Supplemental Materials and Methods

Mouse orthotopic intracranial-injection model

Mice were anesthetized with an intraperitoneal injection of 0.1 ml of stock solution containing ketamine HCl (25 mg/ml), xylazine (2.5 mg/ml), and 14.25 % ethyl alcohol (diluted 1:3 in 0.9 % NaCl). For the stereotactic intracranial injection, the surgical site was shaved and prepared with 70 % ethyl alcohol. A midline incision was made and a 1 mm diameter right parietal burr hole, centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture, was drilled. Mice were placed in a stereotactic frame and 4×10^5 GL261 cells in 2.5 μL saline was intracranially-injected (ic.) with a 26-gauge needle at a depth of 3 mm. The needle was removed and the skin was sutured with 4-0 nylon thread.

Flow cytometry

Single cell suspensions were made from brain, cervical draining lymph node (dLN) or spleen by mashing cells through a sterile 70 μM nylon mesh cell strainer (Fisher Scientific) using the rubber end of a 3 mL syringe into ice-cold PBS (Gibco). Red blood cells were removed by treatment with ACK Lysis Buffer (Lonza) for four minutes on ice. For brain isolates, cells were mixed in a PBS/30% Percoll solution and gently pipetted onto a 70% Percoll cushion. The samples were centrifuged at 1200 × g for 30 min., with no brake activated at the end of the centrifugation. The top layer (composed mostly of tumor cells, neurons and non-myeloid-derived glia) was aspirated, followed by isolation of the leukocyte layer. The cells were washed in cold PBS, twice, to remove any Percoll from the solution. Cells from the brain, dLN and/or spleen

were then quantified in the TC10 Automated Cell Counter (Biorad, Hercules, CA). Cells were incubated in PBS + 2 % bovine serum albumin (Sigma-Aldrich) and stained with: anti-CD4-PB (RM4-5; Ebioscience), anti-CD3-PE-Cy7 (145-2C11; Ebioscience), anti-CD8-PE (53-6.7; Ebioscience) and GITR-APC (DTA-1; Ebioscience) for 30 min on ice. Cells were then permeabilized and fixed overnight at 4°C using the Mouse Regulatory T Cell Staining Kit (Ebioscience) according to manufacturer's instructions and stained with anti-FoxP3-APC (FJK-16s; Ebioscience) for 30 min on ice. Cellular frequency was determined with the LSR II flow cytometer (BD) and Flowjo analysis software (TreeStar, Cupertino, CA).

GL261 cell IDO-specific knockdown

IDO shRNA lentiviral particles containing 3 target-specific constructs or shRNA constructs encoding a scrambled sequence were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). To knock down IDO gene expression, GL261 mouse glioma cells were transduced with control or IDO1-specific shRNA lentiviral particles at a ratio of 5 infectious units of virus per cell in the presence of 8 μg/mL polybrene for 6 hours. The next day, fresh media containing 2 μg/mL puromycin was added to the media for the selection of cells that had stably incorporated shRNA. In order to achieve complete silencing of IDO in GL261 cells, selected cells were expanded and transduced again with control or IDO shRNA lentiviral particles as described above. IDO knockdown was confirmed by analyzing control and IDO_{kd} tumors from $IDO^{-/-}$ mice (Supplemental Figure 1).

GL261 cell IDO1-specific overexpression

Although GL261 cells naturally express very low levels of IDO, they were used as the primary RNA source for cloning purposes. The cDNA encoding mouse IDO was amplified for 40 cycles with the following primer pair: Forward 5'- ATGGCACTCAGTAAAATATCTC -3' and Reverse 5'- CTAAGGCCAACTCAGAAGAGCTTTCTC -3'. The PCR product was further amplified while introducing restriction sites, *KpnI* and *NotI*, suitable for subsequent cloning into the pEF6/Myc,His vector (Invitrogen). The resulting PCR product and vector were restricted with *KpnI* and *NotI* enzymes, agarose gel purified and subsequently ligated using the T4 ligase (New England Biolabs, Ipswich, MA) to generate the pEF6/Myc,His vector encoding mIDO cDNA. To confirm that we had accurately cloned in the full IDO gene, we used in-house sequencing to compare our resulting transcript with that published in the NCBI databased for NM_008324.1, finding 100% overlap. GL261 cells were plated at 80% confluency and transfected with the plasmid encoding IDO using lipofectamine 2000. The following day, 4 μg/mL blasticidin was added to the media for selection of cells that had stably incorporated and expressed the IDO transcript. IDO overexpression in GL261 cells was confirmed at both the RNA and protein level through PCR and Western Blot (see Supplementary Methods) using rabbit polyclonal anti-mIDO1 antibody (ab106134, Abcam, Cambridge, MA), respectively (Supplemental Figure 1).

