

1 **Immune cell regulation in stem cell niche contacting glioblastomas.**

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41 **Abstract**

42 Glioblastomas (GBM) are tumors for which immune-targeted therapies have failed to show
43 clinical benefit and for which few biomarkers provide context for meaningful therapeutic
44 stratification. Radiographic contact of GBM tumors with the lateral ventricle stem cell niche
45 correlates with worse patient prognosis; however, the extent to which proximity to the
46 ventricle impacts antitumor immunity remains unknown. We demonstrate that T cell
47 checkpoint receptor expression is elevated in ventricle-contacting GBM as is the
48 abundance of a specific, suppressive CD32⁺CD44⁺HLAD^{high} myeloid population suggesting
49 a distinct immunoregulatory influence on antitumor immunity in proximity to the lateral
50 ventricle. Phospho-specific mass cytometric profiling revealed extensively impaired immune
51 signaling in ventricle-contacting GBM in response to inflammatory cytokine stimulation,
52 further supporting a suppressive milieu influencing immunity at the lateral ventricle.
53 Collectively, we identify a regulatory impact of ventricle contact on antitumor immunity in
54 the brain, and reveal novel clinically targetable mechanisms of immunomodulation in
55 patients with glioblastoma.

56 **Keywords:** glioblastoma, tumor immune microenvironment, checkpoint receptors, mass
57 cytometry, lateral ventricle.

58 **Significance Statement:** We demonstrate that the immune microenvironment of
59 glioblastoma tumors contacting the lateral ventricle differs from non-contacting tumors. This
60 work connects immune-biology to a radiographically detectable feature, the lateral ventricle,
61 and highlights non-invasive imaging as a means to identify targetable immune features in
62 glioblastoma tumors.

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65 Introduction

66 Glioblastoma (GBM) is the most common primary brain tumor, accounting for up to
67 60% of all brain tumors (1). Despite standard-of-care chemo/radiotherapy, median overall
68 survival remains 15-months post diagnosis (2). While efforts have been made to
69 phenotypically and molecularly characterize GBM tumors and illuminate the surrounding
70 stromal elements impacting gliomagenesis, few cell-intrinsic factors beyond isocitrate
71 dehydrogenase (*IDH*) mutation and O-6-methylguanine-DNA methyltransferase (*MGMT*)
72 promoter methylation have proven effective at stratifying patient outcome and guiding
73 clinical care (3). Additional, less-invasive, risk-stratifying features are urgently needed to
74 make headway in understanding the complex cellular milieu in the tumor and advance
75 therapeutics in the clinic.

76 The majority of GBM tumors present in the cerebrum, and although GBM may arise
77 anywhere within the brain parenchyma, both adult and pediatric patients with primary high-
78 grade gliomas (HGG) that exhibit radiographic contact with the lateral ventricles (LV) have
79 worse prognosis (4,5). This effect is independent of other predictive factors, such as patient
80 age, performance status, or molecular characterization (6). While regional tumor position
81 stratifies prognosis, what factors proximal to the LV contribute to poor prognoses remains
82 unclear.

83 Immune cells comprise a major component of the tumor lesion and play a critical
84 role in controlling tumor growth in both solid and hematologic malignancies. The immune
85 microenvironment within the brain, however, is distinct from peripheral tissue and the
86 antitumor potential of leukocytes in this discrete environment remains poorly characterized
87 (7). Cytometric profiling has demonstrated regional differences in resident immune cell

88 phenotypes under homeostatic conditions, particularly in the LV (8); however, how regional
89 neuro-immunity is impacted in the context of primary tumor lesions in the ventricle remains
90 poorly understood.

91 In this study, we performed multi-dimensional single-cell mass cytometry on 25
92 primary human GBM tumors demonstrating radiographic contact with the lateral ventricle
93 (C-GBM) or located distally from the ventricle (NC-GBM) to comprehensively profile the
94 immune infiltration in tumors within each region. Supervised and unsupervised machine
95 learning approaches including clustering tools FlowSOM (9) and Root-mean-square
96 deviation (RMSD) (10), population identification via Marker Enrichment Modeling (MEM)
97 (11), and patient stratification using Citrus(12) and Risk Assessment Population
98 Identification (RAPID) (13) identified distinct immune subsets enriched in C-GBM and NC-
99 GBM that correlated with patient outcome. Moreover, several targetable immune receptors
100 were elevated in C-GBM tumors suggesting that an immunosuppressive environment lies
101 proximal to the LV. Lymphocytes and tissue-resident microglia were enriched in NC-GBM
102 tumors and correlated with more favorable outcome, whereas anti-inflammatory M2-like
103 monocyte-derived macrophages (MDMs) bearing a distinct $CD44^+CD32^+HLA-DR^+$
104 phenotype and exhausted $PD-1^+TIGIT^+$ T cells were enriched in C-GBM tumors.

105 Phospho-specific mass cytometry revealed broad T cell signaling defects and
106 differential use of myeloid signaling networks employed by C-GBM and NC-GBM
107 leukocytes in response to inflammatory cytokine stimulation. $CD32^+HLA-DR^+$ MDMs were
108 highly responsive to cytokines compared to their $CD32^-HLA-DR^-$ counterparts. Further,
109 STAT3 phosphorylation dominated signaling networks in C-GBM immune infiltrates
110 compared to NC-GBM infiltrates, highlighting a potential STAT3 driven immunosuppressive
111 mechanism in C-GBM tumors. This work highlights differing immune microenvironments

112 within MRI-defined regional tumor classes, suggests distinct, targetable mechanisms of
113 immune dysregulation in GBM tumors in relation to the lateral ventricle, and emphasizes
114 the potential for radiographic image-guided patient stratification methods to inform clinical
115 care.

116 **Results**

117 **1. Peripheral immune cells are abundant in lateral ventricle contacting and non-** 118 **contacting glioblastomas.**

119 We utilized high-dimensional mass cytometry to compare the immune composition
120 of 25 freshly resected glioblastoma tissues either contacting the lateral ventricle (C-GBM;
121 n=13) or distant from the ventricle (NC-GBM; n=12) (**Figure 1a**). All patients expressed the
122 wildtype *IDH1/2* variant. The median overall survival was 366 days (range: 57-1588 days),
123 typical of the disease. A full description of patient characteristics is given in
124 **Supplementary Table 1**. As in prior reports (4), ventricle contact was a predictive factor of
125 outcome. Resected tumor tissue was dissociated into single-cell suspensions and profiled
126 using a 33-marker panel to characterize 13 expert-identified infiltrating immune subsets
127 (**Figure 1b, Supplementary Table 2**).

128 We first compared the tumor immune composition to that of healthy donor peripheral
129 blood mononuclear cells (PBMCs) or resected epileptic brain tissue (**Figure 1c**). Consistent
130 with prior reports, T cell infiltrate in GBM, particularly the CD4 T cells, was similar to
131 epileptic brain, accounting for 11% and 6% of the total leukocyte infiltrate respectively.
132 Other lymphocyte populations—Tregs, CD4⁺CD8⁺ (DPT), CD4⁻CD8⁻ (DNT), natural killer
133 (NK) T cells, $\gamma\delta$ T cells, B cells and NK cells—each made up <5% of the leukocyte fraction
134 in the tumor. CD45^{low}CD64⁺ microglia were the most abundant leukocyte population in the

135 brain accounting for 36% of the immune fraction in GBM tumors. CD45^{high}CD14⁺ peripheral
136 monocyte-derived macrophages (MDMs) were the next most abundant leukocyte
137 population, accounting for 32% of leukocytes in glioma, compared to 4% in PBMC and 5%
138 in epileptic brain, consistent with an extensive peripheral myeloid infiltrate in glioblastomas
139 (14).

140 Considering that the extent of lymphocytic infiltration correlates with more favorable
141 outcomes in peripheral solid tumors (15), we hypothesized that total leukocyte abundance
142 in NC-GBM may drive favorable outcomes observed in this cohort. While the total leukocyte
143 abundance in C-GBM and NC-GBM was higher than epileptic brain, no significant
144 difference was found between the two patient cohorts, accounting for 23% (range: 0.47-
145 61.62%) and 20% (range: 0.38-77.87%) of the tumor mass respectively (**Figure 1d**).
146 Moreover, the ratio of lymphoid cells (T, B, NK) and phagocytes (MDM and microglia) was
147 not significantly different between C-GBM and NC-GBM, suggesting that neither total
148 leukocyte abundance nor bulk lymphocyte abundance fully accounts for differences in
149 survival outcome between C-GBM and NC-GBM patients. However, meaningful differences
150 in the frequency and phenotypes of specific lymphocyte and phagocyte populations were
151 evident in C-GBM and NC-GBM tumors. For instance, the abundance of CD3⁺ T cells was
152 elevated in NC-GBM compared to C-GBM or epileptic brain tissue (**Figure 1e**). By
153 overlaying leukocytes onto t-SNE axes, we identified distinct leukocyte populations in
154 PBMCs, epileptic brain, and GBM tissue. The phenotypes of macrophages in C-GBM and
155 NC-GBM tumors differed, with macrophage populations occupying distinct islands within
156 the t-SNE space (**Figure 1f**), suggesting that phenotypic differences in immune infiltration
157 correlated with ventricle contact status.

158 To further explore the extent of these cohort-level differences in immune abundance,
159 data from 19 GBM patients (9 C-GBM, 10 NC-GBM) was used to investigate the immune
160 composition of C-GBM and NC-GBM tumors. Live CD45⁺ cells were equally sampled from
161 each patient prior to plotting on common t-SNE axes using 33 measured dimensions
162 (**Figure 1g**). Differences in immune cell composition were evident when comparing the
163 distribution of C-GBM and NC-GBM samples across the common axes. FlowSOM
164 clustering on the t-SNE axes defined 38 phenotypically distinct immune cell clusters across
165 the entire patient cohort (**Figure 1g, Supplementary Figure 1a**). The majority of patients
166 were well represented, with between 2 and 17 patients contributing to each cluster
167 (**Supplementary Figure 1b**). Of the 38 clusters, 17 were statistically enriched in immune
168 cells from C-GBM patients, and 17 were enriched in cells from NC-GBM patients. Four
169 clusters (19, 26, 32, 34) were not statistically enriched in either cohort (**Figure 1g, Figure**
170 **1h, Supplementary Figure 1**). Taken together, these data highlight extensive differences
171 in the immune composition of GBM tumors and suggest that while immune infiltration
172 occurs in both C-GBM and NC-GBM, the relative composition of the immune cell fraction in
173 ventricle contacting tumors is distinct from that of ventricle non-contacting tumors.

174 **2. Five immune cell subsets are differentially enriched in ventricle contacting and** 175 **non-contacting glioblastomas.**

176 We next used machine learning to perform unbiased computational analysis of the
177 immune microenvironment of GBM tumors and identify the most distinct immune subsets
178 associated with C-GBM and NC-GBM tumors. Using the Citrus algorithm to compare
179 leukocyte phenotypes and frequencies between our two cohorts (12), we identified 10
180 immune clusters differentially enriched in C-GBM and NC-GBM tumors. Five terminal
181 clusters representing the most phenotypically distinct populations were selected for further

182 study (**Supplementary Figure 2a**). Three Citrus clusters were enriched in NC-GBM
183 (Clusters 1, 2, and 5), while 2 clusters (Clusters 3 and 4) were enriched in C-GBM (**Figure**
184 **2a**). The identities of these clusters inferred from Citrus were confirmed via traditional
185 biaxial gating and computational labeling using marker enrichment modeling (MEM) (11)
186 (**Figure 2b-f**). Cluster 1, enriched in NC tumors, consisted of $\gamma\delta$ T cells and $CD3^+CD4^-CD8^-$
187 T cells (DNT) previously identified in GBM (16). Cluster 2 was a population of resident
188 microglial cells characterized as $CD45^{low}CD64^+CD14^-HLA-DR^+CD32^+$ (**Figure 2c**). Cluster
189 5, also enriched in NC tumors, consisted of lymphocyte populations: CD8 T cells (28%), B
190 cells (21%), NK cells (11%), and CD4 T cells (7%) (**Figure 2f**). In contrast, Clusters 3 and
191 4, which were enriched in C-GBM, were characterized as $CD45^{high}CD64^+CD14^+$, consistent
192 with peripheral monocyte-derived macrophages (MDMs) (17) (**Figure 2d, e**). Expert-guided
193 gating on patient samples not included in the initial analysis (n=6) identified similar immune
194 phenotypes consistent with the Citrus findings and MEM labels (**Supplementary Figure**
195 **2b-d**), and confirmed statistical enrichment of the immune populations identified by the
196 Citrus algorithm (**Supplementary Figure 2e**).

