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A Head Start: CAR-T Cell Therapy for Primary Malignant Brain Tumors

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Opinion statement

Oncology is the midst of a therapeutic renaissance. The realization of immunotherapy as an efficacious and expanding treatment option has empowered physicians and patients alike. However, despite these remarkable advances, we have only just broached the potential immunotherapy has to offer and have yet to successfully expand these novel modalities to the field of neuro-oncology. In recent years, exciting results in preclinical studies of immune adjuvants, oncolytic viruses, or cell therapy have been met with only fleeting signs of response when taken to early phase trials. Although many have speculated why these innovative approaches result in impaired outcomes, we are left empty-handed in a field plagued by a drought of new therapies. Herein, we will review the recent advances across cellular therapy for primary malignant brain tumors, an approach that lends itself to overcoming the inherent resistance mechanisms which have impeded the success of prior treatment attempts.

Introduction

Primary malignant brain tumors (PMBTs) encapsulate a spectrum of favorable and unfavorable prognosis. Within this heterogeneous group, we find the malignancy, glioblastoma (GBM), which unfortunately carries the highest incidence (4.40 per 100,000) and prevalence (9.23 per 100,000) of PMBTs; worse still, it is the subtype that holds the lowest 5- and 10-year survival rates (5.4% and 2.7%, respectively) [1]. Despite stepwise advances in treatment across surgery, radiotherapy, and

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chemotherapy, the median survival remains slightly over a year at 12–15 months [2, 3].

Immunotherapeutic renaissance

In the preceding decade, we have seen a revolution in oncology that harkens back to the foundations of immunotherapy championed by William B. Coley in the 1890s, when he injected inoperable tumors with cultures of erysipelas [4]. Over a century later, this revolution has been met with tremendous success following the expansion of checkpoint inhibitors across hematologic and solid malignancies [5, 6], yet these practice-changing outcomes have not translated into the realm of the neuro-oncologist. At this time, there are no FDA-approved immunotherapies for PMBTs despite many unique therapies currently in clinical trials [7]. Over the last 20 years, we have learned that PMBTs, and GBM in particular, are highly immunosuppressive tumors [7–10] and there are limitations to a safe immune response in the central nervous system [11]. To date, we have learned a substantial amount through several iterative, albeit failed, phase 3 trials evaluating immunotherapy in GBM [12–14]. These results, in short, suggest single-agent anti-PD-1 therapy alone may not be sufficient to overcome the inherent immunosuppressive microenvironment within GBMs.

Fundamentals of T cell receptor signaling

In order to understand the intricacies of why certain CAR T cell therapies may or may not work, we must first look at how T cells traditionally recognize antigen. In contrast to immunoglobulins, found on and secreted by B cells, which can interact and bind target antigens on pathogens and toxins within the extracellular space of a host's body, T cells require antigen presented to them by host cells. These antigens can be derived from infectious organisms, such as intracellular bacteria or viruses, but they can also recognize tumor antigen when presented on the surface of a cancer cell. [15]

The antigen presentation by a host cell or tumor is facilitated by the peptide-binding glycoproteins of the major histocompatibility complex (MHC). There are two classes of MHC molecules—MHC class I and MHC class II—which differ in their structure as well as expression on different cells of the body [15]. While the MHC molecules facilitate the presentation of peptides, in recent years, we have also learned about additional molecules the body uses to present antigen to our adaptive immune system. In one example, a family of CD1 molecules present lipid-based antigens on the surface of host cells, and in another example, MR1 (MHC class I-related molecule) presents metabolite-derived antigens on the surface of host cells [16]. These antigen-binding molecules are loaded with antigen which has been "processed" by the host cell, e.g., broken into short segments which can be bound and presented on the surface of the host cells. Effectively, these antigen-binding molecules present a veritable smorgasbord of intracellular antigens, both from foreign organisms within a cell and from the cells themselves, to the circulating T cells.

T cells recognize these presented antigens through their T cell receptor (TCR). T cells express approximately 30,000 T cell receptors on their surface. Each receptor is approximately 7 nm in size and consist of two different polypeptide chains linked by a disulfide bond [15, 17]. Each chain has a variable and a single constant region. As with antibody genes, the variable domain is encoded in separate pieces that rearrange differently in each T cell to create a potential repertoire of ~ 10^{13} possible combinations [18]. The most prevalent T cell receptor chains are T cell receptor α (alpha, TCRα) and T cell receptor β (beta, TCR β), although there are a minority of T cells which bear an alternative pair of heterodimers, designated T cell receptor γ (gamma, TCRγ), and T cell receptor δ (delta, TCR δ). In contrast to immunoglobulins, each of these heterodimer pairs have only one antigen-binding site and are not secreted. These heterodimers have an amino-terminal variable (*V*) region, a constant (*C*) region, and a short relatively inflexible hinge region. The dimers span the T cell's lipid bilayer with a hydrophobic transmembrane domain and end with a comparably short cytoplasmic tail.

For the sake of this focused review article, we will highlight the most prevalent TCR heterodimer pair, TCR-αβ. This receptor, which in and of itself has no intrinsic signaling capacity, is assembled in the endoplasmic reticulum (ER) where it non-covalently associates with signaling dimers of CD3εγ, CD3εδ, and CD3ζζ [19]. This receptor complex moves to the Golgi for glycosylation before being expressed as a unit on the cell surface [20]. Notably, the cytoplasmic tails of CD3ε, CDδ, and CD3γ each contain one immunoreceptor tyrosine-based activation motif (ITAM), and that of CD3ζ contains three ITAMs. In total, one TCR–CD3 complex is composed of 10 ITAMs [19]. When the TCR-αβ binds to its cognate peptide antigen presenting in an MHC molecule, several subsequent steps occur.

These ITAM sites on the CD3 subunits are then phosphorylated by Src family kinases; the phosphorylated ITAMs subsequently serve as docking sites for a cascade of downstream signaling, including enzymes and adaptor proteins [17].

