Combined cytotoxic and immune-stimulatory gene therapy for primary adult high-grade glioma: a phase 1, first-in-human trial

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Summary

Background High-grade gliomas have a poor prognosis and do not respond well to treatment. Effective cancer immune responses depend on functional immune cells, which are typically absent from the brain. This study aimed to evaluate the safety and activity of two adenoviral vectors expressing HSV1-TK (Ad-hCMV-TK) and Flt3L (Ad-hCMV-Flt3L) in patients with high-grade glioma.

Methods In this dose-finding, first-in-human trial, treatment-naive adults aged 18–75 years with newly identified high-grade glioma that was evaluated per immunotherapy response assessment in neuro-oncology criteria, and a Karnofsky Performance Status score of 70 or more, underwent maximal safe resection followed by injections of adenoviral vectors expressing HSV1-TK and Flt3L into the tumour bed. The study was conducted at the University of Michigan Medical School, Michigan Medicine (Ann Arbor, MI, USA). The study included six escalating doses of viral particles with starting doses of 1×10^{10} Ad-hCMV-TK viral particles and 1×10^{9} Ad-hCMV-Flt3L viral particles (cohort A), and then 1×10^{11} Ad-hCMV-TK viral particles and 1×10^{9} Ad-hCMV-Flt3L viral particles (cohort B), 1×10^{10} Ad-hCMV-TK viral particles and 1×10^{10} Ad-hCMV-Flt3L viral particles (cohort C), 1×10^{11} Ad-hCMV-TK viral particles and 1×10^{10} Ad-hCMV-Flt3L viral particles (cohort D), 1×10^{10} Ad-hCMV-TK viral particles and 1×10^{11} Ad-hCMV-Flt3L viral particles (cohort E), and 1×10^{11} Ad-hCMV-TK viral particles and 1×10^{11} Ad-hCMV-Flt3L viral particles (cohort F) following a 3+3 design. Two 1 mL tuberculin syringes were used to deliver freehand a mix of Ad-hCMV-TK and Ad-hCMV-Flt3L vectors into the walls of the resection cavity with a total injection of 2 mL distributed as 0·1 mL per site across 20 locations. Subsequently, patients received two 14-day courses of valacyclovir (2 g orally, three times per day) at 1–3 days and 10–12 weeks after vector administration and standad upfront chemoradiotherapy. The primary endpoint was the maximum tolerated dose of Ad-hCMV-Flt3L and Ad-hCMV-TK. Overall survival was a secondary endpoint. Recruitment is complete and the trial is finished. The trial is registered with ClinicalTrials.gov, NCT01811992.

Findings Between April 8, 2014, and March 13, 2019, 21 patients were assessed for eligibility and 18 patients with high-grade glioma were enrolled and included in the analysis (three patients in each of the six dose cohorts); eight patients were female and ten were male. Neuropathological examination identified 14 (78%) patients with glioblastoma, three (17%) with gliosarcoma, and one (6%) with anaplastic ependymoma. The treatment was well-tolerated, and no dose-limiting toxicity was observed. The maximum tolerated dose was not reached. The most common serious grade 3–4 adverse events across all treatment groups were wound infection (four events in two patients) and thromboembolic events (five events in four patients). One death due to an adverse event (respiratory failure) occurred but was not related to study treatment. No treatment-related deaths occurred during the study. Median overall survival was 21·3 months (95% CI 11·1–26·1).

Interpretation The combination of two adenoviral vectors demonstrated safety and feasibility in patients with high-grade glioma and warrants further investigation in a phase 1b/2 clinical trial.

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Introduction

High-grade gliomas of astrocytic, oligodendroglial, or ependymal origin, are the most common types of primary brain tumour in adults.[1] High-grade gliomas are associated with poor prognosis, despite standard therapy, leading to a median overall survival of around 14–16 months.[2] Gene therapy for malignant gliomas using viral vectors encoding a direct or conditional tumouricidal approach has been shown to be safe in humans.[3] Important challenges of gene therapy remain

Research in context

Evidence before this study

We searched PubMed to identify both preclinical and clinical studies using "TK AND Flt3L AND Glioma" as the search term. The search encompassed articles published between Jan 1, 1993, and May 23, 2023; no language restrictions were applied to the search. 28 articles were found, all authored by our research group and focused on preclinical models involving rodents, dogs, and marmosets. These articles served as the background information that was submitted as part of our investigational new drug application to the US Food and Drug Administration as the basis for the clinical evaluation of our approach in a phase 1 clinical trial. The progress made in glioma treatment in the past 20 years has been limited. Almost all clinical trials conducted thus far have not demonstrated robust clinical benefits for this disease. Adenovirus-expressing herpes simplex virus 1 thymidine kinase (Ad-TK) has shown the ability to induce infiltration of diverse immune cells, including macrophages and T cells, both in rodent models and clinical settings. However, the immune response against glioma by Ad-TK plus ganciclovir therapy has little anti-tumour activity without additional immunostimulatory strategies. Incorporating the co-expression of FMS-like tyrosine kinase 3 ligand (Flt3L), Ad-TK plus ganciclovir treatment stimulates a potent antitumour immune response in experimental settings.

Added value of this study

To our knowledge, this study is the first-in-human trial using two distinct adenoviral vectors in the treatment of high-grade glioma. It is also, as far as we know, the first study to show extended persistence of HSV1-TK in the peritumoral brain in human patients. This phase 1 trial showed that the dual-vector treatment was safe and tolerable in patients with high-grade glioma when combined with standard-of-care chemoradiotherapy.

Implications of all the available evidence

The strategy used in this trial holds the potential of eliciting targeted anti-glioma immune responses against residual malignant glioma cells that persist after surgical resection. The long-term persistence of HSV1-TK transgene expression suggests that continuous administration of valacyclovir could enhance the cytotoxic effect of Ad-hCMV-TK and improve patient outcomes. Our findings have the potential to improve overall survival and inform future schedules of administration of valacyclovir, and thus warrant further investigation in larger phase 2 clinical trials.

