Single-cell characterization of human GBM reveals regional differences in tumor-infiltrating leukocyte activation

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Abstract

Glioblastoma (GBM) harbors a highly immunosuppressive tumor microenvironment (TME) which influences glioma growth. Major efforts have been undertaken to describe the TME on a single-cell level. However, human data on regional differences within the TME remain scarce. Here, we performed high-depth single-cell RNA sequencing (scRNAseq) on paired biopsies from the tumor center, peripheral infiltration zone and blood of five primary GBM patients. Through analysis of > 45'000 cells, we revealed a regionally distinct transcription profile of microglia (MG) and monocyte-derived macrophages (MdMs) and an impaired activation signature in the tumor-peripheral cytotoxic-cell compartment. Comparing tumor-infiltrating $CD8^+$ T cells with circulating cells identified $CX3CR1^{high}$ and $CX3CR1^{int}$ $CD8^+$ T cells with effector and memory phenotype, respectively, enriched in blood but absent in the TME. Tumor $CD8^+$ T cells displayed a tissue-resident memory phenotype with dysfunctional features. Our analysis provides a large-scale dissection of GBM-associated leukocytes, serving as a reference map of human GBM-TME.

Introduction

Glioblastoma (GBM) is a fatal disease without effective long-term treatment options. The current standard of care consists of tumor resection followed by adjuvant chemoradiotherapy resulting in a median overall survival of only 14 months [1]. One of the hallmarks in GBM progression is the high rate of neovascularization. The GBM-induced aberrant vessels not only nourish glioma cells, but also provide a specialized niche for tumor-associated stromal and immune cells such as monocyte-derived macrophages (MdMs), yolk sac-derived microglia (MG) (together termed glioma-associated macrophages/microglia, GAMs), and peripheral adaptive immune cells. This immune tumor microenvironment (iTME) paradoxically acts in an immunosuppressive manner and promotes tumor progression [2]. For example, clinical trials of systemic T cell checkpoint blockade showed only disappointing results [3, 4], which was attributed in part to the immunosuppressive components of the GBM iTME. The origin of GAMs, infiltration of peripherally derived macrophages across the blood-brain-barrier (BBB) or recruitment of tissue-resident MG to the tumor site, as well as their contribution to gliomagenesis are studied intensively [2, 5-7]. Hence, major efforts have been undertaken to describe the GBM iTME on a single cell level [5, 6, 8]. However, human data on the composition of the iTME in different tumor regions (contrast enhancing tumor center versus peripheral infiltration zone) remain scarce [9, 10].

To study the region-dependent cellular diversity within individual GBMs, we performed single-cell RNA sequencing (scRNA-seq) on patient-matched biopsies from the tumor center and the peripheral infiltration zone of five primary GBM patients. Additionally, peripheral blood mononuclear cells (PBMC) of the same patients were included to explore the transcriptional changes occurring during tumor infiltration of circulating immune cells.

Our analysis revealed a regionally distinct transcription profile of MG and MdMs and an impaired activation signature in the tumor-peripheral cytotoxic-cell compartment. Comparing tumor-infiltrating $CD8^+$ T cells with PBMC-derived, identified $CX3CR1^{high}$ and $CX3CR1^{int}$ $CD8^+$ T cells with effector and memory phenotype, respectively, enriched in blood but absent in the iTME. Tumor $CD8^+$ T cells displayed features of tissue-resident memory T cells and were characterized by an exhaustion phenotype. This work provides a large-scale dissection of glioma-associated cell types complemented by patient-matched PBMCs, revealing an abundance of information about the composition and molecular diversity of the iTME in GBM.

Results

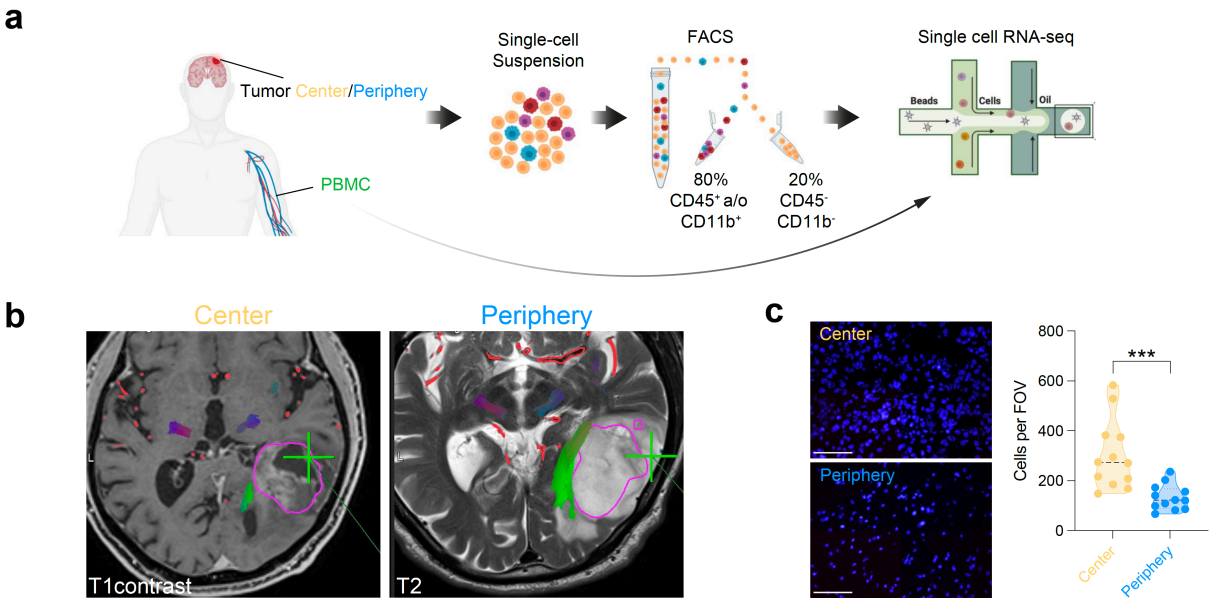

Fig. 1 Single-cell RNA-seq of cells from tumor center, periphery and blood. a Experimental workflow for single-cell analysis of cells isolated from tumor center, periphery and peripheral blood mononuclear cells (PBMC), including fluorescent-activated cell sorting and 3'-scRNA-seq. **b** Axial T1 with contrast (left) and T2 (right) MRI brain in a patient with a left temporal GBM. Fresh tumor biopsies were taken according to neuronavigation (green cross). The tumor center was defined as contrast enhancing, whereas the tumor periphery was defined as T2 hyperintense. **c** Nuclear DAPI staining of resected tissue specimens. 40x magnification (scale bar = 20 µm). n = 3 patients, 4 field of view (FOV) per patient. Statistics: ***$p < 0.001$, two-tailed Mann Whitney U test.

scRNA-seq analysis of paired tumor center, periphery and PBMC samples.

Fresh, neurosurgically resected tissue from five GBM patients were harvested (Supplementary Table 1). According to the 2021 WHO Classification of Tumors of the Central Nervous System [11], in which the term glioblastoma designates only IDH-wildtype grade 4 tumors, we will hence use the term grade 4 glioma, as we included as well IDH-mutant grade 4 tumors (Supplementary Table 1). The tumor center was defined as contrast enhancing, whereas the tumor periphery was defined as T2 hyperintense by magnetic resonance imaging (MRI)-guided, navigated surgical resection (Fig. 1b). Increased cellular density of the center vs. periphery samples was confirmed by nuclear DAPI staining on matched histological micrographs of the resected tissue specimens used for scRNA-seq (Fig. 1c). As outlined in Fig. 1a, we separately processed patient tumor and blood samples and enriched them for immune cells by fluorescence-activated cell sorting (FACS) (Supplementary Fig. 1a and 1b). The three samples per

89 patient (center, periphery and PBMC) were loaded on different wells of a 10x Genomics Chro-
90 mium system for a targeted recovery of 10,000 cells. Due to technical issues cells from the
91 center sample of patient BTB 609 could not be collected.

92 In total we analyzed 45,466 cells that passed initial quality control and filtering, com-
93 prising 8,254 cells from tumor center, 5,954 cells from tumor periphery and 31,258 PBMCs,
94 with 6,354 to 10,957 cells per patient (Supplementary Table 2; Supplementary Fig. 1c-1f). All
95 cells were projected onto a two dimensions *t*-distributed stochastic neighbor embedding (tSNE)
96 [12]. As we observed a good overlap of cells across patients, we chose not to perform any
97 correction for patient-specific effects (Supplementary Fig. 1g). Using hierarchical clustering,
98 the cells were partitioned into clusters (Supplementary Fig. 3a) which were then annotated into
99 eight distinct cell types for the immune subset and five cell types for the CD45 negative subset
100 (Fig. 2a; Supplementary Fig. 3b, g-h; Supplementary Table 2). Notably: the annotation of most
101 of the immune cell types was performed by whole-transcriptome comparison of our cells to a
102 reference dataset of bulk RNA-seq samples of sorted immune cell types from human PBMC
103 (Supplementary Fig. 3c) [13]; the annotation of MdMs and microglia was performed by whole-
104 transcriptome comparison to a dataset of bulk RNA-seq samples of sorted immune cell types
105 from the tumor microenvironment of human gliomas (Supplementary Fig. 3d) [5] and using
106 signature scores defined from scRNA-seq of GAMs (Supplementary Fig. 3e-f) [7]; finally,
107 CD45 negative cells were annotated by whole-transcriptome comparison to a scRNA-seq da-
108 taset of $IDH1^{wt}$ GBM (Supplementary Fig. 3i) [14]. The expression of known marker genes
109 across cell types is shown in Fig. 2b, and genes whose expression is most specific to each cell
110 type are shown in Supplementary Fig. 2.

111 In line with previous work [5, 6, 10], we noted that GAMs accounted for the most fre-
112 quent cell type in the center iTME (on average 44.5% among leukocytes in center vs. 24.7%
113 among leukocytes in periphery), while the T cell compartment accounted for the most abundant
114 immune population in the tumor periphery (Fig. 2d). When comparing phagocytic cell types,
115 we found that MdMs decreased substantially in the glioma periphery, while MG did not exhibit
116 a differential distribution between tumor center and periphery (Fig. 2e).

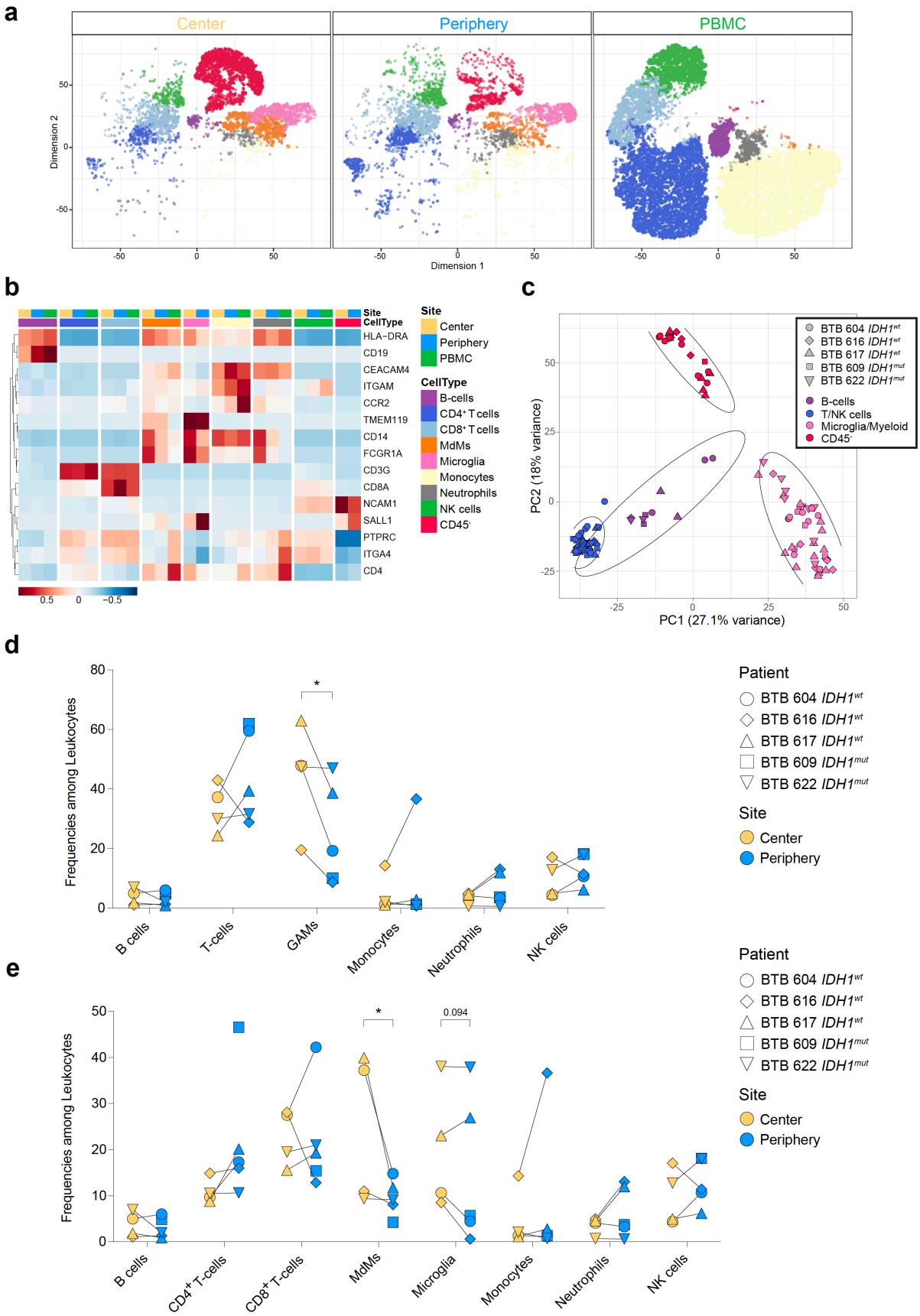

Fig. 2 Single-cell RNA-seq analysis identifies main immune cell populations. a Dimensionally reduced tSNE projection of the scRNAseq data showing the identified main cell clusters. **b** Heatmap displaying averaged and normalized expression values of characteristic cell-type

specific genes used to annotate clusters in (**a**). Columns are ordered by site and cell type, and rows show centered and scaled expression values, hierarchically clustered. **c** Principal component (PC) biplot of pseudo-bulk scRNAseq samples aggregated by patient and cell type. Symbols represent individual patients and cell lineage is displayed by different colors. **d, e** Relative frequencies of immune populations among leukocytes between tumor center and periphery. Symbols represent individual patients and paired samples are indicated by connecting lines. Statistics: *FDR<5%, *diffcyt-DA-voom* method.

