Clinical Cancer Research

Systemic Tolerance Mediated by Melanoma Brain Tumors Is Reversible by Radiotherapy and Vaccination

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Abstract

Purpose: Immune responses to antigens originating in the central nervous system (CNS) are generally attenuated, as collateral damage can have devastating consequences. The significance of this finding for the efficacy of tumor-targeted immunotherapies is largely unknown.

Experimental Design: The B16 murine melanoma model was used to compare cytotoxic responses against established tumors in the CNS and in the periphery. Cytokine analysis of tissues from brain tumor–bearing mice detected elevated TGFβ secretion from microglia and in the serum and TGFβ signaling blockade reversed tolerance of tumor antigen-directed CD8 T cells. In addition, a treatment regimen using focal radiation therapy and recombinant *Listeria monocytogenes* was evaluated for immunologic activity and efficacy in this model.

Results: CNS melanomas were more tolerogenic than equivalently progressed tumors outside the CNS as antigen-specific CD8 T cells were deleted and exhibited impaired cytotoxicity. Tumor-bearing mice had elevated serum levels of TGFβ; however, blocking TGFβ signaling with a small-molecule inhibitor or a monoclonal antibody did not improve survival. Conversely, tumor antigen–specific vaccination in combination with focal radiation therapy reversed tolerance and improved survival. This treatment regimen was associated with increased polyfunctionality of CD8 T cells, elevated T effector to T regulatory cell ratios, and decreased TGFβ secretion from microglia.

Conclusions: These data suggest that CNS tumors may impair systemic antitumor immunity and consequently accelerate cancer progression locally as well as outside the CNS, whereas antitumor immunity may be restored by combining vaccination with radiation therapy. These findings are hypothesis-generating and warrant further study in contemporary melanoma models as well as human trials. *Clin Cancer Res; 22(5); 1161–72. ©2015 AACR.*

Introduction

Brain metastases afflict 20% to 40% of patients with advanced cancer and represent a major source of morbidity and mortality (1). The basic mechanisms constraining immune responses against central nervous system (CNS) tumor antigens, however, remain poorly defined (2). Patients receiving chemotherapy and radiation for high-grade gliomas exhibit impaired T-cell homeostasis (3), and lymphopenia has been identified as a negative prognostic indicator in these patients (4). While it is unclear whether these observations represent sequelae of pathology, treatment effect, or a combination of these factors, location in the immunologically distinct CNS may play an important role in clinical outcome.

Although the presence of the blood–brain barrier, lack of conventional lymphatics, paucity of antigen-presenting cells, and low basal expression of MHC molecules qualify the CNS as immunologically unique, peripheral leukocytes access the brain and orchestrate robust immune responses under inflammatory conditions (5). Unlike peripheral lymphocytes, however, these cells interact with a variety of tissue-resident cells, including astrocytes, neurons, and microglia, which modulate lymphocyte function in a highly context-dependent manner (6). Location within the CNS may be important for immunosuppression in animal models as extracranially implanted gliomas are characterized by lower levels of TGFβ transcription, increased infiltration by CD4 and CD8 T cells, decreased T regulatory cell (Treg) accumulation, and slower growth as compared with intracranial gliomas (7).

To investigate the effects of tumor location on immune function we used B16, a poorly immunogenic murine melanoma cell line that expresses no MHC II and low levels of MHC I (8) and/or a

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Translational Relevance

Brain metastases are a significant source of morbidity and mortality for patients with advanced melanoma, yet little is known about the effects of tumor location on antitumor immunity. The data presented here indicate that B16 melanoma brain tumors are more systemically immunosuppressive than equivalently advanced flank and lung tumors and that focal radiation therapy and tumor antigen–specific vaccination can restore immune function and mediate tumor regression. On the basis of these data, we hypothesize that patients harboring central nervous system (CNS) malignancies may exhibit accelerated systemic disease progression. Furthermore, these findings suggest that combination immunotherapy regimens involving specific vaccination and focal radiation therapy may be active against CNS melanoma.

more immunogenic variant which expresses a class I (H-2Kb) restricted epitope of ovalbumin. We found that brain tumors are more tolerogenic than equivalently advanced tumors located outside the CNS and that mice harboring brain tumors have higher local and circulating levels of TGFβ, although blocking TGFβ failed to mediate tumor regression. Having established intracranial B16 as a challenging model, we tested the induction/expansion of antitumor T cells by vaccination. Vaccination alone had a modest effect on survival, but combination immunotherapy using a recombinant *Listeria monocytogenes* (LM)-based vector and focal radiation therapy (RT) significantly prolonged survival.

Although previous studies demonstrated glioma regression after treatment with RT and PD-1 blocking antibodies (9), we found that LM and RT were superior to anti-PD-1 and RT against established intracranial melanoma. Mechanistically, vaccination combined with focal RT significantly decreased secretion of TGFβ1 from microglia and increased intratumoral polyfunctional CD8 T cell density. On the basis of these data, we propose a mechanism by which microglia in the brain tumor microenvironment mediate systemic immune tolerance and describe how appropriately primed T cells can reverse this effect. These findings may have implications for systemic disease control as well as designing and implementing effective immunotherapies for patients with metastatic brain tumors.

Materials and Methods

Mice, cell lines, antibodies, and vaccines

Female C57BL/6 (Jackson Laboratory) or LY5.2 (NCI) mice (6–8 weeks) were housed in pathogen-free conditions under approved animal protocols (Institutional Animal Care and Use Committee of Johns Hopkins University, Baltimore, MD). OT-1/CD45.2/Rag$^{-/-}$ and Pmel/CD45.2 mice were used as donors for adoptive transfer experiments. Recombinant LM-OVA was constructed in the Lm Δ*actA* Δ*inlB* Δ*uvrAB* background by integrating pPL2-OVA as described (refs. 10, 11; Aduro Biotech). LM-OVA was grown in BHI to mid-log, washed, and stored in PBS/8% glycerol at −80°C in single-use aliquots. For vaccination, LM-OVA was thawed, diluted in PBS to 1×10^7 cfu per mouse (0.1 LD_{50}), and administered by intraperitoneal injection. For Vac-OVA or Vac-GP100, mice received 1×10^6 pfu (0.1 LD_{50}). The G4 hybridoma was used to produce hamster antimurine PD-1 monoclonal antibodies as described at 10 mg/kg (12).

Tumor models

B16-OVA cells were maintained in culture under continuous selection. For intracranial implantation, cells were resuspended at either 1,000 cells/μL for survival experiments or 5,000 cells/μL for immunology experiments. For flank tumor and lung tumor implantation, cells were resuspended at 50 and 500 cells/μL, respectively. Flank tumors were established by injecting 200 μL subcutaneously in the right flank. Lung tumors were established by injecting 200 μL by tail vein injection. Intracranial tumors were established as previously described (9). No cell line authentication was done.

Flow cytometry

Flow cytometry was carried out on a FACSCalibur or LSR II (BD Biosciences). For adoptive transfer experiments, the following antibodies were used: CD45.2 PE, PB (Biolegend), CD8a PerCP, Pac Orange (Invitrogen), CD4 PerCP (BD), IFNγ APC, PE-Cy7 (Biolegend), Granzyme B PE (eBio), TNFα PE (BD), IL2 APC (BioLegend), FoxP3 AF700 (BioLegend), CD11b AF700, PE (eBio), and IL17 PerCP/Cy5.5 (BioLegend). Data were analyzed using FlowJo software (Tree Star).

