

## Supplementary Methods

**Cell lines.** The murine glioma cell lines GL261 (Tumor Bank Repository, National Cancer Institute, Frederick, MD), GL261-5 (a clone with slower *in vivo* growth kinetics)<sup>1</sup>, and human HeLa and HEK293 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium with nutrient mixture F12 (DMEM/F12) (Corning). Murine GSC-005 glioma cells (kindly provided by I.M. Verma, The Salk Institute for Biological Studies, CA)<sup>2</sup> were maintained in DMEM/F12 supplemented with N2 (1x; Invitrogen), fibroblast growth factor-2 (20 ng/ml; PeproTech), epidermal growth factor (20 ng/ml; Promega), and heparin (50 µg/ml; Sigma). The murine breast cancer cell line 4T1.2 expressing the luciferase gene (kindly provided by C. Bartholomeusz, MD Anderson Cancer Center, TX) was maintained in RPMI 1640 medium (Corning). The human glioma-like stem cell lines GSC11, GSC20, GSC7-2, GSC13, and GSC8-11<sup>3-5</sup> were cultured in DMEM/F12 with B27 (1x; Invitrogen), antibiotic-antimitotic (1x; ThermoFisher Scientific), fibroblast growth factor-2 (20 ng/ml), and epidermal growth factor (20 ng/ml). Cultures, except for cultures of GSCs, were supplemented with 10% fetal bovine serum (HyClone Laboratories) and antibiotics (100 µg/ml penicillin, 100 µg/ml streptomycin; Corning). All cells were kept at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Oncolytic adenoviruses.** A previously constructed oncolytic adenovirus, Delta-24-RGDOX<sup>1</sup>, was propagated in human lung carcinoma A549 cells. Virions were collected and purified using the Adenopure kit (Puresyn, Inc.) following the manufacturer's instructions. Viral titers and replication were determined by measuring the plaque-forming units per ml (pfu/ml) using conventional methods. Briefly, HEK293 cells ( $2.5 \times 10^5$ ) were incubated in 24-well plates with serial dilutions of the viral stock. Forty-eight hours later, cultures were fixed with 100% ice-cold methanol for 10 min at -20°C. Cells were stained for hexon expression using an anti-adenovirus polyclonal antibody (Millipore Sigma, AB1056; 1 h, 37°C) followed by secondary staining with a biotinylated anti-goat IgG antibody (H+L, Vector Biolabs, BA-5000; (1 h, 37°C). The Vector Vectastain ABC kit (Vector Biolabs, PK-4000) and ImmPACT DAB peroxidase substrate kit (Vectro Biolabs, SK-4105) were utilized for the visualization of positive cells. Hexon-stained areas were counted under a light microscope (20× objective) in 10 individual fields per well. In wells with viral dilutions showing 5-50 positive cells/field, the viral titer was calculated using the following formula:  $\text{pfu/ml} = [(\text{mean number of positive cells/field}) \times (\text{fields/well})] / [\text{volume virus (ml)} \times \text{dilution factor}]$ . The antibodies used are shown in Supplementary Table 1.

**Viral replication assay.** To determine the replication ability of Delta-24-RGDOX in the adherent cell lines used in this study, we seeded  $1.5 \times 10^5$  cells/well in a 12-well plate and counted the cells after letting them attach overnight to calculate the appropriate amount of Delta-24-RGDOX needed to achieve specified multiplicities of infection (MOIs). Delta-24-RGDOX was diluted in PBS to achieve the specified MOIs and added dropwise to the attached cells, which were cultured in 200 µl of serum-free media for 20 min at 37°C and shaken every 5 min to disperse the virus. The serum-free media was then removed, and the cells were cultured in 1 ml of complete medium. Non-adherent cells were cultured with diluted Delta-24-RGDOX in 200 µl of serum-free media for 20 min at 37°C and shaken every 5 min to disperse the virus, and then 800 µl of complete medium was added. After a 48-h incubation period, adherent and non-adherent cells and supernatant were collected, flash-frozen and thawed 3 times, and then centrifuged at  $500 \times g$  for 5 min. The supernatants were used for viral titration in HEK293 cells, as explained in the "Oncolytic adenoviruses" section above.