197 Examining each cluster more deeply, $\gamma\delta$ T cells in Cluster 1 expressed low levels of
198 CD45RA, CD38, CD43, and CCR4, while DNT cells expressed CD43 and CD44, consistent
199 with an effector T cell phenotype (**Figure 2g, Figure 2h**). Similarly, CD8 T cells present in
200 Cluster 5 expressed high levels of CD43, CD44, and CD45RA indicative of activated
201 CD45RA expressing effector memory T cells (T_{EMRA}) (18). B cells in NC-GBM tumors
202 expressed low levels of HLA-DR and high levels of the inhibitory Fc receptor CD32
203 (Fc γ RII), suggesting impaired antigen presentation capacity. NK cells within Cluster 5 were
204 characterized as $CD16^+CD56^-CD43^+CD44^+CD11b^{low}$, a mature, cytotoxic NK cell
205 phenotype. Finally, microglia enriched in NC tumors (Cluster 2) were characterized as

206 CD45RO^{low}CD11b^{low}HLA-DR⁺CD32⁺CD64⁺CCR4^{low}CD69^{low}PD-L1^{low}, characteristic of
207 activated microglia. In contrast, MDMs within contacting tumors (Clusters 3 and 4)
208 expressed higher levels of CD45RO, CD11b, CD32, and CD44 than their microglial
209 counterparts. The distinguishing characteristics of macrophage populations A (Cluster 3)
210 and B (Cluster 4) included higher expression of CD14 in Cluster 3 and higher expression of
211 the chemokine receptors CXCR3 and CCR4 in Cluster 4 (**Figure 2g, h**), confirming that
212 these populations represent two activated, phenotypically distinct myeloid subsets in the C-
213 GBM microenvironment.

214 Citrus events were then overlaid on patient-specific t-SNE maps (**Supplementary**
215 **Figure 3**) to determine the source of subsampled Citrus clusters and compare the
216 phenotypes and frequencies of immune cells within larger FlowSOM subsets (13). Immune
217 phenotypes identified in the subsampled set of cells were no statistically different than the
218 larger immune populations (p=0.6844) (**Supplementary Figure 3a-d**). Consistent with the
219 abundance of total CD45⁺ populations discussed above, the average number of
220 phenotypically distinct FlowSOM clusters, an estimate of overall immunological diversity,
221 was similar between C-GBM and NC-GBM tumors (23 vs. 22 FlowSOM clusters
222 respectively), as was the number and proportion of clusters represented by Citrus in the
223 entire dataset (**Supplementary Figure 3e**), suggesting that the degree of diversity within
224 the immune infiltrate does not significantly contribute to outcomes associated with ventricle
225 contact status.

226 The frequency and immune phenotype of individual patients' clusters defined by
227 MEM labels (**Figure 2i, Figure 2j, Supplementary Figure 3f, Supplementary Figure 4a-**
228 **b**) reflected the larger patterns identified by Citrus. For example, Patient LC03 who had a
229 C-GBM tumor and the worst overall survival (57 days post resection), possessed abundant

230 frequencies of macrophages corresponding to Citrus cluster 3 (44%; 8/23 clusters) and
231 cluster 4 phenotypes (9.46%; 2/23 clusters). Five percent of the immune fraction in this
232 patient consisted of CD8 T cells (4%) and B cells (1%) with <1% of the microglia and
233 $\gamma\delta$ /DNT populations (Clusters 1 and 2) (**Figure 2i, Supplementary Figure 4a**).
234 Conversely, Patient LC06, who had an NC-GBM tumor and the greatest overall survival
235 (1588 days post resection) included no macrophages in clusters 3 or 4, but had an
236 abundance of microglia (21%), DNT cells (17.87%), $\gamma\delta$ T cells (9.69%), NK cells (2.37%),
237 CD8 T cells (3.39%), and B cells (3.87%), collectively reflecting phenotypes found in
238 clusters 1,2, and 5 (**Figure 2j, Supplementary Figure 4b**). We next sought to compare the
239 phenotypes of all FlowSOM immune clusters in order to identify common immune
240 populations across all patients in our cohort. Comparison of all clusters identified by
241 FlowSOM (455 clusters from all patients) using Root-mean-square-deviation (RMSD)
242 identified populations enriched in C-GBM or NC-GBM tumors overlapping with Citrus
243 phenotypes (**Supplementary Figure 5a**). Across all patients within the cohort, RMSD
244 analysis identified 17 common immune phenotypes across our samples. The majority of
245 RMSD clusters were well represented across the cohort, with RMSD cluster 6 (CD8 T cells)
246 the most represented cluster, and RMSD cluster 12 (microglia) the least represented
247 (**Supplementary Figure 5b**). The abundance of each cluster within each patient sample
248 (**Supplementary Figure 5c**), the statistical enrichment (**Supplementary Figure 5d**), and
249 MEM labels for each immune phenotype (**Supplementary Figure 5e**) were consistent with
250 Citrus findings.

251 Taken together, these data indicate that although individual patients possessed a
252 range of immune cell phenotypes in their tumors, common immune signatures could be
253 identified across the cohort. In particular, microglia and lymphocytes, specifically $\gamma\delta$ T cells,

254 DNT cells, CD8 T cells, NK cells, and B cells were enriched in patients with NC-GBM.
255 Conversely, patients with C-GBM possessed an immune microenvironment enriched in
256 MDMs, revealing starkly contrasting immune microenvironments in C-GBM and NC-GBM
257 tumors.

258 **3. Immune cell frequencies correlate with patient outcome.**

259 We next determined if the relative abundance of immune populations correlated with
260 outcome, similar to observations in peripheral solid tumors (19). Using a Cox proportional
261 hazards model, $\gamma\delta$ T cells and DNT cells (Citrus cluster 1) and macrophages (Citrus
262 clusters 3 and 4) did not significantly correlate with patient outcome (**Figure 3a**). Microglial
263 cells (Citrus cluster 2) and lymphocytes (Citrus cluster 5), however, correlated with
264 improved patient survival. Higher frequencies of microglia cluster 2 (>2%) correlated with
265 more favorable overall survival outcomes (median 560.5d vs 252.0d; $p=0.0405$, HR=
266 0.3782 , CI [0.1414-1.012]) as did lymphocytes (>6%) (median 507d vs 215d; $p=0.0126$,
267 HR= 0.3226 , CI [0.155-0.9015]). Importantly, 5/6 patients in the “microglia high” and 6/7
268 patients in the “lymphocyte high” group presented with NC tumors, consistent with our
269 previous findings (**Figure 2**).

270 To corroborate these results using an orthogonal, unsupervised computational
271 approach, the Risk Assessment Population IDentification (RAPID) workflow was used to
272 directly reveal immune cell clusters whose abundance correlated with patient outcome (13).
273 RAPID identified eight populations that were either strongly ($p<0.05$) or modestly ($p<0.1$)
274 correlated with patient outcome (**Figure 3b**). These phenotypes were stable through
275 iterative runs of RAPID, including repeated subsampling (**Supplementary Figure 6**).
276 Consistent with Citrus results, one statistically significant RAPID metacluster (06)

277 represented a population of CD43⁺ NK cells whose abundance was predictive of favorable
278 outcomes (p=0.0298, HR=0.3554, CI [0.1369-0.9223]). Five out of six patients with high
279 frequencies of these NK cells presented with NC tumors, consistent with Citrus results.
280 Greater frequencies (>1.32%) of RAPID metacluster 20 (CD45^{int/low}CD64⁺HLA-
281 DR⁺CD45RO^{low} microglia) also correlated with longer survival (710d vs 246d; p=0.0218,
282 HR=0.3189, CI [0.1232-0.8253]). All five patients with high abundance of this microglial
283 subset presented with NC-GBM tumors. Conversely, three subsets of
284 CD45^{high}CD64⁺CD32⁺HLA-DR⁺CD14⁺ macrophages correlated with poor prognosis (RAPID
285 metaclusters 14, 17, and 29). For example, Metacluster 17, an MDM population
286 phenotypically similar to Citrus Cluster 4, correlated with roughly 4-fold worse prognosis
287 (median overall survival (mOS) = 113d vs 426d, p=0.004, HR= 3.805, CI [0.7730-18.73]).
288 Critically, patients with the highest frequencies of MDMs presented with ventricle-contacting
289 tumors, providing further evidence that blood-derived macrophages correlate with ventricle
290 tumor contact and worse prognosis.

291 Collectively, these data suggest that infiltration by specific immune cells correlates
292 with survival in glioblastoma and LV contact status. Orthogonal approaches confirmed that
293 computationally identified subsets of microglial cells and lymphocytes correlated with more
294 favorable outcomes whereas increased infiltration of subsets of peripheral macrophages
295 correlated with worse outcome independent of LV contact status. Thus, C-GBM and NC-
296 GBM have distinct immune microenvironments and contrasting patient outcomes that align
297 closely with the expected functional role of the immune cells present in each.

298 **4. The immune microenvironment of lateral ventricle contacting gliomas is enriched**
299 **in inhibitory checkpoint receptor expression.**

300 We next sought to identify targetable immune receptors that may give insight into
301 mechanisms of immunoregulation in tumors proximal to the ventricle and inform clinical
302 decision making. We used machine learning to identify individual cellular features that were
303 enriched in C-GBM or NC-GBM immune infiltrates (**Figure 4a, Supplementary Figure 7a**).
304 Citrus identified elevated expression of 5 markers on 7 immune subsets in C-GBM tumors
305 (**Figure 4a, Supplementary Figure 7a-b**). Of note, the checkpoint receptor PD-1 was
306 elevated on both CD4 and CD8 T cells infiltrating C-GBM tumors, as were CD32, CD69,
307 CD44, and HLA-DR on C-GBM infiltrating peripheral MDMs. Moreover, CD32 and CD69
308 were elevated across multiple phagocytic populations infiltrating C-GBM including MDMs
309 and microglia. Lastly, HLA-DR was elevated on a population of $\gamma\delta$ T cells infiltrating C-GBM
310 tumors (**Figure 4b-c**). Consistent with Citrus results here and in Figure 2, expert-guided
311 biaxial gating confirmed an increased frequency of CD32⁺CD44⁺HLA-DR⁺ macrophages
312 infiltrating C-GBM (**Figure 4d**) compared to NC-GBM.

313 Expert gating strategies confirmed increased frequencies of PD-1⁺ CD4, CD8, and
314 DNT cells within C-GBM tumors (**Supplementary Figure 7c-i, Supplementary Tables 2**
315 **and 3**). Critically, PD-1⁺ T cells infiltrating C-GBM co-expressed the inhibitory receptor
316 TIGIT at a higher frequency in C-GBM (46% vs. 23%), whereas a higher frequency of PD-1⁻
317 TIGIT⁻ T cells were found in NC-GBM (36% vs 17%), suggesting that C-GBM T cells may
318 be more phenotypically exhausted compared to their NC-GBM counterparts (**Figure 4e, f**).
319 Additionally, C-GBM tumors bore increased frequencies of T and NK cells (**Supplementary**
320 **Figure 7c-f**), myeloid subsets (**Supplementary Figure 7g-i**) and B cells (**Supplementary**
321 **Figure 7j**) with an activated phenotype compared to NC-GBM tumors. While CD45⁻ tumor
322 stromal cells in C-GBM and NC-GBM expressed a variety of immune checkpoint
323 receptors/ligands, we found no difference in the frequency of checkpoint positive tumor

324 cells in C-GBM and NC-GBM tumors (**Supplementary Figure 7k**). Interestingly, increased
325 frequencies of T cells expressing several markers (CD27, CD32, CXCR3, CCR7)
326 suggested that these cells in C-GBM tumors possessed a phenotype consistent with
327 immunological memory. Indeed, the frequency of CD45RO⁺CCR7⁺ central memory T cells
328 (T_{cm}) was elevated in C-GBM tumors (**Supplementary Figure 7l**). Further, consistent with
329 Citrus results (**Figure 2**), increased frequencies of CD45RO⁻CCR7⁻ T_{EMRA} CD8 T cells were
330 found in NC-GBM tumors.

