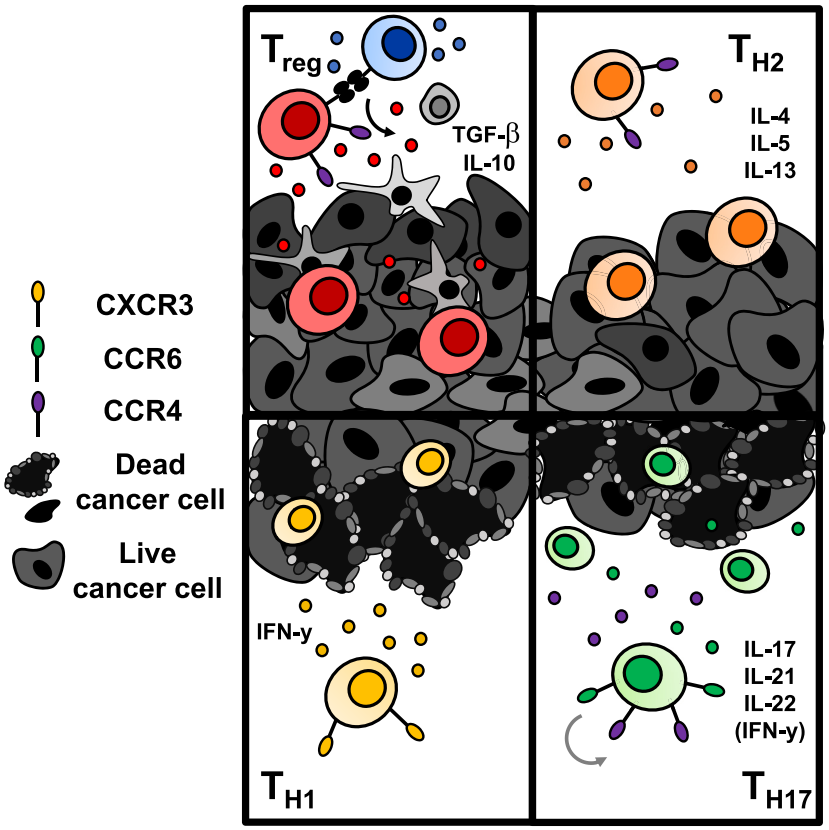

FIGURE 5 | Antitumor immunity of $CD4^+$ T cells is dependent upon the subset to which they are polarized. Regulatory T cells (Tregs) (top left) and Th2 cells (top right) are classically tumor promoting. Tregs downregulate effector T cell responses *via* secretion of suppressive cytokines or engagement of inhibitory checkpoint molecules like CTLA-4 or TIGIT. Th2 cells secrete suppressive cytokines that hinder a Th1-mediated antitumor response. Conversely, transfer of Th1 cells (bottom left) and Th17 cells (bottom right) enhance antitumor responses. Th1 cells produce IFN-γ and enhance $CD8^+$ cell-mediated immunity. Th17 cells produce proinflammatory cytokines that have controversially been implicated in carcinogenesis; however, adoptive transfer of Th17 cells has shown robust immunity in several solid tumors. Transferred Th17 cells have stem-like self-renewal capabilities and enhanced persistence long term over Th1 cells.

antitumor immunity of Th1, Th2, Th17, and Tregs has been thoroughly studied, and emerging reports on the potency of human $CD4^+CD26^{high}$ T cells that possess improved migration, persistence, and multi-functionality underscores the rationale for translating adoptive transfer of $CD4^+$ T cells clinically (135).

The historical understanding of T helper subsets originated with a hypothesis of two opposing helper subsets, termed Th1 and Th2, with distinct functions in promoting cell-mediated or humoral immunity, respectively (136). While both Th1 and Th2 have demonstrated some degree of antitumor efficacy *in vivo*, Th1 cells were shown to induce a $CD8^+$ CTL memory response against antigen rechallenge while Th2 cells did not (137). The mechanism of Th1-mediated immunity relies on their production of IFN-γ, which can augment $CD8^+$ T cell infiltration and macrophage production of nitric oxide to induce tumor cell apoptosis (138). In a recent clinical trial, $CD4^+$ Th1 cells specific for ERBB2IP were successful in regressing a patient's metastatic cholangiocarcinoma (127). Conversely, Th2 cells, as producers of IL-4, have largely been regarded to promote tumor growth because they inhibit the Th1 polarization program and produce suppressive IL-10 (139). Other reports reveal that Th2 cells stimulate tumor necrosis through inhibition of angiogenesis (140). Recently, in a prophylactic myeloma model, adoptive transfer of Th2 cells induced a strong type II inflammatory response at the tumor site and prevented tumor growth *via* M2-macrophages producing arginase (141). However, these cells were transferred into a host deficient in IFN-γ, which may itself support persistence of Th2 cells, so translational relevance of their efficacy is debatable. Also, dissent over the role for Th2 cells in ACT is furthered since arginase activity has previously been correlated with tumor progression (142). This body of work underscores a need to further understand the role of Th2 cells in antitumor immunity.

Th17 cells, characterized by high IL-17 production, play a contested role in tumor immunity but have been shown highly potent in several preclinical ACT models. Th17 cells are phenotypically polarized by the cytokines IL-6, TGF-β, and IL-1β *via* induction of STAT3 and RORγT and are maintained long term by IL-21 and IL-23 (143–146). ICOS costimulation fosters differentiation and expansion of Th17 cells (147) as well as the function of IL-17-producing $CD8^+$ T cells (148). Incorporation of an ICOS costimulatory domain in CAR T cells augments persistence of co-adoptively transferred $CD8^+$ T cells in a humanized model of

mesothelioma (60). In several different cancer models, transfer of Th17-polarized cells enhanced survival and tumor regression superiorly to Th1 or unpolarized $CD4^+$ cells (147, 149, 150). In addition, when expanded *ex vivo* long term, Th17 cells retain their antitumor efficacy while Th1 cells lose tumor control (151). Phenotypically, Th17 cells express more stem-like markers (CCR7, Lef1, TCF7) and fewer exhaustion markers (PD-1, KLRG-1, Tim3) compared with their Th1 counterparts, possibly contributing to longevity (150, 151). Important to the field of CAR therapies, Th17-polarized human meso-CAR T cells exhibit enhanced immunity against mesothelioma versus Th1-polarized cells after both short and long expansion (151). Also, in patients with CLL treated with CD19-CAR T cells, complete responders had CAR T cells with a transcriptomic profile of STAT3/IL-6 signaling, generating a type-17 signature with higher production of IL-17 and IL-22 compared with non-responders (152). Thus, it is truly possible that isolating human PBMCs and polarizing cells to a Th17 phenotype during CAR transduction and activation may generate a therapy with enhanced persistence and thus a long-lived response in patients with solid tumors refractory to treatment with standard bulk CAR T cell preparations.

Despite such preclinical evidence of antitumor potency, adoptive transfer of engineered cells polarized to Th17 phenotype has not been translated yet clinically. The numerous cytokines required to polarize may generate a T cell with enhanced stem-like properties and persistence but also represent a major hurdle halting ease of translation. Our lab has recently described a novel method for isolating potent $CD4^+$ T cells *via* surface expression of CD26, an ectoenzyme with costimulatory properties (130, 135). In work pioneered by Nelson and Bailey, $CD4^+$ T cells expressing high levels of CD26 are polyfunctional, secreting up to five cytokines simultaneously including IL-17 and IFN-γ, and have robust migratory capacity. $CD4^+CD26^{high}$ meso-CAR T cells are highly potent against difficult to treat mesothelioma and pancreatic tumors, and have superior persistence compared with other subsets expressing intermediate or low levels of CD26 (135). Clinical translation of CAR-engineered $CD26^{high}$ cells could support superior trafficking, long-term persistence and cytotoxicity at baseline, which could be further enhanced with fourth-generation CARs; thus, these cells are a strong candidate for overcoming major barriers to successful solid tumor CAR therapies.

T cell memory, persistence, and therapeutic efficacy are tightly related to metabolic state, and within an unfavorable environment such as a solid tumor, their ability to use nutrients for energetic needs may mean the difference between life and death of the cell. Just as different memory or helper subsets have varied capacity to kill solid tumors, T cells armed with a superior metabolic state are more equipped to exert their effector functions and generate long-lasting memory responses against tumor antigens. Therefore, we present metabolic manipulation of antitumor T cells as another approach to generating potent therapies below.

Fine-Tuning Metabolic Fitness

How T cells use energy to survive in the tumor microenvironment has recently gained the interest of cancer immunotherapists. Manipulation of T cell bioenergetics to elicit immunity to solid tumors has shown great promise recently in the preclinical setting. At the fundamental level, it is now clear that lymphocytes engage specific metabolic pathways to best support their functions, intricately regulated by nutrient demand and availability (153). Resting T cells favor energy production through the TCA cycle and fatty acid oxidation (154). Once activated, however, both $CD4^+$ and $CD8^+$ T cells become quickly poised to exert an effector response, and thus upregulate biosynthetic pathways and rely on aerobic glycolysis, where glucose is rapidly consumed and shuttled through glycolysis to lactate to support their proliferation and effector functions (154, 155). Conversely, memory and Treg cells operate using mitochondrial metabolism and fatty acid β-oxidation in a similar manner as naïve T cells (156). Induction of anabolic, glycolytic pathways may augment proliferation and the inflammatory nature of T cells but correlates with poorer persistence *in vivo*, which in adoptive transfer therapy directly associates with a less effective antitumor response (157, 158). As memory-like T cells are most effective in mediating long-term responses to solid tumors, new data implicates that modulation of their metabolism to favor catabolic pathways may generate a lymphocyte population with enhanced antitumor functions *in vivo*.

Yet, complete denial of anabolic pathways is not a quality of successful T cell therapies. In fact, blunting the anabolic pathway in T cells prevents their capacity to lyse targeted antigens. For example, in models of autoimmunity, genetic deletion of *Glut1* prevented effector T cells from causing pathology in inflammatory bowel disease (159). Inhibition of fatty acid synthesis in T cells limited Th17-induced autoimmunity and promoted a Treg signature, notoriously implicated in promoting tolerance to tumors (160). Pharmacologic inhibition of glycolytic enzyme GAPDH with dimethyl fumarate prevented acquisition of effector function in Th17 cells and skewed their polarization *ex vivo* toward a Treg phenotype, reducing autoimmune pathology in models of experimental autoimmune encephalitis (161). Therefore, direct inhibition of glycolysis in T cells is likely to be deleterious for cancer therapies. Augmentation of fatty acid oxidation in $CD8^+$ T cells by treating mice with metformin, on the other hand, promoted memory T cell formation and enhanced immunity to tumor challenge post vaccination (162). Fostering a balance between memory-like metabolism and intrinsic support of glycolysis in CAR T cells may be important for maintaining T cell function and fate within the metabolically restricted tumor microenvironment when the supply of glucose and oxygen is limited (163).

Interestingly, several groups have demonstrated that metabolic manipulation of T cells *in vitro* can benefit antitumor efficacy of transferred cells *in vivo*. Overexpression of glycolytic enzyme phosphoglycerate-mutase 1 limited persistence of transferred $CD8^+$ T cells, while inhibition of glycolysis with 2-deoxyglucose augmented stem memory characteristics like Tcf7 and Lef1 expression, and significantly enhanced survival of tumor-bearing hosts (164). Inhibition of AKT signaling, discussed previously as a method for reducing T cell differentiation *ex vivo*, was also shown to decrease glycolytic function and enhance mitochondrial spare respiratory capacity in $CD8^+$ T cells (165). Moreover, when these AKTi-treated T cells were transferred into mice, they persisted superiorly to untreated cells (165). Similarly,

inhibition of the inositol triphosphate receptor, an important second messenger for calcium release from intracellular storage, in $CD4^+$ T cells *ex vivo* prevented glycolytic initiation due to altered calcium flux, fostered a CM phenotype, and augmented their therapeutic efficacy against established melanoma tumors (166). Interestingly, the integrity of the mitochondria in T cells also profoundly impacts their capacity to mount durable immunity to tumors. For example, Pearce and colleagues showed that mitochondrial morphology is tightly related to T cell metabolism; fused mitochondria, described as tubular and closely associated, were characteristic of memory T cells. Conversely, effector T cells were composed of "fissed" or distinct mitochondria dispersed throughout the cytoplasm (167). Forced mitochondrial fusion and inhibition of fission of T cells *via* pharmaceutical approaches *ex vivo* using M1 and Mdivi-1, respectively, promoted a superior antitumor response once transferred *in vivo* (167).

Emerging data reveal that programming and polarization of $CD4^+$ T cells also critically determines metabolic commitments and modulates their antitumor properties. Recently, the Mehrotra lab reported that *ex vivo* polarized Th1/Th17 hybrid cells upregulate glutaminolysis and rely on oxidative phosphorylation compared with glycolytic-Th1 cells, ultimately supporting their superior antitumor capacity over traditional Th1 or Th17 cells (168). Homeostatic gamma chain cytokines have also been shown to alter the metabolic fate of antitumor T cells. For example, priming T cells with IL-15 (169) or IL-21 (170), previously discussed as a potential method for enhancing their stemness, redirects metabolism away from glycolysis in favor of fatty acid β-oxidation. This bioenergetic signature directly correlates with T cells possessing longer-lived memory responses to tumors and foreign antigen. Thus, it seems that holding back acquisition of full effector glycolytic capacity in CAR T cells *ex vivo* before infusion could greatly enhance persistence of cells in patients, augmenting therapeutic outcomes in solid tumors.

IMPACT OF HOST IMMUNITY

It is possible that targeting solid tumors *via* a single or combination of several known surface antigens, even with the most persistent or metabolically fit T cell subset, will not be sufficient to evoke cures in patients with heterogenous hard-to-treat solid tumors. Thus far, TIL therapies and ICB have shown greater responses in treating these types of tumors, likely through their ability to induce or bolster an endogenous response of exhausted cells against a highly personalized repertoire of neoantigens existing within the tumor (171–173). TIL therapies in melanoma have shown response rates of up to 50% in contrast to previously FDA approved therapies such as interleukin-2 with response rates near 15% at best (174), and melanoma patients with the highest neoantigen load have the best progression-free survival (175). PD-1 blockade success in clinical trials has led to FDA approval for solid tumors such as metastatic melanoma, advanced NSCLC, recurrent or metastatic SCC of head and neck, refractory classical Hodgkin lymphoma, urothelial carcinoma (176), and as second line in MMR/microsatellite instability-high tumors as of May 2017.

As TIL therapies and checkpoint blockade have generated robust results in patients in several solid tumors, it is likely that incorporating the mechanisms of TIL/ICB into CAR constructs may improve their efficacy. Activated CAR T cells within the tumor microenvironment do express high concentrations of exhaustion markers such as PD-1, Tim-3, Lag3, and 2B4 (177). PD-1 expression also contributes to reduced efficacy of transferred cells regardless of tumor specificity (177). Strategies to improve efficacy of PD-1 expressing, exhausted CAR T cells or to rejuvenate host tumor-specific exhausted T cells along with CAR therapy are threefold: (1) genetic removal of PD-1 from CAR T cells, (2) combination PD-1 blockade with CAR infusion, or (3) CAR T cell production of PD-1 blockade within the host. These strategies and our recommendations for designing next-generation CAR therapies with highest efficacy are discussed below.

The first evidence of reducing PD-1 signaling from a CAR T cell was shown through a PD-1 dominant negative receptor, where engagement of PD-1/PD-L1 would not generate a signal (178). The dominant negative receptor enhanced the functionality of CAR T cells and survival of mice treated with meso-CAR against mesothelioma compared with control CAR with the ability to signal PD-1 (178). Recent advances in genome editing using CRISPR/Cas9 technology have permitted removal of PD-1 entirely from T cells, and in two solid tumor models (prostate and glioma) have shown benefits of this intervention for tumor regression (179, 180). While important for efficacy of transferred cells, and likely to be incorporated into more T cell therapies in the near future, removal of PD-1 would not benefit endogenous exhausted cells specific for potentially unknown antigens. In addition, genetic deficiency of PD-1 has been shown to induce terminally exhausted cytotoxic $CD8^+$ T cells; without PD-1, T cells have robust cytokine production and proliferation upon early exposure to antigen, but contract more rapidly and have compromised long-term survival compared with T cells with normal PD-1 expression (181). Thus, genetic removal of PD-1 may not benefit CAR T cell survival long term.

Theoretically, CAR-mediated destruction of tumor cells could also lead to generation of new antigen-specific lymphocytes *via* epitope spreading (**Figure 6**) (182). These newly activated cells are susceptible to suppression within the tumor similar to CAR T cells. To overcome this limitation, PD-1 blockade could be given in combination with or could be encoded by CAR T cells to both support the transferred cells and the endogenous tumor-specific lymphocytes. Preclinically, combination therapies in solid tumors have demonstrated enhanced proliferation, function, and antitumor efficacy of HER2-CAR T cells in breast cancer and sarcoma (183). At the time of writing, clinical trials with such combinations are heavily skewed toward blood cancers (NCT02926833, NCT02706495, and NCT03287817 in DLBCL, NCT03310619 in B cell NHL, and NCT02650999 in DLBCL, follicular lymphoma, and mantle cell lymphoma; ClinicalTrials.gov identifiers). Preliminary results in these hematologic malignancies suggest that PD-1 blockade may enhance CAR T cell persistence and could improve objective responses in patients (184, 185). Thus, there is rationale for combining these approaches to improve persistence of CAR T cells and generate more robust responses.

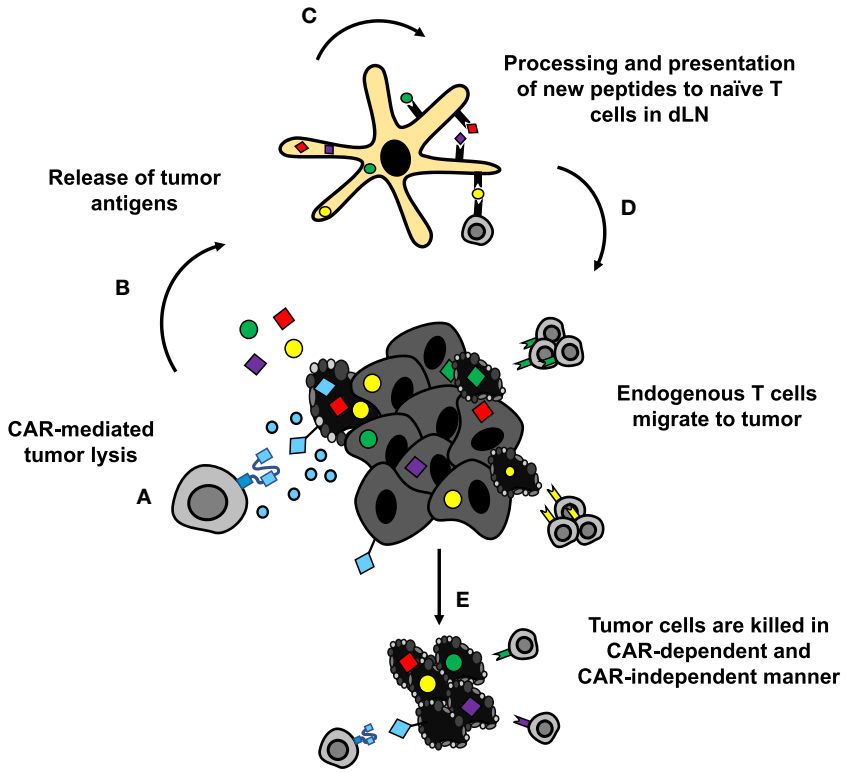

FIGURE 6 | CAR-mediated tumor destruction can synergize with host immunity through epitope spreading. **(A,B)** Chimeric antigen receptor (CAR)-mediated tumor cell lysis induces inflammation, and release of tumor antigens. **(C)** DAMPs from dying cells recruit APCs to tumor site, which take up and process the released antigens for presentation. **(D)** APCs present newly processed tumor antigens to naïve T cells in lymph nodes. Activated T cells migrate to the tumor site. **(E)** Tumor-specific lymphocytes synergize with CAR T cells to eradicate difficult to treat solid tumors.

This principle could be streamlined even further if the CAR T cells produced monoclonal antibodies that inhibit checkpoint molecules themselves. Preclinical evidence in lung and ovarian tumors shows that CAR T cells producing PD-1 blocking antibodies are more therapeutic than control CAR T cells against the same target (186). Importantly, CAR T cell production of PD-1 antibody was more effective than systemic administration of the antibody, which could be related to localized, high dose delivery (186). Similar results were found in a renal cell carcinoma model where production of antibodies to PD-L1 enhanced CAR T cell function, though the results were less dramatic (187). Since both of these studies were conducted in NSG mice, the efficacy of ICB-producing CAR T cells may be even more striking in a host with an intact immune system. These preclinical results were rapidly translated to several clinical trials in China for variety of solid tumors, described in **Table 1**.

THE ULTIMATE CAR T CELL THERAPY

Chimeric antigen receptor T cell therapies exemplify an incredible opportunity—one like Hippocrates described—to take control of healing patients through empowering and redesigning a patient's own T cells to destroy tumor cells. As depicted in **Figure 1**, for a CAR T cell therapy to be more successful in solid tumors, the design should encompass three axes. Illustrated in **Figure 7**, we posit that this therapy would incorporate bispecificity through the CAR construct, generate enhanced potency *via* engineering a superior T cell subset, and revitalize the host immune response through cytokine and checkpoint antibody secretion. First, to enhance specificity, syn-Notch inducible CAR expression upon engagement of a tissue-specific antigen could improve sensitivity of the CAR to target the tissue and reduce off-tumor effects. Secondly, to enhance persistence, trafficking, and self-renewal properties, a CAR-engineered T_{SCM} $CD8^+$ T cell expanded with pharmacologic inhibitors or generated from iPSCs, or either a Th17 cell or a $CD4^+CD26^{high}$ T cell could overcome these limitations of poor quality T cell infusion products. Use of a multipotent T cell may permit adoptive transfer of fewer cells, thereby streamlining and reducing the cost and time investment to generate T cell products for infusion. A lower dose of T cells could reduce risk for severe toxicities and cytokine storms; however, engineering such a potent cell could alternatively be more toxic to patients when infused. Therefore, safety switches or Boolean logic gates should be incorporated to prevent life-threatening adverse events. Finally, taking advantage of the host's response to personalized neoantigens, PD-1 antibody-producing CAR T cells that also produce cytokines like IL-12, IL-15, IL-18, or IL-21 locally in the tumor after engaging a tumor-specific antigen would counteract the highly suppressive environment and synergize the power of the endogenous

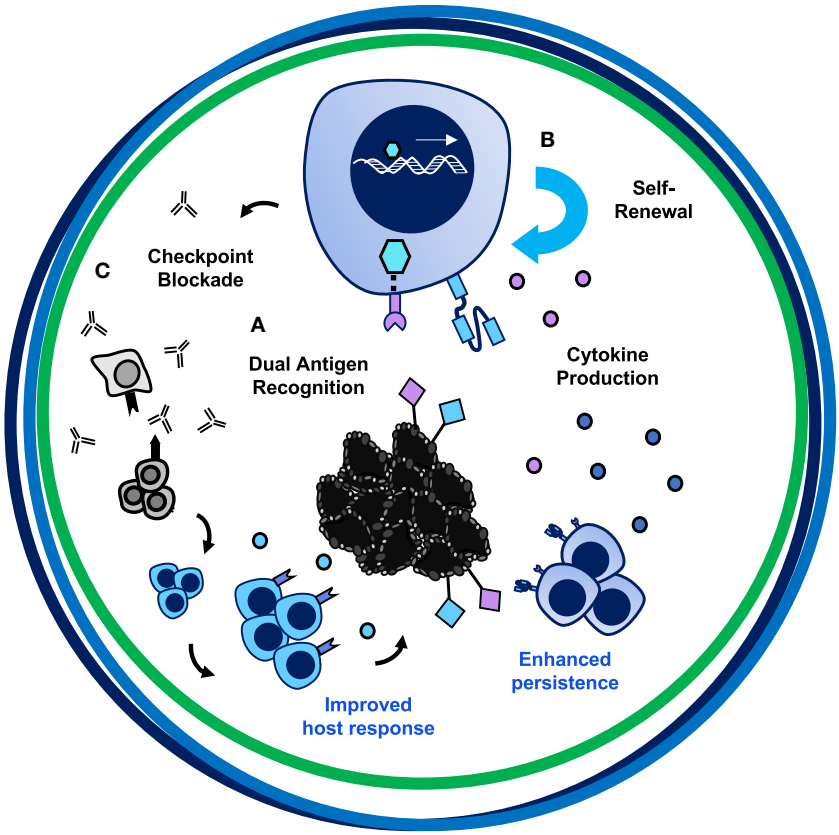

FIGURE 7 | The trifecta of successful chimeric antigen receptor (CAR) T cell therapies in solid tumors. The ultimate CAR T cell therapy has tumor specificity, potent migratory capacity and persistence, and improves the host immune response. **(A)** Bispecificity through syn-Notch technology augments targeting to tumor/tumor-specific tissue. **(B)** Engineering a T cell with enhanced persistence and migratory capacity—such as a Th17 or $CD4^{+}CD26^{high}$ cell—or with self-renewing properties—such as a $CD8^{+}$ T_{SCM} cell—will enhance long-term memory responses to prevent tumor recurrence. **(C)** Secretion of PD-1 blockade and cytokines such as IL-12, IL-15, IL-18, or IL-21 locally could overcome the suppressive tumor microenvironment, reinvigorate the exhausted host immune response to other tumor antigens, and synergize with CAR-specific T cells to destroy large heterogenous solid tumors.

immune response with the genetically redirected CAR T cell response.

Though the combination of these approaches is theoretically appealing, a T cell incorporating the several mechanisms proposed has not yet been engineered. Such a construct may prove difficult to generate without interruption of normal gene function. Use of CRISPR/Cas9 could direct incorporation to a specific location in the genome to enhance efficacy, such as the *TRAC* locus (188). Generating a universal CAR that is not MHC restricted, where infusion products could be mass-produced versus individually developed for each patient, could also make this CAR design feasible. The ease of developing a T cell as proposed is likely to improve over time as academic and industrial facilities expand and commercial-grade production becomes streamlined through automation and improved quality control (189). While it may make for a complex construct, harnessing capabilities of genetic redirection, optimal T cell subsets, and augmented crosstalk to other infiltrating immune cells may be one attainable approach to eradicate heterogenous and therapeutically resistant solid tumors.

CONCLUSION

Adoptive cell transfer with CAR-redirected T cells is a potentially curative approach for patients with previously treatment-resistant tumors. CAR T cells have proven their potency against hematologic cancers evidenced by their recent FDA approvals for B-ALL and DLBCL. For solid tumors, these therapies remain in early development but may require a new approach to enhance their efficacy. Herein, we have presented a combinatorial approach to augment the ability of CAR T cells to overcome challenges they face within the tumor microenvironment. We posit that a future CAR T cell armored with (1) a superior targeting system specific to the tumor and tumor tissue, (2) engineering of a highly potent, persistent, and self-renewing T cell subset, and (3) rejuvenation of the endogenous host response through CAR T cell production of monoclonal antibodies against immune checkpoint molecules will bolster the immune attack on the solid tumor to best reduce toxicity and support a long-lived memory response against targeted antigens and personalized neoantigens. Elegant findings from investigators worldwide will continue moving forward the

solid tumor CAR T cell approach to generate cures for patients with previously therapeutically resistant cancers.

AUTHOR CONTRIBUTIONS

HK and CP conceptualized, wrote, and edited the manuscript. AS, CD, MW, and SM provided feedback and edited the manuscript.

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Oncolytic Viruses Partner With T-Cell Therapy for Solid Tumor Treatment

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Adoptive T-cell immunotherapies, including chimeric antigen receptor-modified T-cells (CAR-T cells), have revolutionized cancer treatment, especially for hematologic malignancies. Clinical success of CAR-T cell monotherapy in solid tumors however, has been only modest. Oncolytic viruses provide direct cancer cell lysis, stimulate systemic immune responses, and have the capacity to provide therapeutic transgenes. Oncolytic virotherapy has shown great promise in many preclinical solid tumor models and the first oncolytic virus has been approved by the FDA for the treatment of advanced melanoma. As monotherapies for solid tumors, oncolytic virotherapy provides only moderate anti-tumor effects. However, due to their complementary modes of action, oncolytic virus and T-cell therapies can be combined to overcome the inherent limitations of each agent. This review focuses on the aspects of oncolytic viruses that enable them to synergize with adoptive T-cell immunotherapies to enhance anti-tumor effects for solid tumors.

Keywords: oncolytic virus, CAR-T cell, bispecific T-cell engager (BiTE), cytokine, chemokine, checkpoint inhibitor

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INTRODUCTION

Clinical use of adoptive cell therapies to treat cancer has gained great interest in recent years, adding new treatment options to the paradigm of surgery, radiotherapy, and chemotherapy. Bolstering a patient's immune system with infused T-cells that have been genetically modified to specifically target tumor cells holds great promise and has demonstrated clinical efficacy in hematologic malignancies (1). These T-cells are genetically modified to express a chimeric antigen receptor (CAR) with an extracellular domain derived from single chain variable fragment (scFv) specific to a target surface antigen on cancer cells and intracellular CD3ζ signaling domain. CARs can be further modified to include co-stimulatory domains like CD28, 4-1BB and ICOS, resulting in a cell that can respond to tumor antigens by proliferating and killing target cells dependent upon target antigen expression (CAR-T cells) (2).

Adoptive cell transfer of autologous CAR-T cells targeting B-cell antigen CD19 have resulted in profound remission in patients with refractory B-cell malignancies. Recently, the first chimeric antigen receptor CAR-T cells, Tisagenlecleucel (3), have been approved by the FDA for the treatment of acute lymphoblastic leukemia. Followed in quick succession by the approval of a second CAR-T cell therapy, Axicabtagene ciloleucel for lymphomas (4). However, the use of adoptive cell therapies for the treatment of solid tumors as a monotherapy has been less successful. Compared to hematological malignancies, clinical outcome in trials utilizing CAR-T cells to target various solid tumors has a much higher rate of patients achieving only stable disease and no response/progressive disease (5). The major barriers to successful CAR-T cell therapies for solid tumors include; lack of tumor specific or downregulation of antigen expression, the immunosuppressive tumor microenvironment which lacks necessary pro-inflammatory

stimulatory molecules and is abundant with inhibitory checkpoint molecules, and physical barriers of the solid tumor mass (6) (**Figure 1A**). These preclinical and clinical trials suggest that CAR-T cells are insufficient to overcome these inhibitory mechanisms as a monotherapy and therefore require additional therapy to enhance their anti-tumor effect.

Oncolytic viruses (OVs) have been designed to selectively replicate in and kill cancer cells. It is well established that OVs can stimulate adaptive immune responses to tumor cells due to the release of tumor associated antigens (TAAs), pathogen-associated molecular patterns (PAMPS), and danger-associated molecular patters (DAMPS) from lysed tumor cells. These responses also shift tumors from cold (immune desert) to hot (inflamed) tumors (7). Once processed by antigen presenting cells (APCs), TAAs can then induce anti-tumor T-cell responses in parallel with anti-viral responses. Based on these unique features, OVs are now considered a cancer immunotherapy agent (7). However, OV treatment alone is still unable to cure bulky and/or metastasized tumors and thus OVs also require additional therapies to enhance their anti-tumor effect. OVs have the added advantage of being able to deliver therapeutic transgenes to further enhance anti-tumor activity of host immune cells ("Armed" OVs; **Figure 1B)**. Although the anti-tumor capacity of OVs including "Armed" OVs has been investigated for decades, OVs are only now being used clinically after the recent approval of talimogene laherparepvec (T-VEC), a herpes simplex-1 (HSV) oncolytic virus expressing granulocyte macrophage colony-stimulating factor (GM-CSF), for the treatment of malignant melanoma (8). Intralesional administration of T-VEC induces a systemic immune response as indicated by reduction in size of untreated lesions (abscopal effect). However, clearance of these lesions was incomplete. Combining T-VEC with immune checkpoint inhibition provides complementary immune stimulation mechanisms as demonstrated in recent case studies (9–11). These clinical results clearly indicate that combination of OVs with another immunotherapy agent has additive anti-tumor effects. Combining OVs with CAR-T cell treatment strategies could function in a complementary and additive manner by overcoming the limitations of each treatment moiety (e.g., limited anti-tumor effects of OV to distant (untreated) sites, limited accessibility/persistence of CAR-T cells at tumor sites). This review will focus on recent developments and applications in OVs that have the potential to synergize with adoptive T-cell immunotherapy.

COMBINATION OF OVS WITH ADOPTIVE T CELL THERAPY

Combinatorial treatment with OVs has been demonstrated to augment the anti-tumor activity of adoptively transferred T-cells (12). In a syngeneic immunocompetent mouse model using B16ova melanoma, it was demonstrated that intratumoral administration of oncolytic vesicular stomatitis virus (oVSV) leads to increased $CD8^+$ T cell infiltration and resulted in 50% survival within 30 days, compared to treatment with heat-inactivated oVSV or mice left untreated whose median survival was approximately 20 days. Similarly, infusion of OT-I (OVA-specific) T-cells resulted in 50% survival within 30 days. To enhance the antitumor effect, the authors combined oVSV treatment with systemic infusion of OT-I T-cells resulting in a more potent anti-tumor response than either single agent treatment, approximately 70% survival at 50 days (13). In a similar model, intratumoral administration of oncolytic adenovirus combined with *ex vivo* activated OT-I T-cells led to increased presence of endogenous $CD8^+$ T-cells resulting in rejection of tumor re-challenge (14). Thus, combining oncolytic virotherapy with adoptive T-cell immunotherapy has proven to be beneficial in immunocompetent mouse models. These results suggest that OVs and T-cell therapy independently and additively function to control tumor growth.

OVs for T Cell Retargeting

One anti-tumor T-cell mechanism relies on the ability of the T-cell to recognize tumor antigens, thereby priming the T-cell to produce a cytolytic effect. Unfortunately, tumor cells are adept at escaping immune surveillance. One mechanism for this escape is the dysfunctional antigen processing of tumor cells through reduced expression of the major histocompatibility complex class I (MHC-I) (15). In heterogeneous solid tumors a tenuous balance is struck in which cytotoxic T-cells can eliminate the most susceptible tumor cells with high expression of target antigens. However, tumors can undergo a process of immune editing by which tumor cells that rapidly divide have increased mutational burden leading to downregulation or loss of target antigens. Once the infiltrated T-cells kill the tumor cells expressing a target antigen the remaining cancer cells can no longer be targeted by the T-cells, resulting in tumor immune escape and outgrowth (16). Even in hematologic malignancy, although CD19 is expressed on essentially all cases of B-cell Acute Lymphoid Leukemia (B-ALL) at clinical presentation, relapses with loss or diminished surface expression of CD19 are increasingly recognized as a cause of CD19.CAR-T cell treatment failure (17). Other clinical data has suggested that T-cell based immunotherapy leads to downregulation of MHC-I through loss of functional β2-microglobulin (18). An advantage of OVs is that MHC expression is induced after OV infection of cancer cells as demonstrated by oncolytic herpes simplex virus (19). Additionally, measles virus induces MHC and costimulatory molecules (20), and reovirus induces MHC-I as well as β2-microglobulin, TAP-1, and TAP-2 to enhance antigen presentation (21, 22). The potential of oncolytic virotherapy to overcome the attenuation of antigen escape induced by T-cell immunotherapy is a benefit of combination therapy.

Bispecific T cell engagers (BiTEs) are molecules consisting of a CD3-scFv linked to another scFv specific for an antigen expressed on the surface of tumor cells. By utilizing these molecules, tumor resident/infiltrated T-cells can be redirected toward additional specific antigens expressed on cancer cells. Blinatumomab is an FDA approved CD19 BiTE for the treatment of relapsed or refractory B-ALL (23) which functions to educate cytotoxic T cells to target malignant B-cells expressing CD19 (24). In a phase III trial comparing Blinatumamab to standard chemotherapy, complete remission rates (34 vs. 16%) and overall survival (7.7 vs.

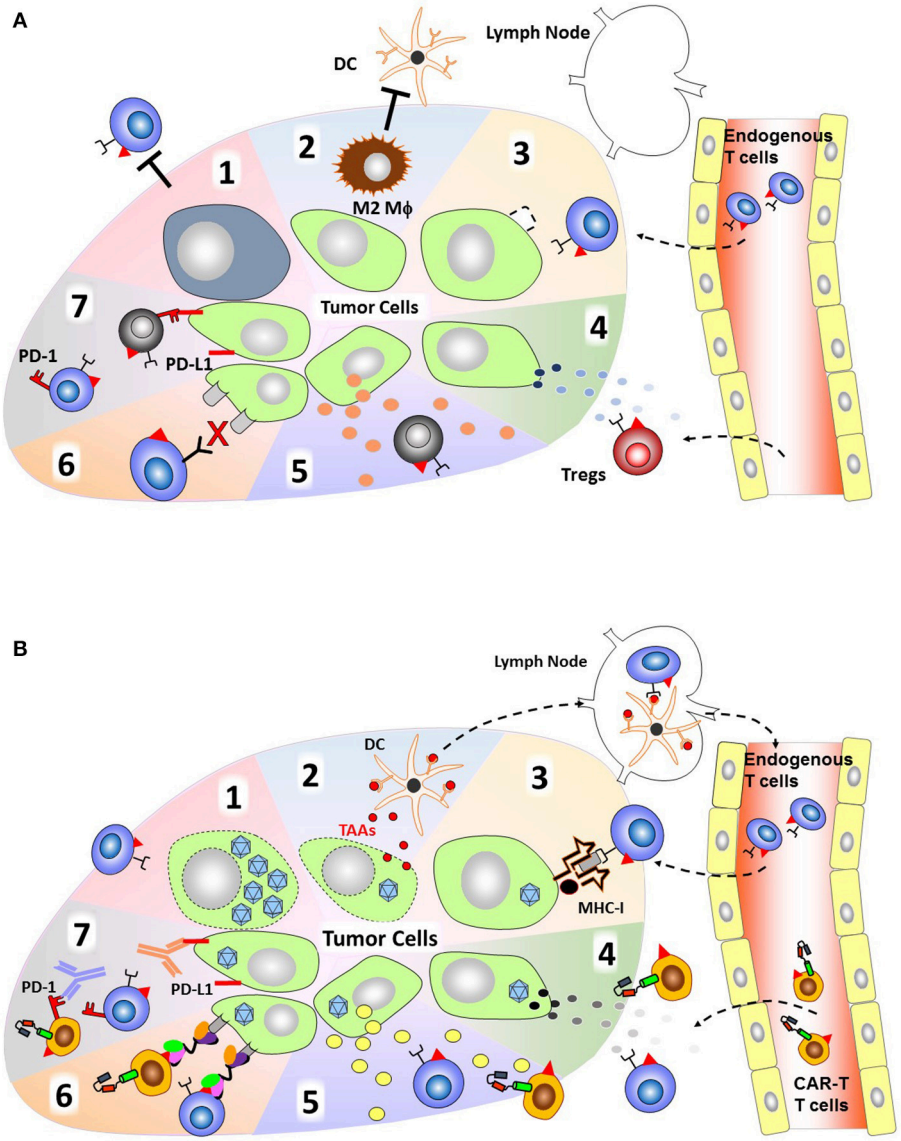

FIGURE 1 | Attributes of OVs to overcome immunosuppression by the tumor microenvironment. **(A)** The immunosuppressive tumor microenvironment. (1) T-cells have poor accessibility to dense, bulky tumors. (2) Presence of immunosuppressive cells such as myeloid derived suppressor cells (MDSCs) and M2 macrophages. (3) Downregulated MHC-I expression resulting in poor antigen presentation/recognition. (4) Tumor cells secrete chemokines attract immunosuppressive cells such as regulatory T-cells (Tregs). (5) Tumor cells can also secrete inhibitory cytokines (e.g., TGF-β, IL-(10) that inhibit cytotoxic T-cell function. (6) Cancer cells often lack tumor specific antigens that can be recognized by endogenous T-cells. (7) Expression of immune checkpoint molecules (e.g., PD-L1) that cause exhaustion upon engagement of cognate receptors on T-cells (e.g., PD-1). **(B)** Mechanisms by which oncolytic viruses can help T-cells to overcome the immunosuppressive environment. (1) Direct oncolysis of tumor cells and increased tumor accessibility by creating space within the tumor mass. (2) Release of DAMPs, PAMPs, and TAAs upon tumor cell lysis that can recruit APCs, and TAAs can be processed and presented to T-cells at lymph node. (3) OV infection can induce expression of MHC-I and β2M. (4) OVs can be engineered to express chemokines to increase infiltration of both endogenous T-cell and CAR T-cell. (5) Express inflammatory cytokines to increase T-cell proliferation at the tumor site. (6) Produce BiTE (Engager) molecules to redirect T-cells to tumor specific antigens. (7) Express Checkpoint inhibitors for attenuating T-cell exhaustion.

4 months) were significantly improved in patients receiving the BiTE. However, due to the short half-life of the BiTE molecule, the drug must be administered by continuous infusion and the vast majority of patients (87%) receiving Blinatumamb had grade 3 or higher adverse events (25). Although there are currently many BiTE molecules in development for clinical use (26), this potential side effect due to systemic and frequent infusion may need to be addressed.

To increase the efficacy of BiTE molecules and decrease unwanted side effects due to constant systemic administration, local constitutive expression of BiTEs at the tumor site would provide stimulation for tumor resident T-cells without systemic

toxicity. To this end, OVs have been used to express various BiTE molecules, providing a retargeting moiety to T-cells together with virus mediated oncolysis. To target tumor cells expressing the EphA2 antigen, an oncolytic vaccinia virus (VV) was engineered to express an EphA2 BiTE, called T-cell engager armed VV (TEA-VV). In an orthotopic lung tumor xenograft model, when human PBMCs were delivered together with the EphA2.TEA-VV, tumor growth was significantly reduced compared to mice receiving only oncolytic VV or unarmed oncolytic VV with PBMCs (27).

Likewise, an oncolytic adenovirus (Onc.Ad) expressing an EGFR-BiTE (Onc.Ad-EGFR.BiTE), derived from cetuximab which is used clinically to treat colorectal (28) and head-and-neck squamous cell carcinomas (29), was able to induce *ex vivo* activated, adoptively transferred T-cell accumulation and proliferation in a subcutaneous model of colorectal carcinoma. Administration of unarmed Onc.Ad provided oncolysis and reduced tumor growth which was significantly enhanced by the addition of the BiTE molecule in the presence of activated T-cells (30). However, this Onc.Ad-EGFR.BiTE combined with transferred unstimulated T-cells required systemic administration of IL-2 and did not clear the tumors, suggesting that additional activation and/or persistence of T-cells at the tumor site is required to lead T-cell dependent anti-tumor effect through the BiTE molecule. The group then tested their Onc.Ad-EGFR.BiTE combined with CAR-T cells targeting another antigen, folate receptor alpha (FR-α) which had been previously tested and shown to be safe but not efficacious in patients with metastatic ovarian cancer (31). Treatment with Onc.Ad-EGFR.BiTE was able to increase FR.CAR-T cell killing, proliferation, and IFNγ production *in vitro*. *In vivo*, Onc.Ad-EGFR.BiTE combined with two administrations of FR.CAR-T cells significantly delayed tumor growth in a xenograft model in which the tumor cells expressed intermediate levels of FR-α and high levels of EGFR. In a second *in vivo* model, the tumor cells expressed low levels of FR-α, and high levels of EGFR, the combination of the Onc.Ad with CAR-T cells resulted in sustained reduction of tumor volume compared to single agent treatments. Additionally, when the Onc.Ad-EGFR.BiTE was combined with an irrelevant CAR-T cell, the presence of the BiTE molecule increased CAR-T cell infiltration and activation markers similar to the FR.CAR-T treatment (32) (**Table 1**). Thus, demonstrating the combination of viral-mediated oncolysis with retargeting of immune cells to secondary targets can produce an additive anti-tumor effect of CAR-T cells.

OVs Expressing Cytokine/Chemokine

Before T cells can perform their cytotoxic functions at tumor sites, they must first home to their target and infiltrate the tumor mass. Chemokines are molecules that serve to draw immune cells to sites of inflammation. It was recently demonstrated that intratumoral administration of an oncolytic type II herpes simplex virus (HSV-2) induces high expression of multiple pro-inflammatory chemokines (i.e., CCL2, CCL3, CCL4, CXCL9, CXCL10, CXCL11) resulting in increased accumulation and persistence of adoptively transferred OT-I T-cells in both immune competent and incompetent models (37).

The suppressive tumor microenvironment is depleted of pro-T cell cytokines which is a significant inhibitory mechanism tumors develop to evade cytotoxic T-cells. OVs can deliver molecules to stimulate T-cells at the tumor site and reverse this anergy. Administration of an Onc.Ad expressing TNFa and IL-2 (Onc.Ad-IL2/TNFa) in five consecutive doses has significant antitumor effect in an immune competent Syrian hamster model of pancreatic cancer (38). Subsequently, this Onc.Ad-IL2/TNFa was combined with mesothelin-CAR-T cells (meso.CAR-T), tested in patients with pancreatic adenocarcinoma or mesothelioma (39), in a preclinical model of pancreatic ductal adenocarcinoma. A single intratumoral administration of the Onc.Ad followed 3 days later with systemic administration of meso.CAR-T resulted in 100% survival after 100 days compared to median survival of 56 days in mice treated with only meso.CAR-T or Onc.Ad-IL2/TNFa. Importantly, the combination of Onc.Ad-IL2/TNFa with meso.CAR-T was able to inhibit the formation of lung metastases. In a syngeneic immunocompetent model, mice treated with murine meso.CAR-T had little tumor control but when combined with non-replicating Ad vectors expressing murine IL-2 and TNFa complete short-term tumor inhibition was achieved. The Ad vectors themselves or combined with an irrelevant CAR-T cell, lead to host immune cell infiltration and caused a reduction in tumor volume, demonstrating the benefit of activating host immune responses for combinatorial treatment (33).

To increase the efficiency of CAR-T cell trafficking and persistence within tumors, production of proinflammatory chemokines and cytokines from the tumor mass has been investigated. An Onc.Ad expressing both IL-15 and RANTES (Onc.Ad-IL15/RANTES) has demonstrated that combining both molecules can have a profound effect on adoptively transferred GD2.CAR-T cells, which have accomplished remission in patients with neuroblastoma (40). Intratumor administration of Onc.Ad-IL15/RANTES increased the infiltration and persistence of GD2.CAR-T cells in a xenograft model of neuroblastoma resulting in significantly enhanced survival (34). This work establishes the potential of utilizing oncolytic viruses armed with proinflammatory molecules to increase the antitumor activity of CAR-T cells that have modest effects on their own.

OVs and Checkpoint Blockade

One of the strongest barriers to successful T-cell therapy for solid tumors is the expression of inhibitory immune checkpoint ligands expressed on tumor cells (41) (**Figure 1A**). These ligands shut down effector T-cell function resulting in their inability to attack and control cancer cells. Antibodies targeting these immune checkpoint molecules can be effective in reversing this T-cell hypofunction which is reflected in the recent, rapid approval of these antibodies for clinical use. These antibodies, however, are associated with systemic toxicities and are only modestly efficacious as monotherapies (42).

Cancer cells upregulate the T-cell inhibitory ligand PD-L1 in the presence of IFNγ, which is produced by activated T cells, and CAR-T cells express PD-1 upon activation (35). Our group has recently demonstrated that a combinatorial Ad vector

TABLE 1 | Preclinical studies combining oncolytic viruses with CAR-T cells.

Virus	Tumor	CAR antigen	CAR endodomain	Dose/mouse	References
Onc.Ad-EGFR BiTE	Pancreatic ductal carcinoma/colorectal carcinoma	Folate receptor alpha (FR-α)	41BB	1×10^7 CAR-T 1×10^9 Onc.Ad/ 1×10^7 CAR-T (2x) 1×10^9 Onc.Ad	(32)
Onc.Ad-TNFα/IL2	Pancreatic ductal carcinoma	Mesothelin (meso)	41BB	1×10^6 CAR-T 3×10^9 Onc.Ad (xenograft)/5×10^6 CAR-T 1×10^9 Onc.Ad (syngeneic)	(33)
Onc.Ad-Rantes/IL15	Neuroblastoma	Ganglioside GD2	CD28 &OX40	1×10^7 CAR-T 1×10^6- 1×10^9 Onc.Ad	(34)
CAdVEC-αPDL1	Prostate, Squamous Cell Carcinoma	Human epidermal growth factor 2 (HER2)	CD28	1×10^6 CAR-T 1×10^7 Onc.Ad	(35)
CAdVEC-IL12p70/αPDL1	Head and neck squamous cell carcinoma	Human epidermal growth factor 2 (HER2)	CD28	1×10^6 CAR-T 1×10^8 Onc.Ad	(36)

expressing a PD-L1 blocking mini-antibody (CAdVEC*PDL1*) enhances the antitumor effect of HER2.CAR-T cells, which were recently reported to be safe in patients with sarcoma (43), against multiple human cancer cells *in vitro* and *in vivo* (35). Local expression of the PD-L1 blocking antibody via CAdVEC*PDL1* treatment proved to be less toxic and provide greater tumor control than systemic administration of PD-L1 antibody. While this combinatorial treatment strategy is effective, providing significant long-term survival advantage, it was not curative in subcutaneous xenograft models. We then utilized our adenoviral vector to deliver a stimulatory cytokine in addition to the PD-L1 blocking antibody since there is ample evidence of oncolytic vectors expressing cytokines enhancing adoptive T-cell therapies as described above. We generated a library of helper-dependent Ads expressing various cytokines (IL2, IL7, IL-12p70, IL15, and IL21) and screened them for their ability to enhance HER2.CAR-T mediated killing of head-and-neck squamous cell carcinoma (HNSCC) targets. We found that only IL-12p70 mediated tumor regression in conjunction with PD-L1 blocking antibody in both HPV positive and negative HNSCC xenograft models. We then generated a single vector expressing IL-12p70 with the PD-L1 blocking antibody which was co-injected with an oncolytic adenoviral vector (CAdVEC*IL12_PDL1*). This treatment combined with HER2.CAR-T cells was able to control both primary and metastasized tumors in an orthotopic model of HNSCC causing lymph node metastasis similar to those seen in HNSCC patients. This superior anti-tumor effect leads to 100% survival of animals treated with the combination of CAR-T cells and CAdVEC*IL12_PDL1* for more than 120 days without xenogenic GVHD after single treatment (36). These results suggest that OVs expressing a checkpoint inhibitor in conjunction with CAR-T cell treatment is effective, but CAR-T cells require additional signals (e.g. cytokine) to maintain anti-tumor effects. We expect that only blockade of PD-1:PD-L1 interaction may lead to over-activation of CAR-T cells and results in immediate exhaustion, but provision of appropriate cytokine (Signal 3) can attenuate this exhaustion. However, the mechanism by which exogenous IL-12p70 (STAT4 activation) leads to long-term anti-tumor effect of CAR-T cells and how blockade of PD-1:PD-L1 interaction contributes to IL-12p70 signaling is still unclear.

CONCLUSION

These preclinical data clearly demonstrate that, although tumors are adept at evading immunotherapies, combining OVs with adoptive T-cell immunotherapeutic strategies can overcome these evasion mechanisms. Based on previous clinical trials with mono-immunotherapy, combining immunotherapy regimens that target different aspects will be necessary to eradicate tumors. OVs provide complementary antitumor mechanisms such as stimulation of innate immune responses, increasing tumor antigen presentation, and direct oncolysis of tumors. Additionally, OVs can provide targeting molecules like bispecific T-cell engagers, stimulatory cytokines, chemokines, and even immune checkpoint inhibitors. However, most preclinical studies combining OVs and CAR-T cells are based on immunodeficient mouse models, and further investigation using immunocompetent models (e.g., humanized mouse) will be needed to understand how host immune responses (e.g., anti-viral response) contribute to this combinatorial therapy.

Armed OVs can be rationally designed to provide T-cells with optimal synergistic molecules for specific tumor targets and therefore represent an ideal platform for targeted cancer therapies. Based on clinical trial data with CAR-T cells for solid tumors, we may be able to identify appropriate molecule(s)

expressed by OVs for each CAR construct and for each target tumor/tissue to maximize the anti-tumor effect of CAR-T cells. Since safety of both agents as monotherapy have been demonstrated in numerous clinical trials, and our data indicate that we can obtain durable responses with 1-2 log lower dosages of each agent used as monotherapy in preclinical models (35, 36), combination of OVs and CAR-T cell therapy may be a safer and more effective treatment in future clinical trials for solid tumors.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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CAR T Cell Therapy for Neuroblastoma

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Patients with high risk neuroblastoma have a poor prognosis and survivors are often left with debilitating long term sequelae from treatment. Even after integration of anti-GD2 monoclonal antibody therapy into standard, upftont protocols, 5-year overall survival rates are only about 50%. The success of anti-GD2 therapy has proven that immunotherapy can be effective in neuroblastoma. Adoptive transfer of chimeric antigen receptor (CAR) T cells has the potential to build on this success. In early phase clinical trials, CAR T cell therapy for neuroblastoma has proven safe and feasible, but significant barriers to efficacy remain. These include lack of T cell persistence and potency, difficulty in target identification, and an immunosuppressive tumor microenvironment. With recent advances in CAR T cell engineering, many of these issues are being addressed in the laboratory. In this review, we summarize the clinical trials that have been completed or are underway for CAR T cell therapy in neuroblastoma, discuss the conclusions and open questions derived from these trials, and consider potential strategies to improve CAR T cell therapy for patients with neuroblastoma.

Keywords: neuroblastoma, pediatric oncology, immunotherapy, CAR T cells, adoptive T cell therapy, clinical trials

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INTRODUCTION

Neuroblastoma is a tumor of childhood arising from neural crest cells. Often diagnosed during the first 10 years of life, it is the most common extracranial solid tumor in childhood and is responsible for 11% of pediatric cancer deaths in patients younger than 15 years of age (1). Approximately 650 patients are diagnosed in the United States with neuroblastoma each year, which accounts for 7.5% of all cancer diagnoses for children younger than 15 years old (2, 3). Clinical presentation and outcomes are extremely variable. Newborns and infants are often incidentally found to have adrenal tumors that spontaneously regress without therapy, while toddlers and older children frequently present with widely metastatic disease that requires multimodal intensive therapy including surgery, chemotherapy, radiotherapy, autologous stem cell transplant, differentiation therapy, and monoclonal antibody-based immunotherapy. Patients with localized disease typically have excellent outcomes, with >90% event free survival (EFS) rates 5 years after diagnosis (4). In contrast, patients with high risk disease (defined by age >18 months, extent of metastases, and histologic and genetic factors such as N-MYC amplification) historically have had poor long term survival prospects, with 5-year EFS of about 50% (5–8). Patients who do survive often suffer long term sequelae from their intense treatment including hearing loss, growth retardation, and

secondary malignancies (9). This population therefore has a desperate need for novel therapies to improve survival and to decrease morbidity.

Antibody-based immunotherapy was recently integrated into frontline protocols for patients with high risk neuroblastoma. A pivotal phase III clinical trial published in 2010 revealed an increase in 2 year EFS from 46 to 66% and overall survival (OS) from 75 to 86% for patients who received adjuvant anti-GD2 monoclonal antibody given with IL-2, GM-CSF, and retinoic acid compared to patients who received retinoic acid alone (6). Incorporation of anti-GD2 monoclonal antibodies into therapy for neuroblastoma has been one of the most successful interventions to improve survival for high risk patients (6, 10–13). This success has firmly established a new paradigm for the treatment of neuroblastoma that includes immunotherapy.

While survival rates have improved since the adoption of anti-GD2 antibodies, ∼50% of patients will relapse and eventually die from their disease (6). Additionally, 20% of patients are refractory to induction therapy at diagnosis and may not ever receive anti-GD2 antibody (14). These patients are in need of more potent and targeted approaches. One such approach is adoptive transfer of chimeric antigen receptor (CAR) T cells, which combine the specificity of an antibody with the cytolytic capacity of T cells in an MHC independent manner (15). CD19 and CD22 CAR T cells have demonstrated remarkable success in children with relapsed and refractory leukemia and lymphoma (16–20). While anti-GD2 monoclonal antibodies have been successful in treating patients with neuroblastoma metastases in their bone marrow, they have generally not been useful as single agents against bulky disease (21). CAR T cells have the potential for increased potency and durability compared to monoclonal antibodies and thus could overcome this challenge. Additionally, while antibodies generally do not penetrate the central nervous system (CNS) (22), CAR T cells are able to cross the blood-brain barrier (23, 24). Relapsed neuroblastoma of the CNS has emerged as a clinical entity since the adoption of anti-GD2 monoclonal antibodies, and CARs could present an answer to this challenging clinical problem (25, 26).

CAR T cells have already shown promise in clinical trials for neuroblastoma with several objective responses seen in early phase studies (27–31). In general, however, CAR T cell activity has not been as robust in neuroblastoma as in hematologic malignancies. There are many challenges in designing CAR T cells against neuroblastoma including suboptimal T cell persistence and potency (27–29), a paucity of tumor specific targets (32, 33), and an immunosuppressive tumor microenvironment (34, 35). However, CAR T cell engineering is accelerating at a rapid pace, with the aim to improve potency and specificity of tumor targeting (36–41). Neuroblastoma is an excellent testing ground for these new therapeutics since immunotherapy has already been validated for these patients. In this review, we will discuss the clinical experience to date with neuroblastoma-directed CAR T cells and the challenges of applying these powerful therapeutics to neuroblastoma patients. As CAR T cell design becomes more sophisticated, these agents are primed to become part of the multimodal approach used to treat patients with high risk neuroblastoma.

CLINICAL EXPERIENCE

Much of the early clinical experience treating children with CAR T cells has been in hematologic malignancies, but neuroblastoma has also been an area of intense investigation, with a steady stream of clinical trials of CAR T cells for patients with relapsed or refractory disease since the early 2000s. Despite preclinical development of CAR T cells against a variety of neuroblastoma associated antigens, only those directed against GD2 and L1-CAM (CD171) have reached clinical trials. **Table 1** summarizes completed and ongoing clinical trials.

GD2

The most-studied tumor associated antigen in neuroblastoma is GD2. GD2 is a disialoganglioside that is highly and nearly universally expressed on neuroblastoma tissue (44) and likely plays a role in tumor immune evasion (45). It is a natural choice as a target for CAR T cell therapy in neuroblastoma based on the success of anti-GD2 monoclonal antibody therapy (6, 10–12).

One of the first CAR T cells products tested in children was a first generation anti-GD2 CAR (containing only the CD3ζ endodomain but no costimulatory domain). In preclinical models, Rossig et al. demonstrated that GD2 was a viable CAR T cell target for neuroblastoma (46). To translate the preclinical promise of anti-GD2 CAR T cells into patients, Pule et al. aimed to treat patients in a manner that could enhance CAR persistence. CAR T cells with first generation signaling domains (CD3ζ only) had previously demonstrated limited persistence in human trials for other indications, indicating that the CD3ζ only intracellular domain was not sufficient for optimal activity (47–49). Rather than endowing the CAR with embedded costimulation, Pule and colleagues generated a T cell product that could receive physiologic costimulation through engagement of a native T cell receptor (TCR).

These researchers drew on experience from clinical trials in which Epstein Barr Virus-specific cytotoxic T lymphocytes (EBV-CTLs) were adoptively transferred to patients with EBV-associated malignancies (50–53). In those trials, T cell persistence of at least 3 months was seen even with relatively low doses of EBV-CTLs. Adding tumor specificity with a CAR construct was a logical next step to take advantage of the longevity of EBV-CTLs. A Phase I trial (NCT00085930) tested this approach by infusing EBV-CTLs co-expressing a first generation anti-GD2 CAR into relapsed and refractory neuroblastoma patients who were seropositive for EBV viral capsid antigen (27).

In this trial, EBV-specific lymphocytes were extracted from eleven patients with refractory or recurrent neuroblastoma, transduced with retrovirus encoding a GD2 CAR molecule (containing the single chain variable fragment (scFv) derived from Dinutuximab, 14g2a), and stimulated *ex vivo* with autologous EBV-transformed lymphoblastoid cell lines (LCLs). This product was called GD2 CAR-CTL. Concurrently, bulk T cells were transduced with the same GD2 CAR but activated through the native TCR with anti-CD3 antibodies (GD2 CAR-ATC). Each patient received between 2×10^7 and 1×10^8 cells/m^2 of both GD2 CAR-CTL and GD2 CAR-ATC. A 12-base pair mutation between the receptor stop codon and the 3′ LTR

TABLE 1 | Summary of CAR T cell clinical trials for neuroblastoma.

Clinical Trial	Study Design	Status	Target	scFv	Signaling domains	Response/toxicity	Location	References
N/A	Phase I: N = 6	Completed	L1-CAM	CE7R	CD3ζ only	PR in 1/11 patients with limited disease burden, no DLT	Seattle Children's Hospital (Washington, USA)	(29)
NCT02311621	Phase I: N = 22	Recruiting	L1-CAM	CE7R	4-1BB.CD3ζ; CD28.4-1BB.CD3ζ	No objective responses, DLT with hyponatremia in two patients, self-limited rash in five patients	Seattle Children's Hospital (Washington, USA)	(42)
NCT00085930	Phase I: N = 19	Active, not recruiting	GD2	14g2a	CD3ζ only	CR in 3/19 patients, PR in 1/19 patients, response correlated with CAR T cell persistence, no DLT	Baylor College of Medicine/Texas Children's Hospital (Texas, USA)	(27); (28)
NCT01822652	Phase I: N = 11	Completed	GD2	14g2a	CD28.OX40.CD3ζ	No objective responses, no DLT	Baylor College of Medicine/Texas Children's Hospital (Texas, USA)	(43)
NCT02761915	Phase I: N = 12	Recruiting	GD2	KM8138	CD28.CD3ζ	Mixed response in 1/12 patients, no DLT	University College London (London, United Kingdom)	(30)
NCT02765243	Phase II: N = 34	Recruiting	GD2	Unknown	CD28.4-1BB.CD27.CD3ζ	PR in 15% of patients, no DLT	Zhujiang Hospital (Guangzhou, Guangdong, China	(31)
NCT03294954*	Phase I	Recruiting	GD2	14g2a	CD28.CD3ζ in invariant NKT cells	N/A	Baylor College of Medicine/Texas Children's Hospital (Texas, USA)	Unpublished
NCT02107963	Phase I	Completed	GD2	14g2a	OX40.CD28.CD3ζ	N/A	National Cancer Institute (Washington, D.C., USA)	Unpublished
NCT01460901	Phase I	Completed	GD2	14g2a	CD3ζ only	N/A	Children's Mercy Hospital Kansas City (Kansas, USA)	Unpublished
NCT03373097	Phase I/II	Recruiting	GD2	14g2a	CD28.4-1BB.CD3ζ	N/A	Bambino Gesu Hospital and Research Institute	Unpublished
NCT02919046	Phase I	Recruiting	GD2	14g2a	CD28.OX40.CD3ζ	N/A	Nanjing Children's Hospital (Nanjing, China)	Unpublished

This table summarizes the completed and ongoing clinical trials of CAR T cell therapy for neuroblastoma patients. Differences in the single chain variable fragments (scFv) and CAR signaling domains are highlighted, and clinical responses are summarized (trials for which no clinical data has been publicly presented are shaded in gray). NCT03294954 uses invariant NKT cells for CAR transduction, as opposed to T cells as in all other listed trials (*). Clinical references are provided. PR, partial response; CR, complete response; DLT, dose limiting toxicity.

allowed for comparison of *in vivo* durability of the two cell types by RT-PCR. There was little to no detection of GD2 CAR-ATCs after 2 weeks, but clear persistence of the EBV specific GD2 CAR-CTLs until on average 6 weeks, demonstrating that costimulation is vital for CAR T cell persistence. Four of the eight patients (50%) with evaluable tumors had a partial or complete response, though all later progressed. Responses included one patient with a complete response of an extradural parietal lesion as measured by MIBG, one patient with a complete response of extensive bone marrow disease, and two patients with significant tumor necrosis confirmed by imaging and biopsies. These data support the hypothesis that ongoing costimulation increases persistence *in vivo* and results in increased efficacy and durability of response. A subsequent study with longer follow up determined that even low levels of persistent cells correlated strongly with slower time to disease progression (28).

While using viral specific CTLs takes advantage of the native TCR machinery with physiologic stimulation, there is some evidence that co-engagement of a CAR and TCR can result in T cell exhaustion and decreased CAR persistence (54). Most CAR constructs now rely on embedded costimulation. The same group from Baylor produced a third generation CAR containing both the CD28 and OX40 costimulatory domains. Preclinical studies demonstrated that incorporation of tandem costimulation domains increased expansion of the engineered T cell product and augmented cytokine release (55, 56), which prompted testing this construct in clinical trials.