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the low efficacy of direct tumour cell killing in situ and insufficient distribution of the transgenic therapeutic products preventing elimination of infiltrating malignant cells.[4,5,6]

Initiation of an effective immune response against cancer requires functional dendritic cells, which are absent from the non-inflamed CNS, possibly underlying the lack of anti-high-grade glioma immune responses (appendix p 24). Treatment with FMS-like tyrosine kinase 3 ligand (Flt3L) recruits dendritic cells into the tumour microenvironment.[7–9] Herpes Simplex Virus type 1-thymidine kinase (HSV1-TK) is an enzyme that phosphorylates the prodrug valacyclovir and converts it into a nucleotide analogue which causes cell death of dividing cells such as in high-grade gliomas.[4,6,10] By combining Flt3L and conditionally cytotoxic HSV1-TK, the infiltrating dendritic cells recruited into the tumour microenvironment become exposed to endogenous glioma antigens (released by HSV1-TK dependent tumour cell cytotoxicity), and become activated by TLR2 agonists, such as HMGB1; these dendritic cells then become capable of inducing specific anti-glioma immune responses against malignant glioma cells that remain post-surgical excision (appendix p 24).[11–13] In pre-clinical studies this approach induced T-cell cytotoxicity and immune memory, and eliminated a large percentage (ie, 50–75%) of tumours in various mouse and rat models of glioblastoma.[13–15] The safety of this dual-vector treatment has been established in rodents, dogs, and marmosets, but not yet in humans.[12,15–17] We hypothesise that the co-administration of adenovirus-expressing HSV1-TK under the control of the major immediate early human cytomegalovirus promoter (Ad-hCMV-TK) and adenovirus-expressing Flt3L under the control of the major immediate early human cytomegalovirus promoter (Ad-hCMV-Flt3L) to the post-resection tumour bed followed by oral valacyclovir can be safely combined with standard-of-care chemoradiotherapy in patients with newly diagnosed high-grade glioma, and will stimulate an anti-high-grade glioma immune response.

See Online for appendix

Methods

Study design and participants

We performed a phase 1, open label, dose escalation trial with a 3+3 design. The study was conducted at the University of Michigan Medical School, Michigan Medicine (Ann Arbor, MI, USA). Consenting adult patients, aged 18–75 years, with newly identified supratentorial brain lesions compatible with a diagnosis of high-grade glioma that was amenable to attempted gross total resection with no previous treatment with gene therapy, chemotherapy, or radiotherapy were enrolled in the study. Disease was evaluated per immunotherapy response assessment in neuro-oncology criteria (iRANO). High-grade gliomas can include glioblastoma multiforme (WHO grade IV); gliosarcoma (WHO grade IV); anaplastic astrocytoma (WHO grade III); anaplastic oligodendroglioma (WHO grade III); and anaplastic ependymoma (WHO grade III). Other key inclusion criteria were Karnofsky Performance

Status (KPS) score of 70 or more; and adequate bone marrow, renal, and hepatic function (absolute neutrophil count ≥1500 cells per mm³; platelets ≥100 000 per mm³; haemoglobin ≥10·0 g/dL; blood urea nitrogen ≤30 mg/dL; creatinine ≤1·7 mg/dL; bilirubin ≤2·0 mg/dL; alanine aminotransferase to aspartate aminotransferase ratio ≤3×upper limit of normal). Exclusion criteria included tumour infiltration of the cerebellum, bilateral tumour spread, spreading to the ventricular system or brain stem, history of primary CNS disease that would interfere with patient evaluation, history of other cancer within 5 years, evidence of other clinically significant disease, pregnancy, or active systemic infection. Detailed information on eligibility is in the appendix (pp 3–4). The sex declaration occurred when the patients registered in the medical record. Provided options for sex at birth were: male, female, and intersex. There was no independent gender determination during the trial.

This clinical trial was reviewed, and ethics approval was obtained from the Institutional Review Board-MED at the University of Michigan School of Medicine (HUM00057130). An investigational new drug application was granted by the US Food and Drug Administration to PRL on April 16, 2011 (BB-IND 14574). All patients provided pre-operative written consent, and definitive enrolment occurred intraoperatively after confirming the presence of malignant glioma through pathology examination. The protocol is in the appendix (p 47).

Procedures

The viral vectors, Ad-hCMV-TK and Ad-hCMV-Flt3L, were produced at the Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, USA. Patients were assigned to six escalating doses of Ad-hCMV-TK and Ad-hCMV-Flt3L (appendix p 17). The starting doses were 1×10^{10} Ad-hCMV-TK viral particles and 1×10^{9} Ad-hCMV-Flt3L viral particles (cohort A; $1{\cdot}1\times10^{10}$ viral particles total), and the doses were escalated to 1×10^{11} Ad-hCMV-TK viral particles and 1×10^{9} Ad-hCMV-Flt3L viral particles (cohort B), 1×10^{10} Ad-hCMV-TK viral particles and 1×10^{10} Ad-hCMV-Flt3L viral particles (cohort C), 1×10^{11} Ad-hCMV-TK viral particles and 1×10^{10} Ad-hCMV-Flt3L viral particles (cohort D), 1×10^{10} Ad-hCMV-TK viral particles and 1×10^{11} Ad-hCMV-Flt3L viral particles (cohort E), and finally, 1×10^{11} Ad-hCMV-TK viral particles and 1×10^{11} Ad-hCMV-Flt3L viral particles (cohort F; 2×10^{11} viral particles total). In our implementation of the 3+3 method, a cohort of three patients is assigned to a dose. If none of the three patients had a dose-limiting toxicity, the next cohort is assigned to the next highest dose. If at least two of the three patients had a dose-limiting toxicity, the trial is halted, and the next lowest dose is selected as the maximum tolerated dose. Patients were assigned to dose cohorts in order of enrolment. Further details of the 3+3 design are in the appendix (p 47). The starting dose of Ad-hCMV-TK was higher than the starting dose of Ad-hCMV-Flt3L because adenoviral vectors expressing HSV1-TK have been used in previous trials up to doses of 2×10^{12}, and Ad-hCMV-Flt3L was being used for the first time in humans.[5] Two 1 mL tuberculin syringes were used to deliver freehand a mix of Ad-hCMV-TK and Ad-hCMV-Flt3L vectors into the walls of the resection cavity with a total injection volume of 2 mL distributed as 0·1 mL per site across 20 locations. Further surgical procedure and injection administration details are in the appendix (p 6).

Two 14-day courses of valacyclovir administered in 2 g doses orally, three times a day, started at 1–3 days and 10–12 weeks after vector administration (appendix pp 6–7). This approach was based on our animal data showing viral transgene expression up to 6 and 12 months after delivery of adenovirus into the brain.[18–20] Dose modification for valacyclovir was implemented as per protocol (if deemed necessary by the investigator and approved by the medical monitor; appendix pp 9, 10, 18–19). Depending on clinical status, dexamethasone was administered before surgery and immediately following surgery at doses individualised to each patient's clinical conditions (appendix p 8). Patients received standard-of-care chemoradiotherapy approximately 15–35 days after viral administration (appendix pp 7–8, 10).

