Autologous CMV-specific T cells are a safe adjuvant immunotherapy for primary glioblastoma multiforme

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BACKGROUND. The recent failure of checkpoint-blockade therapies for glioblastoma multiforme (GBM) in late-phase clinical trials has directed interest toward adoptive cellular therapies (ACTs). In this open-label, first-in-human trial, we have assessed the safety and therapeutic potential of cytomegalovirus-specific (CMV-specific) ACT in an adjuvant setting for patients with primary GBM, with an ultimate goal to prevent or delay recurrence and prolong overall survival.

METHODS. Twenty-eight patients with primary GBM were recruited to this prospective study, 25 of whom were treated with in vitro–expanded autologous CMV-specific T cells. Participants were monitored for safety, progression-free survival, overall survival (OS), and immune reconstitution.

RESULTS. No participants showed evidence of ACT-related toxicities. Of 25 evaluable participants, 10 were alive at the completion of follow-up, while 5 were disease free. Reconstitution of CMV-specific T cell immunity was evident and CMV-specific ACT may trigger a bystander effect leading to additional T cell responses to nonviral tumor-associated antigens through epitope spreading. Long-term follow-up of participants treated before recurrence showed significantly improved OS when compared with those who progressed before ACT (median 23 months, range 7–65 vs. median 14 months, range 5–19; *P* = 0.018). Gene expression analysis of the ACT products indicated that a favorable T cell gene signature was associated with improved long-term survival.

CONCLUSION. Data presented in this study demonstrate that CMV-specific ACT can be safely used as an adjuvant therapy for primary GBM and, if offered before recurrence, this therapy may improve OS of GBM patients.

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Introduction

Therapeutic intervention with cytotoxic T lymphocytes is emerging as a powerful tool for the induction of sustained immune control of malignancies and virus-associated complications (1). Adoptive cellular therapy (ACT) utilizing genetically modified or nonmodified antigen-specific T cells has provided sustained clinical responses in certain clinical settings (2, 3). To date, the therapeutic benefit of ACT has been limited in solid tumors, most likely due to the immune microenvironment and the impact of advanced tumor burden, which is associated with

rapid progression and poor overall survival (OS) (4). In this context, early intervention with immunotherapy following primary diagnosis and first-line treatment offers an attractive alternative to treatment in advanced stages of disease (5). The additional advantage of preemptive therapeutic intervention with ACT, after primary diagnosis, is the reduced T cell–to–target antigen ratio, which should limit the cellular dysfunction associated with chronic T cell activation in the setting of high tumor burden (6).

Despite great success in many aspects of tumor therapy over the past 5 decades, including recent advances in antibody and cellular immunotherapies, dramatically improved outcomes for patients with glioblastoma multiforme (GBM) remain elusive (7, 8). Molecularly targeted therapies and checkpoint inhibitors have thus far failed to offer significant survival benefit beyond the current median OS of 14 to 16 months after initial diagnosis (9–12). As such, the standard of care for patients with GBM remains largely unchanged from surgical resection followed by radiotherapy and chemotherapy with temozolomide. However, evidence of prolonged survival in a subset of patients following either checkpoint inhibition or ACT
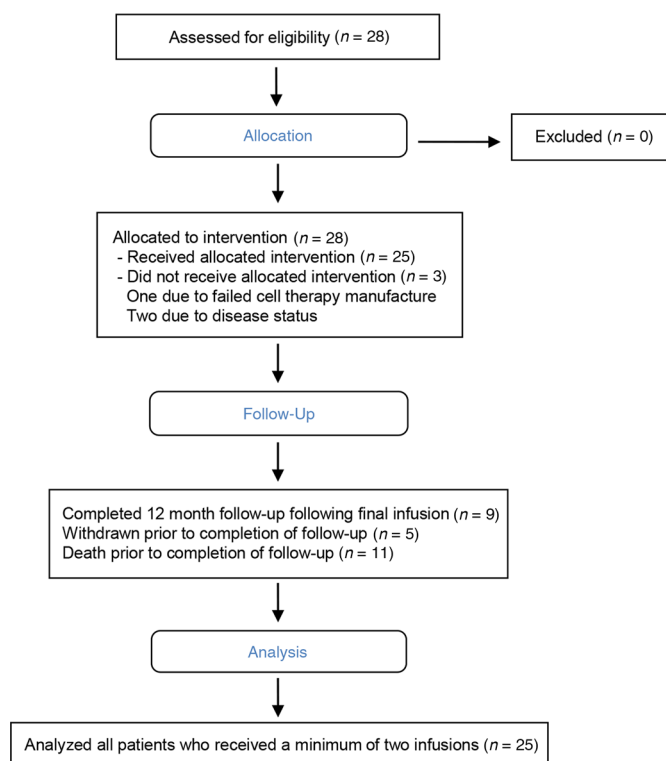
Figure 1. CONSORT diagram showing participant allocation, follow-up, and analysis.

Table 1. Clinical characteristics of GBM patients enrolled in the study

Number of patients	28
Age	Median: 59 (range: 32–74)
Sex	
Female	10
Male	18
GBM location	
Frontal	9[A]
Temporal	7[A]
Parietal	5
Parieto-occipital	4
Occipital	2
Parieto-temporal	1
Thalamic	1
IDH mutation	3 of 28
Methylated *MGMT*	11 of 28
Pre-ACT CTx/RT	28 of 28
During ACT CTx	21 of 25
Post-ACT CTx	16 of 25

[A]One patient described as both temporal and frontal. CTx, chemotherapy; RT, radiotherapy.

provides cause for optimism about the impact of immunotherapy on patient outcomes in the GBM setting (13–17). Considering the aggressive nature of GBM, the high rate of recurrent disease, and the overall poor survival outcomes for these patients, identifying the most appropriate strategies for administering immunotherapy to treat GBM is paramount to its success. We previously completed a phase I clinical trial assessing the safety and potential efficacy of using cytomegalovirus-specific (CMV-specific) ACT to treat patients with recurrent GBM (18). Although the role of CMV in GBM remains controversial (19), the presence of foreign CMV antigens within GBM tissue makes this an attractive target for ACT (20–23). Our initial studies using CMV-specific ACT against recurrent GBM provided encouraging clinical responses, with multiple patients showing prolonged OS (24). To assess whether earlier intervention with ACT improved patient outcomes, we initiated a phase I safety study utilizing CMV-specific ACT to treat GBM following primary resection and chemo/radiotherapy. Here the preemptive administration of CMV-specific ACT before evidence of recurrence was safe and provided encouraging clinical evidence of improved progression-free survival (PFS) and OS compared with the initiation of ACT following disease progression. Furthermore, these patients showed clear evidence of improved CMV-specific T cell immunity following ACT.

Results

Patient characteristics and ACT. Twenty-eight patients diagnosed with primary GBM were recruited for this study (Figure 1, Tables 1 and 2, and Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/

JCI138649DS1). No patient had evidence of progression before recruitment. This cohort was 64% male and 36% female, with a median age of 59 years. Patients were diagnosed with primary GBM, predominantly in the frontal and temporal regions; 11% of patients had isocitrate dehydrogenase (IDH) mutations and 39% had methylation of the methylguanine-DNA methyltransferase gene (*MGMT*) (Table 1).

