**RESOURCE AVAILABILITY**

**Data and Code Availability**

Flow Cytometry Standard (FCS) files generated in this study are freely-available at the Sage Synapse data repository (Synapse ID: syn21038562, https://www.synapse.org/#!Synapse:syn21038562/files/). SYLARAS source code is written in the Python programming language and is available for academic re-use under an MIT license agreement at Github (https://github.com/gjbaker/sylaras).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Mice**

Twelve (12)-week-old female C57BL/6J mice (syngeneic to the GL261 mouse glioma cell line) were purchased commercially from the Jackson Laboratory (Stock Number: 000664, Bar Harbor, ME). Mice were randomly assigned to experimental groups and housed under standard conditions. Animal procedures were documented in a protocol (IS00000178) pre-approved by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School. Mice were housed five per microisolator cage adhering to the guidelines outlined by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the Harvard Center for Comparative Medicine (HCCM).

**Glioma Cells**

GL261 (Glioma 261; RRID: CVCL\_Y003) mouse glioma cells were obtained from the Developmental Therapeutics Program (DTP), Division of Cancer Treatment and Diagnosis (DCTD) Tumor Repository through a material transfer agreement with the Biological Testing Branch (BTB) of the National Cancer Institute (NCI). The cells were determined by the Section of Comparative Medicine at Yale University’s School of Medicine to be free of common viruses and mycoplasma bacteria (MPV, LCMV, TMEV, SENDAI, MVM, MHV, ECTRO, REO, MYCO) on December 11, 2015. The cells were cultured in T75 or T175 tissue culture flasks under humidified conditions in 95% air/5% CO2 at 37°C. Culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.3 mg/mL L-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin. Cells were passaged every 3-5 days depending on initial seeding density.

**METHOD DETAILS**

**Study Design**

To screen the GL261 glioma model with SYLARAS, we randomized a cohort of age-matched C57BL/6J mice (N=48) to one of two treatment arms. In one arm, mice were engrafted with GL261 glioma cells suspended in cell culture media. The other arm served as an experimental control and was injected with vehicle alone to account for neuroinflammation caused by tumor implantation and physiological changes in immune system architecture over time. The two cohorts were then divided among one of three subgroups to be euthanized at 3 time points in tumor progression in test vs. control pairs (n=8 mice per subgroup). Time points were 7-, 14-, and 30-days after tumor engrafted.

**Stereotactic Brain Tumor Engraftment**

Details on the stereotactic engraftment of glioma cells into the mouse brain can be found here: (Baker et al., 2015). Briefly, mice were anesthetized with an intraperitoneal injection of 75 mg/kg ketamine and 0.5 mg/kg dexmedetomidine followed by a subcutaneous injection of 5 mg/kg carprofen and were non-responsive to toe and tail pinch prior to proceeding. Fur above the cranium was trimmed using surgical clippers and the cranium was prepped with povidone-iodine and scrubbed with 70% isopropyl alcohol. Petrolatum ophthalmic ointment was applied to the eyes to prevent drying before the mouse skull was secured in the stereotactic frame. Using a scalpel, a 1 cm midline incision from the frontal bone to the occipital bone was made. The skin above the cranium was retracted with Colibri retractors, and a hole was drilled into the cranium at the stereotactic coordinates: +0.5 mm AP, +2.5 mm ML. A bolus of 3x104 GL261 cells suspended in FBS-free DMEM was then injected 3.0 mm below the surface of the brain using a microliter syringe and a 33G needle. The scalp was sutured with three 3-0 nylon monofilament sutures. Post-operative mice were administered 0.1 mg/kg of buprenorphine hydrochloride subcutaneously and 2.5 mg/kg atipamezole hydrochloride intramuscularly for pain and anesthesia reversal then allowed to recover in fresh cages with access to food and water.