331 Taken together, our data suggests that not only does tumor proximity to the lateral
332 ventricle influence the types of immune cells infiltrating GBM tumors, but also impacts the
333 phenotype, and potential function of these cells. Through multiplex machine learning and
334 expert-guided strategies, we have identified multiple therapeutically targetable checkpoint
335 receptors on distinct immune subsets in GBM tumors.

336 **5. Immune receptor expression correlates with patient outcome.**

337 We next sought to identify which of the differentially enriched immune markers
338 identified by Citrus may help stratify patient outcomes. To do so, we developed the median
339 marker implementation of RAPID (mmRAPID) whereby the median intensity values for
340 select markers in RAPID clusters were correlated with patient outcome (See Methods).
341 Median expression values of 12 immune receptors across 46 immune clusters correlated
342 with patient outcome (**Figure 5a**). Consistent with enriched immune receptor expression in
343 C-GBM tumors (**Figure 4**) and immune abundance correlating with outcome
344 (**Supplementary Figure 6**), a 2-fold increase in PD-1 expression in CD4 T cells
345 (mmRAPID Cluster 9) (**Figure 5b**) and CD8 T cells (mmRAPID Cluster 25) (**Figure 5c**)
346 correlated with 2-fold worse survival (215d vs. 441d and 240d vs 570d, respectively).

347 Importantly, although contact status was not used in the mmRAPID analysis, 6/9 patients
348 with C-GBM tumors had PD-1^{high} CD4 T cell infiltration and 8/9 patients had PD-1^{high} CD8
349 infiltration whereas 2/10 patients with NC-GBM tumors had PD-1^{high} CD4 T cell infiltration
350 and 3/10 NC-GBM patients had PD-1^{high} CD8 T cell infiltration. Further, mmRAPID
351 identified a 2-fold increase in CD32 expression on CD8 T cells (mmRAPID Cluster 24) with
352 a 2.6-fold decrease in patient survival (**Figure 5d**).

353 Consistent with Citrus identification of elevated expression of CD44 on C-GBM
354 infiltrative macrophages, mmRAPID found that 3.5-fold elevated expression of CD44 on
355 macrophages (mmRAPID Cluster 15) correlated with 2-fold worse survival. The majority of
356 patients (5/6) with CD44^{high} macrophages had C-GBM tumors, whereas 9/13 patients with
357 CD44^{low} macrophages had NC-GBM tumors (**Figure 5e**). Elevated expression of CD69 on
358 CD4 T cells (mmRAPID Cluster 9) correlated with poor outcome and lateral ventricle
359 contact, but elevated CD69 expression on microglia (mmRAPID Cluster 40) was associated
360 with more favorable outcomes and NC-GBM tumors (**Figure 5g**). Moreover, mmRAPID
361 identified elevated HLA-DR expression on CD4 T cells, DNT cells, peripheral MDMs, and B
362 cells as predictive of poor outcome and ventricle contact (**Figure 5h-k**). Surprisingly, PD-
363 L1 expression, a feature of NC-GBM enriched microglia (**Figure 2**), correlated with an
364 approximate 3-fold difference in survival outcomes and favored NC-GBM patients
365 (**Supplementary Figure 8a**). Consistent with increased frequencies of CD27⁺ T cells in C-
366 GBM tumors, we found that elevated CD27 expression on CD4, DNT, and CD8 T cells
367 correlated with worse prognosis and favored patients with C-GBM tumors (**Supplementary**
368 **Figure 8b-d**). While CD28 expression on microglia correlated with more favorable
369 outcomes and NC-GBM tumors (**Supplementary Figure 8e**), CD38, CCR4, TIM3, and
370 CD57 were associated with worse outcomes (**Supplementary Figure 8g-i**).

371 Taken together, these data identify a statistical association between immunoreceptor
372 expression, lateral ventricle contact, and patient outcome as identified by two contrasting
373 machine learning tools. Critically, elevated receptor expression tied to contact status and
374 patient outcome identified by mmRAPID was consistent with Citrus identification of
375 enriched immune populations in C-GBM and NC-GBM tumors. Co-expression of multiple
376 predictive markers within the same immune subset suggests that key immune populations
377 (e.g., PD-1⁺CD27⁺ T cells or HLA-DR^{high}CD44⁺ MDMs) may be critical drivers of immunity
378 within the ventricle tumor microenvironment. Paradoxical co-enrichment of activation
379 markers (CD27, CD69, HLA-DR) as well as inhibitory receptors (PD-1) on the same
380 subsets correlating with outcome suggested that these immune cells may be hyper-
381 activated, may possess some functional signaling capacity.

382 **6. Increased signaling capacity in the lateral ventricle tumor microenvironment.**

383 To address the signaling capacity of GBM infiltrating immune cells, we first used
384 mass cytometry to assess the basal levels of 12 phosphoprotein readouts in immune
385 infiltrates from 10 patients in our cohort (5 C-GBM, 5 NC-GBM) or healthy PBMC (**Figure**
386 **6a, Supplementary Table 1 and 4**). Basal levels of phosphorylated STAT1 (p-STAT1)
387 were found only in myeloid cells from PBMC or GBM samples; however, basal levels of p-
388 STAT3 were elevated in CD4 T cells, Tregs, B cells, and myeloid cells in GBM tumors
389 compared to healthy donor PBMCs. Elevated basal STAT3 phosphorylation in myeloid
390 cells in particular is consistent with infiltration of suppressive M2-like macrophages
391 previously described (reviewed in (20)). Further, p-STAT5 levels were slightly increased in
392 myeloid cells in the GBM tumor microenvironment. Ribosomal protein S6 (S6) and nuclear
393 factor κ B (NF κ B) were phosphorylated at baseline in T and B cells, and were highly
394 phosphorylated in myeloid cells. Contrastingly, NK cells exhibited drastically reduced basal

395 S6 and NFκB phosphorylation, suggesting that while NK cells infiltrating GBM tumors may
396 be functionally impaired, T, B, and myeloid populations appear to be functionally
397 competent.

398 Given the co-enrichment of immune activation and inhibitory receptors and
399 correlations with worse survival seen in C-GBM, we next assessed the ability of leukocytes
400 in C-GBM and NC-GBM tumors to respond to cytokine stimulation. Bulk tumor samples
401 were stimulated with cytokines with defined roles in regulating antitumor immunity including
402 interleukin 2 (IL-2), IL-6, and interferon alpha (IFNα). FlowSOM clustering on t-SNE axes
403 compared protein phosphorylation levels to basal signaling states in each of 42 defined
404 immune subsets (**Figure 6b**). Quantification of the signaling responses for each of the 12
405 phosphoproteins within each cluster then identified which signaling networks were
406 responsive to cytokine stimulation. Stimulation with IL-2 led to differential STAT
407 phosphorylation within distinct immune subsets in C-GBM and NC-GBM tumors. For
408 instance, STAT1 phosphorylation was highly refractory to IL-2 stimulation in C-GBM
409 immune infiltrates, including Cluster 12 (a population of CD32⁺HLA-DR^{high} MDMs [see
410 **Figure 4**]). Conversely, Cluster 2 (CD32⁺HLA-DR⁺ MDMs) exhibited phosphorylated
411 STAT1 in response to IL-2 in NC-GBM tumors but not C-GBM tumors. Furthermore, IL-2
412 induced STAT3 phosphorylation in microglia (Clusters 5 and 19), and NK cells (Cluster 38)
413 and STAT5 phosphorylation in CD32⁺HLA-DR^{high} MDMs (Clusters 2 and 4) and B cells
414 (Cluster 41) in C-GBM tumors. Immune infiltrates in NC-GBM tumors, however, exhibited a
415 different pattern of IL-2 mediated STAT3 phosphorylation, as Cluster 8 (microglia) and
416 Cluster 38 (NK cells) failed to phosphorylate STAT3. Similar to C-GBM, Cluster 2 and 4
417 were responsive to IL-2 induced STAT5 phosphorylation, whereas Cluster 41 (B cells) was
418 refractory to STAT5 phosphorylation. The proportion of clusters responding to IL-2 through

419 STAT signaling revealed that immune infiltrates in C-GBM tumors were more significantly
420 responsive to IL-2 through STAT3 (25% of clusters) than NC-GBM infiltrates (12.5% of
421 clusters) (**Figure 6c**). Looking at the entire signaling profile, IL-2 induced several signaling
422 cascades leading from membrane proximal signaling (LCK) to nuclear signaling (NF- κ B) in
423 several immune clusters (e.g. Cluster 2, 4, and 5) while other subsets (e.g. Cluster 41)
424 appeared to be reciprocally impacted by IL-2 stimulation through multiple networks (**Figure**
425 **6d**).

426 Analogous to IL-2, IL-6 stimulation induced differential phospho-signaling networks
427 in C-GBM and NC-GBM immune infiltrates (**Supplementary Figure 9a, b**). STAT3
428 phosphorylation was induced in CD32⁺HLADR⁺ MDMs (Clusters 2 and 21), microglia
429 (Cluster 5), DNT cells (Cluster 15) and B cells (Cluster 41) infiltrating C-GBM, however
430 these subsets failed to elicit a response to IL-6 in NC-GBM. (**Supplementary Figure 9a,**
431 **b**). Interestingly, while IL-6 failed to induce STAT3 phosphorylation in Cluster 2
432 macrophages in NC-GBM patients, STAT5 appeared to be preferentially phosphorylated in
433 this subset. In fact, IL-6 preferentially induced STAT3 phosphorylation across all C-GBM
434 immune subsets, while STAT5 was favored in NC-GBM subsets (**Supplementary Figure**
435 **9c**), highlighting that inflammatory stimuli may have different immunomodulatory effects
436 depending on tumor proximity to the lateral ventricle.

437 Stimulation with IFN α further distinguished immune responses in C-GBM and NC-
438 GBM tumors (**Supplementary Figure 10a**). Interestingly, STAT1 and STAT3 appeared to
439 be phosphorylated in microglia infiltrating C-GBM (Cluster 5) but not NC-GBM. In fact, a
440 STAT1/STAT3-ERK-p38-CREB circuit appeared to be active in this population in C-GBM
441 but not NC-GBM. Moreover, IFN α induced similar inflammatory circuits in CD32⁺HLA-
442 DR^{high} macrophages in either C-GBM or NC-GBM. For example, STAT1, 3, and 4 were

443 induced within Cluster 2 from both cohorts; however, STAT5 was preferentially induced in
444 NC-GBM Clusters 2 and 5 as well as was an AKT-ERK-p38-CREB circuit specifically in
445 Cluster 2 from NC-GBM patients (**Supplementary Figure 10b**), suggesting an active
446 inflammatory response to IFN α in this subset. Similar to IL-2 and IL-6, IFN α stimulation
447 appeared to favor STAT3 phosphorylation over STAT1 in C-GBM patients, as 19% of
448 clusters demonstrated STAT3 responses in C-GBM, whereas only 10% of clusters
449 demonstrated STAT1 phosphorylation, further supporting a role for STAT3 in immune
450 regulation in C-GBM tumors.

451 Together, these data indicate that, not only do immune cells differentially infiltrate
452 ventricle contacting and non-contacting tumors, but the inflammatory milieu within C-GBM
453 may rewire immune signaling networks, drastically altering immune responsiveness to
454 external stimuli. Strikingly, STAT3 phosphorylation appeared to drive much of the cytokine
455 responsiveness in C-GBM tumors regardless of the cytokine stimulation or expected
456 activation of canonical signaling pathways, consistent with our hypothesis that C-GBM
457 tumors possess a distinct STAT3-driven immunosuppressive microenvironment.

458 **Discussion**

459 Significant improvements in molecular and histologic characterization have
460 increased our understanding of neurologic tumors, including glioblastoma. Unfortunately,
461 improved tumor classification has not translated into clinical therapeutics that meaningfully
462 impact patient outcomes. Work over the past decade has focused on tumor-specific
463 characterization and targeting, and only recently has a large impetus been placed on
464 understanding stromal factors that drive gliomagenesis and therapeutic resistance. The
465 immune microenvironment, in particular, constitutes a critical part of tumor lesions and

466 plays a crucial role in regulating tumorigenesis in peripheral and intracranial tumors (21,22).
467 While immune-targeted drugs have proven effective at generating antitumor immunity
468 towards peripheral solid tumors and in some cases mediate complete tumor regression, the
469 same efficacy has not been demonstrated in neurologic tumors (NCT02017717,
470 NCT02667587) (23,24). Early work using immunotherapeutic strategies has, in fact,
471 demonstrated some capacity to elicit antitumor immunity, yet these approaches have not
472 improved GBM outcomes (25-27), highlighting a need for novel, straightforward
473 approaches, such as MRI-guided regional tumor position, to identify appropriate immune
474 targets and patient cohorts to optimize therapeutic benefit.

