Vaccination Elicits Correlated Immune and Clinical Responses in Glioblastoma Multiforme Patients

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Abstract

Cancer vaccine trials have failed to yield robust immune-correlated clinical improvements as observed in animal models, fueling controversy over the utility of human cancer vaccines. Therapeutic vaccination represents an intriguing additional therapy for glioblastoma multiforme (GBM; grade 4 glioma), which has a dismal prognosis and treatment response, but only early phase I vaccine trial results have been reported. Immune and clinical responses from a phase II GBM vaccine trial are reported here. IFN-γ responsiveness was quantified in peripheral blood of 32 GBM patients given therapeutic dendritic cell vaccines. Posttreatment times to tumor progression (TTP) and survival (TTS) were compared in vaccine responders and nonresponders and were correlated with immune response magnitudes. GBM patients (53%) exhibited ≥1.5-fold vaccine-enhanced cytokine responses. Endogenous antitumor responses of similar magnitude occurred in 22% of GBM patients before vaccination. Vaccine responders exhibited significantly longer TTS and TTP relative to nonresponders. Immune enhancement in vaccine responders correlated logarithmically with TTS and TTP spanning postvaccine chemotherapy, but not with initial TTP spanning vaccination alone. This is the first report of a progressive correlation between cancer clinical outcome and T-cell responsiveness after therapeutic vaccination in humans and the first tracing of such correlation to therapeutically exploitable tumor alteration. As such, our findings offer unique opportunities to identify cellular and molecular components of clinically meaningful antitumor immunity in humans. [Cancer Res 2008;68(14):5955–64]

Introduction

Glioblastoma multiforme (GBM) is a uniformly fatal brain tumor responsible for 50% of all primary brain tumors and 21% of all central nervous system tumors in adults (1, 2). GBM patients survive 12 to 18 months, without the possibility of remission or effective treatment (1–3). Survival represents the most objective standard for evaluating GBM therapy, in part because surgical tumor mass reduction does not necessarily correlate with prolonged survival (4–6). Surgical resection followed by radiation and temozolamide chemotherapy (7, 8) remains the most effective established GBM treatment, providing a 15-month median survival, with <28% of patients surviving 2 years. Thus, additional GBM therapies are needed.

Cancer vaccines represent promising additional GBM therapies. Vaccination with cytokine-transfected tumor cells, adoptive transfer of tumor-activated T cells, and administration of antigen-pulsed dendritic cell (DC) vaccines have all been associated with enhanced immunity and/or favorable clinical outcomes (9–18). Tumor antigen-bearing DC vaccines, most commonly composed of autologous DC pulsed with synthetic peptides for non–central nervous system (CNS) tumors, have elicited antitumor CD8 T-cell responses in most subjects, including GBM patients (12, 15, 19–21). Some progress has been made in the identification of GBM antigenic peptides and CTL epitopes (22–24). Nevertheless, vaccinating with undefined tumor cell lysates or mRNA obviates the need for antigen identification while reducing risk of immune evasion due to singe-antigen loss (25).

Initial reports using DC vaccines in GBM patients indicated no serious adverse effects and no evidence of autoimmunity, except that a single patient repeatedly developed peritumoral edema (9–18). Some trials also noted favorable clinical outcomes, including prolonged time to tumor progression (TTP) and/or time to survival (TTS), and radiological evidence of tumor regression. Intriguingly, post-hoc analysis also suggested that GBM chemotherapy might synergize with prior DC vaccination (26).

Relationships between immunosuppressive cytokine (transforming growth factor-β, TGF-β) and shorter survival and between antitumor responsiveness after vaccination and longer survival were reported in two recent DC vaccine trials for GBM (17, 18). Nevertheless, similar relationships were also evident between TGF-β or postvaccine outcomes and prevaccine metrics in these studies, clouding their relevance to vaccination per se. Moreover, previous GBM patient numbers were insufficient to show either definitive benefits from single DC vaccine trials, progressive correlations between immune and clinical responses, or both (9–18). Thus, the variables governing clinical responsiveness after DC vaccination in GBM, as in other cancer patients, remain elusive.

Here, we report immune and clinical outcomes of 34 GBM patients enrolled in a phase II trial, in which autologous tumor lysate-pulsed DCs were given to participants. Cytokine (IFN-γ) responsiveness was assessed after DC/lysate-stimulation of prevaccine and postvaccine peripheral blood mononuclear cells (PBMC) of patients using a qPCR-based method, as previously described (12, 15). Although uncommon in non-CNS cancer vaccine trials, this assay has been used in multiple glioma vaccine trials (12, 15), wherein response metrics strongly correlate with *in vivo* postvaccine responses by a predominant tumor antigen–reactive population of nascent $CD103^+CD8^+$ T cells (27). All GBM vaccine responders previously identified by this assay also exhibited postvaccine expansion of tetramer-reactive $CD8^+$ T cells (22–24), providing qualitative validation in relevant patients.

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Table 1. GBM patient demographic variables

Age (y)	Gender	ICR	Alive	Post-vac Rx	TTS	IFN-γ
59	M	No	No	CPT-11	132	NA
56	M	No	No	—	335	NA
57	F	No	No	—	138	*0.46*
28	F	No	No	CPL/AC, TMX, CCNU	409	0.34
55	M	Yes	No	Gliadel	512	0.48
54	M	Yes	No	—	552	0.5
24	M	Yes	No	TMZ, CCNU, CPT-11	669	0.54
53	M	Yes	No	TMZ, PCV	193	0.6
50	M	Yes	No	TMZ, VP16, THL, CBRX	448	0.64
60	F	No	No	TMZ, GLV	335	0.8
22	M	Yes	Yes	IRESSA/RAPA	1216	0.85
42	M	No	No	TMZ	520	0.81
58	M	Yes	No	—	728	0.85
53	M	Yes	No	TMZ, PCV	375	0.97
47	M	Yes	No	—	189	1.2
58	M	No	No	—	414	1.38
74	M	Yes	No	—	235	1.49
66	M	Yes	No	TMZ	423	1.66
63	M	Yes	No	TMZ, GLV, CIV	464	1.67
37	M	Yes	No	—	227	1.73
42	M	Yes	No	TMZ	330	2.02
39	M	Yes	No	TMZ, AC	662	2.1
59	F	Yes	No	—	455	2.23
55	M	Yes	No	TMZ	341	4.31
64	M	Yes	No	TMZ	942	4.6
65	F	Yes	Yes	IRESSA/RAPA	994	5.2
44	F	Yes	Yes	—	910	5.3
52	M	No	No	TMZ	777	5.95
67	F	Yes	No	TMZ, CCNU	784	7.1
68	F	Yes	No	—	438	9.85
40	M	Yes	No	TMZ, GEF/RAPA	643	13.45
44	M	Yes	Yes	TRCVA	1148	17.1
61	F	No	No	—	664	50
48	F	Yes	Yes	TMZ	1192	51.32

Abbreviations: *M,* male; *F,* female; *ICR,* image-complete surgical resection before vaccination; *TTS,* immediate postvaccine TTS from prevaccine surgery; *IFN-γ,* post–third vaccine enhancement of IFN-γ production (italicized value is post–second vaccine from a patient who expired before the third vaccine); *CPL,* carboplatin; *AC,* accutaner; *TMX,* tamoxifen; *TMZ,* temozolamide; *THL,* thalidomide; *CBRX,* Celebrexr; *GLV,* Gleevecr; *RAPA,* rapamycin; *CIV,* continuous infusion of vincristine; *GEF,* gefitinib; *TRCVA,* Tarcevar (erlotinib).

