



Published in final edited form as:

J Immunother. 2012 June ; 35(5): 385–389. doi:10.1097/CJI.0b013e3182562d59.

Sequential Immunotherapy by Vaccination with GM-CSF Expressing Glioma Cells and CTLA-4 Blockade Effectively Treats Established Murine Intracranial Tumors

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Abstract

Malignant glioma is an incurable disease with a relatively short median survival. Several clinical trials have demonstrated that immunotherapy with vaccination is a safe and possibly effective way of prolonging survival. Antibody-based blockade of CTLA-4 ligation on T lymphocytes is associated with enhanced antitumor immunity in animal models of cancer and in patients with advanced melanoma. We hypothesized that sequential therapy with GM-CSF - expressing whole glioma cell vaccination and CTLA-4 blockade is an effective strategy for treating established intracranial gliomas. GL261 glioma cells were injected into the right frontal lobes of syngeneic C57/BL6 mice. At days 3, 6, and 9 after tumor implantation, mice were treated with subcutaneous injection of irradiated GMCSF-expressing GL261 cells. Mice were also treated with intraperitoneal injection of anti-CTLA-4 monoclonal antibodies (mAbs), either at days 3, 6, and 9 or days 12, 15, and 18. Animals were followed for survival. Splenocytes were harvested at day 22 for use in ELISPOT assays. Early treatment of established intracranial gliomas with high-dose CTLA-4 blockade was associated with increased survival in GL261-bearing mice. Later treatment with anti-CTLA-4 mAbs did not significantly improve survival compared to control-treated mice. Early vaccination followed by subsequent CTLA-4 blockade was associated with significantly improved survival versus either treatment alone and intensified tumor-specific immunity as measured by interferon-gamma ELISPOT. Sequential immunotherapy with GM-CSF-expressing irradiated glioma cells and CTLA-4 blockade synergistically prolongs survival in mice bearing established intracranial gliomas.

Keywords

glioma; vaccine; CTLA-4; GVAX; immunotherapy; GM-CSF

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Financial Disclosure: All authors have declared there are no financial conflicts of interest in regards to this work.

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Introduction

After repeated efforts, immunotherapy for cancer has recently seen advances in the clinical arena. For instance, phase III trials have shown efficacy and increased survival for active cellular immunotherapy in patients with advanced hormone-refractory prostate cancer (1), and the FDA has granted approval for use in these patients. For more than a decade, blocking ligation of the costimulatory molecule cytotoxic T-lymphocyte Antigen 4 (CTLA-4) has been a promising approach by which to augment and expand the host immunity that exists against some cancers (2). In a multicenter Phase III study published in 2010, CTLA-4 blockade with ipilimumab, a humanized monoclonal antibody against CTLA-4, was associated with significantly improved survival in patients with advanced melanoma. The disease-control rate in the ipilimumab group was 28.5% (3). While cancer immunotherapy approaches are beginning to have a clinical impact, much work needs to be done to refine and better understand both these treatments and interactions between the tumors and the immune system.

Malignant glioma may present a particular challenge. These tumors are very aggressive, and average survival is only 15 months from the time of initial diagnosis (4). Furthermore, they are remarkably immunosuppressive, both systemically (5) and in the tumor microenvironment (6). Despite these obstacles, vaccination strategies (7-9) and CTLA-4 blockade (10) have shown efficacy in preclinical glioma models, and phase II clinical studies of peptide (11) and dendritic cell vaccination (12, 13) have demonstrated clinically correlative biological activity.

Combining cancer vaccination with CTLA-4 blockade has been a preclinical strategy for some time (14), and multiple reports show its promise. In theory, as whole tumor cell vaccination augments the antitumor T-lymphocyte response, subsequently treating with CTLA-4 blockade stands to strengthen the response further. We hypothesize that CTLA-4 blockade after vaccination (“sequential immunotherapy”) is a rational paradigm for potent immunotherapy and that this system functions in an intracranial murine glioma model. For vaccination, we employ a platform in which glioma cells are transduced to express high levels of granulocyte-macrophage colony stimulating factor (GM-CSF), irradiated, and then injected subcutaneously, known as GVAX (15).