CMV-specific T cells for ACT were successfully generated from 27 of 28 patients. ACT products contained a median of 79.3% (range 12.0%–97.3%) CD3$^+$CD8$^+$ T cells and 12.8% (range 2.4%–80.2%) CD3$^+$CD4$^+$ T cells (Figure 2A). The remaining cells were predominantly natural killer cells. Evidence of CMV-specific reactivity was detected in all ACT products (Figure 2B), with a median percentage of 40% CMV-specific IFN-γ–producing cells. Patient 2105-12's ACT product displayed low CMV reactivity and failed to meet manufacturing criteria due to low cell yield. CMV reactivity in 25 of the remaining 27 ACT products was dominated by CD8$^+$ T cells, while ACT products from patients 2105-02 and 2105-27 were dominated by CMV-specific CD4$^+$ T cells (Table 3 and Figure 2B). ACT products displayed reactivity against a median of 6 CMV-encoded peptide epitopes (range 1–9) restricted by a median of 3 different HLA types (range 1–7). Patient HLA types and the CMV epitopes encoded in the peptide pool are listed in Supplemental Tables 1 and 2. Three patients, including 2105-12, did not receive ACT. The additional 2 patients, 2105-07 and 2105-19, were withdrawn before ACT commencement due to advanced disease. The remaining 25 patients received a minimum of 2 and maximum of 6 doses of ACT (median 5.5), depending on
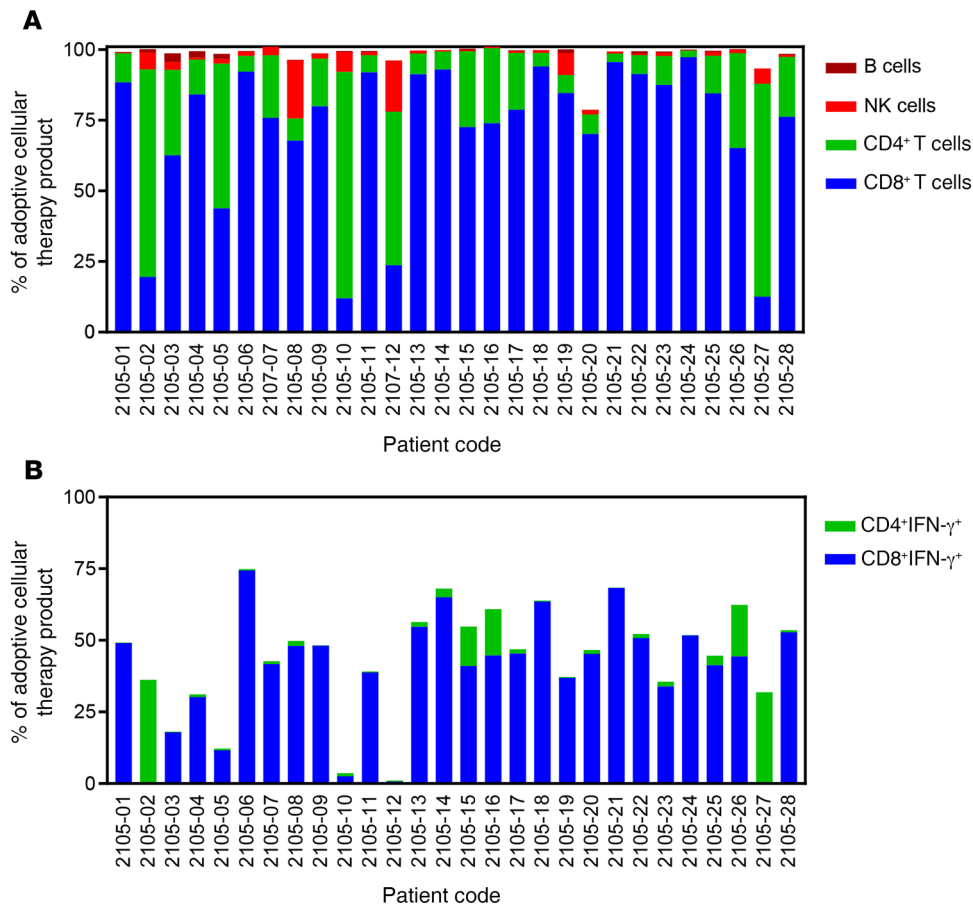
Figure 2. Characteristics of CMV-specific ACT products generated for GBM adoptive immunotherapy. (**A**) The phenotypic characteristics of T cells were assessed using standard TBNK analysis. Data represent the proportion of CD4+ T cells, CD8+ T cells, NK cells, and B cells in the final products. (**B**) T cells were assessed for the intracellular production of IFN-γ following recall with the CMV peptide pool used to generate the cell therapy. Data represent CD8+ or CD4+ T cells producing IFN-γ as a proportion of total viable lymphocytes.

the availability of product, at 2×10^7 cells/m². Patient 2105-25 was withdrawn following 2 doses due to advanced disease. The total ACT dose administered to each patient is provided in Table 3. Twenty of the 25 treated patients commenced ACT infusions before evidence of disease progression, while the remaining 5 patients progressed before the first ACT infusion.

Safety profile and clinical responses to CMV-specific ACT in primary GBM. Prior to recruitment, all patients underwent surgical resection of their primary tumor and had no evidence of relapse. Patients then received standard radiotherapy and chemotherapy, and ACT commenced at a median of 162 days and following diagnosis 56 days after recruitment. CMV-specific ACT was well tolerated in all patients. Adverse events were recorded throughout the trial, with terms and grades assigned using the Common Terminology Criteria for Adverse Events v4.0. Their association with ACT was classified using a 5-point scale: unrelated, unlikely to be related, possibly related, probably related, or definitely related. The majority of adverse events were determined to be related to the natural history of GBM and therefore unrelated to ACT. In addition, no adverse events that were possibly, probably, or definitely associated with ACT were observed. A summary of adverse events that were unlikely to be related to therapy

are provided in Table 4. Patients were monitored for disease progression using magnetic resonance imaging (MRI) during the course of ACT and for 12 months following their final infusion. Representative MRI scans from 4 patients are shown in Figure 3. Of 25 evaluable patients, 5 were withdrawn before completion of follow-up, 5 showed no evidence of tumor progression, and 10 were alive at completion of follow-up (Figure 4 and Table 3). Twelve months after completion of ACT, the PFS and OS of all treated patients were 20% and 52%, respectively. Furthermore, PFS and OS of patients who were offered CMV-specific ACT as adjuvant therapy were 25% and 60%, respectively.

Immunological monitoring following CMV-specific ACT. To assess the impact ACT has on CMV-specific cellular immunity in patients with GBM, peripheral blood mononuclear cells (PBMCs) were assessed for CMV-specific IFN-γ production using intracellular cytokine analysis. We assessed PBMCs isolated before the commencement of ACT (pretherapy, before the first infusion), during ongoing ACT cycles (during therapy, before the third or fourth infusion), in the first 2 months after the completion of therapy (post short term [post ST]; median 1 month, range 0–2), or at least 3 months after the completion of therapy (post long term [post LT]; median 6 months, range 3–12). Samples were selected based on availability and one

