

1 **Single-cell RNA sequencing reveals functional heterogeneity and sex differences of**
2 **glioma-associated brain macrophages**

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13 **Abstract**

14 Brain resident and infiltrating innate immune cells adapt a tumor-supportive phenotype in the
15 glioma microenvironment. Flow cytometry analysis supported by a single-cell RNA
16 sequencing study of human gliomas indicate considerable cell type heterogeneity. It remains
17 disputable whether microglia and infiltrating macrophages have the same or distinct roles in
18 supporting glioma progression. Here, we performed single-cell transcriptomics analyses of
19 CD11b+ cells sorted from murine syngeneic gliomas, indicating distinct activity of microglia,
20 infiltrating monocytes/macrophages and CNS border-associated macrophages. Our results
21 demonstrate a previously immeasurable scale of molecular heterogeneity in the innate
22 immune response in gliomas. We identified genes differentially expressed in activated
23 microglia from glioma-bearing mice of different sex, and profound overexpression of the
24 *MHCII* genes by male microglial cells, which we also observed in bulk human glioma
25 samples. Sex-specific gene expression in microglia in the glioma microenvironment may be
26 relevant to sex differences in incidence and outcomes of glioblastoma patients.

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28

29 **Introduction**

30 Infiltrating immune system cells represent an abundant non-malignant component of the
31 tumor microenvironment (TME). These cells play a pivotal role in tumor progression and
32 modulation of tumor responses to therapy¹. A high number of macrophages within TME have
33 been associated with a poor prognosis in many cancers because those tumor-educated cells
34 suppress anti-tumor immunity, stimulate angiogenesis, and promote tumor invasion².

35 The central nervous system (CNS) is equipped with resident innate immune cells—microglia
36 and CNS border-associated macrophages (BAMs)—consisting of perivascular, meningeal,
37 and the choroid plexus macrophages. Those cells migrate to the CNS early in the prenatal
38 life and maintain a long-lasting population. In malignant gliomas, besides activation of local
39 microglia, circulating monocytes invade the brain from the periphery and differentiate within
40 the tumor; therefore, microglia and infiltrating monocytes/macrophages are commonly
41 referred to as glioma-associated macrophages (GAMs), due to the shortage
42 of immunocytochemical markers allowing their reliable identification³. Transcriptome profiling
43 of bulk CD11b+ cells isolated from human glioblastomas (GBMs) and rodent gliomas showed
44 a mixture of protumorigenic and antitumorigenic phenotypes and did not reveal consistent
45 markers and pathways^{4–6}. Recent reports have demonstrated that GAMs consist of diverse
46 cell populations with likely distinct roles in tumor progression^{7–10}. Dissecting the TME
47 composition and functional heterogeneity of tumor-infiltrating immune cells would extend the
48 understanding of the glioma immune microenvironment and allow to modulate functions of
49 distinct subpopulations for therapeutic benefits.

50 Sex differences in incidence, transcriptomes, and patient outcomes in the adult GBM
51 patients have been reported, providing an explanation for GBM occurrence with a male-to-
52 female ratio of 1.6:1¹¹. The sex-specific outcome can be related to immune function because
53 the efficacy of cancer immunotherapy was found to be sex dependent, with better outcomes
54 in males¹². Transcriptomic studies of male and female microglia demonstrated that in naïve
55 mice, male microglia show enrichment of inflammation and antigen presentation-related
56 genes, whereas female microglia have a higher neuroprotective capacity^{13,14}. Until now, sex
57 differences have been largely unexplored in animal studies on glioma immunobiology.

58 Here, we used single-cell transcriptomics (scRNA-seq) to decipher the composition
59 and functions of GAMs in murine experimental gliomas growing in male and female mice.
60 We demonstrate distinct transcriptional programs of microglia, infiltrating
61 monocytes/macrophages, and CNS BAMs. The identified microglia and
62 monocyte/macrophage signature markers allow for clear separation of these cells within
63 glioma TME. We demonstrate that in the presence of glioma, microglia and

64 monocytes/macrophages activate similar transcriptional networks. However, the
65 transcriptional response of monocytes/macrophages is more pronounced and associated
66 with activation of immunosuppressive genes. A subset of macrophages expresses *Cd274*
67 mRNA (coding for PD-L1). Moreover, we found that in males, both microglia and a
68 substantial fraction of monocytes/macrophages from gliomas express a higher level of the
69 *MHCII* genes, an observation that correlates with an increased number of CD4+ T cells in the
70 male versus female TME. This work demonstrates a cell type heterogeneity and sex-specific
71 differences of immune cells infiltrating gliomas.

72

73 **Results**

74 **Single-cell RNA-seq identifies distinct subpopulations amongst CD11b+ cells from** 75 **naïve and glioma-bearing brains**

76 We employed a murine orthotopic GL261 glioma model because tumors established from
77 GL261 cells recapitulate many characteristics of human GBMs and are frequently used to
78 study glioma immunology, immunotherapy, and in preclinical studies¹⁵. To assess the
79 heterogeneity of GAMs in GL261 gliomas, we performed scRNA-seq on CD11b+ cells sorted
80 from naïve and tumor-bearing brains of male and female mice (two replicates per group)
81 **(Figure 1a)**. The tumor-bearing animals were sacrificed 14 days post implantation. This time
82 point corresponds to a pre-symptomatic stage of tumorigenesis, when tumors are confined to
83 the striatum and restricted to a single hemisphere **(Supplementary Figure 1a-d)**. At that
84 stage, GL261 gliomas show a substantial infiltration of peripheral monocytes/macrophages¹⁶,
85 and no signs of necrosis. Using flow cytometry, we sorted the CD11b+ cells of a particularly
86 high purity (>96%) and viability (~95%) **(Supplementary Figure 1e)**. Inclusion of live/dead
87 cell discrimination during sorting did not result in further improvements of cell viability;
88 therefore, we omitted this step in order to shorten sample preparation time.

89 To resolve the molecular profiles of the sorted cells, we analyzed their transcriptomes
90 using a single-cell RNA-seq technique. After quality control and accounting for technical
91 noise, single-cell transcriptomic profiles for 40,401 cells and 14,618 genes were considered
92 for the analysis (see Methods for details). Unsupervised clustering of each group
93 demonstrated a similar number of clusters between sexes. For naïve female and male
94 CD11b+ cells, 9 and 8 clusters were obtained, respectively, and 13 clusters were identified
95 for CD11b+ cells from tumor-bearing hemispheres, for both sexes. We visually inspected the
96 transcriptomic diversity of computed clusters, projecting the data onto two dimensions by t-
97 distributed stochastic neighbor embedding (t-SNE) **(Figure 1b)**. To characterize the cell
98 identity of the obtained clusters, we applied the immune cell marker panel **(Figure 1c)**

99 created with the literature-based markers (**Supplementary Table 1**)^{3,7,8,10,17-29}. The cellular
100 identities were inferred by identifying significantly overexpressed genes in each cluster. In
101 naïve brains, microglia (MG) comprised the vast majority of all sorted cells (91% in females,
102 90% in males), whereas BAMs constituted 6% of cells in both sexes (**Figure 1d**).
103 Additionally, besides the major microglial clusters, we identified a minor population of
104 premature microglia (pre-MG) expressing markers of microglia (*Tmem119*, *P2ry12*) and
105 genes characteristic for their premature state (*Csf1*, *Crybb1*, *Mcm5*, *Ifit3*)²⁰. Amongst
106 CD11b+ cells from male controls, we found a small subset of monocytes (Mo, *Ly6c2*+,
107 *Ccr2*+), natural killer (NK, *Ncam1*+), and dendritic cells (DC, *Cd24a*+), all of which were not
108 found in female controls.