Adoptive transfer experiments

Spleens and lymph nodes from OT-1, Rag$^{-/-}$, or Pmel mice were collected and homogenized. For wild-type Pmel mice, CD8 T cells were isolated by positive selection (Miltenyi Biotech) and labeled with CFSE (Invitrogen). Cells were resuspended in PBS at 1.25×10^7 cells/mL and then transferred by retro-orbital injection (2.5×10^6 cells) 12 days after implantation of 1×10^4 F10 B16-OVA cells in the brain or flank of mice expressing the congenic marker CD45.1 (LY5.2). Five days after adoptive transfer, brains, draining lymph nodes (DLN), and spleens were collected and homogenized. Brain and flank tumors were excised and tumor-infiltrating lymphocytes (TIL) were isolated using Percoll (Sigma) density gradient centrifugation per manufacturer instructions. Cells were isolated and stimulated with 2 μmol/L H-2K^b–restricted class I epitope SIINFEKL ($OVA_{257–264}$) or the human epitope KVPRNQDWL ($gp100_{25–33}$) in the presence of GolgiStop (BDBiosciences) and then analyzed by FACS.

In vivo cytotoxic T lymphocyte assays

Assays were performed as previously described (13). Splenocytes from wild-type C57BL/6 mice were isolated and divided into two groups. One group was labeled CFSE^{lo} (0.5 μmol/L), whereas a second group was labeled CFSE^{hi} (5 μmol/L) and loaded with SIINFEKL peptide at a concentration of 2 μmol/L. Cells were combined and transferred by retro-orbital injection. For tumor-bearing mice, 1×10^4 F10 B16-OVA cells were implanted in the brain or flank 17 days prior to target transfer. Vac-OVA or LM-OVA were administered as described above. Spleens were harvested from recipient mice 18 hours after target transfer and splenocytes analyzed by FACS.

Radiation therapy

16 Gy was delivered using the small-animal radiation research platform (SARRP; Xstrahl) as previously described (9).

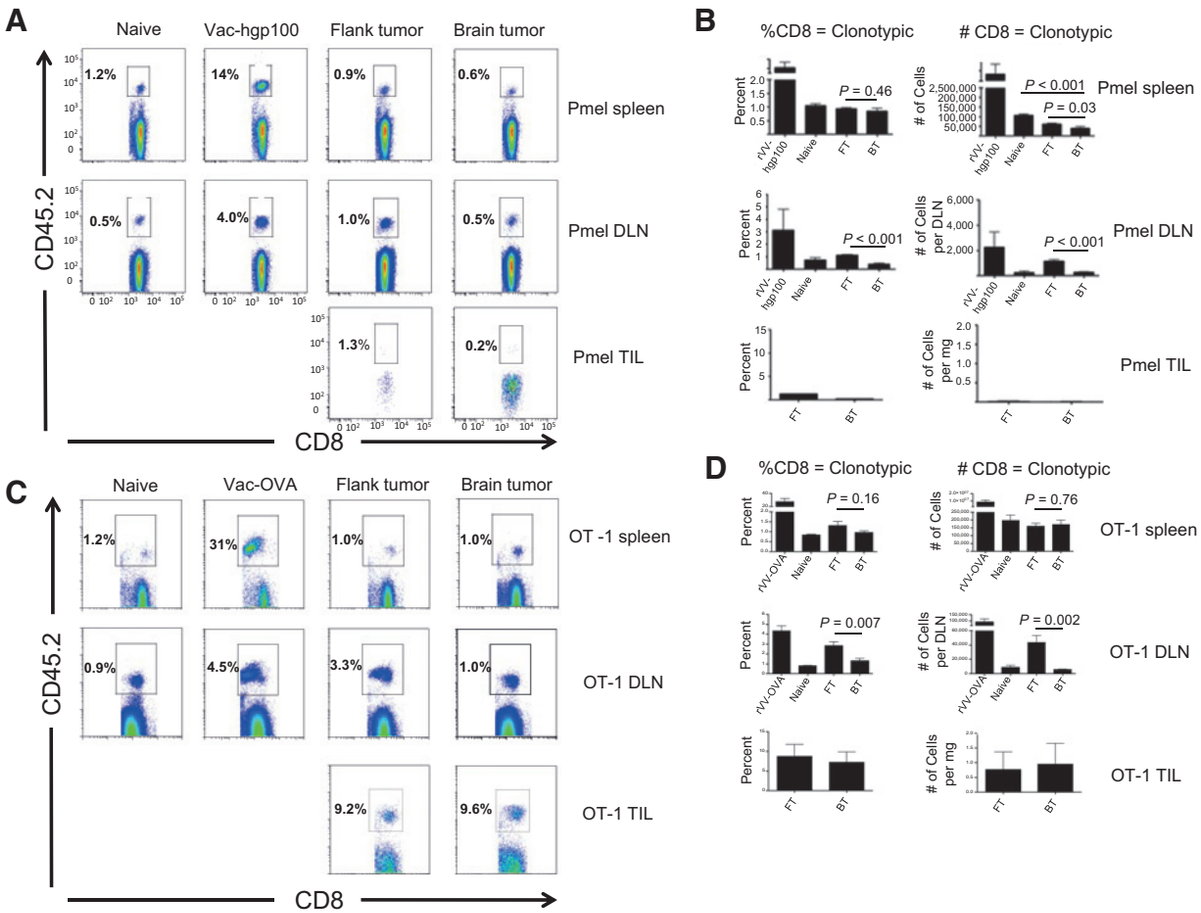


Figure 1.
Adoptively transferred tumor antigen–specific CD8 T cells are tolerized by CNS melanoma. A, representative FACS plots of Pmel (CD45.2$^+$) CD8 T cells isolated from spleens, brain tumor DLN, and TIL. B, summary graphs showing percentages and numbers of Pmel CD8 T cells. C, representative FACS plots of the percentage of OT-1 (CD45.2$^+$) CD8$^+$ T cells isolated from spleens, brain tumor DLN, and TIL, animals bearing B16-OVA tumors. D, summary graphs showing percentages and numbers of CD8 T cells represented by the adoptively transferred OT-1 population. $n = 5$ mice/group, repeated $\times$ 3.

TIL immunophenotyping, pathology, and immunohistochemistry

Two thousand F10 B16-OVA cells were implanted in the left hemisphere of C57BL/6 mice. RT was delivered on day 7, LM-OVA was administered on day 10, and mice were sacrificed on day18. Tumors were excised from surrounding brain tissue and homogenized. TILs were isolated using density gradient centrifugation (Percoll). Cells were stimulated for 4 hours with PMA/ionomycin, washed, stained for CD8, CD4, IFNγ, TNFα, IL2, Granzyme B, FoxP3, and IL17, and analyzed by flow cytometry. For immunohistochemistry, mice underwent transcardial perfusion with 10 mL PBS followed by 4% paraformaldehyde/PBS. Brains were removed and cryoprotected in 30% sucrose/PBS for 48 hours at 4°C, snap frozen, and stored at −80°C before sectioning. Hematoxylin and eosin (H&E) staining was performed by the histology core facility. For immunostaining, slides were washed twice for 5 minutes in PBS and blocked in 5% NGS/PBS for 1 hour. Tissues were incubated with anti-CD3ε antibody (Dako; 1:10 diluted in 3% NGS/PBS) overnight at 4°C and washed in PBS before incubating with goat anti-rabbit secondary (1:1,000) for 1 hour at room temperature.

