Microenvironment and Immunology

Cancer Research

Autologous T-cell Therapy for Cytomegalovirus as a Consolidative Treatment for Recurrent Glioblastoma

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Abstract

Glioblastoma multiforme (GBM) is one of the most aggressive human brain malignancies. Even with optimal treatment, median survival is less than 6 months for patients with recurrent GBM. Immune-based therapies have the potential to improve patient outcome by supplementing standard treatment. Expression of human cytomegalovirus (CMV) antigens in GBM tissues provides the unique opportunity to target viral antigens for GBM therapy. Here, we report findings of a formal clinical assessment of safety and potential clinical efficacy of autologous CMV-specific T-cell therapy as a consolidative treatment for recurrent GBM. From a total of 19 patients with recurrent GBM, CMV-specific T cells were successfully expanded from 13 patients (68.4%), 11 of whom received up to four T-cell infusions. Combination therapy based on T-cell infusion and chemotherapy was well tolerated, and we detected only minor adverse events. The overall survival of these patients since first recurrence ranged from 133 to 2,428 days, with a median overall survival of 403 days. Most importantly, 4 of 10 patients that completed the treatment remained progression free during the study period. Furthermore, molecular profiling of CMV-specific T-cell therapy from these patients revealed distinct gene expression signatures, which correlated with their clinical response. Our study suggests that a combination therapy with autologous CMV-specific T cells and chemotherapy is a safe novel treatment option and may offer clinical benefit for patients with recurrent GBM. *Cancer Res; 74(13); 3466–76. ©2014 AACR.*

Introduction

Glioblastoma multiforme (GBM) is one of the most malignant human adult brain tumors. Current treatment regimes include surgical resection, radio-, and chemotherapy, but patient prognosis remains poor with a median survival after initial diagnosis of less than 15 months (1) and a 5-year survival rate of less than 10% (2). GBM is incurable and inevitably recurs after initial therapy. Median survival for recurrent GBM is 3 to 6 months, and most patients do not survive longer than 1 year (2–4). Although chemotherapy, especially the introduction of temozolomide, has been shown to increase survival to some degree (1), dramatic improvements in outcome for patients with GBM have remained elusive. Therefore, much interest has focused on immunotherapeutic approaches. Strategies under investigation include tumor lysate vaccines, tumor antigen vaccines, and targeting of immunomodulatory molecules (reviewed in ref. 5).

Accumulating evidence indicates human cytomegalovirus (CMV) as a contributing factor to glioma progression (6, 7), and CMV has been suggested as a therapeutic target (8). Although not classified as an oncogenic virus, CMV can increase cellular proliferation, angiogenesis, and immune evasion, thus enabling several hallmarks of cancer (9, 10). Recently, an onco-accessory function of CMV has also been described in a mouse model of glioblastoma using murine cytomegalovirus infection (11). More importantly, CMV antigens and nucleic acids have been detected in histologic sections of GBM but not in surrounding healthy tissue (12–18). Although these histologic findings have been disputed (19–21), more recent studies have confirmed the presence of CMV sequences in malignant cells (22, 23). Vaccination of one patient with dendritic cells pulsed with autologous GBM lysate elicited a CMV-specific immune response, further supporting the presence of CMV antigens in GBM tissue (24). This provides an opportunity to target viral antigens with immune-based therapies. Low levels of CMV antigen expression in tumor cells were found to be associated with longer survival of patients with GBM (15, 16), thus indicating that antiviral therapy could improve GBM prognosis. In addition, recent studies supplementing standard GBM therapy with antiviral valganciclovir treatment for more than 6 months demonstrated a survival benefit for patients with GBM (25, 26).

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We have explored the feasibility and safety of an autologous T-cell–based GBM immunotherapy targeting CMV antigens. We have recently shown that CMV-specific $CD8^+$ T cells in patients with GBM have reduced functional capacity, but that this limitation can be reversed following *in vitro* stimulation. Adoptive transfer of these cells into a single patient with recurrent GBM in combination with standard chemotherapy was associated with long-term disease-free survival (27). Here, we report the findings from a formal clinical assessment of this initial finding as a phase I clinical trial. We demonstrate that autologous CMV-specific T-cell therapy is safe with minimal side effects and may offer clinical benefit for patients with recurrent GBM.

Patients and Methods

Study design, ethics, and patients

This phase I clinical study was designed to assess the safety and tolerability of autologous CMV-specific T-cell therapy for recurrent GBM. This clinical trial was conducted according to Declaration of Helsinki principles and was approved by The QIMR Berghofer Medical Research and Uniting Care Health Human Research Ethics Committees. All participants signed a consent form, which was approved by both ethics committees. This study is registered under the Australia New Zealand Clinical Trial Registry (ACTRN12609000338268). Study completion required a minimum of three T-cell infusions, whereas additional infusions could be administered depending on availability of cells. Infusions consisting of 25 to 40 $\times$ 10^6 autologous CMV-specific T cells in sterile saline were administered in 4 ($\pm$ 2) weeks intervals. The infusions were coordinated with periods of chemotherapy to avoid unwanted side effects. Peripheral blood samples collected before each infusion and at regular intervals after infusion were used for haematologic and immunologic monitoring. Each follow-up visit included vital observations and a quality-of-life questionnaire. Magnetic resonance imaging (MRI) was used to assess baseline tumor load before infusion and at regular intervals after first infusion.

