

Greater chemotherapy-induced lymphopenia enhances tumor-specific immune responses that eliminate EGFRvIII-expressing tumor cells in patients with glioblastoma

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Epidermal growth factor receptor variant III (EGFRvIII) is a tumor-specific mutation widely expressed in glioblastoma multiforme (GBM) and other neoplasms, but absent from normal tissues. Immunotherapeutic targeting of EGFRvIII could eliminate neoplastic cells more precisely but may be inhibited by concurrent myelosuppressive chemotherapy like temozolomide (TMZ), which produces a survival benefit in GBM. A phase II, multicenter trial was undertaken to assess the immunogenicity of an experimental EGFRvIII-targeted peptide vaccine in patients with GBM undergoing treatment with serial cycles of standard-dose (STD) (200 mg/m² per 5 days) or dose-intensified (DI) TMZ (100 mg/m² per 21 days). All patients receiving STD TMZ exhibited at least a transient grade 2 lymphopenia, whereas those receiving DI TMZ exhibited a sustained grade 3 lymphopenia (<500 cells/μL). CD3⁺ T-cell ($P = .005$) and B-cell ($P = .004$) counts were reduced significantly only in the DI cohort. Patients in the DI cohort had an increase in the proportion of immunosuppressive regulatory T cells (T_{Reg} ; $P = .008$). EGFRvIII-specific

immune responses developed in all patients treated with either regimen, but the DI TMZ regimen produced humoral ($P = .037$) and delayed-type hypersensitivity responses ($P = .036$) of greater magnitude. EGFRvIII-expressing tumor cells were also eradicated in nearly all patients (91.6%; CI₉₅: 64.0%–99.8%; $P < .0001$). The median progression-free survival (15.2 months; CI₉₅: 11.0–18.5 months; hazard ratio [HR] = 0.35; $P = .024$) and overall survival (23.6 months; CI₉₅: 18.5–33.1 months; HR = 0.23; $P = .019$) exceeded those of historical controls matched for entry criteria and adjusted for known prognostic factors. EGFRvIII-targeted vaccination induces patient immune responses despite therapeutic TMZ-induced lymphopenia and eliminates EGFRvIII-expressing tumor cells without autoimmunity.

Keywords: epidermal growth factor receptor mutant III, glioblastoma multiforme, immune therapy, temozolomide.

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Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor, and despite surgical resection, radiation, and chemotherapy, median survival remains <15 months.¹ These conventional therapies lack absolute selectivity

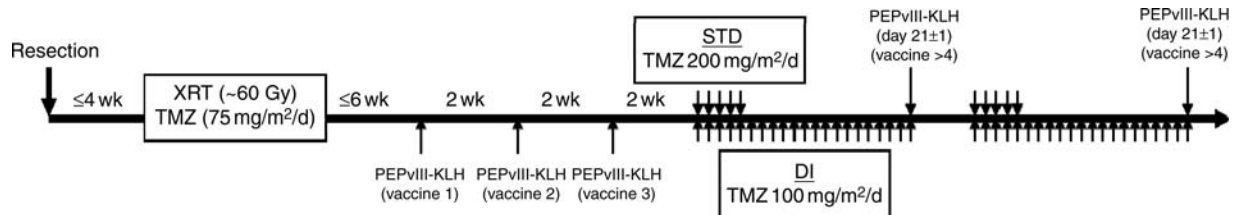


Fig. 1. Treatment protocol schema. After tumor resection and conformal external beam radiotherapy (XRT) with concurrent TMZ at a targeted dose of 75 mg/m², the initial 3 vaccinations of PEPvIII-KLH were given biweekly, starting within 6 weeks of completion of radiation. Subsequent vaccines were given on day 21 \pm 2 during 28-day cycles of TMZ until clinical or radiographic evidence of tumor progression or death. Patients were sequentially assigned to receive TMZ at a targeted dose of 200 mg/m² for the first 5 days of a 28-day cycle ($n = 12$; STD) or at a targeted dose of 100 mg/m² for the first 21 days of a 28-day cycle ($n = 10$; DI).

for tumor cells and are limited by nonspecific damage to normal tissues. Immunologic recognition of tumor-specific gene mutations may allow more precise eradication of neoplastic cells.

The epidermal growth factor receptor variant III (EGFRvIII) is an immunogenic, tumor-specific mutant protein² expressed on the cell surface of about one-third of GBMs^{3,4} and a broad array of other neoplasms.^{5,6} EGFRvIII functions as a constitutively active tyrosine kinase^{7,8} that enhances tumorigenicity⁷ and tumor cell migration⁹ while conferring radiation and chemotherapeutic resistance.^{10,11} Expression of EGFRvIII is also an independent negative prognostic indicator for long-term survival for patients with GBM.³ Thus, EGFRvIII makes an ideal potential immunotherapy target.

