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Vaccination with Tumor Lysate-Pulsed Dendritic Cells Elicits Antigen-Specific, Cytotoxic T-Cells in Patients with Malignant Glioma

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ABSTRACT

The primary goal of this Phase I study was to assess the safety and bioactivity of tumor lysate-pulsed dendritic cell (DC) vaccination to treat patients with glioblastoma multiforme and anaplastic astrocytoma. Adverse events, survival, and cytotoxicity against autologous tumor and tumor-associated antigens were measured. Fourteen patients were thrice vaccinated 2 weeks apart with autologous DCs pulsed with tumor lysate. Peripheral blood mononuclear cells were differentiated into phenotypically and functionally confirmed DCs. Vaccination with tumor lysate-pulsed DCs was safe, and no evidence of autoimmune disease was noted. Ten patients were tested for the development of cytotoxicity through a quantitative PCR-based assay. Six of 10 patients demonstrated robust systemic cytotoxicity as demonstrated by IFN-γ expression by peripheral blood mononuclear cells in response to tumor lysate after vaccination. Using HLA-restricted tetramer staining, we identified a significant expansion in CD8+ antigen-specific T-cell clones against one or more of tumor-associated antigens MAGE-1, gp100, and HER-2 after DC vaccination in four of nine patients. A significant CD8+ T-cell infiltrate was noted intratumorally in three of six patients who underwent reoperation. The median survival for patients with recurrent glioblastoma multiforme in this study ($n = 8$) was 133 weeks. This Phase I study demonstrated the feasibility, safety, and bioactivity of an autologous tumor lysate-pulsed DC vaccine for patients with malignant glioma. We demonstrate for the first time the ability of an active immunotherapy strategy to generate antigen-specific cytotoxicity in brain tumor patients.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive and common primary brain tumor, accounting for 50% of intracranial gliomas and 25% of intracranial tumors in adults (1). GBM diagnosis carries with it a median survival of 12–18 months (with 90–95% of patients surviving <2 years), without the possibility of spontaneous remission (2). Current treatment, consisting of surgical resection followed by radiation therapy and chemotherapy, has not substantially changed the bleak prognosis for GBM patients, despite the efficacy of similar therapies for patients with non-glioma tumors (3, 4). Even the few treatments that have been found to be effective against GBM typically either exhibit only small increases in survival in large population studies or primarily benefit certain patient subpopulations, such as the young (5, 6). The overwhelming majority of patients with malignant glioma, composed of GBM and anaplastic astrocytoma (AA), experience increasingly rapid tumor recurrence after surgical resection or other treatment and eventually succumb to such events. Thus, novel therapies for malignant gliomas and recurrent gliomas, in particular, are desperately needed.

Cancer vaccines represent one novel form of therapy for recurrent malignant glioma (7). The clinical efficacy of therapeutic vaccination for any human tumor, however, remains controversial because consistent tumor destruction or extended life span is not observed in most vaccinated cancer patients (8–11). In contrast, current cancer vaccines do reliably elicit tumor-reactive CTLs in most patients (8–11). Consistent with this finding, we reported previously that therapeutic vaccination with autologous tumor peptide-pulsed dendritic cells (DCs) is sufficient to enhance peripheral tumor-reactive CTL activity and CD8+ T-cell infiltration into tumors *in situ* in newly diagnosed GBM patients (12).

In the above-mentioned study, vaccine generation was constrained by the time required to obtain peptide antigen from each patient's autologous cultured tumor cell line. The generation of such lines is particularly difficult in recurrent patients, whose tumors have been exposed to radiation. We therefore examined the ability of tumor lysate-pulsed DCs to activate peripheral tumor-reactive CTL activity and CD8+ T-cell infiltration into tumors *in situ* in recurrent malignant glioma patients.

Here, we use autologous tumor lysate-pulsed DCs to introduce undefined tumor-associated antigens (TAAs) to the T cells of patients with recurrent as well as newly diagnosed malignant glioma. Three biweekly intradermal lysate-pulsed DC vaccinations were administered to nine patients with recurrent GBM and three patients with recurrent AA. One patient with newly diagnosed GBM and one patient with newly diagnosed AA were also treated.

A potential risk of vaccine therapy, particularly for tumors within essential organs such as the brain, is destructive autoimmunity. Traditionally, the concern over autoimmunity has been addressed by targeting vaccines to specific antigenic epitopes not present on vital host tissues. Vaccines using undefined mixtures of tumor antigens have been reported to support sustained antitumor responses more effectively than peptide-based vaccines. A balance, however, must be struck between specificity and the ability of the tumor to evade responses directed toward a limited set of antigens. In addition, as vaccines for brain tumors become increasingly effective for treating patients, it will be necessary to move from approaches at higher risk for destructive autoimmunity toward those specifically targeting tumor tissue. One such method would be to use glioma-associated antigens. We sought to characterize common tumor antigen epitopes to which malignant glioma patients responded after vaccination with undefined autologous tumor antigen. We recently demonstrated the expression of TAAs in glioblastoma and demonstrated the presentation of some common TAA epitopes in a MHC-restricted fashion (13, 14). We used these findings to determine whether DC vaccination generated T-cell clones that recognized these epitopes.

No significant adverse effects were associated with DC vaccination. One patient was tested for tumor-specific T-cell cytotoxicity through a conventional CTL assay and demonstrated robust cytotoxicity after vaccination. Ten additional patients had pre- and postvaccination CTL assays based on quantitative reverse transcription-PCR detection of IFN- γ expression. Six patients demonstrated significant peripheral cytotoxicity postvaccination based on IFN- γ expression. A subset of patients demonstrated the generation of not only tumor-specific T cells but also tumor antigen-specific T cells after DC vaccination. Six patients underwent reoperation for recurrence, and three of these patients demonstrated significant CD4+ and CD8+ tumor infiltration. We also compared survival rates for the eight study patients with recurrent GBM with those of 26 control patients with recurrent GBM

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who underwent craniotomy at our institution around the same time period. Tumor lysate-pulsed DC vaccination was associated with a 133-week median survival as compared with a 30-week median survival for 26 control patients. This Phase I study demonstrates the feasibility and safety of an autologous peptide-pulsed DC vaccine for malignant glioma.

