

Supplementary Information

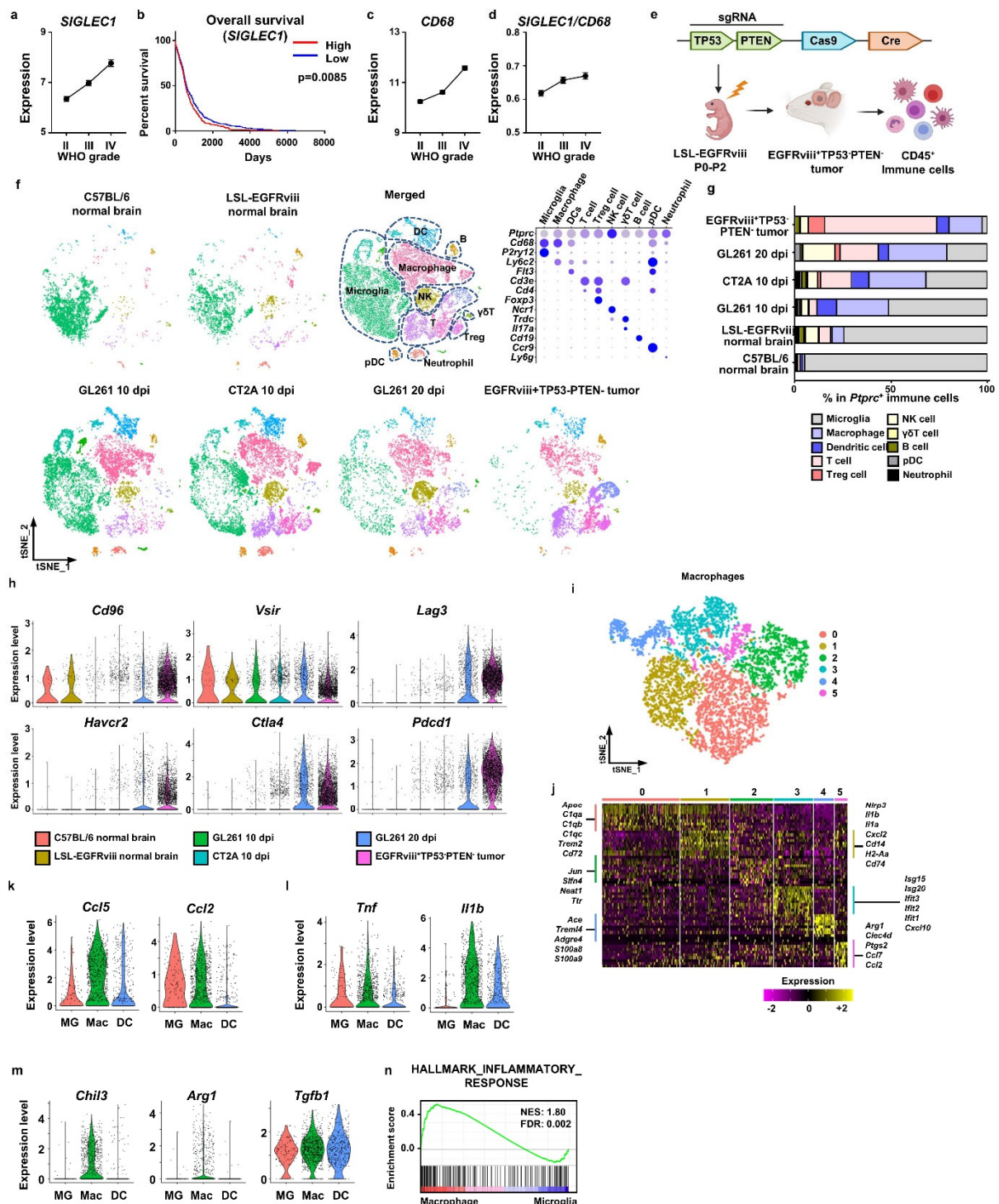
Title: Blood monocyte-derived CD169⁺ macrophages contribute to antitumor immunity against glioblastoma

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Supplementary Figures

Supplementary Figure legends



Supplementary Figure 1. CD169 is expressed in inflammatory macrophages in gliomas.

a-d We analyzed human data from The Cancer Genome Atlas Glioblastoma Multiforme and Low Grade Glioma (TCGA-GBMLGG) databases ($n = 694$). Patients were grouped by their gene expression levels compared to the median. **(a)** *SIGLEC1*, **(c)** *CD68*, and **(d)** *SIGLEC1/CD68* ratio using World Health Organization (WHO) tumor grades. **(b)** Overall survival of patients with high ($n = 347$) or low ($n = 347$) according to higher or lower expression than the median (6.82) of *SIGLEC1*. Differences in survival was measured by log-rank

(Mantel-Cox) test.

