Clinical Cancer Research

# IDO1 Inhibition Synergizes with Radiation and PD-1 Blockade to Durably Increase Survival Against Advanced Glioblastoma

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## Abstract

**Purpose:** Glioblastoma is the most aggressive primary brain tumor in adults with a median survival of 15–20 months. Numerous approaches and novel therapeutics for treating glioblastoma have been investigated in the setting of phase III clinical trials, including a recent analysis of the immune checkpoint inhibitor, nivolumab (anti-PD-1), which failed to improve recurrent glioblastoma patient survival. However, rather than abandoning immune checkpoint inhibitor treatment for glioblastoma, which has shown promise in other types of cancer, ongoing studies are currently evaluating this therapeutic class when combined with other agents.

**Experimental Design:** Here, we investigated immunocompetent orthotopic mouse models of glioblastoma treated with the potent CNS-penetrating IDO1 enzyme inhibitor, BGB-5777, combined with anti-PD1 mAb, as well as radiotherapy, based on our recent observation that tumor-infiltrating T cells directly increase immunosuppressive IDO1 levels in human glioblastoma, the previously described reinvigoration of immune cell functions after PD-1 blockade, as well as the proinflammatory effects of radiation.

**Results:** Our results demonstrate a durable survival benefit from this novel three-agent treatment, but not for any single- or dual-agent combination. Unexpectedly, treatment efficacy required IDO1 enzyme inhibition in non-glioblastoma cells, rather than tumor cells. Timing of effector T-cell infiltration, animal subject age, and usage of systemic chemotherapy, all directly impacted therapy-mediated survival benefit.

**Conclusions:** These data highlight a novel and clinically relevant immunotherapeutic approach with associated mechanistic considerations that have formed the basis of a newly initiated phase I/II trial for glioblastoma patients. *Clin Cancer Res; 24(11); 2559–73. ©2018 AACR.*



## Introduction

Glioblastoma is the most common and aggressive form of malignant brain tumor in adults. While it can occur at all ages, the median age of diagnosis is 64 years old (1). Despite the multimodal treatment of this cancer that includes maximal surgical resection, radiotherapy, chemotherapy with temozolomide, as well as tumor-treating fields, most glioblastoma patients have a recurrence within one year. The median overall survival (mOS) for glioblastoma patients is 15–20 months postdiagnosis (2–5). Potent tumor-induced immunosuppressive mechanisms contribute to poor outcomes, as indicated by inverse correlations between patient survival and the expression of cell death protein-1 (PD-1), its ligand, PD-L1 (6), and/or indoleamine 2,3 dioxygenase 1 (IDO1; refs. 7, 8). While PD-L1, PD-1, and the tryptophan catabolic enzyme, IDO1, suppress immunity through independent, nonredundant molecular pathways (9), each factor is increased as a result of glioblastoma-infiltrating T cells.

Motivated by the recent success of checkpoint inhibitors for treating patients diagnosed with end-stage melanoma (10), non–small cell lung (NSCLC; ref. 11), and renal cell (12) cancers, anti-PD-1 monotherapy was recently evaluated in a large, randomized phase III clinical trial of recurrent glioblastoma patients (13). Single-agent PD-1 blockade had no effect on overall survival, when compared to glioblastoma patients treated with the control

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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**doi:** 10.1158/1078-0432.CCR-17-3573

## Translational Relevance

Glioblastoma is the most common primary malignant brain tumor and carries a dismal prognosis. Recent attempts at treating glioblastoma patients with checkpoint inhibitors, such as the PD-1 mAb, have similarly failed to provide a survival benefit. Our preclinical findings align with these clinical results. A potential mechanism contributing to PD-1 failure is based on our recent work showing that glioblastoma-infiltrating T cells increase the expression of immunosuppressive indoleamine 2,3 dioxygenase 1 (IDO1). To address this possibility preclinically, we tested a novel IDO1 enzyme inhibitor with PD-1 mAb, in the presence or absence of radiotherapy, and found that the three-agent combination cured a substantial fraction of mice with well-established intracranial glioblastoma. In contrast, neither monotherapy nor two-agent combinations conferred a long-term survival benefit. These data serve as strong rationale for initiating a phase I/IIa clinical trial evaluating the treatment of concurrent radiotherapy with PD-1 and IDO1 inhibition in glioblastoma patients.

agent, bevacizumab (anti-VEGF; ref. 14). This disappointing outcome indicates a need to reevaluate the approach for effective application of immunomodulatory treatments in glioblastoma patients, such as the simultaneous inhibition of multiple immune checkpoints, as has been done in preclinical studies targeting CTLA-4 and PD-L1 in mice with intracranial malignant glioma (15, 16). However, when tested in glioblastoma patients, the CTLA-4/PD-L1 treatment combination produced grade III–IV toxicities, drawing into question its safety. A potential alternative to this toxic combination relates to PD-(L)1 pathway treatment that has been relatively well tolerated in patients as a monotherapy, and further united with radiation, which enhances antigen presentation for priming tumor-specific T-cell responses, as well as potently increasing tumor-infiltrating T cells (17, 18). Previous work confirmed a survival benefit for immunocompetent mice with intracranial glioblastoma following dual radiation and anti-PD-1 treatment (19), supporting the rationale for ongoing clinical trials evaluating combination radiation and PD-(L)1 blockade in glioblastoma patients (NCT02336165, NCT02617589, NCT02667587). Although these clinical studies have yet to report results pertaining to progression-free survival and OS, preliminary analyses have demonstrated that radiation and PD-1 blockade is well tolerated in glioblastoma patients at 1 year posttreatment (20).

Despite the benefit of dual therapeutic combinations (21, 22), tumor relapse remains common, which even applies to treating nonadvanced, 7-day-old preclinical glioma with radiation and anti-PD-1 (19). The inability of previous studies to durably control advanced preclinical glioma suggests an additional need for improving survival outcomes in the clinic. Our recent analysis of The Cancer Genome Atlas, in combination with the investigation of humanized mice intracranially engrafted human patient-derived xenograft glioblastoma cells demonstrated that tumor-infiltrating T cells increase immunosuppressive IDO1 levels, which is associated with a poor prognosis in glioblastoma patients (23). Concordant with these results, we hypothesized that the further addition of an IDO1 enzyme inhibitor, when combined with radiation and anti-PD-1 therapy, would synergize with immune-mediated anti-glioblastoma activity. Rationale for this concept is provided by phase I/II clinical trial results indicating control of tumor growth when combining an IDO1 inhibitor with PD-1 blockade in NSCLC patients (24).

Here, we characterized the novel IDO1 inhibitor, BGB-5777, and confirmed its: (i) accumulation in the brain tumor parenchyma after oral delivery as compared with the clinical compound, INCB024360; and (ii) potent ability to inhibit the enzyme conversion of tryptophan into kynurenine, *in vitro and in vivo*. The simultaneous administration of BGB-5777 with radiation and PD-1 blockade-, but neither mono- nor dual-therapy among the three agents, provided durable tumor control in 30%–40% of mice with advanced intracranial glioblastoma. Long-term absence of tumor persisted for $\geq$150 days of posttreatment monitoring and tumors were likely completely rejected as indicated by the absolute quenching of glioblastoma cell bioluminescence, *in vivo*. T-cell depletion studies confirmed the dependence of an immune-mediated component for treatment efficacy, and the results of immunologic rechallenge experiments indicated the maintenance of protective immunity against clonogenic and nonclonogenic glioblastoma. Optimal survival from the three agent treatment required the: (i) inhibition of IDO1 in nonglioblastoma cells; (ii) presence of effector T cells; and (iii) young animal subject age. In contrast to the expectation of an increased rate of natural tumor progression with cancer cells expressing IDO1, our survival effect following the three agent therapeutic treatment, was unexpectedly independent of IDO1 metabolism by glioblastoma cells. Our results provide rationale for the direct translation of this novel trimodal approach for treating glioblastoma patients, while also introducing new mechanistic findings that directly relate to effective use of IDO1 inhibitors with immune checkpoint blockade.

## Materials and Methods

### Mice and cell lines

Four- to 8-week-old C57BL/6 and BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All of the procedures involving animals were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) of *BeiGene*. Six- to 8-week-old or 72-week-old C57BL/6 wild-type (catalog no. 000664) or IDO1$^{-/-}$ (catalog no. 005867) mice were obtained from Jackson Laboratories and maintained in the Northwestern University Center for Comparative Medicine. CT26WT murine colon carcinoma cells were obtained from the ATCC and maintained in DMEM supplemented with 10% FBS. A20 murine B-cell lymphoma were obtained from ATCC and maintained in RPMI1640 supplemented with 10% FBS plus 0.05 mmol/L 2-mercaptoethanol. Unmodified murine GL261 cells were obtained from the NCI (Frederick, MD), IDO1 O/E GL261 (8) murine wild-type tGBM, and IDO1$^{-/-}$ tGBM cells (25), as well as human U87, GBM12 and GBM43 cells (23) were described in our previous studies, while murine unmodified CT-2A and luciferase-expressing GL261 (GL261-luc.) cells were provided by Dr. Thomas Seyfried (Boston College, Boston, MA) and Dr. C. David James, PhD (Northwestern University, Chicago, IL), respectively, and cultured in DMEM supplemented with 10% FBS, in addition to penicillin (100 μg/mL) and streptomycin (100 mg/mL) at 37°C. Glioblastoma cells were not tested for mycoplasma prior to analysis. All products used for cell culture were purchased from Gibco Invitrogen.

