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REVIEW

CAR T cell therapy for pediatric central nervous system tumors: a review of the literature and current North American trials

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Abstract
Central nervous system (CNS) tumors are the leading cause of cancer-related death in children. Typical therapy for CNS tumors in children involves a combination of surgery, radiation, and chemotherapy. While upfront therapy is effective for many high-grade tumors, therapy at the time of relapse remains limited. Furthermore, for diffuse intrinsic pontine glioma (DIPG) and diffuse midline glioma (DMG), there are currently no curative therapies. Chimeric antigen receptor T (CAR T) cell therapy is a promising novel treatment avenue for these tumors. Here, we review the preclinical evidence for CAR T cell use in pediatric brain tumors, the preliminary clinical experience of CNS CAR T cell trials, toxicity associated with systemic and locoregional CAR T cell therapy for CNS tumors, challenges in disease response evaluation with CAR T cell therapy, and the knowledge gained from correlative biologic studies from these trials in the pediatric and young adult population.

Keywords Chimeric antigen receptor · Central nervous system · Pediatric · Cancer · Immunotherapy

1 Introduction

Central nervous system (CNS) tumors are the leading cause of cancer-related death in children [1]. Traditionally, patients have received a range of multimodal therapy including surgery, radiation, and chemotherapy. More recently, molecularly targeted agents have been introduced for a subset of patients with CNS tumors harboring actionable molecular aberrancies; however, these are relatively rare cohorts of tumors and efficacy of such strategies remains variable [2, 3]. Unfortunately, the outcome for many children and young adults with CNS tumors remains dismal, and novel curative options are needed desperately.

Diffuse midline glioma, H3K27-altered (DMG), is a highly infiltrative CNS tumor that is universally fatal and confers a median survival of approximately 1 year [4]. DMG most often localize in the brainstem, thalamus, and spinal cord. Loss of H3K27me3 is the defining feature, most often in the setting of a mutation in H3K27M. Outcomes are largely unchanged since the introduction of focal radiation. Hundreds of clinical trials, including traditional chemotherapy and targeted agents, have been unsuccessful in obtaining a durable cure.

Embryonal tumors (e.g., atypical teratoid rhabdoid tumor (ATRT), embryonal tumor with multilayer rosettes (ETMR), medulloblastoma, and pineoblastoma) account for approximately 450 new diagnoses each year in the United States. Medulloblastoma is the most common subtype and is classified with an integrated histologic and molecular diagnosis (e.g., WNT, Sonic Hedgehog (SHH), Group 3, and Group 4). While the overall survival (OS) rate for patients with medulloblastoma is approximately 80% in patients classified as standard risk and 60% in those classified as high risk, each subgroup harbors distinct clinical demographics, genetics, and outcomes [5–7]. Despite the frequency

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of cure with upfront therapies, there is a paucity of effective treatment strategies for relapsed disease, which has a 2-year OS of 17% and a 5-year OS of 12% [8]. Combination chemotherapy has shown an improvement in outcomes with a median OS of 19 months in the most recent Children's Oncology Group trial [9]. Similarly, upfront therapies may be effective for other embryonal tumors, but the OS is poor following relapse [10].

Ependymoma is a molecularly heterogeneous disease with potentially as many as ten distinct molecular subtypes. The majority of supratentorial ependymoma harbor a ZFTA-RELA fusion, while most posterior fossa tumors are in the PF-A subgroup with 1q gain/6q loss indicating high risk for relapse [11–13]. While the 5-year OS is 80%, ependymoma is a relentless disease that often relapses late, and there is only a 55% event free survival (EFS) 10 years post-diagnosis [14]. Similar to CNS embryonal tumors, therapeutic options for relapsed disease are limited. In pediatric patients with ependymoma, a recurrence leaves patients with an average OS duration of 36.9 months [15].

With the consistent and profound efficacy of cellular therapy against hematologic malignancies such as pediatric leukemia [16], investigators internationally are working to bring the success of genetically modified cellular therapies to children with CNS tumors. Despite being linked by a fixed anatomical space, CNS tumors span multiple biological classes with distinct anatomical, clinical, molecular, and microenvironmental characteristics that may influence the efficacy of cellular therapy. Here, we review the preclinical success of chimeric antigen receptor (CAR) T cells, the preliminary clinical experience of CNS CAR T cell trials in the United States, and the knowledge gained from correlative biologic studies from these trials.

