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CAR T-cell therapy for glioblastoma: recent clinical advances and future challenges

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Abstract

In patients with certain hematologic malignancies, the use of autologous T cells genetically modified to express chimeric antigen receptors (CARs) has led to unprecedented clinical responses. Although progress in solid tumors has been elusive, recent clinical studies have demonstrated the feasibility and safety of CAR T-cell therapy for glioblastoma. In addition, despite formidable barriers to T-cell localization and effector function in glioblastoma, signs of efficacy have been observed in select patients. In this review, we begin with a discussion of established obstacles to systemic therapy in glioblastoma and how these may be overcome by CAR T cells. We continue with a summary of previously published CAR T-cell trials in GBM, and end by outlining the key therapeutic challenges associated with the use of CAR T cells in this disease.

Key words

CAR | EGFRvIII | glioblastoma | immunotherapy | T cells

Brief Introduction to CAR T Cells

Genetic engineering of T cells to express chimeric antigen receptors (CARs) directed against specific antigens has opened the door to a new era of personalized cancer therapy. CARs are artificial fusion proteins that incorporate an extracellular antigen-recognition domain, a transmembrane domain, and an intracellular T-cell signaling domain.[1,2] After a CAR construct is transfected into autologous or allogeneic peripheral blood T cells using plasmid transfection, mRNA, or viral vector transduction, the T cells are infused into the patient to target whichever surface-exposed tumor antigen is specified by the CAR's extracellular targeting moiety, usually in the form of a single-chain variable fragment (scFv).[3,4] Upon CAR engagement of its associated antigen, primary T-cell activation occurs and leads to cytokine release, cytolytic degranulation, and T-cell proliferation.[5] Additional T-cell effector mechanisms and memory responses also occur in a manner dependent on the mechanism of co-stimulation (4-1BB or CD28 in the case of "second generation CARs," or both of these signaling domains for "third generation CARs").[6,7] Thus, if a suitable tumor-associated antigen is identified as a target, CAR T cells specific for that antigen are capable of inducing durable antitumor responses in a human leukocyte antigen–independent manner.[8]

The greatest advances for CAR T cells have occurred in the treatment of hematologic malignancies, with the FDA having approved 2 therapies. Tisagenlecleucel, a CD19-targeted CAR T-cell therapy formerly known as CTL019, was approved for the treatment of patients up to 25 years of age with B-cell precursor acute lymphoblastic leukemia that is refractory or in second or later relapse.[9] Subsequently, axicabtagene ciloleucel, another anti-CD19 CAR T-cell treatment, was approved for large B-cell lymphoma patients who have failed at least 2 prior therapies.[10] Remarkably, both of these CAR products led to durable remissions in patients refractory to standard salvage therapies. With the unprecedented success of CAR T cells in leukemia and

lymphoma, a growing number of preclinical studies and clinical trials have focused on translating this treatment to solid tumors. This review will focus on these efforts with regard to glioblastoma (GBM), the most common primary malignant brain tumor in adults and a near uniformly fatal disease.[11]

Appeal of CAR T Cells for Glioblastoma

As the number of cancer therapeutics approved by the FDA has skyrocketed over the past decade, only 3 new treatments have been approved for GBM since 2005: temozolomide, bevacizumab, and tumor-treating fields (TTFields).[12–14] The lack of progress in bringing novel GBM therapies to the clinic relates to a set of challenges associated with brain tumors in general, as well as biological complexities unique to GBM. The following sections outline several of the most difficult problems encountered in the development of novel treatments for GBM, and how CAR T cells may be capable of overcoming them.

Central Nervous System Penetration

The blood–brain barrier (BBB), composed of continuous tight and adherens junctions between brain capillary endothelial cells, excludes the vast majority of cancer therapeutics from entering the brain parenchyma.[15,16] Even when the integrity of the BBB is disrupted in contrast-enhancing regions of GBM tumor, regions of non-enhancing, infiltrating tumor evident on T2-weighted (T2W) or T2W fluid attenuation inversion recovery (FLAIR) imaging are characterized by an intact BBB and do not receive therapeutically effective drug exposure.[17] Thus, a cure for GBM will be possible only if these regions of tumor are adequately treated.