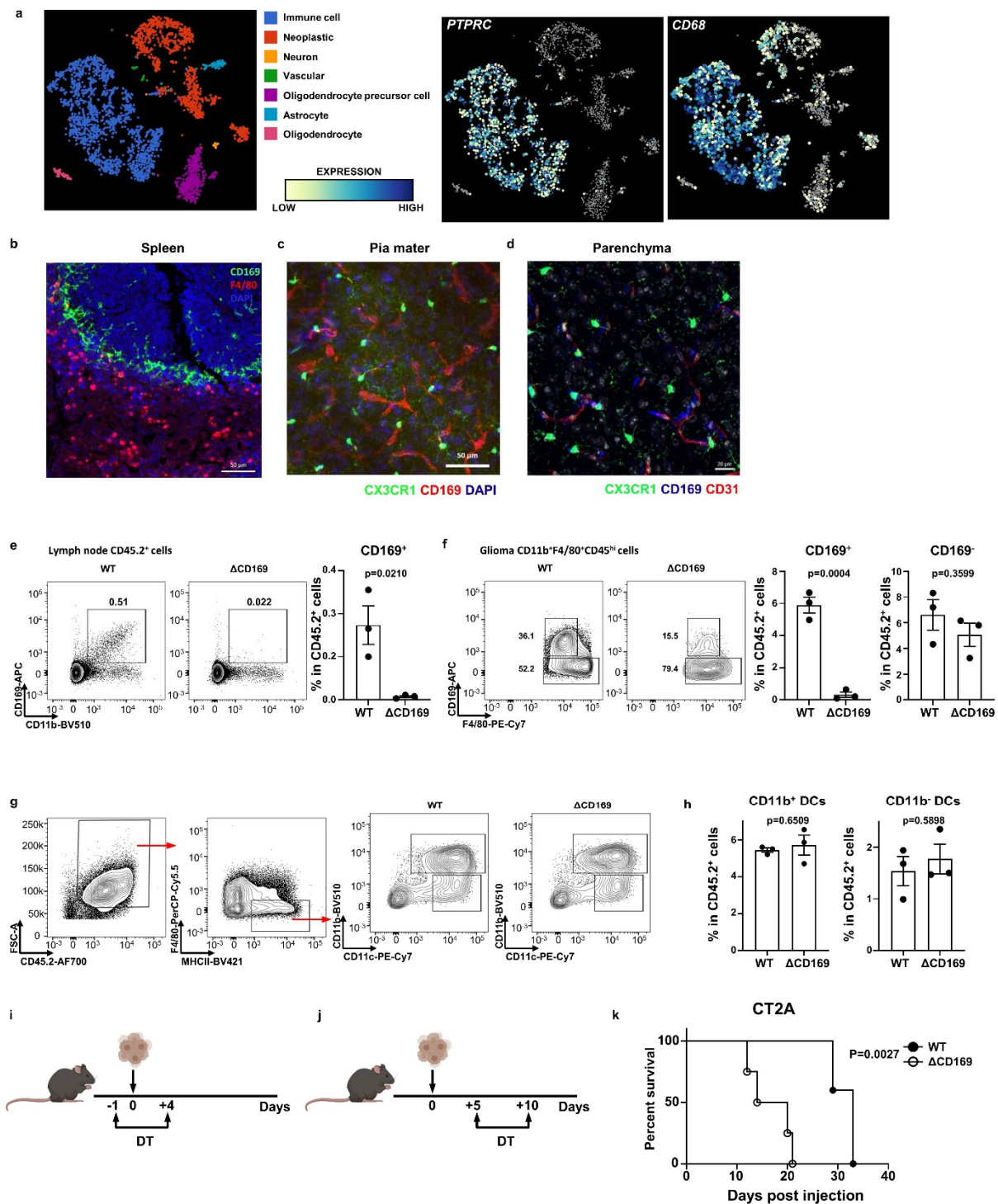
e Experimental scheme of mouse GBM model induced by genetic modification. Gene transfer of small guide RNA (sgRNA) for tumor protein p53 (*Trp53*) and phosphatase and tensin homolog (*Pten*), and gene coding clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9 (*Cas9*) and *Cre-recombinase* into the brain ventricle of newborn mice induced epidermal growth factor receptor variant III (*EgfrvIII*) expression, while ablating expression of the tumor suppressor genes, *TP53* and *PTEN*. CD45⁺ immune cells were isolated from engineered tumors that developed in adult mice. Figures were created with BioRender.com.