2 Preclinical evidence

There has been a rapid increase in preclinical evidence to support the development of early phase CAR T cell trials. The dynamic nature of CNS tumors can lead to intratumoral and intertumoral heterogeneity [17], reflecting the complex interplay of genetic [18], epigenetic [19], cellular, and spatial factors [20] that shape immunosuppressive or immune inert tumor microenvironments (TME). These factors may differentiate tumors even within conventional biologic subgroups, affecting clinical outcomes and optimal therapeutic targeting. The intricate diversity within CNS tumors has so far highlighted multiple mechanisms of resistance for CAR T cell therapies, such as antigen loss [21, 22], cell exhaustion derived from immune checkpoint pathway inhibition [23], and ineffective cell trafficking within the TME [24, 25], making most high-grade CNS tumors unresponsive. To reduce immunosurveillance failure, the attention of researchers has moved to the investigation of different CNS tumor targets, aiming to define versatile combinatorial strategies targeting multiple antigens. In addition, preclinical models have supported the use of locoregional CAR T cell delivery to reduce risk of systemic adverse effects such as cytokine release syndrome (CRS), while improving CAR T cell trafficking and tumor infiltration [26–29].

Given the heterogeneity of high-grade CNS tumors, multiple tumor antigens and their expression have been explored across several tumor types. One of the earliest surface antigen targets identified for pediatric CNS tumors is human epidermal growth factor receptor 2 (HER2/ErbB2) [30]. HER2 is expressed on a wide range of CNS malignancies, with limited expression on normal brain tissue [31, 32]. Multiple publications from independent groups have demonstrated robust preclinical anti-tumor effect *in vitro* and *in vivo* by HER2-specific CAR T cells against a variety of CNS tumors, namely diffuse intrinsic pontine glioma (DIPG), ependymoma, glioblastoma (GBM), and medulloblastoma [30, 33–35]. In more recent studies, patient-derived cerebellar xenograft models of ependymoma also showed significant survival benefit, alone or in combination with azacytidine, following locoregional CAR T cell injection into lateral ventricle of NSG mice [29]. The efficacy of HER2 CAR T cells was also demonstrated against intracranial DIPG xenograft tumors with intravenously administered HER2 CAR T cells inducing impressive regression in tumor burden and extended survival [36]. This group also evaluated the feasibility of delivering CAR T cells into the ventricles of non-human primates (NHPs) with existing reservoirs [34] and observed an increase in interleukin-6 (IL-6) and interleukin-2 (IL-2) in the cerebrospinal fluid (CSF), indicating host immune system activation and recognition of the target by HER2 CAR T cells. Importantly, no severe systemic or local toxicity was observed.

Ephrin type-A receptor 2 (EphA2), a pro-oncogene, is another attractive target that is overexpressed on GBM [37, 38]. EphA2 is involved in cell proliferation, migration, angiogenesis, and invasion [38, 39]. Preclinical evaluation of EphA2-specific CAR T cells has demonstrated tumor regression in multiple orthotopic xenograft mouse models [37, 40, 41]. Early work showcased the potent killing capability of EphA2-CAR T cells against glioma neurospheres *in vitro* [37]. Interestingly, while EphA2 CAR T cells delivered intracranially in a murine model resulted in a complete response, similar efficacy was not observed when EphA2 CAR T cells were administered intravenously. Similar results were shown in medulloblastoma models by a second group where EphA2 CAR T cells orthotopically injected into the lateral ventricle of NSG mice extended survival benefit up to 200 days post-treatment, and repeated intracranial administration of anti-EphA2 CAR T cells demonstrated increased mouse survival up to 500 days [29]. A different

EphA2 single-chain variable fragment (scFv) incorporated into second- and third-generation CAR constructs using CD28 4-1BB co-stimulatory domains alone or in combination demonstrated anti-tumor efficacy in orthotopic glioma mouse models [41]. Notably, administrating lower doses of EphA2 CAR T cells exhibited diminished killing capacity *in vivo*, highlighting dose and location of administration impact efficacy of CAR T cells.

B7-H3, another promising therapeutic target for pediatric and young adult tumors, exhibits high levels of detection in CNS malignancies including ATRT [28], DIPG [42], gliomas [43], and medulloblastoma [44]. In one study, profiling 49 patient-derived gliomas revealed B7-H3 expression in 95.9% samples [26]. In preclinical testing against medulloblastoma and patient-derived DIPG models, B7-H3-specific CAR T cells demonstrated specific production of high levels of IFNγ, TNFα, and IL-2 [44], effectively eliminating neurospheres *in vitro* [45]. Upon intravenous injection into NSG mice, B7-H3 CAR T cells were able to cross the blood–brain barrier and clear tumors from the posterior fossa, significantly extending survival [44]. These findings were corroborated in orthotopic medulloblastoma models, with tumor regression and significant survival benefits following intravenous injection of B7-H3 CAR T cells 28 days post-tumor cell inoculation. Tumor regression and significant survival benefit were observed also against orthotopic glioma models, when B7-H3 CAR T cells were intratumorally delivered 7 days after tumor implantation [26]. Moreover, the activity of B7H3 CAR T cells was highlighted in GBM models, in which CAR T cells injected into the lateral cerebral ventricle of NSG mice weekly for 2 weeks cleared tumors from the frontal cortex [46]. Similar studies confirmed tumor eradication from the caudate nucleus, following intratumor delivery of B7-H3 CAR T cells 7 days post-tumor cell implantation [45]. Given the high expression across a broad range of CNS tumors without expression on normal brain tissue and the above noted preclinical activity, multiple groups have taken B7-H3 into clinical testing [26, 44, 47].