The concept of the CNS as an immune privileged site has been overturned in recent years by several important studies.[18–21] The discovery of lymphatic vessels in the brain[19] and improved understanding of effector T-cell trafficking in the CNS[22] has led to renewed enthusiasm for immunotherapeutic approaches to GBM. Because T cells can penetrate the BBB and infiltrate the brain in a diffuse manner, a successful tumor-associated T-cell response in GBM would obviate the challenges posed by poor drug delivery to the tumor.[23]

A recent trial from our institution demonstrated that after a single peripherally infused dose, epidermal growth factor receptor variant III (EGFRvIII)–directed CAR T cells could successfully traffic to regions of active GBM.[24] In the 7 subjects enrolled on this study who underwent surgical resection following CAR T-cell infusion, post-infusion tumor was analyzed for CART-EGFRvIII infiltration by quantitative (q) PCR. In 2 of these subjects, CART-EGFRvIII DNA sequences were 3 times and 100 times higher, respectively, in brain specimens than in the peripheral blood 2 weeks following CAR T-cell infusion, suggesting effective trafficking and likely expansion of the CART-EGFRvIII cells in situ within active regions of GBM. In one subject, CART-EGFRvIII cells were still detected in the tumor 2 months after infusion. Although there are other challenges associated with the use of CAR T cells for GBM, as described below, successful CAR T-cell trafficking to the tumor following peripheral infusion may overcome the usual therapeutic challenge associated with the BBB. However, the full extent of distribution of peripherally infused CAR T cells throughout the brain, particularly in non-enhancing regions of infiltrating tumor, has yet to be determined. An alternative approach is to administer CAR T cells directly into the CNS via intracavitary or intraventricular infusion.[25]

Obviating the Need for Antigen Presentation and Primary Immune Response

Tumor mutational load (TML), which is associated with the abundance of available neoantigens, plays a key role in tumor immunogenicity across many malignancies.[26–28] While patients with tumors harboring high TML, such as melanoma and non–small cell lung cancer, have enjoyed unprecedented responses to immune checkpoint inhibitors, gliomas carry a substantially lower average TML than these cancers.[29,30] Thus, even as the arsenal of therapies designed to lessen immunosuppression in the tumor microenvironment continues to expand, efficacy of these therapies in GBM is limited by inadequate neoantigen load for T cells to recognize as foreign. The obvious benefit of CAR T cells is that they can be designed to recognize a prespecified tumor antigen, rendering TML less important in the generation of an antitumor immune response.

Even when adequate tumor neoantigen is present, generation of a primary immune response in GBM is also limited by defects in both antigen-specific T-cell receptor signaling and antigen-independent co-stimulatory signaling.[31] Some of these are automatically overcome by adoptive transfer of CAR-expressing T cells. First, the use of CAR T cells eliminates the requirement for antigen presentation. This is critically important in GBM, as these tumors frequently display deficient antigen-processing machinery and inadequate major histocompatibility complex–peptide presentation.[32] Second, the CAR construct includes co-stimulatory domains, obviating the need for stimulation of a primary immune response. Just as glioma cells exhibit ineffective antigen presentation, they also do not express the co-stimulatory molecules required to activate naïve T cells, leading to tumor-specific T-cell ignorance.[33]

Addressing Glioma Stem Cells

Glioma stem cells (GSCs), a population of cells that possess unique molecular signatures and reside within protective niches in the tumor, play a critical role in tumor initiation and persistence in GBM.[34] GSCs are a key cause of treatment failure in GBM due to their intrinsic drug and radiation resistance and ability to repopulate the tumor mass.[35] CAR T-cell therapies have demonstrated efficacy against GSCs in vitro.[36] In addition, human epidermal growth factor receptor 2 (HER2), EGFRvIII, and interleukin-13 receptor alpha 2 (IL-13 Rα2), the leading candidates studied thus far as tumor-associated antigen targets for CAR T cells in GBM, can be expressed by GSCs.[36–38] CAR T-cell therapies

therefore represent an opportunity to eradicate this population of self-renewing, tumor-propagating cells that are vital to therapeutic resistance.

Published Clinical Studies of CAR T Cells in Human GBM

IL-13 Rα2 CAR T Cells

Expression of IL-13 Rα2, present in over 75% of GBMs and associated with activation of the phosphatidylinositol-3 kinase/Akt/mammalian target of rapamycin pathway,[39–41] is linked to increased tumor invasiveness and poor prognosis.[42] Due to its specificity for GBM tumor cells and limited expression in normal brain and other tissues,[43] IL-13 Rα2 has long been recognized as an attractive candidate for CAR T-cell targeting.[44] In a safety and feasibility trial of a first-generation IL-13 Rα2–specific CAR, termed "IL-13 zetakine," repeat doses of autologous CD8+ T cells engineered to express this CAR were administered intracranially to 3 patients with recurrent GBM following gross total tumor (Table 1).[45] In addition, one subject was subsequently treated with direct intratumoral CAR T-cell infusions at a distant site of tumor recurrence. This first-in-human study demonstrated that IL-13 Rα2–directed CAR T cells could be successfully manufactured and delivered to patients with recurrent GBM via an implanted reservoir/catheter system. The CAR T cells were well tolerated, with adverse events such as headaches and transient neurologic deficits being manageable. In addition, promising early signs of antiglioma activity were demonstrated. Patients experienced a rapid increase in necrotic tumor volume by MRI, significant loss of IL-13 Rα2 tumor cell expression, and encouraging duration of overall survival.