475 Here we describe the immune microenvironment in glioblastomas based on regional
476 tumor position, identifying immunomodulatory mechanisms in GBM tumors presenting with
477 radiographic contact with the walls of the lateral ventricle. Complementary machine
478 learning approaches provided supervised and unsupervised approaches that identified
479 phenotypic and functional immune profiles associated with ventricle contact status and
480 patient outcome. Namely, the abundance of lymphocytes and tissue resident microglia
481 were increased in NC-GBM tumors, whereas C-GBM tumors were enriched in anti-
482 inflammatory CD32⁺CD44⁺HLA-DR^{high} M2-like MDMs and exhausted PD-1⁺TIGIT⁺T cells.
483 These observations suggest that C-GBM tumors, and potentially the periventricular space
484 itself, are highly immunosuppressive environments.

485 Several lymphocyte subsets were enriched in NC-GBM tumors including $\gamma\delta$ T cells
486 and CD8⁻CD4⁻ DNT cells, and CD8 T cells, NK cells, and B cells which correlated with
487 improved patient survival. Both $\gamma\delta$ T cells and DNT cells can generate neuro-inflammatory
488 responses during brain pathology, and are associated with antitumor cytotoxicity in
489 glioblastoma (10,28,29). CD45RO⁻CCR7⁻ CD8 T_{EMRA} cells were also enriched in NC-GBM

490 tumors. Although T_{EMRA} cells are terminally differentiated, they possess increased
491 cytotoxicity and tumor-killing capacity (18,30), suggesting that T_{EMRA} cells may contribute to
492 tumor control in NC-GBM tumors. Moreover, NK cells infiltrating NC-GBM tumors bore a
493 $CD56^{low}$ phenotype associated with potent toxicity, whereas $CD56^{high}$ NK cells infiltrating C-
494 GBM tumors suggest a regulatory capacity (reviewed in (31,32)). Infiltration of multiple
495 lymphocyte subsets with cytotoxic potential in NC-GBM suggests a more effective
496 antitumor immune response in the microenvironment, and thus contribute to longer
497 survival. Furthermore, B cells were enriched in NC-GBM. Recent reports have correlated
498 development of B cell enriched tertiary lymphoid structures (TLSs) in tumors with improved
499 patient survival and responsiveness to immunotherapy (33-35); however, the precise role of
500 B cells in NC-GBM and their ability to support TLSs in NC-GBM tumors remains to be seen.

501 In contrast to NC-GBM tumors, lymphoid infiltration into C-GBM was reduced, and
502 lymphocytes in C-GBM tumors bore a hyper-activated, exhausted phenotype characterized
503 by increased expression of CD32, CD69, HLA-DR, CD27, and PD-1 on C-GBM T cells that
504 correlated with worse prognosis. An increased frequency of $PD-1^+$ T cells infiltrating C-
505 GBM co-expressed a second checkpoint receptor, T cell immunoreceptor with Ig and ITIM
506 domains (TIGIT). While the precise mechanism of TIGIT-mediated immune suppression is
507 unclear, evidence suggests that TIGIT serves a compensatory role to PD-1-mediated
508 inhibition, eliciting resistance to PD-1 targeted checkpoint blockade therapy (36). Moreover,
509 TIGIT expression is associated with elevated T cell activation, immune exhaustion, and
510 responsiveness to checkpoint blockade (37-39). Functionally, T cells in both C-GBM and
511 NC-GBM expressed limited Granzyme B and Ki-67, suggesting a lack of effector function
512 and proliferative capacity consistent with immune exhaustion. In contrast to T_{EMRA}
513 infiltration in NC-GBM, $CD45RO^+CCR7^+$ central memory T cell phenotypes (T_{CM})

514 predominated in C-GBM tumors. In fact, several T cell markers correlating with patient
515 outcome (CD32, CD69, HLA-DR, CD27) are associated with T cell memory phenotypes.
516 Whether T_{EMRA} and T_{CM} differentially infiltrate NC-GBM and C-GBM tumors, respectively, or
517 are polarized by microenvironmental factors, and their functional consequences upon
518 arrival remains to be determined. Although, increased frequencies of multiple CCR7⁺
519 lymphocyte populations in C-GBM tumors suggest that a CCR7-CCL19/CCL21 axis may
520 recruit T_{CM} into the periventricular niche. Importantly, T cells in both C-GBM and NC-GBM
521 tumors exhibited basal phosphorylation of both ribosomal protein S6 and NFκB, indicating
522 incomplete exhaustion and retention of some degree of functionality. While lymphocytes in
523 both C-GBM and NC-GBM showed impaired responses to inflammatory cytokines (**Figure**
524 **7**), (arguing for exhaustion), lymphocytes in C-GBM tumors demonstrated moderate
525 responses to inflammatory cytokines, particularly through CREB. It remains to be seen
526 what effects other cytokines and soluble factors may have on T cell signaling, recruitment,
527 and survival in C-GBM and NC-GBM.

528 Tumor contact with the lateral ventricle also impacted phagocytic populations in the
529 tumor microenvironment. A subset of CD45^{low}CD11b^{low/-}HLA-DR^{low}CD14⁻ microglia was
530 enriched in NC-GBM tumors and corresponded to more favorable outcomes, however, no
531 distinct phenotype emerged to illuminate their functional capacity. Microglia in NC-GBM
532 tumors demonstrated limited responsiveness to inflammatory cytokines with S6
533 phosphorylation remaining an active signaling component (**Figure 7**). A key role for
534 microglia lies in maintenance of tissue homeostasis, in large part through phagocytosis of
535 cellular debris and tissue pruning (reviewed in (40)). The phagocytic capabilities of NC-GBM
536 resident microglia and ability to present antigen is unknown, but may support infiltrating
537 lymphocytes in mediating tumor control. In contrast to NC-GBM tumors, two populations of

538 peripheral CD45^{high}CD11b⁺CD14⁺ MDMs were enriched in C-GBM tumors. These
539 populations were distinguished by expression of CD32, CD44, HLA-DR, CD69, and
540 chemokine receptors CXCR3 and CCR4. Along with elevated frequencies of CCR7⁺
541 lymphocytes, these findings further support a role for chemotactic factors in recruiting
542 leukocyte populations into the niche.

543 Functionally, C-GBM infiltrating MDMs expressed CD163 and CD209 (DC-SIGN),
544 indicative of M2-like anti-inflammatory macrophages in the tumor microenvironment (41).
545 MDMs also exhibited high basal expression of phosphorylated STAT3, consistent with M2-
546 like polarization. Interestingly, CD32⁺HLA-DR⁺ macrophages showed greater
547 responsiveness to cytokine stimulation than their CD32⁻HLA-DR⁻ counterparts (**Figure 7**).
548 Upon stimulation, these MDMs utilized different signaling networks in C-GBM and NC-GBM
549 tumors. For example, CD32⁺HLA-DR⁺ macrophages signaled through a STAT 3/4/5-ERK-
550 p38-CREB axis in NC-GBM tumors in response to IL-2 stimulation. While this network was
551 active in C-GBM MDMs, 4-EBP1, S6, and NFκB were also phosphorylated, suggesting
552 some degree of differential macrophage signaling in these regional tumor classes.
553 Similarly, C-GBM MDMs were more responsive to IL-6 stimulation, particularly through
554 STAT3 in C-GBM compared to NC-GBM. Perhaps most striking, CD32⁺HLA-DR⁺ MDMs
555 were highly responsive to IFNα stimulation. While all STAT proteins assessed were
556 phosphorylated following IFNα stimulation in C-GBM and NC-GBM MDMs, NC-GBM MDMs
557 favored an inflammatory ERK-p38-CREB signaling axis, while C-GBM MDMs favored 4-
558 EBP1, S6, and NFκB signaling, further supporting a role for the tumor environment in
559 mediating differential myeloid signaling responses. Recent reports have demonstrated
560 conflicting roles for IFNα signaling in mediating pro- or antitumor responses depending on
561 the chronicity of IFN exposure (see (42)). Our results support a hypothesis that long-term

562 IFN exposure in the ventricular space may negatively impact antitumor immunity, whereas
563 acute inflammatory IFN signaling along with increased lymphocyte infiltrate in NC-GBM
564 supports antitumor immunity and prolonged overall survival in patients with NC-GBM
565 tumors. Importantly, STAT3 phosphorylation played a role in C-GBM immune infiltrates
566 regardless of stimulation condition. This points to STAT3 as a critical, targetable, driver of
567 antitumor immunity in the ventricular space, and suggests the stoichiometry of STAT3 may
568 enforce a regulatory immune signaling axis; however, the ability of STATs to form
569 heterodimeric complexes in response to inflammatory cues in brain tumors, and the
570 resulting functional consequences, is poorly understood.

571 We assessed signaling responses to three inflammatory cytokines that impact
572 antitumor immunity to peripheral solid tumors; however, a complex milieu of cytokines,
573 soluble mediators, and unique parenchymal factors within the brain may further influence
574 antitumor immunity in the brain. Identifying the cellular source--be it tumor-, stem cell-,
575 ependyma-, or immune-derived—and the dynamic interplay between these cell subsets
576 within the microenvironment in mediating antitumor immunity in C- and NC-GBM tumors
577 will be critical to advance our understanding of neuro-oncology and develop novel
578 therapeutics.

579 This work highlights potential immunotherapeutic targeting strategies for GBM
580 patients based on MRI-guided tumor proximity to the lateral ventricle. Future studies will be
581 needed to determine whether patients with NC-GBM or C-GBM tumors may be more
582 amenable to drug combinations targeting lymphocyte or MDM populations respectively.
583 Immuno-oncology agents currently approved or in clinical trials may afford the most
584 immediate benefit, particularly for patients with C-GBM tumors; including agents targeting
585 PD-1 (nivolumab, pembrolizumab), TIGIT (tiragolumab), CD27 (varlilumab), or STAT3 (WP-

586 1066). It remains to be seen, however, which immunotherapeutic combinations will lead to
587 improved outcomes in GBM patients.

588 The tumor immune microenvironment is heavily influenced by tumor tissue-of-origin,
589 particularly in the brain. For instance, brain metastases possess distinct immune
590 microenvironments dependent on tissue of origin (43). Here, we demonstrate that regional
591 position of primary brain lesions, visualized as MRI-guided contact with the lateral ventricle,
592 influenced antitumor immunity in the brain and will be germane to clinical decision making,
593 particularly in patient selection and therapeutic options available to patients with C-GBM vs
594 NC-GBM.

595 **Methods**

596 *Human Specimens*

597 Freshly resected glioblastoma tumor tissues were collected from the Department of
598 Neurosurgery at Vanderbilt University Medical Center between 2014 and 2018 as
599 previously described (13,44,45). Non-tumor bearing brain tissue was collected from
600 temporal lobectomy as standard-of-care treatment for epilepsy from the Veterans' Affairs
601 Medical Center affiliated with Vanderbilt University. Glioblastomas with isocitrate
602 dehydrogenase (IDH) mutations confirmed by standard pathological diagnosis were
603 excluded from this study. All samples were collected with patient informed consent in
604 compliance with the Vanderbilt Institutional Review Board (IRBs #030372, #131870,
605 #181970), and in accordance with the Declaration of Helsinki. Samples were de-identified
606 prior to tissue processing. All patients were adults (age range 40-80 years old) at the time
607 of tumor resection. Extent of resection was classified as gross total resection (GTR) or
608 subtotal resection (STR) independently by a neurosurgeon and a neuro-radiologist. Gross

609 total resection was defined as no significant residual tumor enhancement upon gadolinium
610 enhanced magnetic resonance imaging (MRI) of the brain 24 hours' post-surgery. Tumor
611 contact with the lateral ventricle was confirmed upon inspection of MRI and verified by a
612 neurosurgeon and neuro-radiologist. All patients were considered for postoperative
613 chemotherapy (temozolomide) and radiation according to standard-of-care. Methylation of
614 the O⁶-methylguanine-DNA methyltransferase (MGMT) promoter was determined by
615 pyrosequencing (Cancer Genetics Inc, Los Angeles CA, USA). *IDH* mutation status was
616 confirmed using polymerase chain reaction (PCR). Patient follow-up extended to October
617 2019, noting time to first radiographic progression or tumor recurrence as assessed by a
618 neuro-oncologist and neuro-radiologist or time to patient's death. All deaths were deemed
619 related to tumor progression. Median overall survival (mOS) of the patient cohort
620 presented herein was 366 days. A total of 25 glioblastoma patients from both male and
621 female subjects were included in the present study. A complete list of clinical
622 characteristics can be found in (**Supplementary Table 1**).