Preclinical studies have demonstrated that an EGFRvIII-targeted peptide vaccine is immunogenic and efficacious against established intracerebral tumors.¹² Temozolomide (TMZ), an alkylating chemotherapeutic with significant myelosuppression as its major dose-limiting toxicity,^{13,14} was shown to provide a survival benefit in patients with newly diagnosed GBM.¹ Patients treated with TMZ, especially at dose-intensified (DI) regimens that may be more efficacious,¹⁵ have an increased incidence of opportunistic infections^{1,13} and are immunosuppressed. The profound lymphopenia induced by therapeutic TMZ would be expected to limit vaccine-induced antitumor immunotherapy. However, preclinical data have suggested that a lymphopenic state can induce reactive homeostatic proliferation and enhanced antitumor immune responses,^{16–20} although this is contrary to conventional thinking. We hypothesized that chemotherapeutic approaches that produce greater degrees of lymphopenia, such as DI TMZ regimens, might potentiate immune response to an experimental EGFRvIII-targeted peptide vaccine when compared with other TMZ regimens that induce less lymphopenia. Therefore, we assessed the immunogenicity and potential efficacy of an EGFRvIII-targeted peptide vaccine¹² in the context of standard-dose (STD) (5 of 28 days) and DI (21 of 28 days) TMZ regimens in a sequential phase II, multicenter prospective trial of patients with newly diagnosed, EGFRvIII-expressing GBM.

Patients and Methods

Vaccine Product and Administration

The vaccine is a peptide that spans the EGFRvIII mutation (LEEKKGNYVVTDC) conjugated to keyhole limpet hemocyanin (PEPvIII-KLH; BioSyn Corporation). All vaccines were given intradermally with a granulocyte-macrophage colony-stimulating factor (GM-CSF; 150 μ g/immunization) within 10 cm of the inguinal ligament on alternating sides on day 21 \pm 2 of each 28-day TMZ cycle.

Patient Selection and Clinical Protocol

Adults with newly diagnosed GBM who had gross total resection of their tumor and a Karnofsky Performance Scale (KPS) score of ≥ 80 were eligible for vaccination if tumor cells expressed EGFRvIII by immunohistochemistry (IHC) and they had no radiographic evidence of progression after radiation therapy. The trial design and informed consent were approved by the FDA (under BB-IND-9,944) and the local institutional review boards.

After tumor resection and conformal external beam radiotherapy with concurrent TMZ at a targeted dose of 75 mg/m² (Fig. 1), informed consent was obtained. Between 2 and 4 weeks after completing radiation, each patient had MRI to assess progression. The initial 3 vaccinations of PEPvIII-KLH were given biweekly starting within 6 weeks of completing radiation. Subsequent vaccines were given until clinical or radiographic evidence of tumor progression or death. Patients were sequentially assigned to receive TMZ at a targeted dose of 200 mg/m² for the first 5 days of a 28-day cycle ($n = 12$; STD) or at a targeted dose of 100 mg/m² for the first 21 days of a 28-day cycle ($n = 10$; DI).

Patients were monitored bimonthly by MRI. Progressive disease was defined radiographically according to the Macdonald criteria²¹ or by the development of a new contrast-enhancing lesion > 1 cm at the discretion of the treating neuro-oncologist. Adverse events were defined according to the National Cancer Institute's (NCI) Common Toxicity Criteria (CTC; Version 2.0).

Autoimmunity was assessed by measuring the erythrocyte sedimentation rate, antinuclear antibody titer, and rheumatoid factor levels at baseline, then monthly after the 4th vaccine. Upon tumor progression, further treatment was at the discretion of the patient's treating neuro-oncologist.

Historical Cohort

A historical cohort ($n = 17$) was selected for progression-free survival (PFS) and overall survival (OS) comparisons. All patients in this cohort were adults, had EGFRvIII-expressing primary GBMs, a KPS status $>80\%$, and a resection of $>95\%$ of the original contrast-enhancing tumor volume, and had been treated with radiation therapy and TMZ.³ Patients with tumor progression within 4 weeks of completing radiation therapy were excluded from this historical control cohort.

Immunologic Monitoring and Immunohistochemical Analysis

Delayed-type hypersensitivity (DTH) reactions were assessed within 48–72 h. A positive skintest was defined as >5 mm induration. DTH reactions were compared before TMZ cycle 6 at vaccine 8 so that immune response effects would not be influenced by patients who decided to discontinue TMZ after the 6th cycle, which is the current standard of care.

Serum was analyzed using ELISA. A result was considered positive if it was more than twice background. Humoral responses were compared based on maximum titer obtained.

Immunostaining for EGFRvIII was performed on paraffin-embedded tissue specimens before enrollment and at progression.^{5,6}

Methylguanine Methyltransferase Promoter Methylation Status

Methylguanine methyltransferase (MGMT) promoter methylation was assessed.²² Methylated and unmethylated sequences were quantified using a CEQ 8800 genetic analysis system (Beckman-Coulter). A ratio of methylated-to-unmethylated ≥ 1.0 was scored as methylated.

Lymphocyte and Regulatory T-Cell Counts

Absolute and subset leukocyte counts were quantified by flow cytometry using a direct immunofluorescence, single platform, FDA-approved method in the clinical laboratory at the primary study center (Duke University) and evaluated at vaccine 9, which occurred during TMZ cycle 6 so that maximal differences between groups could be compared.