### Orthotopic intracranial injection model

Mice were intracranially injected at 8, 12, or 72 weeks of age. All surgical procedures were completed in accordance with NIH guidelines on the care and use of laboratory animals. Mice were anesthetized with 0.15-mL solution containing ketamine HCl (90 mg/mkg) and xylazine (10 mg/kg) with an intraperitoneal injection and administered meloxicam (2 mg/kg) through subcutaneous injection for pain management. The surgical site was shaved and prepared with iodine and 70% ethyl alcohol. A midline incision was made, followed by drilling a parietal burr hole 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture. Mice were inserted into a stereotactic frame and $2 \times 10^5$ GL261 or $1 \times 10^5$ CT-2A cells in 2.5 μL saline were intracranially injected with a 22-gauge needle at a depth of 3 mm. After needle removal, the skin was stapled.

### In vivo treatments

For trimodal therapy, mice were treated with whole brain radiotherapy (2 Gy/day × 5 days), intraperitoneal PD-1 mAb or PD-L1 mAb (clone J43 and 10F.9G2, respectively; 500 mg loading dose followed by 200 mg maintenance doses every 3 days for 3 treatments total; BioXCell) and oral gavaged with BID BGB-5777 (100 mg/kg; BeiGene) suspended in ORAplus (Perrigo). For whole brain radiotherapy, the mice were anesthetized with 0.15-mL solution containing ketamine HCl (90 mg/mkg) and xylazine (10 mg/kg) with an intraperitoneal injection. Mice were placed in a lead box that only exposed the head and irradiated at 2 Gy/dose with a cesium-137 source.

For leukocyte depletion, mice were intraperitoneally injected with 200 mg CD4 mAb (clone GK1.5; InVivoMab), CD8 mAb (clone YTS 169.4; InVivoMab), or NK1.1 mAb (clone PK136; InVivoMab) every three days beginning at either 5 or 14 days post-intracranial injection and ending at 28 days post-intracranial injection.

### Tryptophan enzyme assays

Recombinant IDO1 was overexpressed and purified from *E. coli* cells with an N-terminal His tag. The IDO1 enzyme assay with D-tryptophan was carried out using a methodology similar to that described in the literature (26). The reaction mixture contained 50 nmol/L IDO1, 1.3 mmol/L D-tryptophan, 5 mmol/L L-ascorbic acid, 6.25 μmol/L methylene blue, 0.4 mg/mL catalase, and compound (or DMSO) in a buffer containing 50 mmol/L potassium phosphate pH 7.5 and 0.1% BSA. Absorbance at 321 nm was continuously recorded to monitor the formation of N′-formylkynurenine by a FULOstar OMEGA plate reader (BMG LABTECH). The enzyme activity was derived from the slope of the linear increase of absorbance as a function of time from 90 to 150 minutes. The $IC_{50}$ was calculated on the basis of the remaining enzyme activity in the presence of increasing compound concentrations.

The IDO1 enzyme assay with L-tryptophan was performed using the similar methodology as with D-tryptophan except for the following modifications: (i) 50 nmol/L human IDO1 or 20 nmol/L mouse IDO1 and 0.1 mmol/L L-tryptophan; (ii) IDO1 enzyme, compound (or DMSO), L-ascorbic acid, methylene blue, and catalase preincubated for 1 hour before L-tryptophan was added to initiate the reaction. The enzyme activity was derived from the linear absorbance increase within the first hour. Recombinant TDO was overexpressed and purified from *E. coli* cells with a C-terminal His tag. The TDO enzyme assay was performed using the same methodology as the IDO1 assay with D-tryptophan except that 100 nmol/L TDO and 0.5 mmol/L L-tryptophan ($K_m$ concentration) were used for the TDO assay.

### Kinetic measurement

$K_{obs}$ determination was performed using the same methodology as the IDO1 enzyme assay with D-tryptophan except that 150 nmol/L IDO1 was used and the absorbance at 321 nm was measured immediately after the reaction initiation. $K_{obs}$ was calculated based on the relative reaction velocity as a function of time in the presence of increasing concentrations of compounds. $K_i$ and $k_{off}$ were obtained from a linear fit of $K_{obs}$ as a function of compound concentrations.

### IDO1 inhibitors and blood–brain barrier penetration

BGB-5777 was synthesized at Beigene and possessed a purity ≥ 99% as measured by high-performance liquid chromatography. The reference compound, INCB024360, was purchased from WuXi AppTec. BGB-5777 was formulated in 0.5% (w/v) methylcellulose solution.

Six- to 8-week-old male C57BL/6 were fasted overnight, followed by a single oral dose of BGB-5777 at 10 mg/kg or INCB024360 at 50 mg/kg. Three groups of mice were euthanized at indicated time points (1, 2, and 4 hours; $n = 3$/time point) after dosing, respectively. Blood was collected via cardiac puncture, the brain was collected and washed in ice-cold saline and homogenized in ice-cold PBS. The concentration of BGB-5777 and INCB024360 in plasma and brain were determined by LC-MS/MS.

### Pharmacokinetics/pharmacodynamics/tumor growth analysis

Eight-week-old female Balb/c mice were subcutaneously inoculated with $1 \times 10^5$ CT26WT tumor cells. Tumor volumes were measured after becoming visible in two dimensions with calipers and the volumes were expressed in $mm^3$ using the formula: $V = 0.5(a \times b^2)$, where *a* and *b* are the long and short diameters of the tumor, respectively. Animals with tumor volume ranging from 200 to 500 $mm^3$ were randomized into groups with 3 mice per group prior to treatment. Tumor-bearing mice were fasted overnight and followed by oral gavage with BGB-5777. Three groups of mice were treated with 100 mg/kg of BGB-5777 and blood was collected at 0.5, 1, 2, 4, 8, and 24 hours after dosing while tumor tissues were collected at 4 and 24 hours after dosing. Other groups of mice were treated with vehicle (0.5% MC) alone, 25, 50, 100, and 150 mg/kg of BGB-5777 and animals were euthanized at 4 hours after dosing. The concentration of BGB-5777 and kynurenine in plasma and tumors were determined by LC-MS/MS.

Eight-week-old female BALB/c mice were subcutaneously inoculated with CT26WT or A20 tumor cells. Animals were randomized into two groups with 12 mice per group and treated by oral gavage twice daily with vehicle (0.5% MC) or 100 mg/kg BGB-5777. Individual body weight and tumor volume were recorded, twice weekly, with daily monitoring of mice for clinical signs of toxicity for the duration of the study.

### In vitro analysis of IDO1 enzyme activity

Murine unmodified GL261, IDO1 O/E GL261, murine CT-2A, murine WT tGBM or IDO1$^{-/-}$ tGBM cells, as well as human unmodified U87, IDO1 O/E U87, human PDX12 or PDX43 cells, were plated in 12-well plates at a density of $1 \times 10^6$ cells/well and treated with 100 ng/mL mouse or human IFNγ, respectively,

overnight. Twenty-four hours later, cells were washed and treated with 100 ng/mL IFNγ and either 0, 10, 50, or 250 nmol/L BGB-5777 for 48 hours. The media and cell lysates were collected and stored at −80°C for HPLC quantification of Trp and Kyn.

### Flow cytometry and T-cell stimulation

Brain tumor (BT), cervical lymph node (cLN), and spleen were isolated at 3 weeks post-intracranial injection and mashed through a sterile 70-μm nylon mesh cell strainer (Thermo Fisher Scientific) with the rubber plunger end of a 3-mL syringe into ice-cold DMEM. Single-cell suspensions of BT were mixed in a PBS/30% Percoll solution and slowly pipetted onto a 70% Percoll cushion. Samples were centrifuged at 1,200 × *g* for 30 minutes with no brake. The top layer was aspirated and the buffy coat, between the 30% and 70% Percoll layers, was isolated and washed in cold PBS. To stimulate T cells, cell stimulation cocktail (PMA/Ionomycin/Brefeldin A/Monensin; eBioscience) was coincubated with a fraction of the buffy coat isolate for 5 hours in DMEM at 37°C. Suspensions of BT buffy coat, cLN, and spleen were divided into three groups for staining: (i) unstimulated T cells, (ii) stimulated T cells, and (iii) antigen-presenting cells. Cells were incubated with antibodies in PBS + 2% FBS for 30 minutes on ice and according to Supplementary Table S1. Samples were permeabilized overnight at 4°C using Fix/Perm Buffer (eBioscience) and incubated accordingly. Cellular frequency was determined with an LSR Fortessa flow cytometer (BD Biosciences) and Flowjo analysis software (TreeStar).

### RNA isolation and real-time PCR

Total RNA was extracted from freshly dissected tissue samples or cultured glioblastoma cells using the TRIzol Reagent and PureLink RNA Mini Kit (Thermo Fisher Scientific), respectively. One microgram of total RNA was reverse transcribed into mRNA using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was performed on a CFX96 Touch Real-Time PCR Detection System using the default program setting (Bio-Rad). Primers utilized for RT-PCR are described in Supplementary Table S2. The relative quantitation of gene expression was calculated using the $2^{-\Delta\Delta C}{}_t$ method (27) with normalization of the target threshold cycle ($C_t$) values to the internal housekeeping gene (*GAPDH*).

