Featured Article

Clinical Responsiveness of Glioblastoma Multiforme to Chemotherapy after Vaccination

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ABSTRACT

Purpose: **Although the development of immune-based therapies for various cancers including malignant glioma has been heralded with much hope and optimism, objective clinical improvements in most vaccinated cancer patients have not been realized. To broaden the search for vaccine-induced benefits, we examined synergy of vaccines with conventional chemotherapy.**

Experimental Design: **Survival and progression times were analyzed retrospectively in 25 vaccinated (13 with and 12 without subsequent chemotherapy) and 13 nonvaccinated *de novo* glioblastoma (GBM) patients receiving chemotherapy. Immune responsiveness and T-cell receptor excision circle (TREC) content within $CD8^+$ T cells ($CD8^+$ TRECs) was determined in vaccinated patients.**

Results: **Vaccinated patients receiving subsequent chemotherapy exhibited significantly longer times to tumor recurrence after chemotherapy relative to their own previous recurrence times, as well as significantly longer postchemotherapy recurrence times and survival relative to patients receiving isolated vaccination or chemotherapy. Patients exhibiting objective (>50%) tumor regression, extremely rare in *de novo* GBM, were also confined to the vaccine + chemotherapy group. Prior tumor behavior, demographic factors, other treatment variables, distribution of vaccine responders, and patients with high $CD8^+$ TRECs all failed to account for these differences in clinical outcome. Within all GBM patients receiving post-vaccine chemotherapy, however, $CD8^+$ TRECs predicted significantly longer chemotherapeutic responses, revealing a strong link between the predominant T-cell effectors in GBM and tumor chemosensitivity.**

Conclusions: **We propose that therapeutic vaccination synergizes with subsequent chemotherapy to elicit tangible clinical benefits for GBM patients.**

INTRODUCTION

Malignant brain tumors are among the gravest forms of cancer. The most common of these incurable tumors, glioblastoma multiforme (GBM) (grade IV glioma, glioblastoma), carries with it an average survival between 12 and 18 months (with 90–95% of patients surviving less than 2 years), without the possibility of spontaneous remission or effective treatment (1–3). The consistently short survival and absence of effective treatment that make GBM such a devastating disease also render the evaluation of new therapies for this disease relatively rapid and unequivocal. Survival from diagnosis represents the most objective standard for evaluation of GBM therapies, in part because tumor mass reduction (*i.e.*, surgically) does not necessarily correlate with prolonged survival (4–6).

Unfortunately, conventional therapies are remarkably ineffective at improving GBM clinical outcome, despite the ability of conventional therapies to confer significant benefits to patients with non-glioma tumors (3, 7, 8). Even the few treatments effective against GBM typically either exhibit small increases in survival that are evident only from large population studies or primarily benefit certain (*i.e.*, young) patient subpopulations (9, 10). Thus, novel therapies for GBM are needed.

Cancer vaccines represent one novel therapy for GBM (11–13). The therapeutic efficacy of vaccination for any human tumor, however, remains controversial because tumor destruction or extended life span is not observed in most vaccinated cancer patients (14–16). In contrast, current cancer vaccines do reliably elicit tumor-reactive cytotoxic T lymphocytes (CTLs) in most patients (14, 15, 17). The reasons underlying the general clinical failure of cancer vaccines are unknown, but one possibility is that the kinetics of tumor killing by effector CTLs in cancer patients may be too inefficient to keep pace with rapidly growing, mutating tumors *in situ*. Consistent with this notion, it was reported previously that therapeutic vaccination with autologous tumor antigen-pulsed dendritic cells is sufficient to enhance peripheral tumor-reactive CTL activity and $CD8^+$ T-cell infiltration into tumors *in situ* in GBM patients (13). Nevertheless, improvements in overall patient survival were not apparent in this initial study.

Because CTLs induce death in their cellular targets, it is not unreasonable to expect that inefficient CTL killing might either incompletely trigger death pathways in targeted tumor cells or select for CTL-resistant tumor variants. In the first case, vaccine-elicited tumor-responsive CTLs might fundamentally alter tumors by "priming" their apoptotic machinery, much as death receptor ligation (which CTLs can induce) sensitizes cells to anticancer drug-induced apoptosis (18). In the second case,

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CTLs could fundamentally alter tumor cell physiology and/or genetics through selection of immune-resistant variants, a demonstrated phenomenon in cancer patients receiving therapeutic vaccinations (19–21). Either possibility could in theory be exploited by additional therapeutic modalities. Therefore, the clinical insufficiency of cancer vaccines encourages the examination of synergy between vaccination and other therapies and with chemotherapy in particular.

A retrospective examination of the impact of therapeutic vaccination on the efficacy of conventional GBM chemotherapy was thus undertaken. Progression rates and overall survival were compared among 12 vaccine-treated, 13 chemotherapy-treated, and 13 vaccine + chemotherapy-treated *de novo* GBM patients. The results suggest that chemotherapy synergizes with previous therapeutic vaccination to generate a uniquely effective treatment that slows GBM progression and significantly extends patient survival relative to individual therapies. This represents the first evidence that a vaccine-based therapeutic approach may extend survival in a majority of cancer patients and represents a novel treatment strategy that may substantially prolong GBM survival across a wide age range relative to standard radiation plus chemotherapy. Additional independent evidence linked levels of the predominant antitumor effector T cells in GBM patients, $CD8^+$ recent thymic emigrants (RTEs), to chemotherapeutic responsiveness, consistent with a direct influence of antitumor immunity on GBM chemosensitivity.

MATERIALS AND METHODS

Patients and Clinical Variables. All patients suffered from *de novo* GBM (average age, 55 years; range, 32–78 years) and provided informed consent to treatments and associated monitoring.

Patients in the vaccine group underwent craniotomy (five patients underwent one craniotomy before receiving vaccine therapy, six patients underwent two craniotomies, and one patient underwent four craniotomies before receiving vaccine therapy). All of these patients received a course of radiation before vaccination. Four patients in this group also received chemotherapy, and one patient received stereotactic radiosurgery (SRS) before vaccination. After vaccination, five of these patients underwent another craniotomy, and three received additional SRS. None received chemotherapy after vaccination.

All patients in the chemotherapy group underwent craniotomy, radiation, and chemotherapy. Six of these patients underwent a second craniotomy, and five patients received additional SRS. Of note, the longest overall survivor in this group (991 days) suffered from postoperative intracranial abscess requiring multiple surgical procedures for drainage. Intracranial infections in malignant glioma patients are associated with prolonged survival and have been proposed to initiate an antitumor immune response (22).

Patients in the vaccine + chemotherapy group underwent craniotomy (eight patients underwent one craniotomy, and five patients underwent two craniotomies) before receiving the vaccine therapy. All of these patients received radiation therapy. Five patients received additional chemotherapy, and three received SRS. After vaccination, six of these patients underwent another craniotomy, and five received SRS. All patients received chemotherapy after vaccination at the time of tumor progression. Notably, a single patient in this group (surviving >730 days and depicted in Fig. 3*B*) experienced a cutaneous glioblastoma with single lymph node involvement before vaccination and at the site of irradiated tumor cell inoculation for delayed type hypersensitivity testing. These two tumors were removed surgically approximately 1 year prior to chemotherapy and did not recur.

