Durable Therapeutic Efficacy Utilizing Combinatorial Blockade against IDO, CTLA-4, and PD-L1 in Mice with Brain Tumors

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Abstract

**Purpose:** Glioblastoma (GBM) is the most common form of malignant glioma in adults. Although protected by both the blood–brain and blood–tumor barriers, GBMs are actively infiltrated by T cells. Previous work has shown that IDO, CTLA-4, and PD-L1 are dominant molecular participants in the suppression of GBM immunity. This includes IDO-mediated regulatory T-cell (Treg; CD4+CD25+FoxP3+) accumulation, the interaction of T-cell–expressed, CTLA-4, with dendritic cell-expressed, CD80, as well as the interaction of tumor- and/or macrophage-expressed, PD-L1, with T-cell–expressed, PD-1. The individual inhibition of each pathway has been shown to increase survival in the context of experimental GBM. However, the impact of simultaneously targeting all three pathways in brain tumors has been left unanswered.

**Experimental Design and Results:** In this report, we demonstrate that, when dually challenged, IDO-deficient tumors provide a selectively competitive survival advantage against IDO-competent tumors. Next, we provide novel observations regarding tryptophan catabolic enzyme expression, before showing that the therapeutic inhibition of IDO, CTLA-4, and PD-L1 in a mouse model of well-established glioma maximally decreases tumor-infiltrating Tregs, coincident with a significant increase in T-cell–mediated long-term survival. In fact, 100% of mice bearing intracranial tumors were long-term survivors following triple combination therapy. The expression and/or frequency of T cell expressed CD44, CTLA-4, PD-1, and IFN-γ depended on timing after immunotherapeutic administration.

**Conclusions:** Collectively, these data provide strong preclinical evidence that combinatorially targeting immunosuppression in malignant glioma is a strategy that has high potential value for future clinical trials in patients with GBM. *Clin Cancer Res;* 20(20); 5290–301. ©2014 AACR.
Translational Relevance

The use of agents that reverse immunosuppression in tumors is an approach that is gaining clinical traction. Recently, Wolchok and colleagues (2013) demonstrated that the combination of immune checkpoint blockade inhibitors (via CTLA-4 and PD-1 mAb) resulted in an objective response in patients with end-stage melanoma. However, whether a similar type of approach will be effective in patients with intracranial tumors has remained an elusive question. Here, we first extended our previous findings by investigating the role of IDO1 in brain tumors, followed by the presentation of preclinical findings demonstrating a highly effective therapeutic strategy that simultaneously targets immune checkpoints and tryptophan catabolism in brain tumors. Given the current interest for exploring clinical trials utilizing CTLA-4, PD-(L)1, and IDO blockade in patients with brain tumor, these results serve as an important proof-of-concept supporting the future pursuit of this strategy in patients with incurable glioblastoma.

and TGF-β (15–17), as well as the accumulation of regulatory T cells (Treg; CD4+CD25+FoxP3+; refs. 18, 19). The latter component is a potently immunosuppressive subset that in GBM is characterized by the constitutively high level of CTLA-4, GITR and is predominantly represented as being thymus derived (20). On the basis of previous studies in peripheral tumor models demonstrating the impact of IDO on Treg activation (21), expansion (22) and/or recruitment (23), we recently asked whether there was a dominant cell type that required IDO to regulate Treg levels in brain tumors (9). Our original hypothesis assumed that DC-expressed IDO would be required, based on previous work showing their role in Treg modulation (10, 21, 24). However, upon more careful examination, while IDO-expressing DCs control Treg expansion, this mechanism depends on the conversion of CD4+CD25−non-Tregs into CD4+CD25+(FoxP3+) Tregs (25–27). Given that glioma-resident Tregs are primarily thymus derived (20), this likely explains why IDO−/−DC failed to impact Treg levels in brain tumors.

Here, we first extend our previous observations by determining the immunodominance of IDO-competent and IDO-deficient malignant glioma, ultimately revealing that timing of tumor implantation plays a significant role in tumor rejection. Next, we determined the therapeutic impact of inhibiting IDO, alone, or when combined with the current standard-of-care therapy, temozolomide (Temodar), unexpectedly revealing that the enzymatic inhibition of IDO does not play a crucial role in significantly increasing overall survival in malignant glioma. Importantly, we tested the individual and combined impact of inhibiting CTLA-4, PD-L1, and IDO in models of established glioma, demonstrating a robust decrease in tumor-resident Tregs concurrent with increased survival when all three targets were inhibited simultaneously. However, when we tested the impact of a similar strategy in an aggressive intracranial melanoma model, only a modest effect on survival was observed. Collectively, these data significantly enhance our understanding of therapeutically targetable immunosuppressive pathways active in brain tumors.