Regulatory T-cell (T_{Reg}) counts were obtained by staining peripheral blood cell surface antigens (CD25-PE and CD4-PerCpP-Cy5.5; BD) followed by

incubation in permeabilization buffers (eBioscience) and staining with FOXP3-APC (eBioscience). CD25⁺ FOXP3⁺ cells were gated from CD4⁺ lymphocytes and analyzed on a Becton Dickinson FACSCalibur using CellQuest software.

Statistical Analysis

The primary endpoint for this study was to determine if there were differences in immune response between two different TMZ regimens used in conjunction with an experimental PEPvIII-KLH vaccine. The study was powered based on this primary endpoint with 83% power to differentiate between a 10% immune response rate (null hypothesis) and a 50% immune response rate (alternative hypothesis) assuming a 1-sample binomial test conducted at the .05 level of significance with a sample size of 10 patients in each cohort.

The characteristics of the enrolled patient cohorts (A, B, and pooled) were compared with those of the historical cohort using *t*-tests and Cochran–Armitage tests. Paired *t*-tests, a Wilcoxon signed-rank test, or exact McNemar's tests were used within each cohort to assess the change from postradiation baseline in immune responses and leukocyte counts. Between-cohort comparisons of these changes were made using two-sample *t*-tests, Wilcoxon–Mann–Whitney tests, or Fisher's exact tests. Frequencies were used to describe the EGFRvIII-specific antibody responses and DTH reactions. A binomial test was used to assess whether the proportion of patients with EGFRvIII IHC staining changes after vaccination and DTH response after 8 vaccines was significantly different from 0.

PFS and OS were secondary endpoints and were estimated using the Kaplan–Meier method. The Cox proportional hazards model was used to compare both cohorts with respect to PFS and OS and to compare all patients with a historical cohort; adjustments for known prognostic factors, age, and KPS were made.

On the basis of their baseline characteristics and Stupp's nomogram,²³ patients were assigned an expected survival time, which was compared with actual survival time using a binomial test in which the proportion exceeded 0.5.

Adverse events were defined according to the NCI's CTC (Version 2.0). All statistical analyses were conducted using SAS version 9.1 (SAS Institute). A two-sided significance level of .05 was used for all statistical tests.

Results

Study Population

Patients with EGFRvIII-expressing, newly diagnosed GBM were eligible for the study. A total of 12 patients were enrolled to the STD cohort and 10 to the DI cohort for a total of 22 patients. All patients received at least one dose of the vaccine. The mean age of the

study participants was 57 years (range: 41–83 years). Their KPS scores were distributed as follows: 100 (64%), 90 (27%), and 80 (9%). None of the patients required corticosteroids >2 mg dexamethasone/day at the first vaccination.

The median age of the historical cohort was 59 years (range: 37–74). There was no difference in age between the study participants and the historical cohort ($P = .668$; t -test). Patients in the STD cohort had a better KPS than the historical cohort ($P < .0001$); however, such was not the case with the DI cohort ($P = .092$; Cochran–Armitage trend test).

Toxicity and Adverse Events

Toxicity was generally minimal (Table 1), and there were no significant elevations of serum markers of autoimmunity or development of new T2 abnormalities on MR imaging that would be indicative of demyelination. However, 4 patients, all in the DI cohort, had possible allergic drug reactions. One subject was removed from the study without further testing for a presumed severe allergic reaction (DI #6). A second subject, with a similar type of reaction (DI #12), underwent negative allergy testing to PEPvIII and KLH but had evidence of tumor progression prior to subsequent vaccination. Two other subjects had similar reactions, with one being removed from the study due to tumor progression (DI #15) and the second continuing with vaccinations without further allergic reaction (DI #17). All patients, including those who had possible allergic reactions, were included in subsequent immune and survival analysis.

Effects of TMZ on Lymphocytes

The TMZ doses evaluated here are the same as those being evaluated for differences in efficacy in a randomized, phase III trial (RTOG 0525). The effects of TMZ dose were evaluated in a peripheral blood count. All patients receiving the STD 5-day schedule exhibited at least transient grade 2 lymphopenia (<800 cells/ μ L) by the 4th cycle of TMZ (Fig. 2). Grade 3 lymphopenia was observed in only 2 patients with this regimen at any time point. However, sustained grade 2 lymphopenia (<800 cells/ μ L) was induced in all patients receiving the DI schedule by the 4th cycle of TMZ, and by the 6th cycle of TMZ, all patients exhibited a sustained grade 3 lymphopenia (<500 cells/ μ L).

The overall effect of TMZ on lymphocyte counts was seen in both T-cell (CD3⁺) and B-cell compartments (Table 2). The reduction was statistically significant only within the DI cohort ($P = .005$; paired t -test). Although B-cell counts increased in the STD cohort, they were significantly reduced in the DI cohort ($P = .004$; paired t -test).