Materials and Methods

Animals

C57BL/6 mice were obtained from Charles River Laboratories. All animal studies were done in accordance with protocols approved by our Institutional Animal Care and Use Committee.

Cell lines

A syngeneic murine tumor cell line, GL261, was obtained from the National Cancer Institute (Frederick, MD). GL261-GM-CSF cells, intended for use as vaccine, were created via retroviral transduction and infection as previously described (16, 17). ELISA assay confirmed expression and secretion of murine GM-CSF by GL261-GM-CSF cells per manufacturer’s instructions (R&D Systems, Minneapolis, MN). In vitro expression of GM-CSF was measured as 390 (95% confidence interval 287-493) nanograms / 1×10^6 cells / 48 hours. All GL261 cell lines were cultured in RPMI-1640 medium (Mediatech Inc., Manassas, VA) without L-glutamine and supplemented with 10% FCS at 37°C and 5% CO₂.

Lentiviral transduction

GL261 cells used for tumor implantation were infected with recombinant lentivirus that was designed to express firefly-luciferase (ffluc) and the fluorescent protein, mCherry, as a tag for expression of both gene products. The ffluc and mCherry-expressing recombinant lentivirus has been described previously (18). Briefly, after exposure to viral supernatant in cell culture medium with polybrene, infected GL261 cells were washed, cultured, and sorted by their expression of mCherry using fluorescence-activated cell sorting on a custom FACSaria (BD Biosciences, San Jose, CA). Expression of firefly luciferase/mCherry was confirmed by fluorescence-based excitation on a standard inverted microscope.

Tumor implantation

GL261-ffluc cells were trypsinized, washed with cold RPMI-1640 twice, and counted by trypan blue exclusion. Cells were resuspended in RPMI-1640 at a concentration of 3×10^7 cells/mL. 75,000 GL261-ffluc cells were suspended in 2.5 microliters of RPMI-1640 without supplements and carefully mixed with 2.5 microliters of 3% methylcellulose. The resulting 5 microliter mixture was implanted into the right frontal lobes of C57BL/6 mice aged 6-8 weeks using an automated microsyringe (Hamilton Co., Reno, NV) and a stereotactic mouse frame (Kopf Instruments, Tujunga, CA). All intracranial tumor injections utilized the GL261-ffluc line. Tumors were reliably and statistically significantly detectable by bioluminescence at day 6 after intracranial implantation (data not shown).

Vaccination and anti-CTLA-4 treatment

On days 3, 6, and 9 after tumor implantation, appropriate mice underwent subcutaneous injection of 1×10^6 irradiated (30 Gy) GL261-GMCSF cells in a volume of 100 microliters of phosphate buffered saline (PBS). Control mice were subcutaneously injected with 100 microliters of PBS. On the appropriate days after tumor implantation, 100 micrograms of anti-CTLA-4-Ig (9H10 clone, BioXCell, West Lebanon, NH) or isotype control (Hamster IgG₁) were delivered by intraperitoneal injection in 500 microliters of PBS.

IFN- γ ELISPOT assay

Splenocytes were harvested from mouse spleens 22 days after tumor implantation. 1×10^6 splenocytes were stimulated for 48 hours *in vitro* with 1×10^5 irradiated (35Gy) GL261 cells or in RPMI 1640 medium, supplemented with 10% IFCS, 50 μ M 2-ME, 2 mM glutamine, 20 mM HEPES, penicillin-streptomycin in 1-ml tissue culture plates (BD Falcon, San Jose, CA). After stimulation, 1×10^5 splenocytes from mice in each treatment and control group were loaded in triplicate onto Millipore MultiScreen-HA 96-well filter plates coated with anti-IFN- γ mAb (eBioscience, Inc., San Diego, CA). Plates were incubated at 37°C and 5% CO₂ for 24 hours, washed three times with buffer, and incubated with biotinylated anti-IFN- γ monoclonal antibodies for 2 hours at 37°C. Plates were washed 4 times and incubated with Avidin-horseradish peroxidase conjugate for 45 minutes. Plates were washed three times with buffer and, then, twice with PBS before development using BCIP/NBT substrate (Sigma-Aldrich) for 10 minutes. Spots were identified and counted on an AID Version 3.1.1 ELISPOT reader.

