Supplementary Methods

IHC Staining Image Analysis: Antibodies used can be found in Supplementary Table 1. Multispectral hematoxylin/DAB images were captured using a Nikon Eclipse Ci with a Nuance Imaging System (Perkin Elmer). Software was trained to segment cells based on nuclear staining. Known positive controls for each antigen were used to set DAB optical density thresholds, and positive cells contained at both a DAB-positive region and a nucleus. Demographic data for samples included on the TMA are listed in Supplementary Table 2.

Expression Analysis and Visualization: Normalized data was analyzed across datasets and plots generated using the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) and included "Harris" normal brain[1], "Gutman" pilocytic astrocytoma[2], "Paugh" pediatric glioma (GBM only)[3], "Paugh" DIPG[4], and "Loeffler" adult GBM[5].

Autopsy Sample Collection: Research autopsies were performed as quickly as possible after patient death to minimize ischemic time, 6-24 hours post-mortem. Samples were collected and immediately formalin-fixed or flash frozen.

Autopsy vs Surgical Tissue: Confounding effects of tissue ischemic time were evaluated by staining five pHGG samples with CD68, CD163, CD3, and CD8, imaging four regions of each (to control for intratumoral heterogeneity), and comparing to the TMA pHGG samples, collected at the time of resection. Percent area positive is plotted in GraphPad and evaluated by t-test.

Survival Analysis: Percent cells positive data from each TMA spot was averaged for each patient, ranked from highest to lowest infiltration, and a Kaplan-Meier of the upper and lower infiltration plotted vs survival from time of diagnosis. The Mantel-Cox test was used to evaluate differences between the survival curves. For CD68 and CD163, Nanostring normalized count data was also split into upper 50% and lower 50% expression, and included in the survival curves. CD68, CD163: n=20. CD3, CD8: n=9.

Tumor Tissue Nanostring Analysis: Raw data was imported into R using the NanoStringNorm package without normalization. Raw counts were normalized with the DESeq2 package[6] using default

parameters. Normalized counts are reported in for samples above the noise threshold, defined as two standard deviations above the negative control signal. Significance values were calculated using DESeq2 and are reported as "NA" for those genes that did not pass thresholds for independent filtering or for samples that contained outliers using Cook's distance measure. Demographic data for patients included in Nanostring analysis can be found in Supplementary Table 3.

Additional Cell Lines: Validated SU-pcGBM-II was a kind gift from the Monje Lab. LN229, U138, and T98G were purchased from ATCC. Cells were cultured as described in the main text.

Growth Assay: Cells were plated at 1e5 per six-well plate in Neurocult media on day 0 and counted by hemocytometer on days 2, 4, and 7. Media was added as necessary to prevent overgrowth and acidification. Number of live cells was plotted in GraphPad Prism and differences tested by two-way ANOVA.

Luminex assays: DIPG or GBM cells were seeded at 5e5/mL in Neurocult media. After 24 hours, cell-free supernatant was collected and frozen at -80C until analysis. Bio-Rad BioPlex Pro assays were performed on a BioPlex 200 and analyzed using BioPlex Manager software according to the manufacturer's protocols.

Macrophage Nanostring: Following co-culture, all floating cells were aspirated and adherent cells washed and detached with Versene. CD45 positive selection (Miltenyi) was performed to isolate macrophages. RNA was isolated from macrophage pellets using the RNeasy Mini Kit and run with the Nanostring Human Myeloid Panel. Analysis of select genes was performed in nSolver software (Nanostring).

Supplementary References

1. Harris LW, Lockstone HE, Khaitovich P, Weickert CS, Webster MJ, Bahn S. Gene expression in the prefrontal cortex during adolescence: implications for the onset of schizophrenia. *BMC Med Genomics*. 2009; 2:28.

2. Sharma MK, Mansur DB, Reifenberger G, et al. Distinct genetic signatures among pilocytic astrocytomas relate to their brain region origin. *Cancer Res*. 2007; 67(3):890-900.

3. Paugh BS, Qu C, Jones C, et al. Integrated molecular genetic profiling of pediatric high-grade gliomas reveals key differences with the adult disease. *J Clin Oncol*. 2010; 28(18):3061-3068.

4. Paugh BS, Broniscer A, Qu C, et al. Genome-wide analyses identify recurrent amplifications of receptor tyrosine kinases and cell-cycle regulatory genes in diffuse intrinsic pontine glioma. *J Clin Oncol*. 2011; 29(30):3999-4006.

5. Reifenberger G, Weber RG, Riehmer V, et al. Molecular characterization of long-term survivors of glioblastoma using genome- and transcriptome-wide profiling. *Int J Cancer*. 2014; 135(8):1822-1831.

6. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014; 15(12):550.

Supplementary Figure Legends

Supplementary Figure 1: Representative staining of TMAs. For each antibody, an area is shown both in the raw and processed form for control and tumor tissue.

Supplementary Figure 2: Immune infiltration in surgical and autopsy specimens. pHGG tissue from surgery or autopsy was evaluated for infiltraton of CD68, CD163, CD3, and CD8. Bars represent median. Differences were evaluated by Student's t-test, no significant differences found.

Supplementary Figure 3: TMA analysis by percent area. Regions of antigen positivity are plotted as a percent of total area. Bars represent median. Significance evaluated by one-way ANOVA.

Supplementary Figure 4: Transcript expression by various brain tumor types in a public database. A-D) R2 software was used to visualize MAS5.0 normalized gene expression from a standard Affymetrix u133p2 array collected across different datasets for CD68, CD163, CD3E, and CD8A. Datasets include "Harris" normal brain, "Gutman" pilocytic astrocytoma, "Paugh" pediatric GBM, "Paugh" DIPG, and "Loeffler" adult GBM.

Supplementary Figure 5: Transcript expression by pediatric brain tumors. Nanostring analysis of RNA isolated from snap-frozen tissue for the expression of for CD68 and CD163. Bars represent mean. Significance evaluated by one-way ANOVA.

Supplementary Figure 6: Relationship between DIPG patient survival and immune cell infiltration. Samples used for TMA and Nanostring (CD163, CD68, and CD163:CD68) or TMA only

(CD3 and CD8) were ranked for highest to lowest expression of each gene, split into upper and lower groups at the median, and Kaplan-Meier curves plotted. Significance was evaluated by Mantel-Cox test.

Supplementary Figure 7: Expression of immunosuppressive factors by DIPG and GBM cell cultures. A) Expression of soluble immunosuppressive factors was evaluated by luminex in three DIPG cultures (light grey) and 5 GBM lines (dark grey). Significance evaluated by one-way ANOVA with U87 serving as the control for Dunnett's multiple comparisons test, or by T-test when factors were detectable in only two groups. n/d: Not detected. N=3-4, error bars represent SEM. B) Secretion of each factor by grouped DIPG and GBM cultures evaluated by Student's T-test; no differences found. Bars represent mean.

Supplementary Figure 8: Expression of chemokines by DIPG and GBM cell cultures. A) Expression of chemokines was evaluated by luminex in three DIPG cultures (light grey) and 5 GBM lines (dark grey). Significance evaluated by one-way ANOVA with U87 serving as the control for Dunnett's multiple comparisons test, or by T-test when factors were detectable in only two groups. n/d: Not detected. N=3-4, error bars represent SEM. B) Secretion of each factor by grouped DIPG and GBM cultures evaluated by Student's T-test; no differences found. Bars represent mean.

Supplementary Figure 9: Growth rate of cultured DIPG and GBM cells. Cells were plated at equal density and counted over seven days of growth. N=3. Significance evaluated by two-way ANOVA, no differences found.

Supplementary Figure 10: Macrophage transcript expression following co-culture with tumor cell cultures. Significance evaluated by one-way ANOVA with media serving as the control for Dunnett's multiple comparisons test. N=4, error bars represent standard SEM.

Supplementary Figure 11: Flow cytometry of additional cultured GBM cells. Histograms are overlayed with isotype control.

Supplementary Figure 12: NKG2D ligand expression in DIPG. Percent cells positive and representative staining for control and DIPG tumor tissue. Bars represent mean. Significance evaluated by Student's t-test.

