Clinical & Translational Immunology

Australian and New Zealand Society for Immunology Inc.

Clinical & Translational Immunology 2019; e1050. doi: 10.1002/cti2.1050
www.wileyonlinelibrary.com/journal/cti

SPECIAL FEATURE REVIEW

Clinical chimeric antigen receptor-T cell therapy: a new and promising treatment modality for glioblastoma

Michael P Brown[1,2,3], Lisa M Ebert[1,a] & Tessa Gargett[1,a]

[1]Translational Oncology Laboratory, Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, SA, Australia
[2]Cancer Clinical Trials Unit, Royal Adelaide Hospital, Adelaide, SA, Australia
[3]School of Medicine, University of Adelaide, Adelaide, SA, Australia

Correspondence
MP Brown, Translational Oncology Laboratory, Centre for Cancer Biology, University of South Australia and SA Pathology, North Tce, Adelaide, SA 5001, Australia.
E-mail: michaelp.brown@sa.gov.au

[a]Co-senior authors.

Received 30 January 2019;
Revised 15 April 2019;
Accepted 22 April 2019

doi: 10.1002/cti2.1050

Clinical & Translational Immunology
2019; **8**: e1050

Abstract

Chimeric antigen receptor (CAR)-T cell therapy is now approved in the United States and Europe as a standard treatment for relapsed/refractory B-cell malignancies. It has also been approved recently by the Therapeutic Goods Administration in Australia and may soon be publicly reimbursed. This advance has accentuated scientific, clinical and commercial interest in adapting this exciting technology for the treatment of solid cancers where it is widely recognised that the challenges of overcoming a hostile tumor microenvironment are most acute. Indeed, CAR-T cell technology may be of the greatest value for those cancers that lack pre-existing immunity because they are immunologically 'cold', or have a low somatic tumor mutation load, or both. These cancers are generally not amenable to therapeutic immune checkpoint blockade, but CAR-T cell therapy may be effective because it provides an abundant supply of autologous tumor-specific T cells. This is achieved by using genetic engineering to re-direct autologous T-cell cytotoxicity towards a tumor-associated antigen, bypassing endogenous T-cell requirements for antigen processing, MHC-dependent antigen presentation and co-stimulation. One of the most challenging solid cancers is glioblastoma, which has among the least permissive immunological milieu of any cancer, and which is almost always fatal. Here, we argue that CAR-T cell technology may counter some glioblastoma defences and provide a beachhead for furthering our eventual therapeutic aims of restoring effective antitumor immunity. Although clinical investigation of CAR-T cell therapy for glioblastoma is at an early stage, we discuss three recently published studies, which feature significant differences in target antigen, CAR-T cell phenotype, route of administration and tumor response. We discuss the lessons, which may be learned from these studies and which may guide further progress in the field.

Keywords: chimeric antigen receptor, glioblastoma, neurotoxicity, T cells

INTRODUCTION

In both children and adults, glioblastoma is an aggressive primary tumor of the brain that has an almost uniformly fatal outcome. Glioblastoma has been defined as a rare cancer because its incidence is < 6 in 100 000. However, its organ site and dismal prognosis result in a mortality and morbidity burden that is disproportionate to its incidence. In adults, standard multi-modality treatment using maximal safe resection, radiotherapy and chemotherapy extends patient survival beyond a year, but recurrence is virtually inevitable, with short survival times and no effective therapies.

Immune checkpoint inhibitor therapy, which has transformed the care and outlook for so many cancer patients, has proven to be a disappointment for most glioblastoma patients. Glioblastoma tumor cells are hosted by a stroma replete with immunosuppressive myeloid and lymphoid cells but depleted in immunocompetent antigen-presenting cells and lymphocytes.[1] Glioblastoma tumor cells also usually lack the rich repertoire of nonsynonymous somatic gene mutations that generate the tumor neo-epitopes capable of eliciting productive if transient antitumor immunity.[2]

This dire clinical situation supports the rationale for an adoptive immunotherapeutic approach to glioblastoma therapy using CAR-T cells. In this approach, autologous T cells are collected from a patient's blood, expanded in vitro and genetically engineered to express a CAR molecule on the cell membrane using a viral expression vector or electroporation.[3] The CAR consists of an extracellular tumor antigen-binding domain [usually derived from a single-chain variable fragment (scFv) of a monoclonal antibody] fused via a transmembrane linker to intracellular T-cell signalling domains, which activate the cell upon CAR engagement. The signalling domains always include CD3ζ, and if no other signalling domains are present, then this is considered a first-generation CAR. The addition of one co-stimulatory signalling domain, commonly CD28 or 4-1BB, creates a second-generation CAR, and multiple co-stimulatory domains create a third-generation CAR.

Upon infusion of the engineered cells back into the patient, CAR-T cells home to the tumor and mediate targeted destruction of cancer cells. Of note, this approach confers several cell-autonomous functions on the T cells, enabling them to function effectively in a tumor microenvironment, which lacks many key elements required for conventional T-cell recognition and destruction of tumors. Hence, the expression of CARs can bypass such requirements for conventional T-cell activation as tumor cell expression of MHC molecules, functional antigen processing machinery and co-stimulatory molecules.

Recently, rapid developments in the CAR-T cell field have culminated in several published reports of clinical CAR-T cell therapy for patients with poor-prognosis recurrent glioblastoma, with mixed but informative results. We review these early-phase clinical studies and discuss their findings and the implications for the future development of clinical CAR-T cell therapy for this dreadful, challenging disease.

GLIOBLASTOMA: THE DETAILS

Incidence, pathology and classification

Glioblastoma is the commonest, most aggressive brain cancer. It accounted for almost two-thirds of all Australian brain cancers in 2013 with 982 (of 1592) new cases with an estimated age-standardised incidence rate of 4 per 100 000.[4] In the world each year, there are 100 000 new cases of glioblastoma.[5] Despite its rarity, it is a leading cause of cancer burden with 96% of the burden resulting from premature death. Strikingly, the 5-year relative survival (just 4.6% at 5 years) for glioblastoma patients has remained stable over the last three decades.[4]

Gliomas, including the most advanced form, glioblastoma, were believed to arise from the glial cells supporting neurons in the central nervous system (CNS). However, recent evidence indicates that glioblastoma arises instead from neural stem cells within the subventricular zone of the brain.[6] The histological grading of gliomas is categorised according to World Health Organization (WHO) guidelines. The highest grade (grade IV) of glioma is glioblastoma, and the diagnosis depends on observations of high cellularity, atypical glial cells, and microvascular proliferation (MVP), or significant necrosis or both.[7] MVP reflects extensive neo-angiogenesis with highly abnormal, leaky and hyperdilated vessels, which are found around and oriented towards necrosis. Necrosis reflects chronic tissue hypoxia and is another strong predictor of aggressiveness. Indeed, glioblastoma was historically known as glioblastoma multiforme in recognition of

its particularly high inter- and intra-tumoral histological heterogeneity.[8] Although the molecular classification of glioblastoma informs prognosis, it has not yet provided definitive evidence to influence the use of immunotherapy. Finally, surprisingly for an aggressive cancer, glioblastoma does not metastasise outside of the CNS.[7]

Standard treatment and prognosis

There is a clear unmet clinical need for effective therapies at all stages of glioblastoma natural history. Current standard treatment for newly diagnosed glioblastoma patients with good performance status and aged ≤ 70 years employs the Stupp protocol – surgery with 'maximal safe resection' followed by concurrent chemo-radiotherapy with temozolomide (TMZ) and then maintenance TMZ chemotherapy for the next 6 months.[9] This treatment regimen results in a median survival time of 14.6 months.[9] Older patients can still obtain a survival benefit with similar treatment.[10] In two studies, additional survival benefit was gained by patients whose tumors had methylation or silencing of the promoter for the O^6-methylguanine-DNA methyltransferase (*MGMT*) gene, which encodes a DNA damage repair enzyme.[10,11] For patients with multifocal disease, median overall survival (OS) is significantly shorter at 6 months.[12]

That glioblastoma almost invariably recurs illustrates its treatment-resistant nature, and its infiltrative growth pattern leads to most recurrences occurring near the surgical margin within 6–9 months of initial treatment. There is no standard management for recurrent glioblastoma. Attempts to improve outcomes by adding bevacizumab and other agents have not extended survival significantly, so 6-month progression-free survival (6PFS) for recurrent disease remains at ~15%, and OS is generally < 6 months.[13]

Immune Checkpoint Inhibitor (ICI) therapy

Combination ICI therapy (using ipilimumab and nivolumab) has had a revolutionary impact in melanoma and kidney cancer patients, with tumor response rates of at least 40%, which translate to substantially enhanced survival prospects.[14,15] The same combination therapy provides comparable beneficial effects in patients with melanoma brain metastases,[16,17] yet ICI therapy has had little beneficial clinical impact in glioblastoma patients. For example, no survival difference was observed in the Checkmate-143 randomised trial of nivolumab versus bevacizumab in recurrent glioblastoma patients.[18] In other trial arms exploring combinations of nivolumab with ipilimumab, the response rate was a disappointing 7%.[19] In three recently reported studies of neoadjuvant anti-PD1 therapy in recurrent glioblastoma patients, although clinical responses were lacking, evidence on surgical resection specimens of persistent T-cell inflammation and pro-inflammatory cytokine profiles tended to favor an improved prognosis and may suggest a rationale for ICI use in conjunction with CAR-T cell therapy.[20–22]

Although acute ICI sensitivity of tumors with a high mutation burden, because of mismatch repair deficiency has been described,[23,24] recent data suggest that only 3.5% of primary or recurrent glioblastoma patients had a high somatic tumor mutation burden and so may benefit from ICI therapy.[2] Notwithstanding promising case reports,[25–28] formal testing of the sensitivity of this group of patients to ICI therapy is awaited. Therefore, in the bulk of glioblastoma patients with a tumor mutation burden below that typically associated with response to ICI therapy, a strong rationale exists for alternative immunotherapeutic approaches such as CAR-T cell therapy.[3]

CHALLENGES FOR SUCCESSFUL IMMUNOTHERAPY IN GLIOBLASTOMA

Glioblastoma creates a hostile tumor microenvironment and induces systemic immune suppression

Glioblastoma is characterised by a local immunosuppressive environment in both the myeloid and T-cell compartments. There is a striking predominance of $CD11b^+$ myeloid cells, which outnumber all other immune cell types, including T cells.[29] The $CD11b^+$ myeloid cells include brain-resident microglia, myeloid-derived suppressor cells (MDSC) and, to a lesser extent, infiltrating macrophages.[30] These myeloid populations have many immunosuppressive features, including production of IL-10 and TGF-β[30,31] and high-level expression of the T-cell checkpoint molecule PDL1.[31] PDL1 is also broadly expressed in patient glioblastoma tissue.[32] Glioblastoma contains substantial numbers of $FoxP3^+$ regulatory T cells,[33] and intra-tumoral $CD8^+$ T cells have a profoundly

exhausted phenotype characterised by the expression of LAG3, TIGIT, CD39 and especially PD1.[34] Finally, T cells are frequently observed to cluster around blood vessels,[32,35] suggesting either active exclusion or a lack of signals directing them into the tumor.