Statistical Analysis

Mice were followed daily for survival by a blinded observer, and survival was analyzed with Mantel-Haenszel statistics and Kaplan-Meier curves. For ELISPOT analysis, differences in the numbers of spot-forming splenocytes were examined by the Student's t-test. All statistical analyses were performed using GraphPad software (GraphPad, La Jolla, CA).

Results

Early CTLA-4 blockade prolongs survival in syngeneic mice bearing intracranial GL261 tumors

Fecci, et al. have previously demonstrated that antibody-based blockade of CTLA-4 binding eradicates SMA-560 glioma tumors implanted in the brains of Vm/DK mice (10). 100 micrograms of antibody were delivered systemically on days 3,6, and 9 after tumor implantation. While the GL261 model that we employed for this study is associated with similar systemic immune effects as are both SMA-560 cells and human glioblastoma (19), we wanted to examine the impact of CTLA-4 blockade in this system. 75,000 viable GL261-ffluc cells were injected into the right frontal lobes of C57/BL6 mice on day 0 and, on days 3,6, and 9, we delivered 100 micrograms anti-CTLA-4 mAb via intraperitoneal injection. At this schedule and dose, most mice survived long-term, whereas all control mice succumbed by day 50, with median survival of 26 days (Figure 1a). However, when syngeneic mice are treated with 100 micrograms of anti-CTLA4 mAb on days 12, 15, and 18 after tumor implantation, survival is equivalent to that of mice treated with control antibody (Figure 1b). High-dose antibody-based CTLA-4 blockade prolongs survival in mice bearing GL261 tumor when tumors are initially taking and are small, but is ineffective against larger, more established tumors.

Following whole tumor cell vaccination with CTLA-4 blockade enhances antitumor immunity in mice bearing established intracranial GL261 tumors

Subcutaneous and intradermal injection of irradiated whole tumor cells that are engineered to express GM-CSF is an established technique of activating effective antitumor immunity, including against intracranial tumors (20). We hypothesized that T-lymphocytes stimulated by vaccination would be further and more durably activated by subsequent systemic CTLA-4 blockade. Therefore, after vaccinating mice with irradiated GL261-GMCSF cells on days 3, 6, and 9 after intracranial tumor implantation, we systemically delivered 100 micrograms of anti-CTLA-4 mAb on days 12,15, and 18 (Figure 2a). By ELISPOT analysis, a significantly higher number of splenocytes harvested from combination-treated tumor-bearing mice secreted IFN- γ than those taken from mice treated with either vaccination or CTLA-4 blockade as monotherapies (Figure 2b). Interestingly, at this late timepoint (day 22), splenocytes from animals treated with CTLA-4 blockade alone were no more reactive than mice from vehicle-treated controls.

Sequential treatment with whole-cell vaccination and systemic CTLA-4 blockade enhances survival in glioma-bearing mice

Earlier work has demonstrated the potency of combining GM-CSF-expressing whole tumor cell vaccination with CTLA-4 blockade in subcutaneous tumor models. In each of these models, vaccination and anti-CTLA-4-mAb were given concurrently. It is clear that, in murine glioma models characterized by implantation of a low number of tumor cells and relatively short lifespans, early treatment with CTLA-4 blockade can be highly effective on its own, but that there is little impact when therapy is initiated at later timepoints. We hypothesized that delayed CTLA-4 blockade in the setting of established antitumor immunity would prolong survival, in correlation with the strong interferon-gamma Elispot response that we observed. Having demonstrated no significant survival difference vs. control when GL261-bearing mice were treated with 100 micrograms of 9H10 antibody on days 12, 15, and 18 after tumor implantation, we chose this dose for evaluation in our sequential immunotherapy model. When mice were treated sequentially, following early GL261-GMCSF vaccination with CTLA-4 blockade on days 12,15, and 18 was associated with significant improvement in survival (Figure 3, Table 1).