### High-performance liquid chromatography analysis

Serum was isolated from peripheral blood for Trp and Kyn analyses by reverse-phase high-performance liquid chromatography (HPLC) using a Coulochem III detector with a 5041 enhanced analytical cell containing a glassy carbon electrode (+600 mV; ESA) as described previously (28). The mobile phase (pH 4.6) consisted of 75 mmol/L monosodium phosphate ($NaH_2PO_4$), 25 μmol/L ethylenediaminetetraacetic acid (EDTA; disodium salt), and 100 μL/L triethylamine in acetonitrile (6:94 v:v with water). The chromatograms were integrated and quantified using EZChrom SI software (Agilent Technologies). Serum (30 μL) samples were processed in a blinded fashion and mixed with a 0.5% SDS (30 μL) solution, incubated for 15 minutes at room temperature before the addition of 30 μL of 10% sulfosalicylic acid solution, and the proteins were allowed to precipitate on ice for at least 15 minutes. Following precipitation, the samples were centrifuged at 16,100 × *g* for 15 minutes at 4°C. The supernatant was extracted and loaded into a Costar Spin-X centrifuge tube filter (0.22 μm nylon; Corning Inc.) and centrifuged at 12,000 × *g* for 6 minutes at 4°C. The samples were then diluted 1:10 in 0.02 normal perchloric acid ($HClO_4$) prior to analysis. A standard curve was generated daily from the concentrated Trp (9 mg/mL) and Kyn (1 mg/mL) standards, diluted in 0.02 normal $HClO_4$ and held at 4°C until a 20-μL volume was injected into the system. The standards were made using a serial dilution technique so that the standard levels encompassed the expected levels in the serum samples. The standard curve was created using the system software, and samples were not analyzed unless a linear standard curve with $R^2 \geq 0.995$ was achieved.

### Statistical analysis

Data are represented as the mean ± SEM. Lines were fit to enzyme activity plots using linear regression. The statistical significance of the differences in mRNA expression and tumor-infiltrating T-cell response between two groups was determined by Student *t* test or Wilcoxon rank-sum test as appropriate. Differences among multiple groups were assessed using ANOVA with *post hoc* Tukey test, or Kruskal–Wallis test followed by Bonferroni correction as appropriate. Overall survival was defined as the time from glioma cell engraftment until endpoint criteria/death. Survival curves were plotted using the Kaplan–Meier method and compared by log-rank test. Data were analyzed using Prism 6.0 software (GraphPad Software). A *P* value less than 0.05 was considered significant.

## Results

### BGB-5777 potently inhibits IDO1 enzyme activity and is brain penetrant

We tested the uncharacterized IDO1 inhibitor, BGB-5777, for biodistribution and *in vivo* stability. Plotting the reaction velocity of one phase decay over time reveals a half-life ($t_{1/2}$) for BGB-5777 that is more than twice the $t_{1/2}$ as detected for INCB024360; the latter compound of which has been and continues to be evaluated in multiple clinical trials (Fig. 1A and B). Both compounds possess similar inhibitory constants ($K_I$) and IDO1 binding affinities with a half maximal inhibitory concentration ($IC_{50}$) in the low nanomolar (nmol/L) range. Both agents are highly specific for IDO1 and do not inhibit human TDO conversion of L-Trp.

The retention times of BGB-5777 and INCB024360 in the plasma and brain after a single oral dose was compared. Quantification of central nervous system (CNS) BGB-5777 and INCB024360 levels reach their highest concentration of 2.89 μmol/kg and 3.22 μmol/kg, respectively, at 1 hour postadministration (Fig. 1C). BGB-5777 maintains a high brain to plasma ratio of 1.39, 1.39, and 2.08 at 1, 2, and 4 hours posttreatment, respectively, while the brain/plasma ratio for INCB024360 is lower, primarily due to the higher drug concentration in the plasma when compared with the brain. These data collectively demonstrate that the novel IDO1 targeting agent, BGB-5777, possesses a long half-life, high specificity for IDO1 but not for TDO, and effectively penetrates the CNS, supporting further study into determining whether it possesses anticancer properties.

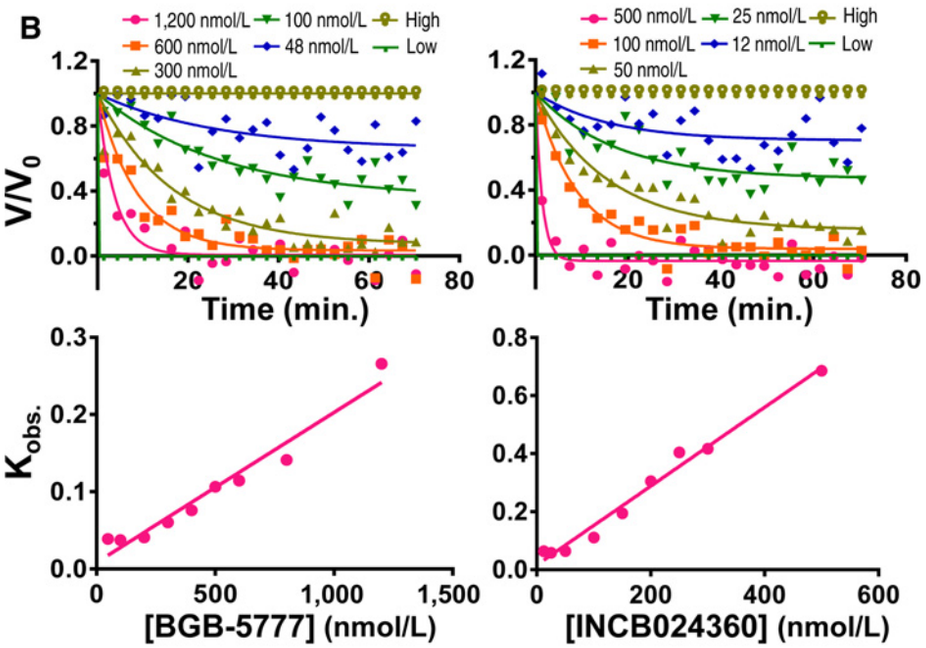
### BGB-5777 and IDO1 enzyme activity in glioblastoma cells

IDO1 is normally expressed at low to undetectable levels in human glioblastoma cells (23), but is rapidly induced by progressively increasing concentrations of human IFNγ (hIFNγ; Supplementary Fig. S1A), a cytokine produced and secreted by

A

| Enzyme | Kinetic measure | BGB-5777 | INCB024360 |
|---|---|---|---|
| hIDO1 | $t_{1/2}$ | 87 min. | 42 min. |
| | $K_I$ | 37 nmol/L | 12 nmol/L |
| | $IC_{50}$ (*L*-Trp) | 39 nmol/L | 16 nmol/L |
| | $IC_{50}$ (*D*-Trp) | 46 nmol/L | 12 nmol/L |
| mIDO1 | $IC_{50}$ (*L*-Trp) | 14 nmol/L | 12 nmol/L |
| TDO | $IC_{50}$ (*L*-Trp) | 1,300 nmol/L | >10 μmol/L |

B




C

| | Time point | Brain (μmol/kg) | Plasma (μmol/L) | Brain/plasma ratio |
|---|---|---|---|---|
| BGB-5777 | 1 h | 2.89±0.62 | 2.26±0.91 | 1.39±0.27 |
| | 2 h | 0.32±0.07 | 0.24±0.07 | 1.39±0.46 |
| | 4 h | 0.06±0.01 | 0.05±0.03 | 2.08±1.83 |
| INCB 024360 | 1 h | 3.22±2.27 | 29.33±19.13 | 0.17±0.15 |
| | 2 h | 4.30±.46 | 4.96±0.48 | 0.87±0.01 |

**Figure 1.**
Biochemical analysis of the IDO1 inhibitor, BGB-5777. **A** and **B,** Kinetic and $IC_{50}$ measurements of pharmacologic IDO1 enzyme inhibitors. Recombinant human and mouse IDO1 and TDO were expressed in and isolated from *E. coli* cells. Human IDO1 (50 nmol/L), mouse IDO1 (20 nmol/L), or TDO (100 nmol/L) were then combined with L-tryptophan (0.1 mmol/L), L-ascorbic acid (5 mmol/L), methylene blue (6.25 μmol/L), catalase (0.4 mg/mL), and IDO1 inhibitor or vehicle only (DMSO), in potassium phosphate buffer (50 mmol/L; pH 7.5) and BSA (0.1%). $IC_{50}$ was calculated on the basis of the remaining enzyme activity in the presence of increasing concentrations of IDO1 inhibitor. $K_{obs.}$ determination was performed using the same methodology as the IDO1 enzyme activity assay with D-tryptophan, except that 150 nmol/L IDO1 was used and sample absorbance was measured immediately after the initiation of reaction. **C,** Blood–brain penetration of BGB-5777 and INCB024360 was evaluated in 6- to 8-week-old male C57BL/6 mice fasted overnight, followed by a single oral dose of BGB-5777 or INCB024360 at 10 mg/kg. The concentration of BGB-5777 or INCB024360 in plasma and brain were determined by the LC-MS/MS method.

activated tumor-infiltrating cytolytic T effector cells. Treatment of human U87 glioblastoma cells with hIFNγ decreases Trp levels from 159.15 nmol/L/mg in unstimulated cells, to 90.93 and 84.97 nmol/L/mg, after coincubation with 100 and 1,000 ng/mL hIFNγ, respectively (Supplementary Fig. S1B). Coincidently, there is a corresponding increase in Kyn concentration that changes from undetectable levels without stimulation, to 42.17 and 49.33 nmol/L/mg after treatment with 100 and 1,000 ng/mL hIFNγ, respectively. The effect of 100 ng/mL hIFNγ treatment is dose dependently inhibited by coincubation of the novel IDO1 enzyme inhibitor, BGB-5777. There is a substantial increase in Trp levels from 54.65 to 131.60 nmol/L/mg, and a decrease in Kyn levels from 85.39 to 35.58 nmol/L/mg, when comparing hIFNγ-stimulated U87 cells without BGB-5777 to the group cotreated with 250 nmol/L BGB-5777, respectively (Supplementary Fig. S1C). Although unmodified murine GL261 and CT-2A glioma cells do not show signs of IDO1 enzyme activity, BGB-5777 effectively inhibits the loss of Trp and accumulation of Kyn in

cell culture supernatants of GL261 cells constitutively expressing murine IDO1 cDNA (GL261 mIDO1 O/E; Supplementary Fig. S1C). The lack of endogenous Trp metabolism among unmodified murine-, but not in human glioma cells, likely reflects biological differences in the regulation of IDO1 expression (Supplementary Fig. S2); raising important considerations for effectively targeting this immunosuppressive mediator when comparing mouse brain tumor models and human patients with glioblastoma.