128 *MG and MdMs display regionally distinct transcription profiles.*

129 To perform a differential expression analysis between tumor sites, we stratified the anal-
130 ysis by annotated cell type and aggregated cells from each patient (see Supplementary Meth-
131 ods). A principal component analysis (PCA) on the aggregated transcriptome data confirmed
132 that the major source of variation was the cell type lineage (Fig. 2c), with notable differences
133 between lymphoid, MG/myeloid cells, and $CD45^-$ cells (PCs 1 and 2). Interestingly there was
134 no clear association between patient *IDH1* status and these or deeper components, suggesting
135 that the iTME seemed independent of *IDH1* status in grade 4 glioma. Moreover, immune cell
136 type differential abundance analysis revealed an equal distribution among *IDH* variants, further
137 supporting this observation (Fig. 2d, e).

138 Differential expression analysis between MG from tumor center and periphery revealed
139 a highly significant downregulation of inflammatory genes in the peripheral MG. This included
140 scavenger receptors (*CD36* and *MARCO*), chemokines (*CXCL3* and *CCL20*) and immune re-
141 ceptors (*IL7R [15]* and *CD109*, a negative regulator of TGF-β signaling [16]) as well as genes
142 involved in cell growth (*CSRP1*) and cell metabolism (*SMPDL3A [17]* and *SDS*) (Fig. 3a and
143 Supplementary Table 3). The latter transcribes for the serine dehydratase, an enzyme catalyzing
144 the dehydration of L-serine/L-threonine to yield pyruvate/ketobutyrate [18]. The downregula-
145 tion of *SDS* in the peripheral MG with reduced metabolization of L-serine to pyruvate could
146 potentially lead to a reduced oxidative phosphorylation in peripheral MG, a metabolic feature
147 described for dysfunctional MG in Alzheimer's disease models [19]. Concomitantly, increased
148 L-serine levels have been associated with the induction of alternative, M2-like microglial po-
149 larization and inhibited secretion of inflammatory factors (TNF-α and IL-1β) [20].

150 Interestingly, we found upregulation of Inhibitor of DNA-Binding 1, also known as In-
151 hibitor of Differentiation 1 (*ID1*) in the peripheral MG, which is well described in GBM pro-
152 gression, treatment resistance and glioma stem cell biology [21]. Recently, new evidence has
153 emerged, linking *ID1* to suppression of the anti-tumor immune response in the myeloid com-
154 partment and promoting tumor progression [22].

155 To further explore the underlying biological processes differing between MG in the two
156 compartments, we conducted a gene set enrichment analysis (GSEA) on the results of the dif-
157 ferential expression analysis using Gene Ontology (GO) database (Biological Processes). This
158 revealed overall a significant downregulation of GO categories involved in antigen processing
159 and presentation via MHC-I and MHC-II in the peripheral MG relative to the center MG, as
160 well as downregulation of amino acid metabolism and TNF-α signaling pathway (Fig. 3b),

161 which further supported our observation of a strong immunosuppressive phenotype in periph-
162 eral MG.

163 When comparing the transcriptional profile of MdMs from the peripheral front to the
164 tumor center, we observed upregulation of proinflammatory genes *MEFV* encoding pyrin [23]
165 and *APOBEC3A,* a cytidine deaminase involved in RNA editing during macrophage M1 polar-
166 ization and response to interferons (IFN) [24]. Moreover, upregulation of *KCNJ2*, a voltage-
167 dependent potassium channel has been shown to regulate macrophage proliferation [25]
168 whereas *GPR132* serves as lactate sensor in the acidic TME and could potentially facilitate
169 MdM migration to the tumor site [26]. *MIR22HG*, a long non-coding RNA (lncRNA) has been
170 associated with tumor suppressive properties in hepatocellular carcinoma, where it has been
171 linked to chemokine signaling pathways and phagosome activation [27] (Fig. 3c).

172 Along with this, we observed downregulation of anti-inflammatory genes in the periph-
173 eral MdMs. This included *RNASE1*, a signature gene of macrophages enriched in immune
174 checkpoint inhibitor (ICI) non-responding melanoma patients [28], *PLTP*, a negative regulator
175 of NF-κB activation [29], *NRP1*, a key gene required for macrophage attraction towards hy-
176 poxic tumor niches and thereby retaining their pro-tumorigenic features [30], and *IL4I1*, a novel
177 metabolic immune checkpoint in the tryptophan/aryl hydrocarbon receptor (AHR) pathway
178 [31] (Fig. 3c). Hence, MdMs might display a proinflammatory phenotype in the glioma periph-
179 ery, however, are less abundant there (Fig. 2e).

180 We observed a marked downregulation of *SDS* in the peripheral MdMs, similar to the
181 peripheral MG population, leading to a presumptive accumulation of L-serine in the peripheral
182 MdMs. In contrast to MG, serine metabolism has been shown to indeed support proinflamma-
183 tory IL-1β cytokine production in macrophages [32]. Together with the reduced tryptophan
184 metabolism through *IL4I1* and downregulated *SLC39A8*, a transmembrane zinc importer whose
185 reduction has been linked to increased IL-6/IL-1β secretion and increased NF-κB signaling in
186 innate immunity [33], these data shed new light on regional differences in the innate im-
187 munometabolism in the iTME of grade 4 glioma.

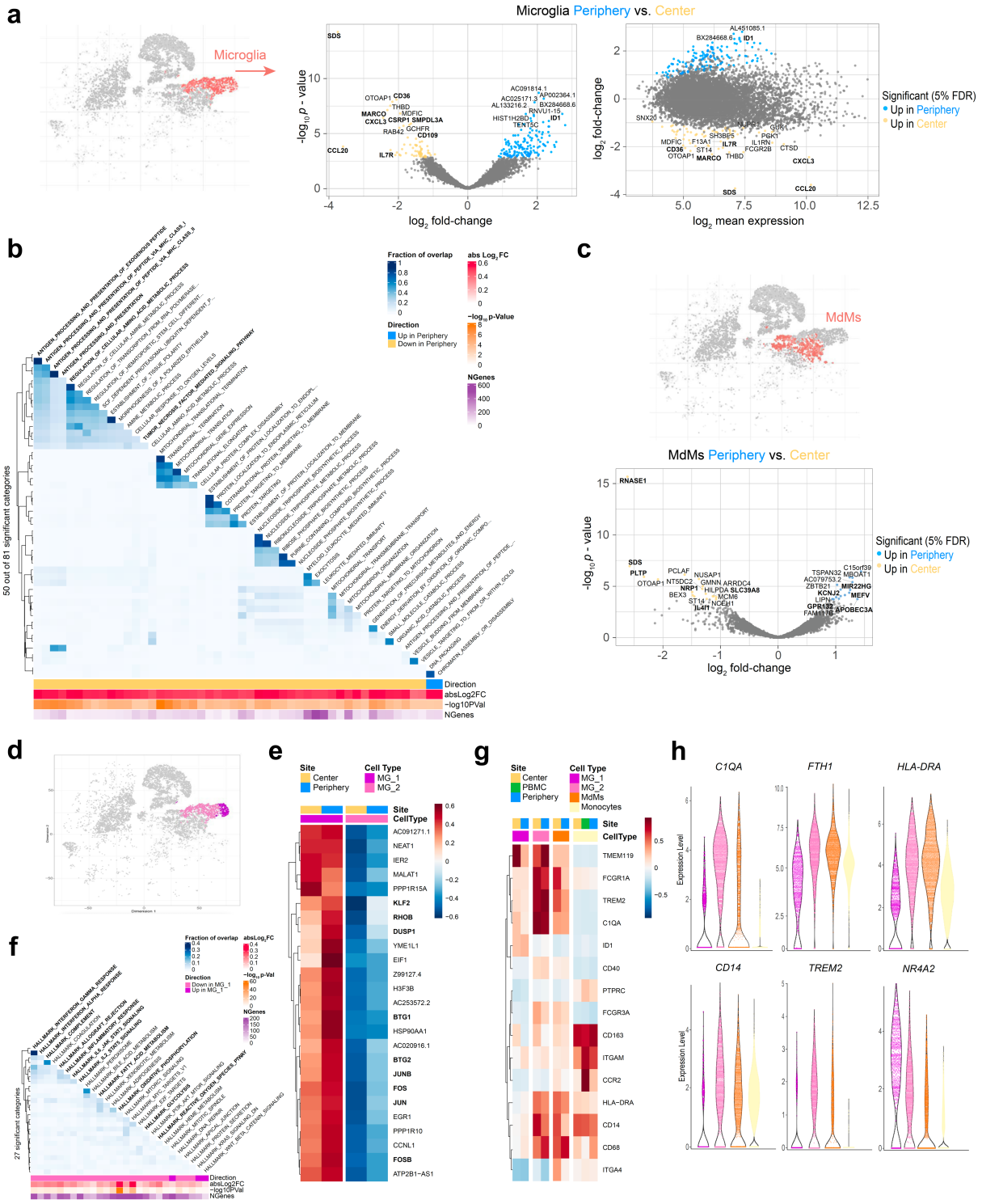

Fig. 3 MG and MdMs display distinct regional transcription profiles. a Microglia cluster highlighted on tSNE map and scatterplots showing differentially expressed genes (FDR<5%, indicated by blue and yellow) in Microglia (MG) cells from tumor periphery versus center. Volcano plot showing *p* value versus fold-change (left) and MA plot showing fold-change versus mean expression (right). **b** Heatmap representation of Gene set enrichment analysis (GSEA) results between peripheral and center microglia using Gene Ontology (GO) collection (Biological Processes). The fraction of overlap between gene sets is calculated as Jaccard coefficient

196 of overlap between the gene sets. **c** Monocyte-derived macrophages (MdMs) cluster highlighted
197 on tSNE map and volcano plot showing statistical significance (FDR<5%, indicated by blue
198 and yellow) versus fold-change of differentially expressed genes in MdMs from tumor periph-
199 ery versus tumor center. **d** Unsupervised hierarchical sub-clustering of the MG population re-
200 vealed two transcriptionally distinct subsets of MG, termed MG_1 and MG_2, displayed on the
201 tSNE map. **e** Heatmap displaying the cluster-specific genes identifying MG_1 and MG_2 sub-
202 clusters. Columns are ordered by site and cell type, and rows show centered and scaled expres-
203 sion values, hierarchically clustered. **f** Heatmap representation of GSEA between MG_1 and
204 MG_2 subclusters using Hallmark collection of major biological categories. **g** Heatmap dis-
205 playing previously described reactivity markers of MG. Columns are ordered by site and cell
206 type, and rows show centered and scaled expression values, hierarchically clustered. **h** Violin
207 plot showing expression levels of selected reactivity markers among mononuclear phagocyte
208 populations.