MATERIALS AND METHODS

Patient Population. Inclusion criteria were a Karnofsky score of ≥60, an age of ≥18 years, lowest possible maintenance dose of glucocorticoid therapy, and the following normal baseline hematological parameters (within 1 week before first vaccination): hemoglobin >9.9 g/dl; total granulocyte count >1,000/ml; platelet count >60,000/ml; BUN <30 mg/dl; creatinine <2 mg/dl; alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase <2× the upper limit of normal; and a prothrombin time and activated partial thromboplastin time no greater than 1.4× control, unless therapeutically warranted.

Exclusion criteria included pregnancy; severe pulmonary, cardiac, or other systemic disease associated with an unacceptable anesthetic or operative risk; presence of an acute infection requiring active treatment; history of an autoimmune disorder; or prior history of other malignancies, excluding basal cell carcinoma and benign tumors. Patients were required to use a medically accepted form of birth control during the study.

There were 14 patients enrolled in the study (4 women and 10 men), with an age range from 28 to 61 years (Table 1). Patients with verified histological diagnoses of recurrent AA (three patients) or recurrent GBM (nine patients) were eligible and enrolled in this study after surgery. Newly diagnosed patients with GBM (one patient) and AA (one patient) were also enrolled.

Autologous Tumor Culture. After surgical resection, tumor samples were processed for tissue culture by mincing them with scissors and passing them through metal meshes of decreasing pore size. The cell suspension was then plated onto tissue culture flasks and grown in DMEM/F10 (Irvine Scientific, Santa Ana, CA) plus 10% FCS (Irvine Scientific) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA).

HLA Typing and Tumor Antigen PCR. Briefly, GBM cells were stained with biotin-conjugated HLA-A2- or HLA-A1-specific monoclonal antibody (US Biological, Swampscott, MA) or biotin-conjugated isotype control antibody. After streptavidin-PerCP (BD PharMingen, San Diego, CA) staining for 30 min, the mean fluorescence intensity of HLA-A2 staining was analyzed by flow cytometry. Tumor antigen expression was examined by reverse transcription-PCR. Primers used for PCR were as follows: (*a*) β-actin, 5′-AATCTGGCACCACACCTTC-TAC-3′ (forward) and 5′-CTTCTCCTTAATGTCACGCACG-3′ (reverse), amplification product = 394 bp; (*b*) HER-2, 5′-TCTGACGTCCATCGTCTCTG-3′ (forward) and 5′-AGGGCATAAGCTGTGTCACC-3′ (reverse), amplification product = 458 bp; (*c*) gp100, 5′-TGGCTCTTGGTCTCAGAAGA-3′ (forward) and 5′-AGGTGCAGTGCTTATGACTT-3′ (reverse), amplification product = 669 bp; and (*d*) MAGE-1, 5′-GCCTGCTGCCCTGACGAGAG-3′ (forward) and 5′-AGGAGAGACCTAGGCAGGTG-3′ (reverse), amplification product = 559 bp.

Preparation of Tumor Lysate. Tumor samples from surgery were processed in the laboratory to produce single-cell suspensions as follows: the surgical specimen was washed thrice in dissection medium (HBSS + 30 μg/ml catalase + 6.6 μg/ml desferoxamine + 25 μg/ml *N*-acetyl cysteine + 94 μg/ml cystine-2HCl + 1.25 μg/ml superoxide dismutase + 110 μg/ml sodium pyruvate + 2.4 μg/ml HEPES + 0.36% glucose + 800 μM $MgCl_2$ + 100 units/ml Fungi-Bact). Then, the specimen was minced with scissors and passed through metal meshes of decreasing pore size (0.38 and 0.14 mm), followed by a nylon mesh with a pore size of 0.21 mm. Cells were lysed by four freeze (on liquid nitrogen)/thaw cycles (room temperature). Lysis was monitored by light microscopy, and larger particles were removed by centrifugation (1900 rpm for 10 min at 4°C). Supernatants were then passed through a 0.2 μm filter, the protein concentration was determined by Bio-Rad protein assay, and aliquots were frozen at −80°C until use.

Preparation of Autologous DCs. Mononuclear cells were isolated using leukapheresis. PBMCs were obtained fresh for each vaccine 7 days before vaccination. A COBE Spectra Apheresis System was used to harvest the mononuclear cell layer. Leukapheresis yielded 10^{10} PBMCs. These cells were allowed to become adherent for 2 h at 37°C in tissue culture flasks at a concentration of 5×10^6 cells/ml in RPMI 1640 (Gibco, Gaithersburg, MD) with 10% autologous heat-inactivated serum. After 2 h at 37°C, nonadherent cells were removed by washing with warm complete medium. To generate autologous DCs, adherent PBMCs were cultured in complete medium for 7 days in the presence of recombinant human granulocyte macrophage colony-stimulating factor (800 units/ml; clinical grade; Immunex, Seattle, WA) and recombinant human interleukin (IL)-4 (500 units/ml; R&D Systems, Minneapolis, MN).

Pulsing of Autologous DCs with Autologous Tumor Lysate. On the day before each of the three DC vaccinations (days −1, 13, and 27), DC cultures containing 10^7-10^8 cells were washed in RPMI 1640 with autologous patient serum supplemented with 50 μg/ml autologous tumor lysate. The DCs were incubated overnight for 18 h at 37°C with tumor lysate on a tissue rotator to facilitate their interaction.

DC Phenotypic Evaluation. After 7-day maturation in granulocyte macrophage colony-stimulating factor and IL-4, DCs were harvested from flasks. Cells were resuspended in PBS containing 2% fetal bovine serum (v/v) and stained with anti-CD14 FITC, anti-HLADR phycoerythrin (PE), and biotinylated anti-CD86, anti-CD83, anti-CD54, and anti-CD40 antibodies (BD PharMingen). Species and isotype-matched monoclonal antibodies were used as controls.