Table 2. Clinical characteristics of individual GBM patients

Patient Code	Age	Sex	Histological GBM type	IDH status	MGMT Status	CXT/RXT before ACT	Treatment during ACT	Treatment following ACT
2105-01	41	M	Temporal	WT	U	XRT/TMZ	TMZ, Surgery	Nil
2105-02	66	M	Parieto-occipital	WT	U	XRT/TMZ	TMZ, Avastin	Nil
2105-03	55	M	Frontal/Temporal	WT	U	XRT/TMZ	TMZ	TMZ
2105-04	75	M	Parieto-occipital	WT	M	XRT/TMZ	TMZ	TMZ, Avastin
2105-05	64	F	Parietal	WT	U	XRT/TMZ	Nil	Nil
2105-06	56	M	Occipital	WT	U	XRT/TMZ	TMZ, Avastin, Lomustine	Nil
2105-07	51	F	Temporal	WT	U	XRT/TMZ	Not applicable	Nil
2105-08	69	F	Temporal	WT	M	XRT/TMZ	TMZ	TMZ
2105-09	40	M	Temporal	WT	U	XRT/TMZ	Nil	Nil
2105-10	74	M	Frontal	WT	M	XRT/TMZ	TMZ, Avastin	Avastin
2105-11	64	F	Parieto-occipital	WT	U	XRT/TMZ	TMZ	TMZ Avastin, XRT
2105-12	67	F	Parietal	WT	M	XRT/TMZ	Not applicable	Not applicable
2105-13	57	F	Parietal	WT	M	XRT/TMZ	TMZ	TMZ, Avastin
2105-14	32	F	Parietal	Mutant	U	XRT/TMZ	TMZ	Surgery, TMZ
2105-15	63	F	Parietal	WT	M	XRT/TMZ	TMZ	TMZ, Avastin, XRT
2105-16	46	M	Frontal	WT	M	XRT/TMZ, TMZ	Nil	Nil
2105-17	61	M	Temporal	WT	U	XRT/TMZ	Nil	Surgery, TMZ
2105-18	50	M	Frontal	WT	U	XRT/TMZ	TMZ	TMZ
2105-19	71	M	Frontal	WT	U	XRT/TMZ	Not applicable	Not applicable
2105-20	45	M	Frontal	WT	M	XRT/TMZ	TMZ	Surgery, TMZ
2105-21	61	M	Parieto-temporal	WT	M	XRT/TMZ	TMZ	Surgery, TMZ, Avastin
2105-22	64	M	Frontal	WT	U	XRT/TMZ	TMZ, Avastin	TMZ, Avastin
2105-23	33	M	Temporal	Mutant	U	XRT/TMZ	TMZ	TMZ
2105-24	60	M	Occipital	WT	U	XRT/TMZ	TMZ	Surgery, TMZ
2105-25	53	M	Thalamic	WT	U	XRT/TMZ	TMZ	TMZ, Avastin
2105-26	65	F	Frontal	WT	U	XRT/TMZ	TMZ	TMZ
2105-27	38	F	Frontal	WT	M	XRT/TMZ	TMZ	Nil
2105-28	41	M	Parieto-occipital	Mutant	M	XRT/TMZ	TMZ	TMZ

TMZ, temozolomide; XRT, radiation.

sample was used for each time point. The frequency of CMV-specific IFN-γ^+CD8$^+$ and IFN-γ^+CD4$^+$ T cells was determined as a proportion of total viable lymphocytes. At all time points following ACT, there was a significant increase in the frequency of CMV-specific T cells in PBMCs (Figure 5A), with a median CMV-specific response of 0.51% before therapy, 0.72% during therapy, 0.80% post ST, and 1.0% post LT. We also assessed changes in the structural composition of the CMV-specific immune response in patients following CMV-specific ACT. To do this, we expanded CMV-specific T cells from PBMCs at different time points and assessed their reactivity against the peptide epitopes recognized by the autologous ACT product and HLA-matched to the patient (Supplemental Tables 1 and 2). Representative analyses from 6 patients are shown in Figure

5B. The remaining patients who were analyzed are shown in Supplemental Figure 1. While there was little change in the dynamics of the CMV-specific T cell response following ACT for some patients (represented by patient 2105-15), in the majority of patients we noted changes in the immunodominance hierarchy of CMV epitope–specific T cell responses that included the expansion of T cells against previously subdominant epitopes.

To further explore changes in the immune landscape following CMV-specific ACT, we next investigated the potential for epitope spreading by assessing T cell immunity to a panel of common tumor-associated antigens (TAAs) (Supplemental Table 3) that have been previously reported to be expressed in GBM (25–31). To assess immunity against these common TAAs,

Table 3. Adoptive cellular therapy product characteristics and patient survival analysis at completion of follow-up

Patient Code	Progression at Treatment	Number of ACT Infusions	Total ACT Dose (×10^7)	CMV-specific CD8+ T cells (%)	CMV-specific CD4+ T cells (%)	TTP (Months)	OS (Months)
2105-01	N	5	18	49.0	0.2	4	5
2105-02	Y	5	20	0.2	36.0	2	5
2105-03	N	3	9.5	17.9	0.2	11	15
2105-04	N	3	9.0	30.2	0.9	No progression	Alive
2105-05	N	5	19	11.6	0.6	8	10
2105-06	Y	6	24	74.4	0.5	2	12
2105-08	N	3	8.7	48.0	1.8	6	10
2105-09	N	3	9.0	48.1	0.1	1	3
2105-10	N	5	21	2.5	1.1	3	Withdrawn
2105-11	N	6	28	38.8	0.3	9	Alive
2105-13	Y	6	20	54.7	1.7	1	13
2105-14	N	4	14	65.0	3.0	7	Withdrawn
2105-15	N	6	18	41.0	13.8	8	Alive
2105-16	N	6	27	44.7	16.2	No progression	Alive
2105-17	N	5	24	45.4	1.5	4	14
2105-18	N	6	24	63.5	0.4	No progression	Alive
2105-20	N	6	26	45.3	1.3	4	Alive
2105-21	Y	6	23	68.3	0.1	0	Alive
2105-22	N	6	30	50.8	1.4	11	15
2105-23	N	6	26	33.9	1.6	No progression	Alive
2105-24	N	6	24	51.7	0.1	6	Alive
2105-25	Y	2	9.8	41.3	3.3	0	Withdrawn
2105-26	N	6	21	44.4	18.0	12	Withdrawn
2105-27	N	3	11	0.0	31.9	4	Withdrawn
2105-28	N	4	16	52.8	0.7	No progression	Alive

[A]Adverse event terms and grades based on the Common Terminology Criteria for Adverse Events version 4.0. [B]Number of patients experiencing each adverse event at the specified grade. All events had an association with the investigational product of "unlikely to be related." [C]Occurred on 2 occasions for this patient. [D]Occurred on 3 occasions for this patient.

we generated 3 pools of overlapping peptides (Supplemental Table 3) and stimulated PBMCs from trial participants with each individual TAA pool. Stimulated PBMCs were cultured for 2 weeks in the presence of IL-2, and then the expanded T cells were assessed for antigen specificity using an intracellular cyto-kine assay. Nineteen of the 25 screened patients displayed strong T cell reactivity against at least 1 of the 3 TAA pools, indicative of the priming of T cell responses against these antigens in trial participants (Figure 6, A and B). While these TAA-specific responses remained stable in a large proportion of the patient cohort, we observed improved T cell reactivity in 9 patients following ACT. In some instances, this constituted an increase in the preexisting response (patients 2105-03, 2105-10, 2105-15, and 2105-28)

while in others (patients 2105-06 and 2105-14) we observed the emergence of responses against a different TAA pool. These observations suggest that in addition to the impact on CMV-specific peripheral immunity in patients with GBM, CMV-specific ACT may demonstrate an additional bystander impact leading to additional T cell responses through epitope spreading.