Glioblastoma can also induce a state of systemic immune suppression. For example, compared to healthy controls, glioblastoma patients have an increased frequency of circulating MDSC,[30] higher levels of serum IL-10[36] and elevated PDL1 expression on blood monocytes.[31] In addition, circulating T cells are impaired in both number and function.[37] The reduction in circulating T-cell frequency has recently been shown to result from sequestration of naïve T cells in bone marrow, because of loss of the surface receptor for sphingosine-1-phosphate (S1P), which is required for bone marrow egress.[38] Interestingly, in a pre-clinical mouse model, restoring surface expression of S1P on T cells greatly improved ICI responses.[38]

Opportunities and limitations for priming of tumor-specific T cells in glioblastoma immunotherapy

The CNS has long been considered an immune-privileged site and, until very recently, was thought to lack the lymphatic vessels that could transport antigen from the CNS into the periphery for T-cell priming in lymph nodes. In 2015, however, functional lymphatic vessels were reported to line the dural sinuses of mice, with potentially analogous structures also observed in human dura.[39] These vessels can transport immune cells from the cerebrospinal fluid (CSF) to the deep cervical lymph nodes, thus supporting an emerging concept that the brain is subject to active immune surveillance and is not immunologically separate from the periphery. Also of note, classical dendritic cells have been identified within glioblastoma tumor tissue[29] and could transport tumor antigen to draining lymph nodes for T-cell priming. Indeed, despite multiple local and systemic immuno-suppressive mechanisms, growing evidence indicates that glioblastoma patients can prime tumor-specific T-cell responses.[40,41]

CLINICAL TRIALS OF CAR-T CELL THERAPY FOR BRAIN CANCERS

Published clinical experience of CAR T-cell therapy for glioblastoma has been limited to the following antigenic targets: IL-13Rα2, erbB2/HER2 and epidermal growth factor receptor deletion mutant variant III (EGFRvIII). These early-phase trials provide clinical proof-of-concept for the safety and utility of CAR-T cell therapy for brain cancer. Details of the patients, CAR-T cell therapeutic products and results are summarised in Tables 1–3, respectively.

Table 1. Patient characteristics

Trial	Patient number	Age (years)	Prior treatment	Disease stage at treatment	Antigen expression in tumor
Brown *et al.*[42]	13 enrolled; 10 products manufactured; 3 treated	Mean 50 (range 36–57)	3/3 Pts, 1L: Surgery, RT, TMZ; 1/3 Pts, post-study and pre-biopsy: RT, BCNU, bevacizumab	High-grade (WHO grade III or IV) recurrent glioblastoma	Pt #1, Low Pt #2, High Pt #3, Intermediate
Brown *et al.*[44]	1	50	1L: Surgery, RT, TMZ; 2L: other investigational therapy	Multifocal recurrent glioblastoma	Intermediate (H Score 100)
O'Rourke *et al.*[49]	10	Median, 59.5 (range 45–76)	10/10 Pts, 1L: Surgery, RT, TMZ; 8/10 Pts, 2L or 3L including bevacizumab, chemotherapy (CCNU and/or carboplatin and/or BCNU), or dendritic cell vaccine	Recurrent glioblastoma; 9/10 Pts, multifocal; 1/10 Pt, deep, unresectable	Median = 71% (range 6–96%)
Ahmed *et al.*[50]	17 treated; 16 evaluated	10 Pts, median 60 (range 30–69); 7 Pts, median 14 (range 10–17)	14/17 Pts, 1L: Surgery, RT, TMZ; 3/17 Pts, 1L: Surgery, RT; 10/17 Pts, 2L-5L salvage therapies; 6/17 Pts, other investigational therapies	Recurrent or progressive glioblastoma	6/17 Pts 1–25% 9/17 Pts 26–50% 1/17 Pts 51–75% 1/17 Pts 76–100%

1L, first-line; 2L, second-line; 3L, third-line; 5L, fifth-line; Pt, patient; RT, radiotherapy; chemotherapy drugs: TMZ, temozolomide; carmustine (bis-chloroethylnitrosourea, BCNU); lomustine (chloroethyl cyclohexyl nitrosourea, CCNU).

Table 2. CAR-T cell characteristics

Trial	Target antigen	Route of administration	CAR-T dose	CAR expression	CAR-T cell phenotype	CAR-T persistence
Brown *et al.*[42]	IL-13Rα2	Intracavitary with Rickham reservoir/ catheter	12 infusions; 1st dose = 10^7 CAR-T cells; 2nd dose 5×10^7 CAR-T cells; doses #3–12 = 10^8 CAR-T cells;	100% after hygromycin selection	Uniformly $CD3^+CD8^+$ $CD45RO^+$ $CD69^+CD95^+$; $CD45RA^-$, $CD62L^-$, $CCR7^-$	Low levels of intra-tumoral T cells detected at w14 after final T-cell infusion in 1/1 tissue samples analysed
Brown *et al.*[44]	IL-13Rα2	Intracavitary with Rickham reservoir/ catheter; intraventricular via catheter to left ventricle	Intracavitary 1st dose = 2×10^6 CAR-T cells, subsequent 5 doses = 10^7 CAR-T cells; intraventricular 1st dose = $2x10^6$ CAR-T cells, subsequent 9 doses = CAR-T 10^7cells	64–81% as detected by truncated CD19 staining	74–90% $CD4^+$T cells; 53–57% central memory T cells ($CD45RO^+$ $CD62L^+$)	CAR-T cells detected in CSF after each intraventricular administration for up to 7 d
O'Rourke *et al.*[49]	EGFRvIII	Intravenous	1.75–5 $\times 10^8$ CAR-T cells	Median 19.7% (range 4.8–25.6%)	NR	CAR-T cells detected in peripheral blood of all Pts up to d14, with loss of detection by d30; CAR-T cells detected in tumor in 5/7 evaluated Pts
Ahmed *et al.*[50]	HER2	Intravenous	5 dose cohorts: $1 \times 10^6/m^2$; $3 \times 10^6/m^2$; $1 \times 10^7/m^2$; $3 \times 10^7/m^2$; $1 \times 10^8/m^2$ T cells	Mean 39% (range 18–67%)	$CD3^+CD8^+$ T cells (mean 71%; range 16–97%); $CD3^+/CD4^+$ T cells (mean 24%; range 0.3–88%)	CAR-T cells detected in peripheral blood with peak levels between 3 hours and 2 weeks; 7/15 Pts had detectable CAR-T cells at 6 w, and 2/6 Pts had detectable CAR-T cells at 12 m; tumor infiltrating CAR-T cells not evaluated

CSF, cerebrospinal fluid; d, day(s); h, hours; m, months; NR; not reported; Pt, patient; w, week(s).

Table 3. Adverse events and response to treatment

Trial	Adverse events: ≥ grade 3	Objective tumor response	Progression-free survival	Overall survival post-treatment
Brown *et al.*[42]	Pt #1, headaches; Pt #2, neurological event [shuffling gait and tongue deviation]	Direct analysis of tumor response not performed. Pts #1 & #3, no tumor recurrence at 60 d+ post-treatment; Pt #2, tumor recurrence at w3	NR	Pt #1, 10.6 m; Pt #2, 8.6 m; Pt #3, 13.9 m
Brown *et al.*[44]	None	CR	7.5 m	NR
O'Rourke *et al.*[49]	3/10 Pts, neurological events [seizure, neurological decline]	9/10 Pts, SD at d28; 1/10 Pts, PD at d28	NR	Median, 8 m (range 3–18 m)
Ahmed *et al.*[50]	No grade 3 AEs; 2 Pts, grade 2 seizures; 1 Pt, grade 2 headache	1/16 Pts, PR for 9 m; 7/16 Pts, SD for 8 w-29 m; 8/16 Pts, PD	3/16 Pts, PFS 24 m+	Median, 11 m (range 4.1–27.2 m)

AEs, adverse events; CR, complete response; d, day(s); m, months; NR, not reported; PD, progressive disease; PFS, progression-free survival; PR, partial response; Pt, patient; SD, stable disease; w, week(s).

IL-13Rα2-specific CAR-T cell therapy

In the first clinical study in humans, Brown *et al.* reported three recurrent glioblastoma patients treated with CAR-T cells specific for the tumor-associated antigen, IL-13Rα2.[42] The IL-13Rα2 antigen is overexpressed in > 60% of glioblastoma cases, is not expressed at significant levels in normal brain, is a poor prognostic factor and is expressed by both stem-like and differentiated glioblastoma cells as well as tumor-infiltrating myeloid cells.[42,43] This was a first-generation CAR construct, which contained only the CD3ζ intracellular signalling domain, expressed after electroporation of plasmid DNA into anti-CD3-activated and IL-2-expanded peripheral blood T cells. Importantly, CAR-T cell products could not be manufactured for 10 of 13 enrolled patients within the required timeframe using this method. The CAR-T cells were delivered intracranially into the tumor resection cavity. Intra-patient dose escalation was performed and patients received up to 12 intracavitary infusions over five weeks with two of the three patients receiving the full treatment course.

This regimen was well tolerated and exhibited an acceptable safety profile with limited and transient adverse events. One patient was hospitalised with grade 3 headache, shuffling gait and tongue deviation, which resolved after a single 10 mg dose of intravenous dexamethasone.

All three patients had magnetic resonance imaging (MRI)-detected brain inflammatory changes at the intracerebral site of T-cell infusion. The degree of brain inflammation appeared to associate with the extent of pre-infusion IL13Rα2 antigen expression, with inflammation most pronounced in the two patients with the highest expression of IL13Rα2 as determined by immunohistochemistry (IHC) and quantitative polymerase chain reaction (qPCR) for mRNA.

In two of the three patients whose tumor response was assessed by post-treatment MRI scans at weeks 3 and 5 and > 60 days, tumor recurrence near the resection cavity was not evident. In the remaining patient who had recurrence, tumor biopsy revealed reduced IL-13Rα2 expression (from pre-infusion levels) suggesting that antigen-positive tumor cells had been eliminated by the infused CAR-T cells. Moreover, these CAR-T cell products, which had expanded uniformly as $CD8^+$ T cells with an activated effector phenotype, displayed limited persistence at the injection site as measured by IHC for T-cell markers and qPCR for the CAR transgene.[42]

In a follow-up study, changes were made to overcome some of the identified deficiencies of the earlier CAR-T cell product. The CAR was re-engineered in a lentiviral vector to encode an additional 4-1BB (CD137) co-stimulatory domain. The transduced T-cell population had been enriched for central memory cells using a two-step immuno-magnetic bead selection by first depleting $CD14^+$, $CD25^+$ and $CD45RA^+$ cells and then retaining $CD62L^+$ cells.[43] To date, a single-patient case report has been published using this new approach.[44] Lympho-depleting chemotherapy was not used in this or the previous study.