Materials and Methods

Mouse and cell lines

C57BL/6 (wild-type; Cat# 000664), IDO−/− (Cat# 005897), Rag1−/− (Cat# 002216), and OT-II (Cat# 004194) mice were obtained from Jackson Laboratories, maintained in the University of Chicago Carlson Barrier Facility and intracranially injected between the ages of 6 and 8 weeks. All mouse strains used in the included studies were on the C57BL/6 background and were provided autoclaved food pellets and water ad libitum. All surgical procedures were completed in accordance with NIH guidelines on the care and use of laboratory animals for research purposes. All studies performed on mice were approved by the Institutional Animal Care and Use Committee of the University of Chicago (Chicago, IL). Mice were euthanized by cervical dislocation. GL261 and B16-F10 cells were obtained from the NCI Frederick National Tumor Repository Lab (no authentication of the cell lines was conducted) and cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum as well as streptomycin (100 mg/mL) and penicillin (100 U/mL) at 37°C in a humidified atmosphere of 95% air/5% CO2. All cell culture products were purchased from Gibco Invitrogen.

Mouse orthotopic intracranial injection model

A detailed description can be found in the Supplementary Materials and Methods section.

Reagents and treatments

A detailed description can be found in the Supplementary Materials and Methods section.

Western blotting

A detailed description can be found in the Supplementary Materials and Methods section.

Flow cytometry and T-cell stimulation

A detailed description can be found in the Supplementary Materials and Methods section.

Statistical analysis

Data were analyzed using Prism 4.0 software (GraphPad Software). Experiments were repeated at least two times each. Data are represented as the mean ± SEM for all figure panels in which error bars are shown. The *P* values represent ANOVA for groups of 3 or more, whereas two-tailed unpaired Student *t* tests were used for paired groups.
A P value of less than 0.05 was considered statistically significant.

Results

The role of IDO and antigen specificity in glioma immunity

The genetic ablation of IDO in glioma cells results in the spontaneous rejection of brain tumors mediated by T cells (9). Previous work demonstrating that the majority of patient GBM specimens are >50% positive for IDO (7) suggests that this tryptophan catabolic enzyme tonically maintains suppression of the antitumor response. To determine the minimum number of IDO-deficient cells in a brain tumor required to induce tumor rejection, we mixed IDO-competent and IDO-deficient GL261 cells at various ratios and tested the effects on survival in IDO1-deficient (IDO1−/−) mice. As shown in Fig. 1A, 100% of glioma-bearing mice with IDO-competent (Vc) tumor cells died with a median overall survival of 24 days. In contrast, glioma-bearing mice with tumors mixed with IDO-competent and -deficient (IDOkd) tumor cells at 3:1, 1:1, or 1:3, resulted in 40% of mice surviving for up to 150 days (P < 0.05, <0.01, and <0.001, respectively). However, even with the survival advantage conveyed by the different ratios of IDO-deficient glioma cells, it was still overall lower when compared with the group of mice intracranially injected with IDO-deficient cells, alone, which resulted in 75% of mice surviving for up to 150 days after intracranial injection (P < 0.001).

To determine the nature and strength of the antitumor response induced by IDO-deficient glioma cells, we established IDO-competent and/or IDO-deficient tumor cells in both cerebral hemispheres of IDO−/− mouse brain to better understand IDO-dependent glioma-induced immunodominance. As shown in Fig. 1B, when mice were simultaneously injected IDO-competent cells on both sides of the mouse brain, 100% of mice died with a median survival of 15.5 days after intracranial injection. Interestingly, when mice were simultaneously injected IDO-competent and -deficient cells in opposite cerebral hemispheres, 100% of mice died with a median survival of 22 days. This was in contrast with the survival benefit imparted when IDO-deficient glioma cells were intracranially injected into both cerebral hemispheres, resulting in 80% of mice surviving up