In vitro expansion of CMV-specific T cells from patients with GBM

CMV-specific T cells were generated by *in vitro* stimulation with synthetic peptide epitopes. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by Ficoll gradient, washed, and resuspended in RPMI-1640 supplemented with 10% FBS (growth medium). The cells were cocultured with autologous PBMCs presensitized with CMV peptides (5 μg/mL; Supplementary Table S1) at a responder to stimulator ratio of 2:1 in growth medium. After 3 days and then every 3 to 4 days thereafter, the cultures were supplemented with growth medium containing 120 IU/mL recombinant IL2 (Komtur Pharmaceuticals). Cells were cryopreserved on day 14, after testing for sterility and CMV-specific T cells using an IFNγ intracellular cytokine assay. Release criteria were $\geq$ 0.1% CMV-specific T cells in total lymphocyte population and >50% cell viability.

Characterization of CMV CTL by intracellular cytokine assay and flow cytometry

PBMCs or cultured T cells were stimulated with peptides corresponding to defined $CD8^+$ T-cell epitopes derived from CMV proteins (1 μg/mL) and incubated in the presence of Brefeldin A for 4 hours. For polyfunctional analysis, cells were incubated with a CD107a-antibody, Brefeldin A, and Monensin for 5 hours. After surface staining for CD8, CD4, and CD3, cells were fixed and permeabilized with cytofix/cytoperm and stained for IFNγ (and IL2 and TNF in polyfunctional assays). Immune monitoring was achieved by surface staining with appropriate peptide-MHC (pMHC) multimers (Immudex) and surface markers, followed by fixation with a transcription buffer set and intracellular staining (all reagents from BD Biosciences, unless otherwise indicated). Stained cells were resuspended in PBS containing 2% paraformaldehyde and acquired using a FACSCanto II or LSR Fortessa with FACSDiva software (BD Biosciences). Postacquisition analysis was conducted using FlowJo software (TreeStar).

Cell sorting and gene expression analysis by Taqman RT-PCR array

PBMCs were revived from cryopreserved stocks and stained with appropriate CMV dextramers, followed by staining for surface markers to allow for separation of monocytes, B cells, and T cells. After filtering the cell suspension through a nylon mesh for removal of cell clumps, total $CD8^+$ T-cell and CMV-specific $CD8^+$ T-cell populations were isolated using a FACS Aria (BD Biosciences) cell sorter. Total RNA was purified using the Qiagen RNeasy Micro Kit. An RNA amount equivalent to 3,000 cells was transcribed into cDNA using the high-capacity RNA-to-cDNA-Kit. The cDNA was preamplified with a custom primer pool, loaded into custom-designed Taqman array cards and run on the Viia7 real-time PCR system (all reagents from Life Technologies). The array cards were designed to contain 91 genes that were described to be regulated in CD8 T cells during CMV infection (28). Data were normalized to housekeeping genes (18S, actin, and ß2-microglobulin) and analyzed using Gene Spring v12.5 software.

Statistical analysis

Mortality and progression-free survival (PFS) were characterized using Kaplan–Meier curves, and the corresponding median survival times were obtained for the intention-to-treat (ITT) and per-protocol (PP) patient populations of 11 and 10 patients, respectively. The ITT patient population included patients who received at least one T-cell infusion and the PP patient population included patients who received at least three infusions. Time to death or censoring was defined as the time between the last follow-up date and the date of the first recurrence. Time to progression or censoring was defined as the time between the date of the subsequent recurrence after the treatment or the last follow-up date and the date of the first recurrence. Left truncations occurred since patients did not receive the treatment at the date of the first recurrence and have been accounted for

Table 1. Characteristics of patients with GBM and treatment history before T-cell therapy

GBM:ID	Age at diagnosis	Gender	Final histology	Time to recurrence (d)	Recurrences before T-cell therapy	Time to T-cell therapy (d)	Number of operations	Gliadel use	XRT/TMZ before T-cell therapy	Additional treatment before T-cell therapy
01	60	M	GBM	300	2	706	2	2	Yes	None
02	49	M	Gliosarcoma	404	1	473	2	0	Yes	Avastin
03	50	M	GBM	208	3	1,572	4	2	Yes	Etoposide, thalidomide
04	72	F	GBM	439	1	519	2	2	Yes	None
05	52	M	GBM	378	1	—	2	0	Yes	None
06[a]	32	F	GBM	1,058	1	2,117	3	1	Yes	Avastin
07	54	M	GBM	143	1	241	2	0	Yes	Carboplatin
08	66	M	GBM	499	1	—	2	0	Not recorded	None
09	74	F	GBM	235	2	499	2	1	Yes	None
10[a]	37	M	AA	1,630	3	—	3	0	No	Avastin
11	21	M	GBM	639	1	—	2	0	Yes	Avastin
12	47	F	GBM	4,552	1	—	2	0	Yes	None
13	57	M	GBM	244	1	318	2	0	Yes	None
14[a]	47	F	GBM	1,105	1	—	2	0	Yes	Avastin
15	60	M	GBM	197	1	283	1	0	Yes	None
16	50	M	GBM	989	1	1,092	1	0	Yes	None
17	23	M	GBM	436	1	—	1	0	Yes	Avastin
18	61	F	GBM	323	NIA	—	2	0	Yes	None
19	41	F	GBM	413	2	704	3	0	Yes	Carboplatin, lomustine, avastin

NOTE: Time to recurrence is calculated from date of first surgery to date of tumor recurrence based on MRI findings. Number of operations refers to tumor debulking surgeries only. Time to T-cell therapy is calculated from date of first diagnosis. Shaded areas indicate patients that did not complete the trial.

