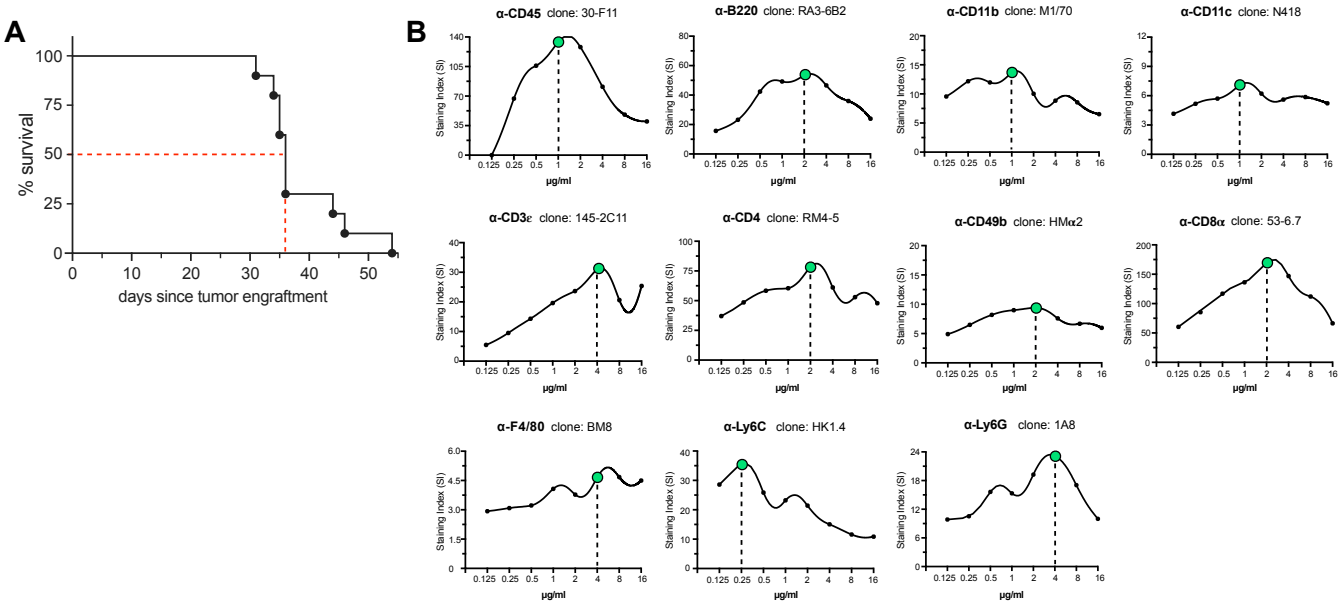
Cell Systems, Volume *11*

Supplemental Information

SYLARAS: A Platform for the Statistical Analysis and Visual Display of Systemic Immunoprofiling Data and Its Application to Glioblastoma

Gregory J. Baker, Jeremy L. Muhlich, Sucheendra K. Palaniappan, Jodene K. Moore, Stephanie H. Davis, Sandro Santagata, and Peter K. Sorger

Content



C

type	name	bead size	threshold	# of events	FSC voltage	SSC voltage
cytometer setup & tracking	CS&T beads	2 μm (dim), 3 μm (mid/bright)	12,910	automated	automated	automated
PMT calibration	Sphero Rainbow Fluorescent Particles	3.0 - 3.4 μm	5,000	50,000	538	390

D

target	clone	conjugate	detection channel	laser line (nm)	PMT intensity	voltage	band pass filter	long pass filter	type	log
CD45	30-F11	V500	AmCyan	405	24,291	302	525/50	505	area	on
B220	RA3-6B2	PerCP/Cy5.5	PerCP/Cy5.5	488	51,789	495	710/50	690	area	on
CD11b	M1/70	BUV737	BUV737	355	39,925	610	740/35	690	area	on
CD11c	N418	AF647	AF647	594	187,488	374	660/20	640	area	on
CD3ε	145-2C11	AF488	AF488	488	59,683	511	525/50	505	area	on
CD4	RM4-5	BV605	BV650	405	60,119	500	670/35	635	area	on
CD49b	HMα2	PE/Cy7	PE/Cy7	488	22,867	604	780/60	755	area	on
CD8α	53-6.7	PE-CF594	PE-Texas Red	488	107,455	440	610/20	600	area	on
F4/80	BM8	PE	PE	488	156,784	519	575/26	505	area	on
Ly6C	HK1.4	APC/Cy7	APC/Cy7	594	55,387	427	780/60	735	area	on
Ly6G	1A8	BUV711	BUV786	405	36,228	650	780/60	750	area	on
live/dead	n/a	eFluor455UV	BUV395	355	29,688	339	450/50	n/a	area	on
CD45 isotype	A95-1	V500	AmCyan	405	24,291	302	525/50	505	area	on
			FSC (25K threshold)			359			area, height, width	off
			SSC			460			area, height, width	off

E

throughput	volume per well (μL)	flow rate (μL/sec)	sample volume (μL)	wash volume (μL)	# of mixes	mixing volume (μL)	mixing speed (μL/sec)	stopping gate
standard	250	1.5	200 (samples and beads)	600	3	50	50	10M (all events)

Fig. S1. Experimental Optimization for 12-color Immunophenotyping of GBM-bearing Mice by Flow Cytometry, Related to Fig. 1.

(**A**) Kaplan-Meier Survival Analysis of the GL261 Mouse Glioma Model. Ten (10) C57BL/6J mice were intracranially engrafted with $3x10^4$ syngeneic GL261 cells at 12-weeks-of-age.