f-h CD45-expressing immune cells were sorted from tumors from the mouse glioma model or normal mouse brain for single-cell RNA sequencing. **(f)** Immune cells were clustered by their expression of the following marker genes: microglia (*Cd68⁺P2ry12⁺*), infiltrated macrophages (*Cd68⁺Ly6c2⁺Flt3⁻*), dendritic cells (DC; *Flt3⁺*), T cells (*Cd3e⁺*), regulatory T cells (Treg; *Cd3e⁺Cd4⁺Foxp3⁺*), natural killer cells (NK; *Ncr1⁺*), $\gamma\delta$ T cells (*Trdc⁺Il17a⁺*), B cells (*Cd19⁺*), plasmacytoid dendritic cells (pDC; *Ccr9⁺*), and neutrophils (*Ly6g⁺*). **(g)** The proportion of immune cells in *Ptprc⁺* cells from each mouse model. **(h)** Expression levels of inhibitory receptors (*Cd96*, *Vsir*, *Lag3*, *Havcr2*, *Ctla4*, and *Pdcd1*) in T cell clusters.

i-j Tumor-infiltrating macrophage clusters (GL261, CT2A, and EGFRvIII⁺TP53⁻PTEN⁻ tumors) were re-clustered by gene expression.

k-n Gene expression of immune cells from EGFRvIII⁺TP53⁻PTEN⁻ tumors. Expression level of **(k)** proinflammatory chemokines (*Ccl5* and *Ccl2*), **(l)** cytokines (*Tnf* and *Il1b*), and **(m)** M2 macrophage markers (*Chil3*, *Arg1*, and *Tgfb1*) in myeloid cell clusters. **(n)** Gene set enrichment analysis in macrophage and microglia clusters.

Source data are provided as a Source Data file.



Supplementary Figure 2. CD169⁺ macrophages are in normal tissues but not in brain parenchyma.

a Cell clusters and expression of *PTPRC* and *CD68* in human GBM (GSE84465).