Figure 1. The rejection of IDO-competent and -deficient brain tumors is context-dependent. A, survival analysis of indoleamine 2,3 dioxygenase knockout (IDO−/−) mice intracranially injected (i.c.) with a total of 4 × 10⁵ GL261 cells transduced with -scrambled shRNA (vector control, Vc), -shRNA specific to IDO (IDO knockdown, IDOkd), or mixed (Vc + IDOkd) at different ratios of cells. **P < 0.01; ***P < 0.001 (n = 5–11/group). B, survival analysis of IDO−/− mice i.c. injected with 4 × 10⁵ Vc or IDOkd GL261 cells in the right (right) and left (left) cerebral hemispheres, simultaneously [Day 0 (D0)], or 4 × 10⁵ IDOkd GL261 were i.c. injected in the right hemisphere (D0), followed by an intracranial injection of 4 × 10⁵ Vc GL261 cells at 7 or 32 days after i.c. (D7 or D32, respectively; n = 4–8/group). **P < 0.01. C, survival analysis of wild-type (WT) or OT-II (CD4⁺ T cells specific to chicken ovalbumin 323-339 I-Ab) mice i.c. injected with 4 × 10⁵ unmodified GL261 (n = 5–7/group). D, the frequency of CD4⁺FoxP3⁺ Treg cells isolated from brain tumors derived from unmodified GL261 cells analyzed at 3 weeks after i.c. Tregs were initially gated on CD3 and CD4. Bar graphs are shown as mean ± SEM (n = 4–5 mice/group). E, survival analysis of WT or OT-II mice i.c. injected with 4 × 10⁵ Vc or IDOkd GL261 cells (n = 3 mice/group). For survival experiments, mice were analyzed for up to 150 days and results reflect the data from two independent experiments.
to 150 days after intracranial injection \((P < 0.001)\). When taking into consideration the results from Fig. 1A, these data collectively suggest that the microenvironment within IDO-competent gliomas is sufficient to induce a coordinated immunosuppressive response that overcomes the antitumor response elicited by completely IDO-deficient satellite tumors in the brain. We next tested whether the prior establishment of IDO-deficient tumors would be sufficient for rejecting IDO-competent tumors. In mice already bearing IDO-deficient tumors, when IDO-competent glioma cells were implanted early (i.e., 7 days after intracranial injection), 100% of mice survived, whereas a later challenge with IDO-competent glioma cells (i.e., 32 days after intracranial injection) led to only 40% of mice surviving \((P < 0.001, \text{respectively})\). These data highlight the contextual nature of the antitumor immune response mediated by IDO-deficient glioma cells and suggest that timing of rechallenge plays a critical role with regard to overcoming tumor-induced immunosuppression and consequent effects on survival.

Given the potent effects of glioma cell-specific IDO-deficiency on the induction of productive tumor immunity, we next wondered whether this effect required antigen specificity or whether the nonspecific presence of CD4\(^+\) T cells was sufficient for increasing overall survival. As expected, Fig. 1C shows that neither wild-type (WT) nor OT-II (antigen restricted CD4\(^+\) T cells to chicken ovalbumin) mice mounted a long-term survival effect when intracranially injected with normal (IDO-competent) glioma cells. Notably, both WT and OT-II mice showed a significant accumulation of Tregs in the brain, although OT-II mice had relatively decreased levels \((P < 0.01)\). When OT-II mice were intracranially injected with IDO-competent or -deficient glioma cells, neither group could mount a long-term (i.e., up to 150 days after intracranial injection) survival response. These data suggest that antigen-specific CD4\(^+\) T cells are required for mediating the antitumor response in the context of IDO-deficient brain tumors.

**IDO expression and combinatorial targeting with temozolomide in glioma**

Previous work from peripheral tumor models has shown that inhibiting IDO with the well-characterized molecular agent, 1-methyltryptophan (1-MT), requires the addition of a chemotherapeutic agent to provide a significant antitumor response \((10)\). To address this, we hypothesized that 1-MT would synergize with temozolomide, the current standard-of-care chemotherapeutic agent for patients with glioblastoma, to achieve a synergistic antitumor-mediated survival benefit. As shown in Fig. 2A and B, neither L1-MT nor D1-MT in glioma-bearing mice significantly impacted overall survival, when compared with control untreated mice with a median survival of 24.5 days after intracranial injection. In contrast, the treatment of mice with temozolomide, alone, led to an increased median survival of 37.5 days after intracranial injection \((P < 0.01)\). To our surprise, neither the addition of L1-MT nor D1-MT increased survival further, versus treatment with temozolomide, alone. However, there was a small but significant survival advantage when D1-MT was coupled with temozolomide, compared with L1-MT with temozolomide, reflected by the median overall survival of 46 versus 35 days after intracranial injection \((P < 0.05)\). Given the surprisingly low level of efficacy upon treatment with 1-MT and temozolomide, we hypothesized that...
additional tryptophan catabolic pathways are present in the context of glioma, given the recent evidence that IDO2 and TDO also play a role in tumor immunity (28, 29). We quantified the expression of IDO1, IDO2, and TDO from normal (IDO-competent) GL261 cell-based tumor lysates at 1 and 3 weeks after intracranial injection from WT, IDO−/−, and Rag1−/− (lack functional T and B cells) mice in Fig. 2C and D. Not surprisingly, when compared with WT mice, IDO expression was unchanged in both IDO−/− and Rag1−/− mice, concordant with data showing that tumors require IDO to suppress tumor immunity, as well as inferring that the tumor is a primary source of IDO expression. Interestingly, IDO2 was expressed in WT mice bearing glioma and that expression was enhanced by peripheral IDO deficiency (P < 0.05) as well as the temporal-sensitivity to the absence of T cells (P < 0.05). Equally, if not more intriguing was the expression of TDO in WT and IDO−/− mice that was potently decreased by the absence of functional T cells (P < 0.05). As far as we know, this is the first reported observation that the immune system plays a role in regulating TDO expression in tumors. Collectively, these data suggest that due to the presence of IDO2 and TDO in glioma, therapeutic intervention against all three mammalian tryptophan catabolic enzymes may require simultaneous blockade to significantly impact brain tumor immunity.