209 *The iTME of grade 4 glioma harbors two transcriptionally distinct MG subpopulations.*

210 Unsupervised hierarchical sub-clustering of the MG population revealed two transcrip-
211 tionally distinct iTME MG subsets, which we termed MG_1 and MG_2, respectively (Fig. 3d
212 and 3e). The MG_1 cluster was highly enriched for the activator protein-1 (AP-1) family of
213 transcription factors including *FOS, FOSB, JUN, JUNB, MAF* and *MAFB* (Fig. 3e and Supple-
214 mentary Table 4), which convey a surveilling phenotype to adult MG, but are also involved in
215 numerous processes including cell growth, differentiation and immune activation [34]. Specif-
216 ically, *FOSB* gene products have been implicated in the excitotoxic MG activation by regulating
217 complement C5a receptor expression [35]. Yet, concomitant upregulation of anti-inflammatory
218 Krüppel-like factor 2 (*KLF2*) [36] and Dual Specificity Protein Phosphatase 1 (*DUSP1*), an
219 inhibitor of innate inflammation by negatively regulating the mitogen-activated protein kinase
220 (MAPK) pathway [37], together with increased expression of anti-proliferative genes like
221 *RHOB, BTG1* and *BTG2* paint a more complex picture of these cells. Particularly, *BTG1* has
222 been identified as an activation-induced apoptotic sensitizer in MG after exposure to inflam-
223 matory stimuli [38], serving as an autoregulatory mechanism and possibly hinting towards an
224 exhausted state in these MG_1 cells. GSEA for differences between MG_1 and MG_2 clusters
225 using the MSigDB Hallmark collection of major biological pathways [39] revealed downregu-
226 lation of many MG effector functions in the MG_1 population including (1) inflammation
227 ("Complement", "Inflammatory Response", "Allograft Rejection", "Reactive Oxygen Species
228 Pathway"), (2) immune cell activation ("IFN-α Response", "IFN-γ Response", "IL6 JAK
229 STAT3 Signaling", "IL2 STAT5 Signaling") and (3) immunometabolism ("Fatty Acid Metab-
230 olism", Oxidative Phosphorylation", "Glycolysis") (Fig. 3f). As we examined the expression
231 of previously described reactivity markers of MG including *C1QA,* Ferritin (*FTH1*), *FCGR1A,*
232 *HLA-DRA, CD14* and *TREM2 [40-43],* and established MG homeostatic genes like *CX3CR1,*
233 *HEXB* and *SPI1* (PU.1), we noted a marked downregulation of these genes in the MG_1 cluster,
234 while the anti-inflammatory transcription factors *NR4A2* [44] and *NR4A1* [45] were highly up-
235 regulated (Fig. 3g, 3h, Supplementary Fig. 4a and Supplementary Table 4). Additionally, while
236 total MG didn't show differences in abundance between tumor sites (Figure 2e), changes could
237 be observed when stratifying for MG subclusters. We noted in 3 of 4 (75%) paired center-
238 periphery samples an increased abundance of MG_1 cells in the tumor periphery. And, the
239 presumably more reactive MG_2 cells concomitantly decreased significantly in frequency in
240 the tumor periphery (Supplementary Fig. 4b). Collectively, these data argue for the non-reac-
241 tive/exhausted phenotype of MG_1.

242 *The tumor peripheral cytotoxic cell compartment exhibits an impaired activation signature.*

243 Next, we investigated the regional differences in the lymphoid compartment composed
244 of $CD4^+$ and $CD8^+$ T cells and natural killer (NK) cells. We observed only very few significant
245 changes in the transcriptomic profiles of $CD4^+$ T cells between tumor center and periphery
246 (Supplementary Fig. 4c and Supplementary Table 3). Yet, comparing peripheral $CD8^+$ T cells
247 with $CD8^+$ T cells from tumor center revealed 110 differentially expressed genes (43 genes
248 upregulated and 67 genes downregulated) (Fig. 4a and Supplementary Table 3). Many down-
249 regulated genes in the peripheral $CD8^+$ T cells associated with canonical IFN responses (*IFI6,*
250 *IFI27, MX1, STAT1, EPSTI1 PARP9, ISG15)* [46] cell proliferation (*STMN1, CENPF, HELLS,*
251 *NUSAP1* and *DNPH1*) and T cell co-stimulation (*CD28, TMIGD2* (CD28H), *TNFRSF4*
252 (OX40), *CD27* and *TNFRSF18* (GITR)) (Fig. 4a). Contrary to our expectations, we saw upreg-
253 ulation of *CTLA4* in the center $CD8^+$ T cells which acts as a negative costimulatory molecule.
254 However, unlike other costimulatory receptors, such as CD27 and CD28*,* CTLA-4 is not con-
255 stitutively expressed on T lymphocytes [47]. but only induced following T cell activation, along
256 with positive costimulatory molecules such as OX40 and GITR. In addition, upregulation of
257 CTLA-4 requires entry into the cell cycle [47]. In line with that, we detected an upregulation
258 of proliferative genes in center $CD8^+$ T cells. In summary, CTLA-4 induction in center $CD8^+$ T
259 cells rather suggested T cell activation than exhaustion, especially since other inhibitory recep-
260 tors like *PDCD1* (PD-1)*, LAG3* and *HAVCR2* (TIM-3) were not differentially expressed be-
261 tween sites. Moreover, we did not observe differential expression of genes involved in $CD8^+$ T
262 cell effector functions like cytotoxicity (e.g., *GZMK, GZMB, KLRG1,* PRF1) or cytokines (e.g.,
263 *CCL5, XCL1, XCL2, IL10*). Yet, we noted upregulation of inhibitory genes (*TGFB1* and *FCRL6*
264 [48]) in the peripheral $CD8^+$ T cells, suggesting that a pool of activated, proliferating and IFN-
265 responsive $CD8^+$ T cells is present in the tumor center, but fails to populate the infiltrative tumor
266 periphery.

267 Similar trends were observed for the peripheral NK cell population with peripherally
268 reduced IFN response (*MX1* and *IFI44L*), and proliferative genes (*STMN1, HELLS, CENPF,*
269 *PTTG1* and *DNPH1*), downregulated stimulatory receptors (*TMIGD2* (CD28H) and
270 *TNFRSF18* (GITR)), and reduced NF-κB signaling (*NFKB1* and *RELB*) (Fig. 4b and Supple-
271 mentary Table 3). Although, we observed upregulation of key genes associated with NK cell
272 effector function in the periphery (e.g., *FCGR3A* (CD16), *FGFBP2, ITGB2, GZMH* and
273 *KIR2DS4*), increased expression of inhibitory receptors like *LILRB1* and *KLRG1*, the latter es-
274 pecially in co-expression with chemokine receptor *CX3CR1*, identified the peripheral NK cells
275 rather to be terminally differentiated with impaired cytotoxic capabilities [49]. This was in line

with the observed abrogated cytokine activity profile in the peripheral NK cells with reduced expression of key factors like *XCL1, XCL2, LTB* and *CKLF*. In summary, our data revealed an impaired activation signature in the peripheral cytotoxic cell compartment.

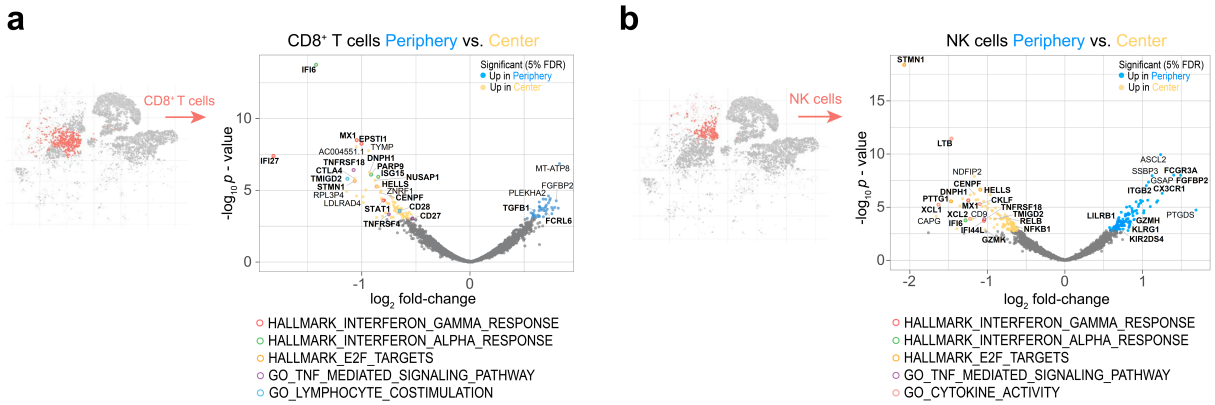

Fig. 4 The peripheral cytotoxic cell compartment exhibits an impaired activation signature. a, b Volcano plots showing differentially expressed genes (FDR corrected p value < 0.05, indicated by blue and yellow) in CD8$^+$ T cells (**a**) and NK cells (**b**) from tumor periphery versus tumor center. Colored rings mark genes belonging to selected GSEA Hallmark or Gene Ontology (GO) pathways as indicated.

286 *CX3CR1 labels a specific CD8$^+$ T cell population in the circulation of grade 4 glioma patients.*

287 Next, we investigated the relationships between circulating CD8$^+$ T cells and those from
288 the tumor milieu and, more specifically, the peripheral, infiltration zone characterized by an
289 abrogated CD8$^+$ T-cell IFN response and activation signature. Strikingly, there were large tran-
290 scriptomic differences between PBMC and periphery CD8$^+$ T cells (Fig. 5a), with 1,417 differ-
291 entially expressed genes (864 genes upregulated in the tumor periphery and 553 genes upregu-
292 lated in PBMC) (Fig. 5b, Supplementary Table 5).

293 Interestingly, one of the key genes upregulated in PBMC CD8$^+$ T cells was the chemo-
294 kine receptor *CX3CR1*, whose expression labelled a specific population among these cells (Fig.
295 5c). Flow cytometry of an additional matched glioma grade 4 patient cohort confirmed an in-
296 creased abundance of CX3CR1$^+$ CD8$^+$ T cells in PBMC compared to almost absent CX3CR1$^+$
297 CD8$^+$ T cells in tumor periphery (Fig. 5d, Supplementary Table 1).

298 Recently, expression of CX3CR1 was demonstrated to distinguish memory CD8$^+$ T cells
299 with cytotoxic effector function [50]. Further characterization of classical central memory (T_{cm})
300 and effector memory (T_{em}) populations by varying surface expression levels of CX3CR1 iden-
301 tified a novel CX3CR1^{int} subpopulation, termed peripheral memory (T_{pm}). T_{pm} cells underwent
302 frequent homeostatic divisions, re-acquired CD62L, homed to lymph nodes, and predominantly
303 surveyed peripheral tissues compared to T_{cm} and T_{em} [51]. In our dataset, the circulating
304 CX3CR1$^+$ CD8$^+$ T cells indeed displayed a core signature of memory CD8$^+$ T cells with effector
305 function, comprising expression of LFA-1 (*IGAL- ITGB2*), *EOMES, SELL* (CD62L), *CCR7^{low},*
306 *CD27^{low}, TBX21^{high}* (Tbet), *IL7R, TCF7, FAS* and *ITGB1,* separating them from circulating
307 *CX3CR1$^-$ CD28^{high}*, *CD27^{high}* and *IL7R^{high}* naive CD8$^+$ T cells (Fig. 5e and Supplementary Fig.
308 5b). The observed high expression of cytolytic molecules *GZMB* (Granzyme B) and *PRF1* (Per-
309 forin 1) in the *CX3CR1$^+$* cells advocated for their cytotoxic effector phenotype (Fig. 5n). Flow
310 cytometric analysis confirmed T_{eff} to be CX3CR1^{high}, with negligible expression levels in the
311 naive CD8$^+$ T cells, whereas the identified memory CD8$^+$ T cells (T_{em} and T_{pm}) were CX3CR1^{int}
312 (Fig. 5f, 5g). Collectively, surface expression analysis of CX3CR1 identified a subset of
313 CX3CR1^{high} T_{eff} and CX3CR1^{int} memory (T_{em}, T_{pm}) CD8$^+$ T cells in the circulation of grade 4
314 glioma patients with potentially elevated tissue surveilling properties in the case of T_{pm}, which
315 are, however, largely absent in the tumor microenvironment.

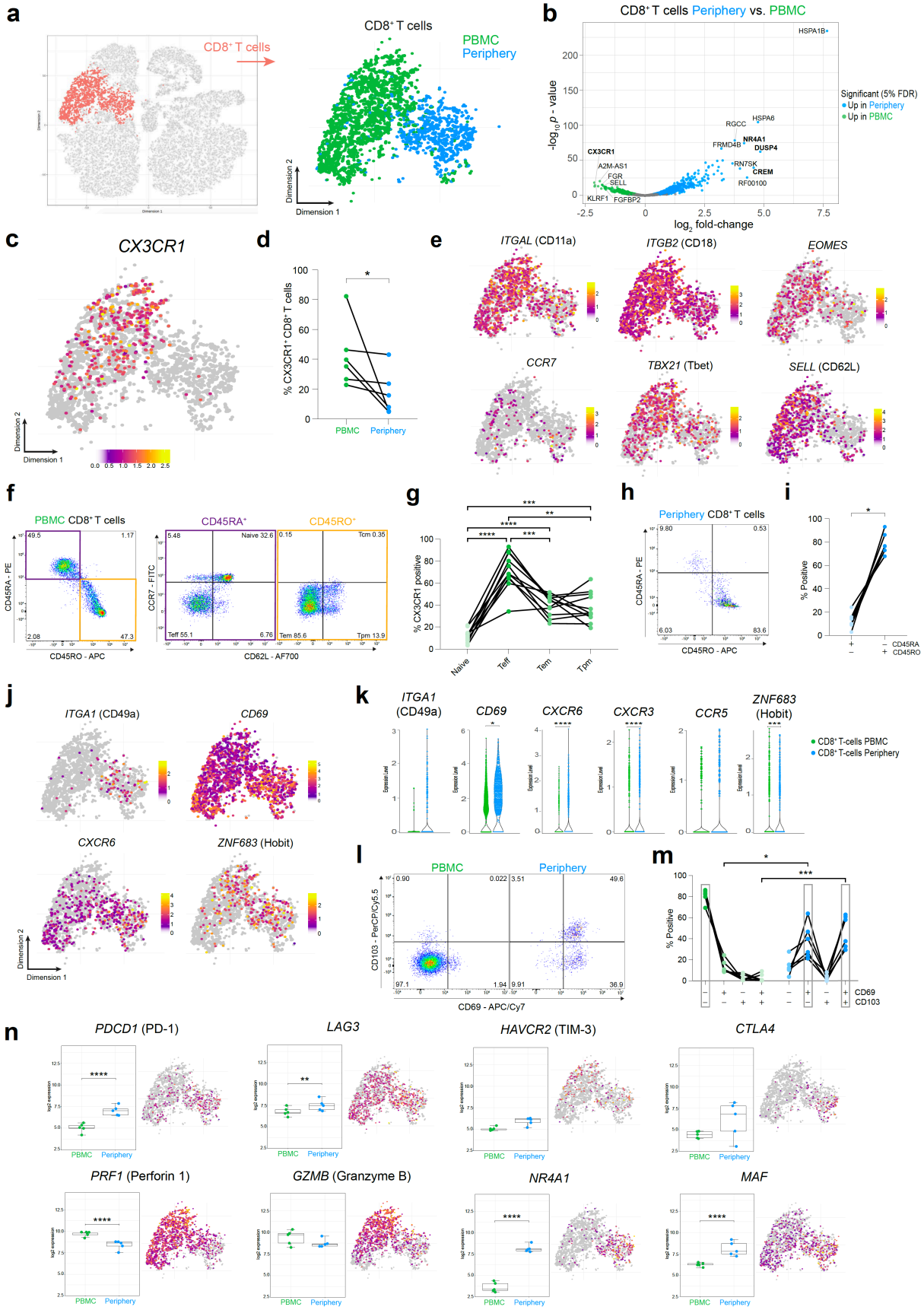

Fig. 5 CD8+ T cells in grade 4 glioma show distinct memory phenotypes depending on site. **a** CD8+ T cell cluster highlighted on tSNE map (left). CD8+ T cell cluster colored by site of origin (right). **b** Volcano plot showing differentially expressed genes (FDR corrected *p* value <