The third generation anti-GD2 CAR was administered to eleven patients with relapsed or refractory neuroblastoma. Patients were treated in one of three cohorts: GD2 CAR T cells alone, GD2 CAR T cells after lymphodepleting chemotherapy, or GD2 CAR T cells after lymphodepleting chemotherapy given with the PD-1 inhibitor pembrolizumab. Patients who received lymphodepletion with or without checkpoint blockade had increased expansion of their CAR T cells and longer CAR T cell persistence. Anti-PD-1 therapy did not appear to dramatically affect these parameters or efficacy. Unfortunately, even after patients received proper lymphodepletion, this CAR was found to have minimal activity with no measurable responses (43). One explanation for the lack of long-term persistence seen in this

trial is tonic signaling of the CAR T cell caused by aggregation of the 14g2a anti-GD2 scFv, leading to T cell exhaustion and limited anti-tumor efficacy (57). T cell exhaustion, which will be further discussed below, has emerged as an important factor that can limit CAR efficacy and is highly dependent on costimulation molecules (57, 58).

Another Phase I trial of anti-GD2 CARs is underway in the United Kingdom (NCT02761915) utilizing an scFv based on a previously described humanized murine antibody KM8138 (59) that is fused to a CD28 costimulatory domain and CD3ζ. Based on promising preclinical data (60), this trial is enrolling children with relapsed or refractory neuroblastoma and evaluable disease in a dose escalation model. Preliminary results presented in abstract form demonstrate minor clinical response by imaging criteria and cytokine release syndrome (CRS) in at least one patient at higher dose levels, but CAR T cell persistence also appears to be limited (30). A fourth generation GD2 CAR (including CD28, 4-1BB, and CD27 costimulatory domains in addition to CD3ζ) is also being tested in a multi-institutional Chinese Phase II trial for high-risk neuroblastoma patients. An abstract presented in 2017 reported 15% of 34 patients with a partial response and no dose limiting toxicities. Two patients had significant tumor regression, one with two bulky lesions that regressed by >90% each and one with a reduction in retroperitoneal tumor dimensions and standardized uptake value (SUV) by PET scan measured 2 months after CAR T cell therapy (31).

Despite mixed results in the early GD2 CAR clinical trials, this target remains an area of intense focus. There are currently many ongoing preclinical studies focused on targeting GD2 as well as five open clinical trials of CAR T cells directed against GD2 for neuroblastoma patients (NCT03373097, NCT02761915, NCT02765243, NCT03294954, NCT02919046). While the experience thus far with GD2 CARs in clinical trials has established safety and feasibility, limited T cell persistence has emerged as a major hurdle to success.

L1-CAM/CD171

Another target of interest in neuroblastoma is L1-CAM, an adhesion molecule that is overexpressed on neuroblastoma. Monoclonal antibody CE7 preferentially binds to a tumor-specific epitope of L1-CAM (61). The mechanism of tumor specificity has not been elucidated, but appears to be glycosylation-dependent (62–64). A first generation CAR containing the CE7 scFv, a CD4 transmembrane domain, and the CD3ζ intracellular signaling domain (CE7R CAR) demonstrated preclinical activity in xenograft models of neuroblastoma (65). A clinical construct was designed to include a selection-suicide fusion protein composed of hygromycin phosphotransferase and thymidine kinase (HyTK), allowing for CAR ablation with ganciclovir in the case of unforeseen toxicity. In a Phase I clinical trial of escalating doses of CE7R HyTK CD8+ CAR T cells, the authors demonstrated safety and observed no off-tumor, on-target toxicity. However, only one of six patients had a significant clinical response. That patient had limited disease burden, whereas the patients with higher disease burden had progressive disease. All patients ultimately died of their disease (29). Similar to GD2, lack of persistence of CAR T cells was also a major limiting factor in this study, which may have been related to the lack of costimulation in the CAR or to immunogenicity of the suicide HyTK protein (66).

To enhance the activity and persistence of L1-CAM directed CARs, the researchers then generated a second generation CAR (2G CE7 CAR) containing a 4-1BB costimulation domain and a truncated extracellular epidermal growth factor receptor (EGFRt) domain in place of the HyTK suicide switch (allowing for an alternative ablation strategy with cetuximab) (67, 68). Reassuringly, there was no significant clinical toxicity in non-human primates treated with 2G CE7 CAR T cells at doses 10–100 times higher than the doses employed in the clinical trial, though these primates did not have antigen positive malignancies (69).

A Phase I trial with the 2G CE7 CAR in rotation with a similar third generation product that also includes a CD28 endodomain is currently underway at Seattle Children's Hospital for recurrent or refractory high risk neuroblastoma patients (NCT02311621). Patients receive anti-L1-CAM CAR T cells in a defined ratio of 1:1 CD4:CD8 T cells. This strategy is based on previous successes of this controlled strategy for CAR T cell treatment of B-ALL and non-Hodgkin lymphoma at the same institution (70–72). Further study is required to determine the utility of a defined CD4:CD8 T cell product as this has not been tested in a randomized clinical trial, and equally impressive response rates have been obtained using non-selected populations of T cells or PBMCs after transduction (16, 17, 19, 20).

In a recently presented abstract, the researchers reported that L1-CAM CAR T cells infiltrate sites of disease in patients but appear to be causing off-tumor toxicity with transient skin rash (where the CAR T cells may colocalize with L1-CAM expressing normal cells) and poorly understood hyponatremia in some patients. Although these toxicities have all been transient and the trial is ongoing (42), the early finding of possible off-tumor, on-target toxicity is a reminder of the difficulty of identifying appropriate CAR-T cell targets (discussed further below).

CHALLENGES IN TARGETING NEUROBLASTOMA WITH CAR T CELLS

Clinical experience thus far with CAR T cells for neuroblastoma indicates that T cell persistence is emerging as a major impediment for the success of these therapeutics. Outcomes have been encouraging but modest, with only a fraction of patients achieving measurable responses and very few patients demonstrating long term persistence of CAR T cells. In order to achieve the level of success that has been seen in hematologic malignancies, the field will have to address this challenge. Additionally, target selection is equally important, as many neuroblastoma targets are also expressed on normal tissues, creating the potential for off-tumor, on-target toxicity as may have been seen with L1-CAM CARs (albeit transiently). There may be a therapeutic window for CAR T cells against highly expressed tumor antigens that exhibit lower levels of expression on normal tissue, so this does not necessarily

preclude these molecules as targets. Finally, as with other solid tumors, a complex, immunosuppressive microenvironment in neuroblastoma tumors presents a barrier for efficacious CAR T cell therapy.

T Cell Persistence and Exhaustion

CAR T cell persistence is essential for durable clinical responses (16, 47, 73–75). Long term follow-up of Baylor's first generation anti-GD2 CAR T cell trial demonstrated that time to disease progression was significantly delayed in patients whose T cells were detectable for longer (27, 28). In the trial of a first generation L1-CAM CAR, the only patient of six with a clinical response had detectable CAR T cells in the blood 56 days after treatment, while patients without objective response had shorter persistence (29).

CAR T cell persistence may be diminished due to T cell exhaustion. T cell exhaustion has primarily been studied in the setting of chronic antigen exposure including for viral infections (76, 77) and cancer (78–81). Exhausted T cells upregulate inhibitory receptors after excessive and continuous stimulation over a matter of days to weeks and exhibit diminished effector functions. T cell exhaustion appears to be partially reversible. This is fundamentally different from T cell senescence, which typically occurs over months to years, is associated with telomere shortening, and represents a terminally differentiated state without potential for reversibility or proliferation (82).

An exhausted CAR T cell phenotype has recently been described in GD2 CAR T cells, driven by antigen-independent tonic signaling (57). Long et al. explored why GD2 CAR T cells containing the 14g2a scFv appeared to be less functional than CD19 CAR T cells. The authors found that unlike the CD19 CAR, the GD2 CAR aggregated on the surface of T cells and subsequently triggered low level tonic signaling in the absence of antigen, which ultimately resulted in T cell exhaustion. Additionally, they demonstrated that integration of the CD28 costimulatory domain into tonically signaling CAR T cells amplified this phenotype, while inclusion of a 4-1BB costimulatory domain protected against T cell exhaustion (57). This finding is in line with clinical studies of CD19 CAR T cells, as those with 4-1BB costimulatory domains demonstrate long term persistence while those with CD28 costimulatory domains do not (16, 18, 19). Our group plans to open a clinical trial of GD2 CAR T cells with a 4-1BB costimulatory domain in early 2019, which will be the first such trial in North America.

Persistence can be affected by factors extrinsic to the CAR molecule. Early CAR T cell trials did not incorporate lymphodepletion prior to CAR T cell infusion, which may have compromised expansion of the engineered T cells (18, 43, 71). Lymphodepleting chemotherapy improves engraftment and efficacy and has become a standard part of CAR T cell regimens (83, 84). The mechanism of increased activity after lymphodepletion is thought to be depletion of regulatory immune cells and/or a reflexive increase in homeostatic cytokines IL-7 and IL-15 that drive CAR T cell proliferation (84–86). Given that endogenous cytokines may increase CAR efficacy, some groups have focused on increasing CAR potency by programming CAR T cells to secrete immunostimulatory cytokines locally (87, 88). Systemic infusion of cytokines is often associated with unacceptable toxicity (89–91), and overexpression of the cytokine receptor does not overcome a dearth of cytokines in the tumor microenvironment (92). Therefore, providing local and inducible cytokine release by the CAR T cells themselves is an attractive strategy. Initial reports have demonstrated improved potency of CD19 CAR when co-expressed with IL-7 (93), IL-12 (94), IL-15 (95), membrane bound chimeric IL-15 (88), and IL-21 (93). Further studies will be required to translate these results clinically and to see if this can be generalized to solid tumors and to neuroblastoma specifically.

Anti-carcinoembryonic antigen (CEA) CAR T cells were engineered to produce IL-12 only after engagement with target antigen by placing IL-12 under the control of a nuclear factor of activated T cells (NFAT) promoter. In a colon cancer model, CEA CAR T cells that expressed inducible IL-12 mediated greater tumor regression and abrogation of antigen negative tumor outgrowth. This effect was likely enhanced by activated macrophages that infiltrated the tumors in response to the locally secreted IL-12. (96). An alternative system combines oncolytic viruses that secrete cytokines IL-15 and CCL5 with anti-GD2 CAR T cell therapy in xenograft models of neuroblastoma in order to increase T cell infiltration and persistence (97).

Shum et al. recently described a system in which a constitutively active IL-7 receptor was co-expressed with a second generation GD2 CAR. This resulted in improved efficacy of GD2 CAR T cells *in vitro* and in a murine xenograft model of neuroblastoma (98). This modification did not lead to malignant transformation in short term assays, an important safety consideration as the IL-7 receptor was derived from a patient with T cell acute lymphoblastic leukemia (99). However, implementation of such a strategy into clinical trials will require caution due to the potential for delayed malignant transformation. These approaches to increase potency and persistence of CAR T cells are beginning to undergo testing in early clinical trials (NCT03635632), and may help to improve efficacy, durability, and ultimately clinical outcomes.

Target Selection and Potential for Toxicity

Choosing an optimal CAR T cell target in neuroblastoma and more generally in solid tumors is a daunting task. Much of the success of CD19 and CD22 CAR T cells hinges on the restriction of these targets to lymphoblasts and normal B cells, which are in large part dispensable with appropriate supportive measures (16–20). An ideal CAR target antigen is highly and homogeneously expressed on tumor cells with minimal expression on vital tissues. Fulfillment of these criteria is difficult for solid tumor antigens, as many antigens are expressed in cells of related origin.

Many antigens overexpressed on neuroblastoma are often present at lower levels in peripheral nerves and/or on other neural tissue (100–102), so an important consideration in the development of anti-GD2 CAR T cells is the potential for off-tumor, on-target toxicity. Anti-GD2 monoclonal antibodies cause pain requiring continuous infusion of narcotics for analgesia (103–106) due to their interaction with peripheral nerves and possibly engagement of the complement system (107). However, clinical trials of CAR T cells targeting GD2 have not resulted in toxicity despite clear signs of on-tumor efficacy (27,

28, 30, 43). Still, due to toxicity concerns, most anti-GD2 CAR T cells for clinical trials have been designed to include a "suicide switch" to allow for rapid ablation.

Oncologists have approached adoptive T cell therapy for solid tumors cautiously due to the overlap of antigen expression with normal tissues. There have been several incidents of off-tumor toxicity in human trials using engineered high affinity TCRs against MAGE-A3 (108) and MAGE-A12 (109) that cross reacted with normal tissue. Additionally, one patient with metastatic colon cancer died after treatment with a HER2-targeted CAR (110). The initial case report of this incident noted that there was pulmonary infiltration by CAR T cells that could be due off-tumor, on-target toxicity. However, that patient was administered a dose of CAR T cells that was found to be 100 times the maximum tolerated dose of CD19 CAR T cells as well as exogenous IL-2. She was found to have very high levels of circulating cytokines and our recent understanding of the toxicities associated with CAR T cells indicates that this was more likely to be caused by CRS than off-tumor, on-target toxicity (111). This is further supported by recent efforts at Baylor College of Medicine to target HER2 on pediatric sarcomas using CAR T cells. In carefully designed dose escalation trials conducted without and with lymphodepletion, anti-HER2 CAR T cells elicited no off-tumor, on-target toxicity but resulted in clinically significant responses including a complete response in a patient with metastatic rhabdomyosarcoma (112, 113).

One possible explanation for the lack of toxicity for both GD2 and HER2 CARs is the differential in antigen density between tumor cells and normal tissue. Antigen density is emerging as an important consideration for CAR efficacy. When our group engineered a CAR against Anaplastic Lymphoma Kinase (ALK) on neuroblastoma, there was a clear correlation between the number of surface molecules of target antigen and ALK CAR T cell efficacy. A threshold number of target molecules was required to elicit effector functions (114). *In vivo*, ALK CAR T cell efficacy was only seen when ALK expression was high on tumor cells. Similarly, in a Phase I trial of CD22 CAR T cells of children with ALL, after initially achieving a complete response, most patients relapsed with leukemia expressing lower levels of CD22 than their pre-treatment samples, apparently below the threshold for CAR efficacy (20). Others have found a similar relationship of CAR efficacy and antigen density in preclinical studies of CARs for targets including CD123, CD20, HER2, EGFR, and CD30 (115–121). This represents a paradigm shift in the field as it opens up potential therapeutic windows for targets expressed at low levels on normal tissue as long as expression on tumor is high (111).

As CARs are engineered to become more potent, they could also become more toxic due to recognition of lower levels of target. While several clinical trials of GD2 CAR T cells containing the 14g2a binder have been carried out without any reports of central or peripheral neurotoxicity (27, 28, 30, 43), one preclinical study of a high affinity GD2 CAR reported neurotoxicity and T cell infiltration in the brains of mice (122). However, studies of a CAR with the same high affinity binder in our laboratory do not cause neurotoxicity (123), calling into question whether the findings were truly due to off-tumor, on-target toxicity.

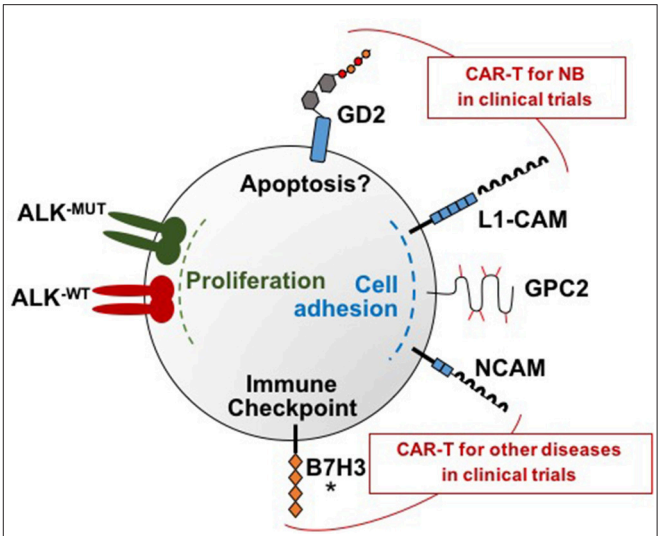

FIGURE 1 | Molecular targets under investigation for CAR T cell therapy for neuroblastoma. There are a total of six neuroblastoma surface targets for which CAR T cells have been developed: GD2, L1-CAM, GPC2, B7H3, and ALK, and NCAM. These targets each have distinct functions that are depicted in this figure. Note that both wild type and mutated ALK are overexpressed on neuroblastoma samples and both can be targeted by the ALK CARs. GD2 and L1-CAM are the two targets currently in clinical trials for neuroblastoma. Clinical trials that include CAR T cells targeting NCAM are ongoing for multiple myeloma and AML but not yet for neuroblastoma. An asterisk marks B7H3 because clinical trials are currently being planned.

The point remains, however, that as CAR T cells are better engineered to target low target antigen density tumor cells, there will be potential for increased toxicity and clinical trials must be conducted carefully.

Novel Targets

In addition to GD2 and L1-CAM, researchers are investigating several novel target antigens for CAR T cell therapy in neuroblastoma, and preclinical data are summarized below. **Figure 1** depicts the targets currently under investigation for CAR T cell therapy for neuroblastoma.

Glypican 2 (GPC2)

GPC2 is a member of the glypican family of proteins (124), and is instrumental for growth and differentiation of axons in the developing nervous system (125, 126). Gene expression-based exploration of the surfaceome of neuroblastoma cells identified GPC2 as a cell surface molecule that is highly expressed in neuroblastoma with low expression on normal tissue, indicating that it may be an ideal candidate for CAR T cell based immunotherapy (32, 33). Retrospective review demonstrated significantly decreased survival in neuroblastoma patients with tumors expressing high levels of GPC2. Bosse et al. generated an anti-GPC2 antibody drug conjugate (ADC) that demonstrated strong antitumor activity in a patient derived xenograft (PDX) mouse model (32).

Concurrently, another group developed CARs containing heavy chain only scFvs against GPC2 with 4-1BB and CD3ζ

endodomains. Anti-GPC2 CAR T cells demonstrated *in vitro* activity and *in vivo* clearance of human neuroblastoma xenografts (127). Though this study needs to be expanded to include a broader array of neuroblastoma cell lines and primary human samples, preliminary data suggest that GPC2 should be further evaluated as a clinical target for CAR T cell therapy in neuroblastoma. These studies also demonstrate the importance and power of a surfaceome approach to identify new targets for CAR T cell immunotherapy, with a <5-year turn-around time from target identification to development of therapeutics with potential for clinical application.

B7-H3/CD276

B7-H3 (CD276) is a checkpoint molecule expressed at high levels on many pediatric solid tumors including neuroblastoma (128–131). It plays a role in immune evasion (132) and metastatic potential (133), and overexpression correlates with poor prognosis in many cancers (134). These characteristics have made B7-H3 an attractive target for immunotherapeutic strategies, and early phase clinical trials with monoclonal antibodies have demonstrated encouraging results in both neuroblastoma and other malignancies (135–137). 8H9, a monoclonal antibody recognizing B7-H3, has been in clinical trials for more than 10 years; an 8H9 radioconjugate is an important element of a regimen for relapsed CNS neuroblastoma (NCT00089245) (135, 136). More recently, early phase clinical trials with a tumor specific anti-B7-H3 monoclonal antibody (MGA271) demonstrated safety and efficacy in adult malignancies (138). Our group has developed an active CAR targeting B7-H3 containing the scFv derived from MGA271 and efficacy is currently being explored in neuroblastoma (139, 140).

Anaplastic Lymphoma Kinase (ALK)

Several groups have identified anaplastic lymphoma kinase (ALK) as a potential oncogene in neuroblastoma (141–143). ALK is a receptor tyrosine kinase and, similar to GPC2, its expression is primarily restricted to the central and peripheral nervous system during fetal development (144). ALK regulates cell proliferation, differentiation, and apoptosis and has been implicated in many signaling pathways including PI3K/AKT, RAS/MAPK, and STAT3 (145). Activating mutations occur almost universally in familial neuroblastoma but also occur in a sizable percentage of sporadic neuroblastoma cases. Additionally, 15–20% of neuroblastoma patients overexpress wild type ALK in the absence of an activating mutation (146).

Anti-ALK CARs with a 4-1BB costimulatory domain were generated using previously described monoclonal ALK antibodies (147). ALK CAR T cells demonstrated *in vitro* activity but had limited efficacy *in vivo* in xenograft models of neuroblastoma (114). Investigations into the reasons for limited CAR efficacy demonstrated that ALK expression on the neuroblastoma cell lines used was below the threshold of antigen expression required for CAR activity. This finding demonstrates the importance of antigen density for CAR T cell efficacy (115–121).

Neural Cell Adhesion Molecule (NCAM/CD56)

NCAM (CD56) is another glycoprotein that is important in neural development and is overexpressed on neuroblastoma (148). Similar to ALK and GPC2, it is overexpressed on tumors of neuroendocrine origin (149). It is also expressed on normal tissues, including most prominently on natural killer (NK) cells. Phase I and II clinical trials had demonstrated a favorable safety profile of anti-CD56 ADCs in solid tumors such as small cell lung cancer (150). The high and homogeneous expression on neuroblastoma and the limited toxicity of antibody-based therapy led one group to develop a CAR directed against CD56. This second generation CAR with a CD28 costimulation domain controlled tumor burden in a xenograft neuroblastoma model, but had only modest effects on survival (151). CD56 CAR T cells are being studied in clinical trials for relapsed multiple myeloma and for relapsed AML (NCT03473496, NCT03473457), though there are not yet published reports of any patient treated. Further investigation into CD56 as a target in neuroblastoma is warranted but off-tumor toxicity will need to be carefully monitored given significant normal tissue expression.

Natural Killer (NK) Cell Adoptive Therapy

NK cells have long been recognized as important in neuroblastoma and killer cell immunoglobulin-like receptors (KIR) haplotypes are strongly correlated to survival (152–154). NK cells lack the specificity of T cells, but they have the capacity to kill infected and malignant cells without the prerequisite priming and sensitization to peptide-MHC complexes on the target cell surface. Instead, NK activity is regulated by a balance of activating and inhibitory receptors (155). Several trials are underway in which neuroblastoma patients receive adoptively transferred *ex vivo* expanded but unmanipulated NK cells (NCT02573896, NCT01857934, NCT02650648, NCT03209869).

Given their importance in control of neuroblastoma, researchers have attempted to augment the anti-tumor effects of NK cells in by imparting them with tumor antigen specific CARs. One group generated patient-derived NK cells expressing a second generation GD2-specific CAR, and demonstrated significant improvement in cytotoxicity against primary patient neuroblastoma cells compared to NK cells without a CAR (156). Similarly, expressing the GD2-CAR in an NK-92 cell line promoted *in vitro* cytotoxicity against neuroblastoma cell lines that were resistant to killing by the parental NK-92 cell line (157). NK cells do not have the same proliferative capacity as T cells, and clinical trials of adoptively transferred NK cells are often marked by short persistence and disappointing anti-tumor effect (158). The persistence of NK cells and invariant NK T cells can be increased by constitutive secretion of IL-15, an approach being studied in clinical trials for children with neuroblastoma at Baylor College of Medicine (NCT03294954) (159, 160).

Engineering a Successful CAR T Cell Product

In contrast to standard chemotherapy or "off the shelf" immunotherapies such as monoclonal antibodies, an important consideration for CAR T cell therapy is the ability to manufacture adequate quantities of a viable, maximally efficacious T cell

product. Some patients have poor expansion and inadequate production of CAR T cells. One group hypothesized that myeloid derived suppressor cells (MDSC) in the apheresis product may interfere with T cell expansion, and found higher proportions of monocytes in PBMC concentrates to inversely correlate with fold expansion of CD19 and GD2 CAR T cells (161). CAR T cell quality is of particular concern for patients who have undergone chemotherapy, radiation, and/or stem cell transplant, all important elements of upfront neuroblastoma therapy. Data presented in abstract form describe T cell fitness in PBMC samples collected at diagnosis and after each cycle of chemotherapy from children with a wide variety of cancers including neuroblastoma. These data suggest that after chemotherapy, patients develop poor CAR T cell potential, defined by a low proportion of naïve T cells, mitochondrial dysfunction, and poor spare respiratory capacity (162). Further study is warranted to understand this phenomenon and whether it ultimately impacts CAR T cell efficacy in patients, as highly active CD19 CAR T cells have been successfully generated from most patients with heavily pretreated ALL (18, 19).

Overcoming Immunosuppressive Tumor Microenvironment (TME)

The immunosuppressive tumor microenvironment (TME) presents a significant barrier to successful CAR T cell therapy for neuroblastoma. Neuroblastoma tumors are intermixed with a suppressive cell population that includes tumor associated macrophages (TAMs) and regulatory T cells (Tregs). Presence of these cells predicts poor outcomes (34, 163). Tumors also express inhibitory ligands such as PD-L1 that dampen T cell responses (164–167). Furthermore, the TME contains an array of soluble factors such as TGF-β and IL-10 that act to directly inhibit T cells (34, 168–171). Finally, physical barriers such as stroma, extracellular matrix (ECM) and tumor associated vasculature prevent tumor infiltrating T cells from easily accessing their target (172–175).

Enhancing Trafficking to Neuroblastoma

For CAR T cell therapy of hematologic malignancies, the majority of malignant cells are located within the hematopoietic system. Solid tumors are not as readily accessible, a fact supported by data from early clinical trials in which GD2 CAR T cells were easily detectable in peripheral blood but rarely seen in post-treatment tumor biopsies (27). Optimal trafficking of T cells occurs when the effector T cells express a chemokine receptor that is complementary to chemokines that are rich in the tumor microenvironment, either excreted by tumor cells or surrounding tumor stroma. Expression of chemokine CCL2 has long been associated with more effective immune responses against neuroblastoma and it is secreted by neuroblastoma cell lines and primary tumor cells (176, 177). However, CAR T cells generated from neuroblastoma patients were found to have very low expression of the corresponding chemokine receptor, CCR2, despite expressing high levels of other chemokine receptors. Transgenic expression of CCR2b on GD2 CAR T cells in a neuroblastoma xenograft model improved kinetics of CAR T cell chemotaxis and greater anti-tumor efficacy (177).

Depleting Suppressive Immune Cells

Assuming adoptively transferred T cells migrate appropriately to a solid tumor, they must circumvent many immunosuppressive factors within the TME. Many researchers are working to overcome this barrier. One strategy involves depleting suppressive immune cells. In a xenograft model of osteosarcoma, Long et al. observed that MDSCs decreased GD2 CAR T cell efficacy. When mice were treated with ATRA, which can induce differentiation of immature myeloid cells to a non-suppressive subtype (178), they had fewer suppressive MDSCs and there was a modest improvement in tumor control and survival (35). Alternatively, CARs themselves can be redirected against TAMs and regulatory T cells. One group took advantage of the dual specificity of CD123 CAR T cells against both Hodgkin lymphoma cells and TAMs. They found that with this strategy, they could target and eliminate TAMs and achieve durable remissions in Hodgkin lymphoma xenograft models (179).

Overcoming Inhibitory Signals

To evade the immune system, tumors express PD-L1, the ligand for PD-1, an inhibitory receptor on T cells. Engagement of this receptor dampens the native immune response (180) and blocking antibodies can "remove the brakes" and prompt an anti-tumor response, leading to success in early phase clinical trials (181–184). Neuroblastoma in particular was found to more frequently express PD-L1 than most other pediatric solid tumors. Additionally, PD-L1 expression [defined as >1% positive in tumor cells by immunohistochemistry, in line with some adult carcinoma scoring systems (185)] in neuroblastoma is associated with inferior survival (167).

PD-L1 upregulation on solid tumors can limit the efficacy of tumor-specific CAR T cells (186). Liu et al. postulated that they could improve anti-tumor control by combining CAR T cell therapy with a "switch-receptor" that would interrupt PD-1 inhibitory signaling. They endowed multiple CAR T cells with an additional chimeric receptor with a PD-1 extracellular domain directly connected to an intracellular CD28 co-receptor to provide costimulation and activation of T cells upon engagement with PD-L1. In all models, the switch receptor augmented CAR T cell function, and importantly, to a greater degree than anti-PD-1 monoclonal antibodies (39).

Interfering With Inhibitory Soluble Factors

When neuroblastoma directed CAR T cells penetrate the suppressive immune milieu, they inevitably encounter suppressive factors including soluble cytokines that can suppress T cell function. These factors can be secreted by tumor cells or by surrounding stromal cells and include TGF-β, IL-10, galectin-1, and galectin-3 (34, 168–171); they represent potential targets to enhance CAR T cell efficacy. TGF-β in particular has importance in the neuroblastoma TME. Elevated levels of TGF-β transcripts in primary neuroblastoma samples were associated with shorter EFS (187), and blockade of TGF-β induced a more potent NK cell response in conjunction with anti-GD2 monoclonal antibody in a neuroblastoma xenograft model (188). T cells engineered to express dominant negative TGF-β receptors have been shown in a number of settings to

improve efficacy of adoptive T cell therapy (189–191). This strategy was recently corroborated in a preclinical CAR model using an anti-prostate-specific membrane antigen (PSMA) CAR (192). PSMA CAR T cells coexpressed with the dominant negative receptor demonstrated increased proliferation, cytokine secretion, exhaustion resistance, persistence, and anti-tumor efficacy. With such pre-clinical promise, this construct has been incorporated into a clinical trial (NCT03089203).

Targeting Tumor Stroma

CAR T cells must penetrate physical barriers within the tumor stromal compartment that augment tumor growth and prevent infiltration of surveilling immune cells. Cancer associated fibroblasts (CAF) are the dominant cell type in the tumor stroma and express fibroblast activating protein-α (FAP) at high levels (173, 174, 193, 194). In a murine model of lung cancer, the efficacy of CAR T cells targeting the Ephrin Receptor tyrosine kinase EphA2 was enhanced by coadministration of anti FAP CAR T cells (195), providing proof of principle that anti-stromal CAR T cells can contribute to successful CAR T cell therapy in the solid tumor setting. Though this CAR has not yet been tested in neuroblastoma models, CAFs derived from primary neuroblastoma samples universally express FAP and enhance tumor engraftment and growth, and thus represent a potential target within the neuroblastoma TME (196).

T cell infiltration into tumors requires degradation of ECM proteins, including heparan sulfate proteoglycans (HSPG) (172). HSPGs are expressed on neuronal tissue during development and neuroblastoma cells are known to express some HSPGs at high levels (127). Activated T cells secrete heparanase to actively break down HSPG (197), but *ex vivo* culture of T cells causes downregulation of heparanase and abrogates their ability to degrade ECM (198). Expression of heparanase in a GD2 CAR T cell significantly improved tumor infiltration and antitumor activity in a neuroblastoma xenograft model (198), validating this as a potential method to improve CAR T cell therapy in stromal-rich tumors.

The immunosuppressive tumor vasculature presents a third physical barrier that may be a viable target to improve CAR T cell therapy. Vascular endothelial growth factor (VEGF) is a proangiogenic factor secreted by tumors, and can directly suppress immune cell infiltration of tumors (175). In a neuroblastoma xenograft model, anti-GD2 CAR T cells co-administered with the anti-VEGF antibody bevacizumab had superior anti-tumor activity over GD2 CAR T cells alone, thought to be primarily related to increased tumor infiltration by T cells (140).

CONCLUSIONS

Immunotherapy with anti-GD2 antibodies has revolutionized the care of neuroblastoma patients, but there is still a great need for novel therapies for the patients with refractory or relapsed high risk disease. Early clinical trials with CAR T cells in neuroblastoma have demonstrated safety and shown some objective clinical responses. They have also provided insight into reasons for limited success, including lack of T cell persistence, difficulty in target antigen selection, and a suppressive tumor microenvironment. These challenges are universal in the CAR T cell field, in particular for solid tumors like neuroblastoma, and there are significant efforts underway to improve upon each of these domains. Successful CAR T cell therapy in neuroblastoma will require rational engineering approaches that address each of the above-mentioned barriers. Many studies presented in this review have encouraging pre-clinical results and thoughtful incorporation of some of these strategies into clinical trials will ultimately validate CAR T cells to treat neuroblastoma and improve patient outcomes.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: RM has a pending patent application for the use of GD2 CAR T cells in H3K27M mutant gliomas.

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MINI REVIEW
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CAR-T Cells and Oncolytic Viruses: Joining Forces to Overcome the Solid Tumor Challenge

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Adoptive transfer of chimeric antigen receptor (CAR)-modified T cells has resulted in unprecedented rates of long-lasting complete responses in patients with leukemia and lymphoma. However, despite the impressive results in patients with hematologic malignancies, CAR-T cells have showed limited effect against solid cancers. New approaches will need to simultaneously overcome the multiple challenges that CAR-T cells encounter in solid tumors, including the immunosuppressive tumor microenvironment and heterogeneity of antigen expression. Oncolytic viruses are lytic and immunogenic anti-cancer agents with the potential to synergize with CAR-T cells for the treatment of solid tumors. In addition, viruses can be further modified to deliver therapeutic transgenes selectively to the tumor microenvironment, which could enhance the effector functions of tumor-specific T cells. This review summarizes the major limitations of CAR-T cells in solid tumors and discusses the potential role for oncolytic viruses as partners for CAR-T cells in the fight against cancer.

Keywords: chimeric antigen receptors (CAR), oncolytic viruses, solid tumors, immunotherapy, immunosuppressive tumor microenvironment, adoptive cell transfer (ACT)

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INTRODUCTION

The recent approval by the US Food and Drug Administration (FDA) of two different CAR-T cell therapies for the treatment of leukemia and lymphoma represents a landmark in the development of cancer immunotherapies. Together with immune checkpoint blockade therapy (1), CAR-T cells are revolutionizing the field of cancer therapy, providing hope for a cure in patients with previously refractory cancers (2–8). However, despite the stunning results of CAR-T cells in patients with hematologic malignancies, this approach has shown little effect in patients with solid tumors. Recent clinical trials demonstrated that CAR-T cells are able to infiltrate the tumor mass and exert antigen-directed activity (9–12). However, with rare exceptions (13, 14), observed responses in patients with solid tumors have been minor and transient.

In order to induce complete responses in patients with solid tumors, CAR-T cells need to overcome several barriers. First, CAR-T cells must traffic from the blood into the tumor, infiltrate the tumor mass, and be able to survive and maintain their effector functions in a tumor microenvironment that is highly immunosuppressed and enriched in stroma. Then, CAR-T cells need to eliminate the totality of the cancer cells, which is extremely difficult due to the heterogeneity of antigen expression in cancer cells and the intrinsic plasticity of tumors that may lead to tumor escape (15, 16). Finally, CAR-T cells are living drugs that can lead to dramatic antitumor responses but can also induce significant toxicities (17–19). New approaches to enhance therapeutic outcome in patients with solid tumors must therefore focus on enhancing potency without increasing toxicity.

Rapid advances in synthetic biology, T cell immunology, and gene editing have fueled the design of next generation CAR-T cells with the potential to overcome some of the hurdles encounter in solid tumors (20). However, it is unlikely that CAR-T cell therapy alone will be sufficient to induce complete responses in the majority of cancers. Combining CAR-T cells with other cancer treatments that have different mechanisms of action and the potential to synergize with T cells may reduce tumor escape and increase the success rates of CAR-T cell therapy. As novel therapies emerge, rational combinations will need to be tested based on an understanding of the mechanisms underlaying tumor resistance to CAR-T cells.

Oncolytic virotherapy is a therapeutic approach to treat cancer that uses native or genetically modified viruses that selectively replicate within cancer cells (21). The field of oncolytic virotherapy has gained renewed attention after the FDA approval of Talimogene laherparepvec (T-VEC), an oncolytic herpes simplex virus type 1 (HSV-1) modified to express GM-CSF (22), and the recent reports of high response rates obtained in patients with advanced melanoma when combining T-VEC with checkpoint blockade (23, 24). Oncolytic viruses (OV) mediate their antitumor effect through a dual mechanism of action, including a direct lytic effect on tumor cells and the induction of anti-cancer adaptive immunity (25, 26). Moreover, OV can be further modified to selectively deliver therapeutic transgenes to the tumor microenvironment to enhance their antitumor potency or boost an antitumor immune response (27). All these characteristics make OV excellent potential partners to synergize with emerging immunotherapies, and several combinatorial approaches are being currently tested in preclinical and clinical trials (26, 28).

This review provides an overview of current barriers that CAR-T cells encounter in solid tumors, summarizes the advances in the field of OV and discusses the preclinical and clinical data that support the clinical testing of OV in combination with CAR-T cells to overcome the solid tumor challenge.

CAR-T CELLS IN SOLID TUMORS: CHALLENGES AND LIMITATIONS

While most of the early trials of CAR-T cells for solid tumors resulted in poor therapeutic outcomes, some case reports of dramatic clinical responses with manageable therapy-related toxic effects provide clear reasons for optimism (13, 14). Recent reports with second generation CAR-T cells suggest that CAR-T cells can traffic, persist, and proliferate in the tumor (9, 10). Moreover, evidence of transient antitumor activity has been observed in patients with difficult-to-treat tumors, such as glioblastoma (13), neuroblastoma (14), pancreatic cancer (12), and sarcoma. Here, we summarize the lessons learned in these clinical trials and discuss the hurdles that CAR-T cells must overcome for effective therapy, focusing on those challenges that OV may help to address.

Trafficking, Proliferation, and Persistence

The ability of tumor-specific T cells to traffic to the tumor, proliferate, and persist is considered critical to achieve an effective anti-tumor response (14, 29, 30). While T cells can actively traffic to sites of disease, often tumors present low levels of inflammation and lack of the chemokines required for migration. Also, physical barriers, such as aberrant vasculature, increased stromal stiffness and high interstitial pressure, may impair T-cell infiltration. Once in the tumor, CAR-T cells must efficiently proliferate, and persist until the entirety of the tumor is eliminated. However, T-cell proliferation and persistence are often hampered due to T-cell intrinsic (T-cell fitness) or extrinsic factors (tumor microenvironment). The requirements for proliferation and persistence can be relaxed in some instances if regional delivery and redosing of CAR-T cells is a therapeutic option (31). For example, in a recent clinical trial, multiple intracranial injections of CAR-T targeting IL13Rα2 mediated a transient complete response in a patient with glioblastoma (13). In this patient, two intracranial CAR-T cell delivery routes were tested: intracavitary and intraventricular. While intracavitary therapy was only able to control growth of the local tumor, intraventricular therapy resulted in a dramatic reduction in the size of all intracranial and spinal tumors. These results highlight the importance of trafficking and administration route to achieve the optimal tumor responses. Developing strategies to enhance trafficking and persistence to increase the therapeutic CAR-T cell input in the tumor would represent a vertical advance in the field.

Tumor Immunosuppression

On arrival to the tumor, CAR-T cells encounter an immunosuppressive environment that prevents T-cells from reaching their full therapeutic potential. The main barriers that CAR-T cells need to overcome once in the tumor include: (i) suppression by immunoregulatory cells, including myeloid-derived suppressor cells (MDSC), tumor associated macrophages and neutrophils, and regulatory T cells; (ii) presence of an array of immunosuppressive molecules, such as IL-10, TGF-β, PD-L1, IDO and arginase-1, and (iii) microenvironment factors, such as hypoxia, low pH and nutritional depletion. These conditions, together with chronic antigen exposure, can lead T-cells to distinct stages of functional dysfunction (32–34). Moreover, the stromal microenvironment can actively exclude T cells from the vicinity of cancer cells (35). Finally, a recent clinical report suggests that the tumor microenvironment can become even more immunosuppressive after CAR-T cell activation within the tumor, probably due to an initial production of IFN-γ (10). Finding ways to prevent or reverse T-cell dysfunction by reverting tumor immunosuppression will be key to improving treatment.

Tumor Escape by Loss or Heterogeneity of Antigen Expression

One of the main limitations in the treatment of solid tumors with CAR-T cells is the absence of cancer-restricted antigens that are uniformly expressed in tumor cells and absent in essential organs. Solid tumors exhibit heterogeneity of antigen expression with regards to intensity and distribution. Tumor escape due to heterogeneity or loss of antigen expression is an emerging threat to CAR-T cells, as it can result in overgrowth of target-deficient tumor cells that are invisible to CAR-T cell therapy (36–38). Preclinical studies have demonstrated that

tumor cells expressing high levels of the targeted antigen are preferentially eliminated by CAR-T cells, whereas those with the lowest expression may survive (39–41). Decreased expression of the targeted antigen after CAR-T cell therapy has been observed in several clinical trials, including those targeting Her2 (9), EGFRviii (10), IL13Rα2 (11), and mesothelin (12). These results demonstrate the potential of CAR-T cells to eliminate antigen-positive tumor cells, but also highlight the importance of designing new strategies to simultaneously target different antigens. Several groups are designing new CAR constructs able to target more than one antigen simultaneously (39, 42, 43). While reducing the risk of escape, these strategies may also result in increased on-target off-tumor reactivity, as most of the targeted antigens can be expressed in healthy tissue at low levels (17–19). An alternative approach would be to find strategies to activate an endogenous immune response that could partner with CAR-T cells to completely eliminate the tumor. Some reports suggest that CAR-T-cell mediated tumor destruction may lead to the release of other tumor antigens that are cross-presented in a process known as epitope spreading (44, 45). This observation requires further investigation, but it could explain how complete elimination of tumor lesions has been achieved even when the tumors did not uniformly express the target (13).

ONCOLYTIC VIRUSES: LESSONS LEARNED IN CLINICAL TRIALS

To date, there are three viruses commercially available for the treatment of cancer: T-VEC approved in the USA, H101 approved in China and Rigvir approved in Latvia, Georgia and Armenia. Several other viruses are in clinical trials and may eventually join this short list of marketed viruses (46). Some of the lessons learned from clinical trials that will drive the design of future therapies include: (*a*) OV can induce a therapeutic benefit in cancer patients, including complete responses, in the absence of severe adverse effects (47–50). Interestingly, some of these complete responses are reached after the virus have been eliminated, suggesting that the complete elimination of the tumor may depend on the activation of an immune-mediated anti-tumor response (48). On line with this observation, a recent clinical trial reported that the overall survival among patients who received a chimeric poliovirus reached a plateau of 21% 1 year after treatment that was sustained for months (51). This plateau in long-term survival is similar to the one observed in Kaplan-Meier curves from cancer patients treated with other cancer immunotherapies and highlights the role of the immune system on the emergence of long-term survivors (52); (*b*) The antiviral immunity constitutes an obstacle against OV as it sequesters or neutralizes viral particles before they reach their target. A major question is how to deliver the virus to the tumor efficiently; (*c*) Virus replication has been detected in tumor biopsies a few days after treatment. However, the ability of OV to survive and spread through the tumor is limited by antiviral T cells (47, 48, 53); (*d*) Tumors treated with OV typically show increased immune cell infiltration, including activated macrophages and cytotoxic T-cells, and pro-inflammatory cytokines (47, 48, 53). Tumor-specific T cells have been detected after treatment with OV (53, 54). While the capacity of OV to expand neoantigen-specific T cells deserves further investigation, the potential of OV for combination with immunotherapies such as immune checkpoint inhibitors has been well-recognized (28, 55–58). Several clinical trials are currently testing the combination of OV with immune checkpoint therapy and initial reports showed promising results (23, 24).

ONCOLYTIC VIRUSES: THE IDEAL ALLIES FOR CAR-T CELLS?

OV have the potential to synergize with CAR-T cells by helping them simultaneously overcome some of the multiple barriers found in solid tumors. First, viruses provide a danger signal that can revert tumor immunosuppression, which could facilitate CAR-T cell trafficking, proliferation, and persistence in the tumor microenvironment. Second, the direct lytic effect of OV on cancer cells results in tumor lysis and release of tumor-associated antigens (TAA), which can induce an anti-tumor adaptive response that could potentially mitigate tumor escape by antigen loss. Third, OV can be armed with therapeutic transgenes that could further enhance the effector functions of T cells. Here, we provide an overview of the biological properties of OV that may be considered when choosing a viral platform for combination with CAR-T cells, and we summarize the recent preclinical strategies that have been explored combining CAR-T cells and OV.

Oncolytic Viruses as Immunotherapy Agents

The immune system is well-equipped to mount an innate inflammatory response to viruses that eventually will induce the infiltration of effector T-cells. In particular, OV have pathogen-associated molecular patterns (PAMPS) detected by pattern-recognition receptors (PRRs) on tumor and epithelial cells as well as macrophages and dendritic cells (59). These PRRs induce danger associated molecular patterns (DAMPs) characteristic of an immunogenic cell death (60, 61). PRRs also signal through NF-kB to induce the expression of cytokines such as TNF-α and IL6, and through IFN Regulatory Factor (IRF) to induce type I interferons and activate caspase 1 that matures IL-1β (62). This pro-immune cytokine environment can facilitate the maturation and function of DC's, macrophages, and epithelial cells that can lead to the recruitment of neutrophils and natural killer (NK) cells, monocytes, and memory T-cells to the site of infection (63–65). Tumor cells dying due to the lytic activity of OV can release TAA. Activated DC's with their MHC loaded with virus and/or tumor epitopes can traffic to the draining lymph nodes to engage specific T-cells and stimulate their proliferation and circulation into the bloodstream. Chemokines of the infected tumors can induce integrin expression on these T-cells and selectin expression on endothelial cells to extravasate them. Under these conditions, T cells can be recruited efficiently to infected tumors, and as discussed above, increased T cell

infiltration is generally detected in tumors of patients treated with OV therapy. Interestingly, viral infection has been shown to induce neoantigen-directed T cell responses (53, 54), which could synergize with CAR-T cells and virus-specific T cells to clear the tumor. A mayor limitation to study the impact of the immune-modulating effects of OV on CAR-T cell therapy is the lack of good animal models. However, it can be hypothesized that following the establishment of a more immunogenic intratumoral milieu, killing of target cells may be more efficient due to cooperation between the effector T-cells.

The ability of OV to induce an anti-tumor immune response is now considered a key mechanism of action to obtain long-term antitumor responses. Therefore, most of the current efforts directed at enhancing the therapeutic potential of OV are focused on improving their capacity to induce a systemic antitumor response.

The Oncolytic Virus Armamentarium

Multiple types of viruses are used in cancer virotherapy, each one of them with its unique properties (**Table 1**) (66). Here we discuss some of the different factors that should be considered when selecting an OV for combination with CAR-T cells. In general terms, viruses that replicate in the cytoplasm (RNA viruses) kill tumor cells faster than nuclear ones (DNA viruses) as they do not need to reach the nucleus of the infected cells. But for the same reason, they offer less opportunities for tumor-selective control. Tumor-selective replication of most oncolytic RNA viruses, such as reovirus, picornaviruses (Coxsackeivirus, Rigavirus), rhabdovirus (Vesiscular Stomatitis Virus [VSV], Maraba Virus), and paramixovirus (Measles Virus, Newcastle disease virus [NDV]), depends on defects of the interferon pathway in tumor cells. Because IFN induction is a central pathway in the innate response to viruses, which potentiates the adaptive T cell responses, the inflammatory response elicited with these viruses is expected to be lower. DNA viruses, such as adenoviruses, have slower replication cycles but are amenable to being controlled in the nucleus of the infected cells using tumor-selective promoters. The presence of an envelope also determines the oncolytic properties of a virus. Enveloped viruses (i.e., Measles virus, NDV, VSV, Herpes simplex virus, and Vaccinia virus) bud from cells and are less "lytic" than naked viruses. The envelope also contributes to the main clearance mechanisms in blood, with complement having a major role for enveloped viruses and antibodies for non-enveloped ones. Size is also an important parameter for the properties of OVs. The smaller the virus, the easier it will be for the virus to penetrate and diffuse throughout the tumor. But a larger virus with a larger genome allows the insertion of non-viral transgenes. Arming OV with therapeutic transgenes offer the opportunity to complement the OV in multiple ways. Among RNA viruses, VSV, Measles virus, and NDV can accept transgenes in contrast to picornaviruses and reoviruses, and for DNA viruses, Adenovirus, Herpes Simplex Virus and Vaccinia virus can be armed with transgenes in contrast to parvovirus. The list of genes that have been included in OV that could be potentially useful for combination with CAR-T cells is long and it has been reviewed recently (67). It includes, among others: (a) inducers of immunogenic cell death (68), (b) transgenes directed to modulate the immune system, such as cytokines (22, 69–71), chemokines (72, 73), co-stimulatory proteins (74–77), bispecific T-cell engagers (BiTEs) (78, 79), and immune checkpoint blockers (80–83), and (c) stroma-degrading proteins that could facilitate the spread of OV and T-cells within the tumors (84, 85). Comparing viruses and transgenes is a very challenging task given the limitations of preclinical immune competent mouse models, where many human viruses present defects in replication and tumors do not edit the immune system in a slow and progressive way as occurs in humans.

Combining CAR-T Cells and Oncolytic Viruses for the Treatment of Solid Tumors

At a preclinical level, several groups have started to test different transgene-armed OV in combination with CAR-T cells (**Figure 1**). Most of these works assessed the antitumor effects of these therapies in NOD scid gamma (NSG) mice, a mouse strain that is completely deficient in adaptive immunity and severely deficient in innate immunity (86). NSG mice allow the engraftment and persistence of adoptively transferred CAR-T cells, and human tumor xenografts allow the replication of the virus and the delivery of the transgene. Therefore, these studies gave important insights in the antitumor effects of combining CAR-T cells with oncolysis and transgene delivery. An important limitation is that the capacity of OV to induce anti-tumor immunity cannot be assessed using these tumor xenografts.

Oncolytic adenoviruses modified to express IL-15 and RANTES (87) or IL-2 and TNF-α (88) have been shown to increase the accumulation and survival of CAR-T cells in the tumor microenvironment. Similarly, with the goal of enhancing the intra-tumoral trafficking of CAR-T cells, a vaccinia virus expressing CXCL11, a CXCR3 ligand, was used to attract effector cells following transfer (89). Another report demonstrated that expression by an oncolytic adenovirus of a BiTE targeting a second tumor antigen could address heterogeneity of antigen expression (40). Combination of a preparation of CAR-T cells with the OV-BiTE induced activation of T cells in the absence of the CAR-targeted antigen or lack of CAR expression (i.e., non-transduced T cell population). In a slightly different approach, combination of an oncolytic adenovirus with a helper-dependent adenovirus expressing a PD-L1 blocking mini-antibody was used to revert T cell dysfunction by preventing PD1:PDL1 interaction (90). Co-expression of IL12p70 and PD-L1 further augmented the therapeutic efficacy of the combination (91). As expected, all these combinations of CAR-T cells and armed-OV resulted in enhanced tumor control and prolonged survival when compared to each agent as monotherapy. An interesting finding by Watanabe et al. is that CAR-T cells as monotherapy failed to control the growth of the primary tumor, while OV could suppress the progression of the primary tumor but mice died from metastatic disease. Combination of CAR-T cells with an OV armed with IL-2 and TNF-a was able to control both the primary tumor and tumor metastasis (88).

Finally, in a totally different and very preliminary approach, CAR-T cells have been used to deliver OV to the tumor (92). Circulating cells such as lymphocytes, monocytes, erythrocytes,

TABLE 1 | Main groups of oncolytic viruses classified by taxonomy families.

Virus family	Genome type	Genome size (Kb)	Replication site	Capsid	Mechanism of selectivity	Armed	Viruses in clinical development (or approved when indicated)
Retroviruses: Murine leukemia virus	dsRNA (requires integration in cell genome as DNA)	8	Nucleus	Enveloped	Requires cell division to enter the nucleus	Transgenes	Toca-511
Picornaviruses: Coxsackievirus A21 ECHO-7 Seneca virus Poliovirus	ssRNA	7.5	Cytoplasm	Naked	IFN sensitive (IFN inhibits protein translation and virus replication but in tumor cells this protein translational block is impaired) Receptor tropism: CVA21 infects by ICAM-1 as primary receptor and DAF as coreceptor.	miRNAs Only small insertions (300bp)	CAVATAK Rigvir (Approved) SVV-001 PVSRIPO
Rhabdoviruses: Vesicular stomatitis virus (VSV) Maraba	ssRNA	15	Cytoplasm	Enveloped	IFN sensitive M protein mutations increase IFN sensitivity	Transgenes	VSV-IFNβ VSV-IFNβ-NIS Maraba MG1-MAGEA3.
Paramyxoviruses: Meales Newcastle disease virus (avian virus)	ssRNA	16-20	Cytoplasm	Enveloped	IFN sensitive Edmonton Measles strain infects via CD46 and SLAMs found in B and T lymphocytes. Appropriate for myeloma.	Transgenes	MV-CEA MV-NIS NDV PV701 NDV MEDI5395
Reoviruses: Mammalian orthoreovirus Type 3 Dearing	dsRNA (in fragments or segments)	22-27	Cytoplasm	Naked	IFN sensitive	No	Reolysin
Parvoviruses: H1 autonomous rat parvovirus	ssDNA	5	Nucleus	Naked	Unknown (active metabolic and regulatory pathways allow replication of this rat virus in human tumor cells).	No	H1-PV Parvoryx
Adenoviruses	dsDNA	36	Nucleus	naked	RB pathway if E1a binding site to pRB is deleted Control of gene expression with tumor selective promoters.	Transgenes	H101 (approved) CG0070 LOAd703 DNX-2401 Telomelysin ColoAd1 ONCOS-102 VCN-01
Herpesviruses	dsDNA	150	Nucleus	Enveloped	Becomes IFN sensitive if viral gamma 34.5 gene is deleted. Depends in high nucleotide metabolism if viral Thymidine Kinase gene is deleted. Control of gene expression with tumor selective promoters.	Transgenes	Tvec (Imlygic) (Approved) HSV1716 G207 RP1
Poxviruses: Vaccinia Virus (VV) Mixoma Virus (of rabbits)	dsDNA	190	Cytoplasm	Enveloped	Becomes IFN sensitive if viral B18R gene is deleted. Depends in high nucleotide metabolism if viral Thymidine Kinase or Ribonucleotide reductase genes are deleted. Depends on EGF-R pathway if VGF gene is deleted.	Transgenes	JX-594 TG6002 GL-ONC1 GLV-1h68

Key parameters for oncolysis are shown. Some examples of viruses in clinical development are included for each virus family. Top RNA viruses, bottom DNA viruses. Ordered by genome size.

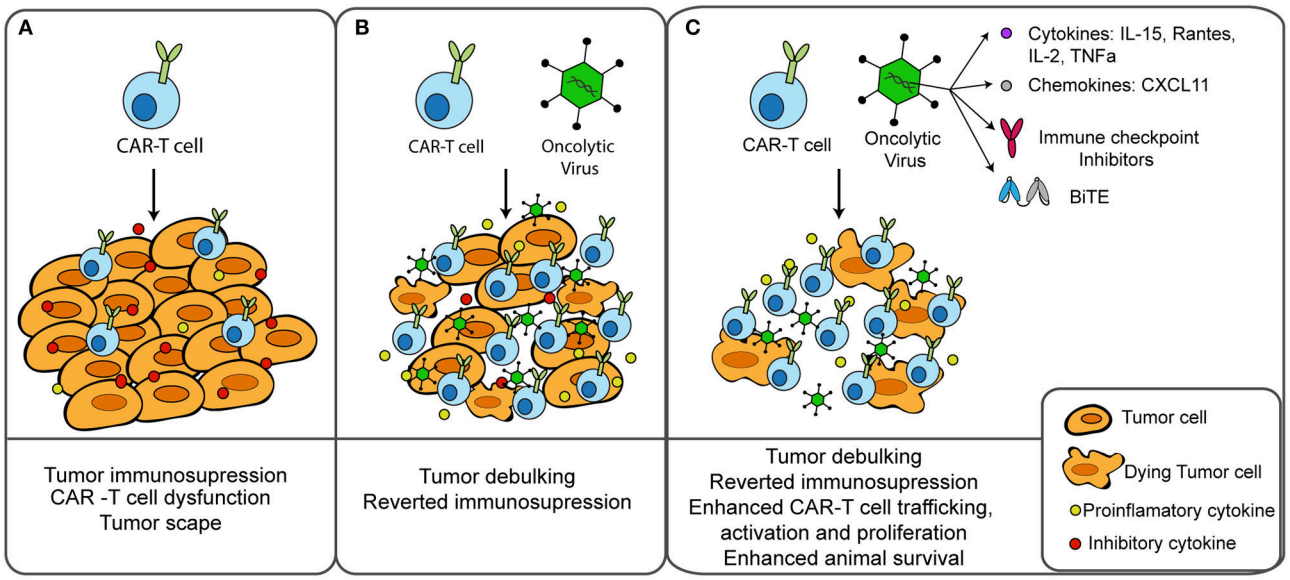

FIGURE 1 | Combination of CAR-T cells and oncolytic virus for the treatment of solid tumors. **(A)** CAR-T cells find several obstacles in solid tumors, including an immunosuppressive environment that can lead to T cell dysfunction and treatment failure. **(B)** Cancer treatment with oncolytic viruses prior to CAR-T cell therapy results in tumor debulking, immunogenic cell death and reverted tumor immunosuppression. **(C)** Oncolytic viruses can be genetically modified to deliver therapeutic transgenes into the tumor microenvironment to enhance T-cell effector functions. Preclinical studies combining CAR-T cells with oncolytic viruses armed with cytokines, chemokines, BiTEs, or immune checkpoint inhibitors resulted in enhanced therapeutic outcomes.

or even platelets can bind viruses and have shown tumor-targeting properties (93–96). Loading OV onto tumor-specific T cells (by adhesion to the T-cell surface) can protect the virus from neutralizing antibodies while retaining its antitumor efficacy after release in the tumor microenvironment (96). OV-tumor delivery by CAR-T cells could enhance virus delivery to the tumor and subsequent oncolysis could attract more CAR-T cells, establishing a positive feedback loop.

REMAINING QUESTIONS AND FUTURE DIRECTIONS

With such a variety of oncolytic viruses it is hard to know which one will be best suited for combination with CAR-T cells. In practical terms, it is difficult to envisage a virus commercially developed solely for the combination with CAR-T cells. Therefore, marketed viruses or viruses under clinical investigation are expected to be the first ones to be used in the clinic in combination with CAR-T cells.

While the general value of the virus to attract T-cells to the tumor is widely accepted (53, 97), practical questions on best delivery routes and dosing schedules are more difficult to predict. Intratumoral administration of the OV provides larger amounts of virus in the injected tumors, but it is technically challenging for visceral tumors or metastases, and non-injected tumor lesions will be less likely to get any virus to change the immunosuppressive microenvironment. Systemic intravenous administration is easier to perform and potentially useful to reach all metastases, but efficient neutralization of the virus in the bloodstream, especially with high titers of neutralizing antibodies raised after the first virus administration, will impose a barrier for repeated delivery. The immune response to the virus may also be very different if the virus is injected intratumorally or systemically. Usually vaccination immunization is performed subcutaneously or intramuscularly as the immune system does not respond aggressively to systemic pathogens, partly due to a lower inflammatory response of liver Kupffer cells compared to tissue-resident dendritic cells and the tolerogenic nature of the liver (98). Therefore, the immune response elicited by an OV replicating in a tumor may be tamed or modulated when the virus has been detected systemically. Timing of the virus and CAR-T cells can also impact the outcome. In principle, the virus should go first to change the immune suppressive tumor microenviroment, induce a direct lytic effect on tumor cells, and create a more appropriate environment that attracts the CAR-T cells. Patient preconditioning should also be considered prior to therapy. Although the immunostimulatory environment generated by the virus may bypass the need to lymphodeplete the patient to promote CAR-T cell expansion, lymphodepletion could still be a good approach to foster virus replication and persistence in the tumor while providing an advantage to the co-administered CAR-T cells (4, 99, 100).

Oncolytic viruses offer a strong inflammatory self-amplification oncolytic mechanism of action that can also result in the release of TAA. However, the ability of OV to induce an anti-tumor immune response is not well-understood. Given the large number of viral non-self-peptides after treatment with OV, it is likely that immune responses to the viral epitopes will dominate the response in a mixture with tumor neoantigens

(101–103). New strategies to increase the immunogenicity of tumor epitopes and reduce the immunodominance of viral antigens are needed to promote epitope spreading (104).

Finally, T cells could also be manipulated to become a better partner for oncolytic viruses. Virus-specific T cells have been used as a platform for CAR expression (105). Virus-specific CAR-T cells retain the ability to recognize both virus-infected and tumor targets through their native and chimeric receptors, respectively. Thus, these T-cells could be ideal for a combined treatment with OV, as the presence of the virus could boost the amplification of CAR-T cells in the tumor. A drawback of this approach is that a faster clearance of the OV will occur.

AUTHOR CONTRIBUTIONS

SG and RA conceptualized, wrote, and edited the manuscript.

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REVIEW
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Expanding the Therapeutic Window for CAR T Cell Therapy in Solid Tumors: The Knowns and Unknowns of CAR T Cell Biology

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A major obstacle for chimeric antigen receptor (CAR) T cell therapy in solid tumors is the lack of truly tumor-specific target antigens, which translates to the targeting of tumor-associated antigens (TAAs) overexpressed on tumors but shared with normal organs, raising safety concerns. In addition, expression of TAAs in solid tumors is particularly heterogeneous. In this regard, it is critical to deeply understand the sensitivity of CAR T cells, especially against low-density targets and the possible therapeutic window of antigen density targeted by CAR T cells. In this review, we discuss the recent findings of mechanisms of antigen recognition through CAR, including immunological synapse formation, and the impact of target antigen density for induction of distinct T cell functions. We also discuss rational strategies to adjust and expand the therapeutic window for effective and safe targeting of solid tumors by CAR T cell platforms.

Keywords: T cell biology, chimeric antigen receptors, immune synapse formation, immunotherapy, cancer immunology

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INTRODUCTION

Chimeric antigen receptor T cell (CAR T cell) therapy has shown significant efficacy in hematological malignancies (1–3). Recently the U.S. FDA approved two types of CD19-targeting CAR T cells, tisagenlecleucel (Kymriah™–Novartis) in leukemia (August 2017) and lymphoma (May 2018) and axicabtagene ciloleucel (Yescarta™–Kite) in lymphoma (October 2017). The compelling success of CD19-specific CAR T cell therapies propels the development of CARs that can induce similar efficacy in solid tumors; however, the process is faced with multiple challenges that must be addressed to achieve sufficient efficacy.

Among the many challenges of CAR T cell therapy in solid tumors, a major obstacle is the lack of truly tumor-specific target antigens, which forces cellular immunologists to target tumor-associated antigens (TAAs) overexpressed on tumors but also expressed on normal tissues and organs, raising safety concerns. For instance, fatal cytokine release syndrome (CRS) has been reported from the targeting of human epidermal growth factor receptor 2 (HER2) with CAR T cells due to the recognition of low-levels of HER2 expressed on the normal cells of lung epithelium (4). Also, carbonic anhydrase IX-specific CAR T cells in renal cell cancer induced liver toxicities (5) and carcinoembryonic antigen (CEA)-specific transgenic T cell receptor (TCR) T cells induced

severe colitis in colon cancer patients (6). In addition, the tumor microenvironment (TME) of solid tumors is particularly immunosuppressive, which prevents effective anti-tumor immune responses. The immunosuppressive TME contains multiple components including physical barriers, such as a dense extracellular matrix; dysfunctional epithelial cells; metabolic checkpoints, such as hypoxia and immunological barriers, such as immunosuppressive cytokines/molecules and immunosuppressive immune cells. To target such tumors effectively, multiple factors impacting efficacy and toxicity must be simultaneously addressed.

In this regard, it is critical to deeply understand CAR T cell biology and multiple factors that can affect the therapeutic window of CAR T cell therapies. In this review, we discuss the recent findings of mechanisms of antigen recognition through CARs, including immunological synapse (IS) formation, impact of target antigen density for induction of distinct T cell functions, and the kinetics of target cell killing. We also discuss rational strategies to adjust and expand the therapeutic window for effective and safe targeting of solid tumors by CAR T cell platforms.

TABLE 1 | Comparison of CAR and TCR T cell biological factors.

FACTORS	TCR	CAR
Components	Heterodimer	Single chain (Dimerized)
Costimulation (e.g., CD28, 4-1BB pathways)	Separated (in trans)	Linked (in cis; 2nd and 3rd generation CAR)
Coreceptor involvement	Yes (CD4, CD8, and CD45)	Yes (CD45, unknown for CD4, and CD8)
Target	MHC/peptide complex	Surface antigen*
Typical affinity of receptor	Lower (K_d:10^{-4} M to 10^{-6} M)	Higher (K_d:10^{-6} M to 10^{-9} M)
Required number of Ag to recognize	One	100 or less**
Hierarchical threshold antigen density for T cell functions	Yes	Yes
Immune synapse formation	Yes (Systematic "bull's eye" structure)	Yes (Disorganized)
Time required to form stable and functional immune synapse	Longer (5–10 min.)	Shorter (<2 min.)
Serial killing	Yes	Yes

**Some of CARs have been developed to recognize MHC/peptide complex.*
***Not tested precisely for target with under 100 target molecules.*

BASICS OF CAR T CELL BIOLOGY

While basic mechanisms by which T cells interact with targets through T cell receptors have been intensively investigated, those of CAR-target interactions are less well understood. As CARs consist of combined parts of the TCR complex and antibodies, it will be valuable to discuss the similarities of CARs to endogenous, unmodified TCR T cells and define distinct differences of CARs to better understanding CAR T cell biology (**Table 1**).