**General Reagents**

ddH20; RPMI-1640 (Corning, Cat. No. 10-040-CV); L-glutamine (Gibco, Cat. No. 25030-081); penicillin-streptomycin (10,000 U/mL) (ThermoFisher Scientific, Cat. No. 15140-163); heat-inactivated (HI) fetal bovine serum (FBS) (Gibco, Cat. No. 16140-071); Dulbecco’s phosphate buffered saline (DPBS) w/o CaCl2, MgCl2 (Corning, Cat. No. 21-040-CV); 5% (w/v) sodium azide (NaN3) (BDH, Cat. No. BDH7465-2); ethylenediaminetetraacetic acid (EDTA) disodium salt dehydrate (C10H14N2Na2O8•2H2O) (Sigma Aldrich, Cat. No. ED2SS); 15 mL polypropylene conical tubes (Falcon, Cat. No. 352097); 50 mL polypropylene conical tubes (Falcon, Cat. No. 352098); 5 mL polystyrene serological pipettes (Corning, Cat. No. 4050); 10 mL polystyrene serological pipettes (Corning, Cat. No. 4100); micropipettes (1000 μL, 200 μL, 20 μL, 10 μL) (Gilson); research plus 12-channel pipette (50-300 µl), (Eppendorf, Cat. No. 3122000060); 0.1-10 µl TipOne natural pipet tips (USA Scientific, Cat. No. 1111-3200); 1.0-20 µl TipOne natural pipet tips (USA Scientific, Cat. No. 1120-1810); 1-200 µl TipOne natural pipet tips (USA Scientific, Cat. No. 1111-1200); 101-1,000 µl TipOne natural pipet tips (USA Scientific, Cat. No. 1111-2820); 40 μm nylon mesh cell strainers (Falcon, Cat. No. 352340); 2 L polyethylene Dewar flask (Nalgene, Cat. No. 4150-2000); sterile cryogenic storage vials (Sigma-Aldrich, Cat. No. V7634); mini vortexer 120V (VWR, Cat. No. 58816-121); polypropylene general-purpose test tube racks (Nalgene, Cat. No. 5930-0020); 96-well reversible microcentrifuge tube rack (Bio Plas, Cat. No. 0091); S1 pipet filler (ThermoFisher Scientific, Cat. No. 9531); 9 L TruCool rectangular ethylene-vinyl acetate foam ice pans (BioCision, Cat. No. BCS-112); 1.5 mL microcentrifuge tubes (USA Scientific, Cat. No. 1615-5500); gel loading tips (Costar, Cat. No. 4853); 60 mm x 15 mm polystyrene tissue culture dishes (Falcon, Cat. No. 353002); 0.4% Trypan Blue solution (Gibco, Cat. No. 15250061)

**Reagents Germane to Mouse Euthanasia, Perfusion, and Tissue Processing**

ketamine hydrochloride injection (VEDCO, NDC: 50989-996-06); xylazine hydrochloride injection (AKORN INC, NDC: 59399-111-50); 0.9% sodium chloride (NaCl) injection, USP (Hospira, NDC 0409-4888-10); 1 mL Norm-Ject® sterile Luer-slip syringes (Henke Sass Wolf, Cat. No. 4010.200V0); PrecisionGlide needles - 26G x ½ (0.45 mm x 13 mm) (BD, Cat. No. 305111); sodium chloride (NaCl) (Sigma Aldrich, Cat. No. S9888); calcium chloride (CaCl2•2H2O) (Sigma Aldrich, Cat. No. C8106); sodium phosphate monobasic (NaH2PO4•2H2O) (Sigma Aldrich, Cat. No. 71505); D-glucose (C6H12O6) (Sigma Aldrich, Cat. No. G8270); sodium bicarbonate (NaHCO3) (Sigma Aldrich, Cat. No. S5761); potassium chloride (KCl) (Sigma Aldrich, Cat. No. P9333); heparin sodium salt from porcine intestinal mucosa (Sigma-Aldrich, Cat. No. H4784); extruded polystyrene foam block (2); Halsted-mosquito hemostat (2) (Fine Science Tools, Cat. No. 13008-12); fine scissors—martensitic stainless steel (2) (Fine Science Tools, Cat. No. 14094-11); Friedman rongeur (Fine Science Tools, Cat. No. 16000-14); Littauer bone cutters (Fine Science Tools, Cat. No. 16152-12); cover-glass forceps (Fine Science Tools, Cat. No. 11073-10); Dumont #5 forceps (2) (Fine Science Tools, Cat. No. 11252-40); Graefe forceps (2) (Fine Science Tools, Cat. No. 11051-10); Masterflex L/S digital pump system with easy-load II pump head, 600 RPM, 115/230V (Cole-Parmer, Cat. No. EW-77921-75); 20 G x 1 1⁄2" aluminum hub blunt needles (Kendall, Cat. No. 8881202363); razor blades (VWR, Cat. No. 55411-050); frosted microscope slides (Fisher Scientific, Cat. No. 12-550-343); 3 mL Luer-Lok® syringes (BD, Cat. No. 309657); PrecisionGlide needles - 23G x 1 (0.6 mm x 25 mm) (BD, Cat. No. 305145); Falcon 3 mL polyethylene transfer pipets (Corning, Cat. No. 357524)