Long-term clinical outcome of ACT in patients with primary GBM. To assess the potential long-term impact of CMV-specific ACT in our cohort of patients with primary GBM, we performed a follow-up survival analysis from the time of diagnosis. Median PFS in the 25 treated patients was 10 months (range 2–65 months; Figure 7A) and median OS was 21 months (range 5–65 months) following diagnosis (Figure 7B). To assess OS and PFS in patients

Table 4. Adverse events with a relationship to the investigational product of greater than "not related"

Adverse Event Term[A]	No. Patients[B]
Grade 1 events	
Upper limb weakness	1
Headache	1
Fatigue	1
Bradycardia	1
Hypertension	1
Hypotension	1
Grade 2 events	
Ataxia	2
Hemispatial neglect	2
Cognitive disturbance	1[C]
Upper and lower limb weakness	2
Dexterity	1
Fatigue	1
Memory impairment	1
Grade 3 events	
Seizure	3
Ataxia	2
Dehydration	2
Cognitive disturbance	2
Upper and lower limb weakness	1
Right-sided hemianopia	1
Headache	1
Psychosis	1
Subdural hemorrhage	1[D]
Dysphasia	1
Fall resulting in injury to head	1
Depressed level of consciousness	1
Thromboembolic event	1
Grade 5 event	
Headache (increasing intensity)	1

[A]Adverse event terms and grades based on the Common Terminology Criteria for Adverse Events version 4.0. [B]Number of patients experiencing each adverse event at the specified grade. All events had an association with the investigational product of "unlikely to be related." [C]Occurred on 2 occasions for this patient. [D]Occurred on 3 occasions for this patient.

Figure 3. Representative MRI scans following CMV-specific ACT. Scans from 4 patients including 2105-02 and 2105-06 who progressed before ACT and patients 2105-23 and 2105-28, who remained disease free during the follow-up period.

Figure 2). Of these, C1 and C3 had 10 and 7 patients, respectively, and were deemed large enough for survival comparisons. Differential expression analysis between the 2 clusters identified 46 genes ($P < 0.05$ and fold change [FC] > 1.5; Figure 8A). For example, higher expression of the key transcription factor eomesodermin (*EOMES*) was strongly associated with C3. Other genes associated with this cluster included T cell signaling and immune regulation molecules cystatin F (*CST7*), killer cell lectin-like receptor subfamily D, member 1 (*KLRD1*), killer cell lectin-like receptor subfamily D, member 1 (*KLRG1*), protein tyrosine phosphatase, nonreceptor type 6 (*PTPN6*), homing markers such as integrin αL chain (*ITGAL*/CD11a), and effector molecule granzyme H (*GZMH*) (Figure 8B). Pathway enrichment analysis highlighted several differentially activated pathways between the 2 clusters — e.g., cell cycle and proliferation-related pathways were significantly enriched in C1, whereas pathways related to natural killer cell–mediated toxicity and immunity were enriched in C3 (Supplemental Figure 3). Conversely, T cells from patients in C1 showed overexpression of IL-23, SELL, and NYB (Figure 8B). Interestingly, these 2 clusters differed significantly in their OS based on 72 months' follow-up from diagnosis ($P = 0.026$; hazard ratio for C1 vs. C3, 8.5; $P = 0.05$; Figure 8C), with C1 showing considerably poorer OS (median OS 21 months) compared with C3 (median OS not reached). The observed difference was maintained after removing the 4 patients who had tumor progression before the first ACT infusion ($P = 0.024$; Figure 8D), and also upon including *MGMT* methylation status and *IDH1* mutation status in a multivariate survival (Figure 8, E and F) and hazard-ratio (Supplemental Figure 4) analysis. In particular, C3 patients with *MGMT* methylation and/or

treated with ACT before evidence of disease progression, we stratified patients based on disease status at the time of the first infusion (Figure 7, C and D). Patients treated with ACT before recurrence ($n = 20$) showed significantly improved OS compared with those who progressed ($n = 5$) before ACT (23 months, range 7–65 vs. 14 months, range 5–19; $P = 0.018$). The 20 patients treated before progression had a median PFS of 10 months (range 4–65 months). The PFS and OS of the intent-to-treat cohort, all treated patients, and patients treated preemptively before progression at 6, 12, and 24 months are listed in Table 5.

