Dendritic Cell Vaccination in Glioblastoma Patients Induces Systemic and Intracranial T-cell Responses Modulated by the Local Central Nervous System Tumor Microenvironment

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Abstract

Purpose: We previously reported that autologous dendritic cells pulsed with acid-eluted tumor peptides can stimulate T cell – mediated antitumor immune responses against brain tumors in animal models. As a next step in vaccine development, a phase I clinical trial was established to evaluate this strategy for its feasibility, safety, and induction of systemic and intracranial T-cell responses in patients with glioblastoma multiforme.

Experimental Design: Twelve patients were enrolled into a multicohort dose-escalation study and treated with 1, 5, or 10 million autologous dendritic cells pulsed with constant amounts (100 μg per injection) of acid-eluted autologous tumor peptides. All patients had histologically proven glioblastoma multiforme. Three biweekly intradermal vaccinations were given; and patients were monitored for adverse events, survival, and immune responses. The follow-up period for this trial was almost 5 years.

Results: Dendritic cell vaccinations were not associated with any evidence of dose-limiting toxicity or serious adverse effects. One patient had an objective clinical response documented by magnetic resonance imaging. Six patients developed measurable systemic antitumor CTL responses. However, the induction of systemic effector cells did not necessarily translate into objective clinical responses or increased survival, particularly for patients with actively progressing tumors and/or those with tumors expressing high levels of transforming growth factor β_2 (TGF-β_2). Increased intratumoral infiltration by cytotoxic T cells was detected in four of eight patients who underwent reoperation after vaccination. The magnitude of the T-cell infiltration was inversely correlated with TGF-β_2 expression within the tumors and positively correlated with clinical survival ($P = 0.047$).

Conclusions: Together, our results suggest that the absence of bulky, actively progressing tumor, coupled with low TGF-β_2 expression, may identify a subgroup of glioma patients to target as potential responders in future clinical investigations of dendritic cell – based vaccines.

Glioblastoma multiforme is the most malignant primary brain tumor of the central nervous system (CNS) and one of the most lethal of adult cancers worldwide. Current therapeutic options for patients with glioblastoma multiforme consist of surgical resection followed by radiation therapy and chemotherapy. Despite this aggressive multimodality approach, patients with glioblastoma multiforme continue to have a poor prognosis, with a median survival of ~1 year and a 5-year survival rate of <2% (1).

An emerging strategy in the treatment of brain tumors involves the stimulation of an antitumor immune response. Immunotherapy is theoretically appealing because it offers the potential for a high degree of tumor specificity, whereas sparing normal brain structures. Several different laboratories have shown that effective immune responses within the CNS can be generated through the use of gene-modified tumor cell vaccines (2–4), the adoptive transfer of immune T cells (5, 6), or the use of dendritic cell–based vaccines (7–11). These results imply that systemic immunity can enter the "immunologically privileged" CNS, selectively identify tumor-associated antigens, and destroy brain tumor cells (12).

Early-phase dendritic cell–based clinical trials for human tumors outside the CNS have shown favorable toxicity profiles

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and therapeutic efficacy in some patients (13–17). Preclinical animal studies (7, 9, 18–20) and phase I clinical trials (8, 10, 11, 21–24) have also shown that dendritic cells pulsed with tumor lysates, cell fusions, RNA, and/or peptides can elicit antitumor immune responses against CNS neoplasms. Although the clinical data to date is too limited to make any conclusions about efficacy, the advantages of dendritic cell–based immunotherapy, along with its documented safety and feasibility, have stimulated further development and testing.

Whereas recent reports using dendritic cells to treat brain tumors have yielded encouraging results (8, 10, 11, 21–23, 25), there are still many practical and theoretical problems to be resolved. For instance, little is known about the best methods for loading dendritic cells with antigens, the optimal dose and route of administration, or how to identify subgroups of patients that are more likely to develop clinical responses. In an attempt to address some of these issues, we translated a successful preclinical model into a phase I dose-escalation study using dendritic cells pulsed with autologous acid-eluted tumor peptides in a uniform population of malignant glioma patients. All patients had the same histopathologic diagnosis of glioblastoma multiforme (WHO grade 4). Primary objectives were to evaluate safety and feasibility, as well as to determine whether dose-limiting toxicity was reached when given as three biweekly intradermal injections. Secondary objectives were to assess this vaccination strategy for its ability to stimulate systemic antitumor CTL responses and to investigate whether induction of such responses correlated with intracranial T-cell infiltration and/or clinical survival. Finally, we sought to identify surrogate variables that might help select subgroups of glioblastoma multiforme patients with the highest likelihood of responding to dendritic cell–based vaccine strategies. Patients enrolled in this study were followed for almost 5 years.

Materials and Methods

Patient eligibility and treatment schedule. Patients with newly diagnosed or recurrent glioblastoma multiforme, who provided written informed consent according to University of California at Los Angeles (UCLA) Internal Review Board guidelines, were eligible. Inclusion criteria were malignant gliomas that were amenable to surgical resection, a Karnofsky performance score of ≥60, and evidence of normal bone marrow function (e.g., hemoglobin ≥10.0 g/d, absolute granulocyte count ≥1,500/μL, and platelet count ≥100,000 K), as well as adequate liver and renal function. Patients must have recovered from all toxicities related to any previous therapy and not have received any radiation therapy for at least 2 weeks, nitrosureas for at least 6 weeks, nor other chemotherapy for at least 4 weeks before entry into the trial. Exclusion criteria included allergies to any components of the dendritic cell vaccine, concurrent or prior corticosteroid use within 2 weeks of initial vaccination; the presence of acute infection requiring active treatment, severe intercurrent medical conditions, known immunosuppressive disease, positive serology for HIV or hepatitis B; history of an autoimmune disease, or prior history of other malignancies. Twelve patients were enrolled sequentially into three cohorts according to a dose-escalation design, with the first three subjects receiving 1×10^6 dendritic cells per vaccination, the second three receiving 5×10^6 dendritic cells per vaccination, and the final six receiving 1×10^7 dendritic cells.

Preparation of autologous dendritic cells. Standard leukapheresis was done at the UCLA Hemapheresis Unit to harvest peripheral blood mononuclear cells for dendritic cell cultures. Blood was drawn as a source of autologous serum for the cell cultures. Patients were supplemented with oral iron and vitamin C throughout the study to prevent anemia. All *ex vivo* dendritic cell preparations were done in the UCLA Jonsson Cancer Center GMP facility under sterile and monitored conditions. Dendritic cells were prepared by culturing adherent cells from peripheral blood with granulocyte macrophage colony-stimulating factor and interleukin-4 (IL-4), using techniques described previously (26). Following culture, dendritic cells were collected by vigorous rinsing, washed with sterile 0.9% NaCl solution, and cryopreserved in individual aliquots with 10% DMSO and 20% autologous serum.

Autologous tumor culture and preparation of acid-eluted tumor-associated peptides. Fresh tumor samples from surgical resection were transported under sterile conditions to the UCLA Jonsson Cancer Center GMP facility and used to establish autologous primary glioblastoma multiforme cell lines, as previously described (27, 28). Cultured tumor cells were harvested and used for acid elution of surface peptides. The median duration of primary tumor cell culture was 5 weeks (range, 2-14 weeks). Tumor-associated surface proteins enriched for MHC class I peptides were isolated by an acid elution protocol as described previously (7, 29). Peptide washes were then lyophilized to complete dryness, resuspended in 0.2 mL dPBS, aliquoted, and frozen at −80°C. A 5-μL sample of each peptide preparation was quantified by microprotein assay (Bio-Rad, Hercules, CA) and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics, Billerica, MA) as previously published (28). Before patient vaccination, tumor peptides were also tested for sterility by Gram stain; *Limulus* amebocyte lysate assay (Bio Whittaker, Walkersville, MD); and routine aerobic, anaerobic, and fungal cultures. A constant amount of peptide (100 μg) was used to pulse dendritic cells for each injection, regardless of the dose of dendritic cells.