APC coculture experiments

A total of 1×10^4 F10 B16-OVA cells were implanted, and RT and LM-OVA were administered as described (day 10). On day 17, mice were sacrificed and serum, brains, spleens, and tumor DLNs were collected. Red blood cells were lysed in spleens and CD11c$^+$ cells were isolated by positive selection (Miltenyi). Monocytes were isolated from brains by density gradient centrifugation, stained for CD11b AF700 (eBio) and CD45 PE (BioLegend), and sorted using a FACSAria (BD). CD11b$^+$/CD45$^-$ mid cells, as well as CD11c$^+$ splenocytes and unsorted DLN cells were plated in a 96-well plate (1×10^4 cells/well). OT-1 CD8 cells were CFSE-labeled (0.5 μmol/L) and plated with APCs at a ratio of 1:5 for CD11b$^+$/CD45 microglia, a ratio of 1:5 for CD11c$^+$ splenocytes, and a 1:1 ratio for DLNs. SIINFEKL peptide was added to the wells (2 μmol/L) and plates were maintained in an incubator for 48 hours. GolgiStop (BDBiosciences) was added for the last 6 hours and supernatants were collected and stored at −80°C. Cells were collected and stained for CD8, CD45.2, and IFNγ and analyzed by FACS. Supernatants and serum samples were analyzed for concentrations of IFNγ, IL2, IL12, granulocyte macrophage

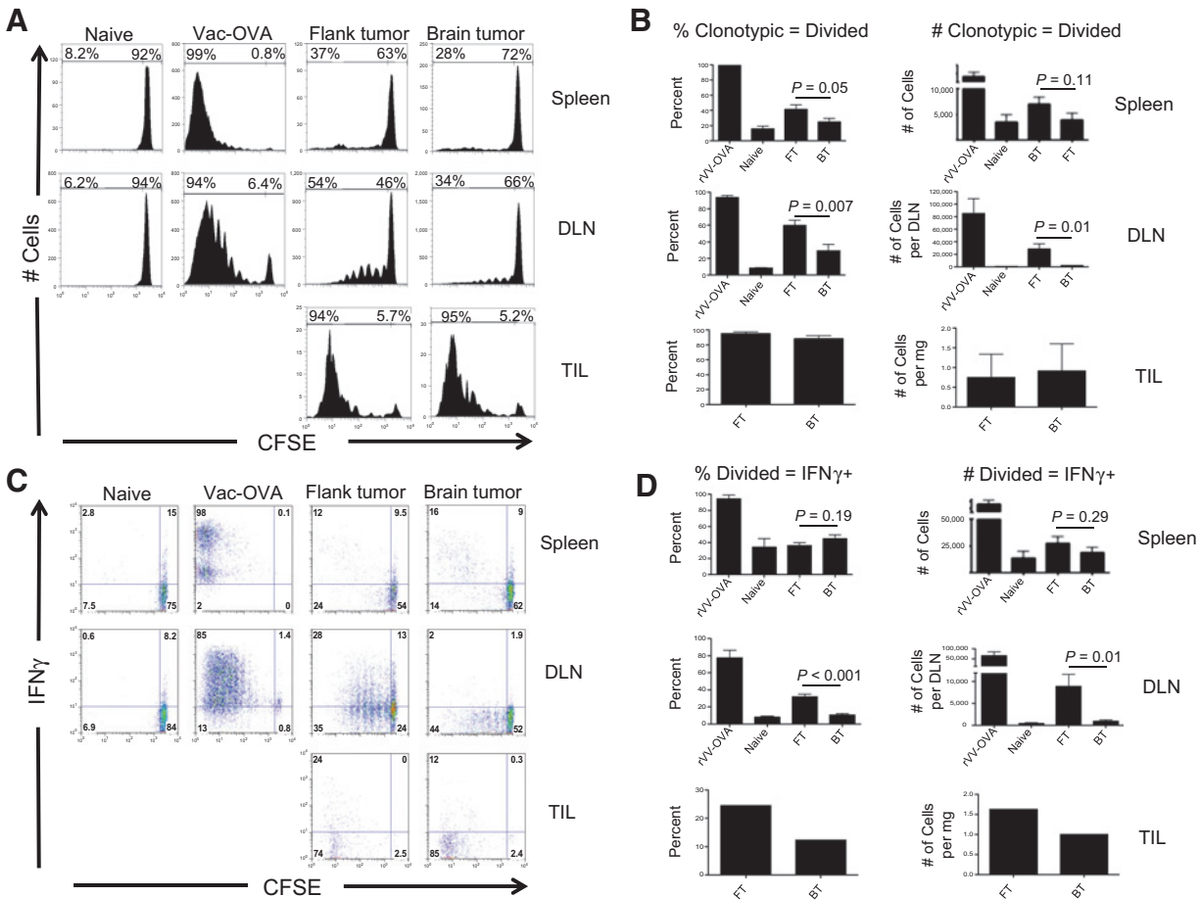


Figure 2.

Tumor-specific CD8 T cells undergo fewer divisions and produce less IFNγ in response to CNS melanoma as compared with equivalent flank tumors. A, representative histograms of CFSE in cancer-specific T cells. B, summary graphs showing percentages and numbers of specific T cells undergoing ≥1 division. C, representative FACS plots of division versus IFNγ. D, summary graphs showing percentages and numbers of divided cells producing IFNγ. $n = 5$ mice/group, repeated × 3 with similar results.

colony-stimulating factor (GM-CSF), IL10, and TGFβ1 by multiplex (Luminex).

Survival experiments and tumor volume analysis

A total of 2,000 F10 B16-OVA cells were implanted in the left hemisphere of C57BL/6 mice. RT was delivered (16 Gy) 7 days after tumor implantation and LM-OVA was administered on day 10. Mice received a LM-OVA boost on day 31. Mice were sacrificed according to protocol upon development of motor deficits or sustained hunched posture. In studies involving post-mortem tumor volume assessment, brains were removed at the time of death and stored in 4% paraformaldehyde/PBS. Tissues were transferred to PFPE (Fomblin, Sigma-Aldrich) and MR images were acquired by the SARRP at Johns Hopkins. Tumor borders were delineated slice-by-slice using ImageJ software and tumor volumes were calculated.

Statistical analysis

Data were analyzed by two-tailed Student *t* test or ANOVA using GraphPad Prism software.

Results

CD8 T cells recognizing an endogenous tumor-associated melanoma antigen are deleted, whereas CD8 T cells recognizing a tumor-restricted neoantigen persist

Antigen-specific tolerance is an early event in tumor progression (14), and prior studies have shown that naïve CD8 T cells specific for the endogenous self/tumor antigen gp100 (Pmel) are rapidly tolerized upon adoptive transfer into C57BL/6 mice with established B16 flank tumors (15). To test whether tumor location affects antigen-specific tolerance, we adoptively transferred Pmel CD8 T cells into mice bearing equivalently sized (Supplementary Fig. S1) B16 flank or brain tumors. Five days after transfer, very few tumor-infiltrating Pmel CD8 T cells persisted in brain or flank tumors (Fig. 1A). However, there was a significantly lower percentage ($P < 0.001$) and number ($P < 0.001$) of adoptively transferred cells in the cervical lymph nodes (brain tumor DLN) compared with flank tumor DLN (Fig. 1B). This effect was systemic, as there were also fewer Pmel CD8 T cells in the spleens of brain tumor–bearing mice as compared with cancer-free ($P < 0.001$) or flank tumor–bearing mice ($P = 0.03$).