**Reagents Germane to Immunolabeling**

Brilliant Stain Buffer (BSB) (BD Biosciences, Cat. No. 563794); TruStain FcX anti-mouse CD16/32 antibody (BioLegend, Cat. No. 101320); fixable viability dye, eFluor 455UV (eBioscience, Cat. No. 65-0868-14); Brilliant Ultraviolet 737-conjugated anti-mouse CD11b, clone: M1/70, isotype: rat DA/HA IgG2b, κ (BD Biosciences, Cat. No. 564443); V500-conjugated anti-mouse CD45, clone: 30-F11, isotype: rat LOU/M IgG2b, κ (BD Biosciences, Cat. No. 561487); Brilliant Violet 605-conjugated anti-mouse CD4, clone: RM4-5, isotype: rat IgG2a, κ (BioLegend, Cat. No. 100548); Brilliant Violet 711-conjugated anti-mouse Ly6G, clone: 1A8, isotype: rat IgG2a, κ (BioLegend, Cat. No. 127643); Alexa Fluor 488-conjugated anti-mouse CD3ε, clone: 145-2C11, isotype: Armenian hamster IgG (eBioscience, Cat. No. 53-0031-82); PE/Cy7-conjugated anti-mouse CD49b, clone: HMα2, isotype: Armenian hamster IgG (BioLegend, Cat. No. 103518); PE-conjugated anti-mouse F4/80, clone: BM8, isotype: rat IgG2a, κ (BioLegend, Cat. No. 123110); PE-CF594-conjugated anti-mouse CD8α, clone: 53-6.7, isotype: rat LOU/M IgG2a, κ (BD Biosciences, Cat. No. 562283); PerCP/Cy5.5-conjugated anti-mouse/human CD45R/B220, clone: RA3-6B2, isotype: rat IgG2a, κ (BioLegend, Cat. No. 103236); Alexa Fluor 647-conjugated anti-mouse CD11c, clone: N418, isotype: Armenian hamster IgG (BioLegend, Cat. No. 117312); APC/Cy7-conjugated anti-mouse Ly6C, clone: HK1.4, isotype: rat IgG2c, κ (BioLegend, Cat. No. 128026); V500-conjugated rat IgG2b, κ isotype control antibody, clone: A95-1, isotype: rat LOU/M IgG2b, κ (BD Biosciences, Cat. No. 560784); fixation/permeabilization solution kit (BD Biosciences, Cat. No. 554714); 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (ThermoFisher Scientific, Cat. No. D1306); 96-well V-bottom, non-treated, polystyrene microplate (Costar, Cat No. 3897); 12-well, V-bottom reagent reservoir (Argos Technologies, Cat. No. B3135); Microseal ‘F’ foil seal (Bio-Rad; Cat. No. MSF1001)

**Reagents Germane to Flow Cytometry**

Sphero rainbow fluorescent particles (3.0-3.4 µm) **(**BD Biosciences, Cat. No. 556291); FACSDiva CS&T research beads **(**BD Biosciences, Cat. No. 655051)

**Preparation of Reagents**

*Supplemented RPMI-1640*

A 500 mL bottle of RPMI-1640 with L-glutamine was supplemented to achieve the following reagent concentrations: 100 U/mL penicillin, 100 U/mL streptomycin, 10% HI-FBS, 0.05% sodium azide, and 0.1% (w/v) EDTA.

*Heparinized Tyrode’s Solution*

A 1 L glass screw-cap storage bottle containing a stirring bar was filled with ddH2O and stirred continuously. The following reagents were then added: 8.0 g sodium chloride, 0.264 g calcium chloride, 0.05 g sodium phosphate monobasic, 1.0 g D-glucose, 1.0 g sodium bicarbonate, 0.2 g potassium chloride, 100 U of heparin sodium. Stored at 4°C.

*Ammonium-Chloride-Potassium (ACK) Lysis Buffer (1X)*

A 1 L glass screw-cap storage bottle containing a stirring bar was filled with ddH20 and stirred continuously. The following reagents were then added: 8.29 g of ammonium chloride, 1.0 g of potassium bicarbonate, 37.2 mg of sodium EDTA. pH adjusted to 7.4. Stored at room temperature.

*Flow Buffer*

1X DPBS containing 0.5% HI-FBS (100-200 mL). Stored at 4°C.

*Flow Buffer + Azide*

Flow Buffer containing 0.05% (w/v) sodium azide (100-200 mL). Stored at 4°C.

*EDTA Solution*

1X DPBS containing 10% (w/v) disodium (100 mL). Ultrasonicated and stored at 4°C.

*Fc Block*

Flow Buffer containing 15 μg/mL mouse monoclonal anti-CD16/32 antibodies (see **Table S2** for preparation details).

*Fixable Viability Dye*

eFluor 455UV fixable viability dye diluted 1.5:1,000 with 1X DPBS (see **Table S2** for preparation details).

**Major Equipment and Parameter Settings**

*Flow Cytometer*

BD LSR II Special Order Research Product (SORP) flow cytometer with BD High Throughput Sampler (HTS) — laser lines and wattages were as follows: 488 nm (20 mw, run at 20 mw); 405 nm (50 mw, run at 50 mw); 594 nm (200 mw, run at 125 mw); 355 nm laser (20 mw, run at 20 mw).

*Plate Washer*

BioTek EL406 automated microplate washer/dispenser — instrument configurations were as follows: plate type = 96-well, W-aspirate, vacuum filtration = false, travel rate = 1 (4.1 & 1.0 mm/sec), delay = 0 msec, z-offset = 55 steps (6.99 mm above carrier), x-offset = 0 steps (center of well), y-offset = 0 steps (center of well), secondary aspirate = no.