In a follow-up trial utilizing a second-generation 4-1BB co-stimulatory IL-13 zetakine CAR, one 50-year-old patient with recurrent multifocal GBM, including leptomeningeal disease, received 6 weekly intracavitary infusions of the CAR T-cell product following surgical resection of 3 of his 5 progressing intracranial tumors (Table 1).[25] Although the locally CAR T-cell treated site remained stable, other intracranial lesions progressed and new spinal lesions developed. The patient was then treated with 10 additional CAR T-cell infusions delivered intraventricularly through a catheter device placed in the right lateral ventricle. Remarkably, in addition to tolerating the infusions without any grade 3 or higher toxicities, this patient experienced regression of all intracranial and spinal tumors lasting for 7.5 months. Although the patient subsequently progressed at new

Table 1 Published human CAR T-cell trials in glioblastoma

CAR Target	CAR Generation[a] (number of subjects)	Biomarker Inclusion Criteria	Mode of Administration	Grade 3/4 Adverse Events Possibly Related to CAR T cells	Efficacy Measures
IL-13 Rα2[25,45]	First ($N = 3$) Second ($N = 1$)	None Tumor IL-13 Rα2+ by IHC[b]	Postresection intracavitary infusions × 12 (catheter device; $N = 3$) Direct intratumoral infusions × 5 (catheter device; $N = 1$) Postresection intracavitary infusions × 6 (catheter device) Intraventricular infusions × 10 (catheter device)	Headache ($N = 2$) Neurologic (shuffling gait, tongue deviation) ($N = 1$) Leukopenia ($N = 1$) Fatigue ($N = 1$) None	Median overall survival ~11 months No tumor recurrence at border of resection cavity Complete response of intracranial and spinal disease lasting 7.5 months
HER2 (virus-specific)[49]	Second ($N = 17$)	Tumor HER2+ by IHC, CMV seropositivity	Peripheral infusions: 1 infusion ($N = 10$) 2 infusions ($N = 4$) 3 infusions ($N = 1$) 4 infusions ($N =$) 6 infusions ($N = 1$)	Lymphopenia ($N = 2$) Headache ($N = 2$) Neutropenia ($N = 1$) Fatigue ($N = 1$) Weakness ($N = 1$) Cerebral edema ($N = 1$) Hydrocephalus ($N = 1$) Hyponatremia ($N = 1$)	Median overall survival ~11 months One patient with partial response more than 9 months Three patients with durable stable disease during 24–29 months of follow-up
EGFRvIII[26]	Second ($N = 10$)	Tumor EGFRvIII+ by RNA-based next-generation sequencing	Single peripheral infusion	Extremity or facial muscle weakness ($N = 2$) Cerebral edema ($N = 2$) Seizure ($N = 2$) LV systolic dysfunction ($N = 1$) Headache ($N = 1$) Intracranial hemorrhage ($N = 1$)	Median overall survival ~8 months One patient remains alive (33 months post CAR T-cell infusion) at time of this review article

[a] First generation: CD3 ξ-chain only.
Second generation: CD3 ξ-chain plus 1 co-stimulation domain (4-1BB or CD28).
Third generation: CD3 ξ-chain plus 2 co-stimulation domains (4-1BB and CD28).
[b] IHC: immunohistochemistry.

locations distinct from his previous tumors, this case report highlights the therapeutic potential for CAR T cells in GBM.

Another important component of the IL-13 Rα2 CAR T-cell trial has been the incorporation of PET imaging to monitor the trafficking of CAR T cells into the brain.[46] Keu and colleagues used PET imaging with [^{18}F]FHBG (9-[4-[18F] fluoro-3-(hydroxymethyl)butyl]guanine) to track IL-13 Rα2 CAR T cells expressing an *HSV1-tk* reporter gene (uptake of this PET tracer is significantly higher in *HSV1-tk* expressing cytotoxic T lymphocytes compared with naïve human T lymphocytes).[46] Although the sample size to date has been small, this approach was safe and feasible, as the study demonstrated a significant increase in [^{18}F]FHBG activity in regions of cytotoxic T-lymphocyte tumor trafficking.

