Long-term Survival in Glioblastoma with Cytomegalovirus pp65-Targeted Vaccination

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Abstract

Purpose: Patients with glioblastoma have less than 15-month median survival despite surgical resection, high-dose radiation, and chemotherapy with temozolomide. We previously demonstrated that targeting cytomegalovirus pp65 using dendritic cells (DC) can extend survival and, in a separate study, that dose-intensified temozolomide (DI-TMZ) and adjuvant granulocyte macrophage colony-stimulating factor (GM-CSF) potentiate tumor-specific immune responses in patients with glioblastoma. Here, we evaluated pp65-specific cellular responses following DI-TMZ with pp65-DCs and determined the effects on long-term progression-free survival (PFS) and overall survival (OS).

Experimental Design: Following standard-of-care, 11 patients with newly diagnosed glioblastoma received DI-TMZ (100 mg/m²/d × 21 days per cycle) with at least three vaccines of pp65 lysosome–associated membrane glycoprotein mRNA-pulsed DCs admixed with GM-CSF on day 23 of each cycle. Thereafter, monthly DI-TMZ cycles and pp65-DCs were continued if patients had not progressed.

Results: Following DI-TMZ cycle 1 and three doses of pp65-DCs, pp65 cellular responses significantly increased. After DI-TMZ, both the proportion and proliferation of regulatory T cells (Tregs) increased and remained elevated with serial DI-TMZ cycles. Median PFS and OS were 25.3 months [95% confidence interval (CI), 11.0–∞] and 41.1 months [95% CI, 21.6–∞], exceeding survival using recursive partitioning analysis and matched historical controls. Four patients remained progression-free at 59 to 64 months from diagnosis. No known prognostic factors [age, Karnofsky performance status (KPS), IDH-1/2 mutation, and MGMT promoter methylation] predicted more favorable outcomes for the patients in this cohort.

Conclusions: Despite increased Treg proportions following DI-TMZ, patients receiving pp65-DCs showed long-term PFS and OS, confirming prior studies targeting cytomegalovirus in glioblastoma. Clin Cancer Res; 23(8); 1898–909. ©2017 AACR.

Introduction

Patients with newly diagnosed glioblastoma have a median survival of less than 15 months, despite maximal tumor resection, high-dose radiation, and temozolomide (TMZ) chemotherapy (1, 2). Novel approaches to therapy are desperately needed. Several groups, including our own laboratory, have demonstrated that human cytomegalovirus proteins are expressed in more than 90% of glioblastomas (3–5). Cytomegalovirus expression has not been detected in surrounding normal brain tissue (3, 4, 6, 7), which provides an unparalleled opportunity to subvert cytomegalovirus antigens as tumor-specific targets. Recent evidence has also demonstrated that cytomegalovirus-specific T-cell immunity can be generated to recognize and effectively kill autologous glioblastoma tumor cells expressing endogenous levels of the immunodominant pp65 antigen (8), providing compelling support for the development of cytomegalovirus-directed immunotherapy for the treatment of glioblastoma.

We have recently demonstrated in a small randomized pilot trial that patients who received cytomegalovirus pp65-specific dendritic cells (pp65-DC) combined with vaccine site preconditioning using tetanus-diphtheria toxoid showed significantly improved progression-free survival (PFS; range, 15.4–47.3 months) and overall...
survival (OS; range, 20.6–47.3 months) compared to controls (9). In addition, in related trials, we have also demonstrated that dose-intensi"fied temozolomide (DT-TMZ) and adjuvant granulocyte macrophage colony-stimulating factor (GM-CSF) can enhance immune responses to tumor-specific antigens in patients with glioblastoma (10).

In this phase I trial, our primary objective was to evaluate the safety and feasibility of vaccinating newly diagnosed patients with pp65-DCs admixed with GM-CSF following host conditioning with DT-TMZ. Secondary objectives were to investigate patient cellular immune responses induced by pp65-DCs admixed with GM-CSF and to determine PFS and OS compared with that expected with standard-of-care. GM-CSF was chosen as an adjuvant to pp65-DCs on the basis of our own experience with GM-CSF-containing DCs (VICTORI trial; ref. 11) and peptide vaccines (ACTIVATE and ACT II trials; refs. 10, 12) and its previously characterized effects on DC viability and differentiation (13). We chose to administer DI-TMZ in this study also based on our prior experience that profound lymphopenia following DT-TMZ, patients with glioblastoma receiving pp65-DCs showed increased pp65 immunity and long-term survival extending beyond predicted rates, fortifying prior studies that target cytomegalovirus antigens in glioblastoma. Randomized studies on the prevention of generated regulatory T cells in the context of temozolomide treatment and cytomegalovirus targeting are under way.

