**SYLARAS PROTOCOL**

**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 (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); purified anti-human CD45R, clone: MB1, isotype: mouse IgG1 (Abnova, Cat. No. MAB16165); Alexa Fluor 488-conjugated anti-human CD8α, clone: AMC908, isotype: mouse IgG2a, κ (eBioscience, Cat. No. 53-0008-80); Alexa Fluor 555-conjugated anti-human CD3δ, clone: EP4426, isotype: rabbit IgG (abcam, Cat. No. AB208514); 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)

**Mice**. Twelve-week-old female C57BL/6J mice syngeneic to the mouse GL261 GBM model were used in this study (Jackson Laboratory, Bar Harbor, ME). All animal experiments were conducted in accordance with procedures preapproved by the Institutional Animal Care and Use Committee (IACUC) and conformed to the policies and procedures of the Center for Comparative Medicine at Harvard Medical School in agreement with the National Research Council’s “Guide for the Care and Use of Laboratory Animals”.

**GBM Cells.** The GL261 GBM cell line was 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).

**Clinical Specimens.** All deidentified human brain tumor resections used in this study were obtained from consented patients under care at Brigham and Women’s Hospital or Dana-Farber Cancer Institute

in association with Institutional Review Board (IRB) protocols: 10-417 or 11-104 (17-000).

**Major Equipment.** BD LSR II Special Order Research Product (SORP) flow cytometer w/ BD High Throughput Sampler (HTS):

* Laser line – power:
  + 488 nm laser – 20 mw (run at 20 mw)
  + 405 nm laser – 50 mw (run at 50 mw)
  + 594 nm laser – 200 mw (run at 125 mw)
  + 355 nm laser – 20 mw (run at 20 mw)

BioTek EL406 automated microplate washer/dispenser:

* In an effort to minimize cell loss, aspiration steps involving 96-well V-bottom microplates were performed using this instrument such that a 50 μL residual volume remained after each aspiration. This factor was accounted for in all reported dilutions. 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

Beckman Coulter Avanti J-26XP centrifuge:

* JS-5.3 anodized aluminum swinging-bucket rotor (Beckman Coulter, Cat. No. 368690)

Bransonic CPXH ultrasonic cleaning bath:

* model 3800

**Reagent Preparation.** supplemented RPMI-1640 (RPMI-1640 with L-glutamine, 100 U/mL penicillin-100 U/mL streptomycin, 10% HI-FBS, 0.05% sodium azide):

* to a 500 mL bottle of RPMI-1640 with L-glutamine were added:
* 5 mL of a 10,000 U/mL penicillin-10,000 μg/mL streptomycin solution
* 50 mL of HI-FBS
* 2.5 mL of a 5% w/v sodium azide solution
* 5 mL of a 10% EDTA solution

heparinized Tyrode’s solution:

* to a 1 L glass screw-cap storage bottle on a stirring plate and containing a magnetic stirring bar and 1 L ddH2O were 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
    - Salts were allowed to completely dissolve prior to storage at 4 °C.

ammonium-chloride-potassium (ACK) lysis buffer (1X):

* to 1 L of stirring ddH20 were added:
  + 8.29 g of ammonium chloride
  + 1.0 g of potassium bicarbonate
  + 37.2 mg of sodium EDTA
* pH was adjusted to 7.4

flow buffer (DPBS + 0.5% HI-FBS):

* to 95 mL of DPBS was added:
* 0.5 mL of HI-FBS
* stored at 4°C

flow buffer + azide (flow buffer + 0.05% sodium azide):

* to 49.5 mL of flow buffer was added:
* 0.5 mL of a 5% w/v sodium azide solution
* stored at 4°C

EDTA solution (1X DPBS containing 10% EDTA):

* to 50 mL of DPBS was added:
* 5 g of disodium EDTA
* placed in ultrasonic bath to facilitate dissolution
* stored at 4°C

Fc block (flow buffer + azide containing 22.5 μg/mL anti-mouse CD16/32):

* refer to accompanying spreadsheet for preparation details (**Supplementary Table 2**).

fixable viability dye:

* eFluor 455UV fixable viability dye was diluted 1.5:1,000 in 1X DPBS
* refer to accompanying spreadsheet for preparation details (**Supplementary Table 2**).