Adverse events (ie, toxicity) were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. Any one of the following occurring within 21 days of dual-vector administration was considered a dose-limiting toxicity: grade 4 toxicity for constitutional symptoms with the exception of fever over 40°C for longer than 24 h, grade 3 or higher neurological toxicity relative to the changes from the pre-treatment neurological status and attributable to the study therapy regimen, grade 3 or higher non-haematological toxicity attributable to the study therapy regimen, and grade 2 or higher autoimmune events.[4,21,22]

Pretreatment evaluation was done within 14 days of surgery and included medical history, physical exam, mini mental state examination, KPS, vital signs, haematology, clinical chemistry, MRI, electrocardiogram, pregnancy test (if applicable), and blood collection (appendix p 5). On the day of surgery (day 0), assessments included vital signs and medication assessments (before surgery), intraoperative biopsy to confirm histological tumour type, and a post-operative MRI to determine the degree of tumour resection. Physicals, medication, adverse event, and mini mental state examination assessments were done on day 2 and weeks 2, 6, 10, 14, and 18. Haematology (complete blood count panel differential, prothrombin time, and partial thromboplastin time) was assessed on day 2. A comprehensive metabolic panel was done on day 2 and weeks 6, 10, 12, 14, and 18. KPS scores and pregnancy tests (if applicable) were assessed on weeks 2, 6, 10, 12, 14, and 18. Blood collection was scheduled at weeks 0, 2, 6, and 10. Survival follow ups were done at 12 and 24 months with overall survival followed up clinically.

Subsequent MRIs, including diffusion-weighted and dynamic susceptibility contrast enhanced images, and resections were performed if indicated clinically. The use of primary prophylaxis for *Pneumocystis carinii* and medically necessary standard-of-care treatment delays were at the discretion of treating oncologists (appendix pp 8–9). Race and ethnicity data were collected from the medical records.

A patient was considered off treatment if the patient withdrew treatment consent, did not complete the second course of valacyclovir, had dose-limiting toxicity, or if the clinical investigator decided it was in the best interest of the patient. A patient was considered off study if the patient withdrew consent to participate in the study or died. If the patient withdrew from the study, the study staff were able to use a public information source (eg, county records) when permissible, to obtain information about survival status only. In patients with a resection sample available from surgery after a recurrence, matched tissues were used to analyse histopathological indices of inflammatory responses using immunohistochemistry and multiplex immunofluorescence (see detailed methods in appendix pp 11–12).

Outcomes

The primary objective was to identify the maximum tolerated dose of Ad-hCMV-Flt3L and Ad-hCMV-TK administered into the tumour bed after maximal safe resection of malignant gliomas. The secondary objectives were to assess the potential benefit of the dual-vector treatment on primary high-grade glioma by measuring overall survival, 12-month and 24-month survival (overall survival was defined from the time of study enrolment [the date of dual-vector injection] to the time of death), as well as investigating the histopathological indices of inflammatory responses as a surrogate marker of immune response in patients in whom a resection specimen was available from a second surgery.

Statistical analysis

Our trial was based on the hypothesis that co-administration of Ad-hCMV-TK and Ad-hCMV-Flt3L viral vectors to any residual malignant glioma cells within the tumour bed, following surgical resection and treatment with valacyclovir in combination with the standard of care post-operative treatment with radiotherapy and chemotherapy (temozolomide), will be safe and can be effectively delivered without interference of standard therapies. Additionally, the combination therapeutic approach might have the ability to potentiate anti-tumour activity in patients with resectable malignant glioma.

As a Phase 1 study, this study was not powered to detect statistically significant differences in measures of clinical responses. The sample size was not defined in the protocol. In principle, given the 3+3 design, we could have recruited between 18 and 36 patients. Because we did not detect a dose-limiting toxicity, but had one patient withdraw and two patients excluded, our final number of patients screened was 21. Recruitment was not time-limited.

The Kaplan-Meier method was used to estimate 12-month and 24-month survival, and median overall survival along with 95% CIs. Post-hoc analyses included: progression-free survival (defined from the time of enrolment to the time of first progression or death using iRANO criteria; appendix p 14); pseudo-progression, correlation of pre-operative diffusion-weighted imaging, and dynamic susceptibility contrast enhanced imaging parameters by MRI with overall survival and progression-free survival using Pearson's coefficient (appendix pp 14–16); persistence of therapeutic transgene expression in patients through the analysis of tumour or serum samples; and serum anti-adenovirus antibody concentrations were correlated with vector dose, overall survival, and intracranial quantitative immune responses measured by immunohistochemistry.

Details regarding the statistical evaluation of the secondary objectives and post-hoc analyses, including immunohistochemistry, multiplex immunofluorescence, neighbourhood analyses, clinical and MRI procedures, and analysis of circulating Flt3L concentrations are in the appendix (pp 13–15). Immunohistochemistry, multiplex immunofluorescence, and Flt3L concentrations were analysed with linear mixed effects models. Progression-free survival was calculated with the Kaplan-Meier method. Clinical and MRI procedures, as well as cell to cell interactions, were analysed using Pearson's correlation analyses. RStudio Version 1.3.1073 was used for statistical analyses except for cell-cell correlations which were analysed in CytoMAP (version 1.4.21).

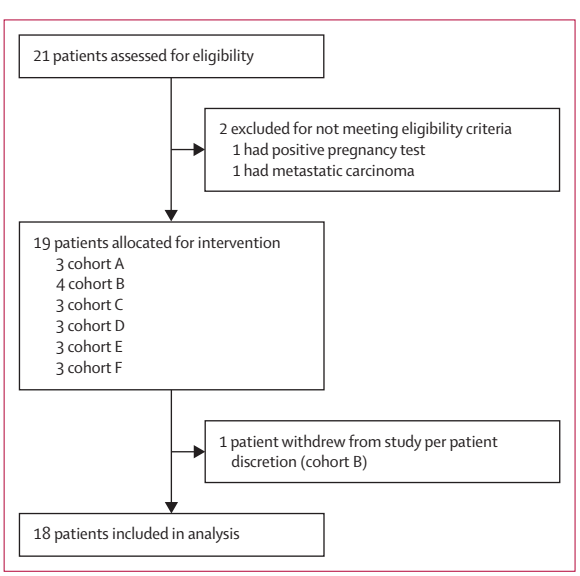


Figure 1: **Trial profile**

Significance was determined by p<0·05. This trial is registered with ClinicalTrials.gov, NCT01811992.

Role of the funding source

The funders of the study played no role in study design, data collection, data analysis, data interpretation or the writing of the report.

