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Enhancing Immunity?to?Glioma: Modulating the Adaptive Immune Response in the Tumor Microenvironment

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Enhancing Immunity to Glioma: Modulating the Adaptive Immune Response in the Tumor Microenvironment

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Neuroscience

by

Joseph Paul Antonios

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ABSTRACT OF THE DISSERTATION

Enhancing Immunity to Glioma: Modulating the Adaptive Immune Response in the Tumor Microenvironment

by

Joseph Paul Antonios

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2016

Professor Linda M. Liau, Co-Chair

Professor Robert M. Prins, Co-Chair

Glioblastoma (GBM) is the most common and lethal of all adult primary malignant brain tumors. For patients diagnosed with GBM, the median survival is 11-14 months despite advances in surgical resection techniques, chemotherapies, and radiation therapy (1). Alternate therapeutic strategies are being actively pursued to target GBM, with various immunotherapeutic modalities designed to generate an anti-GBM immune response showing considerable promise in preclinical models and clinical trials. To more effectively target GBM with these treatments, there has been an increasing appreciation of the numerous mechanisms involved in generating and maintaining the highly immunosuppressive tumor microenvironment in recent years. These studies have described a variety of microenvironmental and systemic factors that promote glioma cell evasion

from the immune system. In light of these, it has become apparent that these factors must be understood and explicitly targeted to mount a successful immune response against GBM.

This thesis describes the utilization of two different immunotherapeutic strategies to target GBM. The first strategy created a novel GBM target by inducing NY-ESO-1 antigen expression with the demethylating agent, decitabine, and targeting that antigen with engineered T cells. Specifically, we utilized human GBM cell cultures to induce expression of the antigen. We evaluated NY-ESO-1 TCR-transduced T cell-mediated GBM tumor cytolysis in a series of *in vitro* cytotoxicity assays. Following this, we examined the application of this therapy using an intracranially-implanted xenograft model. Our studies demonstrated that decitabine could effectively upregulate NY-ESO-1 both *in vivo* and *in vitro*. Engineered T cells were able to induce tumor cytolysis *in vitro* and were able to traffic to and target tumor *in vivo*. Tumor-bearing mice receiving adoptive transfer of these engineered T cells demonstrated significantly increased survival over mice that received non-transduced T cells. By inducing expression of a novel target on GBM, we were able to generate a highly specific, anti-GBM immune response. This strategy represented a clinically translatable therapeutic technique for treating patients with GBM.

The second strategy focused on using existing GBM targets to generate an endogenous immune response in a syngeneic, immune competent mouse model. Briefly, we administered an autologous tumor lysate-pulsed dendritic cell (DC) vaccine to produce a glioma-specific immune response. In our studies, the vaccination appeared to be capable of inducing T cell infiltration into tumors; however, in large, established tumors, this infiltrating response was not sufficient to

increase mouse survival and provide significant therapeutic benefit. We described the role of the negative costimulatory pathway, programmed death-1/ligand-1 (PD-1/PD-L1) in mitigating T cell activation and memory in a series of *in vitro* and *in vivo* studies. We noted that PD-1 blockade with PD-1 mAb was not sufficient to produce a T cell infiltrate. However, when administered with DC vaccination, PD-1 blockade activated the vaccine-generated T cell response in the tumor microenvironment. We found that T cells with PD-1 mAb were able to mediate significant tumor cytolysis when compared to T cells without PD-1 blockade *in vitro*. The adjuvant administration of PD-1 mAb with the DC vaccine resulted in significant survival benefit over DC vaccine alone in mice bearing large, established gliomas. Additionally, this dual treatment resulted in the increased expression of integrin homing and immunologic memory markers on T cells infiltrating tumor. These findings were corroborated in samples from patient GBM, with PD-1 blockade enhancing the T cell-mediated GBM cytolysis. Concerning this strategy, then, these findings provided us with a means to both generate and enhance a tumor-specific response.

While this second strategy proved effective, the mechanism underlying this PD-1/PD-L1-mediated suppression was not fully understood. As such, we proceeded to identify a PD-L1-expressing tumor infiltrating myeloid (TIM) cell population that appeared to dominantly regulate the PD-1/PD-L1 signaling mechanism. Importantly, we determined the role that these cells play in inhibiting the immune response using a series of *in vitro* and *in vivo* studies utilizing TIM depletion and PD-1 mAb treatment strategies. We found that depletion of TIMs in both human GBM cultures and murine glioma abolished PD-1/PD-L1-mediated inhibition of T cell activation. Targeting TIMs with colony stimulating factor-1 receptor inhibitor (CSF-1Ri)

reduced the TIM population significantly and altered the remaining TIMs such that they demonstrated increased expression of chemotactic factors. While treatment with CSF-1Ri in conjunction with DC vaccine did not alter PD-L1 expression on remaining TIMs, we did note that there was increased TIL infiltration with this dual treatment significantly over DC vaccine alone. These findings suggested that TIMs exert inhibitory effects in the tumor microenvironment in a manner not restricted only to the PD-1/PD-L1 signaling mechanism. We found that the combined treatment of CSF-1Ri and PD-1 mAb with DC vaccination both increased TIL infiltration and activation in the tumor microenvironment. These findings were therapeutically relevant, with tumor-bearing mice receiving all three treatments showing a significant increase in survival over mice receiving each treatment alone. The studies outlined herein elucidated the role that TIMs play in dominantly mediating the PD-1/PD-L1 signaling mechanism to restrict TIL activation, as well as the ability to manipulate this population pharmacologically with clinically accessible agents.

In conclusion, this thesis demonstrates two distinct strategies to generate and enhance an immune response against GBM. In our first strategy, we utilized the adoptive transfer of engineered T cells to selectively target an antigen whose expression we artificially induced in GBM. This technique was largely effective. However, we were interested in directly targeting antigens already expressed by GBM. To that end, we described the utility of DC vaccination in generating an immune response. Further, we delineated the inhibitory mechanisms employed by TIMs in the tumor microenvironment and developed a therapeutic adjuvant to administer with DC vaccination. We confirmed the efficacy of these treatments in a series of *in vitro* and *in vivo* animal studies, and we recapitulated these findings in our novel, *ex vivo* human GBM studies.

Together, the studies presented in this thesis represent an innovative approach to understanding and immunotherapeutically targeting the GBM microenvironment.

The dissertation of Joseph Paul Antonios is approved.

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To Carol Kruse, mentor and friend

Table of Contents

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Chapter 1:

Introduction to immunotherapy for glioblastoma: mounting an immune response in the tumor microenvironment

Overview of the Immune Response

The immune system responds to infectious agents and harmful substances over three basic stages: recognition, containment, and elimination of pathogens. When tumor is present, the immune system employs several mechanisms in response. First, innate immunity, dependent on the actions of phagocytic cells (macrophages, histiocytes), granulocytes (neutrophils, basophils, and eosinophils), and dendritic cells (DCs), comes into play. This first line of defense also includes mast cells, derived from the common myeloid progenitors and mediating allergic responses, and common lymphoid progenitor-derived natural killer (NK) cells able to recognize and kill virus-infected and tumor cells. Recognition through pathogen-associated molecular patterns (PAMP) results in a set of responses that drive the immune response to activation of the adaptive immune system. The adaptive immune system is dependent on naïve and effector T-lymphocytes developing in the thymus and B-lymphocytes developing in the bone marrow. This system is mediated by antigen-presenting cells (APCs) that process and present tumor antigens (Ag) on major histocompatibility (MHC) proteins and express co-stimulatory molecules essential to induce an effective immune response. In gliomas, activated DCs (the most potent APCs) migrate to cervical lymph nodes, up-regulate the expression of co-stimulatory molecules, i.e., CD80/86, and activate T cell receptors (TCR), which induce Ag-dependent clonal expansion of cytokine-secreting or cytotoxic T cells tropic to glioma tissue by the virtue of differential expression of chemokine receptors and adhesion molecules. This binding of Ag to TCR results in the proliferation and differentiation of effector T lymphocytes, including cytotoxic CD8+ T cells (CTL), among others. Fully activated CTL attack glioma cells via up-regulation and secretion of FasL, perforin, granzymes and TNF (1).

Immune Suppression in the GBM Microenvironment

The central nervous system (CNS) was once thought to be an immune-privileged site. However, studies have shown that the blood-brain barrier, which restricts small molecule and protein movement into the cerebrospinal fluid (CSF) spaces of the CNS, does not limit CNS entry of immune cells in cancer and other disease states (2, 3). The ability of these cells to sample antigens here, as well as traffic into and out of the CNS has been demonstrated across multiple studies, suggesting that the immune system can mount a T cell-driven response against glioblastoma (GBM) (3-5). Nevertheless, certain barriers do in fact exist in the CNS. The brain environment itself is endogenously immunosuppressive. These limitations are usually important to maintain the integrity of the nervous system. Outside of the CNS, damage to normal tissue associated with an immune response can be tolerated.

However, inflammation in the brain can quickly affect neurologic function. Surrounded by the rigid skull compartment, the brain is unable to tolerate any off-target effects caused by the immune response (6). Indeed, glioma patients are associated with having overall lymphopenia due to the modulatory factors released by the glioma cells (7-10). Elevated levels of immunosuppressive cytokines, including IL-10 and TGFβ, in the relatively acidotic brain tissue do little to promote immunity (11, 12). TGF-β promotes tumor cell migration, angiogenesis and tumor stroma growth (13), and also decreases the number and cytotoxic response of tumor infiltrating T cells, NK cells and macrophages (14, 15). IL-10 downregulates MHC class II expression and is further thought to regulate the Jak/STAT signaling pathway and NF-kB

activity to inhibit inflammation (16, 17). In fact, glioma cells downregulate or defectively express class I and II antigens in proportion to glioma grade (18-20).

In addition to an immune inhibitory CNS milieu, a population of tumor-infiltrating cells appears to limit immune response mounted against tumor and promote tumor progression. This population seems to be largely heterogeneous, with various investigations identifying inhibitory myeloid and monocytic lineage populations, including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and other antigen-presenting cell (APC) populations in the GBM tumor microenvironment (17, 21-31). In our work herein, we describe these cells as tumor-infiltrating myeloid cells (TIMs), a term that encompasses TAMs, MDSCs, myeloid and monocytic lineage cells, and APCs.

While the role of these TIMs has not been fully described, they appear to be directly recruited by the tumor to help promote its progression (32). Modulators secreted by gliomas, including IL-10, TGF-β, and CSF-1, support the proliferation of inhibitory cells such as regulatory T cells and myeloid-derived inhibitory cells (7-10). Murdoch et al. described migration of monocytic cells into tumor through blood vessels dependent on tumor-derived chemo-attractants, including CSF-1, CC chemokines (CCL2 3, 4, 5, and 8), and VEGF (33). The differentiation, proliferation, and migration of monocytes and macrophages occur via colony-stimulating factor (M-CSF or CSF-1) signaling through the tyrosine kinase CSF-1 receptor (CSF-1r) (34-36). Priceman et al. confirmed that macrophages are dependent on CSF-1/CSF-1r signaling for recruitment into tumor, additionally demonstrating their role in promoting tumor growth (37). DeNardo et al. confirmed these findings in the breast cancer model and further showed that CSF-1r blockade

inhibits tumor-infiltrating macrophages associated with chemotherapy treatment, improving treatment outcomes (38). Xu et al. showed that when CSF-1r is blocked with a selective inhibitor, TAM infiltration decreases (39).

There have been several landmark studies evaluating how inhibitory monocytic-lineage cells promote tumor progression (28, 40). Studies showed that they highly express IL-1 and IL-6, with low expression of tumor necrosis factor-a (TNFa) (41, 42). Of note, it was shown that their expression of TNF-a and matrix metalloproteinases (MMP) promoted NF-kB signaling, resulting in increased tumor invasion (43, 44). Lewis et al. also suggested that these macrophage-type cells act to promote angiogenesis and suppress antitumor immune responses (45). Stat-3 signaling was shown blocking the antitumoral response and promoting amplification of regulatory T cells in the tumor microenvironment (46). Depletion of TAMs reversed these findings, such that a tumor lacking TAMs demonstrated slower progression (47). More recent work has identified the relevance of M1/M2 macrophage polarization in these studies. Mantovani et al. suggested that IL-4 and IL-10 in the tumor microenvironment promote the polarization of TAMs to a type II (M2) phenotype (48). The tumor microenvironment has been characterized with the additional expression of IL-6, MDF, TGF-h1, and PGE2 (49). M2-polarized TAMs promote expression of T cell suppressive factors, reduced antigen-presenting capability, and angiogenesis. To contrast, M1-type macrophages support tumor cell killing and promote T cell activation and proliferation (48, 50).

Passive Immunotherapeutic Targeting of GBM

The three-pronged approach to cancer treatment - surgery, chemotherapy, and radiotherapy - has been largely ineffective in significantly increasing median survival for the majority of glioma patients (51, 52). Immunotherapy is a therapeutic modality capable of targeting malignant cells using biological immune mechanisms (53-55). For GBM, this treatment approach is generally divided into two broad categories: passive and active. Passive immunotherapies are characterized by administration of monoclonal antibodies and adoptive T cell transfer. Active approaches, on the other hand, induce an endogenous immune response with the administration of vaccine treatment, such as tumor cell lysate-pulsed dendritic cells or glioma peptide-pulsed dendrite cells) (53). Both these approaches have found success both in the laboratory and the clinic.

The goal of passive immunotherapy is to generate an immune response without the active participation of the patient's immune system. For example, monoclonal antibodies are synthesized to target glioma-specific surface antigens, receptor-ligand pairs, or matrix proteins associated with GBM, with the intent of disrupting cell signaling of GBM cells, opsonizing glioma cells, or enhancing immune function (56-60). This approach has recently seen some significant advancement with the development of convection-enhanced delivery (CED) and trans-nasal therapy to further enhance drug delivery (61-64). More relevant to our discussion is the passive therapeutic approach of adoptive cell transfer - the administration of anti-tumor effector cells directly into the patient (65, 66). Both preclinical and clinical studies have utilized various effector T cells for targeting tumor, including NK and NK T-like CD8+ cells, lymphokine-activated cells (LAK), allo-reactive T cells, and engineered antigen-specific T cells (67-70). The latter, engineered T cells, have shown therapeutic benefit in both preclinical models of glioma (71), as well as in clinical trials in other cancers such as metastatic melanoma (72-74).

Finally, chimeric antigen receptor (CAR) T cells have been employed in studies with considerable success. By joining the variable regions of an antibody with the transmembrane and cytoplasmic signaling domains derived from the TCR ζ-chain and costimulatory molecules such as CD28, CD134, and CD137, CAR T cells can be made to target antigens in an HLA-unrestricted manner and exhibit the cytotoxic activity of T cells (75-80). Together, these therapies represent a clinically relevant approach to generating and introducing an immune response that can selectively target GBM.

Overview of Active Immunotherapy

Active immunotherapy, characterized by vaccine-based treatments, is designed to induce a long-term endogenous immune response. The first attempts at vaccine-based immunotherapy utilized whole-proteins obtained from tumor cell lysate (81, 82). Over the years, active vaccination has been shown to generate a tumor-infiltrating CD8+ T lymphocyte (TIL) response and inflammation capable of promoting increased survival in both preclinical and clinical studies (83-86). Although peptide-based vaccines (87), autologous vaccines with live, attenuated cancer cells (NCT01081223, NCT01290692), and even EGFRvIII Ag vaccines (88-91) have been shown to support an anti-GBM immune response, significant effort has been devoted to the development and application of DC vaccine-based treatments.

DCs are responsible for coordinating immune responses by bridging non-antigen specific innate immunity with the antigen-specific adaptive immunity (92-95). Because of their specialized ability to modulate antigen specific responses through CD8+ cytotoxic T-cells, DCs are prime

candidates to induce anti-tumor immunity. Relevant to tumor immunotherapy, GBM-specific antigens can be used to generate a DC vaccine. Initial studies focused on tumor-eluted peptides (96-98), tumor lysates (99-104), and tumor-derived RNA (105, 106) to generate DC vaccines. In the case of tumor-eluted peptides, autologous tumor cells obtained following surgical resection are used to derive tumor-associated peptides. Monocyte-derived DCs can then be exposed to these peptides and then transferred to patients to stimulate an immune response (98). Liau et al. and Yu et al. demonstrated the feasibility and safety of using tumor-eluted peptide DC vaccination, with increased time to progression and an increase in overall survival noted in treated patients compared to patients receiving standard of care therapy (96-98).

More recently, DCs directly pulsed with autologous tumor lysate have shown promise in extending survival (101). Several Phase I trials have been conducted to test the safety and feasibility of tumor lysate-pulsed dendritic cells, resulting in prolonged clinical time to progression and overall survival in many patients (99-104). There was increased intratumoral infiltration of CD3+ and CD8+ lymphocytes in DC vaccine patients following vaccination (107, 108). Interestingly, varied patient responses were attributed to differences in GBM subtypes of proneural, proliferative, and mesenchymal and their associated genetic signatures (101). The latter – mesenchymal – was both the group with the worse prognosis prior to DC vaccine treatment and with the better survival and time to progression following vaccine treatment. Although it was initially thought that lysate DC vaccine preparations would induce severe autoimmune reactions due to the likely presence of non-GBM specific antigens in tumor lysate, no major adverse events have been reported. Importantly, DC vaccination has been shown to

effectively generate an antigen-specific T cell response, increase immune cell infiltration into tumor, and promote increased survival in both preclinical models and patients.

Modulating the Immune Response in GBM

Studies have demonstrated significant benefit with using adjuvants to immunotherapeutic treatments. Of these, Interleukin-2 (IL-2) has been often utilized alongside both passive and active immune therapies. IL-2 is essential for cytotoxic T-cell proliferation, function, and development *in vitro* (109). Intratumoral adoptive administration of cytotoxic T-cells with IL-2 appeared to increase anti-tumor activity (110). Half of patients treated with IL-2 as an adjuvant to HSV-TK had significant tumor responses. Administration of polyICLC, a TLR-3 ligand, promotes production of pro-inflammatory cytokines and expansion and maturation of killer (NK) cells to enhance anti-tumor immune activity and tumor growth retardation (111, 112). Similarly imiquimod, a TLR-7 agonist, effectively promoted the downregulation of T cell tolerance (113). When administered with melanoma-associated antigen (MAA) peptide-pulsed DC vaccination, there was a significant increase in survival and tumor regression over DC vaccination alone (114).

Nevertheless, the utilization of immunotherapy in a clinical setting is largely hampered by failsafes employed by the immune system to avoid immune over-activation. Generally, to establish an immune response against cancer, effector T cells must be tumor-antigen specific and activated. In order to be activated, a second signal is required in addition to the TCR-mediated antigen-specific signal (115). This activation is mediated by a network of receptor-ligand pairs

that provide stimulatory and inhibitory signaling. When inhibitory, these signals play a necessary function in preventing an autoimmune reaction. However, these mechanisms can be aberrantly utilized in the tumor microenvironment to avoid immune targeting (116). Of these inhibitory signals, two are specifically relevant to GBM: cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and the programmed death 1 (PD-1) receptor (117). CTLA-4 is a coinhibitory receptor that competes with CD28+ receptor binding to mitigate the initial activation of T cells in the periphery (118). Inhibiting CTLA-4 results in objective tumor regression in patients with metastatic melanoma (119, 120). The other, PD-1, is an immune regulatory receptor associated with inhibition of T cells with prolonged antigen exposure, as in cancer or chronic viral infection. The interaction of the PD-1 receptor on T cells with its ligands PDL1 and PDL2 has been correlated to immune suppression (119, 121). Interestingly, the inhibitory PD-L1 signaling by cancer cells has also been noted (117). This direct influence of cancer cell signaling on T cell response makes PD-1 an important subject of interest. Already, some early studies have shown that inhibition of the PD-1 receptor is associated with significantly increased immune response to cancer (119, 122).

Conclusion

With its restrictive skull compartment, and continuous secretion of inhibitory factors, the CNS poses many barriers to mounting an effective immune response to GBM. Despite the intrinsic immune suppressive nature of the CNS, however, GBM is a viable target for immunotherapeutic targeting. Early studies with adoptive cellular therapies and active vaccination strategies have confirmed that GBM can be targeted with immune mechanisms. The safety and feasibility of

these approaches have been confirmed and the mechanisms dictating their efficacy are being described in recent and ongoing studies. Nevertheless, the nature of the tumor microenvironment, inhibitory and suppressive mechanisms utilized by the tumor and associated tumor-infiltrating cell populations, and their respective roles in mitigating the immune therapy-generated response are not fully understood. As our understanding of the mechanisms involved in immune resistance evolves, we will continue to refine our current treatments and devise new ones to specifically target GBM and significantly extend patient survival.

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Chapter 2:

Efficacy of systemic adoptive transfer immunotherapy by targeting selectively-expressed tumor antigens with engineered T cells

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Efficacy of systemic adoptive transfer immunotherapy targeting NY-ESO-1 for glioblastoma

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Background. Immunotherapy is an ideal treatment modality to specifically target the diffusely infiltrative tumor cells of malignant gliomas while sparing the normal brain parenchyma. However, progress in the development of these therapies for glioblastoma has been slow due to the lack of immunogenic antigen targets that are expressed uniformly and selectively by gliomas.

Methods. We utilized human glioblastoma cell cultures to induce expression of New York–esophageal squamous cell carcinoma (NY-ESO-1) following in vitro treatment with the demethylating agent decitabine. We then investigated the phenotype of lymphocytes specific for NY-ESO-1 using flow cytometry analysis and cytotoxicity against cells treated with decitabine using the xCelligence real-time cytotoxicity assay. Finally, we examined the in vivo application of this immune therapy using an intracranially implanted xenograft model for in situ T cell trafficking, survival, and tissue studies.

Results. Our studies showed that treatment of intracranial glioma–bearing mice with decitabine reliably and consistently induced the expression of an immunogenic tumor-rejection antigen, NY-ESO-1, specifically in glioma cells and not in normal brain tissue. The upregulation of NY-ESO-1 by intracranial gliomas was associated with the migration of adoptively transferred NY-ESO-1–specific lymphocytes along white matter tracts to these tumors in the brain. Similarly, NY-ESO-1–specific adoptive T cell therapy demonstrated antitumor activity after decitabine treatment and conferred a highly significant survival benefit to mice bearing established intracranial human glioma xenografts. Transfer of NY-ESO-1–specific T cells systemically was superior to intracranial administration and resulted in significantly extended and long-term survival of animals.

Conclusion. These results reveal an innovative, clinically feasible strategy for the treatment of glioblastoma.

Keywords: cancer, decitabine, engineered T cells, glioblastoma, immunotherapy.

T cell–based immunotherapy is a particularly appealing strategy for the treatment of malignant gliomas due to the inherent high specificity, lytic activity, and ability for T lymphocytes to traffic to distant tumor sites. These T cells could theoretically target infiltrating tumor cells that have permeated into the normal brain parenchyma beyond the reaches of standard surgical resection and are yet unrecognizable by conventional imaging methods.[1,2] While there has been a long-standing interest in applying immunotherapy to primary malignant brain tumors, these efforts have shown little in the way of objective therapeutic efficacy. Part of the difficulty has been: (i) the lack of well-defined immunotherapeutically targetable antigens that are expressed consistently and uniformly by glioma cells, (ii) uncertainty about the ability of immune cells to traffic into the central nervous system, and (iii) systemic and local immune defects in glioblastoma patients that alter the ability of T cells to mediate injury to tumor cells. Here we demonstrate a strategy that can be used to overcome

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many of these critical barriers to effective brain tumor immunotherapy.

The therapeutic use of engineered lymphocytes to target tumor-associated antigens is gaining traction as a viable potential therapeutic modality for cancer. In this study, we combined the selective in vivo induction of New York–esophageal squamous cell carcinoma (NY-ESO-1), an immunogenic tumor-rejection antigen, in glioblastomas with the adoptive transfer of T cells retrovirally transduced to express the NY-ESO-1 T-cell receptor (TCR). We demonstrated that engineered TCR-transduced T cells migrate through the parenchyma of the brain, and elicited enhanced therapeutic benefit after *systemic* delivery in animals harboring well-established intracranial gliomas.

Materials and Methods

Cell Lines and Biological Samples

The human glioblastoma cell lines U-251MG and T98G were obtained from the American Type Culture Collection. The primary glioblastoma cell explant, 13-06-MG, was established from surgically resected tissue of a human leukocyte antigen (HLA)–A*0201+ individual.[3] The human primary melanoma cell lines M407 and 624.38, which express endogenous NY-ESO-1, were supplied by Antoni Ribas. Further details are provided in the Supplemental Methods. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy human volunteers at UCLA Medical Center after leukapheresis. Lymphocytes were isolated by density gradient centrifugation as previously described.[4] Written informed consent and institutional review board approval were obtained for all studies involving human blood and tissues.

NY-ESO-1 TCR-Transduced Lymphocytes

The PG13-based stable retroviral packaging cell line encoding an HLA-A*0201–restricted NY-ESO$_{157-165}$ specific TCR was generated as described[5] and obtained from Dr Paul Robbins (Surgery Branch, NCI/NIH). Briefly, anti-CD3 (clone OKT-3; 50 ng/mL) was used to stimulate human PBMCs for 48 h prior to double transfection with supernatants from the PG13-based retroviral producer cell line, as described.[5,6] The cells were expanded for 3 days in the presence of interleukin (IL)-2 and then rested for 2 days in the absence of IL-2.

In vitro Glioma Cell Treatment with Decitabine

Decitabine (DAC), supplied by Eisai Pharmaceuticals, was reconstituted in dimethyl sulfoxide as a 10-μM stock solution. After allowing tumor cells (10^6 cells/mL) to culture overnight, the cell culture medium was supplemented with 1 μM DAC. The cells were treated again the following day with fresh cell culture medium with or without DAC. On the third day of culture, fresh medium without DAC was placed onto the cells and cultured for additional time periods before assaying.