**Antibody Titration.** Immunolabeling concentrations yielding the highest staining index (SI) for each antibody in the study were determined by first harvesting the spleens of two 12-week-old female C57BL/6J mice placed under terminal anesthesia with a dose of 150 mg/kg of ketamine hydrochloride and 20 mg/kg xylazine hydrochloride diluted in sterile 0.9% NaCl delivered with a 1 mL tuberculin syringe equipped with a 26G needle as a single intraperitoneal (i.p.) injection. Using opposing frosted ends of 2 glass microscope slides, each spleen was gently macerated and rinsed with 4 mL of supplemented RPMI-1640 into a 60 x 15 mm polystyrene petri dish on ice. Splenocytes were aspirated from the dish, dispensed into a 15 mL conical tube, and centrifuged at 350 x g (max RCF) for 10 minutes at 4°C. The cell pellet was resuspended in 8 mL of a 1X ACK lysing buffer and placed on ice for 5 minutes to lyse red blood cells (RBCs). Six (6) mL of flow buffer + azide was added to the tube prior to filtering the cell suspension through a 40 μm nylon mesh into a fresh 15 mL conical tube. Splenocytes were again centrifuged at 350 x g (max RCF) for 10 minutes at 4°C then resuspended in 2 mL of flow buffer + azide. Cell counting was performed using a trypan blue and a glass hemocytometer. Cell concentration was adjusted accordingly to achieve a final concentration of 1x107 cells/mL.

Two-hundred (200) μL of the 1x107 cell/mL splenocyte suspension were added to wells A-H of 11 concentric columns of a 96-well V-bottom microplate using a multichannel pipette followed by centrifugstion at 100 x g (max RCF) for 3 minutes at 4°C. One-hundred and fifty (150) μL of cell supernatant was aspirated from each well followed by resuspension in 100 μL of Fc block (15 μg/mL final concentration). Splenocytes were incubated on ice for 5 minutes prior to centrifugation at 100 x g (max RCF) for 3 minutes at 4°C. One-hundred (100) μL was aspirated from each well before resuspension with 100 μL of target antibodies pre-diluted in Brilliant Stain Buffer to achieve the following two-fold serial dilution series per column for each of the 11 antibodies used in the study: 24, 12, 6, 3, 1.5, 0.75, 0.375, and 0.1875 μg/mL. Splenocytes were immunolabeled on ice for 15 minutes in the dark prior to centrifugation at 100 x g (max RCF) for 3 minutes at 4°C. Two-hundred (200) μL of supernatant were then aspirated followed by resuspension with 100 μL of flow buffer + azide. This washing step was repeated once except that 200 μL (instead of 100 μL) of flow buffer + azide was used in the resuspension step. DAPI was added to each well at a final concentration of 1μg/mL and allowed to incubate for 3-5 minutes prior to data acquisition.

Immunolabeled splenocytes were analyzed on a BD LSR II SORP flow cytometer equipped with a BD HTS for 96-well high-throughput sampling. The following acquisition 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). A total of 10,000 viable singlets were analyzed per well. The median fluorescence intensities (MFIs) of the first (background) and second (first true positive) peaks, and the 84th percentile of the first peak were identified using the layout editor tool of FlowJo software from which a SI for each antibody was calculated according to the following formula: SI=(MFIpos-MFIneg)/[(84%neg-MFIneg)/0.995]. The maximum SI (SImax) of each antibody was used as the target immunolabeling concentration in our longitudinal study.

**Stereotactic Engraftment of GBM Cells into the Mouse Brain.** The reader is referred to (Baker GJ et al. JoVE, 2014 PMID: 26650233) for detailed instruction on how to stereotactically engraft glioma cells into the mouse brain.

