Glioblastoma-mediated Immune Dysfunction Limits CMV-specific T Cells and Therapeutic Responses: Results from a Phase I/II Trial

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ABSTRACT

Purpose: Cytomegalovirus (CMV) antigens occur in glioblastoma but not in normal brains, making them desirable immunologic targets.

Patients and Methods: Highly functional autologous polyclonal CMV pp65-specific T cells from patients with glioblastoma were numerically expanded under good manufacturing practice compliant conditions and administered after 3 weeks of lymphodepleting dose-dense temozolomide (100 mg/m^2) treatment. The phase I component used a 3+3 design, ascending through four dose levels (5×10^6–1×10^8 cells). Treatment occurred every 6 weeks for four cycles. *In vivo* persistence and effector function of CMV-specific T cells was determined by dextramer staining and multiparameter flow cytometry in serially sampled peripheral blood and in the tumor microenvironment.

Results: We screened 65 patients; 41 were seropositive for CMV; 25 underwent leukapheresis; and 20 completed ≥1 cycle. No dose-limiting toxicities were observed. Radiographic response was complete in 1 patient, partial in 2. Median progression-free survival (PFS) time was 1.3 months [95% confidence interval (CI), 0–8.3 months]; 6-month PFS was 19% (95% CI, 7%–52%); and median overall survival time was 12 months (95% CI, 6 months to not reached). Repeated infusions of CMV-T cells paralleled significant increases in circulating CMV$^+$ CD8$^+$ T cells, but cytokine production showing effector activity was suppressed, especially from T cells obtained directly from glioblastomas.

Conclusions: Adoptive infusion of CMV-specific T cells after lymphodepletion with dose-dense temozolomide was well tolerated. But apparently CMV seropositivity does not guarantee tumor susceptibility to CMV-specific T cells, suggesting heterogeneity in CMV antigen expression. Moreover, effector function of these T cells was attenuated, indicating a requirement for further T-cell modulation to prevent their dysfunction before conducting large-scale clinical studies.

Introduction

Cytomegalovirus (CMV) is a B-herpes virus that is very common in humans and leads to asymptomatic infection followed by viral persistence and latency. Seroprevalence is over 60% (1) in adults and is associated with a strikingly high fraction (10% or more) of circulating T lymphocytes targeting CMV (2). Use of tetramers has shown that pp65 is recognized by a high fraction of T cells (3). CMV antigens have shown very frequent expression in glioblastoma (>90%) but not normal brain (4, 5). Previously, patients with glioblastoma treated with an autologous tumor lysate-pulsed dendritic cell (DC) immunotherapy were shown to have robust CMV-specific CD8$^+$ T-cell responses to the pp65 CMV immune-dominant epitope (6), indicating that this epitope was capable of generating immune reactivity in patients with glioblastoma.

The safety and potential clinical efficacy of an autologous CMV-specific T-cell therapy as consolidative treatment for recurrent glioblastoma was previously found to be safe and produced a median overall survival (OS) time of 403 days (7). Whereas some patients showed a small increase in virus-specific T cells in the peripheral blood after the first few infusions, this effect was transient. Although this study provided a proof-of-principle assessment of adoptive T-cell immunotherapy in glioblastoma, a number of limitations in the study design may have contributed to the transient responses: (i) only CMV-specific CD8$^+$ T cells were infused, without the concurrent transfer of CD4$^+$ Th cells, with the latter population being crucial for the maintenance of tumor antigen–specific CD8$^+$ T-cell populations; (ii) T cells were numerically expanded in the presence of IL2, which is also likely to support the expansion of Foxp3-positive regulatory T cells, leading to further inhibition of the expansion, cytotoxicity, and persistence of adoptively infused CMV-specific CD8$^+$ T cells *in vivo*; (iii) the patients included in this study did not receive uniform

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Translational Relevance

Immune effector function of cytomegalovirus (CMV)-specific T cells within patients with glioblastoma, including the tumor microenvironment, was analyzed and showed that their immune activity was significantly blunted, indicating that additional modulation will be required to obtain antitumor effector responses. Only some patients show an antitumor function of autologous CMV-specific T cells despite an adequate CMV-specific response, highlighting the need for prognostic indicators to assist in future patient selection.

chemotherapy prior to T-cell therapy to provide the benefit of *in vivo* clonotypic expansion; and (iv) no tissue correlates were included to evaluate the direct immunologic effects after intravenous administration of CMV-stimulated T cells.

Further support for considering a CMV immunotherapy strategy comes from a phase II clinical trial of patients newly diagnosed with glioblastoma who were treated with autologous DCs pulsed with CMV mRNA pp65. This demonstrated an unprecedented progression-free survival (PFS) time and OS time in which the median survival exceeded 40 months in a small cohort of subjects (8). However, dendritic cell approaches entail complex and expensive processing.

Use of donor-derived antigen-specific T cells is routinely used for immune reconstitution in transplant patients (9, 10) and has been studied in phase I and II trials. To be suitable for clinical applications, the cells used for adoptive T-cell transfer must be virus-specific T cells generated by *in vitro* induction and which were numerically expanded from a small number of precursor cells, over a short time in culture, under highly reproducible conditions, and in accordance with good manufacturing practices (GMP). Most protocols for the generation of virus-specific T cells use peptide-loaded monocyte-derived DCs, artificial antigen-presenting cells (APC), or CMV-infected immature DCs as stimulator cells. However, these protocols are difficult to standardize and often laborious to adapt to GMP conditions. Tetramer expansion approaches are limited by the availability of tetramers for uncommon HLA types and a lack of class II tetramers. Capture of T cells that secrete cytokines in response to stimulation with viral antigens allows for rapid T-cell selection without HLA restriction, but the process is inefficient and produces small numbers of selected cells, impeding the product characterization essential to a later phase clinical study (11).

We developed a GMP-compliant strategy to rapidly generate a single preparation of polyclonal ($CD4^+$ and $CD8^+$) T cells against CMV from the peripheral blood of patients with glioblastoma. CMV-specific T cells can be rapidly produced (10 days) by a single stimulation of donor peripheral blood mononuclear cells (PBMC) with a peptide mixture spanning the CMVpp65 antigen in the presence of the potent prosurvival cytokines IL2, IL4, and IL7. These numerically expanded CMV-specific T cells are highly functional, produce effector cytokines in response to stimulation with CMV antigen, express normal levels of eomes and T-bet, and upregulate expression of CXCR3, a chemokine receptor important for T-cell homing to the central nervous system (CNS). To achieve lymphodepletion in these patients, which would allow for *in vivo* clonotypic expansion, we pretreated the patients with dose-dense temozolomide. In addition to instituting a uniform lymphodepleting preparative chemotherapy regimen prior to T-cell therapy, the infused CMV-specific T cells were evaluated for *in vivo* persistence and effector function in both the peripheral blood and in the glioblastoma microenvironment.

Patients and Methods

Human subjects research

All studies were conducted in accordance with the Declaration of Helsinki. The studies were performed after approval by The University of Texas MD Anderson Cancer Center (MD Anderson) Institutional Review Board (Houston, TX). All subjects gave fully informed and written consent for participation.

Autologous anti-CMV glioma-targeting assessments prior to clinical trial initiation

To clarify the cytotoxicity of the CMV-targeting T cells in patients receiving standard-of-care therapy, 20 patients with glioblastoma were recruited from MD Anderson. PBMCs were purified with Histopaque (Sigma-Aldrich) for density gradient separation. Freshly resected glioblastoma tissue was minced into small pieces using a scalpel, dissociated using a Pasteur pipette, and enzymatic digestion with a mixture of collagenase I and collagenase II (Liberase TM, Roche) for 45 minutes at 37°C. The tumor-infiltrating lymphocytes (TIL) were then isolated by density gradient centrifugation using Percoll (GE Healthcare). After centrifugation, the cells were collected, filtered through a 70-μm Nylon Strainer (BD Biosciences), washed, and counted using a Cellometer (Nexelom Bioscience). Patients were screened for HLA-A2 positivity by flow cytometry using an anti-HLA-A2 FITC Antibody (Thermo Fisher Scientific). HLA-A2–positive patients (13/20) were stained for CMV-specific CD8 T cells using a Dextramer CMV-monitoring PE Antibody (Immudex) that recognizes CMV peptides presented in the context of HLA-A*0201. Autologous CMV-specific T cells were numerically expanded from the peripheral blood of 12 patients (**Table 2**), whereas the matching glioblastoma stem cells (GSC) were generated from tumor tissue as described previously (12). For *in vitro* functional studies against autologous tumor cells, the IFNγ Secretion Assay-Cell Enrichment and Detection Kit (Miltenyi Biotec) was used according to the manufacturer's instructions to enrich the CMV-specific T-cell population.

Chromium release assay

Gamma catch-enriched CMV-specific T-cell cytotoxicity was assessed using the chromium (^{51}Cr) release assay. Briefly, autologous GSC target cells were labeled with ^{51}Cr (PerkinElmer Life Sciences) at 50 μCi/5×10^5 cells for 2 hours. ^{51}Cr-labeled GSC targets (5×10^5) were incubated for 4 hours with serially diluted CMV-specific T cells in triplicate. After centrifugation, the supernatants were harvested and analyzed for ^{51}Cr content.