*Centrifuge and Rotor*

Beckman Coulter Avanti J-26XP centrifuge equipped with a Beckman Coulter JS-5.3 anodized aluminum swinging-bucket rotor

*Ultrasonic Bath*

Bransonic CPXH ultrasonic cleaning bath (model 3800)

**Antibody Titration**

*Preparation of Splenocytes*

To identify optimal immunolabeling concentrations for 11 mouse immune cell lineage antibodies, the spleens of two 12-week-old female C57BL/6J mice were harvested according to the procedure described in the “Lymphoid Tissue Harvesting and Processing” section of the STAR methods. Spleens were gently macerated using opposing frosted sides of two glass microscope slides, then rinsed thoroughly into a single 60 x 15 mm polystyrene Petri dish on ice containing 4 mL of supplemented RPMI-1640. Splenocytes were transferred from the Petri dish to a 15 mL conical tube and centrifuged at 350 x g (max RCF) for 10 minutes at 4°C. The cell supernatant was discarded and the pellet was resuspended with 8 mL of 1X ACK lysis buffer. The tube was incubated on ice for 5 minutes before 6 mL of Flow Buffer + Azide was added to inhibit further lysis. The cell suspension was then passed through a 40 μm nylon mesh into a fresh 15 mL conical tube, centrifuged at 350 x g (max RCF) for 10 minutes at 4°C to pellet the cells, and resuspended with 2 mL of Flow Buffer + Azide. Cell counting was performed using a hemocytometer and Trypan Blue viability dye; splenocyte concentration was adjusted accordingly with additional Flow Buffer + Azide to achieve 1x107 cells/mL.

*Immunolabeling*

Using a multichannel pipette, 200 μL of the 1x107 cells/mL splenocyte suspension was transferred into 11 concentric columns of a 96-well V-bottom microplate on ice (8 rows per column) to test the following 2-fold serial dilution series for each antibody: 24, 12, 6, 3, 1.5, 0.75, 0.375, 0.1875 μg/mL. The plate was then centrifuged at 100 x g (max RCF) for 3 minutes at 4°C. One-hundred and fifty (150) μL of cell supernatant were aspirated from each well using a BioTek EL406 automated microplate washer/dispenser and resuspended with 100 μL of 15 μg/mL Fc Block (see **Table S2** for preparation details). Splenocytes were allowed to incubate on ice for 5 minutes, then centrifuged at 100 x g (max RCF) for 3 minutes at 4°C. One-hundred (100) μL were aspirated from each well and resuspended with 100 μL of antibodies diluted in BSB. Splenocytes were allowed to immunolabel on ice for 15 minutes in the dark. The plate was centrifuged at 100 x g (max RCF) for 3 minutes at 4°C. Two-hundred (200) μL of supernatant were aspirated from each well and resuspended with 100 μL of Flow Buffer + Azide as a wash step. The cells were again centrifuged at 100 x g (max RCF) for 3 minutes at 4°C and washed a second time using 200 μL Flow Buffer + Azide. Immunolabeled cells were incubated in a solution of 1μg/mL DAPI for 5 minutes prior to flow cytometric analysis.

*Data Acquisition*

Immunolabeled splenocytes were analyzed by flow cytometry using a BD LSR II SORP flow cytometer equipped with a BD HTS for high-throughput sampler for the automated acquisition of data from 96-well plates. The following gating strategy was used: (FSC-A vs. SSC-A) → (SSC-H vs. SSC-W) → (FSC-H vs. FSC-W) → (DAPI-A vs. FSC-A) → (CDx vs. count). Ten-thousand (10,000) viable singlets were analyzed per well. A staining index (SI) was then calculated for each antibody at each immunolabeling concentration. Concentrations yielding the maximum SI for each antibody were those used in our SYLARAS screen of the GL261 mouse glioma model.

**Lymphoid Tissue Harvesting and Processing**

*Preparation of Reagents*

The following disposable reagents were gathered before each of three time points in the study: (80) 0.5 mL microcentrifuge tubes, labeled by tissue type, treatment condition, and biological replicate; (64) 60 x 15 mm polystyrene Petri dishes on ice, labeled by tissue type (excluding blood), treatment condition, and biological replicate; (16) 15 mL conical tubes for blood collection, labeled by treatment condition and biological replicate; (16) 3 mL syringes equipped with 23G needles, for bone marrow aspiration, labeled by treatment condition and biological replicate; (16) 1 mL tuberculin syringes equipped with 26G needles for transcardial blood draws, labeled by treatment condition, biological replicate; an single 1 mL tuberculin syringe equipped with a 26G needle for anesthesia administration.

