Engineering the Brain Tumor Microenvironment Enhances the Efficacy of Dendritic Cell Vaccination: Implications for Clinical Trial Design

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Abstract

Purpose: Glioblastoma multiforme (GBM) is a deadly primary brain tumor. Clinical trials for GBM using dendritic cell (DC) vaccination resulted in antitumor immune responses. Herein, we tested the hypothesis that combining in situ (intratumoral) Ad-Flt3L/Ad-TK–mediated gene therapy with DC vaccination would increase therapeutic efficacy and antitumor immunity.

Experimental Design: We first assessed the immunogenicity of tumor lysates generated by Ad-TK (+GCV), temozolomide (TMZ), or freeze/thawing cycles (FTC) in a syngeneic brain tumor model. We also assessed phenotypic markers, cytokine release, and phagocytosis of bone marrow–derived DCs generated by fms-like tyrosine kinase 3 ligand (Flt3L)+IL-6 or by granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL) 4. Inhibition of tumor progression and production of anti-GBM antibodies was assessed following vaccination with (i) tumor cell lysates, (ii) DCs generated with either Flt3L/IL-6 or GM-CSF/IL-4 loaded with either Ad-TK/GCV-, TMZ-, or FTC-generated tumor lysates, or (iii) DCs in combination with in situ Ad-Flt3L/Ad-TK gene therapy.

Results: DCs loaded with tumor cell lysates generated with either Ad-TK/GCV or TMZ led to increased levels of phagocytosis, therapeutic efficacy, and humoral immune response. In situ immunogene therapy in combination with DC vaccination led to brain tumor regression and long-term survival in about 90% of animals, a significant increase when compared with either therapy alone.

Conclusions: Our results indicate that modifying the tumor microenvironment using intratumoral Ad-Flt3L/Ad-TK–mediated gene therapy potentiates therapeutic efficacy and antitumor immunity induced by DC vaccination. These data support novel phase I clinical trials to assess the safety and efficacy of this combined approach. Clin Cancer Res; 17(14); 4705–18. ©2011 AACR.

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults and exhibits a dismal prognosis. Despite therapeutic advances, that is, surgical resection followed by radiotherapy and chemotherapy, the prognosis for GBM is still poor with a median survival of 15 to 18 months postdiagnosis (1). Because GBM diffusely infiltrates into the surrounding brain parenchyma, relapse is almost inevitable even after surgical resection.

Immunotherapy strategies, including vaccination with tumor-associated peptides, autologous tumor cells, or dendritic cells (DC), have been intensively studied in human clinical trials for cancer (2–12). One of the most widely used approaches in cancer immunotherapy is DC vaccination, in which patients’ DCs are cultured and expanded ex vivo, loaded with specific tumor antigens or autologous tumor lysates, and then systemically administered to the patient. Recently, sipuleucel-T (Provenge) became the first antigen-specific, cell-based immunotherapy approach to receive Food and Drug Administration (FDA) approval (13). Sipuleucel-T showed an overall survival benefit to men with castration-resistant prostate cancer in 3 double-blind, placebo-controlled, multicenter trials (14, 15).

Several clinical trials for GBM using DC vaccination approaches have been implemented including vaccination with DCs pulsed with EGFRvIII, a tumor antigen which is expressed in about 30% of human GBM patients (2, 11), or vaccination with DCs loaded ex vivo with autologous tumor lysates (3–10, 12). These therapeutic approaches are designed to facilitate the presentation of brain tumor
antigens to naive T cells, thereby inducing the proliferation of brain tumor antigen–specific cytotoxic T cells. In these studies, DC vaccination induced increased antitumor cellular and humoral immune responses against brain tumors, exhibiting a high safety profile (3, 5–10, 12).

We have developed a combined cytotoxic/immunostimulatory gene therapy using intratumoral injection of adenoviral vectors expressing fms-like tyrosine kinase 3 ligand (Ad-Flt3L) and thymidine kinase (TK) within the tumor mass with concomitant release of tumor antigens and high-mobility group B1 protein (HMGB1), which would potentiate the therapeutic efficacy and antitumor immunity induced by DC vaccination. In situ immunogene therapy in combination with DC vaccination led to long-term survival in about 90% of animals, a significant increase when compared with either therapy alone, indicating that immunogene therapy potentiates DC vaccination–induced therapeutic efficacy and antitumor immune responses. These data support novel phase I clinical trials to assess the safety and efficacy of this combined approach.

Herein, we aimed to test the hypothesis that in situ (intratumoral) gene therapy mediated by expression of Flt3L and HSV1-TK would enhance the therapeutic efficacy of systemic DC vaccination, leading to the design of novel phase I trials for GBM. Recent reports indicate that vaccination efficacy depends on the type of cell death used to prepare the tumor lysates, that is, apoptotic tumor lysates are more immunogenic and elicit greater antitumor effects than necrotic tumor lysates (25, 26). As such, we compared the immunogenicity of tumor lysates prepared by treatment with Ad-TK/GCV, the chemotherapeutic agent temozolomide (TMZ), or freeze/thawing cycles (FTC). Our results show that vaccination with cell lysates prepared using Ad-TK/GCV or TMZ displayed equivalent inhibition of brain tumor progression and were more efficacious than necrotic lysates (FTC). We also compared the in vitro and in vivo phenotypic characteristics of DCs, generated by Flt3L + IL-6 treatment or by granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 treatment, that is, activation markers, cytokine release, and phagocytic ability.