**Tissue Harvesting and Processing.** The following disposable reagents were gathered and labeled before each of our study’s 3 time points:

* 15 mL conical tubes (16)
* labeled: “blood”, <condition>, <replicate> (< > indicates a variable)
* 3 mL syringes equipped with 23G needles (16)
* labeled: “marrow”, <condition>, <replicate>
* 1 mL tuberculin syringes equipped with 26G needles (17)
* (16) labeled: “blood”, <condition>, <replicate>
* (1) unlabeled: used for injectable anesthesia
* 60 x 15 mm polystyrene petri dishes (64)
* labeled: <tissue> (excluding blood), <condition>, <replicate>
* 4 mL of supplemented RPMI-1640 was added to each dish
* 0.5 mL microcentrifuge tubes (80)
* labeled: <tissue>, <condition>, <replicate>

One-hundred (100) μL of a 10% EDTA solution was added to each 15 mL conical tube. Four (4) mL of supplemented RPMI-1640 was added to each 60 x 15 mm polystyrene petri dish with the exception of those labeled “marrow”, to which only 2 mL of supplemented media was added (the other 2 mL of supplemented RPMI-1640 was to be placed into each of the (16) 3 mL syringes). 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 to coat the inner barrel with EDTA by operating the plunger several times. All conical tubes, petri dishes, 3 mL syringes, and microcentrifuge tubes were stored on ice or at 4°C.

Sixteen (16) mice were terminally anesthetized in series with a 150 mg/kg of ketamine hydrochloride and 20 mg/kg xylazine hydrochloride diluted in sterile 0.9% NaCl delivered with the unlabeled 1 mL tuberculin syringe as a single i.p. injection. Refer to accompanying spreadsheet for preparation details (**Supplementary Table 2**). Once non-responsive to both toe and tail pinch, each mouse was pinned ventral side up to an extruded polystyrene foam block by its front and hind paws using four 26G needles and sprayed down with 70% EtOH to prevent fur from entering the dissection cavity. Lymphoid organs were harvested in the following order: blood, thymus, spleen, superficial/deep cervical lymph nodes, bone marrow. *Blood*: after making a “y” incision from the gut to the rib cage to expose the heart, whole blood was aspirated from the right ventricle directly into one of the aforementioned EDTA-coated 1 mL tuberculin syringes. The needle was removed before expelling blood into its respectively labeled 15 mL conical tube stored on ice. Each mouse was then transcardially perfused 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 fully exsanguinate the circulatory system. The method for mouse transcardial perfusion has been described in detail elsewhere (refer to citation 33 of the main text). *Thymus*: Once exsanguinated, each mouse was returned to the extruded polystyrene foam block for thymus excision with small dissection scissors and fine-tipped bent forceps being careful to remove any contaminating adipose and the mediastinal lymph nodes. Thymi were placed into 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 placed into respectively labeled 60 x 15 mm polystyrene petri dishes on ice. *Superficial/deep cervical lymph nodes*: Lymph nodes were dissected under a dissection microscope using small dissection scissors and fine-tipped bent forceps. Excised nodes were placed into respectively labeled 60 x 15 mm polystyrene petri dishes on ice. *Bone marrow*: The right hind limb of each mouse was removed using bone cutters. Musculature and tendons were stripped away from the femur and tibia and the proximal and distal epiphyses of each bone were removed using a single-edged razor blade. Marrow from the two bones was flushed into the respectively labeled 60 x 15 mm polystyrene petri dish on ice using the 2 mL of supplemented RPMI-1640 in the respectively labeled 3 mL syringe. Flushed marrow was gently aspirated and expelled back into the petri dish one time to facilitate cell dissociation.