Final vaccine preparation and dose administration. On the day of each vaccination (study days 0, 14, and 28), cryopreserved dendritic cells were thawed, washed thrice, and pulsed 30 to 60 minutes with 100 μg of autologous glioblastoma multiforme tumor peptides in serum-free RPMI 1640. The available number of peptide-pulsed dendritic cells (identified as unstained large cells) was determined by hemacytometer count using trypan blue. Before administration, peptide-pulsed dendritic cells were washed with saline and the appropriate vaccine dose resuspended in 1 mL of sterile 0.9% NaCl solution. For quality assurance, an aliquot of the final product underwent immediate Gram staining and endotoxin PCR to rule out contamination, and additional aliquots were sent for *Limulus* amebocyte lysate assay and cultures for bacterial and fungal pathogens. The purity and biological signature of each dendritic cell vaccine was also determined by FACScan flow cytometry (BD Biosciences, San Jose, CA). Cells were stained with PerCP-conjugated anti-HLA-DR monoclonal antibody (mAb, for MHC class II, BD Biosciences) and FITC-conjugated anti-CD14-FITC (for monocytes/macrophages; Caltag Laboratories, Burlingame, CA); and >50% of the large cell gate had to express HLA-DR, but not CD14, to be released for patient administration. For a more comprehensive analysis, dendritic cells were also stained with mAb to HLA-A,B,C (MHC class I), CD80 and CD86 (B7 costimulatory molecules), CD40 (tumor necrosis factor receptor superfamily 5), and CD83 (immunoglobulin superfamily). The final 1 mL vaccine dose was drawn into a sterile syringe and given as an intradermal injection (using a 25-gauge needle) in the region below the axillae, with the site of administration alternating between right and left sides for each vaccination. Subjects were monitored for 2 hours post-immunization in the UCLA Clinical Research Center.

Evaluation of clinical status. Toxicity was monitored and graded according to the National Cancer Institute Common Toxicity Criteria. The overall incidence of adverse events was recorded. Evaluation for autoimmune symptoms and neurologic exams were done before and 30 minutes after each vaccination, as well as at all follow-up visits every 8 weeks. Time to tumor progression (TTP) was defined as the interval from surgical resection until the first observation of tumor progression,

as evidenced by magnetic resonance imaging (MRI). Survival time was determined from the date of surgery to date of death due to any cause.

Alamar blue CTL assay. Immunologic monitoring was routinely conducted pretreatment (on day −14), day 35, day 56, day 112, and every 8 weeks thereafter, using 75 mL of blood drawn at each time point. Peripheral blood T cells from patients receiving peptide-loaded dendritic cells were assessed for *de novo* cytotoxic activity against autologous tumor cells using an Alamar blue CTL assay (30). $CD3^+$ T cells were purified by negative selection with specific antibodies (anti-CD14, anti-CD16, anti-CD19; PharMingen, San Diego, CA) and immunomagnetic beads (Dynal Biotech, Inc., Lake Success, NY) as described previously (26). T cells were incubated with 2,500 autologous tumor cells/well at E/T ratios ranging from 80:1 to 5:1, in triplicate. The autologous tumor cells were from the cell lines established from each patient's primary tumor. Controls included wells with T cells alone, tumor cells alone, and medium alone. MHC-restricted cytotoxicity was assessed by the addition of 5 μg/mL anti-β_2-microglobulin antibody (mAb B1G6, Beckman-Coulter, Miami, FL) to some wells. Alamar blue (Biosource International, Camarillo, CA) was added to each well, and the plates were incubated for 20 to 24 hours at 5% CO_2 and 37°C. Following incubation, the Alamar blue fluorescence was read on a Cytofluor 2300 plate reader (PerSeptive Biosystems, Framingham, MA) with excitation at 530 nm and emission at 590 nm. The percentage of lysis was calculated using the formula:

$$\%\ \text{lysis} = 100 \times \frac{\{(F \text{ of targets alone}) - [(F \text{ of effector and target mix}) - (F \text{ of effectors alone})]\}}{(F \text{ of targets alone})}$$

where F = the average fluorescence of the sample wells after the fluorescence of the wells containing medium alone was subtracted. The CTL response was interpreted as "positive" when the percent specific lysis by post-vaccine CTLs (drawn at day 35) was at least twice in magnitude as that of pre-vaccine CTLs (drawn at day −14) at two or more of the effector/target ratios tested.

Immunohistochemistry. Serial paraffin sections of surgical intracranial tumor specimens were cut to 3-μm thickness and stained with anti-human antibodies against CD3, which recognizes all T lymphocytes (1:100 dilution, Biocare Medical, Walnut Creek, CA); CD8 (marker for CTLs), CD4 (marker for helper T lymphocytes, T_H), CD45 (leukocyte marker), and CD45RO (marker for activated lymphocytes) at 1:50, 1:100, 1:300, and 1:50 dilutions, respectively (DAKO Corp., Carpinteria, CA); and transforming growth factor β_2 (TGF-β_2, 1:50 dilution, R&D Systems, Minneapolis, MN). Sections were baked for 1 hour at 60°C, deparaffinized, and endogenous peroxidase activity quenched by treating with 0.5% H_2O_2 in methyl alcohol for 10 minutes. Heat-induced epitope retrieval was done on the slides using 0.01 mol/L citrate buffer (pH 6.0; for CD8 and CD45RO) or 0.001 mol/L EDTA (pH 8.0; for CD3 and CD4) in a vegetable steamer (Black & Decker, Towson, MD); slides were heated for 25 minutes, cooled, and washed in 0.01 mol/L PBS. All slides then were placed on a DAKO Autostainer (DAKO) and sequentially incubated in primary antibody for 30 minutes, then rabbit anti-mouse secondary immunoglobulins (DAKO) for 30 minutes. Diaminobenzidine and hydrogen peroxide were used as the substrates for the peroxidase enzyme. For the negative controls, mouse isotype or rabbit immunoglobulins (DAKO) were used in place of the primary antibodies.

Reverse transcription-PCR. RNA was isolated from frozen tumor samples using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA. For cDNA synthesis, ~1 μg of total RNA was reverse transcribed using the Omniscript RT kit (Qiagen, Valencia, CA). Human TGF-β_2 and IL-10 sequences were then PCR amplified using Taq DNA polymerase (Qiagen). The PCR mixture consisted of 300 μmol/L deoxynucleotide triphosphates, 1.5 mmol/L Mg^{2+}, 10 μmol/L primers, and 2.5 units Taq DNA polymerase/reaction. Primer pairs for TGF-β_2 and IL-10 were purchased from R&D Systems. The expression of TGF-β_2 (product size = 279 bp) and IL-10 (product size = 427 bp) was confirmed by loading a 5 μL volume of each PCR reaction onto 1.5% agarose gels, stained with ethidium bromide. The band intensities were analyzed by densitometry using AlphaEase software (Alpha Innotech, San Leandro, CA). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase expression and statistical analysis was done using Systat software v. 11.