One-hundred (100) μL of a 10% EDTA solution were added to each 15 mL conical tube. Four (4) mL of supplemented RPMI-1640 were added to each Petri dish, with the exception of those labeled bone marrow. Those received 2 mL of media; the other 2 mL were deposited into the respectively-labeled 3 mL syringes for bone marrow aspiration. One-hundred ninety-eight (198) μL of Flow Buffer was added to each microcentrifuge tube. Fifty (50) μL of a 10% EDTA solution was added to each 1 mL tuberculin syringe for drawing blood. Conical tubes, Petri dishes, blood drawing syringes, etc. were kept on ice or stored at 4°C throughout the tissue harvesting and processing procedure.

*Mouse Anesthesia*

At each time point, 8 GBM-bearing mice and 8 age-matched, mock-engrafted mice were terminally anesthetized, one at a time, with 150 mg/kg of ketamine hydrochloride and 20 mg/kg xylazine hydrochloride diluted in sterile 0.9% NaCl and delivered intraperitoneally using a 1 mL tuberculin syringe (See **Table S2** for preparation details). Once non-responsive to both toe and tail pinch, each mouse was pinned ventral-side-up to an extruded polystyrene foam block by their front and hind paws using four 26G needles (one per paw). The abdomen was sprayed with 70% ethanol to disinfect the incision site and obstruct fur from entering the dissection cavity.

*Tissue Excision*

Five (5) lymphoid organs of each mouse were harvested in the following order: blood, thymus, spleen, deep and superficial cervical lymph nodes, bone marrow.

Blood: A “Y” incision was made from the abdomen to the rib cage exposing the heart. Blood was aspirated from the right ventricle into a 1 mL tuberculin syringe. Syringe needles were removed prior to expelling blood from the tuberculin syringe into a respectively-labeled 15 mL conical tube. Mice underwent transcardial perfusion with heparinized and oxygenated (95% O2/5% CO2) Tyrode’s solution at a rate of 4.0 mL/minute for at least 2 minutes in a laminar flow hood to achieve complete exsanguination. More detailed methods on transcardial perfusion of mice can be found here: (Baker et al., 2015).

Thymus: Post-exsanguination, mice were returned to the extruded polystyrene foam block for thymus excision with small dissection scissors and fine-tipped, bent forceps. Care was taken to avoid taking contaminating adipose tissue and mediastinal lymph nodes. Thymi were stored in respectively-labeled 60 x 15 mm polystyrene Petri dishes on ice.

Spleen: Spleens were excised using small dissection scissors and fine-tipped, bent forceps and stored in a respectively-labeled 60 x 15 mm polystyrene Petri dishes on ice.

Superficial/deep cervical lymph nodes: Under a dissection microscope, lymph nodes were removed with small dissection scissors and fine-tipped, bent forceps and stored in respectively labeled 60 x 15 mm polystyrene Petri dishes on ice.

Bone marrow:The right hind limb was removed using bone cutters, then trimmed to isolate the femur and tibia. Proximal and distal epiphyses of each bone were removed using a single-edged razor blade. Bone marrow was flushed into respectively-labeled 60 x 15 mm polystyrene Petri dishes on ice using 3 mL syringes containing 2 mL of supplemented RPMI-1640.

*Tissue Processing*

Spleens, lymph nodes, and thymi were gently macerated using opposing frosted sides of two glass microscope slides, then rinsed thoroughly into respectively-labeled 60 x 15 mm polystyrene Petri dishes on ice containing 4 mL of supplemented RPMI-1640. For cervical lymph nodes, plastic Pasteur pipettes were used to transfer the nodes onto the frosted side of a single microscope slide for maceration. Five (5) mL of ice-cold 1X DPBS was added to each Petri dish and cell suspensions were filtered through clean 40 μm nylon meshes into respectively-labeled 15 mL conical tubes using 10 mL serological pipettes. Tubes were centrifuged at 400 x g (max RCF) for 10 minutes at 4°C and cell pellets were resuspended in 4 mL of a 1X ACK lysis buffer adding using 5 mL serological pipettes. Tubes were allowed to incubate on ice for 5 minutes, centrifuged at 400 x g (max RCF) for 10 minutes at 4°C, and resuspended with varying amounts of Flow Buffer + Azide. The following tissue-specific volumes were used for initial resuspension: 1000 μL (thymus), 1000 μL (spleen), 200 μL (bone marrow), 100 μL (deep/superficial cervical lymph nodes).

Blood samples underwent RBC lysis for 5 minutes on ice by adding 10 mL of 1X ACK lysis buffer to each 15 mL conical tube (or until blood changed from dark burgundy to bright-red). The samples were then centrifuged at 400 x g (max RCF) for 10 minutes at 4°C and resuspended with 200 μL of Flow Buffer + Azide.