T-cell cytokine release assay

CMV-specific T cells were cocultured for 5 hours with either 20 μL/mL of CMV pp65 Peptide Mix (Miltenyi Biotec) or autologous GSC target cells at an optimized effector:target ratio of 5:1, together with CD107a PE-CF594 (BD Biosciences), Monensin (BD GolgiStopTM), and BFA (Brefeldin A, Sigma Aldrich). Cells were also incubated without targets as the negative control and stimulated with phytohemagglutinin (PHA; 50 ng/mL, Sigma Aldrich) as the positive control. Cells were collected, washed, and stained with surface antibodies (CD3-BV650, CD4- APC-Cy7, and CD8-FITC; all from BD Biosciences), fixed/permeabilized (BD Biosciences), and stained with the IFNγ v450 antibody.

Study design and participants

NCT02661282 was an open-label, single-arm 3+3 phase I dose escalation trial conducted at MD Anderson in patients with recurrent glioblastoma followed by a phase II dose expansion trial in patients with recurrent glioblastoma (with a planned resection) and in patients with newly diagnosed glioblastoma. The patients had to be at least 18 years old, have histologically confirmed glioblastoma, a Karnofsky performance scale (KPS) score ≥60, prior radiation therapy and chemotherapy, and be CMV seropositive. For the phase I dose escalation component, patients could have had any number of prior relapses. No study patient was allowed to be receiving steroids for at least 5 days prior to the CMV-specific T-cell infusion. For the expansion cohort with a planned resection, MRI evidence of recurrence was defined as progressive or new contrast enhancement after initial radiotherapy plus concurrent and adjuvant temozolomide treatment, with a minimal volume of 1 cm^3 of contrast enhancement. A minimum period of 12 weeks after concurrent temozolomide treatment and radiotherapy and 2 weeks beyond any prior treatment was required prior to the initiation of study treatment. Participants previously treated with bevacizumab, implanted chemotherapy, or who were receiving other investigational agents or concurrently using optune, were excluded. Patients were required to have adequate renal, hepatic, and bone marrow function, including an absolute neutrophil count (ANC) >1,500 cells per μL, a hemoglobin concentration of ≥90 g/L, and a platelet count >100,000 per μL. Patients with immunodeficiency or with a known history of human immunodeficiency virus or hepatitis were excluded. Pregnant women were not eligible. Approval for the study was granted through the Institutional Review Board at MD Anderson, and all patients had provided written informed consent before study entry.

Procedures

To determine the maximum feasible dose (MFD) or maximum tolerated dose (MTD) of CMV-specific T cells in combination with dose-dense temozolomide, a 3+3 design was used to explore four dose levels of CMV-specific T cells: 5×10^6, 1×10^7, 5×10^7, and 1×10^8 cells. A minimum of 6 patients had to be treated at the MTD or MFD. Each patient in the dose escalation cohort could receive up to four doses of CMV-specific T cells and dose-dense temozolomide, but no intrapatient dose escalation was permitted. A temozolomide lymphodepleting regimen was selected over the more standard lymphodepleting regimen using fludarabine and cyclophosphamide due to concerns regarding the potential CNS toxicity associated with fludarabine. Dose-dense temozolomide was chosen over standard dose temozolomide due to the higher likelihood of inducing grade 3 or 4 lymphopenia with a dose-dense over standard dose regimen, which was compared directly in a randomized phase III clinical trial of patients with glioblastoma (13) and which was shown to enhance antigen-specific immune responses *in vivo* (14, 15). For this trial, no specific threshold value for lymphodepletion was set. For the patients with recurrent glioblastoma in the dose escalation cohort, the median absolute lymphocyte count (ALC) was 1.19 (0.59–2.77) at baseline. The median ALC values on day 22 decreased with cumulative cycles of dose-dense temozolomide exposure. Lymphopenia did not correlate with PFS. Cycles of dose-dense temozolomide could be continued beyond the fourth dose if the patient was receiving benefit from therapy, for up to 12 cycles. Patients who fulfilled eligibility criteria underwent PBMC collection on day 0 via leukapheresis. After leukapheresis, patients began treatment for 21 days with dose-dense temozolomide at a dose of 100 mg/m^2/day. On day 22 (±2 working days), the patients underwent adoptive CMV-specific T-cell transfer (at a dose level assigned by cohort; **Fig. 2A**). The CMV-specific T cells were administered intravenously over approximately 1 minute, followed by a 250 mL saline flush. None of the subjects had concurrently administered temozolomide and adoptive CMV-specific T cells. Patients were pretreated with diphenhydramine and acetaminophen by mouth prior to T-cell infusion. Premedication with steroids was not allowed. Patients then received an additional three cycles of dose-dense temozolomide followed by CMV-specific T-cell infusions at a fixed dose of CMV-specific T cells. Cycles were defined as every 42 days (first four cycles). After they received a total of four cycles of dose-dense temozolomide followed by CMV-specific T-cell infusion, participants could continue on 42-day cycles of dose-dense temozolomide alone until tumor progression was observed, as long as there were no unacceptable toxicities. Responses were assessed by clinical examination and MRI scans every 6 weeks (i.e., after each cycle).

During the phase II component, for patients with recurrent glioblastoma who were undergoing resection, surgery was scheduled on day 30 (±2 working days), which was 7 days after the CMV-specific T-cell infusion. In the postsurgery phase, subsequent cycles of dose-dense temozolomide and CMV-specific T cells were administered on day 22 (±2 working days) for a total of three cycles at the MFD (**Fig. 2B**). Patients started these subsequent cycles 2 weeks after surgery provided that: they had fully recovered from any perioperative complications, KPS score was ≥60, and hematologic and organ function values were adequate to undergo chemotherapy. A patient could remain on dose-dense temozolomide administration until tumor progression occurred, as long as there were no unacceptable toxicities, or until the completion of 12 cycles. The brain MRI obtained within 48 hours after surgical resection was considered the baseline MRI for evaluation of treatment response.

For the phase II expansion cohort in newly diagnosed glioblastoma, patients had to have completed external beam radiotherapy in combination with temozolomide administration within 5 weeks prior to accrual to this study. After a total of four cycles of dose-dense temozolomide followed by CMV-specific T-cell infusion, participants could continue to receive standard dose temozolomide (200 mg/m^2 days 1–5 every 28 days; i.e., 28-day cycles starting in cycle 5) until tumor progression occurred, as long as there were no unacceptable toxicities, or until completion of a total of 12 cycles of treatment (whichever occurred first). Responses were assessed by clinical examination and MRI scans every 6 weeks during the four cycles of dose-dense temozolomide administration combined with CMV-specific T-cell infusions, and every 8 weeks during treatment with standard dose temozolomide (at the beginning of each odd cycle, i.e., cycles 7, 9, and 11). The brain MRI following completion of standard concurrent chemoradiation was considered the baseline MRI for evaluation of treatment response.

Toxic effects were assessed at baseline and before each cycle. Adverse events (AE) were measured according to the NCI's Common Terminology Criteria for Adverse Events, version 4.03. If any treatment-related nonhematologic AE observed was grade >2 (except alopecia, nausea, or vomiting) and/or if platelet counts were $<50 \times 10^9$/L and/or ANC was $<1 \times 10^9$/L, then the temozolomide dose was reduced by one level. Patients who required more than two dose reductions had their treatment stopped. If any treatment-related nonhematologic AE observed was grade 4 (except alopecia, nausea, or vomiting), dose-dense temozolomide treatment was stopped. Treatment was continued for up to 12 cycles of temozolomide or until objective disease progression occurred, intercurrent illness prevented further drug administration, unacceptable AEs occurred, dose delays of more than 6 weeks (or more than two dose delays for the same AE)

occurred, or consent was withdrawn. Patients were replaced who did not receive the CMV product secondary to manufacturing failure, who were unable to complete the first cycle of treatment for reasons other than toxicity or progression, or who, if enrolled in the dose expansion groups, were unable to proceed to surgery.

Generation of CMV-specific T cells

PBMCs from the leukapheresis product were pulsed for 2 hours with peptides whose amino acid sequence overlapped that of the CMV pp65 antigen (JPT) at a concentration of 1.78 μg/mL in cytokine-free medium. Peptide-pulsed cells were cultured for 14 days in equal volumes of RPMI1640 and CLICKS-EHAA media, supplemented with 10% Human AB Serum, 2 mmol/L of GlutaMax, IL4 (10 ng/mL), and IL7 (10 ng/mL) every 3 days. After 14 days of expansion, the frequencies of CMV-specific T cells were determined by intracellular cytokine assay, and the cells were harvested and cryopreserved until used. Clonotypic *ex vivo* expansion of CMV-specific T cells obtained by leukapheresis from patients with glioblastoma could comprise up to 45% of all T cells, but for most subjects, the CMV-specific frequency was less than 5% of the total T-cell composition. Nonetheless, the absolute number of CMV-specific T cells generated during expansion was sufficient to reach the MTD/MFD of 1×10^8 cells.