Epidermal growth factor receptor variant III (EGFRvIII) is derived from a tumor-specific mutation that causes a deletion on the extracellular domain of EGFR, creating a neoepitope on the tumor cells harboring this mutation [48]. This neoepitope tumor specificity makes EGFRvIII an attractive target for cellular therapeutics. Preclinically, EGFRvIII-specific CAR T cells against orthotopic GBM models have shown promising efficacy via either systemic or locoregional injection [49–52]. However, early clinical experience with an EGFRvIII-specific CAR T cell therapy for adult GBM patients indicates that tumor antigenic escape may limit clinical efficacy [21]. Therefore, an augmented CAR design was developed to comprise the antigen binding domain derived from the monoclonal antibody 806, which targets not only EGFRvIII but also an exposed epitope on the overexpressed wild-type EGFR on tumor cells [53, 54]. In addition to demonstrating the efficacy of EGFR806 CAR T cells through intravenous or intracranial delivery against orthotopic GBM models, preclinical studies found low on-target off-tumor toxicity of EGFR806 CAR T cells against tissues expressing normal level of wild-type EGFR (e.g., human astrocytes) [53, 54]. Since EGFR overexpression and EGFRvIII are also detected among pediatric CNS tumors including ependymoma, high-grade glioma (HGG), and medulloblastoma [55–57], EGFR806 CAR T cells have been developed for pediatric clinical studies.

While the development of GD2-specific immunotherapy has primarily focused on neuroblastoma, GD2 is also a prevalent target on pediatric CNS tumors, including ATRT, ETMR, ependymoma, HGG, and medulloblastoma [26, 58]. Mount et al. identified GD2 overexpression on DMG and developed GD2 CAR T cells that have potent efficacy against multiple orthotopic xenograft models with patient-derived DMG when dosed intravenously [58]. Notably, a fraction of the treated mice succumbed to hydrocephalus from peritumoral neuroinflammation during the acute phase of anti-tumor activity, although whether such toxicity was due to on-target off-tumor activity by the GD2-CAR T cells themselves remains undefined [58]. CAR T cells with enhanced affinity to GD2 have been described to exert lethal neurotoxicity in a non-CNS neuroblastoma mouse model because of low amounts of GD2 expression in the mouse CNS [59]. As noted below, this preclinical experience with significant on-target off-tumor toxicity has not yet been observed in the clinical setting with neuroblastoma patients [60], and GD2 remains a potential target for pediatric CNS tumors.

IL-13 receptor α2 (IL-13Rα2) is frequently overexpressed on pediatric CNS tumors but not significantly expressed on normal brain tissues [26, 61–63]. To target IL-13Rα2, one group both developed and optimized a CAR T cell therapy that uses a membrane-tethered IL-13 ligand as the antigen binding domain, which is mutated at a single site (E13Y) to preferentially bind to IL-13Rα2 while reducing the binding affinity to the more widely expressed IL-13Rα1 [64, 65]. With this IL-13Rα2-specific CAR design, termed IL-13-zetakine CAR, intracranially administered CAR T cells were effective in mice with orthotopic GBM tumors [64, 65]. Similar to models mentioned above, the authors compared different delivery routes for the CAR T cells in their orthotopic GBM models and demonstrated that locoregional delivery had superior anti-tumor efficacy compared to intravenous administration. Furthermore, intracerebroventricular injection of CAR T cells provided greater anti-tumor activity compared to intratumoral injection in a multifocal tumor model [65]. These findings, along with the clinical experience in treating adult GBM [22, 66], have informed the development of IL-13-zetakine CAR T cell therapies for pediatric CNS tumor applications.