623 Peripheral blood mononuclear cells (PBMC) were collected from healthy volunteers
624 with written informed consent under IRB protocol #131311 and in accordance with the
625 Declaration of Helsinki. Samples were de-identified prior to processing, and no other
626 information was obtained from healthy individuals.

627 *Tissue Collection and Processing*

628 Fresh tumor tissue was obtained directly from the operating room at Vanderbilt
629 University Medical Center within one hour of resection. Tissues were processed and
630 dissected into single-cell suspensions as previously reported (44,45). Briefly, samples were
631 resuspended in neural stem cell media: DMEM-F12 supplemented with glutamine

632 (Glutamax, Thermo Fisher), 1M HEPES (Gibco), a hormone cocktail consisting of 30%
633 glucose (Fisher), 7.5% sodium bicarbonate (Sigma Aldrich), apotransferrin, insulin,
634 Putrescine Solution, 200 μ M Progesterone, 3 mM Sodium Selenite (Sigma Aldrich), and
635 gentamycin (Fisher). Tissues were minced with razor blades to an approximate diameter of
636 1 mm before enzymatic digestion for 1 hour with collagenase IV (1 ug/ul) and DNase I
637 (0.25 ug/ul) at 37°C and 5% CO₂ with steady shaking. Suspensions were then washed and
638 triturated before filtration twice through 70 μ m and 40 μ m filters. Cell pellets were
639 resuspended in ACK lysis buffer to remove red blood cells (Invitrogen). Cells were washed
640 and resuspended in neural stem cell media supplemented with bovine serum albumin
641 (Sigma Aldrich), heparin, recombinant human FGF (25ug/mL; Stem Cell Technologies),
642 and recombinant human EGF (10 ug/mL; Stem Cell Technologies) in 10% DMSO before
643 long-term cryopreservation (1×10^7 cells/mL) in liquid nitrogen.

644 Healthy donor blood was collected by venipuncture into heparinized collection tubes
645 (Becton Dickinson; 100 mL/donor). Whole blood was diluted 1:4 with PBS before being
646 overlaid onto a Ficoll-Paque Plus density gradient (GE Lifesciences) Blood was then
647 centrifuged at 400g for 30 minutes without a brake. Buffy coats were isolated, washed with
648 PBS, and centrifuged at 500g for 10 minutes. Cell pellets were then resuspended in ACK
649 lysis buffer for 5 minutes, washed, and cryopreserved at 1×10^7 cells/mL in liquid nitrogen in
650 10% DMSO in FBS.

651 *Metal-isotope Tagged Antibodies*

652 All antibodies used for mass cytometry analysis are listed in **Supplementary Table**
653 **S2, Supplementary Table S3, and Supplementary Table S4**. Pre-conjugated antibodies
654 to metal isotopes were purchased from Fluidigm or from commercial suppliers in purified

655 form and conjugated in house using the Maxpar X8 chelating polymer kit (Fluidigm)
656 according to the manufacturer's instructions.

657

658 *Cell Preparation and Mass Cytometry Acquisition*

659 Cryopreserved samples were rapidly thawed in a 37°C water bath and resuspended
660 in complete RPMI supplemented with 10% FBS and 50 units/mL of penicillin–streptomycin
661 (Thermo Scientific HyClone). Cell suspensions were processed and stained as previously
662 described (44,46). Briefly, cells were washed once with serum free RPMI and subsequently
663 stained with ¹⁰³Rh Cell-ID Intercalator (Fluidigm) at a final concentration of 1 uM for 5
664 minutes at room temperature. Staining was quenched with complete RPMI before washing
665 with PBS 1% BSA. Cells were resuspended in PBS/BSA and added to the appropriate
666 antibody cocktail of cell-surface staining antibodies (**Supplementary Table S2,**
667 **Supplementary Table S3, Supplementary Table S4**) and incubated at room temperature
668 for 30 minutes. Samples were washed in 1% PBS/BSA before fixation in 1.6%
669 paraformaldehyde for 10 minutes at room temperature. Cells were again washed in PBS
670 and fixed in ice cold methanol with gentle vortexing before storage at -20°C. On the day of
671 data collection, samples stored at -20°C were washed in PBS/BSA and resuspended in an
672 antibody cocktail of intracellular stains (e.g., granzyme B, VISTA) for 30 minutes. Iridium
673 Cell-ID Intercalator was added at a final concentration of 125 nM and incubated at room
674 temperature for at least 30 minutes. Cells were then washed and resuspended in ultrapure
675 deionized water, mixed with 10% EQ Four Element Calibration Beads (Fluidigm) and
676 filtered through a 40 uM FACS filter tube before data collection. Data were collected on a
677 Helios CyTOF 3.0 (Fluidigm). Quality control and tuning processes were performed
678 following the guidelines for the daily instrument operation. Data were collected as FCS files.

679 *Cytokine Stimulation and Phospho-specific Cytometry*

680 Phospho-specific mass cytometry was performed as previously described (46).
681 Cryopreserved samples were thawed in a water bath as above. Cell pellets were
682 resuspended in complete RPMI (10% FBS, penicillin-streptomycin) and rested at 37°C for
683 15 minutes. Cell suspensions were then washed in PBS and stained in 1 μ M rhodium Cell-
684 ID intercalator in PBS for 5 minutes. Cells were again washed in PBS/BSA and aliquoted
685 equally into cytokine stimulation solutions. Briefly, these conditions included PBS
686 (unstimulated), recombinant human interleukin 2 (20 ng/mL), recombinant human
687 interleukin 6 (20 ng/mL), recombinant human interferon alpha (20 ng/mL) or hydrogen
688 peroxide (10 μ M). Stimulation conditions were allowed to proceed for 15 minutes before
689 immediate fixation in 1.6% PFA in order to halt phospho-protein dissociation. Samples were
690 then washed in PBS/BSA, stained with a cocktail of cell-surface antibodies and fixed in ice
691 cold methanol as above. On the day of collection, samples were stained with a cocktail of
692 intracellular phospho-specific antibodies. Iridium intercalation, resuspension in EQ
693 calibration beads, and sample collection proceeded as above.

694 *Data Preprocessing*

695 Raw mass cytometry files were normalized using the MATLAB bead normalization
696 tool. Files were then uploaded to the cloud-based analysis platform Cytobank. Before
697 automated high-dimensional data analysis, the mass cytometry data were transformed with
698 a cofactor of 5 using an inverse hyperbolic sine (arcsinh) function. Cell doublets were first
699 excluded using Gaussian parameters (Center, Offset, Width, Residual) as reported (46).
700 Intact cells were gated based on DNA content (^{191}Ir and ^{193}Ir). Dead cells were excluded

701 based on rhodium intercalation. Immune subsets were then manually gated using biaxial
702 gating strategies.

703

704 *Dimensionality Reduction and Automated Clustering*

705 T-distributed stochastic neighbor embedding (t-SNE) analysis was performed on
706 each individual patient sample using the Cytobank platform. To avoid down-sampling, all
707 live CD45+ cells were included for each individual patient's t-SNE analysis (range: 1,322-
708 335,303 events) using all immune markers in the antibody panel to generate t-SNE maps, a
709 perplexity of 30, theta of 0.5, and 10,000 iterations. Automated clustering of phenotypically
710 distinct immune cell subsets was then performed for each individual patient using the
711 Cytobank implementation of the FlowSOM clustering tool(9). Briefly, clustering analysis
712 was performed using only the t-SNE1 and t-SNE2 channels, hierarchical consensus
713 clustering, a cluster number of 196, and 10 iterations. Multiple FlowSOM analyses using
714 different iterations of metaclusters (5-50) identified the optimal number of total metaclusters
715 that minimized the variance of each immune marker in each metacluster as previously
716 described (13). For patient-to-patient comparisons where indicated, an equal number of live
717 CD45+ events were down-sampled from each patient file, concatenated together, and the
718 number of FlowSOM clusters was optimized across all patients.

719 *Citrus Clustering*

720 To identify immune populations that may be enriched in abundance in either lateral
721 ventricle contacting or non-contacting glioblastomas, we employed the Cytobank
722 implementation of the Citrus clustering algorithm (12,47). Live CD45+ cells were equally
723 downsampled from 19/25 patient files (478 events/patient) (**Supplementary Table S1**).
724 Patient files included in the analysis were grouped by tumor contact with the lateral

725 ventricle (9 contacting, 10 non-contacting). All immune markers in the Immune Phenotyping
726 Panel (**Supplementary Table S2**) were used to cluster. A nearest shrunken centroid
727 predictive analysis of microarrays (PAMR) model was used to predict enriched cell
728 abundance within each group. The minimum cluster size was set to 5%, with 5 cross
729 validation folds, and a false discovery rate (FDR) of 1%. To determine enriched immune
730 marker expression, the Citrus algorithm was rerun investigating median marker expression.
731 Sixteen immune markers delineating common lymphocyte and myeloid cell subsets were
732 used to cluster events, while the arcsinh expression levels of 17 markers was explored
733 (**Supplementary Table S2**). The maximum number of events per file was sampled. The
734 same analysis settings used for abundance analysis was similarly used for median marker
735 expression (PAMR analysis, 5% cluster size, 5 cross validation folds, FDR=1%). The most
736 terminal clusters of differential abundance between the two cohorts of glioblastoma patients
737 were exported into new experiments for further analysis. Biaxial gating was used to
738 determine each cluster phenotype. Due to down-sampling in the Citrus analysis, and in
739 order to determine from which immune populations each Citrus cluster was sampled, t-SNE
740 analysis was performed on the total number of live CD45+ cells from each patient as well
741 as each patient's individual Citrus clusters. Cells from Citrus clusters were assigned to
742 FlowSOM metaclusters based on 1) position in the overlaid viSNE map, and 2) phenotypic
743 similarity 3) proportion of FlowSOM metaclusters. Further, both abundance of cells with an
744 identified Citrus phenotype and expression of identified markers were validated
745 independently in the entire 25-patient cohort.

746 *Marker Enrichment Modeling*

747 The phenotypes of automatically clustered immune populations generated in Citrus
748 and FlowSOM were identified using Marker Enrichment Modeling (MEM) (11,48). Briefly,

749 clusters generated from FlowSOM or Citrus were exported from Cytobank into R. Within R,
750 MEM labels were generated using all immune markers included in either the immune
751 phenotyping cytometry panel (**Supplementary Table S2**) or the immune checkpoint
752 cytometry panel (**Supplementary Table S3**). As a modification of the original MEM script,
753 herein we compared marker expression within each cluster to a statistical null reference
754 point wherein the magnitude of the median expression value of the null set was defined as
755 0, and the interquartile range (IQR) was defined as the median IQR for all features in the
756 MEM analysis (49). MEM values were then scaled from 0 (no expression) to 10 (high
757 expression) relative to the reference point. Populations were then hierarchically clustered
758 using the hclust package in R based on median marker expression, MEM value, or IQR.
759 MEM labels for each population were confirmed by biaxial gating and used to derive an
760 expert-guided population identity.

761 *Root Mean Square Deviation*

762 To compare intra- and inter-patient phenotypic similarities between automatically
763 clustered immune populations, MEM values were generated for each cluster as indicated.
764 MEM labels were then used to compare each clustered population using the Root-mean-
765 square-deviation (RMSD) calculation in the “MEM_RMSD” function included in the MEM
766 package in R (<https://github.com/cytolab/mem>). Briefly, the “MEM_RMSD” function
767 calculates the square root of the mean squared distance between every MEM value in
768 common for a given pair of cell subsets. These values are then transformed and
769 expressed as a percentage of the maximum RMSD in the analysis. Heatmaps of each
770 hierarchically clustered population based on RMSD score and a matrix of RMSD values
771 were exported from R. MEM labels were generated by concatenating all clusters within a

772 branch of the RMSD hierarchal clustering tree, and an average MEM value for each marker
773 was generated.