109 In tumor-bearing brains, microglia were still the most abundant cell population (64%
110 in females, 65% males), although their proportion decreased due to infiltration of
111 monocytes/macrophages (Mo/MΦ) from the periphery, forming the 2nd main myeloid cell
112 population of the TME (23% in females, 28% in males) (**Figure 1d**). For both sexes, we
113 identified 3 clusters of infiltrating Mo/MΦ that could be further characterized by an
114 inflammatory monocyte signature—Mo (*Ly6c2*^{high}, *Ccr2*^{high}, *Tgfb*^{low})—, an intermediate state
115 of monocyte and macrophage signature—intMoMΦ (*Ly6c2*⁺, *Ccr2*^{high}, *Tgfb*^{high})—, and a
116 differentiated macrophage signature—MΦ (*Ly6c2*^{low}, *Ifitm2*^{high}, *Ifitm3*^{high}, *S100a6*^{high}) (**Figure**
117 **1c**). These results demonstrate the dynamics of monocyte/macrophage phenotypic changes
118 upon infiltration to the TME. We also identified smaller populations of NK cells, DCs, natural
119 killer T cells (NKT), and a minor fraction of B and T cells. Although CD11b+ is generally not
120 expressed on lymphocytes, sparse populations of those cells expressing CD11b+ (>1%) may
121 appear after activation of the immune response^{30,31}. Nevertheless, a vast majority of cells
122 were MG, Mo/MΦ, and BAM; thus, we focused on those major cell types in further analyses.

123 **Assessment of new and known cell type specific markers**

124 To identify the molecular features that distinguish naïve and tumor-associated myeloid cells,
125 we performed further analyses on preselected cell subpopulations. From all the conditions
126 and replicates, we extracted only the cells identified as microglia, monocytes/ macrophages,
127 and BAMs. The combined three cell subpopulations, projected on the two-dimensional space
128 using a Uniform Manifold Approximation and Projection (UMAP) algorithm, formed three
129 separate groups that corresponded to the previously identified cell subpopulations (**Figure**
130 **2a**). This observation demonstrates a predominance of a biological signal over technical
131 artifacts or batch effects. To confirm cell identities, we performed differential expression
132 analyses between these three subpopulations of CD11b+ cells. Among the most highly
133 upregulated genes in each group (see Methods), we found the well-known microglial
134 genes—*Cx3cr1*, *Olfml3*, *Gpr34*, *Tmem119*, *Selplg*, *Sparc*, and *P2ry12*^{18,32}—, monocyte

135 genes—*Ly6i*, *Ly6c2*, macrophage genes—*Ifitm3*¹⁰—, and BAM genes—*ApoE*, *Ms4a7*,
136 *Mrc1*³³. These features confirmed the identity of the distinguished cell populations (**Figure**
137 **2b**). Microglial cell group was best characterized by the expression of *Tmem119*, *Cx3cr1*,
138 *P2ry12*, *Gpr34*, *Olfml3*, and *Sparc* (**Figure 2c**). Some highly expressed microglial genes
139 were found only in a fraction of cells (e.g., the *P2ry13* gene was found in less than 75% of
140 microglial cells); other microglial genes including *Fcrls* and *Cd81* were also expressed in
141 BAMs and *Hexb*, and *Cst3* was expressed both in BAMs and monocytes/macrophages
142 (Mo/MΦ). For Mo/MΦ, we found the specific expression of previously reported genes such
143 as *Ifitm2*, *S100a6*, and *S100a11*¹⁰, as well as novel genes, namely *Lgals3*, *Isg15*, *Ms4a4c*,
144 and *Crip1* (**Figure 2d**). *Ifitm3* was highly expressed by the Mo/MΦ population, but it has been
145 found in a substantial fraction of microglial cells, showing its low specificity towards
146 monocytes/macrophages within glioma TME. Interestingly, among the highly upregulated
147 Mo/MΦ genes, we found markers characterizing discrete subpopulations of the Mo/MΦ. The
148 high *Ly6c2* expression was found in a large cell fraction, which could be further divided into
149 *Ly6c2*^{high}*Ccr2*^{high} monocytes (Mo) and *Ly6c2*^{high}*Tgfb*^{high} monocyte/macrophage intermediate
150 cells (intMoMΦ) (**Figure 2e-g**). The remaining cells resembled differentiated tissue
151 macrophages (MΦ because they lacked the markers of the cytotoxic monocytes *Ly6c2* and
152 *Ccr2* and had a strong “macrophage” signature (*Ifitm2*^{high}, *S100a6*^{high}, *S100a11*^{high}). Notably,
153 we found a population of macrophages (MΦ) expressing a high level of *Cd274* (a gene
154 coding for PD-L1, an immune checkpoint protein), and *Ccl22*, *Ccl5*, which are chemokines
155 important for T-cell recruitment^{34,35}. Such an expression pattern suggests a putative role of
156 those cells in mediating the immunosuppressive response. Additionally, macrophages highly
157 and specifically expressed *Tmem123*, a gene of unknown function in microglia and
158 macrophages, encoding a transmembrane protein Porimin that mediates cell death in Jurkat
159 cells³⁶ and is expressed in mature dendritic cells³⁷.

160 We also examined the expression of genes that have been recently postulated as
161 specific markers of infiltrating monocytes/macrophages within the TME of glioma: *Itga4*⁷, *Hp*,
162 *Emilin2*, *Sell*, and *Gda*¹⁷ (**Figure 2d**). The expression of *Itga4* (CD49d) was generally low
163 and limited mostly to the MΦ subpopulation that resembles the most differentiated fraction of
164 the infiltrating macrophages and expresses a high level of *Cd274* (PD-L1). Low *Itga4*
165 expression in our data set might be explained by an early tumor growth stage (2 weeks after
166 glioma cell implantation) and the high content of cytotoxic monocytes compared to
167 macrophages. In a previous study, samples were collected at later time-points—3 weeks
168 after implantation of GL261 cells and 5 weeks after implantation of shP53-transfected cells⁷.
169 The expression of *Hp*, *Emilin2*, *Sell*, *Gda*, the markers suggested in a recent meta-analysis
170 of bulk RNA-seq data sets and validation at RNA and protein levels¹⁷, was found in the
171 fraction of Mo (*Ly6c2*^{high}, *Ccr2*^{high}). In addition, we examined the expression of *Tgm2* and

172 *Gpnmb*, previously reported as the genes commonly upregulated by GAMs across different
173 glioma animal models and in patient-derived samples in a bulk RNA-seq meta-analysis³
174 (**Figure 2h**). Surprisingly, their expression was limited to the small fraction of Mo/MΦ,
175 demonstrating that bulk RNA-seq results may be biased by genes expressed at a high level
176 by a small subset of cells.