The TCR is a heterodimer of two subunits: a TCRα subunit and a TCRβ subunit. Each subunit contains a variable region domain (V) and a constant region domain (C), which is followed by a transmembrane region. Each V domain contains three complementarity-determining regions (CDRs), which interact with peptide presented on the major histocompatibility complex (MHC). The TCR itself does not possess signaling domains, requiring intracellular signaling to be initiated by the CD3 complex. The CD3 complex consists of three dimers, CD3ζε and CD3δε heterodimers and CD3 ζζ homodimer (7). The CD3γ/δ/ε subunits each consist of a single extracellular immunoglobulin (Ig) domain and an immunoreceptor tyrosine-based activation motif (ITAM), whereas CD3ζ has a short extracellular domain (ECD) and three ITAMs (8, 9). TCR and CD3 subunits form a complex on the T cell surface (TCR-CD3 complex).

CARs are synthetic chimeric proteins that are introduced into T cells to redirect antigenic specificity and enhance cellular functionality (10). CARs typically consist of a single-chain variable fragment (scFv) from a mAb, an extracellular spacer region (termed hinge), a transmembrane domain, CD3ζ signaling domain, and usually one or two costimulatory domain(s) for second-generation or third-generation CARs, respectively (11–14). Atypical constructions of CARs utilize receptor ligands or peptides as the extracellular antigen-recognition domain, such as zetakine CARs—e.g., interleukin-13 receptor alpha 2 (IL13Rα2) zetakine CARs (15). CARs endow T cells with the benefit of directly binding surface antigens via scFv (antibody recognition) in an MHC-independent manner, which allows activity from the same CAR molecule in both CD4 and CD8 T cells and reactivity against patient tumors regardless of histocompatibility. CARs can transmit signals through CD3ζ and costimulatory domains simultaneously to the T cell, which can induce a stoichiometric and potentially ideal activation of T cells.

HOW DO CARS TRIGGER IMMUNOLOGICAL SYNAPSE FORMATION AND TRANSMIT SIGNALING?

T cell activation is mediated through highly organized and dynamic interaction of TCRs with MHC-peptide complexes, referred to as an IS. A matured IS is an aggregation of TCR-based signalosomes that induce T cell responses. The IS is defined by three concentric rings of clustered molecules (**Figure 1A**). The inner circle of an IS is termed the central supramolecular activation cluster (cSMAC), where TCR signaling takes place. The cSMAC contains most of the TCR-MHC-peptide complexes, CD28, PKC-θ, and Lck, whereas peripheral SMAC (pSMAC) contains proteins involved in cell adhesion, such as integrin LFA-1, cytoskeletal linker talin, and ICAM1. Large molecules, such as CD43 and CD45, are excluded from the pSMAC and make up the distal SMAC (dSMAC). Inhibitory and costimulatory molecules, such as PD-1, CTLA-4, and ICOS also are aggregated at the region of IS and play crucial roles in the regulation of T cell activation (16).

Secretion of lytic granules occurs within a variant of IS (namely secretory synapse) between cytotoxic T lymphocytes

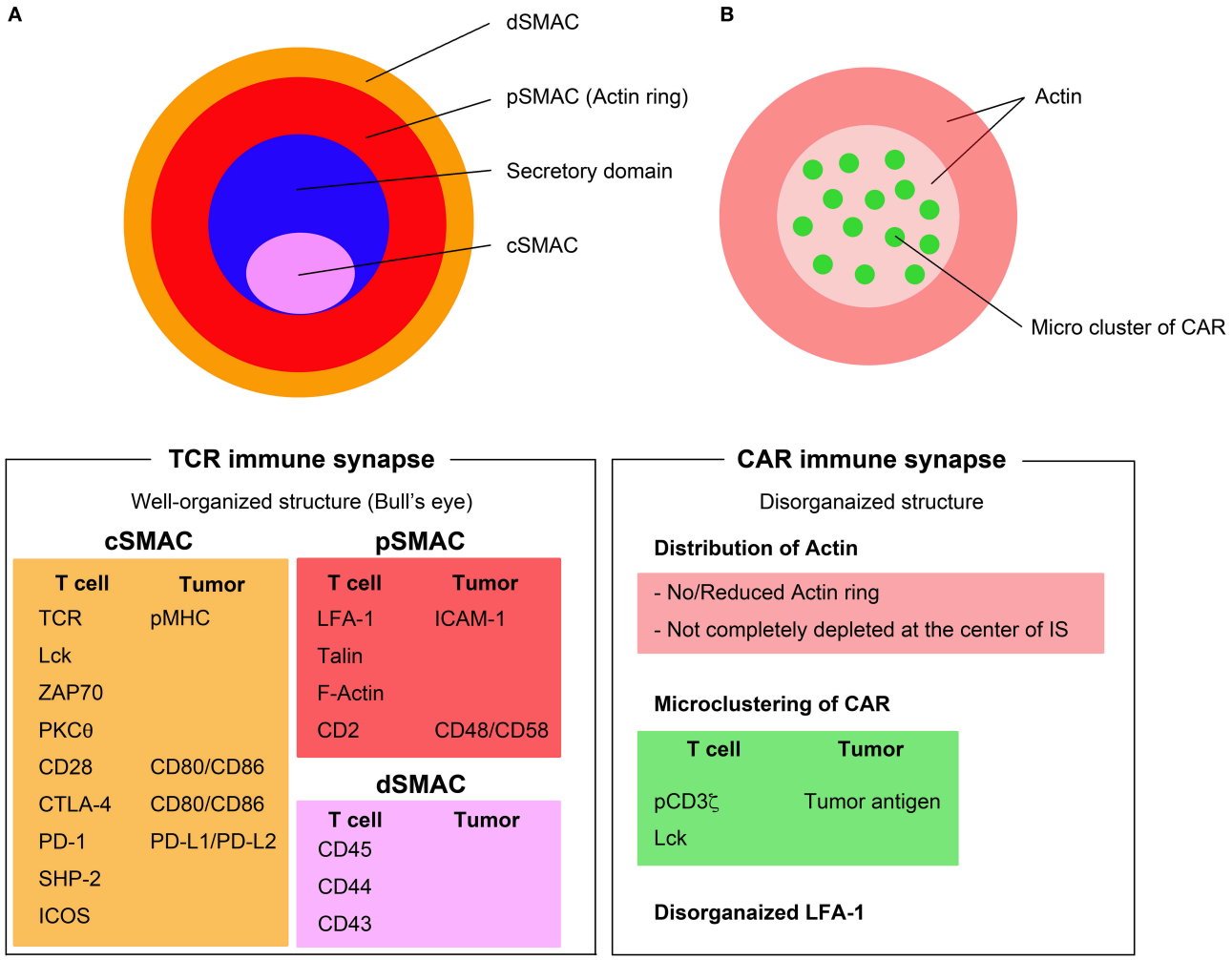

FIGURE 1 | Immunological synapse formation through TCRs and CARs. **(A)** TCR immune synapse shows a well-organized bull's eye structure including the central supramolecular activation complex (cSMAC) (pink), the peripheral SMAC (pSMAC) (red), and distal SMAC (dSMAC) (orange). **(B)** CAR immune synapse (right) displays disorganized structure with no/reduced actin ring and microclusters of CAR/tumor antigen in a disorganized pattern. Major components of the TCR and CAR immune synapse are listed below the figures.

(CTLs) and target cells. The secretary synapse has two separate and distinct domains in cSMAC: one is a signaling domain, which contains the signaling proteins, and another is a secretory domain for exocytosis of cytokines, perforins, and granzymes. Stinchcombe et al. demonstrated that the transient polarization and docking of the centrosome to the plasma membrane, which is controlled by Lck signaling, has an important role in the mechanism of directing this secretion (17–19).

The intracellular signaling downstream of CARs and the mechanisms of the IS formed by CARs have not been extensively studied. It has been demonstrated that CAR clustering, ZAP70 recruitment to IS, and exclusion of CD45 outside of IS occurs between CD19-specific CAR T cells and target cells that is similar to TCR activation. Downstream signaling molecules of the TCR, such as CD3ζ, LAT, Lck, and ZAP70 are phosphorylated after CD19-CAR T cell activation by autologous $CD19^{+}$ B cells (20). In this study, third-generation CAR T cells had a significantly higher phosphorylation status on downstream TCR signaling molecules, and another study demonstrated that third-generation CARs, specifically those incorporating CD28 and 4-1BB costimulatory domains, induced a stronger PI3K/Akt activation when compared to second-generation CAR T cells upon *in vitro* exposure to antigen (21).

The formation of CAR IS has characteristics unlike the structure of TCR IS. The CAR IS does not present a systematic bull's eye structure, which is a characteristic feature of TCR IS. Organization of the actin ring in CAR IS is poor and actin may not be not completely diminished at the center of CAR IS (22). LFA-1 is disorganized and CAR-tumor antigen complexes form microclusters that are randomly distributed at the CAR IS (23) (**Figure 1B**). While TCR IS requires 5–10 min to form the bull's eye structure, the CAR IS might not need to

form these stable structures because the disorganized multifocal pattern of CAR IS is sufficient to rapidly induce significant proximal signaling, which occurs within a short period of time (<2 min). Another important part of IS biology is the delivery of cytotoxic granules, including perforin and granzymes, to the IS mediated by microtubule organizing center (MTOC) (24). The rapid but short duration of proximal signaling of CAR IS also induces rapid MTOC migration to the IS and accelerates the delivery of granules (23). Although the mechanisms of CAR IS have gradually been revealed, it is still unclear whether the differences in CAR IS structure correlate with the efficacy of CAR T cells.

Soluble forms of CAR ligands, such as CD30, mesothelin, and CEA, that exist in monomeric forms cannot trigger CAR signaling (25–27), which is reasonable since they will not induce CAR dimerization. However, CAR T cells can potentially recognize soluble ligands that can exist in oligomeric forms, such as TGF-β, even without cell-cell interaction. Chang et al. recently demonstrated that TGF-β captured by an anti-TGF-β CAR could induce an IS, mimic actin-dependent CAR dimerization, and trigger T cell signaling (28). They also showed that the CAR response to the soluble ligands can be tuned by adjusting the extracellular spacers and the intracellular signaling domains of CARs. These findings reveal mechanisms by which the structures of CARs influence signaling and can also lead to strategies of engineering CAR T cells to overcome tumor immunosuppression by converting TGF-β from a potent immunosuppressive cytokine to a CAR T cell activator.

WHAT IS THE TARGET DENSITY THRESHOLD FOR CAR T CELL RECOGNITION?

It has been demonstrated through fluorescence microscopy that, under optimal conditions, as few as one peptide-MHC complex is sufficient to trigger T-cell activation, IL-2, and TNF-α secretion (29, 30), while a contradictory report suggested that four peptide-MHC complexes are the minimum required amount of agonists for half-maximal activation and calcium flux of $CD4^+$ T cells (31). This high sensitivity of TCR signaling may reflect the unique role of the TCR, which requires the detection of a very rare foreign peptide presented on MHC in the presence of thousands of presented self-peptides. Orchestrated assembly of the receptor complex system may provide such high sensitivity while retaining specificity. The co-receptors CD4 and CD8 also participate in the binding and proximal signaling upon TCR interaction with peptide-MHC. For instance, CD4 acts to reduce the amount of peptide-MHC required from over 30 molecules/target cells to just one molecule (29). Interestingly, TCRs have a hierarchical threshold of antigen density for induction of cell lysis, proliferation, and cytokine production (32), where less antigen density is required for cell lysis than for cytokine production. This phenomenon is observed in the single cell levels but not as a T cell population (33).

To address the question of thresholds for CAR activation, Watanabe et al. investigated the density of CD20 required to activate CD20-specific CAR T cells (CD28 co-stimulation domain) with target cells expressing ∼200–250,000 CD20 molecules per cell (34). Target cells expressing the lowest density of CD20 within the set of the target cells (∼200 molecules/cell) could induce lysis by CAR T cells. This data was consistent with a previous report that CAR targeting a tumor-specific glycoepitope of murine OTS8 that could lyse target cells with similarly low density (∼200 molecules/cell) of target antigen (35). This study also demonstrated that the CAR format is more sensitive than bi-specific T cell engagers (BiTEs) constructed with the same scFv.

Watanabe et al. also demonstrated that the target antigen density that is required to induce T cell proliferation and cytokine production was higher than that required to induce CAR mediated lysis: CD20-specific CAR T cells could lyse target cells with 200 molecules/cell, but cytokine production and T cell proliferation required a higher density of CD20, nearly 5,000 molecules/cell. In addition, recently Walker et al. investigated target antigen density required to activate ALK-specific CAR T cells with the 4-1BB co-stimulatory domain using Nalm6 cells expressing various densities of ALK (∼18,000–450,000 molecules/cell) (36). In this model, production of IFN-γ, IL-2, and TNF-α showed a sharp threshold dependence on tumor antigen density and there was a significantly higher threshold for IL-2 production compared to IFN-γ production. IL-2 production required 60,000 molecules/cell and IFN-γ production required 30,000 molecules/cell to induce a half maximal response. CAR T cells could lyse target cells with the lowest ALK expression in this target cell panel (∼18,000 molecules/cell); however, another panel of target cells with much lower ALK expression will be required to determine the absolute minimal density required for lysis. Liu et al. demonstrated that affinity-tuned anti-HER2 CARs consisting of scFvs derived from high affinity antibodies could degranulate when targeting very low HER2-expressing cells, where the expression was below detection capabilities by flow cytometric analysis (37). Although the number of target molecules was not determined in this study, this result suggests that CARs have a considerably lower threshold of antigen expression for target cell lysis; this threshold may be CAR- or scFv-dependent. There is a one-log difference in the threshold of IFN-γ production between the reports of Watanabe et al. and Walker et al. a discrepancy that might result from differences in the CAR constructs (e.g., affinity of scFv, hinge, co-stimulatory domain) or from the density of CAR expression, two features that could be utilized to more precisely enable control of CAR T cell activation.

These results suggest that CAR T cells can recognize target cells with considerably low levels of target antigen and that they have hierarchical T cell signaling thresholds for cell lysis, proliferation and individual cytokine production. It is likely that each T cell subset or each single T cell has a distinct threshold to be activated, as shown in TCR T cells, but this has not yet been investigated for CAR T cells.

DO CAR T CELLS WORK AS SERIAL KILLERS?

Endogenous T cells and NK cells can sequentially lyse multiple target cells (serial killing), which is likely to be necessary for tumor eradication (38, 39); however, the ability of CAR T cell to mediate serial killing and the kinetics of target cell lysis had not been fully demonstrated until recently. Davenport and colleagues tested the functions of cytotoxic T lymphocytes (CTLs) activated through either the endogenous TCR or an ectopically expressed CAR using a novel transgenic mouse model in which individual T cells co-expressed two antigen receptors (OT-I TCR and anti-HER2 CAR) (40). The authors clearly demonstrated that CAR T cells were serial killers using time-lapse video microscopy; approximately 22% of CAR T cells sequentially delivered a lethal hit to two or three tumor cells and the frequency of serial killing through the CAR was comparable to that through the TCR. Using kinetic analysis of tumor cell killing via a real-time impedance-based assay, the authors showed that CTLs elicited equivalent killing kinetics of target cells regardless of whether recognition occurred through the TCR or CAR for the first 20 h, but CAR-mediated lysis slowed its cytolytic kinetics compared to TCR-mediated lysis after 20 h. This difference in sustained lysis kinetics may be explained by CAR downmodulation after stimulation upon the antigen recognition, which can be ameliorated through TCR-based expression of CAR (41).

HOW DOES CAR AFFINITY AFFECT T CELL FUNCTIONS?

T cell activation is regulated by the interaction between the TCR and MHC-peptide complex and the major factors that have influence on the sensitivity of activation are target antigen density and TCR affinity. One of the major immunosuppressive mechanisms in the cancer microenvironment is failed antigen recognition due to low-affinity TCR and cancer associated peptide-MHC complex interactions (42, 43). TCR affinities to self-derived peptides, such as cancer antigens, are lower than TCR affinities to pathogen-derived antigens (44). Therefore, it is generally more difficult to isolate T cells that have sufficient sensitivity to TAAs than to identify pathogen-derived antigen-specific T cells from patients, and this is the first hurdle of adoptive cell therapies (ACTs) (45).

High TCR affinity is, on the other hand, accompanied by autoimmune responses, which sometimes leads to serious adverse events when patients are treated with ACTs. Although the affinity of TCRs is known to be μM range (K_d:10^{-4}M−10^{-6}M), Zhong et al. reported that T cell antitumor activity and autoimmunity are closely coupled but plateau at a defined TCR affinity of 5–10 μM, which suggests that ACT utilizing supra-physiologic, high-affinity TCRs does not improve efficacy (46, 47).

It has also been reported that a small number of peptide-MHC complexes can achieve a high TCR occupancy since a single complex can serially engage and trigger hundreds of TCRs (48–50). Altogether, this suggests a model where the ideal affinity of TCR should provide interaction sufficiently long enough to transduce proximal signaling but appropriately short to detach and allow as many TCRs to encounter MHC-peptide complexes as possible.

The influence of the scFv affinity on CAR T cell functional response is still incompletely understood. In general, CARs constructed with scFvs possess higher affinity (in the nM range, K_d:10^{-6} M−10^{-9} M) compared to native TCR affinities. Since most TAAs are highly expressed on tumors and at lower levels on normal tissues, it is essential to consider the threshold of the stimulation to yield optimal specificity of CAR-redirected T cell activation since there is a risk that increasing the affinity of CARs will lead to serious adverse effects due to on-target, off-tumor recognition (37, 51). The high affinity of the 4D5 (trastuzumab) scFv may be responsible for the fatal pulmonary toxicity and CRS that was attributed to anti-HER2 CAR reactivity against low level HER2 expression in the normal lung (4). As mentioned before, one strategy to increase the therapeutic index for TAA such as HER2 is affinity tuning of the scFv to generate HER2-specific CAR T cells unable to degranulate in response to normal human primary cells with low level HER2 expression (37).

Similar to native T cells, CAR T cells can also kill multiple target cells in a sequential fashion. However, tumor cells can be eliminated more rapidly when stimulated through CARs than through TCRs because CARs can dissociate from dying tumor cells more rapidly than TCRs (40). Hence, increasing the affinity of CAR T cells may reduce or prevent serial killing, promote T cell exhaustion, and decrease the generation and persistence of central memory and effector phenotype T cells (52), or increase the loss of T cells through activation-induced cell death (53).

SELECTION OF TARGET ANTIGENS FOR SOLID TUMORS

A critical part of adoptive T cell therapy is the selection of the target antigen, in order to deliver sufficient efficacy and minimize toxicity. Some CARs targeting tumor-specific antigens have been developed pre-clinically, including CARs targeting aberrantly glycosylated oncogenes, such as the Tn glycoform of MUC1 (54), and tumor-specific activating forms of integrin (55), and clinically, such as CARs targeting the tumor-specific transcriptional variants EGFRvIII in glioblastoma (56). In the absence of more cancer-specific targets, CAR T cell therapies will most likely continue to target TAAs for solid tumors that also exhibit expression on normal tissues. Indeed, most of ongoing clinical trials of CAR T cell therapies for solid tumors are targeting such TAAs (57).

It is critical to know whether normal tissues express the antigen and its expression levels in order to predict potential toxicities. Several public databases of antigen expression on the normal tissues are available based on gene expression (RNAseq or microarrays) or immunohistochemistry (IHC). However, such technologies contain limitations and pitfalls. For gene expression analysis, antigens expressed by very rare but critical cells may be underestimated. In addition, it may not be possible to distinguish if expressed genes are derived from tissues or from infiltrating

cells. For instance, some databases suggest that human intestine is CD4 positive; however, this expression likely represents infiltrated CD4 T cells rather than intestinal tissues themselves. The accuracy of IHC staining in public databases largely depends on the quality of the antibody, its affinity and the epitope for the antigen. For instance, the cancer-specific Tn glycoform of MUC1 recognized by the high-affinity antibody developed by (58) is extremely rare and unlikely to be identified in public IHC databases. Instead, researchers are more likely discover staining for antibodies developed against the normal glycoform of the antigen; in this case, the broad epithelial expression of normally-glycosylated MUC1 would credential it as an unsafe target for CAR T cells. Similar arguments could be made for the lack of cancer-specific splice variants in public IHC databases, such as EGFRvIII vs. EGFR expression. In addition, false positives and false negatives are problems not yet resolved and the sensitivity of IHC for low-expressing antigens may not be sufficient to select CAR targets for solid tumors. These limitations and pitfalls are well discussed in a previous review (59). New technologies, such as single cell RNA sequencing, may provide more accurate expression profiles that enable researchers to better predict efficacy and toxicity of novel CAR T cells.

STRATEGIES TO EXPAND THERAPEUTIC WINDOW OF CAR T CELL THERAPIES

"Therapeutic window" is a term originally from pharmaceutical toxicology and defined as a range of doses between efficacy and toxicity, achieving the highest therapeutic benefit without resulting in unacceptable toxicity; it is the range between the minimum effective dose (MED) and the maximum tolerated dose (MTD) (**Figure 2A**). Although the pharmacokinetics of engineered replicating cells are largely different from that of drugs, applying the concept of the therapeutic window to the field of ACTs will be valuable for optimizing the therapies. In CAR T cell therapies, targeting antigens expressed exclusively on tumor cells or antigens that are expressed only on non-critical tissues widens the therapeutic window as direct toxicity on vital tissues would not occur (**Figure 2B**). On the other hand, targeting antigens that are expressed in critical normal tissues/cells narrows the window by decreasing MTD (**Figure 2C**).

Determination of the therapeutic window cannot be resolved solely based on the profile of antigen expression. For instance, even in the case of a large differential in antigen expression by tumors and normal tissues, where the antigen is expressed at higher density in tumors, tumors may still be more resistant to CAR T cells than normal tissue due to inherent immunosuppression within the TME that does not exist within normal tissue. In this case, inhibition of T cell infiltration or induction of T cell hypofunction by the tumor would narrow the therapeutic window of CAR T cells by increasing the MED.

Given that truly tumor-specific target surface antigens have as of yet been rarely found, TAAs with shared expression of normal organs may be our only reasonable targets for the foreseeable future; therefore, strategies to expand the therapeutic window of CAR T cell therapy are necessary for the treatment of solid tumors. The possible approaches to expand therapeutic window include: (1) optimizing CAR affinity and sensing, (2) optimizing immunological synapse formation, (3) combination therapies, (4) local delivery of CAR T cells and therapeutic agents, (5) induction of target antigen expression, or (6) other modifications (**Figure 2D**).

OPTIMIZING CAR DENSITY, AFFINITY AND SENSING

Although increasing CAR affinity enables recognition of antigens independent of target density (60), that action may cause serious adverse effects, namely on-target, off-tumor toxicity, and reduce the capability of sequential target tumor killing. Therefore, it is important to address rational strategies to determine ideal CAR affinity. The construction of affinity-tuned scFvs using light-chain exchange technology is one of the most feasible methods to measure the optimal affinities of CARs. With this method, Drent et al. identified that CD38-CAR T cells with ∼1000-fold lower affinity to the original antibody that exhibited optimal proliferation, cytokine production, and cytotoxicity *in vitro* and *in vivo*, but spared normal tissue, compared with high-affinity CAR T cells (61).

Increasing the affinity of scFvs beyond a defined threshold ($K_d < 10^{-8}$ M) does not necessarily induce improved T cell activation (37, 62, 63). For instance, μM affinity CAR T cells exhibited superior cytokine production, expansion and antitumor efficacy, and less systemic off-tumor toxicity compared to nM affinity CAR T cells (64). These reports suggest that CAR T cells with affinities above a defined threshold are not necessarily required, or rather it may be important to generate CARs with varied affinities for the same epitope, and to identify the lowest affinity at which those epitope-specific CAR T cells can exhibit maximal cytolytic, proliferative, and safety potential.

Apart from changing scFv affinity, regulating the level of surface CAR expression is an important factor to induce ideal CAR signaling. CAR T cell function is governed by CAR density as well as target antigen density, where low expression of either can result in limited functionality and sensitivity of CAR T cells (36). On the other hand, continuous signaling (tonic signaling) through CAR can occur depending on the CAR structure and high CAR density, which can induce inferior antitumor effects and T cell engraftment *in vivo* by increasing T cell differentiation, exhaustion and activation induced cell death (AICD) (65, 66). Modifying CAR density while maintaining expression under the threshold required for tonic signaling induction will be required to induce sufficient anti-tumor efficacy and to keep safety potential for each target antigen and CAR construct. Another approach is combinatorial antigen recognition through two different antigens on tumor cells. Split-signaling CAR T cells have been engineered such that CAR-1 drives only the activation signal (signal 1) of CD3ζ and CAR-2 drives only co-stimulation (signal 2) through co-stimulatory molecules, such as CD28 and 4-1BB. Thereby, CAR T cells can be optimally activated only upon recognition of two separate required antigens simultaneously ("AND" logic gated CAR). This approach has been tested in

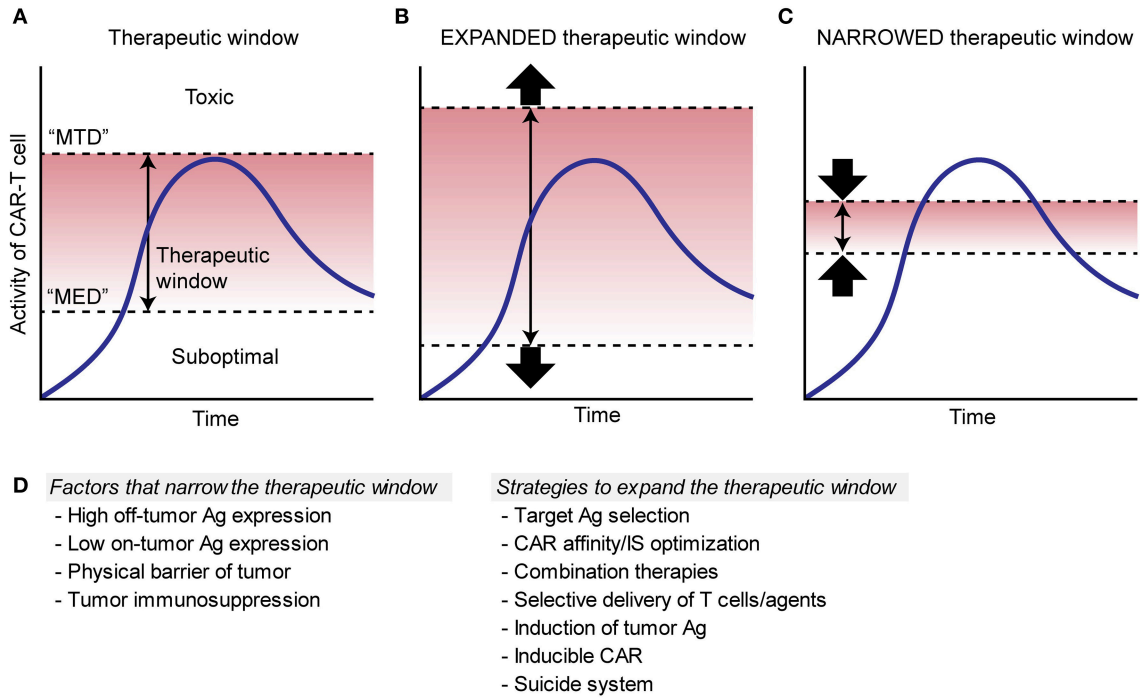

FIGURE 2 | Schema to illustrate therapeutic window of CAR T cell therapies. **(A)**. Therapeutic window (red area) is determined as a range between the minimum effective dose ("MED") and the maximum tolerated dose ("MTD"), where the therapy can achieve the highest therapeutic benefit without resulting in unacceptable toxicity. Blue lines show the kinetics of CAR T cells. **(B)**. Red area shows an expanded therapeutic window with decreased "MED" and/or increased "MTD," which increase a chance for the CAR T cells to induce sufficient efficacy without toxicity. **(C)**. Red area shows a narrowed therapeutic window with increased "MED" and/or decreased "MTD," which can cause toxicity and/or suboptimal efficacy. **(D)**. Factors that can narrow the therapeutic window and possible strategies to expand the window are listed.

CAR T cells with split signals utilizing anti-HER2 and anti-MUC1 CARs (67) or anti-CD19 and anti-PSMA CARs (68) in pre-clinical models. Conversely, CAR T cells can be modified so that CARs can drive full signaling upon recognizing either of two different antigens by expressing two CARs or a single CAR with tandem antigen binding domains ("OR" logic gated CAR) (69–71). Engineering T cells with "AND" logic CARs enables more specific and safer targeting and those with "OR" logic CARs potentially overcomes low target antigen expression and tumor escape by target antigen loss.

The use of adapter molecules to develop a "universal" CAR can be another attractive platform to overcome tumor heterogeneity in antigen expression and to make CAR T cell activities more conditional. Urbanska et al. described a biotin-binding immune receptor composed of an extracellular-modified avidin linked to an intracellular T cell signaling domain which can recognize tumor cells pre-treated with antigen-specific molecules such as mAb, scFv, or other tumor-specific ligands (72). Tamada et al. similarly described anti-FITC CAR which can target those pre-treated with FITC conjugated mAbs, and they demonstrated that anti-FITC CAR T cell activity can be attenuated by injecting FITC-labeled non-specific IgG Ab in a preclinical model (73). This platform enables flexible multiple tumor antigen targeting, which potentially prevents target antigen loss and ease off-tumor toxicity by dividing off-tumor toxicities. This system also enables control of CAR T cell activities by adjusting doses of adopter molecules, or more actively, by quenching CAR by adding the excess amount of non-specific tagged molecules. However, it is still unclear whether each adaptor will induce equal activity when ligated to the acceptor CAR molecule. For instance, it has been revealed that the length and composition of the CAR hinge influences the activity of the T cells and the epitope of the antibody also influences this (whether it is distal or proximal to the target cell membrane) (74–76). It will be needed to address these factors so that they each provide an optimal CAR signal. Another potential problem of this platform to translate to the clinic will be the immunogenicity of the adapter molecules.

OPTIMIZING IMMUNOLOGICAL SYNAPSE FORMATION

As discussed above, the CAR IS is more disorganized when compared to the well-organized bull's eye structure of TCR IS and is characterized by a multifocal pattern of Lck arrangement, decreased actin rings, and diffuse LFA-1 distribution. The remarkable capabilities of CAR IS are virtually instant induction of proximal signaling and rapid delivery of cytotoxic granules mediated by a faster migration of MTOC to the CAR IS compared

to the TCR IS. These superiorities enable CAR T cells to dissociate quickly from destructed tumor cells and to mediate efficient serial killing.

Recently, several studies have reported important findings on how CAR design affects IS formation. Xiong et al. examined the quality of the CAR IS using CD19-specific CAR constructed with either CD28 or 4-1BB co-stimulatory domains and determined that CD28 plus 4-1BB-based third generation CARs are superior to CD28 based second generation CARs, as measured by IS structure, signaling and function (22). In comparison to bi-specific CAR T cells, CARs specific for two glioma-associated antigens, HER2 and IL13Rα2, exhibited significantly higher F-actin accumulation and increased polarization of the MTOC.

The structural characteristics of CAR IS are now in the beginning of elucidation. Although further studies are essential to reveal the correlation between CAR IS structure and the anti-tumor efficacy, modulation of CAR IS would be a great option of CAR T cell therapy if it is possible to increase the efficacy. There have been several attempts to improve the efficacy of CAR T cells by modifying CAR IS through immunomodulatory drugs (IMiDS), such as lenalidomide—a synthetic derivative of thalidomide. Lenalidomide improves CAR efficacy by increasing actin accumulation at the IS and is a promising combinatorial treatment for enhanced CAR activity (77, 78).

COMBINATION THERAPY

Combinatorial approaches may serve as a promising strategy to drive CAR T cell therapy toward solid tumors by overcoming tumor heterogeneity and expanding the therapeutic window. Oncolytic viruses (OVs) are promising agents for the treatment of solid tumors, and an oncolytic herpes virus expressing GM-CSF has been FDA-approved for the therapy of advanced melanoma based on therapeutic benefit in a clinical study (79). OVs can be programmed to specifically target, replicate in, and lyse cancer cells, while sparing normal cells. The release of virus progeny results in an exponential increase of the virus inoculum, which can cause direct tumor lysis while providing danger signals necessary to awaken the immune system (80). Furthermore, OVs can be genetically modified to express therapeutic transgenes selectively in the TME. Their ability to revert tumor immunosuppression while locally expressing therapeutic transgenes provides a rational strategy for combination with CAR T cell therapies. Indeed, we and other researchers reported enhanced CAR T cell efficacy by combining OVs expressing either cytokines (81), chemokines (82), an anti-PD-L1 minibody (83), a BiTE (84), or the combination of them against solid tumors in pre-clinical mouse models. We have shown that an oncolytic adenovirus expressing IL-2 and TNF-α enhanced the efficacy of mesothelin-redirected CAR T cells, which was associated with enhanced T cell infiltration to the tumor bed and reduced metastases (81). Murine TNF-α and murine IL-2 delivered by adenovirus could increase the efficacy of mesothelin-redirected CAR T cells in immunocompetent mice engrafted with highly immunosuppressive syngeneic LSL-$Kras^{G12D/+}$;LSL-$Trp53^{R172H/+}$;Pdx-1-Cre (KPC) mice derived-pancreatic ductal adenocarcinoma (PDA) tumor, whereas multiple injections of anti-mesothelin-CAR T cell monotherapy failed to suppress tumor growth. This combination approach enhanced the efficacy of CAR T cells and did not induce off-tumor toxicity.

Other combinatorial approaches include combination with agonistic antibodies specific for the 4-1BB costimulatory receptor (85), which can directly activate CAR T cells and also can reduce host immunosuppressive immune cells, such as Tregs or MDSCs.

LOCAL DELIVERY OF CAR T CELLS AND THERAPEUTIC AGENTS

Although efficient trafficking of CAR T cells to cerebrospinal fluid in patients with central nervous system (CNS) involved acute lymphoblastic leukemia has been reported (1), the response of primary or metastatic solid tumors in the CNS may be limited by the accessibility of CAR T cells. On the other hand, enhancing the strength of systemically administrated CAR T cells can raise safety concerns, as reported in HER2-redirected CAR T cell therapy. Direct administration of CAR T cells into the tumor bed is an optional route of drug delivery. Priceman et al. demonstrated that intraventricular delivery of HER2-CAR T cells shows antitumor activity against brain-metastatic breast cancer in orthotopic xenograft models, whereas intravenous delivery of HER2-CAR T cells achieved only partial antitumor responses in mice even at 10-fold higher doses compared with local or regional delivery to the brain (86). In confirmation of this administration route, intraventricular administration of IL13Rα2-targeting CAR T cells induced regression of all intracranial and spinal tumors in a patient with recurrent multifocal glioblastoma (87). We have tested intratumoral administration of mRNA-transduced anti-c-Met CAR T cells in patients with metastatic breast cancer (88) in a clinical trial and confirmed feasibility of this approach for clinical use.

Another approach is to engineer CAR-T cells to work only or dominantly in the tumor site. Han et al. developed the "masked CAR" system, which consists of a masking peptide that blocks the antigen-binding site and a protease-sensitive linker. The authors demonstrated that proteases commonly active in the TME (and presumably inactive in normal tissue) can cleave the linker and disengage the masking peptide, which enables CAR T cells to recognize target antigens only at the tumor site (89).

To overcome the immunosuppressive TME, local delivery of cytokines and chemokines, such as IL-18, IL-12, and the combination of CCL19 and IL-7 (90–92) or checkpoint blocking agents (93) by CAR T cells within the TME may help to overcome impediments to T cell infiltration and functionality. These approaches demonstrate enhanced therapeutic efficacy, while avoiding systemic adverse events in pre-clinical models.

INDUCTION OF TARGET ANTIGEN EXPRESSION

As discussed here, target antigen density can govern the efficacy of CAR T cell therapy. In addition, the loss or down-regulation of target antigen is a major cause of tumor escape (94). Induction

or re-induction of antigen expression on target cells may be an attractive approach to expand the therapeutic window. It has been reported that a sublethal dose of radiation can induce the expression of TAAs, such as mesothelin and CEA, on tumor cells (95). Epigenetic control may also modulate target antigen expression; in that vein, an anti-methylation drug, azacytidine (5-AZA), can re-induce CD20 expression on lymphoma cells after treatment, including after treatment with CD20-targeting mAb rituximab (96).

OTHER MODIFICATIONS

Equipping CAR T cells with a suicide system, such as inducible caspase-9 (iCas9) (97, 98) or co-expression of truncated EGFR (99), will enhance the safety of CAR T cells. These systems can induce depletion of CAR T cells by administrating agents that trigger cell-intrinsic apoptosis or cell-extrinsic antibody-mediated depletion of the therapeutic cells. Transfection of T cells with mRNA encoding CAR enables transient expression of CAR (100) and is a technology that is suitable for early phase clinical trials if new antigens are targeted and dose-limiting toxicity may be predicted. Another approach for remote-controlled safety is an inducible CAR system, including a TET-inducible system (101), which enables drug-inducible control of CAR expression.

Lastly, the synthetic Notch (synNotch) system (102, 103) is another attractive platform for diverse and flexible modification of CAR T cells. SynNotch receptors can allow the addition of custom response programs to T cells upon antigen recognition. For instance, synNotch can drive tailored cytokine secretion, biased T cell differentiation, or local delivery of therapeutic payloads, such as antibodies, upon the recognition of the antigen. In addition, synNotch can be utilized to develop sophisticated antigen recognition by CAR T cells based on the Boolean "AND" logic gating.

CONCLUSIONS

The treatment of solid tumors by CAR T cells is complex and multifactorial with a narrower therapeutic window than the targeting of CD19 for the treatment of B cell leukemia and non-Hodgkin lymphoma. Despite a growing list of clinical studies, remarkable responses have been rarely achieved with the exception of a case in glioblastoma with intraventricular delivery of the IL13Rα2 CAR. In this setting, establishment of strategies to expand the therapeutic window is critical. As outlined here, there are several promising approaches to achieve this in the preclinical setting and some of them are currently under investigation in clinical trials. The results of these and future clinical trials will elucidate a more refined path forward for solid tumor treatments.

There remain a lot of unknowns on tumor biology, the TME and CAR T cell biology. Fortunately, powerful tools to address these questions, such as emerging technologies in bioinformatics, mass spectrometry proteomics, mass cytometry, and single cell RNA sequencing, will allow us to access highly multiplexed and precise information on tumors, components of TME and immune cells. Moreover, maturation of technologies in gene-editing, such as the CRISPR/Cas9 system or synthetic biology, such as the synNotch system, will enable flexible design and engineering of T cells. Combining these technologies may lead to breakthroughs for CAR T cell therapies for the treatment of solid tumors.

Finally, several cases with unexpected severe toxicities have been reported when new CAR T cell therapies were first administrated to patients. Unfortunately, current technologies do not allow us to predict all the toxicities in the clinical setting; thus, only clinical trials can currently reveal information on safety and efficacy profiles of adoptive immunotherapies. Continuous development and refinement of preclinical models that can predict toxicity as well as the careful and rational planning and implementation of clinical trials will be crucial for further development of CAR T cell therapies.

AUTHOR CONTRIBUTIONS

KW and SK conceptualized, wrote, and edited the manuscript. ADP and CHJ provided feedback and edited the manuscript.

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REVIEW
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Engineering CAR-T Cells for Improved Function Against Solid Tumors

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Genetic engineering T cells to create clinically applied chimeric antigen receptor (CAR) T cells has led to improved patient outcomes for some forms of hematopoietic malignancies. While this has inspired the biomedical community to develop similar strategies to treat solid tumor patients, challenges such as the immunosuppressive character of the tumor microenvironment, CAR-T cell persistence and trafficking to the tumor seem to limit CAR-T cell efficacy in solid cancers. This review provides an overview of mechanisms that tumors exploit to evade eradication by CAR-T cells as well as emerging approaches that incorporate genetic engineering technologies to improve CAR-T cell activity against solid tumors.

Keywords: chimeric antigen receptor, tumor, cancer, genetic engineering, immunosuppression

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INTRODUCTION

Reconstitution of effective immune function is a major goal of immunotherapies. In the context of cancer, including solid tumors, the complex interaction of various immune cell sub-populations may have to be re-established to obtain adequate tumor control or eradication. Here, the normal functions of T cells to either regulate immune responses or directly kill infected or cancer cells can be exploited and improved by genetic modification. One of the currently most intensely explored methods to enhance T cell function with the aim to improve cancer patient treatment is the introduction of chimeric antigen receptors (CARs) to generate CAR-T cells with greater anti-tumor activity. CARs are synthetic receptors that contain an antigen recognition domain, e.g., a single chain variable fragment (scFv) that binds to a tumor-associated antigen, a hinge region to provide flexibility to the scFv, a transmembrane domain and a signaling domain with or without co-stimulatory domains that activate the cytotoxic functions of the CAR-T cells upon antigen recognition. For example, most CAR constructs use the CD3ζ signaling chain to stimulate cytotoxic CAR-T activity, which mimics the natural biologic T cell activation pathway, with activation of down-stream signal transduction proteins such as ZAP70, NFAT, and PI3K-AKT-mTOR (**Figures 1**, **2**) (1, 2). Analogous to CD3ζ activation following engagement of the T cell receptor (TCR) in non-modified T cells, activation of CD3ζ signaling in CAR-T cells results in production of cytotoxic cytokines (e.g., IFNγ, TNFα) as well as cytokines to recruit and activate additional immune T cells (e.g., IL-2, IL-10, IL-17) (3–6). In addition to TCR engagement, efficient T cell killing requires simultaneous signaling through a co-stimulatory protein. For example, cross-linking of CD28 or the tumor necrosis factor receptor (TNFR) family members ICOS or 4-1BB results in costimulatory signaling in T cells. Therefore, domains of these natural co-stimulatory proteins are also incorporated in many CAR-T cell designs.

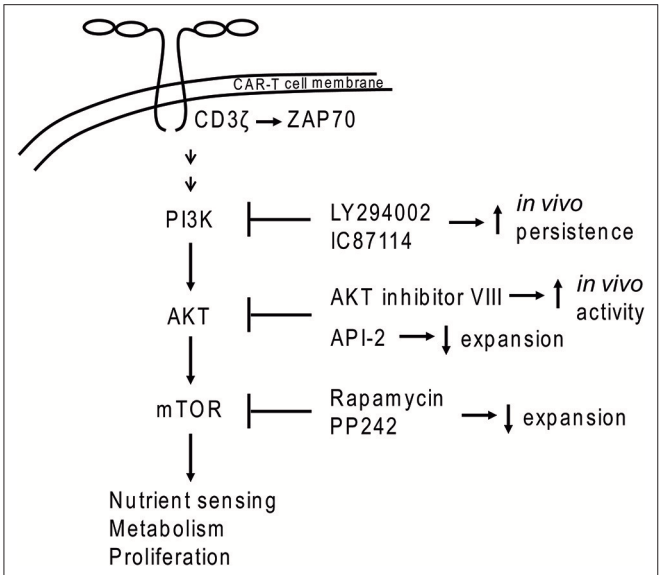

FIGURE 1 | PI3K-AKT-mTOR signaling in CAR-T cells. Dimerization of CAR molecules mimics T cell receptor activation of the PI3K signal transduction cascade. Application of some inhibitors (e.g., PI3K inhibitors LY294002 and IC87114 or the AKT inhibitor VIII) during *ex vivo* expansion led to increased *in vivo* persistence of CAR-T cells.

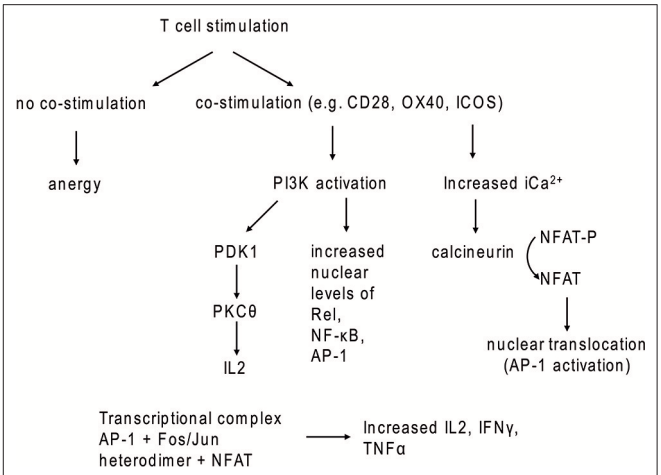

FIGURE 2 | T cell stimulation with and without co-stimulatory signaling. In the absence of co-stimulatory molecules, TCR stimulation leads to anergy.

Application of CAR-T cells in some blood malignancies has generated unprecedented responses in B-cell neoplasms, including leukemia and multiple myeloma (7–20). As a result, many resources world-wide are devoted to the development of CAR-T cells to recognize additional tumor-associated antigens or neoantigens to extend this success to treatment of additional cancers, including solid tumors. Engineering approaches to increase CAR-T cells anti-tumor activity, including T cell infiltration into solid tumors, T cell persistence, recruitment/activation of additional anti-tumor immune cells, can exploit mechanisms tumors employ to create an immunosuppressive niche. As discussed below, tumors secrete cytokines to recruit various tumor-associated cells, which, in turn, secrete anti-inflammatory cytokines and/or express ligands for immune checkpoint receptors, which can block CAR-T cells from infiltrating the tumor as well as cause CAR-T cell exhaustion, thus leading to a general decrease in the anti-tumor activity of T and CAR-T cells. This review provides an overview of pro-tumor cell activities in the tumor microenvironment and explores some of the strategies that may help to increase CAR-T cell persistence and functionality with the aim for improved activity against cancer.

TUMOR MICROENVIRONMENT CHALLENGES TO CAR-T CELL FUNCTION

Tumor cells shape the tumor microenvironment via production and secretion of cytokines that can inhibit T cell function directly or indirectly by recruitment of immunosuppressive cell types (21). Challenges of the tumor microenvironment to T and CAR-T cell activity include hypoxia, metabolic reprogramming conditions, and immunosuppressive signaling through cell checkpoint receptors, all of which serve to protect tumor cells from elimination. As a means of protection of "self," T cells express inhibitory receptors as a concept called checkpoint inhibition. The most widely studied immune checkpoint receptor-ligand interactions are the programmed cell death 1 (PD1)/programmed cell death ligand 1/2 (PD-L1/2), cytotoxic T-lymphocyte antigen 4 (CTLA4)/CD80/CD86, T-cell immunoglobin and mucin domain 3 (TIM-3)/Galectin-9 and phosphatidylserine on surface of apoptotic cells, and lymphocyte-activated gene-3 (LAG-3) / LSECtin (22, 23). Tumors exploit these immune tolerance signaling pathways to induce T and CAR-T cell exhaustion, which is exhibited by loss of proliferative capacity and decreased production of cytokines such as IL-2, TNF-α, and IFN-γ. Furthermore, exhausted T cells express elevated levels of inhibitory receptors, including PD1, CTLA-4, TIM-3, and LAG-3 and higher expression of these receptors was associated with more advanced disease stage in cutaneous T-cell lymphoma patients (24, 25). TIM-3 expression on tumor infiltrating T cells was predictive for poor outcome in renal cell carcinoma patients (26). In addition to T cells, expression of TIM-3, LAG-3, PD1, and PD-L1 was recently demonstrated on B cells, macrophages, natural killer cells, and dendritic cells in effusions obtained from mesothelioma patients (27). While this study evaluated samples from only a small number of patients ($n = 6$), the observation of exhaustion markers on additional immune cells that interact with T cells in order to orchestrate optimal anti-tumor activity may have important implications for control of solid tumors by CAR-T cells.

Several different cell types (e.g., cancer-associated fibroblasts, regulatory T cells, myeloid-derived suppressor cells, and tumor-associated macrophages) comprise the tumor microenvironment and can inhibit T and CAR-T cell function through distinct and overlapping mechanisms (21, 28–32).

Cancer-associated fibroblasts (CAFs) are a major type of stromal cells that occupy the solid tumor microenvironment (33,

34). Activation of fibroblasts by transforming growth factor-β (TGF-β), CXC chemokine ligand 12/stromal cell-derived factor-1 (CXCL12/SDF-1) and IL-6 is common in solid tumors. In contrast to fibroblasts in healthy tissues, CAFs tend to stay in the activated state, through which they may promote tumor metastasis by remodeling the extracellular matrix (ECM) via secretion of matrix metalloproteases (MMP) 2 and 9, which cleave ECM proteins (**Figure 3**) (28). Tumor microenvironments often contain the chemokine CXCL12 and this was shown to be secreted by CAFs in a murine model of pancreatic ductal adenocarcinoma (30). CAFs were also shown to produce CXCL12 in human breast carcinomas and non-small lung cancer (35, 36). Of clinical interest, CXCL12/CXCR4 levels are increased in many cancers, including breast cancer, pancreatic cancer, oral squamous cell carcinoma, ovarian cancer, cervical carcinoma, and gastric cancer (37–45). CXCL12 may serve to prevent adequate T and CAR-T cell penetration into or recognition of the tumor by forming a barrier of $CXCR4^+$ immunosuppressive cells.

Regulatory T (Treg) cells are important for self-tolerance but also contribute to the immune privileged tumor niche by suppression of effector T cell activity. Tregs were shown to home to the bone marrow via CXCL12/CXCR4 signaling in prostate cancer patients with bone metastases (29). The authors postulate that Tregs help create an immunosuppressive niche to aid formation of bone metastases. In an orthotopic mouse model of head and neck squamous cell carcinoma, increased TIM-3 expression was observed on $CD8^+$T cells and Tregs after radiation therapy and PD-L1 inhibition (46). Addition of anti-TIM-3 antibodies to the treatment strategy resulted in increased T cell cytotoxicity and improved survival, but the tumors still relapsed. Depletion of Tregs with an anti-CD25 antibody finally led to rejection of established tumors, presumably by restoration of anti-tumor immunity (46).

Myeloid-derived suppressor cells (MDSCs) inhibit anti-tumor immune function by stimulating the activity of immunosuppressive Tregs, producing reactive oxygen species (ROS) and by secreting anti-inflammatory cytokines like IL-10 and TGF-β. CAR-T cells engineered to express catalase maintained anti-tumor activity in the presence of high H_2O_2 levels and also protected NK cell activity from oxidative stress (31). Blockade of immunosuppressive TGF-β signaling via expression of a dominant negative TGF-β receptor II in CAR-T cells improved CAR-T cell proliferation, cytokine secretion, *in vivo* persistence and tumor control in mouse models of human pancreatic cancer (47). MDSC also express PD-L1, which can cause T and CAR-T cell exhaustion by binding to PD1. Expression of indoleamine-2, 3-dioxygenase (IDO) by MDSCs can also lead to T and CAR-T cell anergy (32). In a murine tumor model, the immune suppressive function of MDSCs was determined to be a result of metabolic reprogramming, where MDSCs that had the highest suppressive activity also had higher glycolysis levels (48). Inhibition of mTOR with rapamycin led to decreased tumor growth via lower glycolytic and suppressive activity of MDSCs. Similarly to CAFs, MDSCs may also be important for tumor metastasis by remodeling the extracellular matrix via production of MMP9 (49).

Tumor-associated macrophages (TAMs) are another type of immune suppressor cells that inhibit T and CAR-T cell function in the tumor niche. Lactate generated by tumor cells as a by-product of aerobic glycolysis can cause polarization of cytotoxic and inflammatory M1 macrophages to TAMs, which are immunosuppressive macrophages that exhibit an M2 phenotype (50). TAMs can recruit Tregs to the tumor microenvironment via secretion of chemokines (e.g., CCL17, CCL18, CCL22) or even induce Treg suppressor activity by secretion of prostaglandin E2 and IL-10 (21). In addition to production and secretion of factors to recruit and stimulate Treg activity, TAMs can also contribute to protection of tumors from eradication by T and CAR-T cells through expression of PD1 ligands PD-L1/PD-L2 or expression of TIM-3 (51, 52). TAM production of nitric oxide was also shown to contribute to resistance against cisplatin, which is commonly used to treat solid tumors (53). Furthermore, resident macrophages were shown to be important mediators of cytokine release syndrome, a sometimes fatal adverse event that limits CAR-T cell therapy, via production of IL-6, IL-1 and nitric oxide (54).

In summary, the immunosuppressive activity of these various tumor-associated cells negatively impact CAR-T cell persistence, penetration into the tumor and overall anti-tumor activity. Possible strategies to overcome these obstacles will be explored in the following paragraphs.

CHOICE OF T CELL POPULATION FOR IMPROVED CAR-T CELL FUNCTION

In vivo persistence of CAR-T cells may be a result of the T cell population(s) selected for CAR modification as well as *ex vivo* CAR-T cell expansion procedures. T cells can be divided into several subsets, including naïve T cells (T_N) ($CD45RA^+CCR7^+$), central memory T cells (T_{CM}) ($CD45RA^-CCR7^+$), effector memory T cells (T_{EM}) ($CD45RA^-CCR7^-$), effector T cells (T_{EFF}) ($CD45RA^+$, $CD45RO^-$ $CCR7^-$ $CCR7^-CD57^+$) and stem cell memory T cells (T_{SCM}) ($CD62L^+CCR7^+CD45RA^+CD45RO^-CD95^+$) (55, 56). Increased anti-tumor activity might be achieved if the optimal T cell populations can be identified to generate CAR-T cells and may even allow decreased CAR-T cell doses for each treatment (57). T cell populations currently used for generation of clinical CAR-T cells often include unselected PBMC. However, high inter-patient variation of T cell function and maturation may contribute to the variable success rates in some settings. Attempts to standardize CAR-T cell production include isolation of T cell subpopulations, such as $CD4^+$, $CD8^+$, $CD62L^+$ (to have high numbers of naïve and T_{CM}) (58). Of interest, $CD8^+$ T_{SCM} were recently used to produce clinical grade CD19-specific CAR-T cells to be tested in a phase 1 trial in patients with B-cell malignancies (59). Another recent study achieved balanced CD4/CD8 ratios with 50% T_{CM} and 46% T_{SCM} from small amounts of blood (60).

It was recently demonstrated that lack of CAR-T cell *in vivo* persistence was due to a lower percentage of naïve T cells (T_N) vs. effector memory T cells (T_{EM}) prior to *in vivo*

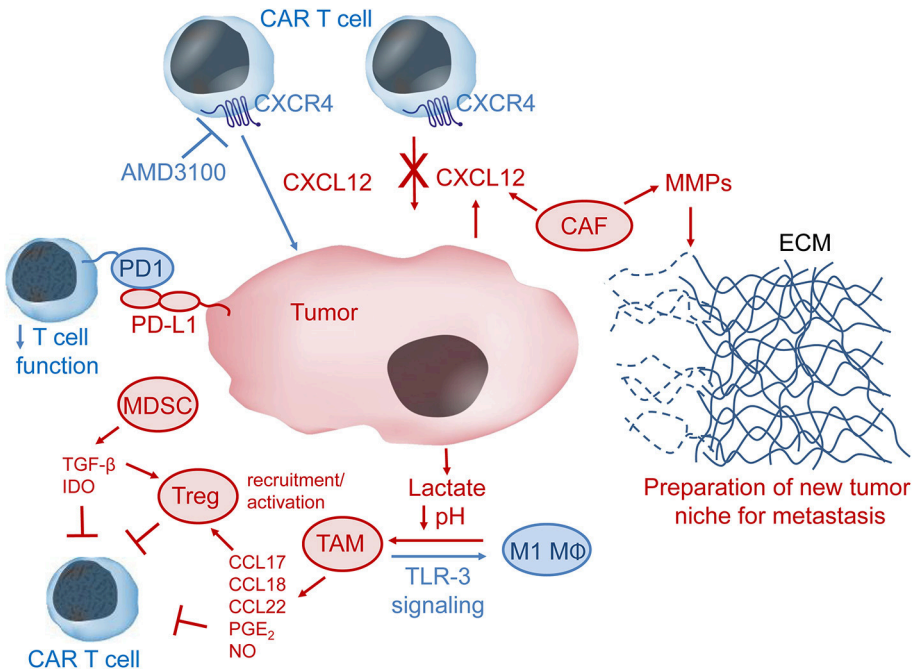

FIGURE 3 | The immunosuppressive tumor microenvironment (TME). CXCL12 in the TME may recruit CXCR4-expressing immunosuppressive cells such as tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), cancer-associated fibroblasts (CAF), and regulatory T cells (Treg) to the tumor niche. CAF can secrete metallomatrix proteins (MMPs) that lead to remodeling of the extracellular matrix (ECM) via degradation of ECM proteins such as collagen, elastin, fibronectin and laminin. ECM remodeling may be important for tumor invasion and metastasis. Lactate produced by tumor cells leads to lower pH in the TME and can facilitate polarization of M1 macrophages (MΦ) to immunosuppressive TAM, which produce several chemokines and other factors, such as prostaglandin E2 (PGE_2) and nitric oxide (NO), that inhibit CAR-T cells either directly or via activation of Treg cells. Stimulation of toll-like receptor 3 (TLR-3) signaling may convert TAM to pro-inflammatory M1 MΦ. MDSC produce and secrete the immunomodulatory factors transforming growth factor β (TGF-β) and indoleamine 2,3-dioxygenase (IDO), which inhibit CAR-T cell anti-tumor activity. Immune checkpoint inhibition, e.g., via interaction of programmed cell death 1 (PD1) on T cells with programmed cell death ligand 1 (PD-L1) on tumor or tumor-associated cells, results in down-regulation of T cell activity. Pharmacologic inhibition of the CXCL12 receptor CXCR4 might help CAR-T cells overcome the CXCL12 tumor barrier and thus increase CAR-T cell anti-tumor activity.

delivery as the proportions of the T cell subpopulations became skewed during *ex vivo* cultivation and expansion (61). It may be important to standardize T cell isolation, *ex vivo* cultivation and expansion in order to achieve robust comparability among studies.

Earlier work showed that the T_{EM} population can be supported by inhibition of the PI3K/AKT/mTOR pathway (62, 63). PI3K inhibition by LY294002 or IC87114 during *ex vivo* expansion improved CAR-T cell *in vivo* persistence without impacting CAR-T cell yield (61). The same study demonstrated that use of inhibitors against AKT (API-2), mTOR (rapamycin, PP242 = Torkinib) or glycolysis (DCA = dichloroacetate) led to decreased numbers of CAR-T cells. In contrast, another study demonstrated that use of AKT inhibitors (Akti-1/2 or AKT inhibitor VIII) during *ex vivo* expansion generated CAR-T cells with greater activity in a $CD19^+$ mouse tumor model without negatively impacting CAR-T cell expansion (64). Differences between these two studies may be due to the distinct properties of the AKT inhibitors employed. Although these concepts were largely developed in the setting of hematopoietic malignancies, they could also impact the effectiveness of CAR-T cell strategies to treat solid tumors.

COMBINATION THERAPIES TO AUGMENT CAR-T CELL ANTI-TUMOR ACTIVITY

One interesting strategy to overcome immune suppression and generate a more robust antitumor immune response is to combine cancer cell specific CAR constructs and monoclonal antibodies that disrupt checkpoint inhibition (e.g., anti-PD1, anti-PD-L1, anti-TIM-3, anti-LAG-3 antibodies) (65). Currently, monoclonal antibody-based high affinity checkpoint inhibitors are tested against PD1 (nivolumab; lambrolizumab; pidilizumab, pembrolizimab, MGD013), LAG-3 (MGD013), and TIM-3 (lirilumab) to support anti-tumor activity of T cells (66–68).

As discussed above, PD1 expression can lead to T cell exhaustion in which the T cell effector functions are compromised and PD1 inhibitors may re-establish the anti-tumor responses by preventing T cell exhaustion. Ibrutinib, a Bruton's tyrosine kinase inhibitor, was shown to down-regulate PD-L1 expression on tumor cells and PD1 in $CD4^+$ and $CD8^+$ T cells via inhibition of STAT3 and also decreased IL-10 production by chronic lymphocytic leukemia (CLL) cells in patients (69). PD1 and CTLA-4 ligand binding were shown to decrease glucose metabolism and inhibit AKT activation. CTLA-4 blocked AKT activation via activated protein phosphatase 2A and PD1

inhibited AKT activation by blocking PI3K activation through CD28 (70). Thus, while inhibition of PI3K activity during *ex vivo* CAR-T cell expansion may improve persistence of the final CAR-T cell product, *in vivo* PI3K inhibition has deleterious effects on CAR-T cell function.

Conversion of pro-tumor TAMs to anti-tumor macrophages (e.g., M1 macrophages) was accomplished *in vitro* and in an *in vivo* murine tumor model via stimulation of toll-like receptor-3 (TLR-3) signaling by administration of poly (I:C) (71). TLR-3 stimulation caused functional changes in TAMs, including increased phagocytic activity and upregulation of CD80 and CD86 expression with subsequent induction of CD4 T cell proliferation and tumor regression (71). The authors postulate that interferon-αβ (IFN-αβ) signaling may control the TLR-3 ligand-induced reversion of TAMs to M1 macrophages as application of anti-IFN-αβ blocking antibodies led to tumor progression even in TLR-3 ligand treated mice. As TAMs are thought to inhibit T and CAR-T cell activity, co-administration of molecules that can diminish the amount of TAMs in solid tumors may improve the efficacy of immunotherapeutic strategies in the setting of solid tumors.

In a murine model of glioblastoma multiforme that expressed epidermal growth factor receptor variant III, the thalidomide-based drug lenalidomide improved *in vivo* CAR-T cell function (lentiviral vector—anti-EGFRvIII-scFv, CD28, 4-1BB, and CD3ζ) with improved proliferation, persistence, and formation of immunological synapses (72). Chronic lymphocytic leukemia (CLL) cells produce IL-10 via STAT3 signaling, which can suppress T cell effector function. Lenalidomide was recently demonstrated to inhibit STAT3 phosphorylation, and thus IL-10 production, in CLL cells by blocking CXCL12-CXCR4-IL-10-STAT3 signal transduction, which reversed suppression of T cell effector function (73). As STAT3 phosphorylation via the CXCL12-CXCR4 signaling axis was also demonstrated in solid tumors, including bladder cancer, breast cancer, and small cell lung cancer, inclusion of the immunomodulatory drug lenalidomide may help increase CAR-T cell function in these settings.

CXCL12 inhibits T cell trafficking into tumors by binding the T cell surface receptor CXCR4 and pharmacologic inhibition of CXCR4 with AMD3100 (plerixafor), a drug used for mobilization of hematopoietic stem cells from bone marrow, led to increased T cell infiltration into tumors and synergistically decreased cancer cell numbers when combined with anti-PD-L1 therapy (30). For example, CXCR4 inhibition with AMD3100 treatment led to increased T cell-mediated antitumor activity with concomitant reduction of Tregs, which resulted in improved survival in ovarian cancer and melanoma immunocompetent mouse models (74). Additional evidence that AMD3100 modulates immunosuppression in solid tumors was recently demonstrated in orthotopic mouse models of malignant mesothelioma (75). The authors reported significantly improved tumor control with a combinatorial therapeutic approach that included simultaneous application of AMD3100 and an immune-activating fusion protein that targets mesothelin, which is expressed on mesothelioma. Decreased Treg infiltration in tumors showed that AMD3100 lowered PD1 expression on $CD8^+$ T cells and converted Tregs into helper-like cells ($CD4^+CD25^-Foxp3^+IL2^+CD40L^+$). While incorporation of AMD3100 into CAR-T cell treatment regimens has yet to be reported, use of AMD3100 to block the CXCL12-CXCR4 signaling axis was found to promote PD1 inhibition in hepatocellular carcinoma and pancreatic cancer (30, 76). Furthermore, inhibition of CXCL12 with the L-RNA-aptamer NOX-A12, which impedes CXCL12 interaction with CXCR4 and CXCR7, led to greater tumor infiltration by T and natural killer (NK) cells with an improved anti-PD1 therapy in a mouse model of colorectal cancer (77).

All-trans retinoic acid (ATRA), a drug commonly used to release the differentiation blockade in acute promyelocytic leukemia, was found to improve the anti-sarcoma activity of a third generation CAR (14g2a scFv, CD28, OX40, and CD3ζ) designed to target $GD2^+$ cells by almost complete elimination of myeloid-derived suppressor cells (MDSC) (78). Similarly, application of the tyrosine kinase inhibitor (TKI) sorafenib increased $CD8^+$ T cell trafficking to tumors as well as anti-tumor activity in a murine tumor model (79). These beneficial effects were at least partially due to sorafenib-induced decrease of MDSC and Tregs in the TME. Thus, sorafenib treatment may also lead to improved activity of CAR-T cells against solid tumors.