Fig. S1 (continued). Median survival (indicated by the intersecting dashed red lines) was 36-days post tumor engraftment. (**B**) 8-point, 2-fold serial dilution titration curves for 11 immune lineage markers using splenocytes from 12-week-old female C57BL/6J mice. Separation index (SI) was calculated as $(MFI_{pos}-MFI_{neg})/[(84\%_{neg}-MFI_{neg})/0.995]$, where MFI_{pos} = median fluorescence intensity of the first positive peak, MFI_{neg} = median fluorescence intensity of the autofluorescence peak, $84\%_{neg}$ = 84th percentile of the autofluorescence peak (Telford et al., Cytometry A. 2009). Adjacent data points were interpolated with cubic splines. Antibody concentrations resulting in maximum SI values (SI_{max}) are indicated by green dots. Data acquisition gating strategy: (FSC-A vs. SSC-A) → (SSC-H vs. SSC-W) → (FSC-H vs. FSC-W) → (DAPI-A vs. FSC-A) → (CD_x vs. count). (**C**) Settings for cytometer setup/tracking and photomultiplier tube (PMT) calibration used to standardize cytometer performance across data acquisition cycles. (**D**) Cytometer run settings. (**E**) High-Throughput Sampler (HTS) configurations.


detection channel:
AmCyan
PCP/Cy5.5
BUV737
AF647
AF488
BV650
PE/Cy7
PE-Tx. Red
PE
APC/Cy7
BV786
CD45 (V500), clone: 30-F11
PRE-compensation
POST-compensation
B220 (PerCP/Cy5.5), clone: RA3-6B2
PRE-compensation
POST-compensation
CD11b (BUV737), clone: M1/70
PRE-compensation
POST-compensation
CD11c (AF647), clone: N418
PRE-compensation
POST-compensation
CD3ε (AF488), clone: 145-2C11
PRE-compensation
POST-compensation
CD4 (BV605), clone: RM4-5
PRE-compensation
POST-compensation
CD49b (PE/Cy7), clone: HMα2
PRE-compensation
POST-compensation
CD8α (PE-CF594), clone: 53-6.7
PRE-compensation
POST-compensation
F4/80 (PE), clone: BM8
PRE-compensation
POST-compensation
Ly6C (APC/Cy7), clone: HK1.4
PRE-compensation
POST-compensation
Ly6G (BV711), clone: 1A8
PRE-compensation
POST-compensation
FVD
PRE-compensation

Fig. S2. Optical Spillover Among an Optimized Panel of 12 Mouse Immunophenotyping Antibodies is Fully Abrogated by Spectral Deconvolution, Related to Fig. 1.

Signal intensity distributions of splenocytes from 12-week-old female C57BL/6J mice immunolabeled with an optimized 11-antibody immunomarker panel then stained with fixable viability dye (FVD). Detection channels of a BD LSR II SORP flow cytometer (columns) are shown pre- and post-compensation. Antibodies (rows) are color-coded according to their target detection channel. Histograms forming the downward diagonal from left to right across the matrix show the placement of the respective channel's compensation gate (blue-green interfaces).