Vaccinated patients were steroid-free during blood collection and vaccinations as described previously (13), and they received three vaccines, 2 weeks apart, of $10-40 \times 10^6$ autologous dendritic cells loaded with either HLA-eluted peptides from cultured tumor cells or 150 μg/ml autologous tumor freeze-thaw lysate, starting approximately 15 weeks after surgery. A fourth identical vaccination followed 6 weeks later only in Phase II trial patients (12 of 25 patients). Serial magnetic resonance imaging (MRI) scans were performed every 2–3 months for all patients and were continuously monitored until mid-2003. For all patients, tumor antigens of uncharacterized composition were obtained from cell cultures (phase IA; Table

Table 1 Demographic and treatment parameters of GBM patient groups

	Vaccine	Chemotherapy	Vaccine + Chemotherapy	Significance (*P*)
Age	53.4 ± 13	55.7 ± 10	54.0 ± 10	0.88*
Karnofsky score after vaccination	84 ± 16		93 ± 9	0.12*
Male (%)	50	38	77	0.14†
Nonsurvivors (%)	100	92	77	0.3†
>2 surgeries before vaccine (%)	58.3		61.5	0.43†
No chemotherapy before vaccine (%)	66.7		61.5	0.58†
Recurrent patients (%)	50 (6/12)	7.7 (1/13)	61.5 (8/13)	0.5‡
No surgery after vaccine (%)	58.3		53.8	1.0†
Days from surgery to vaccine	115 ± 14		121 ± 13	0.94§
Mean survival (mo)	17.9 ± 1.7	15.9 ± 2.1	26 ± 3.7	0.047§
2-yr survival [%] (fraction)	8.3 (1/12)	8.3 (1/12)	41.7 (5/12)	<0.05‖
3-yr survival [%] (fraction)	0 (0/12)	0 (0/12)	18.2 (2/11)	<0.01‖

* Analysis of variance.
† Fisher's exact test.
‡ Binomial distribution, vaccine *versus* vaccine + chemotherapy only.
§ Log-rank test.
‖ Binomial distribution, vaccine + chemotherapy *versus* all other groups. Calculations of % 2- and 3-year survival excluded censored values.

1) or lysates (all other trials; Table 1) originating from a single surgical tumor resection preceding vaccination. Newly diagnosed patients (six patients in the vaccine group and five patients in the vaccine + chemotherapy group) were vaccinated starting approximately 15 weeks after diagnosis. Recurrent patients (six patients in the vaccine group and eight patients in the vaccine + chemotherapy group; $P = 0.5$, binomial distribution) were vaccinated starting from 3–19 months after diagnosis [vaccine group, average, 9.5 ± 5.5 months (range, 4.4–19.1 months); vaccine + chemotherapy group, average, 7.1 ± 4.1 months (range, 3.1–15 months); $P = 0.4$, two-tailed t test]. Recurrent patients contributed less to differences in overall survival between the vaccine + chemotherapy and vaccine groups than did newly diagnosed patients: overall survival of recurrent patients was 899 ± 208 days in the vaccine + chemotherapy group *versus* 557 ± 97 days in the vaccine group ($P = 0.186$, log-rank), whereas overall survival of newly diagnosed patients was 1046 ± 246 *versus* 529 ± 41 days in the vaccine + chemotherapy and vaccine groups, respectively ($P = 0.098$, log-rank).

Cell Isolation and Lysis. Peripheral blood mononuclear cells (PBMCs) were prepared with Ficoll from patients' blood obtained at the time of surgery and/or from banked leukaphereses. $CD4^+$ and $CD8^+$ T cells were purified from PBMCs using MACS bead separation (Miltenyi Biotec, Auburn, CA). $CD4^+$ or $CD8^+$ cells (10^7 cells/ml) were prepared for quantitative real-time PCR (qPCR) by lysis in 100 μg/ml proteinase K (Boehringer, Indianapolis, IN) for 1 h at 56°C, with inactivation at 95°C for 10 min.

Cytotoxic T Lymphocyte Assays. Bulk CTL assay was performed with the JAM assay (23). Pre-vaccine and post-vaccine PBMCs were harvested and adjusted to 8×10^6 cells/ml in RPMI 1640 containing heat-inactivated human AB serum and stimulated with irradiated (11,000 rads) autologous cultured tumor cells at 1×10^6 cells/ml for 6 days in the presence of 20 units/ml recombinant human interleukin (IL)-2. Tumor cells were labeled at 1×10^5 cells/ml in 5 μCi/ml [^{3}H]thymidine for 48 h at 37°C in 5% CO_2. After incubation, target cells in maximum release wells were lysed with 5% SDS in H_2O and incubated in DNase I (Roche) at 20 units/ml final concentration for 10 min. PBMCs (100 μl; maximum, 1×10^7 cells/ml for 100:1 E:T ratio) were added to targets (100 μl; 1×10^5 cells/ml) at various E:T ratios for 6 h. Cells were harvested from plates with a 96-well harvester (Tomtec). CPM were determined using a Microbeta 1450 Trilux liquid scintillation counter (Wallac), and the percentage of target cell lysis was plotted. Progressive or consistent increases in target cell lysis with increasing E:T ratio by post-vaccine PBMCs that was at least 1.5 SDs greater than pre-vaccine lysis was considered a positive response.

Interferon-γ Quantification. Dendritic cells were prepared by incubating loosely adherent PBMCs in RPMI 1640 +10% human AB serum, 500 units/ml IL-4, and 800 units/ml granulocyte macrophage colony-stimulating factor for 8 days at 37°C in 5% CO_2. Dendritic cells (2×10^6 cells/ml) were pulsed with autologous tumor freeze-thaw lysate (150 μg/ml) for 18 h and irradiated. Autologous pre- and post-vaccine PBMCs (1×10^6 cells/ml) were stimulated in 10% human AB serum with 1×10^6 irradiated lysate-pulsed dendritic cells/ml, with IL-2 (300 IU/ml) added on day 2 and a 2-h re-stimulation with 150 μg/ml tumor lysate on day 11. RNA was isolated using Trizol (GIBCO Invitrogen, San Diego, CA) and transcribed using random hexamers. Quantified plasmid DNA standards and cDNAs were amplified using qPCR primers and probes (Qiagen Operon, Alameda, CA) as described previously (24, 25). A ≥1.5-fold increase in CD8-normalized intereron (IFN)-γ production after vaccination indicated a positive response (25). IFN-γ primers were as follows: 5′-AGCTCTGCATCGTTTTGGGTT-3′, forward; 5′-GTTCCATTATCCGCTACATCTGAA-3′, reverse; and 5′-carboxyfluorescein-TCTTGGCTGTTACTGCCAGGACCCA-carboxy-tetramethylrhodamine-3′, probe. Reference (CD8) primers were as follows: 5′-CCCTGAGCAACTCCATCATGT-3′, forward; 5′-GTGGGCTTCGCTGGCA-3′, reverse; and 5′-carboxyfluorescein-TCAGCCACTTCGTGCCGGTCTTC-3′, probe. Reactions were amplified in 25 μl of 10 mM deoxynucleotide triphosphates, 400 nM primers, 200 nM TaqMan probe, and 0.5 unit of platinum Taq polymerase at 95°C for 5 min, 95°C for 30 s, and 60°C for 30 s for 45 cycles and detected on an iCycler (Bio-Rad, Hercules, CA). Patients responsive to TRP-2, Her-2, MAGE-1, or gp100 were identified by post-vaccine increases in IFN-γ production by PBMCs to peptide-pulsed T2 cells (1 μM peptide, 2 h, 37°C) using enzyme-linked immunosorbent assay and/or ELISPOT kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