While prior studies on T cell function have shown as few as one TCR-MHC binding complex is sufficient to trigger tumor necrosis factor alpha (TNFα) and interleukin-2 (IL-2) secretion by $CD4^+$ T cells [21], and as few as three TCR-MHC interactions between a T cell and a host cell are sufficient to trigger cytotoxicity, it is after approximately 8–10 peptide-loaded MHCs are bound to TCR complexes that there is the formation of a stable ring [22]. This confluence is referred to as the immunologic synapse (IS). The IS is defined by three concentric rings of clustered molecules which is often depicted as a "bull's-eye." The inner-most ring is called the central supramolecular activation cluster (cSMAC), where TCR signaling takes place. The cSMAC contains most of the TCR-MHC-peptide complexes, including key signaling proteins like CD4 and CD8, as well as additional proximal signaling proteins like lymphocyte-specific protein tyrosine kinase (Lck), protein kinase C theta (PKC-θ), and CD28. The next ring is referred to as the peripheral SMAC (pSMAC) which contains proteins involved in cell adhesion, such as integrin lymphocyte function-associated antigen-1 (LFA-1), cytoskeletal linker talin, and intercellular adhesion molecule 1 (ICAM1). Large molecules, such as CD43 and CD45, are excluded from the pSMAC and make up the distal SMAC (dSMAC) [20, 23]. Inhibitory and costimulatory molecules, such as programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte-associated protein 4 (CTLA-4), and inducible costimulator (ICOS) also are aggregated at the region of IS and play crucial roles in the regulation of T cell activation. Following activation, these activated T cells rapidly polarize their cytotoxic machinery centered at the IS and aimed toward the site of proximal signaling. Next, microtubules at the rear of the cell which are associated with secretory granules are reoriented toward the microtubule-organizing center (MTOC), where they dock at the IS and are secreted into the synaptic cleft [22]. These cytotoxic granules include perforin and granzymes, which are essential for inducing target cell apoptosis. The entire bull's-eye formation takes on the order 5–10 min to form [24].

During the formation of the IS, the Src kinases-mediated phosphorylation of ITAMs kicks off a cascade of activation leading to the release of Ca^{2+} from the ER. This calcium influx leads to the activation of calcineurin which subsequently dephosphorylates the nuclear factor of activated T cells (NFAT) in the cytosol, thereby inducing its activation and translocation to the nucleus. NFAT as well as other now activated transcription factors (nuclear factor-κB (NF-κB) and activator protein-1 (AP-1)) subsequently induce specific transcription of genes which control T cell proliferation, maturation, and differentiation [25].

Cellular therapy for primary malignant brain tumors

Cellular therapy, particularly chimeric antigen receptor (CAR) T cell therapy, was arguably started in the late 1980s by Dr. Zelig Eshhar [26]. Over the past 20 years, we have seen this concept redesigned (Fig. 1) and implemented with tremendous success in B cell malignancies [27–30]. This success does not appear to be limited to CD19- or CD22-directed therapy, as we see signals emerging of tangible improvements across CD30+ relapsed/refractory Hodgkin or T cell lymphoma and multiple myeloma [5, 31–34]. Herein, we shall review the current and future targets of cellular therapy in GBM.

To place these therapies and targets into perspective, it is essential to understand what composes a CAR as well as how it parallels and differs from a traditional TCR.

CARs have had several revisions to their design since their inception; however, many share similar components. CARs require some interface to act as an antigen recognition domain. Traditionally, this is accomplished using a single-chain variable fragment (scFv), which is formed by linking the variable light (V_L) and variable heavy (V_H) regions of a monoclonal antibody by a short linker [35]. These scFvs can vary dramatically in their affinity, orientation, and number—as groups have begun to string scFvs together in tandem to form bivalent CARs [36]. There are three aspects of scFv-based CARs to highlight here, the first is the ability to target any antigen, provided the amino acid sequence of an antibody with the desired specificity is known. The second notable feature of CARs utilizing an scFv as an interface is their ability to recognize antigen independently of MHC presentation. This prevents tumor escape via the down-regulation of MHC molecules and confers CAR T cells with the ability to recognize non-peptide antigens such as glycolipids or tumor-specific glycosylation patterns [35]. The third element to recognize is the difference in affinity strength between scFvs and TCRs, the former tend to possess high affinities in the nM range (K_d:10^{-6} M – 10^{-9} M) compared with the TCR in the μM range

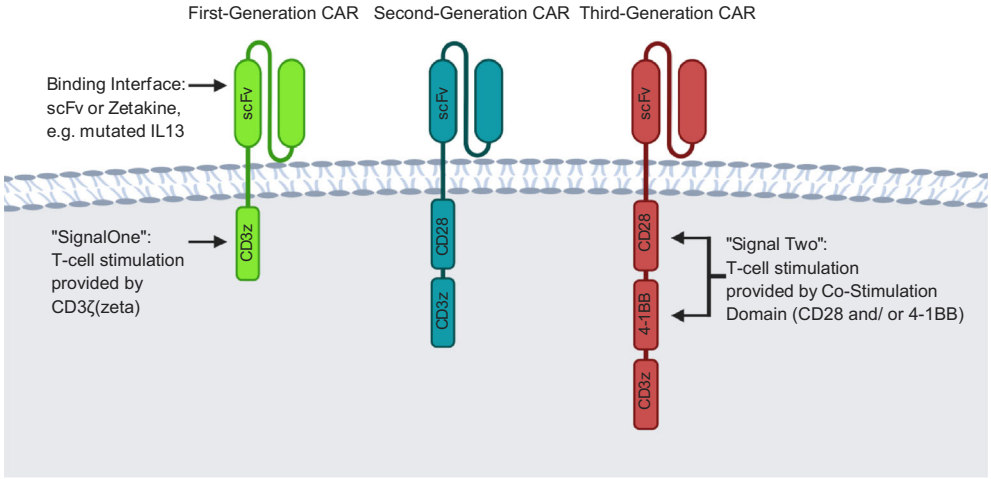

Fig. 1. Diagram of chimeric antigen receptor (CAR) Generations: All CARs are composed of a binding interface; in modern CARs, this is mediated by a single-chain variable fragment (scFv) as denoted. However, this can be replaced by "zetakines" such as the mutated IL-13 or more novel designs with expression of endogenous proteins, such as the CAR constructs expressing the natural killer group 2, member D (NKG2D) receptor. The majority of CARs also include what is traditionally considered "Signal One" of CAR activation by including the CD3z cytosolic signaling domain. Where second- and third-generation CARs differ is with the inclusion of additional cytosolic signaling domains, often considered "Signal Two" of CAR activation. This is most often accomplished with the expression of either the signaling domain for CD28 or 4-1BB in the case of second-generation CARs or the expression of both in third-generation CARs. Figure created with BioRender.com.