Oncoproteins expressed in tumor cells, such as mutant EGFR in non-small cell lung cancer cells (NSCLC), can promote higher expression levels of PD-L1 (80). EGFR inhibition with the TKI gefitinib led to decreased PD-L1 expression in NSCLC tumor cell line models and EGFR-induced PD-L1 expression was shown to be dependent upon ERK1/2 signaling down-stream of EGFR as treatment with the ERK1/2 inhibitor SCH772984 also led to diminished PD-L1 expression (81). A high level of T cell apoptosis was observed upon co-cultivation with PD-L1 expressing tumor cells, and T cell apoptosis was blocked by addition of an anti-PD1 antibody or gefitinib, suggesting that combining targeted TKI therapy with CAR-T cells may increase the persistence of CAR-T cells (81). As an additional indicator for T cell fitness, increased IFNγ production was observed in co-cultivation experiments that included gefitinib. Treatment with the EGFR inhibitor erlotinib also led to decreased PD-L1 expression in tumor cell lines that harbor mutant EGFR but not in cells that have wild-type EGFR, further supporting the link between activated EGFR signaling and PD-L1 expression (82). These authors also performed multivariate analysis of 164 NSCLC patients to compare the tumor pathologic stage (IA, IB, IIA, IIB, IIIA, IIIB), patient age, sex, smoking status, histology (adenocarcinoma vs. squamous cell carcinoma) and *EGFR* status and found *EGFR* mutations and histology to be independent variables for high PD-L1 expression ($P = 0.027$ and $P = 0.046$, respectively).

NOVEL EPIGENETIC APPROACHES TO IMPROVE CAR-T CELL FUNCTION

As discussed above, tumors and tumor-associated cells can inhibit T and CAR-T cell anti-tumor activity via PD1-PD-L1/L2 signaling. Thus, control of PD1 expression on

T/CAR-T cells provides an opportunity for T/CAR-T cells to overcome this inhibitory effect. Gene expression can be modulated via factors that modify chromatin structure, such as histone acetyltransferases and histone deacetylases (HDAC), with open chromatin structures (i.e., containing acetylated histones) available for gene transcription and "closed" chromatin structures (i.e., regions with hypoacetylated histones) as epigenetically silenced genomic regions. Satb1 (Special AT-rich binding protein 1) recruits HDAC1, which leads to transcriptional repression (83). Satb1 was also shown to be important for regulation of PD1 expression and T cell anti-tumor activity (84). Satb1 expression induced by TCR and costimulatory signals inhibited PD1 expression. TGF-β, an immunoregulatory cytokine commonly present in the tumor microenvironment, led to reduced Satb1 expression in T cells and concomitant increase in PD1 expression (84). These experiments were accomplished in unmodified T cells, thus exploration of the role(s), including possible contribution to tonic signaling, Satb1 or other epigenetic modulators may have in CAR-T cells might reveal novel strategies to increase CAR-T cell effectiveness against solid tumors.

The potential for epigenetic modification strategies to improve anti-tumor activity of CAR-T cells is supported by additional studies. The histone deacetylase inhibitor ACY241 was found to reduce tumor cells, Tregs and MDSC as well as PD1 expression on $CD8^+$T cells (85). The functional activity of adoptive T cells in a melanoma tumor model was improved by co-treatment with the histone deacetylase inhibitor LAQ824 (86).

EFFECTS OF VECTOR AND CAR DESIGNS ON CAR-T CELL FUNCTION

In addition to optimizing *ex vivo* CAR-T cell expansion protocols, the choice of a suitable and tailored vector system to deliver improved CAR constructs to T cells may also be important for CAR-T cell anti-tumor activity. In this section, we will thus review different vector and CAR designs and architectures.

As described above, CARs are synthetic receptors composed of a tumor antigen binding domain and an intracelluar CD3ζ-derived effector domain. Accordingly, all components of next generation CARs need to be carefully chosen: **(I)** the scFv or an alternative ligand that ideally exhibits specific targeting with a reasonable on-tumor and low off-target activity; **(II)** the spacer and hinge regions can also impact the antibody/ligand binding avidity and three-dimensional access to tumor antigens; **(III)** the choice of the components of intracellular effector domain is critical to mediate balanced CD3 signaling and T cell persistence; and **(IV)** the combination of I, II and III need to be rationally chosen and experimentally tested for each tumor and tumor antigen, respectively. The features important for optimization of scFv are extensively reviewed elsewhere, thus our discussion will continue with choice of hinge and spacer regions.

The extracellular hinge and spacer component may also influence CAR-T cell persistence and function. Comparison of short (12 amino acids) and long (IgG4 hinge-CH2-CH3 sequence, 229 amino acids) spacers in a murine model demonstrated superior *in vivo* expansion of CAR-T cells containing short spacers (87). The CAR-T cells outfitted with the long extracellular spacer/hinge sequence were depleted *in vivo* via activation-induced cell death independent of tumor antigen recognition and the scFv, mediated by FcγR in the CH2 sequence. Its deletion resulted in CAR-T cells with improved *in vivo* persistence and anti-tumor function (87). This is in line with another report that showed molecular refinements to the CAR spacer could impact multiple biological processes in a solid tumor model, including tonic signaling, cell aging, tumor localization, antigen recognition and superior *in vivo* antitumor activity (88).

In addition, enhanced CAR-T cell functionality was found by ICOS and 4-1BB costimulation, which mediated better functionality and *in vivo* persistence in solid tumor models than 4-1BB CARs (89). However, this depended on design of CAR with the best configuration, i.e., having the ICOS transmembrane domain linked to the ICOS intracellular signaling domain followed by the 4-1BB and CD3ζ domains.

As another important variable, tailored and fine-tuned dosing of the CAR expression is necessary, which is interlinked with the choice of an appropriately designed vector and the number of integrated vector copies. Gammaretro- and lentiviral vectors as well as Sleeping Beauty transposon based vectors are frequently used for this purpose (7, 9, 15, 19, 90). Long terminal repeat (LTR)-driven gammaretroviral vectors exploit strong and compact retroviral promoters within the LTRs, which—in conjunction with a retroviral intron—confer high expression levels in human T cells (91). However, depending on the context, even less expression might be more appropriate. Here interestingly, a self-inactivating (SIN) lentiviral vector outperformed an LTR-driven gammaretroviral vector due to better control and lower CAR expression. Noteworthy, the introduction of an internal ribosomal entry site (IRES) to reduce CAR expression from the LTR-driven gammaretroviral vector also lowered tonic signaling and ligand-independent phosphorylation of the CAR-CD3ζ chain and improved CAR-T cell expansion as compared to CAR-T cells not containing the IRES element (92). CAR-T cell exhaustion can also result from tonic activation of the CAR CD3ζ chain due to clustering of CAR scFv independently of antigen recognition and CAR-T cell exhaustion was found to be increased in CARs that contained the CD28 endodomain as compared to those with the 4-1BB endodomain (89, 93). Interestingly, genetic engineering the 4-1BB CAR to disrupt TRAF2 signaling by mutation of TRAF2 binding sites reduced apoptosis and improved proliferation of these CAR-T cells (92).

To further fine-tune CAR expression to a desired level, the following promoters are frequently used to mediate high (e.g., spleen focus forming virus (SFFV) U3, myeloproliferative sarcoma virus (MPSV) U3) and moderate expression levels (e.g., phosphoglycerokinase (PGK), and elongation factor 1a (EF1a) as house-keeping enzyme promoters) (94–96). In addition to transcriptional control, posttranscriptional regulatory motifs can be included to optimize CAR expression, e.g, miRNA sponges, which act on the posttranscriptional level, can be used to de-target expression from specific cell types and lymphoid subcompartments (97).

A split CAR design was recently described in which the CAR is divided into two sequences, one in which the scFv, transmembrane and co-stimulatory domains are attached to a dimerizer domain and a second that contains the CD3ζ signaling domain attached to a dimerizer domain (98). The CAR is only activated when the CAR binds its antigen and when the small molecule dimerizer is present. This work demonstrated exquisite pharmacologic control of CAR-T cell activity and may increase safety as the activity can be turned on at a specific time, for a set duration, and possibly even the site of action can be controlled (98).

Genetic modification of CAR-T cells to inhibit protein kinase A (PKA) localization to the immune synapse led to improved CAR-T cell trafficking into solid tumors (99). Activation of PKA by prostaglandin E_2 (PGE_2) and adenosine lead to inhibition of the T cell receptor. Expression of a peptide called "regulatory subunit I anchoring disruptor" (RIAD) in CAR-T cells inhibited PKA association with ezrin, and increased the *in vitro* anti-tumor activity of modified CAR-T cells even in the presence of inhibitory molecules such as PGE_2 and adenosine (99). The authors also demonstrated increased tumor-infiltrating capacity and enhanced anti-tumor activity of the RIAD-expressing CAR-T cells in a mesothelin-expressing mouse tumor model.

NOVEL GENE EDITING APPROACHES TO IMPROVE CAR-T CELL FUNCTION

In addition to integrating vector systems, novel gene editing tools enriched CAR-T cell strategies to improve their functionality and versatility. Here, the delivery of designer nucleases, such as zinc finger nucleases, TALENs, megaTALs and CRISPR-Cas9, can be utilized to knock out undesired properties and—in the presence of a carefully designed donor template—to knock in genetic information into so-called "safe harbors" or—by combining both strategies—into the TCR locus. Especially, the latter strategy by knocking out the T cell receptor alpha constant (TRAC) locus has the potential to create off-the-shelf CAR-T cells. Combining this with incorporation of HLA-E, a small and relatively conserved HLA, the NK cell response can be prevented, thus creating universally applicable CAR-T cells. Noteworthy, the so generated designer nuclease-treated TCR-negative CAR-T cells have similar anti-tumor activity as CAR-T cells generated by semi-random lentiviral integration (100). Moreover, by deletion of TRAC and simultaneous incorporation of the CAR at one locus, transgene copy number is controlled and the risk of insertional mutagenesis is potentially lower than that for randomly/semi-randomly integrating viral vectors (100). In a recent bridge to transplantation approach in two infant B-ALL patients, use of TALENs to generate universal CAR-T cells (UCART) by knockout of TRAC coupled with CD52 knockout to endow resistance to the monoclonal antibody Alemtuzumab (Campath), which is used to eliminate $CD52^+$ lymphocytes, was shown to be feasible (101).

Further exploitation of genome editing technologies to improve CAR-T cell anti-tumor activity include knockout of PD1, CTLA-4, TIM-3, and LAG-3. For example, CRISPR-Cas9 was used to attempt generation of universal CAR-T cells with PD1 and CTLA-4 double knockouts (102).

As described above, the CXCL12-CXCR4 signaling axis seems to play important roles in formation and maintenance of the tumor niche. The CXCR4 receptor on T cells is also a coreceptor for HIV entry and CRISPR-Cas9 gene editing/disruption of CXCR4 conferred $CD4^+$ T cell resistance to HIV-1 infection (103). In another approach, electroporation of Cas9:single guide RNA ribonucleoproteins (Cas9RNP) designed to target CXCR4 resulted in loss of high CXCR4 surface expression in about 40% of cells, and these cells could be further enriched by sorting (104).

EMERGING APPROACHES TO OVERCOME TUMOR AND MILIEU IMMUNOSUPPRESSION

The fourth generation of CAR-T cells is known as T-cells redirected for universal cytokine-mediated killing (TRUCKs) (105, 106). This strategy is based on the knowledge that T cell functions and those of cooperating anti-tumor immune cells can be modulated by several cytokines. As some of these cytokines may exhibit systemic toxicity, the inherent CAR-T cell mechanism of action allows localized delivery of potentially dangerous cytokines. Cytokine expression occurs via NFAT signaling upon antigen recognition by the CAR. In their earlier work, Abken and colleagues demonstrated increased anti-tumor efficiency using TRUCKs to deliver IL-12 to the tumor niche. Improved tumor control occurred due to recruitment of anti-tumor macrophages via IL-12 expression (105, 106). This principle can be extrapolated to other cytokines. Interestingly, IL-18 was found to increase human T cell engraftment and persistence in murine xenograft models, while negatively affecting Treg engraftment and suppressive effects (107). Improved tumor control in murine models of leukemia and melanoma were observed employing a CD19 CAR-T cell construct designed to constitutively co-express IL-18 (108). Using the TRUCK strategy to deliver IL-18 resulted in greater anti-tumor activity of CAR-T cells directed against the carcinoembryonic antigen in a pancreatic tumor model (109).

Another strategy that may be useful to improve CAR-T cell anti-tumor activity is implementation of switch receptors that convert pro-tumor into anti-tumor signals. In this regard, transfer of a PD1-CD28 receptor containing a truncated extracellular domain of PD1 and the transmembrane and cytoplasmic signaling domains of CD28 into CAR-T cells resulted in increased CAR-T cell anti-tumor activity and is a promising concept for future clinical investigation (110).

CONCLUSION AND OUTLOOK

Improved understanding of the complex interactions that occur in the solid tumor microenvironment will lead to

improved tailored genetic engineering approaches. For example, exploitation of pro-tumor signaling such as the CXCL12-CXCR4 axis may lead to development of CAR-T cells with navigation systems, exhibiting improved homing to and penetration into solid tumors. A critical point may be the choice of the T cell population selected for CAR-T cell production. Here, novel insight into cell and stem cell biology will guide educated decisions with regard to the choice of the optimal T cell population. As monotherapeutic approaches are seldom effective in tumor control, it may be necessary to target multiple antigens or to explore novel combinations of CAR-T cells and other therapeutic modalities, such as standard chemotherapy and/or radiation therapy, tyrosine kinase inhibitors, epigenetic modulators or other small molecule drugs. This will form a potent arsenal of next generation CAR-T cell strategies to attack solid tumors.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Making CAR T Cells a Solid Option for Solid Tumors

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Adoptive cell therapy with chimeric antigen receptor (CAR) T cells aims to redirect the patient's own immune system to selectively attack cancer cells. To do so, CAR T cells are endowed with specific antigen recognition moieties fused to signaling and costimulatory domains. While this approach has shown great success for the treatment of B cell malignancies, response rates among patients with solid cancers are less favorable. The major challenges for CAR T cell immunotherapy in solid cancers are the identification of unique tumor target antigens, as well as improving CAR T cell trafficking to and expansion at the tumor site. This review focuses on combinatorial antigen targeting, regional delivery and approaches to improve CAR T cell persistence in the face of a hostile tumor microenvironment.

Keywords: immunotherapy, CAR-T cells, solid tumors, cancer, toxicity, cell engineering

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INTRODUCTION

Chimeric antigen receptor (CAR) T cells targeting CD19 for the treatment of relapsed/refractory (r/r) B-cell acute lymphoblastic leukemia (ALL) and lymphoma have led to unprecedented response rates of about 80% in a patient population that up until then had a very poor prognosis (1–7). The FDA approval of CAR T cells for leukemia and then lymphoma in 2017 marked the breakthrough of two converging clinical research fields: CAR T cell immunotherapy and gene therapy. CAR T cells were first conceived by Eshhar and colleagues in 1989 as an enhanced T cell version endowed with an antibody-based recognition domain fused to a CD3zeta signaling domain (**Figure 1A**) (8). Over the years, these so called first generation CAR T cells have experienced an improvement of their anti-tumor potency by adding one (9–13) or two costimulatory domains (11, 14, 15), resulting in second or third generation CAR T cells, respectively (**Figures 1B,C**). Making the impressive potency of CAR T cell therapy available to the more numerous patients suffering from solid cancers has been an endeavor for about a decade now. So far, CAR T cells for solid tumors have not been able to achieve the impressive responses induced in hematological cancers. Identification of unique tumor associated antigens (TAA), CAR T cell trafficking and persistence, as well as the immunosuppressive tumor microenvironment have emerged as the major drawbacks to the success of CAR T cells for the treatment of solid malignancies (**Figure 2**). Multiple approaches aiming at overcoming these hurdles are currently under active investigation.

STATUS OF CLINICAL RESEARCH ON CAR T CELLS FOR SOLID TUMORS

Most Promising Results of CAR T Cell Trials for Solid Tumors So Far

Recapitulating the history of chemotherapy, CAR T cells for the treatment of solid cancers have not yet been able to reproduce the success of their hematological counterparts. Nevertheless, the field has achieved important breakthroughs in the treatment of some solid tumors. In a phase I clinical trial investigating GD2 specific CAR T cells for the treatment of pediatric neuroblastoma, 3 out of 11 patients who had active disease at the time of enrollment achieved a complete remission (16). Encouraging results were also reported from a phase I/II clinical study using a HER2 specific CAR in patients with HER2-positive sarcoma. Of the 17 evaluable patients, all of whom had relapsed or refractory disease, 3 had stable disease and were able to undergo surgery to remove the residual tumor, resulting in complete remission without further treatment (17). In a study conducted by Brown and colleagues, regional, multi-dose treatment with IL13Rα2 specific CAR T cells induced a complete remission in a patient with disseminated glioblastoma (18).

Current CAR T Cell Clinical Trials for Solid Tumors

There are currently over 270 CAR T cell trials registered at the U.S. National Library of Medicine (ClinicalTrials.gov). Of these, about one third are investigating the use of CAR T cells for solid tumor indications. **Table 1** shows selected CAR T cell trials for solid cancers that are currently recruiting patients in the US and Europe. Among the most studied solid tumor targets are EGFRvIII for glioblastoma (NCT03283631, NCT02664363, NCT01454596), GD2 for neuroblastoma (NCT03373097, NCT03294954, NCT02761915) and mesothelin for various epithelial cancers (NCT02792114, NCT01583686). Interestingly, several trials are exploring regional delivery routes of CAR T cell therapy, especially intracranial administration for glioblastoma and other brain tumors (NCT03283631, NCT03500991, NCT01818323, NCT02208362).

FINDING THE RIGHT TARGET ANTIGENS

The ideal target epitope for CAR T cell therapy would be expressed on every tumor cell and crucial for the maintenance and propagation of the malignant phenotype, while being absent on healthy tissues. In practice, the identification of target antigens that are solely present on malignant- but not on healthy cells- has proven rare. Finding suitable TAA has been easier for hematological malignancies than for solid tumors. On-target, off-tumor toxicities of CD19 and BCMA specific CARs, in the form of B cell and plasma cell aplasia, are usually manageable in patients with hematological malignancies. In contrast, on-target, off-tumor toxicities of CARs for solid cancers can lead to fatal outcomes (19). Potential reasons for this may include overlapping antigen expression on epithelial tissues, which most solid tumors originate from, and the spatial confinement of critical sites when targeting solid tumors. Target antigen density on the tumor cells has been shown to positively correlate with CAR T cell functionality, evidenced by activation and cytokine production (20, 21). Thus, finding a target molecule that is highly expressed on the tumor cells is desirable for two reasons- to enhance CAR T cell potency and to avoid on-target, off-tumor toxicities to healthy tissues expressing the target antigen at low levels.

Combinatorial Antigen Targeting

Heterogeneous antigen expression on solid tumors as well as low-level expression of TAA on healthy tissues render it difficult to find well suited targets for CAR T cell therapy of solid tumors. Combinatorial antigen recognition approaches have recently been developed to address these challenges.

"OR" Gate/Tandem CAR

Employing Boolean "OR" logic allows targeting two or more TAAs with a single CAR T cell. In so called tandem CARs the presence of either antigen 1 or antigen 2 is enough to trigger activation (**Figure 3A**). This strategy helps to increase the density of the targetable molecules on the tumor surface and therefore may increase CAR T cell potency. In tandem CAR T cells, effector function is synergistically improved upon co-recognition of both target antigens, while it is still preserved in the presence of only one antigen. Indeed, enhanced antitumor efficacy of dual antigen targeting has been reported in preclinical models for solid and hematological cancers (22–24). Hedge and colleagues designed a HER2/IL13Rα2 tandem CAR for the treatment of glioblastoma. They found the activation characteristics of the HER2/IL13Rα2 tandem CAR to be comparable to those of the corresponding single antigen specificity CAR in the presence of one target antigen. However, when both target molecules were expressed concurrently, heterodimers were induced and a synergistic effect on the CAR T cell activation was observed. Compared to the single antigen specificity CAR T cells, the tandem CAR could delay tumor growth, mitigate antigen escape and improve survival in a glioblastoma mouse model (23). To date it is unclear how the toxicity profile of tandem CAR T cells compares clinically to single antigen specificity CAR T cells. On the one hand, it has been suggested that by endowing tandem CAR T cells with reactivity against two TAA they may display an improved ability to discriminate malignant vs. normal target cells. On the other hand, prediction of potential on-target, off-tumor toxicity sites is rendered more complex, since expression of each of the targeted molecules individually and in combination must be taken into consideration. There is an open phase I clinical trial using a tandem CAR directed against CD19 and CD20 for patients with relapsed/refractory B cell malignancies (NCT03019055). To date, no tandem CAR trials for solid tumors have been opened (according to clinicaltrials.gov).

"AND" Gate CAR

Employing Boolean "AND" logic, CAR T cells can be reprogrammed to activate only in response to target cells expressing two antigens concurrently (**Figure 3B+C**); thereby allowing them to discriminate more safely between malignant

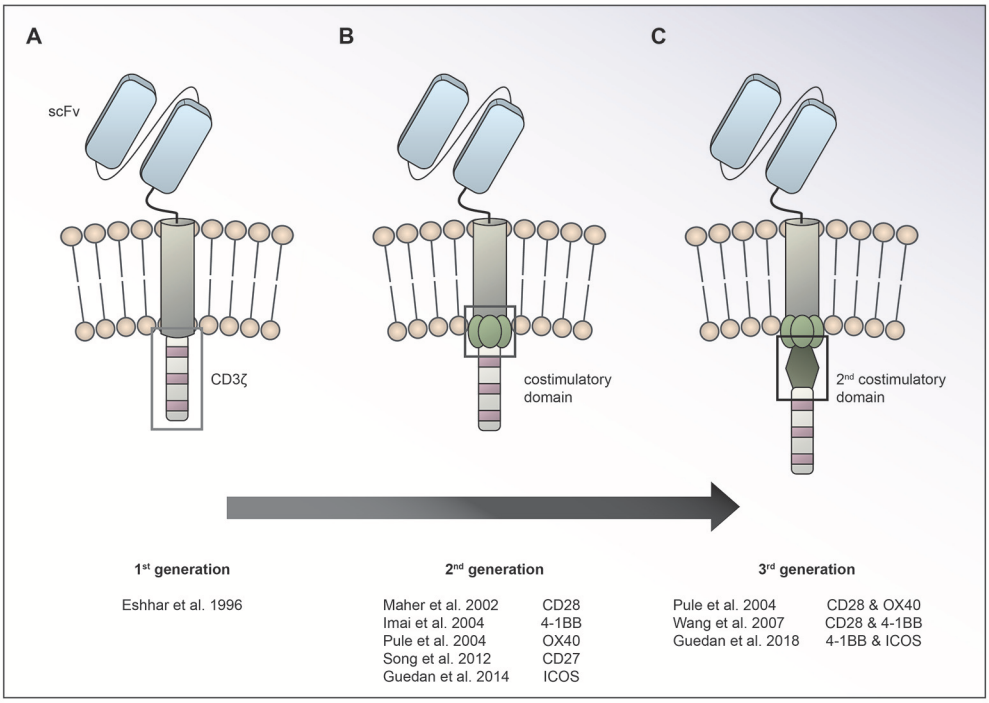

FIGURE 1 | Evolution of CAR design. The basic CAR set up consists of an antigen binding moiety (e.g., scFv based) and a spacer on the extracellular side, a transmembrane domain and domains for T cell activation on the intracellular side. While 1st generation CARs **(A)** contain only a CD3ζ chain for T cell activation, 2nd **(B),** and 3rd **(C)** generation CARs have one or two costimulatory domains incorporated, respectively.

cells and healthy tissues. This can be achieved by engineering T cells to express both, a first-generation CAR that recognizes antigen 1 but induces only inadequate activation, and a chimeric costimulatory receptor that recognizes antigen 2 and allows for full T cell activation by complementing the co-stimulation needed. Kloss and colleagues provided proof of concept that an "AND" gate to regulate CAR T cell activity can be generated by re-associating signal 1 and signal 2 and applied this in the context of a prostate tumor model using PSMA and PSCA antigens (25) (**Figure 3B**). A different approach to generating "AND" gates in T cells is the use of synthetic Notch (synNotch) receptors (26). Sensing of antigen 1 by the synNotch receptor induces transcription of a CAR that is specific for antigen 2 (27) (**Figure 3C**). Use of both of these strategies to generate antigen-sensing circuits resulted in specific efficacy against tumors with dual antigen expression while sparing target cells expressing either antigen alone. Boolean "OR" as well as "AND" gates offer exciting opportunities to enhance efficacy and precision of tumor targeting. By adding a second antigen specificity, on-target, off-tumor toxicities could potentially be prevented (19). The approach of combinatorial antigen targeting may help to overcome the current challenge of identifying suitable target molecules for CAR T cell therapy for solid tumors. However, this promising preclinical data still needs to be validated in clinical studies and the global adoption of one strategy to all different solid tumor entities seems unlikely. One could picture a scenario where combinatorial antigen approaches are exploited to tailor therapy to the individual patient's characteristics. Taking into consideration the tumor entity and stage, one could employ "OR" gates in cases where enhancing anti-tumor efficiency or preventing antigen escape are essential, while using "AND" gates in cases where on-target, off-tumor toxicity is the major concern.

Universal Adaptor CAR

In recent years, several research groups have developed platforms for making universal CARs. The general idea is to have an adaptor CAR that binds to a soluble adaptor which in turn conveys specificity against a certain tumor antigen (28–32) (**Figure 3D**). This approach allows targeting multiple tumor antigens simultaneously through the combined application of the distinct soluble adaptors and thus is an exciting strategy to address solid tumor heterogeneity. An additional advantage of universal CARs is the ability to redirect the CAR T cell to a new target molecule without having to re-engineer the T cell itself by simply switching the soluble adaptor in case of antigen escape or insufficient tumor response. At the same time, the universal CAR platform implies an "ON-switch" system since the soluble adaptor must be administered for the CAR T cell to be able to become active. This feature provides an additional regulatory element with the possibility to attenuate or abolish CAR T cell function by withdrawing the soluble adaptor or even applying a nonspecific adaptor to compete the specific soluble adaptor off.

However, the clinical feasibility of such universal CAR platforms remains to be evaluated. The complex interaction of the different control features this approach provides will have to be examined individually and jointly. The following factors will have to be explored to optimize clinical outcome: number of adoptively transferred universal CAR T cells, dosage regimen of

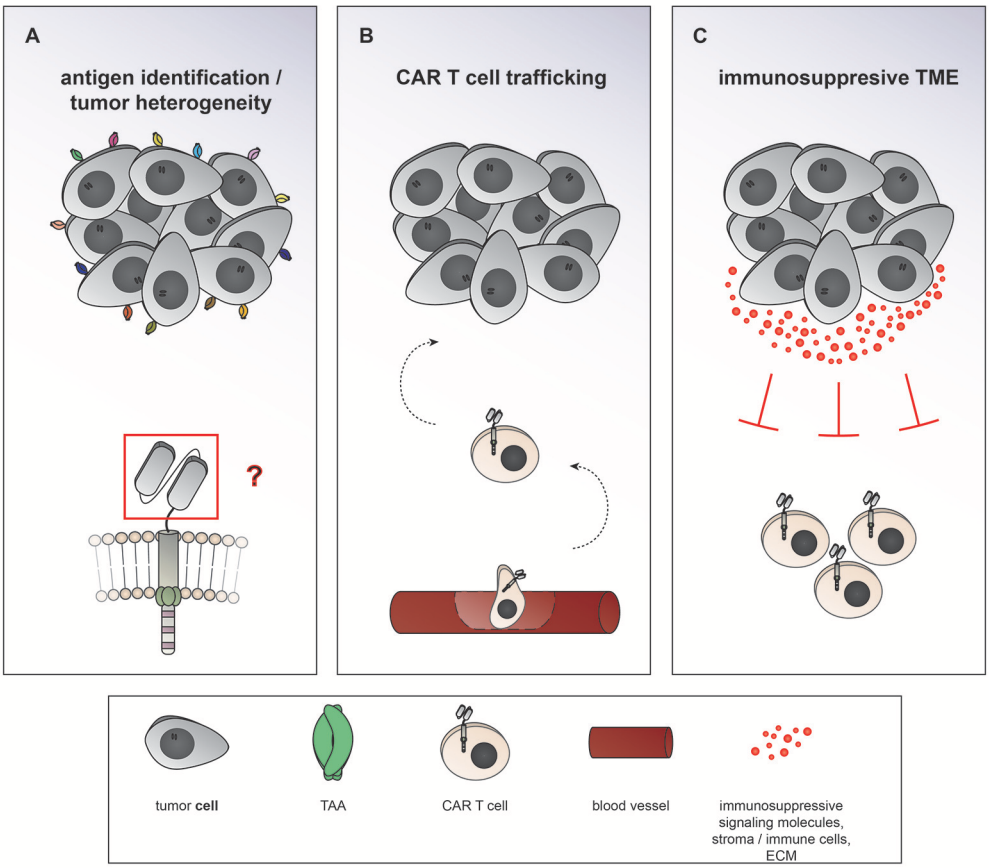

FIGURE 2 | Major hurdles to the efficiency of CAR T cells in solid cancers. **(A)** Heterogeneous expression of tumor associated antigens (TAA) on solid cancers as well as overlapping expression on healthy tissues makes it difficult to find suitable targets of CAR T cells therapy. **(B)** After intravenous application CAR T cells need to traffic to the tumor site, extravasate the circulation, and penetrate the tumor. **(C)** The term tumor microenvironment describes the interplay between the tumor cells themselves and the surrounding blood vessels, stromal cells, immune cells, as well as the extracellular matrix. CAR T cell migration and expansion are inhibited by the immunosuppressive environment of solid cancers.

the soluble adaptor, binding kinetics between the target molecule and the soluble adaptor as well as between the universal CAR T cell and the soluble adaptor. All "ON-switch" CAR T cell platforms entail the additional challenge of deciding when to stop the administration of the CAR activating drug in the case of tumor remission.

GETTING CAR T CELLS TO SOLID TUMORS AND GETTING THEM TO STAY

Insufficient trafficking to and expansion at the tumor site after systemic administration has been identified as a major hurdle to the success of CAR T cell therapy in solid tumors. The mechanisms governing chemotaxis of T cells to the tumor site and the role of the tumor microenvironment in inhibiting CAR T cell migration and expansion have been comprehensively reviewed recently (33–35). Here we will focus on regional delivery as a means to bypass the necessity of T cells trafficking to the tumor and highlight some innovative engineering approaches to improve T cell persistence.

Regional Delivery

To circumvent the challenge of CAR T cells having to traffic into the tumor, several investigators have focused on regional delivery of CAR T cells for the treatment of solid tumors. Preclinical testing has consistently reported significantly lower CAR T cell numbers being required to induce tumor responses and limited or abolished systemic toxicities when a regional administration route is chosen over systemic delivery (36–38). Mesothelin is expressed on a broad range of solid tumors; lung, pancreatic, breast, and ovarian cancer amongst others, and is under active investigation as a target molecule for CAR T cell therapy. The effects of regional delivery of CAR T cells targeting mesothelin in the context of malignant pleural disease have been studied by Adusumilli and colleagues. In a preclinical model of pleural malignancy, they found that intrapleural injection of mesothelin specific CAR T cells improved T cell activation and persistence as well as tumor response compared to intravenous administration of CAR T cells. Importantly, a significantly lower number of CAR T cells was needed for tumor eradication when administered locally as opposed to systemically. Furthermore, the regionally primed CAR T cells were able to traffic to and

TABLE 1 | Selected CAR T cell trials for solid tumors.

	Indication	Lympho-depletion	Route of administration	Distinctive features	Identifier	Center
CD70	Pancreatic/Renal Cell/Breast/Ovarian Cancer, Melanoma	Cyc, Flu	Systemic	IL-2 administration	NCT02830724	NCI
CD171	Neuroblastoma	N/S	Systemic	tEGFR	NCT02311621	Seattle Children's Hospital
EGFRvIII	Recurrent Glioblastoma/-sarcoma	–	Intracerebral	radiolabeling (111In) of CAR T cells	NCT03283631	Duke
	Glioblastoma/-sarcoma	TZM	Systemic	radiolabeling (111In) of CAR T cells	NCT02664363	Duke
	Glioblastoma/-sarcoma, Brain Cancer	Cyc, Flu	Systemic	IL-2 administration	NCT01454596	NCI
ErbB	Head and Neck Cancer	–	Intratumoral	–	NCT01818323	King's College London
FAP	Malignant Pleural Mesothelioma	–	Intrapleural	–	NCT01722149	University Hospital Zurich
GD2	(r/r) Neuroblastoma	N/S	Systemic	iCas9	NCT03373097	Bambino Gesù Hospital, Rome
	Neuroblastoma	Cyc, Flu	Systemic	NK T cells, IL-15 administration	NCT03294954	Texas Children's Hospital
	r/r Neuroblastoma	Cyc, Flu	Systemic	–	NCT02761915	UCL, Great Ormond Street Hospital for Children
GPC3	Pediatric Solid Tumors	Cyc, Flu	Systemic	–	NCT02932956	Texas Children's Hospital
	Hepatocellular Carcinoma	Cyc, Flu	Systemic	–	NCT02905188	Houston Methodist Hospital
HER2(ErbB2)	r/r Pediatric CNS Tumors	–	Intracerebral	tEGFR	NCT03500991	Seattle Children's Hospital
	r/r Glioblastoma	–	Intracerebral	tCD19	NCT03389230	City of Hope Medical Center
	r/r Glioblastoma	–	Intracerebral	–	NCT02442297	Houston Methodist Hospital
	Sarcoma	– / Flu / Cyc, Flu	Systemic	–	NCT00902044	Houston Methodist Hospital
IL13Rα2	Glioblastoma, r/r Brain Neoplasm	–	Intracerebral	tCD19	NCT02208362	City of Hope Medical Center
MET	Melanoma, Breast Cancer	–	Systemic	CAR transfer by RNA electroporation	NCT03060356	UPenn
Mesothelin	Breast Cancer	Cyc	Systemic	–	NCT02792114	MSKCC
	Cervical/Pancreatic/Ovarian/Lung Cancer, Mesothelioma	Cyc, Flu	Systemic	IL-2 administration	NCT01583686	NCI
MUC-16 (ecto)	Recurrent Ovarian/Primary Peritoneal/Fallopian Tube Carcinoma	Cyc, Flu	Systemic and intraperitoneally	IL-12 secreting,tEGFR	NCT02498912	MSKCC
PSCA	Prostate Cancer	–/Cyclo	Systemic	TGF-β resistant CAR T cells	NCT03089203	UPenn
	Pancreatic Cancer	N/S	Systemic	Rimiducid inducible costimulation	NCT02744287	Baylor Sammons Cancer Center
ROR1	Triple Negative Breast Cancer, NSCLC	Cyc, Flu	Systemic	–	NCT02706392	Fred Hutchinson Cancer Research Center

CD, cluster of differentiation; Cyc, cyclophosphamide; EGFRvIII, epidermal growth factor receptor vIII; ErbB, erythroblastosis oncogene B; FAP, fibroblast activation protein alpha; Flu, fludarabine; GD2, disialoganglioside; GPC3, glypican 3; HER2, human epidermal growth factor receptor 2; iCas9, inducible caspase-9 (safety switch); IL13Rα2, Interleukin-13 receptor subunit alpha-2; MET, tyrosine-protein kinase MET (mesenchymal to epithelial transition); MUC-16(ecto), extracellular portion of the glycosylated mucin, MUC16; N/S, not specified; NSCLC, non-small-cell lung cancer; PSCA, prostate stem cell antigen; ROR1, receptor tyrosine kinase like orphan receptor 1; r/r, relapsed/refractory; tCD19/tEGFR, truncated CD19 / EGFR (safety switch); TGF-β, Transforming growth factor beta; TZM, Temozolomide.

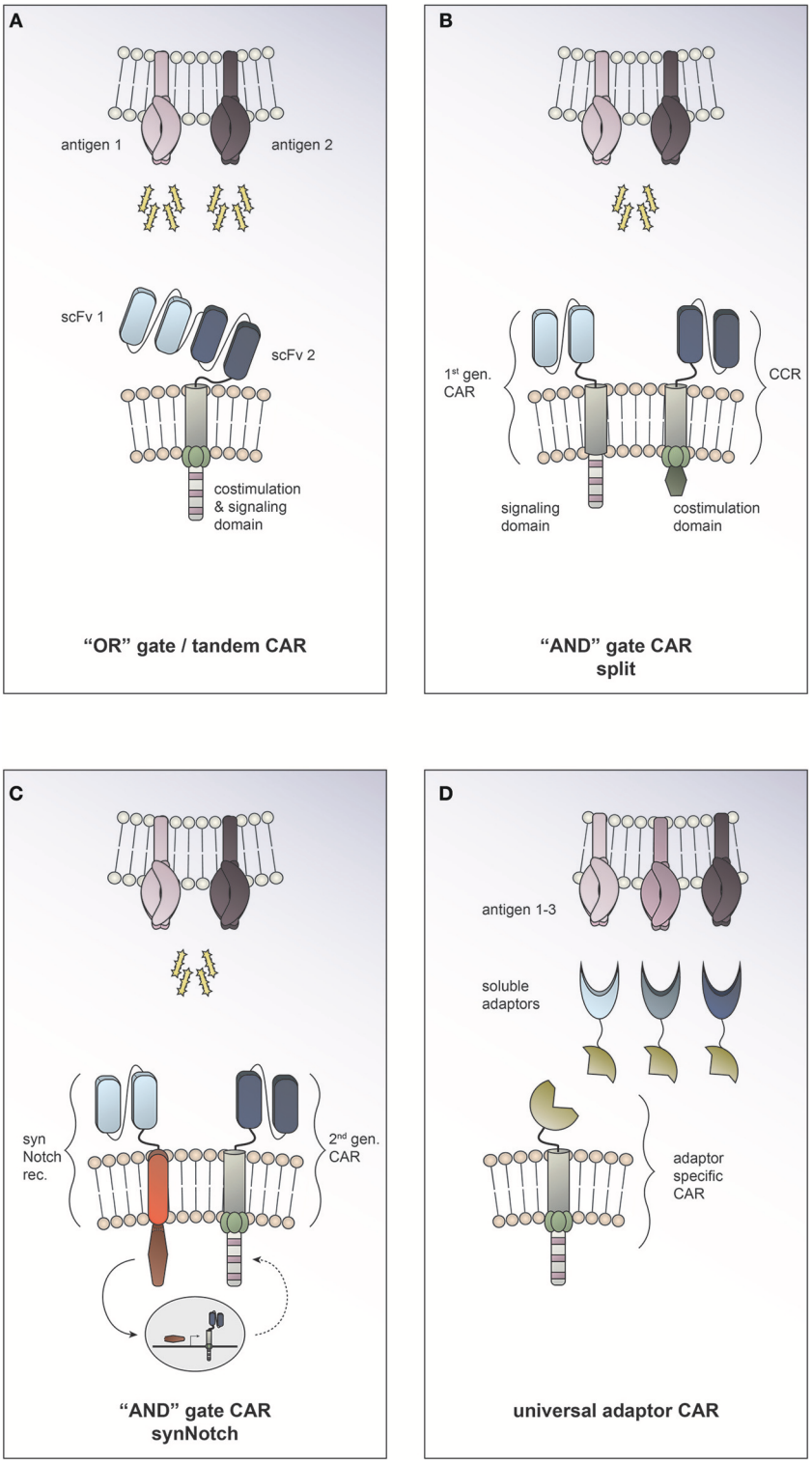

FIGURE 3 | Combinatorial antigen targeting for solid cancers. **(A)** For "OR" gate/ tandem CAR T cells the presence of one antigen is sufficient to trigger effector function, while concurrent expression of both antigens leads to synergistical improvement of activation. **(B+C)** "AND" gate CAR T cells require the presence of either target antigens to efficiently activate. **(B)** The split CAR approach taken by Kloss and colleagues uses a 1st generation CAR that recognizes antigen 1 combined with a chimeric costimulatory receptor (CCR) that provides the necessary costimulation upon encounter of antigen 2. **(C)** In the synthetic Notch (synNotch) approach reported by Roybal and colleagues sensing of antigen 1 by a synNotch transcriptional receptor (synNotch rec.) induces expression of a CAR that is specific for antigen 2. **(D)** Universal CAR T cells can target a variety of different antigens since their antigen specificity comes from the administration of soluble adaptors.

clear tumors at distant sites (37). Based on these promising preclinical data, a phase I trial with regionally delivered anti-mesothelin CAR T cells for malignant pleural disease was initiated (NCT02414269). A preliminary report from this study noted no evidence for toxicity while antitumor activity has been observed. CAR T cells could be detected in the peripheral blood of 6 of the 12 patients treated. Encouragingly, one patient, who had additionally received anti-PD1 checkpoint blockade off protocol, achieved a complete remission as evidenced by PET scan (ASGCT 21st Annual Meeting Abstracts Molecular Therapy, Volume 26, Issue 5, 1–459).

Glioblastoma and brain metastasis are solid tumor entities where regional administration of CAR T cell therapy is actively being explored. Preclinical models have shown antitumor efficiency and safety of intracranial administration of EGFRvIII and HER2 redirected CAR T/NK cells (39–41). To date, the clinical outcomes of 5 patients receiving intrathecally or intracranially delivered IL-13Rα2 targeting CAR T cells for glioblastoma have been reported (18, 42, 43). One patient achieved a 7.5 month lasting complete regression of all intracranial and spinal tumors under continued CAR T cell treatment, which is a remarkable occurrence in this disease.

Further phase I clinical trials investigating intratumoral injection of CAR T cells targeting ErbB for the treatment of locally advanced squamous cell cancer of the head and neck (NCT01818323) (44) and hepatic artery infusion of CEA specific CAR T cells combined with SIRT (selective internal radiation therapy) for CEA positive liver metastasis (NCT02416466) are underway.

A whole new approach to regional delivery of CAR T cells for solid tumors using implantable biopolymer scaffolds has recently been reported by Smith et al. (38). The authors showed that regional delivery and expansion of CAR T cells in biopolymer scaffolds implanted at the tumor site in contrast to systemic administration led to superior antitumor responses in mouse models of pancreatic cancer and melanoma. Furthermore, the simultaneous transfer of CAR T cells and stimulator of INF genes agonist by biopolymer scaffold could extend the immune response to tumor cells not expressing the CAR specific target molecule. Another possible advantage of scaffold-assisted delivery may lie in the ability to protect CAR T cells from the hostile influence of the tumor microenvironment by locally supplying them with growth factors during the initial phase of tumor priming.

Strategies to Improve Persistence

Longer persistence of CAR T cells posttreatment has been associated with better clinical outcome in both patients with hematological and solid cancers (16, 45, 46). The beneficial effect of prior lymphodepletion, including diminution of regulatory T cells, on CAR T cell engraftment has been established (47–49). Rapid *in vivo* expansion of CAR T cells post infusion, often leading to cytokine release syndrome correlates with anti-tumor responses in hematological malignancies and has been frequently observed in clinical trials using CD19- and BCMA-redirected CARs (50, 51). In contrast, CAR T cell trials for solid tumors have not reported outcomes with strong release of proinflammatory cytokines preceding tumor regression. Therefore, it seems likely that insufficient expansion and persistence of CAR T cells in patients with solid tumors is a major cause for the unsatisfying response rates observed so far. Indeed, insufficient engraftment and persistence of solid tumor specific CAR T cells has been reported in several clinical trials. In a study treating melanoma patients with GD2 specific CAR T cells, only 1 out of 6 patients still had detectable CAR T cells beyond 4 months (52). Monitoring of persistence of anti-EGFRvIII engineered T cells in a trial with r/r glioblastoma patients showed rapid reduction of CAR T cell numbers in peripheral blood starting 2 weeks posttreatment (53).

Empowering CAR T Cells to Shape Their Own Cytokine Environment

Cytokine support is a crucial factor for the survival and expansion of T cell therapies. This is particularly true when they encounter hostile conditions as in the microenvironment of solid tumors. Engineering solutions for adoptively transferred T cells have been developed to allow for both, to support themselves with proinflammatory cytokines, and to shield themselves from immunosuppressive cytokines. IL-12 and IL-18 secreting CAR T cells have been shown to persist longer and lead to enhanced tumor responses in preclinical models of solid cancers (54–56). Other investigators have described improved antitumor efficiencies of CAR T cells equipped with constitutive IL-7 and IL-15 signaling, as well as by inducible delivery of IL-15 super-agonist complex by T cells upon encounter of the cognate antigen (57–59).

Taking the reverse approach, the tumor cells' immunosuppressive cytokine signaling can be inhibited or converted into proinflammatory signaling. Overexpression of a dominant negative form of the TFG-β receptor has been reported to increase the anti-tumor potency of CAR T cells against melanoma in a mouse model (60). A phase I clinical trial currently investigates the use of TFG-β resistant CAR T cells directed against PSMA for castrate-resistant prostate cancer (NCT03089203; **Table 1**). By endowing CAR T cells with an inverted cytokine receptor, consisting of the exodomain of the IL-4 receptor fused to the IL-7 receptor endodomain, signaling of the immunosuppressive cytokine IL-4 could be transformed to promote proliferation and anti-tumor efficiency *in vivo* (61).

Engineering approaches that provide CAR T cells with endogenous cytokine support can be categorized into those where interleukins are secreted into the surroundings and those where interleukin signaling is restricted to the CAR T cell itself. Besides providing autocrine stimulation for the CAR T cell itself, secreting approaches may have additional paracrine effects e.g., remodeling the tumor microenvironment and activating by-stander immune cells (55). Yet they come at the risk of causing systemic inflammatory reactions and toxicities, as have been previously reported upon systemic cytokine administration (62). Koneru and colleagues therefore carefully designed their phase I clinical trial of IL-12 secreting MUC-16(ecto) targeting CAR T cells for the treatment of recurrent ovarian cancer by adding an "off-switch" (tEGFR) and administering half the CAR T cell

dose intraperitoneally in order to enhance safety (NCT02498912; **Table 1**) (63).

Targeted CAR Integration Into the T Cell Genome

We have learned from hypothesis driven research and clinical observation that the genomic integration site of the CAR fundamentally impacts the T cell's ability to activate and persist. Targeted insertion of the CAR into the TRAC locus, as opposed to random insertion during conventional CAR T cell manufacturing, enhanced the T cells anti-tumor function in a leukemia mouse model. Delivery of the CAR into the TRAC locus prevented functional exhaustion of the T cells by circumventing tonic CAR signaling, i.e., activation in the absence of the cognate antigen (64).

Fraietta and colleagues recently reported the case of a patient in which the clonal expansion of one single CAR T cell induced remission of chronic lymphocytic leukemia. Further analysis revealed that random insertion of the CAR into the TET2 gene locus had led to disruption of TET2 protein expression in this patient who also had a hypomorphic mutation on their other TET2 allele; the biallelic disruption of TET2 resulted in a central memory state of the T cell clone (65).

Since functional exhaustion and insufficient expansion of T cells have been identified as major shortcomings of CAR T cell therapy for solid cancers, these innovative strategies may help balance some of the challenges encountered. Targeted CAR delivery into the T cell genome holds promise to generate phenotypically more competent cells and thereby enhance their anti-tumor efficiency. However, further research is needed in order to determine feasibility and safety of directed CAR delivery into the T cell genome.

Preventing *Ex vivo* Differentiation and Exhaustion of CAR T Cells

The current CAR T cell manufacturing process requires *ex vivo* activation and expansion of the patient's T cells. This may speed up effector T cell differentiation and functional exhaustion, thereby reducing the potency of the CAR T cell product.

The use of T cell homing nanoparticles has recently been suggested as a new approach to CAR T cell production. T cell homing nanoparticles can reprogram T cells *in vivo*, without the need to remove them from the subject's body. After administration, the nanoparticles deliver CAR encoding DNA selectively to T cells. *In vivo* reprogrammed CAR T cells were as efficient as *ex vivo* manufactured conventional CAR T cells at controlling leukemia progression in a preclinical mouse model (66). The current standard CAR T cell manufacturing protocols requiring *ex vivo* engineering of the adoptive cell product cause time delays, high costs and potentially have a negative impact on the T cell phenotype. However, one of its strength is the long safety record in clinical application. Long-term follow up of patients treated with retroviral engineered CAR T cells has not shown any transformational events in more than 500 patient-years of follow up (67). *In vivo* administration of CAR delivering nanoparticles comes with the risk of unintentional gene transfer into off-target cells. Accidental gene transfer into hematopoietic stem cells represents a major safety concern, since malignant transformation of hematopoietic stem cells causing leukemia has previously occurred in pioneering gene therapy trials (68, 69). Further research is needed to establish the safety profile of gene delivering nanoparticles before they can be translated into clinical application for *in vivo* CAR T cell manufacturing.

Other strategies to avert the negative impact of *ex vivo* culture on the CAR T cells antitumor potency is to make sure CAR signaling starts only post-infusion of the product. The concept of a CAR integrated "ON-switch" was introduced by Wu et al. (70). They designed a split CAR where the functional components of a conventional CAR are dissociated into two parts that only reassemble in the presence of a small molecule. The Tet-OFF CAR platform proposed by Mamonkin and colleagues employs a conditional doxycycline regulated system where the CAR is only expressed upon withdrawal of the drug (71). Both these strategies permit to switch on the CAR expression only post transferal of the adoptive T cell therapy. "ON-switch" concepts for CAR expression combine the advantages of maintaining a more naïve T cell phenotype with the distinguished safety features of *ex vivo* genetic engineering of T cells; thus, for now their clinical translation seems more feasible than *in vivo* CAR T cell engineering.

CONCLUSIONS

CAR T cells for the treatment of solid tumors have made progress for individual target antigens and tumor entities. Broader proof of concept for the efficiency of immunotherapy in solid cancers has been provided by the considerable success of checkpoint blockade. As a "living drug" CAR T cell therapies confer the advantage of potentially life-long tumor surveillance. Lessons learned from the unsatisfying response rates of most pioneering CAR T cell trials for solid tumors have fed back into preclinical development of new concepts to address these hurdles. CAR T cells for solid tumors have passed through the first cycle, form bench to bedside and back. Still there is need for considerable optimization before CAR T cell therapy can advance as a standard treatment option for patients with solid tumors. However, the emerging preclinical and clinical research on identifying suited target antigens as well as improving delivery and persistence of CAR T cells in solid cancer holds promise for wider therapeutic applications.

AUTHOR CONTRIBUTIONS

AS and MM wrote the manuscript and created the figures. Both authors edited the manuscript and approved it for publication.

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Effective Targeting of TAG72^{+} Peritoneal Ovarian Tumors via Regional Delivery of CAR-Engineered T Cells

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Impressive clinical efficacy of chimeric antigen receptor (CAR)-engineered T cell therapy for hematological malignancies have prompted significant efforts in achieving similar responses in solid tumors. The lack of truly restricted and uniform expression of tumor-associated antigens, as well as limited T cell persistence and/or tumor trafficking pose major challenges for successful translation of CAR T cell therapy in solid tumors. Recent studies have demonstrated that aberrantly glycosylated cell surface proteins on tumor cells are amenable CAR targets. Tumor-associated glycoprotein 72 (TAG72) antigen is the sialyl-Tn found on multiple O-glycoproteins expressed at high levels on the surface of several cancer types, including ovarian cancer. Here, we developed a humanized TAG72-specific CAR containing a 4-1BB intracellular co-stimulatory signaling domain (TAG72-BBζ). TAG72-BBζ CAR T cells showed potent antigen-dependent cytotoxicity and cytokine production against multiple TAG72^{+} ovarian cancer cell lines and patient-derived ovarian cancer ascites. Using *in vivo* xenograft models of peritoneal ovarian tumors, regional intraperitoneal delivery of TAG72-BBζ CAR T cells significantly reduced tumor growth, extended overall survival of mice, and was further improved with repeat infusions of CAR T cells. However, reduced TAG72 expression was observed in early recurring tumors, which coincided with a lack of T cell persistence. Taken together, we demonstrate efficacy with TAG72-CAR T cells in ovarian cancer, warranting further investigations as a CAR T cell therapeutic strategy for this disease.

Keywords: chimeric antigen receptor, ovarian cancer, regional intraperitoneal delivery, TAG72, tumor-associated glycoproteins, adoptive cellular immunotherapy, STn, sialyl-Tn

INTRODUCTION

Chimeric antigen receptor (CAR)-engineered T cell therapy in patients with CD19+ B-cell malignancies have demonstrated impressive clinical responses (1, 2), which recently resulted in two landmark FDA approvals for patients with leukemia and lymphoma. These studies have shown that CAR T cells can be optimized to induce durable and complete responses in cancer patients, even under conditions of highly refractory disease. Major obstacles in developing effective CAR T cell therapies for solid cancers are avoiding off-tumor on-target toxicity due to the lack of truly restricted tumor antigens, as well as achieving durable responses that are limited by T cell persistence and tumor trafficking (3, 4). To date, the majority of tumor antigens for directing specificity of CAR T cells have targeted over-expressed proteins, including but not limited to mesothelin, PSMA, PSCA, HER2/neu, EGFR, and IL13Rα2 (3, 5). While the field is still evolving, clinical efficacy of CAR T cells targeting these proteins in solid tumors has been somewhat limited (6), and identification of additional targets as well as addressing limited T cell durability is critically important to the successful translation of CAR T cell therapies.

Aberrantly glycosylated cell surface proteins have long been implicated in tumor development, and have unique glycoprotein signatures that are attractive targets for immunotherapy, including CAR T cells (7, 8). Multiple cancer types including colon, breast, pancreas, and ovarian, are known to over-express aberrantly glycosylated proteins, including the mucins MUC16 and MUC1, and the tumor-associated glycoprotein 72 antigen (TAG72) (9), that differentiate them from normal epithelia. TAG72 is the truncated sialyl Tn (STn) O-glycan carbohydrate hapten located on multiple cell surface O-glycoproteins (10). High expression of TAG72, MUC1, and MUC16 has been shown in ovarian cancer patient tissue samples, with nearly 100% of ovarian cancers identified with simultaneous staining of the three antigens (11). Importantly, approximately 90% of epithelial ovarian cancers are TAG72 positive, indicating its abundance across multiple histological subtypes of ovarian cancer (11).

Several monoclonal antibodies that primarily target the tumor-associated STn have been developed, including the well-studied clone, CC49 (12). CC49 has been subsequently utilized in multiple pre-clinical and clinical investigations using diagnostic imaging and radiotherapy (13–16) and also involved in multiple attempts of antibody humanization (17–20). An early clinical trial of a first-generation CAR T cell targeting TAG72 in colorectal cancer patients demonstrated safety, but with limited anti-tumor responses, likely attributed to the limited T cell persistence and/or anti-idiotype responses from inadequate scFv humanization (21). Given the optimization of CAR T cells in recent years, and the incorporation of intracellular co-stimulatory signaling domains in second-generation CARs that has greatly improved anti-tumor activity, cytokine production and T cell persistence, an evaluation of second-generation CAR T cells targeting TAG72 warrants further investigation.

Here, we describe the generation and anti-tumor efficacy of a second-generation CAR T cell with a humanized anti-human TAG-72 scFv antigen-binding domain and a 4-1BB intracellular co-stimulatory signaling domain (TAG72-BBζ). *In vitro*, TAG72-BBζ CAR T cells demonstrate potent antigen-dependent cytotoxicity against multiple TAG72-expressing human ovarian cancer cell lines and epithelial cells derived from patient ovarian cancer ascites. Furthermore, using *in vivo* peritoneal ovarian tumor models, we show that regional intraperitoneal delivery of TAG72-BBζ CAR T cells eliminate antigen-positive disease and extends overall survival of mice, while intravenous CAR T cell delivery was ineffective in controlling disease. We also demonstrate that repeat regional infusions of CAR T cells promote more durable control of disease compared to single treatment. However, reduced TAG72 expression was observed in early recurring tumors, which coincided with a lack of T cell persistence in our models. Interestingly, late recurring tumors showed re-expression of TAG72, which will require additional mechanistic investigations. These preclinical findings support TAG72-BBζ CAR T cells as a viable therapeutic option for ovarian cancers, and also highlight its broader application for multiple TAG72-expressing solid cancers.

MATERIALS AND METHODS

Cell Lines

The epithelial ovarian cancer line OVCAR-3 (herein referred to as OVCAR3, ATCC HTB-161) was cultured in RPMI-1640 (Lonza) containing 20% fetal bovine serum (FBS, Hyclone) and 1X antibiotic-antimycotic (1X AA, Gibco) (complete RPMI). The epithelial ovarian cancer line derived from metastatic ascites OV-90 (herein referred to as OV90, CRL-11732) was cultured in a 1:1 mixture of MCDB 105 medium (Sigma) and Medium 199 (Thermo) adjusted to pH of 7.0 with sodium hydroxide (Sigma) and final 20% FBS and 1X AA. The epithelial-endometroid ovarian cancer line COV362.4 (Sigma) was cultured in Dulbecco's Modified Eagles Medium (DMEM, Life Technologies) containing 10% FBS, 1X AA, 25 mM HEPES (Irvine Scientific), and 2 mM L-Glutamine (Fisher Scientific) (complete DMEM). The epithelial ovarian cancer line OVCAR-8 (herein referred to as OVCAR8) was a generous gift from Dr. Carlotta Glackin at City of Hope and was cultured in complete RPMI-1640. The epithelial ovarian cancer line SK-OV-3 (herein referred to as SKOV3, ATCC HTB-77) and the colon epithelial cancer line LS 174T (herein referred to as LS174T, ATCC CL-188) were cultured in complete DMEM. DU145-PSCA cells were described previously (22). All cells were cultured at 37°C with 5% CO_2.

DNA Constructs and Lentivirus Production

Tumor cells were engineered to express enhanced green fluorescent protein and firefly luciferase (eGFP/*ffluc*) by transduction with epHIV7 lentivirus carrying the eGFP/*ffluc* fusion under the control of the EF1α promoter as described previously (22). The humanized scFv sequence used in the CAR construct was obtained from a monoclonal antibody clone huCC49 that targets TAG72 (17). The extracellular spacer domain included the 129-amino acid middle-length CH2-deleted version (ΔCH2) of the IgG4 Fc spacer (23). The intracellular

co-stimulatory signaling domain contained was a 4-1BB with a CD4 transmembrane domain. The CD3ζ cytolytic domain was previously described (22). The CAR sequence was separated from a truncated CD19 gene (CD19t) by a T2A ribosomal skip sequence, and cloned in an epHIV7 lentiviral backbone under the control of the EF1α promoter. The PSCA-BBζ CAR construct was described previously (22).

Lentivirus was generated as previously described (22, 24). Briefly, 293T cells were transfected with packaging plasmid and CAR lentiviral backbone plasmid using a modified calcium phosphate method. Viral supernatants were collected after 3–4 days and treated with 2 mM magnesium and 25 U/mL Benzonase® endonuclease (EMD Millipore). Supernatants were concentrated via high-speed centrifugation and lentiviral pellets were resuspended in phosphate-buffered saline (PBS)-lactose solution (4 g lactose per 100 mL PBS), aliquoted and stored at −80°C. Lentiviral titers were quantified using HT1080 cells based on CD19t expression.

T Cell Isolation, Lentiviral Transduction, and *ex vivo* Expansion

Leukapheresis products were obtained from consented research participants (healthy donors) under protocols approved by the City of Hope Internal Review Board (IRB). On the day of leukapheresis, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll-Paque (GE Healthcare) followed by multiple washes in PBS/EDTA (Miltenyi Biotec). Cells were rested overnight at room temperature (RT) on a rotator, and subsequently washed and resuspended in X-VIVO T cell medium (Lonza) containing 10% FBS (complete X-VIVO). Up to 5.0×10^9 PBMC were incubated with anti-CD14 and anti-CD25 microbeads (Miltenyi Biotec) for 30 min at RT and magnetically depleted using the CliniMACS® system (Miltenyi Biotec) according to the manufacturer's protocol and these were termed depleted PBMCs (dPBMC). dPBMC were frozen in CryoStor® CS5 (StemCell Technologies) until further processing.

T cell activation and transduction was performed as described previously (22). Briefly, freshly thawed dPBMC were washed once and cultured in complete X-VIVO containing 100 U/mL recombinant human IL-2 (rhIL-2, Novartis Oncology) and 0.5 ng/mL recombinant human IL-15 (rhIL-15, CellGenix). For CAR lentiviral transduction, T cells were cultured with CD3/CD28 Dynabeads® (Life Technologies), protamine sulfate (APP Pharmaceuticals), cytokine mixture (as stated above), and desired lentivirus at a multiplicity or infection (MOI) of 1 the day following bead stimulation. Cells were then cultured in and replenished with fresh complete X-VIVO containing cytokines every 2–3 days. After 7 days, beads were magnetically removed, and cells were further expanded in complete X-VIVO containing cytokines to achieve desired cell yield. CAR T cells were positively selected for CD19t using the EasySep™ CD19 Positive Enrichment Kit I or II (StemCell Technologies) according to the manufacturer's protocol. Following further expansion, cells were frozen in CryoStor® CS5 prior to *in vitro* functional assays and *in vivo* tumor models. Purity and phenotype of CAR T cells were verified by flow cytometry.

Flow Cytometry

For flow cytometric analysis, cells were resuspended in FACS buffer (Hank's balanced salt solution without Ca^{2+}, Mg^{2+}, or phenol red ($HBSS^{-/-}$, Life Technologies) containing 2% FBS and 1× AA). Cells were incubated with primary antibodies for 30 min at 4°C in the dark. For secondary staining, cells were washed twice prior to 30 min incubation at 4°C in the dark with either Brilliant Violet 510 (BV510), fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein complex (PerCP), PerCP-Cy5.5, PE-Cy7, allophycocyanin (APC), or APC-Cy7 (or APC-eFluor780)-conjugated antibodies. Antibodies against CD3 (BD Biosciences, Clone: SK7), CD4 (BD Biosciences, Clone: SK3), CD8 (BD Bosciences, Clone: SK1), CD14 (BD Biosciences, Clone: MΦP9), CD19 (BD Biosciences, Clone: SJ25C1), CD25 (BD Biosciences, Clone: 2A3), mouse CD45 (BioLegend, Clone: 30-F11), CD45 (BD Biosciences, Clone: 2D1), CD69 (BD Biosciences, Clone: L78), CD137 (BD Biosciences, Clone: 4B4-1), MUC1 (BioLegend, Clone 16A), MUC16 (Abcam, Clone X75 or EPSISR23), biotinylated Protein-L (GenScript USA) (25), TAG72 (Clone, muCC49), Donkey Anti-Rabbit Ig (Invitrogen), Goat Anti-Mouse Ig (BD Biosciences), and streptavidin (BD Biosciences) were used. Cell viability was determined using 4′, 6-diamidino-2-phenylindole (DAPI, Sigma). Flow cytometry was performed on a MACSQuant Analyzer 10 (Miltenyi Biotec), and the data was analyzed with FlowJo software (v10, TreeStar).

In vitro Tumor Killing and T Cell Functional Assays

For tumor killing assays, CAR T cells and tumor targets were co-cultured at indicated effector:tumor (E:T) ratios in complete X-VIVO in the absence of exogenous cytokines in 96-well plates for 24–72 h and analyzed by flow cytometry as described above. Tumor cells were plated overnight prior to addition of T cells. Tumor killing by CAR T cells was calculated by comparing CD45-negative DAPI-negative (viable) cell counts relative to that observed when targets were co-cultured with Mock (untransduced) T cells. For T cell activation assays, CAR T cells and tumor targets were co-cultured at the indicated E:T ratios in complete X-VIVO in the absence of exogenous cytokines in 96-well plates for the indicated time points and analyzed by flow cytometry for specific markers of T cell activation. Frozen, uncultured patient primary ovarian cancer ascites (OAS3, OAS4, and OAS7) were thawed and immediately evaluated in T cell functional assays. A ascites fluid from ovarian cancer patients was obtained from City of Hope National Medical Center (COH) surgical staff in a sterile vacuum container with approval from the COH Institutional Review Board (IRB) and Office of Human Subjects Protection. The COH IRB waived the need for written informed consent as all samples were de-identified and ascites was discard material as previously described (26).