### Translational Relevance

The highly aggressive and therapeutically resistant nature of glioblastoma is evidenced by a median survival of less than 15 months. More precise and efficacious therapies are desperately needed. Several groups have demonstrated that cytomegalovirus proteins are expressed in more than 90% of sampled glioblastomas. Moreover, cytomegalovirus antigen expression is restricted to glioma cells and not surrounding normal brain, providing the opportunity to subvert cytomegalovirus proteins as tumor-specific immunotherapy targets. In this study, we targeted the cytomegalovirus antigen pp65 using dendritic cells (DC) in combination with dose-intensified temozolomide and evaluated patient antitumor immune responses and survival. Despite increases in regulatory T-cell proportions following DI-TMZ, patients treated with pp65-DCs showed increased pp65 immunity and long-term survival extending beyond predicted rates, fortifying prior studies that target cytomegalovirus antigens in glioblastoma. Randomized studies on the prevention of generated regulatory T cells in the context of temozolomide treatment and cytomegalovirus targeting are under way.

### Materials and Methods

#### Patient selection

Patients were enrolled and treated with the study drug in a separate clinical study under an overarching parent protocol. The clinical protocol and informed consent were approved by the U.S. FDA and Institutional Review Board (IRB) at Duke University (Durham, NC) for this study (FDA-IND-BB-12839, Duke IRB Pro00003877, NCT00639639). Eligibility criteria included adults with a histologically confirmed, newly diagnosed WHO grade IV glioblastoma. Patients were eligible if they underwent a gross total resection defined as more than 90% with residual contrast enhancement of less than 1 cm² on post-resection MRI, had a baseline Karnofsky performance status (KPS) score of ≥80, did not require continuous steroid therapy above physiologic levels, and did not receive additional treatments aside from the study therapy. Histopathology of all specimens was initially read as glioblastoma, and diagnosis was reconfirmed by a second board-certified neuropathologist. Both methylguanine methyltransferase (MGMT) promoter methylation and isocitrate dehydrogenase (IDH)-1 and IDH-2 mutation analyses were performed by PCR (16, 17). Given published reports showing high expression of cytomegalovirus viral proteins in more than 90% of sampled primary glioblastoma specimens (3, 4, 6, 7), we elected not to include pp65 staining of tumor tissue as an eligibility criterion for patients enrolled on this trial.

#### Historical controls

A cohort of historical controls at least double the sample size of our study cohort was used to compare survival rates of patients receiving our study drug with similar patients receiving other therapies. Historical controls had histopathology-confirmed primary glioblastoma, with 23 of 23 being negative for the IDH-1 mutation and mixed MGMT methylator phenotype (12 negative, 5 positive, 6 not available). Historical controls were treated similarly to study patients with initial gross total resection and standard 6-week radiation therapy (XRT)/temozolomide. If no progressive disease occurred at this point, historical controls proceeded to receive monthly standard temozolomide cycles (150–200 mg/m²/d × 5 days) and did not receive any other therapies until progression. Those controls who did progress after the completion of standard therapy were offered additional therapies, including bevacizumab, etoposide, irinotecan, CCNU, vorinostat, and heat-shock protein targeted therapies.

#### Study design

Eligible patients underwent initial leukapheresis (apheresis-1) prior to XRT/TMZ for immunologic monitoring and subsequent ex vivo differentiation of autologous DCs. Each patient then completed a 6-week course of conformal external beam XRT to a dose of 60 Gy with concurrent temozolomide at a targeted daily dose of 75 mg/m²/d. Upon completion of standard chemoradiation therapy, all patients were re-imaged with MRI for evidence of progressive disease. Those with evidence of progressive disease or requiring steroid therapy in excess of physiological levels (>2 mg/d of dexamethasone) at the time vaccination was scheduled did not continue on study. At 4 weeks following standard XRT/TMZ, the first DI-TMZ cycle (100 mg/m²/d) was administered over the course of 21 days of a 28-day cycle, and DC vaccine-1 was administered on day 23 ± 1 of that 28-day cycle. For each pp65-DC vaccine, an intradermal injection of 2 × 10⁷ pp65 mRNA-pulsed DCs admixed with 150 μg GM-CSF in 0.4 mL of saline was administered bilaterally in the groin. Following DC vaccine-3, patients underwent a second leukapheresis (apheresis-2) for immunologic monitoring and subsequent ex vivo differentiation of autologous DCs. From DC vaccine-3 and onward, patients received monthly DC vaccines in conjunction with subsequent DI-TMZ cycles every 5 ± 1 weeks for a total of 6 to 12 cycles at the discretion of the treating neuro-oncologist. DCs were

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given on day 23 ± 1 of each 28-day temozolomide cycle for a total of 10 vaccines unless progression occurred. Patients were imaged bimonthly and did not receive any other prescribed antitumor therapy.