*Cell Counting*

Using a P20 micropipette equipped with a gel loading tip, 2 μL of each tissue sample were added to respectively-labeled 0.5 mL microcentrifuge tubes containing 198 μL of Flow Buffer. Ten (10) μL of the resulting 1:100 dilutions were further diluted 1:1 with 0.4% Trypan Blue. Ten (10) μL of these dilutions were used for cell counting with a hemocytometer. Cell yields varied by tissue type: ~3x105 – 1x106 (blood), ~3x107 – 8x107 (spleens), ~6x107 – 8x107 (thymi), ~6x106 – 1.5x107 (deep/superficial cervical lymph nodes), ~9x106 – 1.5x107 (bone marrow). Counts were recorded in an Excel spreadsheet formatted to compute the necessary dilutions to achieve a concentration of 2x107 cells/mL across all samples (**Table S2**). Blood samples typically contained less than 2x106 cells and were not diluted further.

**Immunolabeling**

*Reagent Preparation*

The following disposable reagents were gathered at each time point in the study: (22) 1.5 mL microcentrifuge tubes, labeled in duplicate with antibody names (including the CD45 isotype control) and organized into two duplicate rows of a microcentrifuge rack stored on ice; (1) 12-well, V-bottom reagent reservoir, labeled with antibody names (including the CD45 isotype control); (3) 15 mL conical tubes, one labeled “FVD”, one labeled “Fc Block”, and one labeled “Cocktail”; (1) 96-well, V-bottom microplate.

*Antibody Dilution*

Antibodies (11 immune lineage markers plus CD45 isotype control) were diluted 1:10 with BSB in respectively-labeled microcentrifuge tubes on ice (see **Table S2** for preparation details). A fraction of each working dilution was used in preparing single-color compensation controls. The remaining working dilutions (excluding the CD45 isotype control) were combined into the 15 mL conical tube labeled “Cocktail”, which served as the antibody master mix for multiplex immunolabeling. Antibody stocks and dilutions were kept on ice in the dark.

*Labeling*

Using a multichannel pipette, (100) μL of each lymphoid tissue sample (1x106 cells) were added to respective wells of a 96-well V-bottom microplate according to a predefined plate layout (**Fig. 1**). To obtain enough CD49b+ cells for fluorescence compensation of the CD49b flow cytometer channel, 100 μL of each 200 μL white blood cell suspension was deposited into a common CD49b single-positive control well (G9); the other 100 μL was deposited into the respectively-labeled experimental well. The plate was then centrifuged at 100 x g (max RCF) for 6 minutes at 4°C. Fifty (50) μL of cell supernatant were aspirated from each well using a BioTek EL406 automated microplate washer/dispenser such that a residual 50 μL was left in each well. One-hundred (100) μL of 15 μg/mL Fc Block (see **Table S2** for preparation details) were added to each well of the 96-well plate using a multichannel pipette, mixed by pipetting, and incubated on ice for 5 minutes. The plate was centrifuged at 100 x g (max RCF) for 5 minutes at 4°C and 50 μL of cell supernatant were aspirated from each well. Each of 80 experimental tissue samples received 100 μL of the master antibody mix from the 15 mL conical tube labeled “Cocktail”. One-hundred (100) μL of each single-color antibody control were added to its respective well; 100 μL CD45 isotype antibodies were added to the “ISO” control well, and 100 μL of stock BSB were added to the wells labeled “UNS” and “FVD”.

Cells were allowed to incubate for 15 minutes on ice in the dark before 100 μL of 1X DPBS was added using a multichannel pipette pipetted thoroughly to mix. The plate was then centrifuged at 100 x g (max RCF) for 5 minutes at 4°C. One-hundred ninety-one (191) μL of cell supernatant were aspired from each well and resuspended with 200 μL of 1X DPBS using a multichannel pipette followed by mixing. The plate was again centrifuged at 100 x g (max RCF) for 5 minutes at 4°C. Two hundred (200) μL of cell supernatant were removed from each well.

*Viability Staining*

One-hundred (100) μL of a 1.5:1,000 dilution of fixable viability dye from the 15 mL conical tube labeled “FVD” (see **Table S2** for preparation details) were added to each well of the 96-well V-bottom microplate with the exception of the well labeled “UNS”. This achieved a final staining concentration of 1:1,1000. Cells were then incubated on ice in the dark for 30 minutes. One-hundred (100) μL of 1X DPBS were added to each well and pipetted to wash. The plate was centrifuged at 100 x g (max RCF) for 5 minutes at 4°C. Two-hundred (200) μL of cell supernatant were aspirated from each well and resuspended with 200 μL of 1X DPBS. The plate was again centrifuged at 100 x g (max RCF) for 5 minutes at 4°C followed by the aspiration of 200 μL of cell supernatant.

*Cell Fixation and Short-term Storage*

One-hundred (100) μL of BD fixation/permeabilization solution were added to each well of the 96-well plate and pipetted to prevent cell crosslinking. The plate was then incubated on ice in the dark for 20 minutes. One-hundred (100) μL of Flow Buffer were added to the wells and pipetted to mix before the plate was centrifuged at 100 x g (max RCF) for 5 minutes at 4°C. Two-hundred (200) μL of cell supernatant were aspired from each well and resuspended with 200 μL of Flow Buffer. The microplate plate samples were covered with a Microseal ‘F’ foil seal to prevent dehydration, wrapped in aluminum foil to block light, and stored at 4°C before flow cytometric analysis.