As intratumor antigen heterogeneity is one of the major barriers to successful CAR T cell treatment for solid tumors, including those in the CNS [21, 67, 68], different strategies to simultaneously target multiple tumor antigens have been developed to prevent tumor antigenic escape. For example, a single CAR molecule with two antigen binding domains in tandem has been developed to target HER2 and IL-13Rα2 [69] or EGFRvIII and IL-13Rα2 [70]; tricistronic delivery of three CAR constructs with different specificities has been used to generate trivalent CAR T cells targeting HER2, IL-13Rα2, and EphA2 simultaneously [29, 71]; and these approaches all showed promising efficacy in antigen-heterogeneous CNS tumor preclinical models via locoregional delivery of the CAR T cells [29, 69–71]. Another strategy is based on the "IF/THEN logic gate" to control the specificity of CAR T cells [72], in which a synNotch receptor is applied to bind to a heterogeneous but tumor-specific antigen, such as EGFRvIII, and locally induce the expression of a CAR that is specific to homogeneously expressed antigens. This strategy allows CAR T cells to be locally activated in the tumors while being able to target homogeneous antigens that may not be tumor-exclusive [72]. Moreover, targeting multiple antigens can also be achieved by simpler approaches such as combining CAR T cell products with different antigen-specificities or transducing T cells with a mixture of multiple viral vectors that encode CAR constructs of various specificities [73], which deserve exploration against preclinical models of pediatric CNS tumors.

Although the abovementioned preclinical models provide rationale for anti-tumor activity and tolerability in murine/mammalian models, existing preclinical models poorly recapitulate tumor microenvironment heterogeneity and intrapatient immunological differences. Furthermore, the immune privilege and tumor heterogeneity prevalent in patients may influence clinical efficacy. Given promising preclinical data targeting these antigens, early phase clinical trials are underway to test these constructs in human subjects with pediatric brain tumors.

3 Pediatric trials

3.1 Overview of published and ongoing trials

There has been a relatively rapid translation of preclinical CAR T cell therapy findings into early phase clinical studies in children and adults conducted through multiple academic centers in the United States (Table 1). Current trials have adopted variable approaches, with differences centering mainly on route of delivery, administration of lymphodepleting chemotherapy, and CAR T cell dosing strategy and schedule. While preliminary experience has centered on feasibility and safety, the development of these pipelines has laid a groundwork that will support years of critical advancements [31, 44, 47].

Five clinical studies exploring the safety and efficacy of intravenous administration of CAR T cells targeting

Table 1 North American phase 1 pediatric clinical trial evaluations CAR T cell products

Trial (NCT)	Target	Admin	Dose range	Lympho-depletion (Y/N)	Multi-dose (Y/N)	Toxicity	Status
03500991	HER2	Locoregional	$1–10\times10^7$	N	Y	Headache, transient worsening of neurodeficits	Active, not recruiting
03638167	EGFR	Locoregional	$1–10\times10^7$	N	Y	Not published	Active, not recruiting
04185038	B7-H3	Locoregional	$1–10\times10^7$	N	Y	Headache, N/V, fever	Recruiting
04099797	GD2	IV and Locoregional	$5\times10^6–510^7$/dose ICV, 15 million cells/dose IV	Y	Y	Not published	Recruiting
05768880	B7-H3, EGFR, HER2, IL-13 Rα2	Locoregional	$1–10\times10^7$	N	Y	Not published	Recruiting
04903080	HER2	IV	$8\times10^7/m^2$	Y	N	Not published	Active, not recruiting
05835687	B7-H3	Locoregional	$1\times10^7–1\times10^8$	N	Y	Not published	Recruiting
04196413	GD2	Locoregional	$1\times10^6–100\times10^6$/kg	N/Y	Y	Increased ICP, TIAN	Recruiting
04510051	IL-13Rα2	Locoregional	unknown	Y	Y	Not published	Recruiting

Abbreviations: *N* no, *NCT* National Clinical Trial, *N/V* nausea/vomiting, *IV* intravenous, *ICV* intracerebroventricular, *TIAN* tumor inflammation-associated neurotoxicity, *Y* yes

EGFRvIII, EphA2, HER2, or GD2 have been published to date that include pediatric patients [21, 47, 74–76]. Three of these trials (EGFRvIII, EphA2) predominantly enrolled young adult patients with glioblastoma (GBM). The HER2-targeted CAR T cell trial locoregional delivery with weekly dosing for 3 weeks of 4 weeks cycle with maximum dose level of 10×10^7 cells initially enrolled patients 10–69 years of age, while the GD2-targeted CAR T cell study (NCT 04099797: intravenous (IV) or locoregional (LR) delivery once in 28-day cycles with maximum dose level of 3×10^6 IV and 10×10^7 cells LR) enrolled pediatric patients with DMG.

Additional ongoing trials are evaluating locoregional (or intracranial) delivery of CAR T cells in pediatric patients with CNS tumors with CAR-targeted antigens including IL-13Rα2 (NCT 04510051), HER2 (NCT 03500991), EGFR (NCT 03638167), GD2 (NCT 04196413), and B7-H3 (NCT 04196413 and NCT 05835687) and Quad CAR T product targeting IL-13Rα2, HER2, EGFR, and B7H3 (NCT 05768880) [22, 31, 77]. Locoregional CAR T cell delivery weekly and/or every other week for patients with DIPG to maximum dose of 10×10^7 cells has been tolerable with report of radiographic stability for greater than 12 months [78]. In these studies, patients receive single or multiple infusions of CAR T cells, 1 to 4 weeks apart.