Results

Between April 8, 2014, and March 13, 2019, 21 patients were assessed for eligibility; of these, two were excluded, one because of pregnancy and the other because of metastatic carcinoma, and 19 were allocated for intervention (cohort A n=3, cohort B n=4, cohort C n=3, cohort D n=3, cohort E n=3, and cohort F n=3). One patient withdrew from the study per patient discretion, and the remaining 18 patients with high-grade glioma were included in the analysis; of these patients, eight were female and ten were male (figure 1; table 1; appendix p 20). The data collection cut-off for the study was Jan 14, 2021.

17 (95%) of the 18 tumours were diagnosed as glioblastoma (three [17%] were of the gliosarcoma subtype), and one (5%) was anaplastic ependymoma. All tumours were *IDH* wildtype, and seven (39%) had *MGMT* promoter methylation (table 1; appendix p 20).

Six (33%) patients experienced adverse events that were possibly related to study treatment which ranged from CTCAE grades 1 to 3 (table 2) and consisted of one patient per following toxicities: dyspepsia (grade 1), elevated creatinine (grade 2), dizziness (grade 1), encephalopathy (grade 3), headache (grade 1), seizure (grade 2 and 3), confusion (grade 2), hallucinations (grade 1), acute kidney injury (grade 3), and maculo-papular rash (grade 1).

Ten (56%) patients had serious adverse events (table 3) which ranged from CTCAE grades 2 to 5. There was one death due to a serious adverse event observed in patient 6 which involved the development of pneumocystis pneumonia, which subsequently led to respiratory failure and, the patient's death. This death was not considered to be related to the study treatment. Patient 15 had an intracranial haemorrhage within the tumour bed, which was surgically evacuated, and was given a grade 4 classification. 7 days after the initial surgery and dual-vector treatment, the primary care physician of patient 15 initiated enoxaparin treatment (80 mg every 12 h subcutaneously) for prevention of pulmonary embolism and deep vein thrombosis. 6 days after starting enoxaparin, the patient presented with an intracranial haemorrhage and underwent surgical

	KPS	Dual vector dose cohort	Extent of first surgery*	Diagnosis	*MGMT* promotor methylated	*IDH1* status	Steroids before first surgery*	Subsequent surgery, months from first surgery; $PD^{\%tumor\ if\ \leq 50\%}$ or $PsP^{\%tumor}$			Overall survival, months
								Surgery 2	Surgery 3	Surgery 4	
Patient 1	90	A	Near GTR	Glioblastoma	No	Wild type	No	14·5; $PD^{20\%}$	24·0; $PD^{30\%}$	..	27·5
Patient 2	80	A	GTR	Anaplastic ependymoma	No	Wild type	Yes	13·0; PD	24·9; PD	26·7; PD	52·7
Patient 3	90	A	GTR	Glioblastoma	Yes	Wild type	Yes	17·0; PD	..	..	24·3
Patient 4	90	B†	Near GTR	Glioblastoma	No	Wild type	Yes	..	..	..	..
Patient 5	100	B	Near GTR	Glioblastoma	No	Wild type	Yes	19·4; $PsP^{0\%}$	..	..	26·1
Patient 6	70	B	Near GTR	Glioblastoma	Yes	Wild type	No	..	..	..	5·3
Patient 7	100	B	Near GTR	Glioblastoma	No	Wild type	Yes	13·9; $PsP^{2\%}$	..	..	21·9
Patient 8	90	C	Near GTR	Glioblastoma	Yes	Wild type	No	7·6; $PD^{45\%}$	19·0; $PsP^{5\%}$	..	33·3
Patient 9‡	..	..	..	..	..	..	..	..	..	..	..
Patient 10	70	C	GTR	Glioblastoma	Yes	Wild type	No	..	..	..	9·4
Patient 11	90	C	Near GTR	Glioblastoma	No	Wild type	No	..	..	..	21·0
Patient 12	100	D	GTR	Gliosarcoma	No	Wild type	Yes	..	..	..	11·1
Patient 13	100	D	GTR	Glioblastoma	No	Wild type	No	..	..	..	20·5
Patient 14	100	D	GTR	Glioblastoma	No	Wild type	No	9·2; PD	..	..	21·6
Patient 15	100	E	GTR	Glioblastoma	Yes	Wild type	No	..	..	..	8·1
Patient 16	90	E	GTR	Glioblastoma	Yes	Wild type	Yes	..	..	..	>59·0
Patient 17§	..	..	..	..	..	..	..	..	..	..	..
Patient 18	90	E	GTR	Glioblastoma	No	Wild type	No	..	..	..	16·6
Patient 19	90	F	STR	Gliosarcoma	No	Wild type	Yes	..	..	..	7·1
Patient 20	100	F	GTR	Gliosarcoma	Yes	Wild type	Yes	..	..	..	15·7
Patient 21	100	F	GTR	Glioblastoma	No	Wild type	Yes	16·6; $PD^{50\%}$	28·6; $PD^{20\%}$	..	39·7

KPS=Karnofsky performance status. GTR=gross total resection. STR=subtotal resection. PD=progression of disease. PsP=pseudoprogression. *Initial surgery at the time of enrollment. †Non-evaluable per protocol due to early withdrawal per patient discretion. Dose level participant replaced and participant 4 excluded from survival analysis. ‡Excluded from trial because of positive pregnancy test. §Excluded from trial because of metastatic carcinoma.

Table 1: **Patient demographics, baseline characteristics, and surgery characteristics by patient identification number**

	Grade 1	Grade 2	Grade 3
Dual vector dose cohort A (n=3)			
Nervous system disorders			
Seizure	0	0	1
Dual vector dose cohort B (n=3)			
Nervous system disorders			
Seizure	0	1*	0
Skin and subcutaneous disorders			
Rash maculo-papular	1	0	0
Dual vector dose cohort D (n=3)			
Gastrointestinal disorders			
Dyspepsia	1	0	0
Nervous system disorders			
Headache	1	0	0
Dual vector dose cohort F (n=3)			
Nervous system disorders			
Dizziness	1	0	0
Hallucinations	1	0	0
Encephalopathy	0	0	1*
Investigations			
Creatinine increased	0	1	0
Psychiatric disorders			
Confusion	0	1	0
Renal and urinary disorders			
Acute kidney injury	0	0	1*

There were no grade 4 or grade 5 adverse events possibly related to study treatment and no adverse events possibly related to study treatment reported in dual vector dose cohorts C and E. *Serious adverse events.