**Cytometer Setup and PMT Calibration**

PMT voltages on a BD LSR II SORP flow cytometer were calibrated such that signal intensities corresponding to viable unlabeled splenocytes (well E10) were on scale and to the left of center in each detection channel. To prevent downstream compensation values from exceeding 100%, optical spillover of single-color compensation controls into off-target detection channels was checked to ensure that peak signal intensities occurred in their respective detection channel. Sphero Rainbow Fluorescent Particles (i.e. single-positive (SP) beads) were run at the outset of our study to predefine tolerability ranges for laser intensity, stability, and alignment, and prevent run-to-run variation so that fluctuations in laser emission power between data acquisition cycles could be accounted for with calibration. SP beads were gated using (FSC-A vs. SSC-A) and visualized as biexponential histograms in each detection channel.

**Data Acquisition**

Cytometer setup & tracking was performed using FACSDiva CS&T research beads to optimize and standardize instrument performance across data acquisition cycles. At each acquisition, the 96-well V-bottom microplate was placed in a BD High Throughput Sampler (HTS) affixed to a BD LSR II SORP flow cytometer. The HTS system was programmed to collect events from all wells of the plate in a predefined order. Because fluidic anomalies can impact laser delay stability, wells corresponding to single-positive (SP) beads were run first to check that PMT voltages remained within previously defined tolerability ranges. Optical controls were then run in the following order: UNS, FVD, ISO, single-color compensation controls, experimental samples, SP beads—to again check that PMT voltages were stable over the data acquisition period. Normal C57BL/6J splenocytes were used for all compensation controls except the CD49b single-positive control, which consisted of pooled WBCs from all samples to achieve enough CD49b+ cells for fluorescence compensation. The following acquisition gating strategy was used: (FSC-A vs. SSC-A) → (SSC-H vs. SSC-W) → (FSC-H vs. FSC-W) → (BUV395-A vs. FSC-A) → (CDx vs. count). Histograms were plotted on biexponential scale. Antibodies were detected using the combinations of laser lines, band pass filters, and long pass filters shown in (**Fig. S2C**). Raw data were exported as FCS3.0 files.

**Fluorescence Compensation**

Raw flow cytometry data were spectrally deconvolved using commercial software (FlowJo). Optical controls were imported into the compensation group of a FlowJo workspace. Single-color compensation controls plus cells from the FVD optical-control well (E10) were gated for viable singlets according to the following strategy: (FSC-A vs. SSC-A) → (SSC-H vs. SSC-W) → (FSC-H vs. FSC-W) → (BUV395-A vs. FSC-A). Data in the last plot of the gating strategy (BUV395-A vs. FSC-A) were viewed in contour at the 2% level. Viable singlets were then backgated to FSC-A vs. SSC-A to isolate subsets of viable singlets suitable for fluorescence compensation of each detection channel.

Unimodal CD45 histograms made it difficult to compensate this channel. This was overcome by combining the data from the CD45 isotype control (well E11) with that of the CD45 single-color control (well F9) using FlowJo’s concatenate feature. This resulted in a bimodal distribution which was saved as a FCS3.0 file to the compensation group of the FlowJo workspace and compensated in an otherwise typical fashion. Data corresponding to wells E11 and F9, that were no longer needed and were deleted from the workspace.

Compensation control data were visualized as histograms in their respective detection channels. Histograms were gated at the interface of the first and second signal intensity peaks using FlowJo’s bisector tool. Then, using FlowJo’s compensation tool, data corresponding to the left and right subsets were dragged into the “negative” and “positive” software interface fields. Once this process was performed for each of the 11 immunomarkes plus the FVD control, a new group was created in the workspace and given the name “Cocktail”. Data corresponding to the 80 experimental tissue samples were imported into this group. The compensation matrix was then applied to the “Cocktail” group. The resulting compensated experimental data were gated according to the same strategy as optical control samples to achieve compensated, viable singlets which were exported as new FCS3.0 files.

**Gating Strategy**

Data from compensated viable singlets were visualized as a set of 2,640 histograms (80 experimental tissue samples displayed in 11 immunomarker channels at 3 time points in tumor progression). Histograms were plotted as scalable vector graphics (SVGs) on Logicle scale (Parks et al., 2006) and displayed as a scrolling HTML table. Kernel density estimates (KDEs) generated for each detection channel using data from well E10 (unlabeled control splenocytes) at each time point were superimposed over their corresponding experimental histograms to determine the point at which antibody signal intensities superseded cell-intrinsic autofluorescence. Points at the interface between signal and noise were manually curated and recorded in a .TXT file.