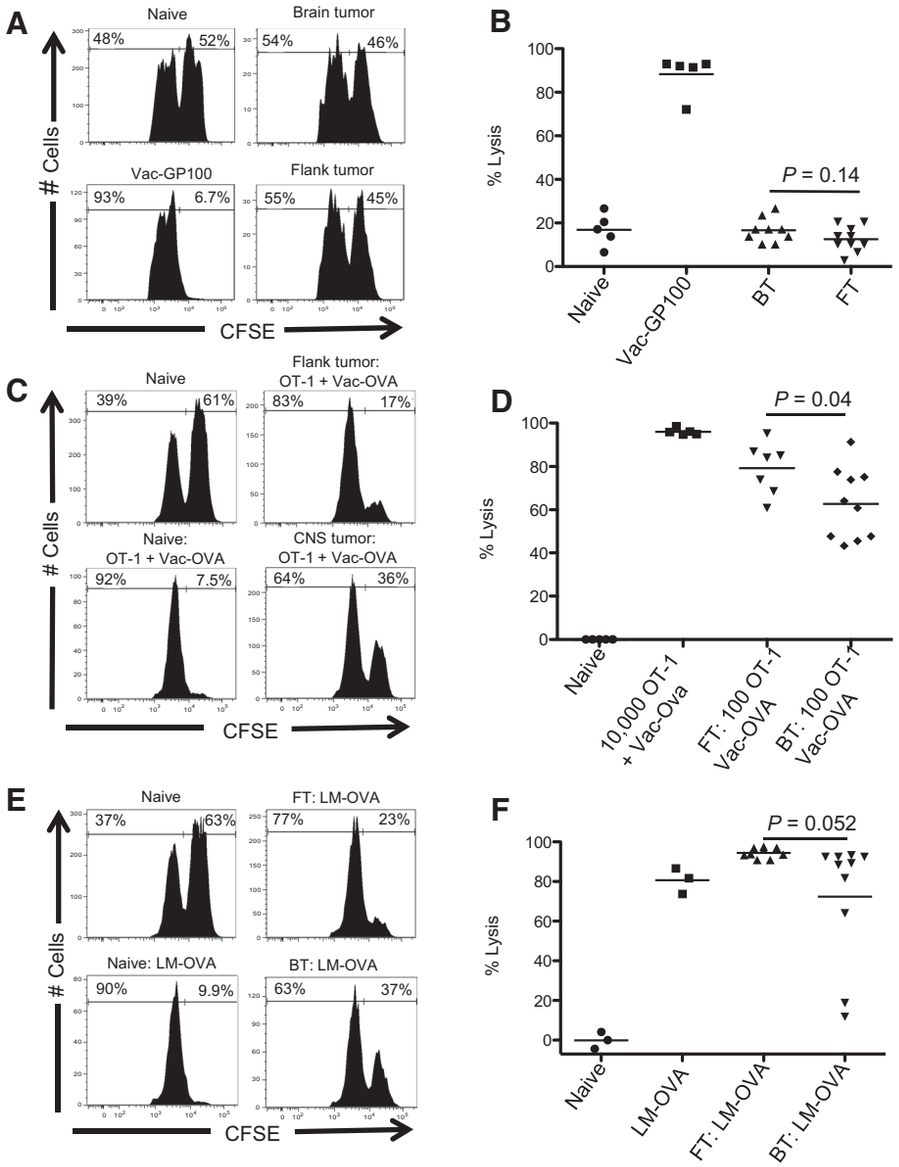


Figure 3.
CNS melanoma impairs a systemic tumor antigen–directed lytic response. A, representative histograms showing numbers of peptide-pulsed ($CSFE^{high}$) and control ($CFSE^{low}$) cells recovered from unvaccinated mice bearing B16-OVA brain or flank tumors. B, summary graphs showing percent target lysis in unvaccinated mice with brain or flank tumors. Each data point represents one animal. C, representative histograms showing OVA-pulsed and control peaks in mice with B16-OVA brain or flank tumors after adoptive transfer of 100 OT-1 cells and vaccination with Vac-OVA. D, summary graphs showing percent target lysis in mice receiving adoptive transfer of 100 OT-1 cells and vaccination with Vac-OVA. E, representative histograms showing OVA-pulsed and control peaks in mice with B16-OVA brain and flank tumors after vaccination with LM-OVA. F, summary graphs showing percent target lysis in mice with B16-OVA brain and flank tumors after vaccination with LM-OVA. Experiments repeated × 2 with similar results. $n = 3$–10 animals per group.

To extend these results to a tumor-restricted antigen, we implanted B16-OVA brain and flank tumors. Here, ovalbumin models a mutated neoantigen, to which pre-existing tolerance is not expected. This model has the advantage that tolerance is unlikely to be primarily deletional, as suggested by previous studies (16). We found a significant decrease in OT-1 percentage ($P = 0.007$) and number ($P = 0.002$) in brain tumor DLNs compared with flank tumor DLNs (Fig. 1C and D). Unlike the Pmel model, however, differences in the spleens did not reach statistical significance (Fig. 1D).

Immunologic tolerance to CNS melanoma antigens is not the result of ignorance

Antigens exit the CNS via cerebrospinal fluid (CSF) drainage along olfactory nerves passing through the cribriform plate and along perivascular spaces, including channels associated with dural venous sinuses (17), en route to the cervical lymph nodes (2). Given that antigens in the CNS parenchyma are poorly recognized by naïve lymphocytes (18), we hypothesized that differences in tumor antigen recognition might underlie the observed differences in brain and flank tumor-bearing animals. To test this hypothesis, we adoptively transferred CFSE-labeled OT-1 CD8 T cells to mice bearing B16-OVA brain or flank tumors. These studies could not be performed with Pmel cells, as so few cells escape deletional tolerance (Fig. 1B). As shown in Fig. 2A, the majority of TIL was divided in both brain and flank tumors, consistent with antigen recognition. OT-1 CD8 T cells in the brain tumor DLN also showed clear evidence of division, with a significant fraction of cells undergoing greater than 4 divisions (Fig. 2A and B). Although division was attenuated in brain tumor DLNs as compared with flank tumor DLNs, these results provide clear evidence of CNS tumor antigen recognition, although we cannot determine whether initial recognition occurred in the DLN or upon entry into the tumor itself. To determine whether brain

tumor antigen recognition impaired acquisition of CD8 T-cell effector function, we analyzed IFNγ production by antigen-specific CD 8 T cells (Fig. 2C) and found a significantly lower percentage ($P < 0.001$) and number ($P = 0.01$) of divided, IFNγ$^+$ CD8 T cells in the DLNs of brain tumor-bearing mice compared with flank tumor–bearing mice (Fig. 2D). Extending these data to a third tumor site, we found that established lung tumors also stimulated IFNγ secretion more readily than brain tumors ($P < 0.001$) and similar to flank tumors ($P = 0.56$; Supplementary Fig. S2).

Antigen-specific cytotoxicity is systemically impaired in animals with CNS melanoma

We next tested the effects of CD8 T-cell priming in the context of a brain tumor or flank tumor on *in vivo* effector function by performing a series of cytotoxic T lymphocyte (CTL) assays in mice bearing either B16-OVA brain or flank tumors. In the absence of tumor antigen–specific vaccination or adoptive T-cell transfer, recognition of tumor antigen was insufficient to confer effector function ($P = 0.14$; Fig. 3A and B). To test whether adoptively transferred, vaccine-primed antigen-specific T cells respond differentially in the context of a brain or flank tumor, we adoptively transferred a physiologically relevant number (~100) of OT-1 CD8 T cells (19) to mice bearing established B16-OVA tumors, then vaccinated with a recombinant OVA-expressing vaccinia-based vaccine (Vac-OVA; ref. 15). In cancer-free mice, targets persisted in untreated animals, whereas essentially all targets were lysed after adoptive transfer of antigen-specific CD8 T cells plus vaccination (Fig. 3C and D). Lysis was attenuated in tumor-bearing mice, and CNS melanoma attenuated CD8 T cell function to a greater degree than flank tumors (Fig. 3D). We next examined whether this relative resistance to vaccination applied to therapy with a novel listeria-based vaccine (20) and found that even in the absence of adoptive transfer, this vaccine strain mediated lysis with a nonsignificant trend toward decreased killing in mice bearing CNS melanoma as compared with mice bearing flank tumors ($P = 0.052$; Fig. 3E and F).