Despite the reductions in CD4⁺ T cells in both cohorts, TMZ treatment did not lead to a significant reduction in the proportion of T_{Reg} in the STD cohort ($P = .134$; paired t -test). Unexpectedly, an increase in

Table 1. Adverse events ($n = 22$)

Adverse event	Patient (symptom)	Grade	Relationship with drug
Constitutional	DI #6 ^a (diaphoresis)	1	Possible
	DI #14 (malaise, fever)	2	Possible
	DI #15 ^a (diaphoresis)	2	Possible
Dermatology/skin	DI #6 ^a (rash)	2	Possible
	DI #7 (itching)	2	Possible
	DI #12 ^a (flushing, rash)	3	Possible
	DI #15 ^a (flushing, rash)	3	Possible
Cardiovascular	DI #17 ^a (rash, itching)	3	Probable
	DI #6 ^a (hypotension)	2	Possible
	DI #12 ^a (chest pain)	2	Possible
Blood/bone marrow	STD #1 (leukopenia)	3	Possible
Gastrointestinal	DI #12 ^a (hypersalivation)	3	Probable
	DI #17 ^a (nausea)	2	Probable

^aPotential allergic drug reactions in DI patients 6, 12, 15, and 17. STD, standard dose. DI, dose-intensified.

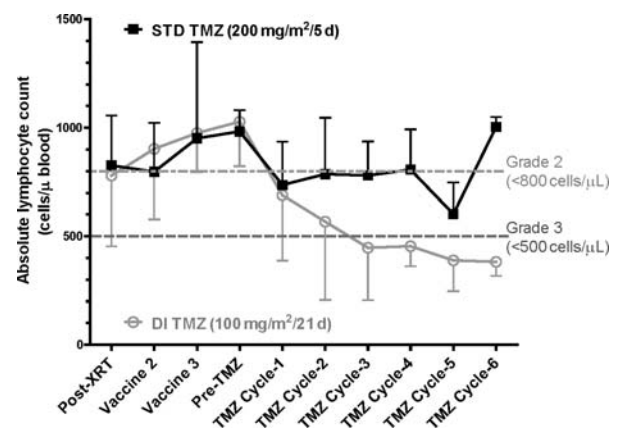


Fig. 2. TMZ-induced lymphopenia. Lymphocyte counts are shown from postradiation baseline through the 6th TMZ cycle in both cohorts. Patients receiving STD TMZ all exhibited transient grade 2 lymphopenia by TMZ cycle 4. Patients receiving DI TMZ all exhibited sustained grade 2 lymphopenia by TMZ cycle 4 and sustained grade 3 lymphopenia (<500 cells/ μ L) by TMZ cycle 6. Mean counts and SD are plotted.

the proportion of CD4⁺ CD25⁺ FOXP3⁺ T_{Reg} from $6.07 \pm 2.34\%$ SD to $8.72 \pm 2.71\%$ SD at vaccine 6 was seen in the peripheral blood within the DI cohort ($P = .008$; Fig. 3) that was significantly different compared with the STD cohort ($P = .002$; t -test).

Immune Responses

Despite all patients having tumors that expressed EGFRvIII, none had evidence of EGFRvIII-specific cellular or humoral immunity prior to vaccination. All 22

Table 2. Lymphocyte changes with TMZ

Cell type (Cells/ μ L)	Group	Postradiation/TMZ baseline (mean \pm SD)	TMZ cycle 6 (mean \pm SD)	Within group paired <i>t</i> -test (<i>P</i> value)	Between group <i>t</i> -test (<i>P</i> value)
CD3 ⁺ T-cells	STD	811 \pm 215	724.5 \pm 14.9	.689	.007
	DI	805.5 \pm 171.5	180.5 \pm 83.6	.005	
B-cells	STD	289 \pm 73.5	375.5 \pm 14.9	.398	.028
	DI	281.2 \pm 72.6	180.83 \pm 77.6	.004	

TMZ, temozolomide; STD, standard dose; DI, dose-intensified.

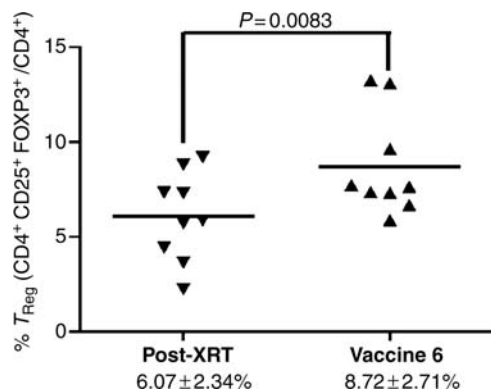


Fig. 3. DI TMZ increases T_{Reg} . Peripheral blood cell surface CD25 and CD4 were stained, followed by intracellular detection of FOXP3. T_{Reg} were defined as CD25⁺ FOXP3⁺ cells within the CD4⁺ T-cell compartment. T_{Reg} increased in the cohort receiving DI TMZ from $6.07 \pm 2.34\%$ postradiation to $8.72 \pm 2.71\%$ ($P = .008$). XRT, external beam radiotherapy.

patients developed EGFRvIII-specific humoral immune responses when analyzed at vaccine 8 (Fig. 4A; $P < .0001$). On the basis of these data, the null hypothesis for the primary endpoint was rejected for both patient cohorts. Unexpectedly, EGFRvIII-specific antibody titers in the DI cohort increased more over time than those in the STD cohort such that mean maximum

antibody titer was significantly higher in the DI cohort (1:634 982) compared with the STD cohort (1:186 521; $P = .037$; Wilcoxon–Mann–Whitney; Fig. 4A). Within the DI cohort, EGFRvIII-specific vaccine titers exceeded 1:2 000 000.