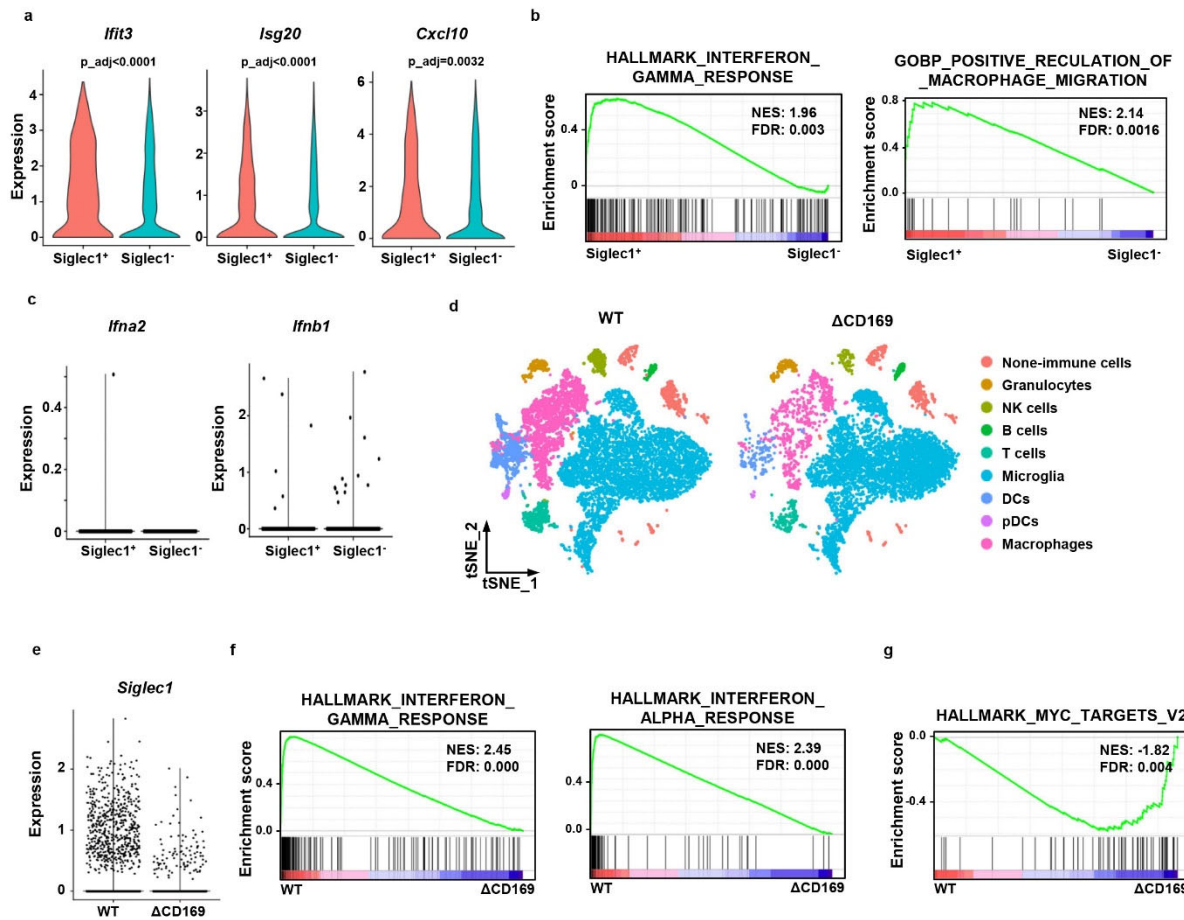
b-d Expression of CD169 in normal **(b)** spleen marginal zone metallophilic macrophages (n = 2), **(c)** brain pia mater (n = 2), and **(d)** perivascular macrophages (n = 2). Data represent two independent experiments. Scale bars indicate **(b-c)** 50 μ m and **(d)** 20 μ m.

e-h GL261 injected WT (n = 3) and CD169-DTR (n = 3) mice were treated with DT at 20 days after GL261 injection. Macrophages in **(e)** lymph nodes and **(f)** glioma, and DCs **(g-h)** in glioma after DT treatment. Data represent 3 independent experiments. Bars indicate standard error of the mean (SEM). P-values were calculated from an unpaired two-tailed *t*-test.

i-j Experimental scheme for the depletion of cells expressing CD169. **(i)** For depletion of CD169⁺ cells before tumor formation, 40 ng/g of DT was intraperitoneally injected at -1 and +4 days of GL261 injection. **(j)** For depletion of CD169⁺ cells after tumor formation, DT was injected at 5 and 10 days after GL261 injection. Figures were created with BioRender.com.

k Survival of mice inoculated with intracranial CT2A cells and intraperitoneal diphtheria toxin (DT; 40 ng per 1 g of mouse body weight). Wild-type (WT, n = 5) and CD169-DTR (n = 4) mice were intraperitoneally injected with DT at -1 and +4 days of CT2A cell injection. Data represent two independent experiments. P-value was calculated with the Log-rank test.

Source data are provided as a Source Data file.