Association of ACT gene expression profile with long-term survival following CMV-specific ACT. Finally, to assess the potential impact of ACT product quality attributes on patient survival, we investigated whether there was any association between T cell gene expression profiles (assessed using the NanoString nCounter gene expression platform) and the OS of patients treated with these products. We performed an unsupervised hierarchical clustering of the ACT product expression profiles, which revealed 4 distinct patient clusters, C1 to C4 (Supplemental
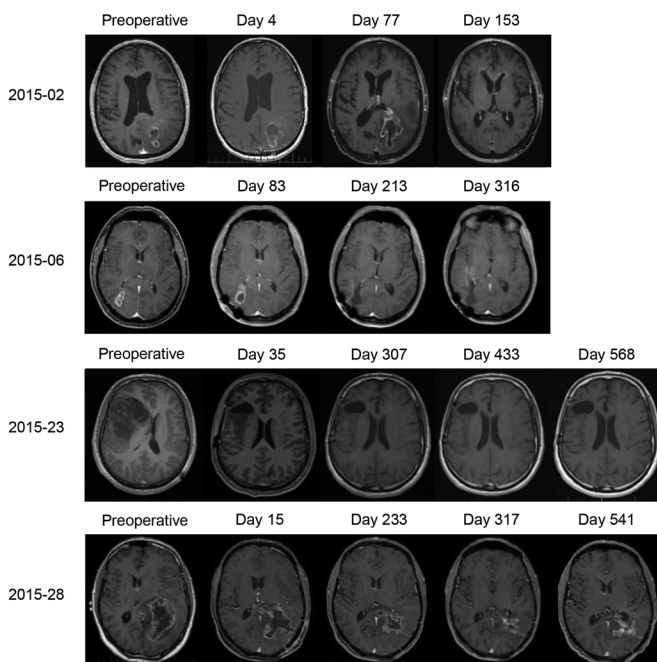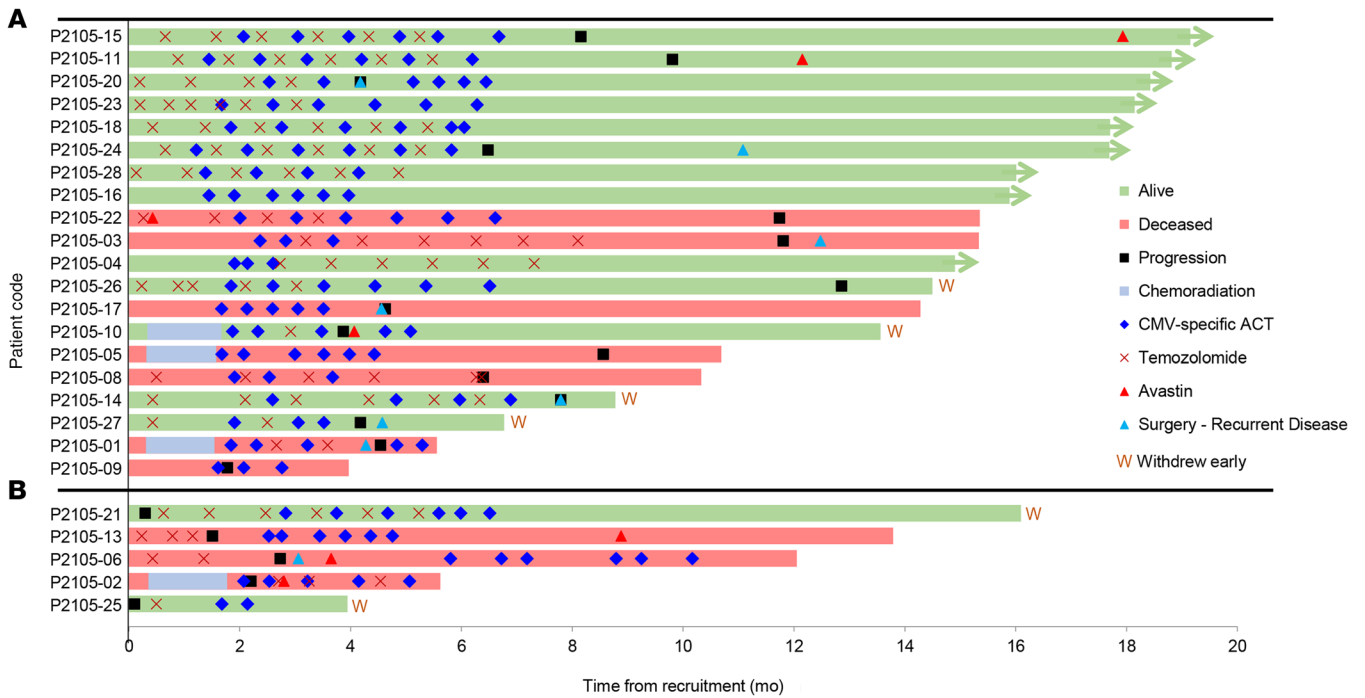
Figure 4. Swimmer plot of clinical course of patients following recruitment. Plot shows timing of ACT infusions and (where relevant), tumor progression, chemo-radiation, temozolomide, Avastin, surgery, withdrawal or death for patients treated with ACT. Panel **A** shows clinical course of patients who were treated with ACT before recurrence, while panel **B** shows clinical course of patients who were treated after recurrence. The key within the plot describes all symbols and color coding.
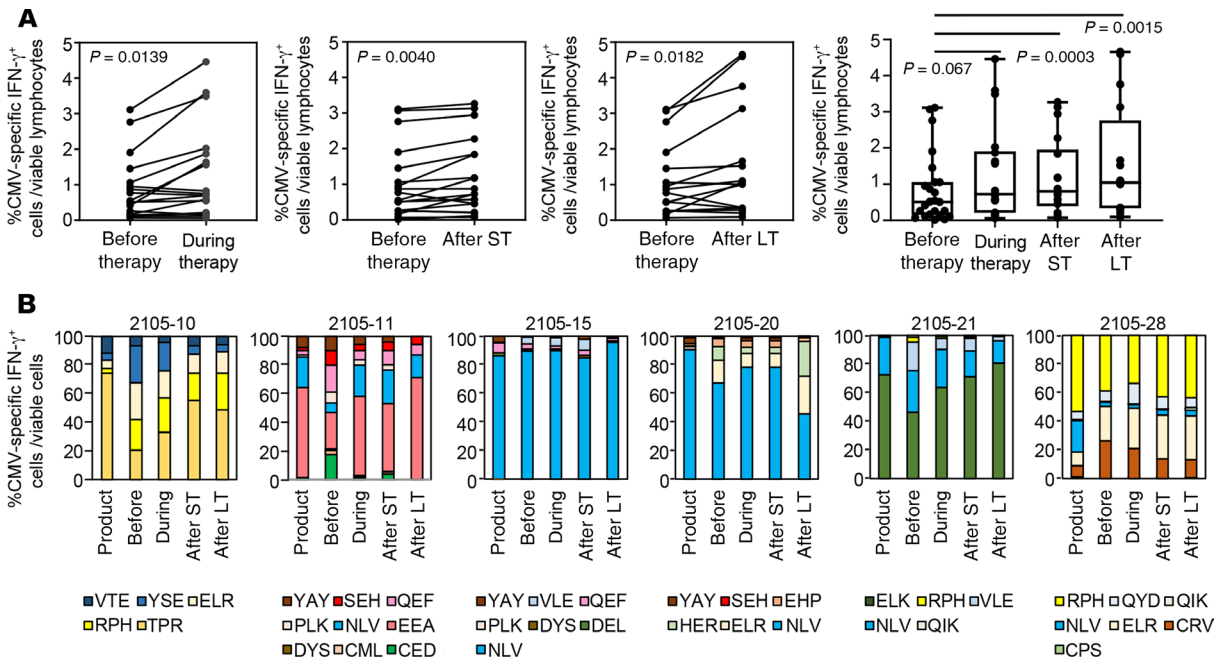

Figure 5. CMV-specific immunological monitoring following ACT. (A) PBMCs isolated from patients pre-ACT ($n = 18$), during ACT ($n = 18$), and either short-term (ST) ($n = 18$) or long-term (LT) ($n = 18$) after ACT were assessed for IFN-γ–producing CMV-specific T cells following stimulation with the CMV peptide pool used to generate the ACT products. Samples were selected based on sample availability and at the time point nearest to the following: first infusion day (pre-ACT), mid-point of infusion (during ACT), a median of 1 month following completion (short-term), and a median of 6 months following completion (long-term). Data represent paired patient analyses of the frequency of CMV-specific IFN-γ–producing cells as a proportion of total lymphocytes. Wilcoxon's matched-pairs signed-rank tests were used to determine significant differences in paired analysis. The box-and-whisker plot represents a summary of all patient responses. Statistical analysis was performed using mixed-effects analysis with Dunnett's multiple-comparisons test. Time points were considered statistically significant when $P <$ 0.05. **(B)** PBMCs isolated from patients at different time points during the clinical trial were stimulated for 14 days with the CMV peptide pool, and then assessed for reactivity against individual HLA-matched peptides contained in the pool. Representative data from 6 donors show the proportion of IFN-γ–producing peptide-specific T cells as a proportion of total CMV-specific T cells. Additional patient data are provided in Supplemental Figure 1.
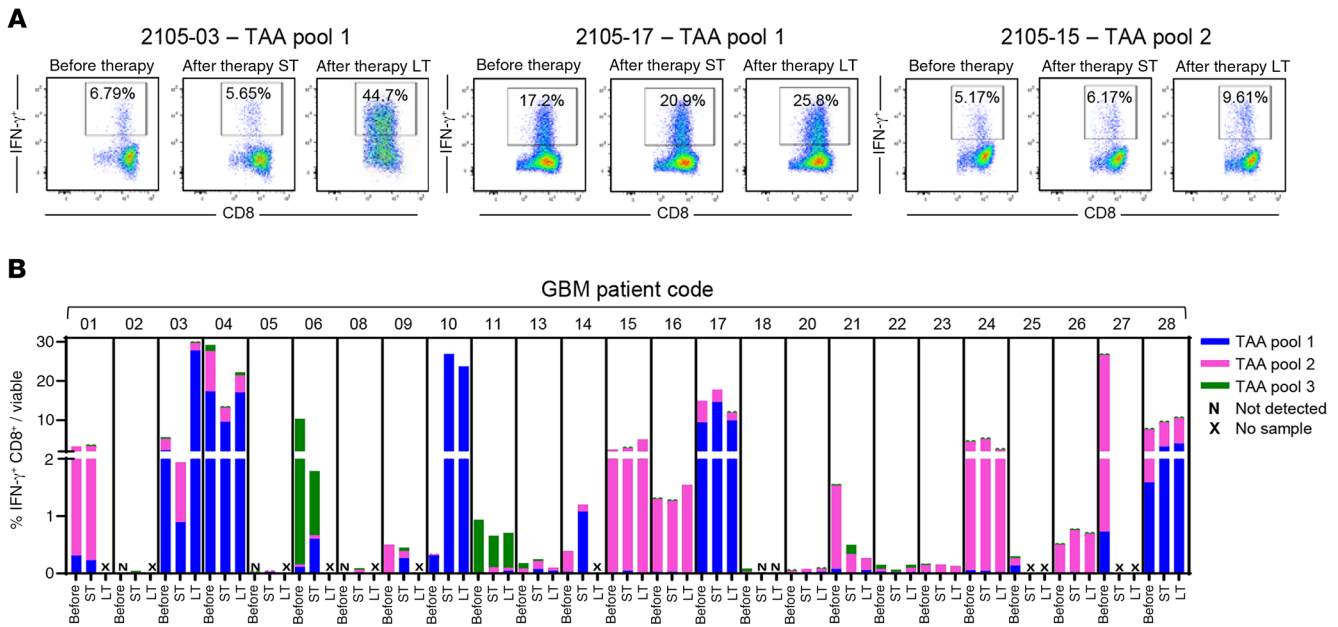
Figure 6. GBM-associated TAA-specific immunological monitoring following ACT. PBMCs isolated from patients at different time points during the clinical trial were stimulated with pools of GBM-associated TAAs, and then cultured for 14 days in the presence of IL-2. T cell cultures were then assessed for reactivity against the peptide pools using an intracellular cytokine assay. (**A**) Representative analysis of T cell responses from 3 different patients. (**B**) Data represent the frequency of IFN-γ+CD8+ T cells in response to each peptide pool. Patients were assessed at 3 time points depending on the availability of samples. Pretherapy (n = 25) was before the first infusion, posttherapy ST (n = 23) and posttherapy LT (n = 20).