### Radiotherapy and PD-1 blockade synergize with IDO1 enzyme inhibition to a yield a durable survival increase against established glioblastoma

Encouraged by the results in Fig. 1, and by the ability of monotherapy with BGB-5777 to slow tumor growth in two independent models of subcutaneously engrafted cancer (Supplementary Fig. S3), we next tested the effects of BGB-5777 in an immunocompetent, syngeneic, and well-established GL261-C57BL6 intracranial model of glioblastoma. BGB-5777, whole-brain radiation (WBRT), and PD-1 mAb treatments were administered singularly, dually, or as a triple combination. Of the six treatments, durable, long-term survival is only observed among mice receiving all three agents, and for which the median OS benefit increases from 25 days in the IgG control group, to 53 days ($P < 0.0001$; Fig. 2A; Supplementary Table S3). There is also a trend for trimodal therapy to be more effective with the inclusion of PD-1 mAb, rather than the alternative pathway inhibitor, PD-L1 mAb ($P = 0.12$; Supplementary Fig. S4). To further investigate the effect(s) of trimodal therapy on intracranial tumor growth, mice with intracranial luciferase-modified GL261 (GL261.luc) were treated as above, with tumor growth and response to treatment monitored biweekly for up to 90 days posttumor cell implantation (Fig. 2B and C). Bioluminescence monitoring shows that animal subjects administered IgG control antibody experience progressive tumor growth, whereas tumor bioluminescence in mice receiving trimodal immunotherapy is undetectable by day 43 post-intracranial administration, suggesting that durable tumor control is due to the eradication of glioblastoma.

### Long-term survival from trimodal therapy requires IDO1 inhibition in nontumor cells and a persistent contribution by CD4+ T cells

Given our previous work demonstrating that IDO1 enzyme activity is primarily mediated by nontumor cells (25), combined with the durable survival benefit mediated by trimodal therapy in mice with glioblastoma cells lacking IDO1 enzyme activity (Fig. 2A; Supplementary Fig. S1C), we next questioned whether animal subjects engrafted glioma cells with high IDO1 enzyme activity would show decreased survival after triple-agent therapy. GL261 IDO1 O/E cells constitutively express high murine IDO1 (Supplementary Fig. S2A) and actively catabolize Trp into Kyn (Supplementary Fig. S1C), which more accurately resembles the endogenous capability of human glioblastoma cells to metabolize Trp into Kyn via IDO1 (Supplementary Figs. S1 and S2; ref. 29). C57BL/6 mice intracranially engrafted GL261 IDO1 O/E cells were treated with trimodal therapy, with or without CD4 and CD8 T-cell–depleting antibodies. The results of this experiment show that, even with high IDO1 enzyme activity exclusively mediated by glioblastoma cells, triple-agent therapy significantly increases median OS from 22 days in the IgG group to 32.5 days ($P < 0.0001$), with 4 of 10 mice possessing a durable survival advantage (Fig. 3A). Importantly, the simultaneous depletion of $CD4^+$ and $CD8^+$ T cells abrogate the long-term survival advantage, despite treatment with trimodal therapy.

Given the striking finding that IDO1 enzyme activity by glioblastoma cells is dispensible for the therapeutic effects of triple treatment, we next investigated the potential contribution of nontumor cell IDO1 on treatment efficacy and responsiveness. Wild-type and $IDO1^{-/-}$ mice were intracranially engrafted with unmodified GL261 cells and treated with IgG control antibodies or the dual combination of radiation and PD-1 blockade. There is no survival difference between wild-type and $IDO1^{-/-}$ mice with glioblastoma and administered IgG antibodies (Fig. 3B). In contrast, while wild-type mice fail to show a durable survival benefit after glioblastoma treatment with dual radiation and PD-1 blockade, the same combination results in a long-term survival advantage in approximately 25% of $IDO1^{-/-}$ mice. Importantly, the triple-agent treatment of IDO1 enzyme inhibitor with radiotherapy and PD-1 blockade facilitates a similar durable survival advantage in both wild-type and $IDO1^{-/-}$ mice (Fig. 3B). These data suggest that nontumor cells are the primary target of IDO1 enzyme inhibition required for trimodal therapy effectiveness.

We next questioned whether specific immune cell subpopulations were selectively important to the overall efficacy of trimodal therapy. Utilizing an early (beginning at 5 days post-GL261 intracranial injection) or late (beginning at 14 days post-GL261 intracranial injection) leukocyte depletion approach, we determined that the early depletion of $CD4^+$ T, $CD8^+$ T, or NK cells, abrogates the durable survival benefit of triple-agent therapy (Fig. 3C). Unexpectedly, when immune cell subsets are neutralized and/or depleted late, or at the same time as trimodal treatment initiation, only the lack of $CD4^+$ T cells abrogates the long-term survival benefit (Fig. 3D). These results suggest the intriguing conclusion that, while $CD4^+$ T, $CD8^+$ T, and NK-cell subsets are required early in the anti-glioblastoma immune response, only $CD4^+$ T cells are continuously required to achieve maximal survival after trimodal immunotherapy.

### Effects of trimodal therapy on the immune response and metabolism in glioblastoma

We examined the impact of trimodal therapy on tumor-infiltrating T cells, immunomodulatory gene expression, as well as Trp, Kyn, and BGB-5777 levels at 1 week after treatment initiation. The triple immunotherapeutic combination significantly decreases the absolute numbers of both Tregs and cytolytic T cells in unmodified GL261 tumors ($P < 0.05$; Fig. 4A). In contrast, trimodal treatment has no effect on Treg levels, but significantly increases absolute numbers of $CD8^+$ T cells, from 160.33 in the IgG group, to 500.78 in the triple-agent therapy group of GL261 IDO1 O/E tumors ($P < 0.05$). Interestingly, high glioblastoma cell IDO1 expression is associated with increases in the number of immunosuppressive Tregs and myeloid-derived suppressor cells (MDSC; Supplementary Fig. S5) regardless of therapeutic intervention and/or inclusion of the IDO1 enzyme inhibitor ($P < 0.05$; Fig. 4A and B), as compared with unmodified glioblastoma tumors.

Gene expression analysis confirms the 12.5-fold increase of IDO1 mRNA levels in GL261 IDO1 O/E tumors as compared with unmodified GL261 tumors (Fig. 4C; $P < 0.01$). Intervention with trimodal therapy does not change the level of IDO1 expression within each GL261 tumor type and as compared with the