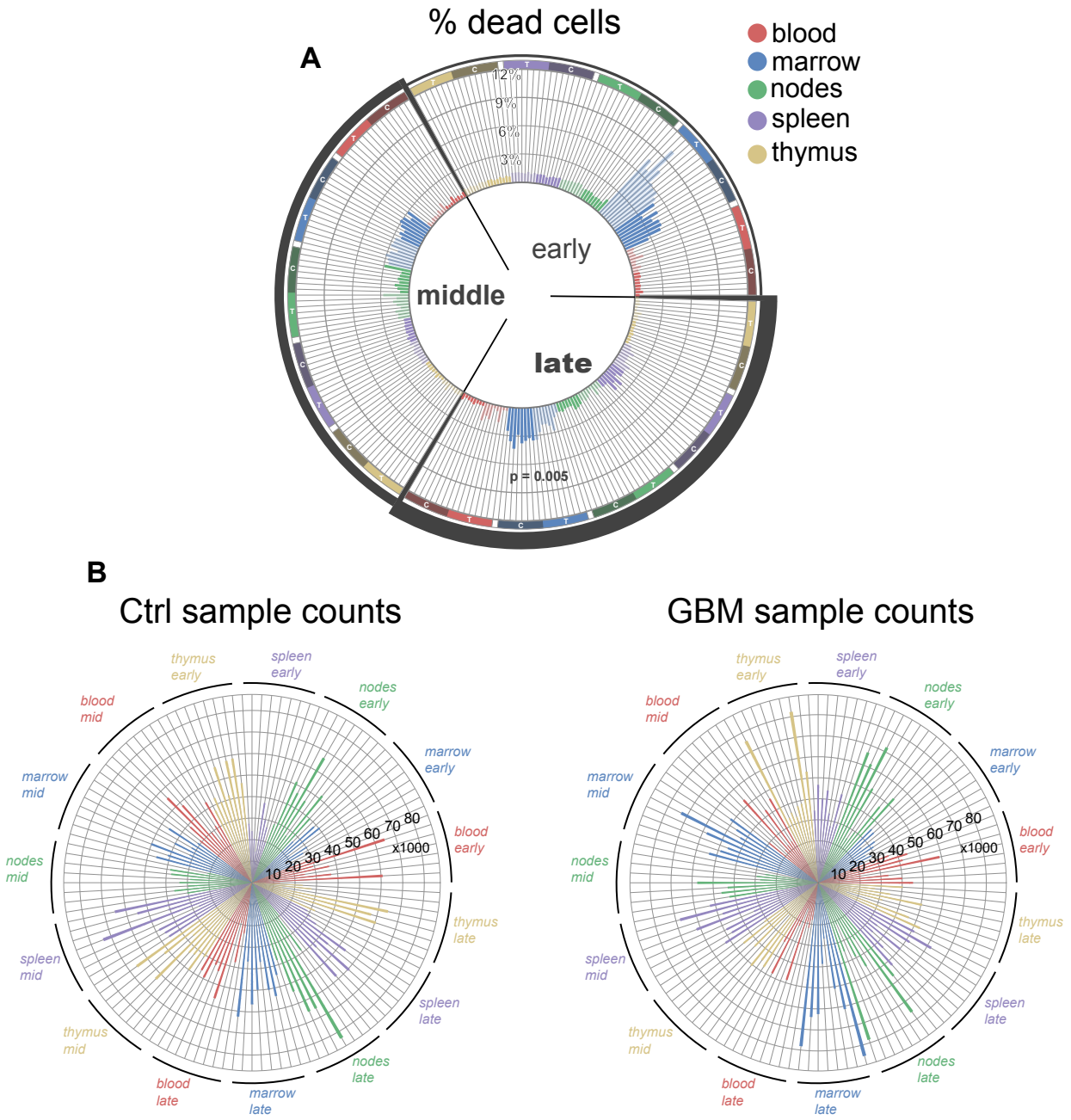
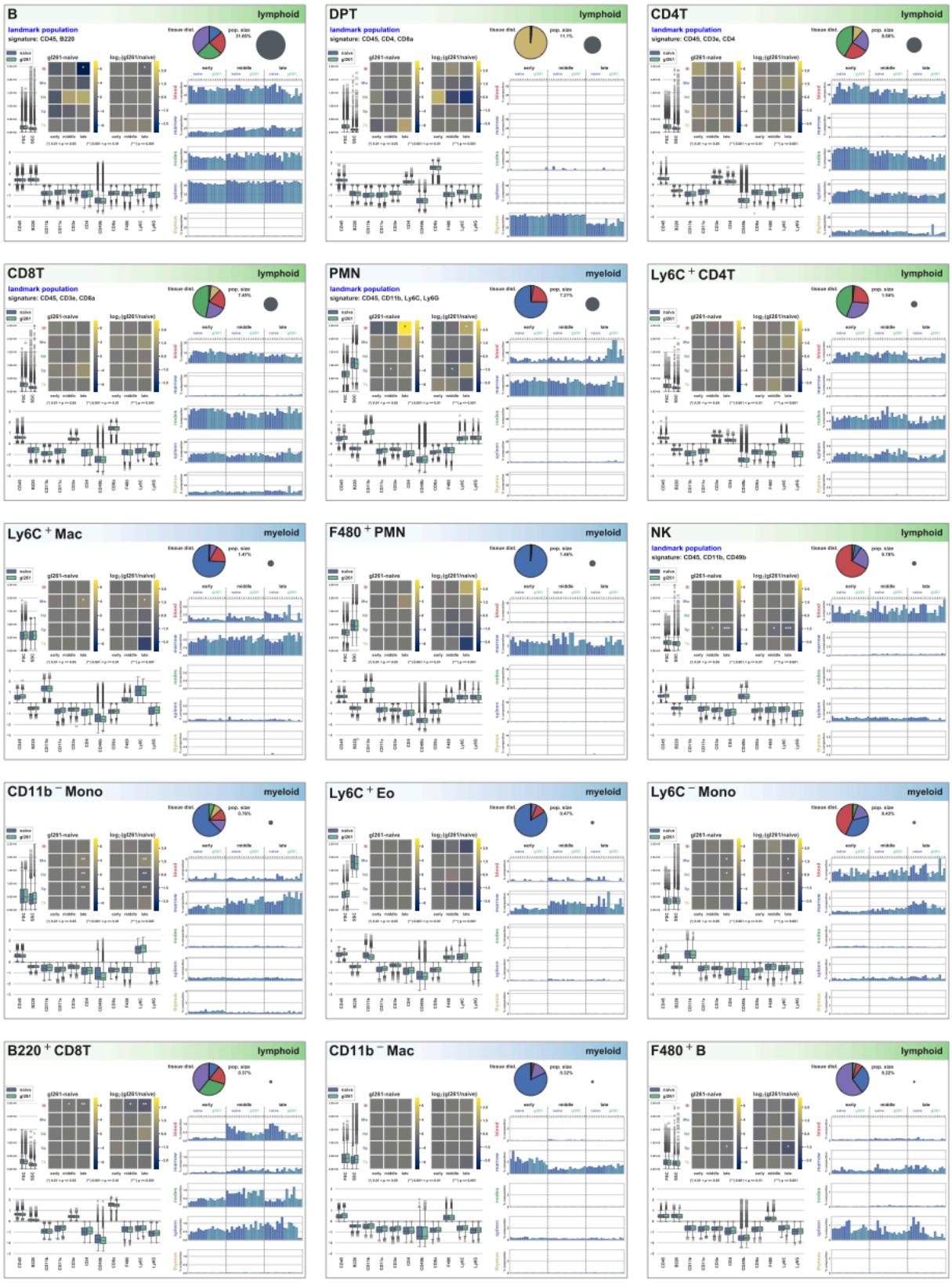


Fig. S3. Cell Viability and Sample Counts for the 240 Lymphoid Tissues Analyzed in this Study, Related to Fig. 1. (**A**) Radial bar chart showing the percentage of dead cells among the dataset's 240 tissue samples. C = control samples, T = GBM samples. Viability only differed between the two groups in the bone marrow at the t = 30-day time point (p = 0.005, two-tailed independent Student's t test, n = 8 mice/group). (**B**) Radial bar charts showing the number of cells in control (left) and GBM (right) mouse tissue samples after weighted random sampling by tissue to balance the number of cells per sample.


B
lymphoid
DPT
lymphoid
CD4T
lymphoid
CD8T
lymphoid
PMN
myeloid
Ly6C + CD4T
lymphoid
Ly6C + Mac
myeloid
F480 + PMN
myeloid
NK
lymphoid
CD11b − Mono
myeloid
Ly6C + Eo
myeloid
Ly6C − Mono
myeloid
B220 + CD8T
lymphoid
CD11b − Mac
myeloid
F480 + B
lymphoid