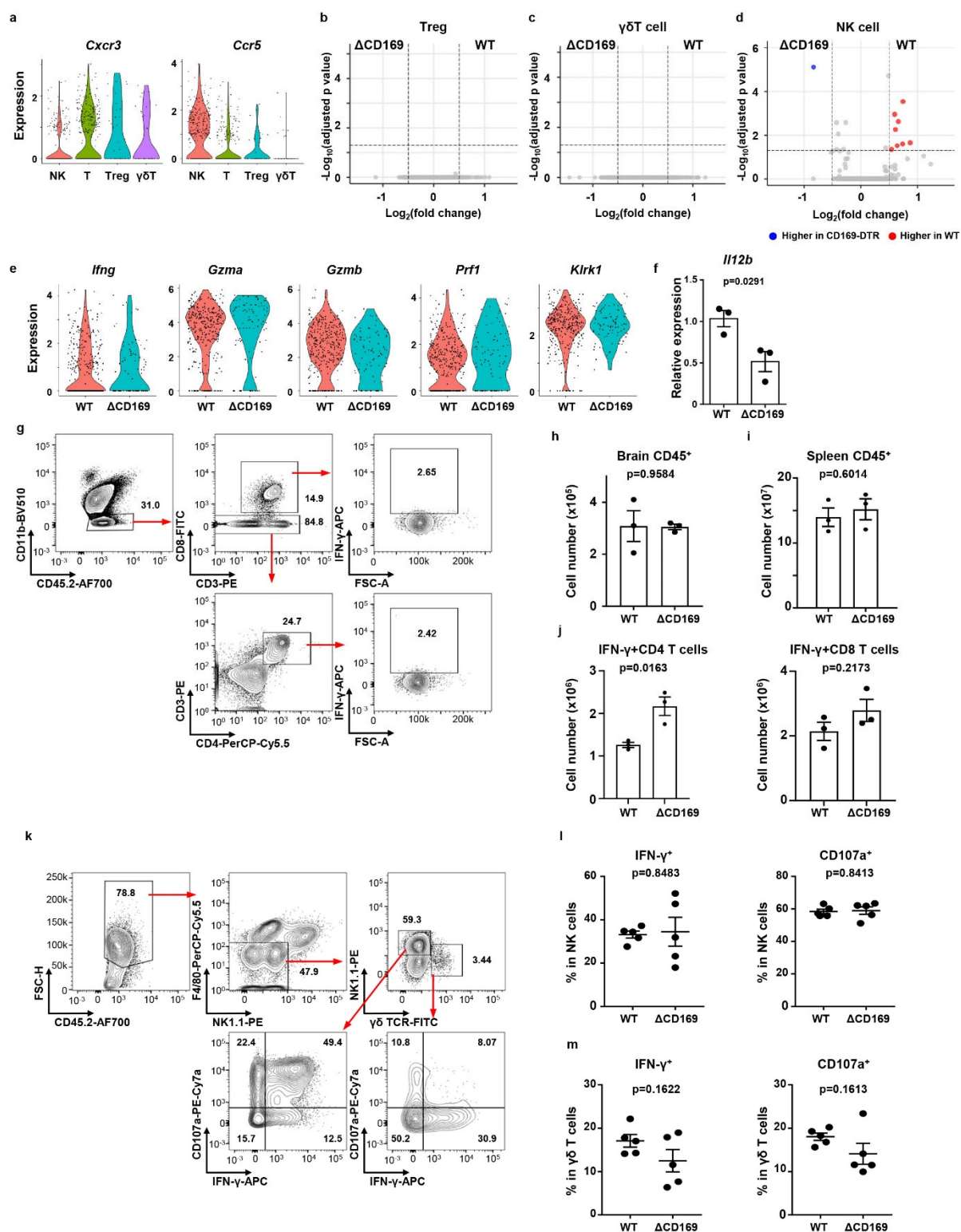
Supplementary Figure 3. CD169 depletion reduces the infiltration and inflammatory gene expression of macrophages.

a-c Macrophages from GL261 tumor samples were clustered by the expression of *Siglec1*. Analysis of **(a)** interferon (IFN)-stimulated gene expression, **(b)** gene set enrichment of IFN- γ response and macrophage migration, and **(c)** type I IFN expression. p_{adj} indicates adjusted p value.

d CD45.2⁺ immune cells from gliomas of WT and CD169-depleted mice were clustered.

e Expression level of *Siglec1* in the macrophage cluster.

f-g Enriched gene sets in macrophages of **(f)** WT and **(g)** CD169-DTR mice.



Supplementary Figure 4. CD169 depletion has limited effects on Treg, $\gamma\delta$ T cells, NK cells, and splenic T cells.

a Expression of chemokine receptors (*Cxcr3* and *Ccr5*) in the lymphocyte clusters.

b-d DEG analysis of WT and CD169-DTR mice for (b) Tregs, (c) $\gamma\delta$ T cells, and (d) NK cells

(Gray dots indicate $|\text{Log}_2(\text{fold change})| < 0.5$ or adjusted p-value > 0.05).

e Expression of genes associated with activation (*Ifng*, *Gzma*, *Gzmb*, *Prfl*, and *Klrkl*) in the NK cell cluster.