3.2 Systemic and neurologic toxicity

The most common side effects reported in the current pediatric and adolescent trials include headaches, nausea/vomiting, and fever. To date, no dose limiting toxicities have been reported [22, 31]. The toxicity profile for those patients with brain tumors appears to be distinct from that described in CAR T cell trials for hematologic malignancies. Specifically, CRS and immune effector cell–associated neurotoxicity syndrome (iCANS) are two systemic toxicities associated with CAR T therapy. Both are well described and have been observed in patients treated for liquid and solid tumors [79–81]. CRS typically presents with a constellation of symptoms that result from immune cell activation and include persistent fevers and hypotension that ultimately requires vasopressor support and may be associated with hypoxia and multi-organ dysfunction. CRS has been responsive to therapies that neutralize IL-6, such as tocilizumab and corticosteroids. Emerging data suggest improvement of CRS with IL-1 neutralization as well [79]. CAR T cell therapy has also been associated with iCANS, which typically includes severe neurologic symptoms including encephalopathy, tremor, aphasia, dysgraphia, apraxia, and seizures, which may progress to cerebral edema in severe cases [81]. The pathophysiology of iCANS is less well understood than that of CRS, and the effectiveness of specific therapies, beyond supportive care and corticosteroids, has not been clearly demonstrated.

Notably, CRS and iCANS appear to be very rare events following locoregional CAR T cell therapy for pediatric patients with CNS tumors. CRS was noted in the first four patients with DIPG or spinal cord DMG treated with GD2 CAR T cells [47]. Within the analysis of these first four patients published, cytokines implicated in CRS, including IL-6, were higher in the plasma following intravenous when compared to locoregional administration of CAR T cell therapy. Within the first three pediatric patients with DIPG treated with locoregional B7-H3 CAR T therapy, CRS and iCANS were not seen; rather, a constellation of neurologic changes including fever, nausea and vomiting, and headache were observed that were distinct from iCANS [78]. Ultimately, the varied experience of neurological sequelae seen by different groups may be due to biological complexity, route of delivery, and off-target effects due to varying antigens.

In contrast to iCANS, patients with CNS tumors receiving locoregional delivery of CAR T therapy develop neurological symptoms that are primarily related to a tumor associated locoregional inflammatory response. This can manifest as a range of neurologic symptoms from worsening of baseline neurological deficits and headaches, to confusion, seizures, and, in severe cases, life-threatening cerebral edema. This entity recently has been described as tumor inflammation-associated neurotoxicity (TIAN), a unique syndrome in patients with CNS tumors treated with immunotherapy [47, 82]. The exact mechanisms of TIAN are still not fully understood, though exciting efforts are ongoing to better understand how electrophysiological signaling of tumor and surrounding CNS tissue may be contributing [40, 82]. Location of a CNS tumor is thought to contribute to the risk of TIAN and the associated structural consequences of local neuroinflammation. As well, the severity of TIAN is thought to be related to the CAR T antigen target, the use of lymphodepleting chemotherapy, and the magnitude of the inflammatory response.

3.3 Correlative analyses to further understand *CAR* T cell activity

Correlative studies in CAR T trials for CNS tumors have employed cerebrospinal fluid (CSF) analysis to gain insights into the therapeutic efficacy and safety of this innovative treatment approach. These studies provide key data to understand these products as CAR T expansion (peak numbers) and CAR T area under the curve (AUC, duration of exposure) are both related to toxicity and activity in prior CAR T therapeutic strategies previously evaluated for non-CNS oncologic diseases [15]. For example, within CD19-specific CAR T therapy, both CAR T cell persistence and expansion

were key factors in both toxicity and response [15]. The current methodologies for studying these aspects of novel CAR T therapies provide opportunities to define exposure of therapy and dosing interval, duration of exposure for disease and temporal nature, and risk of therapy-associated toxicity. Studies of the CSF ongoing as correlative studies within the current trials include the measurement of cytokine levels, assessment of immune cell populations, CAR T cell persistence, and circulating tumor (ct) DNA. These analyses play a crucial role in advancing our understanding of the unique challenges and opportunities presented by CAR T treatment for brain tumors, ultimately contributing to the refinement and optimization of this therapeutic strategy.