177 The third most abundant cell population in our data set were BAMs, marked by highly
178 expressed *ApoE* and *Ms4a7* genes that were recently proposed as universal markers of
179 macrophages residing at the CNS borders³³. *ApoE* and *Ms4a7* were also expressed by
180 Mo/MΦ; however, we found other highly expressed BAM genes (*Mrc1*, *Dab2*, *F13a1*, *Mgl2*,
181 and *Pf4*) to be better for identifying the BAM population (**Figure 2i**). Summarizing, we
182 validated the expression of known markers at the single-cell level and obtained a good
183 agreement of selected microglia and BAM markers in our data set with literature data. In
184 contrast, Mo/MΦ showed substantial heterogeneity that is likely related to their differentiation
185 state. We identified genes characterizing the specific monocyte/macrophage subpopulations.
186

187 **Distinct gene expression profiles of glioma-associated microglia and** 188 **monocytes/macrophages**

189 Distribution of cells according to the conditions (naive vs tumor) revealed separation of
190 functional subgroups of microglia. This separation was further supported by the unsupervised
191 clustering that led to clusters either highly enriched in the cells from naïve brains
192 representing *homeostatic microglia* (Hom-MG) or clusters dominated by cells originated from
193 the tumor-bearing hemispheres representing *glioma-activated microglia* (Act-MG) (**Figure**
194 **3a, Supplementary Figure 3**). This result demonstrates activation of microglia within the
195 TME. Invading monocytes and macrophages displayed glioma-induced gene expression
196 profiles. In contrast to microglia, we did not find any indication of the glioma-induced
197 activation in BAMs because the cells from naïve and tumor-bearing brains distributed evenly
198 and did not show any clusters of cells originating from tumor-bearing hemispheres (**Figure**
199 **3a**).

200 Using microglia and Mo/MΦ scores (defined as an average of expression levels of
201 genes specific to and highly expressed in a given population) (**Figure 3b**), we examined
202 whether the glioma microenvironment alters expression profiles of microglia and macrophage
203 “signature” genes (see for details **Supplementary Table 1**). We noticed a shift towards the
204 lower “microglia signature” score in MG from the tumor-bearing brains compared to MG from
205 the naïve brains (**Figure 3c**). Still, the “microglia signature” in MG from the tumor was strong
206 and distinguishable from Mo/MΦ, allowing for clear separation of the two cell populations
207 (**Figure 3c**). Similarly, the “macrophage signature” score was high and distinctive for the

208 Mo/M Φ population. Additionally, we performed a hierarchical clustering of cells according to
209 the expression of reported microglia and macrophage markers, resulting in clear separation
210 of microglia and Mo/M Φ (**Figure 3d**). This observation indicated that cell identity is retained
211 even under the strong influence of the glioma microenvironment.

212 Previous reports demonstrated that microglia occupy predominantly the tumor
213 periphery, whereas infiltrating monocytes/macrophages are found mostly within the tumor
214 core^{9,38}. Using double immunostaining, we demonstrate that microglia (Tmem119+, Iba1+) adopt an amoeboid morphology in the tumor proximity and localize abundantly at the tumor edge, whereas macrophages (Tmem119-, Iba1+) are observed mostly within the tumor mass for both male and female animals (**Figure 3e, f**).

218 Altogether, we show that microglia undergo glioma-induced activation reflected by upregulation of a number of genes related to cytokine signaling and extracellular matrix degradation. This activation was associated with the slight downregulation in the expression of “microglia signature” genes. Both microglia and monocytes/macrophages retain their cell identity within the TME, and the differential expression of microglia and macrophage-specific genes allows for a clear distinction between these cells.

224 **Transcriptional networks induced in microglia by glioma are present and more** 225 **pronounced in infiltrating monocytes/macrophages**

226 The results presented above demonstrate that microglia and monocytes/macrophages have
227 distinct gene expression profiles. Additionally, we sought to determine whether microglia and
228 infiltrating monocytes/macrophages have common or distinct roles in supporting glioma
229 growth. To answer this question, we examined whether MG and Mo/M Φ activate common
230 or distinct transcriptional networks. We performed this differential expression analysis in two
231 steps. Firstly, we extracted genes highly upregulated in microglial cells from glioma-bearing
232 brains (significantly upregulated genes in Act-MG compared to Hom-MG) (**Figure 4a**).
233 Subsequently, we performed the same analysis for Act-MG and Mo/M Φ cells (**Figure 4b**).
234 This work allowed us to find genes either common or specific for each subpopulation (**Figure**
235 **4c,d**). We found that the majority of genes upregulated in the Act-MG are also expressed by
236 Mo/M Φ , and their expression is usually higher in Mo/M Φ than Act-MG (**Figure 4c**). Among
237 commonly induced genes, we found *Ifitm3* and a group of genes encoding MHCII proteins;
238 *Ifitm3* has been reported as a gene, the expression of which demarcates macrophages from
239 microglia¹⁰. We found that *Ifitm3* was indeed highly expressed in monocytes/macrophages;
240 however, its expression was also induced in glioma-activated microglia, although at the lower
241 level (**Figure 4c, d**). Besides commonly upregulated genes, we also found population-
242 specific expression patterns. Act-MG showed a high expression of *Ccl3*, *Ccl4*, and *Ccl12* (the

243 chemokine-encoding genes), whereas their expression was lower in Mo/MΦ. In contrast,
244 Mo/MΦ were characterized by the high expression of *Ifitm2* and *Ccl5* genes (**Figure 4c**).

245 Next, we performed a Gene Ontology (GO) analysis of biological processes on the
246 two sets of genes—genes significantly upregulated in Act-MG compared to Hom-MG (**Figure**
247 **4e**) and genes significantly upregulated in Mo/MΦ compared to the Act-MG (**Figure 4f**). We
248 found that the glioma-induced expression in MG was specifically enriched in genes related to
249 “cytoplasmic translation” that encode ribosomal proteins, whereas in Mo/MΦ we found
250 specific enrichment in genes related to “drug metabolic process” and “purine monophosphate
251 metabolic process”. All other terms were directly related to the immune function and were
252 largely shared between the genes up-regulated in MG and Mo/MΦ. Both populations showed
253 induction of genes related to “response to bacterium” and “response to interferon-gamma”;
254 however, those terms encompassed the broader number of genes for Mo/MΦ. In addition,
255 Mo/MΦ demonstrated the more pronounced activation of the interferon-related genes
256 because we also identified enrichment of “response to interferon-beta” genes. The genes
257 related to the term “antigen processing and presentation” appeared only in Act-MG; however,
258 the majority of genes contained within this term code for the MHCII protein complex, and are
259 also upregulated in Mo/MΦ.