No patient had DTH responses at the vaccine site after 1 vaccination. When evaluated at vaccine 8, 0 of 5 (0%; CI₉₅: 0%–52.2%; $P > .999$; binomial test) patients had developed DTH responses in the STD cohort. In contrast, after 8 vaccines in the DI cohort, 7 of 8 (87.5%; CI₉₅: 47.4%–99.7%; $P < .0001$) patients had developed DTH responses. Differences between STD and DI cohorts were statistically significant ($P = .005$; Fisher's exact test). In addition, subjects in the DI TMZ cohort had vaccine site DTH responses that were higher at all time points that were statistically significant at vaccine 8 ($P = .036$; Wilcoxon–Mann–Whitney; Fig. 4B). Within the DI cohort, DTH responses exceeded 39 cm.

IHC Analysis of EGFRvIII Expression

Histologic samples were available for EGFRvIII expression analysis by IHC analysis from 12 of 17 recurrent tumors. Eleven (91.6%; CI₉₅: 64.0%–99.8%) of these samples had lost expression of EGFRvIII ($P < .0001$; binomial proportions; Fig. 5).

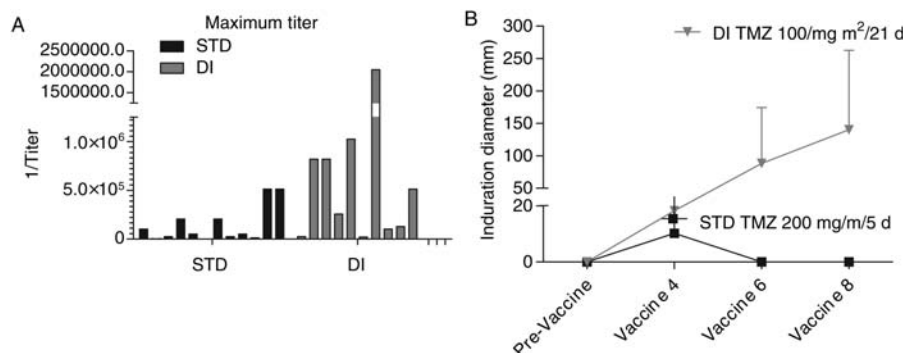


Fig. 4. DI TMZ enhances EGFRvIII-specific humoral and cellular immune responses. (A) ELISA was used to determine EGFRvIII-specific humoral reactivity. Maximum titer is shown. All patients had developed EGFRvIII-specific humoral immune responses when evaluated at vaccine 8. EGFRvIII-specific antibody titers in the DI cohort increased more over time than those in the STD cohort such that the mean maximum antibody titer was significantly higher in the DI cohort (1:634 982) than in the STD cohort (1:186 521; $P = .037$; Wilcoxon–Mann–Whitney). (B) Subjects receiving the DI TMZ regimen for 21 days had vaccine site DTH responses that were higher at all time points than those obtained in the less myelosuppressed patients receiving the 5-day STD TMZ schedule. This was statistically significant when evaluated at vaccine 8 ($P = .036$; Wilcoxon–Mann–Whitney).

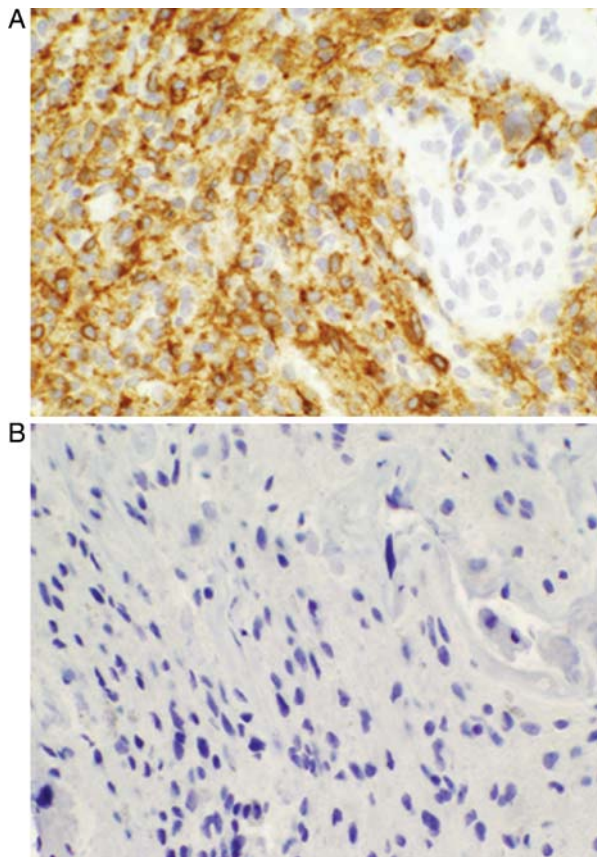


Fig. 5. Representative EGFRvIII IHC of a patient with GBM after EGFRvIII-targeted vaccine. Staining for EGFRvIII before (A) and after (B) the vaccine.