774 *Analysis of phospho-signaling*

775 Analysis of immune cell phospho-signaling under different cytokine stimulation
776 conditions was performed in Cytobank. Baseline phospho-signaling was compared in
777 biaxially-gated populations. Changes in protein phosphorylation under stimulation
778 conditions were normalized to basal signaling in unstimulated automatically clustered
779 FlowSOM populations. Briefly, an equal number of CD45+ events (3523/patient) was first
780 down-sampled from each patient's stimulation condition file before t-SNE analysis as
781 above. All immune phenotypic markers were used to generate the t-SNE plot, while
782 phospho-protein readouts were excluded. FlowSOM clustering analysis was performed on
783 the t-SNE axes and the number of FlowSOM clusters was optimized to minimize variance
784 in marker expression. The median expression value for each phospho-protein readout from
785 each stimulation condition was then compared to the median expression value from
786 unstimulated cells using the median arcsinh transformation. A signaling threshold ≥ 0.2 or \leq
787 -0.2 in arcsinh transformed median phospho-protein expression over baseline was
788 considered a cellular response to each stimulation condition.

789 *Survival Analysis*

790 Correlations of immune subset abundance with overall and progression-free survival
791 were performed using the Risk Assessment Population IDentification algorithm (RAPID) in
792 R (13). Briefly, t-SNE analyses were performed by down-sampling live CD45+ events from
793 each patient file and optimizing the number of FlowSOM clusters as described above. The
794 frequency of cells from each patient within each cluster was then used to stratify patients

795 into high and low cluster abundance based on the interquartile distribution of the subset
796 across the entire cohort. A univariate Cox regression model was then used to estimate the
797 hazard ratio of death and determine statistical significance using in the “survival” package
798 in R. Overall survival (OS) was defined as time from surgical resection to death. Survival
799 time was censored if, at last follow-up, the patient was known to be alive and had not had
800 radiographic tumor progression. Differences in the survival curves were compared using
801 the Cox univariate regression model, reporting a hazard ratio (HR) between the survival
802 curves. Statistical significance α was set at 0.05 for all statistical analyses. RAPID analysis
803 was performed on 10 different t-SNE analyses resampled from each patient. Clusters from
804 each independent analysis that met an HR threshold >1 or <-1 and p-value <0.1 were
805 isolated and RMSD was performed to determine the stability of clusters with similar
806 phenotypes identified by RAPID.

807 A modified version of the RAPID algorithm (mmRAPID) was used to correlate
808 immune receptor expression with patient outcome. Dimensionality reduction was performed
809 using t-SNE on 16 of the clustering markers used in the Citrus median marker analysis
810 (**Supplementary Table S2**). FlowSOM clustering was then performed on the t-SNE axes
811 to minimize the variance in marker expression across all clusters as previously described.
812 The arcsinh transformed median marker expression of 17 markers of interest was used to
813 stratify patients into high and low expression based on the interquartile distribution of the
814 arcsinh transformed values across the entire cohort for each cluster. A univariate Cox
815 regression model was used to estimate the hazard ratio of death as was performed for
816 immune abundance. A similar analysis correlating phospho-signaling to survival outcome
817 was performed using the arcsinh transformed phospho-protein expression values

818 normalized to the unstimulated baseline levels for each patient. Marker expression was
819 validated by biaxial gating and generation of MEM labels for high and low groups.

820 *Statistical Analysis*

821 Statistical analysis was performed in Cytobank as a part of advanced analyses, in R
822 version 3.6.1, or Graphpad Prism version 8.4.3 where indicated. All analyses were graphed
823 in the Graphpad Suite. Outlier analysis was performed before all statistical analyses using
824 the ROUT method with Q=1%. Statistical analysis of 2 groups was performed using a two-
825 sided student's T test with Welch's Correction. Analysis of 3 or more groups was
826 performed where indicated using a one-way or two-way ANOVA with a Tukey or Sidak
827 correction for multiple hypothesis testing, respectively. Immune subset enrichment was
828 statistically determined using a Chi Squared or Fishers Exact Test where indicated.
829 Statistical correlations of immune subset abundance were performed using a two-tailed
830 Pearson Correlation. For all statistical tests unless otherwise indicated, p values of <0.05
831 were considered significant. Graphs show median \pm IQR unless otherwise indicated.

832 **Data Availability**

Datasets analyzed in this manuscript will be made available online, including at FlowRepository (50), for reviewers and at the time of publication. Transparent analysis scripts for datasets in this manuscript (first shown in **Figure 2** and **Figure 5**) will be publicly available on the CytoLab Github page (<https://github.com/cytolab/GBM-IMM01>) with open-source code and commented Rmarkdown analysis walkthroughs.

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843 T.B., R.A.I., and J.M.I. designed the study. T.B., M.J.H., J.S., N.L., and C.E.R.
844 collected data. T.B. and S.M.L. developed data analysis scripts. T.B., S.M.L., R.A.I., and
845 J.M.I. performed mass cytometry data analysis and interpretation. A.M.M. scored MRI
846 images for tumor contact with the lateral ventricle and provided patients' clinical
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850 manuscript.

851 **Declaration of interests**

852 All authors declare no competing interests.

853

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- 1010

1011 **Figure Legends**

1012 **Figure 1: Lateral ventricle contacting and non-contacting glioblastomas are enriched**
1013 **in distinct immune subsets.**

1014 **a)** Representative MRI radiographs of WHO grade IV glioblastoma tumors with confirmed
1015 contact with either of the lateral ventricles (left, C-GBM) or lacking ventricular involvement
1016 (right, NC-GBM). Yellow line indicates the lateral ventricle and arrows indicate the tumor
1017 mass. **b)** Tissue was obtained from patients with radiographically confirmed ventricle
1018 contacting (C-GBM, red) (n=12) or non-contacting (NC-GBM, blue) (n=13) glioblastomas,
1019 temporal lobectomy of parenchymal tissue from epileptic patients (light grey) (n=2) or
1020 peripheral blood mononuclear cells (PBMC, dark grey) from healthy volunteers. Single-cell
1021 suspensions were processed, and mass cytometry was performed before multiple high-
1022 dimensional analysis platforms assessed the immune phenotypes. **c)** Bar graphs
1023 demonstrating the frequency of expert-gated immune populations in epileptic brain (white),
1024 healthy donor PBMC (dark grey), or glioblastoma tumors (light grey). **d)** Bar graphs
1025 demonstrating the total leukocyte infiltration (left) and lymphoid and phagocyte fractions
1026 (right) in epileptic brain (Epi), healthy donor blood (PBMC), C-GBM (C) or NC-GBM (NC).
1027 **e)** Representative biaxial plots demonstrating the frequency of CD45⁺CD3⁺ T cells and
1028 CD45⁺CD3⁻ non-T cells in PBMC, epileptic brain tissue, C-GBM and NC-GBM tumors
1029 respectively. **f)** Representative t-SNE plots of all CD45⁺ leukocytes identified in the blood,
1030 non-tumor bearing brain, or GBM tumors (heat) overlaid onto all CD45⁺ events. Multi-color
1031 overlay represents expert gated populations overlaid onto the t-SNE axes. **g)** Live CD45⁺
1032 cells were merged from all GBM patients (black contour), patients with C-GBM tumors (red
1033 contour), or NC-GBM tumors (blue contour). Overlaid t-SNE plots indicate areas of immune
1034 infiltration unique to LV contacting or non-contacting patients. Enrichment indicates which

1035 computationally gated immune populations were statistically enriched in LV-contacting or
1036 non-contacting gliomas. Heat displayed for various markers indicates phenotypes for major
1037 immune subsets. **h)** Bar graphs demonstrating the frequency of immune cells found within
1038 each computational cluster as a percent of total CD45+ leukocytes. Bars indicate median \pm
1039 IQR. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

1040 **Figure 2: Differential enrichment of five immune phenotypes distinguish ventricle**
1041 **contacting and non-contacting glioblastoma.**

1042 **a)** Citrus clustering of live CD45+ leukocytes in the tumor microenvironment of C-GBM and
1043 NC-GBM tumors revealed differential enrichment of five immune subsets. Representative
1044 biaxial gating of pooled Citrus clusters and Marker Enrichment Modeling (MEM) population
1045 identification classified each cluster as **(b)** $\gamma\delta$ and CD4⁻CD8⁻ DNT cells, **(c)** microglia, **(d)**
1046 and **(e)** macrophages and **(f)** lymphocyte populations of CD8 T cells, B cells and NK cells.
1047 **(g)** Heatmaps of each phenotypic marker used to classify each immune subset reveal the
1048 expression levels of each immune receptor. **(h)** Quantification of the arcsinh transformed
1049 expression level of each immune marker within each subset. Representative t-SNE plot of
1050 all CD45+ leukocytes infiltrating a C- GBM tumor **(i)** or NC-GBM tumor **(j)**. Cell density (left
1051 t-SNE), FlowSOM clustering on the t-SNE axes (middle), and Citrus overlay and
1052 quantification (right) determined the relative frequency of each immune cell subset within
1053 each patient sample. A subset of color-coded MEM labels identified the phenotype and
1054 frequency of key Citrus-identified immune populations. In **(a)**, a regularized regression
1055 model was used as a final step in the Citrus analysis to identify stratifying clusters.
1056 Predictive analysis of microarrays (PAM) stratified immune clusters. A False Discovery
1057 Rate <1% (q) was used to determine significance in all instances.

1058 **Figure 3: Immune subset frequencies correlate with patient outcome.**

1059 **a)** Kaplan-Meier curves indicating overall survival in GBM patients with high vs low
1060 frequencies of Citrus-identified immune populations. **b)** Kaplan-Meier curves for overall
1061 survival in immune subsets stratifying patient outcome identified by RAPID analysis. T-SNE
1062 plots in b indicate the cell density (left), cluster number (middle) and p-value of the hazard
1063 ratio associated with the frequency of each cluster in the entire cohort. Calculated MEM
1064 labels identified key features of stratifying immune subsets.

1065 **Figure 4: Immunosuppressive checkpoint receptors are enriched in ventricle**
1066 **contacting glioblastomas.**

1067 **a)** Representative t-SNE plot indicating the density of all CD45+ leukocytes (left), FlowSOM
1068 clusters on the t-SNE axes, and overlaid immune populations with enriched expression of
1069 indicated immune markers. MEM labels indicate the cellular phenotype in which the
1070 indicated markers were differentially expressed. **b)** Box and whisker plots indicating the
1071 arcsinh transformed median expression values of indicated immune receptors within Citrus-
1072 identified populations of interest. **c)** Histograms of pooled patient Citrus clusters from C-
1073 GBM (red) and NC-GBM patients (blue). **d)** Representative biaxial plot (left) and box and
1074 whisker plots indicating the frequency of CD32⁺CD44⁺ macrophages identified by Citrus. **e)**
1075 Representative biaxial plots and box and whisker diagrams demonstrating the frequency of
1076 TIGIT and PD-1 co-expression in CD4 T cells **(e)** and CD8 T cells **(f)** infiltrating
1077 glioblastoma tumors. In **(a)**, a regularized regression model was used to as a final step in
1078 the Citrus analysis to identify stratifying clusters. Predictive analysis of microarrays (PAM)
1079 stratified immune clusters. A False Discovery Rate of 1% (q) was used to determine

1080 significance in all instances. Bars indicate median \pm IQR. * = $p < 0.05$, ** = $p < 0.01$, *** =
1081 $p < 0.001$, **** = $p < 0.0001$.

1082 **Figure 5: Immune receptor expression correlates with patient outcomes.**

1083 **a)** T-SNE plots representing CD45+ cells pooled from n=19 GBM patients and enumeration
1084 of FlowSOM clusters on the t-SNE axes. Colored clusters indicate clusters identified by
1085 mmRAPID for which expression of the indicated markers stratify patient outcomes. **b-k)**
1086 Kaplan-Meier curves indicating overall survival in immune subsets stratifying patient
1087 outcome identified by mmRAPID analysis. Calculated MEM labels identified key features of
1088 stratifying immune subsets. Box and whisker diagrams plot the arcsinh transformed
1089 median mass intensity for PD-1 expression (**b, c**), CD32 (**d**), CD44 (**e**), CD69 (**f, g**), and
1090 HLA-DR (**h, i, j, k**) in stratified high and low groups.