T-Cell Receptor Excision Circle Quantification. TRECs were quantified in duplicate or triplicate by qPCR using the 5′ nuclease (TaqMan) method, as described previously (26), and detected on an iCycler system (Bio-Rad). qPCR was performed on 5 μl of cell lysate (from 50,000 cells) with primers 5′-CACATCCCTTTCAACCATGCT-3′ (forward), 5′-GCCAGCTGCAGGGTTTAGG-3′ (reverse), and carboxyfluorescein-5′-ACACCTCTGGTTTTTGTAAAGGTGCCCACT-carboxy-tetramethylrhodamine-3′ (probe; MegaBases, Chicago, IL). PCR reactions including 0.5 unit of platinum Taq (Gibco, Grand Island, NY), 3.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 500 nM of each primer, and 150 nM probe were amplified at 95°C for 5 min, 95°C for 30 s, and 60°C for 1 min for 45 cycles. Control β-actin reactions were performed to ensure nucleic acid content, and negative samples were excluded from further analysis. TREC values were adjusted for T-cell purity.

Statistical Analyses. Statistical analyses included two-tailed Mann-Whitney log-rank tests for disease-free and overall survival, binomial distribution probability, and Pearson's correlation coefficients (r values) calculated with SAS and Excel software. Binomial distributions were determined for 2- and 3-year survival frequencies between vaccine + chemotherapy group and other (vaccine or chemotherapy) patient groups. Where indicated, values are included ± SE.

RESULTS

De novo GBM patients (GBM did not arise from malignant progression of initially lower-grade gliomas) were enrolled into one of three vaccine studies conducted from 1998–2001 at a single institution (Maxine Dunitz Neurological Institute), or were given chemotherapy alone, after surgical tumor resection and standard radiation therapy. Vaccinated (vaccine or vaccine + chemotherapy) patients received at least three vaccinations

Table 2 Vaccine trial composition and distinctions

	Phase I A	Phase I B	Phase II
Vaccine	7 patients	3 patients	2 patients
Vaccine + chemotherapy	1 patient	2 patients	10 patients
Antigen source	Tumor line MHC I-elution	Tumor lysate	Tumor lysate
Vaccine course	3; 2 wks apart	3; 2 wks apart	3; 2 wks apart + 1; 6 wks later
Eligibility			
Diagnosis	Newly diagnosed GBM, newly diagnosed AA	Recurrent GBM, AA	Newly diagnosed or recurrent GBM, AA
Karnofsky score	>60	>60	>60
Age (yrs)	>18	>18	>18
Vaccine responsiveness (GBM only)	60%*	60%†	40%†

NOTE. CTL responsiveness was determined from seven testable samples per trial.

Abbreviation: AA, anaplastic (grade III) astrocytoma.

* Bulk CTL assay.

† IFN-γ production by qPCR. Eleven (7 by bulk CTL and 4 by IFN-γ qPCR) of 12 patients and 13 (1 by bulk CTL and 12 by IFN-γ qPCR) of 13 patients were tested for vaccine responsiveness from vaccine and vaccine + chemotherapy groups, respectively.

with autologous tumor antigen-pulsed dendritic cells, starting approximately 15 weeks after surgery and 5 weeks after radiation therapy (Tables 1 and 2; Fig. 1*A*). Patients receiving chemotherapy alone (chemotherapy patients) were treated (with surgery, radiation, and chemotherapy) over the same time interval as vaccinated patients (Table 3). Serial MRI scans were performed every 2–3 months in all patients. Tumor progression and overall survival among vaccine, chemotherapy, and vaccine + chemotherapy groups were monitored and compared (Figs. 1 and 2).

Treatment and demographic variables, including proportion of nonsurvivors, surgery before or after vaccination (where appropriate), chemotherapy before vaccination (where appropriate), gender, and age, were not significantly different among the relevant groups (Table 1). In addition, Karnofsky performance status (KPS) and inclusion of recurrent *versus* newly diagnosed (all *de novo* GBM) patients did not differ significantly between the vaccine and vaccine + chemotherapy patient groups (Table 1). Inclusion criteria for vaccinated *de novo* GBM patients, the pathological type common among the three vaccine trials, were identical among these trials (Table 2). Similarly, antitumor immune response rates were similar for the three vaccine trials (Table 2), suggesting that differences in antigen source and/or vaccine dosing among individual trials did not substantially impact their immunologic efficacy. Moreover, vaccine, vaccine + chemotherapy, and chemotherapy patients exhibited identical recurrence times after initial treatment (Fig. 1*B*), indicating a lack of inherent bias in clinical tumor behavior among all three patient groups before additional treatment. For these reasons, and despite the fact that the trials were not designed to address the issue, we felt that analysis of these patient groups could reveal testable hypotheses pertaining to synergy between vaccination and chemotherapy.

Only the extent of surgical resection and the inclusion of recurrent patients differed significantly between the patient groups. All vaccinated patients received image-complete resections, and both vaccinated patient groups included similar numbers of recurrent patients, whereas a portion of chemotherapy patients received partial resections and, as a group, contained significantly fewer recurrent patients than either vaccine group (Table 1; data not shown). Both of these biases had the potential to produce longer survival in both vaccine groups relative to the chemotherapy group. On the contrary, mean times to progression of chemotherapy patients were similar to those in previous reports (8) and did not differ significantly from those before or immediately after initial treatment in either vaccination group (Table 2; Fig. 1, *B* and *C*). Similarly, overall survival (Table 2; Fig. 2*A*) or survival from therapy initiation for chemotherapy patients was statistically indistinguishable from that of patients receiving vaccine only ($P = 0.66$ for survival from therapy initiation; data not shown). Thus, tumor behavior after initial treatment appeared identical among all three groups, and therapeutic vaccination by itself failed to significantly slow progression or prolong survival relative to conventional GBM chemotherapy (Fig. 2*A*; $P = 0.7$, log-rank test). Exclusion or isolation of recurrent patients did not alter the trend toward increased overall survival in patients receiving post-vaccine chemotherapy but also resulted in too few patients per group to discern any statistical differences (nonrecurrent vaccine + chemotherapy *versus* vaccine, 1046 ± 246 *versus* 529 ± 41 days, respectively, $P = 0.1$; recurrent vaccine + chemotherapy *versus* vaccine, 899 ± 208 *versus* 557 ± 97 days, respectively, $P = 0.2$). Overall survival was also identical between recurrent and nonrecurrent patients pooled from both groups of vaccinated patients ($P = 0.79$; data not shown), inconsistent with a survival bias due to their inclusion.