**Viral-induced cytopathic ability of virus assay.** To determine the virus-induced cytopathic ability of Delta-24-RGDOX, we seeded  $1 \times 10^4$  cells/well in a 96-well plate (75  $\mu$ l media/well). An additional 75  $\mu$ l of media containing the appropriate treatment was then added to the cells. The virus-induced cytopathic ability was measured every 24 h for up to 168 h by quantifying ATP levels using the ViralToxGlo Assay (Promega) according to the manufacturer's protocol.

**IDO inhibitors.** The IDO inhibitors indoximod and 1-methyl-DL-tryptophan (1MT; Sigma-Aldrich) were suspended in PBS containing 3-mm glass balls (Thomas Scientific, #3000), which was rotated overnight to help re-suspend the drug. The clinical grade direct IDO enzyme inhibitor BGB-7204 (BeiGene; kindly provided by D. Wainwright, Northwestern University, IL), whose pharmacokinetic/pharmacodynamics properties have been reported previously<sup>6</sup>, was suspended in Ora-Plus oral suspending vehicle (Perrigo). For *in vitro* use of these inhibitors, DMSO (Sigma) was used as the diluent.

**In vivo studies.** For glioma implantations,  $5 \times 10^4$  GL261-5 or GSC-005 cells/mouse or  $1 \times 10^3$  B16-F10 cells/mouse were implanted into the caudate nucleus of 7- to 10-week-old male or female C57BL/6 mice using a guide-screw system as described previously<sup>7</sup>. The mice were then randomly assigned to experimental groups. Treatment began 7 days after tumor cell implantation. Delta-24-RGDOX adenoviruses ( $5 \times 10^7$  –  $1 \times 10^8$  pfu/dose) were injected intratumorally on days 7, 9, and 11. For 4T1.2 breast tumor implantations,  $1 \times 10^4$  4T1.2 cells/mouse were injected into the right mammary pads of 7- to 10-week-old female BALB/c mice; intratumoral Delta-24-RGDOX ( $1 \times 10^8$  pfu/dose) injections were administered on days 10, 14, 16, 18, and 21. Indoximod (275 mg/kg), 1MT (400 mg/kg), or BGB-7204 (100mg/kg) was administered twice daily, by oral gavage, 5 days/week, from days 7-35 after tumor implantation. Mice surviving 120 days were re-challenged with a new intracranial tumor injection using GL261-5 cells ( $5 \times 10^4$  cells/mouse) on the contralateral side of the initial tumor injection. For the depletion studies, anti-CD4 depletion antibodies or rat IgG2b isotype control antibodies were administered to mice intraperitoneally starting on day 4 after tumor implantation and every 4 days thereafter until day 36. C57BL/6 IDO-knockout (KO) mice (B6.129-Ido1tm1Alm/J; stock no. 005867) were purchased from The Jackson Laboratory and bred in MD Anderson Cancer Center's Research Animal Support Facility. The depletion antibodies used are shown in Supplementary Table 1. All experimental procedures involving the use of mice were done in accordance with protocols approved by the Animal Care and Use Committee of MD Anderson Cancer Center and National Institutes of Health and United States Department of Agriculture guidelines.