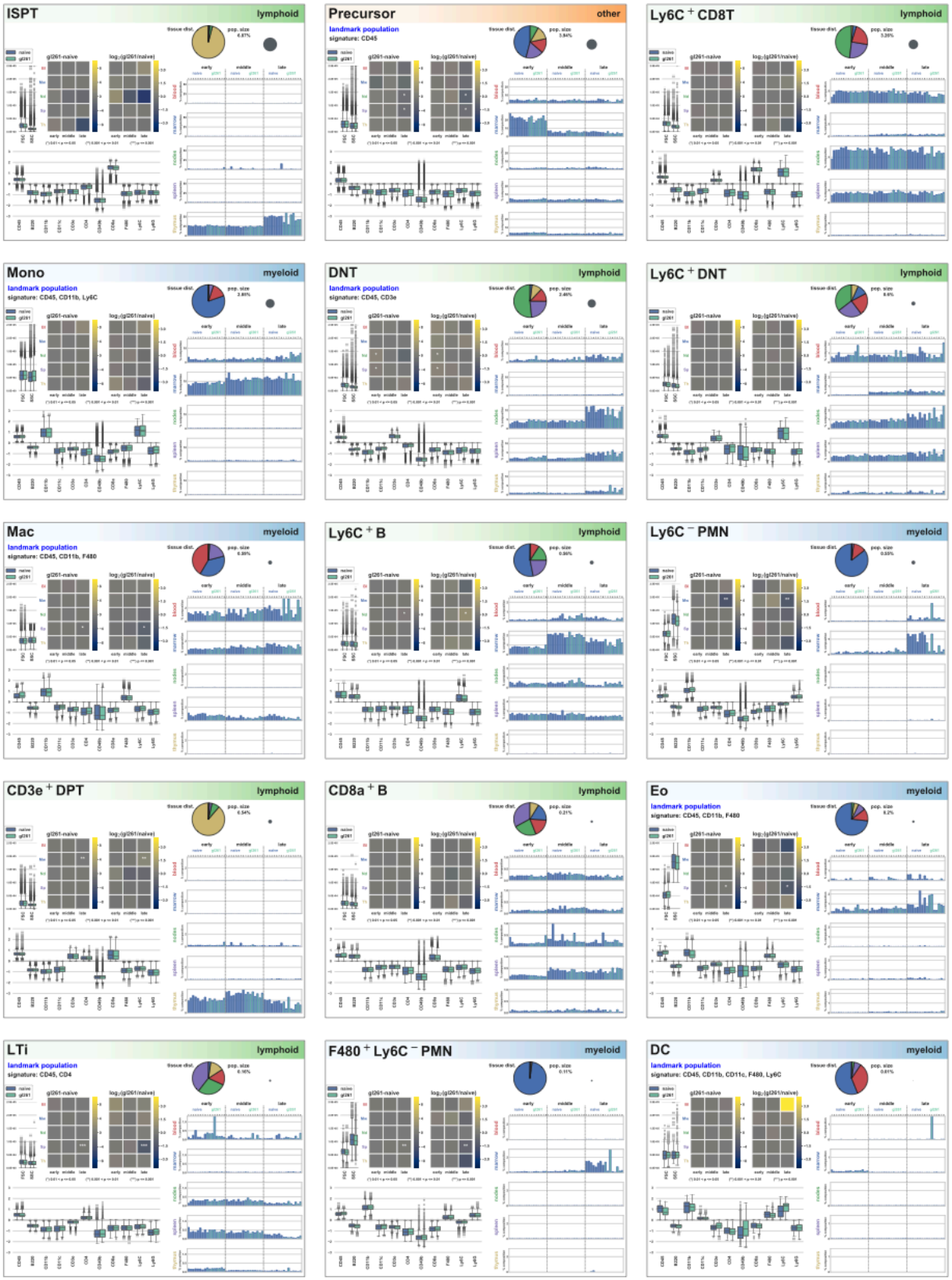
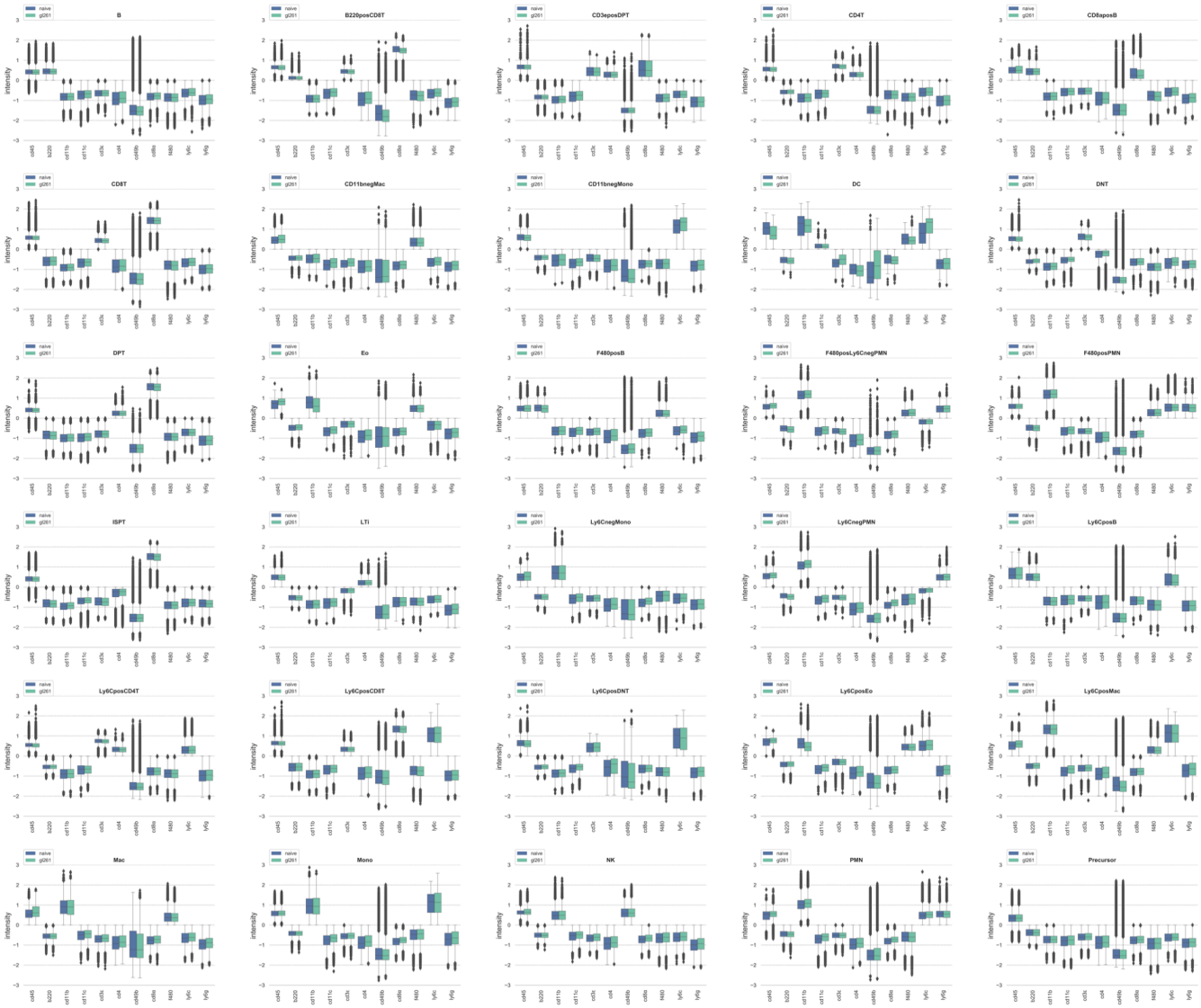