Immune system monitoring

Immune system monitoring was performed on peripheral blood samples collected from patients before and (at multiple time points) after CMV-specific T-cell infusions. In brief, 2×10^5 patient-derived PBMCs were stimulated in a round-bottomed 96-well tissue culture plate with 1 μg/mL of CMVpp65 pepmix for 6 hours at 37°C in cytokine-free medium supplemented with 10% human serum albumin, and 1% L-glutamine. BFA (Sigma, 25 μg/mL) was added for the last 5 hours of culture. The CD107a-PE CF-594 antibody (clone H4A3, BD Biosciences) was added for the entire period of stimulation. Unstimulated or PHA (Sigma, 20 μg/mL) stimulated cells were used as negative and positive controls, respectively. Cells were then harvested, washed, and incubated with BV510-tagged viability dye (Invitrogen) in PBS for 20 minutes at room temperature. Next, cells were washed in flow cytometry buffer containing PBS with 1% heat-inactivated FBS (Gibco BRL) and stained with the following antibodies: CD3-BUV 395 (clone SK7, BD Biosciences), CD8-APC-H7 (clone SK1, BD Biosciences), and CD4-BV785 (clone RPA T4, BioLegend) for 20 minutes in the dark. Cells were then washed, fixed, and permeabilized in Cytofix/Cytoperm Buffer (BD Bioscience), followed by intracellular cytokine staining using the following antibodies: IFN-γ-BV605 (clone B27, BioLegend), TNF-α AF488 (clone Mab 11, BioLegend), and IL2-APC-R700 (clone MQ1-17H12, BD Biosciences). In addition, CMV-specific $CD8^+$ T cells were identified and enumerated using the Dextramer CMV Monitoring Kit (Immudex), which has received a FDA 510(k) clearance for *in vitro* diagnostic use, and which recognizes CMV peptides presented in the context of the following HLA-gene products: A*0101, A*0201, A*0301, A*2402, B*0702, B*0801, and B*3501. Upon dextramer staining, cells were also stained with anti-PD1-BV421 (clone EH12, BD Biosciences) and anti-TIGIT–APC (clone MBSA43, Invitrogen) antibodies for checkpoint markers and CD45RA-BV711 (clone HI100, BioLegend) and CCR7-BV605 (clone G043H7, BioLegend) to define the maturation state of the CMV-specific T cells. Samples were analyzed using an X-20 Fortessa Flow Cytometer (BD Biosciences), and all data were analyzed by FlowJo (TreeStar) software.

Characterization of TILs in glioblastoma

TILs from the brain tissues were prepared by enzymatic digestion using a mixture of collagenase I and collagenase II (Liberase, Sigma) for 45 minutes at 37°C, with repeated dissociation of the tissue by pipetting. The TILs were then isolated by density gradient centrifugation using percoll. Cells were washed in PBS and the frequencies of CMV-specific T cells determined by dextramer staining. The expression of checkpoint markers on TILs was determined by staining for PD1 and TIGIT, and the intracellular cytokine staining was performed using the same antibodies and as described in the immune system monitoring section.

IHC for CMV pp65 antigen

Glioblastomas were formalin fixed and paraffin embedded. Sections were cut at 5 μm, and the IHC analysis was carried out with the Vector Laboratories ABC Kit. The primary antibody for pp65 was purchased from Abcam (ab6503), was diluted at 1:3,200, and incubated on slides overnight at 4°C.

Outcomes

Toxicity was monitored using Bayesian continuous monitoring (16), where the toxicity evaluation endpoint was defined as treatment-related unmanageable toxicity, including grade 3 or 4 AEs that require termination of the treatment during cycle one (6 weeks). A toxicity rate of 30% or higher was considered unacceptable. The prior probability of toxicity was assumed to follow a beta (0.3–0.7) distribution, with 1 patient's worth of information. The toxicity was monitored by a cohort size of four, starting after at least 8 patients had completed toxicity evaluation (within cycle one). The trial would have been stopped if the following statement were true: $\Pr[\text{toxicity rate} > 30\% \mid \text{data}] > 0.90$. The early stopping boundaries for toxicity, shown in the format of (the number of patients with toxicities)/(the number of patients treated) were ≥5/8, 6/12, or 8/16. For the dose expansion cohort of patients with recurrent glioblastoma, a sample size of 10 would give us 80% power to detect a difference of any immunologic effects with the effective size of 0.853, using a one-sided paired *t* test at the significance level of 0.05. For the dose expansion cohort of patients with newly diagnosed glioblastoma, a sample size of 20 ensures that if the trial is not terminated early, a 90% confidence interval (CI) for a 24-month OS rate would have a maximum width of 0.184. Patients who were alive without disease progression or relapse were censored at the time of last contact.

Imaging protocol and imaging response assessment criteria

MRI scans were acquired on a 1.5 or 3.0 Tesla MRI scanner using the standard protocol: axial T1-weighted sequence [T1WI; repetition time (TR), 700 milliseconds; echo time (TE), 12 milliseconds; slice thickness, 5 mm; acquisition matrix 352 × 224]; axial fluid attenuation inversion recovery (FLAIR) sequence (TR, 10,000 milliseconds; TE, 140 milliseconds; slice thickness, 5 mm; acquisition matrix, 256 × 256); and axial post-contrast T1WI, acquired 5 minutes after the contrast injection (TR, 750 milliseconds; TE, 13 milliseconds; slice thickness, 5 mm; acquisition matrix, 384 × 256). The conventional T1WI and FLAIR sequences were used to assess response to therapy via the RANO criteria (17). Contrast-enhancing lesions with bidimensional measurements of >1 cm were considered as measureable (index) lesions, whereas smaller lesions and those with nonenhancing T2/FLAIR hyperintensity were considered to be nonmeasureable (non-index) lesions. Patients were categorized on the basis of best response as having: (i) progressive disease; (ii) stable disease; (iii) partial response; or (iv) complete response.

Statistical analysis

Median PFS and OS times from trial registration were estimated by Kaplan–Meier analyses in the recurrent glioblastoma cohort ($n = 16$) that did not undergo surgical resection. AEs were recorded and tabulated according to type and grade. Mean biomarker expression values were compared using the Mann–Whitney test. Data were analyzed using *R* for Windows, version 3.4.3. with package *survival* version 2.41-3 and GraphPad Prism for Windows, version 6.00.

Data sharing

We will make the data collected as part of this study available to outside investigators, but within the limitations of the Health Insurance Portability and Accountability Act (HIPAA). To maintain compliance with HIPAA regulations, we will execute a data-sharing agreement with the requestor for a limited use dataset as defined by the U.S. Department of Health and Human Services. No biological samples will be released under these proposed mechanisms, due partly due to confidentiality issues and partly due to the limited nature of these samples, which are typically depleted upon analysis. Study protocol and informed consent forms will be made available online upon publication.

Results

CMV-specific T cells are able to target autologous tumor cells in a subset of patients with glioblastoma

Prior to clinical trial implementation, we analyzed the ability of autologous CMV-specific T cells to exert cytotoxic effects on autologous GSC. Of 22 patients with glioblastoma undergoing surgical resection between July 2014 and April 2017, 13 were HLA-A2 positive as determined by flow cytometry. Five of 13 of these patients exhibited a high intratumoral frequency of CMV-specific T cells (intratumoral CMV-specific T cells frequency > peripheral blood CMV-specific T cell frequency) detected using CMV-specific dextramer staining (**Fig. 1A**). Despite the high prevalence of CMV-specific T cells in these patients, when functionality was analyzed, the intratumoral T cells were unable to respond to stimulation with CMV peptides. IFNγ production was partially recovered with nonspecific T-cell stimulation using PHA (**Fig. 1B**). To evaluate the cytotoxic potential of CMV-specific T cells against glioblastoma, we attempted to numerically expand CMV-specific T cells from the peripheral blood samples collected from 12 of 22 patients, and expansion was successful in 11 patients (16.6%–48.5% of IFNγ production in response to CMV peptide stimulation). Autologous GSCs were generated from the glioblastoma to serve as targets. After 14 days of cell expansion, gamma catch was performed to isolate the CMV-specific T cells from the CMV-negative cells. Although the CMV-negative T cells were unable to kill the autologous glioblastoma cells, 5 of 11 patients exhibited a significant CMV-specific cytotoxic response against autologous tumor ($CMV^+ > CMV^-$; **Fig. 1C**). The CMV-specific T cells showed IFNγ production and degranulation (CD107a) in response to the autologous GSCs (**Fig. 1D** and **E**; Pearson correlation: $r = 0.9$; $P = 0.005$). After gamma catch, the CMV^+ T cells exhibited an increase in cytokine release and degranulation in response to autologous tumor, while the CMV^- T cells did not (**Fig. 1E** and **F**). Interestingly, all 11 patients responded to stimulation with CMV peptides regardless of their ability to kill autologous tumor (**Fig. 1G**), suggesting variable presentation of CMV antigens by glioblastoma and the potential employment of additional immune-evading strategies by GSCs. These results supported the clinical utilization of CMV-specific T cells for treatment of glioblastoma, predicting a positive response in about half of the patients.