Thymi, spleens, and cervical lymph nodes were macerated using opposing frosted ends of 2 glass microscope slides which were dipped into the 4 mL of supplemented RPMI-1640 of the respectively labeled 60 x 15 mm polystyrene petri dish to collect as may cells as possible. Plastic Pasteur pipettes were used to transfer lymph nodes onto the frosted end of one glass microscope slide for maceration. A fresh pair of slides were used for each tissue to prevent sample cross-contamination. Five (5) mL of ice-cold DPBS was added to each petri dish prior to filtering each cell suspension through a fresh 40 μm nylon mesh into respectively labeled 15 mL conical tubes on ice using a fresh 10 mL serological pipette for every sample. Filtered samples were centrifuged at 400 x g (max RCF) for 10 minutes at 4°C. Cell supernatants were aspirated and each pellet was resuspend in 4 mL of a 1X ACK lysing buffer using a fresh 5 mL serological pipette for each sample to avoid cross-contamination. Samples were placed on ice for 5 minutes to lyse RBCs. Tissue samples were centrifuged at 400 x g (max RCF) for 10 minutes at 4°C, had their supernatants aspirated, and their cells resuspended in flow buffer + azide using the following volumes: 1000 μL for thymi; 1000 μL for spleens; 200 μL for bone marrow; 100 μL for deep/superficial cervical lymph nodes.

Blood samples were next lysed by adding 10 mL of 1X ACK lysing buffer to each 15 mL polypropylene conical tube. Tubes were placed back on ice for 5 minutes (or until blood color changed from dark burgundy to bright red). Blood samples were then centrifuged at 400 x g (max RCF) for 10 minutes at 4°C, their supernatants aspirated, and their WBC pellets resuspended with 200 μL of flow buffer + azide. Samples were stored on ice.

Two (2) μL of each tissue sample were then added to the 198 μL of flow buffer (a 1:100 dilution) in the respectively labeled 0.5 mL microcentrifuge tubes using a P20 micropipette equipped with a gel loading tip. Ten (10) μL of each 1:100 dilution was further diluted 1:1 with a 0.4% trypan blue solution. Ten (10) μL of the resultant solution was then used to estimate cell number using a brightfield microscope and a glass hemocytometer. Typical cell yields were as follows: 3x105 – 1x106 cells from the blood; 3x107 – 8x107 cells from the spleen; 6x107 – 8x107 from the thymus; 6x106 – 1.5x107 from the combined deep/superficial cervical lymph nodes; 9x106 – 1.5x107 from the bone marrow. Cell counts were recorded in a spreadsheet which returned the volume of additional flow buffer + azide required per sample to achieve a final concentration of approximately 2x107 cells/mL (**Supplementary Table 2**). Because mouse blood typically contained less than 2x106 total cells, the 200 μL volume of each blood sample was simply split between the respective experimental well and the CD49b single-positive compensation control well.

**Immunolabeling.** The following disposable reagents were gathered and labeled before each of our study’s 3 time points:

* 1.5 mL microcentrifuge tubes (12)
* labeled in duplicate: <target antibody or CD45 isotype>
* tubes were placed into a microcentrifuge tube rack on ice
* 12-well V-bottom reagent reservoir (1)
* individual compartments labeled: <target antibody or CD45 isotype>
* 15 mL conical tubes (2)
* one labeled “cocktail”, the other labeled “FVD”
* placed on ice

Dilutions of each antibody (1:10) were prepared using the 12 microcentrifuge tubes. Refer to accompanying spreadsheet for preparation details (**Supplementary Table 2**). From these 1:10 dilutions, antibodies were further diluted in the respective wells of a 12-well V-bottom reagent reservoir to achieve 100 μL volumes at the final antibody concentration to immunolabel the cells of each single-color compensation control. The remaining 1:10 antibody dilutions were used to prepare a master mix of combined target antibodies (excluding the CD45 isotype) placed in the 15 mL tube labeled “cocktail”. All final antibody dilutions were stored on ice in the dark.