Safety and adverse events
All patients were monitored for treatment-related toxicity. Adverse events (AE) and serious AEs (SAE) were graded according to the National Cancer Institute's Common Terminology Criteria for AEs (Version 3.0). Safety checkpoints were defined to halt the study if any 2 patients experienced a drug-related grade IV or irreversible grade III toxicity.

DC vaccine generation
Autologous DCs were generated using the method used by Romani and colleagues (18, 19). After harvest, cells were frozen and assessed for contamination and lineage purity as previously published (20). The 1.932-kB pp65 full-length cDNA insert was obtained from Dr. Bill Britt (University of Alabama-Birmingham, Birmingham, AL), and RNA was generated and transfected as previously reported (9).

Peripheral blood mononuclear cell processing
In addition to leukapheresis, blood draws for pp65 enzyme-linked immunoSpot (ELISpot) assays were performed just prior to vaccination with pp65-DCs. Peripheral blood mononuclear cells (PBMC) were separated from blood collected in ACD tubes within 4 hours of blood collection using Histopaque (Sigma) and stored in liquid nitrogen. On the day of testing, patient PBMCs were rapidly thawed, washed, rested overnight in RPMI-1640 with 10% FBS and additives (21), and processed for absolute cell count and viability on a Guava easyCyte flow cytometer (Merck KGaA).

IFNγ ELISpot
Patient pp65 responses were measured ex vivo by direct IFNγ ELISpot. PBMCs were stimulated overnight with a pool of synthetic peptides spanning cytomegalovirus pp65 (15 mers overlapping by 11 amino acids with >95% purity), kindly provided by Dr. Robert A. Olmsted (Alphavax, Inc.). ELISpot plates coated with mouse IgG1 anti-human IFNγ monoclonal antibody were incubated overnight at 37°C, 5% CO2, washed with PBS/Tween-20, incubated with biotinylated mouse IgG1 anti-human IFNγ for 1 hour at room temperature, washed with PBS, incubated with avidin/peroxidase complex for 1 hour at room temperature, washed, and incubated with substrate (3-amino-9-ethylcarbazole) for 4 minutes at room temperature. Spot enumeration was performed in a blinded fashion by Zellnet Consulting, Inc. using a KS ELISpot reader (Zeiss Inc.), software version KS 4.9.16 using established guidelines (22). Results were expressed as the mean spot-forming cells (SFC)/10^6 PBMC after subtraction of background counts from PBMCs cultured without peptide. Negative values were raised to zero for mean calculations. Positive and negative control PBMCs for pp65 (from Dr. Robert A. Olmsted) were qualified and validated by precision and intermediate precision testing using pp65 standardized peptide pools. The criteria for a valid assay required control wells with cytomegalovirus pp65–positive control > 692 SFC/10^6 PBMC and negative control < 9 SFC/10^6 PBMC.

Treg analysis
PBMC surface antigens were stained with CD4-PE/Cy7 (RPA-T4), CD25-APC (MA251), Ki67-PE (B56), and CD127-PE (hi1-7R-M21; BD Bioscience). After washing to remove unbound antibodies, cells were incubated on ice for 30 minutes in fixation/permeabilization buffer (eBioscience). Cells were washed again with 1× permeabilization buffer (eBioscience), pelleted, and stained with FOXP3-APC (PCH101, eBioscience). Samples were acquired on BD FACS Calibur (BD) and analyzed with Flowjo (TreeStar). Tregs were defined as CD25+FOXP3+ of CD4+ lymphocytes.

Disease progression
Progressive disease was defined radiographically according to the RANO criteria and defined as (i) at least a 25% increase in the longest diameter on an axial image of any enhancing tumor on consecutive CT or MRI images or (ii) the appearance of a new radiographically demonstrable lesion measuring ≥ 1 cm in any 2 perpendicular axial planes (23). Upon tumor progression, patients could undergo stereotactic biopsy or resection for confirmation with additional consent.