3.4 *CAR* T cell detection

The expansion and persistence of CAR T cell following administration to patients with hematologic malignancies is associated with anti-tumor activity [83]. The most common methods of CAR T cell detection following systemic or intraventricular administration include the use of flow cytometry or quantitative polymerase chain reaction (qPCR) techniques, each with its own advantages and limitations. Flow cytometry relies on the binding of fluorescently labeled antibodies to specific surface markers on CAR T cells. When CAR T cells are genetically modified to express unique antigens, this method provides high specificity and sensitivity for detecting and quantifying CAR T cells in a patient's blood or tissue samples [75]. Moreover, the ability to simultaneously analyze multiple antigens expression allows for characterization of the CAR T cell phenotype and of non-CAR T cell populations within the specimen. For example, within the first three patients treated with B7-H3 CAR T cells, the Seattle group showed via flow cytometry on serial CSF biospecimen that circulating CAR T cells in the CSF were detectable post-infusion, but not in peripheral blood [78]. However, the reliability of flow cytometry can be influenced by factors such as sample quality, antibody selection, inter-operator variability, and quantity of cells in the analytic specimen.

Quantitative polymerase chain reaction (qPCR) is performed through amplification of CAR T cell–specific DNA sequences to determine their abundance. qPCR offers high precision and sensitivity, making it suitable for quantifying CAR T cells in clinical samples, even those with relatively low cell numbers [84]. However, qPCR's reliability can be affected by the potential for contamination during sample preparation and the need for rigorous standardization of assay conditions. Furthermore, current techniques assess bulk nucleic acid and are therefore not able to differentiate single cell phenotypes or discern variation in cell populations. CAR detection via qPCR has been important in highlighting preclinical suspicion that locoregional delivery of CAR T cell therapy into the CSF is key for patients with pediatric CNS tumors, due to the protection of the blood–brain barrier. Interestingly, in a study of three patients who received locoregional CAR T cells, CAR T cell DNA via qPCR in the peripheral blood was not detected in any patient at any time point tested, suggesting that locoregional delivery does not lead to systemic circulation of CAR T cells, which may potentially limit systemic toxicity [78].

Within the first four patients with DIPG or spinal cord DMG treated with GD2-CAR T cells published, GD2-CAR T cell expansion and persistence was monitored using *GD2-CAR* transgene qPCR and flow cytometry of cell-surface CAR expression. Flow cytometry–based GD2 CAR T cell detection in blood was limited; however, GD2 CAR T cells were detected in CSF by flow cytometry, particularly following locoregional administration [47].

Antigen loss in malignant cells (i.e., loss of CD19 or CD22) can be monitored through various techniques including flow cytometry of circulating leukemia cells within the blood or bone marrow [85]. However, this is more cumbersome for CNS tumors where surgical approach through biopsy or resection is required to assess for antigen loss, thereby limiting opportunities for such assessment.

The reliability of CAR T cell detection methods depends on the specific goals of the analysis and the careful consideration of their limitations. In clinical practice, combining multiple complementary approaches, such as flow cytometry and cytokine analysis, can enhance the overall reliability of CAR T cell detection and monitoring.

3.5 Cytokine analytics

Cytokine analyses play a crucial role in monitoring patients undergoing CAR T cell therapy, as they provide valuable insights into the immune response and potential side effects associated with this innovative treatment. CAR T cells are engineered to target specific tumor antigens, and their activation within the patient's body can lead to the release of various cytokines, including proinflammatory ones like IL-6, interferon-gamma (IFNγ), and tumor necrosis factor-alpha (TNFα). Current standard incorporates approximately 50 cytokines, which are measured in blood or CSF before and after CAR T treatment for patients as part of most ongoing trials [58]. Enzyme-linked immunosorbent assay (ELISA) is a widely used technique for this purpose [86, 87]. While indirect methods are valuable for assessing CAR T cell function, they may not provide a direct quantification of CAR T cell numbers and can be influenced by patient-specific factors affecting cytokine levels.

One group described the use of CAR T cells targeting the EGFRvIII mutation in adult patients with glioblastoma. The authors demonstrated variations in cytokine levels including IL-6 and IFNγ through systemic CAR T therapy, with more

prominent elevations seen following the first dose administration of CAR T cells [22]. Additionally, in a large cohort of 65 adult patients with recurrence of either high-grade glioma or glioblastoma, elevations of inflammatory cytokines such as IFNγ, CXCL9, and CXCL10 were seen. These patients were treated with locoregional delivery of IL-13-zetakine CAR T cells, and stable disease or better was achieved in 50% of participants [88].