1091 **Figure 6: Immune cells infiltrating GBM tumors are functional and responsive to**
1092 **cytokine stimulation.**

1093 **a)** Heatmaps indicating the arcsinh fold transformed median intensity values of each
1094 indicated phosphoprotein within each manually gated immune subset in healthy donor
1095 PBMC (grey), C-GBM tumors (red), or NC-GBM tumors (blue). **b)** Representative t-SNE
1096 plot indicating the density of CD45+ leukocytes (left), enumerated FlowSOM clusters
1097 (middle) and overlay of expert-gated immune populations onto the clustered t-SNE axes
1098 (right). Representative heatmaps on the t-SNE axes indicate the cluster-specific median
1099 arcsinh-fold change of the indicated phosphoprotein under IL-2 stimulation conditions
1100 compared to basal phosphorylation. **c)** Box and whisker plots indicating the proportion of
1101 clusters in C-GBM or NC-GBM immune infiltrates surpassing the phospho-signaling
1102 threshold (>0.2 arcsinh fold change) in response to IL-2 stimulation. **d)** Graphs indicating

1103 the arcsinh transformed phosphoprotein median intensities for each phosphoprotein in
1104 each of 42 IL-2 stimulated immune clusters. Graphs indicate the median \pm IQR. Black
1105 horizontal lines in d indicate signaling threshold for each arcsinh transformed value. Black
1106 dotted boxes in d indicate clusters for which the arcsinh transformed median mass intensity
1107 surpassed the signaling threshold (± 0.2 -fold change) in either the C-GBM or NC-GBM
1108 cohort). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

1109 **Figure 7: Model of cell signaling networks in GBM immune infiltrates.**

1110 Graphical representation of immune cell signaling networks. For each cytokine stimulation
1111 condition implemented (rows) and each cell population of interest (columns), an aggregate
1112 signaling diagram was generated. Signaling nodes in red indicate active signaling
1113 responses to the indicated cytokine stimuli in C-GBM tumors and blue nodes indicate active
1114 signaling responses in NC-GBM. Purple nodes indicate protein phosphorylation in
1115 response to stimuli in both patient cohorts.

