

Gene Expression Profile Correlates with T-Cell Infiltration and Relative Survival in Glioblastoma Patients Vaccinated with Dendritic Cell Immunotherapy

Robert M. Prins^{1,2,3}, Horacio Soto¹, Vera Konkankit¹, Sylvia K. Odesa¹, Ascias Eskin⁴, William H. Yong⁵, Stanley F. Nelson^{2,3,4}, and Linda M. Liau^{1,2,3}

Abstract

Purpose: To assess the feasibility, safety, and toxicity of autologous tumor lysate-pulsed dendritic cell (DC) vaccination and toll-like receptor (TLR) agonists in patients with newly diagnosed and recurrent glioblastoma. Clinical and immune responses were monitored and correlated with tumor gene expression profiles.

Experimental Design: Twenty-three patients with glioblastoma (WHO grade IV) were enrolled in this dose-escalation study and received three biweekly injections of glioma lysate-pulsed DCs followed by booster vaccinations with either imiquimod or poly-ICLC adjuvant every 3 months until tumor progression. Gene expression profiling, immunohistochemistry, FACS, and cytokine bead arrays were performed on patient tumors and peripheral blood mononuclear cells.

Results: DC vaccinations are safe and not associated with any dose-limiting toxicity. The median overall survival from the time of initial surgical diagnosis of glioblastoma was 31.4 months, with a 1-, 2-, and 3-year survival rate of 91%, 55%, and 47%, respectively. Patients whose tumors had *mesenchymal* gene expression signatures exhibited increased survival following DC vaccination compared with historic controls of the same genetic subtype. Tumor samples with a *mesenchymal* gene expression signature had a higher number of CD3⁺ and CD8⁺ tumor-infiltrating lymphocytes compared with glioblastomas of other gene expression signatures ($P = 0.006$).

Conclusion: Autologous tumor lysate-pulsed DC vaccination in conjunction with TLR agonists is safe as adjuvant therapy in newly diagnosed and recurrent glioblastoma patients. Our results suggest that the *mesenchymal* gene expression profile may identify an immunogenic subgroup of glioblastoma that may be more responsive to immune-based therapies. *Clin Cancer Res*; 17(6); 1603–15. ©2010 AACR.

Introduction

Glioblastoma is a lethal malignant brain tumor with overall survival (OS) rates of less than 3.3% at 5 years (1). Glioblastoma remains one of the diseases for which there is no curative therapy. Despite advances in the identification of potential targets for glioma therapy and recent clinical trials utilizing biological therapies and newer cytotoxic agents (2–4), the prognosis of patients with primary malig-

nant brain tumors remains dismal. This sobering fact underscores the need to rethink conventional approaches to the treatment of malignant brain tumors and to base therapeutic strategies on continuing advances in our knowledge of tumor biology and immunology.

The potential therapeutic benefit of eliciting an antitumor immune response in cancer patients was first suggested decades ago. Immunotherapy is theoretically appealing because it offers the potential for a high degree of tumor specificity, while sparing normal brain structures (5). One such approach uses professional antigen-presenting cells, known as dendritic cells (DC), cocultured with autologous tumor lysate to immunologically target endogenous tumor antigens. Initial studies of DC-based vaccine therapy for malignant gliomas have shown acceptable safety and toxicity profiles (6–14), and multicenter randomized phase II and III studies are currently underway.

Previous preclinical studies (15, 16) strongly suggested that toll-like receptor (TLR) agonists (e.g., imiquimod, poly-ICLC), could enhance DC activation and migration, and stimulate T-cell-mediated antitumor immune responses in murine glioma models. To translate these findings, a phase I clinical trial was initiated to evaluate the adjunctive use of

Authors' Affiliations: ¹Department of Neurosurgery, ²Brain Research Institute, ³The Jonsson Comprehensive Cancer Center, ⁴Department of Human Genetics, and ⁵Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, University of California Los Angeles, Los Angeles, California

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Corresponding Author: Robert M. Prins, UCLA Department of Neurosurgery, David Geffen School of Medicine at UCLA, Center for Health Sciences, Room 74-145 CHS, 10833 Le Conte Avenue, Box 956901, Los Angeles, CA 90095-6901. Phone: 310-825-4207; Fax: 310-206-2093. E-mail: rprins@mednet.ucla.edu

doi: 10.1158/1078-0432.CCR-10-2563

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Translational Relevance

The selective identification of patients who will respond to a particular therapy is of paramount importance, especially for patients diagnosed with malignant glioma. Patients diagnosed with glioblastoma (WHO grade IV) have an expected 5-year survival of less than 3.3%. In this study, we report the results of a phase I clinical trial in which glioblastoma patients were treated with a personalized immunotherapy approach, comprised of autologous tumor lysate-pulsed dendritic cell vaccination. In addition, we utilized gene expression profiling to identify a group of patients with a particular gene expression signature (mesenchymal glioblastoma) that had longer survival following dendritic cell vaccination, compared with contemporary/historic control patients of the same gene expression subgroup, who did not receive vaccination. This signature was associated with inflammatory transcripts and enhanced tumor-infiltrating T lymphocytes. Thus, these results suggest that gene expression signatures may be able to identify an immunogenic subgroup of glioblastoma that could be more responsive to immune-based therapies.

DC vaccination with TLR agonists for its feasibility, safety, and toxicity in patients with newly diagnosed and recurrent glioblastoma. Herein, we report the results of this phase I clinical trial, together with immune monitoring data and novel correlative studies associating OS with gene expression signatures and increased tumor-infiltrating lymphocytes (TIL) for the glioblastoma patients.

Patients and Methods

Patient eligibility

This phase I clinical trial was approved by the University of California Los Angeles (UCLA) Institutional Review Board (IRB) and registered with the National Cancer Institute (NCI) as NCT00068510. Written informed consent was obtained from all the patients. Inclusion criteria were newly diagnosed or recurrent glioblastoma (WHO Grade IV) that were amenable to surgical resection, a Karnofsky performance score (KPS) $\geq 60\%$, evidence of normal bone marrow function (e.g., hemoglobin ≥ 9 g/dL, absolute granulocyte count $\geq 1,500/\mu\text{L}$, and platelet count $\geq 100,000$), adequate liver function (SGPT, SGOT, and alkaline phosphatase ≤ 2.5 times upper limit of normal; bilirubin ≤ 1.5 mg/dL), and adequate renal function (BUN or creatinine ≤ 1.5 times institutional normals) prior to starting therapy. Exclusion criteria included allergies to any components of the DC vaccine, concurrent or previous corticosteroid use within 10 days of initial vaccination, the presence of acute infection requiring active treatment, unstable or severe intercurrent medical conditions (e.g., pulmonary, cardiac, or other systemic disease), known immunosuppressive disease, positive serology for HIV or hepatitis B, history of an autoimmune disease, or previous history of other malignancies.