Progression-free and Overall Survival

In the STD cohort, 9 of 12 patients (75%; CI₉₅: 41%–91%) were alive and lacked evidence of radiographic progression 6 months after vaccination (Table 3, Fig. 6A). In the DI cohort, 9 of 10 patients (90%; CI₉₅: 47%–98%) were alive and lacked evidence of radiographic progression 6 months after vaccination (Table 3). There was no difference in PFS from vaccination ($P = .660$; HR = 0.76; CI₉₅: 0.22–2.64) or histologic diagnosis ($P = .583$; HR = 0.71; CI₉₅: 0.21–2.41) between the STD and DI cohorts after adjusting for age and KPS. However, the study was not powered to detect such differences as suggested by the large confidence intervals.

The median PFS from time of histologic diagnosis for all patients was 15.2 months (CI₉₅: 11.0–18.5 months; Fig. 6A, Table 3). The median PFS from time of vaccination for all patients was 11.8 months (CI₉₅: 8.1–15.6 months). In the historical cohort ($n = 17$), the median PFS from histologic diagnosis was 6.3 months (CI₉₅: 4.1–9.0 months). After adjustment for age and KPS, the risk of progression of vaccinated patients remained significantly less than that observed in the TMZ-treated historical control group ($P = .024$; HR = 0.35; CI₉₅: 0.14–0.87). Because our study subjects were all treated with TMZ during and after radiotherapy, an unintentional study bias may have been introduced by selecting

patients with GBM that possessed the capacity of MGMT methylation, which compromises DNA repair and is associated with longer survival in GBM patients who receive alkylating agents such as TMZ.²² In an exploratory retrospective analysis of study subjects from whom tumor tissue could be obtained ($n = 17$), 7 (41%) had MGMT promoter methylation, which is actually lower than that previously reported and should influence our results negatively.

In the STD cohort, 10 of 12 patients (83%; CI₉₅: 48%–96%) were alive 12 months after vaccination (Table 3). In the DI cohort, 9 of 10 patients (90%; CI₉₅: 47%–98%) were alive 12 months after vaccination (Table 3, Fig. 6B). There was no difference in OS from vaccination ($P = .283$; hazard ratio [HR] = 2.49; CI₉₅: 0.47–13.11) or histologic diagnosis ($P = .385$; HR = 2.0; CI₉₅: 0.42–9.62) between TMZ cohorts after adjusting for age and KPS. However, the study was not powered to detect such differences, again suggested by the large confidence intervals.

The median OS from time of histologic diagnosis for all patients was 23.6 months (CI₉₅: 18.5–33.1 months; Fig. 6B, Table 3). The median OS from time of vaccination for all patients was 19.3 months (CI₉₅: 15.6–30.7 months). In the historical cohort ($n = 17$), the median OS from histologic diagnosis was 15.0 months (CI₉₅: 11.4–19.7 months). Using an unadjusted analysis, the risk of death of study subjects was significantly less than that of the historical controls ($P = .008$; HR = 0.35; CI₉₅: 0.16–0.76). After adjustment for age and KPS, the risk of death of vaccinated patients remained significantly lower than that observed in the TMZ-treated historical control group ($P = .019$; HR = 0.23; CI₉₅: 0.07–0.79).

To explore whether our vaccinated patients had better outcomes than would have been expected by chance according to Curran's recursive partitioning analysis (RPA), we compared our patients' survival with expected survival.²⁴ Of our 22 vaccinated patients, 4 were in class III and 18 were in class IV. Patients in RPA class III have an expected survival of 17.9 months, whereas those in class IV have an expected survival of only 11.1 months. Overall, 20 of our 22 patients (91%; CI₉₅: 71%–99%) exceeded these expectations ($P < .0001$; binomial test).

Finally, to explore whether our vaccinated patients had better outcomes than would have been expected after standard-of-care therapy with TMZ within the same RPA classes, we compared our patients' survival with that reported by Mirimanoff et al.²⁵ on the EORTC 26981/22981-NCIC CE3 phase III randomized trial based on the published nomogram. Overall, 17 of our 22 patients (77%; CI₉₅: 55%–92%) exceeded expectations based on the results of this trial ($P = .008$; binomial test).