Abbreviations: AA, anaplastic astrocytoma; d, days; NIA, no information available; TMZ, temozolomide chemotherapy; XRT, radiotherapy.

[a]Patients that were initially diagnosed with low-grade malignancies.

Table 2. Clinical follow-up of adoptive T-cell therapy of patients with recurrent GBM

GBM:ID	Total cells expanded	Number of cells per infusion	Number of infusions	Time to progressive disease after first infusion (d)	Treatment in addition to T cells	Follow-up since first infusion (d)	Current status (December 31, 2013)
01	26.8×10^7	3×10^7	4	No progression	TMZ	1,447	Alive
02	11.8×10^7	2.8×10^7	4	57	Avastin	330	Deceased
03	7.4×10^7	2.0×10^7	3	135	Avastin, thalidomide	1,134	Alive
04	14.6×10^7	2.9×10^7	4	no progression	TMZ	1010	Deceased[a]
05	Failed	N/A	0	N/A	N/A	N/A	Deceased
06	22×10^7	4×10^7	4	36	Avastin, TMZ	153	Deceased
07	16.8×10^7	3.5×10^7	2	Withdrawn before third infusion	Avastin, carboplatin	35	Deceased
09	13×10^7	2.5×10^7	4	No progression	TMZ	462	Alive
11	13.7×10^7	N/A	0	Withdrawn before infusion	N/A	N/A	Deceased
12	Failed	N/A	0	N/A	N/A	N/A	Deceased
13	15.1×10^7	3.2×10^7	4	34	Avastin, CCNU	100	Deceased
14	10.6×10^7	N/A	0	Withdrawn before infusion	N/A	N/A	Deceased
15	9.2×10^7	2.5×10^7	3	287	None	317	Deceased
16	19.4×10^7	4×10^7	4	143	TMZ, surgery	392	Alive
19	13.7×10^7	3×10^7	3	No progression	Avastin, lomustine	175	Alive

NOTE: Shaded areas indicate patients that underwent venesection but did not complete the trial.

Abbreviations: CCNU, lomustine chemotherapy; d, days; N/A, not applicable; TMZ, temozolomide chemotherapy.

[a]Patient death was not related to GBM.