Statistical analysis. Continuous variables were compared using a paired Student's t test. Categorical variables were compared using the χ^2 or Fisher's exact test. The median survival times, median TTP, and survival curves were determined using the Kaplan-Meier method. The Wilcoxon log-rank test was used to compare curves between study and control groups. All P values are two tailed, and $P < 0.05$ was considered statistically significant.

Results

Patient characteristics. Twelve patients with histologically proven glioblastoma multiforme were enrolled in this phase I trial (Table 1). Seven had newly diagnosed tumors, whereas five had recurrent glioblastoma multiforme. There were seven women and five men, with an age range from 20 to 65 years (mean age of 40 years). Newly diagnosed glioblastoma multiforme patients underwent surgery and a standard course of external beam radiotherapy (up to 6,000 cGy) but no other treatment before dendritic cell vaccination. Recurrent glioblastoma multiforme patients had previous radiation therapy and/or chemotherapy before presenting with tumor recurrence; thus, they underwent surgical resection of their tumors followed by dendritic cell immunotherapy at the earliest feasible date. All patients were treated with concurrent anticonvulsant therapy, were off corticosteroids for at least 14 days before the first dendritic cell vaccination, and did not take any steroids thereafter until they were considered off study because of tumor progression. Thus, all evaluations for toxicity, treatment response, and immunologic monitoring were done in the absence of corticosteroid effects. The median time between surgical resection and the initiation of dendritic cell vaccination was 18 weeks (range, 4-28 weeks). All patients had a baseline brain MRI scan within 1 month before starting the immunotherapy. Because tumor progression in itself was not an exclusion criterion to enrollment in this phase I trial, none of the subjects who were accrued were not treated due to tumor progression per se, although there was one subject who signed the consent but received no vaccine because of steroid dependency.

Primary glioblastoma multiforme cultures and characterization of autologous acid-eluted tumor peptides. Short-term cultures of primary glioblastoma cells were successful in ~80% of all the samples that we attempted. For the intend-to-treat subjects in this trial (n = 15), the tumor cells did not grow sufficiently well in 3 of 15 patients (20%), which left 12 subjects who were actually enrolled and assigned to an experimental cohort. When no cell line could be made, the patients were not subsequently continued on this study. These patients were treated with conventional chemotherapy per standard of care.

As would be expected, autologous tumor cell lines were more consistently established for patients with newly diagnosed tumors than for those with recurrent and previously treated glioblastoma multiforme, as the latter tumors contained a greater amount of necrotic debris and dead cells. Although some variability was observed between patient samples, the cell

Table 1. Patient characteristics

Patient subject no.	Tumor path	Age	Gender	KPS	Dendritic cell dose ($\times 10^6$)	Tumor status at dendritic cell vaccine	Pre-vaccine therapy*	Post-vaccine therapy	Adverse events†
1	GBM‡	33	M	90	1	ND		Reoperation, Temodar	Constipation/diarrhea
2	GBM§	33	M	80	1	PD	Temodar + Accutane, Tamoxifen	Reoperation, CPT-11	Headache, nausea/vomiting, low-grade fever
3	GBM§	23	F	100	1	ND		Tamoxifen, Reoperation, Temodar, Carboplatin	Fatigue, nausea/vomiting
4	GBM§	20	F	90	5	SD		Reoperation	Low-grade fever, constipation/diarrhea
5	GBM†	23	F	100	5	SD		Temodar	Fatigue, myalgia, nausea/vomiting, pain/itching at injection site, lymph node swelling, allergic rhinitis
6	GBM†	42	F	100	5	SD		Temodar + Accutane	Lymph node swelling
7	GBM§	48	M	80	10	PD	"Intra-cellular hyperthermia"	Reoperation, Temodar, Gleevec	Headache, fatigue
8‖	GBM§	55	F	60	10	SD	SRS, Thalidomide, Accutane, Tamoxifen	Temodar	Fatigue
9‖	GBM†	43	F	90	10	ND		Temodar	Erythema at injection site
10	GBM†	65	M	90	10	PD		Temodar	Fatigue
11	GBM†	57	M	90	10	PD		Temodar + VP16	
12	GBM§	43	F	70	10	PD	Temodar, Tamoxifen	CCNU	Seizure, hyponatremia

Abbreviations: SD, stable disease; PD, progressive disease; ND, no measurable disease; KPS, Karnofsky performance score.

*Pre-vaccination therapy refers to additional treatments besides surgery and standard external beam radiation therapy (up to 60 Gy); Temodar, temozolamide; Accutane, isotretinoin; "Intracellular hyperthermia" was an experimental protocol in Switzerland; CPT-11, irinotecan; Gleevec, imatinib mesylate; VP-16, etoposide; CCNU = lomustine; and SRS = stereotactic radiosurgery.

† All adverse experiences related to protocol were of mild severity (National Cancer Institute grades 1 and 2). Adverse events that were of higher severity were determined to be not related to protocol and likely due to tumor progression.

‡ Newly diagnosed glioblastoma multiforme.

§Recurrent glioblastoma multiforme.

‖Patients 8 and 9 received only one injection of 10^7 dendritic cells because insufficient numbers of HLA-DR$^+$/CD-14$^-$ dendritic cells were generated to complete all three injections.

morphology of each cell line was generally uniform and stable throughout the early-passage cultures. The glioblastoma multiforme cell lines were all positive for glial fibrillary acidic protein by immunohistochemistry (data not shown), confirming the maintenance of their glial phenotype. Fluorescence-activated cell sorting analysis and immunohistochemistry for MHC class I was done on the glioblastoma multiforme cell lines and all expressed MHC class I molecules, although at variable levels. Exposing cell cultures to IFN-γ and IFN-α up-regulated both MHC class I expression and the amount of glioblastoma-associated peptides harvested by acid elution, suggesting that the acid-eluted material was indeed associated with MHC molecules (28). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of the acid-eluted material revealed molecular weights between 800 and 1500 daltons, which were compatible with the size of peptides (9-12 amino acids) accommodated by the peptide-binding cleft of MHC alleles.

To prepare a sufficient amount of peptide (100 μg per injection) for this protocol, glioblastoma multiforme cell cultures with at least 10^8 cells was necessary. However, estimating the amount of tumor required for peptide recovery was difficult, as the yield of acid-eluted peptides from primary glioblastoma multiforme cultures was not directly proportional to the number of cells processed. The number of passages required for obtaining sufficient peptide for each patient varied from 3 to 12. Thus, vaccine administration was sometimes delayed by the time required to obtain sufficient peptide antigen (i.e., >4 weeks). For this reason, we are concurrently investigating other methods of dendritic cell pulsing, the clinical results of which will be subsequently compared with those reported here.

Characterization and feasibility of dendritic cell vaccine. Dendritic cells generated from adherent peripheral blood precursors cultured in the presence of cytokines IL-4 and

granulocyte macrophage colony-stimulating factor expressed high levels of MHC class I (HLA-A,B,C), MHC class II (HLA-DR), and B7.2 costimulatory molecules (CD80 and CD86), as well as the absence of CD3 and CD14. Incubation of dendritic cells with acid-eluted tumor-associated peptides resulted in a modest increase in the expression of CD80 (costimulatory molecule B7-1), CD83 (marker for interdigitating dendritic cell/Langerhans cell), and CD86 (costimulatory molecule B7-2), suggesting dendritic cell maturation.