Statistical analysis
For sample size estimation, patients in this phase I study were recruited in a single-arm fashion to evaluate safety and feasibility as the primary endpoint. No a priori power calculations were used for secondary endpoints. Therefore, a final sample size matching that of the prior study (9) was calculated to ensure at least 6 evaluable subjects. For paired comparisons between 2 time points and for fold change comparisons, a Wilcoxon signed rank test was used to determine statistical significance. Patients were enrolled over the course of 2 years and a lock date 4.25 years after the last patient enrolled was applied for survival analysis of censored data (patients who had not progressed and were alive at the time of analysis). PFS was defined as the time from histologic diagnosis at surgery until radiographic or clinical progression and was censored at the lock date if the patient remained alive without disease progression at the time of analysis. OS was defined as the time from histologic diagnosis until death and was censored at the lock date if the patient remained alive at the time of analysis. Median PFS and OS were estimated using Kaplan–Meier methods, and comparisons with historical controls used the log-rank test. Predicted survival utilized known prognostic factors under the recursive partitioning analysis (RPA) classification (24), with observed–expected survival calculated for each patient.

Results
Patient population
A total of 14 patients were initially enrolled in the study (schema shown in Fig. 1). The study therapy was defined as completion of DI-TMZ cycle 1 and DC vaccines 1 to 3 without additionally prescribed therapies. Two patients were excluded from immune response analyses, as they were not eligible for study participation due to treatment with bevacizumab at outside hospitals. One patient discontinued protocol treatment prior to DC vaccine-3 due to disease progression. Primary immune response analysis is based on the 11 patients who received at least 3 pp65-DC vaccinations.

Patients on study had a median age of 55 years and median KPS of 90 at diagnosis (Table 1). To account for the possibility that certain prognostic factors could have selected for more favorable...
patient outcomes, we analyzed primary glioblastoma specimens for IDH-1 and IDH-2 mutations (16) and promoter methylation of MGMT (17). All patient specimens that could be sampled (10 of 11) were negative for IDH mutations. No significant differences in survival were seen in patients with MGMT promoter methylation (5 of 11), although this study was not powered to assess this variable as a prognostic marker for survival. RPA class incorporating KPS and age at diagnosis was used to evaluate expected survival for each patient. Observed–expected survival rates based upon the RPA showed a gain in survival for each of the 11 patients receiving at least 3 vaccines, with a median gain of 30 months for the entire cohort (Table 1).

Toxicity and adverse events

Patients on study were monitored for toxicity and AEs defined according to the National Cancer Institute's Common Toxicity Criteria (Version 3.0). No patients experienced AEs related to the cellular portion of the pp65-DC vaccine, yet one single AE was noted and attributable to GM-CSF administration. This was classified as a severe (grade III) vaccine-related immunologic reaction, which occurred shortly after vaccine-8. Immunologic workup for this patient revealed sensitization to the GM-CSF component of the vaccine and the production of high levels of anti-GM-CSF autoantibodies during vaccination (25). Removal of GM-CSF from the DC vaccine allowed continued vaccination (total of 10 vaccines) without incident for this patient. No other study drug AEs were detected.

Patient survival

Patients in this study administered at least 3 vaccines of pp65-DCs with concomitant DI-TMZ showed significantly increased PFS (Fig. 2A) and OS (Fig. 2B) compared with historical controls ($n = 23$) matched for age, gender, tumor, and standard-of-care treatment. To counter any potential for selection bias of historical

Table 1. Clinical trial patient characteristics

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NOTE: Demographic and prognostics factors for patients with newly diagnosed glioblastoma and corresponding PFS and OS from the time of surgery (histologic diagnosis). Patients shown were those able to complete the predefined study therapy (completion of DI-TMZ cycle 1 and DC vaccines 1–3). Observed and predicted survival times are expressed in months. RPA O-E is the difference between the observed survival and the survival predicted on the basis of the RPA suggested by Curran and colleagues (2A). For class III, the predicted median survival is 17.9 months, whereas for class IV, the predicted median survival is 11.1 months.

Abbreviations: H, Hispanic; NA, tissue not available; O-E, observed–expected survival months; W, white.

*aNo progression.

*bAlive.
controls, a sample size at least double our study cohort was randomly selected from a large database of patients who had resection and were treated contemporaneously over the course of 2 years at our institution. Selection criteria for these controls included identical demographics (age, gender, histopathology-confirmed glioblastoma) and tumor molecular pathology characteristics (IDH-1-negative and mixed MGMT promoter methylation) as well as identical standard-of-care treatment, including gross total resection and standard 6-week XRT/TMZ. Historical controls did not receive any other therapies until progression was documented. Those with progressive disease after the completion of standard therapy were offered additional therapies. Apart from this caveat of additional therapies, patients on our study still showed markedly prolonged PFS and OS compared with matched historical controls (median PFS 25.3 vs. 8.0 months, \( P < 0.0001 \); historical controls median OS 41.1 vs. 19.2 months (95% CI, 14.3–21.3); \( P = 0.0001 \), log-rank test).

