Immune checkpoint blockade for GBM

Glioblastoma Eradication Following Immune Checkpoint Blockade in an Orthotopic, Immunocompetent Model

David A. Reardon\textsuperscript{1,2}, Prafulla C. Gokhale\textsuperscript{3,4*}, Sarah R. Klein\textsuperscript{2*}, Keith L. Ligon\textsuperscript{2,5}, Scott J. Rodig\textsuperscript{6}, Shakti H. Ramkissoon\textsuperscript{5}, Kristen L. Jones\textsuperscript{4}, Amy Saur Conway\textsuperscript{4}, Xiaoyun Liao\textsuperscript{2}, Jun Zhou\textsuperscript{5}, Patrick Y. Wen\textsuperscript{1}, Annick D. Van Den Abbeele\textsuperscript{4,7,8}, F. Stephen Hodi\textsuperscript{2}, Lei Qin\textsuperscript{4}, Nancy E. Kohl\textsuperscript{4+}, Arlene H. Sharpe\textsuperscript{9}, Glenn Dranoff\textsuperscript{2+}, and Gordon J. Freeman\textsuperscript{2}

\textsuperscript{1}Center for Neuro-Oncology, \textsuperscript{2}Department of Medical Oncology, \textsuperscript{3}Belfer Institute for Applied Cancer Science, \textsuperscript{4}Lurie Family Imaging Center, \textsuperscript{5}Center for Molecular Oncologic Pathology, Dana-Farber Cancer Institute/Brigham and Women’s Hospital; \textsuperscript{6}Department of Pathology, Brigham and Women’s Hospital; \textsuperscript{7}Department of Imaging, Dana-Farber Cancer Institute, \textsuperscript{8}Department of Radiology, Brigham and Women’s Hospital, \textsuperscript{9}Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA

*Contributed equally to this work.

+ Current Address:
Nancy E Kohl
Blueprint Medicines
38 Sidney Street
Cambridge, MA 02139

Glenn Dranoff
Novartis Institutes for BioMedical Research
250 Massachusetts Avenue
Cambridge, MA 02139

Running title: Immune checkpoint blockade for GBM

Prior Presentation: Presented in part at the 50\textsuperscript{th} Annual Meeting of the American Society of Clinical Oncology, 2014 and the 19\textsuperscript{th} Annual Meeting of the Society for Neuro-Oncology, 2014.

Keywords: Programmed death-1; cytotoxic T-lymphocyte antigen-4; glioblastoma; immunosuppression; immune checkpoint

Corresponding Author: David A. Reardon, M.D., Clinical Director, Center for Neuro-Oncology; Dana-Farber Cancer Institute; 450 Brookline Avenue, Dana 2134; Boston, MA 02215-5450; telephone: 617-632-2166; fax: 617-632-4773; E-mail: david_reardon@dfci.harvard.edu

Classification: Biological Sciences (Medical Sciences)

Conflict of interest statement:

D. Reardon reports receiving honoraria from Merck, Bristol-Myers Squibb, and Genentech and has served as a consultant for or on the advisory board of Bristol-Myers Squibb, Roche, and Regeneron.

F. S. Hodi reports consulting for Merck and Genentech.
Immune checkpoint blockade for GBM

G. Freeman has served on advisory boards for CoStim, Bristol-Myers Squibb, Roche, Novartis, and Eli Lilly. He has patents/pending royalties with Bristol-Myers Squibb, Roche, Merck, EMD-Serono, Boehringer-Ingelheim, Amplimmune, and Novartis.

A. Sharpe has received sponsored research support from Novartis. She has served on advisory boards for CoStim and Surface Oncology and consulted for Novartis. She has patents/pending royalties from Roche and Novartis, and a reagent license with Pfizer.

None of the other authors have any conflict of interest with the subject matter or materials discussed in the manuscript.

Funding Support: NIH funding support was from NCI U54CA163125 and NCI P50 CA101942 (AS and GF). The authors also gratefully acknowledge the following organizations for funding support: The Ben and Catherine Ivy Foundation; Hope It’s A Beach Thing; and the Rachel Molly Markoff Foundation.
Immune checkpoint blockade for GBM

Abstract

Inhibition of immune checkpoints including cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1) and its ligand PD-L1 has demonstrated exciting and durable remissions across a spectrum of malignancies. Combinatorial regimens blocking complementary immune checkpoints further enhance the therapeutic benefit. The activity of these agents for patients with glioblastoma, a generally lethal primary brain tumor associated with significant systemic and microenvironmental immunosuppression, is not known. We therefore systematically evaluated the anti-tumor efficacy of murine antibodies targeting a broad panel of immune checkpoint molecules including CTLA-4, PD-1, PD-L1, and PD-L2 when administered as single agent therapy and in combinatorial regimens against an orthotopic, immunocompetent murine glioblastoma model. In these experiments, we observed long-term tumor-free survival following single agent anti-PD-1, anti-PD-L1, or anti-CTLA-4 therapy in 50%, 20%, and 15% of treated animals, respectively. Combination therapy of anti-CTLA-4 plus anti-PD-1 cured 75% of the animals, even against advanced, later stage tumors. In long-term survivors, tumor growth was not seen upon intracranial tumor re-challenge, suggesting that tumor-specific immune memory responses were generated. Inhibitory immune checkpoint blockade quantitatively increased activated CD8⁺ and NK cells, and decreased suppressive immune cells in the tumor microenvironment and draining cervical lymph nodes. Our results support prioritizing the clinical evaluation of PD-1, PD-L1, and CTLA-4 single agent targeted therapy as well as combination therapy of CTLA-4 plus PD-1 blockade for patients with glioblastoma.
INTRODUCTION

Outcome for glioblastoma (GBM), the most aggressive primary cancer of the brain, remains dismal. The most effective chemotherapeutic, the alkylating agent temozolomide, extends survival by a modest 2.5 months and results in a median survival of 14.6 months (1, 2). In addition, recently reported phase 3 clinical trials evaluating chemotherapy dose intensification (3), anti-angiogenic therapy (1, 2) and integrin inhibition (3) have failed to improve survival. Innovative treatment strategies to improve outcome for this unmet patient need remain imperative.