Three subjects received three intradermal vaccinations of 1×10^6 acid-eluted peptide-pulsed dendritic cells, and three subjects were treated with three injections of 5×10^6 dendritic cells. For the six patients assigned at the highest dose level (1×10^7 dendritic cells per injection), sufficient numbers of functional dendritic cells for all thre injections were available from a single leukapheresis in only four of the six subjects. Two of the subjects within this high-dose cohort (patients 8 and 9) were treated with only a single vaccination due to insufficient numbers of dendritic cells that met the HLA-DR$^+$/CD-14$^-$ lot release criteria. Of note, patient 8 had prior therapy with thalidomide and patient 9 had previously been treated with Neupogen (r-met-Hu-G-CSF), both of which have potential immunomodulatory effects on dendritic cell phenotype/function (31, 32).

Vaccine safety and toxicity. Dendritic cell vaccinations were well tolerated, with no major adverse events (National Cancer Institute grade 3 or 4) observed in any subject during the vaccine cycles (Table 1). There were no clinical or radiological signs of autoimmune reactions in any patient. Four subjects (patients 2, 3, 4, and 5) showed grade 1 toxicities in the form of low-grade fevers (<100.4°F) and/or flu-like symptoms (e.g., fatigue and myalgia). Three of these patients (patients 2, 3, and 5) also had nausea and vomiting at some point within the first few weeks of the dendritic cell vaccination. Two subjects (patients 5 and 9) exhibited injection site reactions, consisting of erythema, pain, and itching that lasted 48 to 72 hours after the first dendritic cell injection. Two patients (patients 5 and 6) developed lymph node swelling. Patient 5 had palpable axillary and cervical lymph nodes 1 week after the first dendritic cell vaccination, which persisted for 1 month; whereas patient 6 developed supraclavicular lymph node swelling 2 weeks after the first vaccination, which lasted 48 hours. Two subjects (patients 1 and 4) developed diarrhea and constipation, probably due to the supplemental iron tablets that were given to prevent anemia during the trial. There were no treatment-related hematologic, hepatic, renal, or neurologic toxicities. On follow-up MRI scans of the brain, there were no new abnormalities observed following dendritic cell vaccination other than those directly related to tumor growth at the time of tumor recurrence. Cumulatively, these data suggest a low toxicity profile for the acid-eluted glioblastoma multiforme peptide-pulsed dendritic cells at all dose levels tested.

Clinical evaluations. Although this phase I clinical trial was not powered to detect clinical efficacy, tumor response was monitored by clinical and MRI assessments at baseline (within 1 month before therapy), at day 56 post-therapy, and every 8 weeks thereafter as surrogate markers for clinical response and tumor status.

Five of the 12 subjects (patients 2, 7, 10, 11, and 12) had ongoing progressive disease before dendritic cell–based vaccination. Four subjects (patients 4, 5, 6, and 8) had stable gross residual disease, and three patients (patients 1, 3, and 9) had no measurable residual disease at the start of dendritic cell injections. When considering all 12 glioblastoma multiforme patients enrolled in this clinical trial, overall survival was 100% at 6 months, 75% at 1 year, and 50% at 2 years, with two long-term (≥4 year) glioblastoma multiforme survivors. Median TTP was 15.5 months, and median overall survival was 23.4 months. As might be expected, those patients with bulky, progressively growing tumors at the time of initial dendritic cell vaccination, regardless of whether they were newly diagnosed or recurrent, continued to have tumor progression despite active immunotherapy. For this subgroup of patients, the median overall survival was 11.7 months, which is not significantly different from those of historical and concurrent glioblastoma multiforme patients treated at our institution. For the patients with stable tumors or no residual

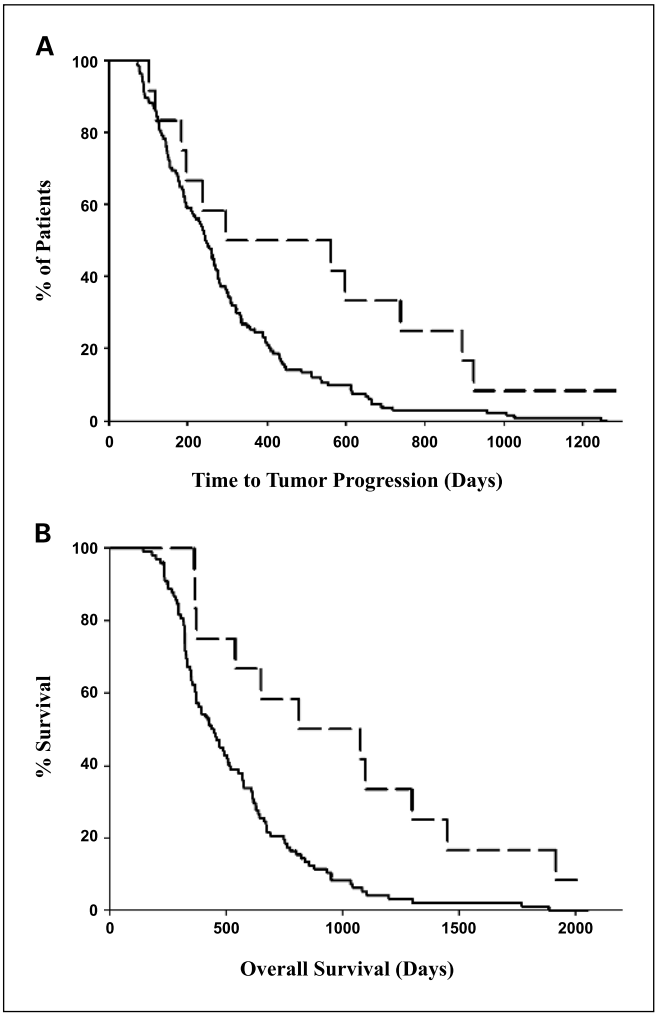

Fig. 1. Kaplan-Meier curves of TTP (*A*) and survival (*B*) between the study group ($n = 12$, *dashed line*) and concurrent control patients with newly diagnosed glioblastoma multiforme (recursive partitioning analysis class III: age <50 years and Karnofsky performance score of ≥ 90) treated at UCLA during the same time period ($n = 99$, *solid line*). Controls represent the population of patients who received surgical resection (not just biopsy) and were off steroids by 14 days after standard radiation therapy. Even when compared with this better overall prognostic class of control patients, the TTP ($P = 0.028$) and overall survival ($P = 0.006$) were significantly longer in the dendritic cell – vaccinated study group.

disease at the time of dendritic cell vaccination, median TTP was 19.9 months. Overall survival times in this group ranged from 18 to >58 months, with a median survival of 35.8 months. This compares favorably even when compared with historical/concurrent data for the best prognostic group of glioblastoma multiforme patients (recursive partitioning analysis class III: age <50 years and Karnofsky performance score of ≥90) treated at UCLA during the same time period, who underwent surgical resection (not just biopsy) and became off steroids within 2 weeks after completion of postoperative radiotherapy (n = 99). In comparison with our study patients, the control population of patients had a median TTP of 8.2 months (P = 0.028) and overall median survival of 18.3 months (P = 0.006; Fig. 1).