Table 2: **Adverse events possibly related to study treatment in evaluable patients (n=18)**

	Grade 2	Grade 3	Grade 4	Grade 5
Gastrointestinal disorders				
Nausea	0	1 (6%)	0	0
General disorders and administration site conditions				
Pain	1 (6%)	1 (6%)	0	0
Infections and infestations				
Urinary tract infection	2 (11%)	0	0	0
Wound infection	0	4 (22%)	0	0
Injury, poisoning, and procedural complications				
Wound dehiscence	0	1 (6%)	0	0
Metabolism and nutrition disorders				
Dehydration	0	1 (6%)	0	0
Nervous system disorders				
Cognitive disturbance	0	1 (6%)	0	0
Dysphasia	2 (11%)	0	0	0
Oedema cerebral	0	0	1 (6%)	0
Encephalopathy	0	1 (6%)*	0	0
Intracranial haemorrhage	0	0	1 (6%)	0
Lethargy	1 (6%)	0	0	0
Right side neglect	0	1 (6%)	0	0
Seizure	1 (6%)*	1 (6%)*	0	0
Syncope	0	1 (6%)	0	0
Confusion	0	1 (6%)	0	0
Renal and urinary disorders				
Acute kidney injury	0	1 (6%)*	0	0
Respiratory, thoracic, and mediastinal disorders				
Respiratory failure	0	0	0	1 (6%)
Vascular disorders				
Thromboembolic event	0	5 (28%)	0	0

Data are n (%). *Possibly attributable to study intervention.

Table 3: **All serious adverse events (n=18)**

evacuation 2 days later. Subsequently, the patient was discharged in a stable condition 3 days after the evacuation. The occurrence of the intracranial haemorrhage was not considered to be related to the study treatment but related to the anti-coagulant treatment. Patient 20 had a grade 2 seizure, grade 3 encephalopathy, and grade 3 acute kidney injury, all of which were possibly related to valacyclovir. None of the other patients had serious adverse events related to the study treatments. However, there were serious adverse events unrelated to the study treatments: grade 3 nausea, grade 2–3 pain, grade 3 syncope, grade 3 dehydration, and grade 5 respiratory failure (patient 6); grade 2 urinary tract infection (two events in patient 20); grade 3 wound infection (patient 12; three events in patient 20); grade 3 wound dehiscence (patient 12); grade 3 cognitive disturbance (patient 16); grade 2 dysphasia (patients 13 and 16); grade 4 cerebral oedema (patient 19); grade 4 intracranial haemorrhage (patient 15); grade 2 lethargy (patient 20); grade 3 right side neglect (patient 16); grade 3 seizure (patient 8); grade 3 confusion (patient 10); and grade 3 thromboembolic events (two events in patient 7; one each in patients 15, 16, and 20)

No dose-limiting toxicity of the dual-vectors was observed, and the maximum tolerated dose of Ad-hCMV-TK and Ad-hCMV-Flt3L vectors was not reached. Other adverse events which were unrelated to study treatments are summarised in the appendix (pp 22–23).

The median overall survival from the time of enrolment was 21·3 months (95% CI 11·1–26·1). 14 patients died due to disease progression, one patient died due to sepsis unrelated to the study, one patient died of acute respiratory distress syndrome unrelated to the study, and one patient died due to an unknown cause. The 12-month overall survival was 72·2% (95% CI 45·6–87·0) and the 24-month overall survival was 38·8% (17·5–60·0; figure 2A). Figure 2B shows major events occurring in patients after treatment. Three of 18 patients were still alive at 3 years, two were still alive at 4 years, and one patient is still alive 59 months after enrolment (patient 16). Progression of disease was observed in all patients. The median progression-free survival was 9·9 months (95% CI 7·3–13·3; post-hoc analysis).

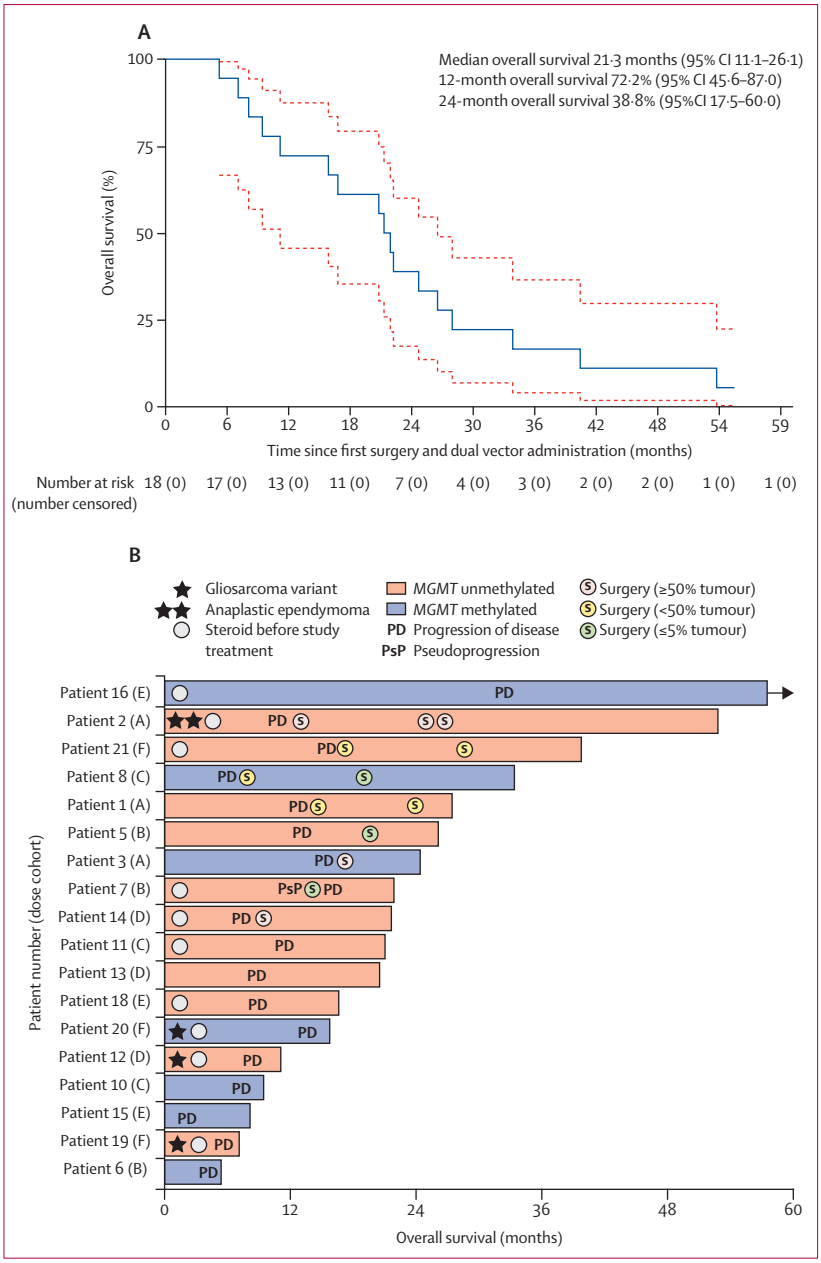


Figure 2: **Overall survival and major events post dual vector treatment**
(A) The solid line is the Kaplan-Meier survival curve and the dashed lines are the 95% CI. (B) Swimmer plots: bars represent each patient's disease progression and overall survival in months . Steroid use at the time of enrolment is indicated. Patient 16 with a rightward arrow was alive at 59 months from start of treatment and is being followed up for survival. Annotations correspond to the respective timing of first progression and pseudoprogression per immunotherapy response assessment in neuro-oncology criteria (if known). Subsequent surgeries with variable proportion of viable tumour is noted for eight of the 18 patients.