260 There are some shared genes that are expressed at a high level in Mo/MΦ compared
261 to their expression levels in Act-MG. The expression of *Cd52*, *Stat1*, *Isg15*, and *Usp18* is
262 mainly upregulated in subpopulations of Mo/MΦ. Proteins encoded by those genes are
263 involved in important immune processes: *Cd52* mediates costimulatory signals for T-cell
264 activation and proliferation³⁹, *Stat1* is a mediator of interferon signaling, *Isg15* stabilizes
265 STAT1 preventing premature termination of an inflammatory response⁴⁰, and *Usp18*
266 maintains microglia in a quiescent state via negative regulation of *Stat1* expression and
267 termination of interferon-induced genes⁴¹ (**Figure 4g**). Such expression patterns may
268 indicate that both microglia and monocytes/macrophages successfully initiate some elements
269 of the immune response; however, such activation is more prominent in
270 monocytes/macrophages. In addition, among genes that were highly expressed in Mo/MΦ,
271 we found *Il1b* coding for an inflammatory cytokine IL-1β along with *Il1rn* and *Il18b* coding for
272 the inhibitors of pro-inflammatory cytokines. These data, together with the high expression of
273 *Cd274* coding for PD-L1 in Mo/MΦ, suggest the specific activation of immunosuppressive
274 pathways in monocytes/macrophages (**Figure 4h**).

275

276 **Sex-related differences in microglial expression of MHCII genes and infiltration**
277 **of CD4+ T cells in gliomas**

278 Sex is an important prognostic marker in GBM patients influencing incidence and disease
279 outcomes^{11,42}. Differences between male and female microglia in naïve mice have been
280 reported^{13,14}. Thus, we sought to determine whether there are sex-related differences in the
281 expression of genes in myeloid populations within the TME. Importantly, the unsupervised
282 cell clustering showed that microglia from glioma-bearing brains, but not from naïve brains,
283 segregate into clusters that are enriched either in cells originating from female or male brains
284 **(Figure 5a, Supplementary Figure 4a)**. Similarly, we noticed the sex-driven cell clustering
285 within the intMoMΦ subpopulation of monocytes/macrophages, pointing to differences in
286 immune cell activation in male and female mice **(Figure 5a, Supplementary Figure 4b)**.

287 The search for differentially expressed genes revealed that in Act-MG from males, the
288 most highly upregulated genes are the genes coding for MHCII (*H2-Ab1*, *H2-Eb1*, *H2-Aa*)
289 and *Cd74*—encoding an invariant MHCII chain that regulates folding and trafficking of the
290 MHCII proteins **(Figure 5b)**. The increased expression of the *MHCII* genes and *Cd74*
291 colocalizes with the male-dominated cell clusters found in the Act-MG, but also in the
292 intMoMΦ population **(Figure 5c)**. This co-localization was not observed in naïve mice
293 **(Figure 5d)**. Accordingly, the cells with the high expression of *MHCII* and *Cd74* genes were
294 enriched in Act-MG and intMoMΦ in males. This enrichment was not observed in other
295 subpopulations: Hom-MG, Mo, MΦ, and BAMs **(Figure 5d)**. Moreover, intMoMΦ in males
296 upregulated *Mif*, encoding a macrophage migration inhibitory factor, an inflammatory
297 cytokine that binds with a high affinity to CD74, inducing a pro-inflammatory response and
298 CCL2-mediated macrophage migration and cell proliferation^{43,44}. The increased expression of
299 *Mif* in cells with the high expression of *MHCII* genes was restricted to the intMoMΦ
300 subpopulation and not detected in the Act-MG **(Figure 5e)**. This observation suggests that
301 although high expression of *MHCII* genes is found in males in both glioma-activated
302 microglia and a substantial fraction of infiltrating monocytes/macrophages, it may exert
303 different effects in those two cell populations. The flow cytometry analysis run on the same
304 cell pool, which was used to perform with the scRNA-seq, showed an elevated number of
305 CD4+ T-cells in tumor-bearing hemispheres from males compared to females. **(Figure 5f)**.
306 We explored the human glioma expression data from The Cancer Genome Atlas (TCGA) to
307 determine whether sex has an impact on the expression of *MHCII* and *CD74* genes. High-
308 grade glioma samples were not discriminated by the expression levels of the selected *MHCII*
309 and *CD74* genes (data not shown), irrespective of *IDH1* mutation status or macrophage
310 content as estimated with the xCell, a gene signature-based method inferring 64 immune and
311 stromal cell types⁴⁵. However, the expression of *MHCII* and *CD74* genes stratified glioma
312 WHO grade II patients into the female-enriched *MHCII*^{low} group and the male-enriched
313 *MHCII*^{high} group **(Figure 5g)**. This observation shows that the differential regulation of genes
314 coding for MHCII complex between sexes is not limited to a mouse glioma model, and those

315 differences could be of clinical relevance. Altogether, we demonstrate that transcriptional
316 responses of microglia to glioma varies between sexes, and microglia from tumor-bearing
317 brains in males exhibit more prominent antigen presentation processes compared to their
318 female counterparts.

319

320 **Discussion**

321 In the present study, we have used cell sorting and single-cell RNA sequencing to dissect the
322 cellular and functional heterogeneity of GAMs. Although it has been known that human
323 glioblastomas, the most malignant primary brain tumors, are infiltrated with resident microglia
324 and bone marrow (BM) derived-macrophages, cell identities and functions of a specific
325 subpopulations could not be inferred from bulk RNA-seq data. GAMs support glioma
326 progression by augmenting tumor invasion, angiogenesis, and inducing
327 immunosuppression², therefore, identifying specific roles of various cells is critical for a cell-
328 specific intervention. Transcriptomic analyses of bulk CD11b+ infiltrates from human GBMs
329 and murine gliomas showed a mixture of profiles characteristic for both pro- and antitumor
330 phenotypes^{4,46}. A commonly used strategy for cell separation based on CD45 expression has
331 been criticized due to upregulation of CD45 by microglial cells under pathological
332 conditions⁴⁷. Studies with genetic lineage tracing showed that BM-derived macrophages
333 accumulate in GL261 gliomas and transgenic RCAS-PDGF-B-HA gliomas, and
334 transcriptional networks associated with tumor-mediated education in brain-resident
335 microglia and recruited BM-macrophages are distinct^{7,38}. Cell type-specific chromatin
336 landscapes established before tumor initiation contributed to transcriptional differences. A
337 study using high-resolution open-skull 2-photon microscopy demonstrated both the presence
338 of microglia and BM-derived macrophages with distinct migratory propensities infiltrating
339 gliomas⁴⁸. Anti-vascular endothelial growth factor A blockade reduced GAM infiltration,
340 particularly BM-derived macrophages⁴⁸.

341 The single-cell resolution achieved in this study clearly demonstrated cellular and
342 functional heterogeneity of GAMs in experimental murine gliomas. We show that at the pre-
343 symptomatic stage of glioma growth, microglia are the main myeloid population within the
344 glioma TME, whereas monocytes/macrophages constitute a quarter and BAMs less than 4%
345 of CD11b+ cells. Using immunofluorescence and double staining for Tmem119 and Iba1, we
346 demonstrated that cell distribution within the tumor is not uniform because, in the tumor core,
347 we found prevailing monocytes/macrophages (Tmem119-, Iba1+ cells), whereas microglia
348 (Tmem119+, Iba1+ cells) occupied the tumor edge and periphery. This result is in agreement
349 with previous reports on mouse experimental gliomas and a single-cell RNA-seq study on
350 matched patient-derived samples from tumor core and periphery^{9,38}.