A durable survival benefit after CTLA-4/PD-L1/IDO blockade in glioma

As an alternative to the combination of 1-MT with chemotherapy, we decided to pursue an approach that utilized 1-MT with CTLA-4 and PD-L1 blockade, instead, given the recent clinical success for end-stage patients with melanoma administered with CTLA-4 and PD-1 monoclonal antibodies (mAb; ref. 30). As shown in Fig. 3A and B, 100% of untreated mice died with a median survival of 29 days after intracranial injection. In contrast, 40%, 60%, and 90% of mice treated with CTLA-4 mAb, PD-L1 mAb, and coadministered CTLA-4 and PD-L1 mAbs, respectively, were still alive at 90 days after intracranial injection, demonstrating
an extraordinary survival benefit in glioma. To determine the impact of 1-MT on this approach, we analyzed mice bearing glioma and administered 1-MT, alone, or in combination with CTLA-4 mAb, PD-L1 mAb, or coadministered with both CTLA-4 and PD-L1 mAbs (Fig. 3C). Excitingly, 100% of mice treated with the triple therapy showed durable survival, when compared with only 20% of mice treated with 1-MT, alone (P < 0.05). To test whether this triple immunotherapy required T cells to mediate the survival effect, we coadministered CTLA-4 mAb, PD-L1 mAb, and 1-MT with CD4- and/or CD8-depleting mAb(s), which resulted in complete abrogation of any survival benefit (Fig. 3D). Similarly, when we treated Rag1−/− mice with the triple therapy, a similar lack of survival benefit was observed, confirming that T cells are required for this approach to be effective. Finally, we tested this effect in IDO−/− mice, given the recent data showing a synergistic benefit of CTLA-4 or PD-1/PD-L1 blockade in B16-F10 cell-based peripheral tumors (31). As shown in Fig. 3E, peripheral IDO deficiency negated the maximal amount of survival benefit, as seen in WT mice, with only 67% of mice that received the triple therapy surviving to 90 days after intracranial injection. Collectively, these data demonstrate that the triple immunotherapy is highly effective at increasing survival in glioma-bearing mice and suggests a complex mechanism that requires peripheral cell expression of IDO to maximize therapeutic benefits.

**Triple therapy against CTLA-4, PD-L1, and IDO decreases Tregs in glioma**

Tregs (CD4+CD25+FoxP3+) are potently immunosuppressive T cells that infiltrate human GBM (19), suppress the cytotoxic effector arm (32), and promote pathogenesis in experimental brain tumor models (18, 20, 33). Therefore, depleting them directly or utilizing immunotherapy to neutralize their presence is a major ongoing goal for improving standard-of-care treatment for patients with GBM. As shown in Fig. 4A, brain-resident Treg levels were decreased by treatment with triple CTLA-4, PD-L1, IDO blockade, but not by 1-MT alone, nor by combinations of 1-MT with CTLA-4 or PD-L1 mAbs versus untreated control mice (P < 0.01). Interestingly, this effect was also seen for the frequency of Tregs expressing high levels of CD44 (P < 0.01), a marker of antigen experience. In contrast, the triple therapy neither affected the frequency of brain-resident cytolytic CD8+ T cells (Tc), nor did it affect the level of antigen-experienced Tcs (Fig. 4B). Moreover, while the triple therapy did not affect the levels of IFN-γ expression in brain-infiltrating CD4+ T cells, when compared with control, it was