We showed the therapeutic efficacy of Flt3L + IL-6 induced DCs loaded with Ad-TK/GCV-generated tumor lysates, in rats bearing established brain tumors. Flt3L is a powerful cytokine that is required for normal development and expansion of DCs in vitro and in vivo (27). Our results show that therapeutic efficacy and induction of antitumor immunity in Lewis rats bearing orthotopic, syngeneic brain tumors was significantly enhanced when DC vaccination was combined with Ad-TK/GCV + Ad-Flt3L gene therapy. These data support the implementation of novel phase I clinical trials for GBM to assess the safety and efficacy of this novel dual therapeutic modality.

Materials and Methods

Adenoviral vectors

We used first-generation, E1/E3-deleted Ad-Flt3L or Ad-TK (described in Supplementary Materials and Methods).

Tumor lysate preparation

CNS1 rat glioma cells were treated with TK/GCV, TMZ, or FTC; described in Supplementary Materials and Methods.

BMDC preparation

Bone marrow was isolated from the femurs of naive, adult Lewis rats as described previously and in Supplementary Materials and Methods. Bone marrow precursors were cultured with either Flt3L and interleukin (IL) 6 or GM-CSF and IL-4. Loosely adherent cells were used for subsequent subcutaneous injections and functional assays as described in Supplementary Materials and Methods.

CNS1 rat glioma model and Ad delivery

All procedures involving live animals were conducted in accordance with protocols approved by the Cedars-Sinai Medical Center’s Institutional Animal Care and Use

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Committee (IACUC). Five thousand CNS1 glioma cells in 3 μL Dulbecco’s modified Eagle’s media (DMEM) were stereotactically implanted in the right striatum of syngeneic Lewis rats as described in Supplementary Materials and Methods. Four or 10 days after tumor implantation, rats received a stereotactic, intratumoral injection of Ad vectors; described in Supplementary Materials and Methods. For survival studies, animals were treated with saline (as a control), 3 × 10⁸ plaque-forming units (pfu) of Ad-Fli3L, 1 × 10⁶ pfu of Ad-TK, or in combination with Ad-Fli3L and Ad-TK.

**Vaccination strategies**

Lysates prepared from 6 × 10⁶ tumor cells were mixed with CpG₂₀₀₆ and injected subcutaneously in the flank 3 times at 7-day intervals as indicated and described in Supplementary Materials and Methods. DC vaccination is described in detail in Supplementary Materials and Methods.

**Acridine orange staining**

Acridine orange was used to detect and quantify the development of acidic vesicular organelles. Briefly, acridine orange moves freely across biological membranes and fluoresces green when uncharged, whereas its protonated form accumulates in acidic compartments and fluoresces bright red. The intensity of red fluorescence is proportional to the degree of acidity. CNS1 tumor cells were stained with acridine orange (1 μg/mL) and analyzed by fluorescence microscopy or flow cytometry as previously described (29) and in Supplementary Materials and Methods.

**Western blotting**

Analysis of cell lysates by Western blotting was conducted as described in Supplementary Materials and Methods. Labeling was conducted using anti-LC-3B antibody, or α-tubulin as a control, followed by horseradish peroxidase (HRP)-conjugated secondary antibody and visualization with enhanced chemiluminescence (ECL).

**Quantification of apoptosis and necrosis**

CNS1 tumor cell lysates were stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) and analyzed by flow cytometry as described by us previously (18, 21) and in Supplementary Materials and Methods.

**Quantification of DC maturation status: measuring expression of cell surface markers and cytokine production**

DCs were incubated with CpG₂₀₀₆ or lipopolysaccharide (LPS) and then assessed for either (i) cytokine production by ELISAs specific for IL-10, IFN-γ, TNF-α, and IL-12 or (ii) expression of cell surface markers OX-62, CD161a, CD3, CD4, CD45, CD45R, CD11c, CD80, CD86, and MHCI by flow cytometry; described in Supplementary Materials and Methods.

**Phagocytic activity of DC**

CNS1 tumor cells stained with Qtracker 655 were treated with the above killing treatments (Ad-TK/GCV, TMZ, or FTC). Dying, labeled tumor cells were incubated Flt3L + IL-6–conditioned DCs labeled with a CD161a-PE antibody. Phagocytosis of tumor cells was assessed by flow cytometry as the percentage of Qtracker 655/CD161a-phycocerythrin (PE)-labeled cells compared with CD161a-PE–labeled cells. Alternatively, microscopy analysis was used to assess the phagocytosis of tumor cell remnants; Qtracker 655 stained tumor cells were cultured with DCs, stained with CD161a followed by an Alexa Fluor 488–tagged secondary antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and samples were analyzed by confocal microscopy, see Supplementary Materials and Methods.

**CTL assay**

Cytotoxicity of lymphocytes against tumor cells was assessed by flow cytometry as previously described (28) and in Supplementary Materials and Methods.

**Detection of anti-CNS1 antibodies**

Serum levels of anti-CNS1 antibodies were measured by flow cytometry, described previously (29) and in Supplementary Materials and Methods.

**Statistical analyses**

Kaplan–Meier survival curves were analyzed using the Mantel log-rank test using Prism GraphPad software (version 3.03). The flow cytometry and ELISA data were analyzed by 1-way ANOVA followed by Tukey’s posttest using NCSS statistical and power analysis software. Differences between groups were considered significant at P < 0.05. All experiments described in our article were conducted twice to confirm the findings.