In post-hoc analyses, pre-operative diffusion-weighted imaging (n=9) and dynamic susceptibility contrast enhanced imaging (n=18) parameters were evaluated. There were weak positive correlations between baseline pre-operative normalised relative cerebral blood volume and overall survival (r=0·53), and between pre-operative normalised apparent diffusion coefficient mean and overall survival (r=0·49). No significant correlations were found between other MRI parameters and survival (overall survival or progression-free survival; data not shown).

Using immunohistochemistry in eight patients for whom a second surgical specimen was available, we found no significant differences between primary and recurrent tumours in CD45$^+$ cells (appendix pp 25–26), or monocytic lineage cells (macrosialin [CD68$^+$] cells; appendix pp 27–28). However, there was a significant increase in CD3e$^+$ cells (T cells) in recurrent tumours (p<0·0001; figure 3A; appendix pp 29–30). There was no difference in CD4$^+$ cells (appendix pp 31–32), but a statistically significant increase of CD8$^+$ cells (p<0·0001; figure 3B; appendix pp 33–34).

Multiplex immunofluorescence analysis showed no significant differences in the proportions of conventional dendritic cells or monocytes, double negative T cells, non-cytotoxic CD4$^+$ T cells, cytotoxic CD4$^+$ T cells, regulatory T cells, macrophages, or other leukocytes between primary and recurrent tumours (appendix p 35). Six of eight patients showed reduced proportions of regulatory T cells in the recurrent tumour microenvironment (appendix pp 35–36). There were significant increases in CD8$^+$ T cells (p<0·0001), plasmacytoid dendritic cells (p<0·0001), double positive T cells (p=0·0022), and cytotoxic CD8$^+$ T cells (p=0·031; figure 3C–F).

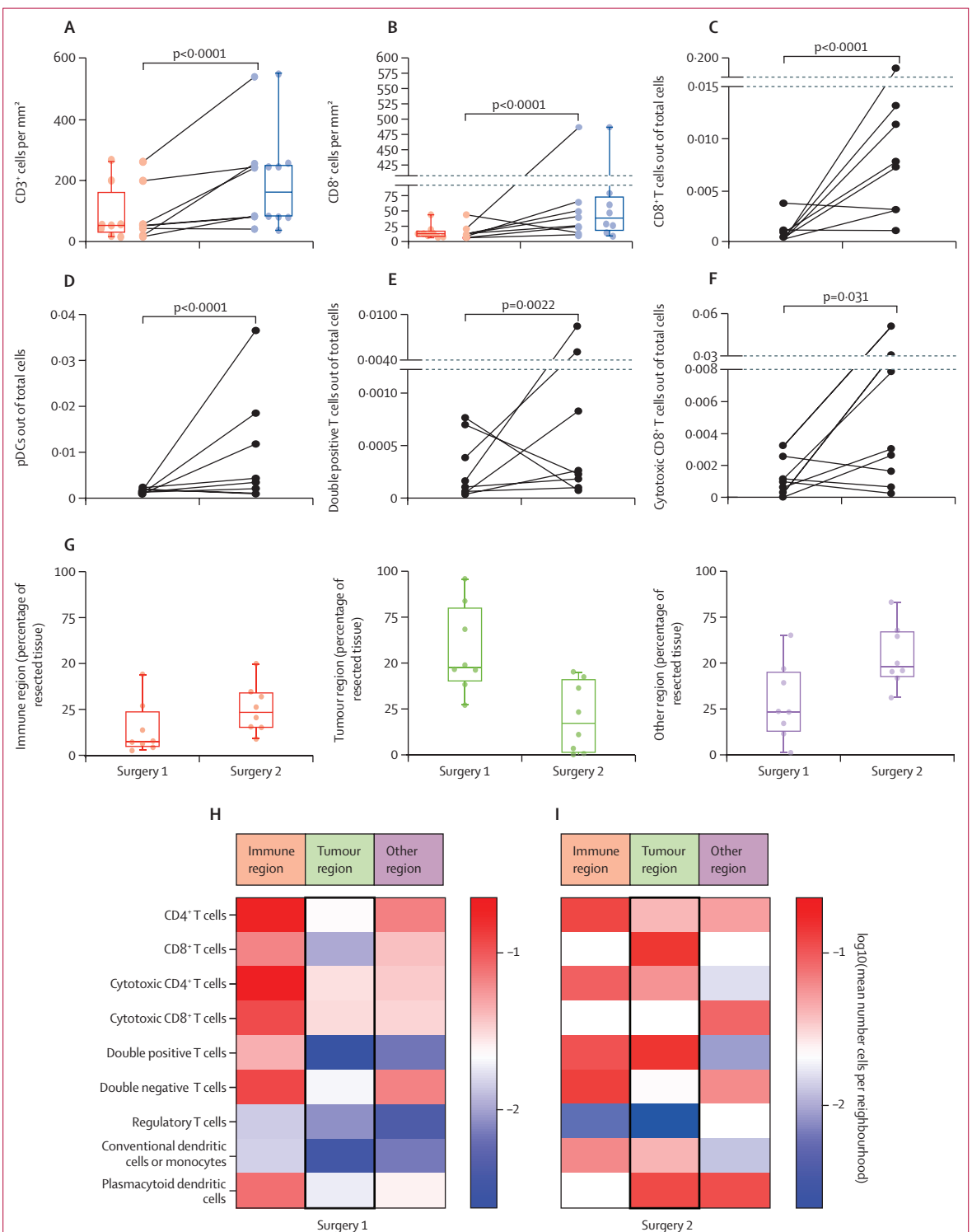
To be able to compare our data with historical controls, we calculated the proportion of CD8$^+$ T cells in the CD3e$^+$ cell compartment for primary and recurrent tumours. Overall, there was a 28% increase in the proportion of CD8$^+$ T cells within the CD3$^+$ compartment between primary and recurrent tumours (n=8). Furthermore, we found increased cell to cell interactions between many T-cell subsets and macrophages in recurrent tumours, and interactions between tumour cells and macrophages decreased (appendix p 37).