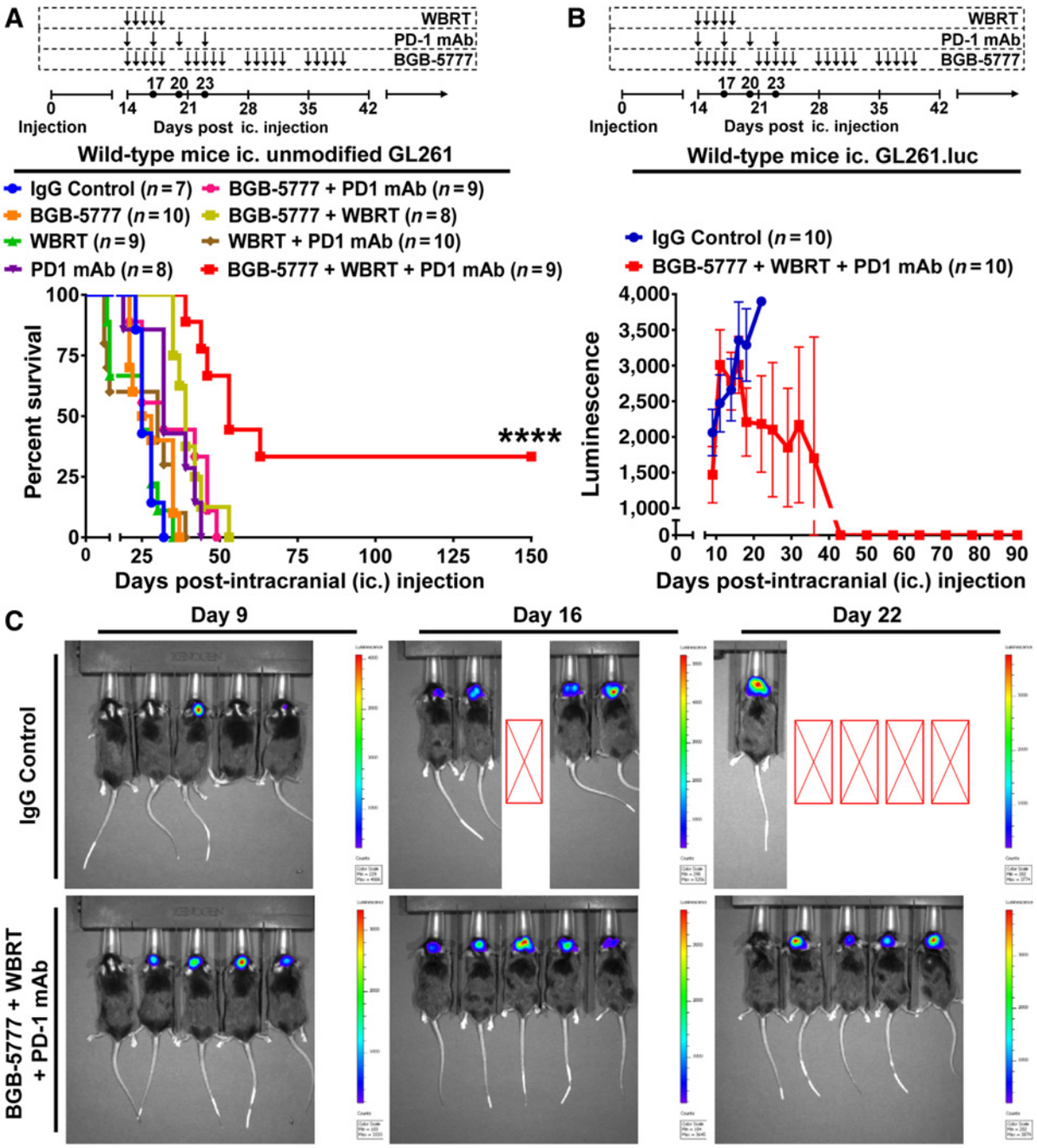



**Figure 2.**

Overall survival and tumor growth of established murine glioblastoma (GBM) simultaneously treated with an IDO1 enzyme inhibitor, radiation, and PD-1 blockade. **A,** Survival analysis for wild-type C57BL/6 mice intracranially engrafted $2 \times 10^5$ syngeneic unmodified GL261 and receiving mono- or combination-therapy with BGB-5777, whole-brain radiation (WBRT) and/or PD-1 mAb beginning at 14 days post-intracranial injection (post-ic.); IgG antibody treatment was included as a control group. **B,** Wild-type mice were intracranially injected with $2 \times 10^5$ GL261 cells expressing luciferase (GL261.luc) and treated with mono- and combination therapies as described above. Average tumor luminescence was determined using an IVIS spectrum imaging station following intraperitoneal (IP) injection of D-Luciferin (150 mg/kg). **C,** Representative IVIS spectrum images of treated mice with intracranial GL261.luc at days 9, 16, and 22 post-intracranial injection. ****, $P < 0.0001$

respective IgG control groups. Similar to IDO1 gene expression, the antitumor T-cell cytokine, IFNγ, is substantially increased in IDO1 O/E glioblastoma regardless of therapy ($P < 0.0001$). In contrast, gene level changes for other factors associated with immunosuppression including TDO, CTLA-4, FoxP3, and/or PD-L1, show nonoverlapping patterns with IDO1 expression in

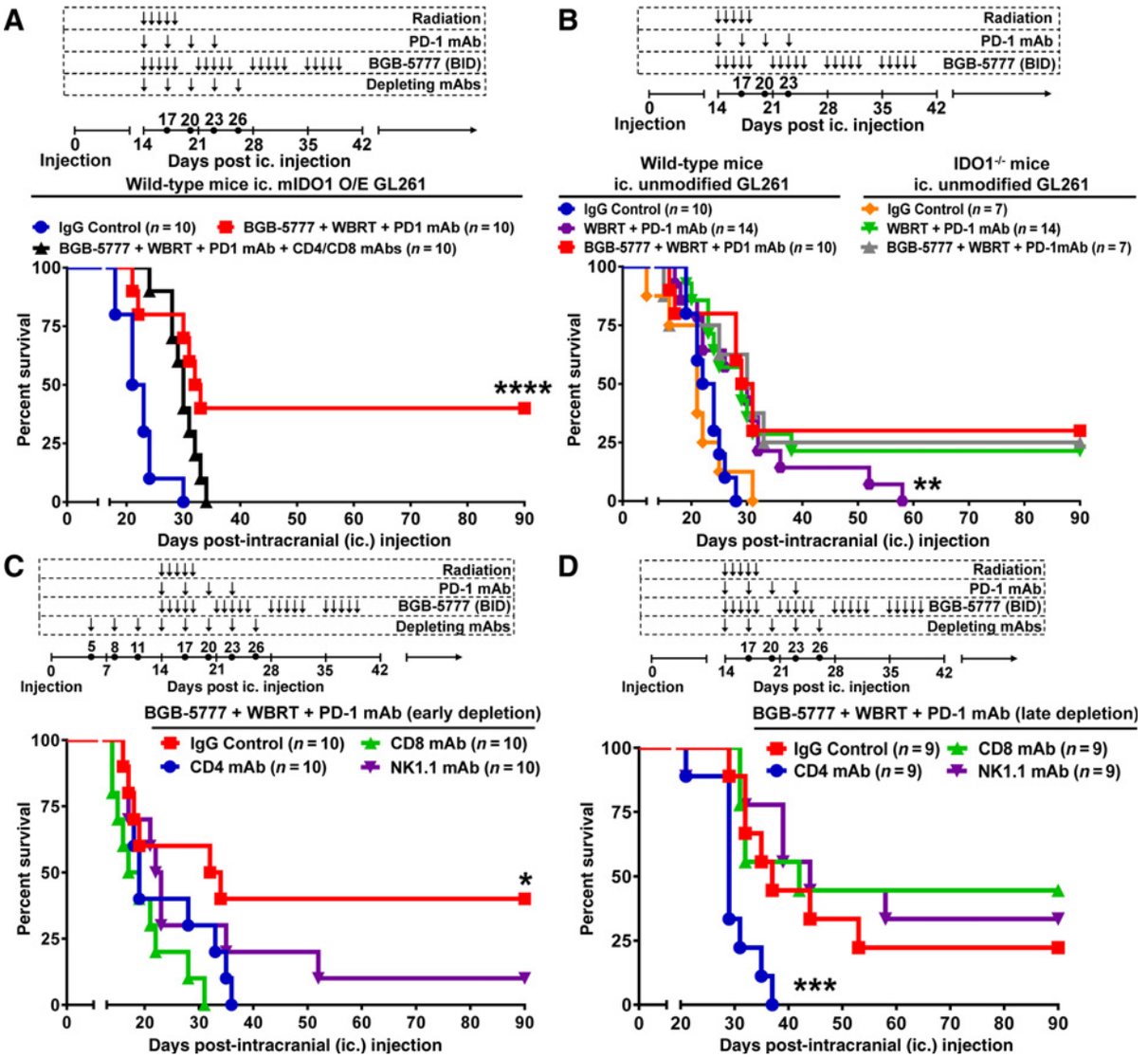



**Figure 3.**

Maximum survival benefit from trimodal therapy requires IDO1 inhibition in nontumor cells and select leukocyte engagement. **A,** Survival analysis of wild-type mice intracranially injected $2 \times 10^5$ GL261 cells expressing murine IDO1 cDNA (GL261 mIDO1 O/E) and receiving trimodal therapy, in the presence or absence of CD4-/CD8-T cell–depleting mAb beginning at day 14 post-intracranial injection. **B,** Survival analysis of wild-type and IDO1 knockout ($IDO1^{-/-}$) mice intracranially injected with $2 \times 10^5$ unmodified GL261 tumors and receiving whole brain radiation (WBRT) and PD-1 mAb treatment, in the presence or absence of BGB-5777 IDO1 inhibitor. Survival analysis of wild-type mice after trimodal therapy, with or without addition of CD4-, CD8- or NK1.1–cell depleting antibodies. **C,** Beginning at day 5 post-intracranial injection or **D,** beginning at day 14 post-intracranial injection (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

glioblastoma, supporting the existence of multiple immune evasion pathways that act independent of IDO1 regulation.

Triple-agent treatment decreases circulating Kyn levels in mice with unmodified GL261 when compared with the IgG control group (Fig. 4D; $P < 0.01$). Also notable, serologic Trp levels do not change due to tumor burden, increased IDO1 metabolic activity in glioblastoma cells, or the presence of triple-agent therapy. Within glioblastoma, trimodal therapy does not change Kyn or Trp levels in unmodified tumors and this outcome is concordant with BGB-5777 treatment administered as a monotherapy (Supplementary Fig. S6). In contrast, GL261 IDO1 O/E tumors possess dramatically increased Kyn levels and a significantly higher Kyn/Trp ratio that is decreased with both trimodal ($P < 0.01$; Fig. 4D) and monotherapeutic IDO1 enzyme inhibitor treatment ($P < 0.05$; Supplementary Fig. S6). Coincident with the effect on intratumoral IDO1 enzyme inhibition, BGB-5777 accumulation is similar between unmodified and IDO1O/E brain tumors (Fig. 4E), confirming a high penetration and retention of BGB-5777 within the brain tumor and independent of IDO1 expression/enzyme activity.