Supplementary Table 1: Antibodies used for immunohistochemistry and flow cytometry

Application	Cell Type	Target	Clone	Manufacturer
IHC	Macrophage	CD68	FA-11	Abcam
IHC	Macrophage	CD163	10D6	Leica
IHC	T cell	CD3	(polyclonal)	Dako
IHC	T cell	CD8	4B11	Leica
IHC	Tumor, Macrophage	PD-L1	015	Sino Biological
IHC	Tumor	MICA/B	6D4	Abcam
IHC	Tumor	ULBP1	(polyclonal)	Atlas
IHC	Tumor	ULBP3	(polyclonal)	Biorybt
IHC	Tumor	ULBP2/5/6	(polyclonal)	R&D
Flow Cytometry	DIPG	HLA-A,B,C	W6/32	Biolegend
Flow Cytometry	DIPG	CD133	clone 7	Biolegend
Flow Cytometry	DIPG	NKG2D-Fc	(n/a)	R & D Systems
Flow Cytometry	DIPG	MICA/B	6D4	Biolegend
Flow Cytometry	DIPG	PD-L1	MIH2	Biolegend
Flow Cytometry	Macrophage	PD-L1	29E.2A3	Biolegend
Flow Cytometry	Macrophage	CD14	M5E2	Biolegend
Flow Cytometry	Macrophage	CD163	GHI/61	BD Biosciences
Flow Cytometry	Macrophage	CD206	15-2	Biolegend

Flow Cytometry	T cell	CD3	OKT3	Biolegend
Flow Cytometry	T cell	CD4	OKT4	Biolegend
Flow Cytometry	T cell	CD8	RPA-T8	BD Biosciences
Flow Cytometry	T cell, NK cell	IFNγ	B27	Biolegend
Flow Cytometry	NK cell	TNFα	MAb11	Biolegend
Flow Cytometry	NK cell	CD16	3G8	BD Biosciences
Flow Cytometry	NK cell	CD56	HCD56	Biolegend

Supplementary Table 2: Characteristics of Patients included in Tumor Microarray

#	Diagnosis	Age (y)	Sex
1	GLIOBLASTOMA MULTIFORME	13.9	F
2	GLIOBLASTOMA MULTIFORME	15.6	M
3	GLIOBLASTOMA MULTIFORME	17.4	M
4	ANAPLASTIC ASTROCYTOMA	1.4	M
5	GLIOBLASTOMA MULTIFORME	7.7	M
6	GLIOBLASTOMA MULTIFORME	13.5	M
7	ANAPLASTIC ASTROCYTOMA	11.5	M
8	ANAPLASTIC ASTROCYTOMA	7.6	F
9	ANAPLASTIC ASTROCYTOMA	7.6	F
10	GLIOBLASTOMA MULTIFORME	6.1	M
11	GLIOBLASTOMA MULTIFORME	17.3	M
12	ANAPLASTIC ASTROCYTOMA	15.2	M
13	GLIOBLASTOMA MULTIFORME	12.6	M
14	GLIOBLASTOMA MULTIFORME	6.9	M
15	GLIOBLASTOMA MULTIFORME	14.3	F
16	GLIOBLASTOMA MULTIFORME	14.3	F
17	ANAPLASTIC ASTROCYTOMA	13.9	F
18	GLIOBLASTOMA MULTIFORME	8.3	F
19	ANAPLASTIC ASTROCYTOMA	15.0	M
20	ANAPLASTIC ASTROCYTOMA	12.3	M
21	GLIOBLASTOMA MULTIFORME	17.4	F
22	GLIOBLASTOMA MULTIFORME	1.5	M
23	GLIOBLASTOMA MULTIFORME	9.1	F
24	ANAPLASTIC ASTROCYTOMA	7.0	M
25	GLIOBLASTOMA MULTIFORME	11.9	M
26	ANAPLASTIC ASTROCYTOMA	17.5	M
27	PILOCYTIC ASTROCYTOMA	3.0	M
28	PILOCYTIC ASTROCYTOMA	5.6	F
29	PILOCYTIC ASTROCYTOMA	9.6	F
30	PILOCYTIC ASTROCYTOMA	12.2	F
31	PILOCYTIC ASTROCYTOMA	8.9	M
32	PILOCYTIC ASTROCYTOMA	7.3	M
33	PILOCYTIC ASTROCYTOMA	3.3	M
34	PILOCYTIC ASTROCYTOMA	11.6	M
35	PILOCYTIC ASTROCYTOMA	17.8	M
36	PILOCYTIC ASTROCYTOMA	1.4	M
37	PILOCYTIC ASTROCYTOMA	2.7	F

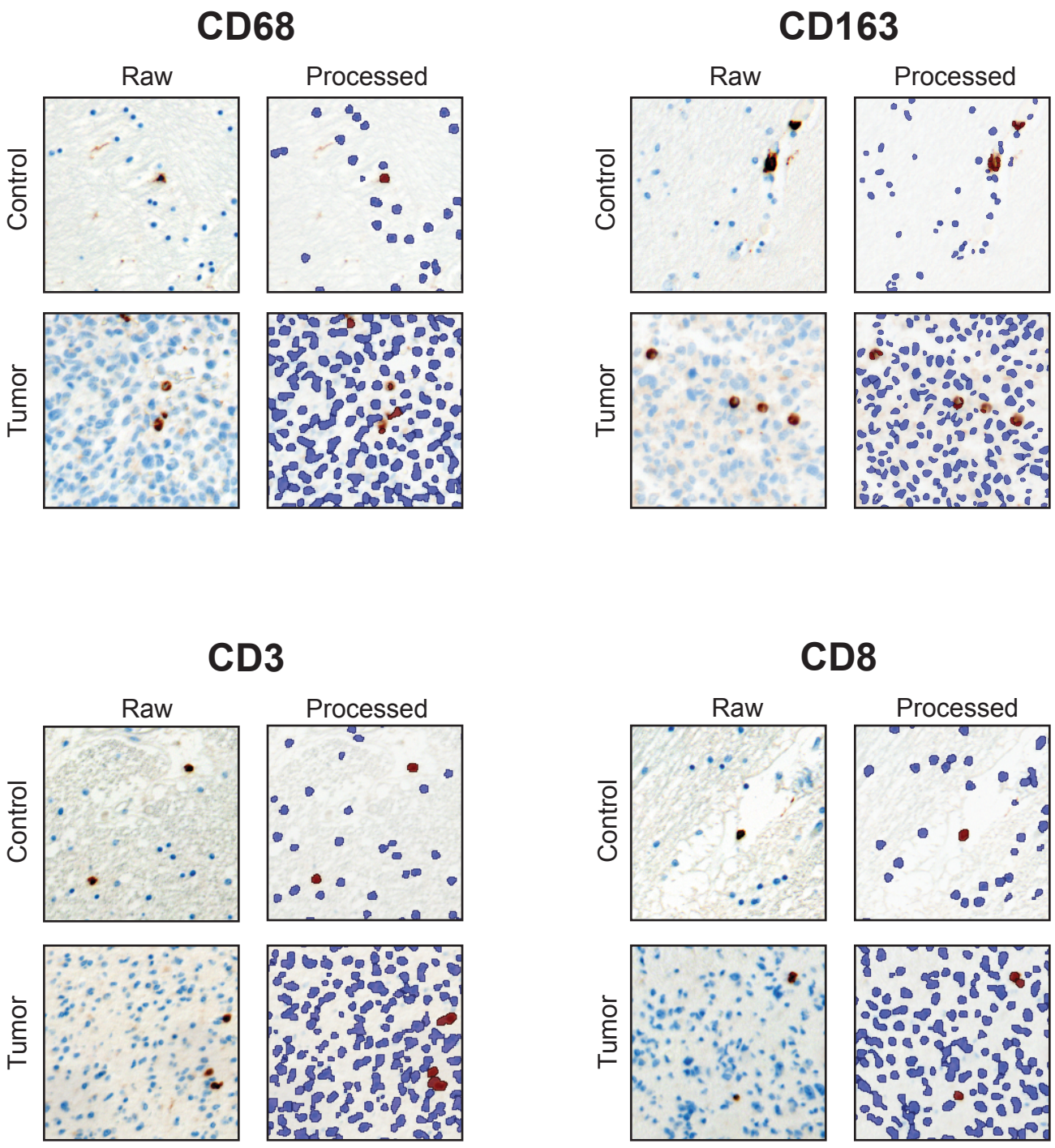
#	Diagnosis	Age (y)	Sex
38	PILOCYTIC ASTROCYTOMA	3.4	F
39	PILOCYTIC ASTROCYTOMA	15.0	F
40	PILOCYTIC ASTROCYTOMA	12.5	F
41	PILOCYTIC ASTROCYTOMA	13.6	F
42	PILOCYTIC ASTROCYTOMA	9.3	M
43	GANGLIOGLIOMA	14.3	M
44	GANGLIOGLIOMA	2.1	M
45	PILOCYTIC ASTROCYTOMA	5.9	F
46	PILOCYTIC ASTROCYTOMA	10.0	F
47	GANGLIOGLIOMA	17.0	F
48	PILOCYTIC ASTROCYTOMA	10.0	F
49	DIPG	7.2	M
50	DIPG	5.7	F
51	DIPG	10.8	M
52	DIPG	10.2	F
53	DIPG	8.1	F
54	DIPG	6.5	F
55	DIPG	11.2	F
56	DIPG	5.5	F
57	DIPG	8.7	F
58	CONTROL	7.2	M
59	CONTROL	5.7	F
60	CONTROL	8.1	F
61	CONTROL	6.5	F
62	CONTROL	11.2	F
63	CONTROL	8.7	F
64	CONTROL	8.2	M
65	CONTROL	1.7	F
66	CONTROL	18.4	M
67	CONTROL	3.1	F
68	CONTROL	7.0	F
69	CONTROL	1.0	M
70	ANAPLASTIC ASTRO (AUTOPSY)	16.3	M
71	GLIOBLASTOMA (AUTOPSY)	12.3	M
72	GLIOBLASTOMA (AUTOPSY)	8.1	F
73	GLIOBLASTOMA (AUTOPSY)	9.4	F
74	GLIOBLASTOMA (AUTOPSY)	7.8	M