Clinical trial

We screened and obtained consent from 65 patients with glioblastoma between July 2016 and December 2019. Forty patients were deemed ineligible during screening, 25 underwent leukapheresis, and 20 patients were treated. Of the remaining 5 patients, 1 had progressive disease before the cell infusion could be administered, and 4 had failure of cell number expansion. For the whole trial (**Fig. 2A** and **B**), CMV-specific T cells could be manufactured for 22 patients with a rate of 26% failed cell number expansions. We were able to produce four doses for only 30% of patients; thus, the remainder received 1–3 doses. The MTD was identified as the MFD of 1×10^8 cells [i.e., no dose limiting toxicities (DLT) occurred at any of the dose levels]. The cut-off date for data analysis was January 2, 2020. **Table 1** shows the baseline demographic and outcome data. The median follow-up time for all patients was 12 months (95% CI, 6–14 months). The longest ongoing duration of response was 39 months. There were no treatment-related deaths; 13 of 16 patients (81%) treated in the dose escalation cohort died from disease progression. The median PFS time was 1.3 months (95% CI, 0–8.3 months), and the 6-month PFS was 19% (95% CI, 7%–52%; **Fig. 2A** and **C**). The median OS time was 12 months (95% CI, 6 months to not reached; **Fig. 2D**), with an estimated 1-year OS rate of 50% (95% CI, 31%–82%) for these patients.

Of the patients treated with the combination of dose-dense temozolomide and CMV-specific T cells, the most common AEs possibly related to dose-dense temozolomide were nausea, constipation, fatigue, and headaches (most grade 2 or lower, except lymphopenia as expected; **Table 2**). There were two incidences of grade 4 thrombocytopenia and neutropenia. There was an incidence of grade 3 seizure and confusion, possibly related to the T-cell infusion. Patient 23 started a new therapy before the completion of cycle 1, was thus not evaluable for DLT, and was replaced in this cohort. There were no treatment-related deaths, and no patients discontinued the study because of drug-related toxic effects.

Imaging response

All patients had baseline MRI prior to treatment with CMV-specific T cells and underwent follow-up MRI for response assessments. Within the dose escalation cohort, 2 patients at dose level one had stable disease that exceeded 147 weeks (37 months). Partial radiographic responses were detected in a patient enrolled in cohorts 2 and 4. One patient had a complete response when treated at dose level 3. This patient's recurrent tumor was resected prior to trial enrollment, and it showed rapid postoperative contrast enhancement that did not correlate with diffusion-weighted imaging ischemia in the immediate postoperative MRI. After the first cycle of CMV-specific T cells, the patient had complete resolution of the gadolinium enhancement (**Fig. 3**). Notably, the long-term responders (>147 weeks) with stable disease and the patients who had either partial or complete radiographic responses were all either positive or indeterminate for O^6-methylguanine-DNA methyltransferase (MGMT) methylation. The radiographic and outcome data suggest that a signal of response was present, especially in patients with glioblastomas that were MGMT methylated. As such, these responses could not be fully attributed to the adoptive T-cell immunotherapy.

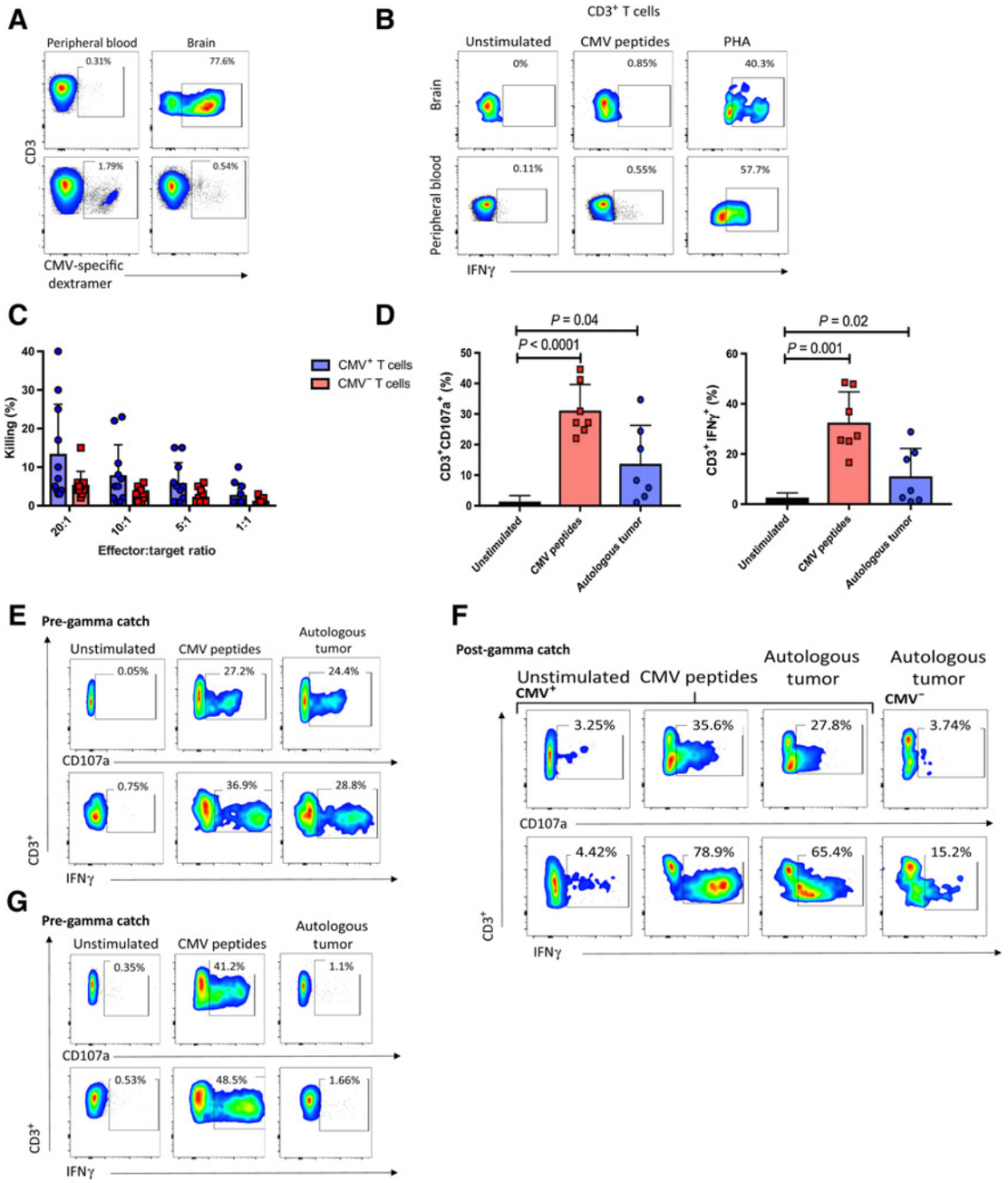

Figure 1.

Patients with glioblastoma receiving standard-of-care therapy had their peripheral blood and their respective glioblastomas analyzed for CMV-specific immunologic responses. **A,** Two representative patient samples depicting the frequency of CMV-specific T cells based on dextramer staining in which a large population of $CD3^+$ T CMV-specific T cells is present or absent in the brain compared with the peripheral blood. **B,** $CD3^+$ T cells isolated *ex vivo* from the glioblastoma microenvironment of a patient with 77.6% of CMV-specific T cells in the brain as shown by dextramer staining, but who did not demonstrate expression of markers associated with immune effector responses such as production of IFNγ in response to CMV peptide stimulation. These T cells were less responsive to PHA stimulation than the $CD3^+$ T cells in the matched peripheral blood. **C,** Peripheral blood CMV-specific T cells were numerically expanded using CMV peptides and the cytokine cocktail for 2 weeks. Gamma catch was performed after 2 weeks to separate the CMV-specific T cells from other T cells. Autologous GSCs were used as targets in a chromium-release killing assay. Of 11 patients, 5 showed increased killing of the autologous GSCs by CMV-specific T cells relative to the rest of the T cells ($n = 11$; $P = 0.045$). **D,** Cytokine release assay depicting production of IFNγ and T-cell degranulation (CD107a) as markers of immune response in the expanded CMV T cells prior to the gamma catch assay. All patient samples responded to CMV peptides; however, similar to the autologous killing assay, only 3 of 8 patients responded to the autologous tumor ($n = 7$). **E** and **F,** Representative (FACS) plots from a patient who had a positive response toward autologous GSCs after CMV-specific T-cell expansion pre- and post-gamma catch assay. The CMV-specific T-cell pellet post-gamma catch is highly responsive to autologous tumor compared with the CMV-negative T-cell pellet and the pre-gamma catch T cells. **G,** Representative FACS plots from a patient who did not show a cytotoxic response toward autologous GSCs after CMV-specific T-cell expansion pre-gamma catch assay.