**Preparation of single-cell suspensions from murine brains and spleens.** Spleens and tumor-bearing brain hemispheres were collected from the mice. Initial suspensions were obtained by cutting the tissue or grinding the organs, filtering them through 100- $\mu$ m cell strainers (Fisher Scientific), and then placing the cell suspension in RPMI 1640 medium (10 ml/sample). All tissues were pelleted by centrifugation ( $500 \times g$  for 7 min at room temperature [RT]). The spleen-derived pellet was re-suspended in Red Blood Cell Lysing Buffer Hybri-Max (Sigma-Aldrich) to lyse the red blood cells according to the manufacturer's instructions. Then, the cell suspension was brought up to 20 ml/sample with RPMI 1640 medium to stop the lysis reaction and washed once in 1x PBS. The brain-derived pellet was washed once with HBSS (Corning), and then the cells were incubated in 8 pg/ml Liberase TM (Millipore Sigma) for 15 min at 37°C, washed again with HBSS, and then resuspended in 40% Percoll (1.130 g/ml; GE Healthcare), which was overlaid on top of 80% Percoll at a 1:1 ratio. The cells were centrifuged for 20 min at  $500 \times g$  at RT with an acceleration of 1 and a deceleration of 0, and the lymphocyte gradient

interphase was collected and washed once with PBS. The cells from the brains and spleens were pelleted by centrifugation at  $500 \times g$  for 7 min at RT and finally re-suspended in FACS buffer (PBS containing 10 mM HEPES, 2 mM EDTA, and 1% fetal bovine serum).

**RNA sequencing and data analysis.** Total RNA was extracted from flash-frozen tumor using the RNeasy Plus Mini Kit (Qiagen). Sequencing was performed by Novogene. RNA quality control was performed to measure quantification using Nanodrop, test RNA degradation or potential contamination using agarose gel electrophoresis, and check for RNA integrity using Agilent 2100 Bioanalyzer System. Library construction was developed from the mRNA of eukaryotic organisms, which was enriched using oligo(dT) beads. This eukaryotic mRNA was then fragmented randomly in fragmentation buffer, and then cDNA was synthesized using random hexamers and reverse transcriptase. After first-strand synthesis, a custom second-strand synthesis buffer (Illumina) was added with dNTPs, RNase H, and *Escherichia coli* polymerase I to generate the second strand by nick translation. The final cDNA library was ready after a round of purification, terminal repair, A-tailing, sequencing adapter ligation, size selection, and PCR enrichment. Sequencing was performed using HiSeq machines (Illumina). Key steps of the RNA-seq data analysis included the evaluation of the quality of the reads by fastqc (fastqc/0.11.8) followed by the removal of the sequencing adapters and unpaired reads by trimmomatic (trimmomatic/0.33)<sup>8</sup>. These trimmed FASTQ files were used to map reads to the mouse genome /ENSEMBL.mus\_musculus.release-75 using the STAR aligner (star/2.6.0b)<sup>9</sup>. Feature counts were extracted from the resulting .bam files by subread (subread/1.6.3)<sup>10</sup>. Estimates of unwanted variations in the raw read counts across samples were determined using the remove unwanted variation (RUV) method on the Galaxy platform, which estimates the factors of unwanted variation using replicate samples<sup>11</sup>. The RUV method uses the empirical Bayes approach to estimate a moderated t-statistic. These estimates were used as batch factors in DESeq2 analyses to determine differentially expressed genes in the group comparisons<sup>12</sup>. For the preparation of heatmaps, the log<sub>2</sub> normalized values for genes with significant adjusted P-values (<0.05) from the DESeq2 analyses were utilized; both rows and columns were hierarchically clustered using  $1 - r$ , where  $r$  is the Pearson correlation, with average linkage. For gene ontology (GO) enrichment analysis, the log<sub>2</sub> fold change (FC) values for genes with significant adjusted P-values (<0.05) from the DESeq2 PBS vs. RGDOX were used; enrichment analyses were performed on <http://www.pantherdb.org/>, with false discovery rate correction. Briefly, the genes with significant fold change are compared against each ontology category in panther database. The values for the genes in each ontology category are compared statistically to the overall distribution of values to look for coordinated shifts across that category. The cutoffs for the ingenuity pathway analyses (IPAs) were P-values  $\leq 0.05$  and log<sub>2</sub> FCs of  $\pm 1$ ; activation z-scores are plotted on graphs. An activation z-score of  $\pm 2$  was considered significant. The expression levels of genes with significant FCs and P-values in the sample group comparisons analysis were overlaid onto the IDO1 network curated from the IPA knowledge base. For the prediction of immune cell compositions as inferred from RNA-seq data, we input transcripts per million for each sample to the seq-ImmuCC platform<sup>13</sup>, and the predicted percentages of various immune cell populations in each sample were plotted in Excel. The gene set enrichment analyses were performed with GSEA\_4.0.3 software, and the whole-genome expression profiles of PBS- and Delta-24-RGDOX-treated tumors were used as inputs. The Treg gene set (Supplementary Data File 1) was curated from the IPA knowledge base, and the MSDC gene set (Supplementary Data File 2) was based on a previous publication<sup>14</sup>.