Within pediatric phase 1 trials, cytokine analysis has become a mainstay of correlative studies, both within CSF and blood, and has aided our understanding in the role of the blood–brain barrier in CAR T delivery and immune activation. BrainChild-01 (NCT 03500991) is an ongoing phase 1 clinical trial at Seattle Children's evaluating repetitive locoregional dosing of HER2-specific CAR T cells to children and young adults with recurrent/refractory CNS tumors. Within initial patient analysis, local CNS immune activation was demonstrated including high concentrations of CXCL10 and CCL2 within the CSF [31]. Within CSF, CCL2 and CXCL10 were the most elevated cytokines within a ~ 50-cytokine panel, whereas serum cytokine levels remained stable through CAR T cell infusions.

Similarly, in the first three patients analyzed from an ongoing trial of locoregional delivery of B7-H3 CAR T cells (NCT 04185038), CSF and serum cytokines were analyzed at several time points. After the initial CAR T infusion, patients had detectable levels of the following analytes in the CSF: CCL2, CXCL10, G-CSF, GM-CSF, IFNα2, IFNγ, IL10, IL12p40, IL12p70, IL15, IL1α, IL3, IL-6, IL7, TNFα, and VEGF [35]. Compared with pre-infusion, patients demonstrated elevations in multiple chemokines and cytokines, including CCL2, CXCL10, GM-CSF, IFNγ, IL15, IL1α, IL-6, and TNFα. Similar to the HER2 targeting trial, the most commonly elevated cytokines in CSF were CCL2 and CXCL10, and there was no fluctuation in cytokine level within serum. While the Stanford group demonstrated changes in cytokines through serial CSF analyses following systemic administration of GD2-targeted CAR T cell therapy, cytokine changes were variable and there was a lack of consistency in this data [47]. These findings have led to an ongoing suspicion that immune activation is specific to the blood–brain barrier and likely locoregional delivery into the CSF is important for treating tumors arising in the CNS.

3.6 Proteomic analytics

Proteomic analysis has emerged as a valuable tool in CAR T cell trials for pediatric CNS tumors, providing insights into the molecular mechanisms underlying treatment response and identifying potential biomarkers for improved patient outcomes. Proteomic analysis takes advantage of the sensitivity of liquid chromatography/mass spectrometry (LC–MS/MS) [89, 90]. At the Fred Hutch Cancer Center, the Paulovich Lab has created analyte panels reflective of the immune state, including immune lineage markers (e.g., CD14, CD45) tested upon both tumor tissue and blood plasma [91]. The current, available panels leverage immuno-multiple reaction monitoring mass spectrometry (immuno-MRM-MS), in which a multiplexed panel of validated antibodies is used to enrich a digested protein lysate for peptides of interest. MRM-MS assays require very little analyte, making them potentially suited to clinical applications in which there are limitations in sample quantity (e.g., needle biopsies or CSF samples). There are many reasons to believe that the CSF, which is proteomically restricted [92] and more proximate to CNS tumors, would be very amenable to this type of analysis as well. An additional advantage is the ability to assay peptides with post-translational modifications representing protein activation states. The assays have demonstrated remarkable reproducibility, sensitivity, and dynamic range, making them an exciting addition to correlative studies within ongoing CAR T trials [93].

Using MRM-MS on serial CSF and serum biospecimens within the BrainChild-03 (NCT04185038) initial analysis, proteins above the lower limit of quantification were detected in 50 and 59 CSF and serum specimens, respectively [78]. In two patients with DIPG, several CSF analytes including markers of macrophage maturation and proteins involved in immune cell recruitment such as CD14, CD163, CD44, CSF-1, CXCL13, and VCAM-1 tracked consistently through therapy. In general, there were fewer protein fluctuations in the serum compared with the CSF, supporting the observation described above for cytokine analyses that inflammatory activity is increased locally in the CNS compared with the systemic circulation following intracranial CAR T cell infusions, likely related to the impact of the blood–brain barrier [78].

By examining the protein expression profiles of CAR T cells, tumor cells, and the tumor microenvironment, we may gain a comprehensive understanding of the complex interactions and signaling pathways involved in CAR T therapy. Proteomic analysis may also allow for the identification of specific proteins associated with treatment success, resistance, or adverse effects, helping to refine CAR T cell therapies for pediatric CNS tumors.

3.7 Evaluating response

While feasibility, tolerability, and safety are becoming increasingly documented, defining overall efficacy and better understanding of which patients may benefit from this therapy remains elusive. The clinical trials to date (Table 1) have demonstrated preliminary tolerability of a range of CAR T cell products, although clinical responses have been observed in only a subset of patients, including in pediatric patients with diffuse midline glioma [47]. Notably, three of

four patients with DMG demonstrated clinical and radiographic improvement when GD2 CAR T cell delivery was transitioned from intravenous infusions to locoregional delivery [47]. While these transient responses highlight the potential benefit of CAR T cell therapy, accurate assessment of response for patients with CNS tumors undergoing immunotherapy remains challenging. As such, novel neuroradiology definitions and cfDNA analyses are currently being evaluated as novel measures of response in patients undergoing CAR T therapy.