DC Functional Assay and Vaccine Administration. For the functional assay, DCs irradiated with 2500 rads were resuspended in RPMI 1640–10% human AB blood phenotype serum at 2×10^5 cells/ml. Allogeneic PBMCs were mixed with DCs. Phytohemagglutinin alone was added to PBMCs as a positive control. RPMI 1640 culture medium alone added to PBMCs constituted a negative control. All assays were performed in triplicate. The assay plate was incubated for 6 days in a 37°C/5% CO_2 incubator. [^{3}H]Thymidine

Table 1 *Patient characteristics, treatment, and general clinical outcome*

Patient no.	Gender	Age (yrs)	Tumor pathology	Pre-vac[a] KPS	Image complete resection	Pre-vac Rx	Survival (weeks)	Adverse events
1	F	37	GBM	100	Yes		A 238[b]	
2	M	46	GBM	100	No	IC BCNU, BCNU	79	
3	M	28	AA	100	Yes	IC BCNU	32	Headache, fatigue
4	M	38	GBM	90	Yes	IC BCNU	238[b]	
5	F	55	GBM	80	Yes	IC BCNU, SRS	183	Erythema
6	F	32	GBM	100	Yes	IC BCNU, BCNU	204[b]	
7	M	34	AA	100	Yes	Antineoplaston, PCV	66	Headache
8	M	56	GBM	60	No		27	Headache, seizure
9	M	44	GBM	90	No	PCV, BCNU, SRS, IA Carbo, IC CCNU	138	
10	M	42	AA	100	Yes	BCNU	244[b]	
11	F	47	GBM	100	Yes	IC BCNU	29	
12	M	49	GBM	90	No	Tamoxifen, VP-16	90	
13	M	61	AA-N	100	Yes		78	Seizures
14	M	57	GBM-N	90	Yes		82	

[a] Pre-vac, prevaccination; KPS, Karnofsky performance score; Rx, treatment; GBM, glioblastoma multiforme; IC, intracranial; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; AA, anaplastic astrocytoma; SRS, stereotactic radiosurgery; PCV, procarbazine, IA, intra-arterial; Carbo, carboplatinum; CCNU, vincristine; -N, newly diagnosed.
[b] Patient still alive.

(1 μCi/well) was added for the final 18 h of culture (*i.e.*, on day 5). Cells were harvested with a Harvester 96-cell harvester (Tomtec, Hamden, CT), and ^{3}H counts were determined with a Microbeta 1450 Trilux liquid scintillation counter (Wallac, Gaithersburg, MD). Patients received 10^7-10^8 tumor-specific, tumor lysate-pulsed DCs s.c. in 0.5 ml of PBS in the deltoid region. Three vaccinations at 2-week intervals were administered to each patient.

Immunohistochemistry. Serial 10-μm paraffin sections of surgical intracranial tumor specimen were stained with mouse antihuman monoclonal antibodies against CD8 (C8/144B clone M7103 at 1:25 dilution), CD45RO (OPD4 clone M0834 at 1:50 dilution), CD20 (L26 clone M0755 at 1:200 dilution), and CD56 (T199 clone M0852 at 1:10 dilution; DAKO Corp., Carpinteria, CA). Primary antibodies were detected using the biotin-peroxidase system (DAKO Corp.).

Cytotoxic T-Cell Assay. A single patient grew sufficient autologous tumor cells to assess cytotoxicity using the JAM assay (15). Briefly, archived PBMCs were stimulated by autologous cultured tumor cells (irradiated with 11,000 rads) for 6 days in RPMI and recombinant human IL-2 (20 units/ml). Tumor cells were labeled as targets in [^{3}H]thymidine (5 μCi/ml) for 48 h at 37°C in 5% CO_2. After incubation, target cells in maximum release wells were lysed with 5% SDS in H_2O and incubated in DNase 1 (Roche, Indianapolis, IN) at a final concentration of 20 units/ml for 10 min. Cells were harvested from plates with a Harvester 96 cell harvester (Tomtec). Tritium ^{3}H counts were determined using a Microbeta 1450 Trilux liquid scintillation counter (Wallac).

***In Vitro* Sensitization, RNA Isolation, and cDNA Synthesis.** Cryopreserved PBMCs from patients 1 week before therapy and 2 weeks after their third vaccination were thawed in complete medium. Cells were plated at 3×10^6 PBMCs and 3×10^5 DCs in 2 ml of medium with 10 μg/ml tumor lysate. IL-2 (300 IU/ml) was added on day 2, and the cells were harvested between days 10 and 13 after initiation of the culture. The harvested cells were then stimulated with tumor cells or autologous PBMCs pulsed with 10 μg/ml tumor lysate for 4 h at 37°C. The cells were collected to extract RNA with RNeasy minikits (Qiagen, Santa Clarita, CA). The RNA was eluted with water and stored at −70°C. For cDNA synthesis, about 1 μg of total RNA was transcribed with cDNA Transcription Reagents (Perkin-Elmer Corp., Foster City, CA) with the use of oligo(dT). cDNA was stored at −30°C until quantitative real-time PCR was performed.

Quantitative Real-Time PCR. Gene expression was measured with the use of the Icycler System (Bio-Rad) as described previously (16). Primers and TaqMan probes (Qiagen) were designed to span exon-intron junctions to prevent amplification of genomic DNA and also to produce amplicons of fewer than 150 bp to enhance the efficiency of PCR amplification. Probes were labeled at the 5′ end with the reporter dye molecule 6-carboxy-fluorescein (FAM; emission max = 518 nm) and at the 3′ end with the quencher dye molecule 6-carboxytetramethyl-rhodamine (TAMRA; emission max = 582 nm). On amplification, probes annealed to the template were cleaved by the 5′-nuclease activity of the Taq polymerase reaction. This process separates the fluorescent label from the quencher and allows release of 1 unit of fluorescence for each unit of amplification. The amount of fluorescence required with each cycle makes it possible to determine the number of cycles necessary to reach a certain amount of fluorescence in a test sample compared with known standard amounts of template provided as a standard curve. DNA standards were generated by PCR amplification of gene products and purification, whereas quantification was determined by spectrophotometry (absorbance at 260 nm). The number of copies was calculated by dividing the total sample weight by the molecular weight of each gene amplicon. Real-time PCRs of cDNA specimens and DNA standards were conducted in 25 μl with 1× TaqMan Master Mix (Perkin-Elmer Corp.). Primers were used at 400–600 nM, and probes were used at 160 nM. Primer sequences and probes were as follows: (*a*) IFN, 5′-AGCTCTGCATCGTTTTGGGTT (forward), 5′-GTTCCATTATCCGCTACATCTGAA (reverse), and FAM-TCTTGGCTGTTACTGCCAGGACCCA-TAMRA (probe); and (*b*) CD8α, 5′-CCCTGAGCAACTCCATCATGT (forward), 5′-GTGGGCTTCGCTGGCA (reverse), and FAM-TCAGCCACTTCGTGCCGGTCTTC-TAMRA (probe). Thermal cycler parameters included 2 min at 50°C, 10 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Standard curves were generated for both IFN-γ and CD8α. PCR efficiency was assessed from the slopes of the standard curves and was between 90% and 100%. Linear regression analysis of all standard curves demonstrated a coefficient of determination (R2) of 0.99 or higher. Standard curve extrapolation of each copy number was performed for both IFN-γ and CD8α. Normalization of sample data was done by dividing the number of copies of IFN transcripts by the number of copies of CD8α transcripts, representing the relevant cell population. The results of IFN-γ fold increase postvaccination compared with prevaccination were presented.