Supplementary Table 3: Characteristics of Patients included in Nanostring Analysis

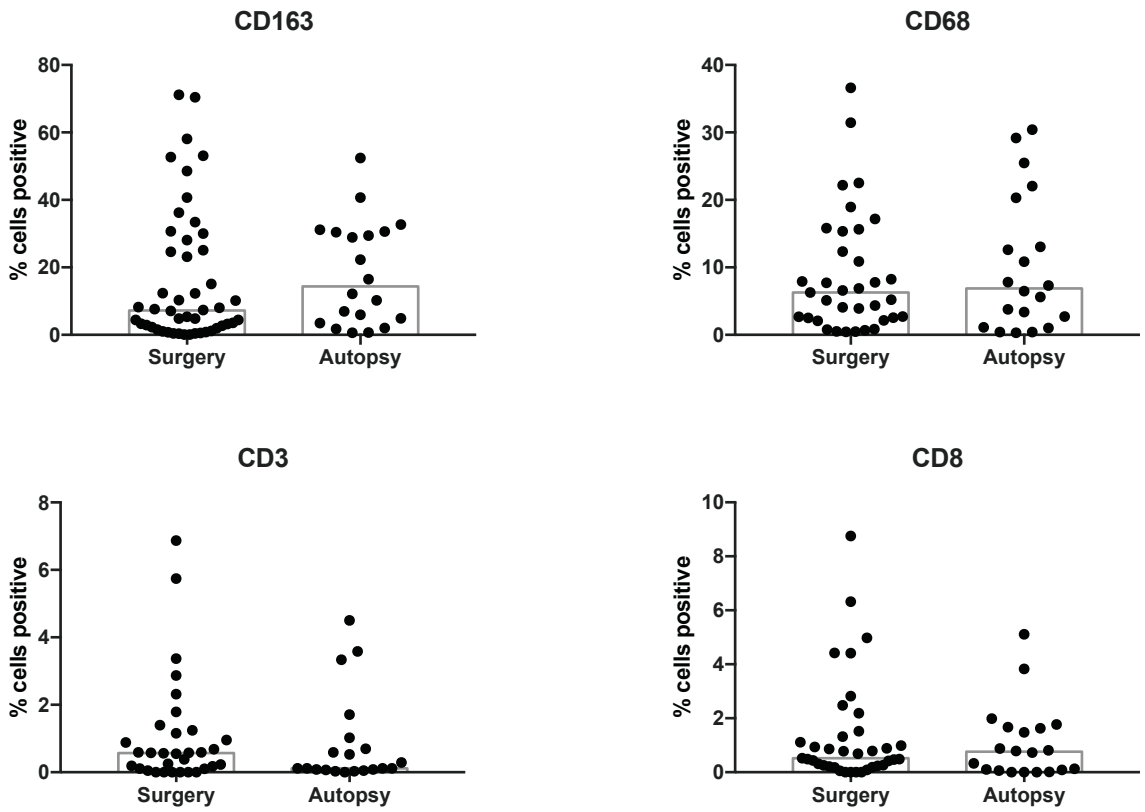
#	Diagnosis	Age (y)	Sex
1	CONTROL	10.9	M
2	CONTROL	8.1	F
3	CONTROL	5.5	F
4	CONTROL	11.2	F
5	CONTROL	8.6	F
6	CONTROL	7.2	M
7	CONTROL	5.7	F
8	CONTROL	6.2	M
9	CONTROL	6.4	F
10	CONTROL	8.0	M
11	CONTROL	12.7	M
12	PILOCYTIC ASTROCYTOMA	2.8	F
13	PILOCYTIC ASTROCYTOMA	6.9	M
14	PILOCYTIC ASTROCYTOMA	9.3	M
15	PILOCYTIC ASTROCYTOMA	8.5	F
16	PILOCYTIC ASTROCYTOMA	3.9	F
17	PILOCYTIC ASTROCYTOMA	3.8	M
18	GLIOBLASTOMA MULTIFORME	3.5	F
19	GLIOBLASTOMA MULTIFORME	2.0	F
20	GLIOBLASTOMA MULTIFORME	7.8	M
21	GLIOBLASTOMA MULTIFORME	0.7	F
22	GLIOBLASTOMA MULTIFORME	15.3	F
23	GLIOBLASTOMA MULTIFORME	5.2	M
24	DIPG	10.9	M
25	DIPG	8.1	F
26	DIPG	5.5	F
27	DIPG	11.2	F
28	DIPG	8.6	F
29	DIPG	7.2	M
30	DIPG	5.7	F
31	DIPG	6.2	M
32	DIPG	6.4	F
33	DIPG	8.0	M
34	DIPG	12.7	M