Using neighbourhood analysis, three distinct regions were identified in all matched surgical samples: the immune region, the tumour region, and the other region. The immune region, defined by a high density of CD45$^+$ cells, increased in proportion in six patients by mean 21·98% (SD 5·6) and decreased in two patients by 18·1% and 23·6%. The tumour region, defined by a high density of transcription factor SOX-2 (SOX2$^+$) cells, decreased in size by mean 36·5% (SD 10·7; n=8; figure 3G; appendix pp 38–40). The other region was characterised by a high density of cells that were both SOX2$^-$ and CD45$^-$, making them unclassifiable using our scheme. Analysis of region composition revealed increased densities of various immune cell types within the tumour region compared with the immune and other regions in recurrent tumours, including CD8$^+$ T cells, double positive T cells, and plasmacytoid dendritic cells (figure 3H–I).

We found HSV1-TK positive cells in five of eight recurrent tumours using immunofluorescence and immunohistochemistry (patients 2, 7, 8, 14, and 21) including a patient who seroconverted (patient 14). Of

the tumours analysed so far, patient 21 exhibited the longest period of expression 17 months after dual vector administration (figure 3J; appendix pp 41–44).

Analysis of circulating serum concentrations of Flt3L shows that the concentrations 2 weeks after treatment were significantly higher than the initial values (p=0·0041;



(Figure 3 continues on next page)

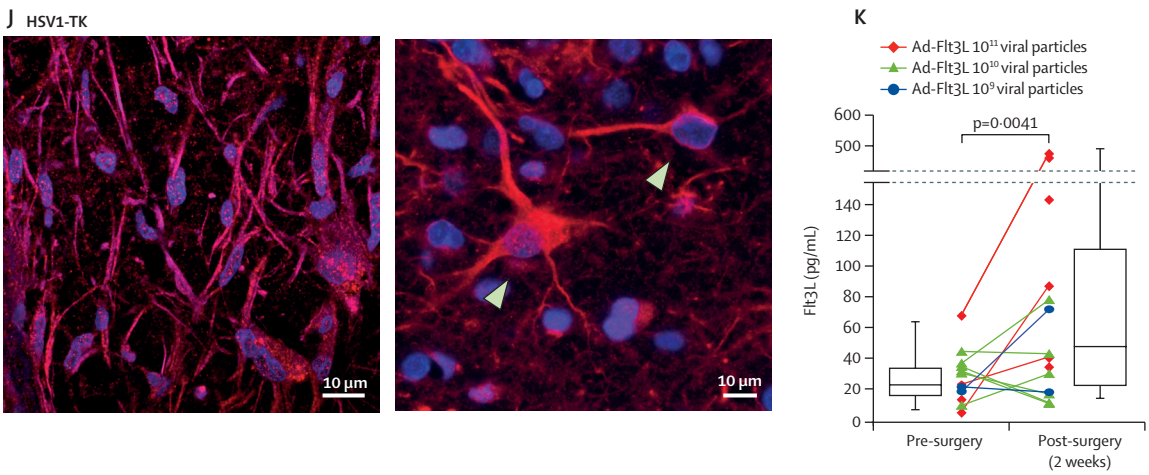


Figure 3: **Infiltration of immune cells and transgene expression post dual vector treatment**
Box plots and line graphs represent the quantification of $CD3^+$ cell numbers (cells per mm^2; A) and $CD8^+$ cell numbers (cells per mm^2; B) using QuPath positive cell detection on high-grade glioma samples obtained pre-TK-Flt3L treatment (surgery 1) and post-surgery upon recurrence (surgery 2). Plots represent mean ±standard error of the mean. The analysis included eight patients (patients 1, 2, 3, 5, 7, 8, 14, and 21). For each marker, a linear mixed effects model was used to compare the number of positive cells (log-transformed) between two surgery procedures. Random effects were included in the linear mixed effects model to correct for correlations between data measured on the same patient. Representative fields of all the patients for $CD3^+$ and $CD8^+$ are shown in the appendix (pp 29–30, 33–34). Multiplex immunofluorescence quantification of $CD8^+$ T cells ($SOX2^-CD45^+CD3e^+CD4^-CD8^+$ [GzB^- and $CD107a^-$]) (C), plasmacytoid dendritic cells ($SOX2^-CD45^+CD3e^-IBA1^-HLA\text{-}DR^+CD11c^-$)(D), double positive T cells ($SOX2^-CD45^+CD3e^+CD4^+CD8^+$) (E), and cytotoxic $CD8^+$ T cells ($SOX2^-CD45^+CD3e^+CD4^-CD8^+$[$GzB^+$ or $CD107a^+$]) (F) out of total cells. Panels C–F display spaghetti plots for surgery 1 samples and surgery 2 samples. The results reported included the cell type rate ratio and p value (adjusted using the Sidak correction method). (G) The tumour microenvironment in high-grade glioma tissues was analysed at primary tumour resection (surgery 1) and at tumour recurrence (surgery 2) using a neighbourhood approach. Box plots display region prevalence in tissues across all eight patients. Individual plots for all patients are in the appendix (pp 38–40). The box plot displays the five-number summary: the minimum (bottom whisker), the first quartile (bottom of the box to the middle line), the median (middle line), the third quartile (the middle line to the top of the box), and the maximum (the upper whisker). (H and I) Heatmaps displaying cluster composition across matched tumour samples at surgery 1 (H) and surgery 2 (I). Cluster composition is the mean number of cells per neighbourhood. Cell type densities within a region relative to other regions are represented from red (highest value) to blue (lowest value) with white being the baseline colour.
(J) Immunoreactivity for HSV1-TK (red) detected by immunofluorescence 17 months following dual vector administration. The left micrograph shows cellular processes immunoreactive for HSV1-TK; two immunoreactive cell bodies are indicated by arrowheads in the right micrograph. Nuclei are shown in blue (DAPI) and the scale bar is 10 µm (patient 21). (K) Circulating concentration of Flt3L in patients 5, 7, 8, 10, 11, 12, 13, 14, 15, 16, 18, 19, 20, and 21 at initial screening and 2 weeks after treatment.

14 patients were included in the analysis, and four patients' samples were not collected; figure 3K). Following vector delivery, there was a significant increase in antibody titres (p=0·0005; appendix p 45), with no correlation between vector dose, overall survival, or intracranial quantitative immune responses measured by immunohistochemistry (data not shown).