One patient (patient 5) had near complete regression of residual tumor, which was seen on MRI 2 months after completion of peptide-pulsed dendritic cell vaccination and before any additional adjuvant treatment. Both the size of the areas of T2W hyperintensity and the contrast-enhancing tumor decreased in this patient (Fig. 2). Although this radiographic change is more likely related to a delayed response to radiation therapy, it is interesting to speculate that dendritic cell–based immunotherapy might have contributed to this clinical response, as this patient also showed significant CTL responses against autologous tumor cells *in vitro*. Serial MRI scans obtained for this patient showed residual tumor after surgery and radiation therapy (Fig. 2A), partial response 2 months post-dendritic cell immunotherapy and before any adjuvant chemotherapy (Fig. 2B), and essentially stable disease at 58 months after initial diagnosis. Interestingly, she is currently alive with no clinical or MRI evidence of tumor recurrence after almost 5 years of follow-up. Although admittedly a select population of patients, the prolonged survival times observed and the immunologic responses obtained in some of these patients support the possibility of an immune-related effect on tumor control.

Systemic antitumor immune responses to dendritic cell vaccination. Systemic tumor-specific cytotoxicity against autologous tumor cells was determined for all patients in this study using conventional CTL assays. Purified $CD3^+$ T cells were tested for *de novo* cytotoxicity without restimulation *in vitro*. Blocking mAb against β_2-microglobulin was added to replicate wells to confirm the MHC-restricted nature of any observed CTL lytic activity. Six patients without preexisting peripheral CTL activity developed peripheral tumor-specific CTL activity post-vaccination (Fig. 3; Table 2).

Whereas the patients who developed systemic antitumor cytotoxicity had significantly longer survival than those who did not (P = 0.04), this survival difference also correlated with the presence or absence of tumor progression at the time of dendritic cell vaccination. The development of a positive CTL response was negatively associated with active progressive disease (as measured by brain MRI). 100% (six of six) of patients who generated positive CTL responses had stable/minimal residual disease burden (stable gross residual disease or no measurable residual disease) at the time of dendritic cell vaccination. Conversely, for the five patients who were experiencing active tumor progression at the time of vaccination, none (zero of five) developed statistically significant cell-mediated CTL responses. These data suggest that glioblastoma multiforme patients with active tumor progression/recurrence may have an impaired ability to mount cellular antitumor

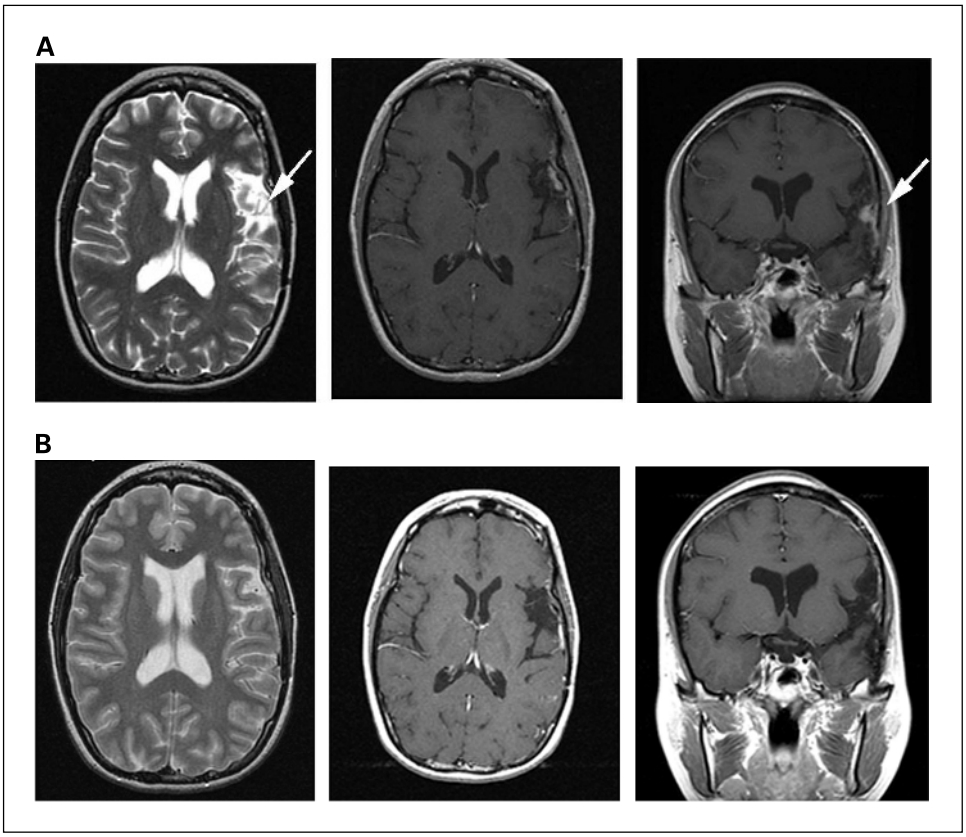

Fig. 2. Objective clinical response of a glioblastoma multiforme patient treated with 5×10^6 peptide-pulsed dendritic cells. Brain MRI scan of patient 5. Axial T2-weighed, axial, and coronal contrast-enhanced T1-weighed MRI scans were done immediately before dendritic cell injections (*A*) and 2 months after completion of peptide-pulsed dendritic cell vaccine series (*B*), before any adjuvant chemotherapy. Note area of T2 abnormality (*arrow, top left*) and contrast-enhancing tumor (*arrow, top right*), which resolved following dendritic cell immunotherapy.

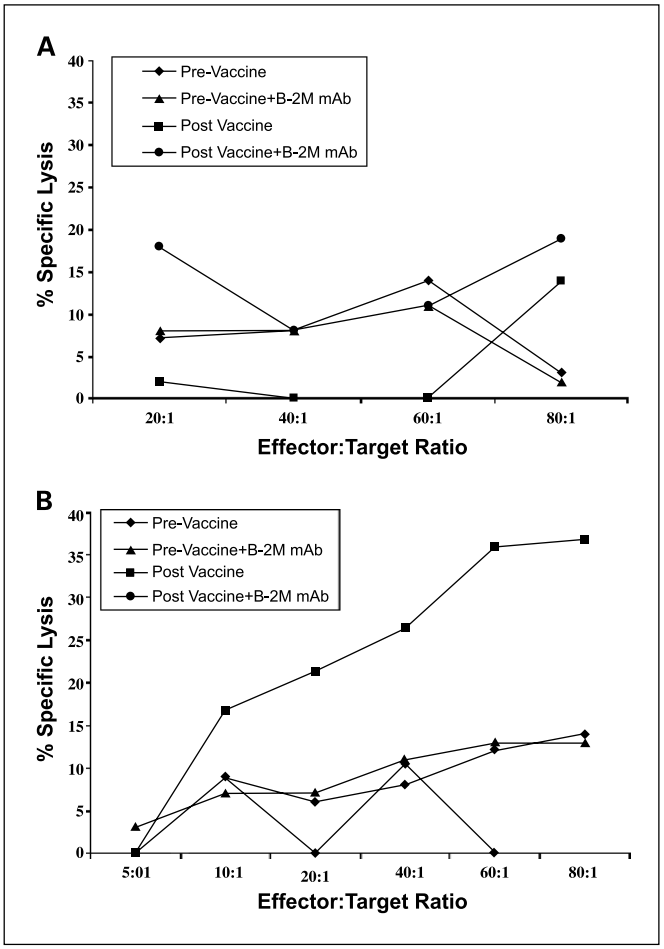

Fig. 3. Peripheral CTL responses to autologous peptide-pulsed dendritic cells. Peripheral blood mononuclear cells from day −14 pretreatment (♦) and day 35 post-vaccine (■) time points tested for *de novo* cytotoxity against irradiated, autologous tumor cells in an Alamar blue CTL assay. *A*, representative *negative* CTL data from a patient who experienced continued tumor progression during the course of treatment (patient 12). The CTL response was not significantly different in the prevaccine and post-vaccine lines. *B*, representative *positive* induction of CTL response in patient 6, who did not have any evidence of disease progression for over 14 months after dendritic cell vaccination. MHC-specific antitumor cytotoxicity was generated, which could be specifically blocked by anti-β_2-microglobulin mAb (●). All assays were done in triplicate.

immune responses, a finding that has been observed previously (33, 34).