Historical dogma purporting immunoprivilege of the central nervous system (CNS) have gradually eroded, with the demonstration of lymphatics within the CNS (4) and growing data supporting a dynamic interaction between the CNS and the systemic immune systems (5). This paradigm shift has contributed to a growing interest in the evaluation of immunotherapeutic approaches for brain tumors including GBM. A variety of vaccination strategies have demonstrated encouraging preliminary results (9). Nonetheless, the dominant immunosuppressive mechanisms exploited by GBM tumors to resist anti-tumor immune attack likely limits improvement in outcome.

Immune checkpoints normally function to reduce or abrogate immune system responses to specific antigens. Among inhibitory checkpoints, CTLA-4 and PD-1 expression markedly increase upon T cell activation (6, 7). CTLA-4 reduces early stages of T cell expansion primarily in systemic lymph nodes by out-competing CD28 for B7 ligand binding (8). In contrast, PD-1 interacts with its ligands PD-L1 and PD-L2 to inhibit T cell receptor–dependent proliferation and cytokine production, alter T cell motility and metabolism, and enhance survival of regulatory T cells primarily within peripheral lymph nodes and regions of inflammation (6, 9, 10). CTLA-4 and PD-1 normally serve as non-redundant, serial, negative regulators to protect normal tissues from damage associated with immune system activation and to foster immunotolerance to prevent autoimmunity.
Immune checkpoint blockade for GBM

Many tumors exploit the normally protective role of inhibitory immune checkpoints as a strategy to evade immune system attack. Tumor infiltrating lymphocytes can express large amounts of PD-1, which may reflect an exhausted phenotype including poor effector function and enhanced expression of other inhibitory receptors (6, 7). PD-L1 and PD-L2 are also expressed by many tumors and in some series have been linked with poor prognosis (8, 9). Among GBM tumors, nearly 90% diffusely express PD-L1 (6), expression of which is linked with loss of the phosphatase and tensin homolog (PTEN) tumor suppressor gene, which occurs in up to 40% of GBM tumors (11, 12).

Individual blockade of CTLA-4, PD-1, or PD-L1 has achieved noteworthy benefit for patients with challenging solid tumors and lymphoid malignancies, including durable tumor regression in up to 25% of advanced melanoma patients (13-15). Combined blockade of CTLA-4 and PD-1 increased the response rate to approximately 70% among advanced melanoma patients (16). The therapeutic benefit associated with these agents for GBM patients has not been determined and limited preclinical GBM data reveal variable results. We therefore conducted a systematic evaluation with blocking CTLA-4, PD-1, PD-L1, and PD-L2 monoclonal antibodies (mAbs) against an orthotopic, immunocompetent GL261 GBM model. In addition to separate blockade of each individual checkpoint, we also evaluated combinatorial regimens with potentially complementary mechanisms of activity. Given the nonredundant regulatory roles of CTLA-4 and PD-1 signaling on T cell activity, and the documented enhanced antitumor effect of combined blockade observed in patients (7), we first evaluated blockade of CTLA-4 with inhibition of either PD-1 or PD-L1. We then evaluated PD-1 blockade with inhibition of either PD-L1 or PD-L2 as a strategy to more effectively suppress overall PD-1 mediated immunosuppression in this model.

Our results reveal that blockade of CTLA-4, PD-1, or PD-L1 alone can eradicate growing GBM tumors within the CNS, including late stage tumors. Anti-tumor activity can be augmented by combinatorial therapy targeting CTLA-4 and PD-1. We also noted that long-term survivors demonstrate immune
Immune checkpoint blockade for GBM

memory responses capable of preventing tumor growth following rechallenge. Finally, we demonstrate that combined CTLA-4 and PD-1 blockade enhances infiltration of effector immune cells while reducing suppressive immune cell subsets within the tumor microenvironment and draining cervical lymph nodes.

METHODS AND MATERIALS

Cell Line, Antibodies and Reagents

Luciferase-transduced GL261 cells (GL261-luc2) were purchased (Perkin-Elmer) in 2014, expanded and frozen without further testing or authentication. Thawed cells were cultured for up to three passages in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and 100 µg/mL G418 at 37°C in a humidified incubator maintained at 5% CO₂ prior to intracranial implantation. Cells were maintained in logarithmic growth phase for all experiments.

The following mouse anti-mouse monoclonal antibodies (mAbs) were generated in specific gene-deficient mice, in the laboratory of Dr. Gordon Freeman: PD-1 - 332.8H3 (mouse IgG1, K); PD-L1 – 339.6A2 (mouse IgG1, K), and PD-L2 – 3.2 (mouse IgG1, K). Mouse anti-mouse CTLA-4 – 9D9 (mouse IgG2b, K), was purchased from BioXCell (West Lebanon, NH). Each of these mAbs blocks interaction with ligand. Isotype controls were purchased from BioXCell and included: MOPC21 (IgG1), MPC-11 (IgG2b), and C1.18 (IgG2a). All mAbs contained less than 2 EU endotoxin/mg protein.