Discussion

In the phase III clinical trial establishing the efficacy of CTLA-4 blockade in patients with advanced melanoma, combining ipilimumab with peptide vaccination did not positively impact disease progression or survival when compared to treatment with drug alone (3). Ipilimumab, alone, did not alter progression-free survival in these patients, but afforded improved overall survival. CTLA-4 blockade appears to have activity in the brain, both in case reports of patients with intracranial metastases (21) and in preclinical models of glioma (10), which we have confirmed here. However, blockade of CTLA-4 ligation shortly after a tumor is implanted may not accurately model advanced or even clinically evident cancer. For instance, when we treated mice with “advanced” brain tumors 2 to 3 weeks after implantation, the impact on survival was modest, and there were no cured mice. Disinhibition of tumor-specific lymphocytes by CTLA-4 blockade at this late time point is insufficient to slow the growth of intracranial gliomas.

While early vaccination (days 3, 6, and 9) significantly improved survival in C57/BL6 mice, there are rare cures in this model of treating established intracranial tumors. However, when GM-CSF-expressing whole tumor cell vaccination was followed by subsequent CTLA-4 blockade, the effect was clearly synergistic, resulting in almost 50% long-term survival and cure. This series of results is consistent with our hypothesis that whole-cell vaccination causes an expansion in the number of circulating activated tumor-specific immune effectors and in the number of tumor-associated epitopes that are identified. Once these tumor-specific lymphocytes are activated, treatment with systemic anti-CTLA-4 mAbs allows them to be effective for a longer period of time. It is mechanistically telling that tumor-specific response, as measured by splenocyte release of interferon- γ after stimulation by glioma cells, was not appreciably affected by treatment with CTLA-4 blockade alone, but was markedly amplified after sequential vaccination and anti-CTLA-4 mAb.

To the best of our knowledge, this is the first report of combined vaccination and CTLA-4 blockade as treatment for intracranial gliomas. This concept was first reported in subcutaneous tumors by van Elsas, et al. (14) who demonstrated that combining anti-CTLA-4 mAbs with irradiated GM-CSF-secreting B16 melanoma cells effectively treated subcutaneous and metastatic B16 melanoma tumors in syngeneic mice.

Furthermore, the same group has examined, in detail, the mechanisms by which combining GVAX and anti-CTLA-4 mAbs affect antitumor immunity, demonstrating increased tumor infiltration by lymphocytes and inversion of the intratumoral $T_{\text{effector}}/T_{\text{regulatory}}$ ratio (22). Blockade of CTLA-4 on both effector and regulatory lymphocyte subsets contributes to the impact of the combination therapy (23).

As mentioned above, a single previous report documents the efficacy of CTLA-4 blockade as treatment against murine intracranial glioma (10). This strategy has yet to be examined in patients with malignant glioma, though a series of patients with melanoma metastases to brain have been treated (24).

We also believe that this is the first preclinical demonstration of the synergistic efficacy of delivering whole tumor cell vaccines prior to CTLA-4 blockade, rather than treating essentially at the same time. It is our proposal that CTLA-4 blockade will be most useful in the context of an immune system that is primed against the tumor. In patients, how long this priming takes is unclear, and it may require several booster vaccinations. In mice, however, maximum antitumor immunity is thought to develop 14-21 days after vaccination. Vaccination can play the role of “primer” in patients with malignant glioma, and a number of clinical trials have demonstrated consistent development of host antitumor immunity after treatment with immunotherapy (11, 12).

Although this sequential treatment schedule has not been reported in the preclinical setting, there are suggestions of powerful impact in selected patients with melanoma and ovarian cancer (25). In a clinical study, the majority of patients with advanced melanoma that were treated with CTLA-4 blockade after GVAX generated “clinically meaningful” antitumor immunity, and histopathologic tumor necrosis in patients with ovarian carcinoma was tightly correlated with intratumoral CD8+/FoxP3+ lymphocyte ratios.

The current study demonstrates that following whole tumor cell vaccination with CTLA-4 blockade can successfully treat murine intracranial gliomas. We believe that sequential vaccination and CTLA-4 blockade is a rational immunotherapeutic strategy that merits rigorous clinical investigation, including in patients with malignant glioma.