351 Microglia and macrophage transcription regulatory networks adapt to changing
352 environments^{49,50}. ScRNA-seq studies on human GBMs suggested that microglia and
353 monocytes/macrophages diminish their signature of origin, impeding clear separation and
354 forming a phenotypic continuum^{8,10}. We performed a detailed analysis of “microglia”
355 and “macrophage” transcriptional signatures, and the unsupervised cell clustering yielded
356 three cell clusters representing microglia, monocytes/macrophages, and CNS BAMs. A cell
357 identity was inferred based on the most highly differentially expressed genes. We discovered
358 that the “microglia signature” is indeed lower in microglial cells within the glioma TME, but the
359 expression of the signature genes is still high and distinguishable from
360 monocytes/macrophages. These observations demonstrate that cell identity is retained even
361 after cell polarization in the presence of a tumor.

362 The expression of cell identity genes does not imply functional divergence. We
363 demonstrate that glioma induces activation of similar transcriptional networks in microglia
364 and monocytes/macrophages; however, the effect is stronger in the latter cell types, likely
365 due to their prevalent localization within the tumor core, in contrast to microglia occupying
366 peripheral tumor regions^{9,38}. At the same time, monocytes/macrophages specifically express
367 numerous genes related to immunosuppression BAMs and do not change their
368 transcriptional profiles in the presence of glioma.

369 Moreover, Act-MG shows high expression of *Ccl3*, *Ccl4*, and *Ccl12* (chemokine-
370 encoding genes), whereas their expression is low in Mo/MΦ. Hence, microglia respond first
371 to glioma and instigate immune cell infiltration and trafficking to the tumor site. *Ccl3* and
372 *Ccl12* are chemokines attracting monocytes, eosinophils, and lymphocytes, respectively.
373 *Ccl12* shares the same *Ccr2* receptor as two other chemokines—*Ccl2* and *Ccl7*—in mice⁵¹.

374 Intriguingly, infiltrating monocytes/macrophages do not form a uniform cell population,
375 and based on the cell expression profiles we distinguished cytotoxic monocytes
376 (*Ly6c2*^{high}*Ccr2*^{high}), intermediate state of monocytes-macrophages (*Ly6c2*^{high}*Tgfb1*^{high}), and
377 differentiated macrophages (*Ifitm2*^{high}, *S100a6*^{high}, *S100a11*^{high}) expressing a high level of the
378 PD-L1 encoding gene (*Cd274*). These observations suggest that monocytes arrive as anti-
379 tumor cells and upon infiltration into the TME, undergo tumor-induced phenotypic
380 differentiation. We found only a partial overlap with the recently proposed macrophage-
381 specific markers in gliomas^{3,7,17}, but the proportion of monocyte/macrophage subpopulations
382 may depend on the particular tumor stage. The discovered subpopulation of Mo/MΦ
383 expressing an inflammatory *Il1b*, along with *Il1rn* and *Il18b*, coding for the inhibitors of pro-
384 inflammatory cytokines, as well as *Cd274* (an immune checkpoint inhibitor), is interesting for
385 its clinical relevance, suggesting that pro-invasive and immunoregulatory functions are split
386 between microglia and macrophages, respectively.

387 The second important finding refers to sex-dependent differences in microglial
388 responses to glioma. Bulk CD11b+ RNA-seq showed that in naïve mice, male microglia
389 express a higher level of *MHCI* and *MHCII* genes and are more reactive to ATP stimulation¹³.
390 This proposal has not been confirmed in the current scRNA-seq study, likely due to technical
391 differences in cell sorting (CD11b+ beads versus flow cytometry) and sequencing methods.
392 Contrastingly, within the tumor-bearing hemispheres, male microglia and intMoMΦ
393 expressed a higher level of genes coding for MHCII components and Cd74, a level of
394 expression that was not found for other cell types. Hence, upon glioma-induced activation,
395 microglia and intMoMΦ in males have higher antigen-presenting capacity than their female
396 counterparts. This increase was associated with the higher infiltration of CD4+ T cells to the
397 glioma TME. CD4+ and perivascular Foxp3+ tumor-infiltrating T lymphocytes are associated
398 with tumor angiogenesis and tumor progression in glioma patients⁵², and their higher ratio
399 over CD8+ cells correlates with unfavorable prognosis in GBM patients⁵³. The analysis of
400 human glioma TCGA datasets demonstrated sex-related differences in *MHCII* complex and
401 *CD74* genes in WHO grade II gliomas, where anti-tumor immunity operates and may
402 influence outcomes. Such differences were not detected in GBMs, irrespective of *IDH*
403 mutation status and macrophage content in the TME. This result could be due to the highly
404 immunosuppressed TME in human GBMs.

405 Although women have an increased susceptibility to autoimmune diseases, men have
406 a higher risk of death for a majority of malignant cancers⁵⁴. Despite the source of sex
407 differences in cancer incidence and survival rate remaining unknown, anti-tumor immunity is
408 an obvious candidate. In the immune checkpoint inhibitor therapy of various cancers, males
409 presented better therapeutic outcome¹². Estrogens mitigate inflammatory responses in
410 microglia⁵⁵, and female microglia have a higher neuroprotective capability^{56,57}.

411 In sum, glioma attracts and polarizes microglia and monocytes that invade the brain
412 from the periphery. Whereas invading monocytes express some inflammation markers, they
413 differentiate into immunosuppressive macrophages within the tumor. Those cells retain their
414 cell-identity signatures, occupy different tumor niches, and present a distinct degree of
415 glioma-induced activation and specific functions. Interestingly, we found the stronger
416 upregulation of genes of the MCHII complex in microglia and more abundant CD4+
417 infiltration in gliomas in males than females. Further studies on glioma immunopathology
418 should explore this issue, ensure correct representation of both sexes and avoid extending
419 findings from single-sex studies to the general population.

420

421 **Methods**

422 **Animals**

423 10-week-old male and female C57BL/6 mice were purchased from the Medical University of
424 Bialystok, Poland. Animals were kept in individually ventilated cages, with free access to food
425 and water, under a 12h/12h day and night cycle. All experimental procedures on animals
426 were approved by the First Local Ethics Committee for Animal Experimentation in Warsaw
427 (approval no 563/2018 and 764/2018).

428 **Implantation of GL261 luc+/tdT+ glioma cells**

429 Mice (12-week-old) were kept under deep anesthesia with 2% isoflurane during surgery.
430 Using a stereotactic apparatus, a single-cell suspension of GL261 luc+tdT+ cells (80 000
431 cells in 1 μ L of DMEM, Dulbecco modified essential medium) was implanted into the right
432 striatum (+1 mm AP, -1.5 mm ML, -3 mm DV) at the rate of 0.25 μ L per minute. To confirm
433 the presence of the tumor, two weeks after implantation, animals received an intraperitoneal
434 injection of 150 μ g luciferin/kg body weight 10 min prior to imaging with the Xtreme *in vivo*
435 bioluminescence imaging system [Bruker, Germany]. The images were acquired at medium
436 binning with an exposure time of 2 min. X-ray images were acquired at the same mice
437 position with the Xtreme equipment. The signal intensity of the region of interest (ROI) was
438 computed using the provided software.