This patient had recurrent and rapidly progressing glioblastoma, which adopted a poor-prognosis leptomeningeal, multifocal pattern of spread.[12] The tumor was *MGMT* promoter-unmethylated. TMZ was given concurrently with post-surgery radiotherapy and as a 6-month maintenance course, which preceded the first CAR-T cell infusion by 16 weeks. In both primary and initial metastatic lesions, there was heterogeneous expression of the IL13Rα2 target antigen, which was lacking in 30% of tumor cells. In all, the patient received 16 loco-regional infusions of two batches of the CAR-T cell product, which contained predominantly $CD4^+$ T cells. No grade 3 or higher adverse events were observed.

During intra-patient dose escalation, CAR-T cell infusions #1-6 were by the intracavitary route. Whereas the treated tumor remained stable for 6 weeks, suggesting local control of this tumor by CAR-T cells, two unresected tumors grew, and four new tumors appeared: two intracerebral, and two metastatic to spinal cord. To improve the prospect of controlling disseminated intra-CNS disease, further CAR-T cell infusions (#7-16) were by the intraventricular route. By cycle 10, all seven tumors decreased by 77–100% in size and continued to complete response (CR) by Response Assessment in Neuro-Oncology (RANO) criteria.[45] The CR held for 7.5 months after the first infusion, and the patient returned to a normal life. Interestingly, the patient received dexamethasone (2–4 mg) during the first three intraventricular CAR-T cell infusions, which were marked by major regression of all metastatic intra-CNS tumors.

Notwithstanding this remarkable clinical response, detectable expansion of CAR-T cells in the cerebrospinal fluid (CSF) was minimal. This

may seem surprising because the peak post-infusion expansion of CD19-CAR-T cells is significantly associated with complete responses in B-cell acute lymphoblastic leukaemia (B-ALL) patients.[46] However, in this study, a higher ratio of peak CAR T-cell expansion to baseline tumor burden was a better predictor of long-term survival than the absolute magnitude of T-cell expansion.[46] This finding is in keeping with the recent demonstration in metastatic melanoma patients that the ratio of reinvigorated circulating T cells to tumor burden was associated with a response to the anti-PD1 monoclonal antibody, pembrolizumab.[47] Minor subpopulations of CAR-T cells and endogenous T cells, myeloid cells, B cells and granulocytes, as well as inflammatory cytokines, peaked 1–2 days after each intraventricular infusion and were detected up to 7 days post-infusion, although no CAR-T cells were detectable in peripheral blood.[44]

Then, four new tumors recurred at non-adjacent sites in this patient, and tumor biopsy at one of these sites indicated decreased IL-13Rα2 expression, suggesting that tumor antigen escape was an important immune evasion mechanism. The transient CR in this patient together with induction by CAR-T cells of inflammatory cells in the CSF raise the possibility that, in the face of heterogeneous target antigen expression, the CAR-T cells may contribute to endogenous antitumor cellular immunity.[48]

EGFRvIII-specific CAR-T cell therapy

In the first clinical study in humans, of CAR-T cells specific for EGFRvIII, O'Rourke *et al.*[49] reported results for the first 10 recurrent glioblastoma patients treated. All patients had poor-prognosis multifocal or deep, unresectable disease, and all also had poor-prognosis *MGMT* promoter unmethylation. For these patients, target antigen expression for trial eligibility was determined in the primary rather than the recurrent tumor specimen and was tested with a validated, RNA-based next-generation sequencing assay, which was considered standard of care at the trial institution. In this assay, target antigen expression was related to wild-type EGFR (WT EGFR) and, given that WT EGFR may have been amplified, EGFRvIII expression varied between 6% and 96% with a median of 71%. To compare, over 2 years in the same institution, of 369 glioblastoma patients, 79 (21%) tested positive for EGFRvIII.

The CAR was a lentivirally encoded second-generation construct with 4-1BB and CD3ζ signalling domains. Patients received a single intravenous CAR-T cell dose without prior lymphodepletion.

No patients in this study had dose-limiting toxicity, CRS, on-target, off-tumor EGFR-directed toxicity, or the neurotoxicity signs and symptoms observed with CD19 CAR-T cell therapy. However, three patients experienced neurological adverse events. One patient with viable glioblastoma and hyponatraemia had seizures and altered mental state, which were treated by anti-epileptics, high-dose corticosteroids and the anti-IL-6 mAb, siltuximab, on day 15 post-infusion. However, the patient's recovery could not be attributed to a single intervention. Given the uncertain aetiology of this patient's seizures, the authors hypothesised that 'localised T-cell activation with an intracranial compartmentalised cytokine release' could be responsible although no evidence was presented to support this contention. A second patient, who had neurological decline at day 15 post-infusion and was treated with high-dose corticosteroids and siltuximab on day 29 post-infusion, was described as having overall a clinical course consistent with progressive disease. A third patient experienced post-operative neurological decline, which was attributed to delayed haemorrhage.

All patients had peak peripheral blood levels of CAR-T cells detected at days 3–10 post-infusion, but CAR-T cells were not detected by day 30 except in one patient at 2 months post-infusion. Seven of 10 patients underwent surgical resection after the CAR-T cell infusion, and of these seven biopsies, five had detectable CAR-T cells, with the highest CAR-T cell levels observed in the four patients who had surgery early after infusion (within 14 days). In addition, expression levels of EGFRvIII in the re-resected tumor specimens were decreased in five of the seven patients, which the authors interpreted as 'on-target effects' of the infused CAR-T cells. In three patients who had repeat surgery, TCRβ CDR3 deep sequencing of re-resected tumor showed in all three patients a denser post-infusion lymphocytic infiltrate, the composition of which was largely different to that found in pre-infusion tumor specimens and in the infusion product itself. In these post-infusion tumor specimens, the cell-equivalent signal from a qPCR assay for the CAR-T transgene revealed an absent signal in one case and in the other two

cases indicated that a distinct minority of tumor T cells were CAR-T cells. Further analysis comparing post-infusion tumor specimens with pre-infusion specimens revealed robust expression of inhibitory molecules (IDO1 and FoxP3, and in some cases, IL-10, PD-L1 and/or TGFβ) and also indicated that most of the post-infusion tumor T-cell infiltrate comprised endogenous regulatory T cells.

Together, these results indicate that intravenously administered CAR-T cells trafficked to the brain tumors and displayed evidence of in situ activation and proliferation mainly of $CD8^+$ CAR-T cells, but also elicited compensatory regulatory mechanisms that reinforced the inhibitory nature of the tumor microenvironment. However, these results also provide a rationale for combining CAR-T cell therapy with immune checkpoint blockade and/or other immune-modulatory therapies.

Tumor response was assessed by MRI at day 28 when all but one patient had stable disease. After 18 months and of the three remaining patients, one patient had stable residual disease and another two patients were alive but with progressive disease.[49]

HER2-specific CAR-T cell therapy using virus-specific T cells

In a phase 1 dose-escalation study, Ahmed *et al.*[50] investigated HER2-specific second-generation (CD28-CD3ζ) CAR-T cell therapy in 17 patients (10 adult and seven children) who had progressive or recurrent $HER2^+$ glioblastoma. HER2 is a well-characterised oncogenic target in breast and other cancers. Baseline peripheral lymphocyte counts were in the normal range for all patients.

Of 41 patients screened for the study, 23 had CAR-T cell products made (12 had HER2-negative tumors and six declined) and 17 had infusions (six had clinical deterioration). Of 17 patients' tumors assessed by IHC, 10 had weak HER2 staining and seven had moderate HER2 staining. Virus-specific T (VST)-cell clones with TCR specificity for Epstein–Barr virus (EBV), cytomegalovirus (CMV) or adenovirus (AdV) were transduced with a retroviral vector to generate the final HER2-CAR-T cell product. The authors hypothesised that the VST cells would receive appropriate co-stimulation after interaction of their native TCRs with latent virus presented by professional antigen-presenting cells. Sixteen patients were CMV-seropositive and of evaluable tumors assessed by IHC, five of 15 and seven of 16 stained for CMV pp65 and CMV IE1 products, respectively. Dose escalation of the intravenously administered CAR-T cells proceeded in five dose cohorts, and six patients received two or more infusions. No dose-limiting toxicity was observed although two patients had grade 2 seizures and/or headaches, which the investigators concluded were probably related to the CAR-T cell infusion.

HER2-CAR-T cells were detected by qPCR in peripheral blood of all patients, with peak levels post-infusion detected at three hours in 15 patients and at 1 and 2 weeks in the other two patients. Seven of 15 tested patients had CAR-T cells detected at 6 weeks, and two of six tested patients had CAR-T cells detected at 12 months but none later than 12 months. The authors concluded that the CAR-T cells did not significantly expand post-infusion but persisted at low levels for up to 12 months. CAR-T cell trafficking and tumor infiltration were not determined.

Tumor response was assessed by MRI and Response Evaluation Criteria in Solid Tumours (RECIST) at 6 weeks post-infusion. With a median follow-up period of 8 months and of 16 evaluable patients, one had a partial response (PR), and seven had a stable disease for 8 weeks to 29 months after the first CAR-T cell infusion. For the entire study cohort, the median time to progression was 3.5 months; median OS was 11.1 months (95% CI, 4.1–27.2 months) after the first CAR-T cell infusion and 24.5 months (95% CI, 17.2–34.6 months) after diagnosis, and three patients remained alive. In a univariate analysis, the only significant factor identified was the absence of pre-infusion salvage therapy in seven patients who had median OS of 27.2 months compared to 6.7 months for patients who had prior salvage therapy. Of these seven patients, one had a PR lasting approximately 8 months, and three were alive 23.7–28.6 months post-infusion.[50]

Given that the HER2-CAR in this study was hosted in CMV-specific T cells among T cells of other viral specificity, it is interesting also to mention a phase 1 study of autologous CMV-specific T-cell therapy as a consolidation therapy for recurrent glioblastoma. Genetic evidence indicates that CMV sequences are present in the majority of diffuse glioma samples, albeit in only a small minority of cells in any individual sample,[51,52] supporting the therapeutic targeting of CMV in glioblastoma.[53] In this phase 1 study,

11 patients received 1–4 intravenous infusions of 2.5–4.0 $\times$ 10^7 CMV-specific T cells. The median OS of the 11 patients who received at least one infusion was 13.4 months, and one patient demonstrated stable disease, remaining progression-free by 4 years after the T-cell infusion. CMV-specific T cells were detected in resected tumor tissue from one patient.[53] These data indicate that CMV-specific T-cell therapy may have clinical anti-glioblastoma activity independently of the presence of tumor antigen-specific CAR.

