

## Supplementary Figure Legends

### Figure S1.

- A) Same as **Figure 1A** except Myeloid CyTOF panel
- B) Same as **Figure 1A** except Myeloid CyTOF panel
- C) Heatmap of median marker expression in clusters shown in **Figure 1D**
- D) Gene expression profiles of GL261 orthotopic tumors (n=2), SB28 orthotopic tumors (n=3), or SB28 cells grown in vitro (n=3) were compared with primary human gliomas from Verhaak et al 2017 by hierarchical clustering.
- E) Gene expression profiles of GL261 orthotopic tumors (n=2), SB28 orthotopic tumors (n=3), or SB28 cells grown in vitro (n=3) were compared with primary human brain tumors and normal brain tissue (n = 13) by hierarchical clustering.
- F) Gene set enrichment analysis (GSEA) comparing interferon gamma signaling pathway activation in bulk mRNA from GL261 tumors relative to SB28 tumors. FDR: False discovery-rate Q-value.
- G) TCGA analysis of human GBM shows an immunosuppressive TME characterized by a higher ratio of M2 TAMs to T cells (CD163:CD3), as compared to other tumor types.
- H) TCGA analysis of human GBM shows an immunosuppressive TME characterized by a higher ratio of PD-L1-positive cells to T cells (PD-L1:CD3), as compared to other tumor types.
- I) TCGA data of human GBM as in **(F)** and **(G)**, shown as a 3D projection.

### Figure S2.

- A) Heatmap of median marker expression in clusters shown in **Figure 2D**.
- B) Alternative gating strategy for TAM subsets based on CD45 and PD-L1 expression, used in **(C)**. Data from the i.c. tumor from **Figure 2E** is shown as a representative sample.
- C) Quantification of marker expression by mass cytometry in TAM subsets gated on CD45 and PD-L1. In intracerebral SB28 tumors, the PD-L1-high CD45-low TAM subset expressed significantly more CD38 than the PD-L1-low CD45-high TAM subset.

### Figure S3.

- A) Heatmap of median marker expression in clusters shown in **Figure 3C**

- B) Heatmap of median marker expression in clusters shown in **Figure 3D**. Scale bar as in (A).
- C) Flow cytometry of splenocytes from representative mice confirming successful depletion
- D) Tumor growth curves of individual mice from ICI depletion cohort

#### Figure S4.

- A) Heatmap of median marker expression in clusters shown in **Figure 4B**
- B) Treatment with huFLT3L expands cDC2s, pDCs, and Tregs in the spleens of mice with SB28-OVA-FL tumors at Day 12 post-inoculation. Volcano plot as in **Figure 4B**.
- C) Heatmap of median marker expression in clusters shown in (B)
- D) Treatment with huFLT3L expands OVA-specific T cells in spleen, corresponding to **Figure 4C**
- E) Treatment with huFLT3L expands Ki67+ OVA-specific T cells in spleen, corresponding to **Figure 4D**
- F) Frequency of OVA tetramer+ CD4 and CD8 T cells (out of CD3+ T cells) in dissociated SB28-OVA-FL tumors at Day 12 post-inoculation.
- G) Frequency of proliferating (Ki-67+) T cells (out of OVA tetramer+ T cells) in dissociated SB28-OVA-FL tumors at Day 12 post-inoculation.
- H) Gating strategy for OVA MHC tetramer+ and Ki-67+ T cells shown in **Figure 4C-D**
- I) T cells were isolated from transgenic OT-I or OT-II mice, or from mice with SB28 parental or OVA-FL tumors. These positive and negative controls confirmed specific staining of OVA MHC tetramer+ T cells using the same reagents shown in (H).
- J) Treatment with huFLT3L expands cDC1 cells in intracerebral but not subcutaneous SB28-OVA-FL tumors. Frequencies of cDC1 cells (CD103+ CD8+ as a frequency of MHCII+ CD3- events) were quantified by flow cytometry in tumor-draining lymph nodes of intracerebral and subcutaneous SB28-OVA-FL tumors +/- huFLT3L treatment. Tissues were harvested on Day 11 and 12 post-inoculation for s.c. and i.c. tumors, respectively.
- K) Gating strategy for cDC1 (CD8+ CD103+ MHC-II+ CD3-) in (J) and anti-SIINFEKL-MHC-I in **Figure 4E**.

#### Figure S5.

A) Kaplan-Meier curves of intracerebral SB28 treated with isotype control IgG (gray), huFLT3L (blue), radiation (XRT, green) or huFLT3L + XRT (purple). Log-rank test P-values and median overall survival are shown.

B) Kaplan-Meier survival curves of intracerebral SB28 treated with isotype control IgG (gray), huFLT3L (blue), or huFLT3L + ICI (brown). Log-rank test P-values and median overall survival are shown.

C) Kaplan-Meier curves of intracerebral SB28 treated with isotype control IgG (gray), double (anti-PD-L1 + XRT, red) or triple-combination immunotherapy (huFLT3L + XRT + anti-PD-L1, black). Log-rank test P-values and median overall survival are shown.

D) Kaplan-Meier survival curves of intracerebral SB28 treated with control IgG (gray), huFLT3L (blue), huFLT3L + PolyIC:LC (pink), huFLT3L + anti-PD-L1 (red), or huFLT3L + CD40 agonist (teal). Log-rank test P-values and median overall survival are shown.

## Figure S6.

A) Heatmap of median marker expression in clusters shown in **Figure 6A-B**.

B) In mass cytometry analysis of cervical lymph nodes at endpoint (Day 27-33), Tregs were expanded by huFLT3L alone and even more when combined with anti-PD-1/anti-CTLA-4 (ICI). Box plot shows frequency of Tregs (Cluster 17: CD3<sup>+</sup> CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup>) as a percentage of CD45<sup>+</sup> cells.

C) Heatmap of median marker expression in Cluster 17 (Tregs), same dataset as **(B)**.

D) Heatmap of median marker expression in clusters shown in **Figure 6C**. No clusters had significantly differential abundance.

E) Heatmap of median marker expression in Cluster 17 (cDC2) and 23 (PD-L1<sup>+</sup> TAMs), same dataset as **Figure 6D**.

F) Heatmap of median marker expression in Cluster 17 (cDC2), same dataset as **Figure 6E**.

G) Changes in the surface immunophenotypes of tumor-infiltrating T-cells in SB28 intracerebral tumors treated with FLT3L, XRT or FLT3L + XRT. Values are transformed medians of mass cytometry intensity measurements.

H) Gating strategy for column graph in **(G)**.

**Figure S7.**

A) The differential abundance analysis from (6D) was repeated using the CATALYST+diffcyt analysis pipeline from Nowicka et al 2019 to compare their performance on an identical dataset (see **Online Supplemental Methods**). The Nowicka pipeline identified a cluster (Cluster 9) that is significantly enriched in RT vs. Ctrl\_IgG. The phenotype of this cluster matches the phenotype of Cluster 23 (PD-L1+ CD206+ F4-80+ TAMs) identified with the FlowSOM+PhenoGraph pipeline and shown in **Suppl Figure S6E**.