439 **Tissue dissociation**

440 Two weeks after tumor implantation, mice with gliomas and naïve animals (controls) were
441 perfused transcardially with cold phosphate-buffered saline (PBS) to clear away blood cells
442 from the brain. Further processing was performed on the pooled tissue from 2 animals per
443 replicate. The tumor-bearing hemispheres and corresponding hemispheres from naïve
444 animals were dissociated enzymatically to obtain a single-cell suspension with a Neural
445 Tissue Dissociation Kit [Miltenyi Biotec] and gentleMACS Octo Dissociator [Miltenyi Biotec]
446 according to the manufacturer's protocol. Next, the enzymatic reaction was stopped by the
447 addition of Hank's Balanced Salt Solution (HBSS) with calcium and magnesium [Gibco,
448 Germany]. The resulting cell suspension was filtered through a 70 μ m and 40 μ m strainer
449 and centrifuged at 300 x g, 4 °C for 10 min. Next, myelin was removed by centrifugation on
450 22% Percoll gradient. Briefly, cells were suspended in 25 mL Percoll solution (18.9 mL
451 gradient buffer containing 5.65 mM NaH₂PO₄H₂O, 20 mM Na₂HPO₄2(H₂O), 135 mM NaCl, 5
452 mM KCl, 10 mM glucose, 7.4 pH; 5.5 mL Percoll [GE Healthcare, Germany]; 0.6 mL 1.5 M
453 NaCl), overlaid with 5 mL PBS and centrifuged for 20 min at 950 g and 4 °C, without
454 acceleration and brakes. Next, cells were collected, washed with Stain Buffer (FBS)
455 [#554656, BD Pharmingen], quantified using an EVE™ Automatic Cell Counter [NanoEnTek
456 Inc., USA], and split for CD11b+ cell sorting and cytometric analysis.

457

458 **Flow Cytometry**

459 Samples were constantly handled on ice or at 4 °C avoiding direct light exposure. First,
460 samples were incubated with LiveDead Fixable Violet Dead Cell Stain [1:1000, L34955,
461 ThermoFisher] in PBS for 10 min to exclude dead cells. Next, samples were incubated for 10
462 min with rat anti-mouse CD16/CD32 Fc Block™ [1:250, #553141, BD Pharmingen] in Stain
463 Buffer [#554656, BD Pharmingen] to reduce unspecific antibody binding. Then, cell
464 suspensions were split and incubated for 30 min with two monoclonal antibody cocktails
465 referred to as myeloid and lymphoid panels. The myeloid panel consisted of: CD45 PE-Cy7
466 [30-F11, BD Pharmingen], CD11b AF700 [M1/70, BD Pharmingen], CD49d FITC [R1-2,
467 BioLegend], Ly6C PerCP-Cy5.5 [AL-21, BD Pharmingen], Ly6G APC [1A8, BD Pharmingen].
468 Lymphoid panel consisted: CD3 FITC [REA641, Miltenyi Biotec], CD4 VioGreen [REA604,
469 Miltenyi Biotec], α -NK-1.1 APC [PK136, Miltenyi Biotec], CD11b PE [M1/70, BD
470 Pharmingen]. Antibodies were titrated prior to staining to establish the amount yielding the
471 best stain index. Samples were acquired using a BD LSR Fortessa Analyzer cytometer. Data
472 were analyzed with FlowJo software (v. 10.5.3, FlowJo LLC, BD). Gates were set based on
473 FMO (fluorescence minus one) controls and back-gating analysis. Percentages on
474 cytograms were given as the percentage of a parental gate (myeloid & lymphoid panels) or
475 the percentage of all alive cells (CD4 in a lymphoid panel). Flow cytometry experiments were
476 performed at the Laboratory of Cytometry, Nencki Institute of Experimental Biology, Polish
477 Academy of Sciences.

478 **CD11b+ cell FACS sorting**

479 Cells were suspended in Stain Buffer [#554656, BD Pharmingen] in a density of 1 mln cells
480 per 100 μ L. For assessment of cell viability during sort, cells were stained with LiveDead
481 Fixable Violet Dead Cell Stain [L34955, ThermoFisher] in PBS for 10 min, and then rat anti-
482 mouse CD16/CD32 Fc Block™ [1:250, #553141, BD Pharmingen] for 10 min, and CD11b+
483 Rat Anti-Mouse FITC [1:800, M1/70, BD Pharmingen] for another 20 min at 4°C. Cells sorted
484 for single cell analysis were stained with CD11b+ Rat Anti-Mouse AF700 [M1/70, BD
485 Pharmingen]. Following staining, cells were washed with Stain Buffer and sorted into 20%
486 FBS in PBS.

487

488 **Single-cell RNA sequencing**

489 Directly after sorting, cell quantity and viability of CD11b+ cells were measured, and a cell
490 suspension volume equivalent to 5000 target cells was used for further processing.
491 Preparation of gel beads in emulsion and libraries were performed with Chromium Controller
492 and Single-Cell Gene Expression v2 Chemistry [10x Genomics] according to the Chromium
493 Single-Cell 3' Reagent Kits v2 User Guide provided by the manufacturer. Libraries' quality
494 and quantity were verified with a High-Sensitivity DNA Kit [Agilent Technologies, USA] on a

495 2100 Bioanalyzer [Agilent Technologies, USA]. Next, sequencing was run in the rapid run
496 flow cell and paired-end sequenced (read 1 – 26 bp, read 2 – 100 bp) on a HiSeq 1500
497 (Illumina, San Diego, CA 92122 USA).

498

499 **Single-cell RNA-seq data preprocessing and normalization**

500 Sequencing results were mapped to a mouse genome GRCm38 (mm10) acquired from the
501 10x Genomics website and quantified using a Cell Ranger v.3.0.1^{58,59}
502 [[https://support.10xgenomics.com/single-cell-gene-](https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/installation)
503 [expression/software/pipelines/latest/installation](https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/installation)]. The total number of cells identified by the
504 Cell Ranger was 41,059 (details in Supplementary Table 2). The median number of detected
505 genes per cell was 1,059, and the median unique molecular identifiers (UMIs) per cell was
506 2,178. Data analysis was performed in R using Seurat v3^{59,60}. Unless otherwise specified in
507 the description, all other quantitative parameters were fixed to default values. To filter out
508 possible empty droplets, low-quality cells, and possible multiplets, cells with less than 200 or
509 more than 3,000 transcripts were excluded from the analysis. Additionally, cells of poor
510 quality, recognized as cells with >5% of their transcripts coming from mitochondrial genes,
511 were excluded from the downstream analysis. After applying these filters, 40,401 cells were
512 present in the data set. Gene expression measurements for each cell were normalized by the
513 total number of transcripts in the cell, multiplied by a default scale factor, and the normalized
514 values were log-transformed ("LogNormalize" method). Following a Seurat workflow, for
515 each replicate the 2,000 most highly variable genes were identified using variance stabilizing
516 transformation ("vst"). To facilitate identification of cell types these gene sets were expanded
517 by adding genes described as having important roles in immune cells (see Supplementary
518 Table 3) and genes involved in cell cycle regulation⁶¹. This extension did not influence our
519 conclusions.