PARTICULAR RISKS OF CAR-T CELL THERAPY FOR BRAIN CANCERS

The brain is held inside the cranium, which is a fixed box with one major outlet, the foramen magnum. Hence, swelling of the brain parenchyma can be accommodated only to a small extent before pressure on vital structures results in neurological symptoms and signs. Indeed, such symptoms and signs often comprise the clinical presentation of glioblastoma. Moreover, rising tumor- and edema-related pressures in the supra-tentorial compartment, which houses the cerebrum, is a common pre-terminal event in glioblastoma patients.

On-target, off-tumor and off-target (cross-reactive) toxicities

Similar concerns relate to autoimmune on-target, off-tumor and off-target (cross-reactive), toxicities, which affect non-tumor tissues bearing the same antigen, or a cross-reacting antigen, respectively. Although neuro-inflammation may be associated with these toxicities, indirect and clinically relevant intracranial mass effects may also occur. Evident examples of severe but reversible on-target, off-tumor neurotoxicity include therapy with autologous T cells expressing high-avidity T-cell receptors (TCRs) specific for the melanoma differentiation antigens, MART-1 and gp100. These transgenic T cells produced uveitis with visual impairment and decreased hearing presumably because of autoimmune reactivity against the same antigens in the pigmented cells of the uveal tract and inner ear. The severity of the neurotoxicity may have been related both to the high-avidity and number of the adoptively transferred transgenic T cells because immunisation against the same antigens had not produced the same adverse clinical effects.[54,55] A clear example of lethal off-target neurotoxicity resulted from TCR-directed adoptive cell therapy in which a MAGE A3-directed TCR cross-reacted with MAGE A12 expressed in brain neurons.[56]

In selecting an antigen target and its binder for glioblastoma CAR-T cell therapy, the abovementioned clinical examples of on-target, off-tumor as well as off-target or cross-reactive toxicities illustrate the importance of prior clinical experience with a non-CAR binder and its antigenic target. For example, we are pursuing clinical application of CAR-T cell therapy in glioblastoma patients with GD2 as the target antigen. In our approach, the scFv of the murine GD2-specific 14g2a monoclonal antibody serves as the CAR-binding moiety. GD2 is a disialoganglioside expressed by tumors of neuroectodermal origin including melanoma, with neuroblastoma showing among the highest levels of expression.[57] High-level GD2 expression is also found in most adult glioblastomas but only at very low level in parts of normal brain.[58–60]

Dinutuximab, which is a 14g2a-derived chimeric mAb, is approved in the United States and Europe as a standard consolidation therapy for neuroblastoma.[61] Its dominant toxicity is peripheral nerve pain, which is believed to be related to the antibody's Fc domain, which is not part of a CAR construct. In fact, of the more than 20 melanoma and neuroblastoma patients who have received GD2-CAR-T cells using the 14g2a scFv, no significant CAR-T cell-related clinical toxicity has been observed and, in particular, no central or peripheral neurotoxicity, thus supporting the safety of this particular CAR-T therapy[62–65] (Gargett T *et al.*, unpublished data). In addition, intra-CNS administration of radio-conjugates of the anti-GD2 antibody, 3F8, was associated with manageable acute toxicities.[57]

The recent discovery of consistent high-level GD2 expression in the rare but lethal childhood primary brainstem tumor, diffuse intrinsic pontine glioma (DIPG), has sparked additional interest in clinical CAR-T cell therapy trials for glioblastoma.[66] Specifically, in the molecularly distinct H3-K27M$^+$ subgroup, which harbours histone H3 gene mutations and comprises 73% of DIPG cases,[67] gene expression is generally dysregulated, including expression of GD2, and virtually all tumors express uniformly high levels of GD2 in contrast to other childhood brain tumors.[66]

On-target, on-tumor toxicity including Cytokine Release Syndrome (CRS) and neurotoxicity

Given the frequent enough observations of 'pseudo-progression', which manifests as tumor swelling and inflammation before later resolution, after ICI therapy and adoptive T-cell therapy for cancer, heightened clinical concern remains for pseudo-progression after CAR-T cell therapy for glioblastoma. This concern also relates to potential on-target, on-tumor toxicities of CAR-T cell therapy and may be exacerbated by properties intrinsic to the CAR-T cell product such as CAR construct, manufacturing method, cell dose or by extrinsic factors such as prior lympho-depleting chemotherapy or route of administration.

In orthotopic murine xenograft models of DIPG and midline glioma, GD2-CAR-T cells eradicated engrafted tumors but lethal on-target, on-tumor neurotoxicity was observed depending on tumor location. When the tumor was located in the pons, several mice were euthanased because of lethal toxicity in one of three independent cohorts. In mice surviving the treatment, tumor clearance was observed and the surrounding tissues appeared grossly normal. In the euthanased mice, a widespread inflammatory infiltrate was observed, mainly involving the brainstem, and was associated with ventriculomegaly because of fourth ventricle compression. Given that minimal normal neuronal cell death was observed, the authors suggested that tumoricidal effects of GD2-CAR-T cells initiated neuro-inflammation and edema resulting in hydrocephalus and death of mice. When diffuse midline glioma was orthotopically implanted in the thalamus of the immunocompromised mice, the GD2-CAR-T cell therapy was associated with tumor swelling or pseudo-progression in this critical location resulting in third ventricle compression and lethal trans-tentorial herniation.[66]

In CD19-CAR-T cell therapy, cytokine release syndrome (CRS), and its frequent accompaniment of neurotoxicity, is a dramatic manifestation of on-target, on-tumor toxicity. Life-threatening or fatal CRS presents with fever, hypotension, coagulopathy and capillary leak, and occurs in up to 8% of cases.[68] The severity of neurotoxicity, which is of unknown aetiology, is associated with the severity of the CRS, and neurotoxicity typically follows onset of CRS by several days. Higher bone marrow $CD19^+$ tumor cell burden, higher CAR T-cell dose, manufacture of CD19-CAR-T cells using bulk $CD8^+$ T cells without CD62L selection, and lympho-depleting fludarabine and cyclophosphamide chemotherapy were independently associated with the development of CRS.[68]

CRS-related neurotoxicity has also been termed CAR-T-cell-related encephalopathy syndrome (CRES)[69] or immune effector cell-associated neurotoxicity syndrome (ICANS),[70] and its grading and associated clinical management algorithms have recently been formalised by consensus.[70] Nevertheless, CRS-related neurotoxicity is related both to tumor burden and the rapidity and magnitude of CD19-CAR-T cell expansion and it, like CRS, is aggravated by pre-existing or concurrent endothelial dysfunction.[68,71–73] Further evidence that T-cell activation plays an important part in the pathogenesis of neurotoxicity is the observation of serious neurotoxicity after treatment with blinatumomab, which is a bi-specific anti-CD3/CD19 T-cell engager.[74]

In contrast, in the currently reviewed clinical studies of glioblastoma CAR-T cell therapy, there were mild-to-moderate clinical or radiological manifestations of what the laboratory studies of tumor biopsies indicated could be on-target, on-tumor brain inflammation associated with the CAR-T cell therapy. However, in none of these studies was there any evidence of CRS, CRES (or ICANS). Potential reasons for the absence of CRS and the strongly related phenomenon of neurotoxicity in these patients include lower tumor burden and the lack of prior lympho-depletion even though the CAR-T cell doses received were in a similar range to the doses administered to the patients with B-cell malignancies. In future, however, it is possible that prior lympho-depletion or alterations in ex vivo culture conditions or selection methods for the CAR-T cells could alter this risk profile and thus affect the propensity of CAR-T cells used in glioblastoma therapy to cause CRS and neurotoxicity.

CAR-T cell design

Design elements of the CAR could also affect the safety and toxicity profile of CAR-T cell therapy for glioblastoma. From clinical studies of CD19-CAR-T cell therapy, the choice of co-stimulatory

domain may significantly affect the function and toxicity of CAR-T cells. Severe and fatal neurotoxicity has been reported after treatment with CD19-CAR-T cells incorporating either 4-1BB or CD28 co-stimulatory domains.[72,73] CD19-CAR-T cells with the CD28 co-stimulatory domain reportedly induce earlier onset CRS relative to their 4-1BB-containing counterparts[69] and have been associated with fatal cerebral edema perhaps because of earlier and higher peak expansion of CAR-T cells, which were derived from cell products containing a greater preponderance of $CD8^+$ T cells and obtained from younger patients with more vigorous cellular immunity.[71] Nevertheless, whether choice of co-stimulatory domain affects neurotoxicity risk has not been formally studied.[72] Other relatively minor modifications to the CAR such as altering the length of the linker between the light and heavy chains of the CAR's scFv moiety can also have a profound impact on the in vivo function of CAR-T cells.[75]

The affinity of a CAR is an important contributor to the in vivo activity of CAR-T cells as illustrated in pre-clinical models of GD2-specific CAR-T cells. Of particular interest is the CAR employing the GD2-specific 14g2a scFv because both the GD2 molecule and its 14g2a epitope are identical in mice and humans, which suggests that results of murine GD2-CAR-T cell experiments might be extrapolated to humans. In spite of GD2 expression in normal mouse brain, second-generation GD2-CAR-T cells were not found in murine brain after adoptive transfer and there was no evidence of brain pathology.[66,76] However, when a high-affinity variant of the 14g2a scFv was created, intracerebral infiltrates of these GD2-CAR-T cells were detected and associated with fatal neurotoxicity in the mice. Similar findings were made when the GD2-CAR-T cells contained a scFv from the GD2-specific mAb, 3F8.[76] 3F8 has the highest reported affinity for GD2 (K_D = 5 nM), whereas 14g2a has significantly lower affinity (K_D = 77 nM).[77] Moreover, although GD2 expression is detected in normal human brain, neurotoxicity has not been observed in multiple CAR-T cell clinical trials in advanced neuroblastoma and melanoma patients using GD2-CARs with the 14g2a scFv.[62–65] Formal testing of erbB2- and EGFR-specific CAR-T cells in pre-clinical models showed that tuning of CAR affinity may yield improved therapeutic ratios of CAR-T cell therapy. For example, lowering CAR affinity reduced toxicity in normal tissues expressing low levels of target antigen while maintaining therapeutic effectiveness against tumors expressing higher levels of target antigen.[78] Together, this evidence suggests that careful CAR design can militate against the dangers of CAR-T cell toxicity in healthy tissues.