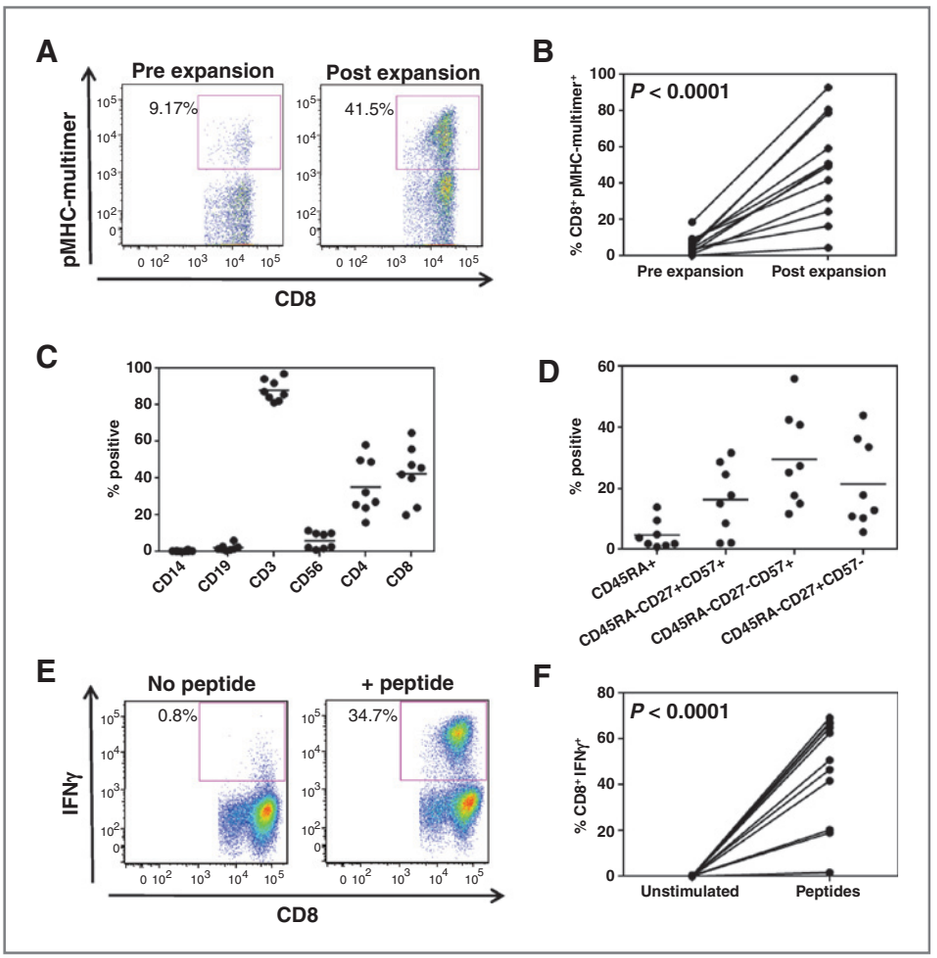


Figure 1. Characterization of *in vitro* expanded CMV-specific CD8$^+$ T cells and clinical response to adoptive T-cell therapy. A, representative FACS plot of pMHC-multimer staining of CMV-specific CD8$^+$ T cells before and after *in vitro* expansion with CMV-derived synthetic peptide epitopes (Supplementary Table S1). B, Pairwise analysis of *in vitro* expanded CMV-specific CD8$^+$ T cells before and after stimulation with CMV-derived synthetic peptide epitopes (data from 12 of 13 patients with GBM are shown; no suitable HLA-peptide multimer was available for one patient). C and D, phenotypic analysis of *in vitro* expanded T cells. These cells were incubated with antibodies specific for various surface markers (CD14, CD19, CD56, CD3, CD4, CD8, CD27, CD57, and CD45RA) and then analyzed using LSR Fortessa with FACSDiva software. E and F, analysis of IFNγ expression by *in vitro* expanded CMV-specific CD8$^+$ T cells measured by ICS assay. E, representative data from one patient with recurrent GBM. F, pairwise analysis of IFNγ expression by CMV-specific CD8$^+$ T cells before and after *in vitro* stimulation. These T cells were incubated with the relevant peptide epitopes (Supplementary Table S2) in the presence of Brefeldin A for 4 hours. Cells were then incubated with antibodies specific for CD3, CD4, and CD8, and then assessed for intracellular IFNγ production.

appropriately. The analyses were performed using SAS Enterprise Guide 4.3 and R 3.0.1.

Results

Patient characteristics

Nineteen patients with recurrent GBM were recruited for this study. Eligibility criteria for the study included (i) age 18 years or above; (ii) geographically accessible for follow-up; (iii) ability to provide informed consent; (iv) Eastern Cooperative Oncology Group performance status 0, 1, 2, or 3; (v) life expectancy of at least 3 months; (vi) positive CMV serology; and (vii) previous histologic diagnosis of GBM (WHO grade IV) and radiologic and/or clinical evidence of tumor progression or recurrence. Four patients had to be withdrawn before venesection due to progressive disease, while insufficient CMV-specific T cells were expanded from two patients due to low precursor frequency or poor cell viability. All patients received standard treatment with maximal safe surgical debulking at primary diagnosis, external beam radiotherapy and chemotherapy (Table 1). CMV-specific T cells were successfully expanded from 13 patients, but two patients had to be withdrawn due to progressive illness and one patient discontinued the intervention after two infusions due to progressive disease. In total, 10 patients completed a minimum of three infusions as required per protocol (Table 2).

In vitro expansion and functional characterization of CMV-specific CD8$^+$ T cells

Autologous CMV-specific T cells were successfully expanded from 13 of 19 patients (Table 2). *Ex vivo* analysis of CMV-specific T cells from patients with GBM using pMHC

Table 3. Safety assessment following adoptive T-cell therapy of patients with recurrent GBM[a]

Adverse event	Number of patients affected (attribution score[b])
Grade 1: mild	
Headache	3 (2)
Fatigue	3 (2)
Visual hallucination	1 (2)
Pyrexia of unknown origin	1 (2)
High blood pressure	1 (2)
Grade 2: moderate	
Abnormal liver function tests	1 (2)
Lymphopenia	1 (2)
Seizure	1 (2)
Anxiety	1 (2)
Grade 3: severe	
Seizure	1 (2)

[a]Severity grade and attribution scores assessed according to the NCI Common Terminology Criteria for Adverse Events. [b]Attribution score: 1, unrelated; 2, unlikely; 3, possible; 4, probable; 5, definite. Adverse event logs were compiled from clinical observation (including vital signs), patient interview, and blood samples taken during infusions and at follow-up visits. Events scored as unrelated were excluded from this table.

multimers showed antigen-specific T-cell frequencies ranging from 0.026% to 18.4% of total $CD8^+$ T cells (median, 4.2%). Following *in vitro* stimulation with HLA-matched CMV peptide epitopes, a significant increase in antigen-specific T cells was observed (range, 4.2%–92.7% of total $CD8^+$ T cells; median, 49.85%; $P < 0.0001$, Fig. 1A and B, and Supplementary Table S2). Phenotypic characterization showed that these T cells were predominantly $CD3^+$ with a combination of both $CD8^+$ and $CD4^+$ T cells (Fig. 1C). The majority of CMV-specific T cells were $CD45RA^-$ $CD27^+$ and/or $CD57^+$ a phenotype characteristic of effector cells (Fig. 1D). Intracellular cytokine analysis revealed that high proportions of these *in vitro* expanded $CD8^+$ T cells expressed IFNγ in response to stimulation with CMV epitopes (Fig. 1E and F). Furthermore, in-depth functional analysis of these T cells from some patients showed that these cells displayed polyfunctional profile and also expressed CD107a (data not shown).