For T cell activation assays on plate-bound antigen, purified soluble TAG72 antigen (BioRad) was plated in duplicate at

indicated TAG72 units overnight at 4°C in 1X PBS in 96-well flat bottom high-affinity plates (Corning). Using a Bradford protein assay, the 20,000 units/mL stock solution of soluble TAG72 antigen was determined to be approximately 1.234 mg/mL of total protein. A total of 10^4 TAG72-BBζ CAR T cells were then added in a fixed volume of 100 μL to each well and incubated for indicated times prior to collection of cells for analysis of activation markers (CD69, CD137) by flow cytometry. Supernatants were also collected for analysis of cytokine production.

Elisa Cytokine Assays

Supernatants from tumor killing assays or CAR T cell activation assays on plate-bound TAG72 antigen were collected at indicated times and frozen at −20°C for further use. Supernatants were then analyzed for secreted human IFNγ and IL-2 according to the Human IFNγ and IL-2 ELISA Ready-SET-GO!® ELISA kit manufacturer's protocol, respectively. Plates were read at 450 nm using a Wallac Victor3 1420 Counter (Perkin-Elmer) and the Wallac 1420 Workstation software.

In vivo Tumor Studies

All animal experiments were performed under protocols approved by the City of Hope Institutional Animal Care and Use Committee. For *in vivo* tumor studies, OVCAR3 and OV90 cells (5.0×10^6) were prepared in a final volume of 500 μl $HBSS^{-/-}$ and engrafted in 6–8 weeks old female NSG mice by intraperitoneal (i.p.) injection. Tumor growth was monitored at least once a week via biophotonic imaging (Xenogen, LagoX) and flux signals were analyzed with Living Image software (Xenogen). For imaging, mice were i.p. injected with 150 μL D-luciferin potassium salt (Perkin Elmer) suspended in PBS at 4.29 mg/mouse. Once flux signals reached desired levels, day 8 for OV90 and day 14 for OVCAR3, T cells were prepared in 1X PBS, and mice were treated with 500 μL i.p. or 200 μL intravenous (i.v.) injection of 5.0 x 10^6 Mock or TAG72-BBζ CAR T cells. In the OV90 tumor model, we tested the impact of repeat treatment with i.p. TAG72-BBζ CAR T cells starting at day 8, followed by treatments at additional indicated days post-tumor engraftment. Humane endpoints were used in determining survival. Mice were euthanized upon signs of distress such as a distended belly due to ascites, labored or difficulty breathing, apparent weight loss, impaired mobility, or evidence of being moribund. At pre-determined time points or at moribund status, mice were euthanized and tissues and/or ascites fluid were harvested and processed for flow cytometry and immunohistochemistry as described below.

Peripheral blood was collected from isoflurane-anesthetized mice by retro-orbital (RO) bleed through heparinized capillary tubes (Chase Scientific) into polystyrene tubes containing a heparin/PBS solution (1000 units/mL, Sagent Pharmaceuticals). Volume of each RO blood draw (approximately 120 μL/mouse) was recorded for cell quantification per μL blood. Red blood cells (RBCs) were lysed with 1X Red Cell Lysis Buffer (Sigma) according to the manufacturer's protocol and then washed, stained, and analyzed by flow cytometry as described above. Cells from i.p. ascites fluid was collected from euthanized mice by injecting 5 mL cold 1X PBS into the i.p. cavity, which was drawn up via syringe and stored on ice until further processing. RBC-depleted ascites was washed, stained, and analyzed by flow cytometry for tumor-associated glycoprotein expression and CAR T cells using antibodies and methods described above.

Immunohistochemistry

Tumor tissue was fixed for up to 3 days in 4% paraformaldehyde (4% PFA, Boston BioProducts) and stored in 70% ethanol until further processing. Immunohistochemistry was performed by the Pathology Core at City of Hope. Briefly, paraffin-embedded sections (10 μm) were stained with hematoxylin & eosin (H&E, Sigma-Aldrich), mouse anti-human CD3 (DAKO), mouse anti-human TAG72 (AB16838, Abcam), rabbit anti-human MUC1 (AB45167, Abcam), MUC16 (AB1107, Abcam). Images were obtained using the Nanozoomer 2.0HT digital slide scanner and the associated NDP.view2 software (Hamamatzu).

Statistical Analysis

Data are presented as mean ± SEM, unless otherwise stated. Statistical comparisons between groups were performed using the unpaired two-tailed Student's *t*-test to calculate *p*-value, unless otherwise stated. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; NS, not significant.

RESULTS

TAG72-CAR T Cells Containing a 4-1BB Intracellular Co-stimulatory Domain Demonstrate *in vitro* Activation Against Purified TAG72

Our first goal was to develop a second-generation TAG72-BBζ CAR construct containing the humanized scFv CC49, the human IgG4 Fc extracellular spacer lacking a CH2 domain (ΔCH2), the CD4 transmembrane domain, the 4-1BB intracellular co-stimulatory domain, and the CD3ζ cytolytic domain followed by a truncated CD19 (CD19t) for cell tracking (**Figure 1A**). We selected this CAR construct based on our recent preclinical investigations demonstrating potent anti-tumor activity of 4-1BB-containing CARs for solid tumors (22, 24, 27). TAG72-BBζ CAR lentivirus was used to transduce human healthy donor-derived peripheral blood mononuclear cells depleted of CD14+ and CD25+ cells (dPBMC), as previously described (22). TAG72-BBζ CAR T cells were enriched during the manufacturing process (based on CD19t+ selection) and were stably expressed on the surface of T cells (**Figure 1B**). CAR T cells expanded *ex vivo* with similar kinetics and comparable CD4:CD8 ratios to Mock (untransduced) T cells (data not shown and **Figure 1C**). Importantly, and as a first measure of CAR T cell activation against TAG72, we demonstrated dose-dependent CD137 expression on the surface of TAG72-BBζ CAR T cells when cultured with plate-bound, but not soluble, purified TAG72 (**Figure 1D**). Similar dose-dependent induction of cell-surface CD69 expression and IFNγ release was observed with plate-bound TAG72 (**Supplemental Figures 1A,B**).

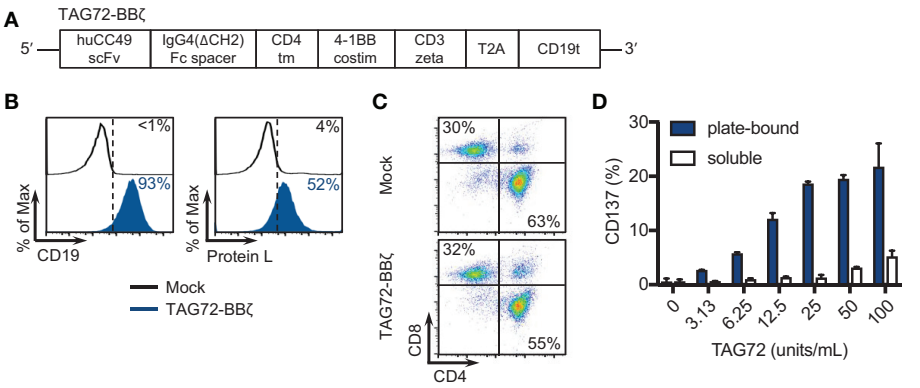

FIGURE 1 | TAG72-specific CAR T cells containing a 4-1BB intracellular co-stimulatory domain. **(A)** Diagram of the lentiviral expression cassette with TAG72-CARs containing the humanized scFv (CC49 clone) targeting TAG72, with a 129 amino acid modified human IgG4 Fc linker (void of the CH2 domain, ΔCH2), a CD4 transmembrane domain, a cytoplasmic 4-1BB costimulatory domain, and a cytolytic CD3ζ domain. A truncated non-signaling CD19 (CD19t), separated from the CAR sequence by a T2A ribosomal skip sequence, was expressed for identifying lentivirally transduced T cells. **(B)** Mock (untransduced) and TAG72-BBζ CAR T cells were evaluated by flow cytometry for CD19t expression to detect lentiviral transduction of CARs (left) or Protein L to detect the scFv (right). **(C)** CD4 and CD8 expression in Mock (top) and TAG72-BBζ CAR T cells (bottom). **(D)** Activation (expression of CD137) was assessed by flow cytometry with *in vitro* stimulated CAR T cells against soluble or plate-bound purified TAG72 antigen for 24 h at indicated protein amounts.

TAG72-BBζ CAR T Cells Effectively Target Ovarian Cancer Cells *in vitro*

We next sought to evaluate cell-surface TAG72 expression on human ovarian cancer cell lines, including SKOV3, OVCAR8, COV362.4, OVCAR3, OV90, as well as the TAG72+ colon cancer line, LS174T. Prior studies have demonstrated expression of TAG72 by immunohistochemistry of ovarian tumor patient samples and by western blotting of human ovarian cancer cell lines (11, 28). By flow cytometry, TAG72 was expressed on OVCAR3 cells (approximately 42%) and to a greater extent on OV90 cells (approximately 90%), with very low levels detected on COV362.4 cells (**Figure 2A**). TAG72 was absent on SKOV3 and OVCAR8 cells. Immunofluorescence staining of tumor cells confirmed TAG72 expression and cellular localization on the cell surface as well as intracellularly (data not shown). Importantly, we observed higher expression of TAG72 on OVCAR3 and OV90 cells harvested from the ascites of tumor-bearing animals as compared to *in vitro* cultured cells (**Supplemental Figure 2**).

To assess antigen-dependent activity of our TAG72-BBζ CAR T cells, we performed co-cultured assays with TAG72-positive and -negative ovarian tumor targets at an E:T ratio between 1:1 and 1:2 to determine their killing potential. After 24 h, antigen-specific T cell-mediated killing activity was evident with TAG72-BBζ CAR T cells relative to Mock T cells (**Figure 2B**). Amongst TAG72-expressing targets, an average of 59% LS174T, 79% OVCAR3, and 67% OV90 cells were killed. After 72 h, killing of the same tumor lines increased to 77, 90, and 97%, respectively. TAG72-BBζ CAR T cells showed minimal killing of TAG72-negative or low expressing SKOV3, OVCAR8, and COV362.4 cells. We further demonstrated the specificity of our TAG72-CAR T cells using a previously described CAR targeting prostate stem cell antigen (PSCA) with the same CAR design (22) (**Supplemental Figure 3**). At 72 h, we observed TAG72-BBζ CAR T cell expansion (2–3 fold) against TAG72-positive tumor cells (**Figure 2C**). Similar tumor killing was observed at lower E:T ratios of 1:10 (data not shown), demonstrating the potent killing ability of TAG72-BBζ CAR T cells. TAG72 is shed from tumor cells in a soluble form (29), which we showed minimally impacted the tumor killing ability of TAG72-BBζ CAR T cells (**Supplemental Figure 4**). We then evaluated cytokine production from CAR T cells as an additional measure of T cell activity. IFNγ and IL-2 cytokine production was observed only when TAG72-BBζ CAR T cells were co-cultured with antigen-positive tumor targets, OVCAR3, LS174T, and OV90 (**Figures 2D,E**). While IL-2 production peaked at early time points (24 h) and was detectable only against OVCAR3 at later time points (72 h), IFNγ levels were more sustained over 72 h.

TAG72-BBζ CAR T Cells Target TAG72-Positive Cells From Ovarian Cancer Ascites *in vitro*

To further confirm TAG72 as an ovarian cancer CAR target and the anti-tumor activity of our TAG72-BBζ CAR T cells, we performed *in vitro* assays utilizing human ovarian cancer ascites from three patients (OAS3, OAS4, OAS7). Freshly thawed ascites from OAS3, OAS4, and OAS7 expressed 62, 80, and 67% TAG72, respectively, by flow cytometry (data not shown), but after 72 h in culture, was reduced to 2, 53, and 19%, respectively (**Figure 2F**), likely reflecting an influence of *ex vivo* culturing conditions on maintenance of TAG72 expression (30). We then evaluated the cytolytic activity of CAR T cells after 72 h of co-culture with freshly thawed patient primary ovarian cancer ascites, and showed potent and selective CAR-mediated killing of the TAG72-positive OAS4 and OAS7 cells, with no detectable anti-tumor activity against the TAG72-negative OAS3 cells (**Figure 2G**). TAG72-BBζ CAR T cells produced IFNγ and IL-2 against OAS4, but not OAS3 and OAS7 cells (**Figure 2H**, **Supplemental Figure 5**).

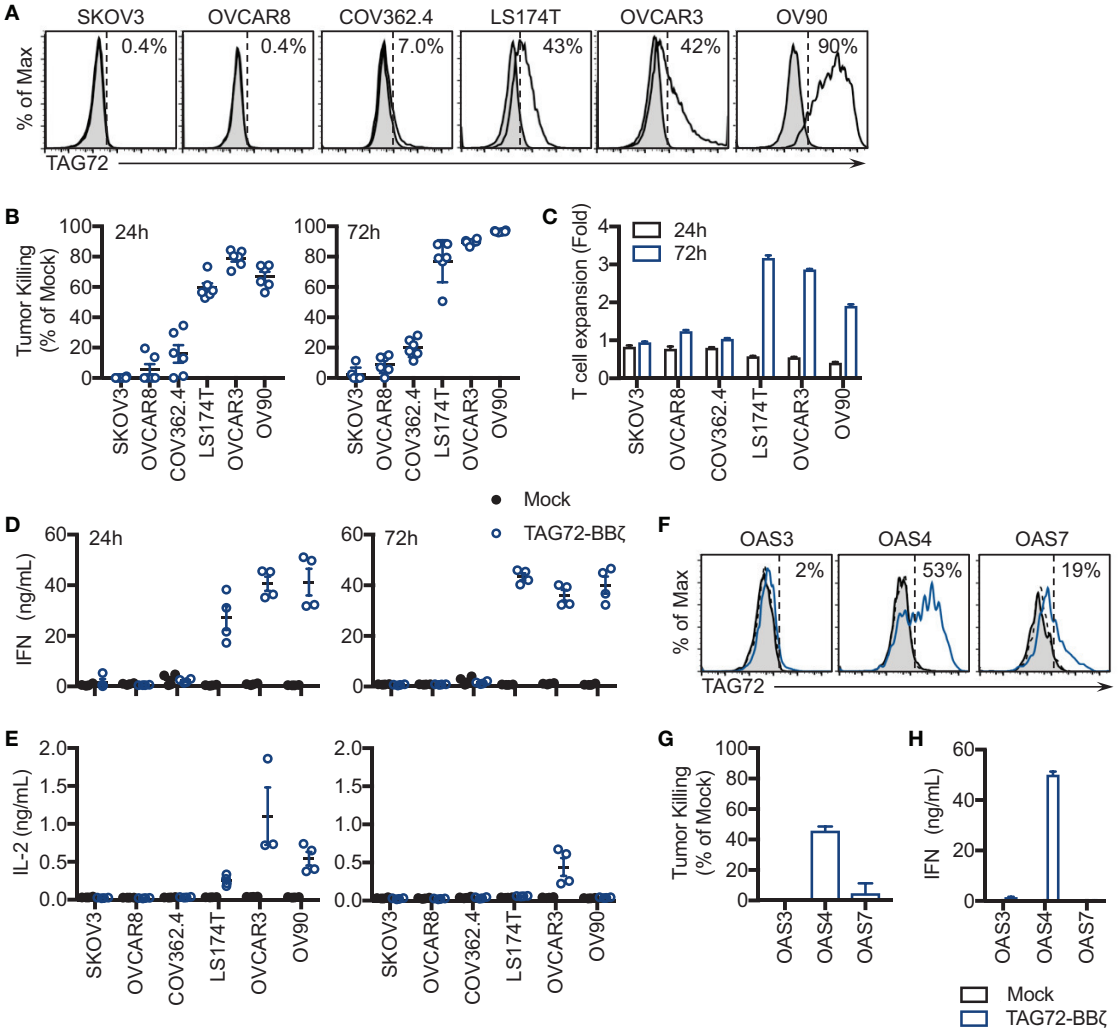

FIGURE 2 | TAG72-BBζ CAR T cells show antigen-dependent cytokine production and tumor killing *in vitro*. **(A)** Flow cytometric analysis of TAG72 surface expression on multiple ovarian and colorectal (LS174T) cancer cell lines. **(B)** Quantification of tumor killing by TAG72-BBζ CAR T cells relative to Mock at an E:T ratio of 1:1, following a 24 and 72 h co-culture with antigen-positive and -negative tumor targets as described in Materials and Methods. **(C)** TAG72-BBζ CAR T cell expansion at 24 and 72 h following co-culture with indicated tumor targets at an E:T ratio of 1:1. **(D,E)** IFNγ and IL-2 levels in supernatant quantified by ELISA from Mock or TAG72-BBζ CAR T cells following a 24 and 72 h co-culture with indicated tumor targets at an E:T ratio of 1:1. **(F)** Flow cytometric analysis of TAG72 surface expression on primary human ovarian cancer cells harvested from patient ascites (OAS) after 72 h in culture. **(G)** Quantification of tumor killing and **(H)** IFNγ production by TAG72-BBζ CAR T cells relative to Mock following a 72 h co-culture with freshly thawed OAS cells at an E:T ratio of 1:1.

Regional Intraperitoneal Delivery of TAG72-BBζ CAR T Cells Exhibits Potent Anti-tumor Activity and Extends Survival in Ovarian Ascites-Bearing Mice

To evaluate the therapeutic potential of our TAG72-BBζ CAR T cells *in vivo*, we first established TAG72+ OVCAR3 tumors in immune compromised NSG mice by intraperitoneal (i.p.) injection, to mimic peritoneal ovarian tumors observed in late-stage human disease. OVCAR3 cells were lentivirally transduced to express eGFP/*ffluc* to allow for tracking of tumor growth via non-invasive optical imaging. At 14 days post-tumor i.p. injection, mice were treated with Mock or TAG72-BBζ CAR T cells (5.0×10^6) by systemic intravenous (i.v.) or regional i.p. delivery (**Figure 3A**). We observed rapid anti-tumor effects in mice treated with TAG72-BBζ CAR T cells via regional i.p. delivery, reaching a maximal anti-tumor response 1–2 weeks following treatment (**Figures 3B,C**). In comparison to regional delivery, i.v. delivery of TAG72-BBζ CAR T cells showed limited anti-tumor responses. Anti-tumor responses in mice were durable for 3–4 weeks, but ultimately tumor recurrences were observed in mice. Regional i.p. delivery of TAG72-BBζ CAR T

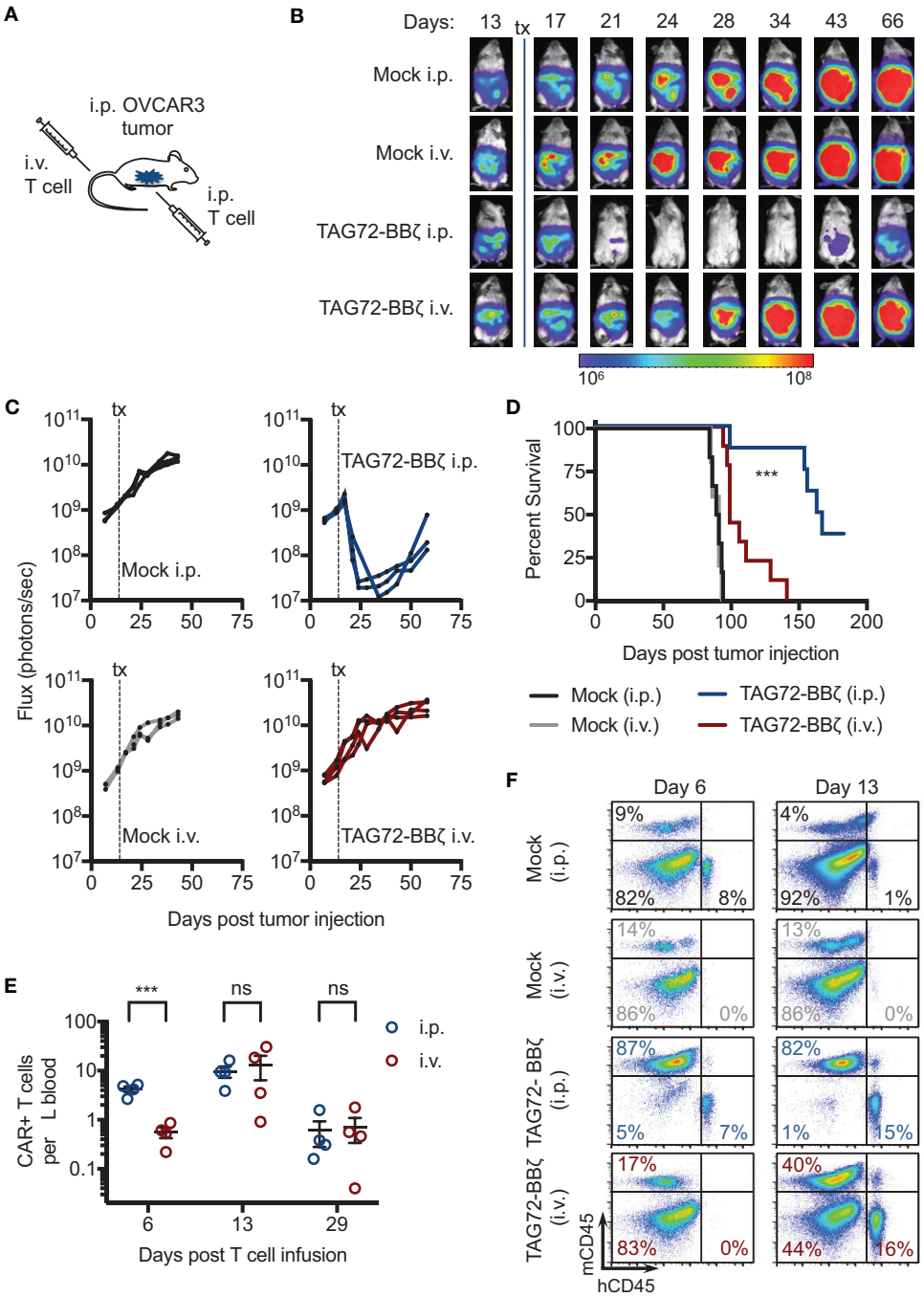

FIGURE 3 | Regional intraperitoneal delivery of TAG72-BBζ CAR T cells significantly reduces tumor burden and extends overall survival of OVCAR3 tumor-bearing mice. **(A)** Schematic illustrating i.p. engraftment of 5.0 × 10^6 OVCAR3(eGFP/*ffluc*) tumor cells in NSG mice, followed by either i.v. or i.p. delivery of 5.0 ×10^6 Mock or TAG72-BBζ CAR T cells on day 14 post-tumor injection. **(B)** Representative bioluminescent flux imaging of mice treated i.v. or i.p. with Mock or TAG72-BBζ CAR T cells. **(C)** Quantification of flux (each mouse) from OVCAR3(eGFP/*ffluc*) tumor-bearing mice treated i.v. or i.p. with Mock or TAG72-BBζ CAR T cells. $N = 3$–4 per group. Data are representative of two independent experiments. **(D)** Kaplan–Meier survival for Mock and TAG72-BBζ CAR T cell treated mice. $N \geq 4$ mice per group. Combined data from two independent experiments. **(E)** Quantification of TAG72-BBζ CAR T cells per μL blood at 6, 13, and 29 days post-treatment. $N = 4$ per group. **(F)** Representative flow cytometric analysis of the frequency of human CD45+ (hCD45) and mouse CD45+ (mCD45) cells in the i.p. cavity of tumor-bearing mice at day 6 and 13 post-treatment. Representative images from two independent experiments. ***$p < 0.001$.

cells significantly extended survival of mice, with limited benefits observed by i.v. delivery (**Figure 3D**).

To address potential differences observed between i.p. and i.v. therapy, we measured CAR T cells in the blood and ascites of mice. Strikingly, appreciable numbers of CAR T cells (huCD45+CD19t+) were found in the blood of mice 6 days post i.p. treatment, with more than 5-fold fewer CAR T cells in the blood of i.v. treated mice at the same time point (**Figure 3E**, **Supplemental Figure 6**). However, we observed equivalent numbers of CAR T cells in the blood of i.p. and i.v. treated mice at later time points, expanding from 1 to 2 weeks, with significant reductions at 4 weeks post-treatment. We then evaluated CAR T cell presence in the ascites of treated mice, and observed CAR T cells at the site of tumors at day 6 post i.p. treatment, with no detectable CAR T cells in i.v. treated mice at the same time point. However, at day 13 post-treatment, similar levels of CAR T cells were observed in mice treated i.v. and i.p. (**Figure 3F**). These data suggest that CAR T cells eventually reached the tumor following i.v. delivery but with delayed kinetics compared with i.p. delivery, which was likely in part responsible for the lack of observed therapy by this route of administration. CD45-negative cells, likely majority being OVCAR3 tumor cells, were significantly depleted in i.p. TAG72-BBζ CAR T cell treated mice, but not i.p. or i.v. Mock T cell or i.v. TAG72-BBζ CAR T cell treated mice. These data support regional intraperitoneal delivery of TAG72-CAR T cells as an effective method of targeting peritoneal ovarian tumors in mice.

Repeat Treatment With TAG72-BBζ CAR T Cells Controls Tumor More Effectively

Based on our findings with TAG72-BBζ CAR T cells in OVCAR3-bearing mice, we next evaluated the OV90 i.p. model, with more uniform TAG72 expression *in vitro* compared with OVCAR3 (**Figure 2A**). We first confirmed effectiveness of regional CAR T cell delivery in this model and showed similarly to the OVCAR3 model, i.p., but not i.v. TAG72-BBζ CAR T cell treatment showed anti-tumor efficacy in the OV90 model (**Supplemental Figure 7A**). Overall survival was only delayed by approximately 25 days in this model with i.p. delivery of TAG72-BBζ CAR T cells (**Supplemental Figure 7B**), likely owing to the aggressive nature of this model. We therefore evaluated whether repeat TAG72-BBζ CAR T cell dosing compared with a single dose improves therapeutic responses (**Figure 4A**). Compared with a single dose of TAG72-BBζ CAR T cells, repeat dosing over the course of 1 month demonstrated more durable anti-tumor responses in the OV90 model (**Figures 4B,C**). When plotted as relative tumor growth kinetics, repeat dosing promoted more extensive tumor regression as well as more durable control of tumors compared with single dosing (**Figure 4D**).

In this study, the overall survival was extended significantly in mice that received repeat doses of TAG72-BBζ CAR T cells (55 day benefit) compared with a single dose (30 day benefit) (**Figure 4E**). Greater T cell numbers were observed in peritoneal tumors of mice with repeat treatment (**Figure 4F**). Importantly, however, we observed reduced numbers, expansion and persistence of CAR T cells in the blood of OV90-bearing mice, compared with the OVCAR3 model (**Supplemental Figures 8A,B**), suggesting that this more aggressive tumor model may also harbor suppressive mechanisms that hamper T cell function and overall CAR T cell efficacy. Collectively, these data demonstrate potent anti-tumor activity of TAG72-BBζ CAR T cells in ovarian cancer xenograft models, and also suggest that repeat dosing of regionally delivered CAR T cells may provide greater control of tumors compared with a single dose.

Tumor Recurrences Following TAG72-CAR T Cell Therapy Show Antigen Escape

One of the major resistance mechanisms to CAR T cell therapy is the tumor antigen heterogeneity that exists in solid tumors that promotes eventual antigen loss or escape (4). While the loss of CAR T cells in our two models preceded tumor recurrences, we next evaluated expression of TAG72 in tumors from Mock and TAG72-BBζ CAR T cell treated mice at various time points pre- and post- therapy. Since TAG72, MUC1, and MUC16 have all been identified as potential targets in ovarian cancer, we first assessed expression of these cell surface antigens on TAG72-negative OVCAR8, and TAG72-positive OVCAR3 and OV90 cells. OVCAR8 appeared to only express low levels of MUC1, and was absent for TAG72 and MUC16, while OVCAR3 expressed all three antigens at varying levels, and OV90 showed low expression of MUC1 and was absent for MUC16 (**Figure 5A**). Therefore, we evaluated the expression of these antigens in OVCAR3 tumors from mice treated with Mock or TAG72-BBζ CAR T cells. At 12 weeks post T cell infusion, tumors from Mock-treated mice showed heterogeneous expression of TAG72 (similar to flow cytometric analysis of the cell line), MUC16, and MUC1 (**Figure 5B**). However, tumor recurrences at early time points from mice treated with TAG72-BBζ CAR T cells showed a dramatic reduction in TAG72 expression, while maintaining expression of MUC16 and MUC1. Similarly, repeat treatment of TAG72-BBζ CAR T cells in the OV90 tumor model also showed a reduction in TAG72 expression in early recurrent tumors following treatment (**Figure 5C**). Interestingly, the expression of TAG72 was detected at high levels in tumor recurrences at later time points, in solid tumors as well as in ascites (**Figures 5C,D**). We further confirmed this finding *in vitro*, showing that residual viable OVCAR3 tumor cells remaining after CAR T cell co-culture expressed lower TAG72, but showed typical TAG72 expression levels on tumor cells that grew out in the absence of CAR T cells (**Figure 5E**). Similar reductions in TAG72 expression were observed with OV90 cells (data not shown). These data suggest that antigen escape plays a key role in tumor recurrences following TAG72-BBζ CAR T cell therapy.

DISCUSSION

In this study, we evaluated a second-generation TAG72-specific CAR T cell with a 4-1BB intracellular co-stimulatory signaling domain in preclinical models of ovarian cancer. TAG72-CAR T cells demonstrated significant anti-tumor activity against peritoneal ovarian tumors when administered via regional

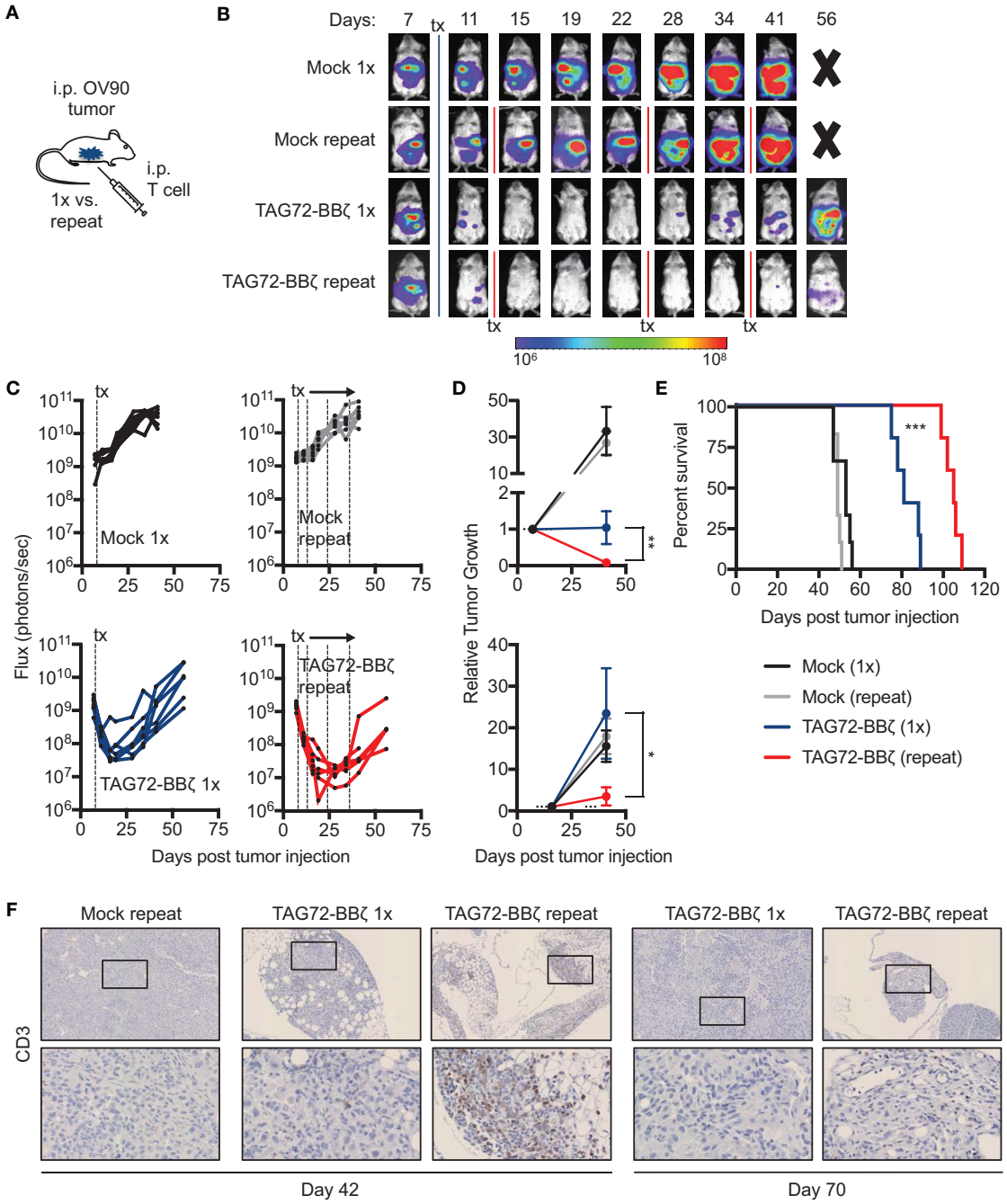

FIGURE 4 | Repeat regional administration of TAG72-BBζ CAR T cells significantly reduces tumor burden and extends overall survival of OV90 tumor-bearing mice. **(A)** Schematic illustrating i.p. engraftment of 5.0×10^6 OV90(eGFP/*ffluc*) tumor cells in NSG mice, followed by either single or repeat i.p. treatment with 5.0×10^6 Mock or TAG72-BBζ CAR T cells on day 8 post-tumor infection. **(B)** Representative bioluminescent flux imaging of mice treated i.p. with a single or repeat treatment of Mock or TAG72-BBζ CAR T cells. **(C)** Quantification of flux (each mouse) from OV90(eGFP/*ffluc*) tumor-bearing mice with single or repeat i.p. treatment of Mock or TAG72-BBζ CAR T cells. **(D)** Top graph: relative tumor growth (flux) from day 7 (one day prior to start of treatment) to day 46 (end of the repeat treatment window). Bottom graph: relative tumor growth (flux) from day 16 (maximum treatment response) to day 46. Fluxes at day 7 or day 16 were normalized to "1" to reflect fold differences in tumor growth compared to day 46. Mann-Whitney test was performed to calculate *p*-values. **(E)** Kaplan-Meier survival for Mock and TAG72-BBζ CAR T cell treated mice. $N \geq 5$ mice per group. **(F)** Histology of human CD3 cells in tumors harvested from single and repeat treated mice at days 42 and 70 post-tumor injection (top: 10X magnification, bottom: 40X magnification). All data are representative of two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

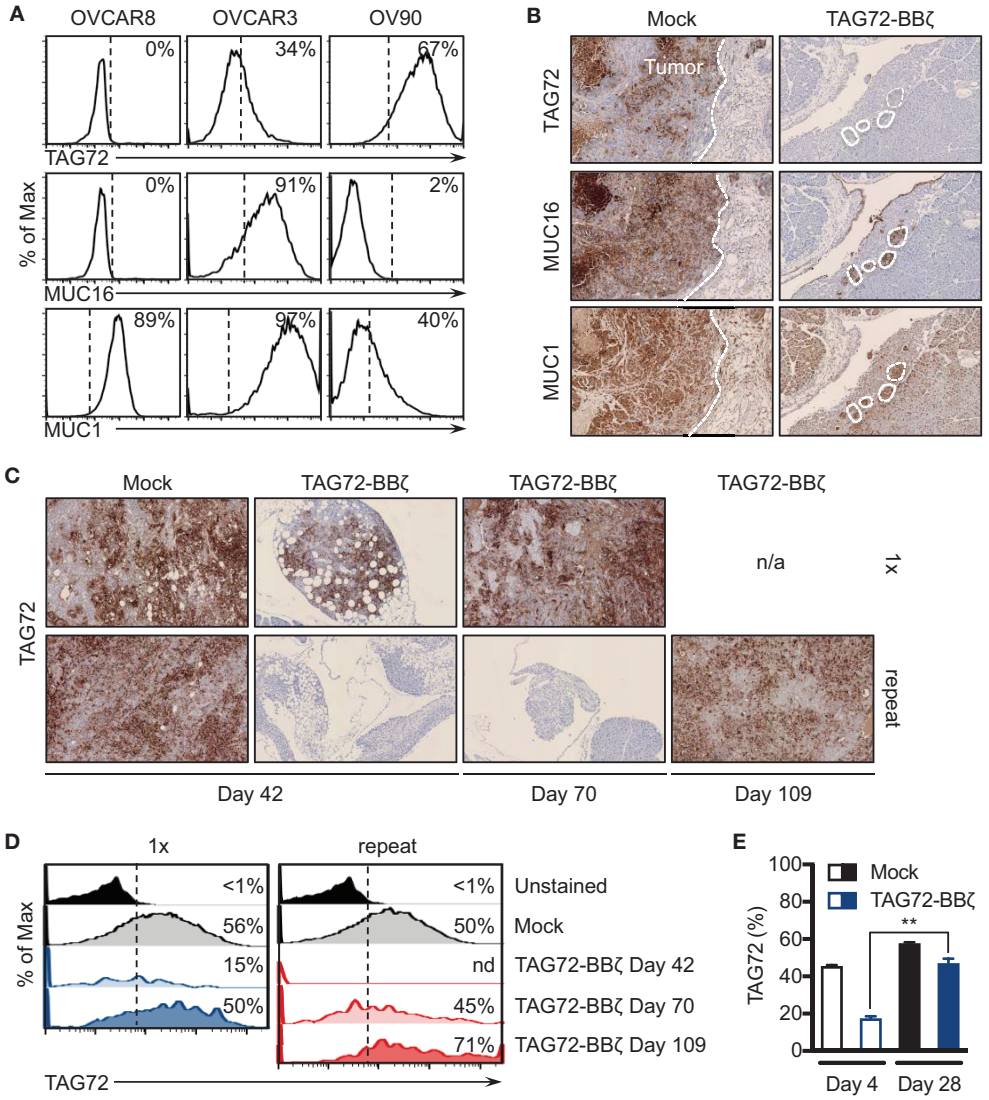

FIGURE 5 | Tumor-associated glycoprotein antigen heterogeneity in ovarian cancer and CAR T cell-mediated antigen escape. **(A)** Flow cytometric analysis of TAG72, MUC16, and MUC1 surface expression on OVCAR8, OVCAR3, and OV90 human ovarian cancer cell lines. **(B)** Histology of TAG72, MUC16, and MUC1 expression in i.p. solid tumors harvested from Mock and TAG72-BBζ CAR T cell treated OVCAR3 tumor-bearing mice at day 99 post-treatment. 10X magnification. **(C)** Histology of TAG72 expression on solid tumors harvested from single and repeat treated OV90 tumor-bearing mice at day 42, 70, and 109 post-tumor injection. 10X magnification. **(D)** Flow cytometric analysis of TAG72 expression in OV90 tumor cells harvested from ascites at indicated time points from mice that received single or repeat i.p. treatment. **(E)** TAG72 expression on OVCAR3 cells at day 4 following co-culture with Mock or TAG72-BBζ CAR T cells (1:10 E:T ratio), and on tumor cells that grew out at day 28. $^{**}p < 0.01$.

intraperitoneal delivery. While we did not directly compare our CAR construct to first-generation TAG72-CARs, which have been previously published (21, 31, 32), substantial data in the field now show superiority of CARs containing co-stimulatory domains compared with first-generation CD3ζ-only CARs (3). Our studies also did not evaluate TAG72-CARs containing CD28 co-stimulation. However, our recent findings with PSCA- and HER2-directed CAR T cells show that while CD28-containing CAR T cells exhibit potent anti-tumor activity in solid tumors, undesirable increases in T cell exhaustion markers, limited persistence, and targeting of tumor cells that express very low levels of antigen may potentiate off-tumor toxicity, compared with 4-1BB-containing CARs (22, 24). Similar findings have been observed by other groups (33–35). In a recent publication detailing the use of TAG72-ζ CAR T cells in the context of colorectal cancer, systemic administration of CAR T cells was well tolerated in patients and demonstrated signs of transient on-target activity (21). However, limited anti-tumor responses in these patients was in part attributed to a lack of T cell persistence with a first-generation CAR construct lacking co-stimulation. In addition to recent *in vitro* work that highlight the potential of TAG72-directed CARs with co-stimulation (36), our current

study demonstrates the anti-tumor activity of second-generation humanized TAG72-BBζ CAR T cells using clinically relevant *in vivo* models of ovarian cancer. While safety of targeting STn antigens (i.e., TAG72) with our CAR T cells was not addressed in the current study, the early clinical experience with first-generation TAG72-CAR T cells (21), along with recent studies using antibody-drug conjugates in non-human primates (37) provides some evidence of safety in targeting these antigens. Further clinical studies are required to evaluate the safety of these new second-generation TAG72-CAR T cells in patients.

Recent work by our group and others have suggested that regional administration of CAR T cells may improve CAR T cell therapeutic efficacy in several solid tumor models (24, 38–40). Our studies also demonstrate potent anti-tumor activity by regional intraperitoneal administration of TAG72-CAR T cells, compared to limited activity with intravenous delivery, using xenograft models of peritoneal ovarian tumors. In our models, i.v. administered CAR T cells did show trafficking to tumor sites at later time points, however, i.p. administered CAR T cells were observed at tumor sites early following treatment, likely driving more immediate anti-tumor responses, compared with systemic delivery of CAR T cells (**Figure 3**). Similar findings were observed previously with CAR T cells targeting peritoneal ovarian tumors (40). We believe that the delayed kinetics in T cell trafficking by i.v. delivery may have insufficiently controlled tumor burden compared with regional delivery, which may be overcome by increasing the dose of i.v. delivered CAR T cells in this model. However, the clinically feasible regional delivery of CAR T cells may provide immediate anti-tumor activity with improved overall therapeutic responses.

Antigen heterogeneity is a major obstacle to the successful translation of CAR T cell therapies for solid tumors. Expression analysis of MUC1, MUC16, and TAG72 on patient samples from various epithelial subtypes of ovarian cancer highlights antigen heterogeneity in this disease and demonstrates the aberrant expression pattern of cell-surface glycoproteins. Although we show antigen-specific targeting and extended survival of mice treated with our TAG72-CAR T cells using two human xenograft peritoneal ovarian tumor models, tumor recurrences were observed in all treated animals. In both the OVCAR3 and OV90 models, tumor recurrences at early time points following CAR T cell treatment were TAG72 low/negative, but maintained MUC16 and/or MUC1 expression. These findings suggest that multi-targeted CAR T cells approaches, which have been developed as either tandem (41, 42) or dual CAR strategies (43, 44) may provide more durable therapeutic responses in tumors with high antigen heterogeneity. Importantly, CAR T cells have already been developed for both MUC16 (40) and MUC1 (45) including a tumor-specific glycoform, Tn-MUC1 (46), and therefore, further exploitation of these targets for treating advanced ovarian cancer is in order. Unexpectedly, we observed TAG72 expression in tumor recurrences at later time points, suggesting that while early resistance mechanisms to CAR T cell therapy may be driven by reduction in tumor antigen density, the absence of CAR T cell selective pressure may have allowed for TAG72 to be re-expressed at later time points. Possible mechanisms include reduced TAG72 expression by downregulation of the enzyme α2,6-sialyl-transferase, transient internalization of TAG72 following exposure to CAR T cells, and pre-existing tumor cells with lower TAG72 that are not targeted by CAR T cells. Similar reductions in tumor antigen density have been observed in leukemia relapses following CAR T cell therapy (47). Additional studies are needed in order to gain a more detailed biological understanding of this observation, which may extend to other tumor antigens including those resulting from aberrant glycosylation. Of note, prior studies have demonstrated that type I and II interferons increase the expression of TAG72 (21, 48), which may also be explored in this setting to increase the tumor antigen density for targeting by CAR T cells.

In the current study, we demonstrated that repeat therapy with TAG72-BBζ CAR T cells increased both maximal therapeutic responses as well as disease control in the OV90 model. While we anticipate that eventual antigen escape-dependent tumor recurrences would have been observed even if repeat treatment continued, it is also plausible that a more optimized CAR T cell with increased persistence may obviate need for repeat therapy. It is noteworthy that our TAG72-CAR T cells also showed significant differences in persistence and expansion in the more aggressive OV90 model, when compared with the OVCAR3 model, suggesting that in addition to antigen escape, other mechanisms may also potentially be limiting the durability of the therapy. For instance, reduced *in vitro* T cell expansion and *in vivo* T cell persistence against OV90 may also be, in part, due to the lower *in vitro* IL-2 production upon TAG72-CAR T cell activation. Therefore, increasing T cell persistence in the solid tumor microenvironment is also imperative, and has been demonstrated recently by several groups engineering CAR T cells with additional supportive cytokines (49–51). However, as recently reported for other advanced tumors (52), improved persistence of T cells within ovarian tumors will be likely be achieved in the context of multi-targeted CAR approaches.

AUTHOR CONTRIBUTIONS

JM and SP conception and design; JM, AK, W-CC, and SP development of methodology; JM, AK, HL, and MR acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.); JM, AK, HL, MR, W-CC, MC, SF, and SP analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis); JM, AK, MR, W-CC, PY, DC, JS, MC, SF, and SP writing, review, and/or revision of the manuscript; JM, AK, MR, W-CC, PY, DC, JS, SF, and SP administrative, technical, or material support (i.e., reporting or organizing data, constructing databases); SF and SP Study supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02268/full#supplementary-material

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REVIEW
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CAR T Cell Therapy of Non-hematopoietic Malignancies: Detours on the Road to Clinical Success

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Chimeric antigen receptor (CAR)-engineered T cells represent a breakthrough in personalized medicine. In this strategy, a patient's own T lymphocytes are genetically reprogrammed to encode a synthetic receptor that binds a tumor antigen, allowing T cells to recognize and kill antigen-expressing cancer cells. As a result of complete and durable responses in individuals who are refractory to standard of care therapy, CAR T cells directed against the CD19 protein have been granted United States Food and Drug Administration (FDA) approval as a therapy for treatment of pediatric and young adult acute lymphoblastic leukemia and diffuse large B cell lymphoma. Human trials of CAR T cells targeting CD19 or B cell maturation antigen in multiple myeloma have also reported early successes. However, a clear and consistently reproducible demonstration of the clinical efficacy of CAR T cells in the setting of solid tumors has not been reported to date. Here, we review the history and status of CAR T cell therapy for solid tumors, potential T cell-intrinsic determinants of response and resistance as well as extrinsic obstacles to the success of this approach for much more prevalent non-hematopoietic malignancies. In addition, we summarize recent strategies and innovations that aim to augment the potency of CAR T cells in the face of multiple immunosuppressive barriers operative within the solid tumor microenvironment. Advances in the field of CAR T cell biology over the coming years in the areas of safety, reliability and efficacy against non-hematopoietic cancers will ultimately determine how transformative adoptive T cell therapy will be in the broader battle against cancer.

Keywords: CAR T cell, immunotherapy, cancer, solid tumor, microenvironment, adoptive cell therapy, non-hematopoietic malignancy

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INTRODUCTION

The use of genetically engineered T cells as a form of cancer therapy heralds a new era of synthetic biology and medicine. Within the past few years, clinical trials using chimeric antigen receptor (CAR) T cells to recognize and eliminate hematopoietic malignancies have demonstrated high rates of response as well as durability of remission that are unprecedented in ALL (1–3), chronic

lymphocytic leukemia (CLL) (4, 5), and refractory B cell lymphomas (6, 7). This culminated in the recent United States Food and Drug Administration approvals of CD19-directed CAR T cells for relapsed/refractory pediatric and young adult ALL and diffuse large B cell lymphoma (DLBCL). While CAR T cell therapy is poised to revolutionize the treatment of leukemias and lymphomas, the field awaits a clear demonstration of efficacy against non-hematopoietic malignancies. The key challenges for these immunotherapies are how to: (I) safely enhance the potency and sustain the function of CAR T cells *in vivo* and (II) develop mechanism-based strategies to increase the resistance of CAR T cells to intrinsic and extrinsic dysfunction. Advances in basic and translational research aimed at improving the safety, consistency and effectiveness of CAR T cells against tumors of non-hematopoietic origin will ultimately determine whether this approach can find wider applications in cancer as well as other diseases.

Adoptive cellular immunotherapy involves expanding T cells from a patient or donor *in vitro*, followed by reinfusion of tumor-specific lymphocytes as cancer therapy. Transfer of expanded tumor infiltrating lymphocytes (TILs) from a subset of individuals with metastatic melanoma has shown potent anti-tumor effects (8, 9). It is likely that TILs target neoantigens within the broad landscape of mutant peptides encoded by *de novo* somatic mutations (10–14). In rare instances, adoptive transfer of autologous T cells targeting antigens encoded by somatically mutated genes has also resulted in clinically meaningful regressions of colon, metastatic bile duct, cervical and breast cancers (15–19). However, this strategy has little effect on other common epithelial malignancies that have lower mutation rates.

Transfer of genetically-redirected T cells bypasses many of the mechanisms involved in immunological tolerance by the creation of antigen-specific lymphocytes independently of intrinsic tumor immunogenicity that is driven at least in part by a high mutational burden. T cells can be directed to novel tumor antigens by introducing genes encoding new antigen receptors, including natural T cell receptors (TCRs) and CARs. CARs are synthetic molecules that combine the effector functions of T cells with the ability of antibodies to detect pre-defined antigens with a high degree of specificity in a non-major histocompatibility complex (MHC) restricted manner (20). These receptors can therefore recognize intact proteins and do not rely on endogenous antigen processing and presentation. CARs are typically comprised of an extracellular domain for tumor recognition and an intracellular signaling domain that mediates T cell activation [reviewed in (21–24)]. The antigen-binding function of a CAR is usually conferred by a single chain variable fragment (scFv) containing the variable heavy (V_H) and variable light (V_L) chains of an antibody fused to peptide linker (20, 25, 26). This extracellular portion of the receptor is fused to a transmembrane domain followed by intracellular signaling modules. First-generation chimeric receptors bearing CD3ζ alone were not sufficient to elicit proliferation or cytokine production in peripheral T cells (27), which likely explains their failure to consistently expand and persist in some of the earliest clinical trials of CAR T cells (28, 29). However, the incorporation of co-stimulatory endodomains into CARs can recapitulate natural co-stimulation (30–32). We and others have demonstrated remarkable rates of complete and durable remission in patients with CLL (4, 5, 33), ALL (1–3), and Non-Hodgkin lymphomas (6, 7, 34) treated with second-generation CD19-directed CARs incorporating 4-1BB or CD28 co-stimulation. Early clinical trials of CAR T cells for the treatment of multiple myeloma have also demonstrated promising results (35–37). Thus, in the setting of hematopoietic malignancies, CAR T cells are emerging as a powerful therapy with the curative potential of allogeneic stem cell transplantation, but without the acute and chronic toxicity of graft-vs.-host disease and conditioning regimens. In contrast, CAR modified T cells are less effective than immune checkpoint blockade and in some cases TIL-based immunotherapy in treating patients with solid tumors to date. In this review, we will discuss the history and current status of CAR T cell therapy for non-hematopoietic malignancies, outline intrinsic mechanisms of T cell potency, describe extrinsic barriers operative in the setting of treating solid tumors, and suggest strategies to enhance the effectiveness of this approach for a variety of these incurable cancers.

HISTORY AND CURRENT STATUS OF CAR T CELL THERAPY FOR NON-HEMATOPOIETIC CANCERS

Initial Clinical Trials of Car T Cell Therapy in Solid Tumors

In early clinical trials of first-generation CAR T cells for solid tumors, safety and therapeutic efficacy were difficult to determine because of the aforementioned poor *in vivo* expansion and persistence of the transferred lymphocytes. These studies included patients with advanced epithelial ovarian cancer or metastatic renal cell carcinoma and targeted the folate receptor or carbonic anhydrase IX (CAIX), respectively (28, 29). A clinical trial of L1-cell adhesion molecule-specific (CD171) CAR T cells for the treatment of metastatic neuroblastoma demonstrated similar results of short-persisting (1–7 days) CAR T cells in individuals with bulky disease, but significantly longer persistence (42 days) in a single patient with limited tumor burden (38). Later trials of first-generation GD2-targeted CAR T cells administered to children with advanced neuroblastoma were more encouraging, with 3 of 11 patients experiencing complete remission, no substantial toxicity observed and sustained therapeutic benefit reported for several subjects (39, 40). Although the results of these trials were encouraging and provided the impetus to incorporate co-stimulatory signaling motifs in addition to CD3ζ, a third-generation CAR specific to the tumor antigen Her2 and integrating CD28, 4-1BB, and CD3ζ signaling moieties resulted in death of a patient with metastatic colon cancer (41). In this case, toxicity was caused by on-target, off-tumor reactivity of the CAR T cells with Her2 on normal lung and/or cardiac tissue (41). This serious adverse event was likely attributed to the infusion of substantially higher numbers of CAR T cells following lymphodepleting chemotherapy compared to most other trials. A second-generation Her2 CAR was also

tested in patients with sarcoma without evidence of toxicity (42). Although there were some indications of anti-tumor activity in this trial, T cell persistence was limited, similar to earlier clinical studies.

Recent Clinical Studies of Car T Cell Therapy in Non-hematopoietic Malignancies

Less dramatic clinical responses have also been observed in recently conducted clinical trials designed for the treatment of solid tumors with CAR T lymphocytes. Although evaluable data are not yet available from many of these studies, there is enough proof-of-concept from successful human studies of CAR T cells in leukemia and lymphoma to establish a concrete platform to treat these other indications. A complete response to CAR T cell therapy of recurrent multifocal glioblastoma was achieved using autologous T cells genetically-redirected to the tumor-associated antigen interleukin-13 receptor alpha 2 (IL13Rα2) (43). Interestingly, multiple intracavitary and intraventricular administrations of IL13Rα2 CAR T cells induced increases in the frequencies and absolute numbers of endogenous immune cells (i.e., $CD3^+$ T cells, $CD14^+$ $CD11b^+$ HLA-DR^+ mature myeloid populations, $CD19^+$ B cells, and few $CD11b^+$ $CD15^+$granulocytes) in association with the elaboration of inflammatory cytokines. This case underscores the possible role of the endogenous immune system in potentiating the anti-tumor activity of engineered CAR T cells and the potential of this approach to safety and dramatically increase quality of life in patients with malignant brain tumors (43).

We have recently generated CARs directed against the epidermal growth factor receptor variant III (EGFRvIII) and used them to gene engineer glioblastoma multiforme (GBM)-specific T cells. We found that we can redirect GBM patient T cells to target glioma tumors via lentiviral transduction with a CAR recognizing EGFRvIII *in vitro*, as well as *in vivo* in murine models (44) and in 10 patients (45) without the systemic toxicity associated with current standard-of-care treatments. In our first-in-human trial of EGFRvIII CAR T cells, we were able to confirm that a single intravenous infusion of these modified lymphocytes resulted in T cell engraftment in the peripheral blood, trafficking to the brain and antigen-directed activity (45). However, we observed that the inhibitory tumor microenvironment ultimately hampers clinical efficacy: following CAR T cell administration, several immunosuppressive factors were upregulated in the tumor environment including programmed death-ligand 1 (PD-L1), tryptophan 2,3-dioxygenase, indoleamine 2,3-dioxygenase, and IL-10. The lack of CAR T cell anti-tumor activity was accompanied by the presence of immunosuppressive regulatory T cells (T_{REGS}) based on their expression of CD4, CD25, and FoxP3. Furthermore, the heterogeneity of EGFRvIII expression was a clear barrier to ongoing clinical responses in this study (45). Thus, adoptive cell therapies for non-hematopoietic malignancies will need to address how to increase both the potency and persistence of CAR T cells in the face of antigen heterogeneity and a strongly suppressive tumor microenvironment (**Figure 1**). This clinical report (45) presents several known obstacles to CAR T cell therapy for solid tumors which are described below in detail.

TUNING CAR T CELL SPECIFICITY AND INTRINSIC FITNESS FOR IMMUNOTHERAPY OF SOLID TUMORS

Tumor Antigen Expression and Heterogeneity

Despite the fact that antigens such as CD19 and B-cell maturation antigen (BCMA) have been successfully targeted by CARs in the setting of hematopoietic cancer, there is an unmet need to identify similarly ideal antigens expressed by solid tumors. A major barrier to the development of CARs for solid tumor indications is, indeed, the identification of tumor antigens that can be targeted safely and effectively [reviewed in (46)]. In an optimal setting, CAR T cells should be directed against a tumor-restricted antigen to avoid on-target, off-tumor reactivity with healthy tissues. The proposed target antigen should be differentially expressed on tumor cells relative to essential normal tissues. In addition, the chimeric receptor must be highly specific for an antigen that is broadly expressed on the majority of cancer cells (46, 47). A variety of tumor-specific and tumor-associated antigens that can be targeted using CAR T cell therapy in non-hematopoietic malignancies have been identified (e.g., EGFR/EGFRvIII, IL13Rα2, Her2, CD171, mesothelin (MSLN), folate receptor alpha, GD2, carcinoembryonic antigen (CEA), chondroitin sulfate proteoglycan 4, c-Met, etc.). Antigens that display high constitutive expression that is tumor-restricted (e.g., chondroitin sulfate proteoglycan 4) may permit the application of CAR T cell therapy to higher proportions of patients and reduce the likelihood of tumor escape (48). However, because most tumor-associated antigens are heterogeneously expressed in tumor tissue, the efficacy of CAR T cells is often limited. Thus, combination therapies incorporating CARs that target multiple antigens will likely be required. There is progress in more safely and specifically targeting non-hematopoietic tumors with CAR T cells, either through creating CAR T cells specific for RNA splice variants or tumor-specific glycans (49, 50), or by generating CAR T cells that are conditionally specific for solid tumors. The latter is achieved by employing sensing and switching strategies in the tumor microenvironment (51–54). In addition to selectively replicating in and killing tumor cells directly, oncolytic viruses armed with payloads (e.g., bispecific T cell engagers, cytokines) may further synergize with CAR T cells to overcome tumor heterogeneity, while simultaneously bolstering anti-tumor activity (55, 56) (**Figure 2**).

Car T Cell Trafficking to Solid Tumors

Following infusion of CAR T cells targeting an appropriate antigen into patients, these lymphocytes are faced with the immediate obstacle of having to successfully localize to the tumor bed. This process is critically dependent on chemokine receptors expressed by the transferred cells and the chemokine gradient

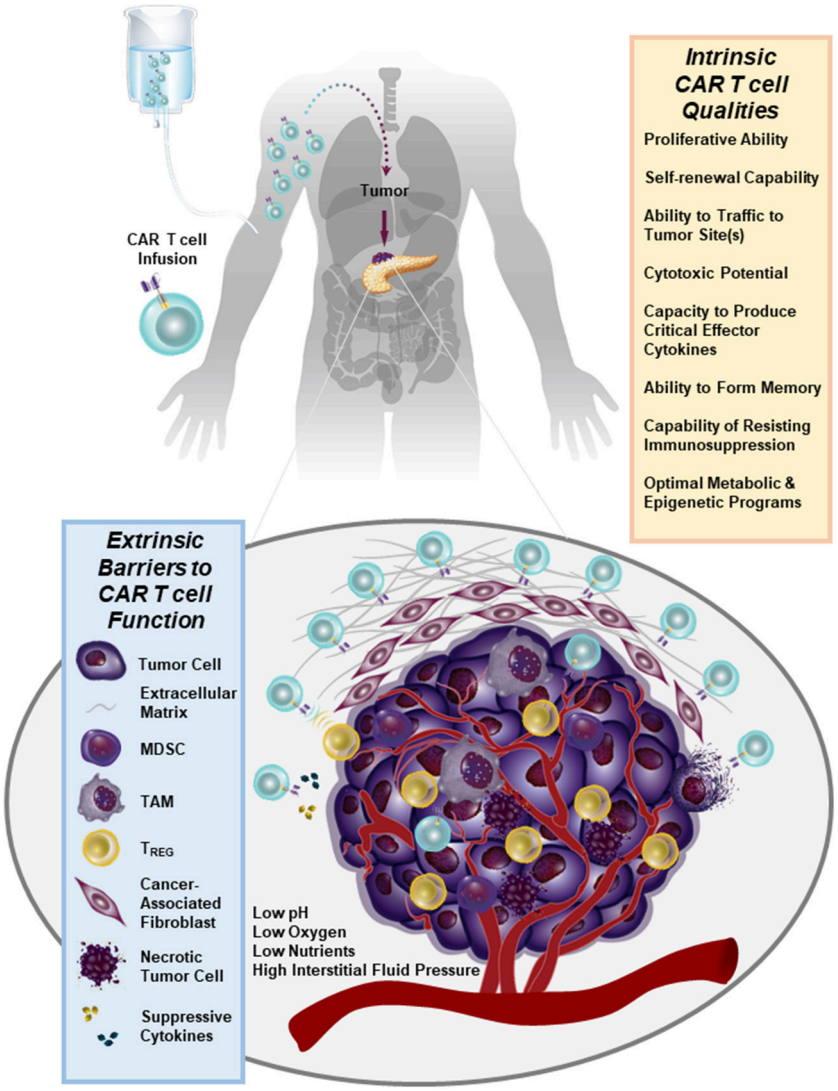

FIGURE 1 | Numerous immunosuppressive barriers present in the solid tumor microenvironment that can hamper the efficacy of CAR T cell therapy are schematically depicted. Several intrinsic qualities of CAR T cells that may impact the anti-tumor potency of these lymphocytes are also listed.

produced by the tumor. This presents a challenge because T cells often do not express the cognate receptors for the chemokines produced by tumors. In addition to this chemokine/chemokine receptor mispairing, tumors produce very small amounts of the chemokines needed for successful trafficking of T cells to the lesion. For example, melanoma cells do not produce sufficient amounts of CXCR3 ligands and this results in inefficient localization of CXCR3 receptor-bearing effector $CD8^+$ T cells to metastatic sites (57). We and others have co-expressed better matched chemokine receptors with CARs which resulted in improved trafficking of CAR T cells and enhanced tumor elimination (58, 59).

Characteristics of Intrinsic Car T Cell Potency

Systematic evaluations of patients with hematologic malignancies responding or not responding to CAR T cell therapy has yielded insights into key determinants of T cell potency that may inform treatment of solid tumors. In CLL, CAR T cells that were particularly effective exhibited robust proliferative capacity as well as long-term persistence *in vivo*. Transcriptomic profiling of patient-derived cell products revealed that CAR T cells from complete-responding patients were enriched in memory related genes, including IL-6/STAT3 signatures, whereas products from non-responding patients upregulated programs involved in effector T cell differentiation, glycolysis, exhaustion, and apoptosis (33). Unexpectedly, there was no association with typical patient- (e.g., age, sex, prior therapy) or disease-related (prior therapies, genetic and other risk profile, tumor burden, etc.) factors with likelihood of response. This makes the important point that cell-intrinsic properties are major determinants of success and failure in CAR T cell therapy (**Figure 1**).