0.05, indicated by blue and green) in $CD8^+$ T cells from tumor-periphery versus PBMC. **c** Expression of *CX3CR1* overlaid on tSNE $CD8^+$ T cell cluster. **d** Frequency of $CX3CR1^+$ $CD8^+$ T cells among all $CD8^+$ T cells in flow cytometry data. **e** Expression of genes associated with memory (upper row) and effector memory (lower row) phenotype overlaid on tSNE $CD8^+$ T cell cluster. **f** Gating procedure applied to identify $CD3^+$ $CD8^+$ naive, T effector cells (T_{eff}), effector memory (T_{em}), peripheral memory (T_{pm}) and central memory (T_{cm}), eluted from PBMCs. **g** Expression of CX3CR1 in $CD8^+$ T cell subpopulations identified in (**f**). **h** Representative dot plot of tumor-periphery $CD8^+$ T cells stained for CD45RA and CD45RO. **i** Quantification of tumor-periphery $CD8^+$ T cells expressing CD45RA or CD45RO. **j** Expression of genes associated with tissue-resident memory (T_{rm}) phenotype overlaid on tSNE $CD8^+$ T cell cluster. **k** Average expression levels of selected T_{rm} markers between $CD8^+$ T cells from PBMC versus tumor-periphery. **l** Representative dot plots of CD69 and CD103 co-expression in $CD8^+$ T cells from PBMC and tumor-periphery. **m** Quantification of CD69 and CD103 co-expression revealed $CD69^-$ $CD103^-$ in PBMC and $CD69^+$ $CD103^-$ and $CD69^+$ $CD103^+$ in tumor-periphery as the dominant phenotypes. **n** Expression of selected markers associated with T cell exhaustion/dysfunction, shown as boxplots between $CD8^+$ T cell from PBMC and tumor-periphery and overlaid on tSNE $CD8^+$ T cell cluster. $n = 6$ donors (**d, i, m**), $n = 11$ donors (**g**). Statistics: Wilcoxon matched-pairs signed rank test (**d, i**); repeated measures one-way ANOVA with post-hoc Šidák's correction for multiple comparisons (**g, m**). For detailed statistical analysis of scRNA-seq expression data, please refer to supplementary methods section. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$, no brackets indicate no significant difference.

341 *CD8$^+$ T cells in the tumor periphery share features with tissue-resident memory T cells (T_{rm})*

342 We next examined the differing transcriptional and surface-specific features between
343 tumor infiltrating and circulating CD8$^+$ T cells. Surface staining for CD45RA and CD45RO,
344 discriminating naive/effector from memory T cells, attributed a predominant CD45RO$^+$
345 memory phenotype to the tumor infiltrating CD8$^+$ T cells (Fig. 5h, 5i). Interrogation of the tran-
346 scriptomic profile of these cells revealed a key marker expression signature consistent with
347 tissue-resident memory T cells (T_{rm}): Expression of cellular adhesion molecules (integrins)
348 *ITGA1* (CD49a) and *ITGAE* (CD103), tissue retention marker *CD69*, chemokine receptors im-
349 plicated in tissue-homing *CXCR3, CXCR6* and *CCR5* [52] and transcription factors, *ZNF683*
350 (Hobit) and *PRDM1* (Blimp1) as well as reduced expression of *TBX21* (Tbet) and *EOMES* [53],
351 strongly suggested a T_{rm} phenotype for these cells (Fig. 5j, 5k and Supplementary Fig. 5b). Co-
352 expression analysis of paired PBMC and tumor periphery samples using flow cytometry
353 showed that CD69$^+$ CD103$^-$ and CD69$^+$ CD103$^+$ cells are the dominant CD8$^+$ T cell populations
354 in the tumor periphery (Fig. 5l and 5m). Combined, these data strongly suggest a T_{rm} phenotype
355 for the CD8$^+$ T cells in the tumor periphery.

356 Previous reports of T_{rm} populating the brain in the aftermath of central or peripheral
357 infections concluded that brain T_{rm} cells surveil the brain tissue and mediate protection by rapid
358 activation and enhanced cytokine production [52]. Indeed, CD8$^+$ T cells in the tumor periphery
359 showed increased expression of genes belonging to costimulatory pathways, including *ICOS,*
360 *TNFRSF4* (OX40) and *TNFRSF9* (4-1BB) (Supplementary Fig. 5c, Supplementary Table 5),
361 albeit accompanied by high levels of inhibitory receptors *PDCD1* (PD-1), *LAG3, HAVCR2*
362 (TIM-3) and *CTLA4* (Fig. 5n). Moreover, expression of genes coding for cytotoxic molecules,
363 including Granzyme B and Perforin 1 were decreased in the peripheral CD8$^+$ T cells, suggesting
364 a compromised killing capacity of these cells. And lastly, CD8$^+$ T cells in the tumor periphery
365 exhibited a transcription factor profile of exhausted T cells with high expression of *NR4A1,*
366 *MAF* and *IRF4* (Fig. 5n and Supplementary Fig. 5d), which have been implicated in T cell
367 dysfunction and exhaustion [54, 55]. Collectively, these data indicate that CD8$^+$ T cells in the
368 glioma periphery share features with T_{rm} cells. However, inhibitory receptor expression, func-
369 tional molecules and transcriptional signature ascribe an exhausted phenotype to these cells.

370 Noteworthy, we observed high upregulation of similar genes in the comparison tumor
371 periphery vs. PBMC for CD4$^+$ T cells as for CD8$^+$ T cells (Fig. 5b and Supplementary Fig. 5e).
372 These included transcription factor family *NRA41-3,* identified as key mediator of T cell dys-
373 function [55], Dual Specificity Protein Phosphatase 2/4 (*DUSP2, DUSP4*) described as nega-
374 tive regulators of mitogen-activated protein (MAP) kinase superfamily and associated with

375 impaired T cell effector activity [56] and T cell senescence [57], and transcription factor *CREM*
376 which has been implicated in IL-2 suppression [58]. These genes could potentially identify pan
377 T cell dysfunction markers within the GBM iTME [59].
378
379 *Interrogation of cell-cell interactions revealed critical role of SPP1-mediated crosstalk be-*
380 *tween MG and lymphocytes in the tumor periphery*
381 We next investigated cell-cell interactions based on ligand-receptor expression levels
382 using the CellChat platform [60]. Considering MG being on average the main innate immune
383 population in the tumor periphery (Fig. 2e), and lymphocytes displaying an impaired activation
384 signature, we focused our analysis on the tumor-peripheral crosstalk between MG and lympho-
385 cytes (Fig. 6a). This revealed SPP1 (Osteopontin) as a leading potential cell-cell interaction
386 mediator between MG and lymphocytes (Fig. 6a and 6b). MG SPP1-mediated signaling was as
387 well among the most significant interactions, when investigating cell-cell communication
388 across all cell types and both sites (Supplementary Fig. 6a and 6b). Further, we found that *SPP1*
389 is mainly expressed by MG rather than glioma cells, contrary to previous reports [61] (Fig. 6c,
390 Supplementary Fig.6c and 6d). MG SPP1 conveys different interactions, depending on the re-
391 cipient cell binding receptor expression profile. NK cells could interact with SPP1 mainly via
392 the integrin complex *ITGA4-ITGB1* (CD49d-CD29) (Fig. 6c), mediating NK cell adhesion and
393 migration [62]. This might facilitate interaction of inhibitory NK receptors KLRB1 and
394 CD94/NKG2A with MG C-type lectin-related ligands and HLA-E, respectively, which could
395 explain the observed impaired activation state of peripheral NK cells.
396 $CD4^+$ and $CD8^+$ T cells exhibited strong interactions with MG *SPP1* as well (Fig. 6b).
397 However, cell-cell communication between MG and T lymphocytes could be mainly mediated
398 via SPP1/CD44 interaction (Fig. 6a and 6c), a ligand-receptor axis recently described to sup-
399 press T cell activation and proliferation [63]. Altogether, cell-cell interaction analysis pointed
400 towards an impaired activation signature in the peripheral glioma-associated immune cells and
401 revealed potentially involved signaling pathways.

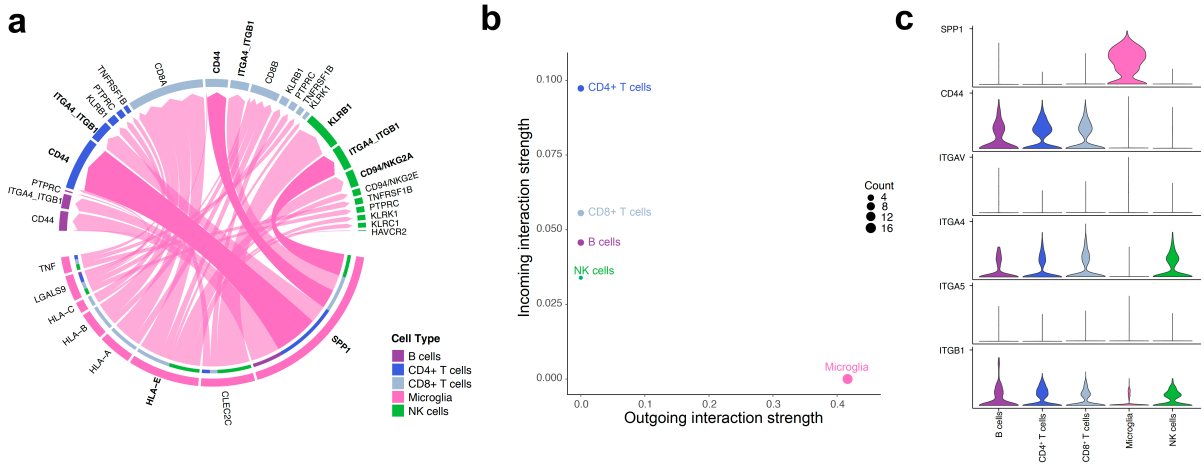

Fig. 6 Cell-cell communication analysis using CellChat reveals critical role for SPP1-mediated crosstalk in tumor periphery. a Chord diagram showing significant interactions from microglia to lymphocyte cell clusters. The inner bar colors represent the targets that receive signal from the corresponding outer bar. The inner bar size is proportional to the signal strength received by the targets. Chords indicate ligand-receptor pairs mediating interaction between two cell clusters, size of chords is proportional to signal strength of the given ligand-receptor pair. **b** Comparison of incoming and outgoing interaction strength allows identification of main senders and receivers. **c** Violin plots showing the expression distribution of signaling genes involved in the inferred SPP1 signaling network.

413 **Discussion**

414 In this study, we combined single-cell RNA sequencing and flow cytometry-based pro-
415 teome analysis to interrogate the regional leukocyte activation signature in patient-matched bi-
416 opsies from contrast-enhancing tumor center, infiltrative peripheral rim, and blood PBMCs of
417 grade 4 glioma patients. Our analyses revealed a distinct, regionally dependent transcriptional
418 profile for most of the investigated cell populations. While peripheral MG and cytotoxic cells
419 predominantly displayed an impaired activation signature, MdMs showed pro-inflammatory
420 traits in the tumor periphery, however, were less abundant there compared to the tumor center,
421 which was reported by others as well [9, 10]. Supplemented with transcriptional and surface
422 proteome analysis of paired PBMC samples, we provide an in-depth characterization of the
423 three main immunological compartments of grade 4 glioma.

424 Previous studies focused on the description of the TME of grade 4 glioma, which also
425 considered regional differences, yet they focused primarily at neoplastic cells rather than the
426 immune compartment [9]. Others investigated the differences in the iTME composition be-
427 tween primary and metastatic brain tumors [5, 6]. Interestingly, the two latter ones reported
428 differences in the iTME composition between $IDH1^{wt}$ and $IDH1^{mut}$ glioma, which we did not
429 observe in our transcriptional data. Of note, both authors included low-grade and even pre-
430 treated recurrent glioma patients into the $IDH1^{mut}$ group, representing a quite heterogenous pa-
431 tient cohort. In this study, we aimed at providing a representative selection of primary, treat-
432 ment-naive grade 4 glioma patients including $IDH1^{wt}$ and $IDH1^{mut}$. Surprisingly, we only found
433 negligible transcriptional iTME differences among these two groups.