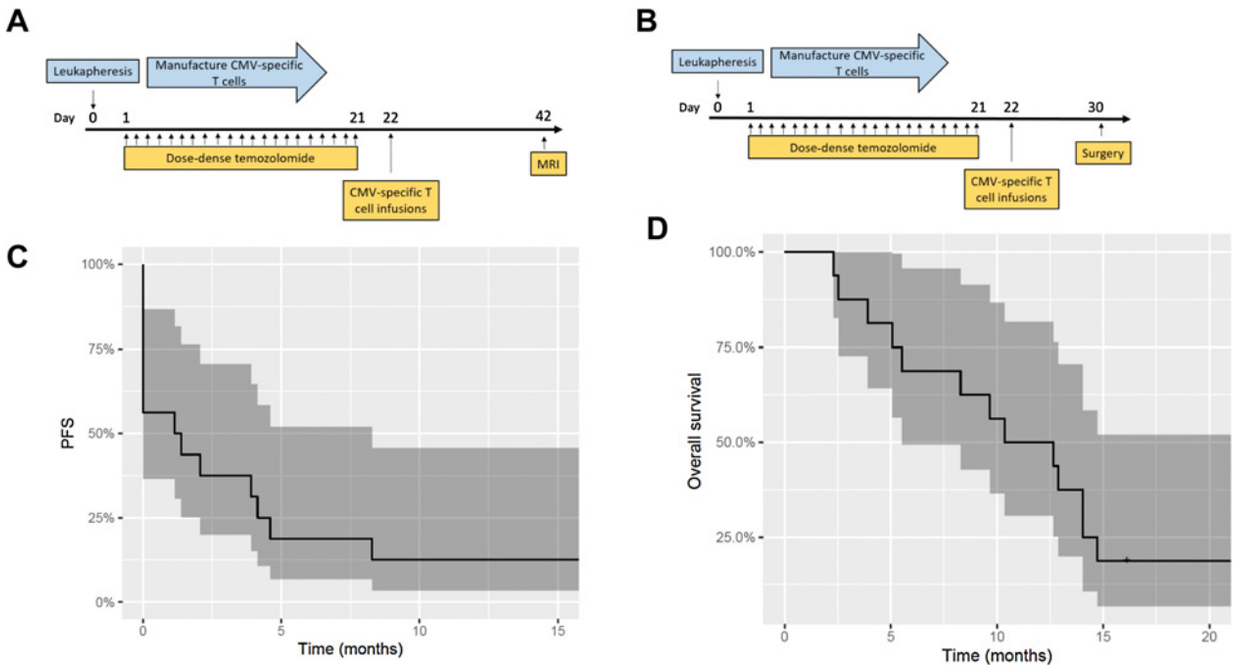

Figure 2.

Design and outcomes of phase I/II clinical trial of autologous CMV-specific T cells in patients with glioblastoma. **A,** Trial schema for the first cycle of treatment for the dose escalation in the cohort of patients with recurrent glioblastoma ($n = 16$). **B,** Trial schema for the first cycle of treatment for the patient with surgically resected recurrent glioblastoma ($n = 1$). **C,** PFS time based on radiographic evidence of tumor recurrence in terms of gadolinium-contrast enhancement seen on magnetic resonance images for patients with recurrent glioblastoma treated with CMV-specific T cells ($n = 16$). **D,** The OS time of patients with recurrent glioblastoma treated with CMV-specific T cells ($n = 16$).

Table 1. Demographic and outcome data of patients with recurrent glioblastoma treated with CMV-specific T cells.

Enrollment number	Age at DX	KPS	Histology	IDH1 mutant	MGMT status	Dose of CMV T cells	Cycles of TMZ	Prior relapses	PFS (weeks)	MRI response	Reason off study	Survival weeks	F/U date
Phase I dose escalation in patients with recurrent glioblastoma with dose expansion													
1	64	70	rGBM	Neg	Pos	5×10^6	4	1	167	SD	CO	182	1/2020
2	59	90	rGBM	Neg	Pos	5×10^6	4	1	169	SD	CO	174	1/2020
3	53	100	rGBM	Neg	Unknown	5×10^6	1	2	0	PD	PD	11	Died
7	61	100	rGBM	Unknown	Undetermined	1×10^7	4	1	36	PR	CO	61	Died
13	27	100	rGBM	Neg	Neg	1×10^7	1	1	0	PD	PD	70+	Lost to f/u
14	28	90	rGBM	Neg	Pos	1×10^7	1	2	0	PD	PD	10	Died
16	44	100	rGBM	Pos	Pos	5×10^7	4	1	20	CR	CO	42	Died
19	52	100	rGBM	Unknown	Pos	5×10^7	2	1	5	SD	PD	22	Died
20	36	90	rGBM	Neg	Undetermined	5×10^7	1	2	0	PD	PD	45	Died
23	50	90	rGBM	Neg	Neg	1×10^8	1	2	0	PD	PD	24	Died
24	28	90	rGBM	Pos	Undetermined	1×10^8	1	1	9	PR	PD	61	Died
25	49	100	rGBM	Neg	Neg	1×10^8	2	1	6	SD	PD	36	Died
27	57	90	rGBM	Neg	Neg	1×10^8	3	1	17	SD	PD	55	Died
29	68	70	rGBM	Neg	Neg	1×10^8	1	1	0	PD	PD	56	Died
30	59	90	rGBM	Neg	Unknown	1×10^8	1	3	0	PD	PD	17	Died
31	40	100	rGBM	Neg	Pos	1×10^8	4	1	18	SD	CO	64	Died
Newly diagnosed glioblastoma cohort treated at the MTD/MFD													
40	53	90	GBM	Neg	Pos	1×10^8	4	0	20	SD	CO	24	11/2019
42	63	80	GBM	Neg	Neg	1×10^8	2	0	19	SD	Note 1	24	1/2020
49	55	90	GBM	Neg	Pos	1×10^8	2	0	9	SD	Note 2	13	12/2019
Patient with recurrent glioblastoma treated at the MTD/MFD undergoing resection													
41	56	90	rGBM	Neg	Indeterminate	1×10^8	1	1	0	PD	PD	22	12/2019

Abbreviations: CO, completion of treatment; CR, complete response; Dx, diagnosis; F/U = follow up; GBM, glioblastoma; N/A, not applicable; Neg; negative; Note 1, physician decision; Note 2: only 2 infusions were available; PD, progressive disease; Pos, positive; PR, partial response; r, recurrent; SD, stable disease; TMZ, temozolomide; unknown, that the testing was not performed; undetermined, testing was done but result could not be determined.

Table 2. Temozolomide and possible T-cell infusion toxicity data in the enrolled glioblastoma patient population ($n = 20$).

AEs possibly related to temozolomide toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Nausea	6	2		
Vomiting	1			
Constipation	5	5		
Dizziness	1			
Fatigue	4	11		
Headache	5		1	
Arthralgia	3			
Anorexia	1	4		
Weight loss	2			
Seizure	2			
Urinary tract infection		1		
Concentration impairment	1	2		
Dry skin	1			
Memory impairment	2	1		
Cognitive disturbance	2			
Insomnia		1		
Anxiety		1		
Depression		1		
Lethargy	1			
Abdominal pain	1			
Decreased platelet count		1		1
Decreased lymphocyte count	2	7	6	
Decreased neutrophil count				1
Maculopapular rash		1		
Mucositis		1		
AEs possibly related to T-cell infusion				
Malaise	1			
Seizure			1	
Confusion			1	

The number of treatment cycles increases the frequency of CMV-specific T cells, but they lack immune function

Because patients with glioblastoma are profoundly immune suppressed, we evaluated whether the CMV-specific T cells were persisting *in vivo* and whether they maintained their immune effector function. CMV-specific dextramer staining was used to detect the percentage of pp65-specfic T cells in the peripheral blood during *ex vivo* expansion, at baseline in subjects (designated preinfusion), and longitudinally during each cycle. Typically, less than 5% of circulating T cells in patients with glioblastoma preinfusion are CMV-specific. To ascertain whether there is an accumulation of CMV-specific T cells in the peripheral blood of patients with glioblastoma after adoptive transfer, CMV-specific dextramer staining was used to detect the percentage of CMV-specific T cells longitudinally (i.e., day 0, 7, 14, and 21) during each cycle (1–4). Only after the fourth cycle on day 21 was there a significant increase in the number of CMV-specific T cells in patients ($P = 0.019$; **Fig. 4A**), but this was not dose specific (Supplementary Fig. S1).

CD107a expression is a marker of cytotoxic T-cell degranulation that becomes upregulated after antigen stimulation, with a concordant loss of perforin (18). As such, the CMV-specific T-cell population identified by dextramer staining was analyzed for CD107 expression. Although this marker was present during *ex vivo* expansion, there was no significant increase in this marker of effector function *in vivo* during adoptive CMV-specific T-cell transfer (**Fig. 4B**). Intracellular cytokine profiling of the CMV-specific T cells showed that there was no significant increase in effector functions of TNFα (**Fig. 4C**), IFNγ (**Fig. 4D**), or IL2 (**Fig. 4E**) *in vivo*. During *ex vivo* expansion of the CMV-specific T cells, they demonstrated markers of effector memory ($CD3^+/CD8^+/CCR7^-/CD45RA^-$), but there was no increase in this phenotype *in vivo* during subsequent cycles, with most of the T cells demonstrating terminal memory ($CD3^+/CD8^+/CCR7^-/CD45RA^+$) and little central memory ($CD3^+/CD8^+/CCR7^+/CD45RA^-$; Supplementary Fig. S2).