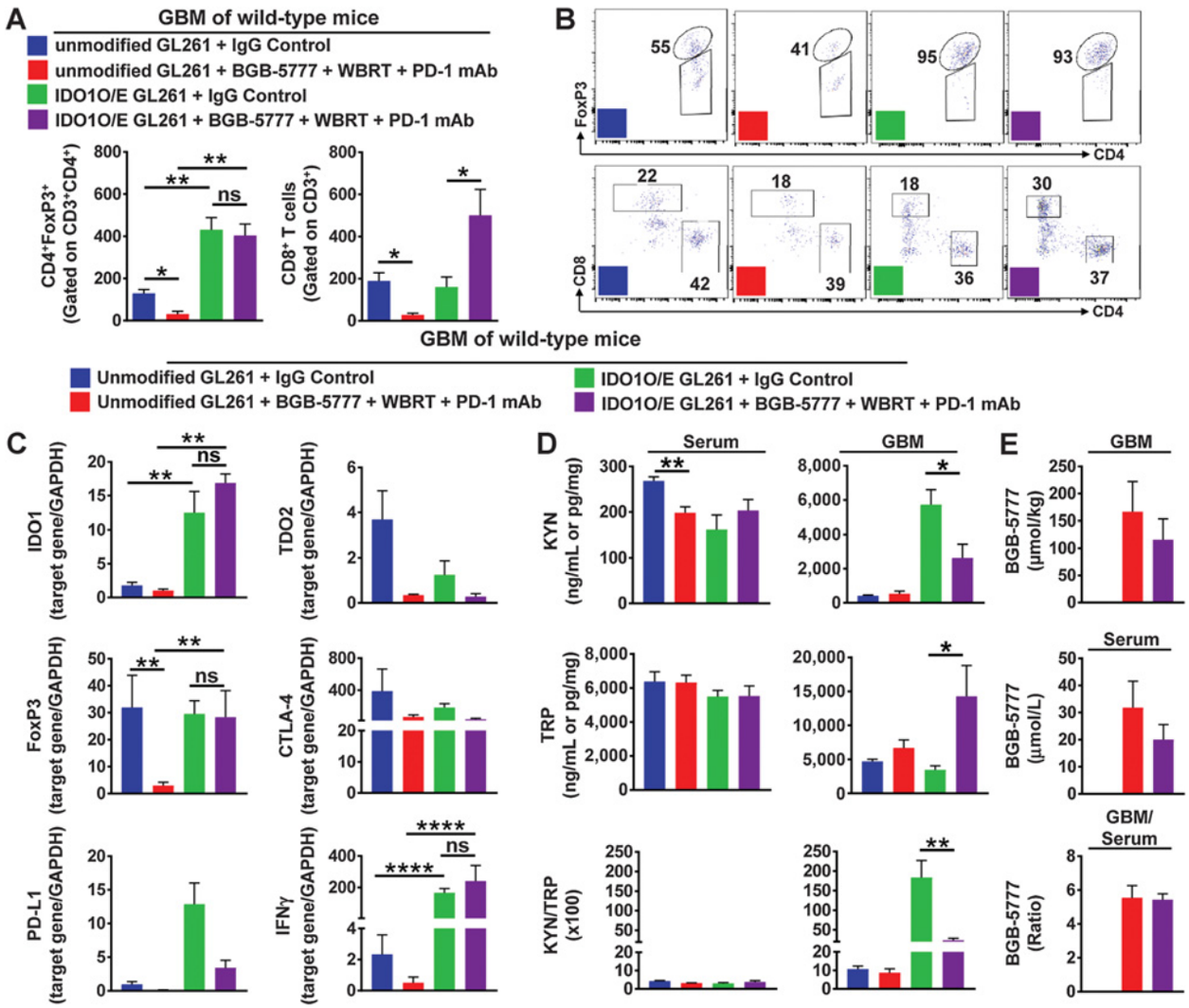



**Figure 4.**

Cellular and molecular analysis of unmodified or IDO1-expressing intracranial glioblastoma (GBM) treated with or without trimodal therapy. Wild-type mice were intracranially injected with $2 \times 10^5$ unmodified GL261 or with GL261 cells expressing murine IDO1 cDNA (GL261 mIDO1 O/E) and treated with trimodal therapy or IgG antibodies. **A,** Absolute numbers of intratumoral $CD3^+CD4^+FoxP3^+$ regulatory T (Treg) and $CD3^+CD8^+$ cytolytic T cells were quantified via flow cytometry ($n = 5$/group). **B,** Gating strategy and representative proportions of Tregs and cytolytic T cells are shown. **C,** mRNA expression levels for IDO1, TDO, FoxP3, CTLA-4, PD-L1 and IFNγ in unmodified and mIDO1 O/E GL261 cell lysates. **D,** Tryptophan (Trp) and kynurenine (Kyn) levels were measured by high-performance liquid chromatography (HPLC) in serum and tumors ($n = 5$/group). **E,** Quantification of BGB-5777 concentration in the same tissue samples analyzed in **D** and determined by liquid chromatography/mass spectrometry (LC-MS/MS). *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; ns, not significant

### Trimodal therapy is less effective with advanced age

Glioblastoma is an age-related disease with a median diagnosis at 64 years old in human patients (1). We previously showed that, 74-week-old mice analogous in age to an adult glioblastoma patient diagnosis, possess a marginally faster glioblastoma mortality rate when compared with a 6- to 8-week-old mouse (30). To determine whether animal subject maturity has a negative effect on the survival benefit provided by trimodal therapy, 8- and 72-week-old mice were treated with BGB-5777, radiotherapy and PD-1 mAb. As shown in Fig. 5A, triple-agent treatment results in a median survival of 31.5 days ($n = 8$) in 72-week-old mice, which is significantly decreased when compared with 8-week-old mice with a survival of 40.5 days ($n = 10$; $P < 0.001$). Importantly, while only 12.5% (1/8) of elderly mice are still alive at 90 days post-glioblastoma challenge, 60% (6/10) of young mice show a durable glioblastoma survival benefit after trimodal therapy, supporting the concept of a negative role for advanced age on immunotherapeutic efficacy of glioblastoma. Gene expression was analyzed for IDO1, IDO2, and TDO levels in the young and aged brain engrafted glioblastoma and treated with triple-agent therapy. The results support our previous observation that advanced age selectively increases nontumor cell IDO1 expression, but has no effect on the alternative Trp metabolic enzymes, IDO2 and TDO (Fig. 5B; ref. 30). The lack of increased IDO1

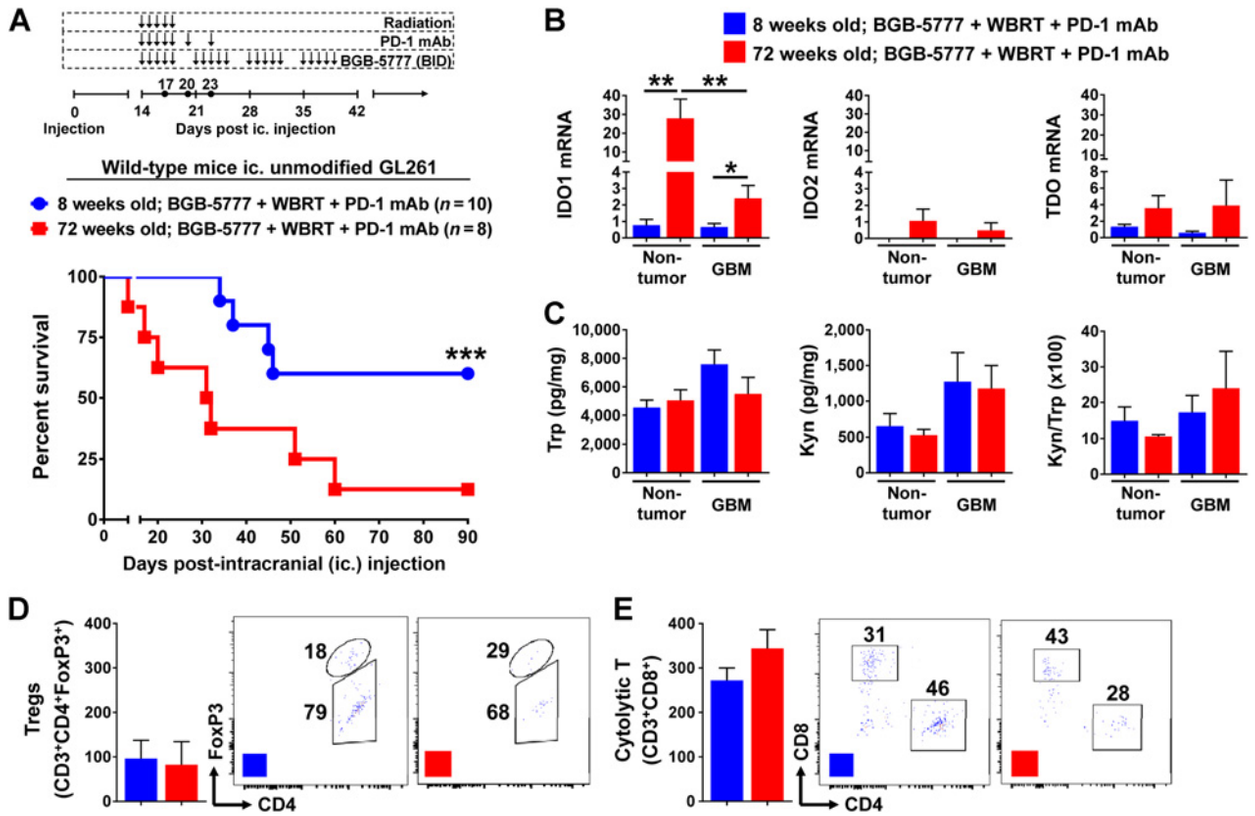



**Figure 5.**

Advanced age decreases the survival benefit of trimodal therapy against glioblastoma (GBM). **A,** Survival analysis of young (8-week-old) and old (72-week-old) wild-type mice intracranially injected with $2 \times 10^5$ unmodified GL261 and treated with BGB-5777, whole-brain radiotherapy (WBRT) and PD-1 mAb (trimodal therapy). Young and old mice were intracranially injected with $2 \times 10^5$ unmodified GL261, treated with trimodal therapy beginning at 14 days post-intracranial injection, followed by GBM and contralateral brain without tumor (nontumor) isolation at 3 weeks post-intracranial injection. **B,** Quantitative gene expression analysis for IDO1, IDO2, and TDO ($n = 5$/group) was performed. **C,** Tryptophan (Trp) and kynurenine (Kyn) levels were analyzed ($n = 5$/group). **D,** Absolute numbers and representative proportions of intratumoral $CD3^+CD4^+FoxP3^+$ regulatory T (Treg; $n = 5$/group) were quantified. **E,** Absolute numbers and representative proportions of $CD3^+CD8^+$ cytolytic T cells were quantified ($n = 5$/group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

metabolic activity in the nontumor cell fraction may reflect IDO1 enzyme inhibitor treatment as part of trimodal therapy (Fig. 5C). There was also no difference in absolute numbers of glioblastoma-infiltrating Tregs or cytolytic T cells among the young and old mice treated with triple-agent therapy (Fig. 5D and E).