HER2 Virus-Specific CAR T Cells

Human epidermal growth factor receptor 2, a receptor tyrosine kinase overexpressed in many human cancers, has also been considered an ideal tumor-associated antigen for CAR targeting in GBM.[47–49] Most recently, 17 patients with progressive HER2+ GBM were treated on a phase I trial with peripheral blood infusions of HER2-specific CAR-modified virus-specific T cells (Table 1).[50] Because safety concerns had been raised by the death of a colorectal cancer patient treated in a previous study with a third-generation HER2-CAR T-cell therapy (composed of a trastuzumab-based antigen-recognition domain and a CD28.4-1BB signaling domain), the investigators in the GBM study utilized a second-generation CAR with an FRP5-based exodomain and a CD28 signaling endodomain. No dose-limiting toxicity was observed, although 2 patients had grade 2 seizures/headaches. HER2-CAR T cells were detected by qPCR in all patients after the infusion, peaking in 15 of 17 patients at 3 hours after the infusion and at 1 week and 2 weeks in the other 2 patients, respectively. At 6 weeks after the infusion, HER2-CAR T cells were present in 7 of 15 patients, with blood levels declining further every month thereafter (with 2 samples remaining positive out to 12 months, but none positive at 18 months). This suggested that the HER2-CAR T cells did not expand after infusion but could persist for up to 1 year at a low frequency. Of 16 evaluable patients, 1 had a partial response lasting for more than 9 months, and 7 had stable disease ranging between 8 weeks and 29 months (with 3 of these remaining free of progression during 24–29 mo of follow-up).

A key aspect of this study was that it relied on the expression of CARs in virus-specific T cells. Using this strategy, virus-specific T cells provide the expected antitumor activity through their CAR but may also receive appropriate co-stimulation following native T-cell receptor engagement by latent virus antigens presented by professional antigen-presenting cells.[50,51] The investigators in this study administered CAR-modified T cells specific for adenovirus, Epstein–Barr virus (EBV), or cytomegalovirus (CMV), the safety of which had been previously demonstrated in hematopoietic stem cell transplant recipients.[52] Among the 17 patients treated, the CAR T cells of all patients contained adenovirus- and EBV-specific T cells, and all CART cells from CMV seropositive patients contained CMVpp65-specific T cells as determined by interferon gamma Elispot assays. Overall, this phase I trial demonstrated the feasibility and safety of peripherally infused virus-specific CAR T cells in GBM and, despite the lack of expansion of the CAR T cells in the blood, displayed encouraging signs of efficacy.

EGFRvIII CAR T Cells

EGFRvIII, resulting from an in-frame deletion of exons 2 to 7, is the most common variant of this receptor observed in human tumors.[53] Approximately 40% of all newly diagnosed GBMs carry amplification of the EGFR gene, and about 50% of EGFR-amplified GBMs contain constitutively active and oncogenic EGFRvIII.[54,55] Prior studies have found that the EGFRvIII alteration is associated with shorter survival in GBM, although recent data suggest that prognosis for these EGFRvIII+ patients may not differ from those with EGFR gene amplification.[56]

The amino acid sequence resulting from the EGFRvIII alteration yields a novel glycine residue at the junction of exons 1 and 8, generating a tumor-specific and immunogenic epitope within the extracellular domain of EGFR. As a result, both vaccine and CAR T-cell therapies against EGFRvIII have been developed.[24,57] In a first-in-human phase I trial performed at our institution, 10 patients with recurrent GBM were treated with a single dose of peripherally infused EGFRvIII-directed CAR T cells (Table 1).[24] Manufacturing and infusion of the CAR T cells was feasible and safe, without evidence of off-tumor toxicity or cytokine release syndrome (CRS). While the study was not designed to evaluate for efficacy, no patients experienced tumor regression (although one patient had residual stable disease lasting >18 mo). Notably, the patients enrolled on this study had especially grim prognoses, as all patients had GBM that was heavily pretreated and O^6-methylguanine-DNA methyltransferase unmethylated at the time of CAR T-cell infusion, and all but one had multifocal disease.