3.8 Neuro-radiology

MRI (magnetic resonance imaging) is the standard diagnostic imaging modality for disease assessment for CNS tumors, although there are limitations in assessing treatment response and guiding clinical decisions in the context of immunotherapy [94]. One significant limitation is its inability to distinguish between true tumor progression and pseudoprogression [95], defined as the temporary increase in tumor size that occurs secondary to many tumor directed therapies, including immunotherapy. Within CAR T trials, this ambiguity in radiographic interpretation can lead to challenges in accurately evaluating treatment response and determining the appropriate course of action [95].

Another limitation of MRI is the lack of specific imaging biomarkers that can reliably predict treatment response to immunotherapy in CNS tumors. Unlike non-CNS tumors where biomarkers such as changes in tumor size or metabolic activity can be assessed through functional imaging modalities like positron emission tomography (PET) [96], CNS tumors often exhibit atypical response patterns to immunotherapy that may not be adequately captured by conventional MRI techniques [97]. In response to these limitations, there has been concerted effort from the Response Assessment in Pediatric Neuro-Oncology (RAPNO) and the Response Assessment for Neuro-Oncology (RANO) committees to address this with specific neuro-radiology criteria and recommendations for immunotherapy trials [98]. The immunotherapy Response Assessment for Neuro-Oncology (iRANO) criteria were developed to provide standardized guidelines for evaluating treatment response in CNS tumors undergoing immunotherapy. iRANO incorporates clinical and radiological features to differentiate between true progression and pseudoprogression, allowing clinicians to make more informed decisions regarding treatment continuation or modification [99].

The iRANO criteria utilize a combination of imaging findings, including changes in contrast enhancement patterns, T2/FLAIR hyperintensity, and clinical parameters such as neurologic deterioration and steroid use, to classify treatment response into four categories: complete response, partial response, stable disease, and progressive disease. These criteria aim to improve the accuracy of response assessment and reduce the likelihood of prematurely discontinuation of tumor directed therapy and are now standard within CAR T cell trials in pediatric brain tumors.

3.9 Cell-free DNA

Cell-free DNA (cfDNA) analysis in cerebrospinal fluid (CSF) is gaining prominence as a non-invasive method for monitoring CAR T cell trials in pediatric brain tumors. This approach allows the detection and molecular analysis of microscopic DNA released by tumor cells and may also be a means to detect CAR T cells in the CSF [100]. The analysis of cfDNA can provide valuable insights into the dynamics of the disease, assess therapeutic response, and potentially identify the emergence of resistance or relapse. In the context of pediatric brain tumors, where neurosurgical procedures to obtain tissue carry additional risks, cfDNA analysis in CSF offers a promising avenue for both diagnosis and for real-time monitoring of treatment effectiveness.

4 Future directions

CAR T cell therapy has had an important impact on clinical outcomes in pediatric leukemia with thus far limited long-term adverse effects. While this treatment strategy for pediatric patients with CNS tumors remains in its infancy, early data raises the potential that it will be equally paradigm shifting. There is a vast amount of information to be leveraged from preclinical modeling and ongoing clinical trials and associated correlative studies. Correlative analyses from clinical trials aim to enhance understanding of determinants of therapy-related toxicity, CAR T cell persistence through mitigation of T cell exhaustion and complementary immune activation pathways required to maximize CAR T cell effector function, and CAR T cell anti-tumor activity. Next-generation CAR T products are also being evaluated, in the hopes that this strategy may enhance response. For example, one current CAR T product targeting EGFRvIII while also secreting T cell engaging antibody molecules (TEAMs) demonstrated a dramatic preliminary radiographic response in three adult patients with recurrent glioblastoma within days of infusion, albeit only durable in one of three patients [76].

Key next steps in the field must include a better understanding of the TME and its optimal modulation before, during, and after CAR T cell therapy, as well as recruitment of endogenous immune cells to enhance anti-tumor activity. This work will include the study of CAR T cells with enhanced effector function through improved cytotoxic activity or anti-tumor payloads, improved migration and tumor chemotaxis, and determining the best combinatorial

partners and their multimodal sequencing. Importantly, CAR T cell efforts for children and young adults with all cancers must remain committed to improving access and equity around the development and especially the implementation and conduct of these trials. While many aspects of these therapies remain unknown, it is abundantly clear that many patients are in desperate need of new therapeutic strategies and that CAR T cell therapy is a promising frontier demanding further exploration.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Competing interests Dr. Vitanza is chair of the Scientific Board for Brain Child Bio. Otherwise, the authors have no financial or nonfinancial interests that are directly or indirectly related to the work submitted for publication.

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