Intracranial Tumor Cell Inoculation

1x10⁵ GL261-luc2 cells, which are syngeneic in C57BL/6 mice,(8) were resuspended in PBS and injected stereotactically into the right striatum of anesthetized, 6-10 week old, female, albino C57BL/6 mice (Jackson Laboratory; Bar Harbor, ME, USA) using a Hamilton syringe and stereotactic frame. Mice were
Immune checkpoint blockade for GBM

euthanized for signs of morbidity due to tumor burden or at 120 days after re-injection if healthy appearing. All animal experiments were approved by the Dana-Farber Animal Care and Use Committee.

In Vivo Treatment and Tumor Assessment

For all studies, mice with enlarging tumor burden defined by increasing bioluminescence signal between days 3 and 6 post tumor implantation were randomized into control and treatment cohorts (eight mice per cohort). Tumor response assessment was done by quantifying bioluminescence in all animals as well as MRI imaging in a subset (see SI Materials and Methods).

First, we determined the anti-tumor effect against a growing, established tumor model. Therapeutic mAbs and isotype controls were administered via intraperitoneal injection beginning on day 6 (500 µg) after tumor implantation with repeat injections every 3 days (250 µg/dose) for a total of eight injections. Control animals received equivalent doses of isotype murine IgG according to the same dosing schedule. No treatment was administered after day 27 following tumor implantation. Using this treatment schedule, we systematically evaluated the anti-tumor activity as measured by survival using mAbs against CTLA-4, PD-1, PD-L1, and PD-L2 as single agent and in combinatorial regimens in separate experiments.

We then addressed whether inhibitory immune checkpoint blockade can have a therapeutic effect against an advanced, later stage tumor model. A similar treatment schema to that detailed above was employed but treatment was initiated on day 14 after tumor implantation. CTLA-4 or PD-1 mAb monotherapy or in combination, as well as appropriate isotype controls, were repeated every 3 days for eight total doses. No further therapy was administered beyond day 35 after tumor implantation.

Re-challenge Experiments
Immune checkpoint blockade for GBM

GL261-luc2 cells (1 x 10^5) were injected intra-cranially into the contralateral hemisphere in a cohort of mice previously treated beginning on day 6 who survived over 100 days as well as five treatment naive CL57BL/6 albino mice. Rechallenged mice were followed for a minimum of 100 additional days and received no additional therapy. Mice were then sacrificed at which point the brain was removed, fixed in 4% formalin, and embedded in paraffin. Two-3 µm sections were stained with hematoxylin and eosin and then examined for microscopic evidence of tumor by two neuropathologists (KLL and SR) blinded to prior treatment.

**Characterization of Immune Response**

Immune response assessment studies were performed on material obtained from euthanized, tumor-bearing animals on day 24 following mAb treatment administered on days 14, 17, 20 and 23. For flow cytometry, brains, spleens, and superficial cervical lymph nodes were harvested, and homogenized using enzymatic (1.5 mg/mL collagenase IV, 200 U/mL DNaseI, HBSS with calcium and magnesium) and/or mechanical tissue disaggregation. Brains cells were resuspended in 25% Percoll Plus (Sigma) for myelin removal and leukocyte isolation. Red blood cells were removed using a Ficoll gradient (GE Life Sciences). The following antibodies were used for flow cytometric analysis of cell surface proteins: anti-CD45 (30-F11; Biolegend), anti-CD3 (17A2; Biolegend), anti-CD4 (RM4-5; Biolegend), anti-CD8 (53-6.7; Biolegend), anti-CD11b (M1/70; Biolegend), anti-CD86 (GL-1; Biolegend), anti-NK1.1 (PK136; Biolegend), anti-Gr1 (RB6-8C5; Biolegend), anti-PD1 (RMP1-30; eBioscience), anti-Tim3 (B8.2.C12; Biolegend) and anti-CTLA4 (UC10-4B9; Biolegend). Dead cells were excluded using the Zombie NIR fixable viability kit (Biolegend). Following surface staining, cells were fixed and permeabilized with the FoxP3 Fixation/Permeabilization Kit (eBioscience). The following antibodies were used for intracellular staining: anti-FoxP3 (MF-14; Biolegend), and anti-Granzyme B (NGZB; eBioscience). Acquisition was performed on an LSR Fortessa.
Immune checkpoint blockade for GBM

SORP HTS flow cytometer (BD Biosciences). Data analysis was performed using FlowJo X 10.7.7r2 (Tree Star).

Immunohistochemistry studies for the detection of CD4 and CD8 positive lymphocytes were performed on separate, snap-frozen and paraffin-embedded portions of the frontal cerebrum according to established methods. Analysis of circulating immunocytokines was performed on serum using the Mouse Chemokine Antibody Array (R&D Systems; Minneapolis, MN) according to manufacturer’s instruction (for further details, see SI Materials and Methods).

**Statistical Analysis**

Survival estimates and median survivals were determined using the method of Kaplan and Meier. A log-rank (Mantel-Cox) test was used to calculate $P$-values derived from statistical analysis of Kaplan-Meier survival curves. A one-way ANOVA followed by a Tukey’s multiple comparisons test was used to determine the statistical significance between two experimental groups in the flow cytometric and serum analysis. $P$-values of less than 0.05 were considered statistically significant (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). Quantitative analysis was performed with Graphpad Prism 6 (Graphpad Software, Inc).

**RESULTS**

**Eradicating established GBM tumors and generating long-term survival**

We first performed a series of experiments to systematically evaluate the anti-tumor activity of blocking individual inhibitory immune checkpoint molecules as well as combinatorial regimens with potentially complementary mechanisms of action (Fig. 1 and Table 1). In our initial experiments, treatment began on day 6 against an established, growing model tumor, GL261-luc2 (Fig. 1A). Intracranial
Immune checkpoint blockade for GBM

bioluminescence of all animals initially increased for 1-2 weeks, consistent with growing tumor burden. Thereafter, bioluminescence decreased in responding mice, consistent with tumor regression. In contrast, nonresponders in each treatment cohort progressively increased luciferase counts (Fig. 2 and Supplemental Fig. S1). Changes in tumor burden were confirmed by magnetic resonance imaging in a subset of treated mice (Fig. 2).