Safety and clinical evaluation of CMV-specific T-cell–based immunotherapy for recurrent GBM

Of the 13 patients for whom CMV-specific T cells were generated, 10 patients received three to four T-cell infusions ($2–4 \times 10^7$ cells/infusion, Table 2). One patient only received two infusions, whereas two patients died before the availability of T cells. Infusions were generally well tolerated and mostly minor adverse events were recorded (Table 3). The toxicity grading was assigned according to the NCI Common Terminology Criteria for Adverse Events. A single serious adverse event (SAE) possibly related to T-cell therapy was recorded (Patient GBM:19). This patient had a generalized seizure within 12 hours of the first T-cell infusion and was hospitalized for 3 days. The patient had a history of seizures before entering into the current trial. The investigators discussed this SAE with the Data Safety Monitoring Committee, and deemed the SAE was unlikely to be associated with T-cell therapy. No further seizures developed in this patient after subsequent T-cell infusions. These analyses indicate that autologous CMV-specific T-cell infusions are a safe treatment for GBM. The median overall survival (OS) of the 11 patients that received at least one infusion was 403 days (range, 133 to 2,428 days; Fig. 2A). The time to progression for all patients after infusion ranged from 108 to more than 1,783 days, with a median of 246 days (Fig. 2B). Of the 11 patients treated with T-cell therapy, four patients remained progression-free (Table 2). Patient GBM:01 showed the longest stabilization of disease with almost 4 years of PFS after the T-cell infusion. This patient had no other treatment subsequent to CMV-specific T-cell therapy and remains disease-free to date.

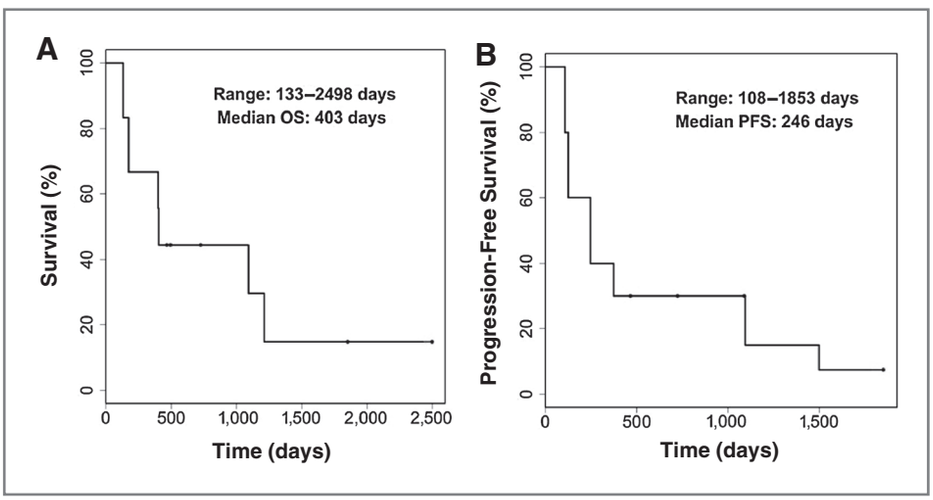
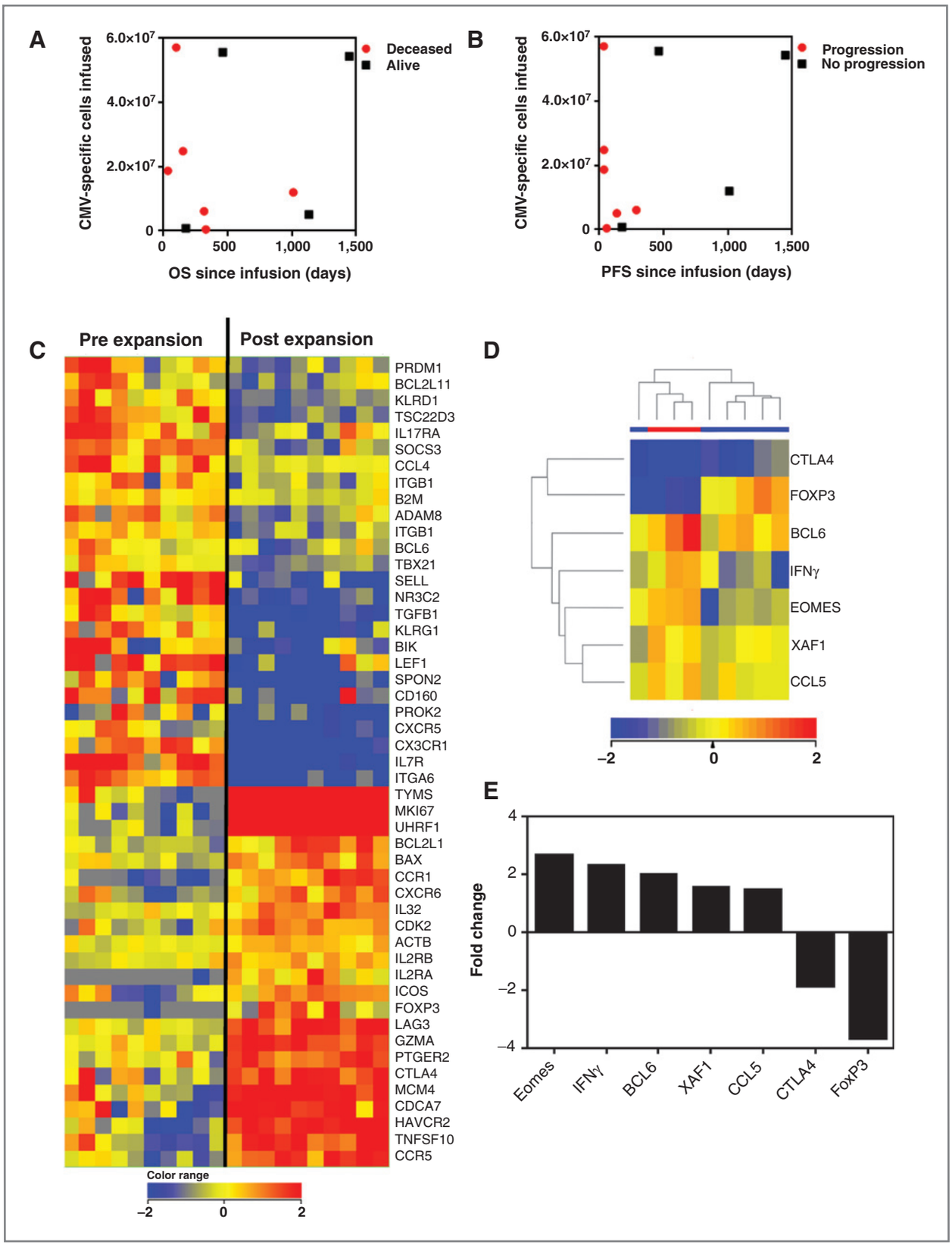