When compared with RPA class predicted median survival (24), we found that 100% of these patients exceeded expected median survival, with a median gain in survival of 30 months (Table 1). In addition, 4 of these 11 patients remained progression-free 59 to 64 months from initial surgery. The one enrolled patient who did not receive at least 3 vaccinations progressed at 5.3 months and died at 9.4 months from diagnosis. With inclusion of this patient in survival analyses, the median PFS and OS were 20 and 33.4 months, respectively.

**Patient cytomegalovirus pp65 immune responses**

Of the 11 patients treated with DI-TMZ and at least 3 vaccines of pp65-DCs, all but one demonstrated an increase in pp65 IFN\(\gamma\) ELISpot following DI-TMZ cycle 1, likely due to the homeostatic expansion of several antigen-specific populations (Supplementary Fig. S2). Tetramer-positive CD8\(^+\) T cells then decreased at vaccine-4 after reinitiating DI-TMZ with cycle 2, similar to the decline in functional pp65-ELISpot responses in Fig. 3B. Due to tetramer detection more so indicates cellular phenotype rather than function, we chose to optimize the pp65 ELISpot assay to monitor pp65 functional responses by directly stimulating PBMCs with the pp65 epitope and measuring IFN\(\gamma\) output with background subtraction of nonspecific IFN\(\gamma\)-secreting cells. Using the pp65 IFN\(\gamma\) ELISpot as a more informative and encompassing assay to detect the expansion and contraction of pp65 responses, we observed that

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Survival rates in patients receiving pp65-DCs and DI-TMZ compared with historical controls. PFS (A) and OS (B) of study patients (n = 11) with newly diagnosed glioblastoma receiving DI-TMZ conditioning and GM-CSF-containing pp65-DC vaccines compared with matched historical controls (n = 23) with newly diagnosed glioblastoma treated with standard-of-care and additional therapies after disease progression. Kaplan–Meier survival curves represent observed rates for DI-TMZ + pp65-DC patients who completed the predefined study therapy. Of all 11 patients, 4 had not progressed and were alive at the time of survival analysis [DI-TMZ + pp65-DCs median PFS = 25.3 months (95% CI, 11.0–\(\infty\)) vs. historical controls median PFS = 8.0 months (95% CI, 6.2–10.8), \( P = 0.0001 \); DI-TMZ + pp65-DCs median OS = 41.1 months (95% CI, 21.6–\(\infty\)) vs. historical controls median OS = 19.2 months (95% CI, 14.3–21.3); \( P = 0.0001 \), log-rank test].

Overall, the extent of pp65 IFN\(\gamma\) increased early on from DC vaccines 1–3 seem to be important for clinical responses in these long-term survivors, as those with extended OS > 40 months showed a much more significant expansion in pp65 responses from baseline (prior to vaccine-1) to pheresis-2 (after 3 vaccines) compared with those with OS < 40 months (post-vaccine-3, \( P = 0.031 \); Fig. 3C). The 2 patients with the greatest fold changes (open circles) showed prolonged OS at 59 months (censored at the time of analysis) and at 41.1 months.

A tetramer analysis was performed for 6 patients, using commercially available pp65 tetramer for available HLA types. The percentage of pp65 tetramer-positive CD8\(^+\) T cells began to increase following DI-TMZ cycle 1, likely due to the homeostatic expansion of several antigen-specific populations (Supplementary Fig. S2). Tetramer positivity did remain elevated after 3 DC vaccinations to pheresis-2. Tetramer-positive CD8\(^+\) T cells then decreased at vaccine-4 after reinitiating DI-TMZ with cycle 2, similar to the decline in functional pp65-ELISpot responses in Fig. 3B. Because tetramer detection more so indicates cellular phenotype rather than function, we chose to optimize the pp65 ELISpot assay to monitor pp65 functional responses by directly stimulating PBMCs with the pp65 epitope and measuring IFN\(\gamma\) output with background subtraction of nonspecific IFN\(\gamma\)-secreting cells. Using the pp65 IFN\(\gamma\) ELISpot as a more informative and encompassing assay to detect the expansion and contraction of pp65 responses, we observed that
Functional responses against the pp65 antigen were boosted by pp65-DC vaccines 1–3 and subsequently diminished by the continuation of monthly DI-TMZ cycles.

Radiographic changes to MRI scans MRI scans were performed for all patients within 3 weeks following completion of standard XRT/TMZ (75 mg/m^2/d). We performed a retrospective assessment, with secondary review by a radiation-oncologist and neuro-oncologist, of MRI scans for patients with shorter survival times. Overall, serial MRI scans for these patients demonstrated the usual trend in progressive features, including increasing FLAIR signal from baseline post-XRT/TMZ scans and increased nodular enhancement on T1-weighted images within the resection cavity, both of which were concerning for progressive disease.