Despite exhibiting initial progression times that were identical to those of the other two groups, GBM patients receiving post-vaccine chemotherapy enjoyed significantly longer times to tumor progression after chemotherapy relative to previous progression times in the same patients or to comparable progression times in patients receiving vaccination or chemotherapy alone (Fig. 1*B*). This pattern is not normally observed in GBM patients, for whom sequential times to tumor progression typically decrease (27, 28). Overall survival (Table 2; Fig. 2*A*) or survival from treatment initiation (data not shown) for GBM patients receiving chemotherapy after vaccination was also significantly prolonged relative to that of patients receiving either vaccine or chemotherapy alone ($P = 0.025$ and 0.047 for vaccine + chemotherapy relative to vaccine group and for vaccine + chemotherapy relative to vaccine and chemotherapy groups combined, respectively; data not shown). This demon-

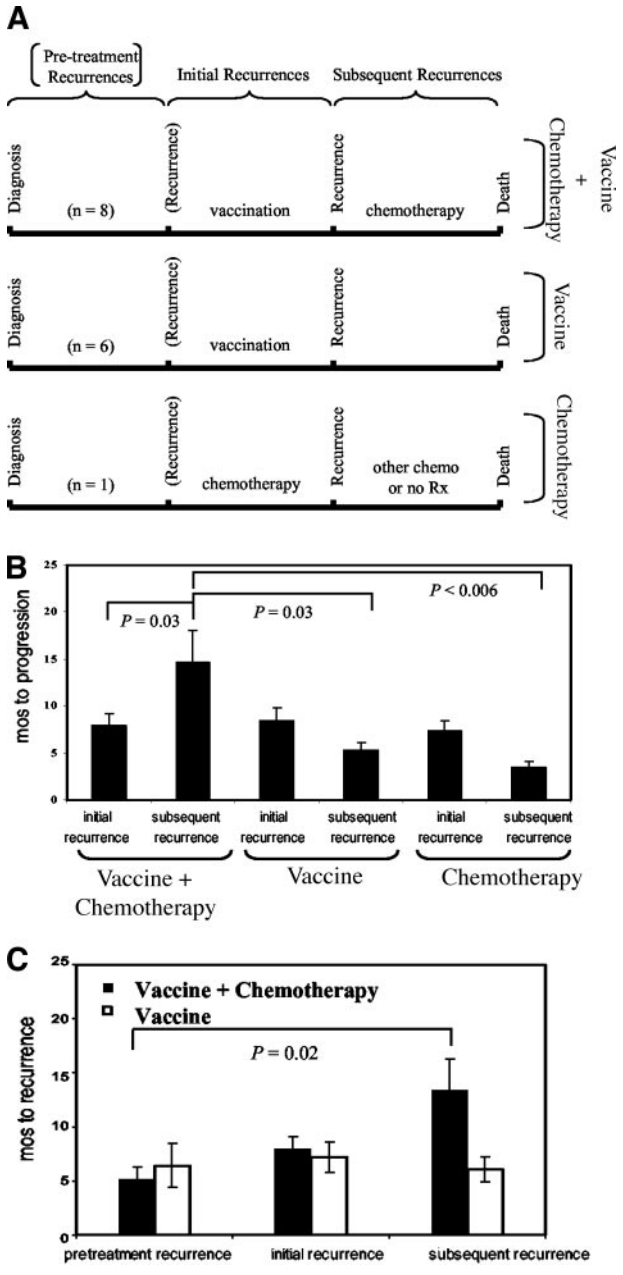

Fig. 1 *A*, tumor progression (recurrence) intervals monitored for each group of GBM patients. Progression times were monitored over intervals before (*Pretreatment Recurrences*) or spanning (*Initial Recurrences*) vaccination or chemotherapy and subsequently thereafter (*Subsequent Recurrences*). All patients ($n = 12$ for vaccine group and $n = 13$ for other groups) experienced initial and subsequent recurrences, whereas only the indicated subpopulations (n) experienced pretreatment recurrences. *B*, initial and subsequent times to tumor progression in vaccine, chemotherapy, and vaccine + chemotherapy groups. Tumor progression was defined as the time from first diagnosis of brain tumor (*de novo* GBM in all cases) to the first new scan enhancement, if verified by subsequent scans or by histology, or time from diagnosis to death due to tumor progression. Initial recurrence times were identical among all three groups ($P > 0.6$). The small difference in subsequent recurrence times between vaccine and chemotherapy groups and the difference between pretreatment and initial recurrence times within the vaccine + chemotherapy group were not statistically significant ($P > 0.07$). *C*, pretreatment and initial recurrence times of recurrent patients in vaccine ($n = 6$) and vaccine + chemotherapy ($n = 8$) groups. Mean times to tumor progression ± SE are shown for each group over specific intervals, as indicated in *B* and *C*. The small difference between pretreatment and initial recurrence times within the vaccine + chemotherapy group was not statistically significant ($P > 0.62$). As for all (recurrent and nonrecurrent) patients as depicted in *B*, the difference in subsequent recurrence times within recurrent patients in the vaccine + chemotherapy group was significant ($P = 0.02$). Significance (P values) was derived from double-sided paired t tests (pretreatment or initial recurrence after vaccine *versus* subsequent recurrence after chemotherapy in vaccine + chemotherapy group) or unpaired double-sided t tests (all other comparisons).

Table 3 Chemotherapy use

Vaccine + chemotherapy	Patient no.	Drug(s)
	1	Gliadel wafers
	2	Temozolamide
	3	Temozolamide
	4	Temozolamide
	5	Temozolamide and irinotecan
	6	Temozolamide
	7	Temozolamide and Accutane
	8	Tamoxifen
	9	Temozolamide and CCNU
	10	Temozolamide and Accutane
	11	Temozolamide and Gleevec
	12	Temozolamide, procarbazine, CCNU, and vincristine
	13	Temozolamide, thalidomide, and etoposide
Chemotherapy		
	1	Temozolamide
	2	Temozolamide
	3	Temozolamide and procarbazine
	4	Temozolamide, carboplatin, vincristine, and procarbazine
	5	Temozolamide, BCNU, and thalidomide
	6	Temozolamide and procarbazine
	7	Temozolamide
	8	Temozolamide and thalidomide
	9	Gliadel wafers, and vincristine
	10	Temozolamide, carboplatin, and vincristine
	11	BCNU and temozolamide
	12	Gliadel wafers and temozolamide
	13	BCNU

NOTE. Temozolomide standard dose is 150–200 mg/m^2 once a day × 5 days every 28 days. BCNU was given 150–200 mg/m^2 intravenously every 6 weeks. Gliadel wafers are a timed-release encapsulation of BCNU.

Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitosourea.

strates that tumors exhibited the same previous clinical behavior regardless of their treatment grouping, inconsistent with the possibility that an inadvertent selection bias resulted in inherently slower progressing tumors in patients receiving chemotherapy after vaccination. In addition, progression times after vaccination were not significantly different from pre-vaccine progression times in recurrent vaccinated patients (Fig. 1*C*). This indicated that increased times to tumor progression were unique to the period of chemotherapy after vaccination and further refuted the possibility of selection bias. The two groups of vaccinated patients were also statistically indistinguishable