TGFβ is elevated in the serum of mice with CNS melanoma

TGFβ family cytokines are pluripotent molecules involved in regulating tissue homeostasis (21), and TGFβ1 has been implicated as a critical driver of melanoma progression (22). To determine whether CNS melanoma is associated with systemically elevated levels of TGFβ, we measured serum levels of TGFβ1. As shown in Fig. 4A, mice with B16-OVA brain tumors had significantly higher levels of serum TGFβ1 than mice with B16-OVA flank tumors ($P = 0.039$). On the basis of these data, we hypothesized that blocking TGFβ signaling might reverse brain

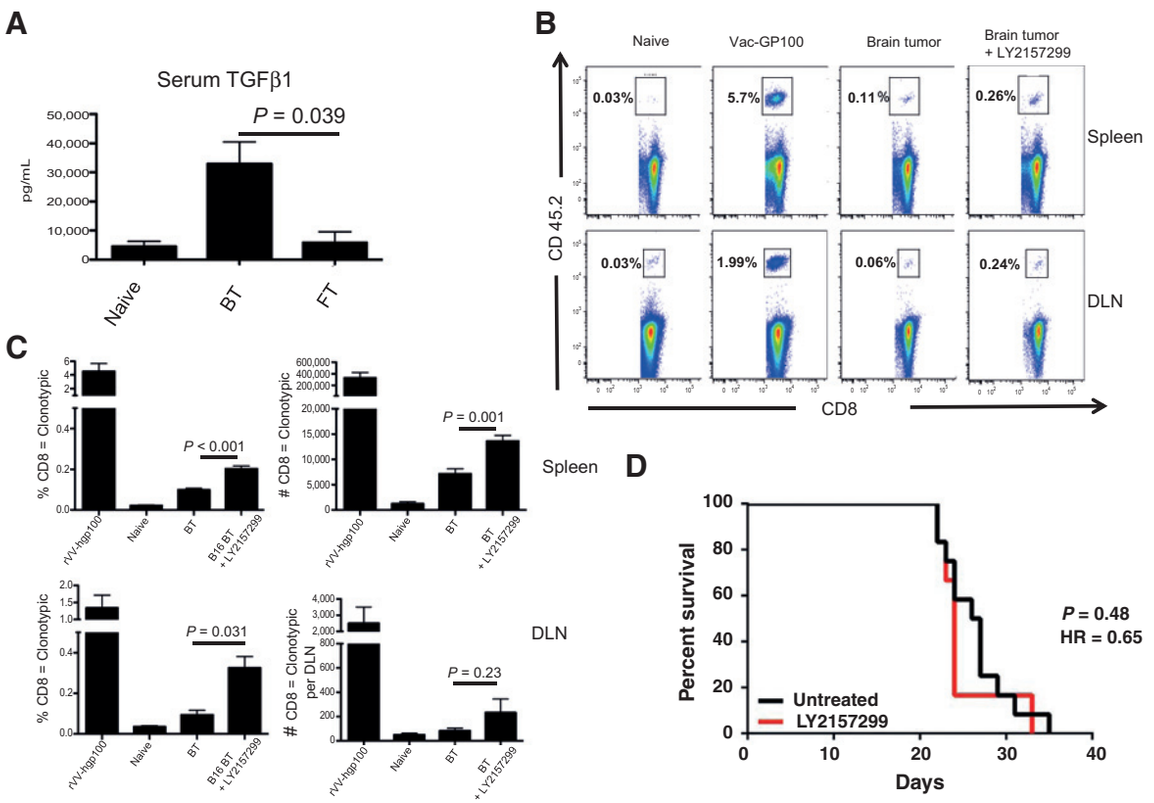


Figure 4.
Elevated TGFβ1 is associated with systemic tolerance in animals with CNS melanoma. A, concentration of TGFβ1 in serum of mice with B16 brain or flank tumors. B, representative FACS plots demonstrating the percentage of CD8 T cells represented by the adoptively transferred Pmel population in the spleen and cervical lymph nodes after treatment with LY2157299. C, summary graphs showing the percentage and number of adoptively transferred Pmel cells recovered from animals with CNS melanoma after treatment with LY2157299. D, survival of animals with CNS melanoma treated with the TGFβ signaling inhibitor LY2157299. A–C, $n = 5$ animals/group, repeated × 2. D, $n = 10$ animals/group, repeated × 1.