In vitro Cytotoxicity Assays

Cytotoxic killing of tumor cells was assessed using the xCELLigence Real-Time Cell Analyzer System (Acea Biotechnology). Target cells (U-251MG or 13-06-MG gliomas, DAC-treated [DAC+] or nontreated [DAC−] were plated at day 0 (10^5 cells/well) in 150 μL of medium. After overnight tumor cell adherence to the well bottom, effector cells (NY-ESO-1 TCR-transduced T cells) were added at effector:target (E:T) ratios of 10:1, 5:1, 1:1, and 1:10. Maximal cell release was obtained by adding 1% Triton X-100 to the wells. Cell index values (relative cell impedance) were collected over 30 h and normalized to the maximal cell index value immediately prior to effector cell plating. The percentage lysis was calculated as a proportion of the normalized cell index at a time point of interest versus the normalized cell index at the point of initial effector cell plating.[7]

In vivo Trafficking Studies

For evaluation of T cell trafficking, female NOD scid gamma-chain deficient (NSG) mice, 6–10 weeks of age, were implanted with U-251MG glioma cells (2.5×10^5 in 2 μL phosphate buffered saline [PBS]) in the right frontal lobe. Further details are provided in the Supplemental Methods. Mice bearing one-week established tumors then received i.p. DAC (10 mg/kg) or control PBS on days 3, 5, and 7 post tumor implant, for a total of 3 doses. Two days following the last DAC injection, intracranial administration of lymphocytes occurred using the same coordinates, but at the contralateral position from the tumor implant. Brains were collected 2 days after adoptive cell transfer (ACT) and processed for immunohistochemistry (IHC).

In vivo Survival Studies

For survival studies, mice were implanted with U-251MG or 13-06-MG glioma cells (2.5×10^5 in 2 μL PBS) as described before. Mice were then randomized into treatment groups ($n = 6$–8 mice/group) and injected i.p. with 10 mg/kg DAC or control as described. NY-ESO-1 TCR engineered T cells or nontransduced PBMCs (10^6/2 μL) were administered intratumorally (i.t.) at the same ipsilateral coordinates, or systemically by i.v. injection (10^7 cells/100 μL). Immediately after ACT, 10^5 IU of IL-2 in 100 μL or 100 μL of PBS 1× was administered i.p. and again at 24 and 48 h post cell transfer for T cell support. The mice were monitored over time for symptoms of disease progression and sacrificed according to institutional guidelines. The UCLA Animal Research Committee approved all experiments. Additional details are included in the Supplemental Methods section. Survival plots were generated using the Kaplan–Meier method and plotted using GraphPad Prism software.

In vivo Cellular Characterization Studies

For analysis requiring harvest of brain and splenic tissue, NSG mice were implanted with U-251MG glioma and received i.p. DAC one-week post-implant as described before. NY-ESO-1 TCR engineered T cells (10^6/2 μL) were administered intravenously or intratumorally as described previously. Animals were sacrificed 72 h after ACT and the tumor-bearing brain hemispheres and spleens were harvested. Infiltrating lymphocytes from brain were obtained using a Percoll gradient. Cells were stained with anti-human CD3 phycoerythrin (PE)-Cy7 (clone SK7; eBioscience), CD4 Alexa Fluor 700 (clone RPA-T4; BD Biosystems), CD8 Pacific Blue (clone SFCI21Thy2D3; BD Biosystems), and TCRvβ13.1-PE (clone IMMU 222; Beckman Coulter).

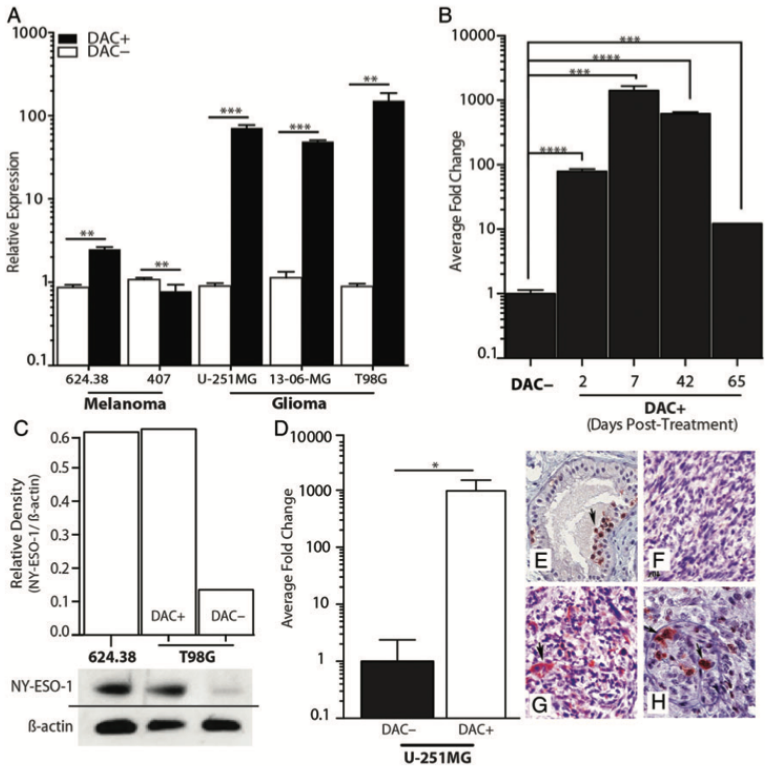

Fig. 1. Upregulation of NY-ESO-1 by DAC treatment in vitro and in situ. (A) Relative expression of NY-ESO-1, with or without DAC (1 μM) treatment for 48 h (DAC+ and DAC−, respectively), was examined in 2 melanoma cell lines, 624.38 and 407, which endogenously express NY-ESO-1. Only slight upregulation was noted when treated with DAC. Glioma cell lines U-251MG, 13-06-MG, and T98G show dramatic upregulation of NY-ESO-1 after DAC treatment (**$P < .01$, ***$P < .001$, Student's *t*-test). (B) Time-course expression of NY-ESO-1 in U-251MG glioma cells after treatment with DAC. Expression measured by quantitative (q)PCR from cells harvested at the indicated time points (***$P < 0.001$, Student's *t*-test). (C) Verification of gene expression at the protein level in 624.38 and T98G glioma cells, with and without DAC treatment by western blot. Relative density (NY-ESO-1/β-actin) is plotted. (D) Mice with intracranial U-251MG tumors were treated with control (DAC−) or DAC (DAC+); tumors were then excised at day 19 post-implant and used either for qPCR or for IHC detection of NY-ESO-1 expression. The relative fold change in gene expression is graphed (*$P < .05$, Student's *t*-test). IHC staining on a positive testes control (E), as well as on brains from DAC− (F) and DAC+ (G and H) is displayed. Data shown are representative of one experiment repeated 3 times with similar findings.

Quantitative Real-Time PCR

Total RNA was isolated with the RNeasy Mini kit (Qiagen) according to manufacturer's protocol. Human glioblastoma cancer cell lines (3×10^6) or tumor tissue specimens (25 mg) were used. Quantitative real-time (RT) PCR was performed with the LightCycler RT-PCR system (Roche), using the LightCycler SYBR green mastermix (Roche). Additional details are provided in the Supplemental Methods.

Western Blotting

Membrane-bound proteins were probed with monoclonal antibodies to NY-ESO-1 (Upstate Biotechnology) and β-actin (Sigma) and then visualized with a secondary antibody conjugated to horseradish peroxidase (1:2500, Jackson ImmunoResearch Laboratories) using a chemiluminescent substrate (Thermo-Scientific). X-ray films were scanned on a BioRad Gel Doc with Image Lab software.

Histology and Immunohistochemistry

Histology and IHC staining were performed as previously published.[8] To evaluate the induction of NY-ESO-1 in orthotopic glioma xenografts, DAC-treated and untreated control mice ($n = 8$/group) were sacrificed on day 21 after implantation. Whole brains were rapidly harvested and placed in formalin overnight prior to being embedded in paraffin. Serial sections (5 μm) were deparaffinized, then either stained with hematoxylin and

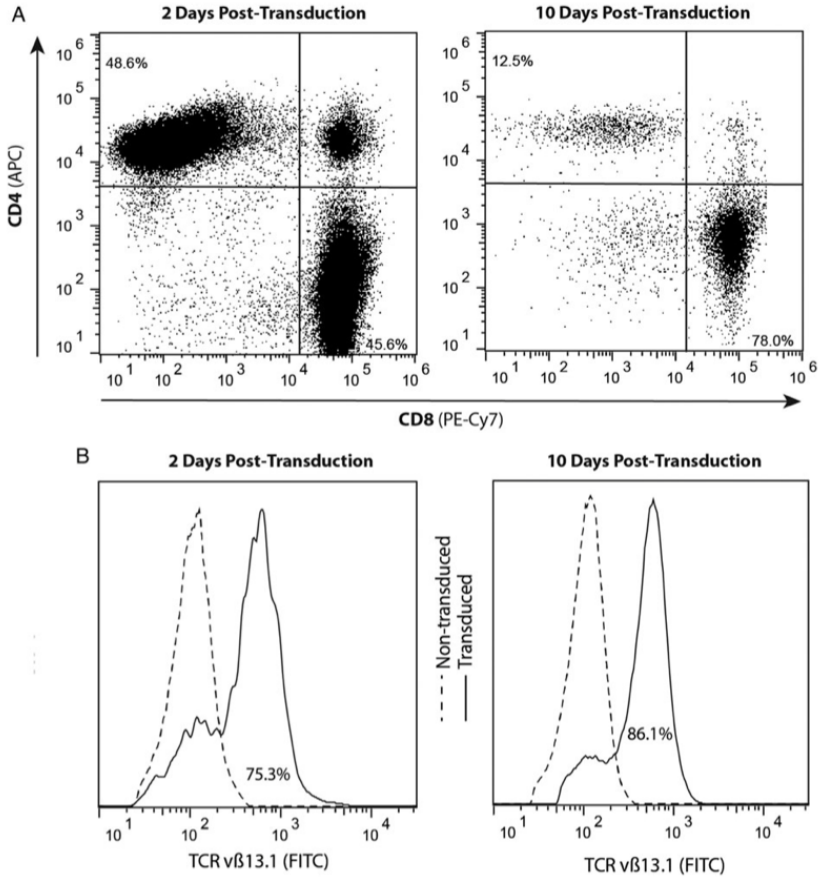

Fig. 2. Retroviral transduction of the NY-ESO-1 TCR generates a high percentage of antigen-specific T cells, over a 10-day culture, prior to ACT. (A) Populations skew toward a CD3/CD8 cytotoxic T cell phenotype over 10 days of expansion. The percentages of CD8+ and CD4+ T cell populations were estimated by flow cytometry at 2 days (45.6% and 48.6%, respectively) and at 10 days (78.0% and 12.5%, respectively) following transduction and expansion in medium supplemented with 300 IU/mL of IL-2. APC, allophycocyanin. (B) Expression of TCRvβ13.1 by CD8+ T cells at 2 days (75.3%) and 10 days (86.1%) posttransduction compared with nontransduced T cells. Data shown are representative of one experiment repeated 4 times with similar findings. FITC, fluorescein isothiocyanate.

eosin or used for IHC with antibodies to NY-ESO-1 (Life Technologies), CD3, or CD8 (both Biocare Medical). Further details are provided in the Supplemental Methods.

Statistical Analysis

Continuous variables were compared using Student's *t*-test. Results comparing more than 2 groups were analyzed by ANOVA followed by Kruskal–Wallis statistics. Values were considered significant at $P < .05$. Median survival times were estimated using the Kaplan–Meier method. Within each glioma xenograft type, study comparisons between treatments were carried out using a log-rank test. A Cox proportional hazards model, stratifying on the glioma xenograft types, was also used to compare the effectiveness of treatments after combining the 2 different xenograft models. SAS 9.3 software was used for all statistical analyses.

Results

Decitabine Induces Durable Expression of NY-ESO-1 in Glioblastoma In vitro and In vivo

In our previous studies, we discovered that the cancer-testis antigen NY-ESO-1 was highly upregulated after a short, low-concentration in vitro exposure to the demethylating agent DAC.[9] To translate these findings to a therapeutic model and

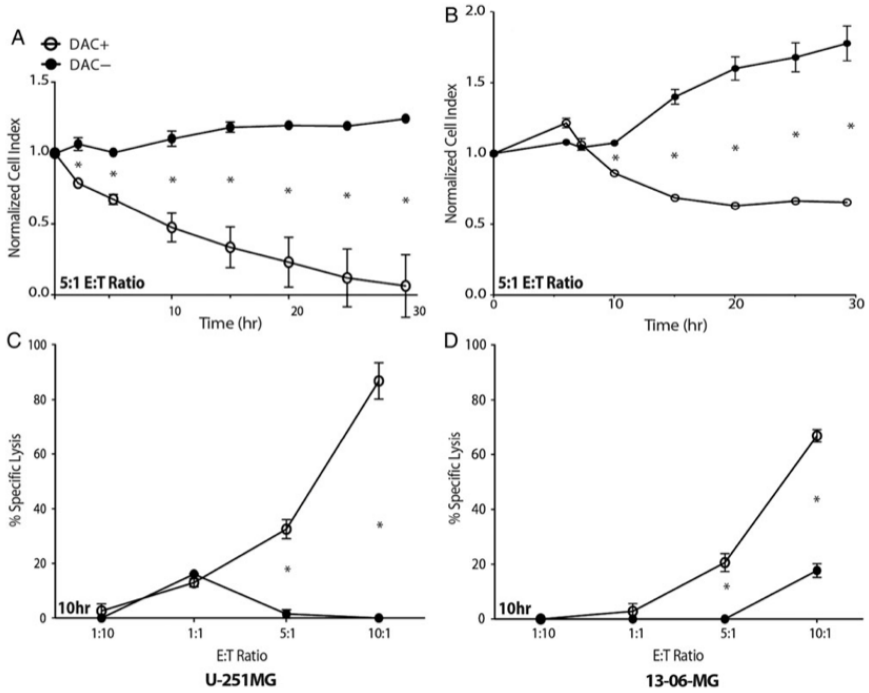

Fig. 3. Glioblastoma cells treated with DAC are susceptible to lysis by T cells engineered to express the NY-ESO-1 TCR. A real-time, impedance-based cytotoxicity assay (xCelligence) was used to evaluate the lysis of (A and C) U-251MG and (B and D) 13-06-MG glioma cells over a 30-h period when coincubated with NY-ESO-1 TCR-transduced effector T cells. (A and B) Representative data for glioma cell lysis at an E:T ratio of 5:1. DAC-treated (DAC+) cells are significantly more susceptible to lysis compared with untreated cells. (C and D) NY-ESO-1 TCR-transduced effector T cells were coincubated with (C) U-251MG and (D) 13-06-MG glioma cells at multiple E:T ratios (1:10, 1:1, 5:1, and 10:1). The percent specific lysis at 10 h is plotted (*$P < .05$, Student's *t*-test). Data shown are representative of one experiment repeated 7 times with similar findings.

assess the generalizability of NY-ESO-1 upregulation in glioblastoma, we examined the extent and durability of NY-ESO-1 upregulation in established glioma cell lines and on primary, surgically resected glioblastoma cells after a 48-h in vitro exposure to DAC. DAC induced rapid, robust expression of NY-ESO-1 in all glioma cells tested at both the mRNA and protein levels (Fig. 1A and C). This short exposure to DAC resulted in durable NY-ESO-1 upregulation that was observed up to day 65 in culture after a single course of DAC (Fig. 1B). NY-ESO-1 expression was also induced in situ in an orthotopic intracranial glioma model, when intracranial glioma–bearing mice (U-251MG) were treated with DAC (10 mg/kg/d, every other day for 3 injections). Quantitative PCR of NY-ESO-1 exhibited a 500-fold increase in NY-ESO-1 expression in tumor tissue isolated from animals treated with DAC compared with a placebo (Fig. 1D). Similarly, IHC staining for NY-ESO-1 protein also demonstrated tumor-restricted expression of NY-ESO-1 in glioma-bearing mice treated with DAC (Fig. 1E–H). Thus, short periods of DAC exposure resulted in rapid and extended upregulation of NY-ESO-1 in vitro and in vivo.

Decitabine Treatment Immunosensitizes Gliomas to T Cell–Mediated Cytotoxicity Prior to Adoptive Cell Transfer

To evaluate whether DAC treatment sensitizes glioma cells to T cell recognition in vitro and in vivo, we transduced the NY-ESO-1–specific T cell receptor clone 1G4 into normal human peripheral blood lymphocytes utilizing a retroviral transduction system.[5,6] Two days following transduction, flow cytometric analysis revealed equal distribution in CD4 and CD8 T cell populations. However, after 10 days of expansion culture containing IL-2, the population shifted predominantly to a CD8+ T cell phenotype (Fig. 2A). A representative analysis is shown in Fig. 2B, which reveals that ~75% and 86% of the CD3+ T cells expressed the NY-ESO-1 TCR at days 2 and 10, respectively, posttransduction.

The antitumor cytotoxic effect of these engineered lymphocytes against DAC-treated and -untreated glioblastoma cells was evaluated using a real-time, impedance-based cytotoxicity assay (Fig. 3) and confirmed with a more conventional assay (Supplementary Fig. S1). NY-ESO-1 TCR-transduced T cells effectively and specifically lysed DAC-treated 13-06-MG and U-251MG

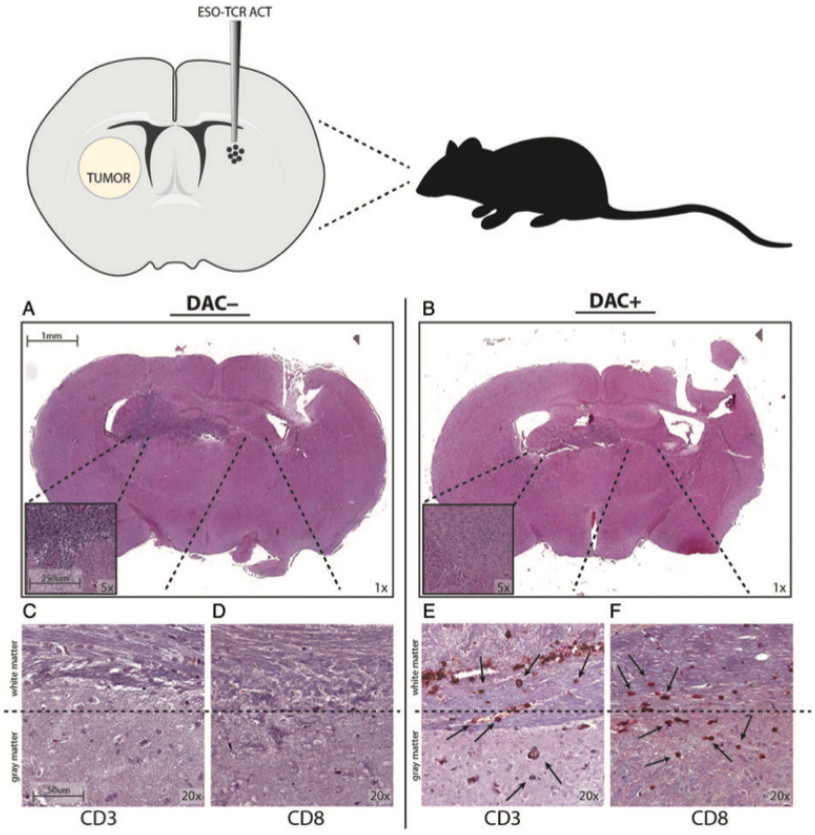

Fig. 4. DAC treatment of intracranial glioma-bearing mice permits trafficking of NY-ESO-1 TCR engineered T cells through the brain parenchyma. Mice with 1-week established intracranial U-251MG were treated with a vehicle control (DAC−) or with 10 mg/kg DAC i.p. × 3 days (DAC+). NY-ESO-1 TCR-transduced T cells were injected into the opposite hemisphere of the brain 2 days following the last DAC administration; 24 h later, the animals were sacrificed and the brains removed, sectioned, and immunolabeled for human CD3 and CD8. (A and B) Coronal sections (mag = 1×) stained with hematoxylin and eosin in mice given (A) control treatment or (B) DAC. (C–F) Injection of NY-ESO-1 TCR-transduced T cells into the hemisphere contralateral to the tumor implantation site at 2 days following the DAC treatment shows migration to contralateral human glioma xenografts. (C and D) DAC− group show lack of T cell infiltration into tumor, while (E and F) DAC+ group show trafficking through white matter (mag = 10×). Scale bar = 1 mm (A and B), 250 μm (A and B inset), and 50 μm (C–F). Data shown are representative of one experiment repeated twice with similar findings.

targets (Fig. 3A–D). DAC-treated U-251MG and 13-06-MG cells were effectively killed at an E:T ratio as low as 5:1, with effects seen as early as 2 h (Fig. 3A and B) and in a dose-dependent manner (Fig. 3C and D). Little cytotoxicity to DAC-untreated cells was observed. Killing of U-251MG continued until almost complete lysis of target cells was achieved by 30 h (Fig. 3A).

NY-ESO-1–Specific T cells Administered Intracranially Effectively Traffic Through the Brain Parenchyma and Exhibit Antitumor Effects

To evaluate the ability of NY-ESO-1–specific T cells to survive and traffic within the brain parenchyma, 2.5×10^5 U-251MG cells were stereotactically implanted into the right frontal lobe and allowed to establish for 7 days. DAC or control treatments were administered to animals systemically via i.p. injection. Subsequently, NY-ESO-1 engineered T cells (1×10^6) were injected into the contralateral hemisphere of the brain. Animals were sacrificed 48 hours after intracranial ACT and the brains were analyzed histologically for evidence of tumor and T cell trafficking. Tumor was readily detected in mice treated with NY-ESO-1 T cells in the absence of DAC (Fig. 4A), while no evidence of residual tumor was found in groups treated with DAC and NY-ESO-1 T cells (Fig. 4B). In mice treated with DAC, human CD3+ and CD8+ T lymphocytes were present at perivascular spaces within white matter (Fig. 4E and F). In

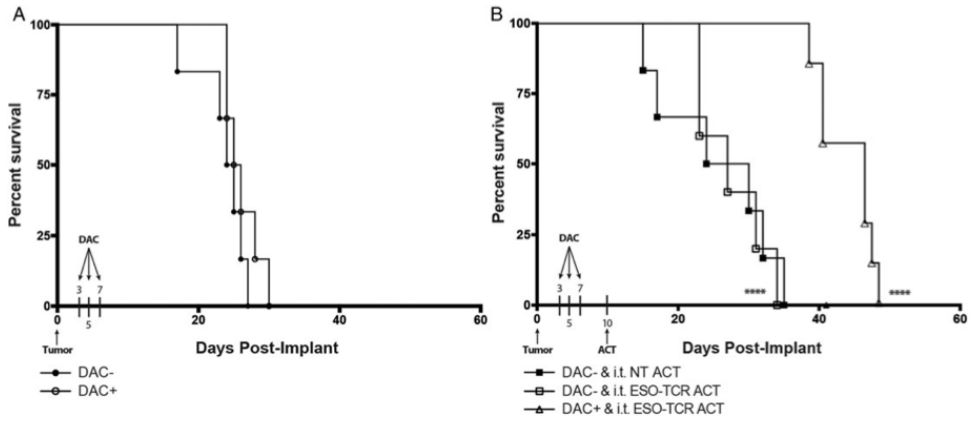

Fig. 5. Extended survival of glioma-bearing mice that received DAC treatment followed by i.t. ACT of NY-ESO-1–specific T cells. (A) Groups of NSG mice ($n = 6$/group) were implanted with 2.5×10^5 U-251MG glioma cells into the brain. Beginning at day 3 post tumor implant, the animals were treated with 10 mg/kg DAC i.p. every other day for a total of 3 doses (DAC+) or left untreated (DAC−). No significant difference in median survival was noted (DAC−, 24.5 d; DAC+, 25.5 d). (B) NSG mice ($n = 6$/group) were intracranially implanted with 2.5×10^5 U-251MG glioma cells. Two groups received i.t. ACT of NY-ESO-1 TCR engineered T cells (ESO-TCR ACT) or nontransduced T cells (NT ACT) (10^6 cells/2 μL) on day 10 in the absence of DAC treatment. One group received NY-ESO-1 TCR engineered T cells (ESO-TCR ACT) (10^6 cells/2 μL) on day 10 following DAC treatment regimen as described before. Significant differences were noted between DAC+ and i.t. ESO-TCR ACT and DAC− and i.t. NT ACT groups (****$P < .0001$, log-rank survival test). Data shown are representative of one experiment repeated 3 times with similar findings.

particular, human T cells were noted within the white matter tracts of the corpus callosum, likely in transit from the contralateral hemisphere. Histologically evident tumor cell masses were found in all mice not treated with DAC. In these animals, T cells could be located only at their implantation site in the brain contralateral to the tumor (Fig. 4C and D). These studies indicate that DAC treatment induced changes that render tumor-specific T cells capable of migrating through white matter tracts toward tumor cells, even from the contralateral side of the brain.