Intracranial T-cell infiltration associated with prolonged survival. The accumulation of tumor-specific T cells locally within tumors has been associated with positive clinical responses in gliomas and other cancers (35–37). We therefore investigated whether induced systemic CTL responses could access the CNS to infiltrate intracranial tumors and whether such intratumoral T-cell infiltration correlated with clinical survival. Eight patients who developed new areas of contrast enhancement on follow-up MRI scans underwent surgical resection/biopsy at some point following dendritic cell vaccination. Four of these patients survived >30 months, whereas three patients died within <12 months of surgical diagnosis. In the reoperated patients with >30-month survival (patients 1, 3, 4, and 9), there was a robust infiltration of $CD3^+$ tumor-infiltrating lymphocytes (TIL) in the specimens at reoperation that was not present in the tumor tissues obtained from the same patients before dendritic cell treatment. The majority of these TIL were $CD8^+/CD45RO^+$ memory T cells, with lesser numbers of $CD4^+$ helper T cells (Fig. 4A and B). In the tumors from patients who died from tumor progression within 1 year (patients 2, 7, and 12), there were no differences in the numbers of infiltrating T cells in the surgical specimens taken from before and after dendritic cell vaccination (Fig. 4C). Specimens from six control patients who did not receive dendritic cell immunotherapy also showed no significant difference in T-cell staining at initial surgery when compared with their reoperation specimens (Fig. 4D).

Similar to our findings with respect to systemic CTL responses, the immunohistochemical detection of enhanced TIL accumulation was associated with the disease status at the time of dendritic cell vaccination. Four of four (100%) patients who showed evidence for increased TIL in the resected tumors had dendritic cell vaccinations during a time of minimal tumor burden, whereas the patients (three of three) who did not show any detectable increase of TIL were experiencing active tumor progression before the course of dendritic cell vaccination. These data suggest that active tumor recurrence and/or bulky residual tumor may negatively influence the ability of T lymphocytes to accumulate within the local tumor microenvironment, which could be associated with decreased clinical benefit from dendritic cell vaccination.

Decreased tumor-associated expression of transforming growth factor-β_2 correlates with prolonged survival. To better elucidate features of the tumor environment that might predict clinical response to dendritic cell–based vaccination, we tested whether the local accumulation of T cells within gliomas was associated with the secretion of immunosuppressive cytokines by the tumors. Tumors from our patients were studied for their expression of TGF-β_2 and IL-10 by reverse transcription-PCR and immunohistochemistry. Although our sample size is small, patients that had detectable TIL (patients 1, 3, 4, and 9) also showed quantitatively lower expression of TGFβ_2 in tumor samples taken before and after dendritic cell vaccination (Fig. 5). All these patients also had relatively prolonged survival (>30 months) compared with those with higher TGF-β_2 expression. On the other hand, there was no obvious correlation between the expression of IL-10 and the infiltration of T lymphocytes within the resected tumors (data not shown). These data suggest that the secretion of TGF-β_2 within the local tumor microenvironment may contribute to the inability of TIL to significantly accumulate within CNS gliomas. This, in turn, may negatively influence the ability to mount a clinically relevant local antitumor immune response in brain cancer patients.

Discussion

In this phase I study, we report the safety, feasibility, and bioactivity of a vaccine comprised of autologous dendritic cells exogenously pulsed with peptides acid eluted from the surface of glioblastoma multiforme cells following surgical resection. Our results showed that the methods for producing and administering this dendritic cell vaccine were feasible and safe in newly diagnosed and recurrent glioblastoma multiforme patients, with no evidence of autoimmune complications observed over a follow-up period of almost

5 years. The treatment was tolerated well, with only minor toxicities (National Cancer Institute grades 1-2). Furthermore, we also showed that it is feasible to treat glioblastoma multiforme patients with up to 1×10^7 dendritic cells pulsed with autologous acid-eluted peptides, although the highest dose caused practical difficulties for some patients. Two patients received only one of the total of three scheduled vaccinations due to difficulty getting enough HLA-DR$^+$/CD-14$^-$ dendritic cells for all three injections at the highest doses.

Six of the 12 patients developed measurable peripheral antitumor T-cell responses, although there was no significant difference in the magnitude of CTL responses among the three dendritic cell dose cohorts tested. Given that no important differences in immune response were seen among the doses, we have no objective evidence to conclude that higher dendritic cell doses are needed, particularly given the practical difficulties of getting enough cells at the highest dose from a single leukapheresis. In the 5 years since this trial was started, we believe that it may be the ratio of tumor peptides to dendritic cells that will be important in future vaccination strategies rather than the absolute number of dendritic cell itself.

One of our patients had MRI evidence of an objective clinical response, which correlated with a robust tumor-specific immune response by CTL assay. However, for the majority of subjects, the detection of CTL responses in the peripheral blood of immunized patients paradoxically was not in itself predictive of objective clinical response and/or prolonged survival. Studies in recent years have begun to dissect out the apparent dichotomy between peripheral CTL and clinical responsiveness (38). It is now appreciated that the systemic peripheral blood antitumor response does not necessarily translate to vaccine-induced responses within the tumor (39, 40). The inability of traditional CTL data to correlate with clinical outcome in this trial and others (10, 40) prompted us to search for alternative surrogate variables of vaccine responsiveness and/or predictors of subgroups of glioblastoma multiforme patients that may most likely respond to immune-based therapies.

In preclinical animal models of experimental intracranial gliomas, we have previously shown that active immunotherapy with dendritic cell–based vaccines is associated with increased CNS T-cell infiltration and prolonged survival (7, 41). Other investigators have reported that the localization of tumor-specific T cells at the tumor site is often a requirement for regression of systemic tumors (42) and even CNS tumors (43). In this clinical trial, we also observed dramatic intratumoral infiltration of CD8$^+$ and CD4$^+$ CTL in some patients following dendritic cell vaccination. However, whether or not the lymphocytes found in the tumor specimens from our vaccinated patients were exerting an actual antitumor effect cannot be determined by our current results, as these TILs may be functionally compromised. To definitively determine whether these TILs have clear antiglioma activity, subsequent studies are currently under way to generate tumor-specific human T-lymphocyte lines from the TILs within the clinical tumor specimens collected from our vaccinated patients. Nevertheless, there was a significant correlation between intracranial T-cell infiltration within the local tumor environment and prolonged survival in our trial patients ($P = 0.047$), suggesting potential functional activity.