PERSPECTIVES FOR FUTURE DEVELOPMENT OF CAR-T CELL THERAPY OF GLIOBLASTOMA

The field of clinical exploration of CAR-T cell therapy for glioblastoma is just opening up. Unlike any other tumor type, except perhaps pancreatic cancer, the challenges facing successful clinical implementation of glioblastoma CAR-T cell therapy appear daunting and on occasions insurmountable. For the most part, the challenges are quite different from those facing the field of leukaemia and lymphoma CAR-T cell therapy. First, CD19 is an example of an ideal CAR-T cell target because it is expressed at uniformly high level on tumor cells and otherwise only on 'dispensable' normal B cells.[79] In contrast, ideal tumor antigens have not been discovered in glioblastoma because antigen expression on normal CNS and other tissues creates toxicity concerns, and expression of identified tumor antigens is heterogeneous. Hence, even though CR rates from CD19-CAR-T cell therapy in B-ALL patients are high, and up to 25% of patients later relapse with CD19-negative disease, additional near-ideal tumor antigens such as CD22 or CD20 are available for B-ALL CAR-T cell therapy and are being tested clinically in combination with CD19-targeting.[80] Second, the relatively common and severe toxicities of CRS and neurotoxicity of CD19-CAR-T cell therapy, which have not been apparent with glioblastoma CAR-T cell therapy, create a narrow therapeutic window for CD19-CAR-T cell therapy, making identification of predisposing pre-infusion factors a priority.[68] Finally, CD19-CAR-T cells operate in a tumor microenvironment in which there is often an abundant supply of co-stimulatory molecules such as CD80 and CD86, which are expressed by B-cell malignancies and which may promote CD19-CAR-T cell function and survival. Conversely, in the glioblastoma microenvironment, expression of co-stimulatory molecules is distinctly lacking.

Although nothing conclusive can be drawn, because the reviewed glioblastoma CAR-T cell

therapy studies were early-stage and small, a number of intriguing observations emerge and there may be some lessons to be learnt.

Considerations for clinical trial design, endpoints and monitoring

It is important to remember that a cell therapeutic product is a package rather than a drug, and that details such as CAR construct design, type of vector, cell manufacturing conditions, cell dose and schedule, route of administration and preparative regimen matter. In addition, details of the patient population (e.g. primary versus recurrent glioblastoma), disease burden (e.g. post-resection), treatment history and glucocorticoid use also matter. In addition, molecular characteristics of glioblastoma such as *MGMT* promoter methylation and *IDH1* mutation status may not just be of prognostic importance but may be also influence CAR-T cell therapy once more is understood about the immunobiology of these genomic alterations. Larger cohorts of patients in early-phase trials may help address the role of these factors.

Clinical trial design matters. In choosing and evaluating efficacy endpoints in early-phase studies, an improvement in OS is desirable, but delaying tumor progression is also clinically meaningful in a disease such as glioblastoma where PFS at 6 months (PFS6) is becoming a benchmark for post-recurrence treatment effects. Similarly, assessments of cancer-related symptoms and quality of life remain important. It is widely recognised that as an imaging modality, MRI can be difficult to interpret, thus limiting the utility of conventional response criteria such as RECIST in glioblastoma both for measuring objective tumor responses and evaluating progression events. Accordingly, Response Assessment in Neuro-Oncology (RANO) criteria include both radiological and clinical criteria such as glucocorticoid use and are becoming the 'industry standard'.[45] Moreover, accounting for phenomena such as pseudo-progression has now formally been incorporated in the new immune or iRANO criteria.[81]

Finally, to enable definitive and sophisticated measurements of immune and tumor responses and biomarkers from surgical specimens after the treatment intervention, and to enable timed correlations with peripheral blood biomarkers, strong consideration should be given to 'window of opportunity' trial designs in primary and recurrent glioblastoma.

Risk mitigation strategies

In none of these early clinical studies of glioblastoma CAR-T cell therapy were any systemic toxic effects such as severe CRS and neurotoxicity observed. However, of particular concern in glioblastoma CAR-T cell therapy are treatment-related effects that would create brain swelling, arising from either on-target, on-tumor pseudo-progression or on-target, off-tumor and cross-reactive toxicities in normal brain. For highly vulnerable patient populations such as children, who have tumors such as DIPG or glioblastoma in critical locations, risk mitigation strategies could include conventional surgical approaches such as extra-ventricular drain (EVD) insertion as a prophylactic measure. Although other measures such as high-dose glucocorticoid therapy and anti-IL-6 antibodies may be used to manage symptoms and downstream toxicities (see next section), genetic engineering of the CAR-T cell itself may be needed to improve its safety.

One method is to incorporate upstream of the transgene in the CAR construct a suicide gene that encodes a homo-dimerisable safety switch called inducible caspase-9 (CaspCIDe).[82] This safety switch is triggered following administration of the bio-inert, small-molecule drug, rimiducid. Rimiducid binds modified FK506 binding domains of CaspaCIDe, thus enforcing chemical-induced dimerisation (CID) of the caspase-9 molecules genetically fused to the FK506 binding domains and inducing apoptosis of the gene-modified cells.[83] Importantly, rimiducid penetrates the human CNS in concentrations high enough to eliminate CAR-T cells expressing CaspaCIDe.[84]

Management of neurotoxicity

Evidence of neurotoxicity was reported in three of the clinical trials, but rarely at a level of higher than grade 1-2. Grade 3 neurological events were observed as follows: Brown *et al.*[42] reported one seizure and one other neurological event (treated with a single glucocorticoid infusion) in their first study. O'Rourke *et al.*[49] reported one seizure and two cases of neurological decline and two of these were classed as possibly CAR-T cell infusion-related and treated with glucocorticoids and the IL-6 neutralising antibody, siltuximab. Ahmed

et al.[50] reported three grade 2 events, two seizures and one headache, which were attributed to the HER2-CAR-T cell therapy.

None of the glioblastoma patients in the reported trials received lympho-depletion before CAR-T therapy. CRS was not observed, and transient peaks in inflammatory cytokines were observed in the CSF of the patient receiving intracranial IL13Rα2-CAR-T therapy and in the serum of patients receiving intravenous EGFRvIII-CAR-T cell therapy. When two EGFRvIII-CAR-T cell patients developed post-infusion neurological symptoms, which were hypothesised to be neurotoxicity from intracranial cytokine release, the trial investigators elected to treat these patients with siltuximab. Although the IL-6 receptor blocking antibody, tocilizumab, is established as a treatment for CD19-CAR-T cell-related CRS, siltuximab was used instead for two reasons. First, how well tocilizumab penetrated the CNS was not known, and tocilizumab-mediated blockade of the IL-6 receptor antibody temporarily increases IL-6 circulating levels, potentially exposing the brain to transiently higher levels of IL-6.[49]

Notwithstanding these results, the predisposition of glioblastoma patients to neurological impairment and seizures poses particular challenges in both the diagnosis of CAR T-cell-related neurotoxicity and the assessment of treatment response.[69,85] Clinically and radiologically, it can be difficult to distinguish the neurological signs and symptoms of true tumor progression from potential on-tumor, on-target (tumor 'pseudo-progression') and off-tumor, on-target neurotoxicities of glioblastoma CAR-T cell therapy, or indeed from other pathologies such as infection or radionecrosis. Depending on their acuity and severity, and aided by the results of neuro-diagnostic investigations such as computed tomography (CT), positron-emission tomography (PET/CT), and MRI perfusion studies, any neurological manifestations will first be managed clinically with the aim of relieving symptoms and preventing critical CNS compromise. Conventional medical approaches include adequate analgesia, increasing corticosteroid doses, modifying anti-convulsant regimens and, in the case of acutely severe space occupying lesions, reducing edema by hyperventilation, intravenous mannitol or bevacizumab. In the prophylactic setting, neurosurgical measures such as an external ventricular drain or a ventriculo-peritoneal shunt may be considered to control untoward effects of swelling. In the acute setting, lesion excision or decompressive craniectomy may also be used to manage swelling.

However, in the event of other clinical or laboratory evidence pointing to systemic cytokine release, for example, early high fever and hypotension,[68] more specific therapies may be required such as the cytokine blockers, siltuximab and anakinra, or drugs such as rimiducid[84] or cetuximab[86] to mediate targeted deletion of CAR-T cells. In particular, although tocilizumab effectively treats CRS, it does not seem to alter the course or severity of subsequent neurotoxicity.[72,73] Hence, the clinical investigation of other approaches such as therapeutic blockade of IL1 receptor by anakinra would be worthwhile as shown in a murine xenotransplantation model of CAR-T cell therapy for leukaemia in which anakinra rather tocilizumab prevented lethal neurotoxicity after the first CRS-related fever.[87]

Customising CAR-T cell therapy for success in glioblastoma

The expectation of effective CD19-CAR-T cell therapy rests on a product that contains numerous and potent CAR-T cells, which persist and have a memory phenotype. Furthermore, in the case of solid tumors, it is expected that CAR-T cells will traffic to and infiltrate the tumor site.

Tumor antigen heterogeneity and antigen escape

Both heterogeneity of target antigen expression and the evolution of antigen-loss variants under selection pressure from CAR-T cells represent a major limitation of CAR-T cell therapy because CAR-T cell targeting has typically been restricted to one or two tumor antigens. For example, documented loss of tumor expression of the IL13Rα2-CAR-T-cell-targeting antigen may have contributed to the fatal relapse of the initially responding patient discussed herein.[44] In another example, although EGFRvIII is a neoantigen expressed exclusively by glioblastoma cells, its expression is heterogeneous and only in 18-21% of glioblastoma patients.[88] Interestingly, EGFRvIII expression was lost in the patients who had repeat surgery on the control arm of the ACTIV glioblastoma vaccine trial, indicating that antigen expression is inherently unstable.[89] Moreover, uncertainty about whether EGFRvIII is a significant negative prognostic factor, or plays a significant

role in glioblastoma stem cells,[49,88] raises questions about whether EGFRvIII is a key driver mutation responsible for initiating and/or maintaining the tumorigenic phenotype. Nonetheless, the finding of intra-tumoral lymphocytic infiltrates comprising mainly endogenous (non-CAR) regulatory T cells after EGFRvIII-CAR-T cell targeting[49] offers the possibility, as shown in an immunocompetent murine model, that protective endogenous immunity could be recruited against antigen-negative tumor variants following CAR-T cell therapy,[90] and particularly if changes in CAR design could limit the effect the immunosuppressive effects of regulatory T cells.[91]

Possibility of CAR-T cell induction of endogenous antitumor immune responses

Although there is growing interest in combinatorial approaches targeting multiple antigens to overcome these limitations,[79] another intriguing outcome of CAR-T cell therapy may be the induction of a vaccine-like effect. This may result from some level of CAR-T cell-induced tumor cell death, which could generate endogenous immune responses via 'antigen spreading' and then lead to complete tumor eradication as demonstrated in pre-clinical models of other cancer types.[92] Evidence for endogenous immune priming in glioblastoma exists with the generation of de novo T-cell responses,[40,41] which can be rescued from adaptive immune resistance by anti-PD1 therapy.[25–28] Another recent example is a clinical trial of patient-specific glioblastoma vaccines comprising multiple 'non-self' neo-epitopes in which vaccine-specific T-cell responses were only found in the two patients who had not received dexamethasone during priming. Vaccine-induced T cells were found in peripheral blood and infiltrating tumor at relapse in these two patients. These T cells had an exhausted phenotype, suggesting that these circulating T cells may be reactivated by therapeutic immune checkpoint blockade.[36]