Prolonged Survival of Tumor-Bearing Mice Treated with Decitabine and Adoptive Cell Transfer of NY-ESO-1 TCR-Transduced Lymphocytes

Finally, we evaluated whether a regimen of DAC chemotherapy prior to NY-ESO-1 targeted immunotherapy extended the overall survival of mice bearing intracranial glioma. Whereas DAC alone did not result in a significant survival differential between treated and untreated groups (Fig. 5A), intratumoral ACT of 10^6 NY-ESO-1–specific T cells following DAC treatment conferred a significant survival advantage for the combined treatment group compared with mice treated with nontransduced T lymphocytes or NY-ESO-1 TCR ACT in the absence of DAC chemotherapy (Fig. 5B). These findings were recapitulated when the same experimental paradigm was also performed with mice bearing a primary, patient-derived glioblastoma cell culture (13-06-MG; Supplementary Fig. S2A). Further sequential improvements in survival were observed with the addition of systemic IL-2 for T cell support (Fig. 6B).

To evaluate whether the route of administration influenced our ability to treat mice bearing an established intracranial glioma, we treated animals with intravenous or intratumoral ACT of NY-ESO-1 TCR engineered T lymphocytes. Surprisingly, the systemic i.v. delivery of these lymphocytes conferred a significantly greater therapeutic benefit than i.t. delivery (Fig. 6C). More than 40% of the animals treated with systemic i.v. ACT completely rejected the intracranial tumor and survived long-term. Again, these findings were recapitulated with similar experiments performed in mice bearing 13-06-MG primary human patient–derived GBM intracranial xenografts (Supplementary Fig. S2B). Thus, the i.v. delivery of NY-ESO-1 TCR engineered T cell adoptive transfer was statistically more effective than i.t. delivery in conferring a survival benefit to mice bearing established intracranial glioma across multiple models ($P < .05$, log-rank test and stratified Cox regression; Supplementary Tables S1 and S2), suggesting that the blood–brain barrier may not be as limiting a factor to the efficacy of brain tumor immunotherapy as previously believed. These studies prove that a course of DAC renders human glioblastomas susceptible to NY-ESO-1–specific T cell adoptive transfer immunotherapy in an in vivo, orthotopic glioblastoma model.

Systemic Administration Route Promotes a CD8-Dominant Tumor-Infiltrating T Lymphocyte Population

In a parallel series of experiments, instead of monitoring animals for survival, animals were sacrificed 72 h following ACT; the tumor-bearing brain hemispheres and spleens were harvested and processed for flow cytometric analysis of T cell infiltration. NY-ESO-1 engineered T cell infiltration into tumor was observed in both i.t. and i.v. treatment models (Fig. 6D). However, mice that received T cells systemically had a significant

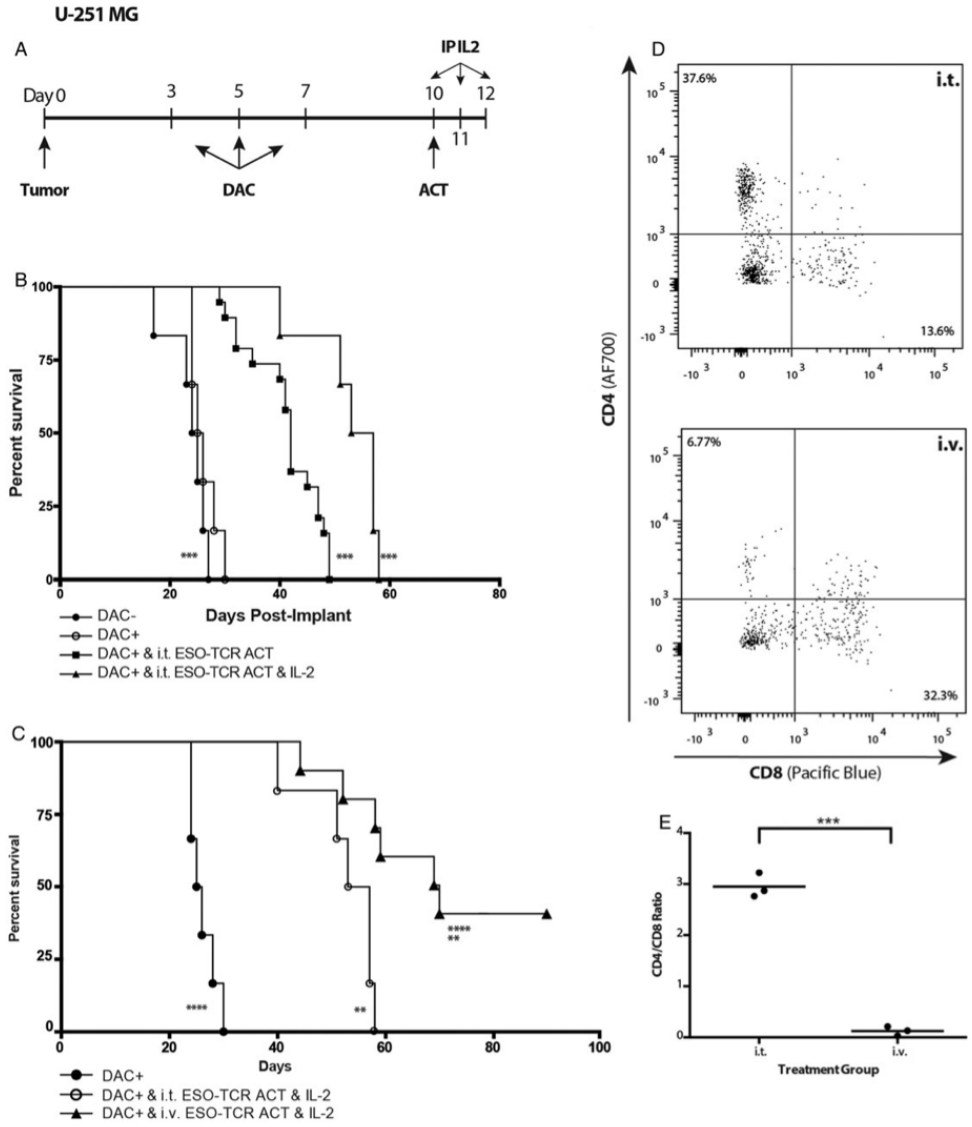

Fig. 6. Systemic IL-2 support and i.v. route of delivery enhance the therapeutic benefit of NY-ESO-1–specific T cell adoptive transfer. (A) Treatment regimen. Mice were intracranially implanted with 2.5×10^5 U-251MG glioma cells. At day 3 post tumor implantation, the animals were treated with 10 mg/kg DAC (DAC+) i.p. every other day for a total of 3 doses (days 3, 5, 7 post tumor implant). Three days following the last injection (day 10 post tumor implant), mice received i.t. adoptive transfer of NY-ESO-1 TCR engineered T cells followed by IL-2 (500 000 IU in 200 μL) or PBS 1× (200 μL) on days 10, 11, and 12 post tumor implant. (B) The mice were then followed for survival. Significant differences were noted between DAC+ and i.t. ESO-TCR ACT and IL-2 and DAC−, as well as between DAC+ and i.t. ESO-TCR ACT and IL-2 and DAC+ and i.t. ESO-TCR ACT groups (***$P < .001$, log-rank survival test and Cox proportional hazards model). (C) Three days following the final DAC injection (day 10 post tumor implant), experimental mice received i.t. or i.v. ACT of NY-ESO-1 TCR engineered T cells (i.t., 10^6 cells/2 μL; i.v., 10^7 cells/100 μL). Treated mice received i.p. injection of IL-2 (500 000 IU in 200 μL) on days 10, 11, and 12 post tumor implant. The mice were then followed for survival. Significant survival differences were observed between DAC+ and DAC+ and i.v. ESO-TCR ACT and IL-2, between DAC+ and i.t. ESO-TCR ACT and IL-2, and between DAC+ and i.v. ESO-TCR ACT and IL-2 (**$P < .01$, ****$P < .0001$, log-rank survival test and Cox proportional hazards model). The survival

increase in the TCRvβ13.1+CD8+ population and a complete reversal of the CD4/CD8 ratio observed (Fig. 6E). Similarly, i.v. delivery resulted in the trafficking of engineered T cells to the spleen. In mice that received i.t. delivery, very few transferred T cells could be found in any lymphoid organs (Supplementary Fig. S3). These findings suggest that i.v. delivery results in the expansion of T cells in vivo, preferential selection for a CD8+ population with a major histocompatibility complex class I–restricted TCR, and CNS homing toward antigen expression.

Discussion

Adoptive cell transfer and other immunotherapeutic modalities have been increasingly successful in the treatment of human cancers, such as malignant melanoma and other malignant solid tumors.[6,10–12] Recent advances in techniques to generate the large numbers of antigen-specific T cells required for effective adoptive transfer have recently made the technique clinically feasible. In these studies, we utilized a retroviral system to engineer NY-ESO-1–specific T lymphocytes for the treatment of glioblastoma. Administration of the hypomethylating agent, DAC, selectively upregulated NY-ESO-1 on intracranial glioblastomas in vivo and allowed for effective ACT immunotherapy with NY-ESO-1 TCR engineered T lymphocytes. NY-ESO-1–specific T lymphocytes could traffic through the normal brain parenchyma and migrate along white matter tracts toward tumor cells in mice only pretreated with DAC. Interestingly, we found that the transfer of T cells by systemic infusion was superior to the local, intracranial delivery of NY-ESO-1–specific T cells in this orthotopic model. These important findings highlight the ability of a demethylating agent to upregulate a well-characterized, immunogenic cancer-testis antigen in the brain, which can be targeted by systemic intravenous delivery of T cells targeting the upregulated antigen.

This strategy overcomes several of the current shortcomings of immunotherapy for glioma. Our treatment regimen induced the expression of a highly immunogenic antigen target in all glioma cell lines tested thus far in vitro and in vivo. This demethylating agent, DAC, induced persistent and durable expression of NY-ESO-1 in situ. This drug is also known to cross the blood–brain barrier, making it amenable to produce the effects on intracranial tumors. Our dosing regimen also is safe and nontoxic enough to be administered repeatedly so that expression of NY-ESO-1 is maintained over time. This dose was calculated to be slightly less than the equivalent FDA-approved dose for patients with myelodysplastic syndrome.[13] Engineered NY-ESO-1–specific T cells have potent antitumor functionality and can be generated relatively easily in large numbers in a short amount of time, which is critical when facing a rapidly progressing disease such as glioblastoma.

Initially, our adoptive transfer model focused on the intratumoral delivery of T cells, a strategy typically employed for intracranial glioma because of the perceived barriers of cellular delivery across the blood–brain barrier. Malignant gliomas rarely metastasize outside of the CNS, making local delivery a logical choice. In this manner, T cells can be delivered directly into tumor-containing areas and do not have to cross the blood–brain barrier. Off-target effects directed against cells elsewhere in the body that endogenously expressed NY-ESO-1, or were induced by systemic administration of DAC, might also be minimized. While we found evidence of efficacy in our model system using the intracranial administration of T cells, only relatively small volumes can be successfully implanted by stereotactic injection, significantly limiting the number of cells that could be injected. The use of larger volumes in the brain can be injurious as well, and lead to reflux of the infusate. This fact becomes increasingly apparent when trying to deliver agents into tumor-bearing areas, where the interstitial pressure is presumably higher due to increased cell density. When extrapolated to a potential human therapy, where $\sim 10^{10}$ cells have been administered in successful trials,[6] the volume requirement to transfer this number of cells becomes untenable for intracranial injection.

To circumvent this limitation, we first evaluated the effects of adding systemic IL-2 to enhance T cell proliferation as well as effector function after adoptive transfer. Careful studies aimed at elucidating the requirements for successful adoptive transfer immunotherapy have essentially deemed such IL-2 support a necessity for successful adoptive transfer immunotherapy.[14] However, with regard to the treatment of intracranial tumors, investigators in the past have been hesitant to administer high-dose IL-2 to patients due to the theoretical concern that the ensuing inflammation could lead to malignant cerebral edema and hemorrhage. However, several large retrospective studies have revealed that the presence of brain metastases posed no additional risk for patients undergoing IL-2 therapy.[15,16] The addition of IL-2 led to enhanced survival of mice treated with the DAC/adoptive transfer paradigm. However, all animals ultimately succumbed to their tumors, even when treated with intratumoral ACT plus IL-2.

As an alternative to intracranial injection, we then decided to evaluate the systemic adoptive transfer of NY-ESO-1 engineered T cells. In melanoma models, adoptive immunotherapy for established tumors in mice would require higher numbers of adoptively transferred cells ($\sim 10^7$) to be effective.[14,17] While the maximum feasible intracranial dose is around $1-2 \times 10^6$ in mice, transfer of this number of cells is easily achieved in the higher volumes afforded by an intravenous injection of the cells into the systemic circulation. Since it is unknown what proportion of these cells might ultimately reach an intracranial tumor target, given different volumes of distribution and requirements for trafficking to a distant tumor target, a dose-to-dose comparison of systemic versus intracranial adoptive transfer becomes less meaningful. Nevertheless, it is notable that in clinical trials of ACT for the treatment of melanoma, clinical responses and regression of brain metastases have

curves are each representative of one experiment repeated 3 times with similar findings. (D) On day 13 post tumor implant, mice were sacrificed and brain tumor–infiltrating lymphocytes harvested and stained by fluorescence activated cell sorting. The histograms depict representative animals after either i.t. (top) or i.v. (bottom) delivery. (E) The tumor-infiltrating lymphocytic population shows a significantly decreased CD4/CD8 ratio in the i.v.-treated group compared with the i.t.-treated group (***$P < .001$, Student's *t*-test).

been demonstrated,[10] indicating that systemically transferred cells are indeed able to traverse a tumor-affected blood–brain barrier. Additionally, clinical trials of ACT against NY-ESO in melanoma and synovial sarcoma demonstrated no toxicities attributable to on-target/off-tumor effects after the systemic infusion of adoptively transferred cells, which was a considerable theoretical concern given that NY-ESO is expressed abundantly in the testis of the adult male, and that fatal toxicities were attributed to such effects in clinical trials of adoptive transfer against other targets.[6,18]

In our studies, the systemic administration of a larger number of antigen-specific lymphocytes, coupled with IL-2 support, demonstrated vastly superior antitumor effects and was able to greatly prolong the survival of tumor-bearing mice to the point that animals were "cured" of their disease. Previous studies have shown that the i.v. adoptive transfer of activated antigen-specific T cells is successful in various disease paradigms where the antigen is present in the CNS.[19,20] However, specific comparisons between i.v. and direct administration of T cells in the setting of a progressively growing glioma have not previously been made. We believe that the survival and proliferation of T cells is likely enhanced in the circulation, given the less hostile environment faced there. Relevant integrin homing markers may help direct these effector cells to sites of tumor and traverse the blood–brain barrier. In addition, i.v. delivery resulted in the selective enrichment of a CD8+ NY-ESO-1 T cell population, suggesting that these cells proliferated in vivo and selectively trafficked to the sites of antigen expression on glioma cells in the brain. Previous attempts to target DAC-upregulated antigens in mouse models of glioma have used only intratumoral administration and have demonstrated only short-lived antitumor effects.[21] In light of the present studies, this was likely due to the limited numbers of cells infused directly into the tumor and the lack of IL-2 support of the adoptively transferred cells. We show for the first time in an orthotopic glioma model that systemic delivery of adoptively transferred T cells is more effective than local i.t. delivery, which may shift our current paradigms for immune-based treatment of this disease.

Overall, our data strongly suggest that the in vivo application of T cell–based immunotherapy can be established by systemic introduction of engineered cells, avoiding the requirement for invasive intracranial injections and their associated risks. The data presented here demonstrate that epigenetic upregulation of NY-ESO-1 by DAC, coupled with NY-ESO-1–specific T lymphocytes, has demonstrable antitumor activity against well-established glioblastomas in mouse models. While NY-ESO-1 is the first antigen we have explored, this same technique could be applied to any number of the antigens induced by DAC treatment either with adoptive transfer of engineered cells targeting multiple antigens or with polyvalent vaccines. A combination of epigenetic modulation and targeted immunotherapy provides a novel systemic treatment strategy for malignant gliomas.

Supplementary Material

Supplementary material is available at *Neuro-Oncology Journal* online (http://neuro-oncology.oxfordjournals.org/).

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Chapter 3:

Vaccination-induced adaptive immune resistance is mediated via a CD8-dependent PD-1/PD-L1 mechanism

Abstract

Dendritic cell (DC) vaccination with autologous tumor lysate has demonstrated promising results for the treatment of glioblastoma in preclinical and clinical studies. While the vaccine appears capable of inducing T cell infiltration into tumors, the effectiveness of active vaccination in progressively growing tumors is less profound. In parallel, a number of studies have identified negative costimulatory pathways, such as programmed death-1 (PD-1/PD-L1), as relevant mediators of the intratumoral immune responses. Clinical responses to PD-1 pathway inhibition, however, have also been varied. To evaluate the relevance to established glioma, the effects of PD-1 blockade following DC vaccination were tested in intracranial (i.c.) glioma tumor-bearing mice. Treatment with both DC vaccination and PD-1 mAb blockade resulted in long-term survival, while neither agent alone induced a survival benefit in animals with larger, established tumors. This survival benefit was completely dependent on CD8 T cells. Additionally, DC vaccine + PD-1 mAb blockade resulted in the upregulation of integrin homing and immunologic memory markers on tumor-infiltrating lymphocytes (TILs). In clinical samples, DC vaccination in GBM patients was associated with upregulation of PD-1 expression in vivo, while *ex vivo* blockade of PD-1 on freshly isolated TILs dramatically enhanced autologous tumor cell cytolysis. These findings strongly suggest that the PD-1/PD-L1 pathway plays an important role in the adaptive immune resistance of established GBM in response to anti-tumor active vaccination, and provide us with a rationale for the clinical translation of this combination therapy.

Introduction

Glioblastoma (GBM) is a devastating disease, for which the diagnosis is associated with an extremely poor prognosis and median survival of 14 months following surgery, radiation, and chemotherapy (1-3). Our group and others have pioneered a DC vaccine-based immunotherapy platform, the results of which have suggested benefit in early phase trials by promoting an endogenous anti-tumoral immune response (4-7). An ongoing randomized, placebo-controlled Phase III clinical trial is now underway based on these results. However, survival in DC vaccine-treated GBM patients has been varied (5). While increased T cell infiltration correlates with survival benefit across subjects, the ability to generate and sustain this response appears to be dependent on factors such as active tumor progression and GBM subtype (4, 8). These findings emphasize the need to more clearly understand the cellular mechanisms by which DC vaccination induces effective tumor-specific immune responses.

A possible explanation for the variability of vaccine efficacy is that the tumor and its microenvironment can adapt to suppress an immune response directed against them. Studies in various cancer models have suggested that checkpoint mechanisms, which exist to promote self-tolerance and protect against autoimmunity, can develop in the tumor microenvironment (9-14). PD-1 /PD-L1 (programmed death-1/ -ligand 1) has been shown to induce functional anergy and limit activation of cytotoxic T cells during long-term exposure to antigen, a phenomenon associated with neoplastic disease (9, 15-17). The upregulation of inhibitory PD-L1 (programmed death-ligand 1) in tumor cells appears to be associated with increased tumor-infiltrating lymphocytes (TILs), a phenomenon readily noted in immunogenic cancers with an

endogenous immune infiltrate (18, 19). Studies in melanoma have frequently shown robust anti-tumor responses in response to PD-1 mAb blockade (20-22). It was first shown that inhibition of PD-1/PD-L1 promotes the anti-tumoral activity of TILs present in B16 melanoma models (23-27). This blockade was dependent on the presence of an infiltrating CD8+ population (21). PD-1/PD-L1-mediated suppression was noted in a glioma model as well. Adjuvant PD-1 mAb blockade combined with external beam ionizing radiation promoted long-term survival in mice when compared to mice that only received radiation alone (28).

Unlike melanoma, however, GBM are not inherently immunogenic and active vaccination is necessary to first generate an intratumoral immune response. In this study, we demonstrated that PD-1/PD-L1 modulates adaptive immune resistance to tumor lysate-pulsed DC vaccine treatment in our murine glioma model. Specifically, we show that this negative costimulatory ligand plays a role in suppressing tumor infiltrating T lymphocyte (TIL) activation, trafficking and memory responses, and that blocking PD-1 can reverse this suppression. Finally, we recapitulated these findings in our patient-derived GBM tissue by a series of novel *ex vivo* studies, further documenting the clinical relevance of the PD-1 mechanism. Thus, these findings suggest that the combination of DC vaccine with PD-1 mAb blockade in human GBM provides a clinically translatable means of promoting an anti-tumoral immune response and attenuating immune suppression.

Methods

Human tissue samples

Paraffin-embedded human GBM tissues from pre- and post-DC vaccine treatments were obtained through the Brain Tumor Translation Resource (BTTR) at UCLA and used for histology and IHC. GBM tissue from freshly resected patient samples was placed in collagenase overnight for digestion and then passed through a Percoll gradient to separate TIL and tumor cell layers, and used for functional *ex vivo* assays.

GL261 lysate preparation

GL261 glioma cells, obtained from Dr. Henry Brem (Johns Hopkins University, Baltimore, MD) were cultured in complete DMEM media. Cells were then harvested and subjected to several freeze-thaw cycles. The suspension was then filtered and the concentration of the lysate obtained then quantified using a Bradford protein assay.

Cell lines

Cells were maintained in complete DMEM (Mediatech, Inc. Herndon, VA), supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA), 1% (v/v) penicillin and streptomycin (Mediatech Cellgro, Manassas, VA) and cultured in a humidified atmosphere of 5% CO_2 at 37°C.

Murine Model

Female C57BL/6 mice, age 6-8 weeks, were obtained from our institutional breeding colony and kept under defined-flora pathogen-free conditions at the Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility of the Division of Experimental Radiation Oncology at the University of California Los Angeles.

Intracranial glioma implantation

Female C57BL/6 mice (6-8 weeks old) were anaesthetized and GL261 glioma cells (2×10^4 in 2 μl PBS) were stereotactically injected at 2.5 mm lateral to bregma at a depth of 3.5 mm below the dura mater with a sterile Hamilton syringe fitted with a 26-gauge needle. The intracranial injection was performed over a 2 min period. Following intracranial tumor implantation, mice were randomized into treatment groups (n=6-16 mice/group).

Bone marrow-derived DC and vaccination model

DCs were prepared from murine bone marrow progenitor cells using a method previously described (53). Mice were treated with subcutaneous injections of lysate-pulsed DC vaccination (1E6 cells/ mouse) on days 3 and 13 post-implantation (elevated tumor burden) or days 0, 7, and 14 (low tumor burden).

In vivo antibody treatments and cell depletions

PD-1 mAb (RMP1-14, BioXCell) was administered i.p. at 250 mg/kg daily on days 3-5 and 13-15. CD4 (GK1.5, BioXCell) and CD8 (YTS 169.4, BioXCell) depleting antibodies were administered i.p. at 200 mg/kg every other day starting four days prior to tumor implantation.

Tissue harvests, immunohistochemistry, and flow cytometry

Spleens, lymph nodes, and tumor-bearing brain hemispheres were harvested from mice on day 16, 72 hours following the second DC vaccination. In cases where sectioning and immunohistochemistry were required, tissue was placed in Zinc Fixative (BD Biosciences, San Jose, CA) for 24 hours and then transferred to 70% ethanol before being embedded in paraffin wax. Murine tissue was stained via immunohistochemical methods with the assistance of the UCLA Translational Pathology Core Laboratory (TPCL) for CD3. Immunofluorescent staining was performed on formalin-fixed paraffin embedded (FFPE) 5μm patient-derived tissue sections as described (21) using CD8 (clone C8/144B; DAKO, 1/5000), PD-1 (clone EPR4877; abcam, 1/50000), and PD-L1 (clone 1-111A, 1:4000, eBioscience). Cy3 and FITC TSA kits (Invitrogen) were used to visualize primary antibody, and nuclei were visualized with 49,6-diamidino-2-phenylindole (DAPI; Invitrogen). Analyses for all tissue was performed using the Vectra inForm analysis software to select and quantify staining and colocalization of markers of interest.

For flow cytometric staining, tumor-bearing brain hemisphere was placed into tissue collagenase overnight. Lymphocytes were isolated from the tumor suspension the following day using 30%:70% Percoll gradient or from spleens using a 70μm mesh cell strainer. Remaining tumor burden was maintained in DMEM media supplemented with 10% FBS and 1% (v/v) penicillin and streptomycin. In experiments requiring analysis of isolated lymphocytes, cells were stained with fluorochrome conjugated antibodies to CD3 (17A2), CD4 (4SM95), CD8 (53-6.7), CD25 (PC61.5), FoxP3 (FJK-16s), Thy1.2 (30-H12), and PD-1 (RMP1-14) obtained from eBioscience. All FACS analysis was performed with the use of an LSRII (BD Biosciences). Gates were set based on isotype specific control antibodies (data not shown). Data was analyzed using FlowJo

software. TILs used in *in vitro* assays were FACS-sorted for CD3+ Thy1.2+ using a FACSAria (BD Biosciences).

nCounter GX Nanostring Analysis

TILs were harvested and then FACS-sorted for Thy1.2+ CD11b- T cells. Sorted cells were then lysed in RLT buffer (Qiagen RNEasy Kit). The gene expression (2,500-10,000 cells/ sample) of approximately 770 genes was quantified via direct binding to tagged probes using the nString Nanostring system.

Real-time cytotoxicity assay

Cytotoxic killing of tumor cells was assessed using the xCELLigence Real-Time Cell Analyzer System (Acea Biotechnology, San Diego, CA). In mice, T cells were initially harvested from tumor bearing brain hemispheres or spleens, and then purified via Percoll gradient and FACS sorting ($CD11b^-$, $CD3^+$). In patient samples, T cells were harvested from freshly resected tumor tissue and then purified. After overnight tumor culture to allow adherence of the cells to the well bottom, T cells were added to tumor cell cultures at a 10:1 T cell to tumor ratio. Cell index values (relative cell impedance) were collected over 15 hours and normalized to the maximal cell index value immediately prior to effector cell plating using RTCA Software 2.0 (Acea Biotechnology, San Diego, CA) as described previously(54). PD-1 mAb (murine, RMP1-14; human, J110, BioXCell) was added to the T cell cultures prior to co-culture at 10ug/mL where necessary.