Response quantification using this assay, in conjunction with safety and outcome analysis, suggest that DC vaccination is safe and may improve GBM clinical and/or treatment responses in proportion to single immune variables.

Materials and Methods

Patients and clinical variables. Informed consent was obtained from all patients, and all procedures were conducted after approval by a local Human Investigations Committee and in accord with an assurance filed with and approved by the Department of Health and Human Services. Forty-four patients were enrolled (13 women, 31 men; ages, 22–74 y), of whom 34 were diagnosed with GBM (10 women, 24 men; ages, 22–74 y; Table 1). The primary safety end point was the number of grade 3 or grade 4 toxicities and was evaluated in all enrolled, including non-GBM, patients.

Primary efficacy end points were TTS and TTP. TTS was evaluated from the date of surgery immediately preceding vaccination to the date of death or last contact (if still living). TTP was evaluated from the same initial surgery date used for TTS to date of progression on magnetic resonance imaging (MRI; ~25% increase in tumor volume), provided progression was verified either histologically or in serial MRI scans (including routine FLAIR, gadolinium-enhanced, and perfusion-weighed MRIs). All diagnostic pathology and scans were subjected to central tumor board review and consensus. Postvaccine immune responsiveness was the primary immunologic end point. Primary efficacy and immunologic end points are presented here only for GBM patients.

GBM patients consisted of 23 recurrent and 11 newly diagnosed, of whom 32 (21 recurrent and 11 newly diagnosed) were evaluable immunologically. Vaccinated patients were corticosteroid-free during all blood collections and vaccinations, as previously described (12). Each of four vaccines consisted of 900 μg autologous tumor lysate/10 to 40 × 10^6 autologous DC. Vaccination started ~15 wk postsurgery. This interval is distinct from "time to treatment" of 971 or 288 d for responders and nonresponders (Table 2), which instead reflects the time from first brain tumor diagnosis to the prevaccine surgery. Time to treatment was distinct, but insignificant between responders and nonresponders due to two responders with extreme disease duration, who did not contribute disproportionately to TTS (227 and 1,192 d) and/or TTP (149 and 109 d).

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Serial MRI scans were performed every 2 to 3 mo, continuously monitored until late 2007. Tumor lysates were derived from a single surgical tumor resection immediately preceding vaccination. Inclusion criteria are as follows: verified histologic diagnosis of malignant brain tumor (inclusion criteria of ≥30 GBM; actual enrolled GBM patient number = 34), Karnofsky score ≥60, age ≥18 y, lowest possible glucocorticoid dose, normal baseline hematology within 1 wk before first vaccination, hemoglobin >9.9 g/dL, total granulocytes >1,000/μL, platelets >60,000/μL, BUN <30 mg/dL, creatinine <2 mg/dL, alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase <2× upper normal limit. Prothrombin and activated partial thromboplastin times were ≤1.4 × control, unless therapeutically warranted. Tumor lysate >1 mg was required to proceed with vaccine manufacturing. Patients were required to practice medically accepted birth control during study. Exclusion criteria are as follows: pregnancy, severe pulmonary, cardiac or other systemic disease associated with an unacceptable operative risk, active treatment of acute infection, history of autoimmune disorder, and inability to give informed consent.

Preliminary safety and chemosensitivity data were reported on the first 12 patients enrolled in this trial in a previous study of patients from multiple vaccine trials (26). Additional safety information and further chemosensitivity data are included here, as they relate specifically to patient safety/serious adverse events (SAE) within this trial, substantiate the previous post-hoc findings in a single uniform DC vaccine trial, and contribute information relevant to the mechanism of the immune/clinical correlation exclusive to this trial.

Preparation of tumor lysate. Surgical tumor specimens were washed 3× in sterile PBS, minced, and passed through 0.38-mm and 0.14-mm pore metal meshes, then through 0.21-mm pore nylon mesh as described (15). Tumor cells were lysed by four freeze-thaw cycles (liquid nitrogen and room temperature). Larger particles were removed by centrifugation (1,000 × *g*, 10 min, 4°C) after verification of universal cell death by Trypan Blue staining under light microscopy. Supernatants were passed through a 0.2-μm filter and concentration determined by Bio-Rad protein assay. Lysate was frozen at −80°C until use.

Table 2. Pretreatment variables and clinical outcomes in responder and nonresponder GBM patients

	Vaccine responders (*n* = 17; 9 recurrent)	Vaccine nonresponders (*n* = 15; 12 recurrent)	Significance
Average age	54 ± 3	49 ± 4	0.151*
Karnofsky score	81 ± 2	84 ± 3	0.210*
Avg time to treatment	971 ± 462	288 ± 46	0.182 †
TTP—recurrent pre-vac (control)	258 ± 102	275 ± 55	0.817 †
TTP—all post-vac	308 ± 55	167 ± 22	0.015 †
TTP—recurrent post-vac	260 ± 85	146 ± 23	0.142 †
TTS—all	642 ± 61	430 ± 50	0.041 †
TTS—recurrent	599 ± 75	401 ± 53	0.067 †
% Image-complete resection	15/17 (88%)	10/15 (67%)	0.148 ‡
% Newly diagnosed	8/17 (47%)	3/15 (20%)	0.064 ‡
% 1st recurrence	7/17 (41%)	9/15 (60%)	0.240 ‡
% 2nd recurrence	0/27 (0%)	3/15 (17%)	0.092 ‡
% ≥3rd recurrence	2/17 (12%)	0/15 (0%)	0.305 ‡
% Prior chemoRx	8/17 (47%)	10/15 (67%)	0.225 ‡
% Postvaccine chemoRx	12/17 (71%)	9/15 (60%)	0.398 ‡
% OR	3/17 (18%)	1/15 (7%)	0.350 ‡
% 1-y survival	14/17 (82%)	10/15 (67%)	0.207 ‡
% 2-y survival	7/17 (41%)	1/15 (7%)	0.030 ‡
% Recurrent 2-y survival	5/9 (56%)	1/12 (8%)	0.029 ‡

NOTE: Vaccine responders exhibited at least 2 SDs above mean prevaccine IFN-γ production after the third vaccination. Calculations of percentage of 1-y and 2-y survival include censored values.