434 We identified a transcriptionally distinct MG subcluster, MG_1, which displayed an
435 anti-inflammatory/non-reactive phenotype. A similar MG subpopulation expressing a compa-
436 rable gene signature has been recently described to be enriched in Alzheimer's disease patients
437 [64]. Additionally, the peripheral cytotoxic cell compartment exhibited an impaired activation
438 state, including a downregulated IFN response signature in $CD8^+$ T cells. Induction of an IFN
439 response state has been described as a consequence of T cell receptor-mediated IFN-γ produc-
440 tion, likely serving as an autocrine response and inducing the proliferative program [46]. Hence,
441 the reduced autocrine IFN-responsive state in the tumor peripheral $CD8^+$ T cells, together with
442 downregulated proliferative and co-stimulatory genes emphasized their impaired activation in
443 the peripheral infiltration zone.

444 By exploring the transcriptional trajectory of $CD8^+$ T cells from the blood circulation
445 into the immunosuppressive TME of the tumor periphery, we uncovered $CX3CR1^{high}$ and
446 $CX3CR1^{int}$ effector and memory $CD8^+$ T cells, respectively, to be highly enriched in the PBMC,

447 but absent in the iTME. Recently, adoptive transfer studies of CX3CR1$^+$ CD8$^+$ T cells in a mel-
448 anoma mouse model significantly suppressed tumor growth [65]. Others identified increased
449 frequencies of CX3CR1$^+$ CD8$^+$ T cells in non-small cell lung and melanoma patients who re-
450 sponded to anti-PD-1 therapy, where these cells exhibited migratory capabilities into the tumor
451 site followed by potent tumor rejection [65, 66]. Thus, the authors proposed T cell CX3CR1
452 expression as a predictor of response to ICI therapy. Therefore, the absence of ICI therapy-
453 responsive CD8$^+$ T cells in the glioma TME could additionally explain the disappointing out-
454 comes of clinical trials using ICI in glioma patients.

455 The observed T_{rm} exhaustion phenotype of the glioma residing CD8$^+$ T cells was re-
456 cently reported as well for tumor-infiltrating PD-1^{high} CD8$^+$ T cells in hepatocellular carcinoma
457 [54]. Whether these glioma-associated CD8$^+$ T cells really possess tumor-specificity requires
458 further study. Particularly in the light of a recent study by Smolders and colleagues who re-
459 ported a consistent brain-resident CD8$^+$ T cell population in a miscellaneous autopsy cohort of
460 patients with neurological disorders excluding brain malignancies (Alzheimer's disease, Par-
461 kinson's disease, dementia, depression, multiple sclerosis), as well as patients with no known
462 brain disease. These brain-resident CD8$^+$ T cells displayed a remarkably consistent T_{rm} pheno-
463 type [67]. The authors further showed high expression of inhibitory receptors CTLA-4 and PD-
464 1 on the brain-resident CD8$^+$ T_{rm} cells, which is in line with the core phenotypic signature of
465 T_{rm} cells from other tissues [68, 69]. Yet, the brain CD8$^+$ T_{rm} cells showed a preserved inflam-
466 matory potential with substantial production of IFN-γ and TNF-α upon *ex vivo* stimulation.
467 They concluded that extensive immune activation with release of highly neurotoxic lytic en-
468 zymes, such as perforin and granzyme B, harmfully impacts the brain parenchyma and should
469 be tightly controlled, whilst maintaining the capability to elicit a fast inflammatory response
470 when a neurotropic virus threatens the CNS [67]. Therefore, inhibitory receptors like PD-1 and
471 CTLA-4 on brain CD8$^+$ T_{rm} cells may support CNS homeostasis by preventing uncontrolled T
472 cell reactivity, and the availability of the receptor ligands may determine their inhibitory effect.
473 While this may represent a well-balanced equilibrium under healthy conditions, the tumor set-
474 ting leads to its disruption with upregulation of inhibitory ligands like PD-L1 on glioma cells
475 and CD86 on GAMs, leading to the dysfunctional state seen in the glioma-residing CD8$^+$ T_{rm}
476 cells.

477 Another study comprehensively showed, that peripheral infections generate antigen-
478 specific CD8$^+$ T_{rm} cells in the brain, mediating protection against CNS infections [52]. These
479 data could implicate that the glioma-associated CD8$^+$ T cells are devoid of tumor-specific reac-
480 tivity, but rather represent a pre-existing T cell population generated after peripheral infections,

481 which acquired a dysfunctional state upon glioma formation. To test this hypothesis, further
482 characterization of these cells is required, including analysis of T cell receptor clonality and
483 tumor-specificity by patient-matched T cell/glioma-sphere co-culture assays.

484 Lastly, our cell-cell interaction analysis revealed signaling pathways between peripheral
485 MG and lymphocytes potentially inducing the observed impaired activation signature. In fact,
486 interaction of NK cell receptor *KLRB1* (CD161) with its C-type lectin-related ligand has been
487 identified lately as a candidate inhibitory receptor on glioma-infiltrating T cells [70] and
488 SPP1/CD44 interaction has been described to suppress T cell activation and proliferation [63].

489 Limitations of the study include the limited patient number, thereby our study was nei-
490 ther designed nor powered to explore differences in neoplastic cells, given the high inter- and
491 intra-patient variability in glioma cells [9]. Importantly, our dataset establishes a starting point
492 for further interrogation and provides an in-depth analysis of the transcriptional landscape of
493 the major immune populations in grade 4 glioma within three important regional compartments.
494 Further, we confirmed the observed phenotype of $CD8^+$ T cells in the blood and tumor periphery
495 by flow cytometry in a cohort of ten additional patients, addressing possible generalization
496 concerns. Together, we provide a novel reference map of leukocyte activation in the TME and
497 blood circulation from grade 4 glioma patients, helping the research community to uncover
498 novel therapeutic strategies to combat this fatal disease.

499 **Methods**

500 *Ethics statement*

501 Human adult GBM tissue samples were obtained at the Neurosurgical Clinic of the Uni-
502 versity Hospital of Basel, Switzerland in accordance with the Swiss Human Research Act and
503 institutional ethics commission (EKNZ 02019-02358). All patients gave written informed con-
504 sent for tumor biopsy collection and signed a declaration permitting the use of their biopsy
505 specimens in scientific research, including storage in our brain tumor biobank (Req-2019-
506 00553). All patient identifying information was removed and tissue was coded for identifica-
507 tion.

508

509 *Glioma tissue dissociation*

510 Resected glioma tissue samples were immediately placed on ice and transferred to the
511 laboratory for single cell dissociation within 2-3 h after resection. Human brain tissue was man-
512 ually minced using razor blades and enzymatically dissociated at 37°C for 30 minutes with 1
513 mg/ml collagenase-4 (#LS004188, Worthington Biochemical Corporation, USA) and 250 U/ml
514 DNAse1 (#10104159001, Roche, Switzerland) in a buffer containing Hank's Balanced Salt
515 Solution (HBSS) with Ca^{2+}/Mg^{2+}, 1% MEM non-essential amino acids (Gibco, USA), 1 mM
516 sodium pyruvate (Gibco), 44 mM sodium bi-carbonate (Gibco), 25 mM HEPES (Gibco), 1%
517 GlutaMAX (Gibco) and 1% antibiotic-antimycotic (Sigma-Aldrich, USA). Cells were filtered
518 and separated from dead cells, debris and myelin by a 0.9 M sucrose (#84100, Simga Aldrich)
519 density gradient centrifugation. Upon ACK-lysis for removal of erythrocytes (#A1049201,
520 Gibco) the now generated single-cell suspension (SCS) was washed, counted and frozen in
521 Bambanker (#BB01, Nippon Genetics, Germany) in liquid nitrogen until use.

522

523 *PBMCs (Peripheral blood mononuclear cells) preparation*

524 Patient blood samples were directly placed on ice and transferred to the laboratory for
525 PBMC isolation. Blood samples were centrifuged to separate buffy coat from plasma and eryth-
526 rocytes, followed by standard density gradient centrifugation protocol (#17144002, Ficoll-
527 Paque PLUS, Cytiva, USA) to isolate PBMCs. PBMCs were frozen in Bambanker (#BB01,
528 Nippon Genetics, Germany) in liquid nitrogen until use.

529

530 *FACS sorting for single cell RNA sequencing (scRNA-seq)*

531 Cryopreserved tumor digests from glioma samples (center and periphery), as well as
532 autologous PBMCs were thawed and washed with excess ice-cold 1xPBS and spun down at

533 350xg for 5 min. Subsequently, the cells were stained with Live/Dead (APC-Cy7 (Near IR), #
534 L34976, Thermo Fischer) and a cocktail of fluorescently-conjugated antibodies CD11b (FITC,
535 clone M1/70, #101206, BioLegend) and CD45 (FITC, clone 2D1, #368508, BioLegend), and
536 large debris were removed with a 40-µm strainer. All samples were acquired on the BD FACS
537 ARIA Fusion III (Becton Dickinson GmbH, Germany). For single-cell RNA-seq experiments,
538 live and single gated cells were sorted into non-immune cell ($CD45^{-}CD11b^{-}$) and immune cell
539 ($CD45^{+}CD11b^{+}$) populations. Both populations were directly sorted into Eppendorf tubes with
540 1xPBS supplemented with 1% BSA for single cell RNA sequencing.
541
542 *Single cell RNA sequencing (scRNA-seq) – Library preparation and sequencing*
543 Single-cell RNA-seq was performed using Chromium Single Cell 3' GEM, Library &
544 Gel Bead Kit v3 (#CG000183, 10x Genomics, Pleasanton, CA, USA) following the manufac-
545 turer's protocol. Briefly, non-immune cells and immune cells were mixed at a defined ratio of
546 1:4. Roughly 8000-10000 cells per sample, diluted at a density of 100–800 cells/µL in PBS plus
547 1% BSA determined by Cellometer Auto 2000 Cell Viability Counter (Nexelom Bioscience,
548 Lawrence, MA USA), and were loaded onto the chip. The quality and concentration of both
549 cDNA and libraries were assessed using an Agilent BioAnalyzer with High Sensitivity kit
550 (#5067-4626, Agilent, Santa Clara, CA USA) and Qubit Fluorometer with dsDNA HS assay
551 kit (#Q33230, Thermo Fischer Scientific, Waltham, MA USA) according to the manufacturer's
552 recommendation. For sequencing, samples were mixed in equimolar fashion and sequenced on
553 an Illumina HiSeq 4000 with a targeted read depth of 50,000 reads/cell and sequencing param-
554 eters were set for Read1 (28 cycles), Index1 (8 cycles), and Read2 (91 cycles).
555
556 *Single cell RNA sequencing (scRNA-seq) - Computational analysis*
557 The dataset was analyzed by the Bioinformatics Core Facility, Department of Biomed-
558 icine, University of Basel. Read quality was controlled with the FastQC tool (version 0.11.5).
559 Sequencing files were processed using the Salmon Alevin tool (v 1.3.0) [71] to perform quality
560 control, sample demultiplexing, cell barcode processing, pseudo-alignment of cDNA reads to
561 the human Gencode v35 reference and counting of UMIs. Parameters *--keepCBFraction 1* and
562 *--maxNumBarcodes 100000* were used.
563 Processing of the UMI counts matrix was performed using the Bioconductor packages
564 DropletUtils (version 1.8.0) [72, 73], scran (version 1.16.0) [74, 75] and scater (version 1.16.2)
565 [76], following mostly the steps illustrated in the OSCA book (http://bioconduc-
566 tor.org/books/release/OSCA/) [75, 77]. Filtering for high-quality cells was done based on

567 library size (at least 2,000 UMI counts per cell), the number of detected genes (at least 700
568 genes detected) and the percentage of reads mapping to mitochondrial genes (larger than 0%
569 and lower than 15%), based on the distribution observed across cells. Low-abundance genes
570 with average counts per cell lower than 0.006 were filtered out. The presence of doublet cells
571 was investigated with the scDblFinder package (version 1.2.0), and suspicious cells were fil-
572 tered out (score > 0.6). After quality filtering, the resulting dataset consisted of UMI counts for
573 15,523 genes and 45,466 cells, ranging from 803 to 9,121 per sample.
574 UMI counts were normalized with size factors estimated from pools of cells created
575 with the scran package *quickCluster()* function [74, 78]. To distinguish between genuine bio-
576 logical variability and technical noise we modeled the variance of the log-expression across
577 genes using a Poisson-based mean-variance trend. The scran package *denoisePCA()* function
578 was used denoise log-expression data by removing principal components corresponding to tech-
579 nical noise. A t-stochastic neighbor embedding (t-SNE) was built with a perplexity of 50 using
580 the top most variable genes (141 genes with estimated biological variance > 0.3, excluding
581 genes with highest proportion of reads in the ambient RNA pool estimated from empty drop-
582 lets), and the denoised principal components as input (5 top PCs). Clustering of cells was per-
583 formed with hierarchical clustering on the Euclidean distances between cells (with Ward's cri-
584 terion to minimize the total variance within each cluster [79]; package cluster version 2.1.0).
585 The number of clusters used for following analyses was identified by applying a dynamic tree
586 cut (package dynamicTreeCut, version 1.63-1) [80], resulting in 10, or 22 clusters with argu-
587 ment *deepSplit* set to 2.
588 The Bioconductor package SingleR (version 1.2.4) was used for cell-type annotation of
589 the cells [81] using as references (i) a public bulk RNA-seq dataset of sorted immune cell types
590 from human PBMC samples [13], available through the *celldex* Bioconductor package; (ii) a
591 bulk RNA-seq dataset of sorted immune cell types from the tumor microenvironment of human
592 gliomas [5] (UMI count matrix and annotation downloaded from
593 https://joycelab.shinyapps.io/braintime/); (iii) a Smartseq2 scRNA-seq dataset of IDH-wild-
594 type glioblastoma tumors [14] (downloaded from GEO accession GSE131928). A microglia
595 and a macrophage signature scores were defined by averaging the center and scaled expression
596 levels of gene lists obtained in [7]. An endothelial score was defined by averaging the center
597 and scaled expression levels of the genes *CDH5*, *VWF*, *CD34* and *PECAM1*. The SingleR high-
598 quality assignments (pruned scores) and the signature scores were used to manually derive a
599 consensus cell type annotation for each cluster.