(K_d:10^{-4} M – 10^{-6} M) [24]. There are alternatives to scFvs as binding interfaces; however, a discussion of alternative antigen receptor-binding domains extends beyond the scope of this review, and they have been extensively reviewed elsewhere [37, 38].

The next component of a CAR to review is the spacer or hinge domain, which connects the scFv (or equivocal binding interface) to the transmembrane domain. The majority of CAR designs utilize either immunoglobulin G (IgG)-based hinges or derivatives of CD8α or CD28 extracellular domains [35]. The domain choices here have not varied dramatically as there is some evidence certain spacers may lead to scFv oligomerization and the promotion of tonic CAR signaling [39]. Conversely, the optimal spacer length has been meticulously evaluated by groups and appears to depend both on the antigen itself and as the position and accessibility of the targeted epitope [40–43].

Following the hinge domain, CARs have a transmembrane domain; this links the extracellular domains of the CAR to the intracellular signaling domains and anchors the receptor to the T cell membrane. Commonly used transmembrane domains have been derived from CD4, CD8α, CD28, and CD3ζ [35], the latter of which is used less frequently after it was found that the CD3ζ transmembrane domain conferred the ability to form homodimers as well as heterodimers with the endogenous TCR-complex [44]. Some groups have started to evaluate variations in this domain; however, it remains largely understudied.

What is arguably the second most critical component to a CAR is what is occurring, under the hood—within the intracellular space. Here, we have the intracellular costimulatory domains, a field ripe with studies, which would require a dedicated in-depth analysis beyond the scope of this review; however, this has been discussed elsewhere [45, 46]. The costimulatory domains found within modern CARs are usually derived from either the CD28 receptor family (CD28, ICOS) or the tumor necrosis factor receptor family (4-1BB, OX40, CD27); these domains have marked differences in their downstream effects on T cell function and persistence. This divergence is highlighted no more starkly than between the use of CD28 and 4-1BB; the former promoted the rapid development of T cell effector functions with an effector-memory phenotype, and dependence on glycolytic metabolism, but conferred limited in vivo T cell persistence. Conversely, costimulation with 4-1BB had led to slower tumor eradication in a murine model of leukemia but increased T cell persistence

ultimately leading to comparable anti-tumor efficacy. Some of the signaling difference with 4-1BB may be tied to an increase in mitochondrial biogenesis, enhanced respiratory capacity, and increased fatty acid oxidation [47–49].

With these basic building blocks, we can touch on the generations of CAR T cells. The initial, or first-generation, CAR T cells included an antigen recognition domain, a hinge, a transmembrane domain, and a CD3ζ intracellular domain, capable of recapitulating "signal 1" of T cell activation. However, first-generation CAR T cells displayed poor anti-tumor efficacy in patients, owing to the limited expansion and persistence of transferred T cells [50–52]. Following this work, efforts were made to include the canonical "signal 2" of T cell activation by way of including intracellular costimulatory domains. Second-generation CARs include the addition of a single costimulatory domain, while third-generation CARs include two costimulatory domains in line with one another along a single receptor in an attempt to harness the benefits from multiple signaling pathways. Notably, second-generation CARs have showed improved cytokine secretion, CAR T cell proliferation, and overall anti-tumor efficacy as reviewed above.

Attempts at improving T cell function further with the advent of third-generation CARs has been mixed and met with challenges. In brief, more signaling is not always better, as studies found the third-generation CARs were more prone to extensive CAR signaling, which was detrimental to T cell functionality, akin to prior reports of tonic CAR signaling hampering CAR T cell performance [42, 53–57].

Lastly, it is important to briefly elucidate the stark differences in CAR signaling when contrasted to a TCR. CAR signaling is faster, albeit less organized, than TCR signaling. While the TCR IS can form in 5–10 min, the disorganized CAR IS forms in under 2 min and rapidly initiates proximal signaling cascades. The comparably quick formation of the CAR IS does not present as the well-structured TCR bull's-eye and typically has poor actin organization. This fast signaling also leads to rapid MTOC migration to the IS and accelerates the delivery of granules. The CAR T cells also detach from dying tumor cells faster, leading to what is referred to as "serial killer" cells which can move from target cell to target cell quickly lysing as they go [22, 24, 58, 59]. Importantly, they do require a higher target density to activate, and this threshold may be different to promote lysis of a target cell vs induction of cytokine production or T cell proliferation [60–62].

Taken together, CARs represent the functional transition of gene engineering to gene therapy. Altering any of the described domains can improve or hinder CAR function. These design elements can also be harnessed to our benefit, potentially helping mitigate toxicity or promoting T cell persistence. These elements will also highlight the need for adequate antigen density, a fact which should be abundantly clear by the end of this review.