Data were adjusted for CD8 mRNA copies on the basic immunological assumption that stimulation with a HLA class I-restricted epitope defines CD8+ T cells as the only relevant population. Because the frequency of CD8+ T cells varies over time in each patient, we believe that it is suboptimal to present data corrected by expression of housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase or β-actin, expressed by any cell, even though this correction has been done in other quantitative real-time PCR applications. We calculated the ratio of IFN-γ mRNA (corrected for CD8 mRNA) obtained from PBMCs stimulated with autologous tumor lysate to that obtained from PBMCs stimulated without tumor lysate. The cutoff value for tumor-specific IFN-γ was derived by analyzing the IFN-γ:CD8 ratios in PBMCs obtained from all patients postvaccination *versus* prevaccination. A cutoff value of 1.5 has been a published standard for evidence of vaccine-related tumor-specific cytotoxic response (16).

Epitope-Specific T-Cell Staining with HLA-Restricted Tetramers. PE-peptide loaded HLA tetramer (tHLA) complexes were synthesized and provided by Beckman Coulter as described previously (17). Recombinant HLA-A*0201 or HLA-A0101 heavy chain containing a biotinylation site and recombinant β2-microglobulin were synthesized and used for refolding of soluble HLA (sHLA) molecules in the presence of a HLA-A*0201 binding peptide. sHLA molecules were prepared for the following epitopes: HLA-A1-restricted MAGE-1 (amino acids 161–169) antigen peptide (EADPTGHSY); HLA-A2-restricted HER-2 (amino acids 369–377) peptide (KIFGSLAFL); and gp100 (amino acids 209–217) peptide (ITDQVPFSV). All peptides were commercially synthesized and purified by gel filtration (Macromolecular Resource, Fort Collins, CO). The refolding reaction was dialyzed and concentrated for purification of correctly refolded sHLA on gel filtration. Monomeric sHLA was biotinylated with BirA (Avidity, Denver, CO) at the heavy chain and separated from free biotin by gel filtration. Biotinylated sHLA was tetramerized by adding avidin-PE (Pierce, Rockford, IL) at a 4:1 molar ratio. The final concentration of tetramer was adjusted to 1 μg/ml. As examined by gel filtration, all tHLAs were without detectable free avidin-PE. After overnight depletion of monocytes, nonadherent PBMCs were resuspended at 10^6 cells/50 μl in ice-cold fluorescence-activated cell-sorting buffer (phosphate buffer plus 5% inactivated FCS; Biofluids), and cells from day 10 CTL cultures were washed and resuspended at 2×10^5 cells/50 μl in cold fluorescence-activated cell-sorting buffer. Cells were incubated on ice with 1 μg of tHLA for 15 min, and incubation was then continued for 30 min with 10 μl of anti-CD8 monoclonal antibody (Becton Dickinson, San Jose, CA). Cells were washed twice in 2 ml of cold fluorescence-activated cell-sorting buffer before fluorescence-activated cell-sorting analysis (Becton Dickinson). Two hundred thousand events were acquired. tHLA staining specificity was previously established by extensive analysis of T-cell clones specific for each of the described epitopes and by comparative analysis of short-term CTL cultures also specific for the above-mentioned epitopes (18).

Statistical Analysis. Data were analyzed with a SAS statistical software package. Means are reported with SD. Continuous variables were compared using Student's *t* test, and categorical variables were compared using χ^2 or Fisher's exact test. Any *P* values less than 0.05 were considered statistically significant. The estimated probability of survival was demonstrated using the Kaplan-Meier method. The Mantel Cox log-rank test was used to compare curves between study and control groups.

RESULTS

Isolation and Characterization of DCs. In this study, each patient's tumor was resected, and the surgical tissue was lysed and used as an antigen source. Mononuclear cells were isolated by Ficoll gradient centrifugation and differentiated into DCs in the presence of IL-4 and granulocyte macrophage colony-stimulating factor. DCs from each study patient were shown to develop high levels of MHC class II and costimulatory molecule B7-2 (CD86) and showed the absence of markers for mature monocytes (CD14) and macrophages