**Results**

**In vitro characterization of tumor lysates to be used for vaccination strategies**

Tumor cell death mechanisms have been implicated in the degree of immunogenicity induced by tumor lysates (25, 26). Thus, we characterized the mechanisms of tumor cell death induced by Ad-TK/GCV, TMZ, and FTC in vitro, which we used to prepare tumor cell lysates for vaccination. Electron microscopy analysis indicated that CNS1 cells treated with Ad-TK/GCV exhibited...
chromatin condensation and cytoplasmic vacuolization, which are typical features of apoptotic cell death (Fig. 1A). FTC-treated CNS1 cells showed typical features of necrosis including cytoplasmic swelling, plasma membrane, and cytoplasmic organelle destruction (Fig. 1A). TMZ induces autophagy in CNS1 rat brain tumor cells, displaying an increased number of autophagic vacuoles in the cytoplasm (black arrow), including autophagosomes, double membrane vacuoles containing organelles (Fig. 1A; white arrow) and autolysosomes vesicular organelles containing digested residual materials (Fig. 1A; white arrowheads). Autophagy was also observed in Ad-TK/GCV-treated tumor cells (Supplementary Fig. S1).

Autophagy is an evolutionarily conserved intracellular degradative process, where cytoplasmic organelles are sequestered into lytic compartments, characterized by the formation of acidic vesicular organelles called autolysosomes (30). To quantify the extent of autophagy induction in CNS1 brain tumor cells, we determined the percentage of cells with accumulated acidic vesicular

Figure 1. Characterization of whole tumor cell lysate to be used in vaccination. A, electron micrographs showing the ultrastructure of CNS1 cells treated with either Ad-TK plus GCV (Ad-TK/GCV) for 72 hours, 3 cycles of FTC, or 200 μmol/L TMZ for 72 hours. A black arrow highlights an autophagosome in CNS1 cells treated with TMZ. A white arrow indicates an autophagosome with a characteristic double membrane structure. White arrowhead identifies autophagolysosome with characteristic residual digested materials. B, acridine orange (AO) staining of CNS1 cells treated with TMZ, Ad-TK/GCV, or serum starvation. Red staining reveals acidic vacuoles corresponding to autophagolysosomes, green indicates nonacidic compartments. C, CNS1 cells were treated with TMZ, Ad-TK/GCV, or starvation in the presence or absence of bafilomycin A1 (bafA1). Cells were stained with AO and analyzed by flow cytometry. The proportions of cells with acidic vacuoles (AO red positive) are shown in representative dot plots. D, CNS1 cells were treated with TMZ, Ad-TK/GCV, or starvation in the presence or absence of bafA1 and analyzed by Western blotting using a primary antibody for LC-3B; α-tubulin was used as a loading control. E, cell death in CNS1 cells treated with TMZ, Ad-TK/GCV, or FTC was assessed by flow cytometry following staining with Annexin V-FITC for apoptosis and PI for necrosis. The proportion of cells in each quadrant is shown in representative samples.
organelles using acridine orange staining (Fig. 1B and C). Acridine orange moves freely across biological membranes and fluoresces green when in an uncharged environment, whereas its protonated form accumulates in acidic compartments and fluoresces bright red (31, 32). The intensity of red fluorescence is proportional to the degree of acidity. As one of the most efficient triggers of autophagy is nutrient starvation (30, 32), serum-starved cells were used as a positive control. Fluorescence microscopy analysis shows accumulation of red fluorescence in the cytoplasm in CNS1 tumor cells treated either with TMZ or Ad-TK/GCV, indicating the presence of acidic compartments (Fig. 1B). Flow cytometry analysis shows that both TMZ and Ad-TK/GCV increased red fluorescence intensity from 9.4% (basal levels) to 35.9% and 36.0%, respectively, comparable with serum-starved cells (42.3%). Increased red fluorescence in CNS1 cells treated with TMZ or Ad-TK/GCV was abolished by treatment with bafilomycin A1, an inhibitor of the fusion of autophagosomes and acidic lysosomes (ref. 33; Fig. 1C). Freeze–thaw lysates did not acquire acridine orange staining. These findings indicate that CNS1 cells treated with TMZ or Ad-TK/GCV exhibit acidic vesicular organelle formation associated with autophagy. Autophagy induction was also assessed by Western blotting of LC3, a specific marker for autophagy (32; Fig. 1D). When cells undergo autophagy, LC3-I is converted to LC3-II (labeled form). However, the increase in LC3-II expression can be associated with increased formation of autophagosomes, or an impaired autophagic degradation (34). To differentiate these 2 possibilities, LC3-II expression was assessed in the presence of bafilomycin A1, which blocks autophagic degradation (33). As shown in Figure 1D, CNS1 cells treated with TMZ or Ad-TK/GCV exhibited further accumulation of LC3-II in the presence of bafilomycin A1, thus supporting the increased LC3-II expression is a consequence of increased levels of autophagosome formation rather than impaired degradation.

The extent of apoptosis and necrosis in CNS1 brain tumor cells was assessed by flow cytometry following Annexin V and PI staining (Fig. 1E). Our results indicate that Ad-TK/GCV induced mainly apoptosis (69.6%), TMZ induced a small population of necrotic cells (18.3%) and minimal apoptosis (3.9%), and FTC induced mostly late apoptosis/necrosis (88.2%; Fig. 1E). Taken together, these data show that Ad-TK/GCV induces apoptosis that is accompanied with autophagy; TMZ induces mainly autophagy, and FTC induces necrosis in CNS1 brain tumor cells.