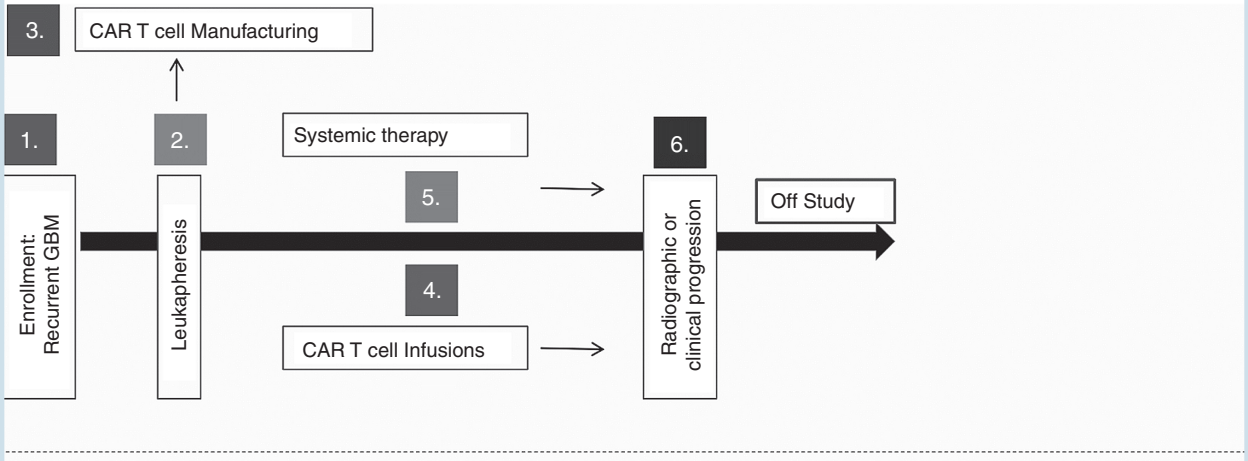
All infused subjects had detectable engraftment of EGFRvIII CAR T cells in the peripheral blood,[24] although the degree of engraftment was considerably lower than what has been observed with CD19-specific CAR T cells bearing the same 4-1BB co-stimulatory domain, lentiviral backbone, and manufacturing process.[58] While this suggests that antigen-driven expansion (as seen in hematologic malignancies with high peripheral blood antigen load) is more robust than expansion attributable to tonic CAR T-cell signaling, it is also possible that lower engraftment levels were observed due to T cells homing to antigen-expressing tissue in the brain. In fact, 7 of the 10 subjects in this study had post-CAR T-cell surgical intervention, allowing for tissue-specific analysis of CAR T-cell trafficking and other "pharmacodynamic" endpoints. In 2 of these subjects, both of whom had their tumors resected within 2 weeks of CAR T-cell infusion, CART-EGFRvIII cells were found at higher concentrations in the brain than in the peripheral blood at the same time point. Because CART-EGFRvIII DNA sequences by qPCR were 3 times and 100 times higher, respectively, in brain specimens than in corresponding blood samples from these patients, there was a suggestion that the CAR T cells had effectively trafficked and expanded in situ within active regions of GBM.

In addition to determining EGFRvIII CAR T-cell trafficking to the tumor, acquisition of posttreatment surgical specimens also allowed for measurement of EGFRvIII target antigen expression and characterization of the tumor immune microenvironment following CAR T-cell infusion.[24] Most of the subjects had specific loss or decreased expression of EGFRvIII in resected tumors following CAR T-cell infusion, with the exception of one patient who had poor CAR T-cell expansion in the blood and no CAR T cells present in the tumor (this patient also experienced early tumor progression). Although it cannot be ruled out that decreased EGFRvIII expression was unrelated to CAR T-cell therapy, as EGFRvIII expression was previously shown to display both spatial and temporal variation,[59] a more recent study demonstrated that the vast majority of EGFRvIII+ GBMs maintain EGFRvIII positivity at recurrence.[56] This suggests that antigen loss was more likely related to successful targeting of EGFRvIII+ tumor cells by CAR T cells. Regarding the tumor microenvironment, in situ phenotypic analyses of nontransduced, polyclonal T cells in post-CAR T-cell surgical specimens demonstrated significant infiltration of regulatory T cells (Tregs), and immunohistochemical stains displayed consistent and significant upregulation of immune checkpoints and other soluble immunosuppressive molecules, including indoleamine 2,3-dioxygenase (IDO) 1, programmed death (PD) ligand 1 (PD-L1), transforming growth factor (TGF)–β, and IL-10.[24] These observations suggest that CAR T-cell targeting of EGFRvIII+ tumor cells induced a compensatory immunosuppressive response in the tumor microenvironment.

Challenges and Future Directions

Tumor Microenvironment

A summary of currently unanswered questions in the use of CAR T-cell therapies for GBM is presented in Fig. 1. One of the most pressing issues is how to address the immunosuppressive GBM microenvironment. Once the CAR T cells arrive in the tumor, the microenvironment presents many obstacles, including tumor-derived soluble factors and cytokines, immunosuppressive immune cells, and physical and metabolic barriers.[60,61] Cytokine networks in the GBM microenvironment include prostaglandin E2, IL-6, IL-10, and



1. Enrollment: Recurrent GBM
-Surgical resection to minimize tumor burden, or prefer measurable tumor to assess response and to provide adequate antigen load?
-Require tissue confirmation that the target antigen is present in the recurrent tumor?
-Maximum steroid dose allowed?