Abbreviations: *Avg time to treatment,* time from histologically confirmed diagnosis of brain tumor until time of prevaccine surgery; *TTP—pre-vac,* prevaccine TTP from GBM diagnosis or last recurrence before prevaccine surgery until time of prevaccine surgery; *TTP—all post-vac* and *TTP—recurrent post-vac,* immediate postvaccine TTP (the same calculation as TTP in Materials and Methods) for all GBM and all recurrent GBM patients, respectively; *TTS—all* and *TTS—recurrent,* immediate postvaccine TTS from prevaccine surgery for all GBM and for all recurrent GBM patients, respectively; *% OR,* proportion of patients exhibiting >50% reduction in tumor volume (objective response) by MRI after vaccination, such reductions were limited to the post-vaccine chemotherapy interval exclusively; *% 2-y survival* and *% Recurrent 2-y survival,* percentage surviving ≥730 d for all GBM patients and for all recurrent GBM patients, respectively (TTP was defined as the time from the day of surgery immediately preceding vaccination therapy to the first new scan enhancement, if verified by subsequent scans or by histology, or time from diagnosis to death due to tumor progression).

Higher response thresholds rendered TTS and TTP progressively higher in all responder relative to nonresponder groups without affecting other prognostic factors. Such thresholds could be defined by median post vaccine IFN-γ enhancement (overall TTS, 656 ± 64 and 429 ± 46 d, $P = 0.024$; overall TTP, 322 ± 56 and 162 ± 22 d, $P = 0.005$ for $n = 16$ responders and $n = 16$ nonresponders; recurrent TTS, 621 ± 81 and 402 ± 49 d, $P = 0.041$; recurrent TTP, 282 ± 94 and 142 ± 22 d, $P = 0.065$ for $n = 8$ responders and $n = 13$ nonresponders, respectively), and 2 SDs above baseline mean IFN-γ production (overall TTS, 729 ± 61 and 415 ± 41 d, $P = 0.002$; overall TTP, 360 ± 64 and 161 ± 20 d, $P = 0.001$ for $n = 13$ responders and $n = 19$ nonresponders; recurrent TTS, 736 ± 29 and 386 ± 44 d, $P = 0.005$; recurrent TTP, 343 ± 116 and 136 ± 19 d, $P = 0.014$ for $n = 8$ responders and $n = 15$ nonresponders, respectively). This trend was evident with progressively higher response thresholds up until approximately the highest quartile (i.e., $n = 8$ responders and $n = 24$ nonresponders; data not shown).

* One-sided *t* test.

† Log-rank test.

‡ Fisher's exact test.

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Preparation of autologous DC. PBMCs were isolated by leukapheresis using a continuous-flow blood cell separator according to manufacturer's instructions (COBE Spectra, Gambro BCT; mononuclear cell collection SOP #306575391), processing 10 L of blood and yielding $\sim 10^{10}$ PBMC. PBMCs were adhered to tissue culture plastic for 2 h, 37°C at 5×10^6 cells/mL in RPMI 1640 + 10% heat inactivated autologous serum (RPMI-10/AS), nonadherent cells were removed, and remaining cells were cultured in RPMI-10/AS containing recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF; 800 units/mL; Immunex) and recombinant human interleukin 4 (IL-4; 500 units/mL; R&D Systems) for 6 d. Autologous DC were not cryopreserved before use, and leukapheresis was repeated for each of the four vaccines.

Pulsing of autologous DC with tumor lysate and vaccine administration. The day before each vaccination (after 6 d in culture), DC cultures containing 1 to 4×10^7 cells were resuspended in 6 mL RPMI-10/AS containing 900 μg autologous tumor lysate, incubated, and rotated 18 h at 37°C. Patients received 1 to 4×10^7 tumor lysate-pulsed DCs s.c. in 1 mL PBS in the deltoid region. All cultured DC exhibited a $CD14^-$, $HLA\text{-}DR^{hi}$, $CD80/86^+$ phenotype and 30 of 30 tested contained <25% (most <10%) $CD83^{hi}$ cells, indicating predominance of immature DC. Release criteria were >70% $CD14^-$, $HLA\text{-}DR^{hi}$ cells, at least 1×10^7 total cells, negative environmental culture, Gram negative test, negative *Mycoplasma* test, and endotoxin of <5 EU/kg. Sample sterility was tested in process by an environmental (sterility) culture and endotoxin test of tumor lysate and DC cultures. DC cultures were tested for sterility 2 d after their initiation, and sterility cultures incubated 5 d in testing laboratory facility, with final results obtained on vaccination day (7 d after DC culture initiation). Gram test, endotoxin, and *Mycoplasma* test were assayed on final vaccine product, which was released only if all three tests were negative. Vaccine of at least 1×10^7 lysate-pulsed viable DCs was prepared in 1 mL sterile PBS, drawn into a tuberculin syringe labeled with two patient identifiers, and delivered directly to administering physician. Three vaccinations at 2-wk intervals were given to each patient, with a fourth vaccination 6 wk after the third.

***In vitro* sensitization and qPCR assessment of cytokine responsiveness.** Autologous DC were prepared by incubating loosely adherent PBMC in RPMI-10/AS, 500 units/mL IL-4, 800 units/mL GM-CSF, 8 d, 37°C, 5% CO_2. 2×10^6 DC/mL were pulsed with 150 μg/mL autologous tumor lysate for 18 h and then irradiated. Autologous prevaccine and postvaccine PBMC (1×10^6 cells/mL) were stimulated in RPMI-10/AS with 1×10^6 irradiated lysate-pulsed DC/mL, with IL-2 (300 IU/mL) added on day 2, and 2 h restimulation with 150 μg/mL tumor lysate on day 11. RNA was isolated using Trizol (Invitrogen) and transcribed using random hexamers and cDNAs were amplified as previously described (28, 29). A ≥1.5-fold increase in reference gene-normalized IFN-γ production (independent normalization performed ± tumor lysate pulsing) after vaccination indicated a positive response, as previously reported (29): IFN-γ primers 5′-AGCTCTGCATCGTTTTGGGTT-3′ (forward), 5′-GTTCCATTATCCGCTACATCTGAA-3′ (reverse), 5′-FAM-TCTTGGCTGTTACTGCCAGGACCCA-TAMRA-3′ (probe); reference (CD8) primers 5′-CCCTGAGCAACTCCATCATGT-3′ (forward), 5′-GTGGGCTTCGCTGGCA-3′ (reverse), 5′-FAM-TCAGCCACTTCGTGCCGGTCTTC-3′ (probe). Amplification variables are as follows: 25 μL, 10 mmol/L deoxynucleotide triphosphate, 400 nmol/L primers, 200 nmol/L TaqMan probe, and 0.5 units platinum Taq polymerase, at 95°C 5 min, 95°C 30 s, 60°C 30 s for 45 cycles, detected on an iCycler (Bio-Rad). Standard curve extrapolation for copy number was performed for both IFN-γ and reference (CD8/β-actin). PCR coefficient of determination (R^2) was 0.99 or higher for all reported values. IFN-γ transcript copies were divided by reference gene copies for normalization.