Vaccination using Ad-TK/GCV- or TMZ-generated tumor lysates induced enhanced antitumor immunity than necrotic tumor cell lysates

To assess whether immunogenicity of tumor cell lysates is dependent on the mechanism of tumor cell death in our model, we compared the therapeutic efficacy of whole tumor cell lysate vaccines generated by treatment of CNS1 cells with Ad-TK/GCV, TMZ, or FTC. In the first paradigm, the animals were vaccinated before tumor cell implantation (Fig. 2A). Lysates were administered subcutaneously 3 times before CNS1 tumor cell implantation into the striatum (Fig. 2A). Vaccination with tumor lysates prepared from any of the 3 methods tested, failed to significantly prolong median survival (Fig. 2B). All tumor lysate vaccines increased the levels of circulating anti-CNS1 antibodies in the serum when compared with saline controls (Fig. 2C). Levels of circulating anti-CNS1 antibodies were significantly higher in rats vaccinated with Ad-TK/GCV- or TMZ-generated cell lysates than in rats treated with FTC-generated cell lysates (Fig. 2C). These findings show that Ad-TK/GCV–treated (apoptotic and autophagic) or TMZ-treated (apoptotic) tumor lysates induce more potent antitumor immune responses than FTC lysates (necrotic).

Flt3L-expanded DCs secrete high levels of T helper cell type 1–polarizing cytokines and elicit potent antitumor immunity

DC differentiation and proliferation can be elicited by either Flt3L or GM-CSF (35–37). Also, recent studies have shown that IL-6 improves expansion of Flt3L-induced DCs (35, 38). To determine whether vaccination with Flt3L-induced DCs elicits antitumor immunity, we compared in vitro and in vivo the characteristics of BMDCs induced by GM-CSF + IL-4 or Flt3L + IL-6 (Fig. 3). Consistent with previous reports (35, 38), expansion of DCs was higher in Flt3L-treated than GM-CSF + IL-4–treated BM cultures; the addition of IL-6 greatly enhanced the proliferation of Flt3L-induced DCs (Supplementary Fig. S2A). Microscopic analysis showed that under both conditions, DCs exhibited long cytoplasmic processes, a typical feature of DCs (Supplementary Fig. S2B). Loosely attached cells conditioned with Flt3L + IL-6 were analyzed by flow cytometry; about 33% of the cells displayed elevated levels of OX-62, expressed by rat conventional DCs (cDC; CD11c+/CD45+CD11b+MHCII+) but not plasmacytoid DCs (pDC; CD3−CD45+CD145R+), and about 94% displayed elevated levels of the natural killer (NK) marker CD161a on their cell surface, a phenotype characteristic of all rat DC subtypes (39). Flow cytometric analysis of cell surface markers revealed that a majority of Flt3L + IL-6–conditioned DCs displayed a characteristic phenotype of cDCs; a very small population of DCs expressed cell surface markers characteristic of pDCs (ref. 39; Supplementary Fig. S3).

Maturation of DCs, with upregulation of MHCII or costimulatory molecules such as CD86 and CD80, is critical to induce the clonal expansion of antigen-specific T-cell populations (40). Thus, we evaluated the maturation status of BMDCs incubated with Flt3L + IL-6 or GM-CSF + IL-4. Flt3L + IL-6–generated DCs expressed CD86 and CD80 (27.3% and 55.1%, respectively); GM-CSF + IL-4–generated DCs expressed higher levels of CD86 and CD80 (80.7% and 78.7%, respectively; Fig. 3A). MHCII expression was similar in both Flt3L + IL-6– and GM-CSF + IL-4–cultured DCs (75.6% and 83.3%, respectively; Fig. 3A). To induce further maturation of DCs, cells were further incubated for 48 hours without cytokines in the presence or absence of activation agents CpG oligodeoxynucleotide
Deprivation of cytokines induced upregulation of CD86, CD80, and MHCII (Fig. 3A). Stimulation by CpG-ODN or LPS further increased the expression of those surface molecules in Flt3L^+ IL-6–cultured DCs. Treatment with CpG-ODN or LPS had little effects on the expression of maturation markers on GM-CSF^+ IL-4–induced DCs (Fig. 3A). These findings indicate that Flt3L^+ IL-6 conditioning maintains BMDCs in a more immature state (lower expression of maturation markers) when compared with GM-CSF^+ IL-4.

The balance between the induction of T helper cell type 1 (Th1) and Th2 CD4^+ T cells determines the efficiency and the type of immune response. Secretion of Th1-polarizing cytokines such as IL-12 and IFN-γ produced by DCs, which promote a shift toward Th1 dominant balance, has been implicated in potent antitumor immunity (40). Thus, we compared the cytokine secretion profile between GM-CSF + IL-4 and Flt3L + IL-6–conditioned BMDCs (Fig. 3B). These findings indicate that Flt3L + IL-6 conditioning maintains BMDCs in a more immature state (lower expression of maturation markers) when compared with GM-CSF + IL-4. The balance between the induction of T helper cell type 1 (Th1) and Th2 CD4^+ T cells determines the efficiency and the type of immune response. Secretion of Th1-polarizing cytokines such as IL-12 and IFN-γ produced by DCs, which promote a shift toward Th1 dominant balance, has been implicated in potent antitumor immunity (40). Thus, we compared the cytokine secretion profile between GM-CSF + IL-4 and Flt3L + IL-6–conditioned BMDCs (Fig. 3B).