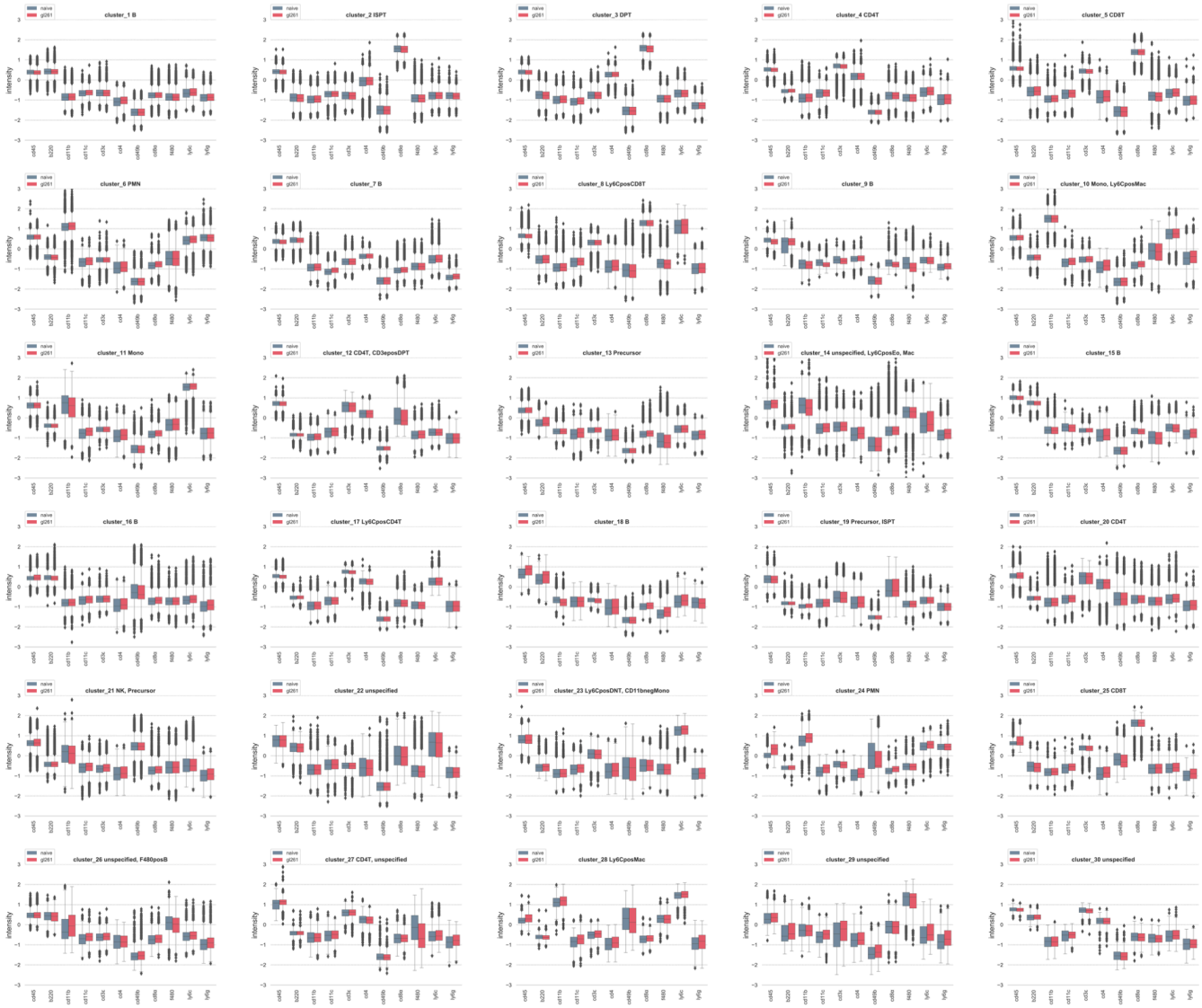
Fig. S4. Dashboards from a SYLARAS screen of the GL261 Glioma Model, Related to Fig. 2.

PDF available for download at: https://www.synapse.org/#!Synapse:syn22249852.

Manual Gating



PhenoGraph


naive
g261
cluster_1 B
cluster_2 ISPT
cluster_3 DPT
cluster_4 CD4T
cluster_5 CD8T
cluster_6 PMN
cluster_7 B
cluster_8 Ly6CposCD8T
cluster_9 B
cluster_10 Mono, Ly6CposMac
cluster_11 Mono
cluster_12 CD4T, CD3eposDPT
cluster_13 Precursor
cluster_14 unspecified, Ly6CposEo, Mac
cluster_15 B
cluster_16 B
cluster_17 Ly6CposCD4T
cluster_18 B
cluster_19 Precursor, ISPT
cluster_20 CD4T
cluster_21 NK, Precursor
cluster_22 unspecified
cluster_23 Ly6CposDNT, CD11bnegMono
cluster_24 PMN
cluster_25 CD8T
cluster_26 unspecified, F480posB
cluster_27 CD4T, unspecified
cluster_28 Ly6CposMac
cluster_29 unspecified
cluster_30 unspecified
intensity
cd45
b220
cd11b
cd11c
cd3c
cd4
cd49b
cd8a
f480
ly6c
ly6g