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**Figure 4. Early [1 week post intracranial injection (wp-i.c.)] therapeutic blockade against CTLA-4, PD-L1 and IDO decreases Treg levels and increases IFN-γ+ Tcs in brain tumors.** Wild-type (WT) mice intracranially injected with 4 × 10⁶ GL261 cells and left untreated or administered D1-MT, CTLA-4 mAb (clone 9H10) and/or PD-L1 mAbs (clone 10F.9G2). The frequency of (A) CD4+FoxP3+ and CD4+FoxP3+CD44+ Tregs, (B) CD8+ and CD8+CD44+ Tcs, or (C) CD4+IFN-γ+ and CD8+IFN-γ+ effector-Tonv and -Tcs, respectively, isolated from the brain, bilateral deep and superficial cervical draining lymph nodes (cLN) and the spleen of glioma-bearing mice at 3 wp-i.c. left untreated, or administered D1-MT, CTLA-4 and/or PD-L1 (n = 5/group). All T-cell populations were initially identified by the expression of CD3+. **P < 0.05; ***P < 0.01.
associated with higher IFN-\(\gamma\) levels in Tc cells \((P < 0.05)\). Taken together, triple blockade of CTLA-4, PD-L1, and IDO in glioma-bearing mice decreases antigen-experienced Treg levels, while coincidently increasing armed cytolytic T cells.

Given the dramatic effects of simultaneous CTLA-4, PD-L1, and IDO blockade on brain-resident Tregs and Tcs in glioma-bearing mice, we hypothesized that, in addition to the effect on overall T-cell frequency, there would also be an effect on the expression of immunomodulatory targets and/or receptors. Both CTLA-4 and PD-1 have previously been shown to be potentially high value targets in experimental mouse models of malignant glioma (34, 35). To understand their expression on T cells in the context of a responsive and productive antitumor response, we analyzed Tregs, Tconv, and Tcs for these molecules at 3 weeks after intracranial injection to determine whether the expression changes commensurately. As shown in Supplementary Fig. S1A, while the triple immunotherapy did not change the expression of PD-1 on either Tregs or Tcs, PD-1 mean fluorescence intensity (MFI) on Tconv increased from 369 \pm 49 in untreated glioma-bearing mice to 790 \pm 181 in mice that received triple immunotherapy \((P < 0.01)\). In contrast, the triple immunotherapy decreased CTLA-4 expression on Tregs from 1,527 \pm 176 in untreated mice to 715 \pm 222 in mice receiving triple therapy, whereas it increased on Tconv from 434 \pm 72 to 1,076 \pm 272 \((P < 0.001,\) respectively; Supplementary Fig. S1B). Collectively, these data suggest that the Tconv subset is particularly sensitive to the effects of CTLA-4/PD-L1/IDO blockade and that as Treg levels decrease due to the effects of triple immunotherapy, the CD4\(^+\) T-cell expression for PD-1 and CTLA-4 changes commensurately.

CTLA-4/PD-L1/IDO blockade targets Tregs and enhances survival from established glioma

Since our previous observation in smaller, less established brain tumors suggested the triple therapy primarily decreased Tregs, we wondered whether this effect would be maintained in larger, more well-established glioma—a time point that coincides with brain tumors that are \(\geq 2\) mm in diameter (9). As shown in Fig. 5A and B, there was a dramatic decrease in Treg levels from 38 \pm 2\% in untreated mice to 5.3 \pm 1\% in mice treated with triple therapy \((P < 0.001)\), which was reversed with the addition of temozolomide back to 39 \pm 4\% \((P < 0.001)\). Importantly, the triple therapy decreased Treg levels, when compared with dual

![Figure 5](image-url)
treatment with CTLA-4/PD-L1 mAbs (P < 0.05), suggesting that all three targets require therapeutic modulation to significantly affect this T-cell subset in glioma. Interestingly, triple therapy also decreased brain-resident Tc levels, when compared with untreated mice, suggesting a possible overall reduction in inflammation mediated by this approach. Importantly, this effect did not translate into differences in the level of CD44^hi^, CTLA-4^+^, PD-1^+^. Aiolos^CD39^- Treg and Tc cells, although it did have an effect on the level of B7L expressed by tumor-infiltrating Tcs (Supplementary Fig. S2A-G). As shown in Fig. 5C, untreated WT mice had a median overall survival of 32 days after intracranial injection with 100% mortality. In contrast, 1-MT alone, increased the median survival to 45.5 days after intracranial injection with 38% of mice alive at 90 days after intracranial injection (P < 0.01). Incredibly, both coadministration of CTLA-4/PD-L1 mAbs or triple therapeutic CTLA-4/PD-L1/IDO blockade resulted in durable survival for 78% of glioma-bearing mice (P < 0.001). Not surprisingly, the concurrent addition of temozolomide to this therapeutic regimen decreased maximal survival, suggesting that active chemotherapy in the context of productive antitumor immunity is an undesirable approach. Interestingly, when the dual and triple therapies were utilized in IDO^-/-^ mice bearing glioma, maximal therapeutic efficacy was unattainable (Fig. 5D), reinforcing our previous observation that peripheral IDO is required for effective immunotherapy in the context of CNS tumors. Ultimately, these data show exciting preclinical results demonstrating the efficacy of either dual or triple immunotherapy utilizing CTLA-4, PD-L1, and/or IDO blockade.