To clarify whether there was an opportunity to modulate the CMV-specific T cells in the setting of glioblastoma-induced immune suppression in the periphery, the adoptively transferred T cells were profiled for expression of PD-1 and TIGIT. Although the expanded CMV-specific T cells and the preinfusion *in vivo* pool expressed both PD-1 and TIGIT, during subsequent infused cycles, expression of these markers was not present (Supplementary Fig. S3), but these cells may have been trafficking to the tumor microenvironment.

CMV-specific T cells are present in glioblastoma tumors but lack effector activity

To determine whether CMV-specific T cells with immune effector function had trafficked to the glioblastoma microenvironment, a patient with recurrent glioblastoma who was scheduled for surgical resection was pretreated with dose-dense temozolomide followed by CMV-specific T cells (**Fig. 2B**). The resected glioblastoma was processed to a single-cell suspension, and the immune cells and PBMCs were analyzed for intracellular immune effector cytokines and surface expression of CD107a. *Ex vivo* CD8 T cells (i.e., unstimulated) from the peripheral blood did not express TNFα, IFNγ, or CD107a, but expression of these markers could be induced by nonspecific immune activation and to a lesser degree, the CMV peptides (**Fig. 5A**). The $CD8^+$ T cells isolated directly from the glioblastoma tumor microenvironment were more refractory to immune simulation and were unreactive to CMV-peptide stimulation (**Fig. 5B**; $n = 1$). The overall frequency of CMV-specific T cells based on dextramer staining within the $CD8^+$ T-cell fraction from either the peripheral blood or the brain in this patient with glioblastoma was relatively minor (**Fig. 5C**). Upon trafficking into the tumor microenvironment, the CMV-specific $CD8^+$ T cells were positive for PD-1 (**Fig. 5D**). In this recurrent glioblastoma specimen, CMV-specific T cells occurred mostly in the tumor vasculature, and these cells were scarce in the tumor microenvironment (**Fig. 5E**). Cumulatively, these data indicate that the CMV-specific T cells within the tumor microenvironment are immunologically dysfunctional. CMV antigen expression was heterogeneous in archival glioblastoma specimens from nontrial participants and typically, in a minority of tumor cells (Supplementary Fig. S4; $n = 34$).

Adoptive CMV-specific T-cell treatment in patients with newly diagnosed glioblastoma

Because we observed a low frequency of CMV-specific T cells in the tumor microenvironment and their immune phenotype was dysfunctional, we analyzed a group of patients newly diagnosed with glioblastoma who had undergone surgical debulking followed by concurrent chemoradiation with temozolomide ($n = 3$) to minimize the immune-suppressive tumor burden. One of these patients was removed from the protocol due to their inability to continue treatment safely without receiving steroids. This patient had significant MRI changes after concurrent chemoradiation, which were consistent with radiation necrosis, and was thus switched to standard temozolomide administration (now on cycle 6) with an ongoing need for steroids. As in the patients with recurrent glioblastoma, PFS and OS were not significantly extended in these patients with newly diagnosed glioblastoma, with only 1 patient demonstrating a durable response, but

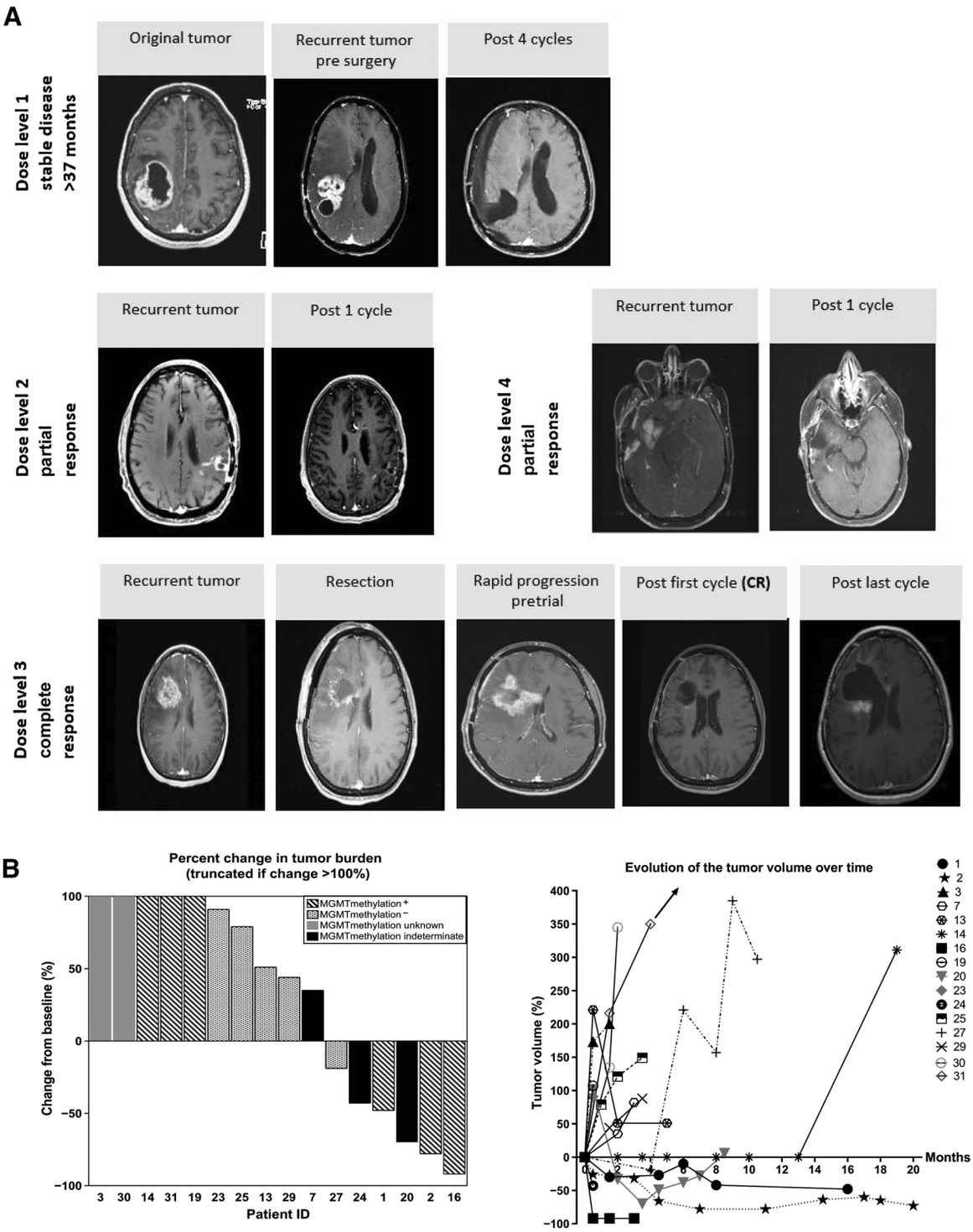

Figure 3.

A, Representative gadolinium-enhanced T1-weighted magnetic resonance images of patients with glioblastoma who had stable disease exceeding 37 months, or partial or complete radiographic responses. **B,** Waterfall plot of radiographic responses of all patients with recurrent glioblastoma treated with CMV-specific T cells and analyzed on the basis of MGMT methylation (top). Volumetric analysis of index gadolinium contrast-enhancing lesion in recurrent glioblastoma patients during treatment with CMV-specific T cells. Each symbol represents a patient cross referenced to **Table 1**.