600 The *findMarkers* function of the scran package was used to find the best markers across
601 annotated cell types (parameters *direction="up"* and *pval.type="any"*). The top 10 markers
602 for each cell type were extracted and pooled to from a list of 68 markers.
603 Differential abundance analysis of identified cell types between tumor sites was per-
604 formed using *diffcyt-DA-voom* method [82]. Differential abundance of cell types was consid-
605 ered to be significant at a false discovery rate (FDR) lower than 5 %.
606 Differential expression between tumor sites, or between PBMC cells and tumor periph-
607 ery cells, stratified by annotated cell type, was performed using a pseudo-bulk approach, sum-
608 ming the UMI counts of cells from each cell type in each sample when at least 20 cells could
609 be aggregated. The aggregated samples were then treated as bulk RNA-seq samples [83] and
610 for each pairwise comparison genes were filtered to keep genes detected in at least 5% of the
611 cells aggregated. The package edgeR (version 3.30.3) [84] was used to perform TMM normal-
612 ization [85] and to test for differential expression with the Generalized Linear Model (GLM)
613 framework, using a model accounting for patient-specific effects. Genes with a FDR lower than
614 5 % were considered differentially expressed. Gene set enrichment analysis was performed with
615 the function camera [86] on gene sets from the Molecular Signature Database (MSigDB, ver-
616 sion 7.4) [39, 87]. We retained only sets containing more than 5 genes, and gene sets with a
617 FDR lower than 5% were considered as significant.
618
619 *Cell chat analysis*
620 The R package CellChat (1.1.3) [60] was used to analyze cell-cell interactions in our
621 dataset (with previously annotated 9 cell types). We followed the recommended workflow to
622 infer the cell state-specific communications (using *identifyOverExpressedGenes*, *identifyOver-*
623 *ExpressedInteractions* and *projectData* with the default parameters). We performed 3 separate
624 analyses, on the center and the periphery subsets and a comparison analysis as described in the
625 official workflow. We visualized the significant interactions for the microglia cluster using
626 *netVisual_chord_gene* and used *plotGeneExpression* to display of the expression of all genes
627 involved SPP1 signaling pathway in the cell populations. Finally, *netAnalysis_signalin-*
628 *gRole_scatter* was used to calculate and visualize incoming and outgoing signaling strength.
629
630 *Flow cytometry analysis of paired PBMC and periphery samples*
631 Cryopreserved samples were thawed and washed with excess ice-cold 1xPBS and spun
632 down at 350xg for 5 min. Cells were resuspended in FACS buffer (PBS plus 2% FBS) and
633 blocked with monoclonal antibody to CD16/32 (Human TruStain FcX, #422302, Biolegend)

634 for 10 min at 4°C before staining with surface antibodies: CD45RA (PE, clone HI100,
635 #304108), CD45RO (APC, clone UCHL1, #304210), CD3e (BV650, clone UCHT1, #300468),
636 CD8a (BV421, clone RPA-T8, #301036), CCR7 (FITC, clone G043H7, #353216), CD62L
637 (AF700, clone DREG-56, #304820), CD69 (APC-Cy7, clone FN50, #310914), CD103
638 (PerCP/Cy5.5, clone Ber-ACT8, #350226) and CX3CR1 (PE/Cy7, clone 2A9-1, #341612). All
639 antibodies were purchased from BioLegend, USA. Cells were stained for 30 min at 4°C, and
640 subsequently washed with FACS buffer. To exclude dead cells Zombie Aqua Fixable Viability
641 Kit (#423102, 1:100, BioLegend) was added. Acquisition was performed on a CytoFLEX
642 (Beckman). Data was analyzed using FlowJo software, version 10.8.1 (TreeStar). Gates were
643 drawn by using Fluorescent Minus One (FMO) controls.
644
645 *Statistical analysis of flow cytometry data*
646 Data analysis and graph generation was performed using GraphPad Prism 9 (GraphPad
647 Prism Software Inc.). Paired comparisons between two groups were performed using Wilcoxon
648 matched-pairs signed rank test. Differences of more than two paired groups were assessed using
649 repeated measures one-way ANOVA test, followed by post-hoc Šidák's multiple comparisons
650 correction. A p value < 0.05 was considered statistically significant. *$p \leq 0.05$, **$p \leq 0.01$, ***p
651 ≤ 0.001, ****$p \leq 0.0001$.

652 **Data availability**

653 The UMI count matrix and cell metadata from the scRNA-seq dataset are available on
654 GEO under accession number GSE197543. The remaining data are available within the Article
655 and Supplementary Information.

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Author contribution

G.H., S. Herter, M.B., S.D. and P.S., conceived and planned the project. J.R. and S. Hogan analyzed the scRNA-sequencing data and performed statistical analysis. P.S. and S.D., performed experiments and interpreted the results. T.S., T.A.M. and M.-F.R. helped coordinating experiments. P.S. wrote the manuscript. G.H. supervised and coordinated the study and critically revised the manuscript. All authors reviewed the paper and approved its final version.

Competing interests

G.H. has equity in, and is a cofounder of Incephalo Inc.

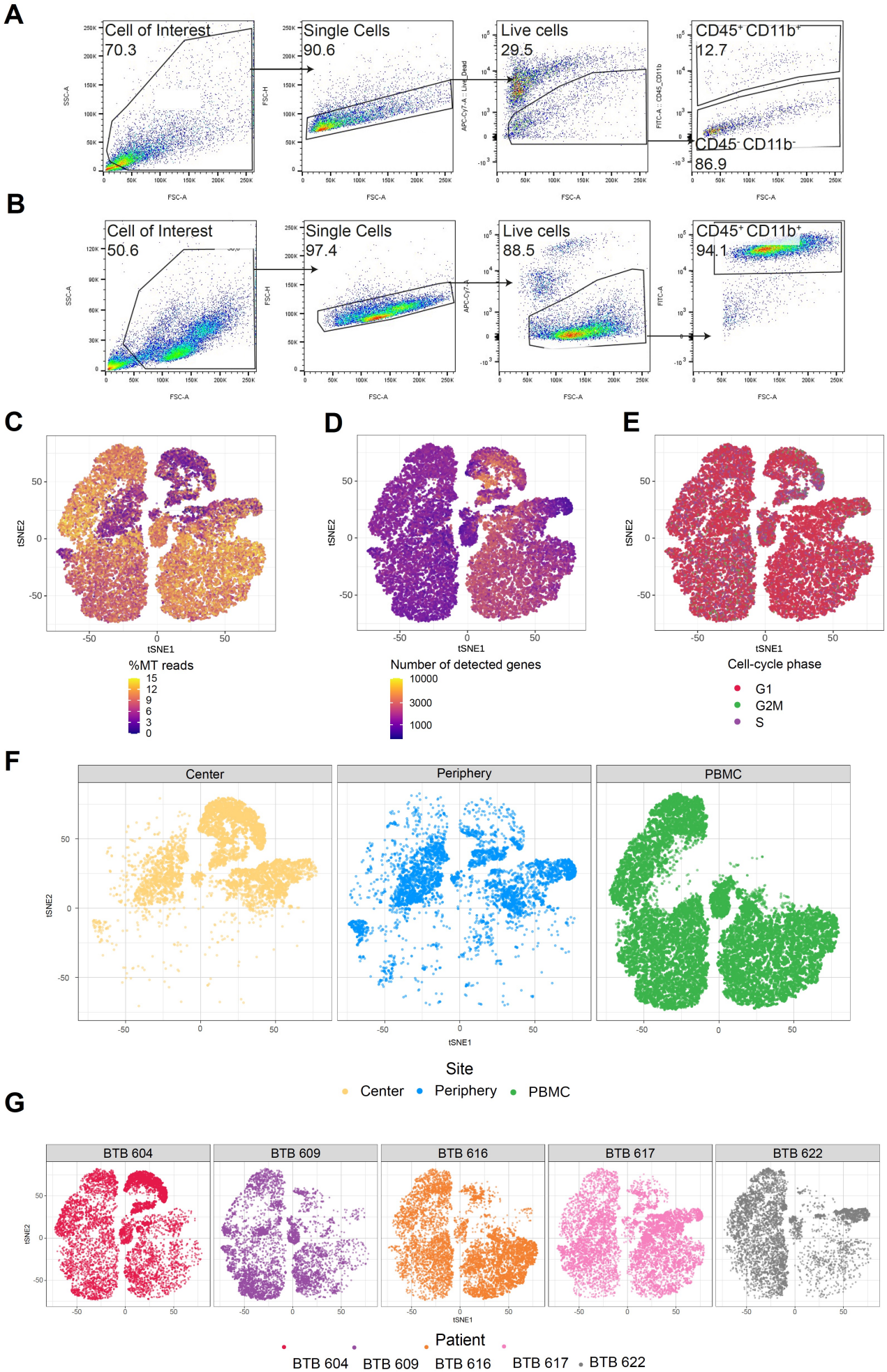

A
Cell of Interest
70.3
Single Cells
90.6
Live cells
29.5
CD45+ CD11b+
12.7
CD45- CD11b-
86.9
B
Cell of Interest
50.6
Single Cells
97.4
Live cells
88.5
CD45+ CD11b+
94.1
C
D
E
tSNE1
tSNE2
%MT reads
Number of detected genes
Cell-cycle phase
G1
G2M
S
F
Center
Periphery
PBMC
Site
Center
Periphery
PBMC
G
BTB 604
BTB 609
BTB 616
BTB 617
BTB 622
Patient
BTB 604
BTB 609
BTB 616
BTB 617
BTB 622

Figure S1. $CD45^+$ $CD11b^+$ immune cells gating strategy and quality control of scRNA-seq data, related to Figures 1 and 2.

(A and B) Gating strategy for paired tumor-derived (A) and PBMCs (B); after debris, doublet and dead cell removal, immune cells were assessed as $CD45^+$ and/or $CD11b^+$.

(C-E) Percentage of mitochondrial (MT) reads (C) number of detected genes (D) and cell-cycle phase (E) overlaid on tSNE representation.

(F and G) tSNE map stratified according to site (F) and patient (G).