Functionally, DCs are characterized by their capacity to present antigen to T cells and induce clonal expansion of antigen-specific T cells. Thus, we assessed the capacity of Flt3L + IL-6–conditioned DCs loaded with Ad-TK/GCV–derived tumor lysates to induce T-cell proliferation. Spleenocytes from tumor-bearing rats were cocultured with CNS1 tumor lysates in vitro in a dose-dependent manner, when produced by Flt3L + IL-6–generated DCs as compared with GM-CSF + IL-4–generated DCs (Fig. 3B). Despite evidence of upregulated production of Th1-polarizing cytokines IL-12 and IFN-γ, we also observed elevated levels of IL-10 production in Flt3L + IL-6–generated DCs. The ratio between IL-12 and IL-10 represents the balance between Th1- and Th2-polarizing cytokine production by DCs (20). Flt3L + IL-6–generated DCs displayed a high Th1 balance when stimulated with either CpG or LPS; however, GM-CSF + IL-4–generated DCs only displayed a high Th1 balance when stimulated with LPS but not CpG (Fig. 3B).

Functionally, DCs are characterized by their capacity to present antigen to T cells and induce clonal expansion of antigen-specific T cells. Thus, we assessed the capacity of Flt3L + IL-6–conditioned DCs loaded with Ad-TK/GCV–derived tumor lysates to induce T-cell proliferation. Spleenocytes from tumor-bearing rats were cocultured with tumor-loaded BMDCs, unloaded BMDCs, or no BMDCs (Supplementary Fig. S4A). T-cell proliferation was only induced when T lymphocytes were cocultured with tumor-loaded DCs (Supplementary Fig. S4B). T cells stimulated by CNS1-loaded DCs effectively killed CNS1 tumor cells in vitro.
Figure 3. Characterization of BMDCs induced by Flt3L + IL-6 or GM-CSF + IL-4. Ten million bone marrow cells were cultured in RPMI conditioned media supplemented with 100 μg/mL Flt3L + 50 μg/mL IL-6 (FL-IL-6), 100 μg/mL Flt3L (FL), or 10 μg/mL GM-CSF + 10 μg/mL IL-4 (GM-IL-4) every 2 to 3 days. Loosely attached cells were harvested at day 7 and B. A, surface molecules of FL-IL-6–induced and GM-IL-4–induced DCs were stained after 7 days culture (prestimulation) or after stimulation with either 100 ng/mL CpG2006, 50ng/mL LPS, or mock (48 hours of stimulation) and then measured by flow cytometry. Overlays from a representative sample are depicted. The proportion of cells positive for each surface marker is shown in representative samples. B, cytokine production from FL-IL-6–induced and GM-IL-4–induced DCs. Immature DCs harvested after 7 days in culture were treated with CpG, LPS, or mock for 48 hours. Supernatant was collected and cytokines were measured by ELISA. *, P < 0.05 vs. mock; ^, P < 0.05 vs. CpG; #, P < 0.05 vs. LPS. C, experimental design to assess therapeutic efficacy of FL-IL-6– or GM-IL-4–generated DC vaccines in rats challenged with brain tumors. Lewis rats were systemically vaccinated 3 times (days −14, −7, and 0) with either FL-IL-6–induced or GM-IL-4–induced DC vaccines (subcutaneously) loaded with Ad-TK/GCV-treated whole tumor lysate, or saline as a control, before CNS1 tumor cell implantation (5,000 cells) in the striatum. D, Kaplan–Meier survival curves of rats treated with FL-IL-6–induced DC vaccine (n = 5), GM-IL-4–induced DC vaccine (n = 5), and saline (n = 5) are shown. *, P < 0.05 versus saline; Mantel log-rank test.
compared with lymphocytes from a naive rat as a control (Supplementary Fig. S4C). Thus, these data show that tumor-loaded BMDCs have the capacity to induce the proliferation of tumor-specific cytotoxic T cells.

While most DC vaccination clinical trials rely on GM-CSF to induce the proliferation and conditioning of DCs, recent studies have shown that Flt3L and IL-6 improves expansion of DCs (38). As such, we assessed the in vivo therapeutic efficacy of systemic vaccination with DCs generated using Flt3L + IL-6 or GM-CSF + IL-4 loaded with Ad-TK/GCV–derived tumor lysates. DCs were delivered 3 times at 7-day intervals before tumor implantation (days −14, −7, and 0). Vaccinated rats were then challenged with CNS1 cells in the striatum at day 0 (Fig. 3C). Rats vaccinated with Flt3L + IL-6–generated DCs completely inhibited tumor growth and exhibited 100% long-term survival, whereas only 60% of rats vaccinated with DCs generated by GM-CSF + IL-4 exhibited long-term survival (Fig. 3D). These data suggest that DCs generated with Flt3L + IL-6 result in vaccines that induce more robust antitumor immune responses than DC vaccines prepared with GM-CSF + IL-4.