**Aryl-hydrocarbon-receptor activity assay.** For the assessment of the activity of aryl-hydrocarbon-receptor (AhR) induced by Delta-24-RGDOX, cells were mock-infected or infected with Delta-24-RGDOX (50 MOIs) and incubated for 48 h. The transcriptional activity of AhR in cell supernatants was quantified using an AhR assay kit (Indigo Biosciences), which utilizes AhR reporter cells expressing a luciferase reporter gene functionally linked to an AhR-responsive promoter. The protocol was performed according to the manufacturer's instructions.

**AhR immunofluorescence.** HeLa cells were seeded in a 96-well black-sided, clear-bottom tissue culture plate (Corning) at a density of  $1 \times 10^4$  cells per well. Cells were mock-treated, treated with kynurenine (150  $\mu$ M; Santa Cruz Biotechnology), or treated with Delta-24-RGDOX (25 MOI) in complete media for 48 h. Then, immunofluorescence staining was performed by following the procedures provided by the antibody manufacturer. Briefly, cells were washed with PBS and fixed with 4% formaldehyde in PBS for 10 min at RT. After the wells were washed 3 times with wash buffer (2 mg/ml BSA in PBS), the cells were permeabilized with 0.2% Triton X-100 for 30 min at RT in a moist chamber. After permeabilization, the cells were washed 3 times and overlaid with the primary antibody against AhR (Santa Cruz Biotechnology) containing 2 mg/ml BSA. After overnight incubation, the cells were washed 3 times and incubated with the FITC-conjugated secondary antibody (Invitrogen, Alexa Fluor 488 goat anti-mouse) for 1 h at RT. The cells were washed 4 times and then stained with DAPI (Invitrogen; 1  $\mu$ g/ml). The cells were then overlaid with mounting medium and imaged using an Axiovert 200 inverted microscope (Zeiss). The cellular and nuclear AhR intensities were quantified using ImageJ software (NIH). The antibodies and their working dilutions are shown in Supplementary Table 1.

**Quantitative real-time polymerase chain reaction.** For the detection of mRNA levels from cultured cells, Trizol reagent was used according to the established routine protocol. For the detection of mRNA levels from freshly dissected tissue samples, the RNeasy Plus Mini Kit (Qiagen) was used. One microgram of total RNA was reverse-transcribed into mRNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on the 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). Primers were purchased from Sigma Aldrich (human IDO, R: TGGAGGAACTGAGCAGCAT, F: TTCAGTGCTTTGACGTCCTG; mouse IDO, R: TTGCGGGGCAGCACCTTTCG, F: CCCACACTGAGCACGGACGG). The RT-PCRs were conducted in a 96-well plate with a holding stage of 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 45 s at 72°C, followed by an infinite hold at 4°C. Relative gene expression was calculated using the  $2^{-\Delta\Delta C_t}$  method by normalizing the threshold cycle ( $C_t$ ) values of the gene of interest to the  $C_t$  values of the internal housekeeping gene.