IL13Rα2-directed CARs

One of the first types of CARs trialed in GBM patients targeted a modified interleukin 13 receptor alpha 2 (IL13Rα2); this monomeric high-affinity IL-13 receptor is a cancer-testis antigen overexpressed in more than 50% of GBM [63, 64]. The first-in-human trial of an IL13Rα2-CAR (IL13-CAR) was accomplished between 2008 and 2011: adults (18–70 years) who were steroid independent with recurrent/refractory unifocal supratentorial grade III or IV glioma whose tumors were amenable to resection were recruited [65]. Treatment included 12 escalating intra-resection cavitary doses of 10^7 to 10^8 IL13-CAR T cells via indwelling Rickham catheter. Overall, in the 3 of 13 patients enrolled for the trial who received treatment, only 2 developed mild grade 3 toxicity (headache), and another developed reversible grade 3 neurotoxicity at the highest cells dose of 10^8 IL13-CAR T cells [65]. For all 3 patients, there was MRI evidence of brain inflammation at the site of T cell infusion, as well as detection of IL13-CAR T cells at tumor sites removed at time of relapse. Notably, post-treatment tumor biopsies showed a significant loss of IL13Rα2 expression, paralleling the experience of CAR T cells in hematologic malignancies [65, 66]. In sum, although this was a small cohort of patients, the three patients treated had a mean survival of 11 months after relapse, with best survival of almost 14 months [65].

The CAR product here is unique when compared with modern or traditional CAR products. The largest difference lies in the binding interface these CARs use: in lieu of an scFv (single-chain variable fragment) as the immunologic binding interface between CAR and tumor, these cells express a membrane-tethered IL13 ligand on their surface. Specifically, this version of IL13 harbors a single-site mutation at E13Y to minimize binding of the CAR to the more readily expressed IL13Rα1/R4α complex on normal tissue [67]. Notably, these CARs also possess several transmembrane and intracellular signaling components not readily seen in recent CAR design [65, 68]. Similarly, their stimulation ex vivo utilized OKT3, an activating mAb targeting CD3,

which has largely transitioned to synthetic CD3/CD28 stimulatory beads. Not only was this process slow, it impacted transduction efficiency [65, 68].

Following this initial attempt at an IL13-CAR, the same group from City of Hope went on to modify their IL13-CAR through several changes before testing this improved version. First, they incorporated a 4-1BB (CD137, BBz) costimulatory domain to provide the canonical "second signal" in T cell activation and a mutated IgG4-Fc linker to reduce off-target Fc-receptor interactions [69]. Building off of prior evidence wherein central memory T cells may be more efficacious as CAR T cells, they enriched their product for central memory T cells [70]. Notably, no lymphodepleting chemotherapy was used in this or the original IL13-CAR study [65, 71]. This "IL13BBz"-CAR was then tested in a patient according to a compassionate use protocol in which he received 16 cycles of intracavitary (cycles 1 through 6) or intraventricular (cycles 7 through 16) infusions of IL13BBz-CAR T cells up to a maximum dose of 10×10^6 CAR T cells [71]. The patient was a 50-year-old man with multiple relapsed O6-methylguanine-DNA methyltransferase (MGMT)-unmethylated GBM and moderate IL13Rα2 staining on tumor (with no staining in 30% of cells, weak intensity staining in 30%) [71]. Infusions were not associated with grade 3 or higher toxicity. The change in route of delivery, from intracavitary to intraventricular, was made after the patient had displayed local control of his disease; however, his disease progressed at areas of non-resected tumor and with new spinal metastasis [71]. After the transition to intraventricular CAR T cell delivery with cycle 7, the patient proceeded to have disease response at all of his residual and metastatic sites through cycle 11 with a decrease in tumor size by 77% to 100% [71]. Through cycles 12–16, the patient went on to have a continued response, and tumors were not measurable by MRI nor detectable by PET. He returned to a normal life with a sustained clinical response for 7.5 months from time of CAR T cell initiation [71]. Unfortunately, he relapsed with disease after cycle 16 at four locations distinct from prior sites. These sites are believed to have decreased IL13Rα2 expression, harkening to what may be considered an increasingly common adaptive resistance mechanism to CAR T cell therapy.

The IL13-CARs have had further preclinical optimization: although these E13Y-mutated IL13-CARs are unique and preferentially bind IL13Rα2 on target tumor, they do have off-target binding to IL13Rα1. As such, to reduce the risk of future off-target, off-tumor toxicity, a dedicated scFv was identified that specifically binds IL13Rα2 without cross-targeting other IL13 receptors. This scFv (scFv47) was demonstrated to effectively target and eliminate IL13Rα2-expressing glioma in murine models with trials in human patients on the horizon [72].

Epithelial growth factor receptor variant III (EGFRvIII)

First described by Sugawa et al. across six primary human glioblastomas in 1990 [73], this gain-of-function mutation results from an in-frame genomic deletion of exons 2 to 7, leading to a ligand-independent receptor with constitutive activity and a novel immunogenic, tumor-specific epitope between amino acids 5 and 274 [74, 75]. EGFRvIII is the most common variant of this receptor observed in human tumors. In newly diagnosed GBMs, nearly 40% carry amplification of the EGFR gene, and of these EGFR-amplified GBMs, approximately 50% contain the oncogenic EGFRvIII [76]. As a result, targeted therapies using small molecular inhibitors, peptide vaccines, and more recently, CAR T cells against EGFRvIII have been developed [77–79].

The latter was evaluated in 10 heavily pretreated patients with recurrent unmethylated-MGMT GBM as part of a first-in-human phase I trial at the University of Pennsylvania [79]. In this study, a single IV dose of 1×10^8–5×10^8 EGFRvIII-CAR T cells were administered, and patients were assessed for response 28 days after CAR T cell infusion. Notably, the cohort's disease expressed a median of 71% EGFRvIII, and 20% of the patients were receiving a minimum of 4 mg dexamethasone or equivalent per day at time of T cell infusion [79]. Following infusion, there were seven grade 3 (neurologic/musculoskeletal) and two grade 4 (cerebral edema) events. Median OS was 251 days with one patient achieving stable disease (SD) for over 18 months [79, 80].