**Mechanism of cell death used to generate tumor lysates determines efficacy of DC vaccination**

Uptake of tumor cell remnants followed by processing and presentation of tumor antigens to naïve T cells by DCs is critical for the induction of adaptive immunity. Thus, we tested whether tumor uptake by DCs depends on the method used for tumor cell killing, that is, autophagy, apoptosis, and necrosis. As shown in Figure 4A, DCs displayed higher levels of phagocytic activity when cocultured with dying tumor cells, as compared with mock-treated cells. Furthermore, Ad-TK/GCV or TMZ treatment increased tumor uptake by DCs when compared with FIC treatment.

We next tested whether the mechanism of tumor cell killing used to prepare the tumor lysates influences the therapeutic efficacy of DC vaccination. Tumor lysate–loaded DCs were administered subcutaneously into Lewis rats 3 times at 7-day intervals before tumor implantation (days −14, −7, and 0). Vaccinated rats were then challenged with CNS1 cells in the striatum at day 0 (Fig. 4C). Rats receiving DCs loaded with Ad-TK/GCV- or TMZ–generated tumor lysates completely inhibited tumor development and animals exhibited 100% long-term survival, whereas only 60% of rats vaccinated with DCs loaded with FTC tumor lysates survived long term (Fig. 4D). Levels of circulating anti-CNS1 antibodies were significantly higher in rats treated with DCs loaded with either Ad-TK/GCV- or TMZ–generated tumor lysates than in rats treated with DCs loaded with FTC tumor lysates (Fig. 4E).

**Combining in situ immunogene therapy and DC vaccination significantly improves antitumor therapeutic efficacy**

DC vaccination strategies have been shown to induce antitumor immune responses in patients with GBM undergoing clinical trials (3, 5–10). In this report, we wished to test the hypothesis that manipulating the tumor microenvironment via in situ immunogene therapy would enhance therapeutic efficacy of DC vaccination. First, we assessed whether DCs loaded with either Ad-TK/GCV– or TMZ–generated tumor lysates in combination with CpG as an adjuvant could elicit regression of an established intracranial tumor mass in the Lewis rat model. We compared therapeutic efficacy of in situ immunogene therapy to DC vaccination in a small tumor model, where tumor-bearing rats were treated 4 days after tumor implantation (Fig. 5A). In the small brain tumor model, Ad-Flt3L + Ad-TK immunogene therapy induced 100% survival, whereas we observed comparable therapeutic efficacy with either the DC vaccine loaded with TMZ-derived tumor lysates or the DC vaccine loaded with TK/GCV–derived tumor lysates in the small tumor model (Fig. 5B).

We next tested whether in situ immunogene therapy and DC vaccination could be used in combination to potentiate therapeutic efficacy in a rigorous, large tumor model, where tumor-bearing rats were treated 10 days after tumor implantation (Fig. 6A). The combination treatment of in situ immunogene therapy with DC vaccination led to long-term survival in about 90% of tumor-bearing animals. These data show a 40% increase in levels of long-term survival when compared with in situ immunogene therapy alone and an 80% increase in levels of long-term survival when compared with DC vaccination alone. The therapeutic efficacy data are corroborated by serum levels of anti-CNS1 antibodies in animals treated with combination therapy, which were increased by about 2.5-fold when compared with either arm of the therapy alone (Fig. 6C).

**Discussion**

DC vaccination approaches for brain tumors have been implemented in human clinical trials (5–10). Several clinical trials for GBM using DC vaccines have shown cellular and humoral antitumor immune responses; and albeit safe, the clinical efficacy of DC vaccination for GBM remains limited (3, 5–10). Our group has shown that in situ Ad-Flt3L/TK–mediated immunogene therapy elicits an influx of DCs, macrophages, CD4+ T cells, and CD8+ T cells into the brain tumor microenvironment (17) and stimulates effective anti-GBM immune response resulting in tumor regression and long-lasting CD8+ T-cell–mediated antitumor immunologic memory in several mouse and rat orthotopic brain tumor models (16, 18, 22–24, 29). On the basis of these data, a phase 1 clinical trial for GBM was recently cleared by the FDA and is slated to commence in 2011.

The combined conditional cytotoxic/immunostimulatory gene therapy approach, utilizes HSV1-TK to kill actively dividing brain tumor cells in the presence of GCV, thereby releasing endogenous brain tumor antigens and also innate immune adjuvants such as HMGB1 (18, 21). HMGB1 belongs to a class of innate immune adjuvants called damage-associated molecular pattern molecules (DAMP) which mediate signaling by binding to a family
of receptors called pattern recognition receptors (PRR), thereby promoting innate and adaptive immune responses (41). DCs express a large repertoire of PRRs and several studies have shown that signaling through PRRs leads to DC activation, which is characterized by high levels of MHC-antigen complexes on the DC cell surface, upregulation of costimulatory molecules such as CD80 and CD86, and the production of cytokines such as IL-12 and IFN-α.