2. Leukapheresis
-Perform at recurrence or earlier in disease course?
-Need for preconditioning, i.e. lymphodepleting chemotherapy?

3. CAR T Cell Manufacturing
-Optimal CAR construct, transduction method, and T cell popultion (e.g. central memory $CD8^+$ T cells?)
-How to minimize time for *ex vivo* manufacturing?

4. CAR T Cell Infusions
-Mode of administration: peripheral versus intracavitary versus intraventricular?
-Number and frequency of infusions?

5. Systemic threrapy
-Which therapies should be added to address immunosuppressive GBM microenvironment?
-Timing with respect to CAR T cell infusions?

6. Radiographic or clinical progression
-Need histopathologic confirmation of tumor progression?
-Need to measure elimination of target antigen-positive glioma cells?
-Are there ways to salvage an initial response?

Fig. 1 Theoretical study schema for a CAR T-cell trial in recurrent GBM. Proposed scientific and clinical questions for future studies are listed for each component.

TGF-β, each of which dampens T-cell proliferation and effector responses.[62] These and other immune inhibitory factors were consistently upregulated in surgical specimens from post-EGFRvIII CAR T-cell infusion patients compared with their pretreatment tumors.[24] Tregs, which comprise up to 30% of infiltrating lymphocytes in GBM and suppress T-cell responses,[63] also pose a challenge for GBM immunotherapy. Similar to cytokines and other soluble immune inhibitory factors that were upregulated after EGFRvIII CAR T cells trafficked to the tumor, post-CAR T-cell infusion tumor specimens from this study also demonstrated a significant influx of Tregs, identified based on coexpression of CD4, CD25, and Forkhead box protein 3.[24] Beyond Tregs, tumor-associated macrophages, microglia, and myeloid-derived suppressor cells are also common in GBM and support tumor cell growth.[61,64,65] In particular, M2-type macrophages play an important role in immune suppressive and tumor supportive actions, including activation of mitogen-activated protein kinase signaling[66] and glioma stem cell stimulation.[67] The extent and mechanisms of adaptive immune resistance in GBM following other types of immunotherapy beyond CAR T cells are unknown, although preclinical studies of dendritic cell vaccines for glioma have suggested that adaptive upregulation of PD-L1 may play a role in mediating treatment failure.[68]

In light of the immunosuppressive milieu encountered by effector T cells in GBM, the efficacy of CAR T cells may be heightened through combinations with small-molecule drugs or checkpoint blocking antibodies, including but not limited to PD-1/PD-L1 inhibitors, anti–TGF-β molecules, and anti–IL-6 antibodies. Specific targeting of immunosuppressive immune cell populations could also be considered. Previously suggested approaches include granulocyte-macrophage colony-stimulating factor neutralization of myeloid-derived suppressor cells,[69] or multiple potential strategies targeted against Tregs, including metronomic chemotherapy,[70] CD25 blockade,[71] anti– C-C chemokine receptor 4 antibodies,[72] or checkpoint inhibitor/immune agonist therapies.[73]

The efficacy of CAR T-cell therapy in GBM is also limited by the markedly stressful metabolic landscape of the tumor. First, hypoxia is a predominant feature of GBM and has been shown to enhance GBM-mediated immunosuppression.[74] Second, nutrient deprivation is typical of the GBM tumor microenvironment. Since neurons and cancer cells rely almost exclusively on glucose metabolism, T cells, which also require increased glucose uptake and glycolysis to support the demands of proliferation and effector function, are starved in the glucose-poor GBM microenvironment.[61] Lastly, low levels of amino acids such as tryptophan, arginine, and lysine can cause protein translation shutdown or autophagy responses in effector T cells.[75] GBM is characterized by high levels of expression of IDO, which catalyzes the conversion of tryptophan into kynurenines.[76] These catabolites mediate induction of apoptosis in effector T cells and amplification of immunosuppression by Tregs.[77] As previously mentioned, IDO was further upregulated in the GBM microenvironment following CAR T-cell infusion in a phase I study of EGFRvIII-directed CAR T cells,[24] suggesting a role for IDO inhibitors in combination with CAR T cells.