Despite strong evidence that local and systemic immune suppression in glioblastoma exists, the reviewed studies demonstrate that functional CAR-T cells can be generated ex vivo. Moreover, in these studies, it is not apparent that medically induced immunosuppression using dexamethasone or TMZ[93] had significant deleterious effects on CAR-T cell function in vivo. Interestingly, at least a daily dose of 4 mg dexamethasone did not prevent a CAR-T cell-mediated complete response[42] although it did prevent priming in response to a neo-epitope vaccine.[36] Follow-up animal studies show that IL-13Rα2-CAR-T cell therapy could be combined with dexamethasone without losing efficacy.[43] These results indicate that CAR-T cell effector activity is surprisingly robust, and may even argue against priming or re-priming effects, which can be perturbed by concomitant glucocorticoid therapy.[94]

CAR-T cell memory phenotype

Although only a single case report, the CR after IL-13Rα2-CAR-T cell therapy suggests that CAR-T cell effector function may be better maintained if the cell product contains a high proportion of CAR-T cells with a memory phenotype. This result is consistent with the 93% CR rate observed after CD19-CAR-T cell therapy for B-cell malignancies in which cell products were made using the same method of enriching memory T cells.[68] Similarly, in this single complete responder, and unlike in these other studies of CAR-T cell therapy in glioblastoma, the cell product contained a preponderance of $CD4^+$ CAR-T cells. Could the activity of cytotoxic $CD4^+$ T cells[95,96] alter the immune context of tumor cell death in a way that might favor vaccine-like effects, as observed after adoptive transfer of $CD4^+$ NY-ESO-specific T cells to a metastatic melanoma patient, who subsequently responded after the development additional melanoma specificities?[48] Do $CD4^+$ CAR-T cells facilitate CAR-T cell re-priming in regional lymph nodes and thus contribute to CAR-T cell survival? These questions await further study.

Need for effective combinatorial therapy

Effective glioblastoma CAR-T cell therapy for long-term disease control may depend on combination with antibodies or drugs that block inhibitory factors such as IL-10, TGFβ or PD1/PDL1, and those molecules particularly associated with hypoxic and necrotic tumors, for example CD39 and CD73. Drugs for these and many other immunosuppressive factors are already in clinical development. For example, in the case of a diffuse large B-cell lymphoma patient refractory to CD19 CAR-T cell therapy, PD1 blockade with

pembrolizumab was reportedly associated with CAR-T cell expansion and tumor response.[97] A phase 1 study of EGFRvIII-directed CAR-T cells combined with pembrolizumab is currently recruiting patients with newly diagnosed, *MGMT*-unmethylated glioblastoma (ClinicalTrials.gov Identifier: NCT03726515). Furthermore, clinical experiments are underway in which gene-editing techniques have been applied to create checkpoint-resistant CAR-T cells (NCT03545815).

CAR-T cell dose and role of lympho-depleting chemotherapy

In each of these four early-phase glioblastoma CAR-T cell studies, intra- or inter-patient dose escalation was employed. Top individual CAR-T cell doses ranged from 1×10^7 to 5×10^8 (Table 2). These cell doses are in a similar range to the adult doses of the US FDA-approved CD19-CAR-T cell therapies, YESCARTA™ (axicabtagene ciloleucel) and KYMRIAH™ (tisagenlecleucel): $2–6 \times 10^8$ CAR-T cells.

In each of these glioblastoma studies, although minimal CAR-T cell expansion was observed, signals of antitumor activity were seen.[42] Therefore, would prior lympho-depletion, which promotes CAR-T cell expansion, be needed to improve glioblastoma CAR-T cell performance? Lympho-depleting chemotherapy is considered essential to the efficacy of CD19-CAR-T cell therapy[98,99] but, particularly with the addition of fludarabine to cyclophosphamide in the preparative regimen, it is associated with heightened risk for severe CRS and neurotoxicity.[68] In the two cited studies,[50,53] VST-cell expansion was lacking. But vigorous VST-cell expansion was observed in recipients of haematopoietic stem cell transplantation who were severely lympho-depleted and who experienced reactivation of the corresponding virus.[100,101] Therefore, if glioblastoma CAR-T cell therapy is otherwise wanting, prior lympho-depletion or viral vaccination or both may be strategies to stimulate the expansion of adoptively transferred T cells.

Route of administration

Intracranial (intracavitary and intraventricular) and intravenous routes of administration were both used in these studies. The rationale for intravenous administration of glioblastoma-targeted CAR-T cells is based on observations of adoptive T-cell therapy in which the transferred T cells were found in CSF or the brain.[98,102,103] In an animal model system, intraventricular rather than intravenous administration of the IL-13Rα2-CAR-T cells resulted in superior antitumor activity.[43] What is the optimal route of administration for glioblastoma patients? Although the IL-13Rα2-CAR-T cell products differed between the two studies,[42,44] it is to be noted that only when IL-13Rα2-CAR-T cells were delivered via the intraventricular rather than intracavitary route was a complete tumor response seen.[42] Loco-regional administration of the CAR-T cell product may also be preferred in order to limit potential systemic toxicities.[44,84]

What might come next: recent pre-clinical studies

Recent pre-clinical studies provide some insight into new strategies that may soon start to be tested in the clinic. One major area of study is the identification and validation of new target antigens, including chondroitin sulphate proteoglycan 4 (CSPG4),[104] podoplanin[105] and CD70.[106] All of these antigens are overexpressed in glioblastoma patient specimens, and CAR-T cells targeting them reduce glioblastoma growth in mouse models.[104–106] Combinatorial approaches are also being explored through the development of CAR-T cells which recognise more than one target antigen. Thus, Ahmed *et al.* have developed tandem CAR-T cells ('TanCAR') that recognise both HER2 and IL13Rα2 via a single CAR molecule incorporating recognition domains for both antigens, as well as 'U-CAR' T cells that recognise HER2, IL-13Rα2 and EphA2 via expression of a tri-cistronic CAR transgene.[107,108] These approaches have the distinct advantage of broadening the specificity of the CAR-T cell product, to address antigenic heterogeneity of tumors and reduce the chance of adaptive resistance to therapy mediated by antigen loss.

Improvements to CAR-T cell manufacturing and integration with standard therapy are also being actively explored. Surprisingly, delivery of a purified $CD4^+$ population of IL-13Rα2-CAR-T cells resulted in enhanced antitumor activity and persistence compared to either purified $CD8^+$ or mixed $CD4^+/CD8^+$ populations.[109] Improvements in activity and persistence of IL13Rα2- CAR-T cells have also been observed when the T cells were engineered to express transgenic IL-15 although

antigen escape was more common.[110] And finally, NKG2D-based CAR-T cells displayed synergistically enhanced antitumor activity when the mice were also treated with radiation, an effect attributed not only to radiation-induced upregulation of NKG2D ligands, but also enhanced CAR-T cell trafficking into the tumor.[111]

The exception proves the rule

> I never make exceptions. An exception disproves the rule.
>
> Sherlock Holmes in *The Sign of the Four*.

The view of the CNS as a primary organ site of immune privilege is breaking down in the face of known extra-CNS immunological reactions associated with such CNS infections as toxoplasmosis, and after the recent discovery of brain lymphatics.[1,39] Furthermore, although glioblastoma creates profound local immunosuppression, evidence is mounting that tumor antigen-specific T cells can both be primed and operate well in the hostile tumor microenvironment. Such evidence includes rare observations of CAR-T cell-mediated antitumor activity,[44] productive glioblastoma vaccine-induced T-cell responses,[95] and marked antitumor activity of immune checkpoint inhibitors.[25–28]

Glioblastoma, like other brain tumors, exerts systemic immunosuppression but does not metastasise beyond the CNS. These observations should encourage more intensive investigations of how CNS-localised immune responses can be generated via intravenous or intracranial routes of administration.

Is it true that adoptive immunotherapy with re-directed T cells obviates 'the need for antigen presentation and stimulation of a primary immune response'?[49] Future studies of lymphocyte trafficking[103] within the CNS and to extra-CNS secondary lymphoid tissues may help our understanding of the priming and re-priming events that govern the reactivity of therapeutic T cells. Moreover, although CAR re-directed T cells may avoid the immediate need for antigen presentation on MHC, in the face of a highly heterogeneous tumor such as glioblastoma, the priming of endogenous T cells directed against tumor antigens may prove to be an important contributor to long-term progression-free survival.

It is possible that glioblastoma CAR-T cell therapy will enable us to establish a beachhead on the shores of our ignorance about the immunobiology of the brain. Clinical CAR-T cell trials that incorporate correlative science studies may extend our scientific understanding and lead us to further ground-breaking clinical applications of glioblastoma CAR-T cell therapy.

ACKNOWLEDGMENTS

We acknowledge support from the Cancer Council SA Beat Cancer Project Hospital Research Support Package, a Cancer Council Early Career Research Fellowship and The Hospital Research Foundation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Lim M, Xia Y, Bettegowda C, Weller M. Current state of immunotherapy for glioblastoma. *Nat Rev Clin Oncol* 2018; **15**: 422–442.
2. Hodges TR, Ott M, Xiu J, *et al.* Mutational burden, immune checkpoint expression, and mismatch repair in glioma: implications for immune checkpoint immunotherapy. *Neuro Oncol* 2017; **19**: 1047–1057.
3. Sadelain M, Riviere I, Riddell S. Therapeutic T cell engineering. *Nature* 2017; **545**: 423–431.
4. Australian Institute of Health and Welfare. Brain and other central nervous system cancers. 2017; Cat. no. CAN 106. Canberra: AIHW.
5. Hanif F, Muzaffar K, Perveen K, Malhi SM, Simjee S. Glioblastoma multiforme: a review of its epidemiology and pathogenesis through clinical presentation and treatment. *Asian Pac J Cancer Prev* 2017; **18**: 3–9.
6. Lee JH, Lee JE, Kahng JY, *et al.* Human glioblastoma arises from subventricular zone cells with low-level driver mutations. *Nature* 2018; **560**: 243–247.
7. Louis DN, Perry A, Reifenberger G, *et al.* The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol* 2016; **131**: 803–820.
8. Perrin SL, Samuel MS, Koszyca B, *et al.* Glioblastoma heterogeneity and the tumour microenvironment: implications for preclinical research and development of new treatments. *Biochem Soc Trans* 2019; **47**: 625–638.
9. Stupp R, Mason WP, van den Bent MJ, *et al.* Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005; **352**: 987–996.
10. Perry JR, Laperriere N, O'Callaghan CJ, *et al.* Short-course radiation plus temozolomide in elderly patients with glioblastoma. *N Engl J Med* 2017; **376**: 1027–1037.
11. Hegi ME, Diserens AC, Gorlia T, *et al.* MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med* 2005; **352**: 997–1003.
12. Patil CG, Yi A, Elramsisy A *et al.* Prognosis of patients with multifocal glioblastoma: a case-control study. *J Neurosurg* 2012; **117**: 705–711.