Using the established tumor model, we compared blockade of CTLA-4, PD-1, and PD-L1 separately (Fig. 1B and C). PD-1 blockade showed the greatest efficacy, with long-term effective cures in 56% of the treated animals, whereas blockade of either PD-L1 or CTLA-4 had less survival benefit. Because CTLA-4 and PD-1 suppress T cell activation nonredundantly, we also evaluated the antitumor effect of CTLA-4 blockade with either PD-1 mAb or PD-L1 mAb. In these experiments, CTLA-4 mAb combined with PD-1 mAb led to an effective cure rate of 75% (Fig. 1B). In contrast, the addition of PD-L1 blockade to CTLA-4 mAb had a negligible effect (Fig. 1C).

We also compared and combined blockade of PD-1, PD-L1, and PD-L2 and found that PD-1 mAb improved survival better than PD-L1 mAb, whereas PD-L2 mAb had no effect (Fig. 1D and E). Of note, combinatorial therapy with PD-1, PD-L1, or PD-L2 did not improve survival compared to single agent PD-1 or PD-L1 mAb therapy.

Of note, long-term (≥100 days) survivors were observed in a subset of mice treated with PD-1, CTLA-4, and PD-L1 mAbs, as well as combinations thereof (Fig. 1B-E and Table 1). These animals appeared healthy and neurologically intact. In addition, there was no evidence of intracranial tumor noted upon histopathologic examination in this subset (data not shown). The median survival of mice treated with any immune checkpoint mAb except anti-PD-L2, as well as combinations thereof, was longer than control mice (Table 1). Among cohorts of mice treated with single agent therapy, median survival was longest among PD-1 mAb treated mice (91 days). Among mice treated with combination therapy, PD-1
Immune checkpoint blockade for GBM

mAb plus CTLA-4 mAb increased survival additively when compared to single agent therapy. This combination achieved the longest survival (median not reached at 141 days, at which point survivors were euthanized) of any treatment cohort in our experiments. In contrast, median survival of the other combination groups were modestly improved compared to controls, but did not differ significantly from mice treated with single agent therapy.

Immunologic memory that prevents recurrence following tumor re-challenge

To examine the development of immunologic memory, long term (> 100 days) survivors of checkpoint therapy for established tumors were challenged with contralateral intracranial injection of 1x10^5 GL261-luc2 cells (Fig. 3A). Thirteen of 14 (93%) long-term survivors following mAbs against PD-1 (n=5), CTLA-4 (n=3) or combination PD-1+ CTLA-4 (n= 6) therapy survived after rechallenge for an additional 120 days (Fig. 3B). When sacrificed, these mice showed no evidence of microscopic intracranial tumor (Fig. 3C). In contrast, four of five treatment-naïve, control mice died approximately 30 days following injection of GL261-luc2 cells.

Advanced, later stage GBM tumors effectively treated with immune checkpoint blockade

In order to evaluate the therapeutic impact of immune checkpoint blockade against an advanced intracranial GBM tumor model, we initiated treatment on day 14 after tumor implantation (Fig. 4A). By day 14, tumors were readily detectable by MRI (data not shown). Treatment was repeated every three days for eight total doses with no further therapy administered after day 35 following tumor implantation. As expected, control animals died from progressive tumor within 28-30 days. In contrast, 1 of 8, 4 of 8 and 7 of 8 animals which were treated with mAbs to CTLA-4, PD-1, or CTLA-4 plus PD-1, respectively, remained alive without evidence of tumor or neurologic compromise >100 days following tumor implantation (Fig. 4B).
Immune checkpoint blockade for GBM

**Intratumoral analysis: enhanced immune effector cell infiltration while decreasing immunosuppressive cells**

We evaluated changes in intratumoral and systemic immune cell subsets in mice treated with mAbs against CTLA-4, PD-1, or combination CTLA-4 plus PD-1 compared to isotype control antibody. In these studies, treatment of advanced disease was initiated on day 14 after tumor implantation as depicted in Fig. 4 and repeated on days 17, 20, and 23 prior to sacrificing the animals on day 24.

Flow cytometry analysis of tumor infiltrating immune cells (Figs. 5 and 6 and Supplemental Fig. S2) revealed an increased number of CD8+ cytotoxic T cells and decreased number of CD4+FoxP3+ Tregs that reached statistical significance for mice treated with CTLA-4 mAb plus PD-1 mAb combination therapy compared to controls (Fig. 5A and B). Single agent CTLA-4 and PD-1 mAb therapy was also associated with a significant reduction of tumor infiltrating Tregs (Fig. 5B). In accordance with these data, the CD8+ effector cell/CD4+FoxP3+ Treg ratio was significantly increased for the combination therapy cohort compared to controls (Fig. 5C). We also noted a significant increase of intratumoral activated NK cells (percent CD86+ gated on NK1.1+ cells)(9, 10) compared to controls for each treatment cohort (Fig. 5D).