**Intracranial melanoma is poorly responsive to CTLA-4/PD-L1/IDO blockade**

GL261 cell-based brain tumors robustly recruit Tregs, which has been shown to be one of the ways that CNS malignancies subvert the antitumor response. However, we were curious as to whether this was a tumor intrinsic phenomenon or whether any malignant intracranial tumor ultimately causes Treg accumulation. As shown in Fig. 6A, WT mice analyzed at 12 days after intracranial injection of GL261 tumor possess 27% ± 7% Tregs in the brain, when compared with B16-F10 tumors that recruit only 2% ± 0.3% Tregs (P < 0.01). This is not a reflection of a difference in overall Treg phenotype, as Tregs isolated from both types of intracranial tumors exhibit the same level of GITR, a prototypical receptor that is highly expressed by Tregs and known to regulate their function in tumors (Fig. 6B; ref. 36). When the GL261 and B16-F10 cell lines were analyzed, in vitro, for PD-1, GITR, and PD-L1, quantifiable differences in GITR and PD-L1 were observed after stimulation with IFN-γ (Fig. 6C). However, whether these differences affect Treg accumulation has yet to be established. To determine the effects of our triple immunotherapeutic approach against intracranial melanoma, we began immunotherapy at 3 days after intracranial injection in WT mice given the known aggressiveness of B16-F10 cells (Fig. 6D). To our surprise, 1-MT alone (P < 0.01), CTLA-4/PD-L1 blockade (P < 0.01), as well as combining all three reagents (P < 0.001) increased overall survival. However, the overall benefit was limited to days, rather than months, as we had observed in GL261 cell-based brain tumors. Moreover, no difference in overall survival was found when a similar approach was used in IDO^-/-^ mice (Fig. 6E), nor when that approach was further combined with GITR and Lag-3 mAbs. These data suggest that the efficacy of inhibiting CTLA-4, PD-L1, and IDO in brain tumors will depend on context and based on the data we have presented here, will be more effective in tumors reliant on Treg accumulation, rather than aggressive tumors known to migrate and evade the immune response by alternative mechanisms.

**Discussion**

Current standard-of-care treatment for patients initially diagnosed with glioblastoma multiforme (GBM) includes surgical resection, radiation, and chemotherapy with Temo-dar (temozolomide). However, this aggressive regimen can leave undesirable side effects, with an average overall survival advantage of only 14.6 months after diagnosis. These grim potential outcomes have served as rationale to develop alternative approaches for treating patients with high-grade primary brain tumors of which immunotherapy is a leading candidate for producing effective, durable and long-lasting patient benefits (32, 37, 38). While these highly promising studies warrant further investigation, it is helpful to also understand the redundant and compensatory immunosuppressive pathways that undermine the efficacy of immunotherapy, as well as general antitumor immunity (39, 40). Among these pathways, IDO plays a central role in regulating immunosuppression, as the genetic ablation of IDO, specifically in glioma cells, leads to the spontaneous and rapid rejection of brain tumors (9). Other high value targets that have been demonstrated to regulate immunosuppression in glioma include CTLA-4 (34), PD-L1 (41), PD-1 (35). However, to the best of our knowledge, no previous study simultaneously targeted these regulatory hubs in the context of malignant glioma.