Statistics

The two-tailed Student's t-test was used to compare continuous variables. Data that necessitated the comparison of more than two groups were analyzed using ANOVA followed by Kruskal-Wallis statistics. Data and data bars in figures represent mean ± SEM. Values were considered significant at $P < .05$. In the cases of survival studies, median survival times were graphed using Kaplan–Meier plots and analyzed using log-rank survival statistics.

Study approval

For human studies, patients provided informed consent for their tissue to be used for research purposes, which was approved by UCLA Medical Institutional Review Board (IRB). For murine studies, mice were handled in accordance with the University of California Los Angeles animal care policy and approved animal protocols.

Results

DC vaccination promotes an anti-tumor, infiltrating T cell response but is ineffective in established intracranial glioma.

We previously noted that established tumor burden and immunosuppressive cytokine levels negatively correlated with immune responsiveness and patient survival after DC vaccination (4). As such, we were interested to examine the critical mechanisms of resistance to vaccine treatment in the face of established tumor burden despite T cell infiltration. To evaluate this question, mice were implanted with 2 x 10^4 murine GL261 glioma cells in the brain and treated with tumor lysate-pulsed DC vaccination. Mice that began receiving DC vaccine on the day of tumor implant, prior to establishment, showed significant survival benefit (**Fig. 1a**). Mice that did not begin receiving DC vaccine treatments until 3 days after tumor implantation or later did not demonstrate increased survival when compared to non-treated controls (**Fig. 1b**). Thus, we showed that DC vaccination was ineffective when there is a larger, established tumor burden, as in humans.

To determine whether vaccination promoted a local immune response in these established tumors, explanted brains were analyzed by immunohistochemical (IHC) staining. DC vaccine-treated tumors showed a robust CD3+ infiltrating population (**Fig. 1c-f**) similar to what was seen in patients' tumors (4), suggesting that DC vaccine treatment elicited a significant intratumoral immune response compared with non-treated controls despite being ineffective at promoting long-term survival in mice with established tumor. To assess whether this tumor-associated lymphocyte population was glioma specific, we evaluated the ability of purified TILs to lyse

cultured GL261 glioma cells in vitro. In a real-time cytotoxicity assay, CD3+ TILs from DC vaccine-treated mice showed 50% cytotoxicity against cultured GL261 glioma cells over 20 hours, whereas splenic lymphocyte controls demonstrated little or no cytotoxicity (**Fig. 1g**). In whole, these findings demonstrated that, despite a lack of survival benefit in mice with established tumor burden, DC vaccination induced a significant anti-tumor immune infiltrate.

Adjuvant PD-1 mAb blockade in the setting of established tumor burden enhances the therapeutic benefit of DC vaccination.

We hypothesized that local immune suppression in the intracranial tumor microenvironment prevented an effective anti-tumor T cell response following DC vaccination. Recent studies have described PD-1-mediated suppression of T cell responses (9, 21). In our mouse glioma model, we found relevant increases in the expression of PD-1 on TILs following DC vaccination in larger, established tumors (**Fig. 2a, b**). There was also a significant increase in PD-1 expression on infiltrating TILs compared with splenic lymphocytes in both control non-treatment and DC vaccinated mice (**Fig. 2b**). In order to gain a more complete understanding of the genetic changes of tumor infiltrating lymphocytes in our mouse model, RNA was isolated from CD3+ TILs of non-treated control and DC vaccine treated mice and quantitative transcriptional changes were assessed. We noted a significant upregulation in PD-1, confirming the FACS data (**Supp. Fig. 1a**). Other T cell markers of immune inhibition, including CTLA-4, TIGIT, ICOS, BTLA, and TGF-β, were downregulated on TILs following DC vaccine treatment (**Supp. Fig. 1b-f**).

Because PD-1 was the only inhibitory marker elevated on TILs following DC vaccination in murine samples, we hypothesized that the PD-L1/PD-1 pathway mediated immune suppression

of the DC vaccine-induced immune response in established tumors. We noted that GL261 glioma cells expressed PD-L1 (**Supp. Fig. 2**). To evaluate the relevance of this finding in our murine model, we first co-cultured TILs harvested from tumor-bearing mice treated with DC vaccination together with intracranially-derived GL261 tumor bulk cells and found that *ex vivo* PD-1 mAb blockade significantly increased GL261 glioma cell cytolysis over control co-cultures of TILs with tumor bulk alone (**Fig. 2c, Supp. Fig. 3**). We then performed *in vivo* PD-1 mAb blockade together with tumor lysate-pulsed DC vaccination in mice with established i.c. GL261 gliomas. When PD-1 mAb blockade was administered concomitantly with DC vaccination, a highly significant survival benefit resulted. Approximately 40% long-term survival was observed selectively in the combination treatment group (**Fig. 2d**). As we described before, there was no therapeutic benefit with DC vaccination alone in these large, established tumors. Similarly, there was no survival benefit noted with PD-1 mAb blockade alone. As such, this data suggested that antibody blockade of PD-1 recovered the therapeutic benefit of DC vaccination in the established i.c. glioma setting.

PD-1 mAb blockade enhances the functional intratumoral CD8+ T cell response

To understand this PD-1 regulatory mechanism and the immune cell subsets critical for the survival benefit seen with the combinatorial treatment, we depleted mice of either CD4 or CD8 cells. As depicted in **Fig. 3a and 3b,** the clinical benefit of a combined DC vaccine and PD-1 mAb blockade was completely dependent on CD8 cells and not on CD4 cells. DC vaccination induced a highly significant increase in the tumor infiltrating CD3+ T cell population, which was also maintained in the combination DC vaccine + PD-1 mAb setting (**Fig. 3c**). Of note, there was no change in the numbers of tumor-infiltrating helper T cell (CD4+) or T regulatory cell (CD4+

CD25+ FoxP3+) populations across treatment groups (**Supp. Fig 4a, b**). A significant proportion of tumor-infiltrating CD3+ cells from DC vaccine and DC vaccine + PD-1 mAb treated mice was comprised of CD8+ T cells. Thus, we evaluated differences in the activation pattern of these T cells between DC vaccine and DC vaccine + PD-1 mAb treated mice. Expression of CD25, the high-affinity subunit of IL-2R, was evaluated. Even though there was an elevation in the percentage of activated CD8+ CD25+ T cells in PD-1 mAb-treated mice, only a minimal number of infiltrating CD8+ T cells could be isolated in the absence of a DC vaccine. In contrast, we observed a significant number of infiltrating T cells in the presence of a DC vaccine; however, without PD-1 mAb treatment, there was not a significant proportion of activated cells. Only in mice treated with both DC vaccination and PD-1 mAb blockade was there a significant increase in both the proportion and number of activated CD8+ CD25+ T cells (**Fig. 3d**). Thus, our findings suggest that the addition of PD-1 mAb blockade to a DC vaccine enhances the migration and activation of T cell responses in a CD8-dependent fashion.

To obtain a more complete understanding of the gene expression differences between TIL isolated from DC vaccine versus DC vaccine+PD-1 mAb-treated animals, we performed an unbiased transcriptional screen of purified TILs. In purified TIL populations isolated from mice treated with DC vaccination and PD-1 mAb blockade, there was increased transcription of the late-activation marker, IL-2Rα, as well as genes associated with activation, homing, and survival (**Supp. Fig. 5a, b**). Furthermore, cell-free supernatants from *ex vivo* co-cultures of TILs and GL261 tumor cells had significantly elevated concentrations of IL-2 and IFN-ɣ in the presence of PD-1 mAb (**Supp. Fig. 6a, b**). These findings suggested that suppression of the PD-1/PD-L1 signaling mechanism via PD-1 mAb blockade activates CD8+ TILs.

PD-1 mAb blockade enhances the trafficking of a tumor-infiltrating memory CD8 T cell population into intracranial gliomas.

Previous work has suggested that expression of the α4, β1 integrin was critical for the trafficking of tumor-specific T cells to glioma (29, 30). Additionally, the development of a memory response with inhibition of PD-1 has been previously described (31, 32). To evaluate this in our model, we compared the pattern of memory and integrin homing markers from splenic T cells and TILs harvested from our DC vaccine and DC vaccine + PD-1 mAb-treated mice. In both treatment groups, there was a population of memory T cells ($CD8^+$ $CD44^+$) in the spleen (**Fig. 4a**) that was not different. However, a significant increase in the percentage of tumor-infiltrating memory T cells was observed with adjuvant PD-1 blockade, such that there was an approximate two-fold increase across treatment groups (**Fig. 4b, c**). We further interrogated CD8+ CD44+ memory cells for the expression of CD62L (L-selectin), a lymph node homing receptor expressed on central memory T cells. There was a significantly elevated proportion of CD8+ CD44+ cells in the spleen from DC vaccine + PD-1 mAb treated mice that expressed CD62L when compared to mice that only received DC vaccination (**Fig. 4d**). This difference was also significant when we looked specifically at tumor-infiltrating cells (**Fig. 4e**). Previous work has demonstrated the role of tumor homing signals, such as the integrin alpha-4 chain (CD49d), to promote T cell trafficking to central nervous system (CNS) tumors (33). In our model, we noted that the concomitant use of PD-1 mAb blockade together with DC vaccination was associated with a statistically elevated proportion of CD8+CD44+ memory T cells that expressed CD49d **(Fig. 4f, g)**. To evaluate how such T cell populations influenced immune memory, we challenged surviving mice from the DC vaccine + PD-1 mAb treatment cohort 60 days after the initial tumor

inoculation with GL261 glioma cells in the contralateral hemisphere. These mice did not receive any additional treatments. When compared to naïve control mice, a significant survival benefit was noted in the long-term survivors from previous DC vaccine + PD-1 mAb therapy (**Fig. 4h**). Such findings suggested that the addition of PD-1 mAb blockade to tumor lysate-pulsed DC vaccination promoted selective intracranial glioma trafficking and immune memory.

Ex vivo PD-1 mAb blockade enhances TIL cytotoxicity against patient GBM cells.

To evaluate whether similar biological principles exist in human GBM patients, we interrogated our clinical trial patient samples using a novel quantitative multiplex immunofluorescence staining panel. There was significant PD-L1 expression in the GBM tumor microenvironment (**Supp. Fig. 7a, b**). Additionally, the percentage of CD8+ TILs that dually expressed PD-1 was elevated following DC vaccine treatment compared with the percentage seen in pre-treatment samples (**Fig. 5a-h**). We noted that this difference was significant across multiple patient samples (**Fig. 5i**). Having confirmed upregulated PD-1 expression on our patient TILs, we next evaluated whether *ex vivo* PD-1 mAb blockade enhanced TIL function in purified, live GBM TILs. To test this, we obtained fresh GBM tissue samples from patients undergoing surgical resection to remove their tumors and harvested CD3+ TILs and autologous tumor cells. When these TILs were co-cultured with the original GBM tumor, we noted some cytotoxicity (**Fig. 5j**). However, when a blocking PD-1 mAb was added to the co-cultures *ex vivo*, there was a significant increase in cytotoxicity across samples. This demonstrated that the PD-1/PD-L1 signaling mechanism suppressed the cytotoxicity of TILs. We confirmed that TILs exerted a tumor-specific immune response, as there was minimal tumor cytolysis when TILs from one patient were co-cultured with GBM from another patient (**Supp. Fig. 8**). Together, these findings

suggested that DC vaccination results in upregulated PD-1 expression in GBM patients and, when blocked *ex vivo*, could restore function in tumor-infiltrating T cells.

Discussion:

In immunogenic cancers, such as melanoma, biologic therapies that incorporate PD-1 blocking antibodies (nivolumab or pembrolizumab) have resulted in extended patient survival in randomized controlled trials (34-42). However, as suggested by Tumeh et al., the significant survival benefit in melanoma patients was dependent on a pre-existing infiltrating population of cytotoxic T cells (21). A study in GBM patients noted that increased T cell infiltrates at the time of resection were predictive of increased survival (43). Based on our previous work, such T cell infiltration is not consistent across GBM subtypes, with non-mesenchymal subtypes exhibiting a lower endogenous lymphocyte infiltration when compared to mesenchymal GBM (8). Although results of ongoing trials examining GBM treatment with PD-1 mAb alone have not yet been published, we hypothesize that clinical efficacy may be varied and dependent on a pre-existing TIL population for PD-1 mAb treatment to work.

Our group previously demonstrated that, despite promoting a T cell infiltrating response, the ability of active vaccination to initiate an immune response and improve survival is less consistent in patients with large, progressive disease (4). Thus, we were particularly interested to understand the mechanisms that would prevent some patients with established and progressive recurrent disease from showing survival benefit. In this study, we effectively recapitulated these clinical findings in our mouse model by vaccinating mice bearing large established i.c. gliomas. We demonstrated that, despite generating a significant localized CD8-dominant tumor-specific T cell response with vaccination, there was no significant survival benefit when mice were vaccinated in the setting of large, progressive i.c. tumors. Importantly, additional vaccine treatments did not provide therapeutic benefit for mice bearing these larger tumors (data not

shown). The corresponding elevation of PD-1 expression with increasing TILs following vaccination suggested that the PD-1/PD-L1 negative costimulatory mechanism of adaptive immune resistance becomes functionally relevant in an environment of elevated tumor burden (44). As such, while the T cell response appears to be crippled by these regulatory mechanisms, it is significantly functionally activated when the PD-1/PD-L1 pathway is inhibited. Thus, by generating an immune response in a non-immunogenic, established tumor, and by activating it with PD-1 mAb blockade, we are able to both generate and maintain effective immunity with therapeutic benefit (**Fig. 6**).

Mice that had been treated with DC vaccine and PD-1 mAb were able to reject a second tumor inoculation without additional vaccination or PD-1 mAb treatments. The relevance of PD-1/PD-L1 to memory T cell generation in the tumor environment has been described previously (31, 45). Interestingly, a significantly elevated proportion of our central memory T cell population in mice vaccinated with adjuvant PD-1 mAb blockade expressed elevated tumor homing markers CD62L and CD49d. These findings suggest that this population may represent a subset of memory T cells that are recent emigrants into the tumor. As they mature to become effector cells, they may downregulate these homing markers. Another possibility is that these non-CD62L and CD49d-expressing memory T cells may represent a resident memory T cell population analogous to those present in barrier tissues such as the epithelium of skin, lung, and gastrointestinal tract (46, 47). These resident cells likewise express memory cell markers, but they are generally thought to be unable to recirculate from tissue to lymph and then back again, an important aspect of the homing phenotype (47). Together, these findings suggest that, despite a large, established non-immunogenic tumor, we are able to generate and maintain an immune response that both

targets existing tumor and prevents tumor recurrence: a hallmark of GBM. It is possible that, in patients, dual vaccination and PD-1 mAb blockade will not only enhance survival benefit with established tumor, but also decrease the likelihood of GBM recurring.

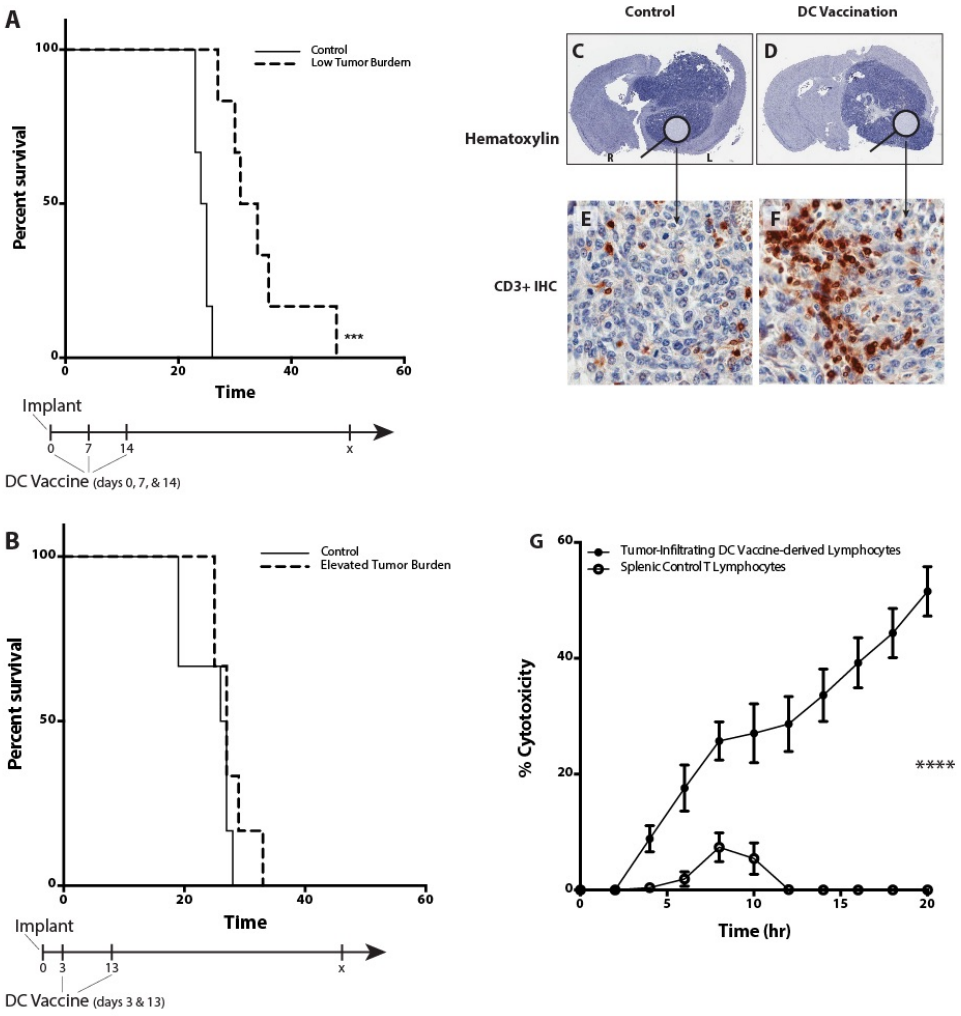
The role of helper T cells and regulatory T cells in tumor-mediated immune suppression has been examined (48-51). Although we cannot rule out that PD-1 mAb blockade does not affect the function of regulatory T cells, we did note in our studies that CD8+ T cells largely mediated our anti-tumoral immune response with no significant changes in CD4+ and T regulatory CD4+ populations across treatment groups. Depletion of CD4 T cells did not provide any added benefit compared with DC vaccine alone. Importantly, the DC vaccine + PD-1 mAb treatment was not improved with the depletion of CD4 T cells. Our flow cytometry data on harvested cells demonstrated that there was no increase in the tumor-infiltrating helper and regulatory T cell populations with DC vaccine treatment, and adjuvant PD-1 mAb did not reduce their proportion in the tumor environment. Therefore, while it is possible that CD4+ cells play some role in the PD-1/PD-L1 mechanism on a broader scale, our results suggest that this is non-contributory in our DC vaccine + PD-1 mAb treatment model.

In conclusion, we demonstrated that the PD-1/ PD-L1 negative costimulatory system mediates adaptive immune resistance to the vaccine-generated tumor infiltrating T cell response in GBM. Specifically, we described the effect of PD-1 blockade on intratumoral effector and memory T cells. We demonstrated drastically improved survival in the in vivo setting following both treatments. Furthermore, mice that survived the initial tumor challenge inoculation following DC vaccine + PD-1 mAb treatment sustained survival benefit over naïve controls when re-challenged

with tumor contralaterally. With this study, we also demonstrated the necessity of a vaccine-generated TIL population in order for the PD-1 pathway to be able to exert an effect within GBM tumors. Although there is probably a broad spectrum of tumor environment-mediated adaptive inhibition, PD-1 is a likely dominant regulatory mechanism in vaccine-induced immune suppression in our model. Future studies are needed to better understand what is likely a heterogeneous array of mechanisms utilized to prevent anti-tumor activity. The interplay of these mechanisms may provide the key to successfully treating GBM using endogenous immune mechanisms.

Figures and Legends:

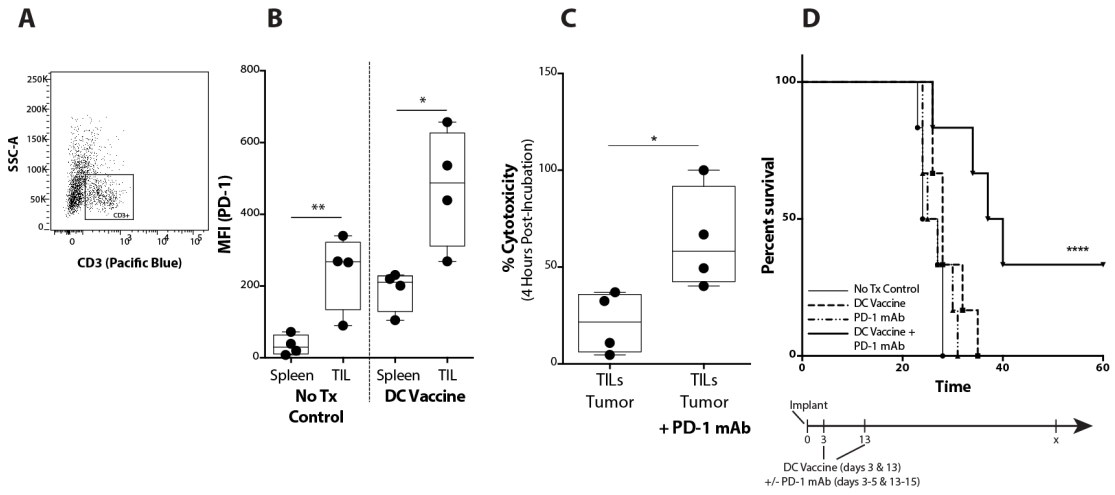
Figure 1



DC vaccination promotes an anti-tumor, but ineffective infiltrating immune response in the established setting. Mice intracranially implanted with GL261 were randomly assigned to receive DC vaccine treatments in **(A)** at time of implant (low tumor burden) or **(B)** once tumors became established (elevated tumor burden). Data shown are representative of one experiment repeated two times with similar findings. **(C-F)** IHC staining with anti-CD3 antibody (red) on brains harvested from these mice (20x magnification). **(G)** CD3+ Thy1.2+ cells were FACS-

sorted from i.c. GL261 gliomas or spleens and plated with GL261 cells for in vitro xCelligence cytotoxicity assay. (n=3) (****$p<0.0001$). Data shown are representative of one experiment repeated two times with similar findings. Each point represents 1 subject (**A, B**) or the average of biological replicate (n=4) (**G**). Survival differences were calculated using log rank statistical tests and graphed using the method of Kaplan-Meier (**A**, **B**) and a Student's t-test was used to calculate statistical significance at individual time points (**G**).

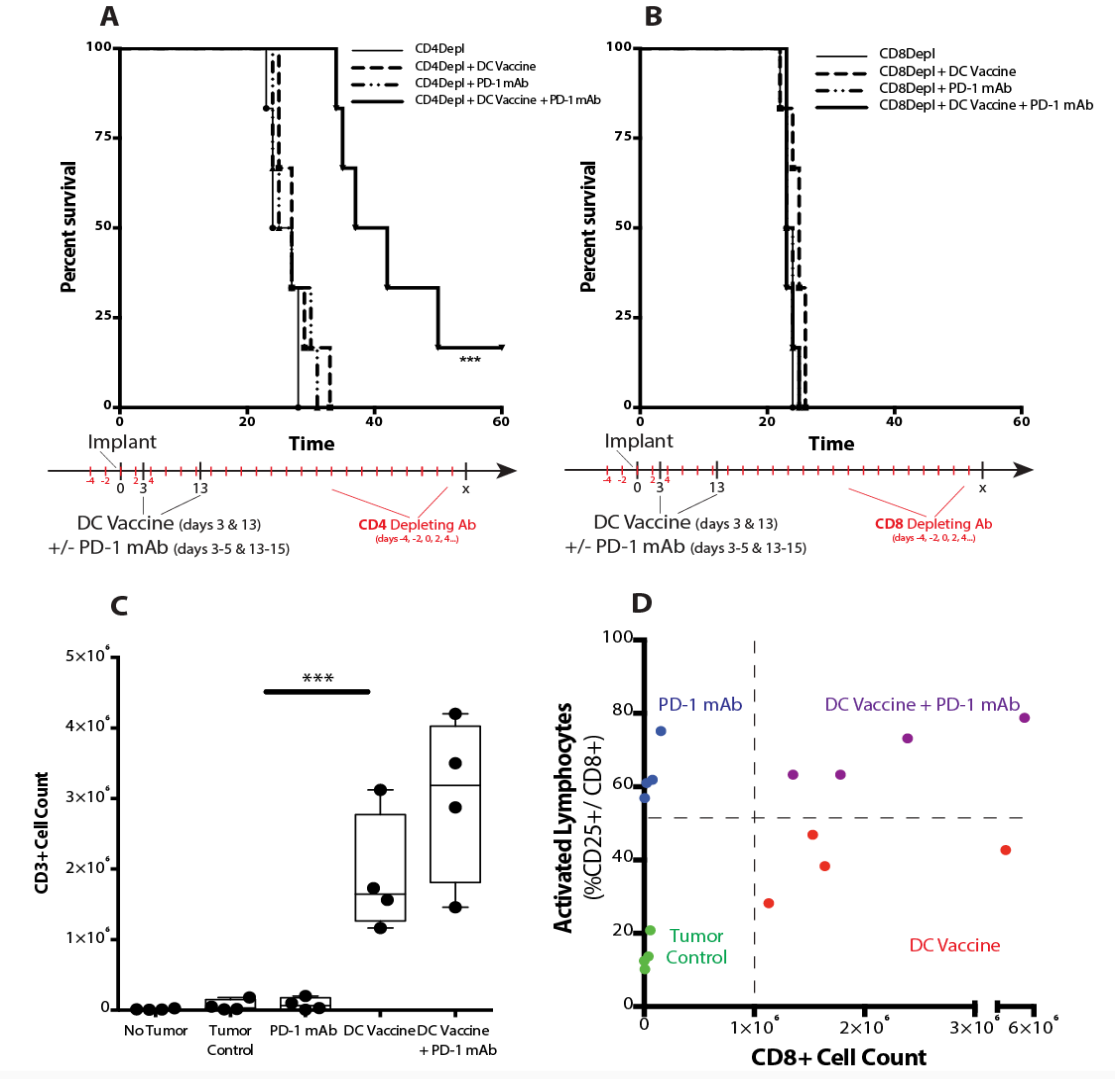
Figure 2



PD-1 blockade rescues the survival benefit after DC vaccination in mice with established tumor burden. **(A)** Splenic and tumor infiltrating lymphocytes (TILs) were gated for CD3 expression and **(B)** Median fluorescence intensity (MFI) of PD-1 expression on CD3+ cells quantified between control non-treatment (No Tx) and DC vaccine treatment groups. (n=4/group). Data shown are representative of one experiment repeated two times with similar findings. FACS-sorted CD3+ Thy1.2+ tumor-infiltrating lymphocytes (TILs) from DC vaccine-treated tumor-bearing mice were co-cultured with intracranially-derived tumor bulk with or without *ex vivo* PD-1 mAb and tumor cytolysis **(C)** at 4 hours was quantified (*$p<0.05$) (n=4/group). **(D)** Mice were randomized into control (tumor-bearing, no treatment), PD-1 mAb, DC vaccine, and DC vaccine + PD-1 mAb) treatment groups. Graphs show evaluation of survival. (n=6/group) (***$p<0.001$). Data shown are representative of one experiment repeated four times with similar findings. Each point represents 1 cell **(A)** or 1 subject (**B-D**). Box-and-whisker plot are used to graphically represent the median (line within box), upper- and lower- quartile (bounds of box), and maximum and minimum values (bars) (**B, C**). A Student's t-test was used to calculate

statistical significance (**B, C**) and survival differences were calculated using log rank statistical tests and graphed using the method of Kaplan-Meier (**D**).