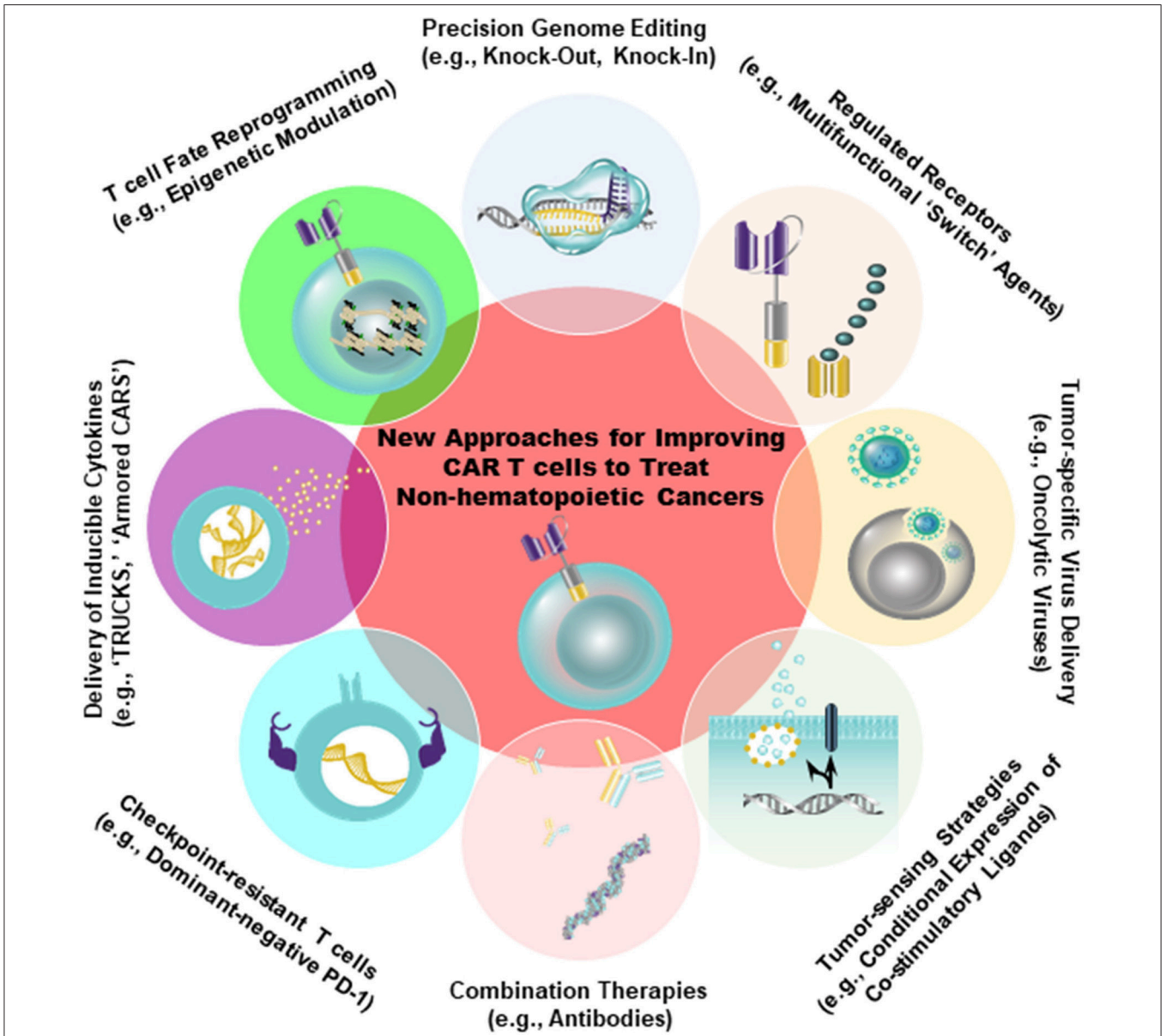

FIGURE 2 | Strategies to improve the safety (e.g., tumor-sensing strategies) as well as to augment the anti-tumor efficacy of CAR T cells are shown. Genetic engineering can be accomplished using viral (e.g., lentiviruses, retroviruses) and non-viral (e.g., CRISPR/Cas9) approaches to endow CAR T cells with gain-of-function or loss-of-function alterations. The overall aim of these approaches is to improve intrinsic T cell fitness and allow these cells to elicit optimal effector activity in the setting of several extrinsic barriers operative within solid tumors, as shown in **Figure 1**.

Generation of Quality Car T Cells

The optimal "seed" population of T cells needed for the generation of CAR T cells that can sustain durable responses against cancer is still a matter of debate. One school of thought is that effector $CD8^+$ T cells producing high amounts of interferon-gamma are most effective at eliminating tumors, while other investigators believe that naïve or early memory $CD8^+$ T cells which differentiate and expand at the tumor site are superior for eliciting long-lasting anti-tumor immunity (60–62). If one assumes a linear model of $CD8^+$ T cell differentiation, naïve T lymphocytes (T_N) are programmed into the earliest identifiable memory T cell stage, stem cell memory (T_{SCM}). This population is thought to give rise to the successive stages of differentiation: central memory (T_{CM}), effector memory (T_{EM}), terminally differentiated effector memory RA (T_{EMRA}), and effector (T_{EFF}) cells (63). Many studies have supported the idea that early memory $CD8^+$ T cells generate the most potent CAR T cells against both liquid and solid tumors. For example, CAR-engineered T_{SCM} cells directed to mesothelin were significantly more effective at regressing established solid tumors compared

to T_{EM} and T_{EFF} cells (63). Retrospective profiling of *ex vivo* $CD4^+$ and $CD8^+$ T cells from CLL patients treated with anti-CD19 CAR T cells revealed that responding and non-responding patients did not differ in their frequencies of T_N, T_{CM}, T_{EM}, or T_{EFF} cells at the time of T cell collection. However, responding patients did exhibit a modest increase in T_{SCM} cells compared to non-responders (33). More significantly, unbiased biomarker analysis revealed that the frequency of apheresed $CD27^+$ $CD45RO^-$ $CD8^+$ T cells from patients responding to CAR T cell therapy was significantly higher compared to non-responder T cells. Notably, this subpopulation of $CD8^+$ T cells possessed functional characteristics of early memory as well as effector T cells (33).

Based on growing pre-clinical and clinical evidence of less-differentiated cells mediating superior anti-tumor efficacy, there is interest in developing ways to conduct large-scale T cell expansion, while simultaneously preserving the functional features of early-memory T cells. Human T cells undergo a series of profound changes with successive rounds of division *in vitro* and *in vivo*. Among these changes are the loss of certain co-stimulatory receptors (e.g., CD28, CD27) and the erosion of telomeres. Depending on the molecular design, co-stimulatory endodomains from these receptors may or may not be incorporated into the CAR. Therefore, culture systems that can prevent telomere loss or potentiate the maintenance of endogenous co-stimulatory receptor expression could restore proliferative potential to conventional effector T cells and presumably increase the functional lifespan of these cells following re-infusion into patients (64, 65). We have recently described a culture system for the production of CAR T cells in 3–5 days, relative to a traditional 9-day process (66). This process allowed us to generate CD19-directed CAR T cells that were less differentiated and, at limited cell doses, significantly more potent against leukemia in an *in vivo* animal model (66). Alternative approaches for reducing CAR T cell differentiation during *in vitro* expansion include inhibition of signaling mediators downstream of the IL-2 pathway such as subunits of Glycogen synthase kinase 3β (60), Protein kinase B (AKT) (67), and Phosphoinositide 3-kinase (68). In addition, replacement of IL-2 with other cytokines such as IL-7 and IL-15 that signal through the γ-common chain receptor (69), but regulate survival and homeostatic T cell proliferation independently of TCR stimulation (70–72) may enhance the *in vivo* expansion and persistence of CAR T cells (73, 74). Genetic reprogramming of induced pluripotent stem cells derived from somatic cells could also be used to generate more naïve-like CAR T lymphocytes for adoptive transfer (75). Finally, in a "bedside-to-bench" study, we demonstrated that unintentional disruption of the gene encoding the methylcytosine dioxygenase TET2 resulted in the massive clonal expansion of CAR T cells that were all derived from a single cell. Furthermore, *TET2*-disrupted lymphocytes exhibited a predominantly T_{CM} phenotype at the peak of the anti-tumor response (76). These findings, along with other recent reports (77–81), underscore the power of epigenetic modulation in effectively re-programming T lymphocyte fate for the generation of CAR T cells with optimal anti-tumor potency (**Figure 2**).

SURMOUNTING TUMOR-MEDIATED BARRIERS TO CAR T CELL THERAPY OF NON-HEMATOPOIETIC CANCERS

A major issue to be addressed for improving the efficacy of CAR T cells against non-hematopoietic malignancies is determining how to effectively enhance the persistence and function of these lymphocytes in toxic tumor microenvironments. CAR T cells are vulnerable to both immunological and metabolic checkpoints as well as other suppressive factors present in the tumor bed. In pre-clinical mouse models, both CAR and TCR transgenic T cells cease to function or die shortly after entering the tumor microenvironment (82, 83). Although repeated infusions of freshly engineered T cells may help to improve engraftment, this approach is not always clinically feasible. Tumor-imposed extrinsic barriers as well as strategies to overcome several of these hurdles for the generation of efficacious CAR T cells to treat solid cancers are described below.

Overcoming Physical Barriers in Solid Tumors

Unlike liquid tumors which do not typically possess physical barriers that would prevent their interactions with CAR T cells, many solid tumors have a formidable barricade that renders these masses inaccessible to invasion by immune cells. This landscape includes stromal cells, immune cells, cancer cells and extracellular matrix (ECM) components (i.e., proteins and glycans). Collagens, fibronectin, laminin, hyaluronan, and proteoglycans heavily contribute to the proliferation of fibrous or connective tissue (desmoplasia). The fibrotic tumor stroma of many solid malignancies, including pancreatic, breast and ovarian cancer is thought to impede effective drug delivery (84–86) and may also prevent infiltration by CAR T cells (**Figure 1**). Accordingly, diffusion of the CAR T cells into tumor tissue was shown to be blocked by the ECM are therefore often trapped (87) and unable to deeply penetrate tumor tissue (88). Desmoplasia combined with high interstitial fluid pressure and rapid tumor cell proliferation also contributes to the collapse of vasculature, which may further impede CAR T cell infiltration from vessels into tumor tissue (89). Tumor vessels may also not possess the receptors necessary for T cell homing and extravasation, including E- and P-selectins, VCAM-1, and ICAM-1 (87). Furthermore, following *in vitro* culture, CAR T cells often lack normal expression of the enzyme heparanase which degrades matrix proteoglycans and potentiates extravasation (90).

Administration of collagenases or hyaluronidase into solid tumors has been shown to enhance ECM breakdown, rendering the tumor more penetrable and thus susceptible to drug and cell-based therapies. Collagenase or hyaluronidase treatment has aided in increased antibody diffusion and chemotherapy uptake in pre-clinical *in vivo* and *in vitro* models of disease (91–94). Alternatively, reprogramming of myeloid cells, which naturally traffic and infiltrate into solid tumors, can effect anti-fibrotic activity and ECM breakdown (95). Depletion of ECM-producing cells (e.g., cancer-associated fibroblasts) can also render solid

tumors more susceptible to therapy (96). In this regard, targeting stromal fibroblasts with anti-fibroblast activation protein (FAP) CAR T cells significantly stalls the growth of multiple types of solid tumors (97). In addition, administration of CAR T cells engineered to overexpress heparanase leads to partial ECM degradation, enhanced T cell infiltration and anti-tumor activity (90). Although these strategies seem promising, the potential negative impact of tumor ECM depletion should not be overlooked. In some studies, ECM reduction can paradoxically accelerate disease progression (98, 99). To avoid this potential negative outcome, direct intracavitary or intratumoral injection relative to intravenous infusion of CAR T cells may circumvent many of the physical barriers described above. In this vein, Klampatsa et al. used intracavitary methods to eliminate mesothelioma cell lines with some success (100), and Adusumilli and colleagues demonstrated that intrapleural administration of CAR T cells was significantly more successful at eliciting anti-tumor activity than the intravenous route (101).

Targeting the Tumor Vasculature and Immune Stimulatory Car T Cell Modifications

In addition to tumor antigens, CARs can be targeted to the tumor vasculature in an effort to restrict blood flow and nutrient supplies to the tumor, which impedes malignant growth and simultaneously increases T cell localization (102). A strategy based on regional infusion of IL-12 secreting CAR T cells directed against VEGFR-2 which is expressed on angiogenic endothelial cells resulted in enhanced accumulation of these lymphocytes and tumor regression in multiple pre-clinical models (103). "Armored CARs" or "TRUCKs" (T cells Redirected for Universal Cytokine Killing) delivering other cytokines such as IL-15 (104, 105) or IL-18 (106) to the tumor microenvironment have also demonstrated superior anti-tumor activity compared to conventional CAR T cells (**Figure 2**). Furthermore, echistatin CARs targeting the angiogenic integrin αvβ3, which is commonly expressed on vascular endothelium of solid tumors (107), increased nanoparticle deposition in tumors (108). These findings indicate that the use of vasculature-targeted CAR T cells may be a potential "lead-in" strategy to enhance delivery of drugs or other adoptively transferred immune cells.

Overcoming Cell-Mediated Immunosuppression in the Solid Tumor Microenvironment

Along with physical barriers, the tumor microenvironment is composed of multiple cellular components and molecular factors that can abrogate the elicitation of effective endogenous anti-tumor immune responses. This immunosuppressive milieu can also severely inhibit the effector functions of adoptively transferred CAR T cells. However, CAR T cell hypofunction is tightly dependent on the tumor microenvironment and in some instances removal of engineered T cells from the tumor restores their functional activity (109). This report as well as other studies (110–112) suggest that favorably altering the toxic tumor microenvironment by directly targeting immunosuppressive cells or engineering T cells to resist tumor-specific inhibitory mechanisms may provide new opportunities to improve CAR T cell function.

Tumor associated macrophages (TAMs) are an immunosuppressive cell type commonly found in solid tumors, and these cells aid in tumor cell survival and growth. While the phenotype of macrophages is pliable and these cells can be programmed to be either tumor-promoting or tumor-suppressive, macrophage function is ultimately dictated by signals from the surrounding tissue-specific niche (113). The tumor microenvironment often pushes macrophages toward a tumor-promoting phenotype (114), and this aids in angiogenesis, growth, immune evasion and metastasis. Therefore, targeting TAMs may improve the efficacy of CAR T cells against solid tumors. Ruella and colleagues recently devised a strategy to deplete tumor-promoting macrophages with macrophage-targeted CAR T cells. This approach was efficacious in a mouse model of Hodgkin lymphoma and led to the establishment of long-term immunological memory (115).

Myeloid derived suppressor cells (MDSCs) are another immunosuppressive cell type found in solid tumors that can dampen CAR T cell function. MDSCs express arginase and indoleamine, which metabolize amino acids that are essential for effector T cell activation and proliferation (116). Accordingly, Burga et al. demonstrated that depletion of $GR1^+$ cells (targeting immunosuppressive tumor-associated neutrophils and MDSCs) augmented the ability anti-carcinoembryonic antigen CAR T cells to reduce colorectal cancer liver metastases (117). MDSCs also produce high levels of reactive oxygen species, which may impair the cytotoxic ability and proliferative capacity of CAR T cells (118). To overcome this oxidative stress, CAR T cells have been modified to express the anti-oxidant enzyme catalase into the local environment and this modification significantly improves their anti-tumor activity (119).

T_{REGS} are well-documented suppressors of T cell function capable of inhibiting anti-tumor activity through multiple mechanisms, including cell-cell contact inhibition, sequestration of IL-2 and the production of immunosuppressive cytokines such as TGF-β and IL-10 (120). Although these cells promote the growth and metastasis of tumors, they are difficult to directly deplete due to the lack of specificity of targeting agents, and the potential to induce autoimmune diseases when global disruption approaches are used (121). Given the high level of TGF-β produced by T_{REGS}, MDSCs, and tumor cells, blocking TGF-β signaling through overexpression of a dominant-negative TGF-β receptor on adoptively-transferred T cells may improve their anti-tumor potency (122, 123). Overexpression of dominant-negative TGF-β receptor II on CAR T cells results in enhanced T cell proliferation, cytokine production, *in vivo* persistence and ability to eradicate tumors in mouse models of aggressive human prostate cancer (124).

Many types of cells including tumor cells, fibroblasts, endothelial cells and immune cells produce the lipid-signaling molecule prostaglandin E2 (PGE_2) by activation of cyclooxygenase (COX)-2 and prostaglandin E synthase. PGE_2 enhances tumor progression by stimulating multiple pathways, including those that mediate angiogenesis and immunosuppression (125). For example, PGE_2 plays a significant role in the suppression of effector T cells and the attraction of

T_{REGS} and MDSCs. PGE_2 and adenosine activate protein kinase A (PKA), which then inhibits antigen receptor –triggered T cell activation. PGE_2 is also known to cooperate with adenosine in the dampening of immune responses mediated by T_{REGS} (126). Recently, Newick et al. engineered CAR T cells to produce a small peptide that inhibits the association of PKA with ezrin, thus reducing the negative effects of PKA on TCR activation (127). This PKA inhibitor ameliorated the immunosuppressive actions of both adenosine and PGE_2, resulting in increased CAR T cell trafficking, tumor cell cytotoxicity, and pro-inflammatory cytokine production (127).

Enhancing the Metabolic Fitness of Car T Cells

Immune cell function and metabolism are impacted by the solid tumor microenvironment. Glucose utilization is heterogeneous within the tumor and associated with perfusion, with lesser-perfused regions of the tumor displaying higher glucose metabolism (128). Both proliferating tumors and effector T cells responding to antigen challenge rely primarily on aerobic glycolysis to fuel expansion, creating competing demands for metabolites within nutrient-poor regions of the tumor (129). This competition for nutrients, metabolites and oxygen (O_2) is thought to impact T cell metabolism, limit T cell-mediated anti-tumor efficacy and contribute to T cell exhaustion and cancer progression (130–132). Stabilization of HIF-1α drives glucose uptake, induces production of S-2-hydroxyglutarate (S-2HG) and consequential epigenetic remodeling as well as increased expression of IL-2, which potentiates $CD8^+$ T cell mediated anti-tumor activity (133, 134). However, under O_2 and glucose limiting conditions, reduction of HIF-1α expression may enhance T cell function (135). In a recent study, $CD8^+$ TILs isolated from clear cell renal cell carcinoma (ccRCC) were shown to exhibit an impaired ability to consume glucose, mitochondrial fragmentation and hyperpolarization, as well as increased production of ROS (136). Because ccRCC develops a unique pathological pseudo-hypoxic response [reviewed in (137)], with increased aerobic glycolysis and vascularization, it is tempting to speculate that the altered tumor microenvironment in ccRCC may have contributed to these observed defects in ccRCC CD8 TIL metabolism (136). Likewise, hypoxic areas within solid tumors are often negatively correlated with patient survival and thought to promote tumor metastasis and resistance to radiotherapy (138–140). Another metabolic checkpoint in the tumor microenvironment regulating immune modulation is amino acid limitation (129). For example, degradation of L-arginine by MDSCs in the tumor microenvironment can lead to reduced expression of CD3ζ and impaired T cell responses (141). In contrast, increased levels of arginine shift T cell metabolism to oxidative phosphorylation and increase central memory differentiation (142).

Activation, growth, proliferation, effector and memory function, and return to homeostasis are linked to the metabolic profile of the T cell (131). T cell subsets differently metabolize nutrients and regulation of nutrient availability can influence T cell differentiation as well as fate (129). Naïve T cells are metabolically quiescent and rely on glucose, fatty acids and amino acids as fuel sources for oxidative phosphorylation (143, 144). T_{CM} cells maintain spare respiratory capacity through oxidation of fatty acids in mitochondria which allows for a rapid recall of the memory response upon antigen re-challenge (145, 146). In contrast, effector T cells, like tumor cells, rely on aerobic glycolysis to provide energy, metabolic intermediates for rapid cell growth and NAD^+/NADH to maintain redox balance (147); although under metabolically challenging conditions $CD8^+$ TILs can partially preserve effector function by catabolizing fatty acids (135). Glutamine is also essential for effector function (148). After conversion to α-ketoglutarate, glutamine can serve as a TCA intermediate or contribute to the citrate pool. Similarly, altering metabolism can impact T cell phenotype; restraining glycolysis, AKT, and mTOR activity or enhancing STAT3 or Wnt/β catenin signaling can arrest T cell development and retain T_{CM} differentiation, which are associated with enhanced T cell persistence and may promote the efficacy of adoptive cell therapy (60, 149–152).

Different types of co-stimulatory endodomains incorporated into a CAR can differentially program T cell metabolism and mitochondrial biogenesis (153). This indicates that the fate of CAR T cells toward memory or effector differentiation can be directed, as cells expressing CARs with 4-1BB signaling domains have enhanced mitochondrial biogenesis and fatty acid oxidation, while CARs with CD28 signaling domains have enhanced aerobic glycolysis (i.e., Warburg metabolism) (153). Therefore, in addition to being able to direct CARs to virtually any cell surface structure on tumor cells, we also have the potential to engineer these lymphocytes to be resistant to the tumor microenvironment by specifying their metabolic program. Alternatively, host pre-conditioning strategies involving the treatment of tumors with HIF blocking agents or metabolic enzymes may represent a promising strategy to limit the metabolic flexibility of tumors as well as the localization of inhibitory immune cells (154). This would allow CAR T cells to function in a more nutrient replete and less suppressive tumor microenvironment.

Engineering Car T Cell Resistance to Immune Checkpoint Inhibitors

Tumors cells can also directly modulate effector T cell activation by expression of inhibitory signals that block T lymphocyte activation and function, thus preventing immune control of tumor growth (155). In addition to secreting immunosuppressive cytokines, tumor cells or other cells in the tumor microenvironment express a number of proteins on their surface that are capable of inactivating CAR T cells. These include PD-1 ligands, PD-L1 (B7–H1), and PD-L2 (B7-DC), all belonging to the B7 receptor superfamily. Other B7 family members, such as B7–H3 and B7–H4, and the unrelated receptors herpes virus entry mediator (HVEM), inhibitory receptor Ig-like transcript-3 and−4 (ILT3 and 4) are also abundantly expressed in the solid tumor microenvironment [reviewed in (156)]. Furthermore, by providing a persistent source of antigen while avoiding clearance, tumors potentially promote T cell exhaustion. As discussed above, checkpoint blockade has been a successful approach to sustain T cell function, and blockade of inhibitory receptors such as T-cell membrane protein-3 (TIM-3), lymphocyte-activation protein-3 (LAG-3), T cell Ig and ITIM domain (TIGIT), cytotoxic T lymphocyte-associated

antigen 4 (CTLA-4), and programmed death-1 (PD-1) or their cognate ligands are being tested in clinical trials to reverse or prevent exhaustion [reviewed in (47)]. The upregulation of these receptors has been previously reported to abrogate the persistence and activity of the anti-tumor response of CAR T cells (155). Accordingly, John et al. reported that combining anti-Her2 CAR T cells and PD-1 blocking antibodies enhances tumor growth inhibition in association with decreased frequencies of $GR1^+$ $CD11b^+$ MDSCs (157). Strategies in which CAR T cells are engineered to secrete immune checkpoint inhibitors such as anti-PD-L1 (110), and -PD-1 (158) antibodies or PD-1-blocking single-chain variable fragments (112) possess the advantage of increasing the local delivery of these agents to the tumor microenvironment, while avoiding toxicities associated with systemic checkpoint blockade. Co-expression of a dominant-negative PD-1 receptor with mesothelin-targeted CAR T cells has also been shown to render these cells resistant to PD-1-induced inhibition and to significantly improve their *in vivo* anti-tumor efficacy following a single administration (155). The Clustered Regularly Interspaced Short Palindromic (CRISPR)/CRISPR associated protein 9 (Cas9) provides a robust and multiplexable genome editing tool that permits knock-out of inhibitory receptors (**Figure 2**). This system can be used to knock-out PD-1 and CTLA-4 on allogeneic universal CAR T cells (159). Finally, it is intriguing to consider the possibility of directing CAR transgenes to specific genomic loci encoding inhibitory receptors using recently developed viral and non-viral technologies (160, 161).

CONCLUDING REMARKS

Many pre-clinical studies indicate that adoptive cell transfer therapy with autologous T cells is a powerful approach for the treatment of cancer. In contrast to the recent FDA approvals of CAR T cells in hematologic malignancies, the effectiveness of this approach for a variety of more common non-hematopoietic cancers is much lower. As was underscored in this review, CAR T cells may hold great promise for the treatment of solid tumors; these malignancies have a high-unmet medical need and are generally considered incurable with present therapies. However, the achievement of complete and durable remissions for patients with non-hematopoietic cancers will require optimization of CAR T cells in the areas of improving antigen targeting, enhancing T cell trafficking, bolstering intrinsic T cell potency and arming these lymphocytes to do battle in the face of multiple immunosuppressive barriers imposed by the solid tumor microenvironment. Both current and future advances in cellular engineering, site-specific genome editing and synthetic biology will undoubtedly bolster the safety, reliability and efficacy of CAR T cell therapy for a variety of diseases. Thus, while there are currently some detours on the road to clinical success, CAR T cells are on the fast track to becoming a potentially curative modality for many different cancers.

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KL, RY, and JF conceptualized, wrote, and edited the manuscript. AB, MD, JM, SL, DD, and BL provided feedback and edited the manuscript.

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REVIEW
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Genetically Engineered T-Cells for Malignant Glioma: Overcoming the Barriers to Effective Immunotherapy

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Malignant gliomas carry a dismal prognosis. Conventional treatment using chemo- and radiotherapy has limited efficacy with adverse events. Therapy with genetically engineered T-cells, such as chimeric antigen receptor (CAR) T-cells, may represent a promising approach to improve patient outcomes owing to their potential ability to attack highly infiltrative tumors in a tumor-specific manner and possible persistence of the adaptive immune response. However, the unique anatomical features of the brain and susceptibility of this organ to irreversible tissue damage have made immunotherapy especially challenging in the setting of glioma. With safety concerns in mind, multiple teams have initiated clinical trials using CAR T-cells in glioma patients. The valuable lessons learnt from those trials highlight critical areas for further improvement: tackling the issues of the antigen presentation and T-cell homing in the brain, immunosuppression in the glioma microenvironment, antigen heterogeneity and off-tumor toxicity, and the adaptation of existing clinical therapies to reflect the intricacies of immune response in the brain. This review summarizes the up-to-date clinical outcomes of CAR T-cell clinical trials in glioma patients and examines the most pressing hurdles limiting the efficacy of these therapies. Furthermore, this review uses these hurdles as a framework upon which to evaluate cutting-edge pre-clinical strategies aiming to overcome those barriers.

Keywords: T lymphocyte, brain cancer, Glioblastoma, TCR - T cell receptor, CAR (chimeric antigen receptor) T cells, Glioma

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INTRODUCTION

Malignant gliomas, including glioblastoma (GBM), are the most common form of malignant primary brain tumors. Among those, GBM represents the most common and aggressive tumors with an average survival rate of 15 months following diagnosis (1). The current standard of care involves maximal safe tumor resection followed by radiotherapy and chemotherapy. Despite advances in cytotoxic therapy regimens, targeted angiogenesis inhibitors and novel therapeutic modalities, such as alternating electric field therapy, patient survival has only improved modestly over recent years (2). GBM may occur *de novo* in multiple types of neuro-epithelial cells, which is diagnosed as primary GBM, or it may arise following the progression or recurrence of low-grade glioma (LGG) into high grade form (HGG), in which case it is diagnosed as secondary GBM.

Primary GBM is more prevalent, confers worse prognosis, and is understood to develop from distinct genetic precursors compared to secondary GBM (3). In addition to the distinction between primary and secondary GBM, malignant gliomas represent the most common mortality and morbidity among pediatric cancers. Especially, high grade gliomas that affect the midline structure of the brain [diffuse midline gliomas (DMG)] are among the poorest responders to existing treatments, due in part to the unique genetic and epigenetic mechanisms driving the development of these tumors (4). The wide differences in tumor etiology and genetic landscape among GBM necessitate different treatment approaches and have resulted in a patient population with an acute need for improved therapy.

The central nervous system (CNS) was once considered an immune privileged site that was spared from the potentially damaging effects of active immune responses (5, 6). However, decades of research into the role of the immune system within the CNS has amended this preconception and allowed for a deeper understanding of how the adaptive immune response can function in the CNS [reviewed in (7)]. Recent studies investigating peptide vaccines and adoptive cell transfer for patients with malignant glioma have demonstrated that systemically administered treatments can, in fact, elicit antigen-specific T-cell responses. Despite these encouraging data, however, therapeutic responses were observed infrequently and had variable durations (8–12). The results of these initial trials underscore the need for continued in-depth research and analysis of the immunotherapeutic approaches for the treatment of glioma patients.

The successes of chimeric antigen receptor (CAR) T-cell therapy in hematological cancers have renewed the hope that durable remissions may become possible for patients with solid cancers, including those with GBM. Brain tumor patients have proven to be a particularly challenging population to treat with immunotherapy as many of the characteristics of a productive immune response, such as edema and widespread inflammatory infiltration, can have a devastating effect when they occur within close proximity to neural tissues. Despite these increased risks, genetically engineered T-cells, such as CAR T-cells, have the potential to improve the survival outcomes for patients. Tumor-targeting CARs are genetically engineered receptors that combine the antigen specificity of antibodies through the use of single chain variable fragments (scFv) with the potent antitumor effects of activated T-cells (13). However, the use of antibody-derived scFv limits antigen selection to surface bound proteins. Therefore, multiple groups, including ours, have begun to evaluate genetically engineered T-cells expressing a physiological form of tumor antigen-reactive T-cell receptor (TCR) in patients where tumor-specific neoantigens are derived from intracellular proteins (14). Regardless of the mode of antigen recognition, genetically engineered T-cell therapy in brain tumor patients has encountered a panoply of challenges. Some of these hurdles may be shared among all solid tumor types, such as antigen heterogeneity and tumor-derived immunosuppression, while other challenges are characteristic to CNS malignancies, such as the absence of professional antigen-presenting cells and the limitations to lymphocyte homing resulting from the blood-brain barrier.

In this review, we will highlight the most recent clinical status of CAR T-cell therapy for malignant glioma and then discuss the major challenges facing CAR T-cell immunotherapy in GBM, including neuroanatomical considerations, barriers to effector T-cell trafficking, immunosuppression in the GBM microenvironment, antigen heterogeneity, off-tumor toxicity, as well as the diverse challenges and opportunities afforded by concomitant therapies in the clinic. Furthermore, we will use these challenges as a framework to evaluate strategies for engineering more effective and specific CAR T-cell therapies for glioma.

CLINICAL EXPERIENCES WITH GBM CAR T-CELL THERAPY

The clinical utility of CAR T-cells targeting CD19 in relapsed and refractory B cell malignancies has proven to be exceptional in these patient populations (15, 16). However, the efficacy of CAR-T therapy in solid tumors has been less evident (17). Despite the complex barriers associated with treating CNS cancers, several early phase CAR T-cell clinical studies provide encouraging data.

GBM-Specific CAR T-Cell Targets

GBM are generally considered to be immunologically cold tumors due in part to the overall low mutation loads of these tumor cells (18). One of the key challenges that has impeded development of CAR therapies for GBM is the limited availability of targetable tumor-specific antigens which do not confer any risk of toxicity toward normal tissues. An attractive mutation resulting in the formation of a common neoantigen in the GBM context is variant III of the epidermal growth factor receptor (EGFRvIII). This truncated receptor is expressed in 20% of newly diagnosed GBM patients and has not been found to be expressed on normal tissues, rendering it tumor-specific (19–21). It is characterized by an in-frame deletion of exons 2–7, which confers ligand-independent constitutive signaling through EGFR that results in cellular proliferation and enhanced resistance to both radio- and chemotherapies. The generation of a glycine at the splice-junction between exons 1-8 provides a surface epitope that can be readily targeted by immunotherapeutic approaches (21).

In a phase I clinical trial, O'Rourke and colleagues treated 10 recurrent GBM patients with a single intravenous infusion of autologous EGFRvIII-specific CAR T-cells. The group observed no objective radiographic response, apart from one patient who presented with stable residual disease for over 18 months. The patients did not suffer any off-tumor toxicities or cytokine release syndrome, providing evidence that systemic infusion of EGFRvIII-CAR T-cells is feasible and safe (12). Importantly, the authors observed significant but transient expansion of the CAR T-cells during the course of treatment and successful infiltration of CAR T-cells in the tumor site, which was ultimately associated with the decrease of EGFRvIII-expressing tumor cells. In addition, the research team noted increased and robust

upregulation of several immune inhibitory molecules, such as programmed death ligand receptor-1 (PD-L1) and indoleamine-2,3-deoxygenase 1 (IDO1). The presence of CAR T-cells at the tumor site is evidence that systemically infused T-cells can be activated and recruited to the brain. While these observations are encouraging, the failure of this therapy to achieve objective clinical responses underscores the potentially debilitating impact of antigen heterogeneity and local immune suppression on CAR therapy which often manifests in the outgrowth of antigen loss variants.

GBM-Associated CAR T-Cell Targets

IL-13 receptor α2 (IL-13Rα2) is a promising non-mutant GBM-associated antigen due to its broad tumor expression and extremely low expression levels in normal brain (22). This monomeric high affinity receptor binds IL-13 but not IL-4 and drives the production of transforming growth factor-β (TGF-β) in the tumor microenvironment (TME) (23). IL-13Rα2 is overexpressed in 75% of GBM patients and is a prognostic indicator for poor patient survival (24). Initial studies by Brown et al. evaluated the effect of repeated intracranial injections of IL13Rα2-targeting $CD8^+$ CAR T-cells in 3 patients with recurrent GBM (25). The treatment was well-tolerated and resulted in transient antitumor activity in two of three patients. However, the authors noted that residual tumor tissue adjacent to the site of injection displayed significantly lower expression of IL13Rα2, implying antigen loss as a result of therapy. The same group subsequently reported a case study where they observed regression of an IL13Rα2-positive multifocal GBM tumor in a patient treated with intraventricular administrations of second generation IL13Rα2-CAR T-cells that also express CD137 intracellular domain as part of the CAR construct (26). The authors observed transient complete response of all cranial and metastatic tumors after repeated infusions. However, the patient eventually succumbed to metastatic recurrent lesions with decreased expression of IL13Rα2, highlighting the importance of developing improved strategies for overcoming acquired immune resistance on a systemic scale.

Another Phase I clinical trial by Ahmed et al. targeting the tumor-associated antigen human epidermal growth factor receptor-2 (HER2) reported the outcome of treating 17 GBM patients with HER2-specific CAR T-cells (27). The authors reported no serious adverse events following the administration of dose-escalating treatments and the observation of clinical benefit in 8 of 17 patients (1 partial response and 7 stable disease). The autologous T-cells used to manufacture CAR T-cells in this study were selected to be virus-specific. Because 16 of the 17 patients tested seropositive for cytomegalovirus, the investigators hypothesized that expression of the CAR construct in virus-specific $CD8^+$ T-cells would optimize the persistence of CAR T-cells if the T-cells were to receive survival and proliferation signals via their endogenous TCR. Unfortunately, the CAR T-cells did not expand and persisted in only low levels in the periphery, suggesting the need to further develop methods of enhancing CAR T-cell survival and expansion *in vivo*.

While the positive safety profiles reported by all four studies are encouraging, these data highlight the substantial challenges facing CAR T-cell therapy for GBM. One key finding from all three recently completed Phase I studies was the low level expansion and persistence of the infused CAR-T-cells. Variable expansion and trafficking of T-cells to the brain tumor site, the dynamic immunosuppressive response mounted by the TME, and antigen loss in post-therapy recurrent tumors may explain some of this lack of expansion and persistence. We will start by investigating each of these sets of challenges in more detail and then review the strategies currently being explored to address them in the setting of malignant glioma.

NEUROANATOMICAL CHALLENGES AND T-CELL HOMING

The efficacy of immunotherapy for malignant glioma relies upon the ability of therapeutic immune cells to reach the brain parenchyma and induce an anti-tumor response. Although adaptive immunity plays a critical role in immune surveillance of the CNS, the CNS has developed mechanisms that tightly regulate entry and activation of innate and adaptive immune cells to limit the potential side effects of neuroinflammation. It is important to recognize that the effects of inflammation, such as edema, cytokine-induced toxicity, and neurodegeneration, can be detrimental to the functional integrity of the CNS. Understanding of the neuroanatomical features that underlie these mechanisms is essential for the successful development and application of genetically engineered T-cells for malignant glioma. The CNS was historically considered a site of immune privilege because neither allografts transplanted in the brain of immune- competent mice nor the inoculation of viral and bacterial pathogens into the brain parenchyma elicited immunological responses (5, 6, 28, 29). These findings were initially attributed to the presence of the BBB, absence of lymphatics, and the relative incompetence of antigen presenting cells in the CNS. However, several decades of research into neuro-inflammatory conditions and clinical oncology have challenged these notions [reviewed in (7)]. It is currently understood that the CNS is neither completely privileged from systemic immunity nor impermeable to activated immune cells (9, 30, 31). Nevertheless, the unique anatomical features of the CNS pose several challenges that impede the ability of T-cells to recognize and respond to antigens within the brain. This section discusses these features and outlines a variety of strategies to overcome these impediments.

Anatomical Considerations of the Immune Response

The CNS can be broadly divided by the areas which are protected by the BBB and those that are not, which has important consequences for the efferent arm of the immune response. The ventricles, meninges, and spinal cord are not protected by the BBB and are bathed in the cerebrospinal fluid (CSF) produced by the choroid plexus (32). The brain parenchyma and its interstitial fluid (ISF) are anatomically separated from both the peripheral bloodstream and the CSF by the BBB. The brain parenchyma lacks conventional lymphatic vessels and

instead relies upon the drainage of tumor antigens and immune cells through the ISF and CSF into the dural, cervical, and nasal lymphatics. Access to these peripheral lymphatics depends upon anatomical location within the CNS. Both the cellular and soluble components of the CSF in the ventricular and subdural spaces can drain efficiently to the peripheral lymphatics (33–35). However, these same components within the ISF of the brain parenchyma are anatomically restricted from reaching the peripheral lymphatic system. Instead, the parenchyma must rely upon the limited exchange of CSF and ISF, termed the glymphatic system, in order for soluble antigens and signaling molecules to reach the peripheral lymphatics (36, 37). The absence of conventional lymphatic access to the parenchyma greatly hinders the afferent arm of the adaptive immune system needed for antigen presentation and the initiation of a systemic immune response to a tumor.

The BBB is a permeability barrier composed of tight junctions connecting endothelial cells with the luminal and abluminal membranes lining the capillaries of the brain (38). Although not an absolute barrier, the BBB restricts the entry of ionic substances, large molecules, and naïve immune cells from the peripheral blood into the brain parenchyma. Lymphocyte entry into the brain parenchyma is tightly regulated (**Figure 1**) by the BBB as well as the glia limitans, which is formed by the fusion of astrocyte processes lining the parenchymal basal membrane along the entirety of the CNS (39). The BBB selectively allows activated but not naïve T-cells to enter the brain (40–42). Therefore, in the absence of inflammation, the brain parenchyma is largely devoid of immune cells. However, it is important to recognize that T-cells can cross the BBB and infiltrate the brain parenchyma given the right circumstances (43–45).

T-Cell Recruitment to the Brain Parenchyma

Although the infiltration of immune cells is heavily restricted, there are a few mechanisms by which a small number of lymphocytes and antigen presenting cells can enter the CNS: (i) via the post-capillary venules into the perivascular space; ii) by extravasation through the choroid plexus of the ventricles into the CSF; or iii) through superficial leptomeningeal vessels into the subarachnoid space (46, 47). We will discuss the first mechanism in detail as it pertains most directly to the recruitment of T-cells into the brain parenchyma.

Recruitment of T-cells into the brain parenchyma is a sequential, coordinated process beginning with the binding of integrins α4β1 and lymphocyte associated antigen-1 (LFA-1) expressed on activated T-cells to the adhesion molecules vascular cell adhesion molecule 1 (VCAM1) and intracellular cell adhesion molecule 1 (ICAM1) on endothelial cells, respectively (39). Adhesion of cells to endothelial cells of the CNS also involves a tissue-restricted adhesion molecule, activated leukocyte adhesion molecule (ALCAM) which binds CD6 on mature T-cells (48). The rolling of T-cells established by these ligand-binding interactions leads to the activation of G protein-coupled receptors on the T-cells, resulting in conformational changes that promote tight binding of integrins to cell adhesion molecules on the endothelium. Following these integrin-adhesion molecule interactions, T-cells traverse through the endothelial lining and reach the perivascular space. Activated T-cells must then cross the glia limitans to enter the brain parenchyma. The entry of T-cells into the brain parenchyma is regulated by matrix metalloproteases (MMPs) secreted by other T-cells (49). Furthermore, factors such as tumor necrosis factor-α (TNFα), IL-12, TGFβ, and IL-6 secreted by astrocytes of the glia limitans in an inflammatory setting additionally regulate entry of activated T-cells across the BBB (50, 51). Similarly, increased expression of cell adhesion molecules in malignant glioma as well as neuro-inflammatory conditions, such as such as multiple sclerosis (MS) and experimental autoimmune encephalomyelitis, was shown to increase infiltration of T-cells into the brain parenchyma (48, 52, 53). Another critical factor that dictates T-cell recruitment into the parenchyma is antigen-specificity. Galea et al. demonstrated that antigen specific $CD8^+$ T-cells can traffic to the site of the brain where cognate antigen is present, while $CD4^+$ T-cells can traffic across the BBB regardless of antigen specificity (38).

Interestingly, it is clear from radiographic imaging of GBM that these tumors regularly disrupt the BBB to an extent that varies within tumors and between patients (54). In particular, glioma cells have been shown to breach the BBB by decoupling vasculature from the astrocytic endfeet that maintain BBB integrity and potentially increase exposure of the tumor to administered therapeutics (55). Nevertheless, BBB is intact in portions where glioma cells infiltrate into the normal brain tissue, and thus novel strategies will be necessary to overcome the tight regulation of lymphocyte trafficking into the brain parenchyma for the success of immunotherapy. From our discussion of CNS anatomy and T-cell recruitment, it is clear that the mechanisms by which T-cells enter the brain parenchyma are complex and require multifaceted considerations of the broader circumstances in the CNS environment. A deeper understanding of the mechanisms underlying T-cell recruitment, especially as it pertains to the heterogeneous settings of malignant glioma, will be required for the development of safe and effective genetically engineered T-cell therapies.

Strategies to Overcome the Unique Neuroanatomical Challenges of the Brain

Regional Delivery

To circumvent the difficulties of CAR T-cell trafficking into the brain parenchyma and to reduce systemic toxicity associated with intravenous delivery, several investigators have initiated clinical trials to study the safety and efficacy of regional delivery of CAR T-cells (**Figure 2**). Regional delivery has been attempted to improve CAR T-cell localization in ovarian cancer, mesothelioma, lung cancer, breast cancer, and squamous cell cancer of the head and neck (NCT02498912, NCT02414269, NCT01818323). Several authors have established the safety and efficacy of intracranial or intrathecal delivery of EGFRvIII and IL13Rα2 CAR T-cells in preclinical models of GBM. Currently, there have been three clinical trials using regional delivery of CAR T- cells as an approach to compensate for poor T-cell

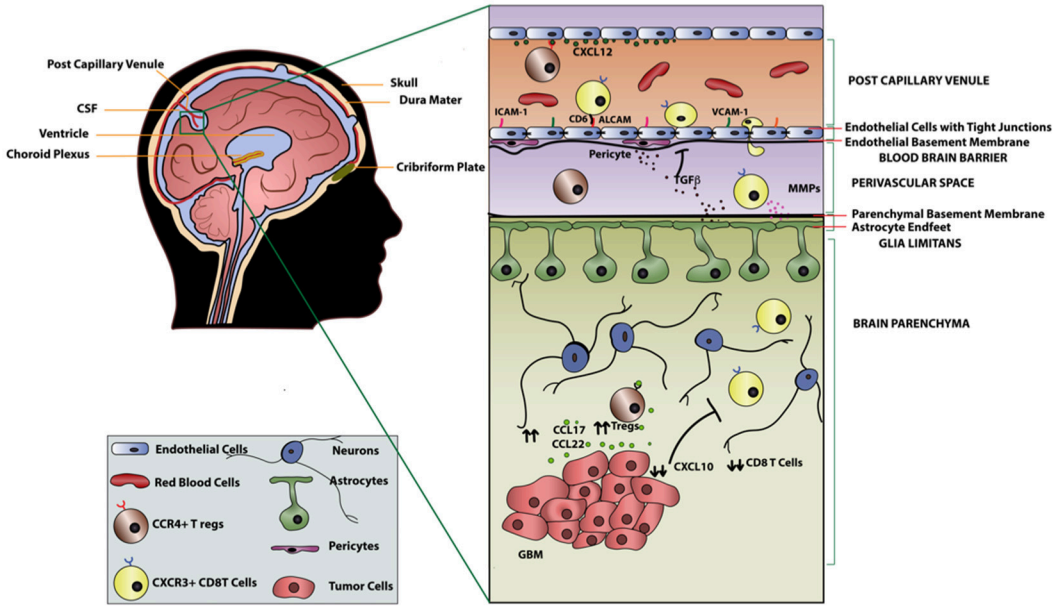

FIGURE 1 | T-cell migration across the blood brain barrier in GBM.

homing and reduce systemic toxicity (25, 26, 56, 57). Yaghoubi et al. treated a GBM patient via intracranial delivery of IL12Rα2-specific CAR T-cells after resection of initial tumor. Tumor regression was observed and T-cells persisted for more than 5 weeks without adverse effects (56). As discussed prior, Brown and colleagues have conducted two clinical trials exploring the local administration of IL-13Rα2 CAR T-cells into GBM patients. In one patient with multiple lesions with meningeal disseminations, who received repeated intraventricular administration of IL-13Rα2 CAR T-cells, persistence of the CAR T-cells was seen in the CSF for at least 7 days after the last intracranial infusion, and the patient had a complete response for 7 months before the tumor recurred. The authors also observed a robust increase in inflammatory cytokine and chemokine induction in the CSF after infusion compared to the baseline levels without observable increase in the peripheral blood (26). Similarly, another ongoing clinical trial providing autologous peripheral blood mononuclear cells transduced with EGFRvIII CAR directly into the tumor site aims to increase the efficacy of CAR therapy and reduce the systemic off-site effects (NCT03283631). While promising, it is important to recognize that intra-CSF delivery of CAR T-cells does not necessarily mean effective delivery to the brain parenchyma, where most glioma tissues reside. Furthermore, the post-infusion persistence of transferred T-cells remains to be elucidated as preclinical studies continue to show varied results (58). The lack of lymphoid organs in the brain to support lymphocyte survival may be one of the factors driving this diminished persistence.

CAR T-Cells Expressing Chemokine Receptors

Efficacy of systemic delivery approaches, such as intravenous infusion, depends on trafficking of CAR T-cells to the tumor site. In addition to adhesion molecules that we discussed earlier in this review, the ability of CAR T-cells to effectively localize to the tumor site also requires expression of chemokine receptors corresponding to chemokine ligands expressed by the tumor (59). Amankulor et al. reported that the T-cell attracting chemokines CXCL9, CXCL10, CCL2, and CCL12 are downregulated in *isocitrate dehydrogenase (IDH)*-mutated gliomas, resulting in the repression of immune cell infiltration (60). On the other hand, CCL17 and CCL22, which promote recruitment of $CCR4^+$ T regulatory cells (Tregs), are upregulated in GBM (61). Interestingly, the expression of CCL2 by some gliomas, which attracts $CD8^+$ T-cells, has been exploited by investigators for adoptive T-cell strategies (62, 63). Another way of improving homing of CAR T-cells to the tumor site is by engineering CAR T-cells that co-express chemokine receptors (**Figure 2**). Our group and others have found that CXCR3, along with its ligands CXCL9 and CXCL10, plays predominant roles in cytotoxic lymphocyte trafficking into the glioma tumor site (45, 64, 65). We have also shown that adjuvant polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (poly-ICLC) provided systemically can promote cytotoxic lymphocyte trafficking into gliomas in an IFN-α and IFN-γ dependent manner through induction of CXCL10 (44).

Additionally, expression of CXCL12 and its receptors, CXCR4 and CXCR7, in the CNS plays important roles in determining whether lymphocytes can gain entry in the CNS under normal and inflammatory conditions. Polarized expression of CXCL12 on the basolateral surface of endothelial cells of the BBB retains CXCR4 expressing leukocytes in the perivascular space and prevents extravasation into the CNS parenchyma under normal conditions (66). During pathological conditions such as multiple sclerosis, polarized expression of CXCL12 is disrupted

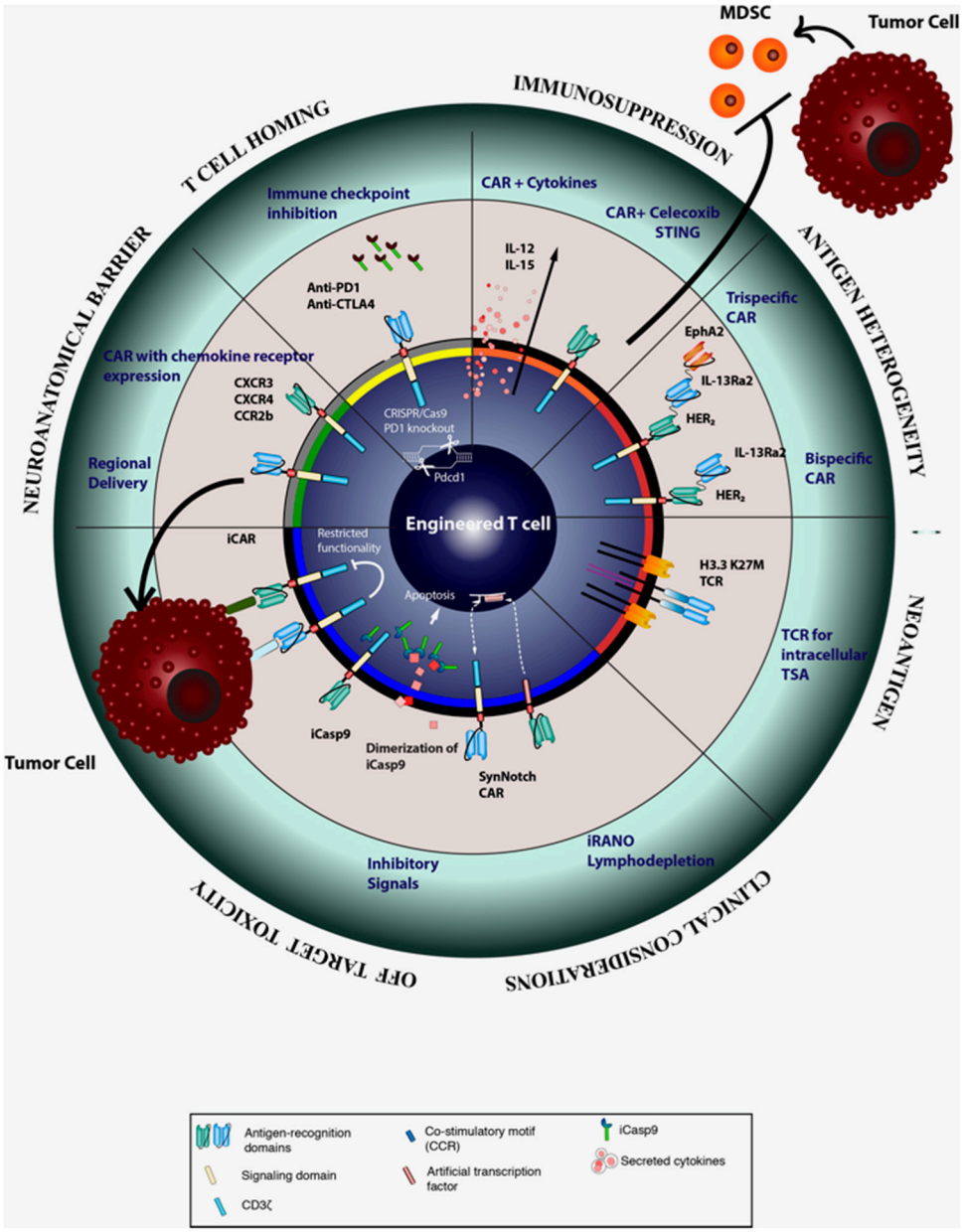

FIGURE 2 | Strategies for improving the efficacy CAR T cell therapy.

by overexpression on the luminal side of the endothelium, thereby resulting in enhanced leukocyte trafficking into the CNS. Klein and colleagues have demonstrated that blockade of CXCR4 on T-cells could facilitate lymphocyte escape from the perivascular space into the CNS parenchyma (67). Additionally, both CXCR4 and CXCR7are highly overexpressed in patient-derived glioma cells (68, 69) and play a critical role in progression of the disease (70). While CXCR4 antagonism inhibited GBM tumor growth in various pre-clinical models (71–73), its direct role in recruitment of T-cells in GBM is yet to be determined.

Focused Ultrasound

In addition to strategies intended to enhance homing to the tumor site, there are therapies aimed at disrupting the BBB that have yet to be tested in the setting of CAR T-cells. High intensity focused ultrasound (HIFU) is a thermal ablation technique that has shown to increase activated TIL migration into solid tumors including breast, liver, pancreas, kidney and bone cancer (74–76). While HIFU is shown to disrupt the BBB, it is also accompanied by some tissue damage (77). Therefore, an alternative approach entitled Focused Ultrasound (FUS), which uses intensities similar to diagnostic ultrasounds, is used along

with microbubbles injected intravenously for regional delivery of drugs and cytokines into the brain parenchyma (78). Chen et al. observed an increase in tumor infiltrating lymphocytes and cytotoxic lymphocytes, in particular, after FUS exposure in the presence of microbubbles (79). Although promising, the possibility of using FUS to increase localization of CAR T-cells to the brain safely needs to be thoroughly researched.

IMMUNOSUPPRESSION IN THE GLIOMA MICROENVIRONMENT

Tumor Cell Intrinsic Mechanisms

The genomic landscape of glioma is complex and encompasses structural rearrangements, mutations in signature oncogenes (*EGFR*, *TP53*, etc.), as well as chromosome modifying proteins such as *ATRX* and *IDH* (19). Among patients with LGG or secondary GBM, mutations within *IDH1* and *IDH2* have been reported in 70–80% of cases (80). The single amino acid change within the isocitrate-binding domain (R132 in IDH1; R140 or R172 in IDH2) confers a gain-of-function mutation leading to the accumulation of the oncometabolite 2-hydoxyglutarate and potential genome-wide epigenetic changes. Our group recently reported that *IDH*-mutant glioma cells are able to influence the tumor immune environment through the suppression of type 1 immune response genes (81). We observed decreased overall expression and activation of signal transducer and activator of transcription 1 (STAT1) and significantly lower levels of the effector T-cell attracting chemokines, such as CXCL10, produced by IDH-mutant glioma cells. Furthermore, a study by Berghoff and colleagues reported a significantly lower rate of T-cell infiltration in *IDH*-mutant vs. *IDH*-wildtype gliomas (82). These studies provide evidence that genetic alterations intrinsic to the glioma tumor cells are able to alter the cellular composition of the TME and aid in immune evasion. Therefore, novel immunotherapeutic approaches need to address the downstream consequences of tumor cell intrinsic mutations in addition to targeting tumor antigens.

In addition to genetic mutations, GBM cells display a vast array of molecular signaling alterations, such as the increased expression and activation of STAT3 (83). Activation of STAT3 results in dynamic transcriptional changes depending on the cellular context (84). In GBM, phosphorylated STAT3 (p-STAT3) has emerged as a major regulator of immune suppression (85). Treatment of GBM patient-derived myeloid cells with the p-STAT3 small molecule inhibitor WP1066 resulted in upregulation of the co-stimulatory molecules CD80 and CD86. Furthermore, in the presence of WP1066, normally unresponsive patient T-cells were shown to proliferate when stimulated with autologous APCs (85). In addition to the immunosuppressive effects of STAT3 in immune cells, work by Wei and colleagues demonstrated that GBM-initiating cells have a constitutively active STAT3 pathway, and that inhibition of STAT3 significantly diminished the ability of these cancer-initiating cells to suppress T-cell expansion and induce Treg recruitment (86). Based on these promising data, WP1066 is being evaluated in a phase I clinical trial for patients with recurrent GBM and melanoma patients with brain metastases (NCT01904123). Inhibition of STAT3 in the GBM microenvironment may significantly contribute to the efficacy of anti-GBM CAR T-cells, and thus the outcomes of this and any future STAT3-targeting clinical trials are highly anticipated.

The cellular arm of the immune system offers a potent, selective, and durable mechanism of protection through the tightly regulated interactions of T-cells and the vast array of peptides presented in the groove of human leukocyte antigen (HLA) molecules. Cytotoxic $CD8^+$ T-cells depend on HLA class I- presented peptides for their activation. A major immune resistance mechanism in GBM is the downregulation of HLA class I expression on tumor cells (87). In certain cases, expression of HLA class I can be restored by treatment with IFN-γ; however, mutations leading to loss of heterozygosity (LOH) of the HLA class I and beta-2 microglobulin regions can result in irreversible downregulation of HLA class I. Our group has previously reported that 41% of analyzed GBM samples showed LOH in the HLA class I region, and this was significantly associated with shorter survival in newly diagnosed GBM patients (88). Downregulation of HLA class I expression can also be the result of changes to the cellular antigen-processing machinery which is involved in stabilizing and promoting the cell surface expression of HLA-I molecules. Tapasin is a protein known to facilitate the binding of peptides to class I molecules, and in its absence, the expression of HLA class I is significantly reduced (89, 90). Thuring et al. reported the significant correlation between tapasin and both HLA-I expression and GBM patients survival time (91). While CAR constructs must target surface antigens, a majority of cancer-specific neoantigens are derived from intracellular proteins. This certainly gives an advantage for TCR-based approaches. However, success of TCR-based approaches will likely require additional therapeutic strategies to ensure sufficient HLA expression levels in the tumor site.

Secreted Factors in the Tumor Microenvironment

In addition to tumor cell intrinsic factors, various other mechanisms have been described that render the GBM microenvironment exceptionally immunosuppressive. These include the recruitment of Tregs and suppressive myeloid cells as well as the upregulation of immune checkpoint molecules and immunosuppressive cytokines [reviewed in detail (92)]. One approach proposed to counteract the immunosuppressive microenvironment is the co-expression of cytokines such as IL-12 and IL-15 by CAR T-cells (93, 94). IL-12 has been shown to enhance $CD8^+$ T-cell activation and to act on surrounding innate immune cells by providing a type I differentiation signal. As a result, pre-clinical models suggest that tumor antigen-specific T-cells engineered to express IL-12, survive longer in the tumor milieu and are more effective at tumor clearance than CAR transgenic T-cells alone (93) As lack of CAR-T cell persistence *in vivo* is another recurring obstacle in both pre-clinical and clinical studies, researchers have engineered CAR-T cells to express the pro-T cell survival cytokine IL-15 in an activation-dependent manner. Krenciute

and colleagues demonstrated that upon recognition of their cognate antigen, T-cells transduced with IL13Rα2-CAR and IL-15 upregulated production of IL-15 which enhanced the cells' effector function and their antiglioma activity *in vitro* and *in vivo* (94).

In addition to utilizing a double transgene strategy, the authors make use of an anti-IL13Rα2 CAR integrating an antibody-derived scFv as opposed to the zetakine-based IL13Rα2-CAR. This scFv-based CAR construct has been shown to exhibit improved antigen specificity as scFv-based CAR-T cells were able to recognize and kill IL13Rα2-expressing but not IL13Rα1-expressing target cells (95). Despite these therapeutic alterations, the authors reported that gliomas recurred in their xenograft model displaying lower expression of IL13Rα2, signifying the critical need that CAR-T cells promote immune responses against multiple tumor antigens.

Another hurdle to be overcome by adoptively transferred CAR T-cells is the high local levels of TGF-β in the TME of GBM (96). Introducing the dominant negative TGF-β type II receptor in addition to the CAR construct when manufacturing CAR T-cells renders them resistant to the effects of TGF-β and has been shown to enhance antitumor activity of the T-cells (97, 98). Furthermore, TGF-β inhibitors and blocking antibodies have been studied extensively pre-clinically. However, their therapeutic efficacy in glioma patients remains unconvincing, likely due to low BBB penetrance, underscoring the possibility that targeting TGF-β alone might not be sufficient to meaningfully impact disease progression (99, 100).

Immunosuppressive Myeloid Cells

Myeloid cells constitute the largest subset of glioma immune infiltrates and can account for up to 50% of the total tumor mass (101, 102). A particular subset of these cells are known as myeloid derived suppressor cells (MDSC) and are generally recognized as a heterogenous population of immature myeloid cells able to support *de novo* gliomagenesis and produce pro-tumorigenic factors within already established tumors (103). Numerous strategies for MDSC depletion and inhibition have been developed, such as the use of the non-steroidal anti-inflammatory drug (NSAID) celecoxib or the administration of STING (stimulator of IFN genes) agonists (**Figure 2**). Our group reported that celecoxib inhibits the production of prostaglandin E2 thus inhibiting the accumulation of MDSCs in the tumor microenvironment (104). The use of celecoxib alone was able to enhance expression of CXCL10 and increase recruitment of cytotoxic lymphocytes to the tumors in a pre-clinical glioma model. Additional experiments demonstrated that intratumoral administration of the STING agonist c-di-GMP was able to relieve the immunosuppressive effect of MDSC *in vivo* (105). As a result of enhanced production of type I cytokines and chemokines, this treatment increased T-cell migration to the tumor site and improved overall survival of tumor-bearing mice. Therefore, the addition of these and other MDSC modulating strategies to traditional adoptive T-cell therapies may provide substantial clinical benefit.

Enhancing CAR T-Cell Function With Immune Checkpoint Inhibitors

Finally, as discussed earlier in this review, both therapeutically administered as well as endogenously activated T-cells are subject to elevated levels of immune checkpoint inhibition in the tumor microenvironment. Recent studies have suggested that tumor-infiltrating lymphocytes within GBM have an increased expression of immune checkpoint molecules such as PD-1, CTLA-4, LAG3, and TIM-3 (27, 106). Similarly, CAR T-cells have also been observed to express immune checkpoint molecules and acquire an exhausted phenotype (107, 108). Upregulation of molecules such as CTLA-4 and PD-1 on T-cells is a natural consequence of T-cell activation and serves the purpose of preventing rampant immune cell reactivity (109). Solid tumors have been shown to co-opt this immune balance mechanism to suppress the local activation and proliferation of T-cells. The ligand for PD-1, PD-L1, is present on both tumor cells and infiltrating myeloid cells. Although a recent study by Nduom et al. reported a median PD-L1 expression of 2.8% in their study of 94 GBM samples, robust induction of PD-L1, which is presumably due to local IFN-γ production, on GBM tissues was observed in the recent EGFRvIII-CAR clinical trial (12, 110).

Blockage of CTLA-4 and PD-1 in murine solid tumor models has led to an increased expression of activation markers by T-cells, such as IFN-γ, IL-2, perforin, and granzyme; furthermore, these treatments have resulted in improved trafficking of activated T-cells to the tumor site (111, 112). While the FDA-approved checkpoint inhibitors are administered systemically, specific blockade of checkpoint molecules within the therapeutic T-cells would mitigate systemic toxicities. Cherkassky et al. developed CAR-T-cells co-transduced with a dominant-negative PD-1 receptor lacking all the intracellular signaling domains (107). Using a pleural mesothelioma model, the authors reported that CAR-T cells expressing the dominant-negative PD-1 receptor controlled the tumor growth more efficiently than the control CAR-T cells, owing to their enhanced survival and ability to evade activation-induced exhaustion. Furthermore, the PD-L1-PD-1 signaling for immunosuppression may take place not only the surface of interacting cells, but may also be mediated by soluble PD-L1 in extracellular vesicles (EVs). A recent report by Ricklefs et al. suggests that GBM-derived EVs, such as exosomes and microvesicles inhibit human T-cell activation and proliferation. This effect correlated with the amount of PD-L1 carried by the EVs and was partially reversed through the use of an anti-PD-L1 antibody (113). Clinical efforts testing the effects of anti-PD-1 therapy alone in patients with recurrent GBM failed to show improved overall survival when compared with other agents (114) (NCT02017717). However, ongoing clinical trials are currently evaluating the use of CAR T-cells with built in CTLA-4 and PD-1 blockade, CAR T-cells in combination with anti-PD-1/PDL1 (115) (NCT03170141, NCT02706405), and the use of CRISPR/Cas9 to disrupt PD-1 in CAR T-cells (NCT03208556) with the aim of increasing CAR T-cells efficacy (**Figure 2**).

CAR T-Cell Persistence and Antigen Specific Memory

As the addition of checkpoint inhibitors may not be enough to re-energize T-cells that are exhausted or drive the persistence of antigen specific memory T-cells to prevent GBM recurrence (116), new strategies are being explored to address these aims. A study by Sengupta and colleagues has investigated the use of a glycogen synthase kinase 3 (GSK3) inhibitor for improving expansion and persistence of the CAR T-cells. GSK3 is constitutively active in naïve T-cells and is inactivated briefly during clonal expansion of the activated T-cell (117). At peak expansion, GSK3 becomes active and results in clonal contraction and ultimately death of the activated T-cell (118, 119). The specific blockade of this protein with small molecule inhibitors results in T-cell expansion and the generation of memory T-cells (120, 121). Sengupta and colleagues reported that IL-13Rα2 CAR T-cells treated with a GSK3 inhibitor showed reduced exhaustion and increased expression of an effector memory phenotype ($CD62L^{lo}/CD45RO^{hi}/CD127^{+}$) (122). Based on the demonstrated protective effects of GSK3 inhibition on activated T-cells, the authors of this study administered the IL-13Rα2 CAR T-cells and GSK3 inhibitor to mice bearing subcutaneous GBM xenografts and demonstrated that mice re-challenged with tumor after initial clearance did not develop new lesions. Furthermore, they identified CAR^{+} effector memory T-cells in the draining lymph nodes and spleens of these animals at 100 days following initial CAR administration.

ANTIGEN HETEROGENEITY, ANTIGEN ESCAPE, AND OFF-TUMOR TOXICITY

Antigen Heterogeneity and Escape in GBM

In addition to being tumor-specific, ideal candidate tumor antigens must be expressed homogenously on the surface of a majority of tumor cells to mediate effective tumor killing. Antigen heterogeneity has been a universal barrier to effective CAR therapy across cancer types, including in the setting of CD19-CAR for leukemia and lymphoma (123). GBM is especially challenging in this regard, as clinical studies for all major tumor-specific and tumor-associated antigens to date have observed outgrowth of antigen loss variants due to substantial heterogeneity within tumors (12, 25–27). Because of its desirability as a tumor-specific antigen and extensive characterization, we will focus on EGFRvIII here as a prototypical example of antigen heterogeneity in GBM.