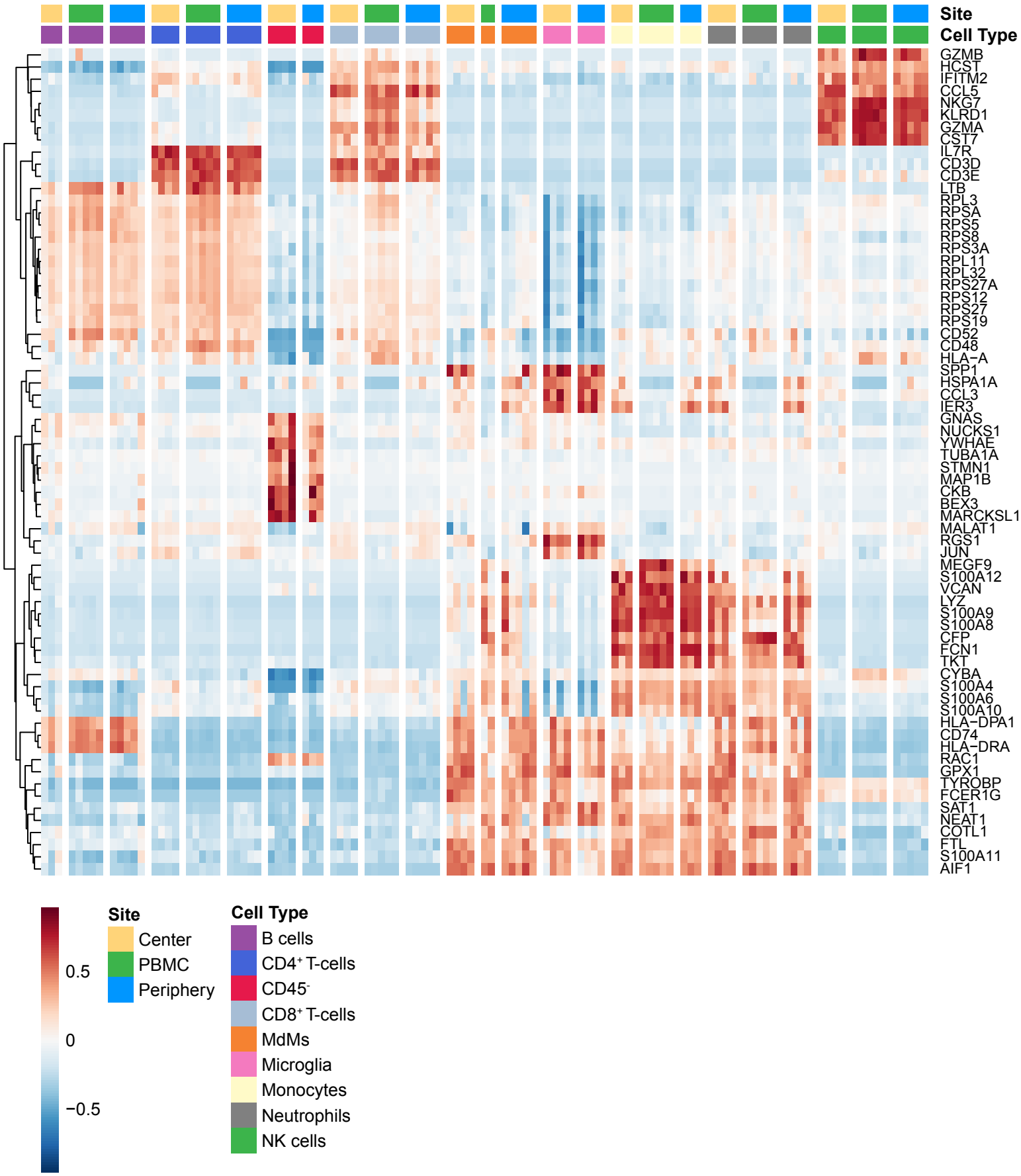

Site
Cell Type
GZMB
HCST
IFITM2
CCL5
NKG7
KLRD1
GZMA
CST7
IL7R
CD3D
CD3E
LTB
RPL3
RPSA
RPS5
RPS8
RPS3A
RPL11
RPL32
RPS27A
RPS12
RPS27
RPS19
CD52
CD48
HLA−A
SPP1
HSPA1A
CCL3
IER3
GNAS
NUCKS1
YWHAE
TUBA1A
STMN1
MAP1B
CKB
BEX3
MARCKSL1
MALAT1
RGS1
JUN
MEGF9
S100A12
VCAN
LYZ
S100A9
S100A8
CFP
FCN1
TKT
CYBA
S100A4
S100A6
S100A10
HLA−DPA1
CD74
HLA−DRA
RAC1
GPX1
TYROBP
FCER1G
SAT1
NEAT1
COTL1
FTL
S100A11
AIF1
0.5
0
−0.5
Site
Center
PBMC
Periphery
Cell Type
B cells
CD4⁺ T-cells
CD45⁻
CD8⁺ T-cells
MdMs
Microglia
Monocytes
Neutrophils
NK cells

Figure S2. Cell type specific gene expression, related to Figure 2.

Heatmap displaying genes whose expression is most specific to each cell type. Columns are ordered by site and cell type, and rows show centered and scaled expression values, hierarchically clustered.

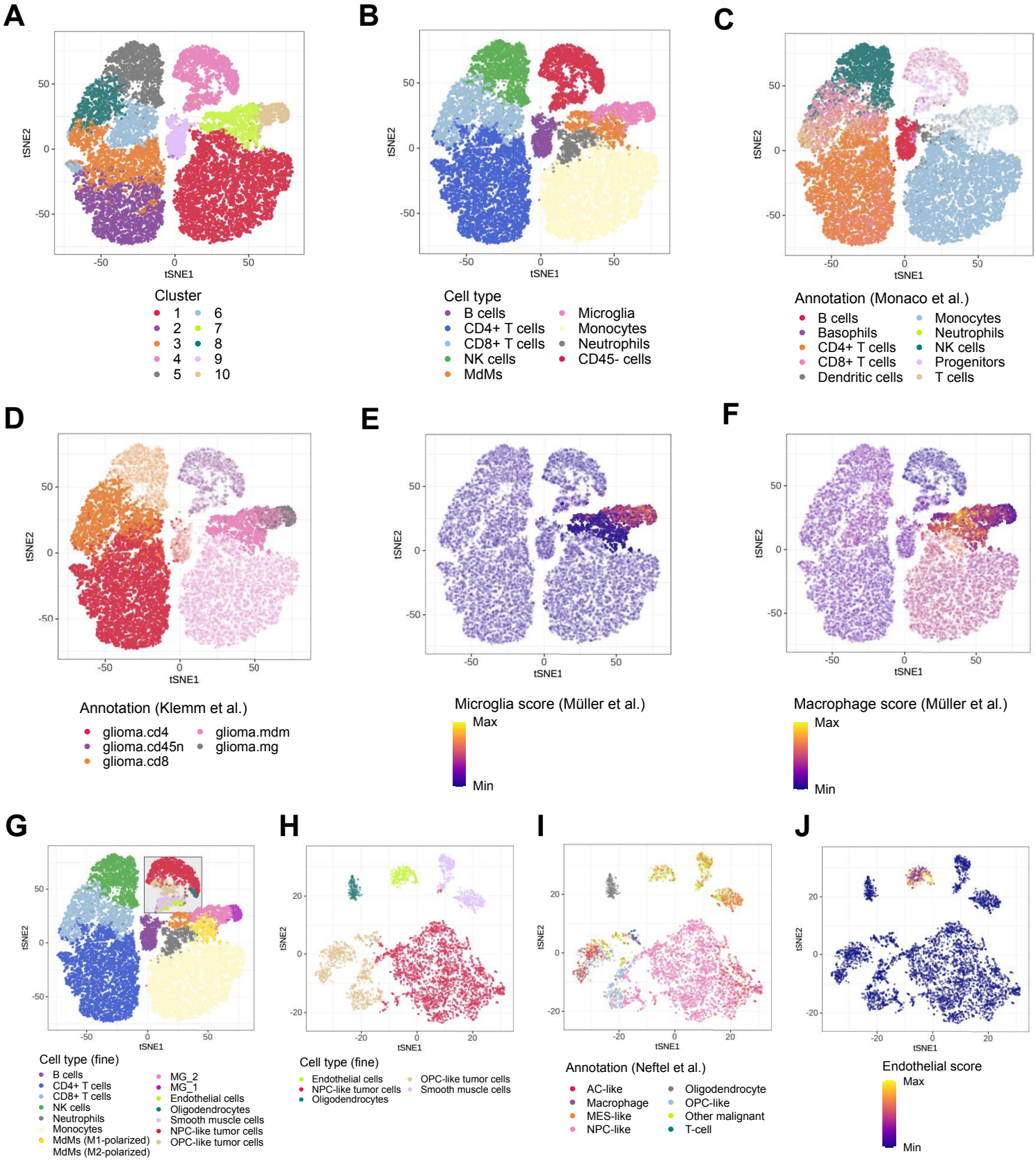

A
tSNE2
tSNE1
Cluster
1
2
3
4
5
6
7
8
9
10
B
Cell type
B cells
CD4+ T cells
CD8+ T cells
NK cells
MdMs
Microglia
Monocytes
Neutrophils
CD45- cells
C
Annotation (Monaco et al.)
B cells
Basophils
CD4+ T cells
CD8+ T cells
Dendritic cells
Monocytes
Neutrophils
NK cells
Progenitors
T cells
D
Annotation (Klemm et al.)
glioma.cd4
glioma.cd45n
glioma.cd8
glioma.mdm
glioma.mg
E
Microglia score (Müller et al.)
Max
Min
F
Macrophage score (Müller et al.)
Max
Min
G
Cell type (fine)
B cells
CD4+ T cells
CD8+ T cells
NK cells
Neutrophils
Monocytes
MdMs (M1-polarized)
MdMs (M2-polarized)
MG_2
MG_1
Endothelial cells
Oligodendrocytes
Smooth muscle cells
NPC-like tumor cells
OPC-like tumor cells
H
Cell type (fine)
Endothelial cells
NPC-like tumor cells
Oligodendrocytes
OPC-like tumor cells
Smooth muscle cells
I
Annotation (Neftel et al.)
AC-like
Macrophage
MES-like
NPC-like
Oligodendrocyte
OPC-like
Other malignant
T-cell
J
Endothelial score
Max
Min

Figure S3. Cross-referencing scRNA-seq data with published datasets, related to Figure 2.
(A, B, G and H) Using hierarchical clustering, identified cell clusters (A) which were then annotated into eight distinct cell types for the immune subset (B) and five cell types for the $CD45^{neg}$ subset (G and H). Grey panel in (G) zooms in on $CD45^{neg}$ subset and is shown in (H).
(C-F) Immune cell types were annotated by referencing to a dataset of bulk RNA-seq samples of sorted immune cell types from human PBMC (C) [1]; MdMs and microglia were annotated by comparing to a dataset of bulk RNA-seq samples of sorted immune cell types from the tumor microenvironment of human gliomas (D) [2] and by using signature scores defined from scRNA-seq of glioma TAMs (E and F) [3]. Clusters are highlighted which were annotated using each respective reference dataset.
(I) $CD45^{neg}$ cells were annotated by whole-transcriptome comparison to a scRNA-seq dataset of IDH1wt GBM [4].
(J) Endothelial score was defined by averaging the center and scaled expression levels of the genes CDH5, VWF, CD34 and PECAM1.

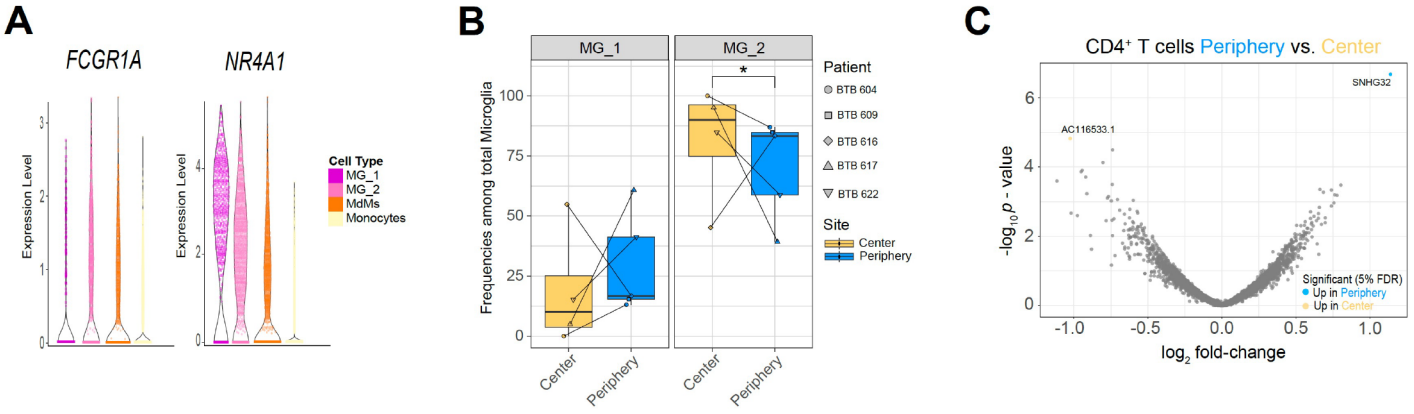

A
FCGR1A
NR4A1
Expression Level
Cell Type
MG_1
MG_2
MdMs
Monocytes
B
MG_1
MG_2
Frequencies among total Microglia
Patient
BTB 604
BTB 609
BTB 616
BTB 617
BTB 622
Site
Center
Periphery
C
CD4+ T cells Periphery vs. Center
SNHG32
AC116533.1
-log10 p - value
log2 fold-change
Significant (5% FDR)
Up in Periphery
Up in Center

Figure S4. Differential abundance of MG subclusters and differential expression between center and peripheral $CD4^+$ T cells, related to Figures 3 and 4.

(A) Violin plot showing average expression levels of selected reactivity markers among mononuclear phagocyte populations.

(B) Frequencies of MG_1 and MG_2 subpopulations among total microglia between center and periphery. Symbols represent individual patients and paired samples are indicated by connecting lines. 3 of 4 (75%) paired samples showed an increased abundance of MG_1 cells in the tumor periphery and decreased frequency of MG_2 cells. Statistical significance was assessed by diffcyt-DA-voom method, *FDR corrected p value < 0.05.

(C) Volcano plot showing differentially expressed genes (FDR corrected p value < 0.05, indicated by blue and yellow) in CD4+ T cells from tumor periphery versus tumor center.