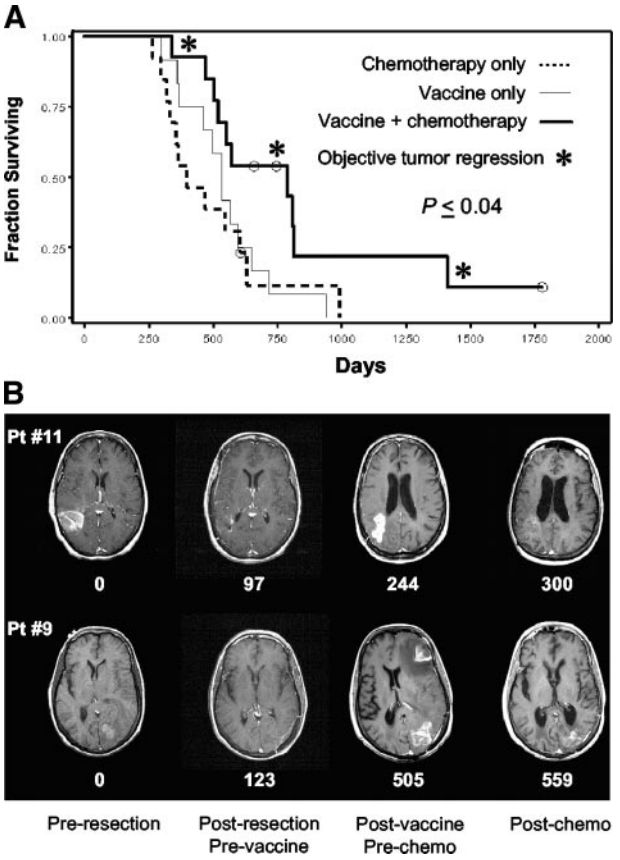

Fig. 2 *A*, overall survival in vaccine, chemotherapy, and vaccine + chemotherapy groups. Overall survival was defined as the time from first diagnosis of brain tumor (*de novo* GBM in all cases) to death due to tumor progression. Kaplan-Meier survival plots with censored values in *open circles* are shown for each group. *Dashed line*, chemotherapy group; *thin solid line*, vaccine group; *thick solid line*, vaccine + chemotherapy group. Survival of the vaccine group was identical to that of chemotherapy group ($P = 0.7$, log-rank test). Survival of vaccine + chemotherapy group was significantly greater relative to survival in the other two groups together ($P = 0.048$, log-rank test), greater than survival in the chemotherapy group alone ($P = 0.028$, log-rank test), and greater than survival in the vaccine group alone ($P = 0.048$, log-rank test). Two of the three patients exhibiting objective tumor regression survived for >2 years (730 days) after diagnosis. *B*, tumor regression following post-vaccine chemotherapy. Relative days after diagnosis are represented by the *numbers* under individual MRI scans, with individual patient scans in each row. Patient 11 recurred 82 days after vaccine initiation; patient 9 recurred 147 days after vaccine initiation, was treated surgically, and recurred 227 additional days (374 days total) after vaccine initiation. An additional patient (patient 10), the longest survivor of those exhibiting objective tumor regression, suffered tumor recurrence 35 days after vaccine initiation and was treated with subsequent chemotherapy, which was followed by objective tumor regression. A complete array of images was not available for this individual, however. All scans except the pre-resection scan for patient 2 were performed after contrast enhancement with gadolinium. Patient numbers can be cross-referenced to Table 3.

with respect to all other treatment variables, including number of craniotomies, proportion of recurrent patients, radiation, SRS, and chemotherapy before vaccination, and they exhibited similar KPS values after vaccine therapy (Table 1).

Importantly for this rapidly fatal disease, 2-, 3-, and 4-year survival were also unique for patients receiving chemotherapy after vaccination. Whereas chemotherapy or vaccination alone resulted in a 2-year survival within the established range for GBM (8%; Table 1), postvaccination chemotherapy resulted in a substantial increase in 2-year survivors (42%; Table 1; $P < 0.05$, binomial distribution). Similarly, no 3- or 4-year survivors were evident after chemotherapy or vaccination alone, but such survivors persisted among post-vaccine chemotherapy patients (Table 1; $P < 0.01$ for 3-year survivors, binomial distribution).

Finally, objective (>50%) regression of tumor mass was observed in 3 of 13 vaccine + chemotherapy patients, and this occurred only after initiation of post-vaccine chemotherapy (Fig. 2, *A* and *B*). A similar regression was also observed in a single grade III malignant glioma patient receiving chemotherapy after vaccination (data not shown). Such dramatic regression of *de novo* GBM was unique to this group and is extremely uncommon in the literature, although a single example of partial GBM regression after post-vaccine chemotherapy has recently been reported (29). In that report, imaging studies suggested either rapid tumor recurrence after vaccination or vaccine-mediated effects (such as inflammation). In this context, it is significant that tumor recurrence in all vaccinated patients in the current study was determined by increased tumor imaging on MRI scans. These increases were probably not related to vaccine-induced inflammation because 33% (4 of 12) of vaccine patients and 46% (6 of 13) of vaccine + chemotherapy patients were biopsied on observation of post-vaccine increases in tumor imaging, and all exhibited histologically verified recurrent tumor (data not shown). This suggests that apparent increases in tumor imaging in our study were not due to vaccine-induced inflammatory responses and instead generally reflected *bona fide* tumor recurrence. This suggests that the specific therapeutic regimen of chemotherapy after vaccination, rather than vaccination alone, elicited tumor regression. In any case, this first demonstration of multiple objective (>50%) regressions of GBM in any adoptive immunotherapy setting, as well as in the treatment of GBM generally, is significant.

The above-mentioned results allowed us to hypothesize that antitumor immunity directly impacts GBM chemosensitivity. Although data refuting inadvertent selection bias were evident, independent means of testing this hypothesis were sought. It was particularly important to rule out that the distinct clinical outcomes in vaccine only *versus* vaccine + chemotherapy groups were not due to differential induction of antitumor immunity, despite our previous demonstration of efficient antitumor CTL response induction using similar vaccine methodology (13).

We initially examined either CTL or type I cytokine (*i.e.*, IFN-γ) responsiveness after vaccination, depending on the particular trial in which patients were enrolled (Table 2). The incidence of responders of either type (CTL+) was distributed equally both within individual vaccine clinical trials (Table 2) and within vaccine and vaccine + chemotherapy groups (Fig. 3*A*; 4 of 11 and 4 of 13 patients tested, respectively; $P = 0.5$, binomial distribution). Vaccine patients combined from both vaccine and vaccine + chemotherapy groups who exhibited enhanced responses to tumor cells or lysate after vaccination exhibited a nonsignificant tendency toward increased overall

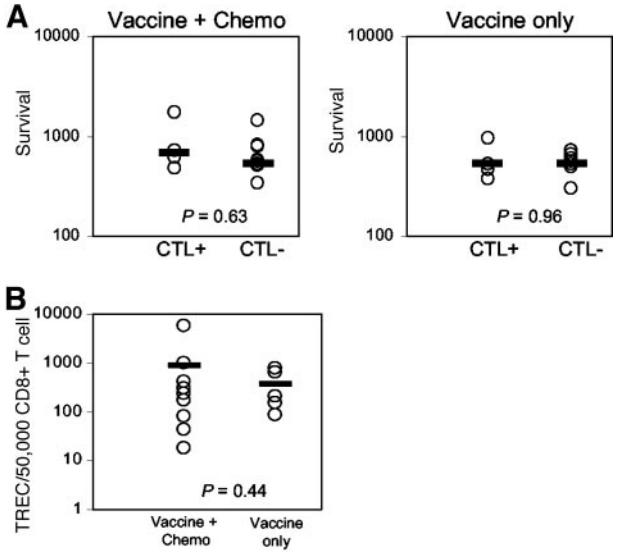

Fig. 3 *A*, CTL or type I cytokine vaccine responders are equally distributed among vaccine and vaccine + chemotherapy groups. *A*, all vaccine responders were separated from nonresponders and plotted against patient survival, which failed to distinguish them. Responders were also equally distributed among vaccine (4 of 12; *right panel*) or vaccine + chemotherapy groups (4 of 13; *left panel*; $P = 0.45$, binomial distribution) and failed to distinguish survival times within these groups (the indicated probabilities were derived from two-sided *t* tests). *B*, TRECs quantified within 50,000 purified $CD8^+$ T cells from PBMCs collected at the time of surgery were determined and plotted for five vaccine and nine vaccine + chemotherapy patients. Purified T cells were unavailable for TREC assay in all other vaccinated patients. $CD8^+$ TREC ranges were statistically indistinguishable between vaccine and vaccine + chemotherapy patients (indicated probabilities were derived from two-sided *t* tests).