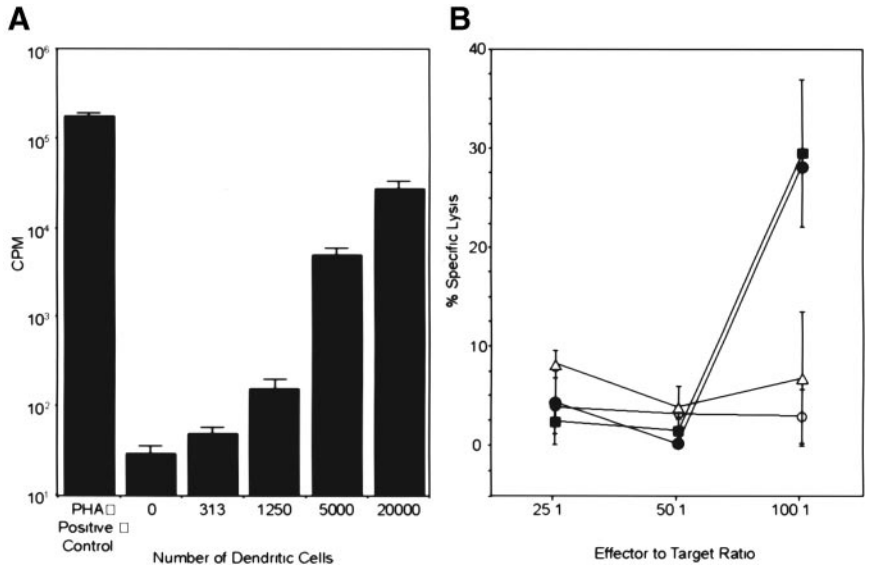

Fig. 1. *A*, dendritic cell (DC) function assay by allostimulation. Irradiated DCs (at the indicated concentrations) or phytohemagglutinin (to 5 mg/ml) was added to 5×10^5 allogeneic peripheral blood mononuclear cells (PBMCs)/ml in medium and incubated for 5 days. Proliferation was assessed by incorporation of [^{3}H]thymidine in PBMCs after 18 h of additional incubation. The graph depicts DC activity from a patient before preparation of vaccine. *B*, peripheral cytotoxicity. PBMCs were collected from patients before and after DC/tumor lysate administration and stimulated *in vitro* for 6 days with irradiated autologous tumor cells in the presence of 10 units/ml interleukin 2. CTL activity was determined by specific fragmentation of ^{3}H-labeled DNA in autologous tumor targets at various E:T ratios using the JAM test. The graph depicts CTL activity from patient 2 six weeks before the first vaccination (○), as well as 1 week before the first vaccination (△), 1 week after the second vaccination (■), and 1 week after the third immunization (●).

(CD11b). DCs were CD54+, CD40+, and phenotypically immature (CD83−). Allogeneic proliferation assays confirmed that the DCs isolated were functional (Fig. 1). Furthermore, sufficient numbers of functional DCs were isolated in all patients in this clinical trial (at least 1×10^7 cells for each of three vaccinations). DCs were differentiated, cocultured with autologous tumor lysate, washed, and administered to patients thrice over the course of 6 weeks.

Safety of Autologous DC Administration. There were no grade III or IV National Cancer Institute Common Toxicity Criteria adverse affects associated with vaccination in this trial. There were no serious adverse events other than death from tumor progression. Three patients complained of transient headaches that were relieved by analgesics on 1, 3, and 5 days after one of the vaccinations (Table 1). Two patients complained of fatigue during the course of the treatment (Table 1). One patient developed erythema at the site of vaccination. Two patients developed generalized seizures 5 and 7 days after vaccination, which resolved with antiepileptic medication. There was no clinical or radiological evidence of autoimmune response in any patient. Autopsies performed on two patients did not show evidence of an autoimmune response. There were some CD45RO+ memory T cells observed in areas of tumor in the brain of one of these patients. CD8+ cytotoxic cells were infrequently observed in the tumors of both patients.

Development of Systemic Cytotoxicity. One goal of this clinical trial was to determine whether *ex vivo* differentiation of DCs and exposure to tumor lysate antigen could induce an immune response after vaccination of malignant glioma patients. Because of previous treatment of patients with radiation, the tumor specimen from only a single patient grew sufficient glioma cells in culture to test cytotoxicity through a conventional CTL assay. Cytotoxicity of PBMCs directed toward autologous tumor cells was assessed in patient 2. This patient demonstrated significant tumor cytotoxicity as early as 1 week after his second vaccination, and this cytotoxicity was sustained after his third vaccination. Ten additional patients underwent measurement of CTL responses through a reverse transcription-PCR-based method to measure IFN-γ mRNA expression. The method controlled for CD8+ mRNA in PBMC samples taken 1 month after the last vaccination as compared with prevaccination samples. Six of 10 patients elicited significant IFN-γ mRNA accumulation in response to autologous tumor lysate after vaccination as determined by at least a 1.5-fold increase in IFN-γ mRNA postvaccination (Table 2).

DC Vaccination Induces the Expansion of Tumor Antigen-Specific Cytotoxic T-Cell Clones. The distribution of HLA-A2 and HLA-A1 and expression of TAAs HER-2, MAGE-1, and gp100 are shown in Table 3. Based on the prevalence of certain TAAs in primary glioma cells, we investigated whether PBMCs from vaccinated pa-

Table 2 *Identification of vaccine-specific CD8+ T cells in PBMCs[a] of patients by tHLA staining and IFN-γ expression by real-time PCR*

PBMCs isolated from glioma patients before and after vaccination with tumor lysate-pulsed dendritic cells were assessed for tumor-specific cytotoxicity by means quantitative PCR for IFN-γ message after *in vitro* exposure to autologous tumor lysate as well as for the presence of antigen-specific CD8+ CTL clones using flow cytometry for HLA-restricted tetramer staining. Tumor-specific cytotoxicity was determined to be positive if IFN-γ message in postvaccination PBMCs was at least 1.5× greater than that in prevaccination PBMCs (bold values; see "Materials and Methods." Numbers in the CTL columns represent the percentage of CD8+ cells that stained positive for antigen-specific tetramer binding.

Patient no.	IFN-γ fold increase Post *vs.* Pre	HER-2-specific CTLs		gp100-specific CTLs		MAGE-1-specific CTLs	
		Pre	Post	Pre	Post	Pre	Post
3	0.96	ND	ND	ND	ND	ND	ND
4	ND	0.3	**0.91**	0.17	**0.49**	0.23	**0.45**
5	**5.32**	ND	ND	ND	ND	0.03	0.02
6	**2.73**	0.37	**2**	0.12	**1.02**	0.08	**0.97**
7	**1.6**	0.5	**1.82**	0.09	**0.77**	ND	ND
8	0.61	0.02	0	0.02	0.02	ND	ND
9	**7.14**	ND	ND	ND	ND	0.01	0.01
10	**1.54**	ND	ND	ND	ND	0.16	**1.47**
12	**1.55**	ND	ND	ND	ND	ND	ND
13	0.57	0.99	0.11	0.55	0.23	0.06	0.02
14	1.02	0.63	0.29	0.85	0.21	ND	ND

[a] PBMC, peripheral blood mononuclear cell; tHLA, peptide loaded HLA tetramer; Post, postvaccination; Pre, prevaccination; ND, not done (due to insufficient cells to perform real-time PCR or HLA-A2/A1 mismatch for specific tetramer analysis).