This assay first quantifies cytokine production in the presence versus absence of lysate, then after versus before vaccination, thereby excluding non–antigen-driven responses. The assay does not exclude the contribution of any particular antigen-responsive cell population, including NKT cells. The use of CD8 as a reference gene was solely to normalize for varying cell levels in response cultures rather than to implicate particular responding subpopulations.

Delayed-type hypersensitivity testing. Single-cell suspensions of autologous tumor cells were prepared from resection samples as described (12, 15), and 2 to 4×10^6 autologous tumor cells from cultures were harvested, washed 3×, and resuspended at 10^7 cells/mL in sterile PBS, with media retained for sterility testing. Cells were irradiated (100 Gy; 10,000 rad) and given at 10^7/mL in sterile PBS. Environmental culture and endotoxin tests were performed on culture media, and Gram stain was performed on tumor cell product just before use. Release criteria were negative environmental culture (incubated 4 d in testing laboratory) and Gram stain, and endotoxin at <5 EU/kg. Delayed-type hypersensitivity (DTH) was given only to the first eight GBM patients vaccinated on the day of vaccination and 2 wk after the third vaccination and read and/or biopsied 7 and 14 d after administration. Robust DTH responses were not observed in any of the eight patients receiving them, despite histologic biopsy examination. DTH lysate testing was not performed due to similarly negative results in patients from previous vaccine trials.

Statistical analysis. Continuous variables were compared using Student's t test and categorical variables compared using Fisher's exact test. Probability of survival was determined with SAS software by the Kaplan Meier method, using two-tailed Mann-Whitney log-rank test exclusively to compare study and control groups. Pearson's correlation coefficients (r values) were calculated in Excel software. Where indicated, values ± SE are included. P values of <0.05 were considered significant.

Results

Safety of autologous DC administration. Forty-four patients, including 10 non-GBM patients, were evaluated for primary safety end point. No grade 3 or grade 4 National Cancer Institute common toxicity criteria adverse effects associated with vaccination were observed. A single patient surviving 784 days experienced a cutaneous glioblastoma with single lymph node involvement and at the site of irradiated tumor cell inoculation for DTH testing. This tumor was judged likely to have derived from the DTH test rather from metastasis of the original brain tumor. The irradiated DTH product did not result in growth of cultured glioma cells after 14 days. Whereas it is possible that the irradiation dose was generally inadequate to kill glioma cells, an audit suggested that tumor cells from this subject may have been more resistant to radiation than those typically observed in GBM, noting that, "while it is also possible that the radiation given for this type of human brain cancer is inadequate to kill all the cells, the occurrence of this SAE in only 1 of the 56 subjects enrolled in this and similar protocols would tend to refute that possibility" (note: at time of audit, 56 patients had been given irradiated autologous tumor DTH upon enrollment in phase I or phase II DC vaccine trials at the authors' institution). Nevertheless, to rigorously refute inadequate irradiation in this patient, 2×10^6 of their irradiated tumor cells were again cultured, resulting in no tumor growth after 14 days but detectable tumor growth in one of three triplicate cultures after 49 days. The SAE was therefore deemed the likely consequence of outgrowth of rare, potentially radiation-resistant tumor cells present in this particular patient. As a precaution against future SAEs, DTH was subsequently discontinued. The patient's two peripheral tumors were removed surgically ~1 year before postvaccine chemotherapy and did not recur. Adverse events and SAEs in all other patients were associated with tumor progression and/or symptoms thereof.

Development of systemic type I cytokine responses. GBM patients are known to exhibit deficits in TCR/CD3 signaling that functionally compromise T-cell responsiveness (30–34), but these deficits are not restored by stimulation through either CD3 or phorbol 12-myristate 13-acetate (PMA)/ionomycin (33). Similar T-cell signaling deficits in cancer patients can, however, be reversed by vaccination (35, 36). This suggests that

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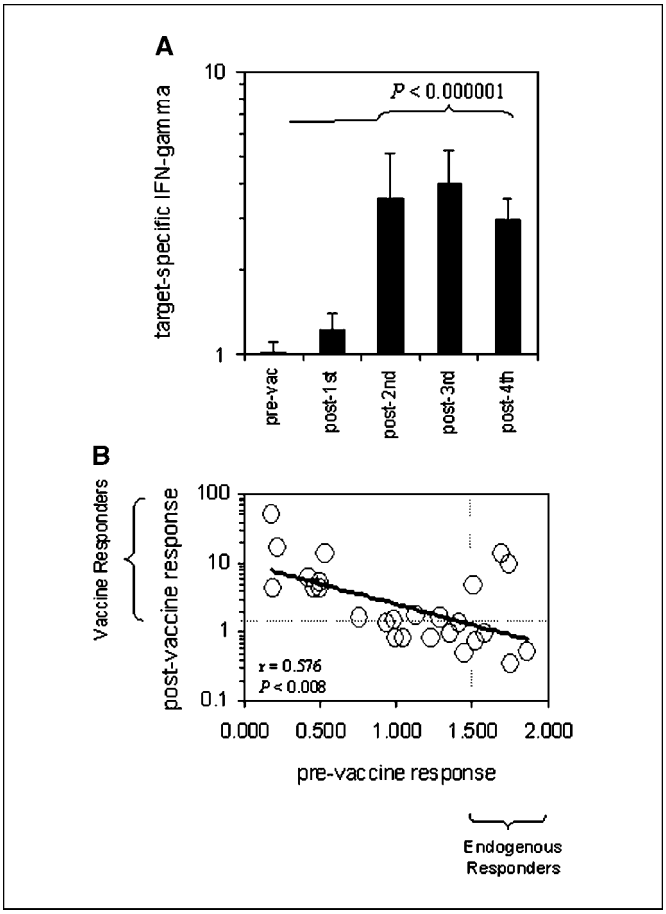