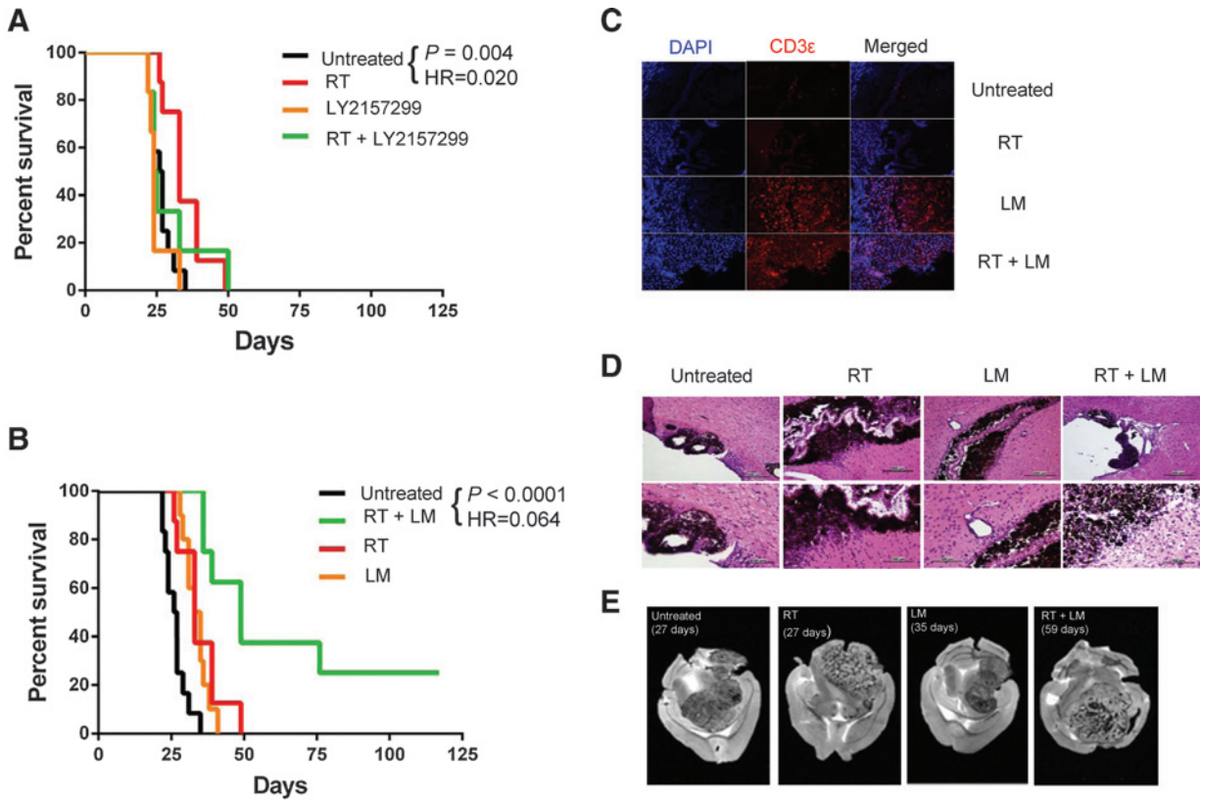
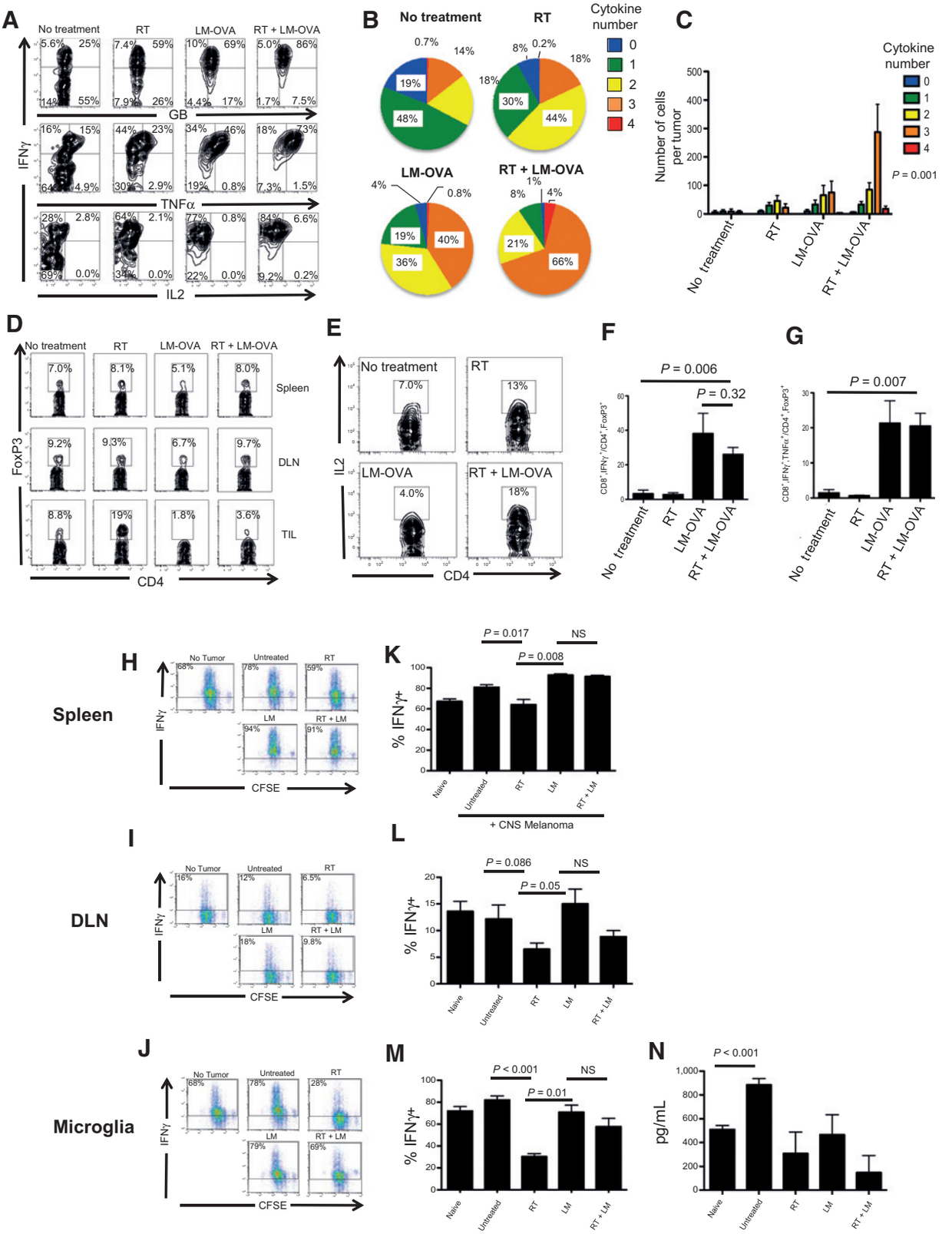


Figure 5.
Combining focal RT with vaccination improves survival in mice with established CNS melanoma. A, combination treatment with focal RT + TGFβ1 signaling inhibition. B, combination treatment with focal RT + LM–based vaccine. C, CD3 immunofluorescence in brain sections from treated mice at 20×. D, representative H&E micrographs of brain tissue from mice with treated brain tumors at 20× (top row) and 40× (bottom row). E, representative *ex vivo* MRI slices from mice with treated brain tumors. Experiments performed × 3 with 10 mice per group, typical results shown.

tumor–mediated tolerance. To test this hypothesis, we returned to the Pmel adoptive transfer model and treated brain tumor–bearing mice with a small-molecule TGFβ signaling inhibitor (LY2157299; ref. 23). We found that inhibiting TGFβ signaling significantly increased the percentage and number of Pmel CD8 T cells in the spleens of brain tumor–bearing mice ($P < 0.001$, $P = 0.001$, respectively) and significantly increased the percentage of Pmel CD8 T cells in the DLN ($P = 0.031$; Fig. 4B and C). Somewhat surprisingly, TGFβ blockade did not increase the number of TIL, nor did treatment affect overall survival (OS; Fig. 4D). Nearly complete blockade of TGF-β signaling was confirmed via Western blotting (data not shown). Treatment with the pan-TGFβ neutralizing antibody 1D11 also did not improve OS in these animals (data not shown). Taken together, these results show that CNS melanoma is associated with elevated TGFβ levels but that blockade alone is not sufficient to alter preclinical outcome.

Combination treatment regimens in animals with CNS melanoma

RT is a standard treatment modality for CNS lesions, and in previous studies using an orthotopic glioma model, we found that combining RT with other immunotherapies had a synergistic effect on OS (9). We thus tested whether the combination of TGFβ signaling inhibition + focal RT delivered using the SARRP could mediate treatment effects in this CNS melanoma model. Unfortunately, this was not the case; although RT enhanced survival significantly (HR, 0.02; $P = 0.004$), TGFβ inhibition did not add to this effect (Fig. 5). Because LM-based vaccination alone appeared to partially reverse brain tumor–mediated tolerance (Fig. 3), we next tested whether LM-based vaccination could prolong survival. Indeed, the LM-based vaccine mediated a relatively modest increase in OS, but the combination of vaccination + RT significantly increased survival, with a number of animals showing long-term nonprogression (Fig. 5B). The addition of TGFβ blockade to this combination regimen (using either 1D11 or LY2157299) did not extend survival compared with LM-OVA + RT (Supplementary Fig. S3), nor did the addition of PD-1 blockade (Supplementary Fig. S4). To explore the mechanism of action of this efficacious combination regimen, we performed immunostaining with anti-CD3ε. The results confirmed the presence of an intratumoral T-cell infiltrate in mice receiving LM alone or LM in combination with radiation therapy (Fig. 5C). Corresponding H&E staining demonstrated a lack of inflammation in untreated tumors and minimal inflammation in tumors treated with RT alone (Fig. 5D). LM vaccination, by contrast, was associated with increased perivascular inflammation, and combination therapy was associated with a marked peritumoral lymphocytic infiltrate. To test whether combination immunotherapy altered patterns of brain tumor progression, brains from representative animals (3 per group) were imaged *ex vivo* with MRI. Tumor borders were