Discussion

TMZ produces a survival benefit in patients with GBM and has become a routine part of their

Table 3. Progression and survival statistical summary

Statistic	STD (<i>n</i> = 12)		DI (<i>n</i> = 10)		Overall (<i>n</i> = 22)		Historical cohort (<i>n</i> = 17)
	Postvaccination	Postsurgery	Postvaccination	Postsurgery	Postvaccination	Postsurgery	Postsurgery
PFS							
Median (mo)	12.1 (7.6, 21.3)	15.9 (10.5, 23.7)	11.6 (8.1, 12.7)	14.9 (11, 15.8)	11.8 (8.1, 15.6)	15.2 (11, 18.5)	6.3 (4.1, 9)
6 mo (%)	75 (40.8–91.2)	100	90 (47.3–98.5)	90 (47.3–98.5)	81.8 (58.5–92.8)	95.5 (71.9–99.3)	58.8 (32.5–77.8)
12 mo (%)	50 (20.8–73.6)	66.7 (33.7–86)	32 (5.8–63.4)	60 (25.3–82.7)	43.3 (22–63)	63.6 (40.3–79.9)	23.5 (7.3–44.9)
24 mo (%)	25 (6–50.5)	25 (6–50.5)	NE	NE	20.3 (5.8–41)	20.1 (5.7–40.7)	5.9 (0.4–23.5)
OS							
Median (mo)	17.4 (13.8, 30.7)	21 (17, 33.1)	>15.7	>18.9	19.3 (15.6, 30.7)	23.6 (18.5, 33.1)	15 (11.4, 19.7)
6 mo (%)	100	100	100	100	100	100	94.1 (65–99.1)
12 mo (%)	83.3 (48.2–95.6)	100	90 (47.3–98.5)	100	86.4 (63.4–95.4)	100	70.6 (43.1–86.6)
24 mo (%)	41.7 (15.2–66.5)	41.7 (15.2–66.5)	NE	NE	46.6 (21.8–68.2)	44.4 (19.8–66.5)	5.9 (0.4–23.5)

95% CI values are shown in parentheses. STD, standard dose; DI, dose-intensified; PFS, progression-free survival; OS, overall survival; NE, not estimable due to insufficient events.

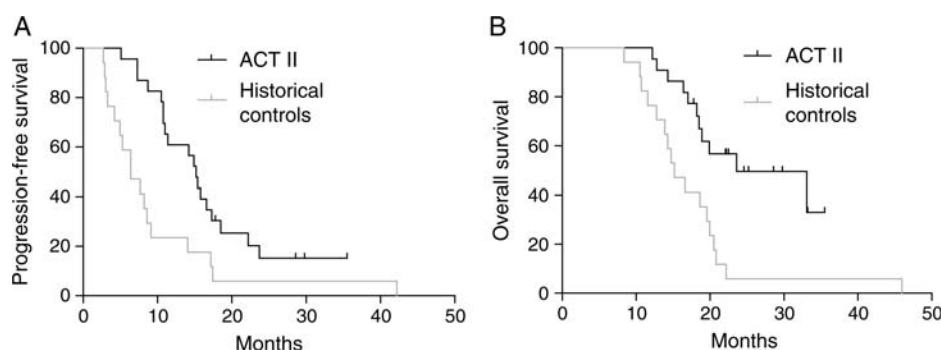


Fig. 6. PFS and OS. (A) The median PFS from histologic diagnosis for all 22 vaccinated subjects (black line) was 15.2 months (CI₉₅: 11.0–18.5 months). In the TMZ-treated historical cohort (*n* = 17; gray line), the median PFS was 6.3 months (CI₉₅: 4.1–9.0 months). The PFS of vaccinated subjects compares favorably with that of the TMZ-treated cohort before (*P* = .006) and after (*P* = .024) adjustment for age and KPS. (B) The median OS from histologic diagnosis for all 22 vaccinated subjects (black line) was 23.6 months (CI₉₅: 18.5–33.1 months). In the TMZ-treated historical cohort (*n* = 17; gray line), the median survival was 15.0 months (CI₉₅: 11.4–19.7 months). The OS of vaccinated subjects compares favorably with that of the TMZ-treated cohort before (*P* = .008) and after (*P* = .019) adjustment for age and KPS.

therapy.¹ As such, more aggressive regimens are now being evaluated.^{15,26} Concurrent use of myelosuppressive chemotherapy during vaccine administration would be expected to suppress the induction of functional immune responses. Despite the profound lymphopenia seen in patients with GBM treated with TMZ and an increase in *T*_{Reg} seen in the DI regimen, our results demonstrate that potent cellular and humoral immune responses can be generated and maintained in patients with GBM receiving serial cycles of TMZ. The significant finding of our study is that both humoral and cellular vaccine-induced immune responses are unexpectedly enhanced by a DI TMZ that induces more profound

and more persistent lymphopenia than the STD TMZ. Although counterintuitive, this is consistent with preclinical studies^{16,18} and findings after adoptive T-cell transfer.¹⁷

An important caveat may be the timing of the vaccine relative to the recovery of lymphocytes after therapeutic TMZ. The dramatic immune responses generated in lymphodepleted patients may be enhanced by homeostatic cytokines that induce proliferation and also reduce the T-cell activation threshold.²⁰ Still, lymphocytes must encounter their cognate antigen and compete for limited amounts of these homeostatic cytokines.²⁰ Thus, B- or T-cells specific for antigens that predominate during this recovery period, like those