There has been much speculation as to why this trial showed a lack of efficacy; however, attention has been given to the adaptive immune resistance mechanisms in the tumor (e.g., immunosuppression), demonstrated by in situ detection of enhanced IDO1, PD-L1, and FoxP3 expression levels of post-treatment GBM biopsies [79, 81]. Loss of surface EGFRvIII expression was also noted in five of the seven patients from whom post-CAR T cell treatment biopsies were obtained [79], although no loss of wild-type EGFR amplification was noted following treatment. These findings parallel the post-treatment analysis from several clinical trials evaluating the EGFRvIII peptide vaccine, rindopepimut, suggesting that although rindopepimut induced a moderate-to-rapid EGFRvIII-specific antibody response in the majority of

patients, the loss of EGFRvIII expression was described in ~59% of GBM tumors post-treatment [81, 82]. Of particular relevance to the evaluation of EGFRvIII-directed CARs, this EGFRvIII antigen loss occurred independent of whether rindopepimut or control was administered, suggesting EGFRvIII loss was not due to antigen negative selection, but rather may be inherent to the natural evolution intrinsic to GBM progression [81].

Another potential explanation for the lack of efficacy may reside in the scFv selected. The scFv incorporated in the first-in-human anti-EGFRvIII CAR study described was derived from a humanized antibody (mAb2173) [80]. This humanized mAb originated from a murine analog (mAb3C10), established by immunization with a synthesized 14-amino-acid peptide (LEEKKGNYVVTDHC) corresponding to the fusion junction of EGFRvIII [83]. This amino acid sequence is the same sequence on which rindopepimut was based and may be subject to the same reduced efficacy if EGFRvIII expression is downregulated or lost [78, 81].

However, there is a second target epitope under investigation, one that shows promising efficacy in preclinical CAR models and is the target for an active CAR trial at the time of writing (Table 1) [87, 100]. The humanized antibody mAb806 targets the extracellular domain (ECD) II of EGFR between amino acids 287 and 302 [101, 102]. This epitope is downstream from the 2173/3C10 target and is inaccessible to the antibody during normal conformational states, which include both the "monomeric, unbound" and the "dimerized, ligand bound" form of the EGFR receptor [102, 103]. However, post-translational modifications reveal a disulfide bridge, which is sterically exposed when EGFR is either overexpressed or in the setting of several common EGFR mutations, including the canonical EGFRvIII in-frame deletion of exons 2 to 7 [102, 103]. The canonical EGFRvIII in-frame deletion, in particular, results in a conformational change in the ECD, which renders the epitope targeted by mAb806 accessible [103, 104]. The benefit to this epitope is that it targets the ubiquitous EGFRvIII and a transitional form found when EGFR is overexpressed [104], which occurs in up to 40% of patients with GBM [76]. Here, loss of one variant of EGFR alone would not result in resistance to targeted therapy; you would need either a loss of the mutated variant or downregulation of wild-type EGFR or loss of both.

HER2-directed CARs

The human epidermal growth factor receptor 2, often abbreviated as HER2/Neu, for its discovery by the Weinberg Lab in a rat neuroblastoma cell line [105], is an established oncogene and member of the HER family [106, 107], frequently amplified or overexpressed in upward of 30% of several solid tumors, as well as up to 80% of GBM specimens [107, 108].

The HER2/Neu receptor exists as a monomer on the cell surface and has no known direct activating ligand; instead, the HER2/Neu receptor is either homo- or heterodimerizes (e.g., with HER1 or HER3) or is found constitutively activated [109, 110].

Notably, HER2/Neu is a tumor-associated antigen (TAA); as such, although it is often upregulated on malignancies, it is not tumor specific and is on normal tissue, including epithelial cells of the gastrointestinal (GI), pulmonary, reproductive, and urinary tracts [111]. Given the relatively strong prevalence among tumors, in 2010, the first cell therapy targeting HER2/Neu was trialed at the Surgery Branch within the National Institutes of Health (NIH) in a patient with metastatic colon cancer utilizing an scFv derived from trastuzumab (Herceptin), named 4D5 [109, 112]. Unfortunately, following non-myeloablative conditioning, this third-generation CAR T cell product was peripherally administered and led to fatal pulmonary toxicity as the cells passed through the pulmonary tree, identifying HER2/Neu expressing pulmonary epithelium and inducing a profound inflammatory response with subsequent respiratory and circulatory collapse [112].

With the tragedy of this first-in-human attempt in mind, a separate group out of Baylor College piloted an alternative HER2-CAR based on an scFv initially described by the labs of Drs. Bernd Groner and Nancy Hynes [113]. Briefly, the HER2-specific murine scFv FRP5 was cloned into a second-generation CAR with a CD28.ζ signaling domain [114–116]. This HER2-FRP5 CAR T product was tested in a recent clinical trial of 19 patients with HER2-positive sarcomas. There were no dose-limiting toxicities (DLTs), and the treatment was well tolerated. Notably, the intravenous (IV) cell doses ranged between 1×10^4 and $1 \times 10^8/m^2$ cells following a dose-escalation model. Of the 17 evaluable patients, four had SD for 3 months post-treatment, and three patients remained in remission at 6, 12, and 16 months at time of publication in 2015, with a reported median OS of 10.3 months [117].

When compared with the single-patient experience at the NIH, this latter study did not use a lymphodepleting

Table 1. Actively recruiting or recently closed CAR T cell trials for primary malignant brain tumors