Tumor Heterogeneity and Antigen Loss

Intratumoral heterogeneity has been described as a root cause of therapy resistance in GBM in general[78] and is perhaps one of the most critical barriers to the long-term efficacy of CAR T cells in this disease. Expression of previously studied CAR T-cell targets in GBM, including IL-13 Rα2 and EGFRvIII, is heterogeneous on both interpatient and intrapatient levels and can vary spatially and temporally.[56,79,80] The importance of spatial heterogeneity is demonstrated by the results of multiple, regionally distinct post-infusion biopsies taken from one subject treated with EGFRvIII CAR T cells.[24] The degree of EGFRvIII expression varied substantially throughout different regions of the tumor, suggesting either that the CAR T cells had varying degrees of efficacy in different tumor locations or, more likely, that baseline pretreatment EGFRvIII expression was spatially heterogeneous. Similar conclusions can be drawn from the randomized, phase III ACT IV trial of rindopepimut,[57] a peptide vaccine targeting EGFRvIII. In this study, patients randomized to rindopepimut displayed a high rate (57%) of EGFRvIII antigen loss in posttreatment tissue. However, the addition of rindopepimut to standard-of-care therapy did not improve outcomes, and loss of EGFRvIII antigen within the rindopepimut-treated group was not associated with clinical benefit. This suggests that successful eradication of EGFRvIII+ cells was accompanied by progression of EGFRvIII– tumor that was present at the time of treatment. Even in patients randomized to standard-of-care therapy without rindopepimut, loss of EGFRvIII expression was demonstrated in over 50% of those who had posttreatment tumor tissue available, underscoring the role of temporal (in addition to spatial) variation in EGFRvIII expression.

Thus, results from both ACT IV and our study of EGFRvIII CAR T cells invoke the important question of whether successful immune targeting of a single antigen will translate into durable clinical benefit, or whether antigen escape will result in minimal clinical impact. The answer may depend on the extent to which CAR therapy can induce indirect tumor killing and/or can trigger "antigen/epitope spreading," a process in which CAR T cells induce the generation of endogenous CD8 T-cell responses against tumor antigens that were not originally targeted by the CAR.[60] This is postulated to occur when CAR T cells destroy their target tumor cells and secrete stimulatory cytokines, resulting in the release of tumor antigens in an immune-activated microenvironment. While one preclinical study supported the possibility of antigen spreading with EGFRvIII CAR T cells,[81] the extent to which this occurs in humans is unknown. If antigen spreading is not occurring, combinatorial targeting of tumor-associated antigens will be required to address tumor heterogeneity. Both bispecific and trivalent CAR T-cell approaches in GBM are currently being pursued.[79,82]

T-Cell Proliferation and Persistence

Since second-generation CAR T cells can amplify in patients after administration, CAR T-cell dosing does not follow classical pharmacokinetic patterns. In hematologic malignancies, for example, a single dose of CAR T cells

is sufficient to induce sustained antitumor response.[83] In these tumors, T-cell amplification in the peripheral blood seems to be required to achieve an effective T-cell to tumor-cell ratio and predicts clinical efficacy.[84] In solid tumors, however, the peripheral blood is not the compartment of therapeutic action, and the effective CAR T-cell dose and frequency/schedule of administration are elusive. With regard to EGFRvIII CAR T cells, maximal detectable trafficking of CART-EGFRvIII cells to the brain coincided with peak engraftment in the peripheral blood, around 1–2 weeks after infusion.[24] However, in some patients CART-EGFRvIII was not detectable in the tumor by 2–3 months post infusion. This raises the question of whether repeated peripheral CAR T-cell infusions may lead to a more persistent expansion of the T cells at the tumor site.

A related issue is whether lymphodepleting preconditioning will lead to improved CAR T-cell expansion and efficacy in GBM. In hematologic malignancies where CAR T-cell therapies have gained FDA approval, it is standard to administer lymphodepleting chemotherapy within 14 days before the planned CAR T-cell infusion.[85] In leukemia, where the peripheral blood disease burden is high, preconditioning with lymphodepleting chemotherapy creates space for the expansion of infused CAR T cells.[83,85]