Figure 1. Type I cytokine responses before and after DC vaccination. *A,* IFN-γ production was quantified according to Materials and Methods 7 d before vaccination (*pre-vac*), 1 wk after the first (*post-1st*) and second (*post-2nd*) vaccines, 5 wk after the third vaccine (*post-3rd*), and 1 wk after the fourth (*post-4th*) vaccine and plotted as mean IFN-γ in presence of autologous tumor lysate over IFN-γ without lysate ± SE for all GBM patients tested. Statistical evaluation was performed relative to pre-vac IFN-γ using ANOVA with the indicated *P* values (post–second and post–third values were also significantly higher by *t* test). Values shown were normalized to reference gene (β-actin and CD8) transcripts but were not normalized to prevaccine IFN-γ levels (n = 26 and included 14 responders and 12 nonresponders). *B,* post–third vaccine IFN-γ enhancement (post–third vaccine presence/absence of tumor lysate divided by prevaccine presence/absence of tumor lysate) was plotted against prevaccine IFN-γ production, and Pearson's correlation coefficient (r) was determined with indicated significance (P). The baseline for prevaccine calculations is IFN-γ production in the absence of tumor lysate with ≥1.5-fold increased production before vaccination constituting an endogenous antigen-directed response. Patients exhibiting high post–third vaccine IFN-γ enhancement with the lowest prevaccine IFN-γ production (<0.8) also exhibited high (>2.0) post–third vaccine IFN-γ production (post–third vaccine presence/absence of tumor lysate).

nonspecifically stimulated responses are not necessarily independent of either immune suppression or vaccine induction in cancer patients and, therefore, may represent poor baselines over which to detect either of these phenomena. For this reason, nonspecific stimuli, such as CD3 or PMA/ionomycin stimulation, were not used as controls, and incubation with DC ± lysate, which independently controls for nonspecific effects of DC expressing irrelevant antigens, was deemed most appropriate.

PBMCs from patients were collected 7 days before vaccine administration on the day of initial leukapheresis (prevaccine time point), 7 days after each of the first two vaccinations (first and second postvaccine time points), 5 weeks after the third vaccination (third postvaccine time point), and 1 week after the fourth vaccination (fourth postvaccine time point).

Using a qPCR-based assay, antigen-directed IFN-γ production was found to progressively increase after vaccination in GBM patients, reaching statistical significance after two vaccinations and maximal levels after three vaccinations (Fig. 1*A*). A >1.5-fold enhancement relative to prevaccine IFN-γ production detected using similar qPCR assays has been reported as evidence of a positive vaccine response in cancer patients (28, 29). By these criteria, 17 of 34 GBM patients exhibited a positive vaccine response after three vaccinations (vaccine responders), 14 showed no such responsiveness, and 3 could not be tested. Of the three untested after three vaccines, one expired before receiving the third vaccine, but exhibited a marginal 1.5-fold enhancement after the first vaccine and a reduced (0.46-fold) response after the second vaccine (italicized value in Table 1). This patient is included as a nonresponder (n = 15).

Seven of 32 tested patients (22%) exhibited >1.5-fold increased IFN-γ production in the presence of antigen before vaccination (Fig. 1*B*), consistent with endogenous antitumor responses. In addition, five patients (16%) exhibited ≥1.5-fold decreased IFN-γ production to lysate-pulsed relative to unpulsed DC, consistent with antigen-directed response suppression. Nevertheless, these five patients did not reveal consistently worse clinical outcomes than other nonresponders (417 versus 502 days TTS; 174 versus 151 days TTP; both $P > 0.05$, log-rank test).

There was no age bias in the frequency of endogenous responses, but their magnitudes were significantly higher in younger (<53 years) GBM patients (1.27 ± 0.45-fold increase with lysate in younger versus 1.02 ± 0.11-fold in older; $P < 0.005$, ANOVA) and also correlated inversely with age in all combined GBM patients ($r = -0.46$; $P < 0.01$). Patients with ≥1.5-fold increased IFN-γ production before vaccination were not, however, more likely to exhibit ≥1.5-fold enhanced IFN-γ production after vaccination (20% and 25% of nonresponders and responders, respectively; $P = 0.594$, Fisher's exact test). In fact, endogenous response magnitudes correlated inversely with postvaccine responses (Fig. 1*B*). It is unlikely that this inverse correlation was an artifact of the postvaccine response calculation being based on baseline IFN-γ production because (*a*) >85% of patients exhibiting post–third vaccine enhancement also exhibited >1.5-fold increased IFN-γ production without baseline normalization (i.e., ±lysate; not shown) and (*b*) endogenous responses also correlated inversely with prevaccine disease duration ($r = -0.647$, $P = 0.02$). Analysis of independent clinical variables, such as tumor load, could further substantiate this, but these data raise the possibility that prior immune exposure of tumors may preclude subsequent immune responses to them. Indeed, data from experimental glioma models suggest that T-cell activity initially slows glioma progression but subsequently renders them increasingly aggressive *in vivo* (not shown).

Survival and TTP after DC vaccination. Prognostic factors for GBM, including patient age, Karnofsky performance status, and prior treatment duration and history, were all statistically identical between responders and nonresponders (Table 2). Nevertheless, there tended to be more newly diagnosed patients within vaccine responders (Table 2), prompting verification of clinical trends (TTS and TTP) in recurrent patients separately, as outlined in Fig. 2*A*.

TTS in vaccine responders was 642 ± 61 days, significantly longer than 430 ± 50 days in nonresponders (Table 2; Fig. 2*B*),

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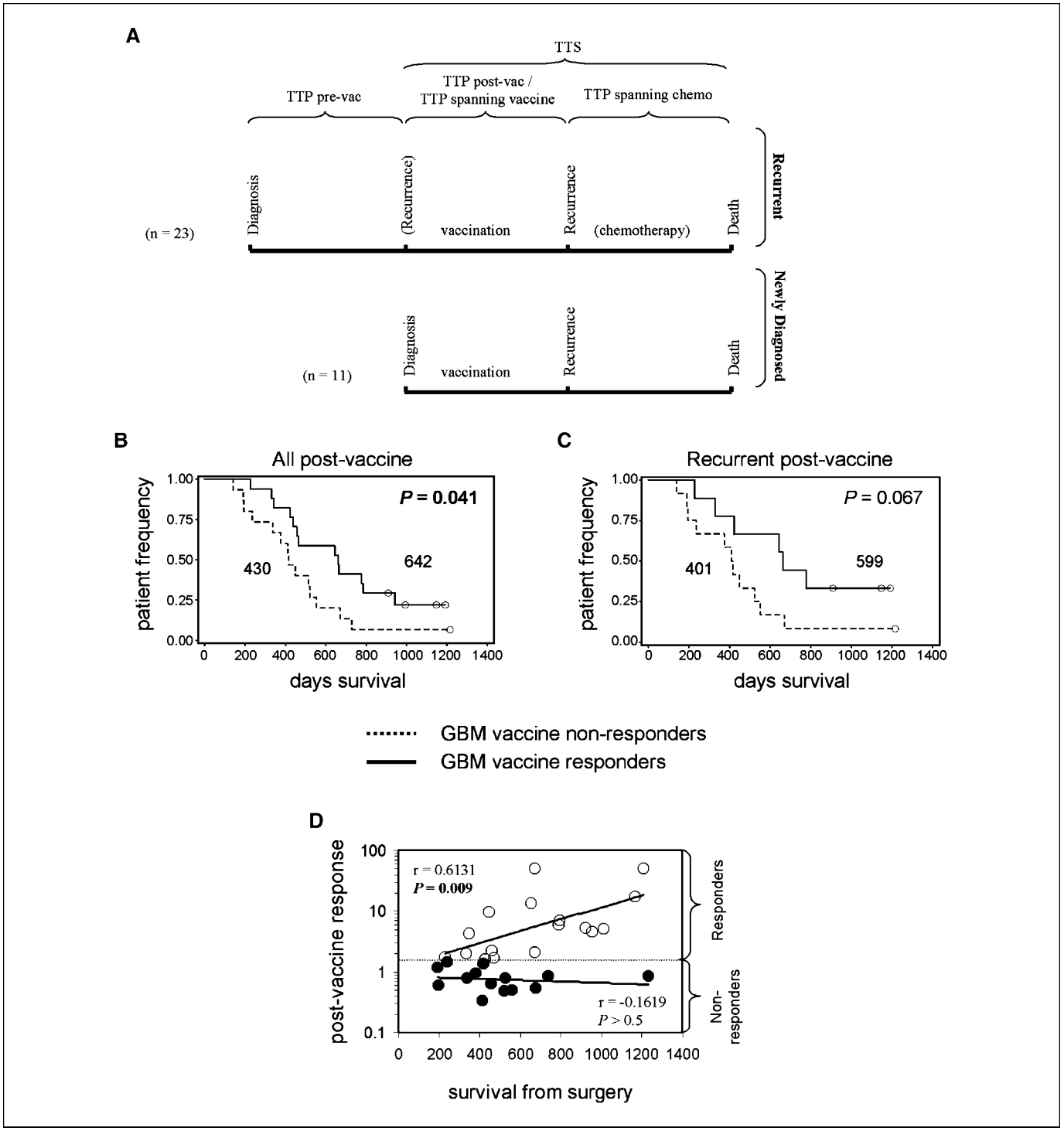