Figure 2. A, OS of patients with GBM treated with autologous CMV-specific T cells since first recurrence. B, PFS following CMV-specific T-cell adoptive immunotherapy (determined from the date of the first T-cell infusion).



Immunologic and molecular analysis of CMV-specific T-cell therapy

To assess the efficiency of T-cell therapy, we first determined the effect of antigen-specific T-cell frequencies on survival. However, there was no correlation between the number of CMV-specific T cells transferred and the OS and PFS (Fig 3A and B). *Ex vivo* longitudinal analysis of CMV-specific T cells showed that although in some patients there was a small increase in the precursor frequency of CMV-specific T cells, the number of antigen-specific T cells returned to baseline after the completion of adoptive immunotherapy (Supplementary Fig. S1A and S1B). The functional profile of these T cells also remained unchanged during and after the completion of T-cell therapy (Supplementary Fig. S1C).

We next explored the possibility that gene expression profiling of T-cell therapy may help to distinguish patients that might benefit from adoptive immunotherapy. To test this hypothesis, we used a custom array that allowed quantitative expression analysis of different categories of genes relevant for T-cell function (Supplementary Table S3). This expression profiling revealed that 47 of 91 genes were significantly changed in CMV-specific $CD8^+$ T cells following *in vitro* expansion (Fig. 3C and Supplementary Fig. S2A and S2B). This gene expression profile was consistent with a signature of activated T cells and was further confirmed by specific antibody staining and flow cytometry (Supplementary Fig. S2C).

To identify gene expression patterns that might be of prognostic value, we divided our patient cohort into two groups: (i) patients with GBM who had short PFS (<100 days) and (ii) patients with GBM who either remained progression-free within the study period or developed progressive disease after more than 100 days. In-depth analysis of gene expression data revealed that these two groups of patients showed significant differences in the expression of seven genes, including T-cell transcription factors (*EOMES, BCL6, and FOXP3*), cytokine/chemokines (*IFNG* and *CCL5*), and checkpoint markers (*CTLA4* and *XAF1*; Fig. 3D and E). These analyses suggest that expression profiling of T-cell therapy may provide clues on the potential therapeutic benefit of adoptive immunotherapy.

CMV-specific T cells are present in GBM tumor tissue and show a distinct phenotype compared with peripheral blood

Ex vivo analysis of tumor-infiltrating antigen-specific T cells can provide some critical insights on the immune control of malignant cells. In our study, GBM:16 patient who received four infusions of CMV-specific T cells developed progressive disease 4 months after the completion of T-cell infusions and then underwent surgical tumor resection. We isolated T cells from the resected tumor tissue and were able to detect CMV-specific $CD8^+$ T cells (Fig. 4A, top). However, the majority of these antigen-specific T cells failed to express multiple cytokines, including IFNγ, TNF, IL2 and showed poor cytotoxic activity as assessed by CD107a mobilization following stimulation with CMV peptides (Fig. 4A, bottom). Furthermore, staining of CD103 as a marker for tissue resident T cells (T_{rm}) revealed that although approximately one third of $CD8^+$ T cells in the tumor tissue were T_{rm}, none of the CMV-specific T cells expressed CD103 (Fig. 4B). The frequency of CMV-specific T cells in the tumor tissue was approximately 4-fold lower when compared with T cells circulating in peripheral blood at different time points before (d0), during (d35), and after T-cell therapy (d78 and d121, Fig. 4C). Tumor-infiltrating CMV-specific T cells expressed higher levels of PD-1, TIM-3, and CTLA-4 and lower levels of transcription factors T-bet, Eomes, and LEF-1 (Fig. 4C). These observations suggest that tumor-infiltrating antigen-specific T cells in this patient with GBM displayed poor functional capacity and increased expression of inhibitory receptors when compared with T cells from peripheral blood. Similar expression patterns of PD-1, CTLA-4, TIM-3, and transcription factors were detected in the global $CD8^+$ T-cell population (Supplementary Fig. S3). We further detected almost 5-fold higher levels of regulatory T cells (CD4+CD25+FoxP3+) than in peripheral blood, which is consistent with an immunosuppressive environment in tumor tissue (Fig. 4C).