survival (Fig. 4*A*; $P = 0.23$, log-rank test), which was somewhat more pronounced when survival from vaccine initiation was examined ($P = 0.08$; data not shown). Similarly, increased overall survival of CTL-responsive patients receiving vaccine + chemotherapy relative to CTL nonresponders receiving vaccine alone approached but did not achieve statistical significance (Fig. 4*A*; $P = 0.05$, log-rank test). Nevertheless, survival time from vaccine initiation of CTL responders in the vaccine + chemotherapy group was significantly higher than that of CTL nonresponders in the vaccine group ($P = 0.022$, log-rank test; data not shown). All other treatment/responder subgroups exhibited less suggestive survival differences (Fig. 4*A*; $P > 0.06$, log-rank test). The average age of CTL responders between groups was also similar (55 ± 6 and 50 ± 17 years for vaccine and vaccine + chemotherapy groups, respectively; $P = 0.63$, two-tailed *t* test). Thus, although we cannot rule out a partial influence of vaccine responders on the most objective measure of GBM outcome (overall survival), neither distinct frequencies nor ages of vaccine responders alone could account for the increased overall survival observed between vaccine and vaccine + chemotherapy groups.

The inability of immunologic vaccine responders to significantly predict clinical outcome in cancer is common in vaccine trials (14, 15, 30, 31) and could stem from the likelihood that conventional CTL and cytokine response assays may not directly measure activities of the most clinically relevant immune effectors. In this context, it is particularly notable that levels of $CD8^+$ RTE T cells measured by TREC analysis (26, 32, 33), which allow accurate prediction of clinical outcome in vaccinated and unvaccinated GBM patients (34), largely account for age-dependent GBM clinical outcome and dominantly influence vaccine responses in GBM patients (34). Moreover, most resting cells capable of binding to any of several tumor-associated antigens in GBM patients were shown to be $CD8^+$ RTEs, and these cells dominated *in vivo* responses to such antigens after therapeutic vaccination (34). We therefore examined whether differential $CD8^+$ TREC levels, as markers for what are likely the most clinically relevant immune effectors in GBM, accounted for overall survival differences between vaccine and vaccine + chemotherapy groups.

Distribution of patients with $CD8^+$ TRECs above the median value (216 molecules/50,000 $CD8^+$ T cells) was equivalent between vaccine and vaccine + chemotherapy groups (Fig. 3*B*; two of five and five of nine patients analyzed, respectively; $P = 0.25$, binomial distribution). Similarly, $CD8^+$ TREC values did not differ between these groups (Fig. 3*B*; $P = 0.44$, two-tailed *t* test). Patients combined from both vaccine and vaccine + chemotherapy groups (14 patients for whom $CD8^+$ TREC data were available) who exhibited high $CD8^+$ TREC levels also exhibited overall survival similar to patients with low $CD8^+$ TREC levels (Fig. 4*B*; $P = 0.79$, log-rank test). Similarly, high $CD8^+$ TRECs failed to predict longer overall survival within the vaccine + chemotherapy group (Fig. 4*B*; $P = 0.36$, log-rank test). High $CD8^+$ TRECs also failed to predict longer survival from vaccination in general or within the vaccine + chemotherapy group ($P = 0.41$ and 0.58, respectively; data not shown). Similar analysis could not be performed for the vaccine only group due to a more limited set of obtainable $CD8^+$ TREC data. Thus, by these analyses, there was no evidence of skewing of patients with high $CD8^+$ TREC levels and no evidence that antitumor activity mediated by $CD8^+$ RTEs or other immune effectors was responsible for longer survival in patients receiving vaccine + chemotherapy.

Although segregation of patients based on either vaccine responsiveness or median $CD8^+$ TREC values failed to reveal statistically different overall survival times, CTL responders receiving post-vaccine chemotherapy did exhibit longer survival from vaccination than nonresponders without subsequent chemotherapy ($P = 0.022$; data not shown), and both IFN-γ response magnitude and $CD8^+$ TRECs exhibited significant correlations with overall survival times from the combined vaccination group patients ($r = 0.77$ and $P < 0.05$ and $r = 0.936$ and $P < 0.01$, respectively, Pearson's correlation; data not shown). Thus, although these aspects of cellular immunity could not account for significant differences in overall survival between the vaccine and vaccine + chemotherapy groups, the involvement of immune processes in postvaccination chemotherapy responses could not be altogether discounted. This suggested that more focused analysis of patients receiving post-vaccine chemotherapy might reveal an association between immune and chemotherapeutic responsiveness.

Because times to recurrence were increased specifically after chemotherapy in vaccine + chemotherapy patients, we considered the increase in time to recurrence relative to previous