RNA isolation and semi-quantitative PCR

Total cellular RNA from 2 week post-intracranial-injected mouse brains (V_c vs. IDO_{kd}) or normal, V_c (control for IDO_{kd})-, IDO_{kd}-, pEF6 (control for IDO_{over}-) and IDO_{over}- GL261 cell lines were isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the

manufacturer's protocol. Equivalent amounts of RNA were reverse-transcribed with the iScript cDNA Synthesis Kit (Bio-rad Laboratories, Hercules, CA). Semi-quantitative PCR was performed using 5 uL Taq PCR Master Mix, 1 uL forward (5'-ATGGCACTCAGTAAAATATCTC -3') primer, 1 uL reverse (5'-CTAAGGCCAACTCAGAAGAGCTTTCTC -3') primer, 2 uL RNase-free H_2O and 1 uL input cDNA per reaction. Amplification was performed using the iCycler Thermal Cycler (Bio-Rad Laboratories) under the following conditions: 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C, followed by 5 min at 72°C. PCR products combined with 2 uL 6X DNA Loading Dye (Fermentas Life Sciences, Glen Burnie, MD) and ran on a 2% agarose gel with the GeneRuler 100bp DNA ladder. The gel was visualized in the ChemiDoc XRS Universal Hood II (Bio-rad Laboratories).

Western blotting

Normal-, IDO_{kd}-, pEF6 (Control)- and IDO_{over}- GL261 cells were centrifuged and resuspended in M-PER (ThermoScientific) with HALT protease inhibitor cocktail (ThermoScientific) for protein extraction. Protein concentrations were quantified by Coomassie Plus (Bradford) Protein Assay (ThermoScientific) and 70 µg of protein extract was used per lane for SDS-PAGE on a 7.5% acrylamide gel (Bio-Rad). Transfer to a PVDF membrane was completed and the membrane was submerged in blocking buffer (4% Milk TBS-T) overnight. Anti-indoleamine 2, 3-dioxygenase 1 (IDO1) antibody [Abcam ab106134 (specific for IDO1 and does not recognize IDO2) (1:25 4% Milk-TBS-T)] was applied for one hour. The membrane was washed 3 × 10 minutes with TBS-T and secondary antibody Donkey anti-Rabbit IgG HRP [Santa-Cruz (1:5000 4% Milk-TBS-T)] was applied alongside StrepTactin-HRP (Bio-Rad

[1:5000 4% Milk-TBS-T]). After ~5 hours, the membrane was washed 6 × 10 minutes with TBS-T. The chemiluminescence reaction was completed using the Immun-Star WesternC Chemiluminescence kit (Bio-Rad) and the membrane was imaged. The membrane was washed, then immersed in Restore Western Blot Stripping Buffer (ThermoScientific) for 15 minutes and placed in blocking buffer for one hour. Monoclonal β-Actin antibody [Sigma (1:3000 4% Milk-TBS-T)] was used to control for protein loading followed by Donkey anti-Mouse IgG HRP [Santa-Cruz (1:5000 4% Milk-TBS-T)].

Histology and quantitation of tumor volume

$IDO^{-/-}$ mice were ic. 4×10^5 V_c or IDO_{kd} GL261 cells and euthanized at 1-, 2- or 3- weeks post-ic. For each mouse group, four - five systematically random sampled sections throughout the injection site, with a minimum of 80 μm between each section, were analyzed (n=3). Sections were dehydrated through ascending concentrations of ethanol, stained with hematoxylin and eosin (H&E) to visualize tumor size and intracranial localization, cleared in Xylenes (Fisher-Scientific; Houston, TX), mounted with Permount (Fisher-Scientific), cover-slipped and viewed using the CRi Pannoramic Scan Whole Slide Scanner (3DHISTECH; Caliper Life Sciences, Hopkinton, MA). Photomicrographs were recorded in an online database and the tracing of tumor size was performed using the automated volume calculator built into the Free Pannoramic Viewer (3DHISTECH).