### Trimodal therapy is effective in multiple glioma models, induces strong immunologic memory, and is limited by systemic chemotherapy

Throughout our investigation, we primarily evaluated the treatment effects of BGB-5777 with radiotherapy and anti-PD-1 in the GL261/GL261-derivative glioblastoma model(s). To address whether trimodal treatment is potentially generalizable as an anti-glioblastoma therapy, CT-2A tumors were established in syngeneic C57BL6 mice and subsequently treated as before. Similar to our results for GL261 tumors, trimodal treatment significantly increases overall survival as compared with the IgG control group (35.5 vs. 20 days, respectively; $P < 0.001$) with durable tumor control achieved in >25% of mice (Fig. 6A).

To determine whether trimodal therapy leads to sustained protection from tumor recurrence, mice that survived the original GL261 inoculation after treatment with triple-agent therapy, were reinjected with GL261 cells into the contralateral brain hemisphere, opposite of the original tumor challenge site. One-hundred percent of mice rechallenged with GL261 cells demonstrated a complete rejection/control of the glioblastoma, as evidenced with the ≥90-day survival benefit (Fig. 6B). Unexpectedly, >50% of mice rechallenged with CT-2A cells also showed a durable survival benefit to the initial treatment and presumed rejection of GL261 tumors, suggesting some form of cross-reactive immunologic protection initiated by the original tumor cell–immune system encounter and orchestrated by the trimodal treatment approach (Fig. 6B).

Given that the current standard-of-care adjuvant treatment in glioblastoma patients involves both radiation and chemotherapy with temozolomide, we next tested the effect of systemic temozolomide treatment with trimodal therapy, for determining whether this agent would further enhance antitumor efficacy. For this purpose, mice with intracranial GL261 received trimodal therapy, alone, or combined with temozolomide. The systemic

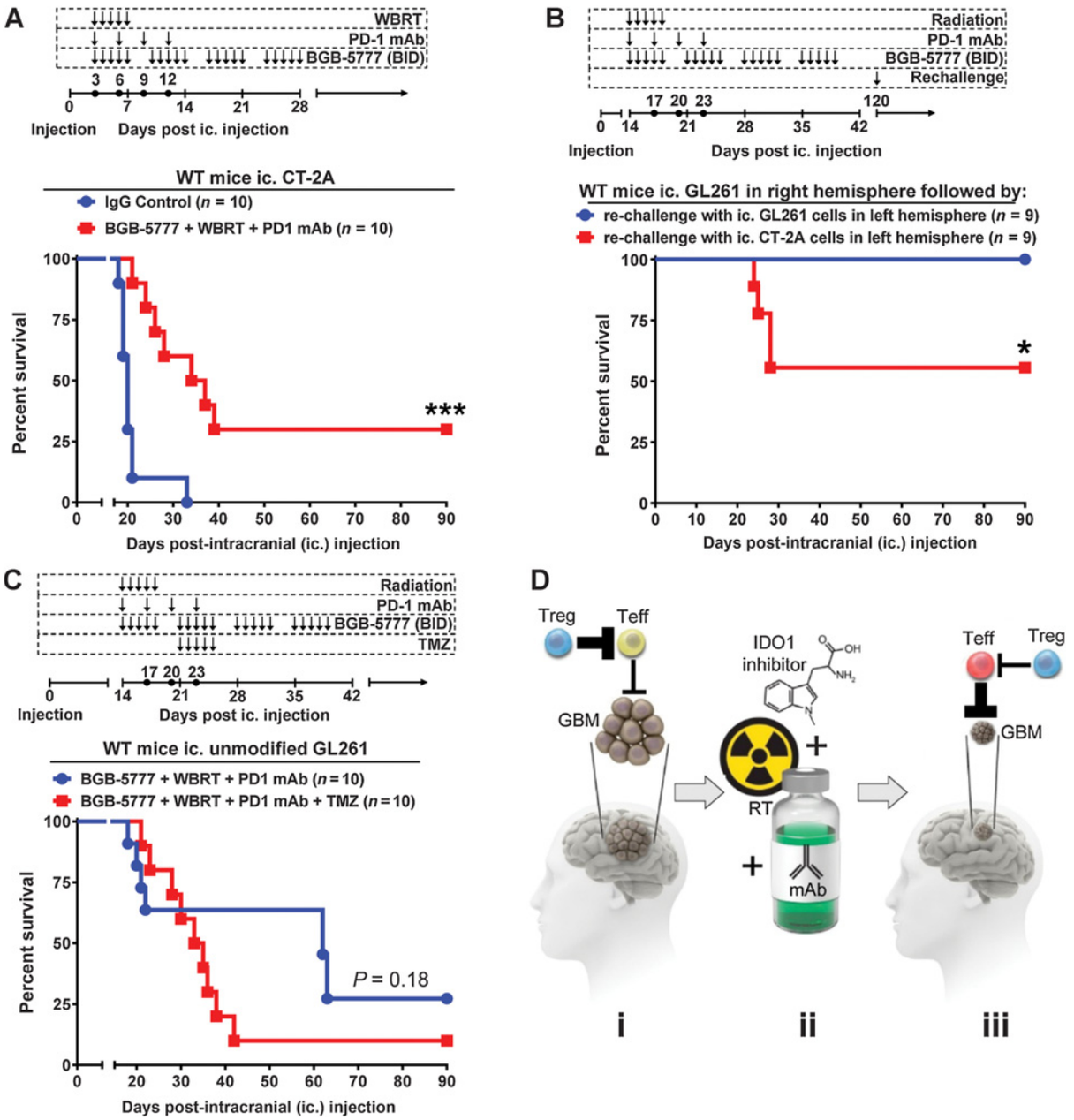



**Figure 6.**

Trimodal therapy is effective against multiple glioblastoma (GBM) models, induces strong immunological memory and is limited by systemic temozolomide (TMZ). **A,** Survival analysis of wild-type mice intracranially injected $1 \times 10^5$ syngeneic CT-2A glioma cells and administered trimodal therapy initiated at 3 days post-intracranial injection. **B,** Wild-type mice were intracranially injected with $2 \times 10^5$ GL261 in the right brain hemisphere and treated with BGB-5777, whole-brain radiotherapy (WBRT) and PD-1 mAb. Mice surviving for 120 days were rechallenged with either $2 \times 10^5$ unmodified GL261 or $1 \times 10^5$ CT-2A cells in the left brain hemisphere. **C,** Treatment strategy and survival analysis of wild-type mice intracranially injected with $2 \times 10^5$ unmodified GL261 cells and treated with trimodal therapy, with or without systemic administration of TMZ. **D,** Model for IDO1 inhibition synergizing with radiotherapy and PD-1 blockade to modulate the antitumor immune response against GBM. (i) The glioblastoma is associated with an impaired antitumor immune response due to potently immunosuppressive mechanisms, such as regulatory T cell (Treg) inhibition of antitumor effector T (Teff) cells. (ii) Host treatment with concurrent IDO1 enzyme inhibition, radiotherapy (RT) and systemic PD-1 mAb (iii) limits the ability of immunosuppressive cells to inhibit Teff cell antitumor activities (*, $P < 0.05$; ***, $P < 0.001$).

administration of temozolomide decreased the proportion of long-term survivors to just 10%: a 3-fold reduction when compared with trimodal agent treatment administered in the absence of temozolomide (Fig. 6C).

A proposed model based on the preclinical data presented above (Fig. 6D) suggests that glioblastoma patients with an unproductive immune response due to potent intratumoral immune suppression (i), may be converted into a productive

antitumor state with trimodal therapy (ii), which aims to limit immune evasion mechanisms and thereby enhance T effector (Teff) cell control of glioblastoma (iii).

## Discussion

In this study, we investigated a novel three-agent therapeutic approach for treating established glioblastoma. The rationale for testing this innovative treatment strategy was based, in part, on our recent findings showing that infiltrating T cells upregulate immunosuppressive IDO1 expression in human glioblastoma. On the basis of work related to the best-in-class IDO1 enzyme inhibitor, INCB024360, demonstrating a favorable safety profile combined with promising tumor control when combined with PD-1 blockade in NSCLC patients, rationale for testing this approach was recently strengthened (24). We hypothesized that the further combination of dual immune checkpoint inhibition with radiotherapy would provide increased T-cell infiltration and/or effector functions in glioblastoma, providing for optimal immune system control of glioblastoma. Our investigation began by characterizing a new IDO1 enzyme inhibitor, BGB-5777, and established its selectivity and effectiveness for inhibiting IDO1-mediated metabolism, as well as its ability to penetrate and accumulate in the brain (Figs. 1 and 4). When used to treat large, intracranial, advanced glioblastoma, BGB-5777 monotherapy had no effect on overall survival. Similarly, monotherapeutic radiation or PD-1 blockade, as well as all dual therapies including radiotherapy combined with PD-1 mAb, failed to durably increase overall survival in animal subjects with advanced glioblastoma. However, the trimodal treatment approach resulted in a durable survival benefit for significant proportions of animal subjects and for which there was no indication of tumor recurrence (Figs. 2, 3, 5 and 6).