FlowSOM

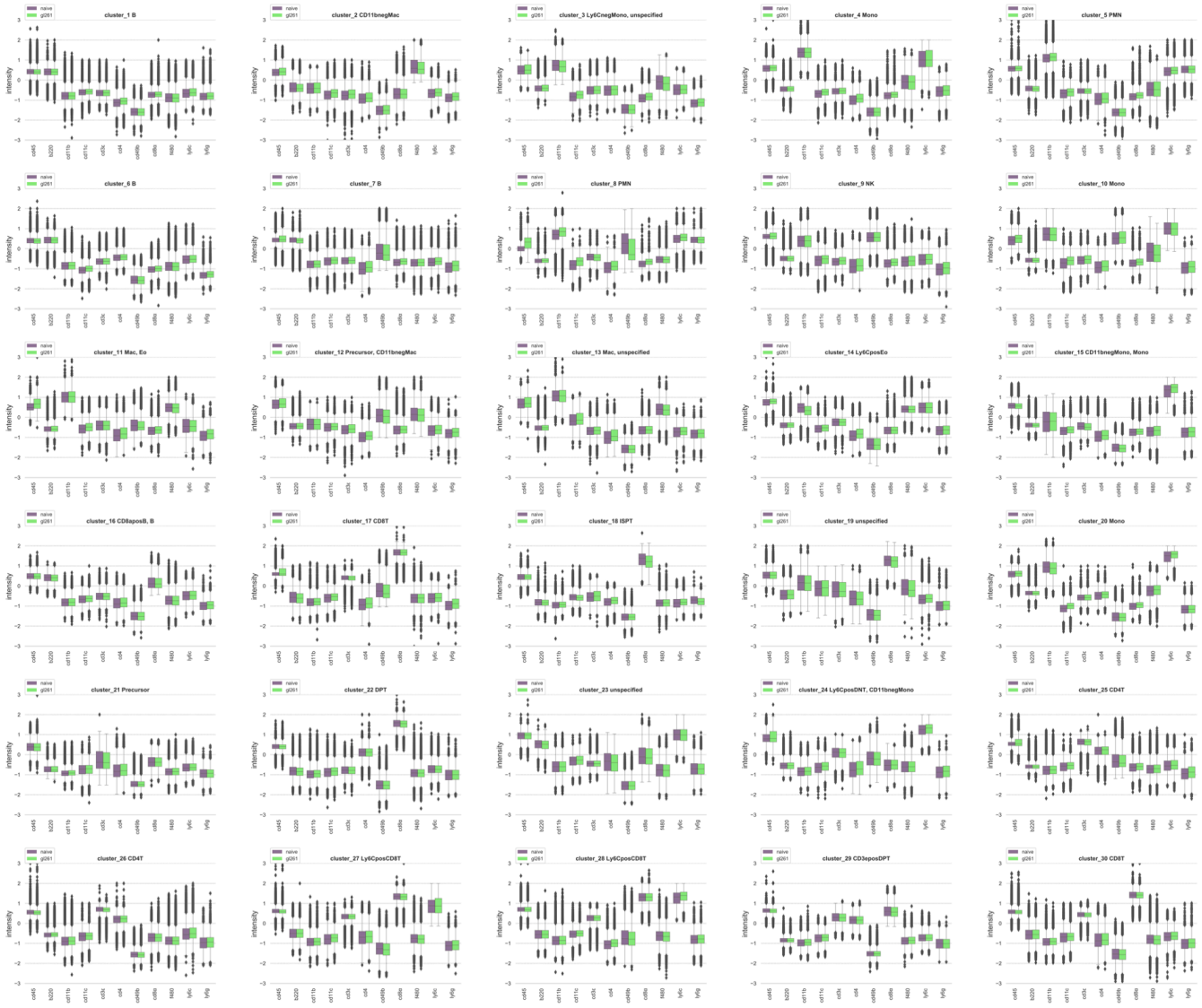
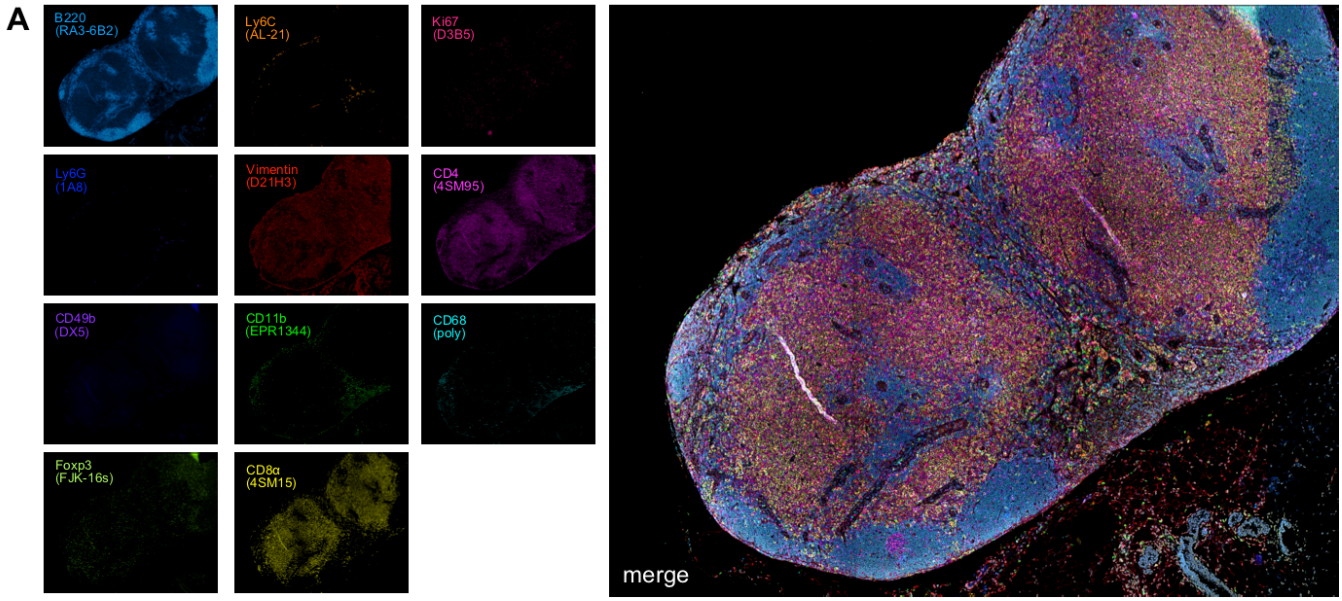


Fig. S5. Antigen Expression by Different Mouse Immune Cell Subsets Identified via Prior Knowledge and Unsupervised Clustering Algorithms, Related to Fig. 4.