One-hundred (100) μL aliquots of each cell suspension were added to the wells of a 96-well V-bottom microplate using a multichannel pipette according to the plate diagram shown in (**Fig. 1**). The plate was then centrifuged at 100 x g (max RCF) for 6 minutes at 4°C. Fifty (50) μL of supernatant was aspirated and cells were resuspended with 100 μL of Fc block (15 μg/mL final concentration) the total volume of which was determined using the spreadsheet (**Supplementary Table 2**). Cells were blocked on ice for 5 minutes before being centrifuged at 100 x g (max RCF) for 5 minutes at 4°C. Fifty (50) μL of cell supernatant was aspirated. The 80 experimental tissue samples were resuspended with 91 μL of the combined target antibodies from the tube labeled “cocktail”. The 100 μL volumes in the 12-well V-bottom reagent reservoir were added to the respective single-color compensation control wells; CD45 isotype antibodies were added to the “ISO” well. Cells in the “UNS” and “FVD” wells were resuspended with 91 μL of Brilliant Stain Buffer. The microplate was allowed to incubate on ice in the dark for 15 minutes before adding 100 μL of DPBS to each well and mixing thoroughly with a multichannel pipette. The plate was then centrifuged at 100 x g (max RCF) for 5 minutes at 4°C followed by aspirating 191 μL from each well. Two-hundred (200) μL of DPBS were added to each well using a multichannel pipette and mixed thoroughly by pipetting. The plate was centrifuged at 100 x g (max RCF) for 5 minutes at 4°C followed by a 200 μL aspiration of supernatant. Fixable viability dye was diluted 1.5:1,000 in 1X DPBS in the 15 mL conical tube labeled “FVD” according to the spreadsheet (**Supplementary Table 2**). One-hundred (100) μL of the diluted FVD were added to each well (a 1:1,000 final dilution) except for the “UNS”, which was resuspended with 100 μL of DPBS only. Cells were incubated on ice in the dark for 30 minutes. One-hundred (100) μL of DPBS were then added to each well and with a multichannel pipette and pipetted thoroughly to wash. The plate was centrifuged at 100 x g (max RCF) for 5 minutes at 4°C followed by a 200 μL aspiration, addition of 200 μL of DPBS using a multichannel pipette, and through mixing. The plate was centrifuged at 100 x g (max RCF) for 5 minutes at 4°C followed by a 200 μL aspiration.

One-hundred (100) μL of a fixation/permeabilization solution were next added to each well and immediately resuspended with a multichannel pipette to prevent cell-to-cell crosslinking. Cells were incubated on ice in the dark for 20 minutes followed by the addition of 100 μL of flow buffer and thorough mixing with a multichannel pipette. The plate was centrifuged at 100 x g (max RCF) for 5 minutes at 4°C followed by a 200 μL aspiration and resuspension with 200 μL of flow buffer. A Microseal ‘F’ foil seal was applied to the top of the 96-well microplate to prevent dehydration, wrapped in aluminum foil to block light, and stored at 4°C prior to data acquisition by flow cytometry.

**PMT Calibration.** Detection channel PMT voltages were calibrated such that the signal intensity distribution corresponding to background autofluorescence of FVD-labeled splenocytes was on scale and to the left of center. FVD-labeled single-color compensation control splenocytes were run to verify that the assigned PMT voltages were compatible with the dynamic range of each immunomarker’s expression profile (i.e. that immunopositive cells were on scale). To prevent downstream compensation values from exceeding 100%, optical spillover of each single-color compensation control into off-target detection channels was checked to ensure that peak signal intensity occurred in the target detection channel. Sphero Rainbow Fluorescent Particles (single-positive beads) were run to predefine tolerability ranges for laser intensity, stability, and alignment before initiating our study so that changes in laser emission power could be monitored and accounted for between successive data acquisitions to prevent run-to-run variation. Single-positive beads were gated according to the following strategy:

* FSC-A vs. SSC-A: on single-positive beads
* biexponential histograms of all detection channels
* An interval gate was placed around the peak in each detection channel to define a narrow tolerability range to calibrate to between runs.