Target	Combination and/or conditioning	Dosing method	Study	Reference
IL13Rα2	Ipilimumab, nivolumab	Intra-cavitary, intraventricular	NCT04003649	[84]
	None	Intra-tumor, intra-cavitary, intraventricular,	NCT02208362	[85]
	± Anti-PDL1 antibody	N/A	NCT03423992	[86]
EGFRvIII	None	N/A	NCT03618381	[87]
	Pembrolizumab	Intravenous	NCT03726515	[88]
	± Anti-PDL1 antibody	N/A	NCT03423992	[86]
HER2	None	Intra-tumoral, intraventricular	NCT03389230	[89]
	None	Intra-cranial	NCT02442297	[90]
	None	Intra-cavitary, intraventricular	NCT03500991	[91]
	None	Intra-cranial	NCT03383978	[92]
	± Anti-PDL1 antibody	N/A	NCT03423992	[86]
B7-H3	None	Intra-cavitary, intraventricular	NCT04185038	[93]
	Temozolomide	Intra-tumoral, intraventricular	NCT04077866	[94]
	± Anti-PDL1 antibody	N/A	NCT03423992	[86]
CD70	Cyclophosphamide, fludarabine, aldesleukin (high-dose)	Intravenous	NCT02830724	[95]
CD133	± Anti-PDL1 antibody	N/A	NCT03423992	[86]
EphA2	± Anti-PDL1 antibody	N/A	NCT03423992	[86]
	None	Intravenous	NCT02575261 (Completed, not reported)	[96]
CD147	None	Intracavitary	NCT04045847 (Completed, not reported)	[97]
Chlorotoxin	None	Intra-tumoral, intraventricular	NCT04214392	[98]
PDL1	Cyclophosphamide, fludarabine	Intravenous	NCT02937844 (Status unknown)	[99]

chemotherapy before CAR T infusion; they also gave a 2-log lower maximum dose of cells, utilized modern ex vivo T cell stimulation with anti-CD3/CD28 beads (contrasted with the use of OKT3 mAb), and used a second-generation CD28-CAR [112, 117]. Structurally, this latter CAR recognizes a discontinuous epitope within residues 11–169 of the mature human HER2 protein, which faces away from the cell surface, whereas the HER2-4D5 CAR binds to the juxtamembrane region of HER2 within residues 529–627 of the ECD [118, 119]. The CAR field has recognized that the effective activation of CAR T cells can depend on the location or affinity of the binding epitope [120]. In fact, this was investigated with the HER2-4D5 CARs, where the use of a lower-affinity scFv resulted in equivocal anti-tumor activity with reduced CAR T cell activation against HER2-expressing normal tissue [121]. The baseline affinities of 4D5 and FRP5 scFvs are 0.15 nmol/L and 6.5 nmol/L, respectively, and in light of this difference in scFv affinity, one can speculate the HER2-FRP5 CARs may be less likely to develop on-target, off-tissue toxicity when presented with moderate levels of HER2 [109, 113].

Lastly, this HER2-FRP5 CAR has been subsequently evaluated in patients with GBM. One major change in

this second HER2-FRP5 CAR study is in the T cells selected for CAR transduction. Preclinical studies with this CAR showed challenges in maintaining proliferative capacity and long-term activity in vivo; similarly, in the initial phase 1 trial of this CAR, only four of thirteen evaluable patients had detectable CAR in their peripheral blood at 3 months post-infusion [117]. In order to augment CAR T cell persistence, the group from Baylor opted to utilize T cells specific for antigens associated with chronic viral infection (e.g., Epstein-Barr virus, EBV, or cytomegalovirus, CMV) as the effector cells for CAR manufacturing. Based on prior evidence, using these viral-specific cytotoxic T cells will not only recognize their tumor target through their transgenic CAR but will inherently recognize viral epitopes through their native receptors and may survive longer in vivo than T cells without virus specificity due to the intermittent antigen stimulation [122]. These cells were used in a phase 1 dose-escalation study conducted at Baylor College of Medicine, Houston Methodist Hospital, and Texas Children's Hospital, in which 17 patients with progressive HER2-positive GBM were enrolled to receive 1 or more IV infusions of autologous virus-specific HER2-FRP5 CAR T cells (1×10^6/m2–1×10^8/m2) without prior lymphodepletion [123]. Similar to the initial HER2-FRP5 CAR T cell trial in patients with HER2-positive solid tumors, the cell infusion was well tolerated without DLT. At time of publication in 2017, of the 17 evaluable patients, 1 had a partial response (PR) for more than 9 months, 7 had SD for 8 weeks to 29 months, and 8 progressed after CAR infusion [123]. The virus-specific HER2-FRP5 CAR T cells were detected in the peripheral blood for up to 12 months post-infusion in two of six evaluable patients, compared with zero out of five evaluable patients in the initial HER2-FRP5 CAR T cell trial. Median OS was 11.1 months from the first CAR infusion and 24.5 months from diagnosis [123]. Further trials are planned to identify the optimal cell dose, route of administration, and need for lymphodepletion.

Novel CAR designs

The future is burgeoning with new CAR T cell designs; herein, we summarize a select array of novel targets and CAR constructs against PMBTs.

EphA2 Erythropoietin-producing hepatocellular carcinoma A2 (EphA2) receptor is a tyrosine kinase overexpressed on GBM and promotes a GBM cell migration [124]. EphA2 is an attractive target for the immunotherapy of GBM as it has limited-to-no expression on normal tissue [124]. EphA2-specific second-generation CAR T cells using an EphA2-specific scFv (4H5) carrying a CD28-ζ endodomain effectively eliminated EphA2-positive glioma cells and glioma-initiating cells in vitro and in an orthotopic murine severe combined immunodeficiency (SCID) model of GBM, suggesting this as a potential target for future cell therapy [125].

B7-H3 (CD276) B7-H3, a type I transmembrane protein, is encoded by chromosome 15 in humans and is expressed with one of two extracellular isoforms, 4IgB7-H3 and 2IgB7-H3 [126]. B7-H3 is broadly overexpressed by multiple tumor types, making B7-H3 an attractive target not only for PMBTs but a variety of other primary malignancies as well [126, 127]. Notably, B7-H3 is highly expressed on pediatric solid tumors, including sarcomas, medulloblastoma, and in diffuse intrinsic pontine glioma [127–129]. B7-H3 CAR T cells have been evaluated in several solid tumor preclinical models, including pancreatic, ovarian, and various pediatric cancers [126, 128].