A variety of EGFRvIII CAR variations have been tested pre-clinically, with alterations in number as well as type of co-stimulatory domains and these studies have demonstrated effective and specific tumor lysis in murine and patient-derived tumor models (124–129). However, as discussed earlier, the first clinical study testing a second-generation EGFRvIII CAR failed to demonstrate efficacy and instead highlighted the strong adaptive capabilities of GBM cells to escape the surveillance of CAR T-cells by eliminating or altering antigen expression over time (12). Pre-clinical studies have also demonstrated that EGFRvIII seems to evade T-cell based targeting approaches due to the vast heterogeneity in its expression on tumor cells (130). Active amplification and rearrangement of EGFR can be found throughout GBM tumors, regardless of EGFRvIII status. Moreover, EGFRvIII-positive subpopulations may give rise to EGFRvIII negative clones which can subsequently re-express EGFRvIII after undergoing epigenetic modification. The survival of antigen loss variants and the relative ease of reacquiring EGFRvIII may contribute to the consistent recurrence of GBM tumors following EGFRvIII-CAR T-cell therapy. Separate mechanisms guided by the same principles may underlie antigen loss and tumor recurrence in the settings of other GBM CAR antigens.

An important barrier to the pre-clinical evaluation of antigen loss in EGFRvIII-CAR T-cell therapy has been the absence of $EGFRvIII^{+}$ GBM patient-derived cell lines and syngeneic mouse models that effectively recapitulate the dynamics of EGFRvIII heterogeneity in patient tumors and allow for accurate prediction of long-term EGFRvIII-CAR T-cell therapy success in the clinic. Recently, several groups have sought to overcome this barrier by engineering novel cell lines and pre-clinical models to better reproduce the heterogeneous nature of EGFRvIII and some glioma-associated antigens (24, 131). Similar methodologies will need to be explored for modeling other candidate antigens undergoing pre-clinical evaluation for CAR T-cell therapy.

Combinatorial Approaches Utilizing Tumor-Associated Antigens

Antigenic profiling of GBM has revealed a vast availability of tumor-associated antigens that may be targetable with immunotherapy (132), yet development of CARs specific for those novel antigens is hindered by safety concerns with regards to systemic and on-target off-tumor toxicity. Several tumor-associated antigen targets have been exploited for the development of CAR. Ephrin type A receptor 2 (EphA2), IL-13Ra2, and HER2 represent promising tumor associated antigens that have been targeted both pre-clinically and clinically using CAR T-cell therapy in the setting of GBM (23, 133, 134). Efficacy for monovalent CAR T-cells targeting each of these antigens has been established in pre-clinical models (135–138). Multiple groups have attempted to address the hurdle of antigen heterogeneity and escape in GBM by engineering combinatorial approaches that simultaneously target multiple GBM-restricted antigens at once (**Figure 2**). Hegde, Grada, and colleagues have developed a tandem CAR combining the recognition of IL-13Rα2 and HER2, based on mathematical modeling that predicted 90% tumor killing in GBM patients with this antigen combination (139, 140). This group went on to demonstrate superior efficacy *in vivo* for the tandem CAR (tanCAR) construct over bivalent CAR targeting the same antigens and observed that, in contrast to bivalent CAR, IFNγ and IL-2 secretion from $tanCAR^{+}$ T-cells was higher than simply an additive effect of two monovalent CARs (141). Unfortunately, tumors did eventually recur in all groups after antigen clearance and tanCAR T-cells were shown to develop comparable increases over time in PD-1 and LAG3, although not TIM3. Based on the analysis of antigen variability across GBM patient cell lines, Bielamowicz and colleagues have

since developed a tri-cistronic CAR transgene encompassing IL-13Rα2, EphA2, and HER2, which they called universal CAR (UCAR) (142). The authors reported increased cytolytic potential for UCAR$^+$ T-cells over bivalent CAR$^+$ T-cells, which was at least partially due to a smaller and more highly organized immune synapse. Although using the trivalent UCAR$^+$ T-cells resulted in significantly increased survival, tumors did recur in some mice between 40 and 60 days after the first T-cell injection following loss of all three antigens. Repeated observation of antigen loss begs the question of how many antigens must be targeted at once for critical mass to occur and drive the complete remission of malignant glioma (141). Further combinations of tumor-associated and tumor-specific antigens remain to be developed for targeting GBM while preventing acquired immune resistance in the form of antigen loss.

Mitigating Off-Tumor Toxicity

One of the most important risks associated with CAR T-cell therapy is on-target off-tumor toxicity, particularly in the case of T-cells targeting tumor-associated antigens. With the exception of EGFRvIII, all of the GBM antigens that are currently being evaluated clinically may be expressed at low-levels on normal tissues, which can result in substantial toxicity. The risk of on-target toxicity increases with affinity of the engineered T-cells to their antigen targets, as well as the potency of the T-cells and antigen expression level on normal tissues (143). In a trial of high dose HER2-CAR T-cell for metastatic colon cancer, one patient died of respiratory failure after low levels of HER2 were engaged on the lung epithelium, but subsequent studies using modified and lower affinity HER2-CAR T-cell have not led to any additional case reports which suggests that these modifications may improve safety (143, 144). A high avidity TCR engineered to target the melanoma associated antigen A3 (MAGE-A3) was tested in a Phase I clinical trial and despite showing strong antitumor effects in most patients, this treatment led to the death of three of the patients receiving the highest dose regimens. This TCR was known to recognize another MAGE-A family member, MAGE-A12, with 10-fold higher affinity. After the death of these patients, MAGE-A12 expression was subsequently found on a subset of neurons in these patients and control brains by histopathological examination (145); these findings underscore the need for stringent characterization of CAR binding and cross-reactivity in normal tissues. Neurotoxicity, characterized by endothelial activation and increased permeability of the BBB, is also a concern for CAR therapy as it was observed in a patient following CD19-CAR T-cell therapy (146). In a pre-clinical murine model, while CAR T-cells targeting GD2 demonstrated a marked efficacy in DIPG xenograft models, peritumoral neuroinflammation during the acute phase of antitumor activity resulted in hydrocephalus that was lethal in a fraction of animals (147). Furthermore, fatal encephalitis resulting from low-level antigen expression on the cerebellum was recently observed following GD2 ganglioside CAR T-cell therapy for neuroblastoma (148). Cytokine release syndrome (CRS) is also an important risk of CAR T-cell therapies that must be managed in the clinical setting. However, none of the existing published trials of CAR T-cells targeting GBM antigens have resulted in CRS or elevated peripheral cytokine levels. Management of off-tumor effects, neurotoxicity, and the potential of CRS remain essential considerations in the development of novel CAR T-cell therapy for human trials.

Several novel approaches have been generated for engineering CARs to limit off-tumor and systemic toxicities that might have promising applications in the context of GBM (**Figure 2**). One important method that has yet to be explored in CNS cancers is the introduction of a latent suicide switch such as inducible caspase-9 (iCASP9) enzyme, which can be used to direct T-cell apoptosis following the administration of a small-molecule drug (149). One benefit of this strategy is the ability to rapidly deplete administered T-cells to resolve cases of CRS and acute tissue toxicity in the clinical setting. In a clinical trial utilizing iCASP9$^+$ alloreplete T-cells after stem cell transplantation where graft vs. host disease was detected, the administration of a small molecule homodimerizer eliminated 85–95% of circulating T-cells within 30 min (150). Employing such an approach in the CNS will require utilizing small molecule drugs with ample ability to cross the BBB where prevention of toxicity to normal brain tissue is warranted. Inhibitory CAR T-cells (iCARs), which target a tumor antigen but co-express an off-switch that is stimulated by a normal tissue-derived cognate antigen, has also been proposed to minimize allogenic CAR T-cell activation in the context of CD19-CAR (151). Roybal, Morsut, and colleagues have recently developed a novel system utilizing a synthetic Notch receptor whose activation drives the transcription of a second generation CAR (Syn-Notch CAR).The goal of this circuit is to prevent any CAR T-cell activation without the separate and sequential engagement of two cognate antigens which may be derived from either the tumor or the tissue microenvironment (152, 153). They reported that Syn-CAR$^+$ T-cells failed to become activated in the absence of either antigen and demonstrated superior tumor-killing efficacy over bivalent CAR$^+$ T-cells (153). An alternative strategy to mitigate off-tumor toxicity is a switch-mediated CAR that uses an antigen-specific antibody-based molecule which specifically binds the administered SwitchCAR$^+$ T-cells. The binding of these antibody-based switches drives immunological synapse formation between SwtichCAR T-cells and tumor cells in a dose-dependent manner in xenograft models of CD19$^+$ and CD20$^+$ hematological malignancies, respectively (154, 155). This methodology has also been effective in targeting HER2$^+$ breast cancer (156). Despite the plethora of methods being explored for CAR engineering for mitigation of on-target off-tumor and systemic toxicity, these strategies have yet to be evaluated in the context of GBM.

Comparing Efficacy of CD4$^+$ and CD8$^+$ CAR T-Cell Subsets in Glioma

While most of the clinical trials we have discussed thus far have used a mixture of CD4$^+$ and CD8$^+$ T-cells (12, 26, 27) or CD8$^+$ T-cells alone (25), there have been recent reports that CD4$^+$ CAR T-cell subsets, in particular, may promote antitumor efficacy. Pre-clinical models, including a model of GBM, utilizing CD4$^+$ cells

transduced with a tumor-specific CAR have been found to aid in tumor-killing by other T-cell subsets as well as to lyse tumor cells directly (157, 158). In the setting of CAR T-cell therapy for solid tumors, the existence of $CD4^+$ subsets has been found to increase CAR T-cell activity and persistence *in vivo* (58, 159). A recent pre-clinical study directly compared efficacy of a second generation IL-13Rα2 CAR transduced into patient-derived $CD8^+$ or $CD4^+$ T-cells and found $CD4^+$ CAR T-cells demonstrated enhanced tumor killing and persistence compared with $CD8^+$ and a mixed $CD4^+/CD8^+$ population in a xenograft model of GBM (159). The $CD4^+$ CAR T-cells in this study secreted more IFNγ and IL-2 than $CD8^+$ T-cells, while the $CD8^+$ CAR T-cells more readily began expressing exhaustion markers.

TCR Approaches for Targeting Malignant Glioma

Because CAR targets are limited to surface expressed antigens, the abundance of tumor-specific neoantigens derived from intracellular proteins has driven the development of TCR-based approaches (**Figure 2**). The histone H3 position 27 lysine to methionine substitution (H3.3 K27M) mutation is shared across 70% of diffuse intrinsic pontine glioma (DIPG) patients and a majority of DMG patients (160). It results in a global decrease of methylation at H3K27me3 and results in the suppression of polycomb repressive complex 2 (PRC2) and altered gene expression (161). Overall survival in DIPG patients with this mutation is shorter compared with patients harboring wild type H3.3 (160). We recently identified an HLA-A*02*:01-restricted epitope which includes the H3.3 K27M mutation, and we cloned cDNA of TCR α- and β-chains from this clone for transduction into T-cells. In this report, we showed that T-cells transduced with this TCR specific for H3.3K27M efficiently killed $H3.3K27M^+$ glioma cells *in vitro* in an antigen- and HLA-specific manner (14). Furthermore, these TCR transduced T-cells also suppressed the progression of intracranial glioma xenografts in mice when used in adoptive transfer studies. These data are the basis for an upcoming Phase I clinical trial administering adoptively transferred T-cells with our transduced H3.3 K27M TCR. While the H3.3 K27M TCR has been effective in murine models of $H3.3K27M^+$ malignant glioma, the effectiveness of TCR approaches in patients may require assurance of HLA class I expression, as discussed earlier. Nonetheless, this strategy remains a potent tool for targeting immunogenic epitopes which are not surface expressed but can be routinely presented by HLA Class I.

In order to develop effective TCR-based therapeutic approaches targeting antigenic heterogeneity of malignant glioma, additional novel tumor-specific neoantigens will need to be identified. A variety of deep sequencing and *in silico* HLA docking approaches have been employed with the aim of identifying neoantigens that can be effectively targeted by CAR and TCR approaches (162–164). These immunogenomics approaches are especially relevant in the context of GBM as 20–30% of recurrent GBM have been found to exhibit a hypermutator phenotype and may provide a rich supply of antigens for achieving complete patient response (162).

Re-discovering Glioma Antigens for CAR T-Cell Therapy

In addition to EGFRvIII, EphA2, IL-13Ra2, and HER2, several other tumor-associated antigens have previously been explored as targets for GBM therapies in preclinical models (165–167). CD70 is found to be highly expressed in both primary and recurrent LGG and GBM, particularly in association with wild-type IDH expression (168). It has been shown to play an important role in recruiting immunosuppressive myeloid cells to the tumor microenvironment and CD70-CAR T-cells have demonstrated remarkable efficacy in patient xenograft and syngeneic murine tumor models (169). Chondroitin sulfate proteoglycan 4 (CSPG4) represents another emerging target for GBM CAR T-cells with high expression of this antigen in two-thirds of GBM patient specimens with little expression on normal tissues (170). GBM neurosphere engraftment in nude mice followed by the infusion of a third generation CSPG4-CAR T-cell demonstrated lasting efficacy and minimal antigen escape, at least partially due to the upregulation of CSPG4 on tumor cells by microglia-derived TNF-α in the tumor microenvironment.

While there is currently a limited number of tumor-specific antigens being targeted in GBM, this list can be expanded through the identification and analysis of tumor-specific post-translational modifications of glioma surface proteins. In particular, novel glycosylation patterns on proteins expressed by tumor cells may allow for the specific targeting of these cells, as in the case of the unique mucin 1 (MUC1) glycoepitopes that are highly expressed in a variety of cancers (171–173). Adoptively transferred T-cells stimulated against MUC1 have demonstrated promising results in clinical trials for breast and ovarian cancers (174–176). Based on the experience of MUC1, the identification and targeting of post-translational modifications of surface expressed proteins may constitute an important strategy for developing novel CAR T-cell therapies in GBM.

PITFALLS AND OPPORTUNITIES FOR BUILDING ON GLIOMA IMMUNOTHERAPY

Radiographic Imaging and Pseudoprogression

In the assessment of treatment response, clinicians rely on radiographic imaging data to interpret changes in tumor size and composition (177). In particular, enhanced regions on contrast-enhanced T1-weighted images are indicative of changes to BBB permeability resulting from tumor proliferation and angiogenesis. Recognizing that the mechanisms behind immunotherapeutic response and recurrence may complicate the interpretation of radiographic information, the Immunotherapy Response Assessment in Neuro-oncology Working Group (iRANO) has proposed new guidelines to facilitate assessment of immunotherapeutic response and address the issue of pseudoprogression following immunotherapy (178). Following treatment with immunotherapy, radiographic lesions may spread beyond incipient tumor margins and include new distal and local radiographic lesions. These changes to images after immunotherapy are inherently ambiguous and may represent

immune infiltration of TME, worsening tumor burden, or a mixed pathology. Radiographic pseudoprogression is transient in nature but can result in the premature termination of potentially beneficial immunotherapeutic treatments and the skewing of clinical trials toward potentially less responsive patients if left unrecognized. iRANO has proposed that clinicians consider pseudoprogression for any apparent radiographic progression within the first 6 months following the beginning of an immunotherapeutic regimen, in the absence of neurological decline, and that indications of progressive disease is confirmed only after follow-up imaging session before the patient is reclassified. Moving forward, there is a considerable need for alternative imaging techniques to be validated, such as magnetic resonance spectroscopy (MRS), perfusion and diffusion MRI, as well as PET scanning for distinguishing tumor progression from immune infiltration (179–183). In addition to improving the criteria by which radiographic images are assessed, clinicians are also encouraged to gather biopsy specimens of lesions whenever possible in order to rule out pseudoprogression and ensure that patients are given a full opportunity to benefit from immunotherapy regimens.

Dexamethasone Administration

While genetically engineered T-cell based immunotherapy is focused upon the development of strong adaptive responses against tumor tissue in the CNS, clinical treatment of GBM often requires the administration of corticosteroids such as dexamethasone to prevent the onset of neurological symptoms associated with peritumoral edema (178). In preclinical models, dexamethasone treatment is associated with a dose-dependent decrease in lymphocyte infiltration of tumor tissue and the inhibition of T-cell maturation in the CNS by a suppressive population of monocytes (184). Furthermore, dexamethasone treatment can impede the maturation of dendritic cells and decrease their antigen presentation ability in an already immunosuppressive tumor environment. While much of these data are restricted to patients receiving high doses of corticosteroids, it is clear that the necessary administration of dexamethasone may present a substantial hurdle to some GBM patients receiving T-cell based immunotherapy unless these issues are addressed. Brown and colleagues recently addressed the question of dexamethasone in CAR T-cell therapy in a xenograft model of GBM. They found that while high-dose dexamethasone completely inhibited CAR T-cell antitumor effects, low-dose dexamethasone did not diminish antitumor effects mediated by CAR T-cell in mice (185). Dexamethasone administration will need to be considered on a patient-by-patient basis and weighed against potential and observed clinical benefit from immunotherapy. The maximum dose of dexamethasone that will not undermine therapeutic response to CAR T-cell therapy remains to be defined in the glioma setting. Ongoing and prospective CAR T-cell therapies for malignant gliomas will need to consider alternative ways to manage the symptoms of progressive disease without corticosteroids, such as through the use of the anti-angiogenesis antibody-based drug, bevacizumab. Additional methods may be required to overcome the immunosuppressive and anti-homing effects of corticosteroid treatment, including alternative delivery routes, more potent CAR T-cells, and the combined strategies for addressing immunosuppressive microenvironment that we have described.

Lymphodepletion and Cytotoxic Therapy

Even though cyclophosphamide and fludarabine have been most widely used for lymphodepletive conditioning regimens prior to CAR T-cell therapies, we focus our discussions on a possibility for the usage of an alkylating chemotherapy agent, temozolomide (TMZ), because this is a part of the current standard-of-care alongside radiotherapy and surgical resection for patients with malignant glioma (186). As TMZ is a potent inducer of lymphopenia, it has drawn interest for use as a pre-conditioning agent before adoptive cell therapy (187–189). It is currently understood that the induction of lymphopenia is a necessary precondition for CAR T-cell therapy as it upregulates and eliminates endogenous competition for homeostatic gamma chain cytokines, such as IL-7, IL-15, and IL-2, to enhance CAR T-cell persistence (190), although lymphopenia in GBM patients treated with standard-of-care TMZ + radiation therapy did not induce compensatory upregulation of IL-7 or IL-15 (188). Suryadevara and colleagues recently used a pre-clinical mouse model of GBM treated with EGFRvIII-CAR T-cells to demonstrate that dose-intensified TMZ lymphodepletion can durably enhance CAR T-cell efficacy and persistence, while standard dose TMZ was transient and did not have significantly different effect from vehicle (189). Furthermore, they showed that dose-intensified TMZ lymphodepletion significantly increased the ratio of CAR T-cell:Treg over that with the standard dose of TMZ. Notably, TMZ and other cytotoxic therapy may be able to produce synergistic effects with CAR T-cell therapy, and there is active ongoing research to improve protection of CAR T-cells from the cytotoxic effects of these therapies (129, 191). These preclinical studies, however, need careful interpretations considering the difference in the dose and duration of therapies between humans and mice.

Conventional fractionated radiotherapy also has a profound lymphodepleting effect due to the large volume of blood that perfuses the human brain and can be affected by radiation (187, 188, 192). It has been associated with the recruitment of Tregs and MDSC, resulting in increased production of TGF-β, IL-10, and angiogenic factors in the TME (193). However, it has been hypothesized that radiotherapy might also play a positive role for CAR T-cell therapies. Radiotherapy can result in release of danger signals, such as HMGB1 and HSP70, which activate the innate and adaptive immune systems, in the context of GBM cell lines (194, 195). The cytotoxic effects of local radiotherapy also lead to the phagocytosis of tumor cells, which in turn can induce maturation of dendritic cells and enhance presentation of tumor antigens (196). In murine models, whole brain radiotherapy resulted in upregulation of MHC Class I and increased infiltration of $CD8^+$ and $CD4^+$ T-cells into the tumor microenvironment (197), although murine models do not allow recapitalization of fractionated radiation therapy in humans. Radiotherapy has been explored extensively (198) in combination with checkpoint blockade but relatively little in the area of CAR

T-cells. However, Weiss and colleagues recently developed an NKG2D-based CAR T-cell for use in a preclinical mouse model of GBM and demonstrated improved efficacy and persistence when CAR T-cell therapy was combined with sub-therapeutic dosages of radiotherapy (199). They concluded this synergistic effect was a result of NKG2D ligands released in the TME following radiation. Importantly, while the authors did not observe any off-tumor toxicity, NKG2D ligand expression is not restricted to GBM tissue and could theoretically result in toxicity. As is the case for TMZ, careful interpretation of these preclinical studies is needed considering the relatively short duration of therapy regimens in mice.

CONCLUSION

Glioma immunotherapy continues to present unique challenges due to anatomical barriers associated with the CNS and the intrinsic danger of eliciting an immune response in close proximity to neural tissue. In this review, we have discussed the most recent clinical outcomes utilizing CAR T-cells to target glioma, as well as the strategies being explored to address emerging impediments to these treatments. Limited engraftment and survival of the infused T-cells due to difficulty homing to the tumor site is a substantial problem in genetically engineered T-cell therapies for malignant glioma. Complex anatomical barriers make drainage of antigens and immune cells from the brain parenchyma into the periphery difficult and may mitigate peripheral lymphocyte activation against tumor antigens. Moreover, the homing of T-cells is limited by the BBB and an immunosuppressive TME. Altering the expression patterns of chemokines and their receptors in an effort to enhance T-cell homing to the brain tumor site have shown promise in pre-clinical studies, but these remain to be tested in the clinical setting.

In addition, the heterogeneous display of tumor antigens has resulted in tumor escape and recurrence of malignant gliomas. However, as additional tumor-associated antigens are explored for combined targeting, concerns about on-target off-tumor and systemic toxicities are warranted. Creative solutions to the combined challenges of safety and antigen heterogeneity have emerged in recent pre-clinical studies, as discussed in this review. In addition to enhancing the CAR T-cells efficacy against multiple tumor antigens, mounting evidence supports the need for combining engineered T-cells with modulators of the highly immunosuppressive TME. Recent data discussed here clearly suggest a potential for synergy of CAR T-cells with other treatments targeting the mechanisms of glioma immunosuppression. In addition to these combined strategies, engineering CAR T-cells which also express pro-survival cytokines may aid in overcoming local immunosuppression.

Several questions remain regarding the optimal delivery method and post-treatment care of GBM patients. Another challenge for clinicians designing and executing GBM clinical trials remains the administration of corticosteroids as means of avoiding the neurological symptoms of edema. The establishment of corticosteroid dosing guidelines for glioma patients receiving T-cell therapies, and the consideration of alternative interventions are likely to maximize the efficacy of CAR T-cells in the clinical setting. Despite the number of hurdles facing the use of genetically engineered T-cells for glioma immunotherapy, novel pre-clinical strategies addressing each of these hurdles continue to present opportunities for clinical progress. Creative and mindful bioengineers will need to work closely with clinical and surgical experts in order to drive forward the field of immune-oncology, both on the bench and at the bedside.

AUTHOR CONTRIBUTIONS

All authors contributed to the concept, review of the literature, writing, and editing the manuscript. BH contributed to drawing the figures.

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REVIEW
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CAR T Cells for Solid Tumors: New Strategies for Finding, Infiltrating, and Surviving in the Tumor Microenvironment

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Chimeric antigen receptor (CAR) T cells, T cells that have been genetically engineered to express a receptor that recognizes a specific antigen, have given rise to breakthroughs in treating hematological malignancies. However, their success in treating solid tumors has been limited. The unique challenges posed to CAR T cell therapy by solid tumors can be described in three steps: finding, entering, and surviving in the tumor. The use of dual CAR designs that recognize multiple antigens at once and local administration of CAR T cells are both strategies that have been used to overcome the hurdle of localization to the tumor. Additionally, the immunosuppressive tumor microenvironment has implications for T cell function in terms of differentiation and exhaustion, and combining CARs with checkpoint blockade or depletion of other suppressive factors in the microenvironment has shown very promising results to mitigate the phenomenon of T cell exhaustion. Finally, identifying and overcoming mechanisms associated with dysfunction in CAR T cells is of vital importance to generating CAR T cells that can proliferate and successfully eliminate tumor cells. The structure and costimulatory domains chosen for the CAR may play an important role in the overall function of CAR T cells in the TME, and "armored" CARs that secrete cytokines and third- and fourth-generation CARs with multiple costimulatory domains offer ways to enhance CAR T cell function.

Keywords: chimeric antigen receptor, solid tumors, T cell, adoptive T cell immunotherapy, engineered T cells

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INTRODUCTION

The use of chimeric antigen receptor (CAR) T cells is gaining traction as one of the most promising advances in cancer immunotherapy. A CAR T cell is a T cell that has been genetically engineered to express an antigen-specific, non-MHC restricted receptor, composed of the single-chain variable fragment (scFv) of an antibody fused to a transmembrane domain and an intracellular signaling domain (1, 2). CARs are introduced to T cells using a plasmid or viral vector, e.g., adenovirus, retrovirus, or lentivirus, of which lentivirus has become the most common method of transducing human T cells (3). mRNA electroporated CAR T cells can also be made, with the advantage of transient CAR expression for easier evaluation of toxicity. Other nonviral vectors for integrating genes include synthetic DNA or mRNA transposon systems, termed Sleeping Beauty, in which a transposon vector can be stably integrated into the genome via a transposon plasmid with a mobilizing transposase protein (4). Importantly, the Sleeping Beauty system has been shown to be less mutagenic than retro- or lenti-viral vectors, because its genomic integration appears to be

largely random, while retro- and lenti-viral vector integration is often biased toward transcriptional sites (5). The earliest first generation CARs contained only the CD3ζ signaling domain, while second generation CARs contain an additional costimulatory signaling molecule, such as 4-1BB, CD28, CD27, OX40, ICOS or RIAD, and some third- and fourth- generation CARs with two or more signaling domains have been developed as well (1, 6).

To date, the success of the CAR T cell has largely been in hematological malignancies (7, 8). A CAR targeted to the B cell antigen CD19 was first used successfully to treat chronic lymphoblastic leukemia (CLL) (9). In August 2017, the FDA approved the use of CART19 (Kymriah) to treat pediatric relapsed or refractory acute lymphoblastic leukemia (ALL) and in October of the same year, another CD19-targeting CAR (Yescarta) was approved by the FDA for adult relapsed or refractory large B cell lymphoma (10). Additionally, the European Medicines Agency (EMA) also approved the use of both these drugs in June of 2018 (11). However, despite extensive research, CAR T cell therapy for solid tumors has not been nearly as successful. Why is it more challenging to target solid tumors with CAR T cells? While there likely are numerous undiscovered reasons, the known barriers in solid tumors can be broken down into three simple categories: finding, getting into, and surviving in the tumor. This review will briefly characterize these three challenges, as well as the most recent research strategies that address them. It will focus particularly on strategies to mitigate tumor antigen heterogeneity and escape, to increase T cell trafficking and extravasation to tumor sites, and to encourage T cell proliferation in the tumor. It will address the evolving understanding of T cell activation, signaling, and the relationship between T cell memory and exhaustion phenotypes, all of which are critical for the development of more effective CAR T cells against solid tumors. Finally, research on the future of the CAR T cell, including the advent of universal CAR T cells using novel gene-editing techniques such as CRISPR/Cas9, and strategies to improve antigen-binding, optimize T cell signaling, and decrease immunogenicity, will be described.

FINDING THE TUMOR: TUMOR ASSOCIATED ANTIGENS, EXPRESSION LEVEL, AND SUSCEPTIBILITY TO CAR T CELLS

The first major difference between solid tumors and hematological tumors is that it is more difficult to find an ideal target antigen. Unlike cancers such as ALL or CLL in which the tumor cells universally express the B-cell marker CD19, solid tumors rarely express one tumor specific antigen. For most solid tumors, it is more common to find a tumor associated antigen (TAA) where the antigen is enriched on tumors but also expressed at low levels on normal tissues (see **Table 1**). This is the case for many frequently targeted TAAs for solid tumors, including CEA, ERBB2, EGFR, GD2, mesothelin, MUC1, and PSMA (1, 14, 18).

Lack of tumor antigen specificity increases the potential risk of significant on-target off-tumor toxicity. This was the case for a patient with metastatic colon cancer who received an infusion of CAR T cells targeted to the antigen HER2 (ERBB2) and died 5 days later (58). The cause of death was attributed to low levels of HER2 on the epithelial cells of the lung, which were attacked by the CARs. Another example of on-target, off-tumor toxicity was found with a high affinity anti-GD2 CAR for neuroblastoma, in which low levels of GD2 in the brain resulted in fatal encephalitis (59). These catastrophic events underscore the importance of finding a safe TAA, given the possibility that even low levels of the target antigen on normal tissues can result in significant toxicity. These acute responses also highlight that the binding affinity of a CAR is related to both safety and efficacy, and that higher affinity is not necessarily better. An *in vivo* study found that CAR T cells targeting ICAM-1, a marker associated with many solid tumors including thyroid cancer (but also expressed on many normal tissues as an adhesion marker), was safer and more effective when bearing CARs with micromolar affinity than with those with higher, nanomolar affinity (39, 40). Additionally, the authors found that the CAR with lower affinity showed less exhaustion and enhanced proliferation *in vivo*. In another approach to limiting CAR toxicity, one group interested in treating colorectal cancer created a CAR targeting GUCY2C, a receptor that is conserved in at least 95% of metastatic tumor at tenfold greater levels, but is not targeted by T cells when expressed in normal epithelial tissues because it is restricted to luminal membranes (33). The CAR was shown to be safe and effective in both immunocompetent mice with metastatic tumors and human xenograft models. Antigens that are aberrantly or overexpressed on tumors but are also expressed on normal tissues can thus be cautiously explored to serve as targets for solid tumors and their metastases.

Suicide genes [reviewed by (60)] are genes coexpressed with the CAR construct that can induce cell death when activated by an agent such as a drug or antibody. Suicide genes have been integral to improving the safety of CAR T cells, particularly as they move into clinical trials. These genes include inducible caspase 9 (iC9) and truncated EGFR (tEGFR or EGFRt) (**Figure 1**), which can trigger antibody-mediated cell death, and herpes simplex virus thymidine kinase (HSV-TK), which disrupts DNA replication and also induces apoptosis via Fas-mediated cell death (68).

Many groups have used immunoproteomics to discover TAAs using autoantibodies against immunogenic antigens expressed by tumor cells (either on the surface or in the cytosol) (69). These antigens may be entirely unidentified proteins (neoantigens) or peptides that are mutated from the wild type (neoepitopes) (70). A few examples of TAAs identified using proteomics include the markers PSMA1, LAP3, ANXA3, and maspin, which were identified by one group as biomarkers for colon cancer (71). Other novel potentially targetable TAAs include olfactomedin 4, CD11b, and integrin alpha-2, which were found to be overexpressed in colorectal cancer with liver metastases (72). Neoantigens can also be found using DNA or RNA sequencing or whole exome screening to identify somatic mutations in tumors (73–75). A study using whole

TABLE 1 | Common solid tumor associated target antigens, most recent CAR constructs, and the stage of testing they have reached.

Target TAA	Solid tumors expressing target TAA	Type of CAR	Clinical trials*	Phase
CD44v6	(Metastasized) colon cancer, soft tissue sarcoma (STS), possible marker for many metastasizing tumors (12, 13)	28ζ CAR-CIK/ HSV-TK suicide gene	Preclinical	–
CAIX (carbonic anhydrase IX)	Metastatic clear cell renal cell carcinoma (ccRCC) (14, 15)	$CD4_{TM}$-γ	Study stopped	I/II
CEA (carcinoembryonic antigen)	Ovarian, gastrointestinal, colorectal, hepatocellular carcinoma (HCC) (16–18)	CD3ζ	NCT02959151 NCT02850536 NCT02349724 NCT03267173	I/II Ib I Early I
CD133	Ovarian, glioblastoma (GBM), HCC (17–19)	BBζ –	NCT02541370 NCT03423992	I/IIa I
c-Met (Hepatocyte growth factor receptor)	Breast (50%), melanoma, HCC	BBζ mRNA	NCT01837602 NCT03060356	Early I Early I
	(20)	c-Met/PDL-1	NCT03672305	Early I
EGFR (epidermal growth factor receptor)	NSCLC, GBM, sarcoma, malignant pleural mesothelioma (MPM) (79.2%), retinoblastoma, glioma, medulloblastoma, osteosarcoma, Ewing sarcoma (21–23)	28/BBζ α-CTLA-4/PD-1 IL12 BBζ/EGFR806/ tEGFR suicide gene	NCT03152435 NCT03182816 NCT03542799 NCT03638167 NCT03618381	I/II I/II I I I
EGFRvIII (type III variant epidermal growth factor receptor)	GBM (24–67%), glioma, colorectal, sarcoma, pancreatic	– tEGFR suicide gene – – BBζ+pembrolizumab	NCT03283631 NCT02844062 NCT01454596 NCT03267173 NCT03726515	I I I/II Early I I
	(16, 24)	–	NCT03423992	I
Epcam (epithelial cell adhesion molecule)	HCC, lung, ovarian, colorectal, breast, gastric, stomach, esophogeal, pancreatic, liver, prostate, gynecological cancers, nasopharyngeal carcinoma	– – 28ζ – –	NCT02915445 NCT03563326 NCT03013712 NCT02729493 NCT02725125	I I I/II I/II I/II
	(16, 25)			
EphA2 (Erythropoetin producing hepatocellular carcinoma A2)	GBM, glioma (26, 27)	–	NCT03423992	I
Fetal acetylcholine receptor	Osteosarcoma, rhabdomyosarcoma (28)	CD3ζ	Preclinical	–
FRα (folate receptor alpha)	Ovarian (90%), urothelial bladder carcinoma (14)	4SCAR (4th gen)	NCT03185468	II
GD2 (Ganglioside GD2)	Neuroblastoma, melanoma, osteosarcoma (100%), rhabdomyosarcoma (13%), Ewing's sarcoma (20%), cervical	3rd gen/inducible Caspase-9/IL-15 28ζ/OX40/iC9/VZV iC9 C7R (IL-7 receptor) 4SCAR – – –	NCT03721068 NCT01953900 NCT03373097 NCT03635632 NCT02765243 NCT02919046 NCT02761915 NCT03356795 NCT03423992	I I I/II I II I/II I I/II I
	(29–32)	– 4SCAR/IgT	NCT03356782	I/II
GPC3 (Glypican-3)	HCC, squamous cell carcinoma (SCC)	 – BBζ/tEGFR – – – – BBζ 3rd gen –	NCT02959151 NCT03084380 NCT02932956 NCT02905188 NCT02876978 NCT02715362 NCT03130712 NCT03198546 NCT03146234 NCT03302403	I/II I/II I I I I/II I/II I N/A N/A
	(17)	–		

(Continued)

TABLE 1 | Continued

Target TAA	Solid tumors expressing target TAA	Type of CAR	Clinical trials*	Phase
GUCY2C (Guanylyl cyclase C)	Metastatic colorectal (33)	?	Preclinical	–
HER1 (human epidermal growth factor receptor 1)	Lung, prostate (1, 34)		Preclinical	–
HER2 (human epidermal growth factor receptor 2) (ERBB2)	Breast (25–30%), ovarian (25–30%), osteosarcoma (60%), GBM (80%), medulloblastoma (40%), gastric, MPM (6.3%), sarcoma, pediatric CNS	BBζ/tCD19	NCT03696030	I
		–	NCT02713984	I/II
		HER2-AdVST + oncolytic adenovirus	NCT03740256	I
		–	NCT02442297	I
		–	NCT03500991	I
		3rd gen	NCT03198052	I
		28ζ	NCT00902044	I
		aE7	NCT03267173	Early I
	(23, 24, 35–38)	BBζ/tCD19 T_{CM}	NCT03389230	I
		–	NCT03423992	I
		–	NCT02792114	I
ICAM-1 (Intercellular adhesion molecule 1)	Thyroid (60%) (39, 40)	3rd gen	Preclinical	
IL13Rα2 (interleukin 13 receptor α2)	Glioma, GBM (41, 42)	–	NCT03423992	I
		BBζ/tCD19	NCT02208362	I
IL11Rα (interleukin 11 receptor α)	Osteosarcoma (28)	28ζ	Preclinical	
Kras (Kirsten rat sarcoma viral oncogene homolog)	Lung adenocarcinoma (30%), pancreatic (43)	–	Preclinical	
Kras G12D	Pancreatic ductal adenocarcinoma (PDA), colorectal, lung (44)	ACT	Clinical	
L1CAM (L1-cell adhesion molecule)	Ovarian (45)	28ζ	Preclinical	
MAGE	NSCLC (MAGE-A3/6), metastatic melanoma (70% MAGE-A1-5) (46, 47)	TCR-directed therapy		
MET	MPM (67%) (48)	28ζ	Preclinical	
Mesothelin	PDA (up to 100%), MPM (85%), Ovarian (70%), lung adenocarcinoma (53%, advanced; 69%, early stage), GBM	–	NCT02930993	I
		?	NCT02959151	I/II
		PD-1/TCR KO	NCT03545815	I
		αCTLA-4/PD-1	NCT03182803	I/II
		–	NCT01583686	I/II
		αPD-1	NCT03030001	I/II
		PD-1 KO	NCT03747965	I
		–	NCT03198052	I
		αPD-1	NCT03615313	I/II
		–	NCT03267173	Early I
		–	NCT03356795	I/II
		BBζ	NCT02792114	N/A
	(49–52)	28ζ	NCT02414269	I
		MCY-M11	NCT03608618	I
MUC1 (mucin 1)	HCC, NSCLC, pancreatic, breast, glioma, colorectal, gastric (17)	αCTLA-4/PD-1	NCT03179007	I/II
		–	NCT02587689	I/II
		± PD-1 KO T cells	NCT03706326	I/II
		± PD-1 KO T cells	NCT03525782	I/II
		–	NCT03198052	I
		–	NCT03267173	Early I
		–	NCT03356795	I/II
		4SCAR-IgT	NCT03356782	I/II
		–	NCT03633773	I/II

(Continued)

TABLE 1 | Continued

Target TAA	Solid tumors expressing target TAA	Type of CAR	Clinical trials*	Phase
MUC16 ecto (mucin 16)	Ovarian (18, 53)	TCR-directed CAR	Clinical Preclinical	
NKG2D (natural killer group 2 member D)	Ewing's sarcoma, osteosarcoma, ovarian (18, 54)	NK-CAR CAR	Clinical Preclinical	
NY-ESO-1	Liposarcoma (>89%), neuroblastoma (82%), synovial sarcoma (80%), melanoma (46%), ovarian (43%), breast (46%), GBM, NSCLC (47, 55, 56)	TCR-CAR ACT/TCR-directed therapies	Preclinical Clinical	
PSCA (prostate stem cell antigen)	Pancreatic, prostate (57)	–	NCT03198052 NCT03267173	I Early I
WT-1 (Wilms tumor 1)	Ovarian (17)	–	Preclinical	

**Recruiting/not yet recruiting studies listed.*

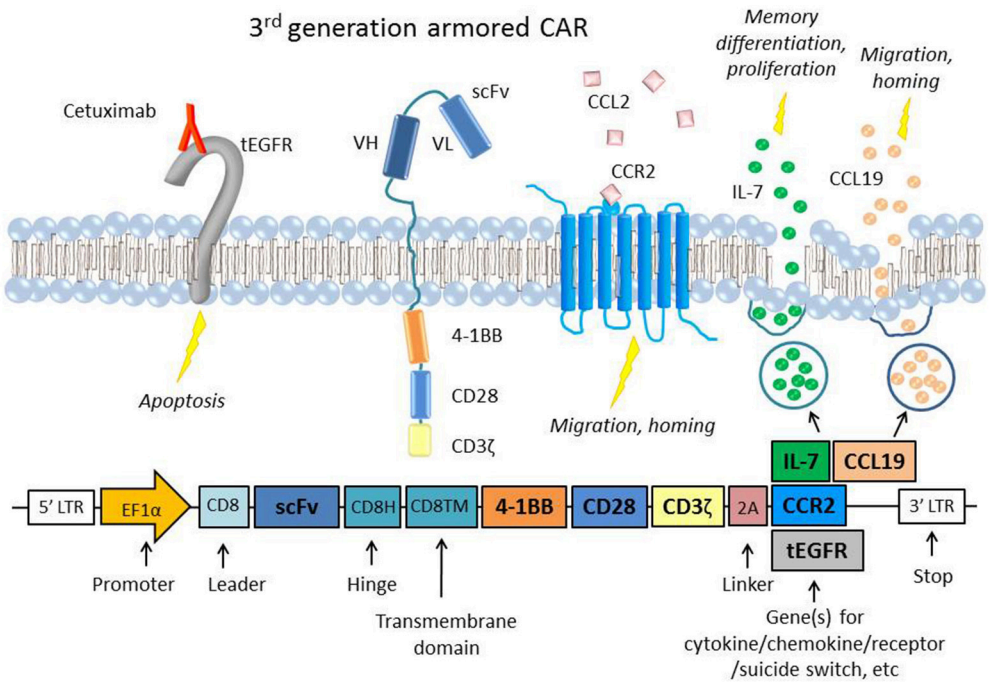

FIGURE 1 | A representative figure of an armored 3rd generation CAR in a T cell and a schematic of the transgene, which includes the extracellular scFv, two intracellular costimulatory domains (4-1BB and CD28), the ζ chain, a 2A linker, and the gene of interest to be coexpressed (61, 62). Examples of "armor" added to the CAR T cell are the CCR2 receptor (63), which has been shown to increase T cell migration and homing to the tumor site (64, 65) or constitutive secretion of the cytokine IL-7 and chemokine CCL19, which are important to memory differentiation and T cell migration, respectively (66). CARs that constitutively secrete IL-12 have also been used in several studies to boost survival and cytotoxicity (67). Also depicted is an example of an inducible suicide gene, tEGFR, which consists of the truncated transmembrane and extracellular portion of the EGFR protein. When targeted by the antibody Cetuximab, the receptor triggers apoptosis in the cell, providing a safety switch to protect against potential toxicity (68). Inducible caspase 9 (iC9) and HSV-TK are other common suicide genes that have been coexpressed with CARs.

exome sequencing of melanoma samples found multiple mutated epitopes in 5 of 8 patients, as well as the presence of T cell clones reactive to 8 of the 9 neoepitopes (73). Another study used whole genome sequencing to identify somatic mutations in glioblastoma multiforme (GBM) samples, and found neoepitope-specific tumor infiltrating lymphocytes (TILs) in all five studied patients (70). Whole exome sequencing for neoantigen prediction was recently employed in a long term study of PDA patients, and the authors found that greater numbers of neoantigens, combined with greater numbers of $CD8^+$ TILs, correlated with increased survival (76). Many of the neoantigens with lasting immunogenicity in long term survivors

were contained within the tumor-associated MUC16 antigen; with metastatic progression, loss of MUC16 clones was seen, indicating a role for the loss of those neoantigens in tumor progression and metastasis.

Some studies have explored the use of CD40 agonists to boost T cell immunity to solid tumors (CD40 is expressed on dendritic cells and other antigen-presenting cells (APCs) and binds CD40 ligand on T cells to stimulate immune response) (77). Using CD40 agonists to augment T cell response to weakly immunogenic tumor antigens or cold tumors is particularly useful in cancers such as PDA that tend to lack mutational burden and often have no baseline immunogenicity. In a murine model of spontaneous PDA (KPC mice), combining chemotherapy with CD40 agonists showed T cell infiltration and neoantigen-specific response and tumor regression (78). The data was consistent with the hypothesis that CD40 activated pre-existing tumor-reactive TILs, showing that priming can overcome suboptimal T cell reactivity to antigen and induce an immune response with subsequent tumor control. These studies using neoepitopes show that tumors can induce secondary immune responses against previously unrecognized antigens, and that endogenous immunity to neoantigens may control tumor spread. This bears significance for adoptive T cell therapy including that which is CAR-based. Many of these methods to screen for neoepitopes rely on identifying pre-existing TCR reactivity and, thus, rely on the inherent immunogenicity of neopeptides; however, identifying neoepitopes and using CAR T cells to target them could theoretically bypass this issue because the scFv of a CAR does not rely on MHC presentation.

Solid tumors tend to display a large degree of antigen heterogeneity. Many tumors have only a subset of cells that express the target antigen. Even in the setting of a uniformly expressed TAA, there is the possibility of antigen loss or antigen escape, where the target antigen disappears from the surviving tumor (79). This has already been observed with CD19 negative relapses in leukemia post CAR19 T cell transfer, and the mechanisms are not well characterized (80). One study discovered a splice mutation that resulted in a form of CD19 that was missing the specific epitope targeted by the CD19 CAR (81, 82). In a phase I study using an EGFRvIII-specific CAR to treat GBM, a single dose of the CAR T cells resulted in downregulation of the EGFR/EGFRvIII receptor and appeared to promote T cell resistance, although administration was shown to be safe and potentially effective (83). In a glioma model, an IL13Rα2 specific CAR T cell that also had transgenic expression of IL-15 successfully killed tumor, proliferated, and produced cytokine *in vivo*; however, recurrent tumors demonstrated IL13Rα2 downregulation (84). Dual or tandem CARs, which recognize two antigens rather than one, have been created to address both antigen heterogeneity and the threat of antigen loss. Such dual CARs have entered clinical trials in hematological malignancies targeting CD19/CD20 and CD19/CD22 [(85); NCT03241940]. For solid tumors, a CAR specific for both HER2 and MUC1 had promising *in vitro* results in a breast cancer model, and a dual-target CAR specific for HER2 and IL13Rα2 showed greater success than single-target CARs in a xenograft glioma model (86, 87).

Also relevant to antigen heterogeneity is the concept of epitope spreading [reviewed by (88)], a phenomenon in which a different epitope of a previously tolerated antigen becomes targeted by T cells. In the context of CAR T cell therapy, this means that even if a tumor does not uniformly express the originally targeted antigen, lysis of some cells by CARs might release tumor-specific neoantigens or epitopes that would be processed and presented by APCs to TILs to induce a secondary immune response against the tumor. Evidence for epitope spreading has been shown in melanoma, where TILs reactive to tumor neoantigens were discovered after vaccination with melanoma antigens (MAGE) (89). Another study using a viral-based vaccine for MUC1 and IL-2 induced epitope spreading and correlated with improved survival of patients with NSCLC (90), and a case study using mRNA electroporated mesothelin CARs displayed an immune response that suggested epitope spreading in two patients with MPM and metastatic pancreatic cancer (91). In a mouse pancreatic cancer model with tumors of low mutational burden and no predicted neoepitopes, introduction of the neoantigen ovalbumin (OVA) spurred a memory immune response leading to tumor clearance and no evidence of antigen escape, while the same tumors provoked no T cell response in immune competent mice without ovalbumin (92). Further understanding and inducing epitope spreading has significant potential to bolster the effectiveness of CAR T cells, especially in tumors with high heterogeneity, low mutational burden, and evidence of antigen escape.

For traditional CAR T cells, the target antigen must be expressed on the cell surface in order to engage with a T cell. However, only about 1% of total cellular proteins are actually expressed on the cell surface, meaning that a huge number of potential tumor target antigens are not available to a CAR T cell (62). Recently, to open the doors to targeting intracellular antigens with CAR T cells, Patel et al. (93) showed success in an *in vivo* myeloma study with a CAR/TCR hybrid that recognized the antigen NY-ESO-1 in the context of HLA-A2. These TCR-CARs were shown to effectively bind an HLA-A2$^+$ T cell artificially engineered to express NY-ESO-1. TCR-CARs that recognize antigen in combination with MHC can thus recognize both extra- and intra-cellular antigens in the way that wild-type or modified TCRs can. Walseng et al. (62) also created a TCR-CAR composed of a soluble TCR directed against either the melanoma-associated antigen MART1 or TGFβR2 (a neoantigen peptide) joined to a CAR signaling component. The result was a versatile receptor that bound antigen in an MHC-I restricted manner, but with signaling and killing similar to that of a CAR. They demonstrated that this construct could be transduced not only into T cells but also into a NK cell, with successful *in vitro* killing.

TUMOR INFILTRATION

Even when a target antigen for a solid tumor is identified, a CAR T cell must be able to reach the tumor site. In hematological cancers, circulating CAR T cells in the bloodstream have already reached their destination. In solid tumors, there are multiple barriers that a CAR T cell must surmount in order to reach the

tumor site [for full reviews of the tumor microenvironment, see (94, 95)]. Chemokine-receptor mismatch can prevent migrating lymphocytes from following a chemotactic gradient. Surface markers like selectins on endothelial cells that bind circulating lymphocytes and induce signaling cascades for subsequent extravasation into sites of inflammation are necessary, as are the corresponding receptors on T cells. Additionally, physical barriers such as cancer associated fibroblasts (CAFs) and abnormal vasculature at the tumor site can block T cell entry (95).

The presence of blood vessels known as high endothelial venules (HEVs) are hypothesized to be critical for T cell infiltration and have been associated with tumor regression in cancers such as melanoma. However, these blood vessels are distorted and immature in many solid tumors, particularly at the core of the tumor where the fewest TILs are found (96). Anti-angiogenic therapy targeting VEGF, CD276, or endothelin B receptor has been shown to normalize tumor vasculature and could be used in combination with targeted therapy like CAR T cells to increase tumor infiltration (97, 98). Notably, one study that performed qPCR on melanoma lesions observed that high HEV density positively correlated with the number of genes encoding for chemokines known to recruit TILs, including CCL2, CCL5, CXCL9-13, CCL19, and CCL21 (99). In colorectal cancer, expression of CXCL9, 10 and 11 were positively correlated with the presence of $CD8^+$ and $CD4^+$ TILs and with post-operative survival (100).

Given the importance of chemokines in lymphocyte migration and homing, varying methods have been used to deliver chemokines intratumorally to attract TILs. One study employed a vaccinia virus to deliver the chemokine CXCL11 intratumorally in a subcutaneous mouse model of MPM and observed significantly increased levels of T cell infiltration and anti-tumor efficacy after intravenous mesothelin-directed CAR T cell injection (101). The same group also developed a CXCL11/mesothelin CAR that increased intratumoral levels of CXCL11 but did not improve anti-tumor activity. The investigators hypothesized that this was due to chronic chemokine secretion inducing hypofunction in the T cells, and/or the anti-angiogenic effects that CXCL11 can exert on its surroundings. However, another study that engineered "armored" mesothelin CAR T cells that constitutively expressed both the cytokine IL-7 and the chemokine CCL19 showed complete tumor regression and prolonged survival in a solid tumor mouse model (**Figure 1**) (66). The study also showed that lymphodepletion before CAR T cell injection decreased efficacy, suggesting that IL-7 and CCL19 recruited endogenous anti-tumor TILs as well. CAR T cells have also been transduced to express chemokine receptors with beneficial results, as in the case of lentivirally engineered mesothelin CAR/CCR2 T cells that displayed greater than 12-fold increased homing and tumor regression in subcutaneous human MPM tumors and a GD2/CCR2b CAR T cells that showed greater than 10-fold increased homing in neuroblastoma tumors *in vivo* (**Figure 1**) (64, 65).

Another promising approach to augment CAR T cell infiltration into tumor sites is the development of a CAR targeting FAP (fibroblast activation protein), which is expressed on multiple types of stromal cells that are associated with nearly all epithelial tumors (102). FAP has been shown to play a role in epithelial-to-mesenchymal transition (EMT) in pancreatic ductal adenocarcinomas (PDA) among other tumor types (103). In one study, where human MPM tumor samples and fibroblast samples were shown to be positive for FAP by immunohistochemistry, FAP CAR T cells efficiently killed MPM cells *in vitro*. The same CAR T cells inhibited tumor growth and lengthened the survival of immunodeficient mice with intraperitoneal (IP) tumor xenografts (104). However, another study showed little efficacy of a FAP CAR in a syngeneic mouse model using multiple tumor types and observed lethal toxicity, which was attributed to FAP expression on bone marrow-derived stem cells (BMSCs) (105). The authors reported that this may have been due to the use of mouse tumor lines with limited FAP expression, while robust FAP staining by IHC was observed in multiple human tumor samples, indicating that human tumor cell lines may have been better targets for the study.

One method of entirely circumventing the hurdle of suboptimal T cell homing (and also potentially avoiding on-target off-tumor toxicity) is regional/local CAR T cell administration, which has already been tested in patients with solid tumors with varying degrees of success. One phase 0 study that enrolled patients with metastatic breast cancer demonstrated that intratumoral administration of mRNA c-Met CAR T cells was safe and resulted in tumor cell death, and showed other signs of anti-tumor inflammation including macrophage recruitment (20). Recently, in a study using a xenograft mouse model of human breast cancer metastatic to the brain, intracranial and intratumoral administration of HER2-specific CAR T cells showed improved antitumor activity compared with intravenous delivery, with complete tumor eradication and 100% survival even after tumor rechallenge (106). Another study showed that regional delivery of a HER2-BBζ CAR T cell cleared medulloblastomas in NSG mice and required a significantly lower dose than intravenous delivery (107). The same CAR in nonhuman primates with HER2 positive medulloblastomas showed no toxicity after intraventricular delivery. A mouse study using a CEA CAR for peritoneal carcinomatosis (colorectal cancer metastasized to the peritoneal cavity) showed that regional intraperitoneal (IP) delivery resulted in better antitumor response than intravenous delivery, even after tumor rechallenge and at distal tumor sites (108). Finally, a study of intracavitary administration of pan-ErbB/IL-4 CAR T cells targeting patient derived MPM xenografts in SCID mice showed tumor regression or cure in all mice (23).

TIL SURVIVAL IN THE TUMOR MICROENVIRONMENT

Once a CAR T cell finds its way into the tumor, the battle is far from over. The tumor microenvironment (TME) has been extensively characterized as hostile for T cells [see (95, 109), and (110) for reviews of the tumor microenvironment and the different cell types it comprises]. The glycolytic metabolism of tumor cells renders the environment hypoxic, acidic, low

in nutrients, and prone to oxidative stress (1, 109). In an inflammatory environment, tumors cells often upregulate ligands such as programmed cell death ligand 1 (PD-L1) and Galectin-9 that bind to inhibitory receptors on T cells (see **Table 2**). The tumor microenvironment also relies on stromal cells like cancer associated fibroblasts (CAFs) and suppressive immune cells, including myeloid-derived suppressor cells (MDSCs), tumor associated macrophages (TAMs), tumor associated neutrophils (TANs), mast cells, and regulatory T cells (Tregs) (**Figure 2**) (95). These cells and tumor cells secrete soluble factors like vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ), which contribute to abnormal tumor vasculature, promote anti-inflammatory polarization of TAMs and other immune cells, and are implicated in EMT (116). They also produce reactive oxygen species (ROS) and molecules like lactate, indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), soluble Fas, and adenosine, which contribute to the suppression of the T cell immune response (**Figure 3**) (117, 121).

When they are activated, effector T cells generally switch from oxidative phosphorylation (oxphos) to glycolysis to facilitate faster proliferation, while memory T cells and Tregs rely on oxphos and fatty acid oxidation (FAO) when activated [for a full review on T cell activation and metabolism see (122)]. However, both of these metabolic resources are limited in the TME because glucose is depleted by tumor cells, leaving glycolytic T cells nutrient-deprived. The lack of glucose results in lowered AKT/mTOR signaling, downregulation of the glucose receptor Glut1, and reduced capacity for glycolysis (120). Low oxygen concentrations in the TME limit oxphos as well. Overall, this results in significant depletion of both major sources of T cell nutrients. In a study of resected tumor tissue from 54 patients with clear cell renal carcinoma (ccRCC), $CD8^+$ TILs showed very low levels of activation and proliferation, and although T cell Glut1 remained expressed, TILs did not uptake glucose (123). The study also observed that mitochondria (crucial for T cell activation), undergo remodeling during glycolysis, lose function and release detrimentally high levels of ROS. This comprehensive study is one of many to show the effects of TME hypoglycemia on suppressing T cell activation via glycolytic and mitochondrial pathways.

The hypoxic conditions in the TME provide particular challenges for memory T cells, the metabolisms of which rely heavily on oxygen. Some studies have recently begun to identify and test modifications to CAR T cells to improve their function in low-oxygen conditions. Kawalekar et al. (6) found that BBζ CAR T cells had increased mitochondrial spare respiratory capacity (SRC) compared with 28ζ CAR T cells, resulting in greater metabolic efficiency even in nutrient-poor, oxygen depleted conditions such as the TME. Because the BBζ costimulatory has been implicated in promoting memory-associated metabolic pathways such as fatty acid oxidation as well as increased persistence (further discussed under "Intracellular signaling pathways of the CAR" below), the increased SRC observed in these T cells was hypothesized to aid their survival in low-oxygen conditions.

One approach designed to protect T cells from the oxidative stress inflicted by ROS in the TME was the design of a CAR T cell coexpressing catalase, an enzyme that reduces hydrogen peroxide to water and oxygen (124). The authors tested both CEA and HER2 CAR T cells *in vitro* and found that CAR-CAT displayed a reduced oxidative state and improved proliferation and cytotoxicity compared with CAR alone. Another study harnessed the hypoxia associated with the TME to develop a CAR coexpressing the oxygen-sensitive domains of HIF1α, a transcription factor that is stabilized in response to hypoxia (125). *In vitro*, the strategy enabled very low CAR expression at normal oxygen levels, but highly increased levels of CAR expression together with HIF1α in hypoxic conditions. While this approach does not address the detrimental effects of low oxygen or ROS in the TME, it does provide proof of concept for a novel type of safety switch that uses the hypoxic TME to a therapeutic advantage.

T cell exhaustion [reviewed by (111)] is characterized by chronic antigen exposure that spurs loss of effector and memory phenotypes, inability to produce cytokines like IFNγ, TNFα, and IL-2, and upregulates expression of inhibitory receptors (IRs) that further shut down effector functions upon binding to inhibitory ligands or soluble factors in the TME (**Table 2**) (109).

TABLE 2 | Some inhibitory receptors and their known ligands [from Wherry et al. (111), unless cited in table].

Inhibitory receptor	Full name	Ligand(s)
A2AR	Adenosine 2A receptor	Adenosine
CTLA-4	Cytotoxic T lymphocyte antigen-4	CD80, CD86
CD160	Cluster of differentiation 160	MHC Class I, herpesvirus entry mediator (HVEM) (112)
LAG-3	Lymphocyte activation gene 3	MHC Class II
PD-1	Programmed cell death 1	Programmed cell death ligand 1 (PD-L1), PD-L2
TIM-3	T cell immunoglobulin-3	Galectin-9 (Gal9), phosphatidylserine (PtdSer), high mobility group protein B1 (HMGB1), Ceacam-1 (113)
TIGIT	T cell immunoglobulin and ITIM domain	PVR (CD155) >> PVRL2 (CD112), PVRL3 (113)

Checkpoint Blockade

One of the most popular and successful strategies to combat T cell exhaustion is the use of checkpoint inhibitors, in which either an IR or its ligand is blocked with an antibody. Drugs targeting PD-L1 (atezolizumab), PD-1 (pembrolizumab, nivolumab), and CTLA-4 (ipilimumab) have been used independently and in combination with CAR T cell therapy with success in many patients [reviewed in (126)]. Currently, atezolizumab and pembrolizumab are used to treat metastatic NSCLC and are being actively studied for use in other solid tumors as well. Pembrolizumab was recently approved by the FDA for first line use in combination with chemotherapy in lung cancer (127, 128). Nivolumab has shown significant responses in a phase

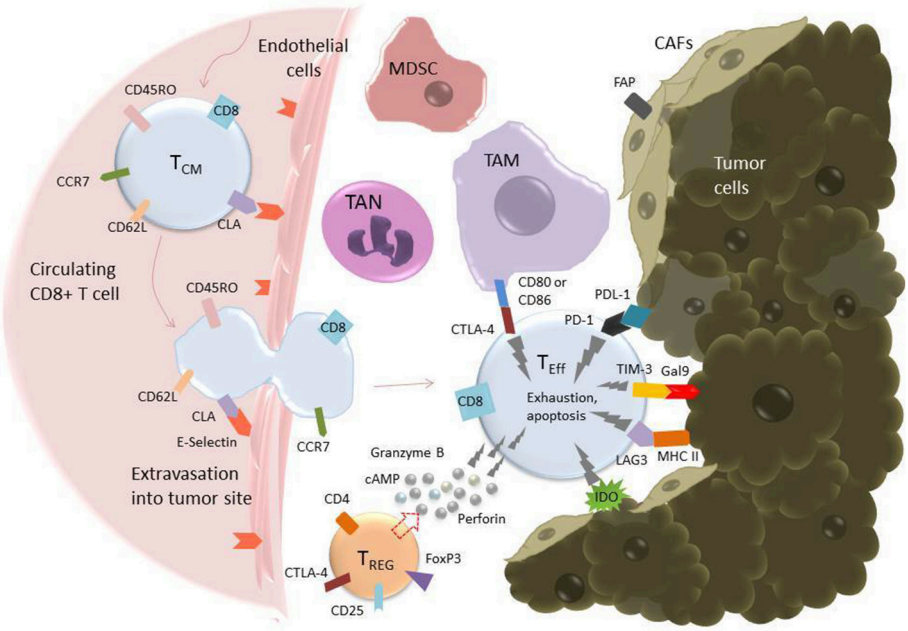

FIGURE 2 | T cell extravasation into the TME and subsequent exhaustion mediated by inhibitory ligands on tumor and tumor-associated cells. Endothelial cells experiencing inflammation express adhesion molecules including selectins, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule (ICAM-1). P- and E-Selectins (the latter shown in the figure) bind cutaneous lymphocyte antigen (CLA), a specially glycosylated form of P-selectin glycoprotein ligand 1 (PSGL-1) that is expressed on activated T cells (114). VCAM-1 binds very late antigen-1 (VLA-4) and ICAM-1 binds lymphocyte function-associated antigen-1 (LFA-1) (115). Upon binding endothelial cell ligands, T cells undergo tethering and rolling before adhering to the endothelium and transmigrating through it as shown. Once in the tumor microenvironment, T cells are in an environment full of tumor-associated, immunosuppressive cells including tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), T-regulatory cells (Tregs), and cancer-associated fibroblasts (CAFs) (95). These cells express inhibitory molecules, including CD80/CD86, which bind the inhibitory receptor CTLA-4 (pictured), and secrete soluble factors that suppress or cause apoptosis in T cells. CAFs also serve as a physical barrier between T cell and tumor cell. Additionally, tumor cells themselves express ligands such as Gal9 and PDL-1, which bind to the T cell inhibitory receptors TIM-3 and PD-1, respectively. All these factors serve to promote an "exhausted" phenotype in the T cell, characterized by upregulation of inhibitory receptors such as PD-1, TIM-3, TIGIT, and LAG-3, loss of CCR7, CD62L, and CD45R0, loss of cytotoxicity, and apoptosis (111).

I/II trial with HCC, among others (17). Ipilimumab was shown to lengthen the survival of metastatic melanoma patients in a phase III study from 2010, and it has shown promising results in mouse mesothelioma models as well as in many other preclinical studies (129, 130). Ipilimumab has also been used in combination with VEGF inhibitors to treat metastatic melanoma in phase I trials. In one study, anti-CTLA-4 therapy combined with anti-VEGF antibodies resulted in an increase in anti-tumor response resistant to the immunosuppressive effects of the ligand galectin-1 (131). Another study by the same authors showed ipilimumab and anti-CTLA-4 therapy resulted in humoral immunity to galectin-3, which is also a suppressive tumor ligand (132).

Preclinically, combining CARs with checkpoint blockade antibodies has shown promising results. CAR T cells have also been engineered to secrete checkpoint inhibitor antibodies themselves. Anti CAIX CAR T cells engineered to secrete anti-PD-L1 antibodies showed significantly improved activity compared to standard CAR T cells, with increased cytokine production and immune cell recruitment as well as significantly reduced tumor size in a human ccRCC mouse model (14). In another study, a CAR19 T cell designed to constitutively secrete anti-PD-1 also showed enhanced anti-tumor activity in a $CD19^+$ lung cancer xenograft model, with increased T cell proliferation and cytotoxicity, and prolonged survival (133). A similar study also used MUC16-ecto targeting CARs secreting anti-PD-1 scFvs in syngeneic and xenograft mouse models of PD-$L1^+$metastatic ovarian cancers, and showed superiority over CAR T cells plus PD-1 checkpoint inhibitors (134). Along a similar line of thinking, an anti-PD-L1 CAR has shown *in vitro* cytotoxicity (135); it has yet to be seen whether these CARs might be successful *in vivo*, either alone or as adjuvant therapy. CAR T cells engineered to secrete PD-1, CTLA-4, or PD-L1 antibodies have gone to clinical trials for MUC1, EGFR, EGFRvIII, and mesothelin expressing cancers (136).