Preparation of autologous tumor lysate

Fresh tumor samples from surgical resection were transported under sterile conditions to the UCLA-Jonsson Cancer Center GMP facility and used to generate autologous tumor lysate, as previously described (8, 17). Tumor tissue was minced, digested in collagenase (Advanced Biofactors) and Dnase-1 (Dornase- α ; Genentech) for 8 to 12 hours at room temperature. To generate lysates, tumor cell suspensions were subjected to 5 freeze-thaw cycles, centrifuged for 10 minutes at $800 \times g$, and the cell-free supernatants were obtained. Protein concentrations of each tumor lysate were determined by a Bio-Rad DC protein assay (Bio-Rad Corp.), and lysates with 100 μg of measured protein were used to pulse DC for each injection.

Preparation of autologous dendritic cells and pulsing with glioma lysate

Monocyte-derived DCs were established from adherent peripheral blood mononuclear cells (PBMC) obtained via leukapheresis performed at the UCLA Hemapheresis Unit. Blood was additionally drawn as a source of autologous serum for the DC cultures. All *ex vivo* DC preparations were performed in the UCLA-Jonsson Cancer Center GMP facility under sterile and monitored conditions. Dendritic cells were prepared by culturing adherent cells from peripheral blood in RPMI-1640 (Gibco) and supplemented with 10% autologous serum, 500 U/mL granulocyte macrophage colony stimulating factor (GM-CSF; Leukine; Amgen) and 500 U/mL of interleukin 4 (IL-4; CellGenix), using techniques described previously (8). Following culture, DCs were collected by vigorous rinsing and washed with sterile 0.9% NaCl solution. The purity and phenotype of each DC lot was also determined by flow cytometry (FACS-can flow cytometer; BD Biosciences). Cells were stained with fluorescein isothiocyanate-conjugated CD83, phycoerythrin-conjugated CD86 and PerCP-conjugated HLA-DR monoclonal antibodies (BD Biosciences). Release criteria were more than 70% viable by trypan blue exclusion, and more than 30% of the large cell gate being CD86⁺ and human leukocyte antigen-DR⁺ (HLA-DR⁺). One day before each vaccination, DC were pulsed (cocultured) with 100 μg of tumor lysate overnight, washed, and the final product was tested for sterility by Gram stain, mycoplasma, and endotoxin testing prior to injection.

Treatment schema

Newly diagnosed glioblastoma patients underwent surgery and a standard course of external beam radiotherapy with concurrent temozolomide chemotherapy prior to DC vaccination (4). These patients were given 3 biweekly DC vaccinations following standard chemoradiation and before adjuvant temozolomide treatment. Recurrent glioblastoma patients had previous radiation therapy and chemotherapy prior to presenting with tumor recurrence, so they underwent surgical resection of their tumors followed by DC immunotherapy after they had recovered from surgery and were tapered off perioperative steroids. This ranged from 7 to 30 weeks after surgery.

Vaccine administration

On the day of each DC vaccination, a 1 mL vaccine dose was drawn into a sterile tuberculin syringe and administered as an intradermal injection (using a 25-gauge needle) in the arm region below the axilla, with the side of administration rotated for each vaccination. Subjects were monitored for 2 hours postimmunization in the UCLA General Clinical Research Center. Eligible patients initially 3 intradermal injections at biweekly intervals. If patients did not develop any toxic side effects from the experimental treatment and had stable disease for more than 3 months, they received booster injections at the same dosage of tumor lysate-pulsed DC concurrently with either 5% imiquimod cream (Aldara, a TLR-7 agonist) or poly-ICLC (Hiltonol, a TLR-3 agonist). Because of initial safety/toxicity concerns of experimental allergic encephalomyelitis (EAE; ref. 18) resulting from the combined use of DC vaccination and TLR agonists, these immune response modifiers were used only in the booster phase of the protocol, after patients had shown acceptable toxicity profiles to DC-lysate vaccinations alone. Booster vaccinations were given at 3-month intervals in between 28-day cycles (5 days on/23 days off) of temozolomide for up to 10 boosters or until tumor progression. For those receiving imiquimod as adjuvant, patients applied 5% imiquimod cream topically over the DC vaccination site 1 day prior to each vaccination cycle, immediately after DC vaccination, and then daily for an additional 3 days postvaccination. For patients in the poly-ICLC cohort, intramuscular injections of 20 µg/kg of poly-ICLC were administered immediately prior to each DC injection at the vaccine injection site. All patients had a baseline brain MRI scan within 1 month prior to starting the immunotherapy and every 2 months thereafter or when clinically indicated.

Patient assessment

Toxicity was monitored and graded according to the NCI Common Toxicity Criteria. The overall incidence of adverse events was recorded. Neurologic examinations were performed before and 30 minutes after each vaccination, and at all follow-up visits. Time to tumor progression (TTP) was defined as the interval from surgical resection until the first observation of tumor progression, as evidenced by MRI or clinical deterioration. Tumor progression was also considered to be nonreversible neurologic progression, permanently increased steroid requirement (applies to stable disease only), or early discontinuation of treatment. OS time was determined from the date of surgery at the time of initial diagnosis of glioblastoma to date of death.

Flow cytometry and cytometric bead array

PBMC from patients enrolled on this clinical trial (pre- and postvaccination) and PBMC from normal volunteers were thawed in warmed RPMI + 2% FBS, washed and stained for the expression of CD3, CD4, and CD25 (all from BD Biosciences), followed by the intracellular labeling of Foxp3 (eBioscience). Stained cells were acquired on a

BD FACS Calibur flow cytometer and analyzed by FloJo software. The frequencies of CD3⁺CD4⁺Foxp3⁺ and CD3⁺CD4⁺CD25⁺Foxp3⁺ PBMCs were compared. For cytokine analysis, serum from patients enrolled on this clinical trial was thawed and incubated with the cytometric bead array (CBA) Human Th1/Th2 Capture Beads (BD Biosciences), washed and subjected to analysis on a BD FACS Calibur flow cytometer together with cytokine standards. Quantitative assessment of cytokine levels was accomplished with a Microsoft Excel-based CBA software program.