For the 4 long-term survivors, MRI scans at baseline post XRT/TMZ, post-vaccine-3, and post-vaccine-6 showed a trend of (i) stable or steadily FLAIR signal and edema during recovery from XRT and standard 6-week temozolomide therapy, (ii) no new T1 contrast enhancement along resection margins or additional sites, and (iii) gradual collapse of the resection cavity (Fig. 4A). Interestingly, in 2 patients with extended OS at 60.7 and 46.5 months (patients 4 and 7), after repeated DC vaccination, satellite hyperintensities that did not enhance with contrast appeared a considerable distance from the resection site (Fig. 4B). Notably, these lesions did not appear in any of the patients with <40-month OS, were not originally present following XRT/TMZ, and were calculated to be outside of XRT high-dose radiation fields, thus unlikely to represent radiation-induced damage. Because of limitations of this retrospective analysis, the clinical significance of these lesions was not determined, whether these changes represent satellite sites of immune-activation related to DC vaccination or present as coincidental age-related changes in this population. Nonetheless, they remain an interesting feature of patients receiving repeated vaccination with pp65-DCs.

Treg reconstitution with temozolomide-induced lymphopenia Following DI-TMZ cycle 1, the proportion of Tregs among CD4⁺ T cells increased (P = 0.001; Fig. 5A). Treg proportions also steadily increased from pheresis-2 to vaccine-7 (Fig. 5B). Interestingly, Treg proliferation, as assessed by Ki67 staining, increased following the initiation of DI-TMZ cycle 1 (P = 0.002; Fig. 5C) and then progressively declined between vaccine-1 and pheresis-2 when DI-TMZ was held. Following lymphodepletion, Tregs have been shown to reconstitute quite early in response to an increased pool of homeostatic cytokines (26–28). Here, Tregs exhibited proliferation following DI-TMZ lymphodepletion. Once DI-TMZ cycle 2 was initiated, a steady rate of proliferation was noted and persisted with subsequent DI-TMZ cycles (Fig. 5D).

As Treg proportions increased significantly following DI-TMZ cycle 1 (Fig. 5), both peripheral CD4⁺ and CD8⁺ numbers decreased (Fig. 6A and C). Although Treg proportions remained elevated throughout sequential pp65-DCs from vaccine-1 to vaccine-3, CD8⁺ numbers and CD8:Treg ratios steadily increased amidst this high proportion of Tregs (Fig. 6A and B). Conventional CD4⁺ and CD4:Treg ratios were seemingly unaffected by sequential pp65-DCs (Fig. 6C and D).

Discussion This study corroborates prior studies targeting cytomegalovirus pp65 in newly diagnosed glioblastoma and demonstrates that cytomegalovirus DC vaccines can increase pp65 immunity despite lymphodepleting and even DI-TMZ while leading to unexpectedly prolonged PFS and OS. In our prior study, we demonstrated that patients randomized to pp65-DCs with tetanus-diphtheria (Td) toxoid vaccine site preconditioning had significantly improved PFS and OS compared to the control cohort (OSTd = 20.6–47.3 months vs. OSunpulsed DC = 13.8–

Figure 3. Patient pp65 ELISpot responses following DI-TMZ and sequential pp65-DC vaccination. A, pp65 antigen-specific T-cell responses as measured by IFNγ ELISpot ex vivo. Before-and-after pp65 ELISpot following 3 vaccinations of pp65-DCs from vaccine-1 to pheresis-2 (mean ± SEM SFC/10⁶ PBMC) in all patients (n = 11) are shown after stimulation with 138 15-mer peptides overlapping by 11 amino acids spanning the entire pp65 gene (P = 0.019; Wilcoxon signed rank). B, kinetics of pp65 ELISpot throughout continuous pp65-DC vaccination and intervening DI-TMZ cycles. Timing of DI-TMZ cycles are shown as detached lines. C, fold changes in functional pp65 ELISpot from baseline pp65 reactivity prior to vaccine-1. Fold increases are stratified by patient OS > 40 months (n = 6) and OS < 40 months (n = 5). Post-vaccine-1: mean 131 versus 3.75 (P = 0.03); post-vaccine-2: mean 2.20 versus 5.34 (P = 0.03); post-vaccine-3: mean 5.45 versus 9.79 (P = 0.03); Wilcoxon signed rank). ELISpot 0 values normalized to [0 + 1] for calculation of fold change from baseline.
**Figure 4.**
MRI changes in long-term survivors with sequential pp65-DC vaccination. 