Figure 4. Comparative efficacy of Flt3L-IL-6-induced DC vaccines loaded with Ad-TK/GCV, TMZ, or FTC tumor lysates. A, phagocytic activity of FL-IL-6-induced DCs. DCs labeled with CD161a-PE were cocultured for 5 hours with Qtracker 655-labeled CNS1 tumor cells that were treated with Ad-TK/GCV, TMZ, or FTC. Phagocytosis of tumor cells by DCs was assessed by flow cytometry; representative dot plots and corresponding percentage of double-labeled cells are shown. Data are represented as the percentage of double-stained versus CD161a-PE-positive cells, *, P < 0.05 versus mock; ^, P < 0.05 versus F/T 1-way ANOVA followed by Tukey’s test. B, confocal micrograph shows a representative DC (green, CD161a) that engulfed tumor cell remnants (red, Qtracker 655); nuclei were labeled with DAPI (blue). The large square in the top left is a maximum projection of the confocal Z stack. The images to the right and below are reconstructed vertical sections along the fine green lines shown on the maximum projection. C, Lewis rats were systemically vaccinated with DC vaccines (subcutaneously) loaded with tumor lysates prepared by Ad-TK/GCV, TMZ, or FTC treatment, or saline as a control, 3 times before tumor cell implantation (days −14, −7, and 0) with 5,000 CNS1 glioma cells in the striatum. D, Kaplan-Meier survival curves of rats treated with FL-IL-6-induced DC vaccines loaded with either TK/GCV lysate (DC-TK/GCV; n = 5), TMZ lysate (DC-TMZ; n = 5), or FTC lysate (DC-F/T; n = 5), or unloaded DCs or saline as controls, are shown. *, P < 0.05 versus saline; Mantel log-rank test. E, serum levels of anti-CNS1 cell antibodies at the time of tumor implantation were measured by flow cytometry. *, P < 0.05 versus saline; ^, P < 0.05 versus DC loaded with FTC lysate; 1-way ANOVA followed by Tukey’s test.
The production of these cytokines is directly involved in priming Th1-biased immune responses (40). We have previously shown that HMGB1 is released from dying brain tumor cells in response to treatment with Ad-TK/GCV or TMZ, and acts as an endogenous TLR2 agonist to activate bone marrow-derived, brain tumor-infiltrating DCs (18). The other arm of our therapeutic strategy involves expression of Flt3L within the tumor microenvironment. Flt3L recreates a missing immune circuit from the brain, by inducing the expansion and migration of DCs into the brain tumor milieu where they encounter and phagocytose newly released endogenous brain tumor antigens (16, 18, 22–24, 29).

Herein, we tested the hypothesis that combining Ad-Flt3L/TK in situ immunogene therapy with peripheral DC vaccination, would lead to enhanced therapeutic efficacy in a syngeneic brain tumor model. Our data show that the therapeutic combination, that is, in situ immunogene therapy with DC vaccination led to long-term survival in 90% of rats bearing large, syngeneic brain tumors; showing about 40% increase in survival compared with Ad-Flt3L/TK immunogene therapy alone and about 80% increase in survival compared with DC vaccination alone. Our results also showed enhanced antitumor humoral immune response, that is, about 2.5-fold increase in the levels of circulating antitumor antibodies compared with either treatment alone.

The enhanced therapeutic efficacy observed could result from expression of Flt3L within the brain tumor microenvironment, which could enhance the trafficking of systemically delivered DC vaccines to the draining lymph nodes, where they present tumor antigens to naive T cells and induce the clonal expansion of tumor-specific CTLs. To this effect, it has been previously shown that Flt3L elicits recruitment of DC populations, including fully differentiated DCs in lymphoid organs (40) and traffic of subcutaneously delivered DC vaccines to the inguinal draining lymph nodes in mice (42). We showed that intracranial delivery of Ad-Flt3L leads to circulating Flt3L in rat serum (19), thus supporting hypothesis that Flt3L could also act systemically and enhance the trafficking of DCs to draining lymph nodes. Increased trafficking of systemically delivered DCs to draining lymph nodes could lead to enhanced priming of antitumor immune responses, inducing increased levels of tumor regression and long-term survival as observed in this study.

An additional explanation for the increased therapeutic efficacy of in situ immunogene therapy in combination with DC vaccination is that DAMPs, that is, HMGB1, are known to enhance the activation status of DCs and facilitate Th1 immune responses (40). We have previously shown that treatment of intracranial brain tumors with TK/GCV results in increased levels of circulating HMGB1 in the sera of mice (18) and rats (21). Therefore, HMGB1 released into the systemic circulation could further activate systemically delivered DCs, thus enhancing their ability to prime an adaptive, antitumor immune response.

When used to treat small, established intracranial tumors, DC vaccination exhibited higher levels of long-term survival (~30%) when compared with treatment of large intracranial tumors (~10% long-term survival). These data suggest that the efficiency of a DC vaccination approach is associated with the degree of tumor burden in the host. Furthermore, our data show that DC vaccination is highly efficacious (100% long-term survival) when administered before tumor implantation, suggesting that DC vaccines would be more effective at preventing tumor recurrences after initial surgical debulking, chemotherapy, and radiotherapy.

Effective uptake and loading of tumor-associated antigens onto MHC complexes of DCs and expansion of DC subgroups that can efficiently prime naive T cells play a critical role in the effectiveness of DC vaccination. Therefore, it is critically important to optimize the preparation of tumor cell antigens and DCs. In this study, we compared the immunogenicity and levels of phagocytosis of apoptotic, autophagic, and necrotic tumor cells lysates. In line...
with previous evidence (26, 43, 44), our data indicate that triggering autophagy and/or apoptosis to generate tumor cell lysates increases the immunogenicity of tumor cells and enhances the delivery of tumor-associated antigens to DCs. Furthermore, we have previously reported that treatment of GBM cells with Ad-TK/GCV or TMZ results in the release of the endogenous TLR2 ligand HMGB1 from GBM cells (18). Autophagic GBM cells have also been shown to