Discussion

The survival of patients with recurrent GBM remains poor despite use of all currently available cytotoxic therapeutics (1, 29–32). Over the last decade, immune-based therapies have emerged as possible tools for the treatment of recurrent GBM (33–35), and exploratory studies have shown improved PFS and OS (36–38). In 2002, Cobbs and colleagues demonstrated expression of the CMV proteins IE-1 and late antigen in GBM tumor biopsies, which were later confirmed by other groups (10, 12–14, 21). Further studies have suggested that CMV-encoded proteins such as viral IL10 and US28, a G-protein–coupled receptor-like protein, may act as tumor promoters in GBM (10, 39, 40). The presence of CMV in GBM has generated considerable interest, especially the potential targeting of the viral proteins using immune-based therapies (18). We have shown previously that CMV-specific T cells from the majority of patients with GBM display reduced multifunctional potentiality and that *in vitro* stimulation of these T cells can improve their functional profile

Figure 3. Molecular analysis of *in vitro* expanded T cells and correlation to clinical outcome. A and B, correlation of the CMV-specific T-cell infusion dose with OS and time to progression. Data represent the correlation between the numbers of adoptively transferred CMV-specific T cells received by each patient, as determined using the intracellular IFNγ analysis, and the OS since first recurrence and time to progression following the first T-cell infusion, respectively. C, gene expression profiling of CMV-specific T cells before and after *in vitro* expansion. Heatmap of 47 of 91 significantly different genes is shown. This analysis is based on T cells from 10 patients with GBM enrolled in the study. Each column represents one patient. Data were normalized to housekeeping genes (18S, actin, and ß2-microglobulin) and analyzed using Gene Spring v12.5 software. D, heatmap of selected genes that were differentially expressed in T cells used for adoptive immunotherapy in patients with short (<100 days) or long-term (>100 days) PFS. Blue line above heatmap, patients <100 days; red line, patients >100 days. E, fold change expression analysis of selected genes in T-cell therapy from short or long-term progression-free surviving patients.