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MRI scans (FLAIR and T1-weighted with contrast) of 4 long-term survivors receiving pp65-DCs and DI-TMZ (patients 2–5). Repeat MRI scans demonstrate steadily decreasing FLAIR hyperintensity and stable or decreasing contrast enhancement with collapse of the resection cavity. **B**, Satellite FLAIR hyperintense lesions appearing after several vaccinations with pp65-DCs in 2 patients with prolonged OS (patients 4 and 7). These lesions were not originally present at the post-XRT/TMZ scan and were calculated to be outside the range of XRT high-dose radiation fields. Presentation of these lesions was first detected after vaccine-4 and -7, and their signal persisted through vaccine-10.
41.3 months, $P = 0.013$; ref. 9). In the current study, targeting cytomegalovirus with pp65-DCs and DI-TMZ again resulted in long-term PFS and OS for patients with newly diagnosed glioblastoma, with 4 of 11 patients who received at least 3 vaccinations remaining progression-free at 59 to 64 months following surgery. This outcome exceeded observed survival in both arms of our prior small randomized trial (9), observed PFS and OS of a matched historical control cohort, and expected median survival using RPA class (24). Furthermore, the survival outcomes in these patients are unlikely to be strictly attributable to DI-TMZ, as the recent phase III RTOG 0525 study demonstrated no survival benefit of the same DI-TMZ regimen over standard (STD)-
temozolomide across all subgroups of patients with newly diagnosed glioblastoma including RPA class, MGMT status, and extent of resection (29).

Our study suggests that DI-TMZ is more efficacious if used in concert with antigen-specific vaccination for glioblastoma. T-cell responses were expanded by pp65-DC vaccines in an antigen-specific manner following DI-TMZ. From vaccine-1 to vaccine-3, all but one patient showed significant expansion in pp65 immunity. Suspending repetitive DI-TMZ cycles during these first 3 pp65-DC vaccines proved beneficial, as pp65-specific immune responses continued to increase during this period. The extent of pp65 IFNγ increases early on from DC vaccines 1–3 was relevant to patient survival, as those with extended OS > 40 months showed a much more significant expansion in pp65 responses after 3 DC vaccines compared with those with OS < 40 months. However, with the reintroduction of DI-TMZ after pheresis-2, pp65 responses diminished, elucidating that repeated monthly DI-TMZ cycles may

Figure 6.
Sequential pp65-DC vaccination expands peripheral CD8\(^+\) T cells and CD8:Treg ratios but does not affect conventional CD4\(^+\) counts or CD4:Treg ratios. A, CD8\(^+\) T-cell counts in the peripheral blood of patients decrease following DI-TMZ cycle 1 from pheresis-1 to vaccine-1 (\(P = 0.020\)) and steadily increase with sequential vaccination of pp65-DCs from vaccine-1 to vaccine-3 (\(P = 0.002\)). B, CD8:Treg ratios steadily increase following sequential pp65-DCs (vaccine-1 to vaccine-3, \(P = 0.004\)). C, Conventional CD4\(^+\) T-cell counts in the peripheral blood of patients dramatically diminish following DI-TMZ cycle 1 (\(P = 0.037\)) and do not increase following sequential pp65-DC vaccination. D, CD4:Treg ratios decrease following DI-TMZ cycle 1 (\(P = 0.002\)) but are not affected by sequential pp65-DCs (A–D, mean ± SEM; Wilcoxon signed rank).
be detrimental to maintaining IFNγ activity with later vaccination of pp65-DCs. This can most notably be appreciated via the kinetics of pp65 responses in the 4 long-term survivors from vaccine-4 to vaccine-10.

This is not unexpected given the known effects of temozolomide on peripheral lymphocyte counts in the blood (10, 30). Thus, our results highlight that the timing of vaccination with pp65-DCs in relation to DI-TMZ or any lymphodepleting drug is an important consideration in generating robust immune responses.

All 11 patients received at least 7 vaccines of pp65-DCs. The kinetics of pp65 vaccine responses showed a significant decline at vaccine-4. This diminished response may have been a result of (i) natural contraction in pp65 reactivity following a one month latency between vaccine-3 and vaccine-4 or (ii) a possible detrimental effect of DI-TMZ cycle 2 depleting pp65-specific effector T cells. Following vaccine-5, when monthly DI-TMZ and monthly pp65-DCs were administered, we observed a slight upward trend in mean pp65 reactivity at vaccine-7, mostly driven by increases in 5 of the 11 patients. Since all patients received at least 7 vaccines, this mean increase was not a result of patient dropout. In addition, at this stage of the vaccination schedule, the memory repertoire of patient pp65-specific T cells may have expanded and adopted a more resilient phenotype to DI-TMZ lymphodepletion. The recruitment of DNA damage repair mechanisms has been described in memory CD8⁺ T-cell populations expressing the DNA repair enzyme MGMT (31). Such expression may have played a role in memory T-cell kinetics at this point in the vaccination schedule amidst monthly DI-TMZ. Nonetheless, studies are underway to investigate mechanisms underlying such memory T-cell resistance to temozolomide.