CD45 signal intensity distributions were unimodal with no discernable local minima. Thus, for each combination of time point and tissue, a common CD45 gate was curated by pooling the corresponding samples, computing Q25 – [1.5 \* [Q75 - Q25]], then rounding to the nearest multiple of 5 (where Q25 and Q75 were the first and third quartiles of the Logicle-transformed data, respectively). The .TXT file of gate points was used to update the HTML table with vertical lines at the curated gate points for confirmation or refinement through an iterative process by updating the .TXT file and re-plotting.

Compensated viable singlets and the curated histogram gate points both underwent Logicle data transformation (Parks et al., 2006). The transformed gate points were subtracted from each of the transformed signal intensity values of corresponding distributions. This caused the gate points to become zero and autofluorescence signal intensities to become negative valued. A spurious population of CD49b+ granulocytes were identified in blood samples. This was a suspected artifact caused by the interaction between residual CD49b+ platelets and Ly6G+ neutrophils in blood samples (Barnard et al., 2005) and countered by only considering CD49b immunomarker status when the immunophenotype of an immune cell was otherwise consistent with that of an NK cell (e.g. CD45+, CD49b+, CD11b+). The background-adjusted data were initially saved as .TSV files and later converted to FCS3.0 using a helper script. These files served as input into the SYLARAS software and have been deposited to the Sage Synapse data depository (Synapse ID: syn21038562, https://www.synapse.org/#!Synapse:syn21038562/files/).

**t-CyCIF**

5μm-thick coronal sections of GL261-bearing mouse brain sections were cut from formalin-fixed, paraffin-embedded (FFPE) tissue blocks. Tissues were mounted on glass microscope slides and iteratively immunolabeled through multiple rounds of 4-color immunofluorescence (i.e. Hoechst, AF488, AF555, AF657) using the t-CyCIF method (Lin et al., 2018). Briefly, tissue sections underwent nuclear counterstaining for 30 minutes at room temperature through incubation with Hoechst dye (10 mg/mL stock) diluted 1:5,000 in 1X PBS. Slides were washed thoroughly in 1X PBS then incubated in Odyssey Blocking Buffer at room temperature for 1 hour to reduce non-specific antibody binding. Autofluorescence background was imaged before each immunolabeling cycle to increase signal-to-noise in the final images. Antibodies were used against the following targets: Ly6C, CD8α, CD68, CD45R/B220, CD4, CD49b, Ly6G, Foxp3, CD11b, Ki67, Vimentin (See **Table S2** for antibody details). Immunolabeling was performed at 4°C overnight in opaque and humidified chambers. Tissues were washed thoroughly in 1X PBS and temporarily coverslipped in 1X PBS containing 10% glycerol before imaging with a CyteFinder slide scanning fluorescence microscope (RareCyte, Seattle, WA, USA) using a 40X (0.6NA) objective and 2x2 binning. After imaging was complete, slides were submersed in Coplin jars containing 1X PBS so the coverslip would fall away from the microscope slide. Antibody fluorophores were quenched after each round of imaging by incubating the tissues in a 3% H2O2 solution diluted in 1X PBS and containing 20 mM NaOH for 2 hours at room temperature in the presence of intense fluorescent light.

One-hundred sixty-eight (168) 400x300µm imaging fields were acquired during each imaging cycle. Imaging fields across cycles were aligned and registered on Hoechst signal using ImageJ’s Multistack Registration Plugin. Registered images were segmented on Hoechst-stainednuclei using ImageJ’s Analyze Particles function. The segmentation mask of each cell was dilated by three pixels to capture antibody signal within the cell cytoplasm and at the plasma membrane. The median immunomarker signal intensities of ~9x104 cells from within the brain tumor microenvironment were then computed and analyzed with SYLARAS software.

**Software**

*FACSDiva*

version 8.0

*FlowJo*

version 10.3.0

*Python*

version 3.6.1

*PhenoGraph*

version 1.5.2

*FlowSOM*

version 1.14.1

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical tests were performed using documented and validated statistical functions in the SciPy.stats library for statistical computing (https://docs.scipy.org/doc/scipy/reference/stats.html). Specific tests and sample sizes are indicated at their point of reference either in the main text or related figure legends. Hypothesis tests were two-tailed and performed using independent observations except for the binomial tests for cluster enrichment associated with data shown in Fig. 6C. A statistical significance threshold was chosen for all tests at an FDR-adjusted p-value (q-value) of less than 0.05. Spearman’s rank-order tests for correlation were used under the assumption of monotonically-related ordinal data. Coefficients of determination (R2) are reported for all regression analyses. Agglomerative hierarchical clustering was performed using the unweighted pair group method with arithmetic mean (UPGMA) linkage algorithm and Euclidean distance metric. Tissue-weighted random sampling of flow cytometry data was performed by assigning a weight to each cell in the data according to the following formula: 1/(ω x Ni), where ω is the number of unique tissue types and Ni is the number of events associated with the ith tissue (e.g. blood, marrow, nodes, spleen, and thymus).

**ADDITIONAL RESOURCES**

Additional information related to SYLARAS: www.sylaras.org.