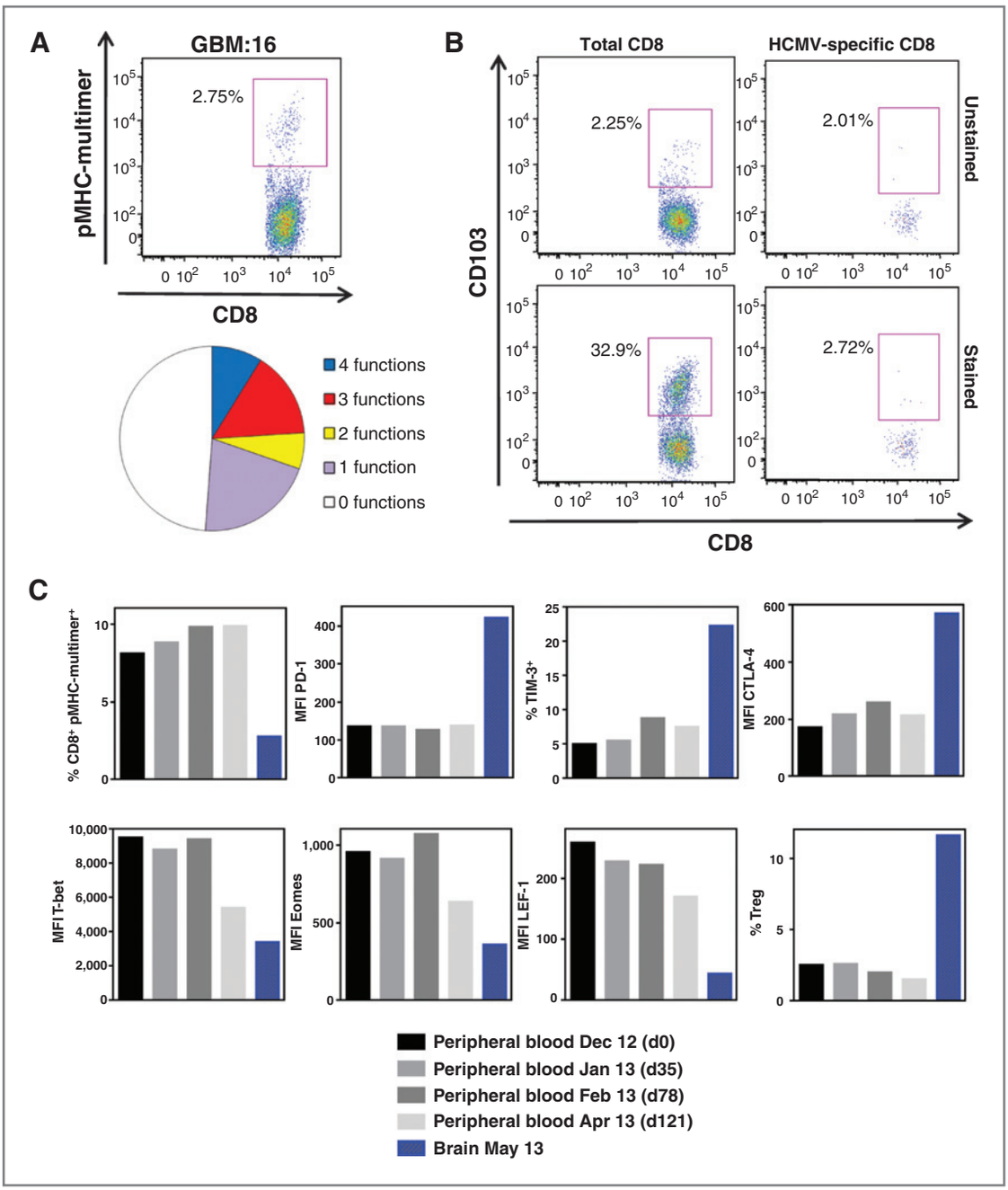


Figure 4. *Ex vivo* analysis of tumor-infiltrating and peripheral blood circulating CMV-specific CD8$^+$ T cells from a patient with GBM. A, e*x vivo* HLA-peptide multimer staining and polyfunctional analysis of tumor-infiltrating CMV-specific T cells from patient GBM:16. B, expression of CD103 (a marker of tissue-resident T cells) on total CD8$^+$ and CMV-specific T cells in tumor-infiltrating lymphocytes. C, longitudinal comparative phenotypic analysis of peripheral blood circulating and tumor-infiltrating CMV-specific CD8$^+$ T cells and CD4$^+$ Tregs. Tumor-infiltrating lymphoid cells and PBMC were incubated with HLA-peptide multimers, antibodies specific for CD3, CD4, CD8 and specific markers (as indicated on the *y*-axis of each box), and then analyzed using a LSR Fortessa with FACSDiva software. Postacquisition analysis was conducted using the FlowJo software.

(27). Adoptive transfer of these T cells into one patient with recurrent GBM was shown to be safe with possible clinical benefit.

In the present study, we report the outcome of the first clinical trial for adoptive immunotherapy using CMV-specific T cells in patients with recurrent GBM. We recruited 19 patients with recurrent GBM and of these 11 patients received multiple infusions of autologous *in vitro* expanded CMV-specific T cells. A number of important conclusions can be drawn from this study. First, adoptive transfer of

CMV-specific T cells was completely safe with minimal toxicities. Although the CMV-specific T-cell therapy was provided in combination with standard therapies (Table 2), we did not observe any deleterious impact of these therapies on the adoptively transferred CMV-specific T cells. More importantly, patients did not experience any severe side effects, and the only recorded SAE was deemed unrelated to the treatment.

Second, clinical follow-up analyses showed that CMV-specific immunotherapy was coincident with disease stabilization and prolonged PFS in some patients. The median OS in our study was >57 weeks (range, 19–346 weeks) with a median PFS of >35 weeks (range, 15.4–254 weeks). Most importantly, 4 of the 10 patients who completed T-cell therapy remained progression-free. Although promising, these observations will require confirmation in a formal phase II randomized clinical trial. Interestingly, the positive effects of antiviral therapy in the GBM setting have also recently emerged from a randomized, placebo-controlled study investigating the use of the antiviral drug valganciclovir for the treatment of primary gliomas (25).

Third, we were unable to see any correlation of antigen-specific T-cell frequencies following adoptive immunotherapy and clinical outcome. Although some patients showed a small increase in virus-specific T cells in the peripheral blood following the first few infusions, this effect was transient. Furthermore, phenotypic and functional analysis showed no link between the clinical response and antigen-specific T cells in the peripheral blood. Although the reason for this lack of correlation is unknown, it is probable that the tumor microenvironment and disease burden may affect T-cell function and influence the clinical response to adoptive immunotherapy (41, 42). Indeed, preliminary data from a single patient (GBM:16) who developed progressive disease soon after the completion of adoptive immunotherapy showed that although antigen-specific T cells were detected in the tumor, the majority of these cells lacked multifunctional potentiality and had undetectable expression of CD103, which is a crucial marker for tissue residence. In addition, these T cells showed increased expression of checkpoint inhibitory receptors when compared with the circulating effector cells from peripheral blood, potentially reflecting the impact of the tumor microenvironment. We further detected higher amounts of regulatory T cells, which might contribute to immunosuppression in GBM tissues (43). These observations were supported by gene expression analysis of antigen-specific T cells used for adoptive immunotherapy. Indeed, increased prolonged PFS was coincident with lower expression of checkpoint inhibitory receptors and increased expression of T-cell transcription factors crucial for T-cell function. It is important to emphasize that these observations will require more in-depth analyses in a larger cohort of patients within a randomized phase II clinical study.

Taken together, adoptive immunotherapy of patients with recurrent GBM with CMV-specific T cells is safe and may provide long-term clinical benefits. These studies provide an important platform for a formal assessment of adoptive T-cell immunotherapy in both therapeutic and prophylactic settings. CMV-specific T-cell–based immunotherapy should be considered as a consolidative treatment following primary diagnosis of GBM for the prevention of recurrent disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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