Boxplot distributions of Logicle-transformed antigen expression by various immune cell subsets from control (naïve) and GBM-bearing (gl261) mice identified through manual gating (top), PhenoGraph clustering (middle), and FlowSOM clustering (bottom). PDF available for download at: https://www.synapse.org/#!Synapse:syn22263977.



See figure legend below for link to high-resolution TIFF.

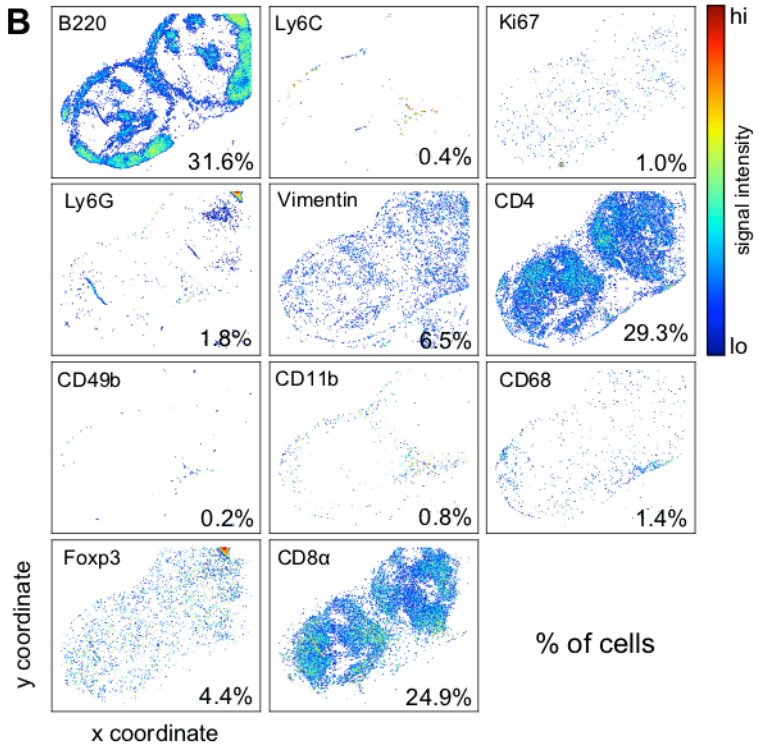
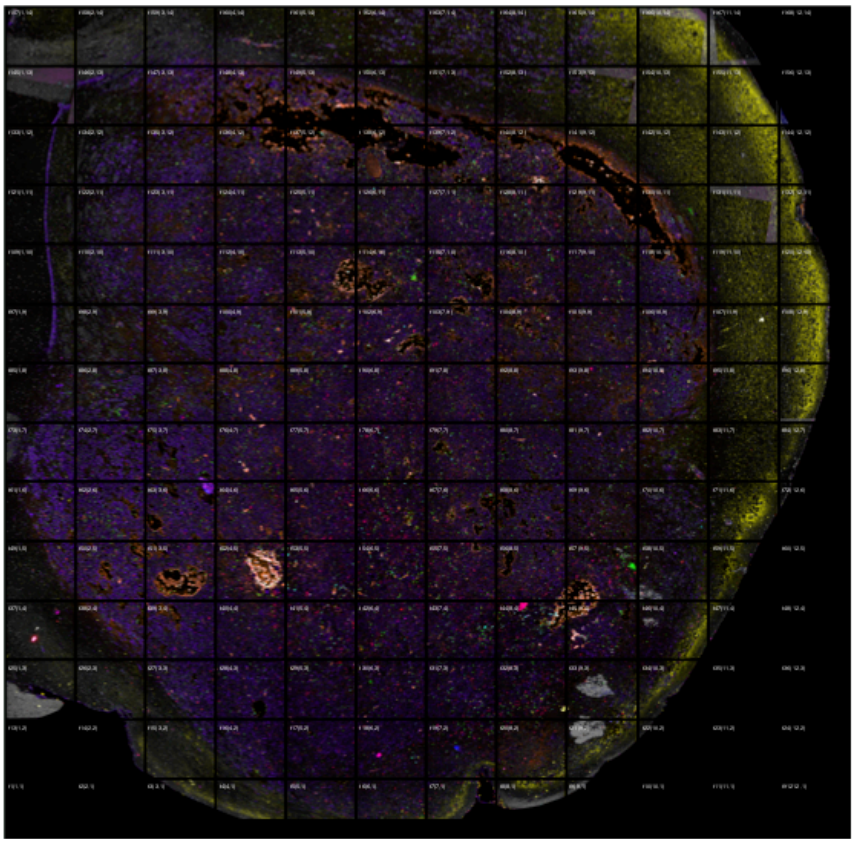


Fig. S6. Validation of a 12-channel t-CyCIF Antibody Panel, Related to Fig. 7.

(**A**) 11 antibodies validated on inguinal lymph node FFPE tissue sections from a C57BL/6 mouse; individual channels (left) and composite image (right) are shown.