Our previous studies characterize how increased Treg fractions govern cellular immune defects in patients with glioblastoma and underscore the deleterious effects of Tregs on vaccine-mediated immunotherapy (32, 33). During recovery from lymphodepleted states, remnant T-cell pools undergo homeostatic expansion through proliferation (14, 27, 28, 34). Tregs represent a portion of the depleted T-cell repertoire and not only proliferate early in this recovery but do so rapidly in response to high levels of the host cytokine IL2 (26–28, 35). In the present study, patients exhibited an increase in Treg proportions as a result of increased proliferation following a single cycle of DI-TMZ. This is not surprising, as previous studies demonstrate increased Treg proportions in patients with glioblastoma following STD-TMZ (35) and DI-TMZ regimens (10). After the initial peak, Treg proliferation gradually declined from vaccine-1 to pheresis-2 whereas pp65 responses simultaneously increased, indicating that while DI-TMZ still resulted in elevated Treg fractions, this did not appear to limit the induction of pp65 antigen-specific cellular responses. Elevated Treg proportions throughout sequential pp65-DC vaccination did not seem to affect peripheral CD8⁺ numbers, as CD8⁺ counts and CD8/Treg ratios steadily increased during this interval. Expansion of CD8⁺ T-cell numbers and functional pp65 responses may have been resilient to Treg suppression immediately following this peak, as provision of an antigen-specific vaccine in the context of lymphodepletion might have superseded homeostatically expanding Tregs (36–38). However, our findings raise the question if targeting Tregs during points of maximal proliferation could further enhance pp65 T-cell responses. A potential strategy previously published by our group employs monocolonal antibody blockade of the IL2 receptor α (IL2R α/CD25) to effectively deplete Tregs without impairing effector pp65 T-cell responses, and this is under further investigation in our 2-arm phase I study (NCT00626483; ref. 35).

In conclusion, our study results confirm our prior findings that cytomegalovirus pp65 represents a targetable axis in newly diagnosed glioblastoma resulting in patient survival far exceeding that of predicted outcomes and observed rates in historical controls. Patients in our study showed not only extended median PFS (25.3 months) and OS (41.1 months) compared with age-matched patients in the RTOG 0525 trial (n = 422) who similarly received 21-day DI-TMZ cycles following the 6-week standard chemoradiation therapy (median PFS and OS, 6.7 and 14.9 months). Although eligibility criteria in our study differed from those in the RTOG trial (partial and total resection, KPS ≥ 60), patients shared similar characteristics with respect to age, MGMT methylation status, and RPA class. We recognize that in our study, the efficacy of DI-TMZ alone was not directly compared with DI-TMZ and pp65-DC vaccination, but given the results from the RTOG trial, DI-TMZ used independently is unlikely to improve outcomes in this patient population according to recent data (29). However, our prior clinical study demonstrated that DI-TMZ-induced lymphopenia, when provided with antigen-specific vaccination, resulted in superior immune responses compared with STD-TMZ dosing (10). Here, using pp65 as a different antigen in glioblastoma, we were able to validate these prior findings, demonstrating that DI-TMZ is best utilized in concert with a vaccine to generate robust antigen-specific immunity. What remains to be determined is whether this combinatorial regimen is strictly dependent on antigen specificity, a question that is best suited for future randomization studies. Overall, our results strengthen prior findings from other trials targeting cytomegalovirus and provide evidence for the association between pp65 targeting in glioblastoma and long-term survival.

Disclosure of Potential Conflicts of Interest
K.A. Batch is a co-inventor on a patent entitled, "Tetanus toxoid and ccl3 improves dc vaccines," which is owned by Duke University. D.D. Bigner is an employee of and has ownership interest in Istari Oncology, and has ownership interest in Five Prime Therapeutics and Celldex. D.A. Mitchell reports receiving commercial research grants from Immunomic Technologies, Inc. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: K.A. Batch, E.A. Reap, G.E. Archer, L. Sanchez-Perez, J.E. Herndon II, P. Healy, R.E. McLendon.
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.A. Batch, E.A. Reap, G.E. Archer
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