Immunohistochemical staining

Serial paraffin sections of pretreatment tumor specimens were cut to 3-µm thickness and stained with anti-human antibodies against CD3 (DakoCytomation) and CD8 (DAKO Corp.). Sections were baked for 1 hour at 60°C, deparaffinized, and endogenous peroxidase activity quenched by treating with 0.5% H₂O₂ in methyl alcohol for 10 minutes. Heat-induced epitope retrieval was performed on the slides using 0.01 mol/L citrate buffer, pH = 6.0 (for CD3, CD8) in a vegetable steamer (Black & Decker); slides were heated for 25 minutes, cooled, and washed in 0.01 mol/L phosphate buffered saline. All slides were then placed on a DAKO Autostainer (DAKO Corp.) and then sequentially incubated in primary antibody for 30 to 60 minutes and in rabbit anti-mouse secondary immunoglobulins (DAKO Corp.) 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 Corp.) were used in place of the primary antibodies. Positive labeling was evaluated and scored by a board-certified neuropathologist (W.H.Y.) in a blinded fashion.

Microarray studies

Of the 23 glioblastoma patients, 16 patients had sufficient residual tumor tissue for microarray molecular analysis at the end of the trial. Total RNA was purified from pretreatment, fresh frozen tumor samples by the RNeasy mini kit (Qiagen) and collected as part of the IRB-approved research protocol. cRNA was generated, quantified, and hybridized to U133 Plus 2.0 arrays at the UCLA DNA Microarray Facility by standard Affymetrix protocols. Cell intensity files (CEL files) were normalized by the Celsius Microarray Database (19), with robust multichip average from Bioconductor (version 2.10) relative to 50 samples of the same platform. The hierarchical clustering (HC) classification for each glioma was determined by a gene voting strategy as described previously (20, 21). Briefly, the mean value of each probeset was evaluated from all samples within the U133 Plus 2.0 platform by the 377 gene probeset list and assigned to an HC group (21). Tumors were assigned to an HC group when the number of probes above the normalized mean was greater than 30% of a given probeset. The OS of patients on this phase I clinical trial was compared with the OS of patients from a collection of samples previously assigned to HC groups (21).

Statistical analysis

TTP and OS curves were determined by the Kaplan–Meier method. The log-rank (Mantel–Cox) test was used to compare curves between study and control groups. All *P* values are 2-tailed, and *P* < 0.05 was considered statistically significant. Statistics were analyzed by GraphPad Prism software.

Results

Patient characteristics

Twenty-three patients with histologically proven WHO grade IV (glioblastoma) were enrolled in this protocol (Table 1): 15 had newly diagnosed tumors, whereas 8 had recurrent disease. There were 16 men and 7 women, with an age range of 26 to 74 years (mean age of 51 years).

DC preparation and phenotype

DCs were generated from adherent PBMC cultured in the presence of 500 U/mL GMP-grade IL-4 and 500 IU/mL of GM-CSF for 1 week prior to harvest, as reported previously (8). All final autologous tumor lysate-pulsed DC preparations consistently contained a high percentage of viable large, granular cells and were free of contamination. Our DC preparations expressed high levels of MHC class I (HLA-A,B,C), MHC class II (HLA-DR), B7.2 costimulatory molecule (CD86), and CD40, but lower expression of CD14 and CD80 (Supplementary Table 1). These DC preparations were partially mature, with less than 45% of the large cells expressing HLA-DR and CD83, as might be expected for a protocol without a dedicated maturation step. Overnight incubation with tumor lysates induced some DC maturation, as evidenced by an increase in the median fluorescence intensity of CD83 (data not shown), similar to previously reported findings (22).

Safety and toxicity

DC vaccinations were well tolerated, with no major adverse events (NCI Common Toxicity Criteria grade 3 or 4) observed in any subject during the vaccine cycles (Table 1). There were no clinical or radiological signs of EAE or other autoimmune reactions in any patient. There were anecdotal cases of transient increased T2/FLAIR and enhancing lesions on MRI after DC vaccination, which may have suggested inflammatory responses post-DC vaccination, particularly in the *mesenchymal* gene-clustered cohort of patients (Fig. 1). However, these MRI changes resolved in due course and did not require surgical intervention. The appearance and disappearance of such MRI findings, presumed to be related to vaccination and neuroinflammation, was noted in 3 of our patients (GBM1-2, 1-3, and 5-4). These 3 patients were in the *mesenchymal* subgroup and are still alive more than 5 years from the initial diagnosis of glioblastoma. Nausea/vomiting, headache and fatigue, diarrhea, low-grade fever, and pain/itching at the injection site were the most common symptoms associated with the treatment (Table 1). Local lymphadenopathy was observed in one patient, temporally coinciding with the expansion of

human cytomegalovirus (HCMV)-specific T-cell expansion (23). In patients who concomitantly received 5% imiquimod cream or poly-ICLC with DC vaccination in the booster phase, no new additional toxicities were reported. Two patients consistently reported transient fevers ($\geq 103^\circ$ F) with each DC + poly-ICLC injection. Cumulatively, these data suggest a low toxicity profile for autologous tumor lysate-pulsed DC plus TLR agonists at all DC dose levels tested.

Systemic cytokine responses and regulatory T-cell populations following DC vaccination with TLR agonists

Others have assessed systemic immune responsiveness from autologous tumor lysate-pulsed DC vaccination by either delayed type hypersensitivity skin testing (6, 12) or by restimulating PBMC with lysate-pulsed DC *in vitro*, followed by assessment of IFN- γ (10, 12). However, the correlations with clinical outcome have not been consistent. In this trial, we elected to assess for more global systemic cytokine responses and changes in regulatory T-cell (Treg) frequency that may be induced by our vaccination strategy.

Peripheral blood changes in the frequency of CD3⁺CD4⁺Foxp3⁺ T cells were compared before and after DC vaccination for patients with available pre- and posttreatment PBMC. We observed that glioblastoma patients on this clinical trial possessed increased frequencies of peripheral blood CD3⁺CD4⁺Foxp3⁺ or CD3⁺CD4⁺CD25⁺Foxp3⁺ lymphocytes compared with normal volunteers (Fig. 2A). However, at the time points measured, there were no relevant changes in the frequency of this lymphocyte population after immunotherapy that statistically correlated with clinical outcome (data not shown).

To assess the cytokine microenvironment after DC vaccination with and without the addition of TLR agonists, we performed CBAs from patient serum during the time course of the clinical trial to evaluate Th1- and Th2-type cytokine levels. Detectable increases in serum TNF- α and IL-6 were observed after DC vaccination (Fig. 2B; Supplementary Fig. 1A). However, the serum cytokine levels were variable between patients and the magnitude of changes did not seem to correlate with clinical outcome. Log-fold elevations in serum TNF- α and IL-6 were observed after booster DC vaccinations with either 5% imiquimod cream or 20 μ g/kg poly-ICLC (Fig. 2C; Supplementary Fig. 1B). To assess whether the Th1/Th2 cytokine balance might be relevant, we calculated ratios of each Th1-type cytokine with Th2-type cytokines to generate an effector/regulatory cytokine ratio (Fig. 2D). However, such information was also not significantly associated with the clinical outcome (data not shown), although our sample numbers may have been too small to detect statistical significance.