A number of unexpected mechanistic findings arose during our study that further inform our understanding of IDO1 and its role in malignant glioma. Comparison of treatment effects in mice with intracranially engrafted unmodified GL261 versus GL261 modified to possess high IDO1 expression/metabolism, support the hypothesis that trimodal treatment efficacy is not dependent on tumor cell IDO1 expression and/or activity (Fig. 4C and D). Similarly, trimodal therapy had no effect on intratumoral Treg levels in glioblastoma with high tumor cell IDO1 expression (Fig. 4A and B), even though enzyme activity was substantially inhibited (Fig. 4D). This result is supported by a reciprocal experiment that we previously conducted whereby the stable knockdown of mouse glioblastoma cell IDO1, led to the lack of immunosuppressive Treg recruitment and the spontaneous rejection of intracranial tumors when engrafted into immunocompetent, but not T-cell–deficient mice (8, 25). These findings therefore raise the intriguing and controversial hypothesis that IDO1 in tumor cells regulates Treg accumulation and suppression of the glioblastoma immune response via a nonenzyme-dependent mechanism, which was previously demonstrated to play a critical role in mouse plasmacytoid dendritic cells (pDC; ref. 31). Regardless of the mechanism that glioma cell IDO1 utilizes to regulate Treg accumulation, neither the increased expression of IDO1 (Fig. 4C), nor the extra accumulation of intratumoral Tregs (Fig. 4A and B), were associated with a negative survival impact on trimodal therapeutic efficacy (Figs. 2A and 3A). In addition, it was previously discovered that radiation leads to increased PD-L1 expression (32). In contrast, our results suggest a negative regulation of PD-L1 expression by trimodal therapy in IDO1 O/E glioblastoma (Fig. 4C). This effect appears to be independent of both the enhanced $CD8^+$ T-cell infiltration (Fig. 4A and B) and lack of change in IFNγ expression (Fig. 4C); both of which are potent drivers of PD-L1 expression *in vitro* (33).

A major finding of our study relates to the dependence of nontumor cell IDO1 for the synergistic benefit with radiation and PD-1 antibody treatment (Fig. 3B). This result contrasts with our previous work evaluating the effects of CTLA-4/PD-L1 inhibition and its negative impact when host IDO1 is absent (15, 25). These dichotomous discoveries highlight important considerations for host cell IDO1 origin and its beneficial or maladaptive association with immunotherapeutic interventions. Previous work in a non-CNS model of cancer identified that, pDCs express IDO1 in tumor draining lymph nodes and potently suppress T-cell responses to presented antigens, *in vitro* (34). Further investigation demonstrated that the IDO1 inhibition of dendritic cells is responsible for the antitumor effects in non-CNS models after treatment with different forms of chemotherapy, *in vivo* (35). These collective observations suggest the hypothesis that pDCs are the primary target(s) of IDO1 enzyme inhibition in the context of trimodal therapy. Given the previous localization of $IDO1^+$ pDCs in lymph nodes, as well as their capability to potently activate Tregs (36), it will be interesting to determine whether they retain their immunosuppressive role in lymph nodes, or are required to take up residence within the CNS, in future modeling of glioblastoma.

The early presence of T cells, and to a lesser extent NK cells, were required to mediate the durable survival advantage of triple therapy with IDO1 inhibitor, RT, and PD-1 mAb (Fig. 3C and D). In contrast, the late depletion of T cells and/or NK cells showed a surprising difference in the effect on overall survival. While $CD8^+$ T and NK cells were dispensible for the long-term survival benefit when depleted at the late time point, a requirement for $CD4^+$ T cells persisted throughout the early and late time points, suggesting that this subset is temporally indispensable for the long-term regulation, support, and/or maintenance of effector anti-glioblastoma T-cell function(s). This finding is interesting, primarily based on our previous work demonstrating that both $CD4^+$ and $CD8^+$ T cells are required at the late time point for long-term survival mediated by CTLA-4 and PD-L1 blockade against advanced glioblastoma (15). The distinct requirements for different T-cell subsets to achieve glioblastoma control with different immunotherapeutic strategies, suggest multiple mechanisms of action that ultimately contribute to the same derivative survival benefit. This may also help to explain the association between trimodal therapeutic treatment of unmodified GL261 tumors and the unexpected decrease of glioblastoma-infiltrating $CD8^+$ T cells (Fig. 4A). Previous work in glioblastoma (37), as well as other inflammatory settings (38) demonstrate that, PD-1 blockade selectively enhances activation and proliferation of $CD4^+$ T cells. The results from our immune cell depletion studies may therefore reflect the unique importance of $CD4^+$ T cells to mediate CNS tumor immunity during PD-1 pathway intervention.

As far as we know, this study is the first to demonstrate a major disadvantage of advanced age on the beneficial effects of combination immunotherapy for treating glioblastoma (Fig. 5A). This is a clinically relevant consideration given that the median age of a glioblastoma diagnosis is at 64 years old (1). The primary factor associated with the decreased therapeutic effectiveness was enhanced IDO1 expression, which we and others have previously

shown to increase with age in the brain (30, 39). In-line with our previous observations, IDO2 and/or TDO expression did not differ, or was not expressed, in glioblastoma isolated from wild-type mice (Fig. 5B), further highlighting the important and age-dependent role of IDO1 in nontumor cells. These data raise several questions that relate to the cellular source(s) of IDO1 expression during aging (40), the age-related impact of IDO1 on immunotherapeutic responsiveness, and whether the age-associated enhancement of CNS IDO1 expression contributes to glioblastoma cell initiation in adults. On the basis of our analysis demonstrating greater immunotherapeutic efficacy in young mice, we also consider the clinical possibility that the combination treatment of IDO1 inhibitor, radiotherapy, and PD-1 blockade will have an especially prominent benefit in younger adults with glioblastoma and/or children with malignant brain tumors.

Another novel aspect of our investigation found that, animal subjects intracranially-engrafted GL261 and treated with trimodal therapy, completely resisted a 4-month rechallenge with the same cell line intracranially injected into the contralateral brain hemisphere. Surprisingly, the immunologic memory to GL261 also conveyed a durable survival advantage when rechallenged with the more aggressive and highly immune checkpoint inhibitor–resistant, CT-2A glioma model (Fig. 6B). This finding potentially suggests that, either the enhancement of shared glioma cell antigens facilitate immune-mediated protection and/or the trimodal therapy mediates epitope spreading that provides protection against nonclonal derivative glioma cells as they relate to the initial challenge. Regardless of the mechanism, the translational implications of this finding suggest that, if the simultaneous treatment of IDO1 inhibition, RT, and PD-1 blockade is able to achieve durable immunologic control of initial glioblastoma outgrowth, endogenous rechallenge events, likely as a result of mutation-driven tumor evolution, may be further controlled by an intact immune system.

An important consideration for the effective clinical translation of our findings relates to the observation that only a subset of mice benefit from simultaneous IDO1 inhibition with radiotherapy and PD-1 blockade. Several possibilities may help to explain the lack of universal (i.e., 100% survival) benefit. The simplest possibility hypothesizes that the treatment strategy is more effective against smaller, less established glioblastoma. While this possibility is reasonable, we consider it unlikely given the confirmation of similar bioluminescence intensities among all intracranial tumors at the time of treatment initiation at 14 days post-engraftment (Fig. 2C). Another possibility is that a behavioral component, such as dominance, social interaction, or feeding/drinking behavior, provides an adaptive advantage for mediating a beneficial response to immunotherapy (41). It is also tempting to speculate that variance in dietary tryptophan intake (42), exercise (43), an impact on body temperature (44), or a combination of these factors contribute to the therapeutic fitness. At this time, we are exploring multiple forms of behavioral conditioning that may help to improve response rates to immunotherapy, with preliminary findings demonstrating actionable considerations to future clinical trials.

Here, we have characterized a novel, pharmaceutical-grade, highly selective IDO1 enzyme inhibitor, BGB-5777, and its ability to inhibit the conversion of Trp into Kyn at nanomolar potency, without affecting the inhibition of other professional mammalian tryptophan catabolic enzymes (i.e., TDO). We further showed that, BGB-5777 effectively crossed the blood–brain barrier and accumulated in the central nervous system, supporting its potential to serve as a future agent targeting IDO1 enzyme activity in malignant brain tumors. The translationally relevant, trimodal combination of BGB-5777, RT, and PD-1 blockade significantly increased the median overall survival in mice with large established tumors through a T-cell–dependent antitumor mechanism. Our data further suggested that the inhibition of Trp catabolism in glioblastoma cells was dispensible for Treg accumulation and the therapeutic efficacy mediated by triple-agent treatment. In contrast, a surprising conclusion of this work shows that, therapeutic synergy with IDO1 enzyme inhibition is primarily mediated by targeting non-glioblastoma cells. Animal subject age and concurrent temozolomide usage also played a critical role in therapeutic responsiveness. At this time, we are translating these findings into a phase I/IIa clinical trial in newly diagnosed glioblastoma patients.

## Disclosure of Potential Conflicts of Interest

R.V. Lukas is a consultant/advisory board member for Abbvie, Arbor, AstraZeneca, and Ziopharm, reports receiving speakers bureau honoraria from American Physician Institute, EBSCO, and Medlink Neurology, and has received travel support from Roche. No potential conflicts of interest were disclosed by the other authors.

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## Acknowledgments

E. Ladomersky is supported by PHS grant number T32CA070085. D.A. Wainwright is supported by PHS grant number R00NS082381, R01NS097851 awarded by the NIH/NINDS, U.S. Department of Health and Human Services; a Robert H. Lurie Comprehensive Cancer Center – Zell Scholar Program of the Zell Family Foundation Gift; Wade F.B. Thompson/Cancer Research Institute (CRI) Clinic and Laboratory Investigation Program (CLIP) Investigator; the Curing Kids Cancer Foundation; the IDP Foundation of the Robert H. Lurie Comprehensive Cancer Center and the Lou and Jean Malnati Brain Tumor Institute at Northwestern Medicine. We thank the Northwestern University Center for Advanced Microscopy (generously supported by NCI CCSG P30 CA060553 awarded to the Robert H. Lurie Comprehensive Cancer Center) and the Northwestern University Flow Cytometry Core Facility (generously supported by Cancer Center Support Grant, NCICA060553) for their technical support of microscope image acquisition and flow cytometry, respectively. We thank Mr. Michael Gallagher for his illustration expertise that led to the creation and rendering of Fig. 6D.

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Received November 29, 2017; revised January 21, 2018; accepted February 27, 2018; published first March 2, 2018.

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