We also characterized tumor infiltrating T cells (TILs) for expression of immunoinhibitory receptors CTLA-4, PD-1, or PD-1 and TIM-3 coexpression (PD-1+/TIM3+). The latter represent a particularly exhausted CD8+ T cell population within tumors.(11, 12) Although CTLA-4 expressing TILs were comparable among treatment groups and controls (data not shown), the numbers of PD-1 positive (Fig. 6A and B) and PD-1+/TIM3+ TILs (Fig. 6C) were markedly lower among mice treated with either PD-1 or CTLA-4 plus PD-1 mAbs compared to controls. Of note, the PD-1 staining mAb used is not blocked by the PD-1 treatment mAb and do not share the same epitope (Supplemental Fig. S3). Moreover, myeloid-derived suppressor cells (MDSC; CD11b+Gr1+) in tumors trended downward for each treatment cohort but only achieved statistical significance for the combination cohort compared to controls (Fig. 6D).
Immune checkpoint blockade for GBM

Immunohistochemical analysis confirmed enhanced CD4 and CD8 infiltrates in general, which were most striking following combination PD-1 plus CTLA-4 mAb therapy (Supplemental Fig. S4).

**Systemic immune system changes induced by immune checkpoint blockade**

Changes in lymphocyte subsets in draining cervical lymph nodes followed similar trends as observed among tumor infiltrating lymphocytes (Fig. 5 and 6 and Supplemental Fig. S5). Specifically, cytotoxic CD8⁺ and CD8/Treg ratio were increased, whereas FoxP3⁺ Tregs and PD-1⁺/TIM3⁺ lymphocytes were decreased among combination-treated animals compared to controls. In contrast, lymphocytes isolated from the spleens of CTLA-4 and combination-treated mice had higher percentages of Tregs and PD-1⁺/Tim3⁺ T cells (Fig. 5 and 6).

Activated NK cells were increased in both the cervical lymph nodes and spleens among mice receiving checkpoint blockade in a pattern similar to that observed in tumors (Fig. 5D). In contrast, MDSCs decreased in the tumor but increased in both cervical lymph nodes and spleens of treated animals (Fig. 6D). The significance of changes observed systemically versus only in the tumor requires further investigation.

We also evaluated the effect of immune checkpoint blockade on serum concentration of 24 chemokines in our model of advanced GBM (Supplemental Fig. S6 and Supplemental Table 1). We detected significant increases in chemokines CCL9 (MIP-1γ), CCL6 (C10), CCL11 (Eotaxin), CXCL12 (SDF-1), and CCL8 (MCP-2) in the serum of single agent and/or combination-treated animals. Changes in other evaluated chemokines did not achieve statistical significance.

**DISCUSSION**

Therapeutics targeting immune checkpoint mediators such as CTLA-4 and PD-1 have achieved profound benefit across a growing number of cancer indications yet their value for patients with GBM, the most
Immune checkpoint blockade for GBM

common and deadliest CNS malignancy, remains unknown. Furthermore, preclinical studies to date vary significantly with regard to methods and reagents, leading in turn to a wide spectrum of outcome and uncertain conclusions. We therefore performed a series of experiments that incorporated novel and strategic considerations in order to clarify the potential value of inhibitors targeting CTLA-4 and PD-1 signaling for GBM.

As a first step, we utilized immune checkpoint blocking mAbs that accurately reflect reagents currently used in the clinic for patients. Current clinical efforts to inhibit CTLA-4 among cancer patients use a human mAb that blocks interaction with B7 ligands and has an Fc that can enlist antibody dependent cytotoxicity (ADCC) to deplete CTLA-4 expressing Tregs (13). In contrast, currently approved human PD-1 mAbs are designed to block interaction with ligands but have an Fc that does not enlist ADCC.(13) To best model this in mice, we used mouse CTLA-4 and PD-1 antibodies with the same properties to treat orthotopic, intracranial GBM tumors in immunocompetent mice. This contrasts with most preclinical experiments including those previously reported for GBM(14-19) which use rat or hamster antibodies in mice and are limited in duration by the development of mouse anti-rat antibodies. Notably, the use of syngeneic mouse mAbs in our study should reduce anti-antibody responses, allow for longer treatment and more closely model the human clinical experience. Among the immune checkpoints targeted in our experiments, only PD-L1 was detectable on GL-261 cells (Supplemental Fig. S7) indicating that the observed therapeutic benefit of PD-1 and CTLA-4 mAbs was due primarily to effects on immune cells rather than the tumor itself.

Second, in order to assess which single agent and combination targets provide the greatest anti-tumor benefit, we systematically blocked CTLA-4, components of PD-1 signaling including PD-1, PD-L1, and PD-L2, as well as combinations with predicted complementary benefit. Among mice with established intracranial tumors, we noted that single agent PD-1 blockade achieved long-term survival among 50% of
Immune checkpoint blockade for GBM

treated mice, whereas 15-20% of animals treated with CTLA-4 or PD-L1 mAbs alone were long-term survivors.

Long term survivors in our study were effectively cured as none of these animals had evidence of viable tumor on histopathologic evaluation upon sacrifice. Although other groups have evaluated single agent CTLA-4, PD-1, or PD-L1 blockade in GBM models, evidence of antitumor benefit varied considerably. For example, CTLA-4 blockade achieves high (≥ 80%) (14, 17), intermediate (20) or low/non-existent (18, 19) rates of long-term survival. Similarly, blockade of either PD-1 or PD-L1 results in either high (20) or little (16) clinical benefit. The variability of therapeutic benefit observed across these studies likely reflects differences in experimental design such as choice of GBM model, choice of blocking mAb, and timing/dosing of mAb administration. In contrast, a distinguishing feature of our study was that all of these variables were controlled, allowing consecutive experiments to specifically assess the relative impact of each immune checkpoint molecule. Of note, our experiments also uniquely evaluated PD-L2 blockade for GBM, although the lack of observed therapeutic benefit may be due to absence of PD-L2 expression by GL261 (Supplemental Fig. S7). Nonetheless, our results suggest that single agent immune checkpoint blockade should prioritize targeting PD-1 for clinical translation among GBM patients, although CTLA-4 and PD-L1 blockade also warrant clinical investigation.