520 **Identification of myeloid cells**

521 Having two biological replicates for each sex and condition (female control, female tumor,
522 male control, male tumor), data from corresponding samples were integrated using a Seurat
523 v3 approach⁵⁹. First, 2000 integration anchors (i.e., cells that are mutual nearest neighbors
524 between replicates) were found. These anchors were then used as input to the data sets
525 integration procedure. Integrated data were scaled, and unwanted sources of variation,
526 namely total number of counts per cell, percentage of transcripts coming from mitochondrial
527 genes per cell, and cell cycle effect were regressed out, as described in a corresponding
528 vignette [https://satijalab.org/seurat/v3.0/cell_cycle_vignette.html]. Data dimensionality
529 reduction was performed using a principal component analysis (PCA), and the first 30
530 principal components were used in the downstream analyses. For each condition separately,

531 the expression profiles were then clustered using an unsupervised, graph-based approach
532 with the resolution parameter set to 0.3. Clustering results were visualized using two-
533 dimensional t-Distributed Stochastic Neighbor Embedding (t-SNE)⁶². Based on expression of
534 the reported/canonical markers, the clusters dominated by myeloid cells in four conditions
535 were identified and further analyzed.

536 **Comparative analysis**

537 The comparative analysis was based on the raw counts but limited to the previously selected
538 profiles and genes (see above). For such a data set, a new set of the 2,000 most highly
539 variable genes was identified using variance stabilizing transformation ("vst"), and this set
540 was further expanded by adding the genes involved in cell cycle regulation. Computation of
541 expression estimations, regression of the unwanted variation, and data dimensionality
542 reduction were performed as described above. Next, the expression profiles were clustered
543 using the same approach as above, but with a resolution parameter set to 0.6. After
544 clustering, data were visualized using two-dimensional Uniform Manifold Approximation and
545 Projection (UMAP)⁶³. Based on expression of reported/canonical markers of myeloid cells,
546 clusters with cells of interest (microglia, macrophages, and BAMs) were identified. Further
547 analysis of the microglia cluster revealed that some sub-clusters cells originated in a
548 significant majority from tumor samples. In contrast, there were no sub-clusters so strongly
549 dominated by cells originated from control samples. Based on that observation, two subsets
550 of microglial cells with distinct transcriptional profiles were identified: homeostatic microglia
551 (Hom-MG) and activated microglia (Act-MG).

552 Differentially upregulated genes (signature genes) were found for each of the identity classes
553 of interest. Significantly upregulated genes between compared groups were found using a
554 Wilcoxon Rank Sum test implemented in Seurat v3 (min.pct = 0.25, only.pos = TRUE).
555 These genes were subsequently used for the functional analysis and characterization of the
556 identified clusters. Gene Ontology analysis was performed using the clusterProfiler
557 package⁶⁴.

558 **Analysis of TCGA data**

559 The normalized expression values for low- and high-grade gliomas were downloaded from
560 The Cancer Genome Atlas (TCGA) website (RNASeqV2 set available on 07/05/19). Sample
561 annotations and *IDH1* mutation status were obtained from Ceccarelli and colleagues' study⁶⁵.
562 The content of immune cells was computed with xCell pre-calculated scores downloaded
563 from the xCell website⁴⁵. The genes encoding MHCII and CD74 proteins were selected
564 based on the literature^{24,66,67}. The expression profiles were clustered using hierarchical
565 clustering. Significance of the clustering was computed using Fisher's exact test. Separation

566 of glioma samples was done for each grade separately. Neither WHO grade IV nor grade III
567 glioma samples were significantly discriminated by the expression levels of the selected
568 genes (data not shown).

569

570 **Immunohistochemistry on brain slices**

571 For tissue collection for histology, mice were anesthetized and transcardially perfused with
572 PBS and 4% paraformaldehyde (PFA). Tissues were dissected, and the brain was post fixed
573 in 4% PFA overnight and then placed in 30% sucrose for 2 days, embedded in Tissue-Tek
574 O.C.T Compound, and 10 μm cryosections were cut and stored at -80 °C. Cryosections were
575 blocked in PBS containing 10% donkey serum in 0.1% Triton X-100 solution for 2 hours and
576 incubated overnight at 4 °C with goat anti-AIF-1/Iba1 antibody (Novus Biologicals;
577 Centennial, Colorado, USA; dilution 1:200, 3% donkey serum, 0.1% Triton X-100) and rabbit
578 anti-TMEM119 antibody (Synaptic Systems; Goettingen, Germany, dilution 1:500, 3%
579 donkey serum, 0.1% Triton X-100). Next, sections were washed in PBS, incubated with a
580 donkey anti-goat Alexa Fluor 647 and donkey anti-rabbit Alexa Fluor 488 (Invitrogen;
581 Waltham, Massachusetts, USA; dilution 1:1000, 3% donkey serum, 0.1% Triton X-100) for 2
582 hours at room temperature. Nuclei were counter-stained with DAPI (0.1mg/mL), and images
583 were obtained using a Leica DM8000 fluorescent microscope.

584

585 **Data availability**

586 Bam files and Seurat v3 processed gene expression matrix for each condition can be
587 downloaded from the NIH GEO database (TBD).

588

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760 **Figure legends**

761 **Fig. 1. Identification of immune cell populations in control and tumor-bearing brain**
762 **hemispheres. a** Scheme of the experimental workflow. **b** t-SNE plot demonstrating
763 clustering obtained for each group (female control, female tumor, male control, male tumor),
764 2 biological replicates were combined. Clusters annotations: MG – microglia, pre-MG –
765 premature microglia, Mo – Monocytes, intMoMΦ – intermediate Monocyte-Macrophage, MΦ
766 – macrophages, BAM – CNS border-associated macrophages, DCs – dendritic cells,
767 Ncam1+/- Ncam1 positive cells, NK – natural killer cells, NKT – natural killer T cells, B cells –
768 B lymphocytes, T cells – T lymphocytes. **c** Expression of “signature” genes selected from the
769 immune marker panel for identification of a cluster cell type (Suppl. Table 1). **d** Pie charts
770 demonstrating distribution of the identified cell types across samples.

771 **Fig. 2. Transcriptomic characterization of main myeloid subpopulations. a** Projection of
772 cells combined from clusters identified as microglia, monocytes/macrophages (Mo/MΦ), and
773 BAMs from all groups. **b** Top 10 differentially expressed genes for the three main identified
774 cell populations. **c-g** Expression level of novel and previously identified “signature” genes for
775 microglia (**c**), macrophages (**d**), microglia and macrophages in gliomas (**e**), and BAMs (**f**). **g**
776 Feature plots depict distribution of the expression of genes discriminating
777 monocytes/macrophages (Mo/MΦ), Monocytes (Mo), Monocyte-Macrophage intermediate
778 (intMoMΦ), and Macrophage (MΦ) subpopulations. **h** Density plots demonstrate the
779 expression level of markers discriminating Mo/MΦ subpopulations. **i** UMAP plot shows
780 clusters of Mo/MΦ subpopulations.