WT *IDH1* performed considerably better than the corresponding C1 patients. These observations strongly support an association between transcriptional profiles of ACT products and OS of patients. The 3 remaining patients — P2105-27 (C2), P2105-09 (C4), and P2105-17 (C4) — had the poorest outcomes (all deceased within 21 months of diagnosis) and the ACT product profiles of these patients clustered separately from C1 and C3 (Supplemental Figure 2).
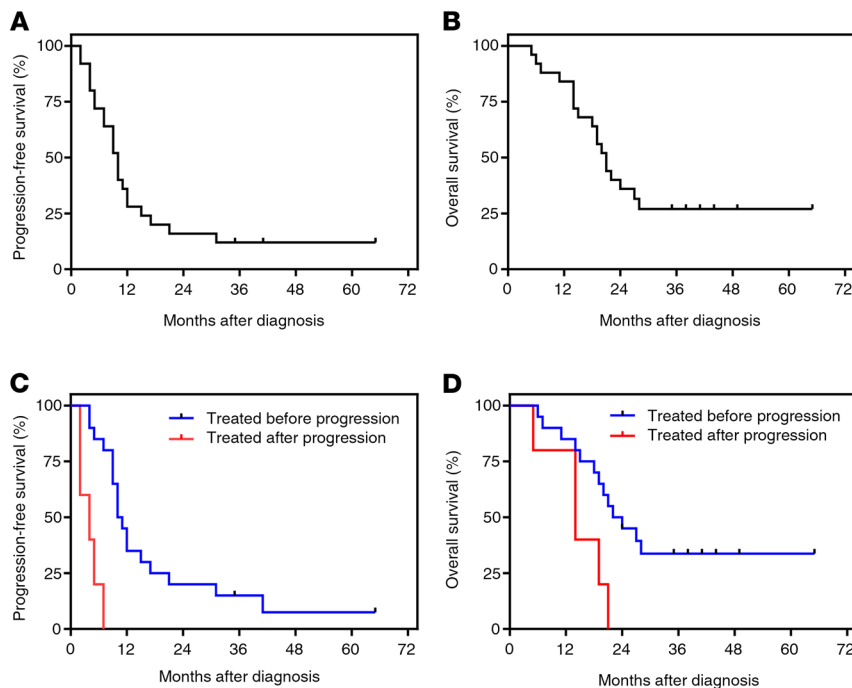
Figure 7. Long-term progression-free and overall survival of patients with GBM following CMV-specific ACT. Progression-free (**A**) and overall (**B**) survival analysis was performed for all 25 patients treated with CMV-specific ACT. Patients were stratified based on their time of progression, either before therapy (n = 5) or after therapy (n = 20), and progression-free (**C**) and overall survival (**D**) were determined. All survival calculations were performed from the time of initial diagnosis.

Table 5. Longer-term survival characteristics in all patients from primary diagnosis

		6 months (%)	12 months (%)	24 months (%)	Median
Intent to treat (*n* = 28)	PFS	71	32	17	10
	OS	89	82	36	21
Treated (*n* = 25)	PFS	72	28	16	10
	OS	92	84	36	21
Preemptive (*n* = 20)	PFS	80	35	20	10
	OS	95	85	45	23
Therapeutic (*n* = 5)	PFS	20	0	0	4
	OS	80	80	0	14

Discussion

Despite the progress made in treating many cancer types in recent decades, significant improvement in the OS of patients with GBM since the introduction of temozolomide has remained elusive (32–35). These observations have recently been reinforced by disappointing results using PD-1 checkpoint blockade to treat GBM (9, 12). Nevertheless, evidence from a number of studies has demonstrated the potential of exploiting the immune system to augment OS in patients with GBM (14, 36–38). In the current single-arm open-label study, we have harnessed the association of CMV with GBM tissue and used CMV-specific ACT to directly target this disease. We have demonstrated the strong safety profile of this therapy, accompanied by potential for favorable outcomes in patients following primary tumor resection. In a cohort of 25 patients treated with ACT, we demonstrated a median PFS of 10 months and OS of 21 months following diagnosis, which compares favorably to the standard-of-care outcomes of 5.9 to 7.3 months and 14.6 to 17.3 months, respectively (34, 39–41). We also demonstrated improvement in peripheral cellular immunity to CMV and TAAs expressed in GBM following ACT, which is indicative of the potential of CMV-specific ACT to modulate global immunity to GBM.

Due to the rapid progression of GBM, early immunotherapeutic intervention in addition to tumor debulking with surgery, radiotherapy, and chemotherapy is likely to provide the most effective means to augment OS in patients. We previously reported the use of autologous CMV-specific ACT in 11 patients with recurrent GBM, and recently provided an update on 4 of these patients who have demonstrated impressive OS of up to 9 years following treatment (24). However, due to the advanced stage of recurrent disease, we were unable to provide treatment for 8 patients recruited to this previous study (18). Thus, the current study was designed to assess the safety and potential efficacy of CMV-specific ACT following primary diagnosis, providing treatment before relapse, while tumor burden is low. We have demonstrated the feasibility of our current approach, evidenced by the capacity to generate cell therapy from 27 of 28 recruited patients, and the treatment of 20 patients before relapse. However, the rapid progression typical of GBM was apparent in our cohort and precluded the treatment of all patients before progression. Two patients experienced tumor progression that prohibited them receiving ACT and a further 5 received treatment after the progression of their disease. Other strategies, including "off-the-shelf" allogeneic cellular therapies, may be considered to treat patients who rapidly progress following

primary treatment. Allogeneic antigen-specific ACT has been successfully used to treat virus-associated lymphomas and posttransplant infectious complications (42, 43).

This early intervention approach has been the focus of many immune interventions recently developed for GBM. Recent observations have shown that the application of checkpoint inhibition in the neoadjuvant setting before surgery can significantly improve outcomes compared with an adjuvant approach in recurrent GBM (14, 36). Similarly, recent studies have applied personalized neoantigen vaccines for patients with GBM, administered within 6 months following primary diagnosis, with median OS of 16.8 and 29 months observed in 2 separate studies (37, 38). CMV-targeted vaccine strategies have also recently focused on treatment following primary standard of care, with encouraging survival results (44). These observations provide promise for early immunological intervention for GBM treatment. It should be noted that, like our study, these vaccine studies were single-arm open-label trials.