The presence of TIL in only a subset of the dendritic cell–vaccinated patients led us to postulate that the local CNS/tumor microenvironment may modulate immune responsiveness and provide insight into possible factors that can differentiate subgroups of glioblastoma multiforme patients who will most likely benefit from immune-based therapies. Immunomodulatory factors secreted by gliomas, such as TGFβ_2, PGE_2, and IL-10, are known to negatively influence T-cell functions (44, 45). In our study, intratumoral infiltration of T lymphocytes was negatively correlated with the expression of the immunosuppressive factor TGF-β_2. TGF-β_2 is

Table 2. Summary of immunologic and clinical data

Patient subject no.	Dendritic cell dose ($\times 10^6$)	Tumor status at initiation of dendritic cell treatment	↑ Systemic CTL activity*	TGF-β_2 expression†	Intracranial T-cell infiltration‡ (post-vaccination)	TTP (mo)	Overall survival (mo)
1	1	ND	+	1.68	++++	18.1	30.2
2	1	PD	–	5.78	–	8.4	11.4
3	1	ND	+	0.66	+++	27	38.9
4	5	SD	+	1.08	+++	19.9	35.8
5	5	SD	+	0.25	NA	No progression	›58.0
6	5	SD	+	1.89	NA	14.5	25.6
7	10	PD	–	5.63	–	6.6	11.9
8	10	SD	–	2.54	+	16.5	21.2
9	10	ND	+	0.32	++++	28.5	›48.4
10	10	PD	–	3.16	NA	9.1	18.0
11	10	PD	–	2.33	NA	5.7	12.0
12	10	PD	–	6.88	–	3.2	7.3

Abbreviations: SD, stable disease; PD, progressive disease; ND, no measurable disease; NA, not available because no surgery was done post-vaccination.
*"Positive" CTL response was set as % specific lysis post-vaccine (d 35) ›2× magnitude of % specific lysis pre-vaccine (d −14) at two or more E/Tratios.
†TGF-β_2 expression is presented as quantitative units relative to primer controls and normalized to glyceraldehyde-3-phosphate dehydrogenase expression.
‡Scored by semiquantitative assessment of number of CD8$^+$ TILs.

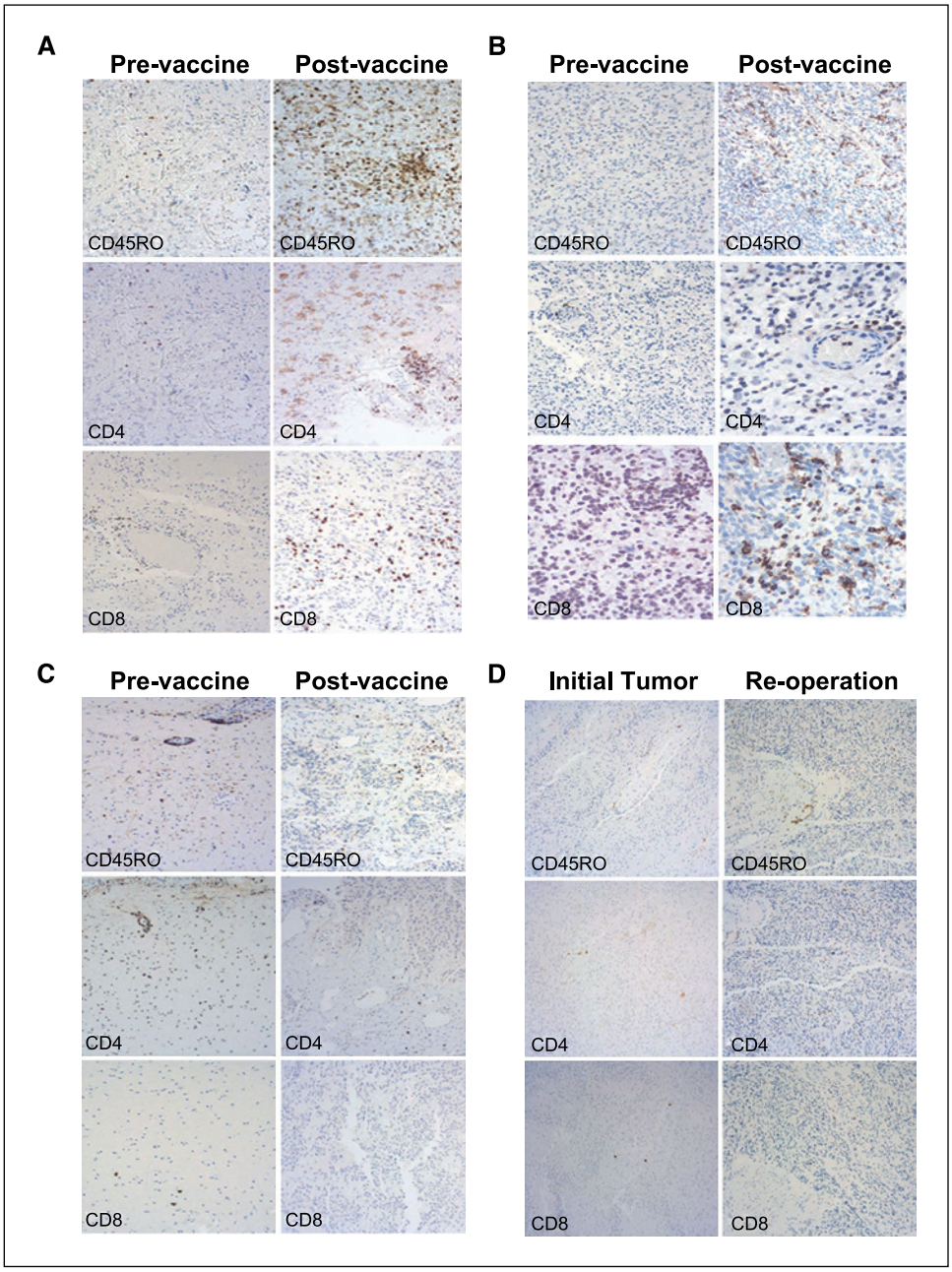

Fig. 4. Increased infiltration of T lymphocytes into glioblastoma multiforme after dendritic cell vaccination. Immunohistochemical characterization of infiltrating T cells in intracranial tumor at initial surgery, before vaccination (*pre-vaccination*) and at reoperation after dendritic cell vaccination (*post-vaccination*). Of the eight patients in the trial who underwent reoperation for tumor progression after dendritic cell vaccination, four patients (*patients 1, 3, 4, and 9*) with prolonged survival (≥2.5 years) tended to have increased intratumoral infiltration of $CD45RO^+$ memory T cells, $CD4^+$ helper T cells, and $CD8^+$ cytotoxic T cells at reoperation post-vaccination (patients 1 and 3, seen in *A* and *B*), while the two patients (*patients 2, 7, and 12*) with shorter survival times (<1 year) had no discernable increase in T-cell infiltration following dendritic cell vaccination (*patient 2, seen in C*). Control patients who did *not* receive dendritic cell immunotherapy also showed no significant difference in T-cell staining at initial surgery versus reoperation (*D*). Immunoperoxidase method, H&E counterstain. Original magnification, ×200.

a multifunctional cytokine that is intimately involved in the suppression of antitumor immune surveillance (46, 47). Like increased TIL, decreased expression of TGF-β_2 has previously been associated with improved survival in glioblastoma multiforme patients (37). To our knowledge, this is the first clinical study to directly correlate the findings of decreased TIL with increased TGF-β_2 in the same human glioma specimens during an active immunotherapy protocol. Collectively, we found that increased intracranial T-cell infiltration and/or low levels of immunosuppressive TGF-β_2 cytokine expression within the local tumor environment may be prerequisites for antitumor efficacy in response to our vaccination strategy. In the future, with advances in the detection of prognostic biomarkers and new developments in molecular/cellular immunoimaging (42), these potentially important predictive factors of vaccine responsiveness could conceivably be determined noninvasively via cerebral spinal fluid sampling and/or *in vivo* neuroimaging techniques.