Supplementary Table 4: Characteristics of cell cultures used in this study

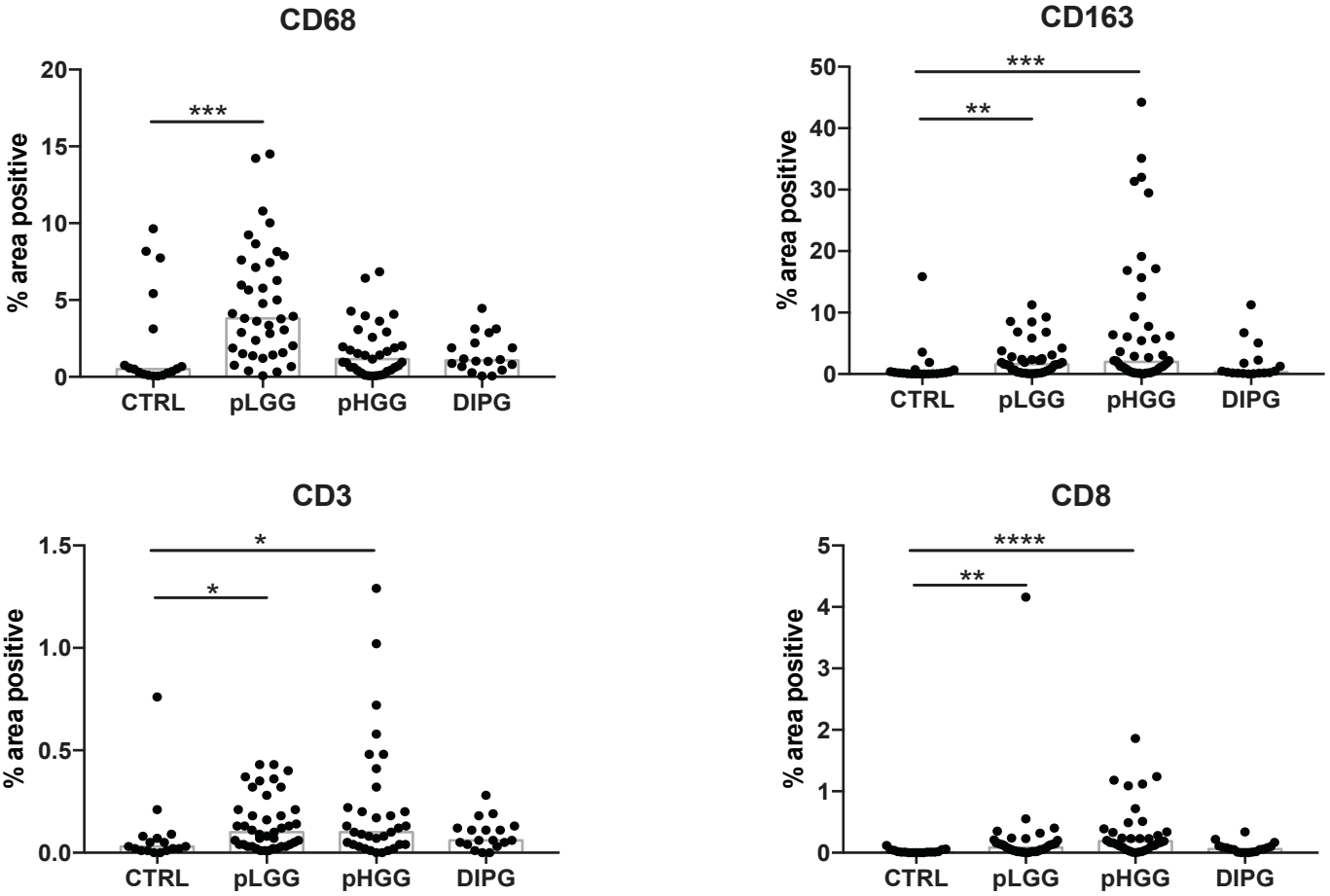
Name (Diagnosis)	Source	Media	Morphology	Histone Mutation
SU-DIPG-IV (DIPG)	Monje Lab (Stanford)	Neural Stem Cell (Neurocult)	90% Adherent, neuronal/fibroblast	H3.1-K27M
SU-DIPG-XIII (DIPG)	Monje Lab (Stanford)	Neural Stem Cell (Neurocult)	90% Neurosphere	H3.3-K27M
SU-DIPG-XVII (DIPG)	Monje Lab (Stanford)	Neural Stem Cell (Neurocult)	90% Neurosphere	H3.3-K27M
U87MG (GBM)	ATCC	Neural Stem Cell (Neurocult)	90% Neurosphere	Wild Type
SU-pcGBM-II (GBM)	Monje Lab (Stanford)	Neural Stem Cell (Neurocult)	50% Neurosphere 50% Adherent	Wild Type
LN229 (GBM)	ATCC	Neural Stem Cell (Neurocult)	90% Adherent	Wild Type
U138 (GBM)	ATCC	Neural Stem Cell (Neurocult)	90% Neurosphere	Wild Type
T98G (GBM)	ATCC	Neural Stem Cell (Neurocult)	50% Neurosphere 50% Adherent	Wild Type