A
No treatment
RT
LM-OVA
RT + LM-OVA
IFNγ
GB
TNFα
IL2
B
No treatment
RT
LM-OVA
RT + LM-OVA
Cytokine number
0
1
2
3
4
C
Cytokine number
Number of cells per tumor
P = 0.001
No treatment
RT
LM-OVA
RT + LM-OVA
D
FoxP3
Spleen
DLN
TIL
CD4
E
IL2
CD4
F
P = 0.006
P = 0.32
G
P = 0.007
H
Spleen
IFNγ
CFSE
No Tumor
Untreated
RT
LM
RT + LM
I
DLN
J
Microglia
K
P = 0.017
NS
P = 0.008
% IFNγ+
Naive
Untreated
RT
LM
RT + LM
+ CNS Melanoma
L
P = 0.086
NS
P = 0.05
M
P < 0.001
NS
P = 0.01
N
P < 0.001
pg/mL

delineated in each slice and volumes were calculated using ImageJ software by a blinded observer (Fig. 5E). To test for differences in morphology, we identified the dorsal–ventral midplane of each tumor and calculated the ratio of tumor volumes superior and inferior to this plane. Although no differences were observed with RT, LM vaccination appeared to constrain tumor growth, with untreated tumors and tumors treated with RT alone exhibiting more variable morphology at the time of death (Supplementary Fig. S5A). In addition, we found that there was no difference in tumor volume between groups at the time of death (Supplementary Fig. S5B).

Combination therapy is associated with polyfunctional CD8 T cells, an increased Teff to Treg ratio, and increased APC function

To elucidate the immunologic mechanisms by which focal RT and systemic LM mediate treatment effects in CNS melanoma, mice with established CNS disease were treated with RT, LM, or the combination of RT and LM. Tumors were harvested 17 days after implantation, and endogenous TILs were analyzed for cytokine production by flow cytometry. As show in Fig. 6A, RT or LM alone generated a modest increase in Granzyme B, IFNγ, and TNFα production, while with combination therapy, the majority of cells produced Granzyme B, IFNγ, and TNFα (Fig. 6B). In addition to stimulating cytokine production, combination therapy increased the density of tumor-infiltrating polyfunctional CD8 T cells ($P = 0.001$ by two-way ANOVA; Fig. 6C).

Accumulation of Tregs is an important mechanism of immunosuppression in cancer (24), so we next evaluated the effects of this regimen on peripheral and intratumoral Tregs. In the periphery, we observed a modest decrease in the percentage of CD4 T cells expressing FoxP3 with LM vaccination (Fig. 6D). Similarly, LM vaccination decreased the percentage of Tregs within the tumor. Conversely, RT approximately doubled the percentage of CD4 TIL–expressing FoxP3, consistent with prior data (25). Combination therapy resulted in an intratumoral Treg profile similar to LM alone, while favorably increasing the percentage of CD4 T cells producing IL2 compared with either RT or LM monotherapy (Fig. 6E). In several human cancer types, the intratumoral Teff/Treg ratio correlates with clinical outcome. Indeed, we found that LM-based vaccination significantly increased this ratio, but RT did not add further to this effect, suggesting that increases in Teff/Treg were not the sole mechanism. To further elucidate the mechanism(s) underlying the combination treatment effect, we tested whether the combination regimen affected the ability of various APC populations to present relevant tumor antigens *ex vivo*. We also quantified concentrations of the proinflammatory cytokines IFNγ, IL2, GM-CSF, and IL12 as well as the inhibitory cytokines IL10 and TGFβ in the supernatants of OT-1 CD8 T cells cocultured with either splenic dendritic cells, tumor DLNs, or microglia (Supplementary Fig. S6). We found that microglia isolated from mice with brain tumors secreted significantly more TGFβ compared with microglia from naïve animals and that combination therapy significantly decreased TGFβ secretion from microglia (Fig. 6). Interestingly, focal RT alone decreased antigen presentation by microglia and splenic APCs, with a trend toward decreased presentation in the tumor DLN as well (Fig. 6), whereas LM-based vaccination largely corrected this APC dysfunction in both distant (splenic) and local sites.

Discussion

Brain metastases are a negative prognostic indicator in patients with metastatic melanoma (26), despite the fact that most patients succumb to systemic disease progression rather than neurologic compromise (10). One potential explanation is that metastasis to the brain is a relatively late event and thus a harbinger for widely disseminated disease. This hypothesis is challenged, however, by the finding that patients presenting with isolated melanoma brain metastases have shorter life expectancies than patients presenting with visceral metastases or synchronous brain and visceral lesions (27). A hypothesis consistent with these clinical data is that brain metastases accelerate systemic disease progression, potentially through an immune-mediated mechanism. Immunosuppression has been extensively studied in patients with high-grade gliomas, but little is known about the systemic immunologic effects of metastatic brain tumors.

Using a B16 model, we demonstrated that brain tumors inhibit cellular immunity to a greater degree than flank or lung tumors in mice. Pmel CD8 T cells were more readily deleted in mice with brain tumors compared with equivalent B16 flank tumors, OT-1 T cells underwent fewer divisions, and daughter cells produced less IFNγ in response to brain tumors compared with flank tumors. Of note, OT-1 TILs were divided in both brain and flank tumors, consistent with the hypothesis that naïve T cells have limited access to the CNS in the absence of inflammation (6) and that tumor antigens originating in the CNS are presented in secondary lymphoid organs. Furthermore, although restimulation of OVA-directed cells in the CNS is sufficient to restore effector function in a CD8-mediated EAE model (28), our data indicate that dividing TIL produce less IFNγ in brain tumors than in flank tumors. Although caution is warranted in interpreting these pooled TIL data; this observation indicates that tolerance acquired upon priming in secondary lymphoid organs may persist in the brain tumor microenvironment.

Impaired cellular immunity has long been suspected in patients with glioblastoma and may be compounded by interventions such as chemotherapy and steroids (4, 29). Our data demonstrate that tumor location within the CNS may be an independent mediator of systemic immunosuppression. We used a series of *in vivo* CTL experiments to explore the functional significance of this finding. Tumor-reactive lymphocytes characteristically

Figure 6.

Combination therapy with LM-based vaccination and focal RT is associated with polyfunctional CD8 T cells, an increased Teff to Treg ratio, and improved APC function. A, FACS plots demonstrating the effects of combination therapy on cytokine production from adoptively transferred, tumor-infiltrating CD8 T cells. B and C, percentages and numbers, respectively, of CD8 TIL producing single and multiple cytokines. D, quantification of Treg ($FoxP3^+$, $CD4^+$) in treated animals. E, quantification of CD4 TIL producing IL2. F and G, Teff to Treg ratios in TIL, with effectors defined as $IFN\gamma^+$, or IFNγ TNFα double positive, respectively. H–J, CFSE dilution of OT-1 T cells cultured with pulsed APC from spleen, DLN, and microglia ($CD11b^+$ $CD45^-$mid). K–M, summary graphs of the percentage of OT-1 responders producing IFNγ when cocultured with the indicated APC populations. N, TGFβ1 concentrations in supernatants from J, M. A–G repeated × 2, $n = 3$–5 animals, group; H–M repeated × 1, $n = 10$ animals/group.

express the exhaustion markers PD-1, LAG-3, and TIM-3 (30), so it was not surprising that tumor antigen processing was insufficient to stimulate a cytotoxic response. This finding is important, however, as it demonstrated that antigen recognition on B16 tumors was insufficient to mediate significant cytotoxicity. We next applied immunologic pressure by adoptively transferring a physiologically relevant number of OT-1 cells and vaccinating with Vac-OVA. Here, we found that mice with B16-OVA brain tumors exhibited impaired target lysis, indicating that the presence of a CNS tumor may blunt systemic responses to some immunotherapies.