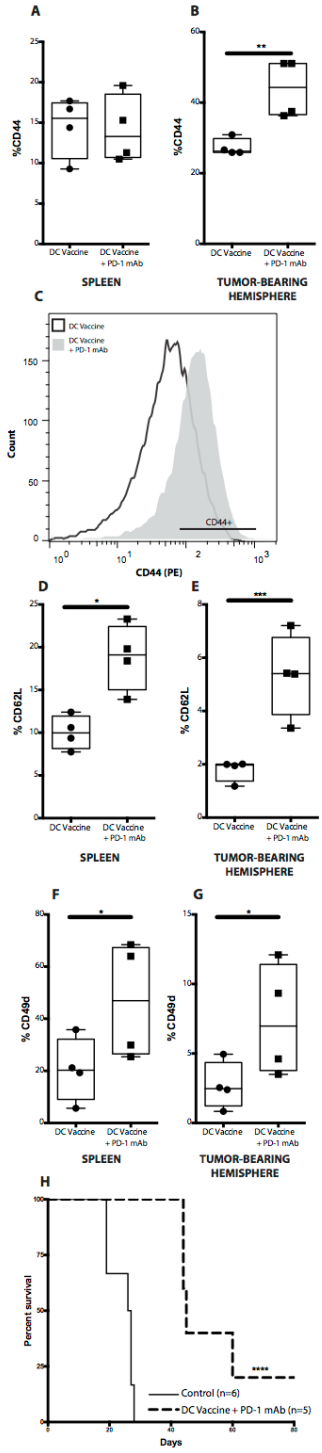
Figure 3



PD-1 mAb blockade enhances the intratumoral CD8 T cell response. Survival of mice from control (tumor bearing, no treatment), PD-1 mAb, DC vaccine, and DC vaccine + PD-1 mAb treatment groups when **(A)** CD4 cells or **(B)** CD8 cells were depleted is shown. (n=6/group) (***p<0.001). **(C)** Absolute number of CD3+ T lymphocytes isolated from tumor-bearing cerebral hemispheres and analyzed by flow cytometry. **(D)** The absolute CD8+ count was plotted against **%** CD25+ CD8+ activated lymphocytes and compared across different treatment groups.

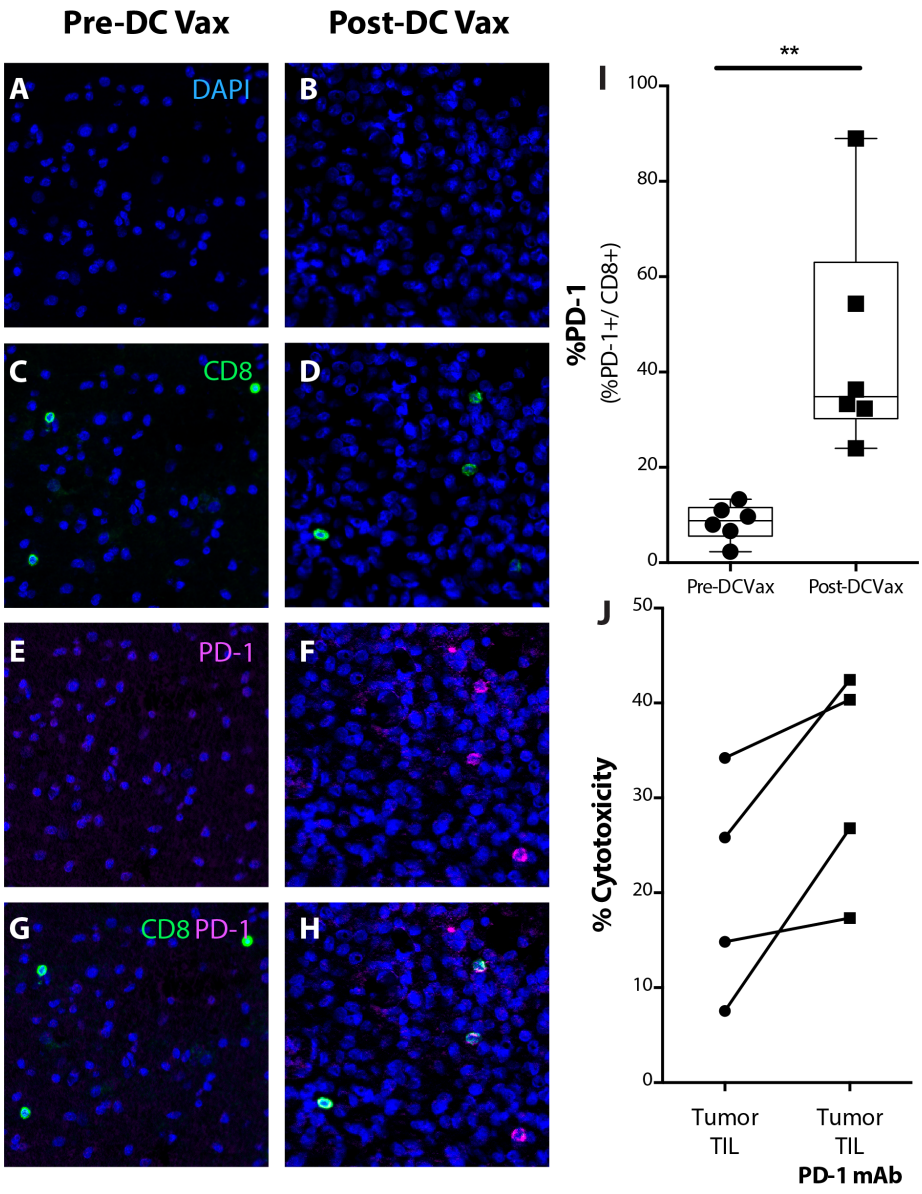
(n=4/group) (**p<0.01, ***p<0.001, ****p<0.0001). Data shown are representative of one experiment repeated four times with similar findings. Each point represents 1 subject (**A-D**). Box-and-whisker plots were used to graphically represent the median (line within box), upper- and lower- quartile (bounds of box), and maximum and minimum values (bars) (**C**). Survival differences were calculated using log rank statistical tests and graphed using the method of Kaplan-Meier (**A**, **B**) and the Student's t-test used to calculate statistical significance at individual time points (**C**).

Figure 4



PD-1 mAb blockade increases the population of T cells expressing memory and tumor homing markers. **(A-C)** Splenic lymphocytes and tumor-infiltrating lymphocytes (TILs) from DC vaccine +/- PD-1 mAb were stained with CD8+ CD44+ cells and analyzed by flow cytometry. (n=4/group) (**p<0.01). **(D, E)** CD62L+ cells (gated from $CD8^+$ $CD44^+$ cells) were analyzed by flow cytometry. (n=4/group) (*p<0.05, ***p<0.001). **(F, G)** CD49d+ cells (gated from $CD8^+$ $CD44^+$ cells) were analyzed by flow cytometry. (n=4/group) (*p<0.05). Data shown are representative of one experiment repeated two times with similar findings. **(H)** Survival of long-term DC vaccine + PD-1 mAb survivors challenged with GL261 glioma cells in the contralateral brain was monitored and compared with naïve control mice. (Control, n=6; DC vaccine + PD-1 mAb, n=5) (****p<0.0001). Data shown are representative of one experiment repeated three times with similar findings. Each point represents 1 subject (**A, B, D-G**). Box-and-whisker plots were used to graphically represent the median (line within box), upper- and lower-quartile (bounds of box), and maximum and minimum values (bars) (**A, B, D-G**). Statistical analyses were performed by a Student's t-test (**A, B, D-G**) and Kaplan-Meier method (**H**).

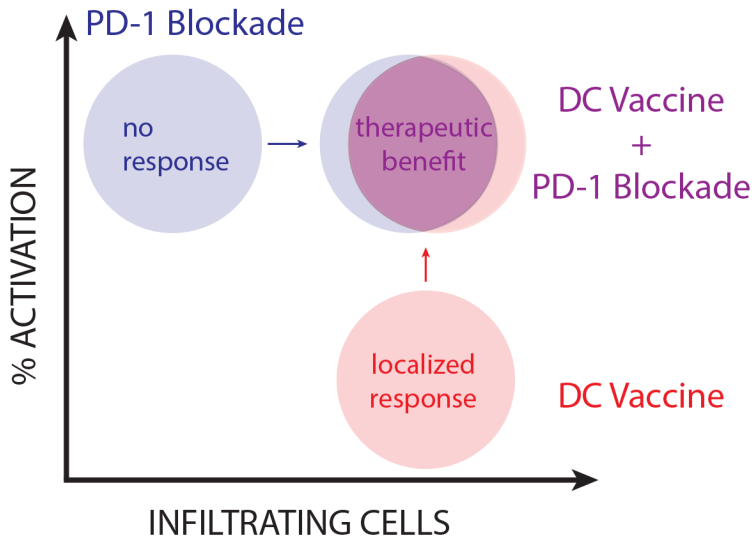
Figure 5



Ex vivo PD-1 blockade enhances TIL cytotoxicity against human GBM. **(A, B)** 4',6-diamidino-2-phenylindole (DAPI), **(C, D)** CD8, **(E, F)** PD-1, and **(G, H)** CD8 and PD-1 co-staining is shown across pre- and post-DC vaccine samples from a representative glioblastoma (GBM) patient (40x magnification). **(I)** Percent PD-1 expression on CD8+ TILs across pre- and post-DC vaccine

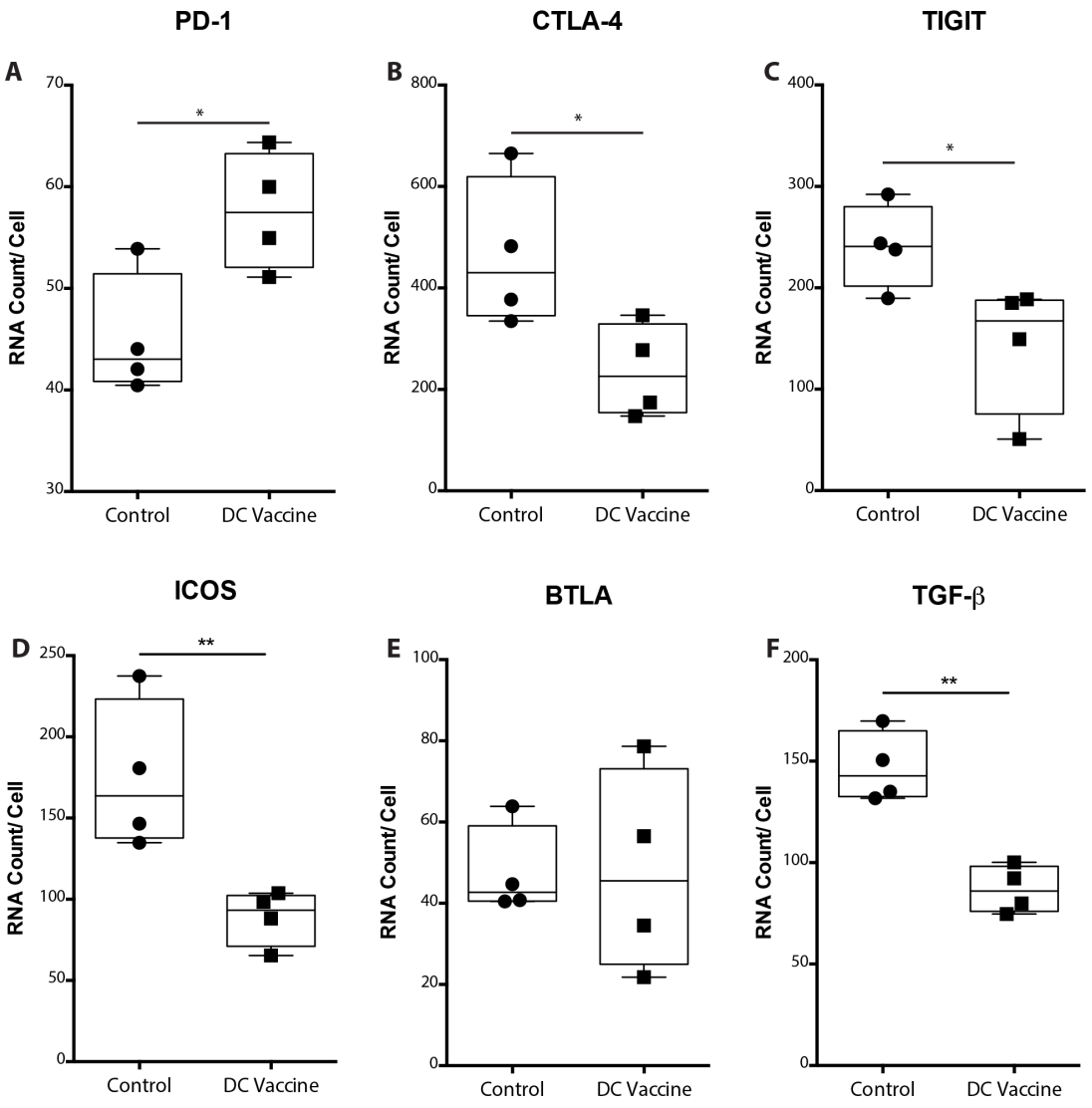
treatment patient samples was quantified (n=6) (**p<0.01). **(J)** TIL cytotoxicity against human GBM at 15 hours with and without PD-1 mAb blockade shown (n=4). Each point represents 1 subject (**I, J**). Box-and-whisker plots were used to graphically represent the median (line within box), upper- and lower- quartile (bounds of box), and maximum and minimum values (bars) (**I**). Statistical analyses were performed using the Student's t-test (**I**).

Figure 6



Therapeutic benefit for i.c. glioma is dependent on TIL infiltration and activation. DC vaccination promotes activation of a significant tumor-infiltrating lymphocyte (TIL) population, which is then further activated in the presence of PD-1 mAb blockade.

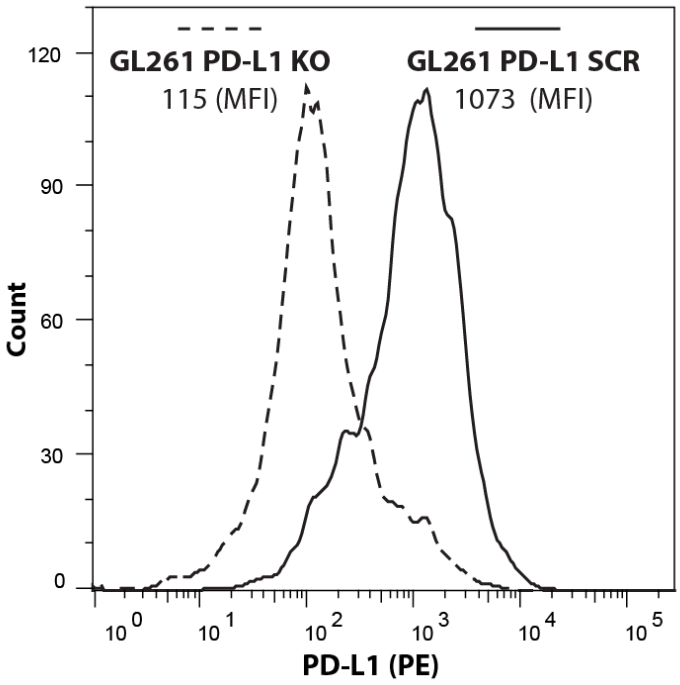
Supplemental Figure 1



Transcriptional regulation of negative costimulatory molecules on purified CD3+ TIL from animals with and without DC vaccine. FACS-sorted CD3+ Thy1.2+ tumor-infiltrating lymphocyte (TIL) expression of **(A)** PD-1, **(B)** cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), **(C)** T cell immunoreceptor with Ig and ITIM domains (TIGIT), **(D)** Inducible T-cell COStimulator (ICOS), **(E)** B- and T-lymphocyte attenuator (BTLA), and **(F)** TGF-β in non-treated control and DC vaccine-treated tumors was quantified by Nanostring. (n=4) (*$p<0.05$,

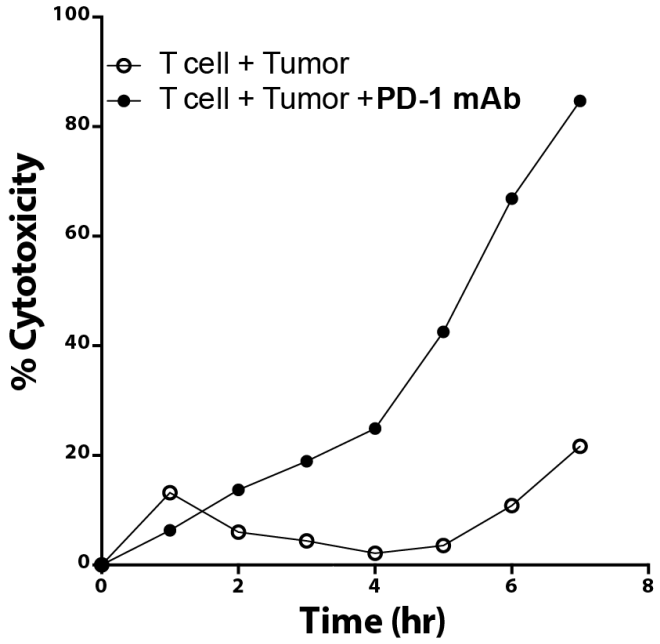
p<0.01). Each point represents 1 subject (A-F**). Box-and-whisker plots were used to graphically represent the median (line within box), upper- and lower- quartile (bounds of box), and maximum and minimum values (bars) (**A-F**). Statistical analyses were performed by the Student's t-test (**A-F**).

Supplemental Figure 2



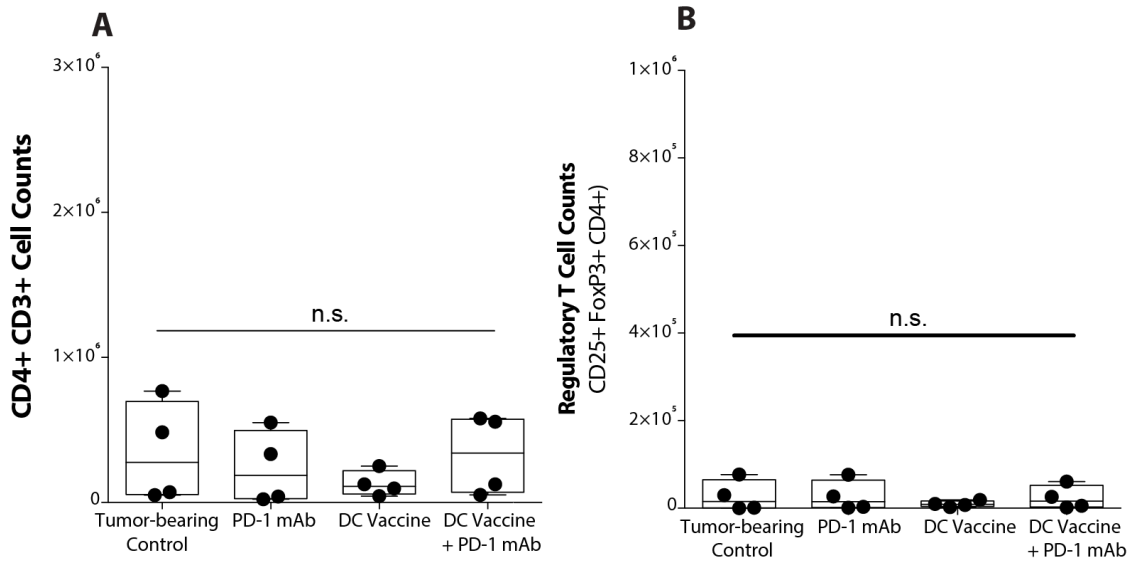
GL261 glioma cells express PD-L1. Median fluorescence intensity (MFI) of PD-L1 on GL26 PD-L1 knock-out (KO) and scrambled control (SCR) cells is shown.

Supplementary Figure 3



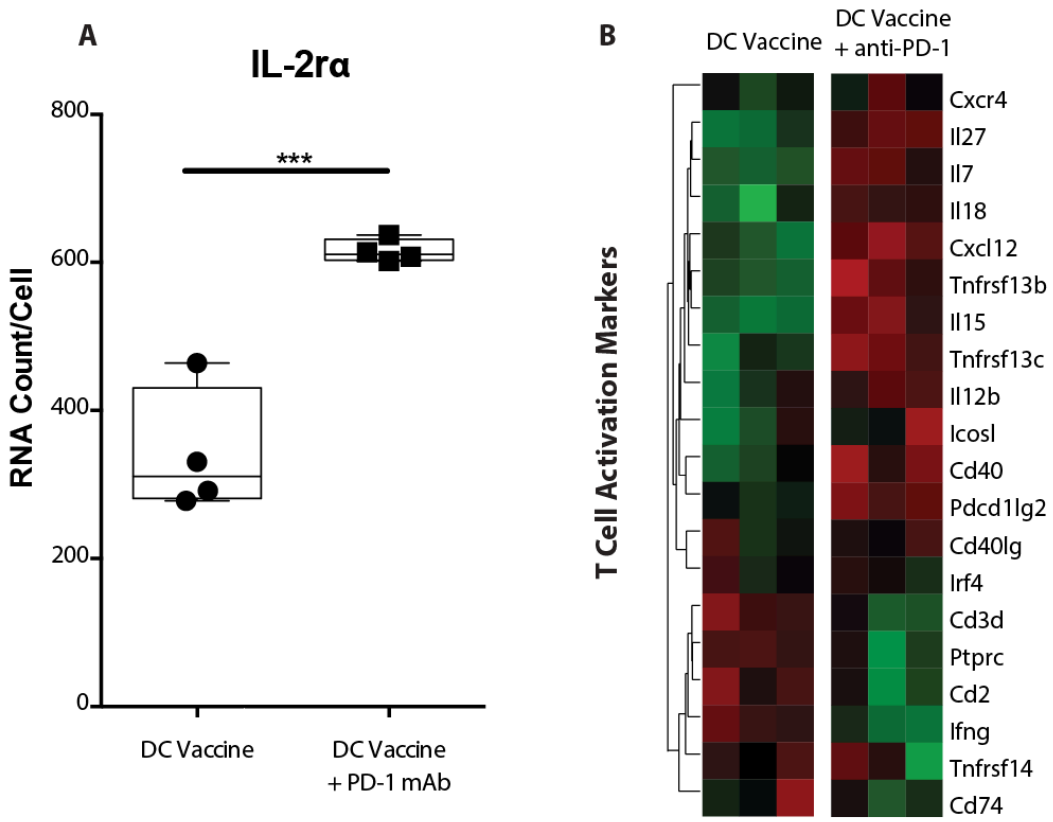
PD-1 mAb enhances TIL cytotoxicity in vitro. FACS-sorted CD3+ Thy1.2+ tumor-infiltrating lymphocytes (TILs) from DC vaccine-treated tumor-bearing mice were co-cultured with intracranially-derived tumor bulk with or without *ex vivo* PD-1 mAb and tumor cytolysis over time was quantified. Data shown is from a representative experiment which has been repeated four times with similar findings.