Supplementary Figure 1



Supplementary Figure 2



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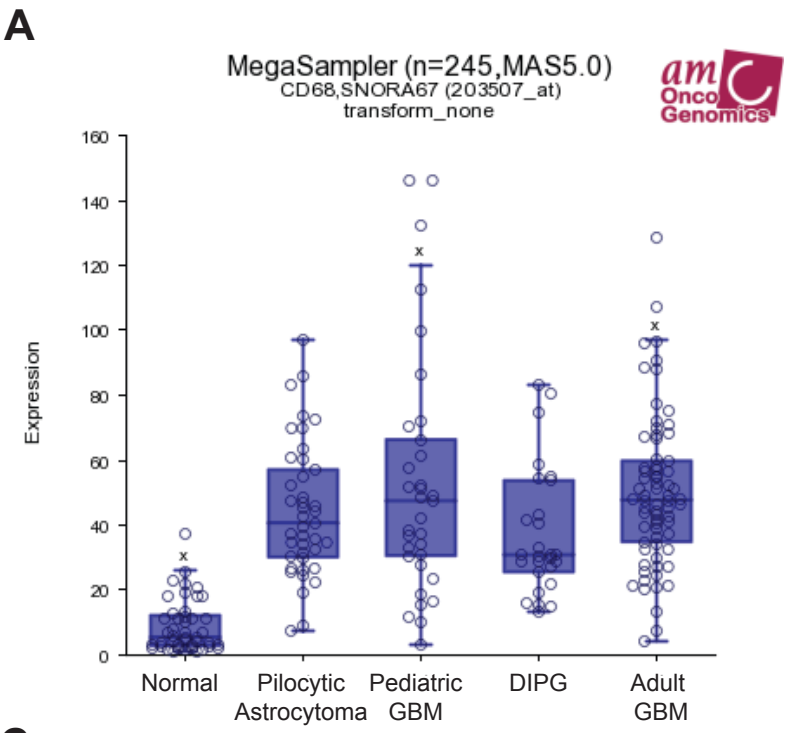
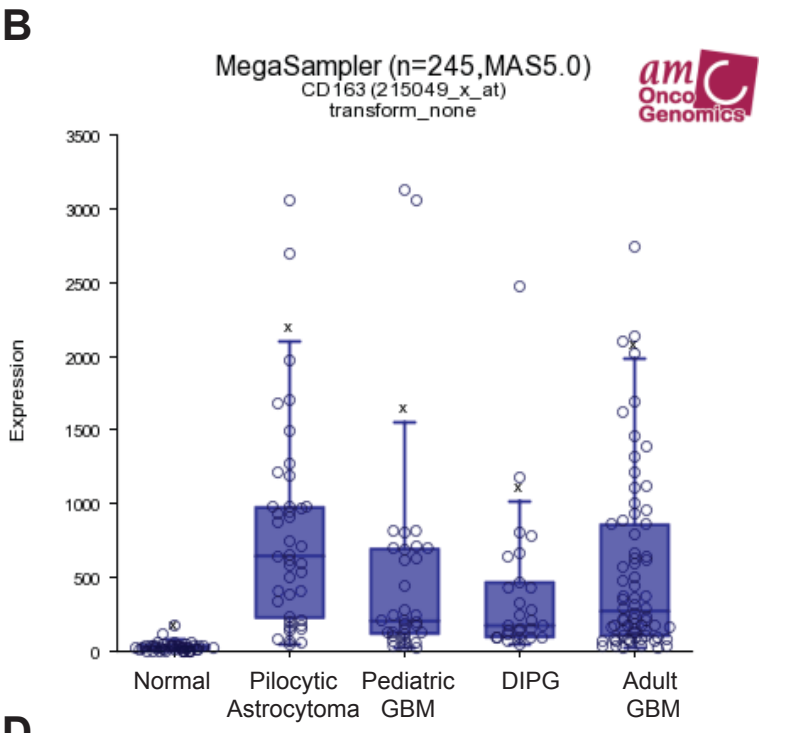
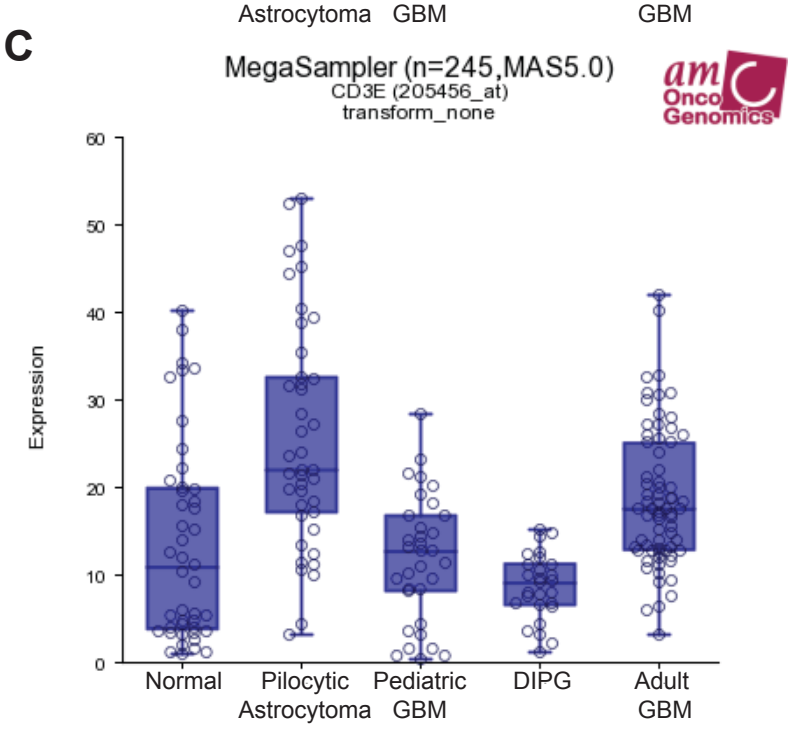
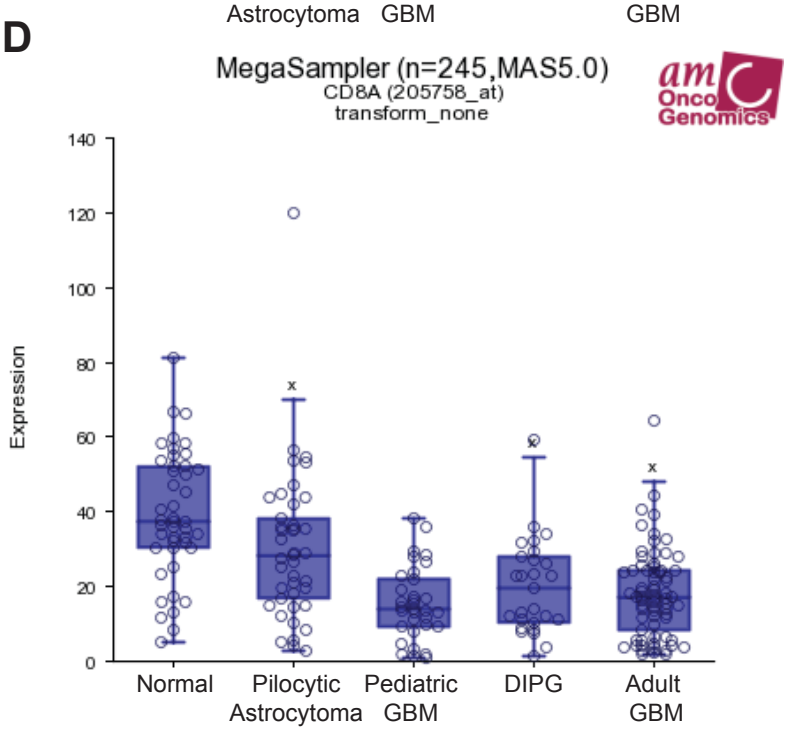
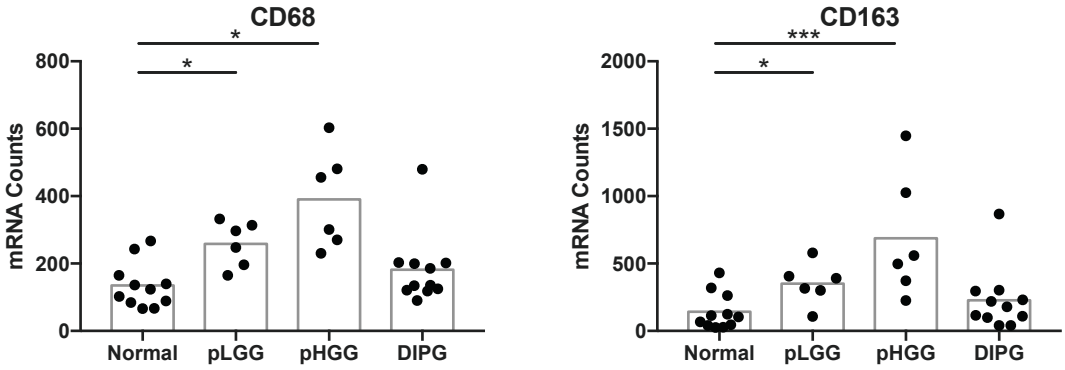