Dysfunctional myeloid cells have been identified as key mediators of immunosuppression in patients with cancer (31) and M2-differentiated microglia may be drivers of glioma progression (32). TGFβ is a pleotropic cytokine that induces immunosuppression and drives tumor progression in several solid tumors, including melanoma and glioma (8, 33). TGFβ has also been shown to enhance IL4-mediated, M2 microglial activation (34). Our data indicate that microglia isolated from mice with B16-OVA brain tumors express significantly higher levels of TGFβ than microglia from naïve mice. We found that this elevation in TGFβ was also systemic, as B16-OVA brain tumor–bearing mice had significantly elevated serum levels of TGFβ1 compared with tumor-free or flank tumor–bearing mice. Furthermore, we found that TGFβ signaling blockade rescued deletional tolerance in the Pmel adoptive transfer model. On the basis of these data, we suspect that CNS melanoma may drive microglia into an alternatively activated phenotype characterized by TGFβ expression. However, TGFβ blockade in this model was unable to mediate a significant antitumor effect, either alone, combined with RT or with LM-based vaccination. Those data are perhaps somewhat contradictory to recent studies demonstrating that blocking TGFβ prior to hypofractionated radiation enhances preclinical responses (35), and additional work will be required to determine whether these differences reflect the use of different TGFβ blocking agents, the location of the tumor, or the cancer model under study. While our studies focused on microglia and antigen-presenting cells in the tumor DLNs and spleen, it is clear that tolerance to tumor antigens is mediated by a number of additional myeloid cell types, particularly myeloid-derived suppressor cells (MDSC; ref. 31). MDSC have been described in a CNS glioma model (36); and additional work will be required to address the role of MDSC populations in this model, both in the systemic tolerance mediated by implanted CNS tumors and in the response of those tumors to RT, vaccination, or combination regimens.

Live-attenuated LM vaccines have demonstrated efficacy in several preclinical cancer models (37, 38) and safety in phase I and II clinical trials (39, 40). The ability of LM to generate adaptive T-cell–mediated immunity is based on its intracellular lifecycle and propensity to infect $CD8^+$ dendritic cells (DC), where bacterial antigens are processed through both MHC class I and II pathways (40). Liau and colleagues have previously reported that a different strain of LM delays progression of intracranial B16 tumors (41). In our studies, LM-based vaccination restored CTL activity in a majority of B16 brain tumor–bearing mice; however, a trend remained toward impaired CTL function compared with flank tumor–bearing animals. Interestingly, treatment with LM vaccines increased lysis in mice with brain or flank tumors as compared with tumor-free mice. This effect was not observed with a vaccinia-based vaccine and suggests that LM may have been superior to vaccinia in boosting a low level of T-cell priming that occurred upon recognition of antigen on tumor cells. These data are consistent with the notion that brain tumors may be more systemically tolerogenic than flank tumors but also show that a potent vaccine may reverse CTL tolerance. Both vaccine platforms, however, failed to completely abrogate brain tumor–mediated tolerance, indicating that combination therapy may be required to achieve maximum efficacy.

RT has been associated with a mix of proinflammatory and inhibitory immunologic effects (15, 42) but may have particular utility in potentiating the activity of immunotherapy (43). Demaria and colleagues showed that RT in combination with FLt3-ligand impairs growth of irradiated tumors as well as tumors outside the radiation field (44, 45) and that local RT combined with cytotoxic T lymphocyte antigen-4 (CTLA-4) blockade inhibits metastasis in a breast cancer model (46). Newcomb and colleagues showed that radiation therapy combined with GVAX generates long-term survival and protective immunity in an orthotopic glioma model (47), and our group demonstrated that combining focal RT with PD-1 blockade prolongs survival of mice with orthotopic gliomas and protects against flank tumor rechallenge (9). Consistent with these data, in some studies, we found that while anti-PD-1 alone or in combination with LM-OVA vaccination had no effect on survival, the combination of RT + anti-PD-1 produced a small percentage of long-term survivors (data not shown). The translational relevance of these findings should be interpreted with caution given that PD-1 or CTLA-4 blockade alone have a modest effect on B16 progression (48), whereas multiple clinical trials have proven efficacy of checkpoint blockade in human melanoma. Furthermore, immune checkpoint blockade may represent a form of vaccination in humans; CTLA-4 blockade has been shown to boost T-cell responses to shared tumor antigens (49), and responses to PD-1 blockade may be associated with recognition of mutated tumor antigens (50). Additional experiments in other melanoma models are warranted to more clearly delineate the role of checkpoint blockade in the setting of tumor antigen-specific vaccination.

We found that combining focal RT with LM-OVA vaccination significantly prolonged survival over either monotherapy. Combination therapy stimulated tumor infiltration by polyfunctional CD8 T cells and increased Teff to Treg ratios, an immune profile that has been associated with tumor regression (24, 51). Furthermore, combination treatment with focal RT and LM-OVA altered the cytokine profile of microglia, reducing TGFβ1 secretion to levels indistinguishable from naïve microglia. Consistent with previous reports, RT increased intratumoral Treg density (52). Conversely, LM-OVA decreased the percentage of Tregs both within the tumor and peripherally. Combination therapy had mixed effects: focal RT abrogated the systemic reduction in Tregs stimulated by LM-OVA vaccination while maintaining a favorable intratumoral Teff to Treg ratio. These data suggest a therapeutic mechanism by which LM vaccination decreased the number of Tregs locally and systemically and bolstered APC function, whereas focal RT promoted polyfunctionality of CTLs and increased intratumoral Teff density. Translation of these studies to patients with CNS melanoma should be tempered by two additional caveats: first, vaccine monotherapy has generally not been as clinically effective as predicted by animal models, and second, although cancer vaccines can add to or synergize with immune

checkpoint blockade in several animal models (53), human studies combining either CTLA-4 blockade (54) or PD-1 blockade (55) with peptide vaccines have not clearly shown additional clinical benefit.

Yet, on the basis of these data, it is reasonable to hypothesize that development of a melanoma brain metastasis curbs immunologic pressure against tumor antigens and accelerates systemic disease progression. This model affords a plausible explanation for why brain metastases carry a grave prognosis, even in the setting of stable CNS disease. Going forward, it will be important to verify the relevance of these findings in other, contemporary, melanoma models as well as in human disease and determine whether CNS tumor burden correlates with the degree of immunosuppression. If so, prompt and aggressive treatment of metastatic brain lesions with immunotherapy, or possibly other modalities, may be critical not only for preserving neurologic function but also for restoring cellular immunity and delaying systemic disease progression.

Disclosure of Potential Conflicts of Interest

T.W. Dubensky Jr and P. Lauer have ownership interest (including patents) in Aduro Biotech. J.M. Taube reports receiving a commercial research grant from and is a consultant/advisory board member for Bristol-Myers Squibb. M. Lim reports receiving commercial research grants from Accuray, Aegenus, Arbor Pharmaceuticals, Celldex, and Bristol-Myers Squibb and is a consultant/advisory board member for Accuray and Bristol-Meyers Squibb. C.G. Drake reports receiving commercial research grants from Aduro Biotech and Bristol-Meyers Squibb; has ownership interest (including patents) in Compugen, NexImmune, Potenza Therapeutics, and Tizona Biotech; and is a consultant/advisory board member for AZ MedImmune, Bristol-Meyers Squibb, Compugen, Genentech, Merck, Potenza Therapeutics, and Tizona Biotech. No potential conflicts of interest were disclosed by the other authors.

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