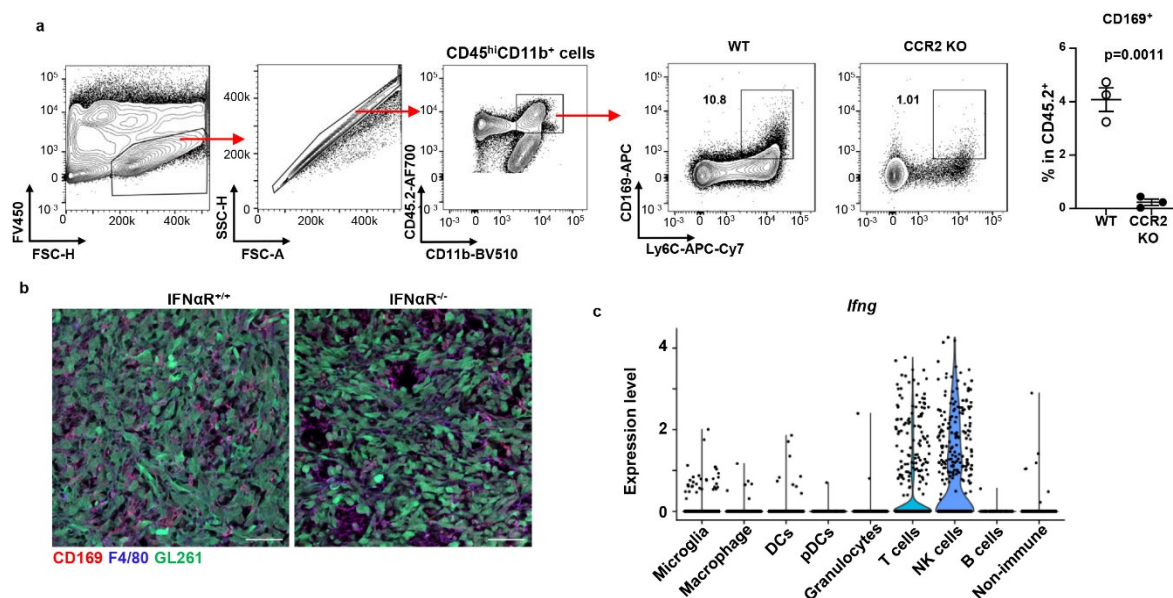
f Expression of *Il12b* mRNA was measured in glioma of biologically independent WT (n = 3) and CD169-DTR (n = 3) mice.

g-j Flow cytometry of immune cells in the brain and spleen of WT (n = 3) and CD169-DTR (n = 3) mice. **(g)** Gating strategy for IFN- γ -expressing CD4 and CD8 T cells in gliomas. The number of CD45⁺ immune cells in **(h)** brain and **(i)** spleen of mice. **(j)** IFN- γ ⁺ CD4 and CD8 T cells in the spleen.

k-m Expression of IFN- γ and CD107a in NK cells and $\gamma\delta$ T cells in brain tumors of WT (n = 5) and CD169-DTR (n = 5) mice were analyzed by flow cytometry at 10 dpi. **(k)** Gating strategy for NK cells and $\gamma\delta$ T cells that expressed IFN- γ and CD107a. IFN- γ ⁺ or CD107a⁺ **(l)** NK cells or **(m)** $\gamma\delta$ T cells in gliomas were analyzed.

Data are presented as mean values $\pm$ SEM. All p-values were calculated by unpaired two-tailed *t*-test. Data represent two independent experiments.