Figure 2. TTS in vaccinated patients. *A,* pretreatment and posttreatment clinical metrics for newly diagnosed and recurrent GBM patients. Previous diagnosis of brain tumor with current GBM diagnosis defined recurrent patients, for whom prevaccine disease duration was assessed from the date of first brain tumor diagnosis to the date of surgery immediately preceding vaccination. TTS was defined in all patients from the date of surgery immediately preceding vaccination to date of death or last contact (if still living). For all new or recurrent GBM diagnoses, the date of surgery immediately preceding vaccination was considered the date of recurrence/diagnosis (day 0 for TTP calculations) due to the fact that a definitive histologic diagnosis was obtained only upon examination of surgical specimens at this time. TTP was defined on the basis of tumor recurrence on MRI (~25% or greater increase in volume), verified by sustained recurrence on serial MRI and/or histologic confirmation by a neuropathologist. Intervals spanning treatments were assessed from date of initial recurrence before either vaccination or chemotherapy to the date of the next sequential recurrence or to time of death, as depicted. All immunologically evaluated vaccinated GBM patients ($n = 32$; *B*) or immunologically evaluated recurrent GBM patients only ($n = 21$; *C*) were separated into two groups based on enhanced IFN-γ production (after third vaccine), and Kaplan-Meier analysis was performed for TTS (time from surgical resection immediately preceding vaccination to time of death). Vaccine responders (exhibiting high IFN-γ enhancement ≥1.5-fold above the mean prevaccine IFN-γ production level) are depicted by solid line, nonresponders (exhibiting low IFN-γ enhancement) by dashed line, with censored values in open circles, and mean survival times included next to each survival curve. Significance (*P*) was determined by log-rank test. *D,* IFN-γ enhancement levels for vaccine responders (*open circles*) and nonresponders (*closed circles*) were plotted against TTS. Exponential trendlines were generated, Pearson's correlation coefficients (*r*) determined, and significance (*P*) assessed for each group separately, as shown.

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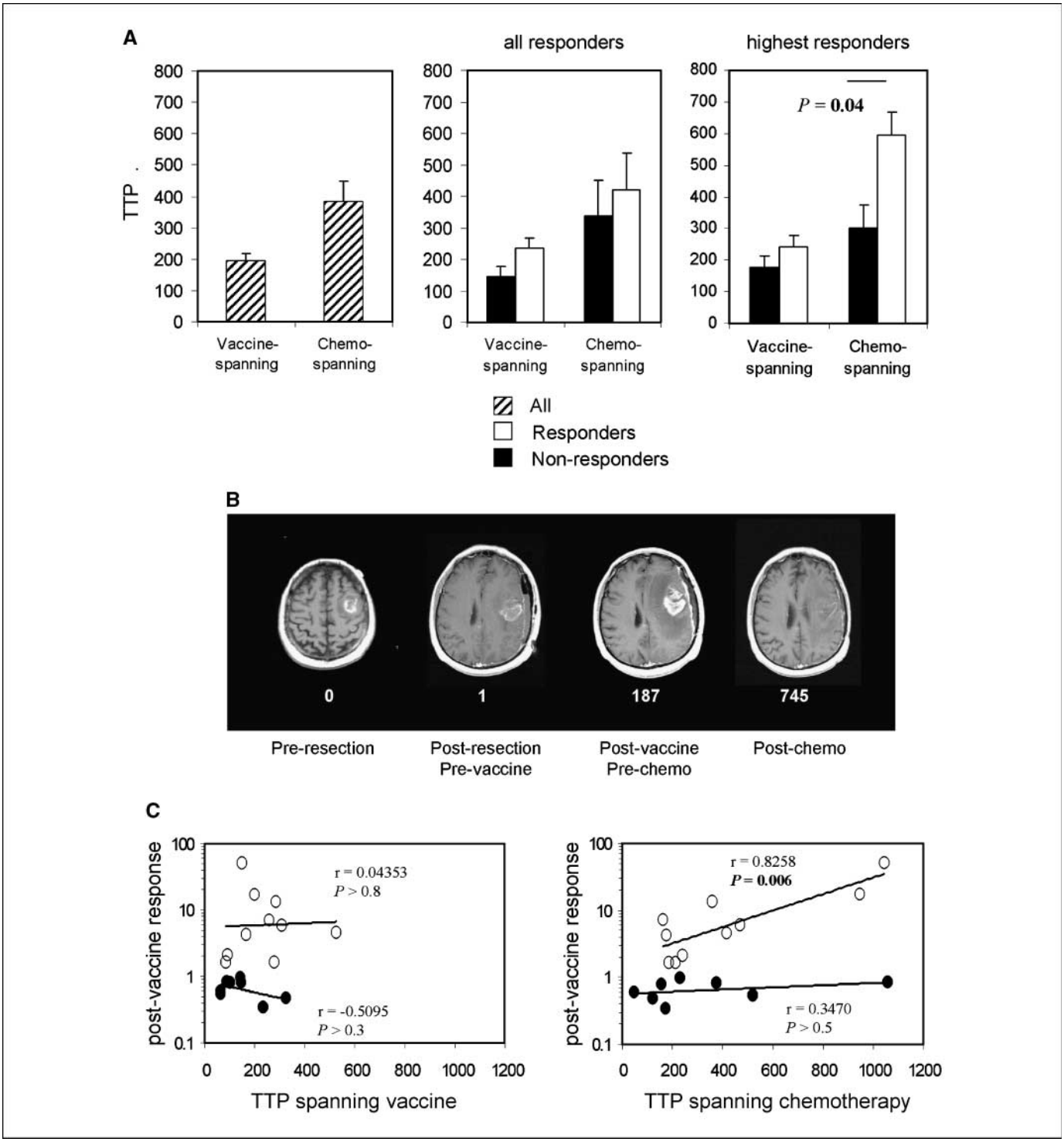