Table 3 *HLA-A1 and HLA-A2 and tumor antigen distribution in patients*

HLA-A1 and HLA-A2 typing was determined by flow cytometry analysis using specific antibody staining. HER-2, gp100, and MAGE-1 expression on tumor cells was determined by reverse transcription-PCR.

Patient no.	HLA-A1	HLA-A2	HER-2	gp100	MAGE-1
1	−[a]	−	+	−	−
2	+	−	−	−	+
3	−	−	−	+	−
4	+	+	+	+	+
5	+	−	+	−	−
6	+	+	+	+	+
7	−	+	+	+	−
8	−	+	−	−	−
9	+	−	−	−	−
10	+	−	+	−	+
11	−	−	+	−	+
12	−	−	+	+	+
13	+	+	+	+	+
14	−	+	+	+	−

[a] −, negative, +, positive (see "Materials and Methods.").

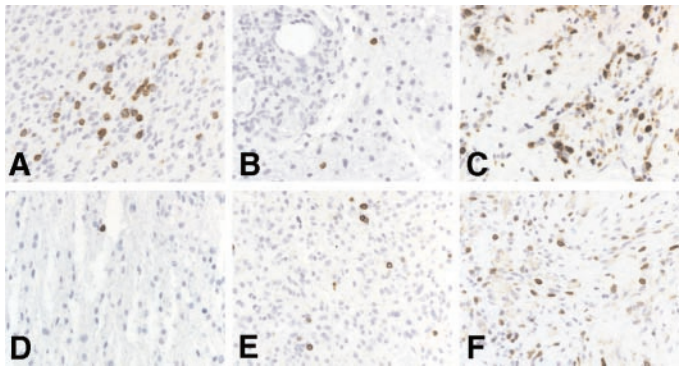

Fig. 3. Immunohistochemical characterization of infiltrating cells in intracranial tumor before and after dendritic cell vaccination. *A*, intratumoral CD8+ cells, prevaccination. *B*, intratumoral CD45RO+ cells, prevaccination. *C*, intratumoral CD8+ cells in tumor periphery, postvaccination. *D*, intratumoral CD45RO+ cells in tumor periphery, postvaccination. *E*, intratumoral CD8+ cells in central area of scar surrounded by tumor, postvaccination. *F*, intratumoral CD45RO+ cells in central area of scar surrounded by tumor, postvaccination.

tients contained CTL clones specific for these antigens including gp100, HER-2, and MAGE-1. Using HLA-restricted tetramer staining, we identified a significant increase in the number of CD8+ antigen-specific T-cell clones after DC vaccination in four of nine informative patients (Table 2). All three positive, informative patients also demonstrated cytotoxicity based on IFN-γ mRNA expression of PBMCs in response to tumor lysate. Fig. 2 shows the tetramer analyses of the four positive patients.

Intratumoral T-Cell Infiltration. We then investigated whether these immune responses could access intracranial tumor. We analyzed tumor tissue for immune cell infiltration from patients who underwent surgical resection after radiologically identified disease progression. We identified CD45RO+ memory T cells and CD8+ cytotoxic T cells in surgical specimens isolated from three of six patients vaccinated with tumor lysate-primed DCs. This T-cell infiltration was not present in tumor tissue resected from the same patients before vaccination therapy. Patient 2 developed tumor recurrence as shown by magnetic resonance imaging with a gadolinium-enhancing mass 3 months after his last vaccination. He underwent re-resection of the mass. There was a moderate number of CD45RO+ memory T cells present in the surgical specimen, whereas there were few cells observed in the surgical specimen from his operation before vaccination (Fig. 3). There was also significant CD8+ cell infiltration in the postvaccination surgical specimen, which was infrequently detected in his surgical specimen before vaccination (Fig. 3).

DC Vaccination Is Associated with Prolonged Survival. A further objective of this study was to determine the clinical response to DC vaccination. Therefore, we compared survival in our patient populations with age, gender, and disease-matched controls, who had not received DC therapy. Study patients with recurrent GBM were included in a survival analysis. Therefore, the four patients with AA, a patient with newly diagnosed GBM, and a patient with recurrent