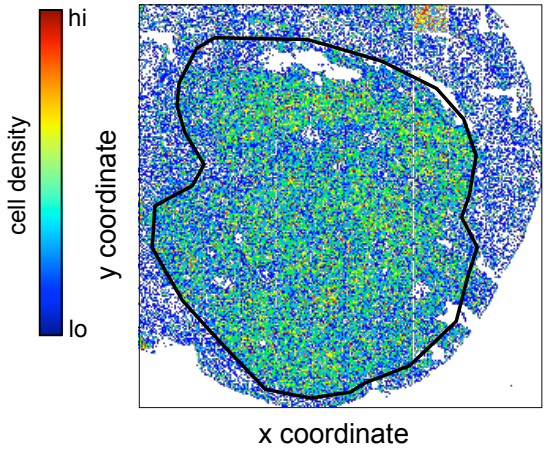
Fig. S6 (continued). High-resolution TIFF available for download at: https://www.synapse.org/#!Synapse:syn22249837. (**B**) X and Y coordinates of cells from the micrograph shown in (A) color-coded according to antibody signal intensity; the percentage of total cells immunoreactive to each antibody is shown.

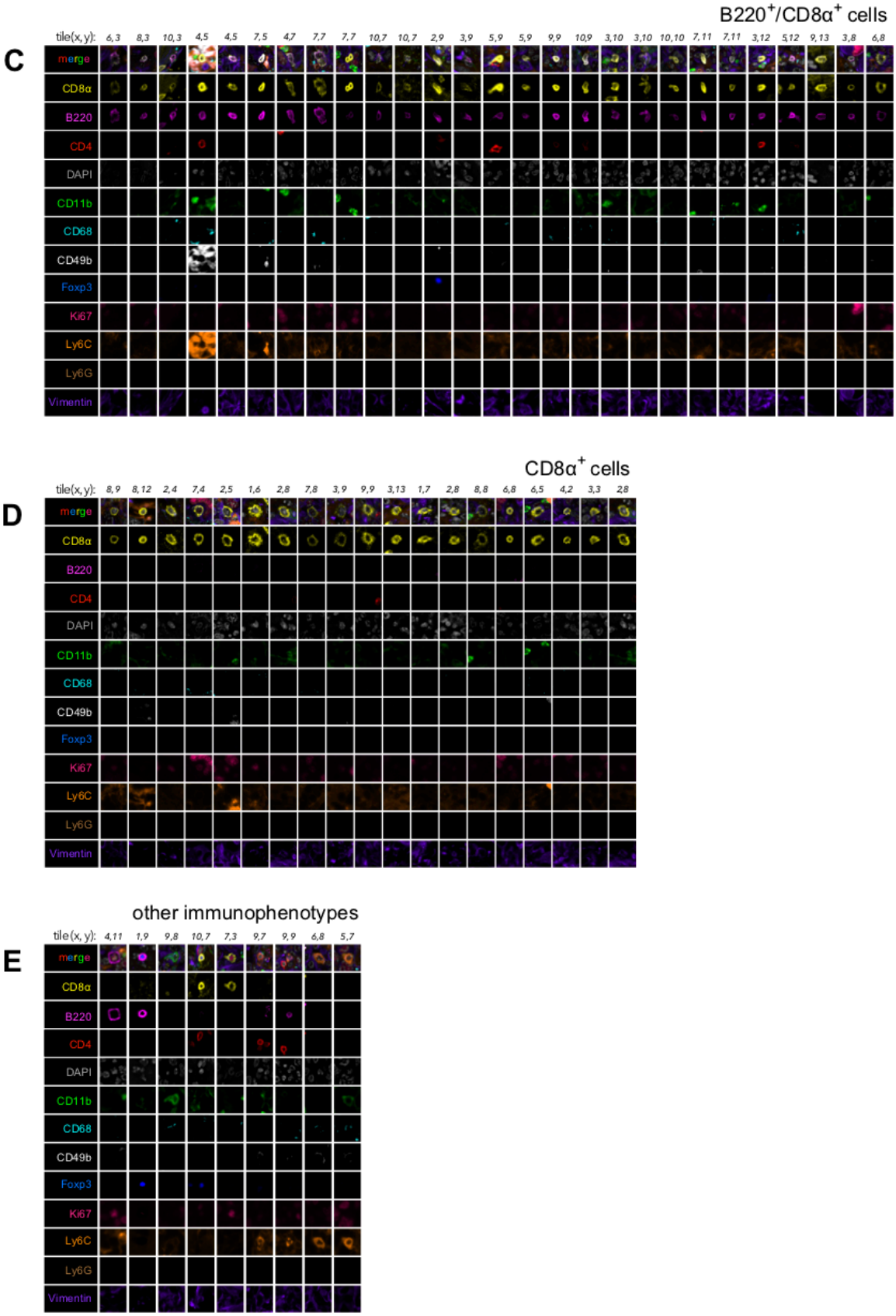
A



See figure legend below for link to high-resolution TIFF.

B





See figure legend below for link to high-resolution TIFFs.

Fig. S7. Survey of Glioma-infiltrating Lymphocytes by 12-channel t-CyCIF, Related to Fig. 7.

(**A**) A 12-channel (11 antibodies plus nuclear counter stain), 168-tile (400μm x 300μm fields of view) mosaic image taken at 40X of the tumor-ipsilateral mouse brain hemisphere bearing GL261 glioma 36-days after engraftment. Tile numbers and grid coordinates are indicated in increasing order from the bottom-left to the top-right of the image. Immunomarker colors are as in (C-E). High-resolution TIFF available for download at: https://www.synapse.org/#!Synapse:syn22249837. (**B**) X and Y coordinates of ~$9x10^4$ cells extracted from the image shown in (A). Data points representing individual cells are colored according to cell density. Black perimeter outlines the tumor/brain parenchyma interface. (**C**) Examples of B220/CD8α double-positive cells. Tile coordinates are provided for each image for cross-referencing with the image shown in (A). (**D**) Examples of CD8α single-positive cells. Tile coordinates are provided for each image for cross-referencing with the image shown in (A). (**E**) Examples of other, lower abundance, immunophenotypes identified in the late-stage GL261 tumor microenvironment. Tile coordinates are provided for each image for cross-referencing with the image shown in (A). High-resolution TIFFs of (C-E) available for download at: https://www.synapse.org/#!Synapse:syn22249837.