Dose escalation

A typical dose escalation scheme was performed with autologous tumor lysate-pulsed DC vaccination, using 1,

Table 1. Patient characteristics

Patient ID	Tumor pathology	Age	Gender	KPS	OS (mo)	HC type	Dose (10%)	Adjuvant	Prevaccination Tx	Postvaccination Tx	Related adverse events
GBM1-1	GBM	39	M	90	33.83	*	1	Imiquimod	Temozolomide	Temozolomide, isotretinoin, celecoxib, reoperation, stereotactic radiosurgery	Fatigue, nausea/vomiting, diarrhea
GBM1-2	GBM	39	M	90	>88.87	Mes	1	Imiquimod	Temozolomide	Temozolomide, isotretinoin, CCNU, Gliadel	Fatigue, arthralgia, low-grade fever
GBM1-3	GBM	34	M	90	>91.3	Mes	1	Imiquimod	Temozolomide	Temozolomide, isotretinoin	Lymphadenopathy, injection site reaction, low-grade fever, myalgia
GBM1-4	rec. GBM	61	M	70	18.57	.	1	None	Temozolomide, thalidomide, isotretinoin, Newcastle virus		
GBM1-5	GBM	58	M	70	10.3	Pro	1	None	Temozolomide	Irinotecan, bevacizumab	Shingles
GBM1-6	GBM	63	F	80	>37.6	Mes	1	Imiquimod	Temozolomide	Temozolomide	
GBM1-7	rec. GBM	41	F	80	10.93	Mes	1	None	Irinotecan, bevacizumab	Irinotecan, bevacizumab	
GBM1-8	rec. GBM	34	M	100	>40.5	PN	1	Poly-ICLC	Erlotinib, temozolomide, ANG, CCNU, celecoxib, tamoxifen	CCNU, celecoxib, tamoxifen	
GBM1-9	GBM	50	M	90	>9.03	.	1	Poly-ICLC	Temozolomide		
GBM5-1	GBM	40	F	80	17.97	.	5	None	Temozolomide, isotretinoin	CCNU, gefitinib, rapamycin, carboplatin	Injection site reaction
GBM5-2	rec. GBM	54	M	80	17.3	.	5	None	Temozolomide, isotretinoin, CCNU	Carboplatin	Allergic rhinitis, itching at injection site, pruritis

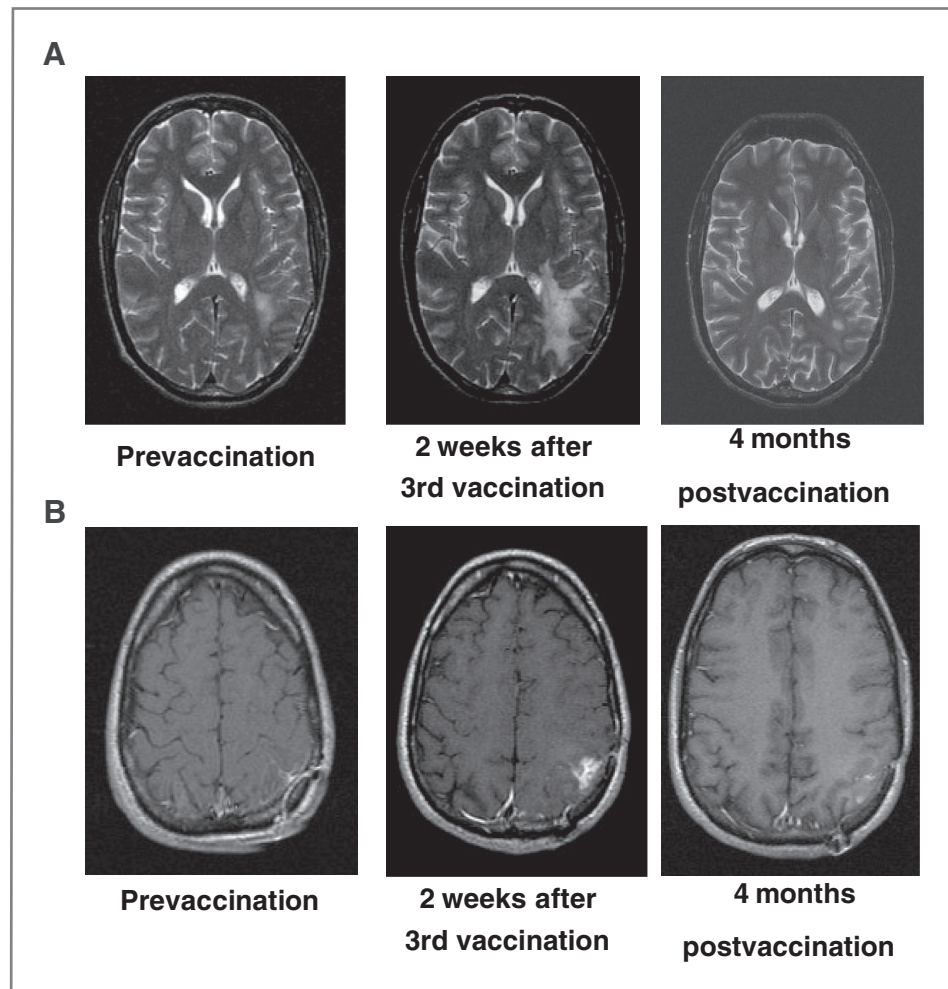
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Table 1. Patient characteristics (Cont'd)