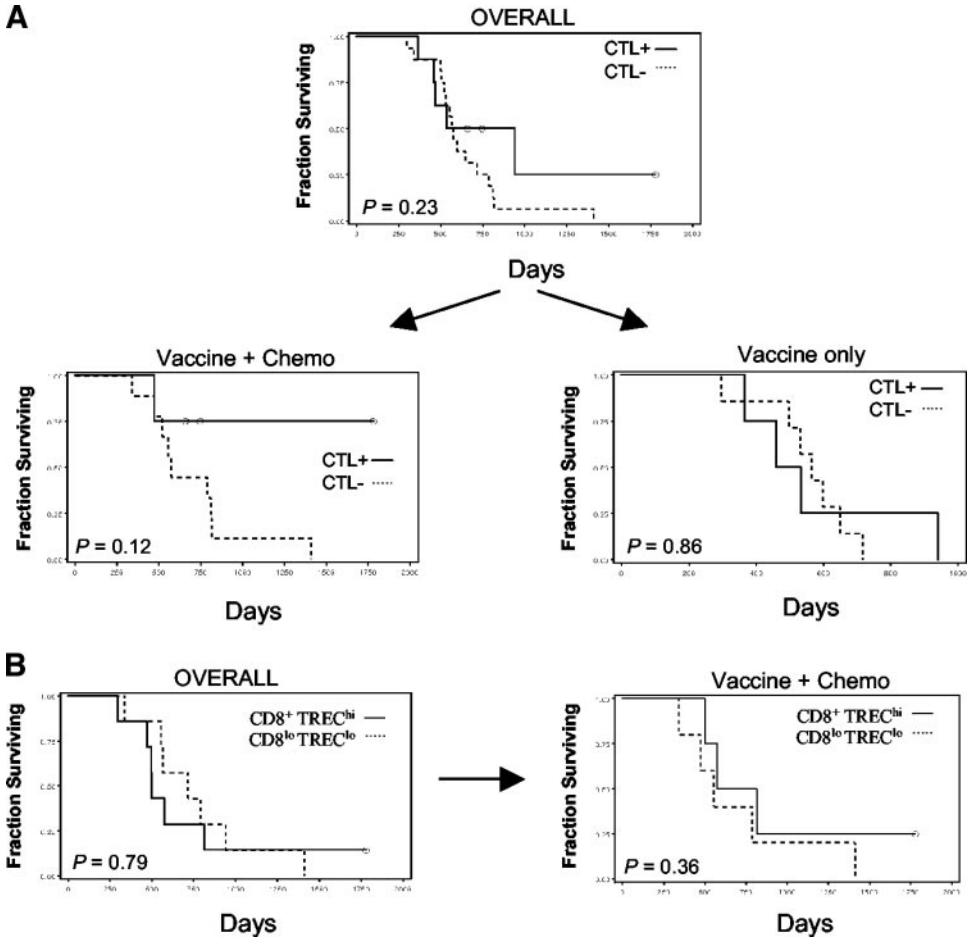

Fig. 4 *A*, vaccine responsiveness fails to account for different survival between vaccine and vaccine + chemotherapy groups. CTL or type I cytokine vaccine responders were separated from nonresponders, and Kaplan-Meier plots for patient survival were generated. Responders failed to exhibit distinct survival in general (*top panel*) or within either the vaccine (*bottom right panel*) or vaccine + chemotherapy group (*bottom left panel*). *B*, all vaccinated patients were separated based on the median TREC level quantified within 50,000 purified $CD8^+$ T cells from PBMCs collected at the time of surgery (216 molecules/50,000 $CD8^+$ T cells). Patients with high $CD8^+$ TRECs failed to exhibit distinct survival in general (*depicted panels*) or within vaccine + chemotherapy groups (data not shown; $P = 0.9$, log-rank). Similar analysis of patients receiving vaccine only was not meaningful due to insufficient subgroup sizes ($n = 3$ and $n = 2$ for low and high $CD8^+$ TREC levels, respectively). Purified T cells were unavailable for TREC assay in all other vaccinated patients. All indicated probabilities were derived from log-rank analysis.

recurrence time (*i.e.*, after vaccination) in the same patient as a quantitative indicator of chemotherapeutic responsiveness. This parameter was also normalized with respect to prior tumor behavior in the same patient, obviating the need to compare only *de novo* GBM patients and justifying expanding the analysis to all GBM patients receiving post-vaccine chemotherapy. We thus compared chemotherapeutic responses to quantifiable aspects of immune responsiveness (IFN-γ response magnitude and $CD8^+$ TREC levels) and to a strong established prognostic factor for GBM, patient age. This analysis was performed in all patients from whom these data were available.

In all GBM patients receiving post-vaccine chemotherapy, patient age correlated inversely with chemotherapeutic responsiveness ($r = -0.56$; $P = 0.04$; Fig. 5*A*), confirming that such responsiveness was age dependent and that this population adhered to established clinical trends (9). IFN-γ response magnitude, on the other hand, failed to correlate with chemotherapeutic responsivenss ($r = 0.44$; $P > 0.05$; Fig. 5*B*), whereas $CD8^+$ TRECs correlated extremely well with GBM chemotherapeutic responsiveness ($r = 0.96$; $P < 0.003$; Fig. 5*C*). In addition, higher $CD8^+$ TREC levels, but neither age nor CTL responsiveness, predicted significantly greater chemotherapeutic responses (Fig. 5, *D*–*F*; average 12.58 ± 5.63 month increase *versus* 1.42 ± 1.75 month decrease with $CD8^+$ TRECs below median; $P = 0.005$, log-rank). This relationship was not simply a function of an independent influence of age on thymic $CD8^+$ RTE production because $CD8^+$ TRECs correlated more strongly to chemotherapeutic responsiveness than did patient age (Fig. 5, *A* and *C*) and uniquely predicted increased times to progression (Fig. 5*D*), consistent with previous studies (34).

Taken together, these findings suggest that GBM tumors are recognized and acted on *in situ* by cellular immune components. Overall, such activity may result in a fundamental alteration of GBM tumors that renders them increasingly sensitive to DNA-altering chemotherapy, despite the inability of vaccination by itself to confer overt clinical benefits to patients.

DISCUSSION

Although originating from distinct clinical studies not designed to address synergy between vaccination and chemotherapy, several criteria help validate comparison among the three patient treatment groups and, in particular, between the two vaccine patient groups analyzed here. First, the overwhelming prognostic factors for glioma clinical outcome are tumor type, tumor grade, and patient age. All three groups were composed only of patients with identical types and grades of tumor (grade IV astrocytoma = GBM) and exhibited statistically identical

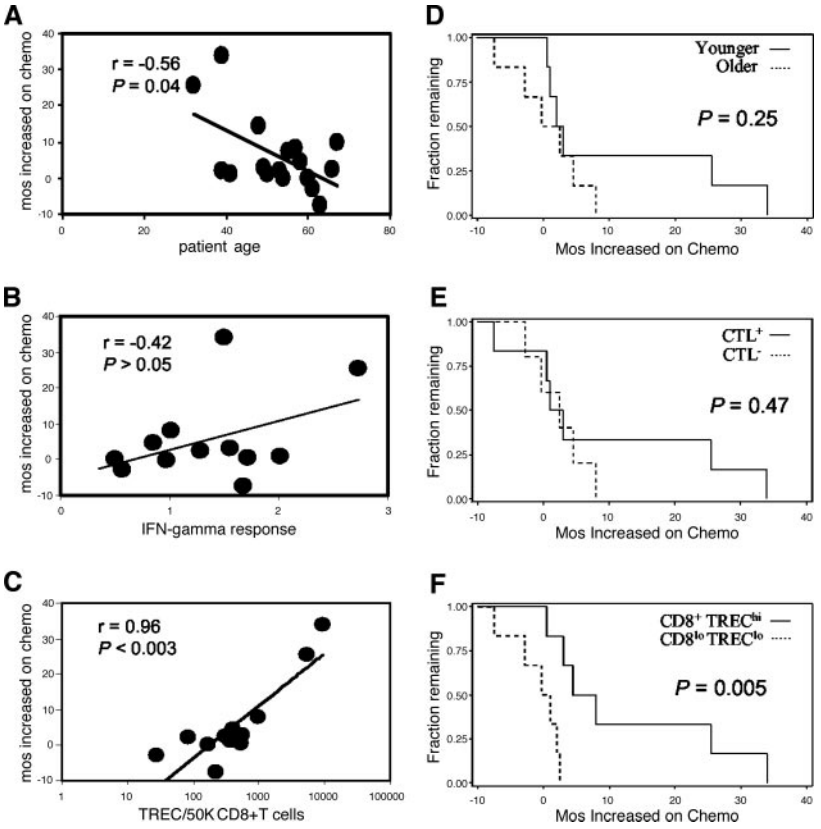

Fig. 5 $CD8^+$ TRECs are strongly associated with chemotherapeutic responses after vaccination. Patient age (*A* and *D*), type I cytokine (IFN-γ) response magnitude (*B* and *E*), or TRECs quantified within 50,000 purified $CD8^+$ T cells from PBMCs collected at the time of surgery (*C* and *F*) were correlated with the increase in time to tumor progression (time to recurrence after chemotherapy minus time to recurrence after vaccination in the same patient; *A*−*C*). Median values for these same three variables were used to subdivide the patient population, and Kaplan-Meier survival analyses were conducted (*D*−*F*). Data were derived from all vaccinated GBM patients (*de novo* and secondary GBM) for whom chemotherapeutic response and age ($n = 17$), IFN-γ response magnitude ($n = 12$), or TREC results ($n = 12$) were available. A related variable, time to tumor progression after chemotherapy divided by time to tumor progression after vaccination, also correlated significantly with $CD8^+$ TRECs ($r = 0.80$; $P < 0.01$) and patient age ($r = -0.67$; $P < 0.05$) but failed to do so with IFN-γ response magnitude ($r = -0.40$; $P > 0.05$).