Among combinatorial regimens, administration of CTLA-4 plus PD-1 mAb therapy was most active and resulted in marked increase in both the percentage of long-term survivors as well as median overall survival, compared to controls or monotherapy with either agent. Approximately 75% of animals treated with this combination were long-term, tumor-free survivors. These findings are consistent with a recent clinical trial that observed a 2-3-fold increase in durable responses among advanced melanoma patients treated with combined CTLA-4 and PD-1 mAb therapy compared to single agent historical data, although higher rates of immune related adverse events were noted with combined therapy (21). Our study is the
Immune checkpoint blockade for GBM

only one to also evaluate combinatorial targeting of PD-1 plus PD-L1, as well as PD-L1 plus PD-L2, as strategies to more effectively block PD-1–mediated immunosuppression. We noted that these combinations offered no increased therapeutic benefit compared to single agent therapy. Whether these results are applicable to other cancer models will require further study. In addition, we observed modestly increased benefit of CTLA-4 plus PD-L1 blockade as has been reported(20). Finally, in our experiments there were no clinically apparent adverse events among responding mice treated with immune checkpoint blockade, including combinatorial therapy. Treated mice demonstrated normal activity and did not develop weight loss or exhibit evidence of neurologic or hormonal deficits, although detailed, organ-specific, histopathologic or laboratory examinations were not performed.

The reason for the greater efficacy of PD-1 blockade relative to PD-L1 or CTLA-4 in this GBM model, as well as combinations thereof, remains to be determined, but several factors may contribute. First, the immunoinhibitory mechanisms of PD-1 and CTLA-4 are inherently distinct (21) and most active on distinct cells in different locations. Because B7 ligands are primarily expressed in lymphoid tissue, CTLA-4 blockade is thought to be most important in lymphoid organs, particularly on Tregs that express high levels of CTLA-4. In contrast, PD-1/PD-L1 interactions are thought to predominate in non-lymphoid tissues with PD-1 expression on CD8- T cells within the tumor microenvironment considered particularly important. In addition, PD-L1 expression in glioblastoma tumors may be upregulated by several factors including IFN-γ, VEGF, and oncogenic changes. We confirmed PD-L1 expression by GL-261, and a recent report demonstrates significant PD-L1 expression by most glioblastoma tumors (22). This expression pattern may mean that T cells can enter CNS tissue pre-coated with PD-1 mAb or require less PD-1 than PD-L1 mAb in situ for effective blockade. Finally, PD-L2 can inhibit T cell activation and would be impacted by PD-1 but not PD-L1 blockade. PD-1 blockade may therefore promote anti-tumor immune activity to a greater extent compared to CTLA-4 or PD-L1 inhibition. All of these factors may contribute to variable anti-tumor responses associated with administration of different immune checkpoint
Immune checkpoint blockade for GBM

blocking antibodies across tumor models and potentially between individually treated patients in the clinic.

In addition, we also demonstrated that CTLA-4 or PD-1 mAb therapy alone and in combination were effective against advanced, later stage, intracranial GBM tumors. In fact, the percentage of long term survivors among animals treated beginning 14 days after tumor implantation, was comparable to that achieved when treatment was initiated six days after implantation. Successful treatment of late stage tumors is of particular relevance to GBM patients who are often confronted clinically with large, unresectable and aggressively growing tumors. Based on our systematic evaluation of combinatorial regimens against both established and advanced GBM models, our results support prioritizing combinatorial blockade of CTLA-4 plus PD-1 for clinical development among GBM patients, while combination treatment targeting CTLA-4 plus PD-L1 may also warrant clinical evaluation.

An exciting capability of immunotherapy is its ability to exert a dual phase therapeutic benefit that initially includes effective treatment of existing tumors, followed by prevention of future relapse by successful induction of tumor specific immune memory responses. Another strategic aspect of our experiments was demonstration that increased survival was also associated with evidence of tumor-specific immunologic memory. Specifically, intra-cranial rechallenge experiments revealed no evidence of tumor growth in all but one long-term surviving animal following initial CTLA-4, PD-1, or CTLA-4 plus PD-1 mAb combination therapy. Prior studies demonstrated induction of memory responses capable of rejecting flank tumor rechallenge (16, 18, 19), however, in our hands, tumors grew in only 50% of injected C57BL/6 mice who underwent flank injection of GL261-luc2, indicating that the flank may not be a reliable site to evaluate the induction of antitumor immune responses. In contrast, over 90% of mice developed tumors when either GL261 or GL261-luc2 was injected intracranially. Our study uniquely demonstrated that induced systemic immune memory responses are capable of rejecting tumor re-
Immune checkpoint blockade for GBM challenge in the CNS. The latter finding is particularly relevant to GBM given that relapse universally occurs within the CNS and very rarely occurs systemically.

Another key aspect of our experiments was a detailed investigation of changes among immune cell populations within the GBM microenvironment, draining cervical lymph nodes and the spleen, as well as changes in circulating immunocytokines. The degree of therapeutic benefit for single agent as well as combination immune checkpoint blockade was associated with reproducible changes in immune cell subsets. Changes in the brain and cervical lymph nodes trended similarly in most cases, whereas changes in the spleen were more variable. Treated tumors draining cervical lymph nodes had an increase in the infiltration of effector CD8+ cells and activated NK cells. Simultaneously the percentage of immunosuppressive lymphocytes including Tregs, PD-1+ lymphocytes, PD-1+/TIM-3+ exhausted T cells, and MDSCs decreased. Our results are consistent with previous studies of checkpoint blockade,(14, 16, 19, 20) and were marked in animals treated with combination CTLA-4 plus PD-1 mAb therapy. We also observed a systemic increase in several interferon-γ inducible chemokines including CCL9 (MIP-1γ), CCL6 (C10), CCL11 (Eotaxin), and CXCL12 (SDF-1).