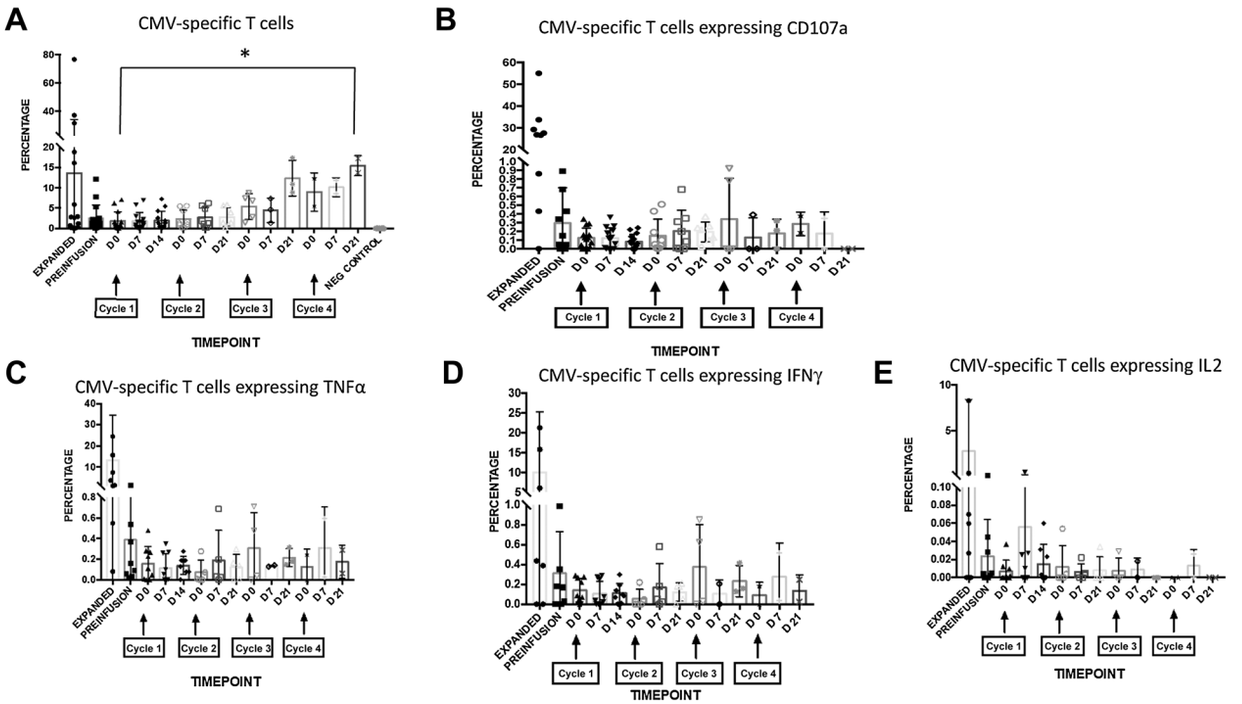

Figure 4.

Peripheral blood monitoring of patients with glioblastoma enrolled during the dose escalation phase of the clinical trial. Each symbol represents a patient data point at a designated timepoint of monitoring. **A,** The frequency of CMV-specific T cells based on dextramer staining was increased after four cycles on day 21 relative to day 0 of the first cycle (*, $P = 0.019$). The *in vivo* CMV-specific T cells did not show increases in CD107a surface expression (**B**), intracellular TNFα (**C**), intracellular IFNγ (**D**), or intracellular IL2 (**E**), with subsequent administration of CMV-specific T cells.

with the confounder that the tumor showed MGMT methylation (**Table 1**). There was no clinical indication for re-resection in these three subjects, and thus CMV-specific T cell infiltration was not available for analysis.

Discussion

Prior clinical trials targeting CMV in patients with glioblastoma have suggested clinical responses (7, 8, 19). Notably, these studies were conducted with small numbers of subjects for whom unintended biases may have not been sufficiently accounted, including prognostic markers such as IDH1 mutation and MGMT methylation. Our *in vitro* data from patients with glioblastoma undergoing resection show that CMV-specific T cells can effectively kill autologous tumor in a subset of CMV-seropositive patients, but not all. Although a DC vaccine may be able to activate hypofunctional T-cell responses, the impact of the immune suppressive tumor microenvironment would remain as a confounder for effective cytotoxicity. These results also indicate that CMV seropositivity does not guarantee a response to CMV-specific T-cell therapy, and they suggest heterogeneity in CMV antigen expression among patients with glioblastoma. In this clinical study, because temozolomide was used as the lymphodepleting agent to allow for clonotypic *in vivo* T-cell expansion, MGMT methylation status was identified as a confounder for radiographic responses and outcome. The two subjects that responded the best in the clinical trial were both MGMT promoter methylated. This was not entirely surprising because dose-dense temozolomide can induce radiographic responses in patients with recurrent glioblastoma that was previously treated with temozolomide (20, 21). To rechallenge with temozolomide at first tumor recurrence in MGMT promoter–methylated patients is a reasonable treatment option with proven clinical benefit (21). In addition, both of these patients were at the stage of first tumor recurrence and may have experienced less bone marrow suppression from other treatment modalities.

The prior use of temozolomide and other cytotoxic chemotherapies before trial participation was probably a key contributor to the cell number expansion failure. We used a rapid expansion protocol in which PBMCs are loaded with CMV pepmix, thereby relying on the monocytes and B cells in the leukapheresis product to act as the APCs. This protocol is very robust in numerically expanding CMV-specific T cells from healthy donors regardless of MHC class. However, we found that the procedure failed for up to a quarter of the patients with glioblastoma treated with temozolomide or other cytotoxic chemotherapies. We believe that this may be related to the cytotoxic effect of temozolomide on T cells, B cells, and monocytes in these patients, which can then negatively affect the quality of the starting product and thus the *ex vivo* expansion of the CMV-specific T cells. An alternative strategy would be to use DCs, but this would increase the time and expense, with the former being a problem for patients with rapidly progressing glioblastoma.

A number of key findings from this study indicate the limitations of adoptive T-cell immunotherapy targeting CMV in patients with glioblastoma. Despite an overall increase in the number of CMV-specific T cells in the peripheral circulation with each administered cycle, with only the last cycle being statistically significant, these CMV-specific T cells lacked expression of CD107a (LAMP-1, a marker for CD8 T-cell degranulation) after stimulation and of markers that would reflect immune-effector responses such as TNFα, IFNγ, and IL2.

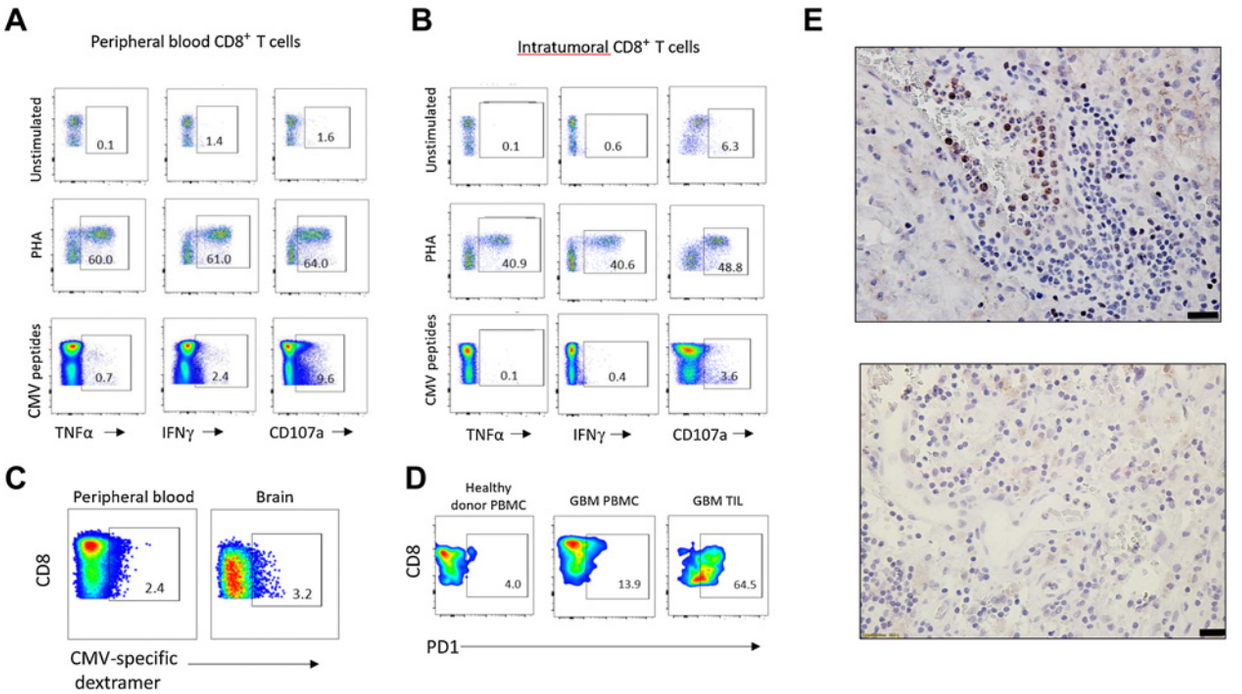

Figure 5.

Patient with recurrent glioblastoma (GBM) treated preoperatively with dose-dense temozolomide and CMV-specific T cells. Both the peripheral blood and the tumor were analyzed for immunologic responses. **A,** $CD8^+$ T cells from the peripheral blood of patients with glioblastoma were analyzed for intracellular production of TNFα and IFNγ as markers of effector responses and CD107a surface expression, for cytotoxic T-cell degranulation. In *ex vivo* unstimulated CD8 T cells, immune effector activity was low (top). Upon stimulation with PHA and ionomycin, these immune responses could be reconstituted (middle) but they were refractory to CMV-specific peptides (bottom). **B,** $CD8^+$ T cells isolated *ex vivo* from the glioblastoma microenvironment did not demonstrate expression of markers associated with immune effector responses (top), were less able to respond to PHA stimulation than the matched peripheral blood $CD8^+$ T cells (middle), and were refractory to CMV peptide stimulation (bottom). **C,** The number of CMV^+-specific T cells based on dextramer staining were a minor population of the total $CD8^+$ T-cell population present in the peripheral blood and brain. **D,** PD-1 expression was upregulated in the CMV-specific CD8 T cells in the peripheral blood of patients with glioblastoma relative to that of normal donors and was maximally expressed within the glioblastoma tumor microenvironment. **E,** IHC staining for pp65 (top) and the isotype control demonstrating CMV-specific immune cells in the perivascular region but not infiltrating other regions of the tumor. Scale bar, 20 μm; magnification, 400×.