Supplementary Figure 4



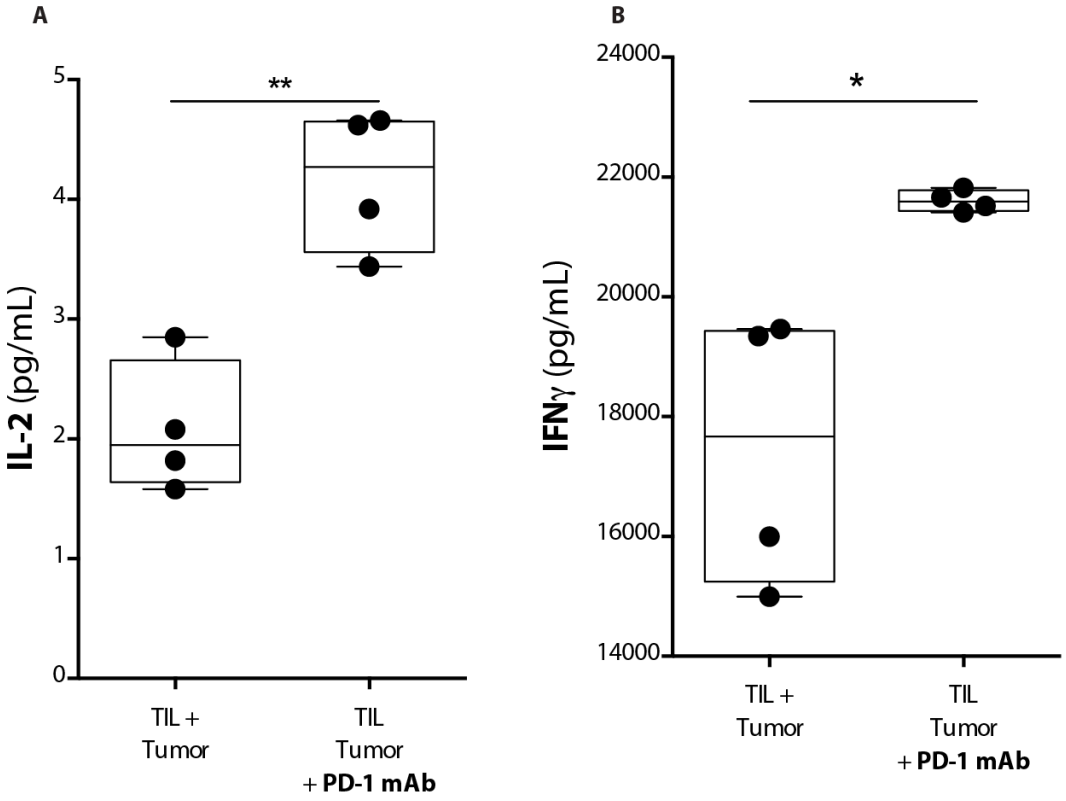
CD4 helper and T regulatory cells are not modulated by a PD-1 mechanism in established tumors. No significant differences in **(A)** Helper (CD3+ CD4+) T cell counts or **(B)** T regulatory cell (CD4+ CD25+ FoxP3+) counts were noted across treatment groups. (n=4/group). Each point represents the average number of cells isolated from the tumor-bearing hemisphere of each animal. Box-and-whisker plots are used to graphically represent the median (line within box), upper- and lower- quartile (bounds of box), and maximum and minimum values (bars) (**A, B**). Statistical analyses were performed using a Student's t-test (**A, B**).

Supplemental Figure 5



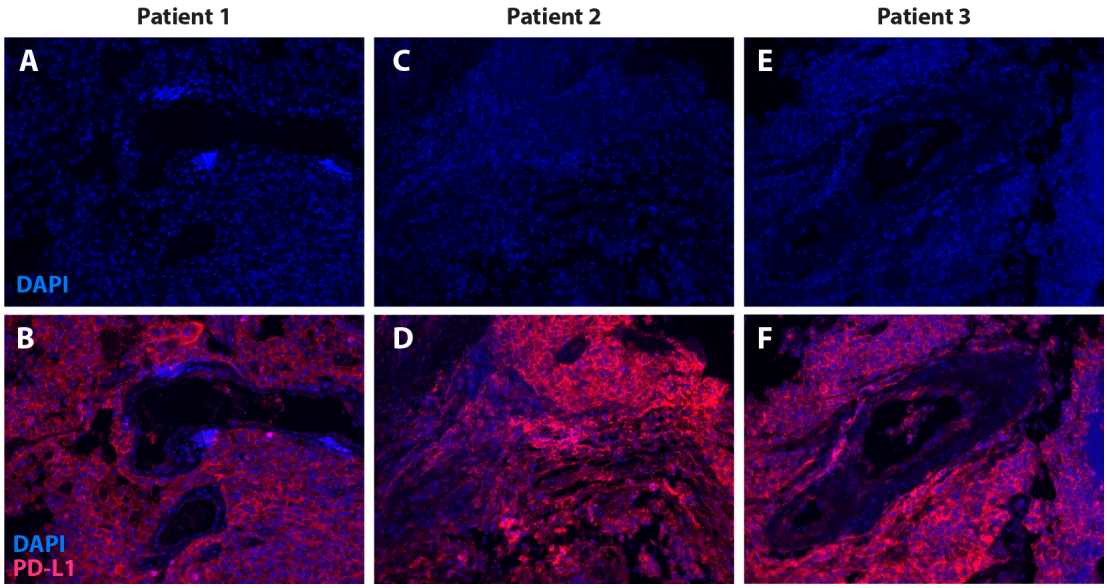
Transcription of T cell activation, homing, and survival markers is increased when PD-1 mAb is given together with DC vaccination. Pathway-based gene clustering of FACS-sorted CD3+ Thy1.2+ tumor-infiltrating lymphocytes (TILs) demonstrates differences in **(A)** the late activation marker, IL-2R ✔, as well as **(B)** cytokines, homing markers, and cell survival transcriptional regulation. (n=4/group) (***$p<0.001$). Each point represents the gene transcription of cells isolated from one animal (**A-D**). Box-and-whisker plots are used to graphically represent the median (line within box), upper- and lower- quartile (bounds of box), and maximum and minimum values (bars) (**B-D**). Statistical analyses were performed using a Student's t-test (**B-D**).

Supplemental Figure 6



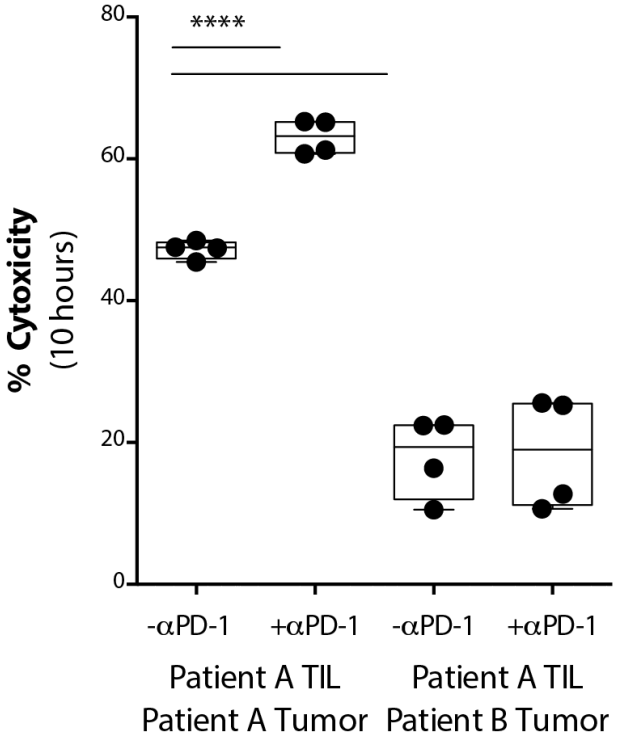
PD-1 mAb enhances TIL function. The levels of **(A)** IL-2 and **(B)** IFNɣ (pg/mL) in supernatants following tumor-infiltrating lymphocyte (TIL) co-cultures with GL261 glioma cells with or without PD-1 mAb was analyzed using a multi-analyte array (*$p<0.05$, **$p<0.01$) (n=4/group). Each point represents 1 biological replicate (**A, B**). Box-and-whisker plots were used to graphically represent the median (line within box), upper- and lower- quartile (bounds of box), and maximum and minimum values (bars) (**A, B**).

Supplementary Figure 7



PD-L1 expression in the GBM tumor microenvironment. **(A, C, E)** 4',6-diamidino-2-phenylindole (DAPI) and **(B, D, F)** programmed death-ligand 1 (PD-L1) co-staining is shown across three glioblastoma (GBM) patients (40x magnification).

Supplemental Figure 8



TIL tumor-specific cytotoxicity is observed in human ex vivo *GBM cultures.* Patient A TIL cytotoxicity against Patient A and Patient B GBMs at 10 hours with and without PD-1 mAb blockade (+/- αPD-1) is shown (****$p<0.0001$) (n=4/group). Each point represents 1 biological replicate. Box-and-whisker plots were used to graphically represent the median (line within box), upper- and lower- quartile (bounds of box), and maximum and minimum values (bars). Statistical analyses were performed using a Student's t-test.

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Chapter 4:

Immunosuppressive tumor-infiltrating myeloid cells mediate adaptive immune resistance via PD-1/PD-L1 mechanism in glioblastoma

Abstract:

While active vaccination for glioblastoma (GBM) appears promising clinically, mechanisms of immune resistance may account for the variable responses seen in patients. In the present study, we identified a PD-L1-expressing tumor infiltrating myeloid (TIM) cell population that expanded in response to vaccination. The presence of these TIMs restricted the activation and tumor-cytolytic function of the vaccine-induced tumor infiltrating lymphocytes (TILs) *in vitro* and decreased survival in our *in vivo* murine glioma model. These immunoregulatory effects were mediated via the PD-1/PD-L1 mechanism, and our studies indicated that the majority of PD-L1 signaling in the tumor environment is contributed by TIMs rather than by tumor cells themselves. While PD-1 blockade partially reversed these effects, targeting TIMs directly with a colony stimulating factor-1 receptor inhibitor (CSF-1R*i*) altered TIM expression of key chemotactic factors to promote TIL infiltration after vaccination rather than inhibiting it. Although neither treatment showed significant therapeutic effects in isolation, combined, CSF-1R inhibition and PD-1 blockade both increased the number of TILs and their activation in the murine dendritic cell (DC) vaccination studies as well as in *ex vivo* GBM patient studies. Together, these studies elucidate the role that TIMs play in mediating adaptive immune resistance in the GBM microenvironment and provide evidence that they can be manipulated pharmacologically with agents that are clinically available.

Introduction:

Glioblastoma (GBM) is a particularly challenging cancer that, despite advances in standard and immune therapies, continues to have a poor prognosis (1-4). Active vaccination strategies have shown enough promise (1, 5) that a randomized, Phase III clinical trial utilizing dendritic cell (DC) therapy in GBM patients is currently underway. In previous studies, however, extended survival associated with this treatment was variable, with evidence suggesting that persistent residual or progressive disease may impair the beneficial anti-tumor response.

Because most GBM are not inherently immunogenic, we hypothesize that an active vaccination strategy might be required to first generate an intratumoral immune response. Recent findings suggest, though, that this response may be subsequently mediated by the adaptive immune resistance caused by PD-1/PD-L1 signaling (6, 7). We recently showed that in large, established tumors, the PD-1/PD-L1 mechanism dominantly inhibited the vaccine-generated anti-tumor immune response (6). Although PD-1/PD-L1 signaling has been documented in GBM (8-10), early studies have suggested that tumor cells themselves are the dominant expressers of PD-L1 (7, 11). However, PD-L1 is known to be expressed by other cells of monocytic lineage that are frequently present in the tumor microenvironment. Schultheis at al. suggested in their study of neuroendocrine carcinomas that tumor-infiltrating macrophages increased their PD-L1 expression following increasing numbers of tumor-infiltrating lymphocytes (TILs) (12). Tumor cells in these neuroendocrine carcinomas consistently did not express PD-L1. These findings have been corroborated in other cancer models, including ovarian (13, 14) non-small cell lung, pancreatic, renal cell, colorectal, and prostate cancers (15-17) as well as chordomas (18).

Tumor infiltrating myeloid cell populations have been evaluated in GBM. Bingle et al. reported that the presence of a large number of infiltrating cells they termed tumor associated macrophages (TAMs) was prognostic of poor survival in patients (19). In murine models of cancer, selective removal of these cells appears to lead to markedly depressed tumor growth rates (20, 21). The infiltration of TAMs appears to increase in response to treatments such as radiation and chemotherapy (22, 23). Other studies have identified inhibitory myeloid cell populations in established disease that expand following immunotherapies (22-24) and thereafter promote tumor progression (25-35). These treatments induce colony stimulating factor-1 (CSF-1) secretion from tumors, which promotes the influx of myeloid cells into tumors. Once there, they increase expression of T cell inhibitory factors, such as PGE_2, TGFβ, and IL-10, and promote tumor progression (22, 23, 36-38).

In this study, we identified that, following vaccination, PD-L1 is dominantly expressed on an expanded GBM TIM population. We showed that PD-1 blockade rendered tumor-specific T cells insensitive to the immunoregulatory effects of TIMs. We also showed that, while treatment with CSF-1R*i* in our vaccinated GBM model did not fully deplete TIMs nor abolish PD-L1 expression on TIMs, it did promote increased cytokine and chemokine signaling by TIMs to support an increased TIL influx. Thus, we found that combined PD-1 mAb and CSF-1R*i* treatment adjuvant to vaccination significantly enhanced anti-tumoral responses in both our murine glioma model and our *ex vivo* GBM patient samples.

Materials and Methods:

Cell lines and Human Specimens

All murine and human glioma tumor cells were cultured using complete DMEM (Mediatech, Inc. Herndon, VA) with supplementary 10% FBS (Gemini Bio-Products, West Sacramento, CA), and 1% (v/v) penicillin and streptomycin (Mediatech Cellgro, Manassas, VA). Cells were then maintained in a humidified atmosphere of 5% CO_2 at 37°C conditions. The Brain Tumor Translation Resource (BTTR) at UCLA provided paraffinized human GBM tissue pre- and post-DC vaccine treatment. Fresh tumor was obtained from newly diagnosed GBM patients immediately following resection and digested in collagenase for 24 hours. Percoll gradient was used to isolate tumor cell and lymphocyte fractions.

Mice

Female C57BL/6 mice (6-8 weeks old) were obtained from Division of Experimental Radiation Oncology at the University of California Los Angeles and housed in a defined flora and pathogen free vivarium as defined by the Association for Assessment and Accreditation of Laboratory Animal Care. Animal treatment was compliant with the University of California, Los Angeles animal care policy and approved animal protocols.

Intracranial glioma implants

Mice were anesthetized and GL261 glioma cells (2×10^4 in 2 μl PBS) were stereotactically injected with a sterile Hamilton syringe fitted with a 26-gauge needle. The intracranial injection ensued over a 2 min period and was positioned 2.5 mm lateral to bregma at a depth of = 3.5 mm

below the dura mater. Following intracranial tumor implantation, mice were randomized into treatment groups (n=6-12/group).

Bone marrow-derived DC and vaccination

GL261 glioma cells were cultured and expanded in complete DMEM media. Cells were then harvested and exposed to 3-5 freeze-thaw cycles. Lysate concentration was quantified using a Bradford protein assay (Bio-Rad). DCs were prepared from murine bone marrow progenitor cells and pulsed with GL261 lysate as previously described (47). DCs (1×10^6 cells/mouse) were then administered subcutaneously at 4 sites on dorsal aspect of mouse midbody on days 3 and 13 following tumor implantation.

In vivo treatments and depletions

Anti-PD-1 mAb (RMP1-14, BioXCell) was administered i.p. at 250 mg/kg daily on days 3-5 and 13-15. Ly6-C (Monts 1, BioXCell) and CD8 (Lyt 2.1, BioXcell) depleting antibodies were administered i.p. at 200 mg/kg every other day starting four days prior to tumor implantation. The CSF-1R*i* (PLX3397, Plexxikon) was administered daily by oral gavage at 50mg/kg for the duration of the experiment starting on the day of tumor implantation.

Tissue harvests, immunohistochemistry, and flow cytometry

Tumor-bearing brain hemispheres were harvested from mice 72 hours following the second DC vaccine treatment and placed in collagenase solution overnight to digest the tissue. Lymphocytes were then isolated using 30%:70% Percoll gradient. Fluorochrome conjugated antibodies to mouse CD3 (17A2), CD4 (4SM95), CD8 (53-6.7), CD25 (PC61.5), FoxP3 (FJK-16s), Ly6-C

(HK1.4), GR-1 (RB6-8C5), CD45.2 (104), CD11b (M1/70), CD11c (N418), F4/80 (BM8), CSF-1R (AFS98), Thy1.2 (30-H12), PD-1 (RMP1-14), and PD-L1 (MIH5) were obtained from eBioscience. Fluorochrome conjugated antibodies to human CD3 (HIT3a), CD8 (SK1), and CD11b (ICRF44) were obtained from eBioscience as well. All FACS analysis was acquired with a FACS LSRII (BD Biosciences) and sorting was performed with the use of an FACSAria (BD Biosciences). Gates were set based on f minus one (FMO). Data was analyzed using FlowJo (Treestar) software. Sorted lymphocytes were placed into culture using RPMI supplemented with 10% FBS and 1% (v/v) penicillin and streptomycin.

In cases where sectioning and immunohistochemistry were required, tissue was placed in Zinc Fixative (1x, BD Biosciences) for 24 hours and then transferred to 70% ethanol before being embedded in paraffin wax. Murine tissue was stained via immunohistochemical methods with the assistance of the UCLA Translational Pathology Core Laboratory (TPCL) for CD8 (4SM15, 1:100, eBioscience) and CD11b (M1/70, 1:100, eBioscience). Human tissue was stained via immunohistochemical and immunofluorescent methods at our facility using CD8 (C8/144B, 1:5000, Dako), CD163 (GHI/61, 1:1000, eBioscience), PD-1 (EPR4877; abcam, 1/50000), PD-L1 (1-111A, 1:4000, eBioscience), and GFAP (6F2, 1:100000, Dako). Analysis for all tissue was performed using the Vectra 3.0 (PerkinElmer) quantitative pathology imaging system and inForm (PerkinElmer) analysis software to select and quantify staining and colocalization of markers of interest.

TIL:TIM Transwell Assay

Thy1.2- CD11b+ TIMs and Thy1.2+ CD3+ TILs were FACS-sorted from tumor-bearing hemispheres of DC vaccinated mice. TIMs were cultured in 24-well plates at 100,000 cells/well in RPMI media supplemented with 10% FBS, 1% (v/v) penicillin and streptomycin, and 100 IU/mL IL-2. In wells requiring addition of TILs, 0.40 μm pore polycarbonate membrane transwell inserts were first placed into the wells. TILs were then added to the transwells at TIL:TIM ratios of 1:1 and 10:1. In wells lacking TIL co-culture, IFNɣ was directly added to media at a concentration of 100 or 1000 IU/mL. In wells where IFNɣ blockade was required, 50 μg/mL of IFNɣ mAb (XMG1.2, BioXCell) was added to the culture media. All cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C conditions for 24 hours following plating before performing cellular analysis.

Quantitative Transcriptional Profiling

Tumor-infiltrating leukocytes from murine brain tumor-bearing hemisphere tissue were sorted for Thy1.2- CD11b+ and Thy1.2+ CD3+ cells using a FACSAria flow cytometer and RNA was then obtained using an RNEasy acquisition kit (Qiagen). RNA samples were analyzed using the nCounter GX Nanostring Analysis system (Nanostring Technologies), which allows for exact quantification of RNA expression via direct binding to tagged probes sampling approximately 770 genes (48, 49). Data was then analyzed using the Nanostring nSolver Analysis software (Nanostring Technologies).

xCELLigence real-time cytotoxicity assay

In human *ex vivo* cytotoxicity studies, CD3+ TILs and CD11b+ TIMs from patient glioma were FACS-sorted and placed into culture with patient GBM (CD3- CD11b-) cells. In murine *in vitro*

cytotoxicity studies, Pmel-1 gp100-specific T cells were placed into culture with GL261-gp100 glioma cells and FACS-sorted CD11b+ TIMs from tumor-bearing brain hemispheres of vaccinated mice. Tumor cytolysis was assessed using the xCELLigence Real-Time Cell Analyzer System (Acea Biotechnology) (50, 51). In both human and murine studies, the tumor cell:TIL:TIM ratio was 1:10:1. Where indicated, T cell media was supplemented with 10uM anti-PD-1 Ab (BioXCell) for 1 hour on ice prior to the addition of T cells to xCelligence assay. Where indicated, TIMs were cultured with 10μM CSF-1R*i*.

In vitro activation of Pmel-1 T cells

Spleens and lymph nodes were harvested from Pmel-1 TCR transgenic mice. The organs were processed and a single-cell suspension was obtained and then cultured with human IL-2 (100IU/mL, NCI Preclinical Repository, Developmental Therapeutics Program) and hgp100 (25-33) peptide (1 μg/mL, NH2-KVPRNQDWL-OH, Biosynthesis. Inc.) in XVIVO-15 (Lonza) and 2% FBS. After 72 hours, cells were washed with PBS 1x and cultured in fresh media with 100 IU/mL IL-2 for an additional 3 days prior to use in cytotoxicity assays.

CRISPR Knock-Out

CRISPR knock-out (KO) of PD-L1 in the GL261-gp100 cell line was performed as described previously (52). Guide sequences for knockout were as follows: A1 (GTA TGG CAG CAA CGT CAC GA) and A3 (TCC AAA GGA CTT GTA CGT GG). Scrambled control line was also generated using the sequence (GCA CTA CCA GAG CTA ACT CA). Transduced cells were purified using puromycin selection followed by FACS sorting of PD-L1(-) cells from the puromycin-resistant population.

Results:

GBM-infiltrating TIMs inhibit T cell-mediated tumor cytolysis via the PD-1/PD-L1 axis

Our group has previously shown that the PD-1/PD-L1 negative costimulatory system reduces the efficacy of vaccination-induced immune responses in established tumors (6). To understand the mechanisms underlying these cellular interactions, we examined pre- and post-vaccinated patient GBM samples and found that PD-L1 expression predominantly co-localized with CD163+ cells of the myelomonocytic lineage (**Fig. 1a-f**). Interestingly, not only was the population of TIMs in vaccinated established tumors expanded (**Fig. 1g**), but there was an increase in the percentage of TIMs expressing PD-L1 following vaccination (**Fig. 1h**). TIMs were the dominant contributors of PD-L1 in the tumor microenvironment (**Fig. 1i**).

In order to interrogate the effect of TIMs on the functional activity of TILs, tumor cells and TILs from freshly resected GBM were cultured with or without TIMs from the same patient specimen. In the presence of TILs, TIMs significantly upregulated PD-L1 expression (**Fig. 1j**). Cytolysis of tumor cells by the TILs was significantly reduced in the presence of TIMs, but addition of a PD-1-blocking mAb to this TIM-TIL-tumor co-culture recovered cytolysis to levels identical to cultures without TIMs (**Fig. 1k, l**). Interestingly, in the absence of TIMs, PD-1 blockade did not provide any additional TIL cytolytic benefit, suggesting that the functional PD-1/PD-L1 interaction was predominantly between TIMs and TILs (**Fig. 1l**).

In the murine GL261 glioma model, we identified a CD11b+ myelomonocytic population that similarly expanded after vaccination in large, established tumors (**Fig. 2a**) and demonstrated

significantly greater PD-L1 expression compared to glioma cells (**Fig. 2b**). These CD11b+ cells were further characterized as non-T lymphocyte (Thy1.2-), monocyte lineage (CD11b+ and Ly6-C+), CSF-1R+, and F4/80+ cells (**Fig. 2c, d**) (**Supp. Fig. 1; Supp. Fig. 2a, b**). To evaluate whether PD-1/PD-L1 also suppressed cytotoxic TIL activity in the murine model, we co-cultured CD11b+ cells sorted from intracranial GL261-gp100 glioma cells and Pmel-1 gp100-specific T cells. As seen with human GBM, the presence of TIMs significantly reduced the T cell cytolytic ability (**Fig. 2e, f**) and this could be recovered with PD-1 mAb treatment, but PD-1 blockade did not have any apparent effect on tumor cytolysis in the absence of TIMs. To confirm that PD-L1 expression by tumor cells did not suppress T cell-mediated tumor cytolysis, we created GL261-gp100 glioma cells in which the PD-L1 gene was disrupted. Cytolysis of control, PD-L1-sufficient GL261 cells was not different when compared to PD-L1-deficient CRISPR GL261-gp100 cells, regardless of PD-1 blockade *in vitro* (**Supp. Fig. 3a, b**). This data suggested that the PD-1/PD-L1 pathway was not primarily mediated by tumor cells, but rather that TIMs used this pathway to inhibit T cell-induced tumor cytolysis in glioma.

TIM PD-L1 expression is an adaptive immune response to vaccine-induced TILs

Given recent findings in other cancers (11, 39), we hypothesized that TILs induced PD-L1 expression on GL261 glioma-infiltrating TIMs. The addition of increasing concentrations of IFNɣ to TIMs cultured *in vitro* resulted in proportional increases in PD-L1 expression on TIMs (**Fig. 3a**). Supernatants from TIM-TIL transwell co-cultures were analyzed using a multi-analyte cytokine assay, which demonstrated significantly increased IFNɣ levels with increasing numbers of TILs (**Fig. 3b**). As a result, TIMs in these cultures demonstrated that increased TIL count resulted in increasing PD-L1 expression (**Fig. 3c**). Neutralizing IFNɣ signaling with mAb

treatment in these TIM-TIL co-cultures completely abolished this TIL-related PD-L1 upregulation on TIMs (**Fig. 3c**). In vivo, post-vaccination TIM expansion and PD-L1 upregulation were significantly reduced when CD8+ T cells were depleted in vaccinated mice (**Fig. 3d, e**). This demonstrated that TIM upregulation of PD-L1 was an adaptive resistance mechanism to the anti-tumor actions of vaccine-induced IFNγ-secreting CD8+ TILs.