Patient ID	Tumor pathology	Age	Gender	KPS	OS (mo)	HC type	Dose (10 ⁶)	Adjuvant	Prevaccination Tx	Postvaccination Tx	Related adverse events
GBM5-3	GBM	26	M	90	81.4	PN	5	Imiquimod	Temozolomide, isoretinoin, CCNU	Carboplatin, irinotecan, bevacizumab, dasatanib, simvastatin, rosiglitazone, procarbazine, CCNU, cyclophosphamide	Injection site itching, dryness and pruritus, lymphadenopathy, nausea, diarrhea, vomiting, fatigue
GBM5-4	GBM	43	M	90	>59.0	Mes	5	Imiquimod	Temozolomide	Temozolomide	
GBM5-5	GBM	45	F	90	34.97	PN	5	None	Temozolomide	Irinotecan, bevacizumab, CCNU, erlotinib	
GBM5-6	rec. GBM	53	M	80	22.33	Mes	5	Poly-ICLC	Temozolomide, Gliadel	Irinotecan, bevacizumab	Nausea, heartburn, constipation
GBM10-1	rec. GBM	58	F	70	28.93	PN	10	None	Temozolomide, irinotecan, paclitaxel		Headaches, nausea/vomiting
GBM10-2	GBM	70	F	100	23.0	.	10	None	Temozolomide	p-EGFR/p-ErbB2 inhib, everolimus, carboplatin, bevacizumab, CCNU	
GBM10-3	GBM	50	M	90	36.33	.	10	Imiquimod	Temozolomide	Irinotecan, bevacizumab	Dermatitis, rash
GBM10-4	GBM	59	M	80	52.6	Pro	10	Imiquimod	Temozolomide, isoretinoin	Irinotecan, bevacizumab, carboplatin, CCNU	Anorexia, abdominal pain
GBM10-5	GBM	64	M	90	13.63	Mes	10	None	Temozolomide	Bevacizumab, CCNU	
GBM10-6	GBM	66	M	90	37.73	Mes	10	Imiquimod	Temozolomide	Irinotecan, bevacizumab, CCNU, etoposide, procarbazine, tamoxifen	Fatigue, left shoulder pain/arthritis
GBM10-7	rec. GBM	74	F	60	17.07	Mes	10	None	Temozolomide	Temozolomide	
GBM10-8	rec. GBM	52	M	60	16.23	PN	10	None	Temozolomide		Upper lip blisters, rash

Abbreviation: Tx, treatment.

Figure 1. MRI changes after DC vaccination. Transient increase in MRI T2/FLAIR lesions (A) and contrast enhancement (B) observed in a primary, newly diagnosed glioblastoma patient following DC vaccination (patient GBM5-4). Axial T2/FLAIR (A) and T1/contrast (B) MRI scans taken at 2 weeks prevaccination, 2 weeks postvaccination, and 4 months later.



5, and 10 million DC administered intradermally. A fixed amount of lysate (100 μ g) was added to the DC and incubated overnight prior to injection. The patient characteristics and survival data for each dose cohort are outlined in Supplementary Table 2. In this dose escalation trial, there was no relationship between increasing DC dose and toxicity or specific adverse events of any kind. There were also no DC dose-dependent differences in immunologic responses tested. As seen in Supplementary Table 2, the median OS was actually longer in the 1 million DC dose cohort compared with the higher dose cohorts. However, these differences in OS were not statistically significant, given the small sample size in each dose cohort and age differences between groups.

Survival analysis

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 prior to therapy), and every 8 weeks thereafter as surrogate markers for clinical response and tumor status. Objective clinical data are summarized in the following text and are listed in Table 1. When considering all 23 glioblastoma

patients enrolled in this clinical trial (newly diagnosed and recurrent patients), the median TTP was 15.9 months. The median OS time, taken from the date of initial surgical diagnosis of glioblastoma, was 31.4 months. OS from the time of initial diagnosis at 1, 2, and 3 years was 91%, 55%, and 47%, respectively. If we include only those who received the DC vaccine in the newly diagnosed setting ($n = 15$), the median OS is 35.9 months, with a mean follow-up time of more than 4 years, and 1-, 2-, and 3-year survival rates of 93%, 77%, and 58%, respectively. For recurrent patients that enrolled in our vaccine trial ($n = 8$), the median OS was 17.9 months from the time of initial glioblastoma diagnosis. OS was significantly longer for those who received DC vaccination at initial diagnosis compared with those who enrolled in this trial at the time of recurrence ($P = 0.03$; Supplementary Fig. 2).

Microarray gene expression profiling

Because gene expression patterns have been shown to be highly correlated with survival in various cancers, we investigated whether the genetic signature of glioblastomas (20) was associated with clinical outcome in this DC immunotherapy trial. In patients where pretreatment tumor