13. Field KM, Simes J, Nowak AK, *et al.* Randomized phase 2 study of carboplatin and bevacizumab in recurrent glioblastoma. *Neuro Oncol* 2015; **17**: 1504–1513.
14. Wolchok JD, Chiarion-Sileni V, Gonzalez R, *et al.* Overall survival with combined nivolumab and ipilimumab in advanced melanoma. *N Engl J Med* 2017; **377**: 1345–1356.
15. Motzer RJ, Tannir NM, McDermott DF, *et al.* Nivolumab plus ipilimumab versus sunitinib in advanced renal-cell carcinoma. *N Engl J Med* 2018; **378**: 1277–1290.
16. Long GV, Atkinson V, Lo S, *et al.* Combination nivolumab and ipilimumab or nivolumab alone in melanoma brain metastases: a multicentre randomised phase 2 study. *Lancet Oncol* 2018; **19**: 672–681.
17. Tawbi HA, Forsyth PA, Algazi A, *et al.* Combined nivolumab and ipilimumab in melanoma metastatic to the brain. *N Engl J Med* 2018; **379**: 722–730.
18. Filley AC, Henriquez M, Dey M. Recurrent glioma clinical trial, CheckMate-143: the game is not over yet. *Oncotarget* 2017; **8**: 91779–91794.
19. Omuro A, Vlahovic G, Lim M, *et al.* Nivolumab with or without ipilimumab in patients with recurrent glioblastoma: results from exploratory phase I cohorts of CheckMate 143. *Neuro Oncol* 2018; **20**: 674–686.
20. Cloughesy TF, Mochizuki AY, Orpilla JR, *et al.* Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma. *Nat Med* 2019; **25**: 477–486.
21. Schalper KA, Rodriguez-Ruiz ME, Diez-Valle R, *et al.* Neoadjuvant nivolumab modifies the tumor immune microenvironment in resectable glioblastoma. *Nat Med* 2019; **25**: 470–476.
22. Zhao J, Chen AX, Gartrell RD, *et al.* Immune and genomic correlates of response to anti-PD-1 immunotherapy in glioblastoma. *Nat Med* 2019; **25**: 462–469.
23. Le DT, Durham JN, Smith KN, *et al.* Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* 2017; **357**: 409–413.
24. Yan L, Zhang W. Precision medicine becomes reality-tumor type-agnostic therapy. *Cancer Commun (Lond)* 2018; **38**: 6.
25. Bouffet E, Larouche V, Campbell BB, *et al.* Immune checkpoint inhibition for hypermutant glioblastoma multiforme resulting from germline biallelic mismatch repair deficiency. *J Clin Oncol* 2016; **34**: 2206–2211.
26. Johanns TM, Miller CA, Dorward IG, *et al.* Immunogenomics of hypermutated glioblastoma: a patient with germline POLE deficiency treated with checkpoint blockade immunotherapy. *Cancer Discov* 2016; **6**: 1230–1236.
27. Roth P, Valavanis A, Weller M. Long-term control and partial remission after initial pseudoprogression of glioblastoma by anti-PD-1 treatment with nivolumab. *Neuro Oncol* 2017; **19**: 454–455.
28. Erson-Omay EZ, Henegariu O, Omay SB, *et al.* Longitudinal analysis of treatment-induced genomic alterations in gliomas. *Genome Med* 2017; **9**: 12.
29. Hussain SF, Yang D, Suki D, Aldape K, Grimm E, Heimberger AB. The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses. *Neuro Oncol* 2006; **8**: 261–279.
30. Gabrusiewicz K, Rodriguez B, Wei J, *et al.* Glioblastoma-infiltrated innate immune cells resemble M0 macrophage phenotype. *JCI Insight* 2016; **1**: e85841.
31. Bloch O, Crane CA, Kaur R, Safaee M, Rutkowski MJ, Parsa AT. Gliomas promote immunosuppression through induction of B7-H1 expression in tumor-associated macrophages. *Clin Cancer Res* 2013; **19**: 3165–3175.
32. Berghoff AS, Kiesel B, Widhalm G, *et al.* Programmed death ligand 1 expression and tumor-infiltrating lymphocytes in glioblastoma. *Neuro Oncol* 2015; **17**: 1064–1075.
33. Heimberger AB, Abou-Ghazal M, Reina-Ortiz C, *et al.* Incidence and prognostic impact of FoxP3$^+$ regulatory T cells in human gliomas. *Clin Cancer Res* 2008; **14**: 5166–5172.
34. Woroniecka K, Chongsathidkiet P, Rhodin K, *et al.* T-cell exhaustion signatures vary with tumor type and are severe in glioblastoma. *Clin Cancer Res* 2018; **24**: 4175–4186.
35. Yang I, Han SJ, Sughrue ME, Tihan T, Parsa AT. Immune cell infiltrate differences in pilocytic astrocytoma and glioblastoma: evidence of distinct immunological microenvironments that reflect tumor biology. *J Neurosurg* 2011; **115**: 505–511.
36. Kmiecik J, Poli A, Brons NH *et al.* Elevated CD3$^+$ and CD8$^+$ tumor-infiltrating immune cells correlate with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor microenvironment and at the systemic level. *J Neuroimmunol* 2013; **264**: 71–83.
37. Woroniecka KI, Rhodin KE, Chongsathidkiet P, Keith KA, Fecci PE. T-cell dysfunction in glioblastoma: applying a new framework. *Clin Cancer Res* 2018; **24**: 3792–3802.
38. Chongsathidkiet P, Jackson C, Koyama S, *et al.* Sequestration of T cells in bone marrow in the setting of glioblastoma and other intracranial tumors. *Nat Med* 2018; **24**: 1459–1468.
39. Louveau A, Smirnov I, Keyes TJ, *et al.* Structural and functional features of central nervous system lymphatic vessels. *Nature* 2015; **523**: 337–341.
40. Liu Z, Meng Q, Bartek J Jr, *et al.* Tumor-infiltrating lymphocytes (TILs) from patients with glioma. *Oncoimmunology* 2017; **6**: e1252894.
41. Valentini D, Rao M, Meng Q, *et al.* Identification of neoepitopes recognized by tumor-infiltrating lymphocytes (TILs) from patients with glioma. *Oncotarget* 2018; **9**: 19469–19480.
42. Brown CE, Badie B, Barish ME, *et al.* Bioactivity and safety of IL13Rα2-redirected chimeric antigen receptor CD8$^+$ T cells in patients with recurrent glioblastoma. *Clin Cancer Res* 2015; **21**: 4062–4072.
43. Brown CE, Aguilar B, Starr R, *et al.* Optimization of IL13Rα2-targeted chimeric antigen receptor T cells for improved anti-tumor efficacy against glioblastoma. *Mol Ther* 2018; **26**: 31–44.
44. Brown CE, Alizadeh D, Starr R, *et al.* Regression of glioblastoma after chimeric antigen receptor T-cell therapy. *N Engl J Med* 2016; **375**: 2561–2569.

45. Wen PY, Macdonald DR, Reardon DA, *et al.* Updated response assessment criteria for high-grade gliomas: response assessment in neuro-oncology working group. *J Clin Oncol* 2010; **28**: 1963–1972.
46. Park JH, Riviere I, Gonen M, *et al.* Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. *N Engl J Med* 2018; **378**: 449–459.
47. Huang AC, Postow MA, Orlowski RJ, *et al.* T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. *Nature* 2017; **545**: 60–65.
48. Hunder NN, Wallen H, Cao J, *et al.* Treatment of metastatic melanoma with autologous $CD4^+$ T cells against NY-ESO-1. *N Engl J Med* 2008; **358**: 2698–2703.
49. O'Rourke DM, Nasrallah MP, Desai A, *et al.* A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med* 2017; **9**: eaaa0984.
50. Ahmed N, Brawley V, Hegde M, *et al.* HER2-specific chimeric antigen receptor-modified virus-specific T cells for progressive glioblastoma: a phase 1 dose-escalation trial. *JAMA Oncol* 2017; **3**: 1094–1101.
51. Bhattacharjee B, Renzette N, Kowalik TF. Genetic analysis of cytomegalovirus in malignant gliomas. *J Virol* 2012; **86**: 6815–6824.
52. Ranganathan P, Clark PA, Kuo JS, Salamat MS, Kalejta RF. Significant association of multiple human cytomegalovirus genomic Loci with glioblastoma multiforme samples. *J Virol* 2012; **86**: 854–864.
53. Schuessler A, Smith C, Beagley L, *et al.* Autologous T-cell therapy for cytomegalovirus as a consolidative treatment for recurrent glioblastoma. *Cancer Res* 2014; **74**: 3466–3476.
54. Yeh S, Karne NK, Kerkar SP, *et al.* Ocular and systemic autoimmunity after successful tumor-infiltrating lymphocyte immunotherapy for recurrent, metastatic melanoma. *Ophthalmology* 2009; **116**: 981–989.
55. Seaman BJ, Guardiani EA, Brewer CC, *et al.* Audiovestibular dysfunction associated with adoptive cell immunotherapy for melanoma. *Otolaryngol Head Neck Surg* 2012; **147**: 744–749.
56. Morgan RA, Chinnasamy N, Abate-Daga D, *et al.* Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. *J Immunother* 2013; **36**: 133–151.
57. Suzuki M, Cheung NK. Disialoganglioside GD2 as a therapeutic target for human diseases. *Expert Opin Ther Targets* 2015; **19**: 349–362.
58. Mennel HD, Bosslet K, Geissel H, Bauer BL. Immunohistochemically visualized localisation of gangliosides Glac2 (GD3) and Gtri2 (GD2) in cells of human intracranial tumors. *Exp Toxicol Pathol* 2000; **52**: 277–285.
59. Wikstrand CJ, Fredman P, Svennerholm L, Bigner DD. Detection of glioma-associated gangliosides GM2, GD2, GD3, 3′-isoLM1 3′,6′-isoLD1 in central nervous system tumors in vitro and in vivo using epitope-defined monoclonal antibodies. *Prog Brain Res* 1994; **101**: 213–223.
60. Yuki N, Yamada M, Tagawa Y, Takahashi H, Handa S. Pathogenesis of the neurotoxicity caused by anti-GD2 antibody therapy. *J Neurol Sci* 1997; **149**: 127–130.
61. Dhillon S. Dinutuximab: first global approval. *Drugs* 2015; **75**: 923–927.
62. Pule MA, Savoldo B, Myers GD, *et al.* Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* 2008; **14**: 1264–1270.
63. Louis CU, Savoldo B, Dotti G, *et al.* Anti-tumor activity and long-term fate of chimeric antigen receptor positive T-cells in patients with neuroblastoma. *Blood* 2011; **118**: 6050–6056.
64. Heczey A, Louis CU, Savoldo B, *et al.* CAR T cells administered in combination with lymphodepletion and PD-1 inhibition to patients with neuroblastoma. *Mol Ther* 2017; **25**: 2214–2224.
65. Gargett T, Yu W, Dotti G, *et al.* GD2-specific CAR T cells undergo potent activation and deletion following antigen encounter but can be protected from activation-induced cell death by PD-1 blockade. *Mol Ther* 2016; **24**: 1135–1149.
66. Mount CW, Majzner RG, Sundaresh S, *et al.* Potent antitumor efficacy of anti-GD2 CAR T cells in H3-$K27M^+$ diffuse midline gliomas. *Nat Med* 2018; **24**: 572–579.
67. Buczkowicz P, Hoeman C, Rakopoulos P, *et al.* Genomic analysis of diffuse intrinsic pontine gliomas identifies three molecular subgroups and recurrent activating ACVR1 mutations. *Nat Genet* 2014; **46**: 451–456.
68. Hay KA, Hanafi LA, Li D, *et al.* Kinetics and biomarkers of severe cytokine release syndrome after CD19 chimeric antigen receptor-modified T-cell therapy. *Blood* 2017; **130**: 2295–2306.
69. Neelapu SS, Tummala S, Kebriaei P, *et al.* Toxicity management after chimeric antigen receptor T cell therapy: one size does not fit 'ALL'. *Nat Rev Clin Oncol* 2018; **15**: 47–61.
70. Lee DW, Santomasso BD, Locke FL, *et al.* ASTCT consensus grading for cytokine release syndrome and neurologic toxicity associated with immune effector cells. *Biol Blood Marrow Transplant* 2019; **25**: 625–638.
71. Poh A. JCAR015 in ALL: a root-cause investigation. *Cancer Discov* 2018; **8**: 4–5.
72. Gust J, Hay KA, Hanafi LA, *et al.* Endothelial activation and blood-brain barrier disruption in neurotoxicity after adoptive immunotherapy with CD19 CAR-T cells. *Cancer Discov* 2017; **7**: 1404–1419.
73. Santomasso BD, Park JH, Salloum D, *et al.* Clinical and biological correlates of neurotoxicity associated with CAR T-cell therapy in patients with B-cell acute lymphoblastic leukemia. *Cancer Discov* 2018; **8**: 958–971.
74. Topp MS, Gokbuget N, Stein AS, *et al.* Safety and activity of blinatumomab for adult patients with relapsed or refractory B-precursor acute lymphoblastic leukaemia: a multicentre, single-arm, phase 2 study. *Lancet Oncol* 2015; **16**: 57–66.
75. Ruella M, Maude SL, Engels B, *et al.* Clinical efficacy of anti-CD22 chimeric antigen receptor T cells for B-cell acute lymphoblastic leukemia is correlated with the length of the Scfv linker and can be predicted using xenograft models. *Blood* 2017; **130**: 807.