We next evaluated the corresponding influence of TIMs on TILs. Tumor-bearing mice treated with DC vaccine, PD-1 blockade, or a Ly6-C mAb that depletes TIMs (**Supp. Fig. 4**) were assessed for TIL infiltration and activation. Although TIM depletion alone did not promote an infiltrating T cell response, when it was combined with DC vaccination the TIL population following vaccination was significantly increased (**Fig. 4a**). Activation markers in the CD8+ subset of these cells were also significantly elevated, comparable to levels observed with PD-1 blockade (**Fig. 4b**). However, PD-1 blockade in addition to DC vaccine + Ly6-C depleting antibody in tumor-bearing mice did not provide any additional increase in T cell activation over DC Vaccine + Ly6-C depletion alone. These results demonstrated *in vivo* that TIMs were indeed the dominant source of PD-1/PD-L1-mediated T cell suppression and that blockade of PD-1 was functionally similar to depletion of TIMs.

*PD-1 mAb and CSF-1R*i *together maximally enhance the vaccine-generated immune response*

CSF-1R blockade has been shown to be a clinically translatable therapy that reduces the infiltrating TIM population in other cancers, as opposed to Ly6-C mAb, which is a mouse mAb not intended for clinical use (23). For our studies, we chose a small molecule CSF-1R inhibitor (CSF-1R*i*, PLX3397, Plexxikon) that crosses the blood-brain barrier and has been well tolerated

in human clinical trials (24, 40). While CSF-1R*i* treatment in unvaccinated mice did not significantly alter the infiltrating T cell response, we showed that CSF-1R blockade in vaccinated glioma-bearing mice led to reduced infiltrating TIM populations (**Supp. Fig. 4**) and significantly increased TIL infiltration (**Fig. 4a**). When compared to the earlier experiments with DC vaccine + Ly6-C TIM depletion, these effects were more profound. Although DC vaccine + CSF-1R*i* increased overall TIL infiltration above that induced by DC vaccine treatment alone, it did not significantly alter TIL activation (**Fig. 4b**). This led us to hypothesize that CSF-1R*i*-treatedTIMs affected TILs through mechanisms other than PD-L1, as TIMs continued to express stable levels of PD-L1 after treatment with CSF-1R*i* (**Fig. 4c**).

To understand the increase in the CD3+ TIL population seen with adjuvant CSF-1R*i* treatment, we used a quantitative transcriptional profiling assay to document the gene expression profile of TIMs from mice treated with the DC vaccination and DC vaccine + CSF-1R*i*. An elevation in chemotactic, apoptotic, and Jak/STAT signaling factors following CSF-1R*i* treatment was observed, supporting our finding of increased TIL infiltration (**Fig. 4d-f**). Interestingly, IL-10, which has been shown to mediate the Jak/STAT signaling pathway and NF-kB activity to further inhibit inflammation, as well as regulate antigen-specific T cells in the chronic viral model (41, 42), was also significantly downregulated with CSF-1R*i* treatment (**Fig. 4g**).

Based on our findings, CSF-1R*i* and PD-1 blockade appeared to influence two independent aspects of the anti-tumor immune response. CSF-1R*i* promoted an increased density of the TIL population generated by the DC vaccine treatment and PD-1 blockade activated the TIL population. When combined, these two adjuvants were able to synergistically enhance the DC

vaccine treatment. With murine cells, maximal tumor cytolysis was achieved with combination therapy when compared either treatment alone (**Fig. 5a**). *In vivo*, DC vaccine with PD-1 blockade significantly increased survival over DC vaccination alone, as did DC vaccine with CSF-1R*i* (**Fig. 5b**). However, DC vaccine with CSF-1R*i* and PD-1 blockade together significantly increased long-term survival of tumor-bearing mice over mice receiving DC vaccination with either treatment alone. Most importantly, similar effects were observed in our *ex vivo* GBM patient tumor samples. When patient GBM-derived TILs were co-cultured with autologous patient GBM TIMs and tumor cells, TIL-mediated tumor cytolysis was enhanced with either PD-1 blockade or CSF-1R*i* alone versus non-treated controls. However, we saw a further significant increase in TIL-mediated tumor cytolysis with PD-1 mAb and CSF-1R*i* combined when compared to either treatment alone (**Fig. 5c, d**).

Discussion:

We have previously established that vaccination is necessary to generate an infiltrating immune response in a non-immunogenic cancer such as GBM, and that the interaction of PD-1-expressing TILs with PD-L1 in the tumor microenvironment reduces its efficacy. (6) Our initial hypothesis of PD-1/PD-L1 checkpoint inhibition was that tumor cells mediated this mechanism to restrict the vaccine-generated immune response. However, our data indicates that TIMs are responsible for PD-L1 expression within the tumor microenvironment across our patient GBM tissue samples and murine glioma tissue. PD-L1 that is expressed on a non-tumor cell population, as opposed to the tumor cells themselves, within the tumor microenvironment is an important distinction. This distinction suggests that direct, restricted targeting of GBM TIMs alone, instead of systemic blockade of inhibitory mechanisms, may be a more effective and efficient treatment option for patients with GBM. It will likely prove necessary to overcome the effects of this cell population to sustain a successful anti-tumor immune response.

We were able to isolate endogenous infiltrating populations of TILs in patient GBMs. When these TILs were placed into co-culture with GBM tumor cells at a sufficient effector-to-target ratio, there was significant tumor cytolysis, confirming that these TILs have the capacity to exerting a tumor specific response. Given that these tumors progress to the point of diagnosis, these endogenous TILs are not able to mediate significant-enough tumor cytolysis and halt tumor progression. In our clinical and pre-clinical studies, dendritic cell vaccination appears capable of boosting the numbers of glioma-infiltrating TILs. However, we found that there is a reciprocal TIM expansion associated with this treatment. In response to TIL-secreted factors, TIMs expand

and inhibit TIL activation and tumor cytolysis directly via the PD-1/PD-L1 signaling pathway The role played by PD-L1 on this immune-responsive TIM population suggests that endogenous adaptive immune resistance may be key in to prevent successful immune mediated suppression of these tumors.

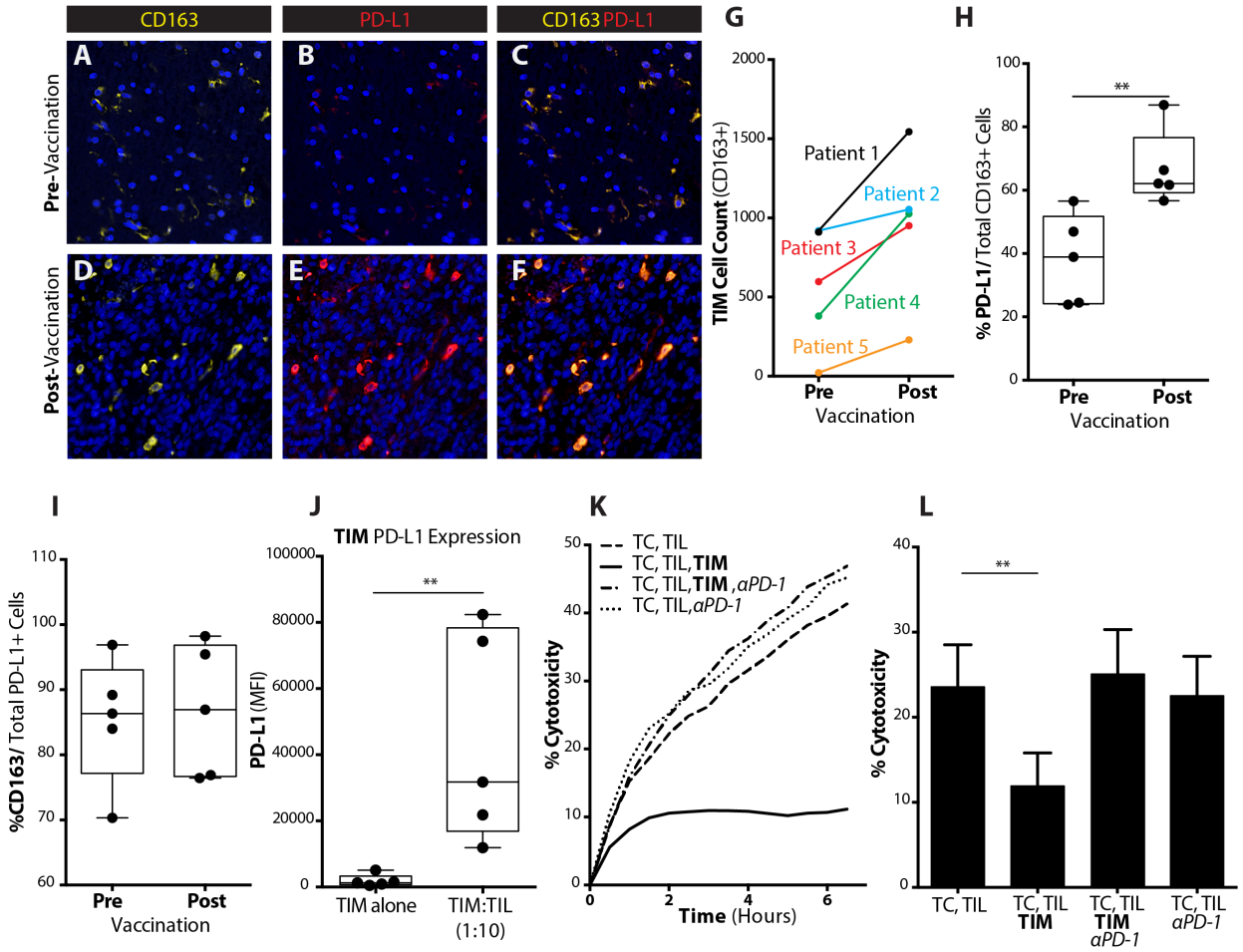
Our work with CSF-1R*i* gave us further insight that TIMs have multiple roles in the tumor microenvironment. Although CSF-1R inhibition did not fully deplete the TIM population, nor alter TIM PD-L1 expression, it did alter the gene expression signature of these cells. TIMs from mice treated with DC vaccine and CSF-1R*i* showed elevated chemokine, cytokine, and Jak/STAT transcripts. The sum total effect of these pathways may be to enhance TIL recruitment and expansion. Future therapies might be utilized to transform these inhibitory cell populations to support a continued anti-tumor immune response.

Interestingly, PD-1 mAb or CSF-1R*i* alone as adjuvants to DC vaccination promote significantly increased survival. The evidence suggests that each treatment is targeting a different aspect of the anti-tumor immune response (**Fig. 6**). With PD-1 mAb, there is increased activation of TILs generated by vaccination. With CSF-1R*i*, there is an expansion of the TIL population following alteration of the TIM phenotype. Independently, each adjuvant enhances one aspect of the DC vaccine-induced immune response. Together, however, these two treatments may both activate and expand the TIL population such that there is significant increase in tumor cytolysis and survival.

The immediate relevance of these adjuvant treatments was confirmed with both elevated tumor cytolysis in *ex vivo* patient GBM cultures and significantly prolonged survival in our preclinical murine glioma model. This study represents the first time that both PD-1 mAb and CSF-1R*i* have been used together to enhance the active vaccination strategy in the glioma model. Currently, there are two PD-1 mAbs approved for clinical use (43-46), and PLX3397, the CSF-1r inhibitor we utilized in these studies, is currently in Phase III clinical trials. As such, we consider the findings in this study to be directly applicable to clinical investigations and propose that such adjuvant treatments be directly applied to our vaccine-treated GBM patient population. In immunogenic cancers that do not need an active vaccination strategy to generate an immune response, such as melanoma, this combination treatment may provide an exciting avenue for therapy by both enhancing and activating immunity in the tumor microenvironment.

FIGURE LEGENDS

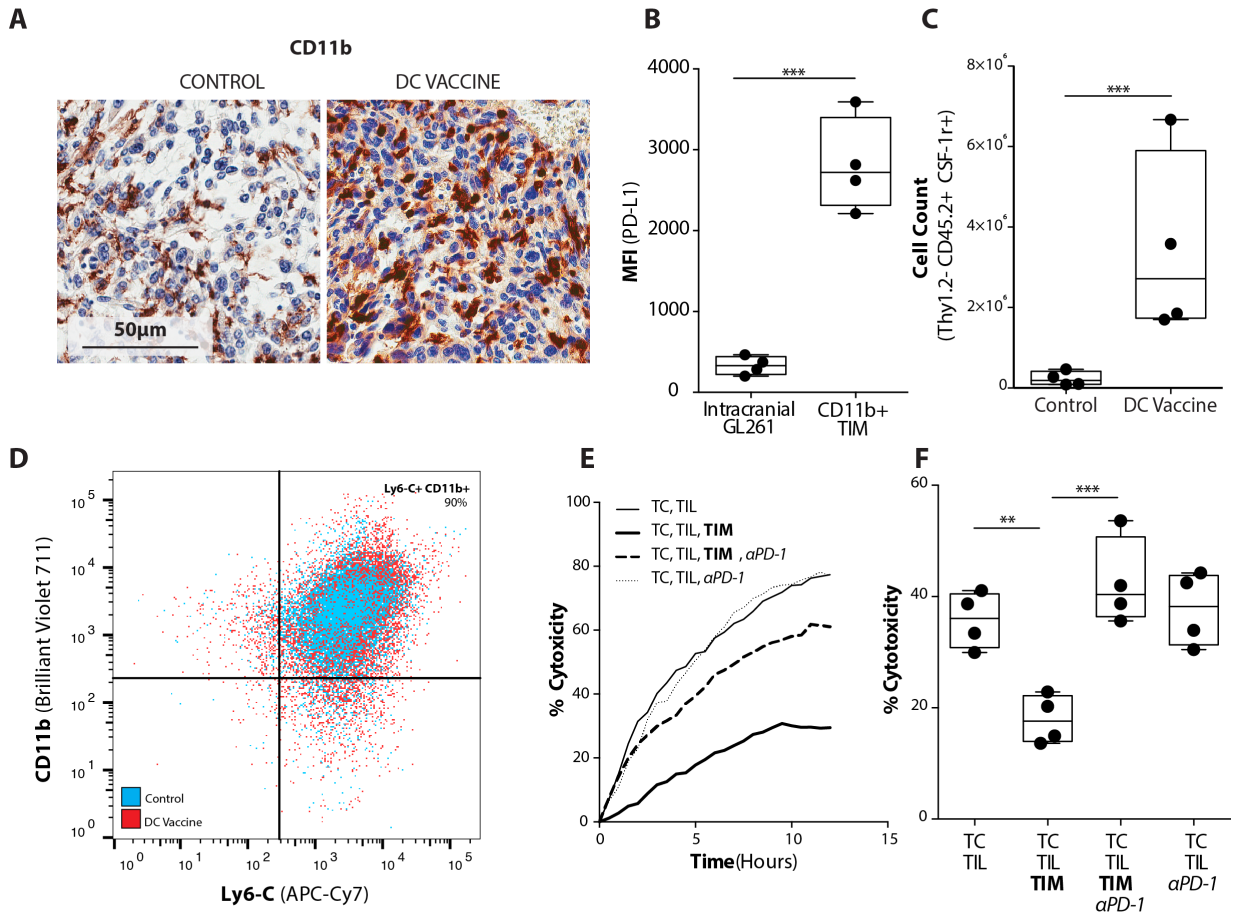
Figure 1



GBM TIMs expand to inhibit vaccine-induced T cell-mediated tumor cytolysis via PD-1/PD-L1 regulatory pathway. **(A, D)** CD163, DAPI, **(B, E)** PD-L1, DAPI, and **(C, F)** CD163, PD-L1, and DAPI co-staining is shown across pre- and post-DC vaccination samples from a GBM patient. **(G)** CD163+ cell count across pre- and post-DC vaccine treatment patient samples was quantified; (n=5). **(H)** The percent expression of PD-L1 on CD163+ cells was quantified; (n=5) (**p<0.01). **(I)** The percent of PD-L1+ cells in the tumor microenvironment that were CD163+

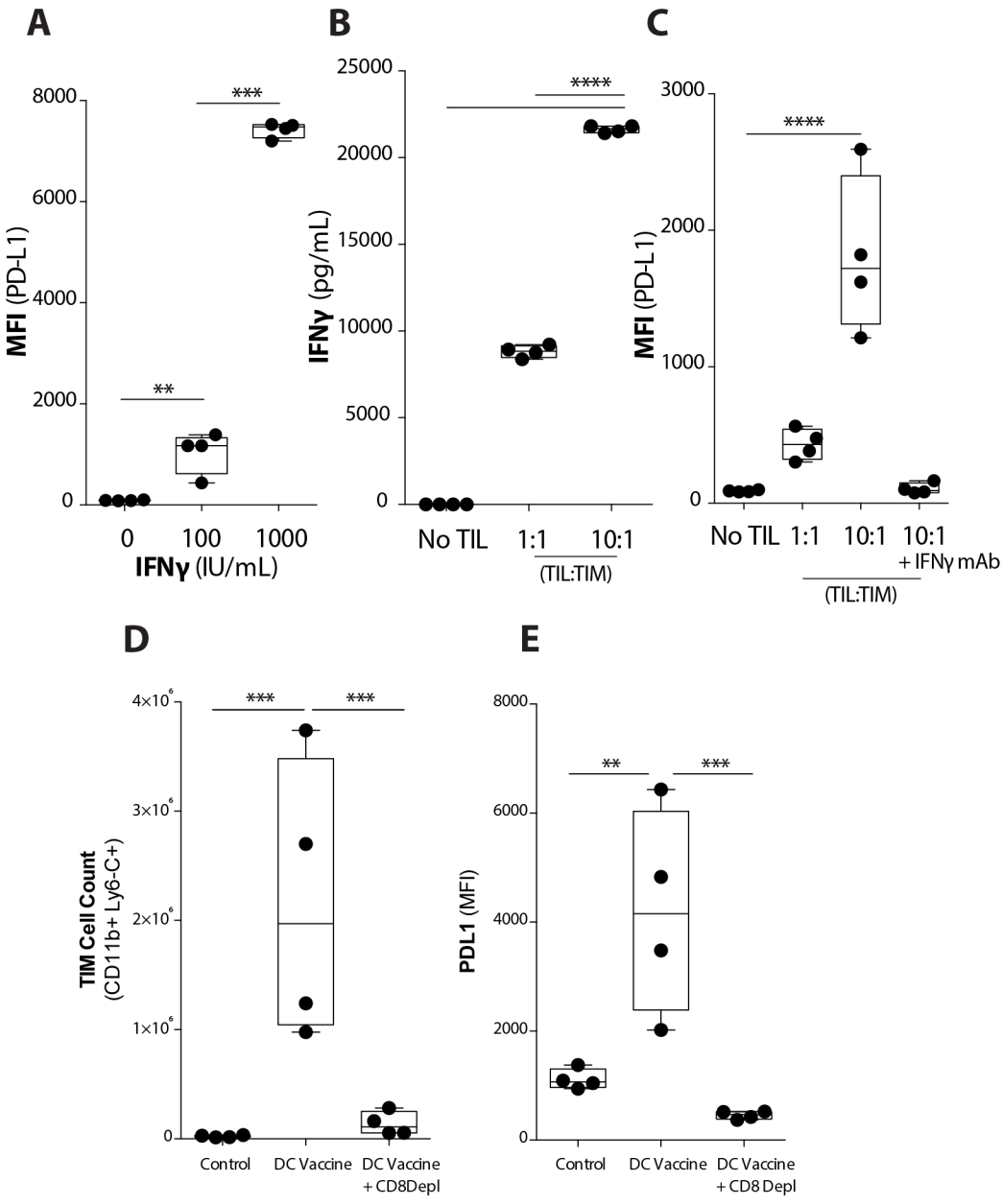
was quantified; (n=5). **(J)** PD-L1 expression on CD11b+ TIMs in the absence or presence of CD3+ TILs from freshly resected GBM shown; (**p<0.01). **(K)** TIL cytolysis of tumor cells (TC) over time in the absence or presence of TIMs or PD-1 mAb shown for freshly resected GBM. **(L)** GBM tumor cell cytolysis at 4 hours in the absence or presence of TIMs or PD-1 mAb shown; (n=11) (**p<0.01).

Figure 2



Murine glioma TIMs expand in response to vaccination to inhibit T cell-mediated tumor cytolysis. **(A)** CD11b IHC staining of non-treatment control and DC vaccinated tumor-bearing mice. **(B)** PD-L1 expression on FACS-sorted intracranial tumor (CD11b- CD3- cells) and TIMs (CD11b+ CD3-) quantified; (n=4/group) (***p<0.001). Flow cytometry characterization of **(C)** Thy1.2-, CD45.2+, CSF-1R cell absolute number (n=4/group) (***p<0.001), and **(D)** representative scatter plot of percent CD11b+, Ly6-C+ TIMs from tumors of control and DC vaccinated mice. **(E)** TIL cytolysis over time of tumor cells (TC) from freshly resected GBM in the absence or presence of TIMs or PD-1 mAb. **(F)** TIL-induced tumor cell cytolysis at 4 hours in the absence or presence of TIMs or PD-1 mAb; (n=4) (**p<0.01, ***p<0.001).

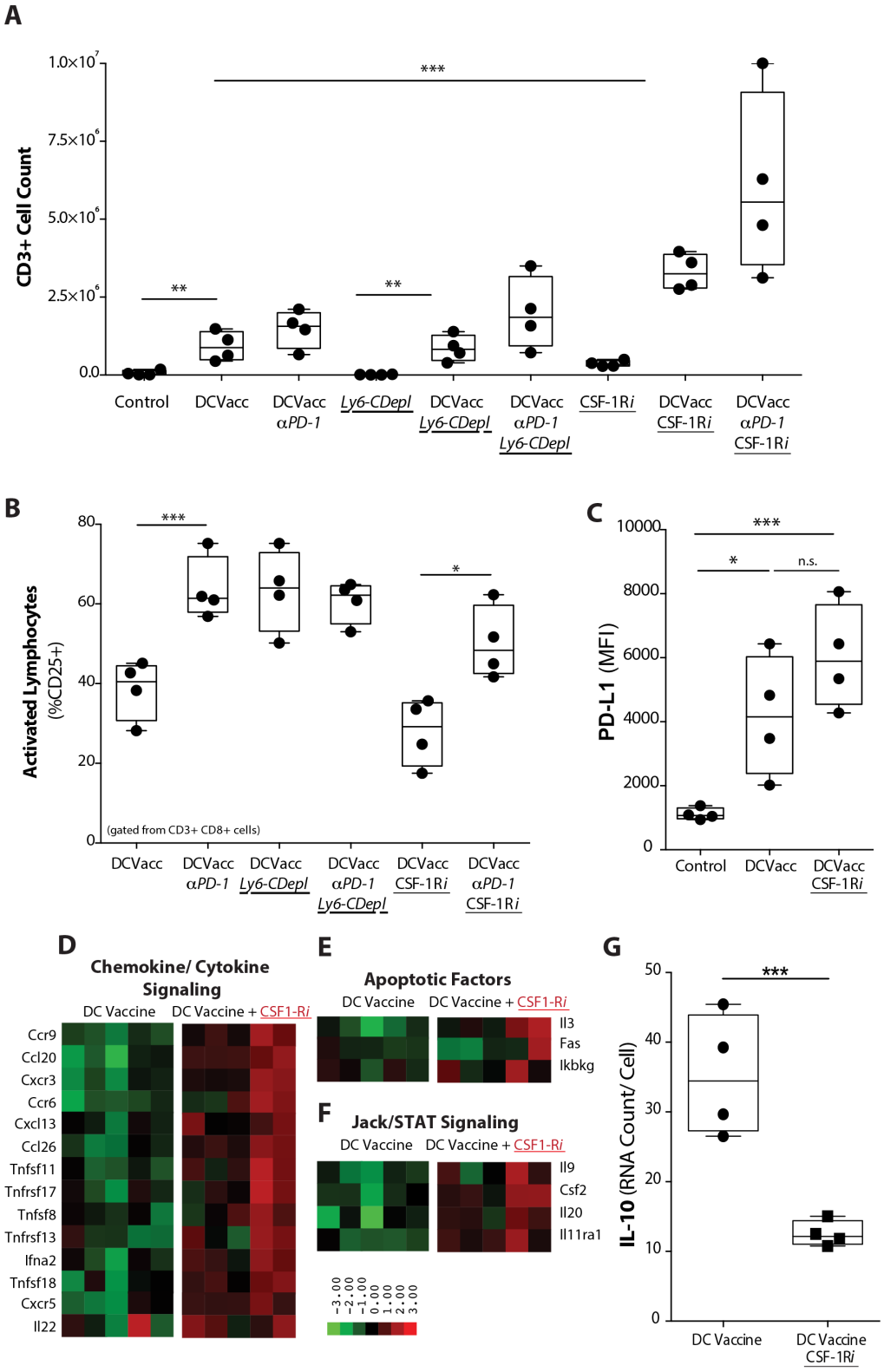
Figure 3



TIMs upregulate PD-L1 in response to vaccine-induced TIL population. **(A)** Upregulation of PD-L1 on TIMs in the presence of increasing IFNɣ was quantified with flow cytometry; (n=4/group) (***p<0.001). **(B)** IFNɣ present in TIL-TIM co-cultures *in vitro* was quantified using a Luminex assay; (n=4/group) (****p<0.0001). **(C)** Upregulation of PD-L1 on TIMs in the presence of