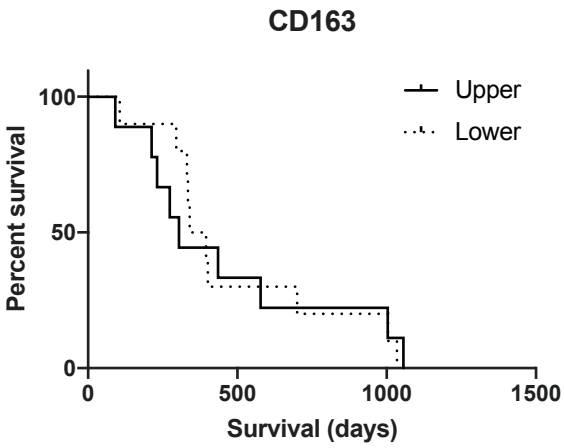


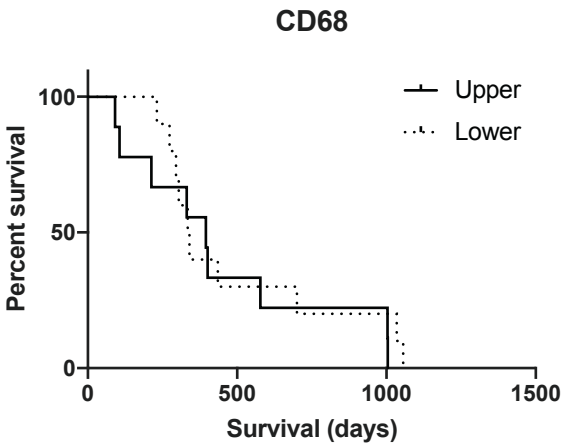


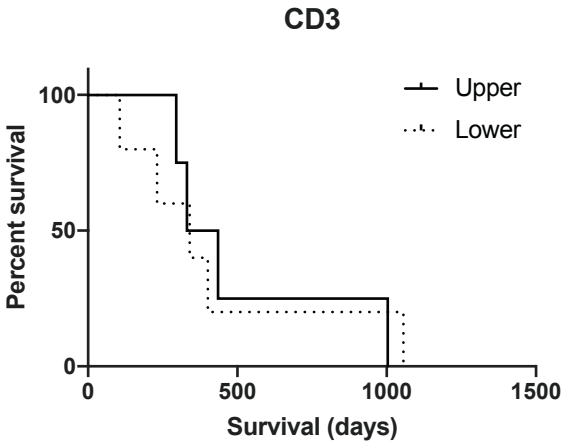
Supplementary Figure 4

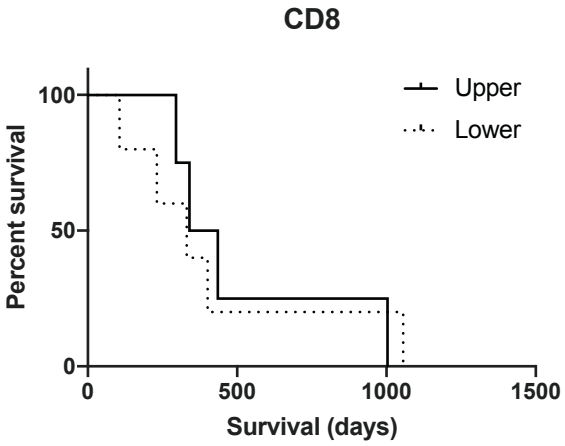


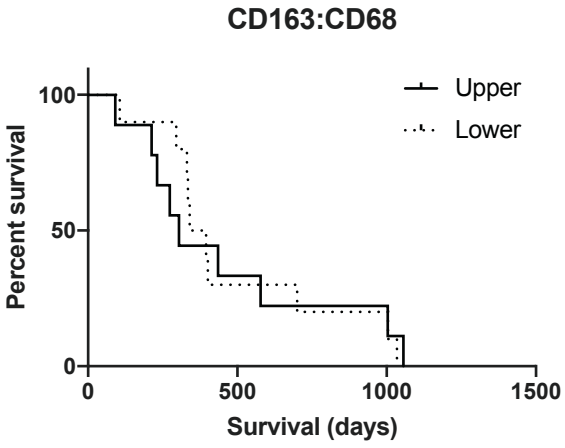