Figure 6. Therapeutic efficacy of in situ immunogene therapy and DC vaccination in a large intracranial GBM model. A and B, Lewis rats were implanted with 5,000 CNS1 GBM cells. After 10 days, rats were treated either with (A) subcutaneous Flt3L + IL-6-induced DC vaccine loaded with Ad-TK/GCV tumor lysate, (B) in situ (intratumoral) Ad-TK, in situ Ad-TK + Ad-Flt3L, or a combination of in situ Ad-TK + Ad-Flt3L gene therapy plus DC vaccination. Anti-CNS1 antibodies were measured 1 day after the second DC vaccine administration (day 18). C, Kaplan–Meier survival curve of rats treated with in situ Ad-TK + Ad-Flt3L combined with DC vaccination (n = 10), in situ Ad-TK + Ad-Flt3L (n = 10), DC vaccine (n = 10), or saline (n = 7).

*, P < 0.05 versus saline; ^, P < 0.05 versus Ad-TK + Ad-Flt3L; Mantel log-rank test. D, serum levels of anti-CNS1 antibodies measured by flow cytometry. Histograms show the fluorescence intensity of CNS1 cells labeled with serum from non-tumor-bearing rats (isotype control; gray histogram) or with serum from rats from each of the experimental groups (unfilled histograms). *, P < 0.05 versus saline; 1-way ANOVA followed by Tukey’s test.
releases HMGB1, without causing lysis of the cell membrane and classical necrosis (45). Previous reports showed that DCs loaded with purified autophagosomes from autophagic tumor cells induced tumor-specific immune responses (26), suggesting that autophagosomes contain a wide range of tumor-associated antigens and immune adjuvants, that is, HMGB1 (46). There is experimental evidence that suggests that intratumoral delivery of a recombinant cytotoxin composed of Pseudomonas exotoxin fused to IL-13 into mice bearing human xenografts causes cell death not only by apoptosis but also by necrosis. Furthermore, Kawakami and colleagues showed that delivery of the IL-13-PE cytotoxin induced phagocytes may play a role in cytokinin-mediated tumor regression (47). In addition, it has been previously shown that the fusion of the recombinant Pseudomonas exotoxin to a model tumor antigen may enhance vaccine potency (48).

GM-CSF combined with IL-4 has been previously used to generate DCs from both murine and human bone marrow progenitor cells (30). Flt3L has been introduced as an alternative means to generate DCs (35, 37, 49) and recent reports showed that Flt3L combined with IL-6 enhances the expansion of Th1-polarizing DCs, a requirement for the efficient induction of antitumor immune responses (50). Thus, we compared the characteristics of GM-CSF + IL-4- and Flt3L + IL-6-generated DCs to establish the optimal parameters for ex vivo expansion of DCs to be used in the vaccination paradigm described. Our results showed that ex vivo conditioning with Flt3L resulted in higher levels of DCs when compared with GM-CSF + IL-4; these results are in line with results previously obtained with canine DC cultures (49). Levels of Th1-polarizing cytokines such as IL-12 and IFN-γ were higher when DCs were cultured with Flt3L + IL-6. These data are in line with evidence that Flt3L induces DCs that preferentially secrete Th1-polarizing cytokines, when compared with GM-CSF cultured DCs (35). Consistent with these results, inhibition of tumor progression and therapeutic efficacy of DC vaccination was higher when we used Flt3L + IL-6-generated DCs. Addition of IL-6 in combination with Flt3L further enhanced the proliferation of BMDCs. Flt3L + IL-6–induced DCs displayed decreased levels of cell surface markers CD80, CD86, and MHCI, and exhibited high phagocytic capacity consistent with an immature phenotype.

The advantage of combining immunogene therapy (Ad-Flt3L/Ad-TK) with DC vaccination is that gene therapy can be administered into the tumor bed/cavity at the time of surgical resection to immediately initiate antitumor immune responses while the autologous DC vaccine with autologous tumor lysate is being prepared ex vivo. After the DC vaccine preparation is completed, it can be administered using the appropriate vaccination regime to enhance the antitumor immunity and therapeutic efficacy. In summary, the data presented show that immunogene therapy not only elicits tumor regression and antitumor immunity but also potentiates the therapeutic efficacy elicited by DC vaccination and support the implementation of novel phase I clinical trials to assess the safety and efficacy of this combined therapeutic strategy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Grant Support
Our work was supported by NIH/National Institute of Neurological Disorders & Stroke (NIH/NINDS) grants 1R21 NS054143; 1R01 NS052465; and 1R01 NS057711 to M.G. Castro; NIH/NINDS grants 1R01 NS054193 and 1R01 NS061107 to P.R. Lowenstein; and F32 NS0503034 to G.D. King. The Bram and Elaine Goldsmith and the Medallions Group Endowed Chairs in Gene Therapeutics to P.R. Lowenstein and M.G. Castro, respectively. The Linda Tallen & David Paul Kane Foundation Annual Fellowship, The Drown Foundation, and the Board of Governors at CSMC.

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Received April 8, 2011; revised May 16, 2011; accepted May 17, 2011; published OnlineFirst June 1, 2011.

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Immunotherapy for Brain Cancer


www.aacrjournals.org Clin Cancer Res; 17(14) July 15, 2011 4717

Published OnlineFirst June 1, 2011; DOI: 10.1158/1078-0432.CCR-11-0915

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