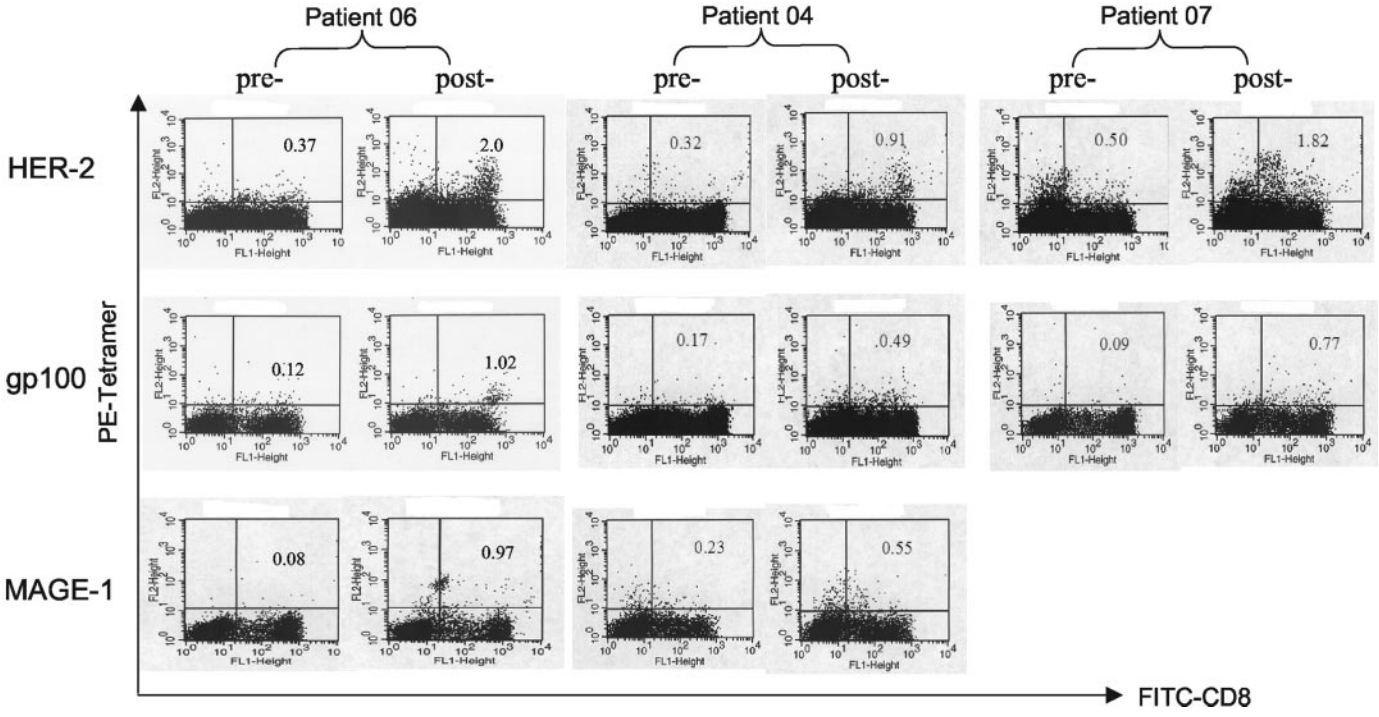

Fig. 2. Identification of tumor-associated antigen-specific T cells in four vaccinated patients' peripheral blood mononuclear cells (PBMCs). PBMCs were stained with HLA-A2-restricted tetramers for HER-2, gp100, and HLA-A1-restricted tetramer for MAGE-1 (*Y* axis), and then cells were stained for the CD8 (*X* axis). The *number* shown in the plots indicates the percentage of tumor-associated antigen-specific T cells in whole PBMC population. Data of three representative patients are presented.

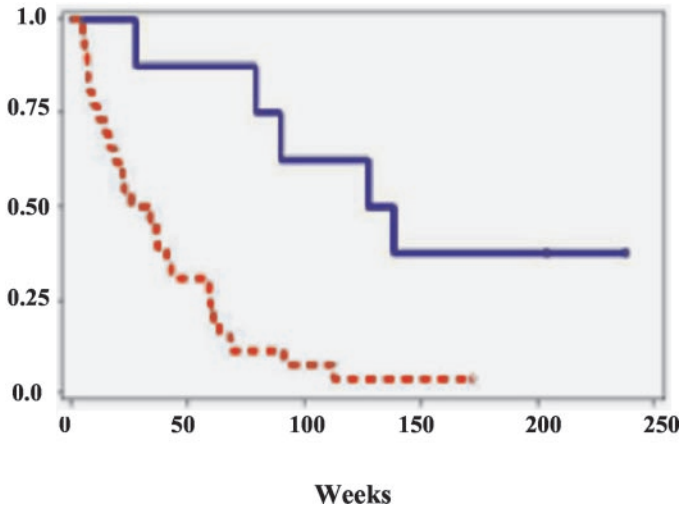

Fig. 4. Kaplan-Meier survival curve of study group ($n = 8$; *solid line*) and control group ($n = 26$; *dashed line*) of patients with recurrent glioblastoma multiforme from time of second craniotomy ($P = 0.003$, log-rank test).

GBM who received only two vaccinations because of noncompliance were excluded from the survival analysis. There were 8 patients in the study group and 26 in the control group with recurrent GBM who underwent craniotomies at our institution. Control patients underwent repeat craniotomy by the same surgeons as the study group and had completed external beam radiation therapy of 60 Gy. They met all inclusion criteria for the DC immunotherapy trial, including a Karnofsky score of ≥60, but were treated before the trial opening (within 2 years) or chose not to participate. There were no statistically significant differences between the study and control groups for age (44.0 ± 9.1 *versus* 53.0 ± 13.8 years; $P = 0.09$) or percentage of patients with image complete resections (50% *versus* 58%; $P = 1.0$). The median survival for the study and control groups was 133 and 30 weeks, respectively. The Mantel Cox log-rank test revealed that the survival curves for the two groups were significantly different ($P = 0.0013$; Fig. 4.).

DISCUSSION

Previous immunotherapeutic treatments for brain tumors used passive, adoptive, and nonspecific strategies that yielded limited benefits (19). These treatments included the intrathecal or intratumoral administration of autologous lymphocytes, IL-2, lymphokine-activated killer cells, or IFNs (20–23). The absence of a significant antitumor effect in these prior studies may have been due to the nonspecific immune response that these approaches generate. Active immunotherapy requires the presentation of tumor antigens by antigen-presenting cells with the goal of inducing an antigen-specific T-cell response.

To investigate the potential of this therapy in a clinical setting, we used DC-based vaccination protocols in two Phase I clinical trials. Through these trials, we demonstrated the safety and bioactivity of this immunotherapy approach in patients with malignant brain tumors. The first trial involved nine patients with newly diagnosed glioma (seven patients with GBM and two patients with AA), who were treated with a series of three intradermal vaccinations of DCs pulsed *ex vivo* with autologous tumor cell surface-derived peptides isolated by means of acid elution (12). The second clinical trial involved 14 patients, 12 of whom had recurrent disease (9 patients with GBM and 3 patients with AA), whereas 2 patients were newly diagnosed (1 patient each with GBM and AA). These patients received DC vaccines that had been pulsed *ex vivo* with whole tumor lysate, which we hypothesized could serve as an optimal source of as yet unidentified immunogenic tumor antigens.

In this Phase I trial, patient tumors were resected, and tumor lysate from surgically removed tumor was used as an antigen source. We reasoned that this approach could have the following advantages: (*a*) it could provide multiple antigens for recognition by specific T cells. Gliomas are heterogeneous tumors among patients as well as within an individual patient, and there are no known defined TAAs expressed by all glioma cells. (*b*) Tumor lysate does not require growing autologous cultured tumor cells, which is difficult with irradiated tumor tissue. (*c*) Cultured GBM cells have the risk of expressing artifactual antigens as a result of culturing. (*d*) Targeting multiple antigens could be advantageous and obviate immune selection of single antigen-loss tumor variants.