Acknowledgments

William Curry Financial Support: Amos Medical Faculty Development Program of the Robert Wood Johnson Foundation; Physician Scientist Development Award, Executive Committee on Research, Massachusetts General Hospital

Glenn Dranoff Financial Support: NIH CA143083

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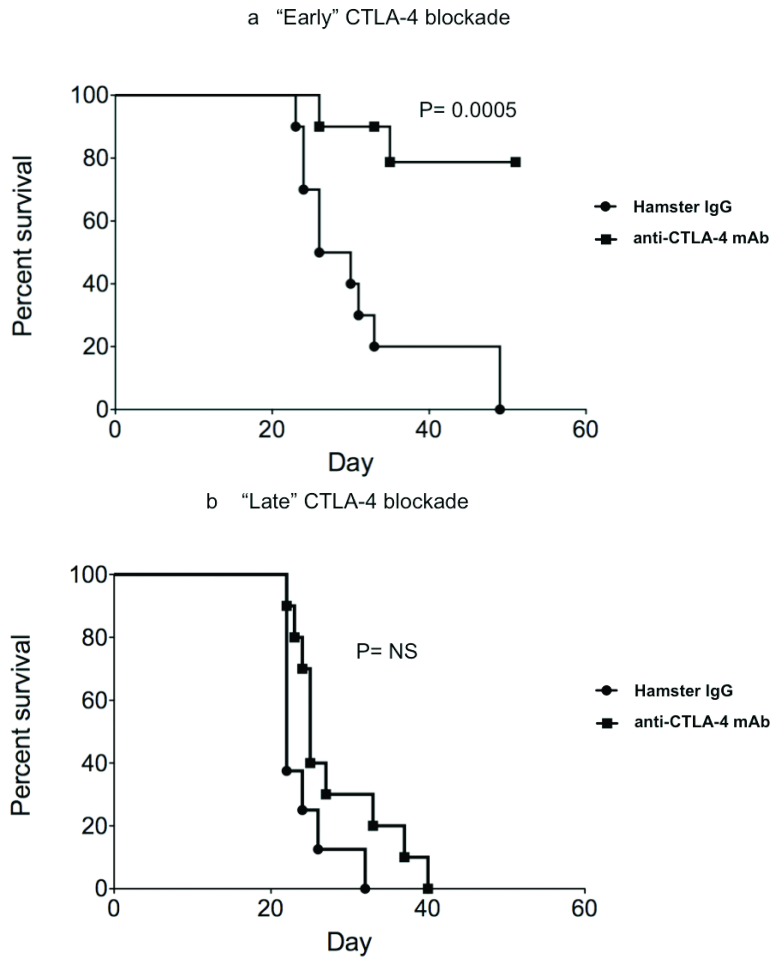


Figure 1. CTLA-4 blockade effectively increases survival in mice bearing recently established intracranial GL261 gliomas, but is less effective when delivered at later timepoints and at lower doses. (A) Intraperitoneal injection of 100 micrograms of anti-CTLA-4 mAb days 3, 6, and 9 after tumor implantation was associated with prolonged survival in C57/BL6 mice bearing intracranial gliomas. (B) CTLA-4 blockade did not significantly improve survival in mice with malignant glioma when antibody was administered on days 12, 15, and 18 after intracranial tumor implantation. (Each experiment documented in figure was performed at least twice).