**Western blotting.** For the detection of protein expression, cell lysates were prepared using RIPA lysis buffer (20 mM HEPES pH 7.0, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 5 mM sodium pyrophosphate, 80 mM  $\beta$ -glycerophosphate, 50 mM NaF, and 0.1% SDS) plus freshly added protease inhibitor cocktail (1x; Sigma-Aldrich), the proteasome inhibitor MG-132 (1  $\mu$ M; Calbiochem), and phosphatase inhibitor cocktail 3 (2.5 mg/ml; Sigma-Aldrich). Cell lysates were flash-frozen in liquid nitrogen and thawed 3 times and centrifuged at 13,000 rpm for 10 min at 4°C. Supernatants were collected, and the concentration of protein was measured using Bradford reagent (Bio-Rad). DTT (50 mM; ThermoFisher) and NuPAGE LDS sample buffer (ThermoFisher) were added to 10-15  $\mu$ g of total protein. The samples were heated at 95°C for 5 min, run on 4-20% Novex Tris-Glycine gels

(Invitrogen), and then transferred to a PVDF membrane (ThermoFisher) and probed with the primary antibodies overnight at 4°C and then subjected to secondary antibody staining for 1 h at RT the next day. Protein bands were visualized using Western Lightning Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer). The antibodies and working dilutions used are shown in Supplementary Table 1.

**Liquid chromatography–mass spectrometry.** For the detection of IDO activity, the kynurenine and tryptophan levels of flash-frozen brain tumors from mice in the different treatment groups were measured by the Mass Spectrometry Facility at the University of Texas Medical Branch, Galveston, TX. Liquid extraction was used to extract polar metabolites from the frozen brain tissue. Briefly, 400 µl of 80% methanol was added to the frozen tissue. Zirconia/silica beads (1-mm diameter) were added to the tube. The tissue was broken down using a bead beater by pulsing the tissue for 45 s. The resulting solution was kept on ice for 30 min to precipitate the proteins. Next, 400 µl of chloroform was added, and the tube was pulsed in the bead beater for an additional 45 s and kept on ice for 15 min. Then, 200 µl of water was added to induce phase separation. The tube was centrifuged, and the upper aqueous phase containing the amino acids/amines was carefully transferred to another tube. Of the 500-µl aqueous phase, 250 µl was dried under vacuum. The dried metabolites were resuspended in 40 µl of buffer used for the derivatization of the amino acids/amines. Of this, 10 µl was taken for further derivatization and analysis by liquid chromatography–mass spectrometry (LC-MS). Standard calibration curves of known concentrations of amino acids, including tryptophan and kynurenine, were made. Before analysis by LC-MS, 5 µM of stable isotope-labeled amino acids, including tryptophan and kynurenine, in 10 µl were added to each sample. The concentrations of kynurenine or tryptophan in the analysis sample were then calculated using linear regression on the standard concentrations by plotting the area ratio (the ratio of the peak area of analyte to the peak area of the internal standard) and the known concentrations. Concentrations of kynurenine or tryptophan were normalized to tumor mass.

**Flow cytometry analysis.** For the analysis of cell surface protein expression, single cell suspensions from murine brains and spleens in a 96-well round-bottom plate were first blocked in anti-mouse CD16/CD32 Fc Block diluted with FACS buffer and then washed once with 250 µl of cold PBS. The cells were then incubated in 100 µl of Fixable Viability Stain 780 (1:1000; BD Biosciences) at 4°C in the dark for 30 min and then washed once with 250 µl of cold PBS. Then, the cells were incubated in 100 µl of primary antibody solution diluted in FACS buffer. After incubation at 4°C in the dark for 30 min, the cells were washed once with 250 µl of cold PBS. For the analysis of intracellular proteins, cells were stained with the eBioscience FOXP3/Transcription Factor Staining Buffer Set (Invitrogen) following the manufacturer's instructions. The cells were finally re-suspended in 0.3 ml of FACS buffer containing 123count eBeads Counting Beads (Invitrogen) to acquire an accurate output for absolute cell count. The stained cells were analyzed using the FACSCelesta flow cytometer (BD Biosciences). FlowJo software, version 10 (FlowJo, LLC), was used for the analysis. To control for the technique and to arrange population gates accurately, we generated fluorescence-minus-one samples for each antibody using pooled spleen cells from brain tumor-bearing mice in different treatment groups. The antibodies and working dilutions used are shown in Supplementary Table 1.