The isolation of tumor lysate provided a patient-specific and tumor-associated mixture of potential T helper and CTL epitopes whose characterization or purification was not required. Mononuclear cells were isolated through leukapheresis, yielding approximately 2.5×10^7 DCs after *ex vivo* differentiation in the presence of IL-4 and granulocyte macrophage colony-stimulating factor. This yield of DCs was 25-fold greater than that used in our first Phase I trial for patients with newly diagnosed tumors (12). Allogeneic proliferation assays confirmed that the DCs isolated were functional, and sufficient numbers were isolated in all patients to administer three intradermal vaccinations.

The vaccinations were well tolerated with no significant toxicities, similar to reports from other DC immunotherapy trials (8–11). Mild toxicities included three episodes of transient headache after vaccination. Two patients complained of chronic fatigue. To date, no clinical or radiological features of autoimmune disease have been detected in our patients, nor have other DC immunotherapy trials reported any significant autoimmune disease (8–11). Autopsies of two patients confirmed the presence of tumor with some infiltration by CD45RO+ memory T cells. There was a paucity, however, of CD8+ cytotoxic T cells in both autopsies. No evidence of autoimmune response was observed in the autopsied brains. Continued evaluation will be necessary to determine whether autoimmunity may develop as a late complication of DC vaccination.

With our previously published tumor peptide-pulsed DC therapy, cytotoxic immunity was detected in four of nine patients (12). Here, vaccination with tumor lysate-primed DCs elicited significant cytotoxic responses against tumor in 6 of 10 patients, as determined by quantitative PCR analysis of IFN-γ message in restimulated PBMCs (Table 2). These results demonstrate that the use of unselected tumor-derived antigenic preparations can effectively induce antitumor cytotoxicity. However, the accumulation of IFN-γ message does not necessarily mean that it will be translated into functional protein. To further determine whether T-cell clones to specific antigen were being generated from DC vaccination, we sought to analyze the presence of CTL clones that recognized TAA epitopes through the use of tetramers. We previously demonstrated the presence of MHC class I-restricted CTLs, which recognized the TAA TRP-2, in a subset of glioma patients treated with DC vaccinations (13). We subsequently investigated the expression in glioma of TAAs related to breast carcinoma and melanoma, including HER-2, gp100, and MAGE-1. We demonstrated that of 43 primary GBMs analyzed, HER-2, gp100, and MAGE-1 mRNA was expressed in 81.4%, 46.5%, and 39.5% of respective samples (14). Of significance was our finding that among tumors derived from the 14 patients treated with lysate-pulsed DC vaccinations, HER-2, gp100, and MAGE-1 mRNA was expressed at 71.4%, 50%, and 50%, respectively. Twelve of 14 (85.7%) patients expressed at least one of these antigens, with 4 tumors expressing all three antigens (HER-2, MAGE-1, and gp100; Table 3). Nine patients

expressed at least one of these antigens in an MHC class I-restricted manner. Of these patients, four demonstrated positive epitope-specific CTLs after vaccination. Each of these positive, informative patients also demonstrated strong posttreatment antitumor cytotoxicity, as determined by quantitative PCR measurement of IFN-γ message in restimulated PBMCs (Table 2). Despite the identification of these antigens on glioma cells and the presence of specific CTL clones within vaccinated patients' PBMCs, however, the exact contribution of these antigens to the antitumor cytotoxic response remains unclear. It is possible that the therapeutic effects observed may result from the additional presentation of as yet uncharacterized immunogenic tumor antigens. The ability to use HER-2, MAGE-1, and gp100 as markers for assessing antigen-specific immune responses, however, as opposed to the use of nonspecific, tumor-derived, mixed antigenic preparations in which individual immunogenic targets may be highly diluted, provides us with a powerful tool to monitor the efficacy of immunotherapeutic protocols.

We identified CD45RO+ memory T cells and CD8+ cytotoxic T cells in surgical specimens isolated from three of six patients vaccinated with tumor lysate-primed DCs. The intratumoral infiltration of CD45RO+ T cells is similar to the infiltration seen in delayed-type hypersensitivity sites and in regressing tumors in a DC immunotherapy trial for patients with melanoma (10). The intratumoral presence of these previously activated and/or memory T cells suggests that DC vaccination may elicit activated T cells with specific homing or expansion properties. The patients who underwent reoperation were, by definition, treatment failures. The presence of cytotoxic T cells in these tumors suggests that the presence of radiological recurrence does not indicate the absence of a cytotoxic immunological response, but rather a shift in the homeostasis of tumor growth overcoming T-cell killing.

Of significance was our finding that patients treated with DC vaccinations demonstrated markedly prolonged survival compared with historical controls (Fig. 4). These findings indicate the possible therapeutic relevance of the observed enhancement in tumor-specific cytotoxicity and confirm the viability of DC therapy for glioma. Patients with recurrent GBM treated with DC vaccinations appear to have prolonged survival compared with patients who undergo craniotomy and conventional treatment. Leukapheresis was used to isolate 25-fold more DCs for vaccination than we were able to generate for our trial of patients with newly diagnosed GBM and AA (12). This augmented number of DCs used for vaccination may increase the T-cell response and may account for the robust increase in survival of recurrent GBM patients in this trial compared with our Phase I DC trial for patients with newly diagnosed malignant glioma. The use of tumor lysate in this trial rather than acid-eluted MHC-associated peptide from cultured GBM cells also represented a divergence from the previously reported trial.

A Phase II study is under way to reassess the role that DC therapy may have in prolonging survival. Okada *et al.* (24) have used an alternative autologous DC strategy to treat recurrent GBM, and the results of this Phase I study are pending. Physician selection and patient bias cannot be completely ruled out in this study, although age, gender, and degree of resection were not significantly different in the study and control groups. Selection bias can only be eliminated by a randomized Phase III study. The successful induction of systemic tumor and antigen-specific cytotoxicity associated with intracranial cytotoxic T-cell infiltration establishes this immunotherapy strategy as a model to help to understand the mechanisms underlying adaptive immunity against glioma.

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