Through the mixture of IDO-competent and -deficient glioma cells, we began our investigation by asking the simple question: which proportion of IDO-deficient cells induces an antitumor immune response that results in a durable survival advantage? The data indicate that mice with brain tumors composed of >50% IDO-competent glioma cells mice had a shorter overall survival, when compared with those tumors with a composition of ≥50% IDO-deficient glioma cells. This observation is concordant with previous immunocytotoxic analysis in human GBM, demonstrating that the majority of GBM cases are >50% positive for IDO in tumor cells (7). Interestingly, when IDO-competent and -deficient brain tumors were independently established in contralateral cerebral hemispheres, the IDO-competent tumors were capable of abrogating any survival effect normally attributable to IDO-deficient tumors. This was not simply due to the burden of tumor cells intracranially injected into the mouse brain,
since when IDO-deficient glioma cells were dually injected, the majority of mice survived and lived for up to 150 days after intracranial injection. Rather, this may reflect the difference in the programming and/or recruitment of stromal cells that IDO-deficient glioma cells recruit to induce antitumor immunity. Importantly, the factors recruited by IDO-deficient glioma cells during the priming phase leading to antitumor immunity are sufficient to cause rejection of IDO-competent tumor cells, with a diluted level of effectiveness if challenged after priming has already occurred. Finally, given our previous data suggesting that CD4\(^+\) T cells are required to reject IDO-deficient tumors based on the lack of long-term durable survival in CD4\(^-\) mice (9), we determined the relevance of antigen specificity in this survival mechanism. As expected, OT-II mice bearing CD4\(^+\) T cells universally antigen-restricted to chicken ovalbumin, a non-glioma expressed protein, were incapable of rejecting both IDO-competent and -deficient brain tumors. Collectively, these data suggest a critical role for IDO in suppressing immune-mediated glioma rejection, we next asked whether inhibiting the IDO pathway via 1-MT would be sufficient to recapitulate our observations with genetic silencing. On the basis of previous studies demonstrating that, 1-MT alone, does not lead to tumor rejection but that, 1-MT in
combination with chemotherapy leads to productive tumor immunity (10), we chose to study the treatment of glioma with 1-MT in combination with temozolomide. We found that both levorotary (L) and dextrorotary (D) stereoisomers of 1-MT were ineffective at increasing overall survival from glioma burden when administered at 2 mg/mL in the drinking water. However, when both D1-MT and L1-MT were combined at 5 mg/mL they showed a significant antitumor effect, possibly reflecting a higher dosing requirement due to poor blood–brain barrier permeability. Interestingly, when either L1-MT or D1-MT were coadministered with temozolomide under the dosing regimen used in this study, neither combinatorial regimen increased survival when compared with mice treated with temozolomide alone. This suggests that, in contrast with peripheral tumor models, the synergism between IDO inhibition and glioma-induced death may differ in sensitivity toward this therapeutic regimen. Alternatively, the lack of synergistic antitumor effect could be due to repeated and frequent dosing with temozolomide, which may abrogate the establishment of a productive immune response. Another possible explanation includes compensation due to kynurenine production by IDO2 and/or IDO1. In support of this hypothesis, we found that all three mammalian tryptophan catabolic enzymes, IDO1, IDO2, and TDO, were expressed in brain tumors. Recent evidence suggests that both IDO2 and IDO contribute to the regulation of immunity (42) and/or progression of glioma (43).

Given that 1-MT and temozolomide did not produce beneficial results when compared to temozolomide alone, we hypothesized that coupling IDO inhibition with the regulation of other powerful immunomodulatory mediators would produce a synergistic survival response in glioma-bearing mice. Data from peripheral tumor models previously demonstrated that combining CTLA-4 and PD-1 and/or PD-L1 inhibition is an attractive method for reducing immune suppression and/or reactivating productive antitumor response (44–46). On the basis of this literature, we wondered whether this approach would also yield benefits in the context of aggressive neoplasms within the central nervous system (CNS), a site normally considered to be relatively immune privileged. Much to our surprise, the simultaneous therapeutic inhibition of CTLA-4 and PD-L1 at 1 week following glioma cell implantation led to a remarkably high survival rate of 90% in glioma-bearing mice. Notably, this survival was durable over a 90-day period of observation. The addition of 1-MT to CTLA-4 and PD-L1 blockade was also associated with a high survival rate of 100% in glioma-bearing mice. Notably, the survival benefit induced by triple immunotherapy was completely abrogated when the immune system was depleted for CD4+ and/or CD8+ T cells. This implies that there is a coordinated mechanism of action between both T-cell subsets to carry out effective glioma immunity. However, the temporal requirement after therapeutic initiation for each T-cell subtype, which cells they must interact with in the tumor microenvironment and whether those interactions mediate direct or indirect antitumor effects, have yet to be determined.