Although recent results with neoadjuvant checkpoint inhibition in patients with GBM is promising, the limited mutational burden and poor CD8+ T cell infiltrate in GBM tissue suggests that augmentation of the T cell response to GBM will be critical for the successful application of immunotherapy (45, 46). Furthermore, this will be dependent on the selection of appropriate antigenic targets. Current chimeric antigen receptor approaches have focused on a number of potential targets, including EGFRVIII, IL-13Rα2, and HER2, with promising early results (13, 15, 47). In addition to CMV antigens and neoantigens, TAAs have also been investigated as targets for immunotherapy in GBM (25, 48, 49). Interestingly, in our study we noted the potential effect of targeting CMV on the T cell response to other TAAs expressed in GBM. Although the impact of these changes on outcome was inconclusive, they provide evidence of a bystander effect or epitope spreading, which has been proposed to broaden the tumor-specific T cell response, with likely implications for long-term tumor control. Similar observations have previously been made in other settings of immunotherapy, including the use of Epstein-Barr virus–specific T cell therapy to treat B cell lymphomas (50). These observations may also provide an opportunity for dual targeting of viral and tumor antigens, to improve efficacy of immunotherapies against GBM. A recent phase I clinical trial in the GBM setting used HER2-CMV bispecific CAR T cells; these displayed a good safety profile and resulted in clinical benefit (51). Augmentation of cell therapy approaches with checkpoint inhibition could provide further benefit to clinical outcome.

Our current study provides clear evidence of the robustness of our protocol for the generation of autologous ACT from patients with GBM, and the safety and feasibility of using ACT in an adjuvant setting to preemptively treat GBM before relapse. We also provide clear evidence of the impact CMV-specific ACT can have on peripheral immunity in this cohort. In particular, we observed specific differences in transcriptional profiles at gene and pathway levels between T cells of clusters C1 and C3 that showed considerable difference in OS. Of particular importance, this study provides evidence that the differential effects observed following the administration of T cells with distinct profiles are independent of other known risk factors in GBM, including unmethylated *MGMT*. These transcriptional differences could
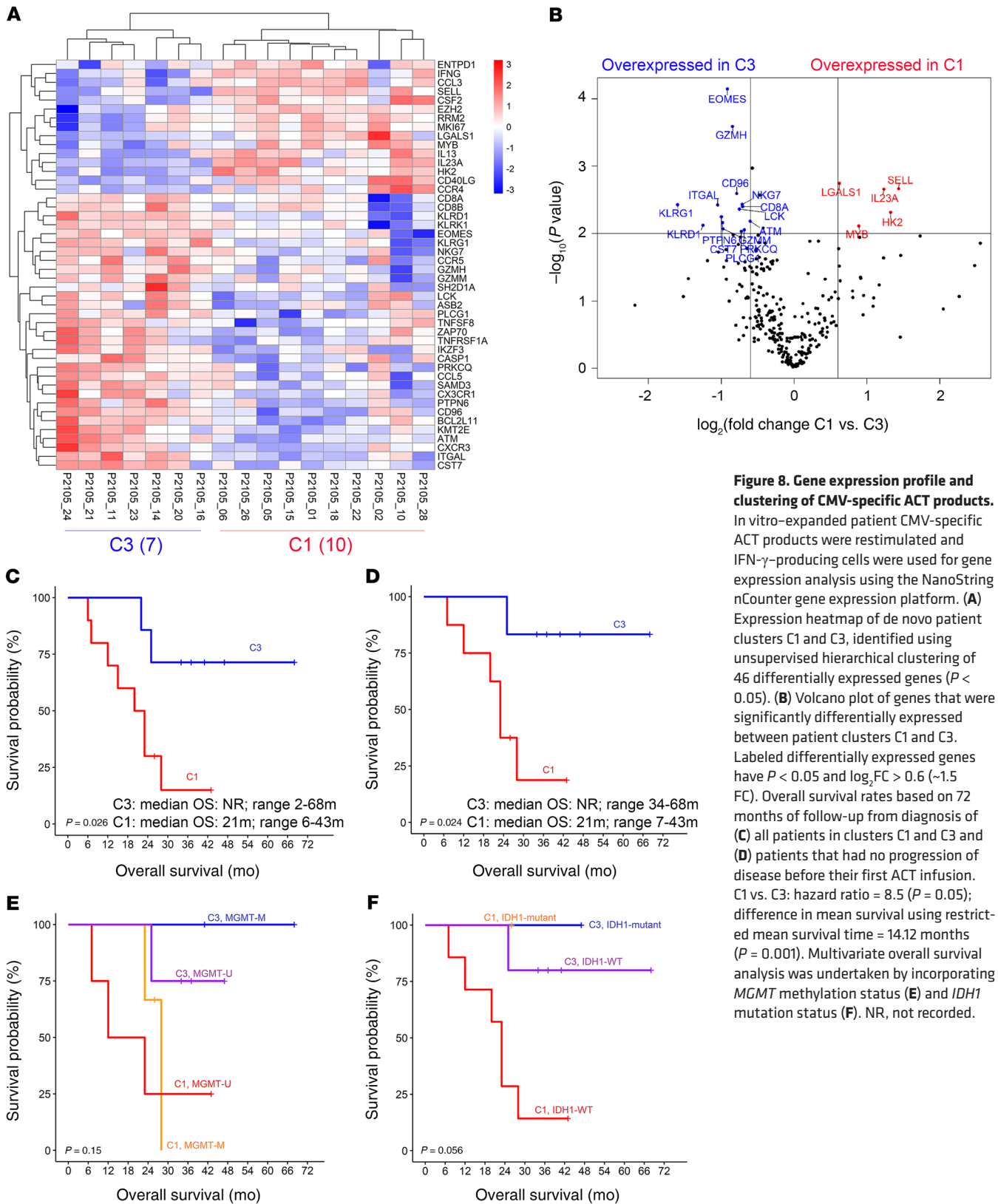
Figure 8. Gene expression profile and clustering of CMV-specific ACT products. In vitro–expanded patient CMV-specific ACT products were restimulated and IFN-γ–producing cells were used for gene expression analysis using the NanoString nCounter gene expression platform. (**A**) Expression heatmap of de novo patient clusters C1 and C3, identified using unsupervised hierarchical clustering of 46 differentially expressed genes ($P < 0.05$). (**B**) Volcano plot of genes that were significantly differentially expressed between patient clusters C1 and C3. Labeled differentially expressed genes have $P < 0.05$ and $\log_2 FC > 0.6$ (~1.5 FC). Overall survival rates based on 72 months of follow-up from diagnosis of (**C**) all patients in clusters C1 and C3 and (**D**) patients that had no progression of disease before their first ACT infusion. C1 vs. C3: hazard ratio = 8.5 ($P = 0.05$); difference in mean survival using restricted mean survival time = 14.12 months ($P = 0.001$). Multivariate overall survival analysis was undertaken by incorporating *MGMT* methylation status (**E**) and *IDH1* mutation status (**F**). NR, not recorded.

be leveraged to functionally manipulate specific pathways and develop T cells that are more effective in treating GBM. Overall, the data presented here must be interpreted with caution and 2 major limitations should be borne in mind. First, observations

from this study need to be reproduced in larger controlled trials conducted concurrently at multiple clinical centers. Second, bias in the selection of patients enrolled in this study may have influenced the clinical outcome observed in the patients. In spite

of these limitations, this study provides the impetus to develop combination or bispecific approaches targeting CMV and TAAs to further enhance the efficacy of ACT for GBM.