**Data Acquisition.** Cytometer setup & tracking was performed using FACSDiva CS&T research beads to optimize and standardize instrument performance prior to each data acquisition. The 96-well V-bottom plate containing the 80 immunolabeled experimental tissue samples and 16 optical controls was then loaded into a BD HTS affixed to a BD LSR II SORP flow cytometer. The gating strategy used at each acquisition was as follows:

* FSC-A vs. SSC-A: on all events (minus RBCs/debris)
* SSC-H vs. SSC-W: doublet discriminator
* FSC-H vs. FSC-W: doublet discriminator
* BUV395-A (FVD) (detected with 355 nm laser off of a 450/50 band pass filter) vs. FSC-A: on FVD negative cells (i.e. viable cells)
* biexponential histograms of all detection channels
  + laser line, band pass filter, long pass filter, antibody detected:
* 405 nm, 525/50, 505, V500-CD45
* 488 nm, 710/50, 690, PerCP/Cy5.5-CD45R/B220
* 355 nm, 740/35, 690, BUV737-CD11b
* 594 nm, 660/20, 640, Alexa Fluor 647-CD11c
* 488 nm, 525/50, 505, Alexa Fluor 488-CD3ε
* 405 nm, 670/35, 635, BV605-CD4
* 488 nm, 780/60, 755, PE/Cy7-CD49b
* 488 nm, 610/20, 600, PE-CF594-CD8α
* 488 nm, 575/26, 505, PE-F4/80
* 594 nm, 780/60, 735, APC/Cy7-Ly6C
* 405 nm, 780/60, 750, BV711-Ly6G

Samples were run in the following order:

1. SP beads (PRE): to check that PMT voltages were within previously defined tolerability ranges prior to data acquisition.
2. unstained control splenocytes (UNS)
3. unstained control splenocytes labeled with FVD (FVD)
4. control splenocytes labeled with FVD and CD45 isotype control antibodies (ISO)
5. single-color compensation controls stained with FVD (control splenocytes were used for each antibody except CD49b, in which case WBCs were used due to the increased abundance of CD49b+ cells in blood)
6. experimental samples (16 mice x 5 tissue samples=80 total)
7. SP beads (POST): to check that PMT voltages remained stable over the acquisition period (fluidic anomalies can impact laser delay stability)

Raw data were exported as FCS3.0 files upon completion of each data acquisition session.

**Spectral Deconvolution and Data Preprocessing.** FlowJo software was used to spectrally deconvolve raw flow cytometry data. Data from 15 of the 16 optical control wells (UNS was excluded) were imported into the *compensation group* of a new FlowJo *workspace*. CD45 signal intensity distributions were invariably unimodal making it difficult to objectively define a compensation gate for the CD45 single-positive control alone. Thus, the data corresponding to well E11 (ISO) was merged with that of F9 (CD45 single-color compensation control) using FlowJo’s *concatenate* feature. The merged data were saved as a new FCS3.0 file and imported into the *compensation group* of the current *workspace*. The original CD45 single-positive compensation control and ISO samples were then deleted from the *workspace*. The merging procedure resulted in a bimodal distribution and the ability to objectively define a CD45 compensation gate between its two peaks. The merged CD45 file plus the other 10 single-color compensation controls and the FVD well (E10)—which served as the compensation control for the FVD itself—were gated for viable singlets according to the following strategy:

* FSC-A vs. SSC-A: on all events (minus RBCs/debris)
* SSC-H vs. SSC-W: doublet discriminator
* FSC-H vs. FSC-W: doublet discriminator
* BUV395-A (FVD) vs. FSC-A (viewed as a contour plot at the 2% level): on FVD negative cells (i.e. viable cells)
* backgate to FSC-A vs. SSC-A: on total viable singlets (or a subset for scarce populations)

Once gated, viable singlets of each compensation control sample were visualized as histograms in their respective detection channel. Signal intensity values of each histogram were bisected at the interface of the penultimate and ultimate modes of each signal intensity distribution using FlowJo’s *bisector tool*. Its *compensation* tool was then opened and the subsets to the left and right of the bisection were dragged into fields labeled *negative* and positive, respectively. The process was repeated for all 11 antibodies plus the FVD. Next, a new *group* in the *workspace* window was generated and titled “cocktails” to which the 80 experimental samples were imported. The finalized compensation matrix was then applied to the “cocktails” group. Viable singlets from each experimental sample were gated in the same way as the single-color compensation controls according to the first 4 steps of the gating strategy outlined above then exported as new FCS3.0 files; the aggregate of such files from each of the study’s 3 time points served as input into our computational data analysis algorithm.