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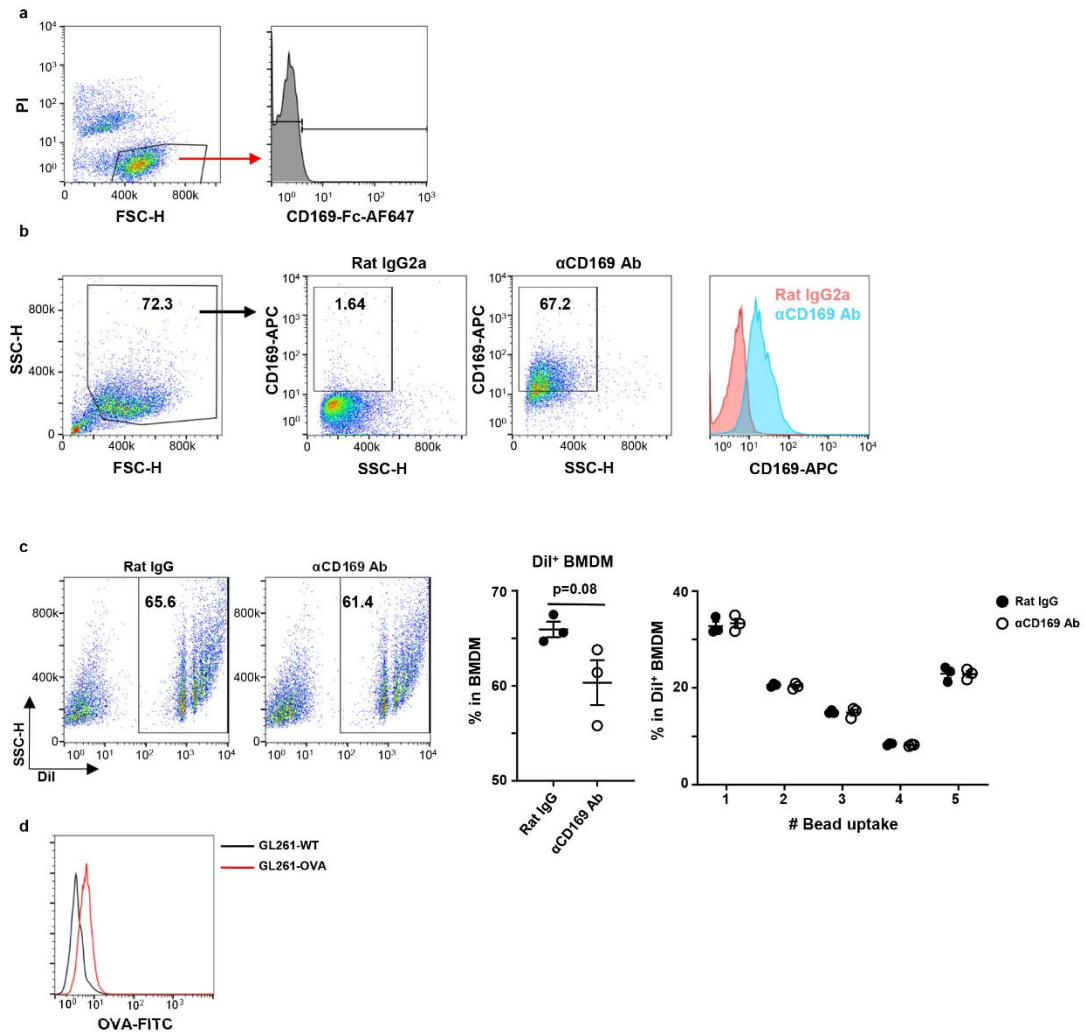
Supplementary Figure 5. Tumor infiltration of CD169⁺ macrophages is regulated by IFN- γ but not by IFN- α signaling.

a WT (n = 3) and CCR2 knockout (KO) (n = 3) mice were injected with GL261 and analyzed by flow cytometry 15 days after tumor injection. Bold numbers in plots indicate the percentage of gated cells. Bars indicate SEM. P-values were calculated by unpaired two-tailed *t*-test. Data represent two independent experiments.

b WT (n = 3) and IFNαR KO (n = 3) mice were injected with GL261-GFP and analyzed 10 days after tumor injection. Scale bar indicates 50 μm. Data represent two individual experiments.

c Expression of *Ifng* in immune cells isolated 10 dpi of GL261 injection were analyzed.

Source data are provided as a Source Data file.



Supplementary Figure 6. Interaction between CD169 on macrophages and CD169 ligand on apoptotic GL261 cells promotes phagocytosis

a Gating strategy for analysis of CD169-ligand on GL261 tumor cells.

b Gating strategy for analysis of CD169 expression on BMDMs.

c BMDMs were treated with anti-CD169 blocking antibody (Ab) or Rat IgG2a isotype control Ab and co-cultured with fluorescently labeled latex bead. Bead uptake by BMDM was analyzed by flow cytometry. Data represent two independent experiments. Bars indicate SEM. P-values were calculated from an unpaired two-tailed *t*-test.

d Expression of OVA in WT GL261 and GL261-OVA cell lines.

Source data are provided as a Source Data file.