Supplementary Figure 5



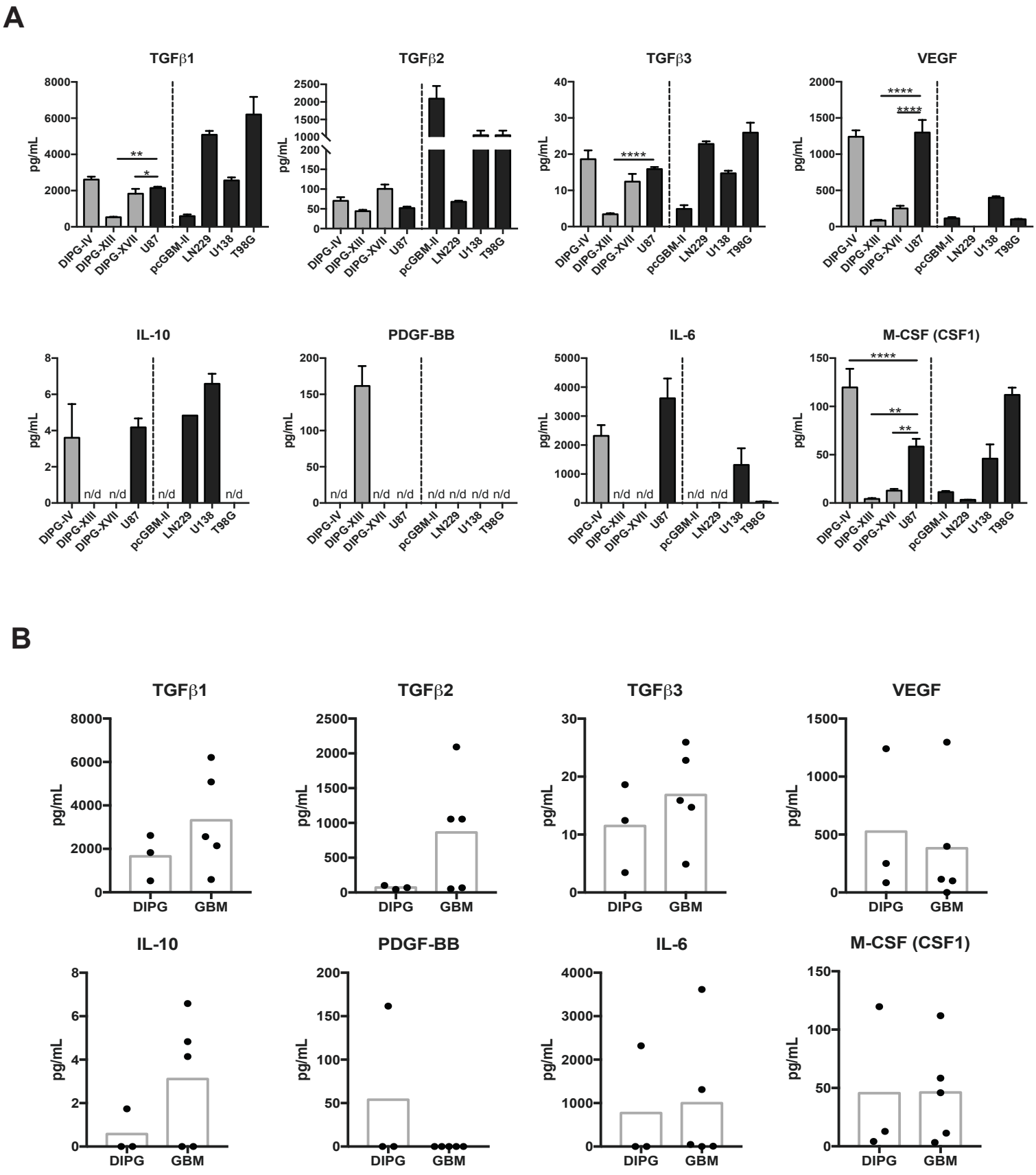




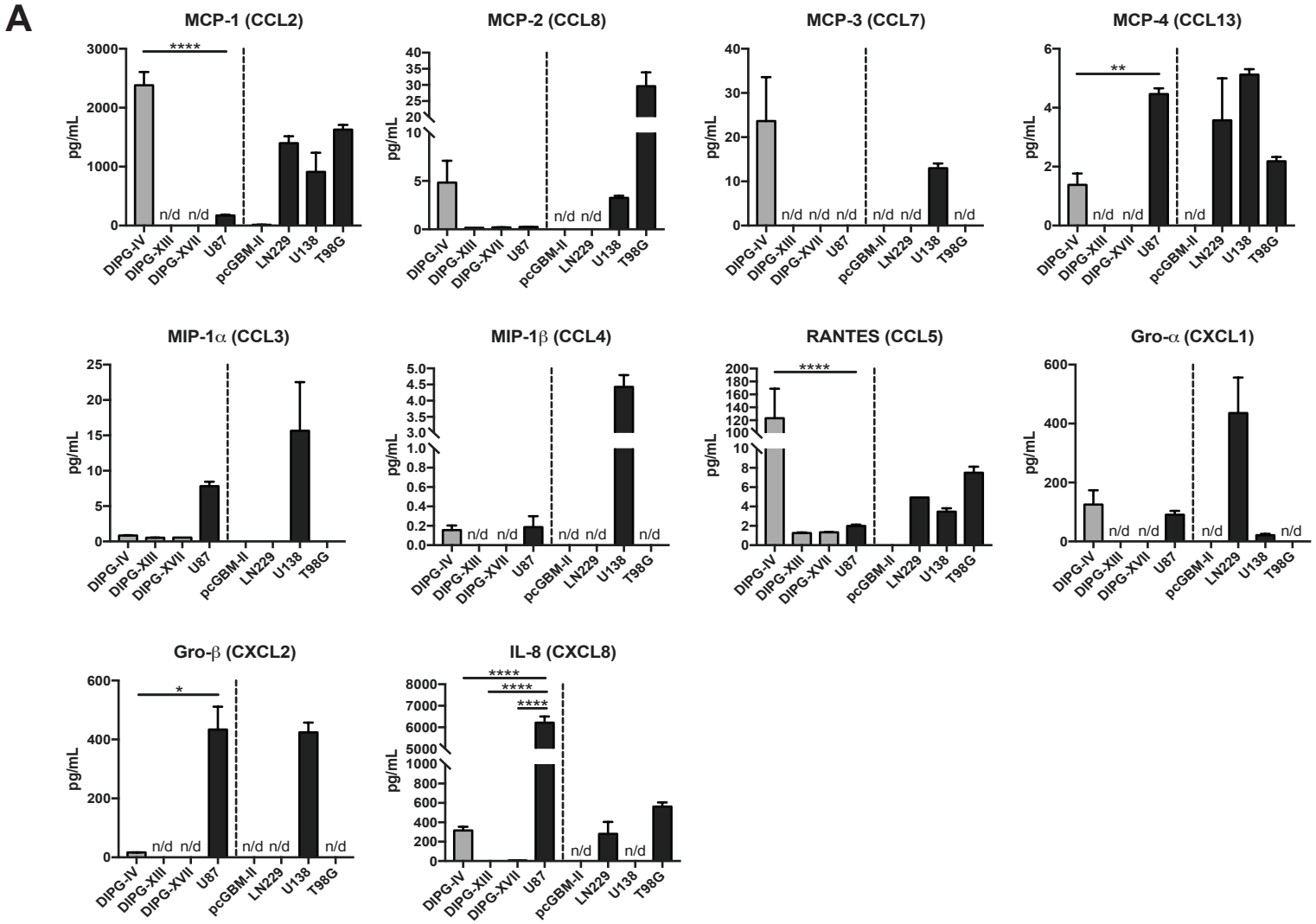


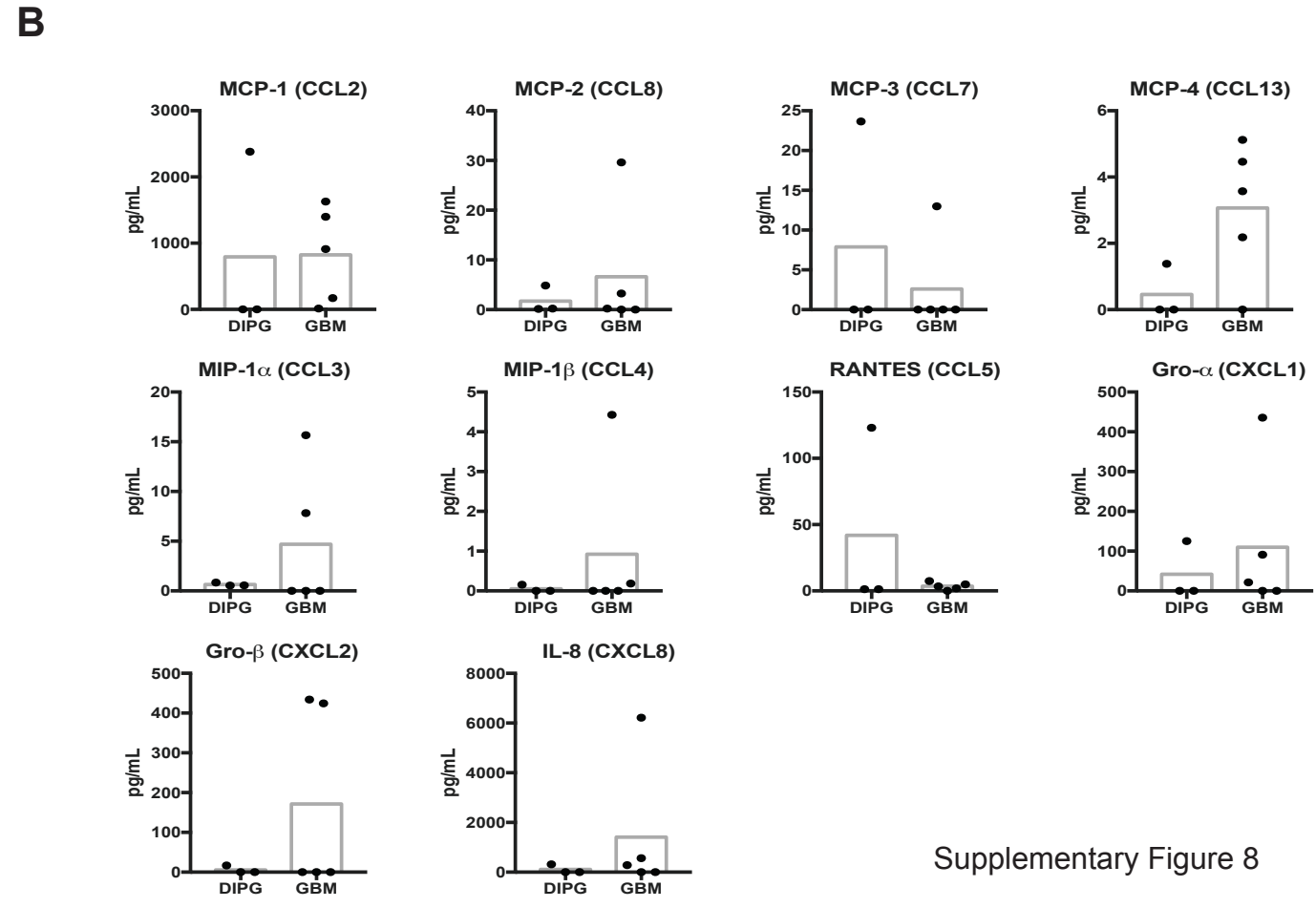


Supplementary Figure 6

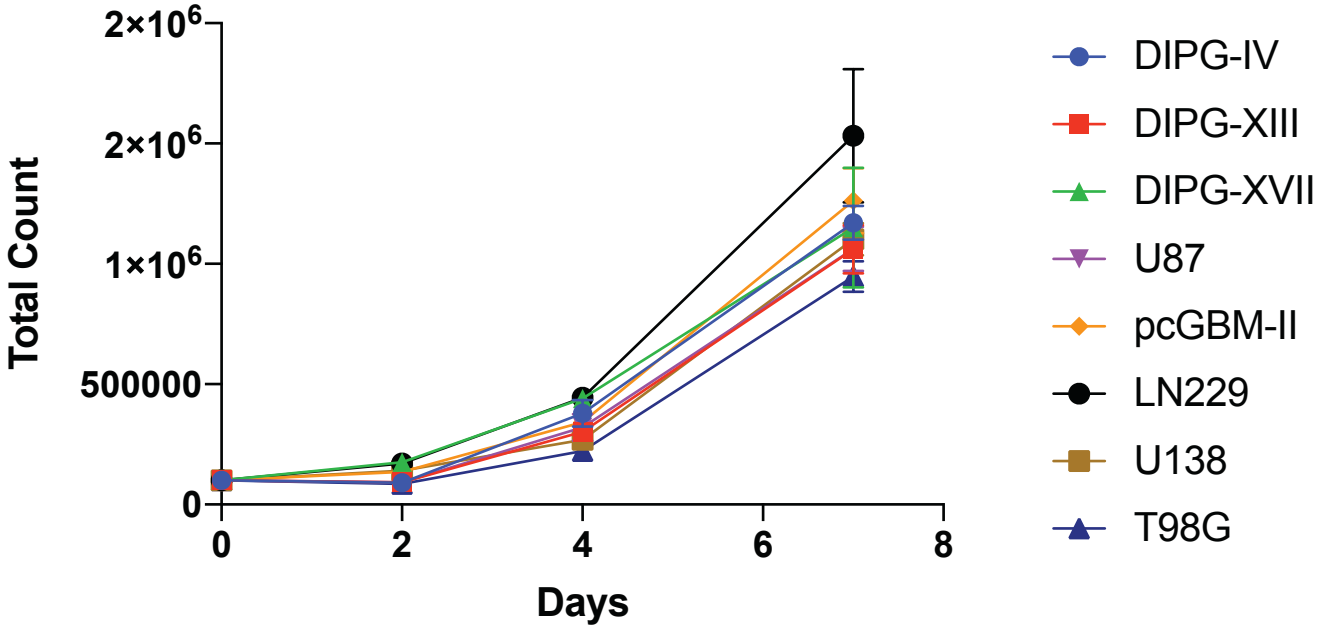


Supplementary Figure 7