Figure 3. *A,* progression times were monitored over intervals spanning vaccination (*vaccine-spanning,* 195 ± 24) or postvaccine chemotherapy (*chemospanning,* 383 ± 64) for all patients considered together (*left*), all vaccine responders and nonresponders (*middle*; mean vaccine spanning TTP, 261 ± 46 d for $n = 11$ responders and 146 ± 32 d for $n = 8$ nonresponders; $P = 0.072$), and highest vaccine responders (*top quartile*) versus all other responders and nonresponders (*right*; mean vaccine-spanning TTP, 252 ± 27 d for $n = 6$ responders versus 178 ± 37 d for $n = 13$ others, $P = 0.116$; mean chemotherapy-spanning TTP, 400 ± 57 d in these responders versus 260 ± 42 d in all others, $P = 0.048$). Patients analyzed in this manner ($n = 18$) experienced recurrences separating vaccination and postvaccine chemotherapy. Significance (P) was assessed by log-rank. A similar trend was evident when patients were separated by 2 SDs above mean prevaccine IFN-γ production, provided endogenous responders were first excluded from the analysis: mean vaccine spanning TTP was 232 ± 29 d in these six responders versus 162 ± 32 d in all eight others ($P = 0.163$), whereas mean chemotherapy spanning TTP was 383 ± 63 d in these responders relative to 218 ± 36 d in others ($P = 0.057$). *B,* tumor regression after postvaccine chemotherapy. Relative days after diagnosis are represented by numbers under individual MRI scans. All scans were performed postcontrast enhancement with gadolinium. Similar regressions from two additional patients enrolled in this phase II trial have been reported elsewhere (26). *C,* progression times as in *A* were monitored and plotted against IFN-γ enhancement magnitudes for vaccine responders ($n = 10$; *open circles*) and nonresponders ($n = 8$; *closed circles*) experiencing recurrences separating vaccination and postvaccine chemotherapy. Exponential trendlines were generated, Pearson's correlation coefficients (r) were determined, and significance (P) was assessed for each group separately, as shown.

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representing an increase of ~7 months. TTP was 308 ± 55 days in vaccine responders, significantly longer than 167 ± 22 days in nonresponders, representing an increase of over 4.5 months (Table 2). TTS and TTP in all vaccinated GBM patients also compared favorably to comparable patients treated with non-vaccine therapies at our institution over the span of the trial (see TTP spanning chemotherapy below). Similarly, TTS and TTP were 599 ± 75 and 260 ± 85 days, respectively, in recurrent GBM vaccine responders compared with 401 ± 53 and 146 ± 23 days for TTS and TTP, respectively, in recurrent nonresponders (Table 2; Fig. 2*C*). Although trends were not significant in recurrent patients, their separation according to higher response levels rendered TTS and TTP progressively higher in all responder groups, including recurrent patients (Table 2, *legend*). In addition, 41% of statistical vaccine responders survived at least 2 years, significantly higher than 7% of nonresponders, and these percentages, which closely parallel analogous metrics in post-hoc analyses (26), were statistically identical in recurrent GBM patients (Table 2). Thus, vaccine responders exhibited more favorable clinical outcomes relative to nonresponders, and this seemed dependent on response levels.

Correlation of vaccine-elicited responses and posttreatment survival. TTS correlated logarithmically with postvaccine IFN-γ response magnitudes exclusively in responders (Fig. 2*D*). This degree of correlation required >11 vaccine responders for statistical detection, substantially exceeding responder numbers in previous GBM vaccine trials and explaining its novel detection here. This is consistent with the notion that vaccine-elicited type I cytokine responses by T cells may proportionally improve GBM survival.

TTP after postvaccine chemotherapy. To follow up initial reports of synergy between DC vaccination and subsequent chemotherapy (26), we examined TTP spanning vaccination alone (the same variable as postvaccine TTP) and spanning postvaccine chemotherapy intervals in 19 GBM patients with recurrences separating these two events (8 responders, 11 nonresponders). A 188-day increase in mean TTP was observed during the postvaccine chemotherapy interval relative to the vaccine interval in these 19 patients (Fig. 3*A*). In addition, at least three patients (one nonresponder, two responders) exhibited complete regressions exclusively within the postvaccine chemotherapy interval (Fig. 3*B*; Table 2), with a fourth responder exhibiting regression over an interval spanning both vaccination and chemotherapy. The increase, however, was of equal magnitude in responders and nonresponders.

Given the direct correlation between vaccine and clinical responses (TTS), we considered whether higher vaccine responders experienced prolonged TTP spanning vaccination and/or chemotherapy. Intriguingly, and although objective vaccine responders and nonresponders each exhibited a 186-day to 190-day increase in TTP spanning chemotherapy (Fig. 3*A*), a significant differential, exclusively evident within the interval spanning postvaccine chemotherapy, was observed between responders exhibiting 5-fold or greater postvaccine IFN-γ enhancement (i.e., top quartile responders) and all others. A similar trend was evident when patients were separated based on lower responses, provided endogenous responders were first excluded (Fig. 3, *legend*).

Direct correlation between vaccine responses and clinical outcomes is not generally evident in non-CNS cancer vaccine trials (20, 37–42). We therefore considered whether vaccination might provide immune-correlated clinical benefits by increasing efficacy of glioma-specific treatments as a by-product of immunoediting (43). We therefore examined the relationship of vaccine responses to TTP spanning vaccination alone or to TTP spanning postvaccine chemotherapy. As in other cancer vaccine trials, there was no correlation between vaccine response magnitudes and TTP spanning vaccination alone in either responders or nonresponders (Fig. 3*C*). Remarkably, however, a correlation between vaccine responses and TTP spanning chemotherapy was observed (Fig. 3*C*), which was stronger than that between vaccine responses and TTS (i.e., in Fig. 2*D*). This suggests that vaccine-induced responses may elicit therapeutic benefits primarily by sensitizing tumors to chemotherapy.