76. Richman SA, Nunez-Cruz S, Moghimi B, *et al.* High-affinity GD2-specific CAR T cells induce fatal encephalitis in a preclinical neuroblastoma model. *Cancer Immunol Res* 2018; **6**: 36–46.
77. Cheung NK, Guo H, Hu J, Tassev DV, Cheung IY. Humanizing murine IgG3 anti-GD2 antibody m3F8 substantially improves antibody-dependent cell-mediated cytotoxicity while retaining targeting in vivo. *Oncoimmunology* 2012; **1**: 477–486.
78. Liu X, Jiang S, Fang C, *et al.* Affinity-tuned ErbB2 or EGFR chimeric antigen receptor T cells exhibit an increased therapeutic index against tumors in mice. *Cancer Res* 2015; **75**: 3596–3607.
79. Ebert LM, Yu W, Gargett T, Brown MP. Logic-gated approaches to extend the utility of chimeric antigen receptor T-cell technology. *Biochem Soc Trans* 2018; **46**: 391–401.
80. Majzner RG, Mackall CL. Tumor antigen escape from CAR T-cell therapy. *Cancer Discov* 2018; **8**: 1219–1226.
81. Okada H, Weller M, Huang R, *et al.* Immunotherapy response assessment in neuro-oncology: a report of the RANO working group. *Lancet Oncol* 2015; **16**: e534–e542.
82. Gargett T, Brown MP. The inducible caspase-9 suicide gene system as a "safety switch" to limit on-target, off-tumor toxicities of chimeric antigen receptor T cells. *Front Pharmacol* 2014; **5**: 235.
83. Di Stasi A, Tey SK, Dotti G, *et al.* Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* 2011; **365**: 1673–1683.
84. Debinski W, Tatter SB. Convection-enhanced delivery for the treatment of brain tumors. *Expert Rev Neurother* 2009; **9**: 1519–1527.
85. Titov A, Petukhov A, Staliarova A, *et al.* The biological basis and clinical symptoms of CAR-T therapy-associated toxicites. *Cell Death Dis* 2018; **9**: 897.
86. Turtle CJ, Hanafi LA, Berger C, *et al.* CD19 CAR-T cells of defined $CD4^+$:$CD8^+$ composition in adult B cell ALL patients. *J Clin Invest* 2016; **126**: 2123–2138.
87. Norelli M, Camisa B, Barbiera G, *et al.* Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat Med* 2018; **24**: 739–748.
88. Weller M, Kaulich K, Hentschel B, *et al.* Assessment and prognostic significance of the epidermal growth factor receptor vIII mutation in glioblastoma patients treated with concurrent and adjuvant temozolomide radiochemotherapy. *Int J Cancer* 2014; **134**: 2437–2447.
89. Weller M, Butowski N, Tran DD, *et al.* Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. *Lancet Oncol* 2017; **18**: 1373–1385.
90. Sampson JH, Choi BD, Sanchez-Perez L, *et al.* EGFRvIII mCAR-modified T-cell therapy cures mice with established intracerebral glioma and generates host immunity against tumor-antigen loss. *Clin Cancer Res* 2014; **20**: 972–984.
91. Suryadevara CM, Desai R, Farber SH, *et al.* Preventing Lck activation in CAR T cells confers Treg resistance but requires 4-1BB signaling for them to persist and treat solid tumors in nonlymphodepleted hosts. *Clin Cancer Res* 2019; **25**: 358–368.
92. Slaney CY, von Scheidt B, Davenport AJ, *et al.* Dual-specific chimeric antigen receptor T cells and an indirect vaccine eradicate a variety of large solid tumors in an immunocompetent, self-antigen setting. *Clin Cancer Res* 2017; **23**: 2478–2490.
93. Grossman SA, Ye X, Lesser G, *et al.* Immunosuppression in patients with high-grade gliomas treated with radiation and temozolomide. *Clin Cancer Res* 2011; **17**: 5473–5480.
94. Arbour KC, Mezquita L, Long N, *et al.* Impact of baseline steroids on efficacy of programmed cell death-1 and programmed death-ligand 1 blockade in patients with non-small-cell lung cancer. *J Clin Oncol* 2018; **36**: 2872–2878.
95. Keskin DB, Anandappa AJ, Sun J, *et al.* Neoantigen vaccine generates intratumoral T cell responses in phase Ib glioblastoma trial. *Nature* 2019; **565**: 234–239.
96. Patil VS, Madrigal A, Schmiedel BJ, *et al.* Precursors of human $CD4^+$ cytotoxic T lymphocytes identified by single-cell transcriptome analysis. *Sci Immunol* 2018; **3**: eaan8664.
97. Chong EA, Melenhorst JJ, Lacey SF, *et al.* PD-1 blockade modulates chimeric antigen receptor (CAR)-modified T cells: refueling the CAR. *Blood* 2017; **129**: 1039–1041.
98. Maude SL, Frey N, Shaw PA, *et al.* Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* 2014; **371**: 1507–1517.
99. Lee DW, Kochenderfer JN, Stetler-Stevenson M, *et al.* T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet* 2015; **385**: 517–528.
100. Leen AM, Myers GD, Sili U, *et al.* Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med* 2006; **12**: 1160–1166.
101. Heslop HE, Slobod KS, Pule MA, *et al.* Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood* 2010; **115**: 925–935.
102. Hong JJ, Rosenberg SA, Dudley ME, *et al.* Successful treatment of melanoma brain metastases with adoptive cell therapy. *Clin Cancer Res* 2010; **16**: 4892–4898.
103. Yaghoubi SS, Jensen MC, Satyamurthy N, *et al.* Noninvasive detection of therapeutic cytolytic T cells with ^{18}F-FHBG PET in a patient with glioma. *Nat Clin Pract Oncol* 2009; **6**: 53–58.
104. Pellegatta S, Savoldo B, Di IN, *et al.* Constitutive and TNFα-inducible expression of chondroitin sulfate proteoglycan 4 in glioblastoma and neurospheres: implications for CAR-T cell therapy. *Sci Transl Med* 2018; **10**: eaao2731.
105. Shiina S, Ohno M, Ohka F, *et al.* CAR T cells targeting podoplanin reduce orthotopic glioblastomas in mouse brains. *Cancer Immunol Res* 2016; **4**: 259–268.
106. Jin L, Ge H, Long Y, *et al.* CD70, a novel target of CAR T-cell therapy for gliomas. *Neuro Oncol* 2018; **20**: 55–65.
107. Hegde M, Corder A, Chow KK, *et al.* Combinational targeting offsets antigen escape and enhances effector functions of adoptively transferred T cells in glioblastoma. *Mol Ther* 2013; **21**: 2087–2101.

108. Bielamowicz K, Fousek K, Byrd TT, *et al.* Trivalent CAR T cells overcome interpatient antigenic variability in glioblastoma. *Neuro Oncol* 2018; **20**: 506–518.
109. Wang D, Aguilar B, Starr R, *et al.* Glioblastoma-targeted $CD4^+$ CAR T cells mediate superior antitumor activity. *JCI Insight* 2018; **3**: e99048.
110. Krenciute G, Prinzing BL, Yi Z, *et al.* Transgenic expression of IL15 improves antiglioma activity of IL13Rα2-CAR T cells but results in antigen loss variants. *Cancer Immunol Res* 2017; **5**: 571–581.
111. Weiss T, Weller M, Guckenberger M, Sentman CL, Roth P. NKG2D-based CAR T cells and radiotherapy exert synergistic efficacy in glioblastoma. *Cancer Res* 2018; **78**: 1031–1043.