Table 2 Current ongoing trials of CAR T-cell therapies for glioblastoma*

NCT# and Institution	Study Name	Phase	Target	Delivery	Additional Features
NCT02844062 Beijing Sanbo Brain Hospital, China	Pilot study of autologous anti-EGFRVIII CART cells in recurrent glioblastoma multiforme	I	EGFRvIII	Intravenous	Lymphodepleting chemotherapy: Cyclophosphamide 250 mg/m^2 days 1–3 Fludarabine 25 mg/m^2 days 1–3
NCT03170141 Shenzhen Geno-immune Medical Institute, China	4SCAR-IgT against glioblastoma multiforme	I/II	EGFRvIII	Intravenous Intracavitary	Lymphodepleting chemotherapy: Cyclophosphamide 250 mg/m^2 days 1–3 Fludarabine 25 mg/m^2 days 1–3 Use PD-1/PD-L1 antibody-producing T cells (IgT) designed to address tumor microenvironment in addition to direct tumor cell killing
NCT02442297 Baylor College of Medicine	T cells expressing HER2-specific chimeric antigen receptors for patients with glioblastoma (iCAR)	I	HER2	Intracavitary	Patients must undergo surgical tumor resection
NCT01109095 Baylor College of Medicine	CMV-specific cytotoxic T lymphocytes expressing CAR targeting HER2 in patients with GBM (HERT-GBM)	I	HER2	Intravenous	First cohort of 17 patients published[49]
NCT02664363 Duke University	EGFRvIII CART cells for newly diagnosed GBM (ExCeL)	I	EGFRvIII	Intravenous	Newly diagnosed residual disease at least 2 cm Leukapheresis occurs prior to standard radiation and chemotherapy, and CART cells are administered during post-radiation temozolomide
NCT0328331 Duke University	Intracerebral EGFRVIII CART cells for recurrent GBM (INTERCEPT)	I	EGFRvIII	Intratumoral via convection enhanced delivery	CART cells are infused immediately following stereotactic radiosurgery
NCT0220937 University of Pennsylvania, University of California San Francisco	Autologous T cells redirected to EGFRvIII with a chimeric antigen receptor in patients with EGFRvIII+ glioblastoma	I	EGFRvIII	Intravenous	First cohort of 10 patients published[26]
NCT0145459 National Cancer Institute	CART-cell receptor immunotherapy targeting EGFRvIII for patients with malignant gliomas expressing EGFRvIII	I/II	EGFRvIII	Intravenous	Lymphodepleting chemotherapy: Cyclophosphamide 60 mg/kg days 1–2 Fludarabine 25 mg/m^2 days 1–5 Given with intravenous aldesleukin (IL-2)
NCT0293844 Beijing Sanbo Brain Hospital, China	Pilot study of autologous chimeric switch receptor modified T cells in recurrent glioblastoma multiforme	I	PD-L1	Intravenous	Lymphodepleting chemotherapy: Cyclophosphamide 250 mg/m^2 days 1–3 Fludarabine 25 mg/m^2 days 1–3 CAR contains the extracellular domain of PD-1
NCT02208362 City of Hope Medical Center	Genetically modified T cells in treating patients with recurrent or refractory malignant glioma	I	IL-13 Rα2	Intracavitary Intraventricular	First cohort of 3 patients published,[45] as well as case report of complete response[25]

*Table data acquired from clinicaltrials.gov on November 27, 2017; does not include solid tumor studies with a glioma arm (NCT02713984 and NCT02617134).

These preconditioning regimens have also been shown to deplete Tregs and activate the innate immune system.[86] While the latter 2 of these effects may have theoretical benefit in GBM, none of the studies of CAR T cells reported in GBM to date have used preconditioning,[24,25] and this issue has yet to be adequately tested in solid tumors in general. In addition, patients with recurrent GBM are often "lymphodepleted" to begin with, due to the effects of standard radiation and temozolomide,[87] further complicating this question.

Finally, there are novel methods of CAR engineering and other approaches to modifying T-cell activation that are currently being studied to improve T cell function in situ. These include, but are not limited to (i) genetic modification of the T cells to express chemokine receptors and improve trafficking to the tumor,[88] (ii) design of hypoxia-induced CAR expression to alleviate hypoxia in the tumor microenvironment,[89] and (iii) engineering of CAR T cells to secrete pro–T-cell survival cytokines such as IL-12.[90]

Conclusions

In summary, while the exploration of CAR T-cell therapy in GBM has just begun, early results have demonstrated feasibility, safety, and even signs of efficacy using this approach. The challenges ahead are numerous, including augmentation of CAR T-cell tumor infiltration, optimization of infusion dosing and frequency, modulation of the immunosuppressive tumor microenvironment, and, perhaps most important, addressing the marked molecular heterogeneity inherent to GBM. In addition, there are challenges associated with the use of immunotherapy more generally in this disease, such as management of concomitant steroid use, differentiation between true tumor progression and radiographic pseudoprogression,[91] and identification of potential biomarkers of response. These will also need to be addressed in any CAR T-cell study for GBM. Finally, IL-13 Rα2, EGFRvIII, and HER2, while representing the CAR targets for the first-in-human CAR T-cell studies reported in GBM, are only a few of the potential antigens that are being explored for CAR targeting in this disease. We direct readers to a recent review by Rodriguez and colleagues for a detailed discussion of the various targets being explored.[44] Clinical investigations in GBM are already under way for CAR T cells targeting ephrin-A2 (NCT02575261) and EGFR (NCT02331693); and other novel antigens, such as CD70, have recently been discovered.[92] Results of these studies and others are eagerly anticipated (Table 2).

Conflict of interest statement. C.H.J. is an inventor of intellectual property licensed by the University of Pennsylvania to Novartis.

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