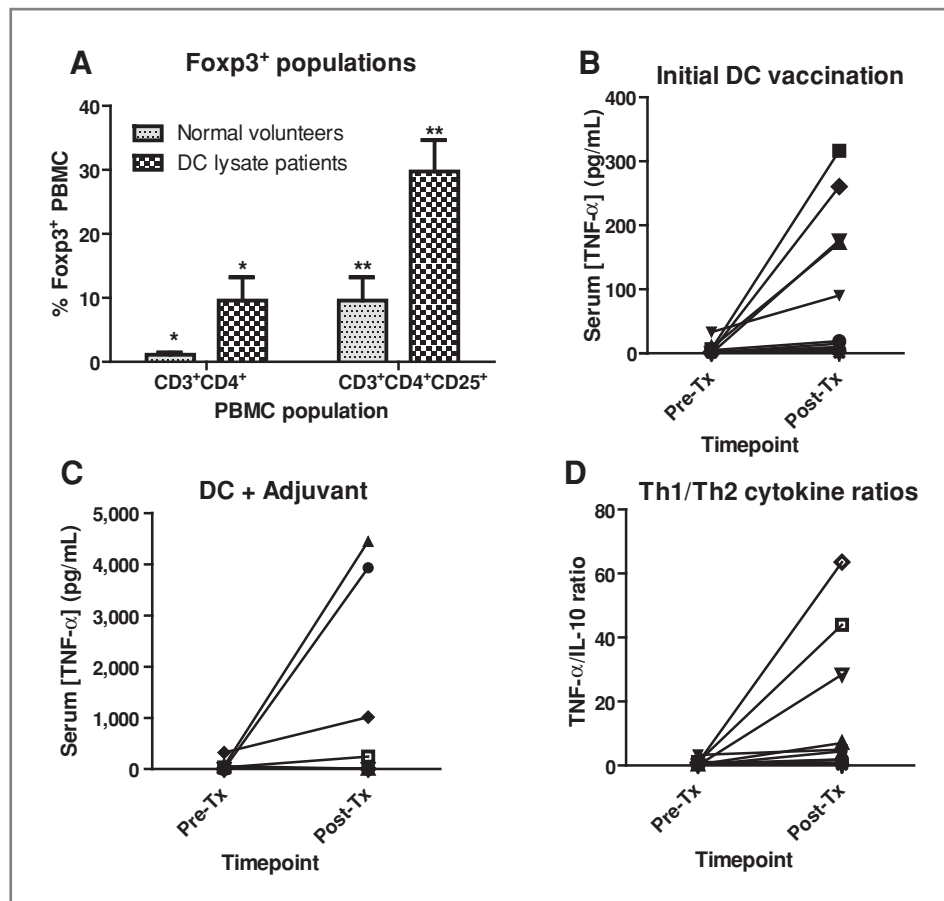


Figure 2. Peripheral blood immune monitoring data. A, PBMCs from normal volunteers and DC trial patient prevaccination timepoints were thawed and stained for the expression of CD3, CD4, and CD25, followed by the intracellular labeling of Fosp3. Stained cells were acquired on a BD FACS Calibur flow cytometer and analyzed by FloJo software. The frequencies of CD3⁺CD4⁺Fosp3⁺ and CD3⁺CD4⁺CD25⁺Fosp3⁺ PBMCs between normal volunteers and glioblastoma patients enrolled in this trial are compared. *, $P = 0.04$; **, $P = 0.01$. B and C, serum cytokine responses, measured prevaccination and day 14 postvaccination, after the initial course of DC vaccination (B) or after booster DC vaccinations with either 5% imiquimod or poly-ICLC (C). Serum from patients enrolled on this clinical trial was thawed, labeled with CBA antibody-coated beads, washed, and subjected to analysis on a BD FACS Calibur flow cytometer together with cytokine standards. Quantitative assessment of cytokine levels was accomplished with a Microsoft Excel-based CBA software program. D, Th1/Th2 cytokine ratios. Raw cytokine data for serum TNF- α and IL-10 at each timepoint were divided to generate a Th1/Th2 ratio. Tx, treatment.

samples were available, we performed microarray-based gene expression classification as previously published (20, 21). As shown in Figure 3, gene expression profiling of our pretreatment tumor samples produced the typical proneural (PN), proliferative (Pro) and *mesenchymal* (Mes) hierarchical clusters, using probesets previously described by our group (20, 21). Furthermore, we validated these hierarchical clusters using the UCSF-Genentech and TCGA probesets (24, 25), which yielded similar gene expression signatures for our DC lysate patients (data not shown).

The *mesenchymal* gene expression signature is defined by overexpression of many inflammatory-associated genes. Thus, we hypothesized that there might be a difference in the clinical outcome of patients on our trial that could be linked to the local microenvironment of the original tumor. To control for any selection bias that might have been introduced by the requisite eligibility criteria for patients receiving the DC vaccine (i.e., subjects needing

to be alive and off steroids long enough for vaccine preparation and administration), we eliminated any control patients that died within approximately 250 days of initial diagnosis for the purposes of our comparative analysis. We also stratified for patients who received radiation alone versus radiation plus concurrent temozolomide chemotherapy after initial surgical resection, and found no statistical difference in these 2 groups when the early progressors (OS < 250 days) were eliminated. As shown in Figure 4, patients enrolled on our trial with the *proneural* gene expression signature had an OS that was indistinguishable from a set of 60 contemporary *proneural* tumors analyzed from UCLA and 3 other institutions (ref. 21; $P = 0.664$; Fig. 4A). In contrast to this, patients in our DC vaccine trial with *mesenchymal* gene expression signatures had a significantly extended survival compared with 82 concurrently collected tumors that were found to have these same gene expression signatures ($P = 0.0046$;

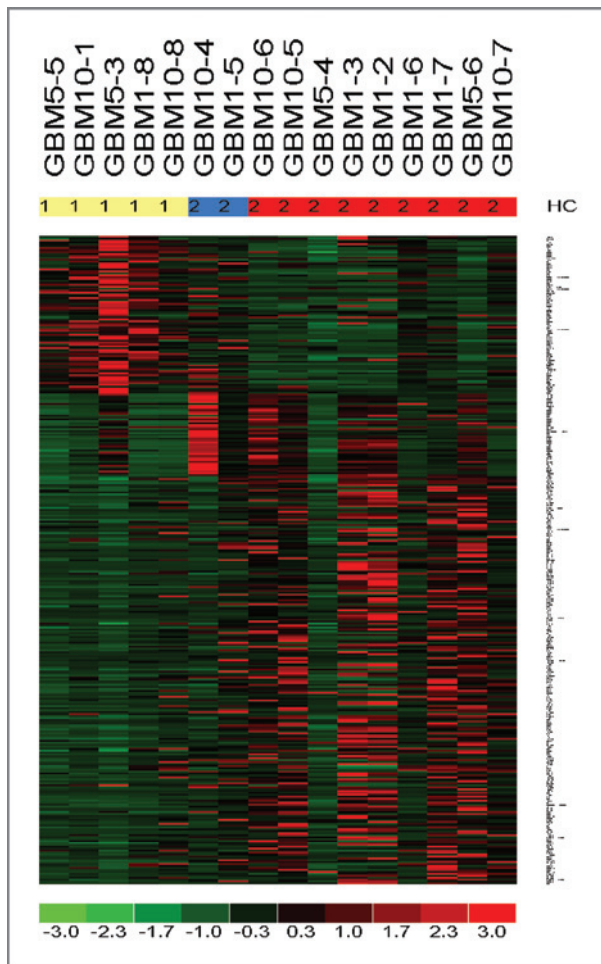


Figure 3. Microarray-based, expression profiling of pretreatment glioblastoma samples from DC vaccine patients. Total RNA was isolated from frozen, surgically resected tumors and subjected to global gene expression classification by Affymetrix human U133 Plus 2.0 microarray chips. Sufficient fresh-frozen tissue was available for extraction of high-quality RNA (without amplification) in 17 of the cases. PN (HC1, yellow legend), Pro (HC2A, blue legend), and Mes (HC2B, red legend) gene expression signatures were identified by probesets previously published (21). Heat maps were created by the dChip microarray software program.

Fig. 4B). Although these data are not intended to represent efficacy, such information is noteworthy because glioblastoma patients with *mesenchymal* gene expression patterns typically have the worst prognosis and are the most refractory to current therapies (21, 24, 25).

Gene expression signature and tumor-infiltrating lymphocytes

The density and location of T lymphocyte accumulation within certain solid tumors have been associated with extended survival (26, 27), and recent evidence suggests that such a correlation may exist in malignant glioma (28). However, an association with the subtype of tumor or treatment modality has not been addressed.