In GBM, one group in China designed third-generation B7-H3-CAR T cells composed of a B7-H3-specific scFv [126]. This CAR effectively controlled disease in vivo in a orthotropic patient-derived xenograft (PDX) model of GBM [126]. Another group, in a collaboration across multiple sites in the USA, generated an array of scFvs targeting B7-H3 largely by way of a yeast display library; from this, several second-generation 4-1BB-CARs were tested against cell lines expressing B7-H3. The CAR, CD276.MG-4-1BB-z (B7-H3 CAR), which produced the largest quantity of cytokines in response to tumor challenge, was then evaluated in murine models of medulloblastoma, osteosarcoma, Ewing sarcoma, and leukemia [128]. The B7-H3 CAR was effective in eliminating disease in a majority of these in vivo models; however, they found the results were not as striking with a erythromyeloid leukemia cell line discovered to have comparably less B7-H3 surface density. On review of other cell lines, as well as genetically modified tumor lines expressing different amounts of B7-H3, there appeared to be a correlation with site density and B7-H3 CAR efficacy, suggesting this B7-H3 CAR is largely dependent on high surface expression of target antigens, and simply, the reduction in target density is sufficient to blunt response to CAR T cell therapy [128].

CSPG4 Chondroitin sulfate proteoglycan-4 (CSPG4), also known as high molecular weight melanoma–associated antigen (HMW), is a well-characterized type I transmembrane cell surface proteoglycan first identified on human melanoma cells and is naturally expressed on normal human syncytiotrophoblast cells in the developing placenta [130]. CSPG4 interacts with α4β1 integrins to directly modulate cell adhesion, motility, and, in malignancy, metastasis [130]. Subsequent studies described high expression of CSPG4 on adult and pediatric solid tumors, as well as a variety of PMBTs [130–133]. Notably, CSPG4 has a restricted distribution in normal tissues, with its only other major expression noted on developing pericytes—potentially conferring the ability to not only target a tumor but its supporting neovasculature—and can largely be considered a placenta/TAA [130, 132].

Several groups have evaluated CSPG4 as a target for antibody or immunotherapy in vitro, as well as across in vivo murine and canine models [131–134]. More recently, there has been interest in assessing CSPG4 as a target for CAR T cell therapy, particularly in GBM, where CSPG4 is expressed in upward of 70% of GBM specimens, as well as on tumor-associated vessels and GBM neurospheres with little intra-tumor heterogeneity [132, 134]. In a study from Baylor and the University of North Carolina, a CSPG4-directed CAR (CSPG4-CAR) was constructed from an scFv (mAb763.74) out of the lab of Dr. Soldano Ferrone [134]. This scFv was placed into both second- and third-generation CAR constructs [134]. The second-generation 4-1BB CAR generated the largest cytokine response in vitro and was selected for in vivo testing using a murine model of GBM [134]. Here, the group found the CSPG4-CAR eliminated GBM in their murine model and did not result in loss of target antigen following therapy. In fact, they discovered the inflammatory response of the activated CSPG4-CAR T cells interacting with local microglia-generated substantial amounts of tumor necrosis factor alpha (TNFα) which, in turn, resulted in upregulation of CSPG4 expression on tumor cells [134]. This discovery, when taken into context of tumor escape from CAR T cell therapies by means of antigen loss or downregulation, suggests CSPG4 may be an ideal target for future cell therapy in GBM and other malignancies.

CD70 CD70 (Cluster of differentiation (CD)70) is a type II transmembrane protein and member of the TNF receptor family. CD70 is the only ligand for CD27, a glycosylated transmembrane protein in the TNF receptor family, and their interactions play a key role in providing costimulation to developing lymphocytes [135]. In this way, CD70 expression is highly restricted to activated T/B cells and a small percentage of mature dendritic cells—notably, expression has also been described on a variety of hematologic and solid tumors, including GBM—where expression of CD70 was associated with poor survival and an increase in M2 macrophage infiltration, suggesting CD70 may play a role in glioma progression [135, 136].

One of the initial CD70-specific CARs was a first-generation CAR described circa 2011 at Baylor and consisted of the full-length CD27 as the antigen-recognition domain [137]. This chimeric CD27-CAR engaged tumor targets expressing the CD70 ligand and resulted in CD70-specific lysis of CD70-positive tumor cell lines as well as CD70-positive tumor in a murine SCID xenograft model [137].

Another group at the NIH tested seven second- or third-generation anti-CD70 CARs with binding moieties from the human CD27 [138]. The CD27 fused with 4-1BB and CD3-ζ showed the highest degree of in vitro functionality and could cure NSG mice bearing CD70-positive human tumors [135, 138]. This group, in collaboration with the University of Florida and several groups in China, went on to evaluate this CD70-CAR in gliomas. They found glioma-associated CD70 expression was predominantly overexpressed in two subgroups of patients, isocitrate dehydrogenase (IDH) wild-type low-grade gliomas and mesenchymal GBMs [135, 138]. They went on to find CD70-CAR was able to generate potent anti-tumor response against CD70-positive gliomas in xenograft and syngeneic animal models [135, 138].

Based in part on these results, at the time of writing, there is a phase I/II clinical trial underway using a second-generation, anti-human CD70/4-1BB/CD3-ζ CAR in CD70-expressing malignancies [139].

CD133 CD133 is a pentaspan transmembrane glycoprotein that is overexpressed in various solid tumors, including over 50% of upper GI malignancies, as well as up to 60% of GBMs [140].

Although a CD133 CAR has not been tested in human subjects with GBM, there is experience with a CD133-CAR in China [141]. An initial case report came out in 2017 describing the use of a CD133-CAR T cell

therapy in a 52-year-old female with refractory, metastatic cholangiocarcinoma. The second-generation 4-1BB CAR utilizes a CD133-specific scFv (mAbHW350341.1) [141]. The patient received two initial infusions of an EGFR-CAR T cell product, reaching a PR for 8.5 months. Unfortunately, she progressed and went on to receive the CD133-CAR T cells, which maintained her disease for 2 months before she developed progressive disease. Notably, in the 10 days after CD133-CAR infusion, she developed grade 3 skin toxicity, including severe dermatologic, mucosal, and GI toxicity described as a confluent rash and sporadic pinpoint hemorrhages. Her serum cytokines showed rapid elevations of TNF-α, IL-6, and CRP, for which she was given etanercept as well as methylprednisolone—the combination of which reversed her symptoms [141].