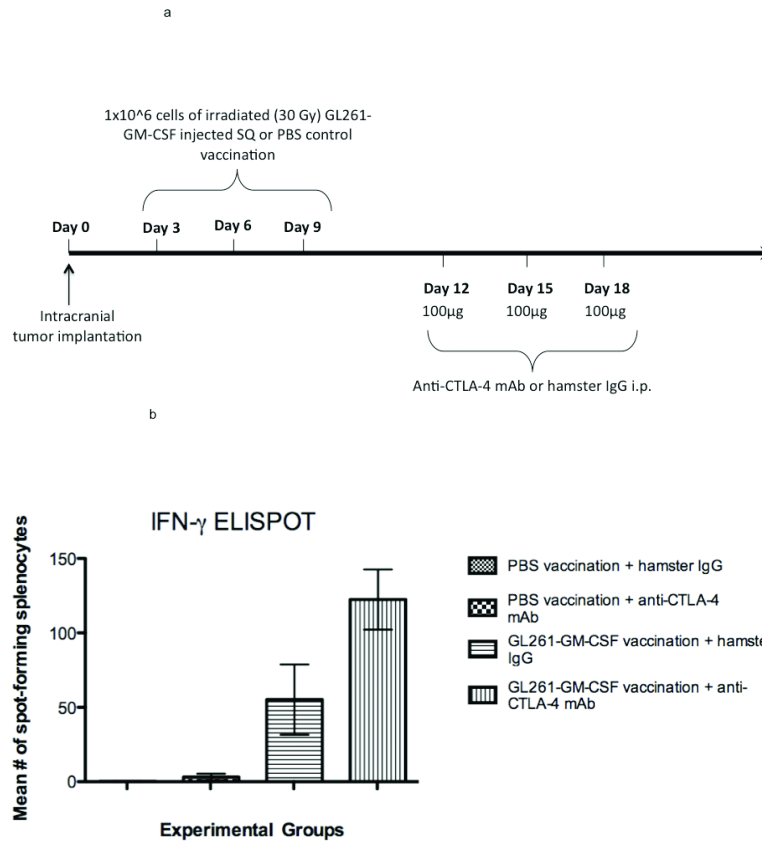


Figure 2. Interferon-gamma ELISPOT. (A) Treatment schedule for sequential combined immunotherapy with GM-CSF-expressing GL261 cells and anti-CTLA-4 mAb. Intracranial implantation of 75,000 GL261-ffluc cells into the right frontal lobe occurred on day 0. (B) Splenocytes harvested from mice treated with sequential vaccination and CTLA-4 blockade were significantly more likely to express interferon-gamma in response to stimulation by irradiated GL261 cells than were mice treated with either vaccination ($p=0.03$) or CTLA-4 blockade alone ($p=0.002$)

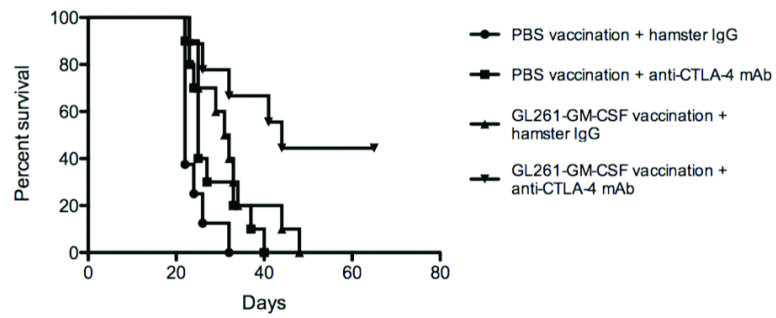


Figure 3. C57/BL6 mice bearing established intracranial GL261 tumors had better long-term survival after treatment with sequential vaccination and CTLA-4 blockade (9H10 antibody given on days 12, 15, and 18) than did mice treated with either as monotherapy (figure is representative of 3 experiments, treating 8-10 mice per group).

Table 1

Survival analyses after sequential immunotherapy. Mantel-Haenszel analysis demonstrates significant survival differences between groups. CTLA-4 blockade at days 12,15, and 18 alone did not significantly improve survival over control, though there was a trend towards longer survival. Following vaccination with CTLA-4 blockade was significantly better than either modality alone.

Comparison Group A	Comparison Group B	Mantel-Haenszel Survival	Significance
PBS Vaccination + hamster IgG	PBS Vaccination + anti-CTLA mAb	p=.053	Trend
PBS Vaccination + hamster IgG	GL261-GMCSF vaccination + hamster IgG	p=.005	Yes
PBS Vaccination + hamster IgG	GL261-GMCSF vaccination + anti-CTLA-4 mAb	p=.0008	Yes
PBS Vaccination + anti-CTLA-4 mAb	GL261-GMCSF vaccination + hamster IgG	p=.218	No
PBS Vaccination + anti-CTLA-4 mAb	GL261-GM-CSF vaccination + anti-CTLA-4 mAb	p=.004	Yes
GL261-GM-CSF vaccination + hamster IgG	GL261-GM-CSF vaccination + anti-CTLA-4 mAb	p=.032	Yes