Markers of effector and central immunologic memory were also not enhanced over time. One could postulate that the effector CMV-specific T cells were trafficking to the tumor microenvironment or were sequestered in the bone marrow (22) and were thus absent from the peripheral circulation. However, the overall frequency of CMV-specific CD8 T cells in the glioma microenvironment was low, and immune profiling revealed that these T cells also lacked effector function. Because the T cells were administered 7 days before surgery, we could not exclude the possibility that the T cells trafficked to the tumor microenvironment at an earlier time and mediated an effector response. Yet, their expression of markers of immune exhaustion such as PD-1 further supports the contention that these T cells are dysfunctional, with an exhausted phenotype that may be refractory to reinvigoration with immune checkpoint inhibitors (23). In addition, the CMV-specific T cells were only found in the perivascular regions of the tumors and were not dispersed throughout the tumor parenchyma. Cumulatively, these data indicate that the CMV-specific T cells generated in this study lack sufficient immunologic function to exert anti-glioblastoma activity. It is possible that other types of immunotherapy strategies may generate sufficient CMV-specific responses because DC vaccination may increase the polyfunctional phenotype of the T cells (24). Nevertheless, the heterogeneity of CMV pp65 antigen expression may be a significant limitation, even if sufficient anti-CMV T-cell responses are generated, as was demonstrated by tumor recurrence upon targeting of the EGFR variant III (15).

To determine whether there might be a response signal of CMV-specific T cells in patients newly diagnosed as having minimal residual disease and who were not receiving steroids, we enrolled a small cohort of these patients ($n = 3$) in the study, but there was no signal of significant response. Thus, this does not appear to be a strategy that can supplant therapeutic control of immune exhaustion. A key logistic issue with this study was that patients were required to be CMV seropositive to permit our numerical expansion of their CMV-specific T cells. Typically, at least 60% of the human population has been exposed to CMV (25). In our study, 63% of the patients screened were seropositive for CMV. Further confounding efficient enrollment was the issue of generating sufficient product to administer to the patients. With a rate of 26% of failed T-cell expansions, only approximately 74% of screened patients could ultimately receive the planned total of four therapeutic cycles, despite extensive clinical efforts to limit the use of steroids, which could impact T-cell expansion and effector functions. CMV-specific T-cell expansion failure was not an issue for normal donors using this strategy. Patients with glioblastoma are lymphopenic (26, 27). Thus, we concluded that numerical cell expansion failures were intrinsic to the patients' T cells, which were hypofunctional, partly owing to prior chemotherapy and the immune-suppressive

environment generated by the cancer. Currently, there are no good assays to predict the cell number expansion capabilities of patient products, and thus the investigators need to emphasize this possibility during the consent process of every patient. Our study also clarifies the need for an optimized cell number expansion strategy that potentially involves genetic modifications able to render these T cells resistant to immune exhaustion, before one proceeds with the next generation of clinical trials.

Disclosure of Potential Conflicts of Interest

B.J. O'Brien reports receiving commercial research grants from Monteris. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The study was designed by the investigators, who oversaw all data collection and interpretation. The funding agencies, The Gateway for Cancer Research, The MD Anderson Glioblastoma Moonshot, and the Brooks Foundation, had no role in the data analysis, interpretation of the results, or writing of the article.

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References

1. Bate SL, Dollard SC, Cannon MJ. Cytomegalovirus seroprevalence in the United States: the national health and nutrition examination surveys, 1988-2004. Clin Infect Dis 2010;50:1439–47.
2. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med 2005;202:673–85.
3. Manley TJ, Luy L, Jones T, Boeckh M, Mutimer H, Riddell SR. Immune evasion proteins of human cytomegalovirus do not prevent a diverse CD8+ cytotoxic T-cell response in natural infection. Blood 2004;104:1075–82.
4. Cobbs C, Harkins L, Samanta M, Gillespie G, Bharara S, King P, et al. Human cytomegalovirus infection and expression in human malignant glioma. Cancer Res 2002;62:3347–50.
5. Mitchell DA, Xie W, Schmittling R, Learn C, Friedman A, McLendon RE, et al. Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma. Neuro Oncol 2008;10:10–8.
6. Prins RM, Cloughesy TF, Liau LM. Cytomegalovirus immunity after vaccination with autologous glioblastoma lysate. N Engl J Med 2008;359:539–41.
7. Schuessler A, Smith C, Beagley L, Boyle GM, Rehan S, Matthews K, et al. Autologous T-cell therapy for cytomegalovirus as a consolidative treatment for recurrent glioblastoma. Cancer Res 2014;74:3466–76.
8. Mitchell DA, Batich KA, Gunn MD, Huang MN, Sanchez-Perez L, Nair SK, et al. Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. Nature 2015;519:366–9.
9. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science 1992;257:238–41.
10. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med 1995;333:1038–44.
11. Feuchtinger T, Matthes-Martin S, Richard C, Lion T, Fuhrer M, Hamprecht K, et al. Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. Br J Haematol 2006;134:64–76.
12. Pollard SM, Yoshikawa K, Clarke ID, Danovi D, Stricker S, Russell R, et al. Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. Cell Stem Cell 2009;4:568–80.
13. Gilbert MR, Wang M, Aldape KD, Stupp R, Hegi ME, Jaeckle KA, et al. Dose-dense temozolomide for newly diagnosed glioblastoma: a randomized phase III clinical trial. J Clin Oncol 2013;31:4085–91.
14. Sampson JH, Aldape KD, Archer GE, Coan A, Desjardins A, Friedman AH, et al. Greater chemotherapy-induced lymphopenia enhances tumor-specific immune responses that eliminate EGFRvIII-expressing tumor cells in patients with glioblastoma. Neuro Oncol 2011;13:324–33.
15. Sampson JH, Heimberger AB, Archer GE, Aldape KD, Friedman AH, Friedman HS, et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. J Clin Oncol 2010;28:4722–9.
16. Thall PF, Simon RM, Estey EH. Bayesian sequential monitoring designs for single-arm clinical trials with multiple outcomes. Stat Med 1995;14:357–79.
17. Wen PY, Macdonald DR, Reardon DA, Cloughesy TF, Soresnsen AG, Galanis E, et al. Updated response assessment criteria for high-grade gliomas: response assessment in neuro-oncology working group. J Clin Oncol 2010;28:1963–72.
18. Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods 2003;281:65–78.
19. Soderberg-Naucler C, Peredo I, Stragliotto G. Valganciclovir in patients with glioblastoma. N Engl J Med 2013;369:2066–7.
20. Perry JR, Belanger K, Mason WP, Fulton D, Kavan P, Easaw J, et al. Phase II trial of continuous dose-intense temozolomide in recurrent malignant glioma: RESCUE study. J Clin Oncol 2010;28:2051–7.
21. Weller M, Tabatabai G, Kastner B, Felsberg J, Steinbach JP, Wick A, et al. MGMT promoter methylation is a strong prognostic biomarker for benefit from dose-intensified temozolomide rechallenge in progressive glioblastoma: the DIRECTOR trial. Clin Cancer Res 2015;21:2057–64.
22. Chongsathidkiet P, Jackson C, Koyama S, Loebel F, Cui X, Farber SH, et al. Author correction: sequestration of T cells in bone marrow in the setting of glioblastoma and other intracranial tumors. Nat Med 2019;25:529.
23. Woroniecka K, Chongsathidkiet P, Rhodin K, Kemeny H, Dechant C, Farber SH, et al. T-cell exhaustion signatures vary with tumor type and are severe in glioblastoma. Clin Cancer Res 2018;24:4175–86.

24. Reap EA, Suryadevara CM, Batich KA, Sanchez-Perez L, Archer GE, Schmittling RJ, et al. Dendritic cells enhance polyfunctionality of adoptively transferred T cells that target cytomegalovirus in glioblastoma. Cancer Res 2018;78:256–64.
25. Staras SA, Dollard SC, Radford KW, Flanders WD, Pass RF, Cannon MJ. Seroprevalence of cytomegalovirus infection in the United States, 1988–1994. Clin Infect Dis 2006;43:1143–51.
26. Chongsathidkiet P, Jackson C, Koyama S, Loebel F, Cui X, Farber SH, et al. Sequestration of T cells in bone marrow in the setting of glioblastoma and other intracranial tumors. Nat Med 2018;24:1459–68.
27. Fecci PE, Mitchell DA, Whitesides JF, Xie W, Friedman AH, Archer GE, et al. Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma. Cancer Res 2006;66:3294–302.