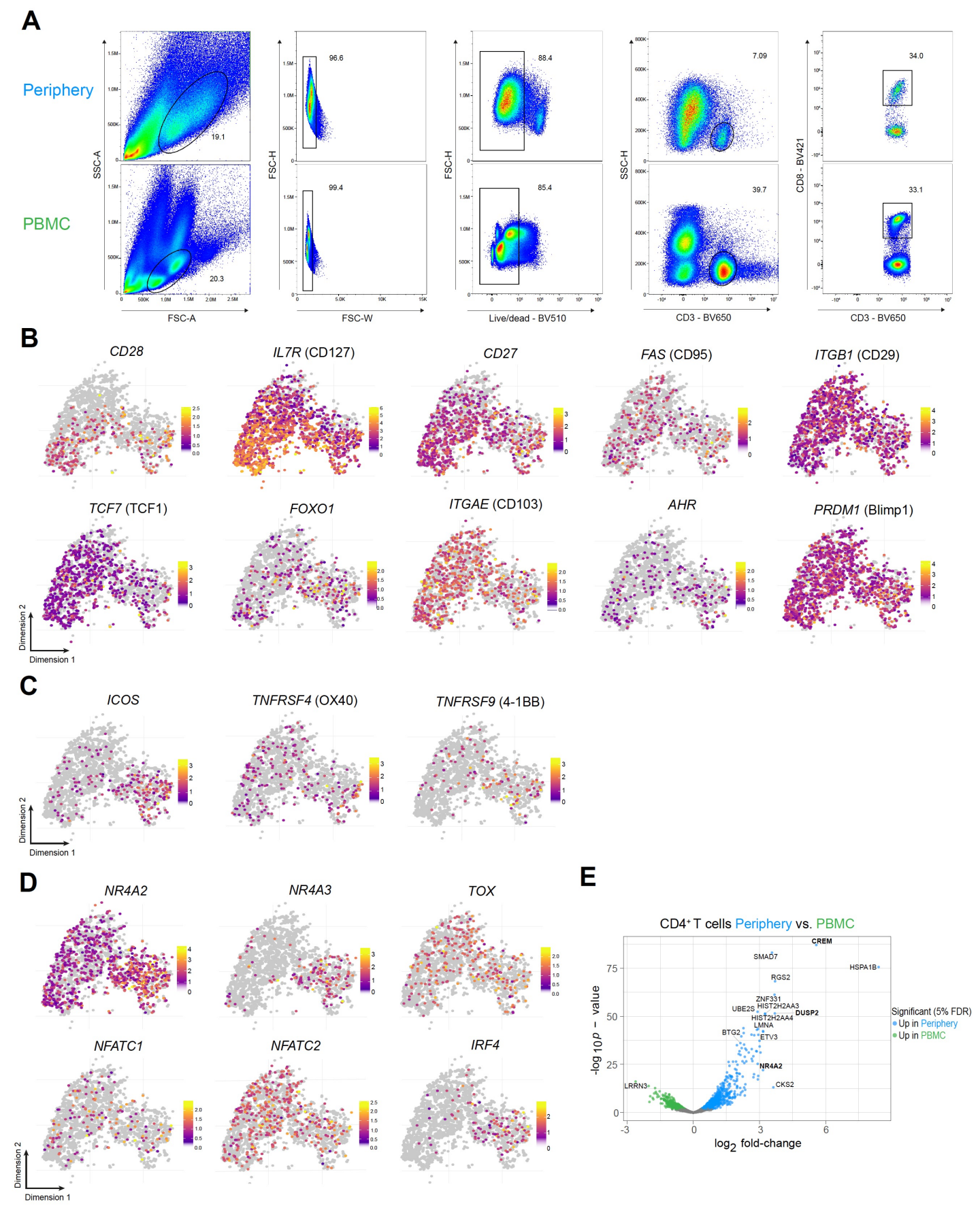
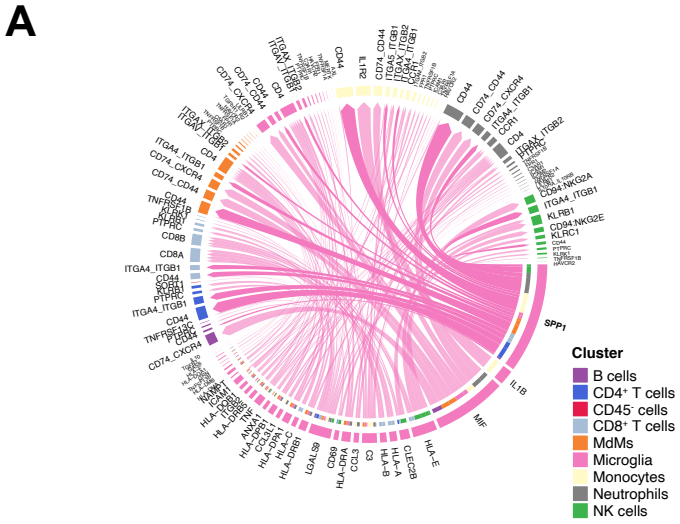

A
Periphery
PBMC
SSC-A
FSC-A
19.1
20.3
FSC-H
FSC-W
96.6
99.4
Live/dead - BV510
88.4
85.4
SSC-H
CD3 - BV650
7.09
39.7
CD8 - BV421
34.0
33.1
B
CD28
IL7R (CD127)
CD27
FAS (CD95)
ITGB1 (CD29)
TCF7 (TCF1)
FOXO1
ITGAE (CD103)
AHR
PRDM1 (Blimp1)
Dimension 1
Dimension 2
C
ICOS
TNFRSF4 (OX40)
TNFRSF9 (4-1BB)
D
NR4A2
NR4A3
TOX
NFATC1
NFATC2
IRF4
E
CD4+ T cells Periphery vs. PBMC
-log 10p − value
log2 fold-change
CREM
SMAD7
HSPA1B
RGS2
ZNF331
HIST2H2AA3
UBE2S
DUSP2
HIST2H2AA4
LMNA
BTG2
ETV3
NR4A2
CKS2
LRRN3
Significant (5% FDR)
Up in Periphery
Up in PBMC

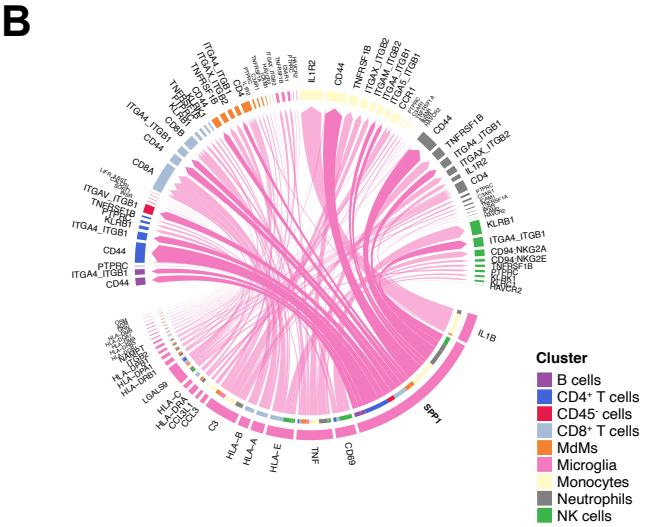
Figure S5. CD8$^+$ T cell phenotype is site-specific, related to Figure 5.

(A) Gating strategy for paired tumor-periphery and PBMC cells; after debris, doublet and dead cell removal, CD8+ T cells were identified as CD3+ CD8+ events.

(B-D) Single-cell expression of markers associated with naive/memory (CD28, IL7R, CD27, FAS, CD29, TCF, FOXO1) and tissue-resident memory (ITGAE, AHR, PRDM1) (B) T cell co-stimulation (C) and T cell exhaustion/dysfunction (D) overlaid on tSNE CD8+ T cell cluster.

(E) Volcano plot showing differentially expressed genes (FDR corrected p value < 0.05, indicated by blue and green) in CD4+ T cells from tumor-periphery versus PBMC.


A
Cluster
B cells
CD4⁺ T cells
CD45⁻ cells
CD8⁺ T cells
MdMs
Microglia
Monocytes
Neutrophils
NK cells
SPP1
IL1B
MIF
HLA-E
CLEC2B
HLA-A
HLA-B
C3
CCL3
HLA-DRA
CD69
LGALS9
HLA-DRB1
HLA-C
HLA-DPA1
HLA-DPB1
CCR1
ANXA1
TNF
CD74_CXCR4
CD74_CD44
CD44
IL1R2
ITGAV_ITGB1
ITGAV_ITGB8
ITGA4_ITGB1
CD8A
CD8B
PTPRC
TNFRSF1B
KLRB1
CD94:NKG2A
KLRC1
CD94:NKG2E
ITGA5_ITGB1
ITGAX_ITGB2
ITGAX_ITGB1
CD4


B
Cluster
B cells
CD4⁺ T cells
CD45⁻ cells
CD8⁺ T cells
MdMs
Microglia
Monocytes
Neutrophils
NK cells
SPP1
IL1B
TNF
CD69
HLA-E
HLA-A
HLA-B
C3
CCL3
CCL4
HLA-DRB1
HLA-DRA
HLA-DPA1
HLA-DPB1
LGALS9
CD44
IL1R2
TNFRSF1B
ITGAX_ITGB2
ITGA4_ITGB1
ITGAX_ITGB1
CCR1
CD4
KLRB1
CD94:NKG2A
CD94:NKG2E
ITGA4_ITGB1
CD8A
CD8B
ITGAV_ITGB1
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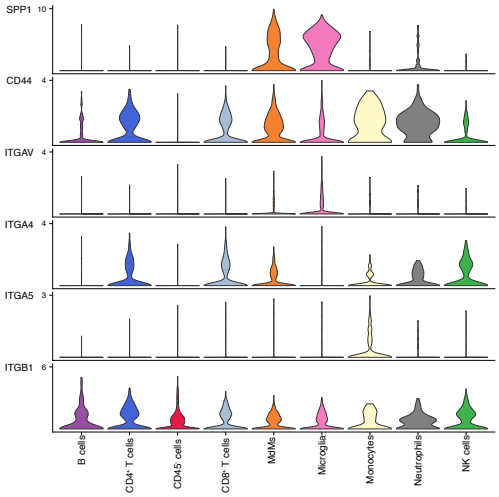
ITGA4_ITGB1
CD44
PTPRC
ITGA4_ITGB1
CD44


C
SPP1
CD44
ITGAV
ITGA4
ITGA5
ITGB1
B cells
CD4⁺ T cells
CD45⁻ cells
CD8⁺ T cells
MdMs
Microglia
Monocytes
Neutrophils
NK cells

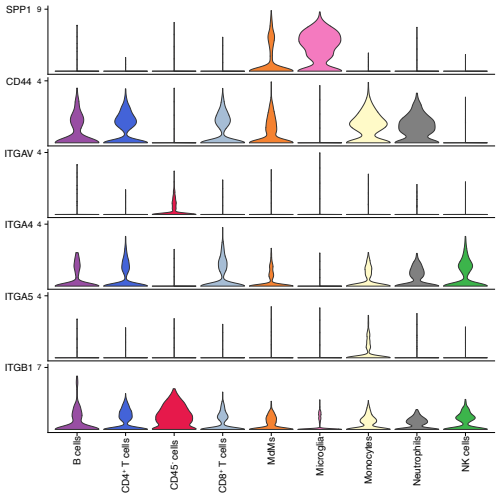

D
SPP1
CD44
ITGAV
ITGA4
ITGA5
ITGB1
B cells
CD4⁺ T cells
CD45⁻ cells
CD8⁺ T cells
MdMs
Microglia
Monocytes
Neutrophils
NK cells

Figure S6. Cell-cell communication analysis using CellChat, related to Figure 6.

(A and B) Chord diagram showing significant interactions from microglia to all cell clusters in center (A) and periphery (B). The inner bar colors represent the targets that receive signal from the corresponding outer bar. The inner bar size is proportional to the signal strength received by the targets. Chords indicate ligand-receptor pairs mediating interaction between two cell clusters, size of chords is proportional to signal strength of the given ligand-receptor pair.

(C and D) Violin plots showing the expression distribution of signaling genes involved in the inferred SPP1 signaling network in center (C) and periphery (D).

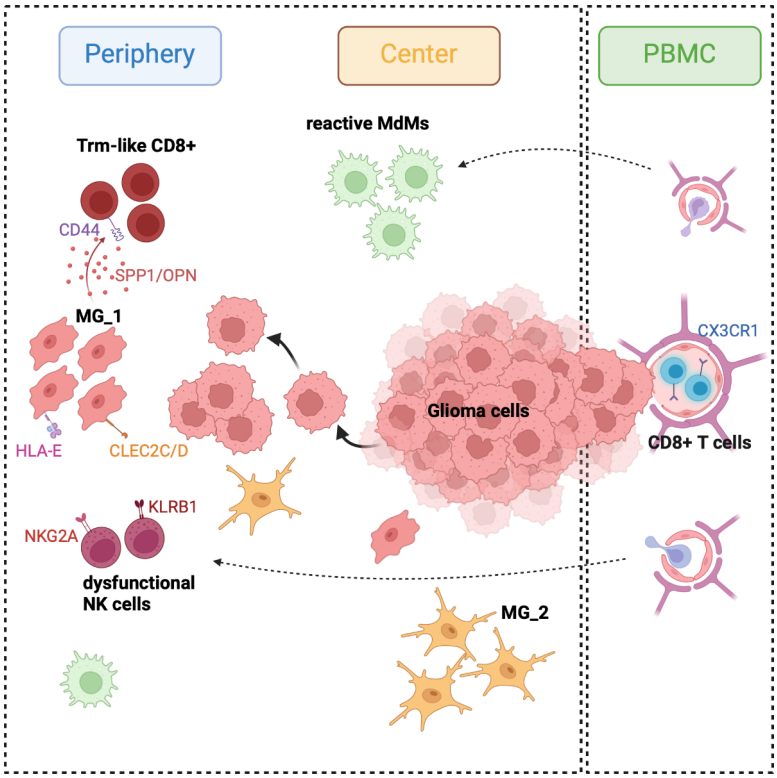

Periphery
Center
PBMC
reactive MdMs
Trm-like CD8+
CD44
SPP1/OPN
MG_1
HLA-E
CLEC2C/D
Glioma cells
CX3CR1
CD8+ T cells
KLRB1
NKG2A
dysfunctional
NK cells
MG_2

Figure S7. Graphical abstract. Proposed schematic of grade 4 glioma-associated immune cells in the three regional compartments. Created with BioRender.com.

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