By virtue of its immunomodulatory properties, there has recently been renewed interest in targeting TGF-β in experimental therapies of human malignant glioma, with several new small molecule inhibitors of TGF-β receptors that are currently being used in preclinical studies (46, 48). Our results suggest promise for using such TGF-β inhibitors in conjunction with active dendritic cell vaccination strategies for possibly synergistic antitumor effect. Unlike the up-regulation of TGF-β_2, however, we did not find any correlation between IL-10 expression and survival.

Regarding the clinical outcome of our vaccinated patients, the number of patients entered into this study was not

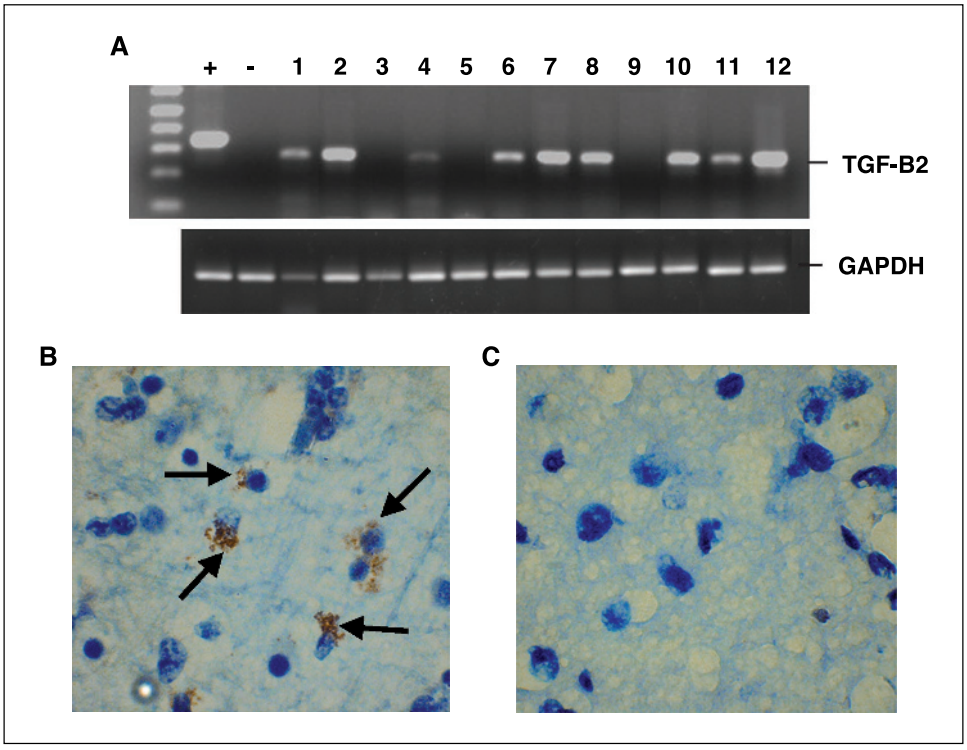

Fig. 5. TGF-β_2 expression in glioblastoma multiforme tumor specimens. *A*, analysis of TGF-β_2 mRNA in tumor tissues isolated from glioblastoma multiforme patients enrolled in the trial. Of note, samples with little or no detectable TGF-β_2 expression (*lanes 1, 3, 4, 5*, and *9*) were associated with patients with positive CTL responses, increased CNS T-cell infiltration, and/or prolonged survival (≥2.5 years) following dendritic cell – based vaccination. (Lane numbers correspond to patient numbers in Table 2). Immunohistochemistry analysis showing (*B*) positive cytoplasmic TGF-β_2 protein expression (*arrows*) in tumor specimen from nonresponder (patient 2, overall survival = 11.4 months), compared with (*C*) negative TGF-β_2 protein expression in tumor sample from potential responder (patient 3, overall survival = 38.9 months). In the TGF-β_2-negative tumor samples, adjacent paraffin-embedded sections were stained for T cells and found to be infiltrated by $CD8^+/CD45RO^+$ TIL. Immunoperoxidase method, hematoxylin counterstain. Original magnification, ×400.

powered to statistically measure efficacy. Nevertheless, the observed 50% 2-year overall survival rate is high, even when compared with the best prognostic groups using recursive partitioning analysis classification for glioblastoma survival (age <50 years and Karnofsky performance score of ≥90). In our study, two patients (patients 5 and 9) are still alive to date and are out to >58.0 and >48.4 months, respectively. For these two long-term survivors, temozolamide (Temodar) was used adjunctively, as patients were allowed to continue with conventional chemotherapy after completion of the dendritic cell injections. Recent evidence has suggested a potential role for temozolamide in synergizing with immunotherapy strategies by either selecting for $CD8^+$ T-cell receptor excision circles (a marker of recent thymic emigrants) or selectively depleting $CD4^+/CD25^+$ T regulatory cells (49).

As with other trials for glioblastoma, age was a prognostic indicator in our cohort of patients. The patients who had longer survival and CNS T-cell responses tended to be younger than those who did not. Recent findings that the reduced thymic T-cell output that accompanies aging is associated with impaired antitumor immunity in glioblastoma patients (50) also support our observation that younger patients may be more likely to develop enhanced migration of tumor-specific T cells into tumors after dendritic cell vaccination.

In our study patients, the ability to elicit a systemic CTL response was negatively correlated with disease burden, as none of the patients with active disease progression at the time of dendritic cell vaccination showed the induction of specific cell-mediated antitumor responses via *in vitro* CTL assays. Furthermore, only those patients whose brain tumors expressed low levels of TGF-β_2 were able to show intratumoral T-cell accumulation and/or objective clinical response. Our data suggest that patients with active bulky tumor residual/progression and elevated TGF-β_2 secretion may harbor greater systemic immune dysfunction, as well as a more profound immunosuppressive milieu within the CNS tumor microenvironment, which may limit the ability of dendritic cell vaccination to induce systemic CTL responses and/or generate local CNS T-cell antitumor responses. The local accumulation of CNS TIL at reoperation correlated better with survival than the magnitude of the systemic CTL response, suggesting that infiltration of brain tumors by systemically activated CTL may be required for antitumor efficacy (43).

Overall, these results could have major implications for patient selection in future studies using immunotherapy for brain tumors. Our data provide further evidence on the feasibility, safety, and *in vivo* bioactivity of autologous peptide-pulsed dendritic cells in patients with glioblastoma multiforme. Although some prolonged survival times have been observed in this select population of patients, proof of clinical benefit remains to be established in future multicenter phase II clinical trials. Nevertheless, this trial provides useful information for future trial design. As with any other targeted treatment modality for glioblastoma, immunotherapy may have potential clinical efficacy if given to the appropriate subgroup of patients and/or if given in combination with other immune pathway modulators, such as TGF-β antagonists. The results of our current and ongoing clinical trials will hopefully help to define which subgroups of patients may respond to tumor vaccination strategies, which in turn would lead to further optimization and refinements of dendritic cell – based immunotherapy with the ultimate goal of developing novel therapeutic vaccines for brain cancer patients.

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