Dominant negative genes for IRs have also been successfully introduced to CAR T cells in many preclinical studies, as in the case of a mesothelin CAR T cell (with either a CD28 or 4-1BB costimulatory domain) overexpressing dominant negative PD-1 (137). The authors observed tumor clearance with the dominant negative PD-1 CARs, while repeated doses of PD-1 blocking antibody in combination with either the mesothelin-28ζ or mesothelin-BBζ CAR was able to prevent growth but not eradicate the tumor. These results show that a genetic built-in resistance to checkpoint inhibition has advantages over a

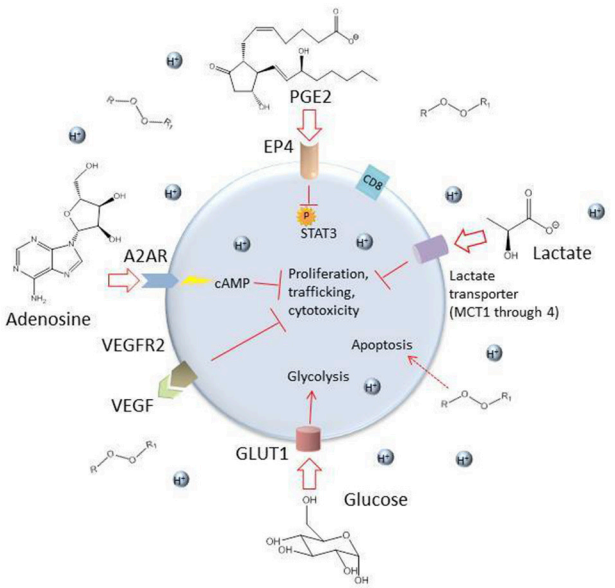

FIGURE 3 | Some inhibitory soluble factors and molecules secreted by tumor cells and tumor-associated cells such as MDSCs, TAMs, TANs, CAFs, and Tregs. High levels of lactate and an acidic environment are generated because of the tumor cells' preferential use of glycolysis, which impairs T cell function (111). The hypoxic environment also limits oxidative phosphorylation, a metabolic requirement for central memory T cells. High levels of reactive oxygen species (ROS) are generated by tumor cells and by induced mitochondrial dysfunction in T cells, which can be toxic to the cell. The soluble factors VEGF, TGFβ, indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), and adenosine are secreted by tumor and tumor-associated cells and can have damaging effects on T cells (109, 117). Adenosine enters the T cell through the receptor A2AR and stimulates production of cyclic AMP, which inhibits T cell proliferation, trafficking and cytotoxicity (118). PGE2 enters through the receptor EP4 and inhibits phosphorylation of STAT3, dampening proliferation, development of favorable memory phenotype, and cytotoxic function in T cells (119). Competition with glycolytic tumor cells for glucose results in downregulation of the glucose receptor GLUT1 because of decreased AKT/mTOR signaling and consequently, the T cell's metabolic capacities are further diminished (120).

blocking antibody that must be repeatedly administered and may cause resistance. Recently, CRISPR/Cas9 technology has been used to knock out the gene for the IR itself, which has been done for both PD-1 and LAG-3 in CD19-BBζ CAR T cells. In both cases, tumors were eradicated in mouse xenograft models using the IR knockout CAR T cells (138, 139). This approach has recently been translated to solid tumors as well: CRISPR/Cas9 was used to knock out PD-1 in T cells while simultaneously transducing them with a CD133-specific CAR, and the resulting PD-1 KO CARs showed improved tumor control in a mouse glioma model compared with control CD133 CARs (140).

With the optimization of gene editing methods, CRISPR/Cas9 edited CARs are already moving into clinical trials: a PD-1 knockout CD19 CAR has is being studied in a phase I clinical trial (NCT03298828).

Switch receptors are designed to mitigate the effects of inhibitory ligands on T cell function while simultaneously enhancing T cell activity. In a switch receptor, the ligand-binding external IR domain is fused to the cytoplasmic signaling domain of an activating molecule. For example, a PD-1/CD28 switch receptor was engineered into mesothelin-BBζ or PSCA-BBζ CAR T cells, and both switch-receptor CARs performed significantly better than wild type CARs at eradicating tumor in xenograft NSG mouse models (141). In a breast cancer model, the investigators engineered a MUC1 CAR that coexpressed a cytokine switch receptor (4/7ICR) with an IL-4 receptor extracellular domain fused to an IL-7 intracellular signaling domain (142). The 4/7ICR MUC1 CARs proliferated, suppressed tumor growth *in vivo* and did not show markers of exhaustion, while MUC1 CARs without the switch receptor did. PSCA CAR T cells that also contained a 4/7ICR switch receptor proliferated and killed better in the presence of IL-4 and showed significantly improved tumor reduction compared to T cells with the CAR alone in NSG mice with subcutaneous pancreatic cancer (57).

Similar to switch receptors are bispecific T cell engagers (BiTEs), which also subvert suppressive signals from the TME by binding both a tumor ligand and a T cell marker (i.e., CD3). Recently, a humanized EGFRvIII-specific scFv linked to an anti-CD3 scFv showed significant control of glioma xenografts and prolonged survival of mice (143). Potentially, the use of bispecific antibodies in conjunction with CAR T cells could play a role in recruiting TILs and in deterring immunosuppressive signals. Another strategy is to have a fusion protein bind not to the T cell but to the tumor itself. Recently, a PD-L1/TGFβR2 fusion protein was developed for use with TGFβ expressing urothelial carcinoma (144). In this study, a PD-L1 antibody fused to a TGFβ receptor was able to accomplish both blockade of PD-L1 on the tumor and binding of TGFβ to attenuate its presence in the TME. Excitingly, the authors also observed a greater presence of chemokines like CXCL11 in the tumor as well as antigen-specific killing by T cells. With the right target tumor ligands, BiTEs could be a promising strategy to augment the function of CAR T cells in solid tumors.

Understanding the metabolism and transcriptional profiles of exhausted or exhausting TILs has significant impacts on the success of therapies like checkpoint blockade and could lead to the production of more functional CAR T cells via metabolic reprogramming. In a mouse melanoma model, one group showed that promoting fatty acid catabolism in vaccine-induced $CD8^+$ TILs using a PPARα agonist combined with anti PD-1 therapy significantly improved anti-tumor activity (145). Another group showed that CTLA-4, PD-1, and PD-L1 blockade increased glucose concentrations in the TME to favor glycolysis in T cells, improving their function in a mouse sarcoma model (120). These studies provide clues into the roles of ligands like PD-L1 on tumor metabolism, in addition to their known inhibitory effects on T cell IRs.

Despite the success of PD-1 therapy in treating NSCLC and melanoma, as well as its use in multiple other clinical trials, it is inadequate to characterize markers like PD-1 as solely inhibitory. Much of what we know about PD-1 in the context of exhaustion comes from chronic viral infection models, and it has become clear that PD-1 can in fact be a marker of activation and positive prognosis if expressed on certain subsets of T cells in cancer,

while some subsets of exhausted T cells may have low PD-1 expression (111, 141). In the same study by Zhang et al. (145) that found a synergistic effect of PD-1 blockade with fatty acid catabolism in a melanoma model, hypoxia-driven hypofunction in $CD8^+$ TILs was accompanied by lower PD-1 expression (but increased LAG-3 expression), and increased PD-1 signaling was hypothesized to be associated with metabolic reprogramming from glycolysis to FAO in low glucose environments like the TME. Another study suggested that PD-1 was in fact not linked to exhaustion, and that prolonged antigen exposure alone could cause T cells to become exhausted (146). In a recent analysis of varied NSCLC patient samples, a population of $CD8^+$ TILs with high PD-1 expression did not appear exhausted, and genes involved in cycling and proliferation such as Ki-67, as well as genes involved in trafficking and metabolism, were upregulated (147). The PD-1 high TILs also showed greater glucose, lipid and fatty acid uptake than patient TILs with lower PD-1 expression. This data challenges the understanding of PD-1 being solely an inhibitory receptor and sheds new light on Zhang et al.'s observation that an increase in PD-1 expression results in lower capacity for glycolysis. Further showing a role for PD-1 outside of exhaustion, a study comparing $CD4^+$ TILs in 34 patients with metastatic melanoma, grouped into young vs. old, showed that the younger patients had a greater percentage of memory T cells that expressed PD-1, Ki-67, and HLA-DR (another activation marker), compared with age matched controls; these memory and activation phenotypes were less distinct in older patients (148). However, supporting the hypothesis of PD-1 as a marker of hypofunction, but not necessarily of terminal exhaustion, one study demonstrated that mesothelin/BBζ T cells that had high PD-1 expression and a hypofunctional phenotype in an vivo human mesothelioma model recovered the ability to produce cytokines and had lower PD-1 expression after 24 h out of the tumor (126). These data show that PD-1 can have highly variable functions which likely depend on T cell phenotype, metabolism, tumor type, and other factors in the TME, and also helps explain why only a fraction of patients respond to PD-1 blockade even when their tumors have high PD-L1 expression.

Transcription Factors

Transcription factors such as T-box transcription factor TBX21 (T-bet) and Eomesodermin (Eomes) are involved in determining T cell fate and their discovery has led to further insight into the mechanisms of T cell exhaustion. T cells high in Eomes and PD-1 have been shown to be terminally exhausted, while those with high T-bet and medium PD-1 levels appear to retain proliferative potential despite displaying other classically defined features of exhaustion (111). Hypoglycemia and hypoxia in the TME have been shown to decrease T-bet expression in TILs that also lose effector functions (145). As an example of Eomes' role in CAR T cell exhaustion, a case study with a patient with refractory diffuse large B cell lymphoma (DLBCL) who received CAR19 T cell therapy combined with PD-1 blockade showed decreased Eomes as well as decreased PD-1 levels in peripheral blood CAR T cells (149). The patient had a clinically significant response to the treatment, indicating that PD-1 blockade improved the efficacy of the CAR T cells.

Other transcription factors and signaling cascades have been investigated as biomarkers to predict T cell function and patient prognosis after adoptive transfer of CAR T cells. A study using IL-18/CEA CAR T cells to treat immunocompetent mice with advanced pancreatic carcinoma showed that constitutive secretion of the proinflammatory cytokine IL-18 resulted in CARs with high T-bet in conjunction with low levels of the transcription factor FOXO1 and showed improved antitumor efficacy (150). In an analysis of CAR19 T cells derived from patients with CLL, the circulating CARs from complete responders had upregulated genes associated with memory differentiation status, including IL-6 and STAT3, and were observed to lose function upon IL-6/STAT3 blockade (119). Nonresponders, on the other hand, had upregulated genes associated with more of an effector phenotype as well as glycolysis, exhaustion and cell death by apoptosis. Finally, the paper showed that $CD8^+$ cells that were CD27 positive and PD-1 negative were positively predictive of response to CAR19 T cell therapy. Investigating the role of these biomarkers in CAR T cell response for solid tumors and whether they have an impact on patient survival may elucidate the transcriptional profiles of functional CARs.

Differentiation and Memory

Naive, central memory and effector memory, and terminally differentiated effector T cells all have distinct markers of differentiation. Differential expression of CCR7, CD62L, CD25, CD45RA, CD45R0, CD95, and IL-7Rα, among others, can identify subsets of T cells (151, 152). Tregs, $CD4^+$ T cells that express CD25, CTLA-4, and FOXP3, are another distinct subset that inhibits T cell effector function; studies have shown that with CAR T cell therapy, Treg presence lowers CAR antitumor activity. Checkpoint blockade targeting CTLA-4 may be one way to address this problem. Other surface markers like IL-2Ra and KLRG1 have been shown to be associated with effector-like phenotypes, while IL-7Rα and the chemokine receptor CXCR3 are associated with memory-like cells (153). Cytokines such as IL-2, IL-12, IL-27, and IFNγ are also traced to effector-differentiated T cells, while IL-10, IL-21, IL-7, IL-15, and TGFβ are associated with a memory phenotype. Genes such as T-bet, Id2, Blimp-1, Batf and Stat4 have been associated with effector phenotypes, while Id3, Bcl-6, Tcf-7, Stat3, Foxo1, and Eomes are all proposed to be upregulated in memory-like T cells (150, 153).

Gattinoni et al. (154) describe a human stem cell memory T cell (TSCM) population that expresses both the classical markers of naive cells as well as certain memory cell markers including CD95 and IL-2Rβ, which the authors determined to be crucial identifiers of TSCM. These cells were found in 2–3% of circulating blood lymphocytes of healthy donors, and could also be induced from naive T cells by culturing them in the presence of a glycogen synthase kinase 3β (GSK3β) inhibitor. (Inhibition of GSK3β has been described to stabilize β-catenin and halt differentiation to effector T cells while promoting memory characteristics). These TSCM cells demonstrated increased proliferation in response to the cytokines

IL-7 and IL-15 compared with effector memory T cells, while still retaining their phenotype.

The memory phenotype has consistently been shown to favor T cell survival, proliferation, and prolonged presence of TILs at tumor sites. However, only a few studies focusing on T cell differentiation and memory up to this point have been done in solid tumor models. Still, valuable lessons can be learned from models of ACT, GVHD, or hematological malignancies. For example, a study of ACT in both NSG mice and nonhuman primates showed that purified TCM cells persist better than standard T cells, and form stable memory pools (155). Cieri et al. (156) published an *in vitro* culture protocol for inducing a stem cell memory-like phenotype in T cells using IL-7 and IL-15 during CD3/CD28 bead activation of naive precursor cells. When transduced with a transgenic TCR, these T cells displayed memory-associated qualities including proliferation, cytokine production, and expression of CD45R0 upon exposure to target antigen, while retaining markers indicative of naive T cells including CD45RA and CD62L. These T cells were very similar to those described by Gattinoni et al. (154) above, except for the expression of CD45R0. The authors found enhanced proliferation in these cells compared with cells expressing a central memory or effector memory phenotype, and this translated to increased expansion and cytotoxicity compared with other subsets of T cells in a mouse GVHD model. The results of these experiments suggest that, as stated by the authors, using naive cells for ACT may significantly improve clinical outcomes. Similarly, using IL-7/IL-15 with naive T cells during transduction and culture conditions to produce more TSCM CAR T cells is a promising strategy for the creation of CAR T cells that both kill and proliferate better in the body. In one such study for solid tumors, T cells bearing a third-generation GD2 CAR (signaling domains for CD28 and OX40), as well as an inducible caspase 9 suicide gene, were activated with CD3/CD28 stimulation along with a variety of cytokines, with the hope of identifying conditions to promote a memory phenotype (157). Addition of IL-7 and IL-15 led to the greatest antigen-specific cytotoxicity *in vitro* along with the highest percentage of stem cell memory and central memory subsets as identified by CD45RA, CCR7, and CD95. The authors predicted better proliferation, survival, and antitumor activity of GD2-CD28-OX40 CAR T cells cultured in IL-7/IL-15.

Clinically, work has been done to determine biomarkers associated with memory that are predictive of response as well as to actually manufacture CAR T cells with optimized differentiation status for infusion into patients. In a study examining T cell memory in ovarian carcinoma patients, it was found that increased presence of $CD8^+$ effector memory cells, as well as the chemokine CXCL9, was significantly associated with long-term survival (158). The authors also implicated the signaling proteins STAT5B, PLCγ1, and NFATc2 as being relevant to survival, with lower levels of these signals correlating with hypofunctional T cells and shorter survival times in patients.

In the study by Fraietta et al. (119) discussed above, the authors looked at both the original unmodified T cells from CLL patients and the corresponding CAR transduced infusion products. All sets of T cells from responding patients showed markers associated with early memory, non-exhausted T cells. As glycolysis is a hallmark of effector/exhausted T cell metabolism, and the T cells of nonresponders displayed upregulated genes for exhaustion and glycolysis, the authors used a glycolysis inhibitor while manufacturing CAR T cells and observed increased numbers of memory CAR T cells along with enhanced proliferation upon exposure to antigen. Blocking glycolysis is another approach that could be used in solid tumor-targeting CAR T cells to push formation of memory T cells during activation and transduction, particularly given (119) evidence that the initial differentiation status of a patient's apheresed T cells may significantly affect the efficacy and persistence of the infusion product. Memory CAR T cells have been manufactured for clinical use already in hematopoetic malignancies such as leukemia; Wang et al. (155) used a protocol using magnetic separation to select $CD8^+$ $CD45RA^+$ $CD62L^+$ TCM cells to transduce with a CD19 CAR while culturing with IL-2 and IL-15, and several variations of these CARs (which have CD28 costimulatory domains) are being tested in a phase I study. Also for leukemia, a GMP protocol for manufacturing CAR T cells highly enriched for TCM and TSCM phenotypes has been recently developed (159) in which, on average, 50% of the T cells were TCM and 46% TSCM. The authors reported that the results were consistently achieved even with very few T cells available to start. The use of CAR T cells enriched for TCM and TSCM has reached the clinic even in solid tumors: the GD2-CD28-OX40 CAR manufactured in IL-7/IL-15 (157) is currently in a phase I trial to treat patients with sarcoma, osteosarcoma, neuroblastoma, and melanoma (NCT02107963).

Tissue Resident Memory Cells

Another memory T cell subset that may be of special importance, especially in treating solid tumors, are tissue resident memory cells (Trms), reviewed in (160). Trms have been shown to permanently reside at sites of prior infection or inflammation and quickly respond to pre-recognized antigen, recruiting other immune cells, and increasing the local anti-tumor immune response at a very early stage (161). $CD8^+$ Trms are characterized by high surface levels of CD103 and the activation marker CD69 and low CD62L and CCR7, and it is believed that TGFβ and IL-15 are both important soluble factors that promote T cell differentiation to a Trm phenotype. Despite having many memory markers, Trms secrete high levels of cytokines such as granzyme B and perforin. Interestingly, Chang et al. (153) and Wakim et al. (162) found that Trms do not tend to have high T-bet as other memory T cells do. Mackay et al. (163) describe downregulation of T-bet, but with necessary residual activity, as one of the factors driving skin Trms, in addition to downregulation of Eomes.

While a significant amount of this research has been done on Trms in skin, some data shows that analysis of Trms across various tissues obtained from humans retain similar phenotypes, particularly in $CD8^+$ Trms (164). One examination of the transcriptional profiles of Trms in multiple tissue types showed that the gene Hobit ("homolog of Blimp1 in T cells") was upregulated, and together with Blimp1 was a driver of

a Trm phenotype (165). CD103 was not expressed on all Trms described, which has also been shown in other tissues including the brain (162). Interestingly, the genes Hobit and Blimp1 were also upregulated in activated NK cells, suggesting a similar signaling pathway during activation of both Trms and NK cells. Other studies offer support for this parallel, including one by Lotem et al. (166) that reported regulation of activation and proliferation in both mature $CD8^+$ T cells and NK cells by the transcription factor Runx3. High levels of Runx3 have been shown by several studies to decrease $CD4^+$ and increase CD103 expression on T cells, biasing them toward a cytotoxic $CD8^+$ Trm phenotype. In a study by Cruz-Guilloty et al. (167), Runx3 also was reported to drive an increase in Eomes expression and granzyme B and perforin secretion in differentiating $CD8^+$ T cells, while T-bet expression peaked early, at around 2 days, and then decreased over a week of differentiation. The authors also reported Runx3 to regulate CD103 in resting NK cells. While ongoing research is required to parse out more information on Trms, induction of a Trm phenotype using TGFβ and IL-15, or via a genetic engineering, could be a powerful way to improve upon ACT or CAR T cell efficacy. In a study of TILs in lung cancer patients, transcriptome analysis showed that CD103 and other Trm-linked markers were significantly increased in the patients with high numbers of TILs; moreover, having a higher percentage of Trms was reported to predict better survival (168). In a study using an orthotopic head and neck cancer model in mice, a cancer vaccine was more successful after induction of Trms, and Trms were still detectable at the tumor site 90 days later (169). Additionally, Trms protected against tumor re-grafting even when recruitment of additional effector T cells was blocked, showing that Trms alone can mount a successful antitumor response and tumor rejection upon rechallenge. These data show that Trms may be critical to successful tumor infiltration and protection against tumor relapse, and the induction of a Trm phenotype is likely to increase therapeutic outcomes.

Overcoming Other Immunosuppressive Factors in the TME

Administration of cytokines to polarize the tumor mileu to be more hospitable to T cells and improve CAR T cell recruitment and functionality has been tested in both preclinical and clinical trials. Local delivery of IL-12, which induces inflammatory immune cell recruitment, augmented the anti-tumor activity of adoptively transferred anti-VEGFR-2 CAR T cells and led to prolonged survival of mice bearing five different subcutaneous tumor types (170). In the study, treatment of IL-12 plus VEGFR2 CAR T cells, but neither alone, reduced VEGFR2-positive intratumoral MDSCs, providing strong support for the combination of IL-12 with CAR T cells. Due to positive responses like these, CAR T cells that constitutively secrete cytokines, termed "armored" CARs [reviewed by Yeku et al. (171)] have been created to enhance T cell infiltration and function in solid tumors (**Figure 1**). Particularly, the cytokine IL-12 has been an attractive tool for this. In a mouse xenograft model of ovarian cancer, MUC16 CAR/IL-12 T cells lengthened survival and showed increased persistence and tumor cytotoxicity (53). More recently, in a syngeneic mouse model of peritoneal carcinomatosis (metastasized from ovarian cancer), IP-delivery of MUC16 CAR/IL-12 T cells was found to confer longer survival, even when administered to mice with significant disease progression (67).

Some other strategies to boost CAR T cell function in the TME include inhibiting suppressive soluble factors, like adenosine, IDO1, and VEGF, and protecting against the immune suppression of non-tumor cells in the TME like MDSCs, TAMs, and stromal cells. In a study using HER2 CAR T cells in a syngeneic tumor model, blockade of the adenosine 2A receptor significantly improved the efficacy of the CAR T cells by enhancing activation and cytokine production (118). Additionally, the authors reported that PD-1 blockade further augmented the T cell immune response. Another study demonstrated significant slowing of tumor growth in a xenograft colon cancer model by combining blockade of IDO1 (negatively correlated with patient survival in colon cancer) with EGFRvIII CAR T cell transfer (172). VEGF blockade has been successful in solid tumors such as melanoma, and VEGF-targeted CARs have shown efficacy in multiple preclinical solid tumor models (131, 132, 170).

Increasing antitumor response can also involve either depleting anti-inflammatory cells in the TME or inducing more inflammatory phenotypes in other immune cells. Research in mouse breast cancer models has suggested that targeting TAMs may be effective for treating progressive cancer, as TAMs were associated with more anti-inflammatory activity and tumor immune evasion (173). Another study demonstrated that in murine ovarian cancer models, macrophages were associated with resistance to VEGF blockade. When macrophages were depleted, survival was prolonged, and in macrophage deficient mice, resistance was not observed unless macrophages were reintroduced into the tumors (174). TAMs are, therefore, a highly active subset of immune cells that seem to promote tumor survival and immune evasion. In a subcutaneous mouse model of ovarian cancer, tumor rejection by HER2 CAR T cells was shown to require the presence of M1 (inflammatory) macrophages and IFNγ receptors on stromal cells, demonstrating that tumor-specific attack by T cells, even functional ones, may not be enough to clear tumors; stromal cell targeting (for example, with FAP CARs) and recruitment of other types of inflammatory immune cells may be necessary (175). Depleting MDSCs can also improve T cell responses, as shown in a study with a GD2 CAR in which the CAR T cell alone had no anti-tumor activity in a xenograft sarcoma model, but in combination with MDSC reduction using all-trans retinoic acid, led to significant antitumor activity (176). Noman et al. (177) demonstrated *in vivo* that hypoxia in the TME plays a significant role in upregulation of PDL-1 on MDSCs and on their subsequent suppression of TILs. PDL-1 upregulation was determined to be dependent on HIF1α, and PDL-1 blockade prevented T cell suppression by MDSCs. In another study (described in section Tumor Infiltration) using a CEA CAR, blockade of PD-L1 positive MDSCs and Tregs in the TME augmented CAR T cell anti-tumor function (108).

Intracellular Signaling Pathways of the CAR

It is also important to study the signaling pathway of the CAR itself, particularly how different costimulatory domains may affect T cell activation, metabolic needs, differentiation pathways, and the propensity to exhaust. Adding a costimulatory molecule to the original CD3ζ cytoplasmic domain revolutionized the functionality of the CAR T cell; now, there is a broad array of signaling molecules that can be used. The most common costimulatory molecules are 4-1BB and CD28, and depending on the CAR and tumor type, many studies focus on one or the other. Some have studied adding a third costimulatory domain, like ICOS or OX40. Some studies have demonstrated little significant advantage of one design over another. In a study comparing 4-1BB vs. CD28 in an EphA2 CAR, both CARs displayed equally potent antitumor activity in a xenograft mouse glioma model, and creating a third-generation CAR with both domains did not improve T cell performance over the second-generation CARs (27). In the study described in section Tumor Infiltration that used mesothelin/IL-7/CCL19 CARs to treat murine mesothelin-expressing PDA, there was also no difference between 4-1BBζ and CD28ζ CARs (66).

Many studies implicate 4-1BB as promoting superior differentiation phenotype and persistence. A recent study used phosphoproteomics to report on the kinetics of the 4-1BB vs. CD28 domains in CAR T cells. The authors found that 4-1BBζ CARs and CD28ζ CARs signaled through the same intermediates, but CD28ζ CARs had more and faster changes in protein phosphorylation, which seemed to drive them toward an effector phenotype. On the other hand, 4-1BBζ CARs were shown to express more memory-related genes and performed better *in vivo* than their CD28ζ CAR counterparts (178). Another study comparing 4-1BB and CD28 signaling in a PSCA CAR to treat patient derived prostate cancer xenografts found 4-1BB to be superior to CD28, with 4-1BBζ CARs leading to less exhaustion and better antigen selectivity (however, *in vitro* killing was equal between the two CARs) (179). In the aforementioned study of a regionally delivered HER2 CAR in xenograft models of brain-metastasized breast cancer, 4-1BBζ CARs also showed superior proliferation and less exhaustion than CD28ζ CARs (106). The evidence for 4-1BB preferentially expressing memory markers so far has been borne out clinically: an *ex vivo* study showed that in both CD19 and mesothelin CARs across multiple donors' T cells, 4-1BB promoted better proliferation, central memory differentiation, and greater levels of fatty acid oxidation and mitochondria generation than CD28, while CD28 was linked to increased glycolysis and an effector phenotype (6). Other 4-1BB based CARs from *in vivo* studies described in this review include HER2 CARs in models of medulloblastoma and gastric cancer (92,30); GD2 CARs in models of neuroblastoma and patients with melanoma (29, 30); mesothelin CARs in preclinical models of mesothelioma (64); and FAP CARs used in models of tumor associated stroma (102). Clinically, data is rare so far for solid tumors, but a case study described by Brown et al. (42) showed tumor regression induced by T cells expressing a 4-1BBζ IL13Rα2 CAR in a patient with glioblastoma.

Numerous studies have engineered effective CARs that signal through the CD28ζ domain, many of which target the same antigens and are used in similar disease models as 4-1BBζ-signaling CARs. These include humanized HER2 CARs that were shown to have a central memory phenotype in the context of treating breast cancer xenografts (35); IL13Rα2 CARs that showed proliferation and cytotoxicity in a mouse model of glioblastoma (41); and FAP CARs in IP mouse models of MPM (104). Other CARs mentioned in this review that use the CD28ζ costimulatory domain include L1CAM CARs for ovarian cancer in mice (45), MET CARs for MPM (48), MUC16 CARs in mouse models of ovarian cancer and peritoneal carcinomatosis (53, 67), and NKG2D CARs in Ewing's sarcoma models (54). Studies have also used CD28 with dual CARs, such as HER2/MUC1 bispecific CARs in *in-vitro* breast cancer models and HER2/IL13Rα2 CARs in xenograft glioma models (86, 87). Clinically, a HER2/CD28ζ CAR was used to treat progressive glioblastoma in a phase I trial that showed efficacy in some patients (38).

Finally, third generation CARs have also been studied in preclinical and clinical settings. A recent study comparing third generation GD2 CARs to treat *in vivo* models of neuroblastoma found 4-1BB/CD28 CARs to be superior to CD28/OX40ζ CARs in terms of activation, exhaustion, and *in vivo* antitumor efficacy (180). Successful *in vivo* studies using 4-1BB/CD28 third generation CARs include an ICAM-1 CAR for a mouse model of thyroid cancer (40), a GPC3 CAR in a patient derived xenograft of model of HCC (32), and a VEGFR2 CAR against multiple tumor types *in vivo* (161). However, the study that observed fatal toxicity with the use of a FAP CAR in syngeneic mice used a 4-1BB/CD28ζ third generation CAR, and the case report (mentioned in section 1) of a patient death was after administration of HER2 4-1BB/CD28ζ CAR T cells (58). A third generation mesothelin CAR using ICOS/4-1BBζ showed significantly better tumor control and better T cell persistence than ICOSζ or 4-1BBζ CARs alone in a mesothelin-expressing pancreatic xenograft NSG mouse model (181). This study also provided significant insight into the signaling pathways that may be required for optimal CAR T cell activation and differentiation. Lower surface CAR expression corresponded to less tonic signaling (signaling in the absence of antigen), which is linked to exhaustion and has been observed in both CD28ζ and 4-1BBζ CARs. Additionally, the authors found that ICOS/4-1BBζ CARs only performed better than second generation CARs when ICOS was proximal to the transmembrane domain. In the clinic, GD2 CARs with CD28/OX40 costimulatory domains are currently in phase I trials for neuroblastoma (31).

The site of gene integration has also been shown to have significant impact on CAR function. A study with a CD19/28ζ CAR used CRISPR to insert the CAR under the control of the TCR promoter (at the TRAC locus), while simultaneously knocking out the TCR via insertion of the CAR gene (182). The results indicated enhanced proliferation, more memory cells, and much less exhaustion. The TRAC locus CAR was also hypothesized to have reduced tonic signaling that would push T cells toward terminal differentiation and exhaustion.

Future Directions and Conclusions

CAR T cell therapy remains extremely expensive, and patients with severely depleted immune systems may not have viable T cells for autologous CAR T cell generation; additionally, concerns about immunogenicity of certain CAR designs may render therapy less effective in patients that develop an immune response to the CAR. New approaches are needed to make CAR T cells not only functional, but also more efficient and accessible.

Technology such as CRISPR/Cas9 as a highly specific and efficient method of genome editing has become translatable to patients in the past few years. Beyond its use in generating IR knockout CARs, CRISPR/Cas9 has been used to knock out or replace the native TCR in CAR and TCR engineered T cells, which has been shown to provide higher antigen sensitivity and specificity (183). In addition to its implications for T cell function, the use of CRISPR is extremely promising in the field of CAR T cell therapy because it can be used to knock out HLA as well as the endogenous TCR, meaning CARs can be made from allogeneic cells without the threat of cross reactivity and GVHD or rejection. This could dramatically reduce the cost, time and resources required to generate CAR T cells for every patient (184). This has recently been done successfully with a TCR and HLA class I double knockout CAR19 (185). Investigators also developed a CAR that knocked out Fas as well as the TCR and HLA-1 genes, which showed enhanced antitumor activity *in vivo* against a leukemic cell line, with longer survival than unmodified CAR T cells. Thus, CRISPR can be used not only to knock out inhibitory receptors, but also to knock out the TCR and HLA to generate universal or "off the shelf" CARs (186). Moreover, these modifications can be accomplished simultaneously with high precision and efficiency.

Universal CARs have also been developed using other systems of genome editing, including transcription activator-like effector nucleases (TALENs), which create double-stranded breaks in DNA for efficient gene knockouts. Recently, TALEN-mediated editing was used to knock out the TCR-α chain in CAR19 T cells. The subsequent universal CAR T cells, which were from allogeneic donors, induced remission in two infants with B cell ALL (187). Before the advent of CRISPR/Cas9, zinc finger nucleases (ZFNs), proteins that recognize three base pairs at a time to bind to DNA, were also used to remove surface expression of molecules like HLA from allogeneic T cells (185, 188). (189) used ZFNs to disrupt both the TCR β- and α-chain genes while also lentivirally transducing the T cells to express a WT-1 recognizing TCR. This led to superior *in vivo* antitumor activity and eliminated off-target reactivity.

In some cases where the scFv is murine-derived, there is the potential for the development of anti-mouse antibodies that could reject the CAR T cells. Many studies have adopted the use of a humanized scFv, and these humanized CARs have also gone to clinical trials (8). However, humanizing the scFv is a long and onerous process and few fully humanized sequences are currently known. Thus, some recent studies have proposed alternatives to the scFv antigen-recognition domain. One of these alternatives is an affinity molecule from the type III domain of human fibronectin (Fn3), which is similar to the scFv of an antibody but is smaller and has a less complex structure without disulfide bonds, enabling easier *in vitro* generation of specific binding domains (190). Additionally, its smaller size may enable the Fn3 to recognize epitopes that scFvs cannot. Fn3 domains specific for CD20, CEA, EGFR, IGF-1R, and VEGFR2 have been developed. A VEGFR2-specific Fn3 CAR with a CD28ζ costimulatory domain showed *in vitro* antigen-specific T cell activation and cytotoxicity, and another Fn3 CAR engineered to target EGFR with both CD28 and 4-1BBζ costimulatory domains showed efficacy that was comparable to a traditional CAR against a xenograft lung cancer model (191, 192). Another alternative to the scFv is the use of antibody mimetic proteins, such as designed ankyrin repeat proteins (DARPins), synthetic proteins mimicking naturally occurring ankyrin membrane proteins that can be generated with antigen-binding specificity and are smaller and more stable than scFvs. Recently, a HER2-specific DARPin CAR was shown to perform as well as a traditional HER2 CAR *in vivo* against a human ovarian cancer cell line (193).

Significant research has been done with CAR T cells in terms of identifying target antigens, avoiding toxicity, improving CAR T cell trafficking and entry into the tumor site, and promoting better signaling, less exhaustion, and memory phenotypes in solid tumors. Additionally, combination therapy with checkpoint inhibitors, armored CARs, and suppression of other inhibitory factors in the TME has been shown to aid in CAR T cell efficacy in solid tumors, with some of these approaches already being used in clinical trials. Solid tumors pose a wide array of challenges that hematological malignancies do not, hence the need for multi-pronged strategies in addressing them. However, it is clear that our understanding of the TME is increasing at a rapid rate. As the signaling pathways between T cells and other TME cellular components, as well as the intracellular signaling cascades specific to CAR T cell activation and exhaustion, become further understood, CARs hold the promise for greater success in treating solid tumors.

AUTHOR CONTRIBUTIONS

MM did the primary research and wrote the manuscript. EM oversaw the preparation of the manuscript and edited the final draft.

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REVIEW
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Genetic Modification Strategies to Enhance CAR T Cell Persistence for Patients With Solid Tumors

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Immunotherapy with chimeric antigen receptor (CAR) T cells offers a promising method to improve cure rates and decrease morbidities for patients with cancer. In this regard, CD19-specific CAR T cell therapies have achieved dramatic objective responses for a high percent of patients with CD19-positive leukemia or lymphoma. Most patients with solid tumors however, have experienced transient or no benefit from CAR T cell therapies. Novel strategies are therefore needed to improve CAR T cell function for patients with solid tumors. One obstacle for the field is limited CAR T cell persistence after infusion into patients. In this review we highlight genetic engineering strategies to improve CAR T cell persistence for enhancing antitumor activity for patients with solid tumors.

Keywords: CAR T cell, immunotherapy, persistence, genetic modification, solid tumor

INTRODUCTION

Patients with relapsed or refractory solid tumors have poor outcomes and novel treatments are needed to improve survival and decrease morbidities resulting from conventional treatments. Immunotherapy with chimeric antigen receptor (CAR) T cells is a promising strategy to improve these outcomes. Generally, CARs consist of three components: (i) a genetically engineered receptor capable of recognizing a tumor associated antigen (TAA) in an HLA-independent manner, (ii) an activation domain derived from the CD3ζ chain of the T cell receptor complex, and (iii) costimulatory domain(s) to potentiate activation initiated via CAR-CD3ζ signaling (**Figure 1A**). CARs are categorized as 1st generation if the construct has no costimulatory domain, 2nd generation when incorporating 1 costimulatory domain, and 3rd generation when incorporating two costimulatory domains (**Figure 1A**) (1). CAR T cells require three signals for optimal effector function: (i) activating signal induced by CAR recognition of tumor antigen and subsequent signal transduction through CD3ζ, (ii) costimulation provided via one or more domains engineered into the CAR construct, and (iii) stimulatory cytokines for continued growth and effector function (**Figure 1B**).

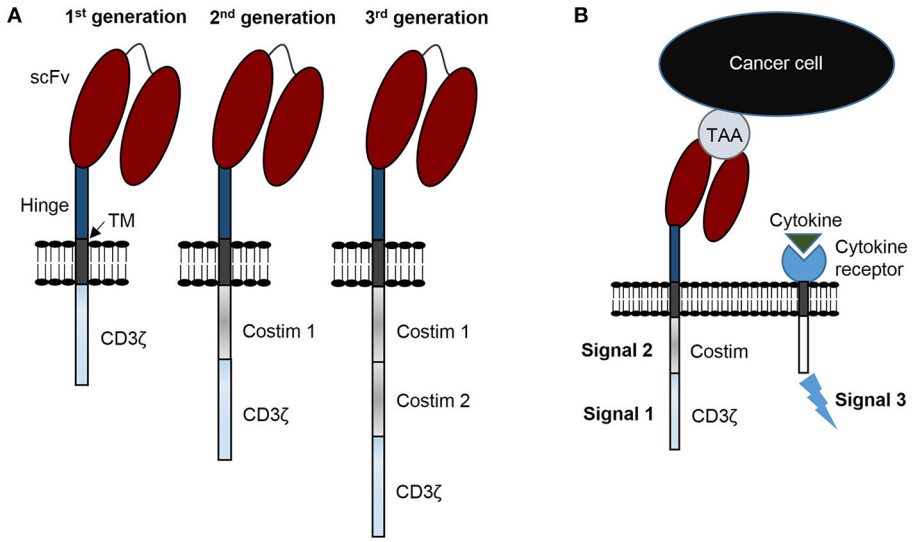

FIGURE 1 | CAR terminology: components, generations, and signals. **(A)** CARs consist of 3 components: a single chain variable fragment (scFv) that recognizes tumor associated antigen (TAA), costimulatory domain(s), and a CD3ζ domain. CARs are designated 1st generation with no costimulatory domain, 2nd generation with 1 costimulatory domain, and 3rd generation with 2 costimulatory domains. **(B)** CAR T cells require 3 signals for optimal function: signal 1 is CD3ζ induced activation, signal 2 costimulation, and signal 3 functional augmentation via stimulatory cytokine(s). TM, transmembrane domain.

While CAR T cell therapy offers promise to improve outcomes for patients with difficult to treat cancers, strategies must be improved to benefit large numbers of patients suffering from relapsed and refractory solid tumors. Several obstacles need to be addressed to realize this goal, including selection of optimal TAAs (2–4), T cell homing to sites of malignant disease (5, 6), T cell penetration into solid masses (7, 8), and overcoming the immunosuppressive tumor microenvironment (9–11). Enhancing persistence of adoptively transferred T cells is another vital challenge for successful treatment of cancer (12–14). Multiple strategies exist to enhance CAR T cell persistence against solid tumors including pre-treatment with cytoreductive chemotherapy (15), optimized T cell culture conditions (16), T cell selection procedures (17, 18), and combinatorial treatment approaches (19–24). This review is focused on novel genetic engineering strategies to enhance CAR T cell persistence and antitumor activity against solid tumors. While promising preclinical data is available, only few of these approaches have been evaluated in early phase clinical studies.

NOVEL COSTIMULATION STRATEGIES TO IMPROVE PERSISTENCE AND ANTI-SOLID TUMOR ACTIVITY

Second generation CAR constructs with CD28 or 41BB costimulatory endodomains are most frequently utilized to generate CAR T cells. Comparison of CD28 vs. 41BB to enhance the function of CAR T cells has been reviewed previously (25). Investigators are now actively exploring CARs encoding alternative costimulatory endodomains or strategies to provide costimulation with a 2nd molecule expressed in CAR T cells.

Recent findings demonstrate that CAR T cell persistence can be enhanced against solid tumors by transducing CD4 and CD8 T cells with CARs encoding different costimulatory domains (**Figure 2A**) (26). This was demonstrated by separately transducing CD4 and CD8 T cells with mesothelin-specific CARs containing either a CD28, 41BB, or ICOS costimulatory domain. After separate transductions, CD4- and CD8-CAR T cells were mixed and infused to determine the optimal combination for enhanced persistence against lung cancer *in vivo*. Strikingly, the best combination for enhanced persistence of CD8-CAR T cells resulted from mixing CD4.ICOS- and CD8.41BB-CAR T cells before injection. CD4.ICOS-CAR T cells persisted *in vivo* regardless of the costimulatory domain expressed by CD8-CAR T cells. On the other hand, CD4-CAR T cells expressing either a CD28 or 41BB costimulatory domain had minimal persistence under any condition, clearly demonstrating that the costimulatory domain of CD4-CAR T cells affects persistence of both CD4- and CD8-CAR T cells in this model. Based on these data, the authors generated a 3rd generation ICOS.41BB-CAR, which also led to enhanced persistence of both CD4- and CD8-CAR T cells *in vivo*, and greater antitumor activity compared to 2nd generation CAR T cells. Given that CD4 T cells provide signals to enhance persistence and effector function of cytotoxic CD8 T cells, this concept is highly relevant for development of future CAR T cell trials for patients with solid tumors. Furthermore, evidence demonstrates that, at least in some models, CD4-CAR T cells targeting solid tumors persist longer *in vivo* and result in superior antitumor activity compared to CD8-CAR T cells (27). Given that optimal costimulatory domains may be different for CD4- and CD8-CAR T cells, CD4-CAR T cells can directly kill cancer cells, and single vectors can be used to make 3rd generation CARs for enhancing persistence

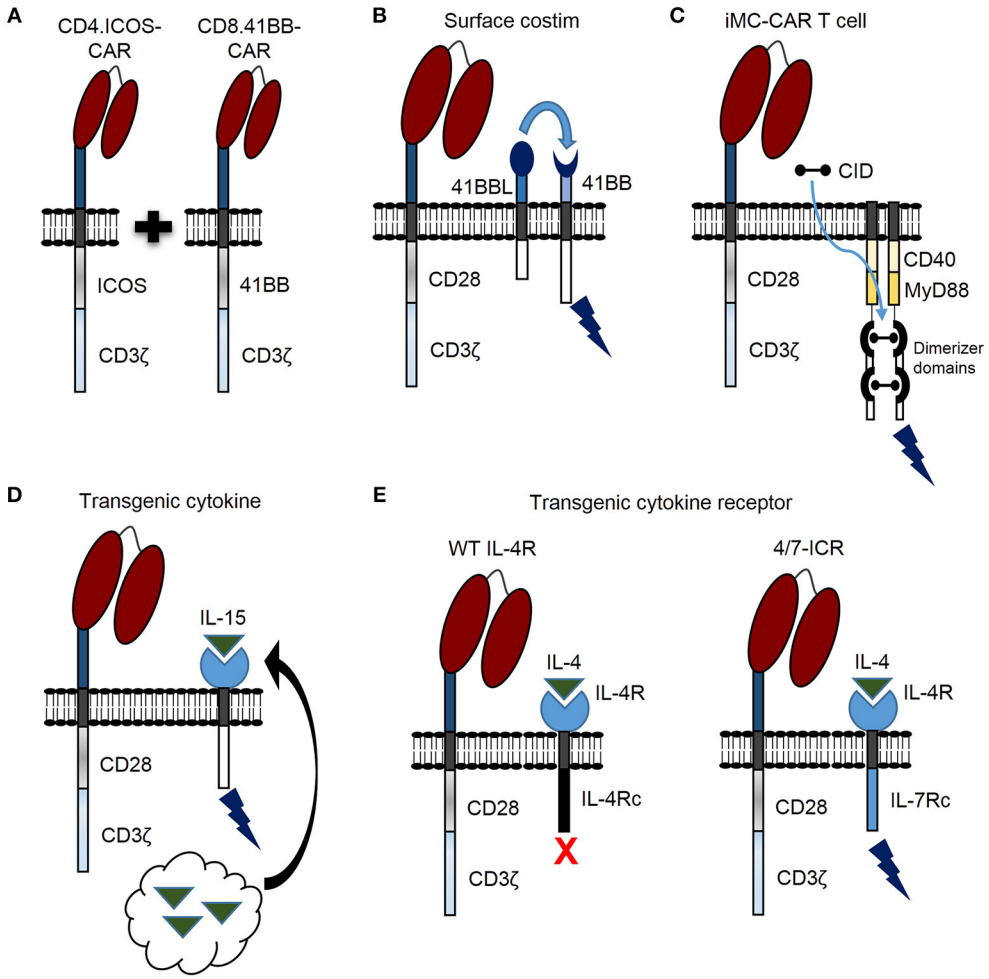

FIGURE 2 | Novel genetic modifications to enhance CAR T cell persistence against solid tumors. **(A)** CAR T cell persistence against solid tumors can be enhanced by transducing CD4 and CD8 T cells with different CAR costimulatory domains. **(B)** CAR T cell activation leads to 41BB receptor (41BB) expression. CAR T cell persistence can be enhanced by constitutive expression of 41BB ligand (41BBL), which interacts with the 41BB receptor in an autocrine or paracrine manner to provide additional costimulatory signal (surface costim). **(C)** Triggering CAR T cell costimulation with a chemical inducer of dimerization (CID) drug is another promising approach. A molecule, which consists of two dimerizer domains and costimulatory domains derived from MyD88 and CD40 (iMC) can be activated by CID and enhance CAR T cell persistence. **(D)** Constitutively expressed cytokine (i.e., IL-15) can be engineered by tethering cytokine to the cell membrane (not shown) or secreted by CAR T cells (black arrow) to enhance persistence. **(E)** The inverted IL-4/IL-7 chimeric cytokine receptor (4/7-ICR) contains an IL-4 receptor extracellular domain (IL-4R) fused to an activating IL-7 cytoplasmic domain (IL-7Rc). While IL-4 binding to wild type (WT) IL-4 receptor leads to CAR T cell inhibition, IL-4 binding to 4/7-ICR enhances persistence and effector function.

of both CD4- and CD8-CAR T cells, future studies focused on optimizing costimulation for both CD4- and CD8-CAR T cells should benefit patients with solid tumors.

Providing costimulation through a 2nd molecule is another promising strategy to enhance persistence, antitumor activity, and safety of CAR T cells for patients with solid tumors. For example, activated T cells express the 41BB receptor, and investigators have shown that CD28-CAR T cells expressing 41BB ligand on the cell surface (**Figure 2B**) endows T cells with superior effector function in comparison to T cells expressing a traditional 3rd generation CD28.41BB-CAR (28). Expressing other tumor necrosis factor superfamily ligands on the T cell surface, such as CD40 ligand, has also resulted in improved CAR T cell function (29).

Triggering CAR T cell costimulation with a drug is another promising approach. For example, investigators designed a molecule, which consists of two dimerizer domains and costimulatory domains derived from MyD88 and CD40 (iMC) (**Figure 2C**) and can be activated by a chemical inducer of dimerization (CID) (30). While initially, this molecule was used to activate dendritic cells, two subsequent studies, as delineated in the next section, have highlighted the benefit of iMC in CAR T cells (31, 32). Of note, costimulation is only activated in the presence of CID, providing a safety mechanism to limit T cell activation.

Using 1st generation prostate-specific antigen (PSCA)-CAR T cells containing an iMC domain (PSCA-iMC-CAR) and PSCA-positive pancreatic cancer cells *in vitro*, Foster and colleagues

demonstrated that CID induced costimulation resulted in greater CAR T cell cytokine secretion, proliferation, and antitumor activity compared to the same cells without CID. *In vivo*, PSCA.iMC-CAR T cells were used to treat mice harboring pancreatic cancers plus/minus weekly CID. Indeed, CID administration enhanced PSCA.iMC-CAR antitumor activity, and 21 days post T cell injection CAR T cells were detected in the blood and tumors from mice treated with CID, but not in those treated without CID.

Separately, Mata and colleagues generated HER2-specific CAR T cells incorporating an inducible costimulatory domain (HER2.iMC-CAR) containing both MyD88 and CD40 signaling regions, (32) and demonstrated that HER2.iMC-CAR T cells have enhanced effector function compared to 2nd generation HER2.CD28-CAR T cells utilized in clinical trials (33). Importantly, in repeat stimulation assays, which model CAR T cell persistence against solid tumors *in vitro*, HER2.iMC-CAR T cells expanded 100–1,000 fold greater than 2nd generation HER2-CAR T cells when targeting multiple different HER2-positive solid tumor types. *In vivo*, HER2.iMC-CAR T cells had significantly greater antitumor activity that led to an enhanced survival benefit compared to 2nd generation HER2-CAR T cells, and controlled solid tumors at a very low T cell dose. Furthermore, repeated induction of costimulation via multiple doses of CID led to superior antitumor activity and a survival advantage for mice bearing HER2-positive solid tumors.

In summary, these results demonstrate that non-traditional costimulatory domains can enhance CAR T cell persistence and antitumor activity against solid tumors, and an inert drug can be injected to adjust CAR T cell activation over time.

TRANSGENIC CYTOKINES ENHANCE CAR T CELL EFFECTOR FUNCTION AGAINST SOLID TUMORS

In addition to modifying costimulatory domains, genetic modification can be used to endow CAR T cells with the ability to express stimulatory cytokines. Interleukin (IL)-15, IL-12, and IL-18 are three examples under active exploration. Constitutively expressed IL-15 can be engineered by tethering IL-15 to the cell membrane (34) or secreted by modified T cells (**Figure 2D**) (35). In this regard, IL-13Rα2-CAR T cells modified to secrete IL-15 (IL-13Rα2.IL-15-CAR) demonstrated superior proliferative capacity and antitumor activity *in vitro* and *in vivo* against high grade glioma compared to IL-13Rα2-CAR alone (35). Both IL-13Rα2- and IL-13Rα2.IL-15-CAR T cells had comparable *in vivo* antitumor activity up to 4 weeks; however, after 4 weeks IL-15 expressing CAR T cells had greater activity indicating that IL-15 improved T cell persistence over a prolonged period of time. Indeed, IL-15 expressing CAR T cells were detected *in vivo* for a significantly longer period of time compared to CAR alone. Intriguingly, in mice treated with IL13-Rα2.IL-15-CAR T cells, tumors recurred at late time points and the majority of relapsed tumors no longer expressed IL-13Rα2, implicating antigen loss as a tumor escape mechanism in this model. This predicts that despite the benefits of improving CAR T cell persistence against solid tumors, antigen loss variants can occur, and strategies to target solid tumors in future clinical trials may require targeting multiple tumor antigens (36, 37). Clinically, transgenic IL-15 expression is actively being explored to improve expansion, persistence and antitumor activity of GD2-CAR invariant natural killer cells for the treatment of patients with neuroblastoma (NCT03294954). Results from this trial should provide insight regarding the impact of constitutively secreted IL-15 to enhance persistence and function of adoptively transferred CAR modified cells, and determine safety in the clinical setting.

IL-12 is another promising cytokine under active exploration to enhance CAR T cell persistence and effector function in both preclinical models (38–40) and a phase I clinical trial for patients with solid tumors (NCT02498912). To enhance CAR T cell activity against ovarian cancer, 2nd generation $MUC16^{ecto}$-specific CAR T cells were modified to secrete IL-12 ($MUC16^{ecto}$.IL-12-CAR) (40). *In vivo* $MUC16^{ecto}$.IL12-CAR T cells demonstrated superior antitumor activity and were detected in the peripheral blood of treated animals, while the same CAR T cells without IL-12 were not detected at any time point, indicating that constitutive IL-12 secretion increased CAR T cell persistence against ovarian cancer. A clinical trial is underway investigating $MUC16^{ecto}$.IL-12-CAR T cells for patients with $MUC16^{ecto}$-positive tumors (NCT02498912), and results should shed light on the possibility of translating this technique to treat a broad range of patients afflicted with solid tumors.

CAR T cells genetically modified to secrete IL-18 exhibit superior antitumor activity against solid tumors compared to 2nd generation CAR T cells in pre-clinical models. Chmielewski and Abken compared 2nd generation CEA-CAR T cells containing a CD28 costimulatory domain to CEA-CAR T cells modified to secrete IL-18 (CEA.IL-18-CAR) under control of a nuclear factor of activated T cells (NFAT)-IL-2 minimal promoter (41). Placing cytokine secretion under control of the NFAT-IL-2 promoter creates an inducible system, whereas cytokine is only secreted upon T cell recognition of its target antigen, theoretically limiting cytokine secretion to the tumor environment. In an immune-competent model of bulky CEA-positive pancreatic cancer, a single injection of CEA.IL-18-CAR T cells led to prolonged survival compared to mice treated with 2nd generation CEA-CAR. Prolonged survival and enhanced antitumor activity were attributed to a pro-inflammatory environment induced by CAR mediated IL-18 secretion. Compared to tumors treated with 2nd generation CEA-CAR, tumors obtained after CEA.IL-18-CAR treatment demonstrated an increased quantity of pro-inflammatory natural killer cells and M1 macrophages, and a decreased quantity of anti-inflammatory M2 macrophages, regulatory T cells, and CD103-positive dendritic cells. Other groups have shown enhanced antitumor activity by genetically modifying T cells to secrete IL-18 (42, 43), and this strategy merits further exploration to enhance CAR T cell activity against solid tumors.

Stimulatory cytokine pathways can also be constitutively activated without the need for cytokine induced stimulation, thus providing T cell survival signals when no cytokine is in the milieu. To enhance expansion, persistence and antitumor activity of 2nd generation GD2-CAR T cells against neuroblastoma,

investigators modified CAR T cells with a constitutively active IL-7 cytokine receptor (C7R) that lacks the IL-7 receptor extracellular domain (44). C7R modified CAR T cells were able to proliferate and kill neuroblastoma cells in serial killing assays to a greater degree than GD2-CAR T cells alone. Impressively, at a low T cell dose GD2.C7R-CAR T cells had substantial antitumor activity *in vivo* against metastatic neuroblastoma. Comparatively, GD2-CAR T cells had limited antitumor activity at the same low T cell dose. Improved antitumor activity resulted in enhanced survival that was secondary to increased expansion and persistence of C7R expressing CAR T cells. Mechanistically, GD2-CAR T cells expressing C7R had greater cell division and reduced apoptosis compared to GD2-CAR alone, effects attributed, in part, to increased BCL2 expression.

While CAR T cell transgenic cytokine production and signaling offer a promising strategy to improve persistence and function of CAR T cells for patients with solid tumors, safety concerns exist. T cell autonomous growth (45) and cytokine induced toxicity can occur (46), and these concerns need to be considered for treating patients. Notably, for both IL-13Rα2.IL15-CAR and MUC16^{ecto}.IL-12-CAR T cells, IL-15 or IL-12 production was low at baseline and increased multiple fold in an antigen specific manner, indicating that elevated cytokine levels should be limited to the local tumor environment. Additionally, both aforementioned CARs were modified with a "safety switch" that proved capable of efficiently eliminating gene modified cells. IL-13Rα2.IL-15-CAR T cells were modified with an inducible caspase 9 (iC9) safety switch that initiates apoptosis in the presence of CID, and these T cells were efficiently eliminated by CID induced iC9 activation. Importantly, the iC9 safety switch was demonstrated to be effective in the clinical setting (47) making it a viable safety mechanism for future trials. MUC16^{ecto}.IL-12-CAR T cells were modified with a truncated epidermal growth factor receptor that can be targeted by cetuximab, a clinically available monoclonal antibody.

As discussed previously, IL-18 secretion in CEA-CAR T cells was under control of an NFAT-IL-2 minimal promoter, which enables T cells to secrete IL-18 only in the presence of target antigen. For CEA.IL-18-CAR T cells, IL-18 was secreted at high levels in the presence of CEA-positive pancreatic cancer cells, but only minimal IL-18 was detected when T cells were exposed to CEA-negative pancreatic cancer cells. Although this strategy demonstrated benefit in this and other pre-clinical studies (48), initial experience in humans was less promising. In one clinical study, the adoptive transfer of *ex vivo* expanded tumor infiltrating lymphocytes transduced to secrete IL-12 under the control of an NFAT-IL-2 minimal promoter induced significant toxicities attributed to high serum IL-12 levels in patients with melanoma (49).

In regard to safety for C7R modified CAR T cells, these demonstrated no cell autonomous growth, and included an iC9 safety switch that led to efficient GD2.C7R-CAR T cell elimination when treated with CID. Alternate gene modification strategies can also be utilized to enhance the safety of CAR T cells by enabling full CAR T cell activation only in the presence of 2 TAAs. Examples include chimeric costimulatory receptors (50, 51) and synthetic Notch receptors (52). Another novel method is to simultaneously induce CAR T cell STAT5 signaling, analogous to signal provided by IL-2, IL-7, or IL-15, and STAT3 signaling, analogous to signal provided by IL-21, in an antigen dependent manner. In this regard, Kagoya and colleagues developed a "new generation" CD19-CAR containing a CD28 costimulatory domain, a truncated IL-2Rβ domain to activate STAT5, and a C-terminus YXXQ motif to activate STAT3 (53). Importantly, STAT5 and STAT3 activation occurred in an antigen dependent manner and the new generation CAR enhanced proliferation of both CD4- and CD8-CAR T cells *in vitro* and *in vivo* compared to 2nd generation CAR T cells with either CD28 or 41BB costimulatory domains. In an *in vivo* solid tumor model using A375 melanoma cells modified to express CD19, new generation CAR T cells persisted to a greater degree in tumors and peripheral blood of treated animals, and had greater antitumor activity compared to 2nd generation CAR T cells.

Together, these findings demonstrate that while safety concerns exist, strategies can be implemented to eliminate genetically modified cells in the setting of unacceptable toxicity, limit full CAR T cell activation to sites of tumor expressing two or more TAAs, or deliver cytokine signals to CAR T cells via activation of STAT5 and STAT3 in an antigen dependent manner.

In summary, studies demonstrate that CAR T cells can be safely engineered to express transgenic cytokines or constitutively active cytokine receptors, which impart CAR T cells with enhanced persistence and antitumor activity against solid tumors. Active and future clinical trials implementing these techniques will guide strategies to improve outcomes for patients with solid tumors.

TRANSGENIC CYTOKINE RECEPTORS TO OVERCOME TUMOR SECRETED INHIBITORY MILIEU

Overcoming effects of inhibitory cytokines is another strategy to enhance CAR T cell persistence and function for treating patients with solid tumors. Switch receptors are one way to transform inhibitory cytokine signals into a stimulus, and thus increase T cell persistence and function (**Figure 2E**) (54, 55). Effectiveness of this strategy was demonstrated against pancreatic cancer using a PSCA-specific CAR engineered with an inverted IL-4/IL-7 chimeric cytokine receptor (4/7-ICR) (56), which contains an IL-4 extracellular domain fused to an activating IL-7 intracellular domain. PSCA-CAR T cells initially killed PSCA-positive pancreatic cancer cells, but only PSCA.4/7-ICR-CAR T cells killed and expanded in the presence of both pancreatic cancer cells and the inhibitory cytokine IL-4. This held true *in vivo* where PSCA.4/7-ICR-CAR T cells exhibited enhanced antitumor activity and greater expansion compared to CAR T cells without 4/7-ICR. Importantly, once PSCA-positive solid tumor cells were eliminated, PSCA.4/7-ICR-CAR T cells no longer expanded, indicating no cell autonomous growth and a positive safety profile. This strategy was also utilized to target breast cancer using 2nd generation MUC1-CAR T cells (57). MUC1.4/7-ICR-CAR T cells demonstrated enhanced expansion

and antitumor activity *in vitro* and *in vivo* compared to MUC1-CAR alone, demonstrating that the 4/7-ICR enhances CAR T cell persistence and function against multiple solid tumor types in the presence of the inhibitory cytokine IL-4.

Transgenic cytokine receptors can also be combined to impart CAR T cells with the ability to transform multiple inhibitory signals into different types of T cell stimuli. Transgenic cytokine receptors were previously developed to overcome the inhibitory effect of transforming growth factor β (TGFβ), a potent immunosuppressive cytokine secreted by multiple solid tumor types. Dominant negative TGFβ receptors (DNR) enable T cells to avoid effects exerted by TGFβ (58, 59), and DNR transduced tumor-specific T cells were recently shown to safely persist in patients years after T cell infusion (60). TGFβ receptor II extracellular domains can also be fused with stimulatory intracellular domains such as the 41BB endodomain (TGFβ/41BB). In a recent publication 1st generation PSCA-CAR T cells were modified to express both TGFβ/41BB and 4/7-ICR, and dubbed SmarT cells (61). SmarT cells demonstrated the ability to recognize PSCA antigen on pancreatic cancer cells through the CAR, induce costimulation via TGFβ induced activation of 41BB, and initiate cytokine signaling through 4/7-ICR in the presence of IL-4. Triple genetic modification enabled SmarT cells to specifically recognize PSCA-positive pancreatic cancer cells, expand, persist, and kill tumor cells in an immunosuppressive TGFβ and IL-4 rich environment. Importantly continued SmarT cell expansion and antitumor activity were dependent upon both PSCA antigen positivity and cytokine induced stimulation through the transgenic receptors. *In vivo*, SmarT cells expanded, persisted, and eliminated pancreatic tumors expressing PSCA, TGFβ, and IL-4, with minimal expansion/persistence at tumor sites expressing PSCA only. Once PSCA-positive, TGFβ, and IL-4 secreting solid tumors were eliminated, SmarT cells contracted, further demonstrating safety of this approach.

In conjunction with enhancing CAR T cell persistence and effector function, transgenic cytokine receptors can be used for selectively enhancing growth of transduced CAR T cells during the T cell manufacturing process. In this regard, a 4αβ chimeric cytokine receptor was developed, consisting of an extracellular IL-4Rα domain paired with an endodomain derived from the shared IL-2/IL-15 β chain (62). This strategy was demonstrated to be effective for generating CAR T cells to target multiple solid tumor types in pre-clinical studies (20, 63), and is under active investigation in a clinical trial for patients with head and neck squamous cell carcinoma (64) (NCT01818323).

In summary, transgenic cytokine receptors can be utilized to avoid immunosuppressive effects of inhibitory cytokines, transform inhibitory signals into activating signals, and to selectively grow transduced solid tumor specific CAR T cells.

CHALLENGES AHEAD

We have described novel genetic modification strategies to enhance the expansion and persistence of CAR T cells for patients with solid tumors. The next frontier in this regard is to overcome limitations imposed by current gene modification techniques, and endow CAR T cells with even greater functional capacity. CAR T cells face many obstacles including homing to and penetrating into solid masses, overcoming tumor antigen escape, surviving within the hostile tumor environment of low pH, high lactate and adenosine, hypoxia, and numerous other immunosuppressive factors including inhibitory stromal cells. Realistically, current engineering strategies allow up to 3 genetic modifications to enhance CAR T cell function. While individual genetic modifications demonstrate efficacy to combat many of the listed challenges, the field is tasked with developing new strategies to combine a greater number of mechanisms for enabling individual CAR T cell products to overcome the potent solid tumor inhibitory environment. One intriguing strategy is to physically load CAR T cells with nanogel "backpacks" capable of delivering relatively large quantities of protein upon CAR recognition of target antigen (65). In such a paradigm, CAR T cells could be physically loaded with proliferative cytokines, leaving room to utilize genetic modifications to overcome other obstacles, such as targeting multiple solid tumor antigens. Another promising strategy is to utilize targeted gene editing and insert CAR constructs into T cell inhibitory loci (66, 67). For instance, CAR DNA could be inserted into the adenosine 2a receptor locus (68), decreasing CAR T cell immunosuppression in an adenosine rich milieu. As these, and other novel strategies are implemented, we envision CAR T cells capable of safely persisting long term and overcoming a greater number of tumor immune evasion strategies.

CONCLUSIONS

Several strategies have been developed to enhance CAR T cell expansion, persistence and antitumor activity by introducing novel costimulatory domains, cytokine genes, and constitutively active or inverted cytokine receptors into T cells. While there are safety concerns regarding autonomous cell growth and cytokine induced toxicity using these approaches, encouraging efficacy and safety data from preclinical studies supports continued preclinical testing and evaluation in humans. Thus, we remain hopeful that optimized CAR T cells will eventually improve outcomes and decrease toxicities for patients suffering from solid tumors.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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