Because the Mes expression signature includes numerous genes associated with inflammation, and tumor-specific T

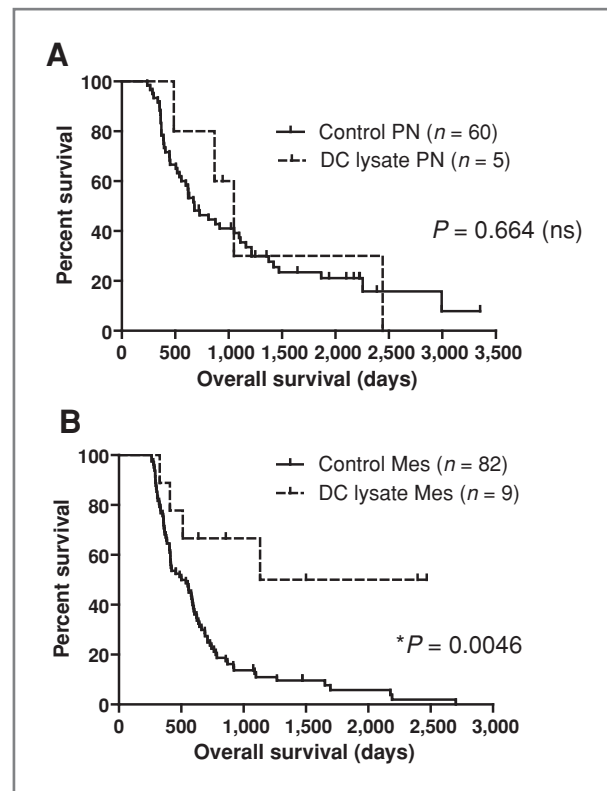


Figure 4. Extended survival in DC-vaccinated patients with *mesenchymal* gene expression signatures, but not in patients with a *proneural* signature. The OS time of DC vaccine patients expressing a (A) PN gene signature ($n = 5$) or (B) Mes gene signature ($n = 9$) was compared with the survival generated from a control, multi-institutional dataset of PN ($n = 60$) or Mes glioblastomas ($n = 82$; solid lines) previously published by our group (21). To accurately account for the potential bias associated with the time delay needed to generate the DC vaccine, we omitted control patients that experienced early progression (<250 days). PN comparison: $P = 0.664$ (not statistically different, ns); Mes comparison: $P = 0.0046$ by the log-rank (Mantel-Cox) test calculated in GraphPad software.

cells are known to be attracted to proinflammatory signals, we evaluated whether patients on our DC trial with *mesenchymal* gene expression signatures also had increased TILs. As shown in Figure 5, tumors with a *mesenchymal* gene expression signature had significantly increased CD3⁺ and CD8⁺ TILs compared with PN tumors ($P = 0.006$). Although our sample size is small, we also found qualitatively increased CD3⁺ and CD8⁺ TIL density after DC vaccination in tumors resected at recurrence (Fig. 5B). In post-DC-vaccinated tumors resected/biopsied at the time of recurrence, increases in CD3⁺ and CD8⁺ TILs were associated with the *mesenchymal* gene expression profile, but not necessarily with the dose of DC given. Such findings point to a potential mechanism by which distinct glioblastoma tumor subtypes might be differentially responsive to immune-based therapies.

Discussion

In this phase I study, we report the safety, feasibility, and bioactivity of a vaccine comprised of autologous DC pulsed

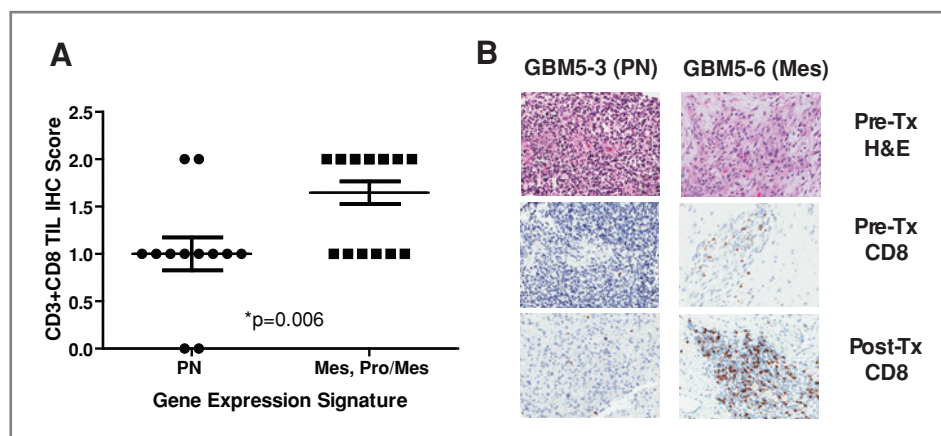


Figure 5. Increased density of CD3⁺ and CD8⁺ lymphocytes in Mes gene expression groups compared with PN tumor sections. A, 3- μ m paraffin-embedded adjacent tissue sections from DC-vaccinated patients were stained separately with CD3 and CD8 antibodies and scored in a blinded fashion by a neuropathologist (W.H.Y.). The immunohistochemical (IHC) scores were compared between samples known to be PN ($n = 5$) versus Mes tumor samples ($n = 9$). *, $P = 0.006$ by 2-tailed t test calculated in GraphPad software. B, representative hematoxylin & eosin staining and CD8 IHC staining (pre- and post-DC vaccination) of a PN and Mes glioblastoma showing increased CD8⁺ TILs in the Mes glioblastoma. Original magnification, $\times 400$. Tx, treatment.

with autologous tumor lysate as an adjuvant following surgical resection with standard chemoradiotherapy. Unlike our previous reported DC vaccination strategy (8) and those reported by other groups (6, 9–13, 29), we included "booster" vaccinations with the innate immune response modifiers, 5% imiquimod (Aldara), or poly-ICLC (Hiltonol) based on our preclinical studies suggesting that proinflammatory innate immune signals could enhance DC activation, trafficking to lymph nodes, and the priming of antitumor antigen-specific T lymphocytes (15). There were no dose-limiting toxicities and no detectable differences in safety or efficacy among the 3 DC dose levels tested. Of note, there was a significant difference in the average age of patients in the 10 million DC cohort compared with the other dose cohorts, which could influence the difference in OS. However, another possible hypothesis is that this trend in the data was a reflection of a dilutional decrease in antigens available for presentation by DC at the highest DC dose cohort (10×10^6 cells), given that the quantity of lysate was fixed (at 100 μ g per dose) despite the increased DC cell dose.

The concomitant administration of 5% imiquimod or poly-ICLC with DC vaccination was also found to be safe and did not result in any additional toxicity or adverse events. To our knowledge, this is the first report of the use of TLR agonists in conjunction with DC vaccination strategies in brain tumor patients. Because TLR agonists were used only in patients in the booster phase, it is unclear whether or to what extent the addition of the TLR agonists contributed to the potential efficacy and OS of these patients. Furthermore, imiquimod and poly-ICLC are two different biological agents, targeting different TLRs. Imiquimod activates TLR-7 whereas poly-ICLC activates TLR-3, but both induce proinflammatory cytokine secretion. These complexities make it somewhat difficult to determine how

these innate immune modifiers actually contributed to our study endpoints. Nevertheless, this study establishes the safety of these TLR agonists in conjunction with glioma lysate-loaded DC, and further phase II studies directly comparing these TLR agonists at the time of initial vaccination (not only in the booster phase) are currently underway.