Discussion

Our phase 1 trial showed that the dual-vector treatment expressing HSV1-TK (Ad-hCMV-TK) and Flt3L (Ad-hCMV-Flt3L) was well tolerated, with no dose-limiting toxicity attributable to the administration of adenoviral vectors. There were occasional mild to moderate toxicities that were possibly related to valacyclovir treatment. However, we cannot definitively conclude whether the toxicity of valacyclovir was increased or decreased compared with its toxicity when given alone, because our sample size is too small to make this determination. A study of adenovirus injected into the tumour determined the dose-limiting toxicity to be 2×10^{12} viral particles.[5] Thus, it is recommended that trials using adenoviral vector doses remain one log below this dose.

Although our study was not powered for survival analysis, the median overall survival of 21·3 months (95% CI 11·1–26·1) is promising, especially considering that glioblastoma has a known historical overall survival rate of 14·6 months (95% CI 13·2–16·8); although, high complexity centres report overall survival of 19·6 months (17·8–21·2).[2] One patient is still alive 59 months after vector administration (patient 16). The range of survival at 2 years in four previous trials using Ad-TK and prodrug was 25·21–42·86%, and the range of overall survival was 10·66–25·3 months.[4,5,6,21,23] Future directions might consider the addition of immune checkpoint blockade or methods to reduce the strong immunosuppressive tumour microenvironment, or both. The increase in circulating antibody titres after administration of viral vectors suggests that resection cavity injection of adenoviral vectors has the potential to prime or bolster the systemic humoral immune response, or both. Interestingly, persistent HSV1-TK immune reactivity in brain tumour samples was also detected in a patient who seroconverted (appendix pp 43–45).

Multiplex immunofluorescence and immunohistochemistry from matched tumours showed increased

CD8+ T cells in all patients but in the patient with anaplastic ependymoma (patient 2; figure 3B), suggesting a potential differential response to therapy compared with glioblastoma. The increase in plasmacytoid dendritic cells was consistent with our preclinical findings.[7,24] Although some studies have reported an increase in CD8+ T cells at recurrence in response to standard-of-care,[25] the magnitude of increase seen in our trial was higher. For example, Mohme and colleagues[25] found a 5% mean increase in CD8+ cells within the CD3+ compartment between primary and recurrent glioblastoma, which is less than the increase seen in our multiplex immunofluorescence. We believe that CD8+ cells are beneficial to the patient and contribute to an effective anti-glioma immune response. Nevertheless, it is crucial to validate this assumption in larger phase 2 or 3 trials. These trials will provide a clearer understanding of the effect of enhanced immune infiltration on patient outcomes and survival. By quantifying the subsets of immune cells and conducting a cell neighbourhood analysis, we showed that proportions of immune cell subsets vary differently than those in comparable studies on high-grade glioma, and immune infiltration occurs both around and within regions densely populated with tumour cells. We believe these differences could be due to the administration of our viral vectors. However, our cell to cell interaction data suggest that immunosuppressive macrophages could reduce the therapeutic impact of the increased number of CD8+ T cells and plasmacytoid dendritic cells, as described in 2021.[26]

Since the development of gene therapy in the 1980s there have been close to 60 viral gene therapy trials for brain tumours.[27] Viral vectors have been used to deliver conditional cytotoxicity, oncolysis, and cytokines; viral vectors have also been used in combination with checkpoint inhibitors.[27] In gene therapy trials, clinical outcomes (ie, overall survival) have been less impressive than expected, with only small numbers of patients displaying long-term survival. In a 2022 publication up to six injections of intratumoural oncolytic HSV-G47Δ were used for the treatment of residual or recurrent glioblastoma in a phase 2 trial that reported promising 1-year survival rates.[28]

A strategy to reduce potential off-target toxicity is to regulate transgene expression. The results of a phase 1 trial by Chiocca and colleagues[29] showed the feasibility and tolerability of a regulated form of IL-12 injected into the resection cavity walls of patients with recurrent high-grade glioma. Furthermore, pseudoprogression has been reported in various trials using oncolytic virus or viral vectors.[22,28,30,31] Although the incidence of pseudo-progression appears lower in our trial, a proper confirmation will need to await larger phase 2 or 3 comparative trials.

Limitations of our phase 1 study, which are shared by other early phase trials, are the low sample size, the absence of a proper control group, and the absence of in vivo detection methods for transgene expression and immune infiltration. Correlative studies have yielded more promising results, with several indicating an increase in immune cells within areas of the tumour microenvironment that had previously been treated with gene therapy vectors, potentially transforming so-called immunologically cold tumours into hot ones.[32] Cold tumours are devoid of immune and inflammatory cells and hot tumours are enriched in these cell types. Our analysis provides evidence of a comparable transition from a cold to a hot tumour in this trial.

Our approach to high-grade glioma treatment using gene therapy exhibits several novel characteristics: the use of two distinct adenoviral vectors, the recruitment of plasmacytoid dendritic cells to the tumour micro-environment, and the extension of the administration of valacyclovir. A rationale for extending the administration of valacyclovir even further in future trials is based on preclinical, and now clinical data demonstrating that adenoviral vector mediated transgene expression can last up to 17 months.[18–20] Additionally, it could be equally important to consider multiple vector administrations, as described in a 2022 study.[28] Our finding of persistent HSV1-TK expression coupled with promising survival data and evidence of recruitment of immune cells, suggests that this treatment might have potential for the management of the disease and warrants further investigation in a phase 1b/2 clinical trial.

Contributors

MGC and PRL conceptualised the study and study design. YU, DO, LJ, JH, OS, DL, AM, SH-J, WA-H, TH, KS, and KM did patient enrolment and treatment. SCP, APL, PM, and SV did neuropathology. MMK, DRW, and TL did radiotherapy. MLV, MEJW, SMF, AC, DZ, VNY, PD, MGC, and PRL did the exploratory immunological and histopathological analysis. YU, MLV, MEJW, SMF, AC, MGC, and PRL did the figures. YU, MLV, MEJW, SMF, AC, DA, RK, MGC, and PRL curated the data. YU, LZ, MLV, and MEJW did the statistical analysis. YU, LJ, AM, MLV, MEJW, SMF, AC, LZ, MGC, and PRL did the analysis and data interpretation. YU, SMF, MGC, and PRL wrote the original draft of the manuscript. All authors thoroughly reviewed the data, made valuable contributions to the development of the manuscript, and provided their approval for the final version to be published. YU, MLV, MEJW, SMF, AC, LZ, MGC, and PRL verified the data. MGC and PRL fund raised.

Declaration of interests

We declare no competing interests.

Data sharing

The trial protocol is provided in the appendix. Data for this study can be made available upon request and approval by the study principal investigator and subject to appropriate data transfer agreements. Requests should be directed to PRL.

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