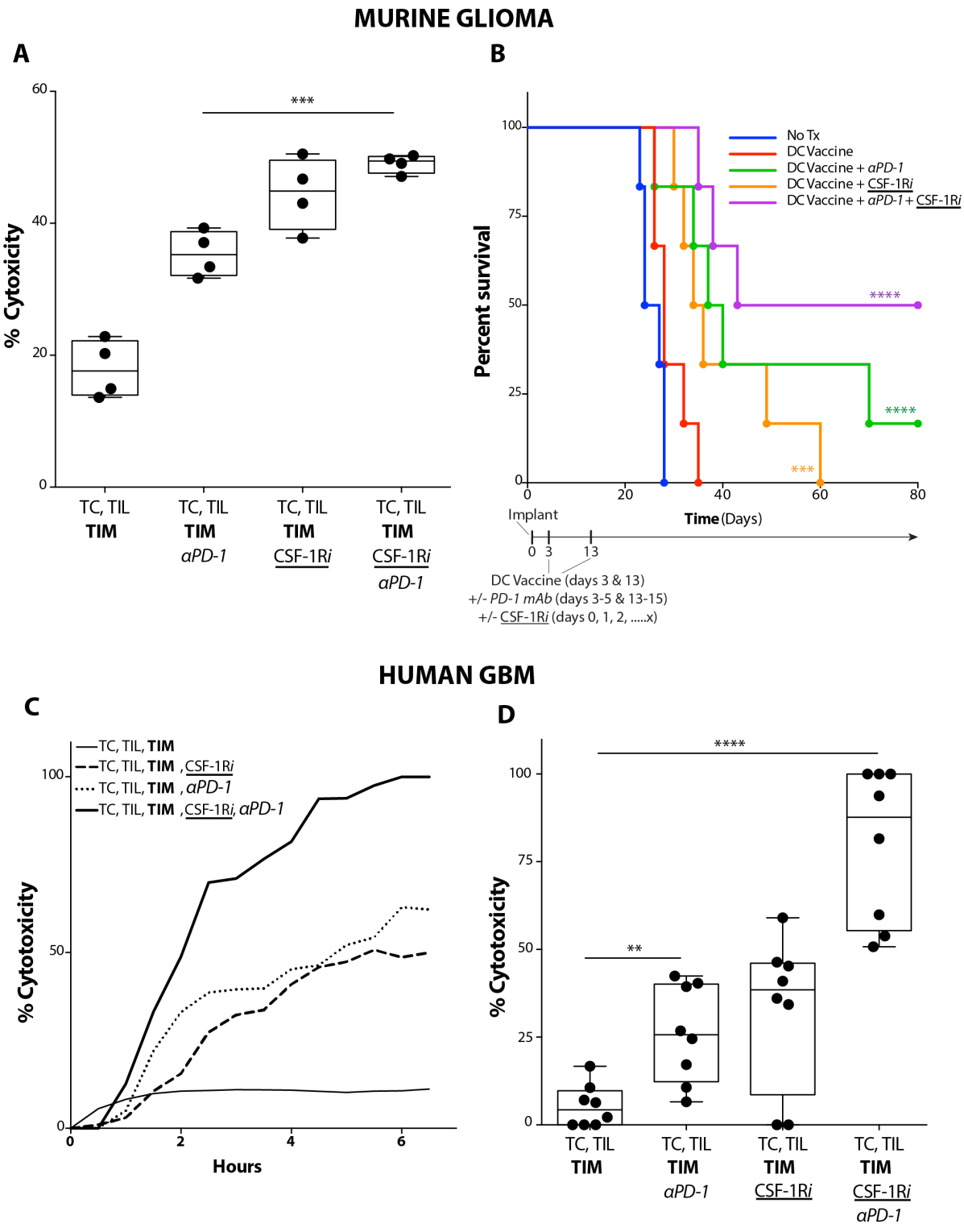
increasing concentrations of TILs or blockade of IFNɣ (IFNɣ-*i*) was quantified with flow cytometry; (n=4/group) (****p<0.0001). **(D)** CD11b+ Ly6-C+ TIM cell count and **(E)** PD-L1 expression in non-treatment control, DC vaccinated, and DC vaccinated + CD8 mAb depletion tumor-bearing mice; (n=4/group) (**p<0.01, ***p<0.001). Statistical analyses were performed using the Student's t-test **(A-E)**.

Figure 4


A
CD3+ Cell Count
1.0×10^7
7.5×10^6
5.0×10^6
2.5×10^6
0.0
***
**
**
Control
DCVacc
DCVacc αPD-1
Ly6-CDepl
DCVacc Ly6-CDepl
DCVacc αPD-1 Ly6-CDepl
CSF-1Ri
DCVacc CSF-1Ri
DCVacc αPD-1 CSF-1Ri
B
Activated Lymphocytes (%CD25+)
80
60
40
20
0
***
*
(gated from CD3+ CD8+ cells)
DCVacc
DCVacc αPD-1
DCVacc Ly6-CDepl
DCVacc αPD-1 Ly6-CDepl
DCVacc CSF-1Ri
DCVacc αPD-1 CSF-1Ri
C
PD-L1 (MFI)
10000
8000
6000
4000
2000
0
***
*
n.s.
Control
DCVacc
DCVacc CSF-1Ri
D
Chemokine/ Cytokine Signaling
DC Vaccine
DC Vaccine + CSF1-Ri
Ccr9
Ccl20
Cxcr3
Ccr6
Cxcl13
Ccl26
Tnfsf11
Tnfrsf17
Tnfsf8
Tnfrsf13
Ifna2
Tnfsf18
Cxcr5
Il22
E
Apoptotic Factors
DC Vaccine
DC Vaccine + CSF1-Ri
Il3
Fas
Ikbkg
F
Jack/STAT Signaling
DC Vaccine
DC Vaccine + CSF1-Ri
Il9
Csf2
Il20
Il11ra1
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00
G
IL-10 (RNA Count/ Cell)
50
40
30
20
10
0
***
DC Vaccine
DC Vaccine CSF-1Ri

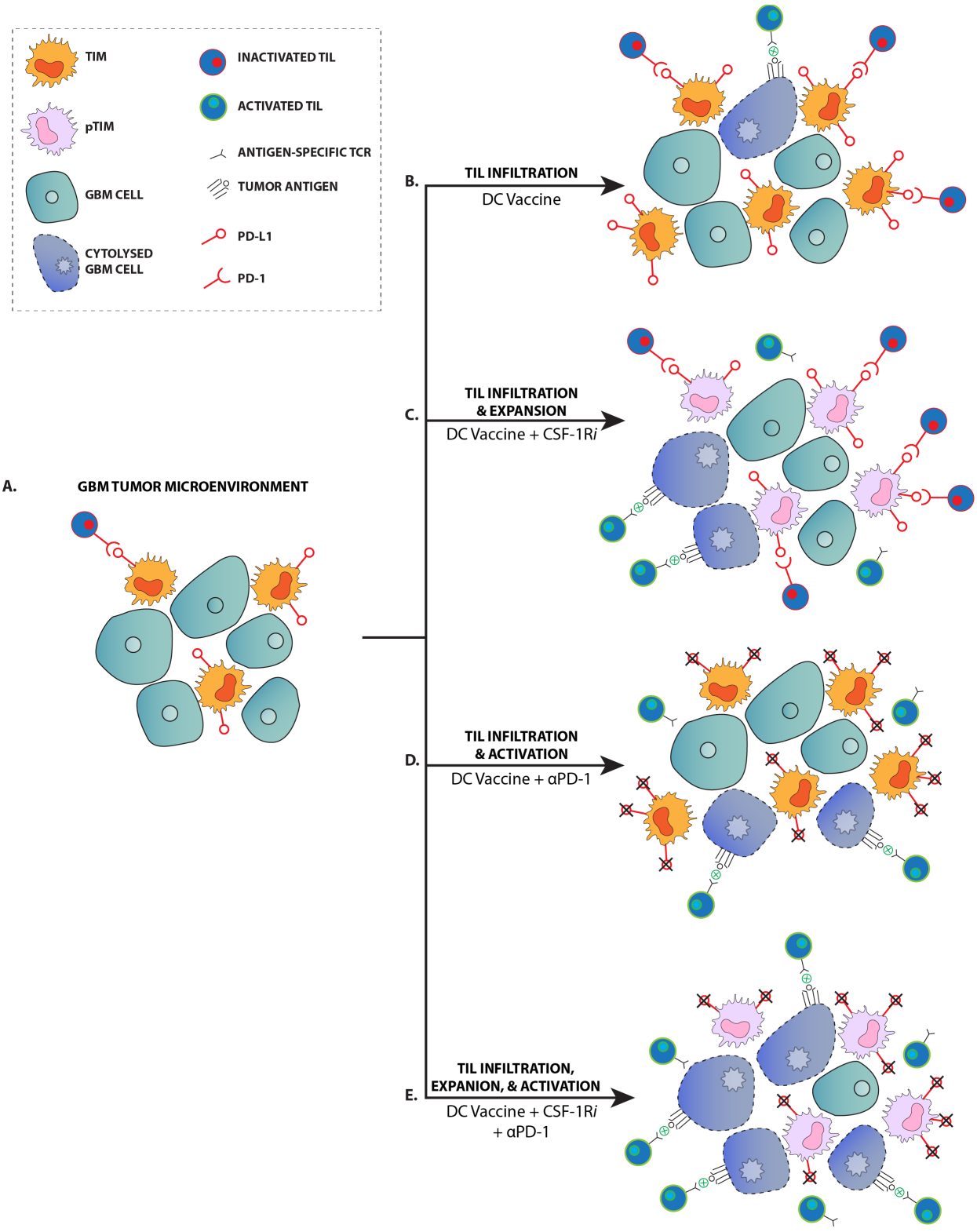
TIM-mediated PD-1/PD-L1 regulatory mechanism reduces T cell infiltration and activation in tumor. **(A, C)** TIL count (CD3+) and **(B, D)** activation (%CD25+/ CD8+ CD3+) in tumors from mice treated with DC vaccine and PD-1 mAb, along with **(A, B)** Ly6-C depleting mAb (Ly6-CDepl) or CSF-1R*i*; (n=4/ group) (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). **(c)** PD-L1 expression across treatment groups (n=5/group) (**p<0.01, ***p<0.001). Unbiased ranking and Nanostring quantification of **(d)** chemokine/cytokine signaling, **(E)** apoptotic, and **(F)** Jak/STAT signaling pathway factors on CD11b+ TIMs from tumor-bearing mice receiving DC vaccination alone or with adjuvant CSF-1R*i* treatment; (n=5/group). (**G**) Quantification of IL-10 expression by CD11b+ TIMs from tumor-bearing mice treated with DC vaccine or DC vaccine + CSF-1R*i* is shown. (n=4/group) (***p<0.001).

Figure 5


MURINE GLIOMA
A
% Cytoxicity
***
TC, TIL TIM
TC, TIL TIM αPD-1
TC, TIL TIM CSF-1Ri
TC, TIL TIM CSF-1Ri αPD-1
B
Percent survival
No Tx
DC Vaccine
DC Vaccine + αPD-1
DC Vaccine + CSF-1Ri
DC Vaccine + αPD-1 + CSF-1Ri
****
****
***
Time (Days)
Implant
DC Vaccine (days 3 & 13)
+/- PD-1 mAb (days 3-5 & 13-15)
+/- CSF-1Ri (days 0, 1, 2, ...x)
HUMAN GBM
C
TC, TIL, TIM
TC, TIL, TIM, CSF-1Ri
TC, TIL, TIM, αPD-1
TC, TIL, TIM, CSF-1Ri, αPD-1
% Cytotoxicity
Hours
D
****
**
% Cytotoxicity
TC, TIL TIM
TC, TIL TIM αPD-1
TC, TIL TIM CSF-1Ri
TC, TIL TIM CSF-1Ri αPD-1

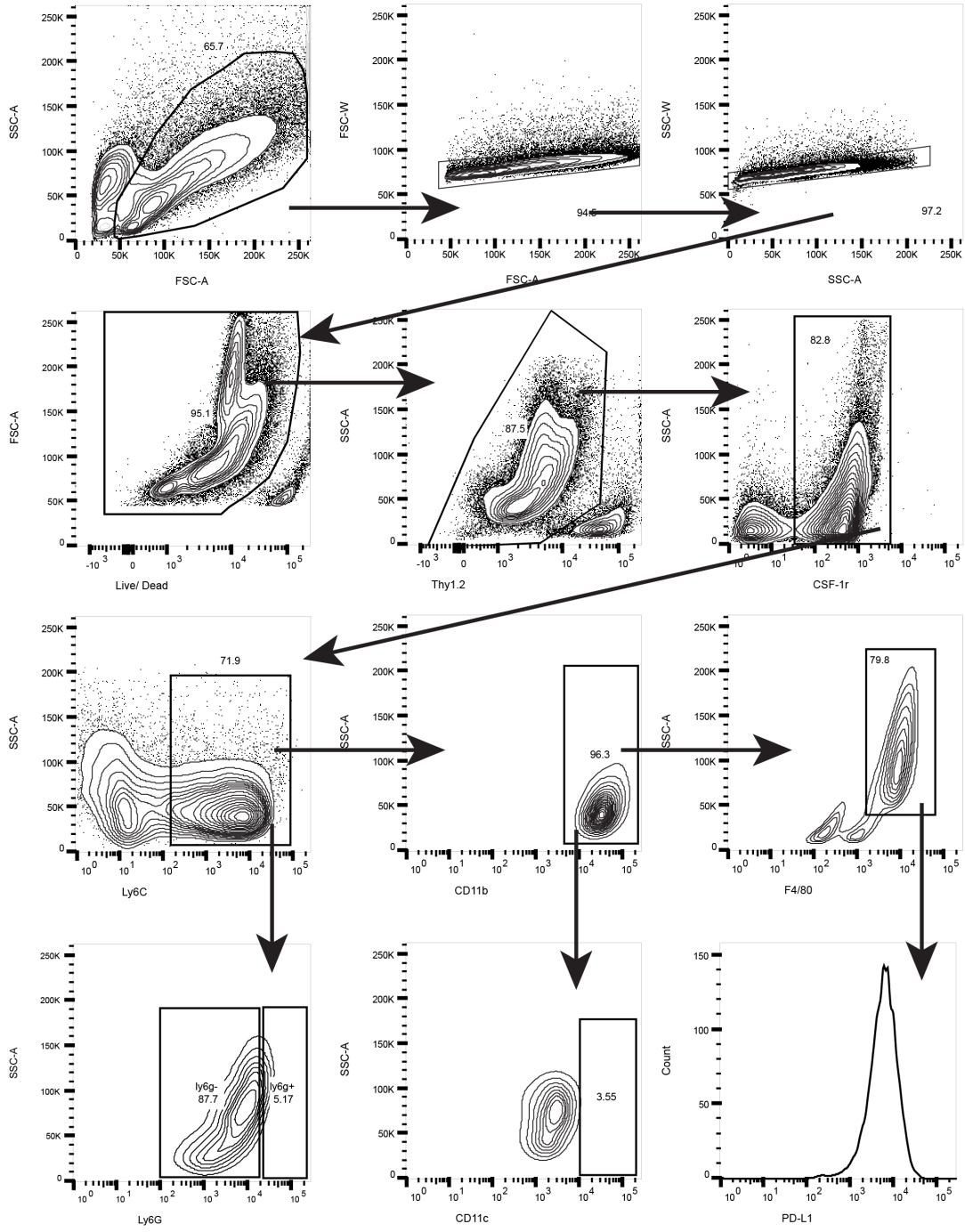
*Combination treatment with PD-1 mAb and CSF-1R*i *maximally enhances vaccination-induced immune response in both murine glioma and* ex vivo *human GBM.* **(A)** Murine GL261 tumor cell (TC) cytolysis at 4 hours following co-culture of TC, TIL, and TIM with PD-1 mAb or CSF-1R*i* treatment; (n=4/ group) (***p<0.001). **(B)** Mice were randomized into control (tumor-bearing, no treatment), DC vaccine, DC vaccine + PD-1 mAb, DC vaccine + CSF-1R*i*, and DC vaccine + PD-1 mAb + CSF-1R*i* treatment groups. Graph shows evaluation of survival; (n=6/group) (****p<0.0001). **(C)** Human GBM tumor cell (TC) cytolysis over time following co-culture of TC, TIL, and TIM with PD-1 mAb or CSF-1R*i* treatment is shown for one patient. **(D)** Human GBM tumor cell (TC) cytolysis at 4 hours following co-culture of TC, TIL, and TIM with PD-1 mAb or CSF-1R*i* treatment; (n=8/ group) (**p<0.01, ****p<0.0001).

Figure 6


TIM
pTIM
GBM CELL
CYTOLYSED GBM CELL
INACTIVATED TIL
ACTIVATED TIL
ANTIGEN-SPECIFIC TCR
TUMOR ANTIGEN
PD-L1
PD-1
A.
GBM TUMOR MICROENVIRONMENT
B.
TIL INFILTRATION
DC Vaccine
C.
TIL INFILTRATION & EXPANSION
DC Vaccine + CSF-1R*i*
D.
TIL INFILTRATION & ACTIVATION
DC Vaccine + αPD-1
E.
TIL INFILTRATION, EXPANION, & ACTIVATION
DC Vaccine + CSF-1R*i* + αPD-1

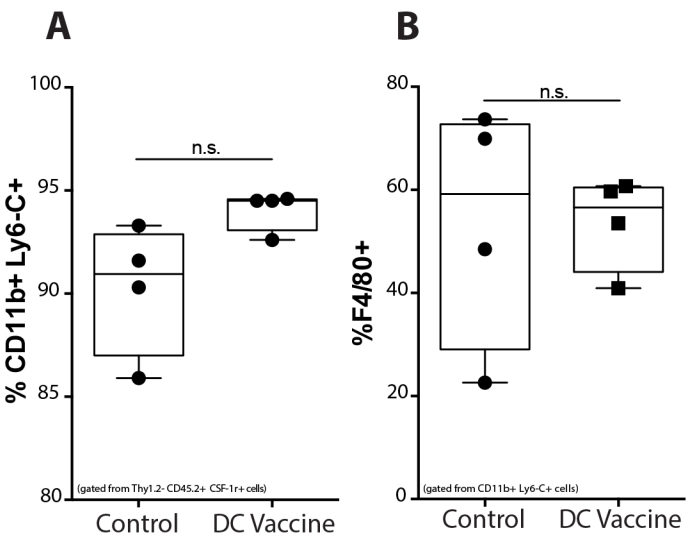
Dual treatments enhance vaccine-induced immune response. Vaccination induces a TIL response that is further enhanced with CSF-1R*i* and PD-1 mAb treatment. DC vaccination creates an infiltrating immune response that is **(A)** lacking in the non-treatment setting. **(B)** While DC vaccine treatment is able to generate an immune response, it is largely ineffective due to inactivation of TILs via the PD-1/PD-L1 signaling mechanism. **(C)** Treatment with CSF-1R*i* results in expansion of the TIL population, increasing the potential for TIL-tumor cell interaction and tumor cytolysis. **(D)** While, PD-1 mAb treatment does not increase the TIL population over what is generated by DC vaccination, it does promote activation of TILs and subsequent tumor cytolysis. **(E)** PD-1 mAb and CSF-1R*i* together promote the expansion and activation of the DC vaccine-generated TIL population such that there is a maximal tumor cytolysis.

Supplementary Figure 1



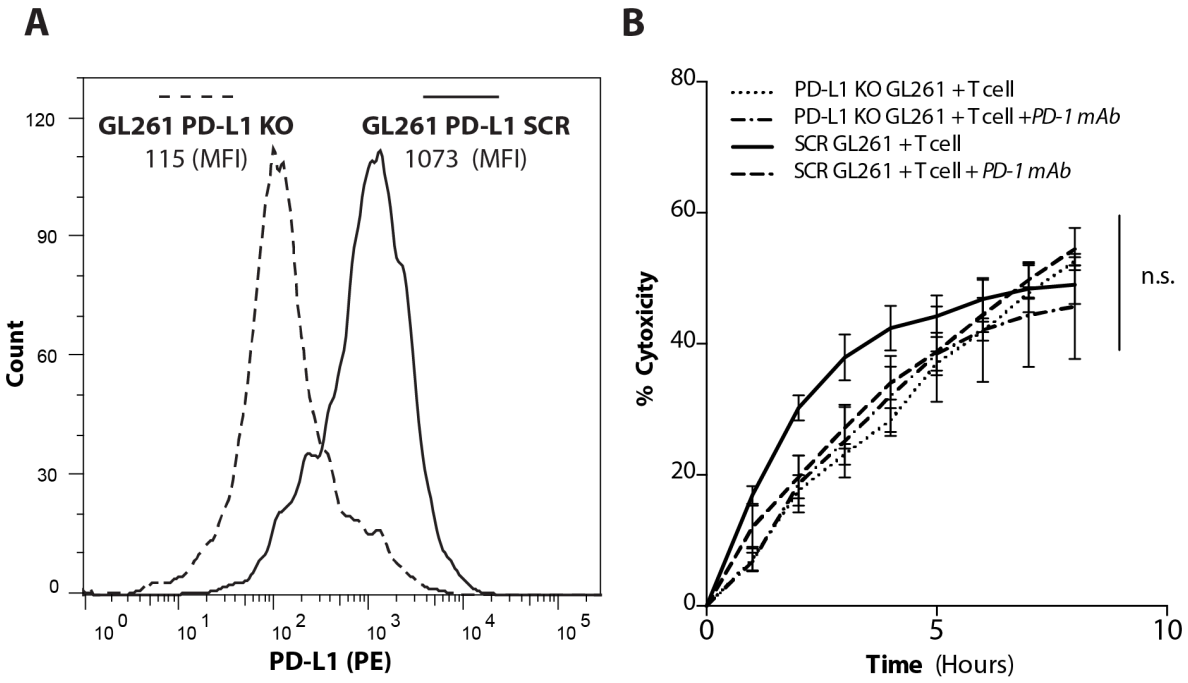
Gating strategy for TIMs. The gating strategy for TIM characterization is shown using FACs plots.

Supplementary Figure 2



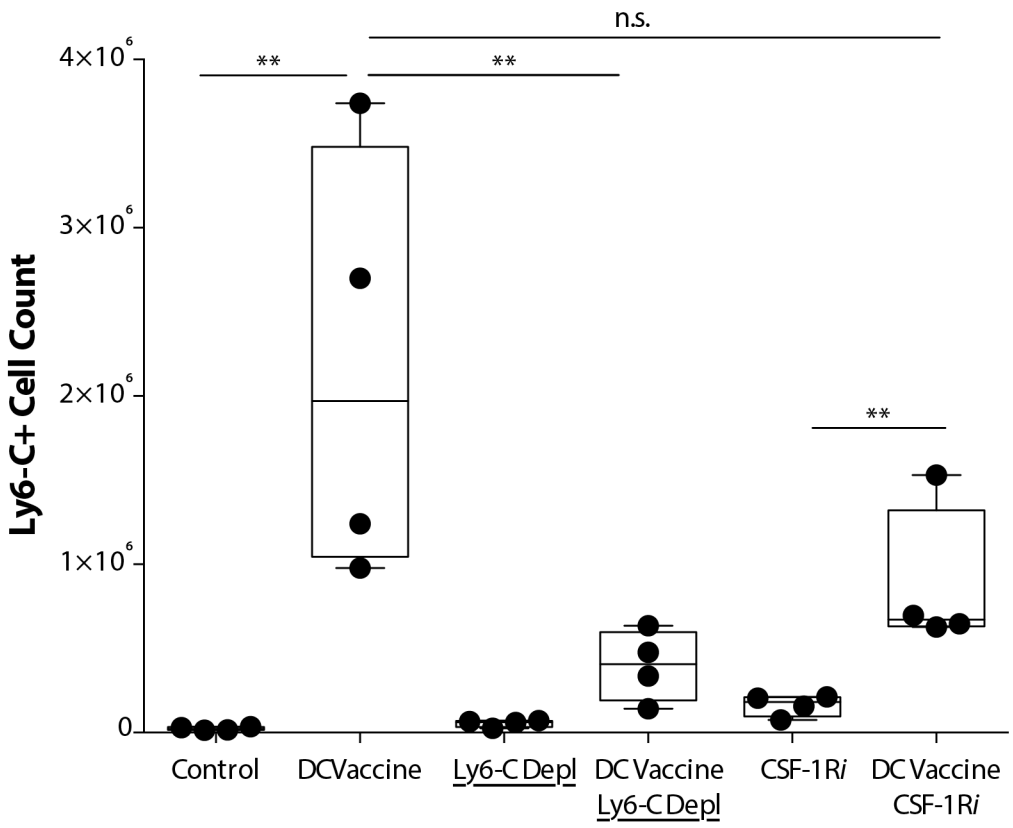
Characterization of TIMs. The proportion of Thy1.2- CD45.2+ CSF-1r*i*+ cells that were **(A)** CD11b+ Ly6-C+ and **(B)** F4/80+ are shown here for both control non-treatment and DC vaccine treatment groups (n=4/group).

Supplementary Figure 3



Tumor cells do not significantly contribute to PD-1/PD-L1 inhibition. **(A)** GL261-gp100 PD-L1 knock-out (KO) and scrambled control (SCR) expression of PD-L1 shown. **(B)** Tumor cytolysis by Pmel-1 T cells over time in the presence of PD-L1 KO or SCR tumor cells is shown. (n=4/group).

Supplementary Figure 4



*TIMs are depleted with Ly6-C mAb, but only reduced with CSF-1R*i *treatment.* TIMs cell count (Ly6-C+) is shown following treatment with Ly6-C mAb or CSF-1R*i* adjuvant to DC vaccination.

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