Although the number of glioblastoma patients entered in this phase I clinical trial was not powered to measure efficacy, the clinical results of this trial are still noteworthy. The median OS from the time of initial surgical diagnosis was 31.4 months for all glioblastoma patients ($n = 23$) treated in this study, including both those enrolled as newly diagnosed and recurrent tumor patients. For those treated in the newly diagnosed setting, the OS was 35.9 months; and the OS was 17.9 months for those who received vaccination at recurrence. In addition, we have had 3 patients surviving more than 6 years to date. Such statistics are compelling in the face of the expected median survival for this disease, which is currently still reported as approximately 14 months for newly diagnosed patients that receive standard surgery, radiation, and temozolomide chemotherapy (4, 30, 31). This compares favorably even when compared with published data for the best clinically defined prognostic group of glioblastoma patients (recursive partitioning analysis; RPA class III: age < 50 years and KPS ≥ 90), whose 2-year survivals were 40% and 29% for RPA III and IV patients, respectively, following treatment with standard radiation and temozolomide (31). Such data are also favorable compared with other recent brain tumor DC-based vaccine trials without booster injections and TLR adjuvants, where the OS was reported as 21.4 months (mean, 11 newly diagnosed and 23 recurrent glioblastoma patients; ref. 10) and 9.6 months (median) in a recurrent glioblastoma population (6).

Glioblastomas are primarily identified by histologic features assigned to cytologically malignant, mitotically active, necrosis-prone tumors established by the WHO (32). Such histologic features are generally associated with patient survival, together with performance status, extent of surgical resection, and age. Yet, histologically identical tumors can behave in different ways: a situation that may underlie the biology of this heterogeneous disease. More recently, extensive genetic profiling of these tumors has been able to identify molecularly classifiable subgroups of glioblastoma (i.e., PN, Pro, and Mes subtypes; refs. 20, 21, 24, 33–38), which can better predict survival than conventional histopathologic analysis. Such new classification techniques are of interest so that patients can be more appropriately stratified for new treatment strategies (20).

The Mes subgroup of glioblastomas typically have a poorer prognosis than the more common PN subgroup (21, 24). However, in our study, patients with the *mesenchymal* gene expression signatures had significantly extended survival compared with a large, multi-institutional cohort ($n = 82$) of glioblastoma samples of the same molecular subgroup treated with various other therapies. No such survival difference was observed in patients from this clinical trial with *proneural* signatures, compared with other control glioblastoma subjects of the PN subgroup ($n = 60$). Admittedly, such comparisons with concurrent and historic controls are not meant to imply efficacy, because this phase I trial did not have a prospectively matched, placebo-controlled arm. Although some prognostic factors, such as age and Karnofsky performance status, were relatively matched in our comparison groups, the extent of surgical resection was not directly compared between the patients in this trial and our concurrent/historic controls. Because we need adequate amounts of tumor (>2 g) to generate the autologous vaccines, tumor resectability was taken into account in the eligibility criteria. Therefore, it is possible that the extent of surgical resection may have been greater in our DC-vaccinated patients compared with concurrent/historic controls, which could have influenced our survival results. Nevertheless, the median OS (31.4 months) of our DC-vaccinated patients is still noteworthy, when compared with large series of glioblastoma patients who underwent gross total tumor resections and were treated with concomitant chemoradiotherapy, where the median survival was reported to be 18.6 months (31).

It is unclear whether the extended survival of our patients with *mesenchymal* gene expression signatures is a direct result of the vaccine effects, or good responses to follow-up therapies after failing the vaccine. Because Mes signatures represent glioblastoma subgroups that are more resistant to conventional therapy, it can be speculated that DC vaccination somehow makes these tumors more susceptible to subsequent treatments (39). Because adjuvant temozolomide treatment was coordinated into the schedule of the DC booster vaccinations, this is a difficult distinction to make from our study design. Nevertheless, our results suggest that *mesenchymal* gene

expression signatures express elevated inflammatory gene transcripts and possess an increased density of tumor-infiltrating CD3⁺ and CD8⁺ lymphocytes compared with glioblastomas expressing other genetic signatures. As such, we hypothesize that the expression of inflammatory genes (e.g., IL-1R, TNF- α signaling factors, and chemokines) may facilitate the priming and trafficking of tumor-specific T cells into the tumor parenchyma, which might be enhanced by DC vaccination and innate immune response modifiers. Hence, the *mesenchymal* gene expression signature may have a direct impact on the bioactivity of the vaccine itself, irrespective of postvaccine therapy. Prospectively designed, randomized, multicenter phase III clinical trials will be required to validate such hypotheses and proof of clinical benefit remains to be established.

Overall, the results reported here may provide novel insights for prospective patient selection in future immunotherapy studies and lend additional credence for the ability of genetic expression signatures to impart relevant data for personalized cancer treatment. On the basis of the results of this phase I trial, we will continue developing more advanced clinical trials with this particular approach. We currently are planning a randomized, multicenter phase II/III clinical trial of DC vaccination for newly diagnosed glioblastoma (DCVax-Brain), which will hopefully help to further define which subgroups of patients may respond to tumor vaccination strategies. This in turn may lead to further optimization and refinements of related trials of DC-based vaccines for patients with glioblastoma, with the ultimate goal of developing novel immunotherapeutic strategies for brain cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Timothy Cloughesy, MD, and Albert Lai, MD, PhD for helpful discussions and comments.

Grant Support

This work was supported in part by NIH/NCI grants K01-CA111402 and R01-CA123396 (to R.M. Prins), R01 CA 112358 (to L.M. Liao), the Philip R. and Kenneth A. Jonsson Foundations, the Neidorf Family Foundation, the Ben & Catherine Ivy Foundation, and Northwest Biotherapeutics, Inc. R.M. Prins is the recipient of the Howard Temin NCI Career Development award and STOP Cancer Career Development award. Flow cytometry was performed at the UCLA Jonsson Comprehensive Cancer Center (JCCC) Core Facility and gene expression was performed in the JCCC Gene Expression Shared Resource, which are supported by the NIH award CA16042. Microarray analysis was supported by the Carson Foundation. Tissue acquisition and IHC was supported by the UCLA Brain Tumor Translational Resource (BTTR), whereas the General Clinical Research Center was supported by M01-RR00865.

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Received September 23, 2010; revised November 19, 2010; accepted November 23, 2010; published OnlineFirst December 6, 2010.

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