age ranges. In addition, a less robust and more subjective prognostic factor, KPS, which was not followed rigorously in chemotherapy patients, was not statistically different between vaccine and vaccine + chemotherapy patients after vaccination. This suggests that, among vaccinated patients, there was no bias in a prognostic factor that was obtained near the time of segregation into vaccine only or vaccine + chemotherapy subgroups. Finally, and perhaps most convincingly, all three groups exhibited identical tumor recurrence times after initial therapy (vaccination or chemotherapy), suggesting that tumors from patients in any one group behaved clinically like those from any other before further treatment. Thus, no prognostically or empirically defined bias in patient composition or tumor behavior among the three treatment groups was evident before secondary chemotherapy.

However, a potential bias between chemotherapy and all vaccinated patients was possible due to a portion of chemotherapy patients experiencing partial surgical resection of their tumors, whereas all patients receiving vaccinations had image-complete resections. In addition, the chemotherapy only group included significantly fewer recurrent patients than either of the vaccine groups, which included similar proportions of recurrent patients. Although the clinical benefit of complete surgical resection has not been scientifically addressed by design, it is thought to provide a statistically significant survival increase (35). This increase is minimal, however, and has only been evident from meta-analyses of hundreds of patients in any study. We therefore do not expect a proportion of incompletely resected patients within an already limited population to account for the relatively large difference in survival between chemotherapy and vaccine + chemotherapy patients. This expectation is supported by the fact that survival was statistically identical between chemotherapy and vaccine (without subsequent chemotherapy) patient groups, the latter of which, like the vaccine + chemotherapy group, was composed entirely of patients with image-complete tumor resections.

Both recurrent and nonrecurrent GBM patients are grouped together in most of our analyses. Because overall survival from initial diagnosis of *de novo* GBM, rather than survival from treatment initiation, was examined, this grouping results primarily in treatment timing differences between recurrent and nonrecurrent patients. Arguments against a bias due to treatment timing differences, as well as sensitization to vaccination by prior treatment, include the fact that each vaccine group contained statistically similar proportions of recurrent patients (Table 1) and that recurrent patients exhibited identical overall survival relative to nonrecurrent patients pooled from both vaccinated patient groups (see Patients and Clinical Variables in "Materials and Methods," and "Results"). The fact that increased survival or times to recurrence were observed and that this was confined to only one of the vaccine groups also refute that recurrent patient inclusion meaningfully affected the clinical outcome data. Thus, the only obvious potential biases in patient composition among the three treatment groups are statistically unlikely and directly refuted by empirical clinical data, although we cannot formally exclude this possibility in a com-

bined, nonrandomized study of so few patients. We nonetheless felt justified in comparing tumor progression and overall survival among three treatment groups of *de novo* GBM patients with identical prognostic and pretreatment factors.

Vaccinated patients receiving subsequent chemotherapy exhibited significantly delayed tumor progression and longer survival relative to those receiving vaccinations without subsequent chemotherapy or those receiving chemotherapy alone. Improved clinical outcome appeared dependent on the specific combination of therapeutic vaccination followed by chemotherapy. These observations suggest a substantial therapeutic slowing of GBM progression and extension of overall survival for GBM patients. It is impossible to exclude the possibility that vaccination and chemotherapy simply had additive, rather than synergistic, effects. It is notable, however, that incrementally favorable clinical outcomes were not evident with vaccination alone, and those seen following post-vaccine chemotherapy appeared to markedly surpass those in previous vaccine studies as well as those in even the most hopeful analyses of GBM chemotherapy, including multiple regimens in predominantly younger, potentially more chemosensitive patients (8). We therefore suspect that vaccination does not simply mimic a second regimen of chemotherapy but instead contributes unique benefits to the patient that are not evident with either vaccination in the absence of subsequent chemotherapy or chemotherapy alone. As such, this allows us to hypothesize that this specific treatment combination conferred significantly increased survival to a population of treated cancer patients, a unique outcome for a vaccine-based therapy. A controlled prospective analysis is thus warranted to rigorously test the prediction that combinatorial immune/chemotherapy is superior to either vaccine therapy or standard chemotherapy alone and represents the best available treatment in a larger population of GBM patients.

Both clinical outcome and chemotherapeutic responsiveness are known to be age-dependent processes in gliomas in general (3, 9, 36). Age-dependent glioma clinical outcome is critically impacted by the production of $CD8^+$ T cells in the thymus in mice, and a directly related parameter, TREC concentration within $CD8^+$ T cells, largely accounts for age-dependent prognosis in GBM patients (34). This raises the possibility of a similar immune impact on additional age-dependent properties of GBM, such as responsiveness to chemotherapy. In support of this, we observed a stronger correlation between $CD8^+$ TRECs and chemotherapeutic responsiveness than between age and chemotherapeutic responsiveness, and $CD8^+$ TREC levels predicted a significant increase in such responsiveness. This close relationship suggested that clinical responsiveness to chemotherapy is similarly impacted by the production and/or function of newly emigrated $CD8^+$ T cells. Because levels of such T cells were shown to predominantly mediate antitumor immune responsiveness after vaccination of GBM patients (34), this constitutes evidence favoring the hypothesis that antitumor immunity impacts GBM chemosensitivity.

Thus, a cellular immune variable related to effector activity appears not only to account for the strongest prognostic factor in GBM (age; Ref. 34) but also to be closely linked to chemotherapeutic responsiveness of these tumors. Because age is the single most dominant factor influencing the outcome of most human tumors, it will be additionally important to determine whether cellular immune processes similarly influence clinical outcome and chemotherapeutic efficacy in distinct human tumors. If so, the clinical expectations associated with immune-based cancer therapies would be substantially broadened.

It has been unclear whether the failure of therapeutic cancer vaccines for most patients stems from the prevention of vaccine-elicited CTL responsiveness to tumor cells or from the inability of responding CTLs to counteract net tumor expansion (17). In light of this, it is tempting to speculate that vaccination induces systemic activation of antitumor CTLs that then infiltrate and attempt to kill tumors. Although such attempts by tumor-reactive CTLs alone may fail to prevent tumor regrowth, immune effector activity related to $CD8^+$ RTEs may nonetheless constrain recurrent tumors by presensitizing them to alternative, drug-inducible apoptotic pathways. In fact, *in vitro* studies have documented that sublethal death receptor signaling of the type elicited by CTLs can enhance the sensitivity of human tumor cells to multiple anticancer drugs (18). Thus, the vaccine protocols used here appeared to elicit immune effector responses that were linked to fundamental alterations in tumors (manifested as sensitivity to chemotherapy), but these responses by themselves were insufficient to counteract net tumor expansion. Although the basis of this insufficiency remains unknown, these findings justify closer examination of the role of immune effectors after therapeutic cancer vaccination, as well as assessment of the molecular consequences of sublethal and lethal immune effector activity on tumors.

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