Figure 1

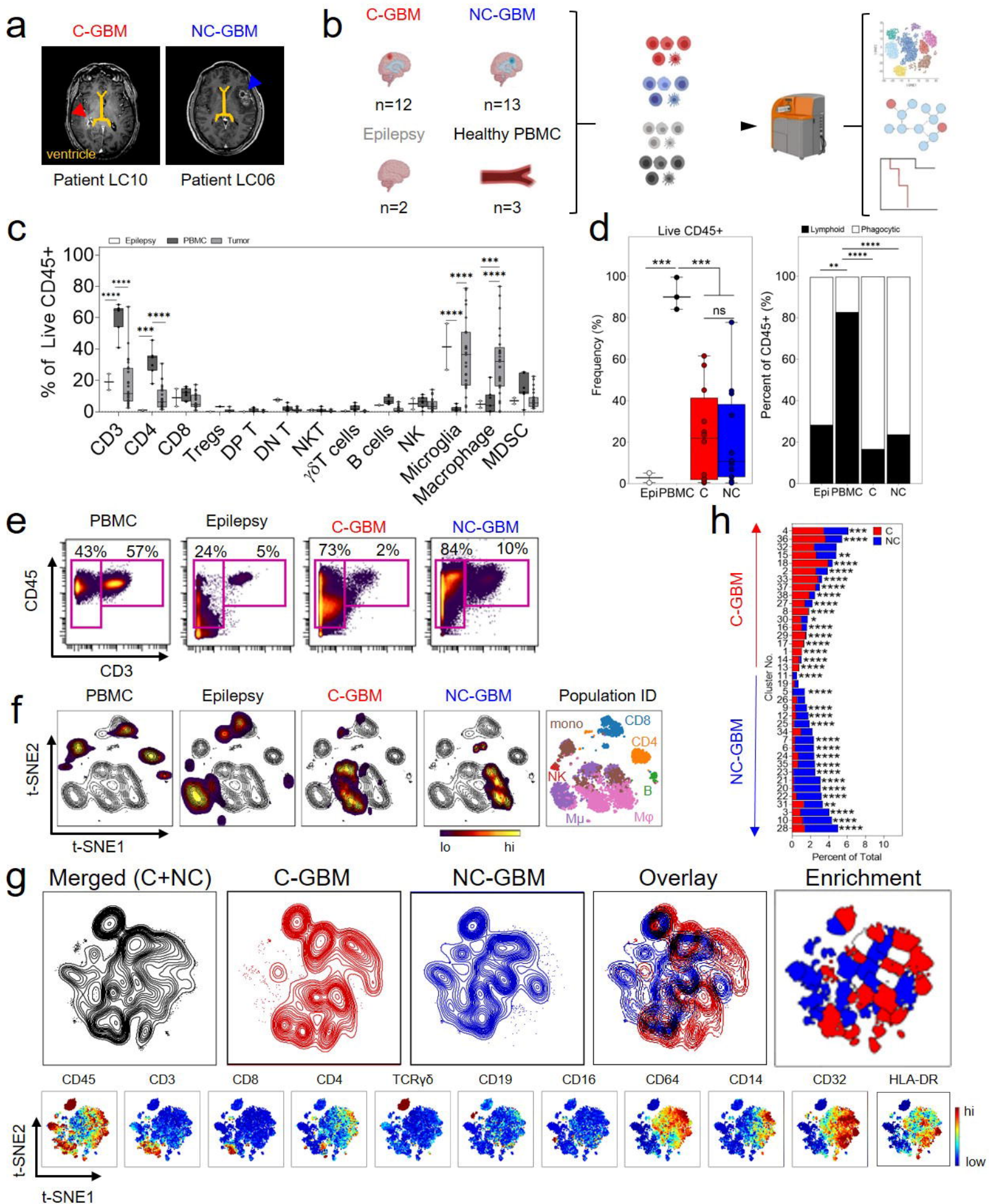


Figure 2

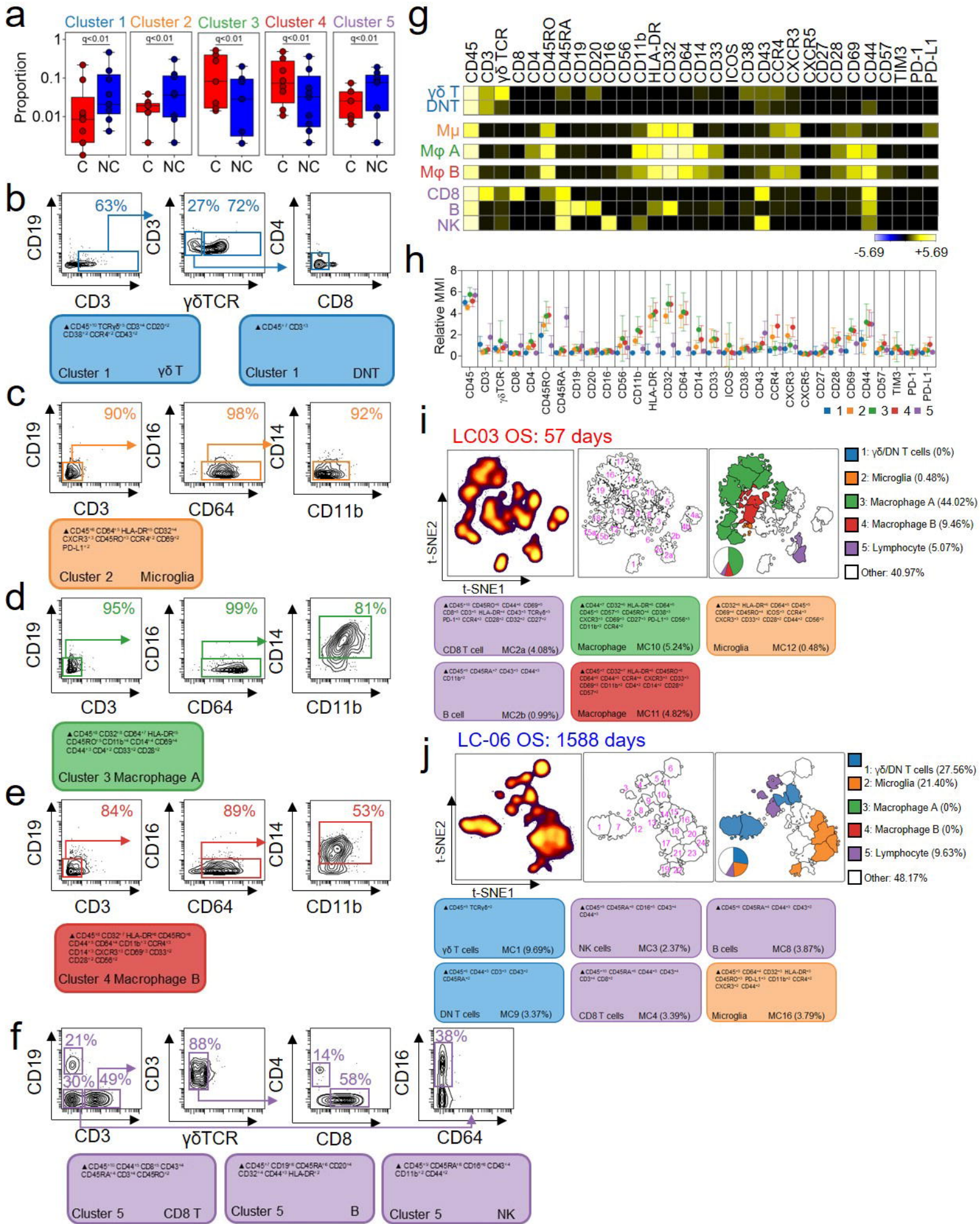


Figure 3

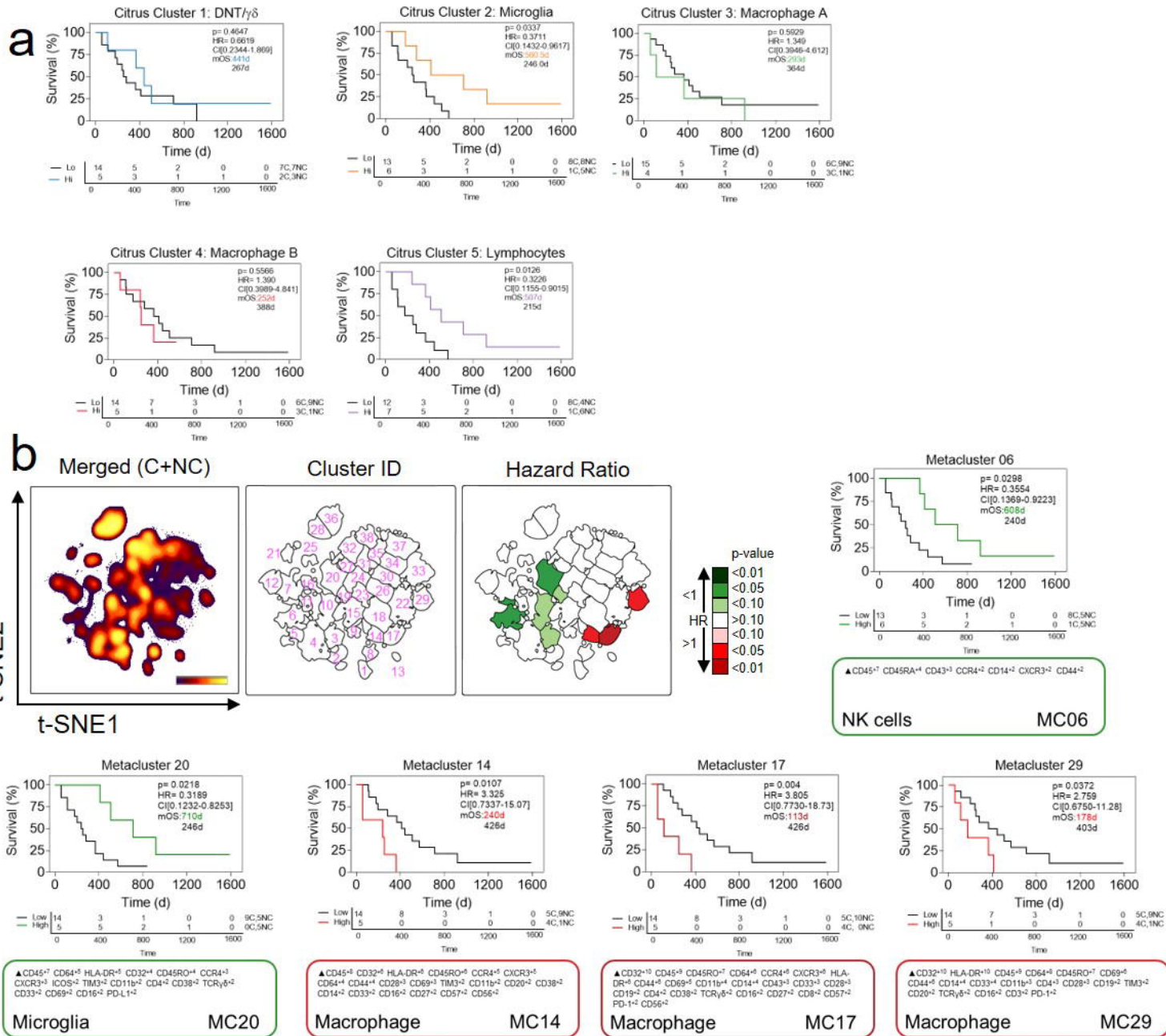


Figure 4

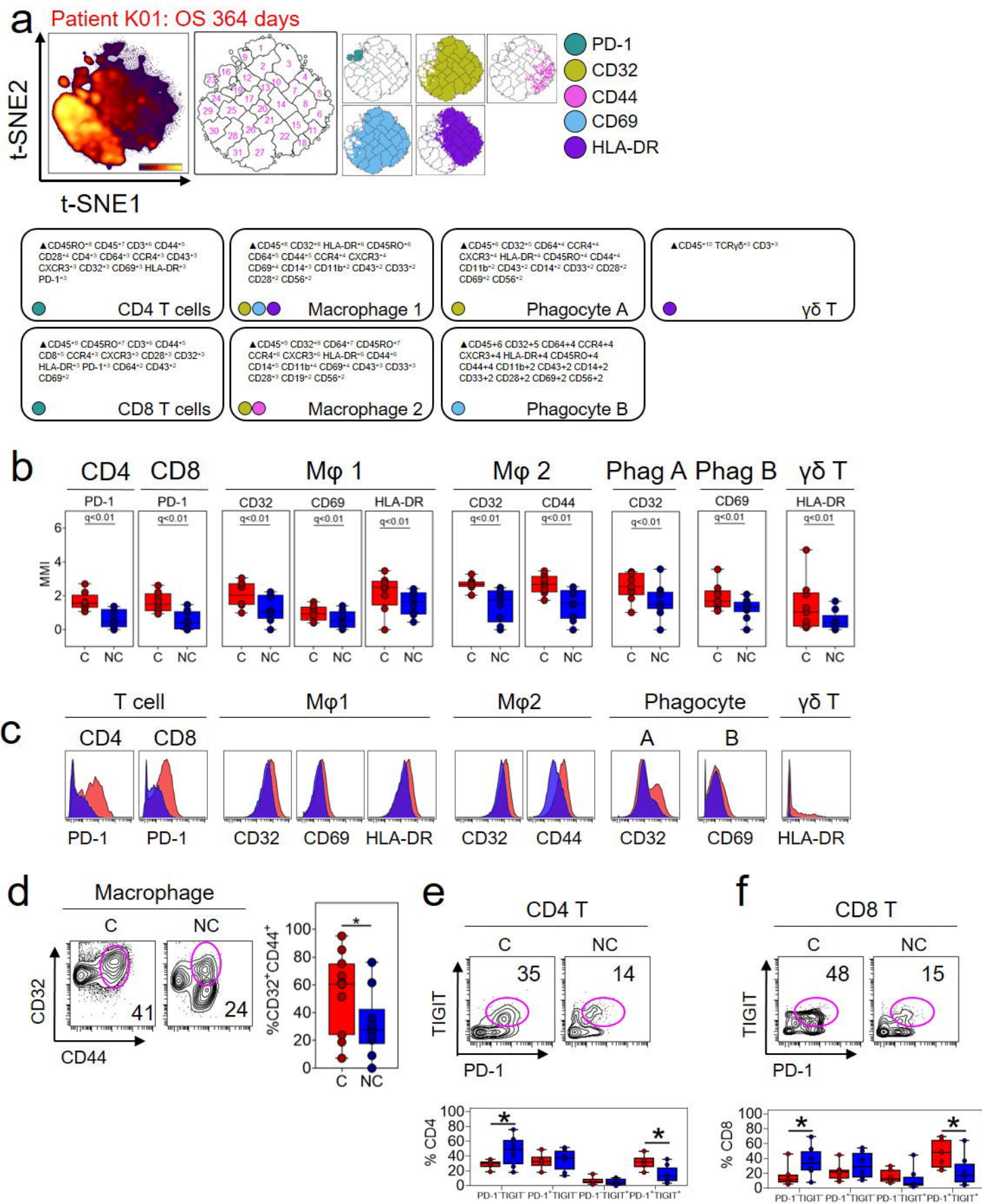


Figure 5

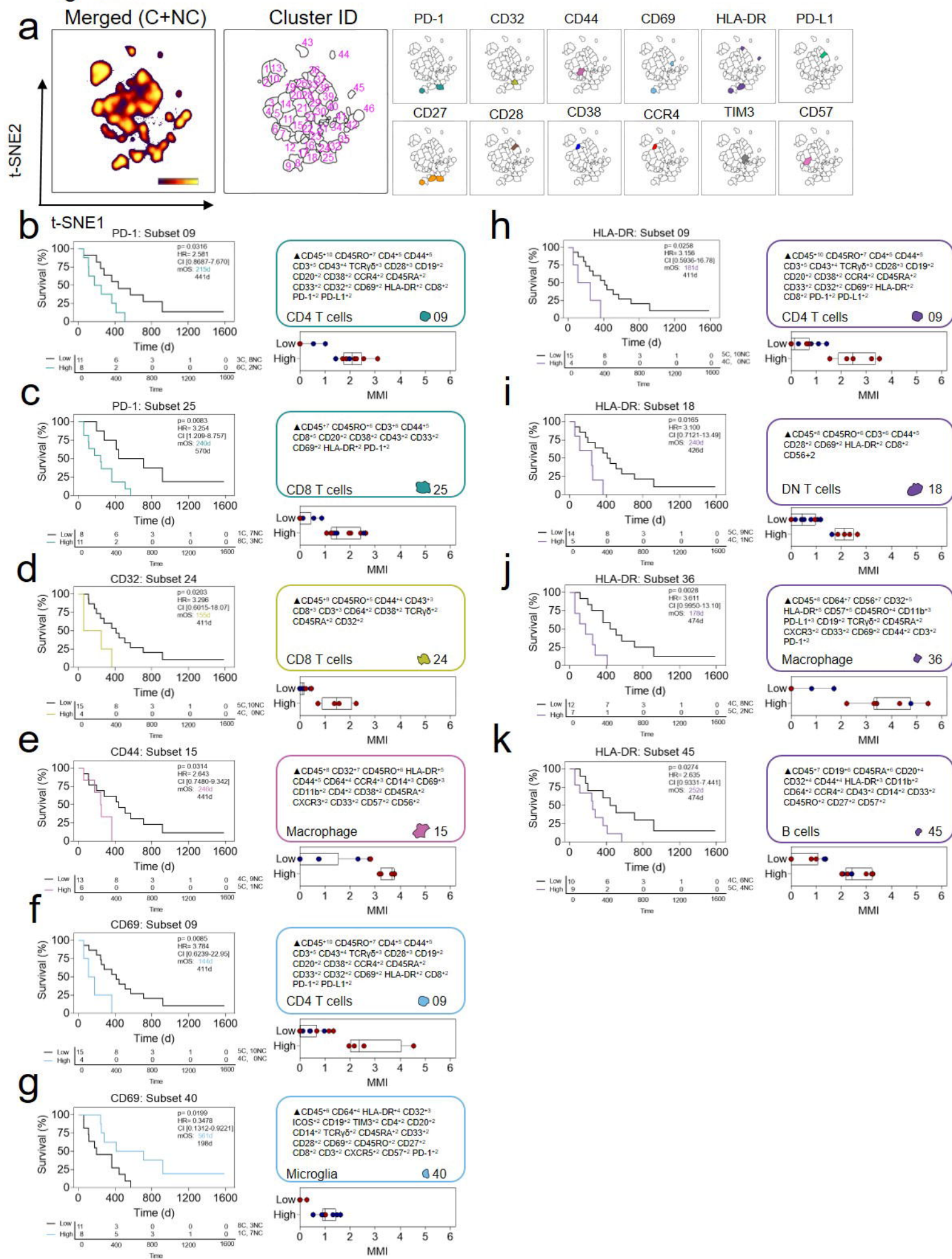


Figure 6

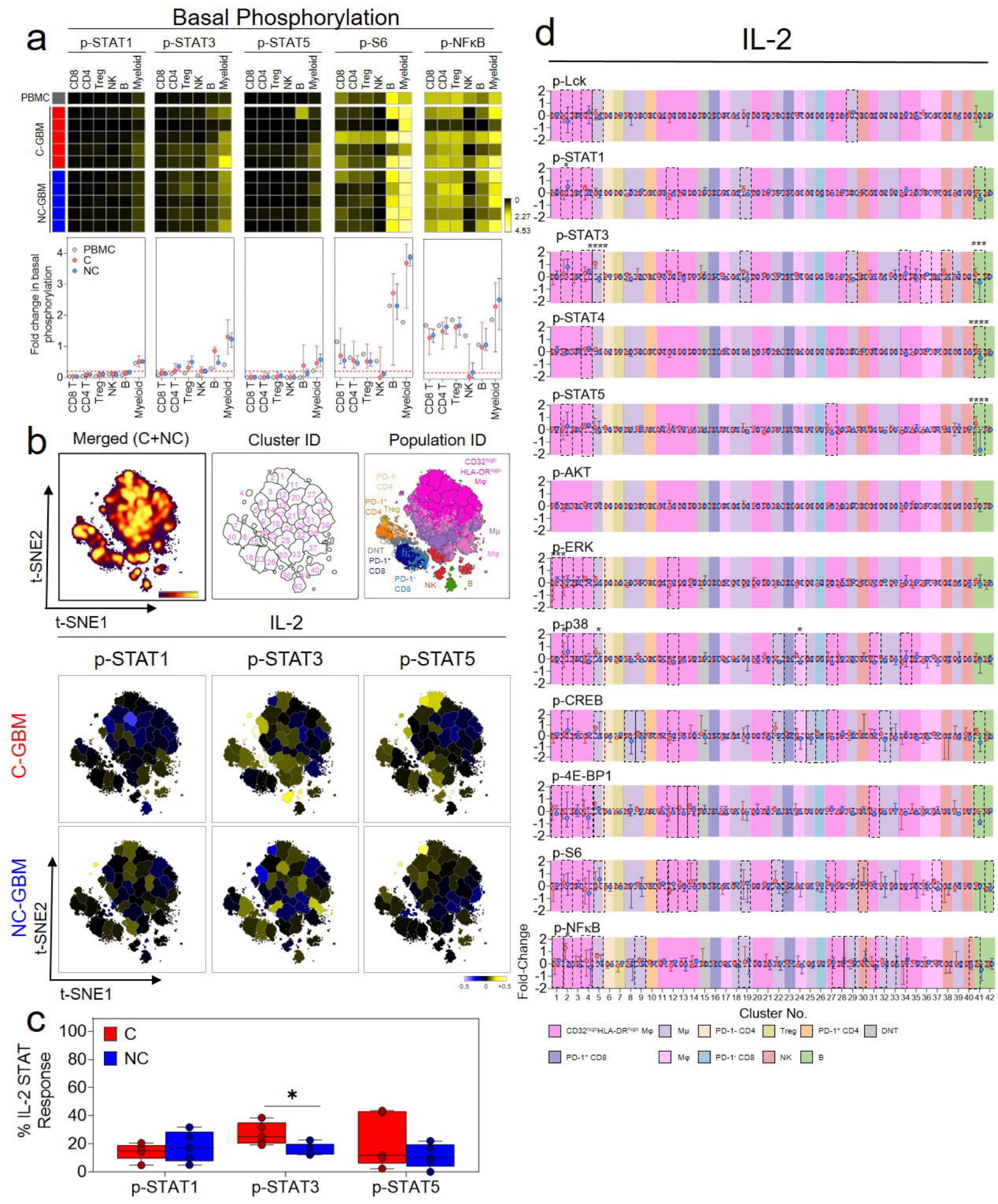


Figure 7