Methods

Patient recruitment and study design. Patients were eligible for the study if they had histological diagnosis of primary GBM (WHO grade IV), Eastern Cooperative Oncology Group performance status of 0, 1, or 2, life expectancy of greater than 6 months, and were CMV-seropositive. The detection of CMV antigens in tumor tissue was not assessed. Patients included in the study were HLA typed (Supplemental Table 1) and received up to 6 intravenous infusions of in vitro–expanded T cells at a dose of 2×10^7 cells/m^2 body surface area every 2 to 4 weeks. Patients continued standard-of-care treatment during the course of immunotherapy. Where possible, administration of ACT was scheduled to fall between chemotherapy treatment weeks to avoid concurrent infusions. Each participant was monitored for safety, disease progression, and immune reconstitution for 1 year after the completion of ACT. Long-term survival analysis was performed for up to 65 months following diagnosis.

Manufacture of CMV-specific T cells for ACT. The protocol used to manufacture CMV-specific T cells for ACT has been described recently (52). Briefly, PBMCs were harvested from peripheral blood within 24 hours of venesection. One-third of the PBMCs were then incubated with a clinical-grade custom CMV peptide pool for 1 hour, washed, mixed with the remaining PBMCs (JPT Technologies; Supplemental Table 2), and then seeded in G-Rex10 culture flasks (Wilson Wolf) at a density of between 2×10^6 and 5×10^6 cells/cm^2. Cells were grown in culture medium containing recombinant IL-21 from day 0, with recombinant IL-2 added every 2 to 3 days thereafter. On day 14, ACT products were harvested and frozen in 1 mL single-dose aliquots in Albumex 4 (CSL Behring) containing 10% dimethyl sulfoxide (WAK-Chemie Medical). ACT products were phenotypically and functionally characterized using Multitest 6-color TBNK Reagent (BD Biosciences) and intracellular cytokine assay, and microbial testing was performed as previously described (52).

Intracellular cytokine assay. ACT products, PBMCs, or laboratory-cultured T cells were stimulated with the custom CMV peptide pool, individual HLA-matched epitopes in the CMV peptide pool (Supplemental Table 2), or TAA pools (Supplemental Table 3). Cells were cultured for 4 hours in the presence of GolgiPlug and GolgiStop (BD Biosciences) and anti-CD107a–FITC (BD Biosciences, clone BerACT8), then washed and stained with anti-CD8–PerCP-Cy5.5 (eBioscience, clone RPA-T8) and anti-CD4–PE-Cy7 or anti-CD4–Pacific Blue (BD Biosciences, clone RPA-T4), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), washed again and stained with anti–IFN-γ–Alexa Fluor 700 (clone B27), anti-IL-2–PE (clone MQ1-17H12), and anti-TNF–APC (clone Mab11) (all from BD Biosciences). Cells were washed, then resuspended in PBS and acquired using a BD LSRFortessa with FACSDiva software (BD Biosciences). Postacquisition analysis was performed using FlowJo software (Tree Star).

Gene expression profiling of CMV-specific ACT products. To enrich CMV-specific CD8+ and CD4+ T cells from the ACT products, the IFN-γ Secretion Assay Detection Kit (Miltenyi Biotec) was used according to the manufacturer's instructions. Briefly, ACT products were thawed, then 10% of the cells were coated with a pool of CMV peptides (Supplemental Table 2) for 1 hour. These stimulators were

washed and incubated with the remaining ACT product cells for 3 hours. Subsequently, the cells were washed with cold buffer (2 mM EDTA and 0.5% bovine serum albumin in PBS) and labeled with IFN-γ catch reagent on ice. ACT products were then resuspended in warmed culture medium and incubated for 45 minutes at 37°C, using a rotor wheel on low speed to maintain the cells in suspension. Products were washed again with cold buffer, and then labeled on ice with FITC-conjugated IFN-γ detection antibody, anti-CD8–PerCP-Cy5.5 (eBioscience, clone RPA-T8), anti-CD4–Pacific Blue (BioLegend, clone RPA-T4), and LIVE/DEAD Fixable Near-IR Dead Cell Stain (Molecular Probes). CD4+ and CD8+ IFN-γ–secreting cells were sorted using a FACSAria III (BD Biosciences) and RNA was extracted from sorted cells (RNeasy Mini Kit, QIAGEN). Gene expression analysis was conducted using the NanoString nCounter gene expression platform (NanoString Technologies) according to the manufacturer's instructions. A custom code set consisting of genes involved in T cell biology, immune regulation, and immune cellular markers was used. The samples were scanned at maximum scan resolution on the nCounter Digital Analyzer and gene expression data normalized by housekeeping gene expression to identify differentially expressed genes. De novo patient clusters were identified using unsupervised hierarchical clustering of NanoString expression profiles of ACT products that were infused into trial participants. Of the 25 treated patients, sufficient CMV-specific ACT product was available for NanoString profiling of 20 products. After removing genes with low expression, evaluation of the number of clusters ($k = 2–5$) resulted in $k = 4$ patient clusters, C1 to C4, with the best intracluster homogeneity (minimum sum of squared error of Euclidean distances). Of these clusters, C1 and C3, which contained 17 of the 20 patients, were used for survival and differential expression analysis.

Statistics. OS and PFS were calculated from the date of recruitment to the date of radiologically documented progressive disease or the date of death. Patients who were alive and without evidence of progressive disease were censored at their last follow-up. Prism 8 software (GraphPad) was used to generate Kaplan-Meier plots and calculate median survival and percentage survival at different intervals. Mixed-effects analysis with Dunnett's multiple-comparisons test was performed in Prism 8 software and used to assess the significance of changes in CMV-specific T cell frequency in PBMCs following ACT. Statistical significance was defined as P less than 0.05 (2-sided).

For clustering analysis, OS was calculated from the date of diagnosis to the most recent follow-up (December 2019), and OS curves with log-rank, t test, P value, and median survival were plotted for patients in clusters C1 ($n = 10$) and C3 ($n = 7$) using the ggsurvplot package in R v3.2. A second set of OS curves was plotted after excluding the 4 patients in these clusters who had disease progression before their first ACT infusion. ACT product genes that were differentially expressed between the 2 largest patient clusters, C1 and C3, were computed using a t test followed by Bonferroni's correction. A volcano plot using the corrected t test P values and log$_2$ fold changes was plotted using R, to highlight the most significantly differentially expressed genes ($P < 0.05$ and log$_2$FC > 0.6, corresponding with an approximately 1.5-fold change in expression levels) between the 2 patient clusters. Pathways that were differentially enriched between clusters C1 and C3 were computed based on the differentially expressed genes between C1 and C3, using the InnateDB platform (https://www.innatedb.com). In addition, the pathways that were

enriched within each of these clusters C1 and C3 were computed using the full NanoString panel of genes that were overexpressed in these clusters (i.e., genes that showed higher median expression in C1 or C3 compared with their median expression across all patients).

Study approval. This prospective study was conducted according to the principles of the Declaration of Helsinki and approved by the QIMR Berghofer Medical Research Institute Human Research Ethics Committee and UnitingCare Health Human Research Ethics Committee. This study was registered under the Australian New Zealand Clinical Trial Registry (ACTRN12615000656538), and all patients provided written informed consent before commencing participation in the trial.

Author contributions

RK, CS, and DW designed this study. RK, CS, DW, KEL, JPM, GRA, and SS contributed to drafting of the manuscript. CS, KEL, JPM, GRA, SR, PC, AP, LB, LLT, and SS conducted experimental studies and analyzed the data. MAN, BM, and KKM were responsible for clinical trial monitoring, ethics approval, and patient recruitment. DW, MAN, BM, and KKM also contributed to clinical data analysis.

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