This CD133-CAR was subsequently tested in a larger trial of 23 patients with CD133-positive relapsed GI malignancies [140]. Patients in this trial who had non-HCC diagnosis were given lymphodepleting chemotherapy prior to CAR T cell infusion. Following an initial dose-escalation scheme, which saw no DLTs, the accepted CART-133 cell dose was 0.5-to-2 × 10^6/kg and was administered in 15 patients. At the time of publication in 2018, the duration of responses in all patients ranged from 9 to 63 weeks, with three patients achieving a PR and 14 patients with SD for 9 weeks to 15.7 months [140]. Seven of the 23 patients experienced a grade III toxicity (hyperbilirubinemia, leukopenia, anemia, nausea), with one grade IV event (leukopenia) [140].

Based on the experience of the CD133-CAR in human subjects with GI malignancies, it appears this may be a reasonable CAR to test in patients with CD133-positive PMBTs.

NKG2DL Natural killer group 2, member D (NKG2D) receptor is a type II lectin-like transmembrane protein expressed by innate and adaptive immune cells, including natural killer (NK) cells, CD8+ T cells, and invariant NKT cells [142, 143]. The NKG2D ligands (NKG2DLs) are highly expressed in GBM, and groups are evaluating whether this may be a target for CAR therapy [144].

In a subcutaneous xenograft murine model of GBM, a second-generation 4-1BB CAR containing the ECD of NKG2D (NKG2D-BBz CAR) was tested in vitro, where effector cells efficiently lysed glioblastoma cells and efficiently eliminated xenograft tumors in vivo without significant treatment-related toxicity [144]. Of note, NKG2DL overexpression has been reported in glioblastoma stem cells, suggesting the NKG2D-BBz CAR may also be an effective means to target the elusive glioblastoma stem cell population [144].

Novel therapeutic strategies

Multi-targeted CARs One approach to overcome the therapeutic barriers of interpatient variability, tumor heterogenicity, and antigen modulation is in the development of multi-targeted CARs. Several groups have started to investigate this multi-targeted approach; one collaborative investigation in Texas has yielded a single tricistronic transgene capable of encoding each respective CAR co-targeting HER2, IL13Rα2, and EphA2 [145]. This CAR utilized the IL13Rα2-binding IL-13 mutein, HER2-FRP5 scFv, and EphA2-specific scFv 4H5, as previously reviewed in this paper. Expression of each CAR was evaluated on the T cell product (UCAR) from patients with GBM and subsequently tested in vitro against the patients' respective autologous tumors, revealing the UCAR product displayed enhanced anti-glioma activity when compared with CAR products expressing only one or two of the CAR transgenes [145]. The UCAR products were then tested in an orthotopic PDX murine model. In this model, the UCAR T cells mediated significantly larger anti-tumor effects compared with single- or double-expressing CAR products. Furthermore, in mice with recurrent tumors, the tumors retained expression of the three antigens, however, at markedly lower levels than pretreatment [145]. Again, these findings suggest simply the presence of antigen is not sufficient to elicit a CAR response, and adequate target density is necessary.

CARs with endogenous cytokine support Another approach with mounting interest is to incorporate the transgenic expression of cytokines essential for T cell activation or homeostasis into CAR vectors. In doing so, one may maintain T cell persistence, potentially heightening a cellular response to lower levels of target antigen.

In one preclinical study, second-generation CD28-CAR T cells comprised of the IL13Rα2-targeting scFv47; the transgenic expression of IL-15 improved T cell persistence and anti-glioma activity [146]. However, the gliomas that recurred had downregulation of IL13Rα2 expression, highlighting an all-too-familiar pattern of CAR T cell resistance despite the improvement in CAR T cell survival.

Conclusion/summary

Across oncology, we have seen a revolution in cancer treatments, none of which, to date, carry more potential for durable cures than immunotherapy. Here, we attempted to survey the variety of CARs trialed in human subjects with PMBTs and highlighted both their respective novelties and pitfalls. A solution to some of these hurdles may be found in the work already accomplished and reviewed here.

We know from the studies targeting HER2/Neu that the route of administration as well as scFv-binding affinity needs to be considered, particularly for TAA with normal tissue expression. The trials targeting IL13Rα2 offer some insight; here, we have evidence that intracranial/intraventricular delivery is feasible and may serve as the optimal approach for PMBTs as we target not only a resected tumor bed but any distal CNS metastasis. IL13Rα2-targeted therapies also highlight the incremental improvement in clinical outcomes seen with the use of newer technologies, e.g., CD3/CD28 beads for ex vivo T cell activation. As this space develops, our approach to CAR T cell therapy may also change in ways unforeseen to us now. Importantly, from the work targeting EGFRvIII and IL13Rα2, we acknowledge antigen loss or downregulation on target tumors is not an isolated resistance mechanism seen with CD19- or CD22-directed CAR T cells, and targeting a single antigen may insufficient to achieve long term responses. Fortunately, there is a silver lining, as the simple evidence of antigen loss or antigen downregulation following GBM-directed cell therapies suggest these therapies are mediating anti-tumor activity.

Distilling what we know, one could reason future CARs for PMBTs may need multiple co-targets and to include endogenous cytokine support to maintain not only a local cellular response but CAR T cell persistence. We should encourage a continued push with regard to the development of novel ex vivo production techniques, minimizing the time between lymphocyte collection and CAR T cell administration. Finally, we should engage with molecular biophysicists to confer on the optimal binding site and avidity with which to facilitate maximum on-target CAR cytotoxicity, while minimizing off-target effect. In short, we have a substantial amount to learn from the CAR experience in PMBTs already accomplished and should use this knowledge as we design the next generation of cellular therapies.

Compliance with Ethical Standards

Conflict of Interest

Nicholas Tschernia declares no potential conflicts of interest relevant to this article were reported.
Simon Khagi declares no potential conflicts of interest relevant to this article were reported.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

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