**Analysis of splenocyte stimulation in co-cultures with target cells.** Target cells were seeded and treated with the specified agents. Four hours later, mouse interferon gamma (IFN $\gamma$ ; 100 units/ml; ProSpec) was added to the cultures. Forty-eight hours after viral infection, the cells were detached with 2 mM EDTA in PBS, fixed with 1% paraformaldehyde, and cleaned with lysine (0.1 M) wash solution.

A total of  $2 \times 10^4$  fixed cells were seeded in 96-well round-bottom dishes. For immune cell activation, pre-fixed target cells were co-cultured with splenocytes ( $5 \times 10^5$ /well) in RPMI 1640 medium containing 100 µg/ml penicillin (Corning), 100 µg/ml streptomycin (Corning), and 55 µM beta-mercaptoethanol (Gibco) for 48 h. Then, the concentration of IFN $\gamma$  or interleukin-2 (IL-2) in the supernatant was assessed with standard ELISA (IFN $\gamma$  or IL-2 DuoSet ELISA, R&D Systems) according to the manufacturer's instructions.

**Histopathological staining.** Tumor-bearing mouse brains were fixed in 10% buffered formalin for 24 h, transferred to 70% ethanol, and then embedded in paraffin for slide sectioning. Paraffin-embedded sections of the mouse brain tumors were deparaffinized at 60°C for 1 h and rehydrated with xylene and ethanol following conventional procedures. For hematoxylin and eosin staining, brain tumor sections were stained with Harris hematoxylin (Fisher Scientific) and Eosin-Y solution (Fisher Scientific) and mounted with Cytoseal 60 (Thermo Scientific). For CD3 immunohistochemistry, antigens were retrieved from the brain tumor sections by exposing the slides to 10 mM citric acid (pH 6.0) inside a steamer for 30 min. The slides were then incubated in 3% hydrogen peroxide in 100% methanol for 10 min at RT to quench endogenous peroxidases. The sections were then subjected to blocking with 5% goat serum in PBS for 1 h at RT followed by incubation with a primary rabbit monoclonal anti-CD3 antibody overnight and then incubation with a biotinylated anti-rabbit IgG secondary antibody diluted in 1% goat serum. The Vector Vectastain ABC kit (Vector Biolabs, PK-4000) and ImmPACT and DAB Peroxidase Substrate Kit (Vector Biolabs, SK-4105-Reagent 1) were utilized to visualize positive cells. Images were captured using an Aperio ScanScope slide scanner, which was also used to measure high-grade tumor areas. Specific antibody information is given in Supplementary Table 1.

**Statistical analyses.** Experiments involving the groups included in the quantitative analyses were performed at least in triplicate. GraphPad Prism 9 was used to perform all statistical analyses and generate all graphs for the *in vitro* and *in vivo* experiments. To determine statistical differences between 2 groups, we performed a two-tailed Student t-test; to determine statistical differences among 3 or more groups, we performed an ordinary one-way ANOVA. The animal survival curves were plotted according to the Kaplan–Meier method. Survival rates in the different treatment groups were compared using the log-rank test, and to account for the presence of long-term survivors, we calculated the difference in restricted mean survival Qme (RMST) of the two groups, Delta-24-RGDOX (arm 0) and Delta-24-RGDOX+IDOi (arm 1), using the R package survRM2 version 1.0-4. At the truncation time tau we used the last point examined as default<sup>15,16</sup>. The statistical analyses of RNA sequencing data are described in the “RNA sequencing and data analysis” section above. *P*-values < 0.05 were considered significant.

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