Discussion

DC vaccination in this study, as in previous trials, was nontoxic and safe, eliciting type I cytokine responses with typical immune kinetics in GBM patients. In addition, several patients exhibited endogenous immune responses before vaccination, refuting the notion that GBM patients are universally immune suppressed and/or refractory (44–46). Curiously, these endogenous responders were not more likely to exhibit vaccine-enhanced responses of similar or higher magnitudes, suggesting that prior antitumor responses failed to boost stronger secondary responses in these patients. This is consistent with a recent report that DC vaccination effectively primes, but poorly boosts, antiglioma responses in mice (47), although endogenous responses were not directly considered in this report. Moreover, endogenous responses correlated inversely with vaccine-enhanced IFN-γ responses, consistent with the notion that endogenous responsiveness may proportionally diminish vaccine responses. This did not seem to be an artifact of the vaccine response calculation, because pretreatment disease duration also correlated inversely with endogenous responses.

Vaccine responders exhibited statistically significant increases in posttreatment TTP and TTS. This did not seem to be due to patient bias, because pretreatment prognostic factors were not significantly different between vaccine responders and nonresponders. Nevertheless, newly diagnosed patients were overrepresented among responders (Table 1), potentially skewing outcomes. In this context, recurrent responders also exhibited the same trend toward greater TTS and TTP, as well as significantly increased 2-year survival, relative to nonresponders (Table 2). That newly diagnosed GBM patients would exclusively receive radiotherapy before vaccination, coupled with reduced power to detect treatment effects of a given magnitude in fewer recurrent patients, together may account for the small decrease in TTS and TTP observed in recurrent patients. In this context, higher vaccine response thresholds rendered TTS and TTP significantly higher in recurrent and nonrecurrent responders. Taken together, the data are consistent with more favorable outcomes in vaccine responders due to treatment alone.

Most patients in this study also received postvaccine chemotherapy. It was therefore notable that, in patients experiencing postvaccine progressions, TTP was significantly longer spanning postvaccine chemotherapy relative to TTP before chemotherapy. This increase was not confined to vaccine responders, a fact that could indicate greater clinical efficacy of chemotherapy relative to vaccination in study patients, or selection of a patient subpopulation with distinct clinical disease course relative to the general population. Refuting the former, TTS of 43 GBM patients treated with chemotherapy over the same period at the same institution was significantly lower than that of the 34 vaccinated

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GBM patients in this study (389 ± 37 versus 547 ± 42 days, respectively; P = 0.032, log-rank test). Thus, vaccination seems more effective than chemotherapy alone, and other factors may influence this increase.

As with recurrent patients, generally, the shorter TTP in patients evaluated for TTP over the postvaccine chemotherapy interval may have been due to the combined effects of the lack of radiotherapy immediately before vaccination and decreased power to detect treatment effects in this patient subgroup. Nevertheless, we could not formally exclude the possibility that postvaccine chemosensitization may be most relevant for a subgroup of patients with distinct clinical disease courses. Separating the highest quartile of vaccine responders from other patients, however, revealed a significant increase only in TTP spanning chemotherapy in responders. In addition, at least four objective tumor regressions occurred exclusively within the postvaccine chemotherapy interval (Table 2), with the most dramatic regressions being vaccine responders (Fig. 3*B* and patient 9 in Fig. 2 of ref. 26). Taken together, these data suggest that the highest antitumor T-cell responses preferentially enhance GBM chemosensitivity, a notion consistent with the tight correlation between an endogenous immune variable and clinical chemotherapeutic responsiveness in GBM (26). That the increase in postvaccine TTP was specific to the postvaccine chemotherapy interval only when the highest responders were considered may be related to an insensitivity of the methods used to define progressions. Notably, RECIST criteria of defining tumor progression, which would undermine detection of transient and/or low-level progressions (16), were not used. Nevertheless, inclusion of transient progressions/inflammation was likely prevented by the requirement of histologic and/or serial MRI verification of tumor progression. Thus, we favor the notion that the dependence of differential increase on high response levels was due to relative sensitivity of our methods to define immunologic responses, a higher threshold wherein immune responses influence tumor chemosensitivity relative to other clinical variables, reduced power to detect clinical correlations due to fewer patients in this analysis, or a combination of these factors.

Whereas others have reported correlations between T-cell and clinical responses after cancer vaccine administration, these were nonprogressive, primarily based on qualitative rather than quantitative response status, and sometimes required data compiled from multiple types of immune assays (40, 41, 48, 49). Thus, the predominant trend is a lack of correlation between postvaccine clinical course and any single immune variable (20, 37–39, 42). The significant progressive (logarithmic) correlation between TTS and postvaccine IFN-γ enhancement in our trial is thus unique and allows us to propose that single-vaccine response metrics can quantitatively reflect inhibition of tumor progression by T cells capable of tumor destruction in GBM patients. Intriguingly, this was traceable, through both correlation with, as well as preferential influence of, high vaccine responses on TTP specifically within the postvaccine chemotherapy interval. These findings are consistent with the notion that chemosensitization of GBM tumors may be among the most direct and clinically beneficial effects of vaccine-induced immune activity. Mechanistically, this is most consistent with DC vaccination inhibiting GBM progression primarily by altering their intrinsic responsiveness to treatment rather than by net tumor destruction via CTL killing. Similar effects might be masked in non-CNS tumors that exhibit greater baseline chemosensitivity, in tumors with treatment-induced drug resistance, or in the absence of postvaccine chemotherapy or other immune-influenced treatment variables. This might explain the lack of progressive correlation between postvaccine immune responses and clinical outcome in previous cancer vaccine trials (20, 42). These findings underscore the need to monitor secondary treatment responsiveness in cancer vaccine trials generally.

In conclusion, analysis of this phase II trial suggests that DC vaccination elicits antitumor type I immune responses in most GBM patients, which may be limited by prior exposure of tumors to similar endogenous immune responses. Furthermore, the data suggest that vaccine responders enjoy improved clinical outcomes and that response magnitudes in these patients correlate progressively with TTS and TTP after postvaccine chemotherapy. Finally, our findings validate postvaccine chemosensitization of GBM by vaccine-induced T-cell responses. It should be noted that these findings, although promising, require verification in larger groups of patients treated in randomized trials. Nevertheless, based on this promise, it is anticipated that screening vaccine candidates for markers predicting vaccine responsiveness (27), combining vaccination with recently improved chemotherapeutic regimens (7, 8), and rendering vaccines accessible to more patients may together help maximize vaccine-mediated clinical benefits for GBM.

Disclosure of Potential Conflicts of Interest

C.J. Wheeler: Patent holder. K.L. Black: Ownership interest, Immunocellular Therapeutics, Ltd. J.S. Yu: Commercial research grant, MGI Pharma; employment and ownership interest, Immunocelluar Therapeutics, Ltd.

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