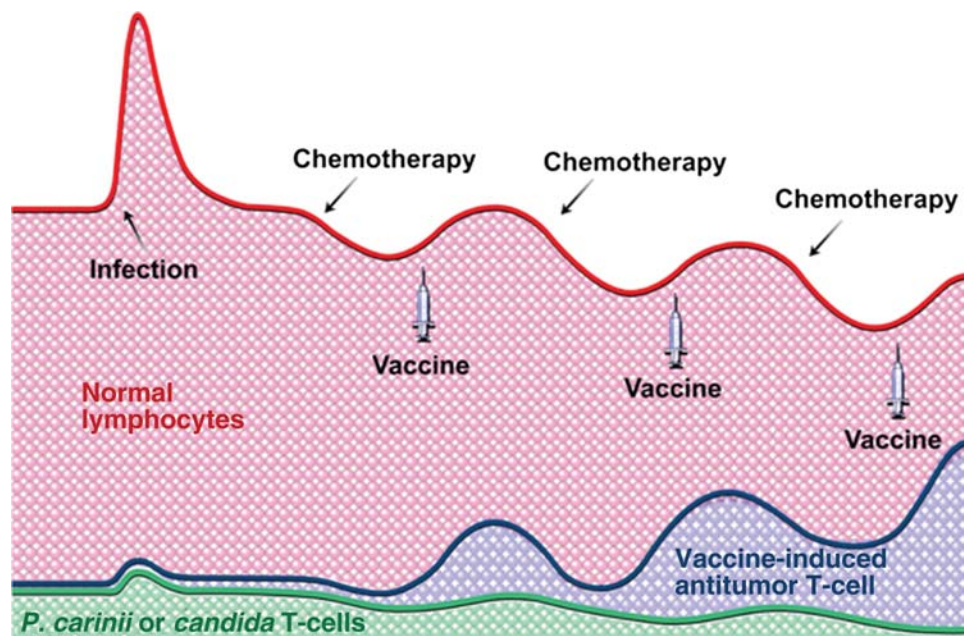


Fig. 7. Homeostatic lymphocyte proliferation enhances vaccine-induced immunity. Following the periods of chemotherapy-induced lymphopenia, there is a homeostatic proliferation of the host's remaining lymphocytes. Lymphocytes that encounter their cognate antigen, eg, in the form of a vaccine, during this recovery differentiate directly into effector cells capable of rapid and intense response to antigen and have a competitive advantage. These lymphocytes become disproportionately overrepresented in the recovering population. Lymphocytes recognizing other pathogens such as *P. carinii* compete less well and are reduced in number, leading to opportunistic infections.

provided in the form of a vaccine, have a competitive advantage (Fig. 7).

Although prior studies have shown that continuous and prolonged TMZ reduces the number of T_{Reg} when defined as $CD4^+ CD25^+$ T cells,¹³ our results show that T_{Reg} levels are actually increased by DI TMZ. This difference between our results and the previous observation may be explained by the failure in the prior work to use a more specific marker for T_{Reg} like FOXP3.

We also found that vaccination eliminated cells expressing the EGFRvIII antigen and was associated with significantly longer PFS and OS than expected. We suspect that the loss of EGFRvIII in the tumor is secondary to immunologic recognition and clearance of EGFRvIII positive cells, although this is speculative. The loss of EGFRvIII expression could be related to treatment with radiation or TMZ or due to endogenous tumor mutations. However, similar findings were observed in preclinical murine models after administration of this vaccine that did not receive radiation or TMZ.¹² Although targeting a tumor-specific antigen such as EGFRvIII lessens the risk of inducing autoimmunity, tumor heterogeneity and persistence of EGFRvIII-negative cells may ultimately limit this approach. Multi-antigenic vaccines may serve as an alternative; however, their use in combination with TMZ may increase the risk of autoimmunity.¹⁹

Contributors

J.H.S., K.D.A., G.E.A., A.D., A.H.F., H.S.F., M.R.G., R.E.M., D.A.M., D.A.R., R.Sa., W.S., J.J.V., D.D.B., and A.B.H. were responsible for the conception and design of the study. J.H.S., K.D.A., A.D., A.H.F., H.S.F., M.R.G., R.E.M., D.A.R., R.Sa., W.S., J.J.V., D.D.B., and A.B.H. were responsible for the provision of study materials or patients. J.H.S., K.D.A., G.E.A., M.R.G., D.A.M., R.Sa., R.Sc., W.S., D.D.B., and A.B.H. were responsible for the collection and assembly of data. J.H.S., K.D.A., G.E.A., A.C., M.R.G., J.E.H., D.A.M., R.Sa., R.Sc., W.S., D.D.B., and A.B.H. were responsible for data analysis and interpretation. J.H.S., G.E.A., A.C., J.E.H., D.A.M., R.Sa., D.D.B., and A.B.H. contributed to writing the manuscript. All authors gave their final approval to the manuscript.

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