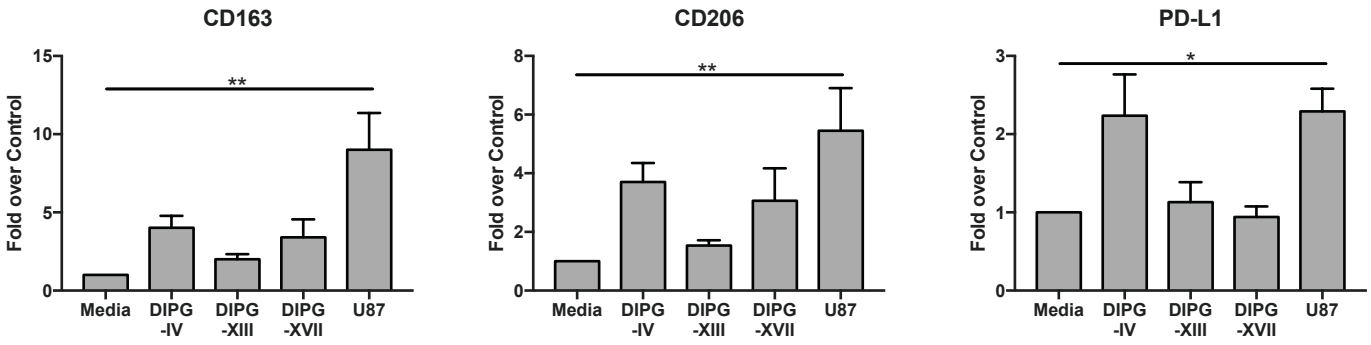




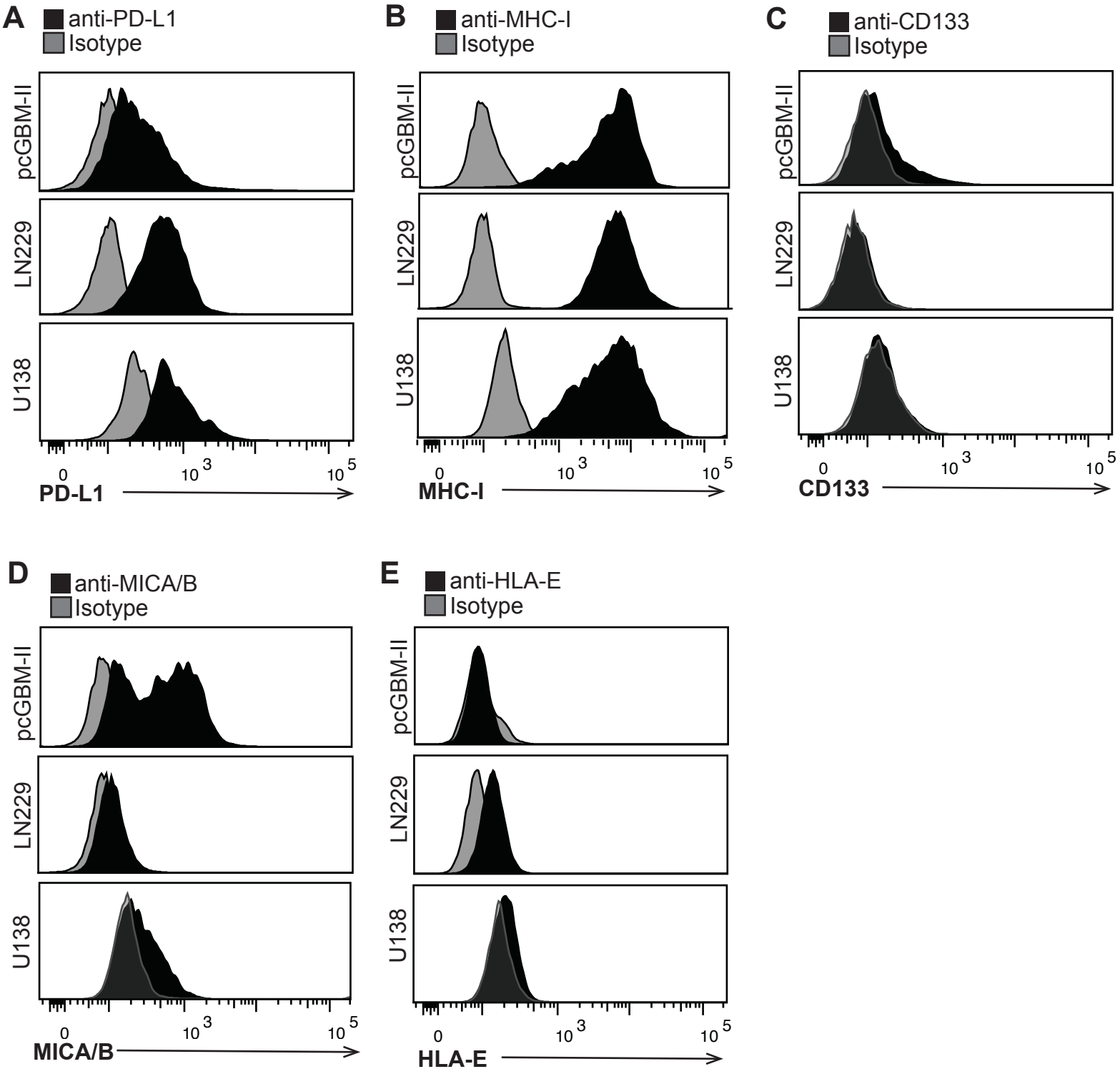
Supplementary Figure 8



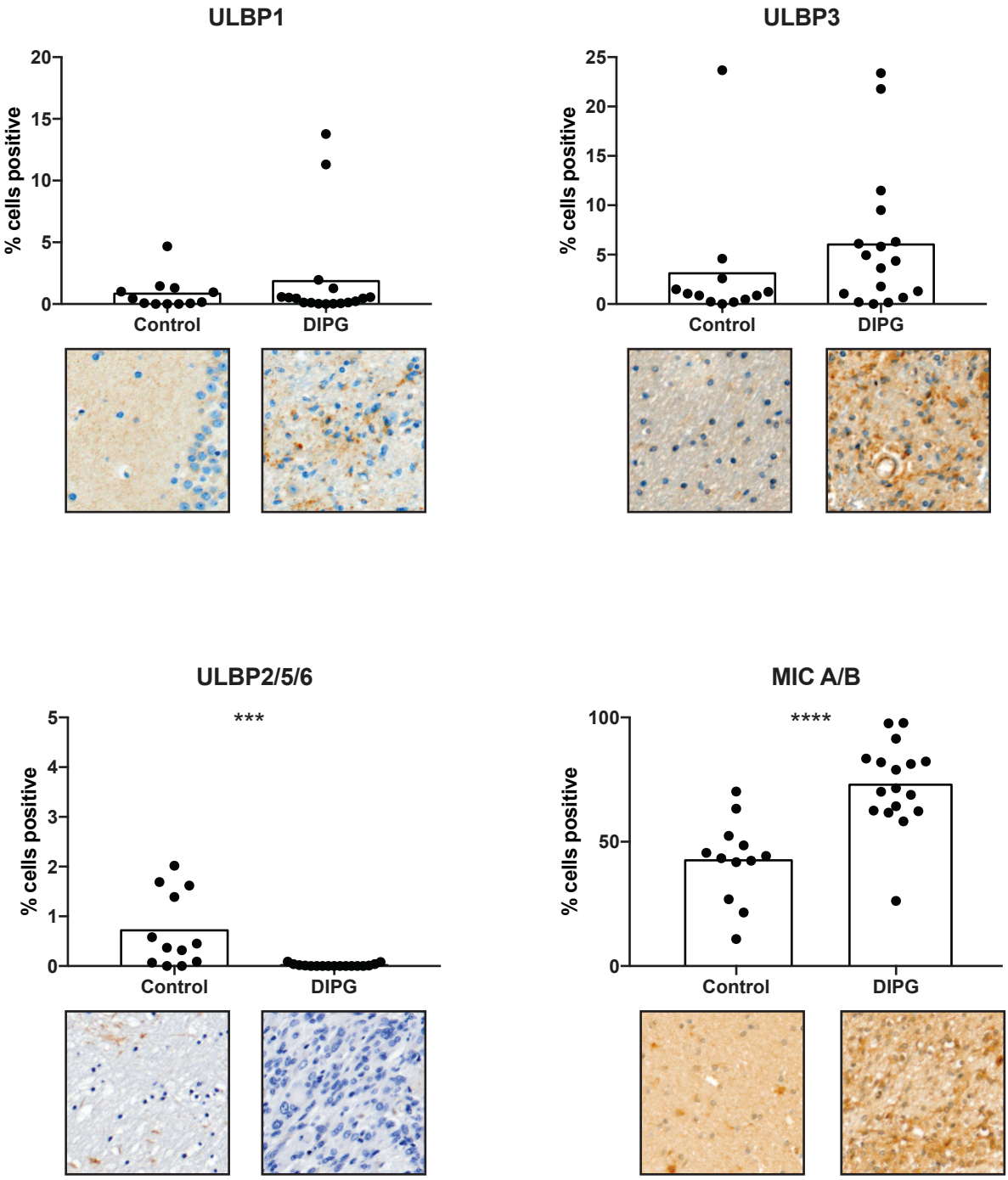
Supplementary Figure 9



Supplementary Figure 10



Supplementary Figure 11



Supplementary Figure 12