In conclusion, we demonstrated that systemic administration of CTLA-4, PD-L1, and PD-1 inhibitors can improve survival for intra-cranial GBM tumors. Single agent blockade achieved durable survival benefit in a subset of tumor-bearing animals that was most robust with PD-1 mAb therapy, but dual blockade of CTLA-4 plus PD-1 exhibited the greatest anti-tumor benefit, CTLA-4 plus PD-L1 had modest activity, while PD-L1 with either PD-1 or PD-L2 revealed no additive benefit. Significant therapeutic benefit was achieved against advanced, later stage tumors and was associated with specific systemic antitumor immunologic memory responses that prevented CNS relapse upon intracranial rechallenge. Mechanistically, tumor and cervical lymph node infiltration of effector immune cells was enhanced, whose activity is likely augmented by a concurrent decrease in immune cells capable of inhibiting anti-tumor responses, in animals with improved survival. Our findings support the clinical evaluation of immune checkpoint blockade for GBM patients and prioritize targeting CTLA-4, PD-L1, and PD-1 separately and in combinatorial regimens for clinical development.

REFERENCES
Immune checkpoint blockade for GBM


Immune checkpoint blockade for GBM


Figure Legends

Figure 1. Immune checkpoint blockade improves survival against an established intra-cranial GL261-luc2 (1x10^5 cells) glioblastoma tumor model including long-term tumor free survival (A). Monitoring and treatment schema and Kaplan-Meier survival curves following immune checkpoint blockade using mAbs against CTLA-4 and PD-1 (B), CTLA-4 and PD-L1 (C), PD-1 and PD-L1 (D), and PD-L1 and PD-L2 (E). Each experiment includes eight mice per cohort and IgG isotype controls.

Figure 2. Regression of growing GL261-luc2 tumors following inhibitory immune checkpoint blockade. Representative MRI findings and bioluminescence of a control mouse treated with isotype control IgG and a responding mouse treated with CTLA-4 plus PD-1 mAb therapy. Following intracranial implantation of GL261-luc2 cells on day 0 (1 X 10^5 cells), mice were treated on days 6, 9, 12, 15, 18, 21, 24 and 27. MRI scans were obtained on days 6, 21 and 35 along with bioluminescence quantitation. On day 21 mice treated with isotype control IgG as well as those treated with CTLA-4 plus PD-1 combination mAb therapy had increased T2WI signal abnormality and increased bioluminescence counts consistent with tumor growth. Control animals died from progressive tumor by day 30 and therefore did not have repeat imaging on day 35. Animals responding to combinatorial immune checkpoint blockade had decreased size of FLAIR signal abnormality on MRI and loss of fluorescence signal consistent with regressing tumors.

Figure 3. Checkpoint blockade survivors reject intra-cranial tumor re-challenge consistent with anti-tumor memory immune responses. (A) Monitoring and treatment schema. (B) Kaplan-Meier survival
Immune checkpoint blockade for GBM

curves of long-term survivors (>100 days) initially treated with the indicated mAbs or treatment-naive controls following intra-cranial inoculation of 1x10^5 GL261-luc2 cells. No treatment was given after tumor re-challenge. (C) Histopathologic examination of post-mortem brains of re-challenged mice and treatment-naive controls. Representative images from long-term (≥ 100 days) survivors after re-challenge reveal some small focal areas of chronic vacuolated parenchymal injury and macrophage immune cell infiltrate at site of initial tumor implantation but no residual tumor.

Figure 4. Immune checkpoint blockade improves survival against an advanced, later-stage intra-cranial GL261-luc2 glioblastoma tumor model including long-term tumor free survival. (A) Monitoring and treatment schema for advanced disease model and (B) Kaplan-Meier survival curves following inhibitory immune checkpoint blockade using mAbs against CTLA-4 and PD-1.

Figure 5. Immune cell infiltrates within intracranial tumor, draining cervical lymph nodes (cLN), and spleen following checkpoint blockade. Tumor was established with advanced disease and treated as described in Figure 4 with isotype control IgG or mAbs against CTLA-4, PD-1 or the combination of CTLA-4 plus PD-1. Leukocyte populations were prepared from tissues on Day 24. (A) CD8^+/granzyme B^+ effector T cells as a percentage of live CD45^+CD3^+ cells. (B) CD4^+/FoxP3^+ Tregs as a percentage of live CD45^+CD3^+ cells. (C) The ratio of effector CD8 T cells to Tregs. (D) CD86^+ cells as a percentage of NK1.1 cells. Graphs include values for individually analyzed mice, and the mean ± SEM of nine mice per treatment group. One-way ANOVA was used to determine statistical significance (*P<0.05; **P<0.01; ***P<0.001)