One unexpected finding from our study was that CTLA-4, PD-1 ± IDO blockade in mice deficient for peripheral IDO (i.e., non-glioma derived) showed decreased overall survival, when compared with WT mice. This is a somewhat paradoxical observation given the recent finding that host-derived IDO plays a critical role in antitumor immunity when coupled with either CTLA-4 or PD-1 blockade (31). However, it is important to keep in mind that what is often observed in the CNS, regardless of the presence or absence of neoplasm, does not recapitulate an identical response, peripherally. This is likely due, in part, to the presence of the blood–brain barrier, lack of a developed lymphatic system, the normally low expression of MHC class II, as well as the different stromal cells within the CNS parenchyma.

We found that, regardless of early or late blockade for CTLA-4, PD-L1, and IDO, Treg levels were overall decreased. Interestingly, the late administration of immunotherapy also induced a decrease in brain tumor-infiltrating Tcs. This latter observation has potentially important implications related to the overall inflammatory state in the brain. Theoretically, any immunotherapy will generate some degree of inflammation associated with the production of proinflammatory cytokines and cell death associated with tumor rejection. Thus, a therapy that is effective for killing tumor cells, but recruits fewer leukocytes to the CNS, is highly desirable. This is particularly notable since the common way to decrease inflammation in the CNS for patients with GBM is by administering Dexamethasone, a glucocorticoid that will likely marginalize an active T-cell–mediated response. Thus, while overall survival is similar between dual and triple therapeutic approaches, the additional advantage of decreased inflammatory cell infiltration warrants further investigation.

Our investigation found that CTLA-4/PD-L1 mAb ± 1-MT treatment in the context of intracranially injected GL261 (glioma) cell-based brain tumors resulted in a highly effective and durable survival advantage, while the intracranially injected B16-F10 (melanoma) tumor model, showed a dramatically reduced level of efficacy. Other notable differences included a significantly decreased level of Treg infiltration in B16-F10 cell-based tumors with higher levels of IFN-γ-inducible GITR and B7-H1 expression. Interestingly, there was no difference in survival when B16-F10 cells were intracranially injected in IDO−/− mice, again highlighting the contextual difference between eradicating peripheral tumors as previously shown (31) with the data we have included here. Given the capability that both GL261 and B16-F10 cells express IDO and consequently modulate antitumor immunity (9, 47), it will be interesting to determine whether both types of tumors express similar levels of IDO, IDO2, and TDO.

In summary, we have extended our previous observations delineating the impact of IDO in brain tumors, demonstrated that all three mammalian tryptophan catabolic enzymes are present and have found a potent method to induce the rejection of primary tumors by virtue of CTLA-4 and PD-L1 blockade. By coupling this therapeutic regimen...
with 1-MT, we were able to reduce Treg and Te levels in established brain tumors with similar levels of overall survival and durable efficacy. Since clinical-grade analogs are available for all three agents that we tested, this strategy has high therapeutic value for patients with GBM. Whether this approach will be equally effective for those patients that are initially diagnosed versus those who present with recurrent tumors has yet to be explored and is difficult to model experimentally. Also, our data suggests that coconmitant administration of CTLA-4/CD-11/IDO blockade and temozolomide is not advantageous. Accordingly, we plan to determine whether staggering the treatments avoids therapeutic abrogation in the future. Ultimately, this work serves a proof-of-concept that this type of approach works and is relevant for treating patients with incurable malignant glioma.

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

Authors’ Contributions
Conception and design: D.A. Wainwright, M. Dey, I.V. Balyasnikova, J.W. Kim, J. Qiao, M.S. Lesniak
Development of methodology: D.A. Wainwright, J.W. Kim, M.S. Lesniak

References


Correction: Durable Therapeutic Efficacy Utilizing Combinatorial Blockade against IDO, CTLA-4, and PD-L1 in Mice with Brain Tumors

In this article (Clin Cancer Res 2014;20:5290–301), which was published in the October 15, 2014, issue of Clinical Cancer Research (1), the phrase "...whereas two-tailed unpaired Student t tests were used for paired groups," within a sentence in the statistical analysis section, generated confusion among readers. The authors have provided clarification, and the new paragraph should read as follows:

Data were analyzed using Prism 4.0 software (GraphPad Software). Experiments were repeated at least two times each. Data are represented as the mean ± SEM for all figure panels in which error bars are shown. The P values represent ANOVA for groups of 3 or more, whereas two-tailed unpaired Student t tests were used for comparisons between two groups. A P value of less than 0.05 was considered statistically significant.

The authors regret this error.

Reference


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Durable Therapeutic Efficacy Utilizing Combinatorial Blockade against IDO, CTLA-4, and PD-L1 in Mice with Brain Tumors

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