Figure 6. Suppressive immune cell infiltrates within intracranial tumor, draining cervical lymph nodes (cLN) and spleen following checkpoint blockade. Mice were treated and cells prepared as in Figure 5. (A) Expression of PD-1 on T Cells gated for live CD45^+CD3^+ cells. Isotype control antibody staining is shown on the right. Representative flow cytometry contour plots from each tissue of all treatment groups. Percentages of CD45^+CD3^- T cells that are (B) PD-1^+ and (C) PD-1^+/TIM3^-. (D) CD11b^-/Gr1^- myeloid derived suppressor cells as a percentage of live CD45^+ cells. Graphs include values for individually analyzed mice, and the mean ± SEM of nine mice per treatment group. One-way ANOVA was used to determine statistical significance (*P<0.05; **P<0.01; ***P<0.001)
Table 1. Outcome by treatment cohort for individual (A) and aggregate (B) experiments against an established glioblastoma model.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Treatment mAb</th>
<th># Treated</th>
<th># long-term survivors (% of cohort)</th>
<th>Median Survival (days)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B</td>
<td>Isotype control</td>
<td>16</td>
<td>0</td>
<td>27.5</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>CTLA-4</td>
<td>16</td>
<td>4 (25)</td>
<td>40.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>PD-1</td>
<td>16</td>
<td>9 (56.3)</td>
<td>&gt;146</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>CTLA-4 + PD-1</td>
<td>16</td>
<td>12 (75)</td>
<td>&gt;146</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>1C</td>
<td>Isotype control</td>
<td>8</td>
<td>0</td>
<td>24.0</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>CTLA-4</td>
<td>8</td>
<td>0</td>
<td>31.0</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td>PD-L1</td>
<td>8</td>
<td>2 (25)</td>
<td>32.0</td>
<td>0.0012</td>
</tr>
<tr>
<td></td>
<td>CTLA-4 + PD-L1</td>
<td>8</td>
<td>3 (37.5)</td>
<td>30.0</td>
<td>0.0016</td>
</tr>
<tr>
<td>1D</td>
<td>Isotype control</td>
<td>8</td>
<td>1 (12.5)</td>
<td>25.5</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>PD-1</td>
<td>8</td>
<td>3 (37.5)</td>
<td>35.5</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>PD-L1</td>
<td>8</td>
<td>2 (25)</td>
<td>32.0</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>PD-1 + PD-L1</td>
<td>8</td>
<td>3 (37.5)</td>
<td>33.5</td>
<td>NS</td>
</tr>
<tr>
<td>1E</td>
<td>Isotype control</td>
<td>8</td>
<td>0</td>
<td>27.0</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>PD-L1</td>
<td>8</td>
<td>1 (12.5)</td>
<td>32.0</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>PD-L2</td>
<td>8</td>
<td>0</td>
<td>27.5</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>PD-L1 + PD-L2</td>
<td>8</td>
<td>1 (12.5)</td>
<td>35.5</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*p-value reflects comparison to isotype control cohort
NA: not applicable; NS: not significant
David Reardon / Figure 1

A. Established glioblastoma model (↓ = intracranial implantation; ↓ = treatment; ◦ = bioluminescence measurement)

B. Percent survival vs. days after implantation for different mAb therapies:
- PD-1 + CTLA-4
- PD-1
- CTLA-4
- IgG Control

C. Percent survival vs. days after implantation for different mAb therapies:
- PD-L1 + CTLA-4
- PD-L1
- CTLA-4
- IgG Control

D. Percent survival vs. days after implantation for different mAb therapies:
- PD-1 + PD-L1
- PD-1
- PD-L1
- IgG Control

E. Percent survival vs. days after implantation for different mAb therapies:
- PD-L1 + PD-L2
- PD-L1
- PD-L2
- IgG Control
David Reardon / Figure 2

Day 1: GL261-luc2 implantation (1x10^5 cells)

Day 6: MRI #1
Treatment #1

Days 9, 12, 15, 18
Treatment #2, 3, 4, 5

Day 21: MRI #2
Treatment #6

Days 24, 27
Treatment #7, 8

Day 35: MRI #3

Isotype IgG control therapy

CTLA-4 + PD-1 mAb combination therapy

Color Scale
Min = 5.00e4
Max = 5.02e6

Radiance (p/sec/cm^2/sr)
David Reardon / Figure 3

A. Re-challenge experiment:
- Intracranial implantation (↓)
- Treatment (↓)
- Bioluminescence measurement (●)
- Contralateral brain GL261-luc2 implantation (▼)

B. Percent survival over time:
- Prior mAb therapy:
  - PD-1 + CTLA-4: 5/6 survivors
  - PD-1: 5/5 survivors
  - CTLA-4: 3/3 survivors
  - Treatment naïve: 1/5 survivors

C. Prior mAb therapy:
- Treatment naïve
- CTLA-4 mAb
- PD-1 mAb
- CTLA-4 + PD-1 mAb
David Reardon / Figure 4

A Advanced glioblastoma model (↓ = intracranial implantation; ⇓ = treatment; • = bioluminescence measurement)

Days

B

mAb therapy

Percent survival

# Long-term survivors

Days after implantation

PD-1 + CTLA-4 7/8
PD-1 4/8
CTLA-4 0/8
IgG control 0/8
David Reardon / Figure 5

A

Brain

Percent CD8+ Gzm B+ T cells

Spleen

Percent CD8+ Gzm B+ T cells

B

Percent CD4+ FoxP3+ T cells

C

Ratio of CD8+ Gzm B+/CD8+ T cells

D

Percent CD8+ of NK1.1+ cells

Author Manuscript Published OnlineFirst on November 6, 2015; DOI: 10.1158/2326-6066.CIR-15-0151

Downloaded from cancerimmunolres.aacrjournals.org on June 22, 2017. © 2015 American Association for Cancer Research.
Cancer Immunology Research

Glioblastoma Eradication Following Immune Checkpoint Blockade in an Orthotopic, Immunocompetent Model

David A. Reardon, Prafulla C. Gokhale, Sarah R. Klein, et al.

Cancer Immunol Res  Published OnlineFirst November 6, 2015.

Updated version  Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-15-0151

Supplementary Material  Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2015/11/06/2326-6066.CIR-15-0151.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.