781 **Fig. 3. Tumor-derived microglia and macrophages form separate cell populations. a** A
782 UMAP plot demonstrates the distribution of CD11b+ cells from naïve and tumor-bearing
783 mice. **b** Distribution of microglia and Mo/MΦ “signature” gene scores (presented as an
784 average of expression of the selected genes). **c** Density plots of microglia and Mo/MΦ scores
785 across MG and Mo/MΦ populations demonstrating no overlap of a specific “signature”
786 between the two cell populations. **d** Cell hierarchical clustering according to the expression of
787 reported macrophage markers demonstrates bimodal cell distribution. **e, f**
788 Immunohistochemical staining for microglia (Iba+, Tmem119+) and Mo/MΦ (Iba+, Tmem119-
789) shows the localization of specific immune cells within the tumor and its surroundings in
790 male (**e**) and female (**f**) animals; a dashed line marks the tumor edge; scale – 100 μm.

791 **Fig. 4. Functional analysis of glioma-activated microglia in comparison to tumor-**
792 **infiltrating monocytes/macrophages. a–b** Heatmaps show significantly upregulated genes
793 in homeostatic microglia (Hom-MG) vs activated microglia (Act-MG) (**a**) and Act-MG vs
794 Mo/MΦ (**b**). Differentially upregulated genes in those cells have been tested further. **c** Scatter

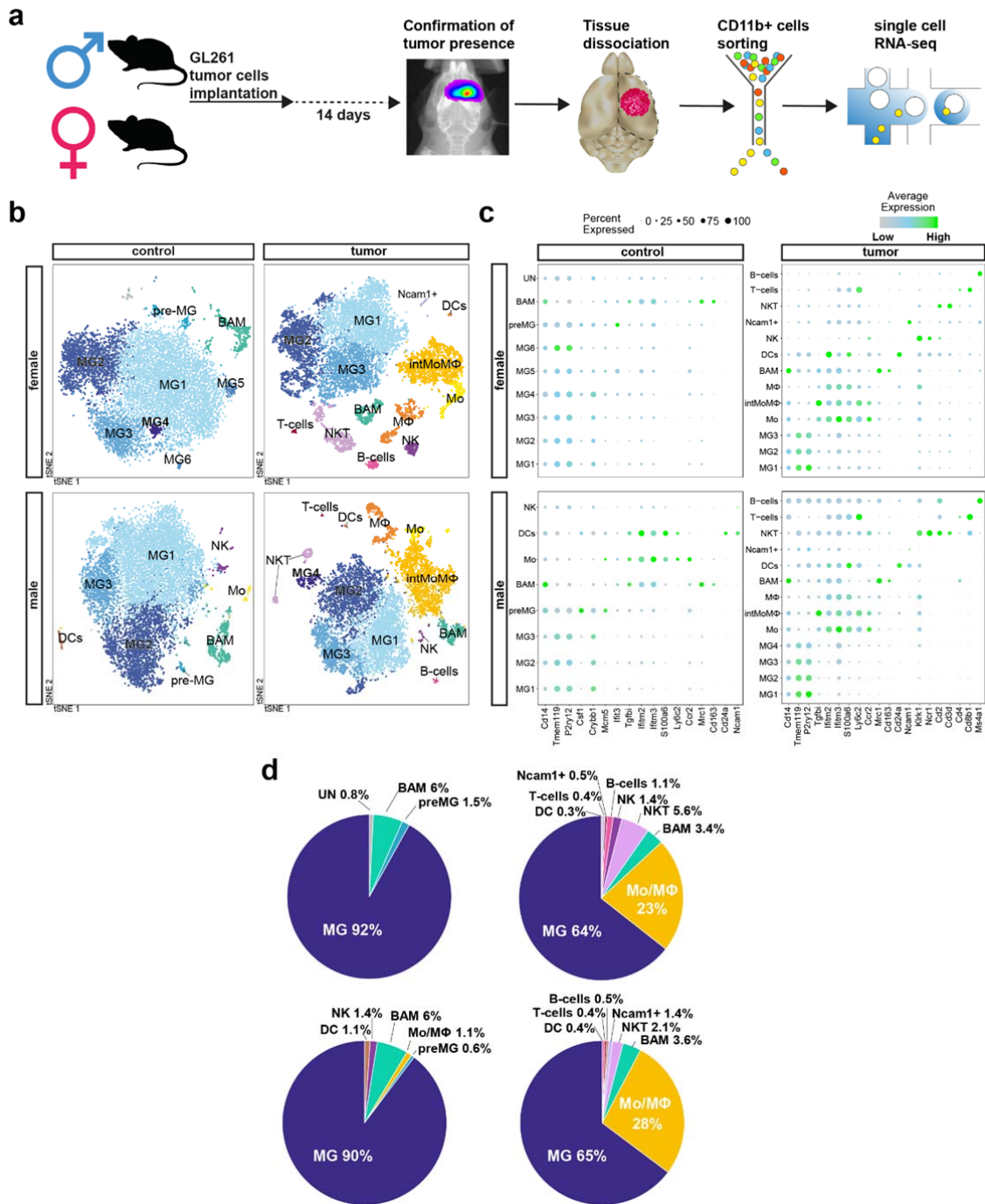
795 plot depicts levels of differentially upregulated genes in Act-MG and Mo/M Φ . **d** Heatmap
796 shows the comparison of expression of top 25 upregulated genes in Act-MG and Mo/M Φ .
797 Gene Ontology analysis of biological processes for genes upregulated in **(e)** Act-MG
798 compared to Hom-MG and **(f)** for genes upregulated in Mo/M Φ compared to the Act-MG. **g-h**
799 Expression levels of selected genes expressed specifically in distinct subpopulations

800

801 **Fig. 5. Expression of *MHC II* and *Cd74* genes is more abundant in microglia and**
802 **monocytes/macrophages from gliomas in males. a** UMAP plots demonstrate the
803 distribution of male and female cells across cell clusters and reveals sex-enriched areas. **b**
804 Volcano plots depict differentially expressed genes across sexes in Act-MG and Mo/M Φ
805 infiltrating gliomas. **c** Expression of the most highly upregulated genes from males. **d** Density
806 plots show enrichment of male cells in *MHCII*- and *Cd74*-high expressing gene populations
807 of Act-MG and intMoM Φ . **e** Violin and density plots demonstrate that *Mif* upregulation is
808 limited to the intMoM Φ *MHCII*^{high} cells. **h** Flow cytometry analysis shows the increased
809 infiltration of CD4⁺ cells to male TME compared with female TME. **g** Normalized log₂ RNA-
810 seq counts for *MHCII* complex genes from TCGA WHO grade II glioma patients' data set
811 show significant differences between male and female glioma patients (Fisher's exact test).

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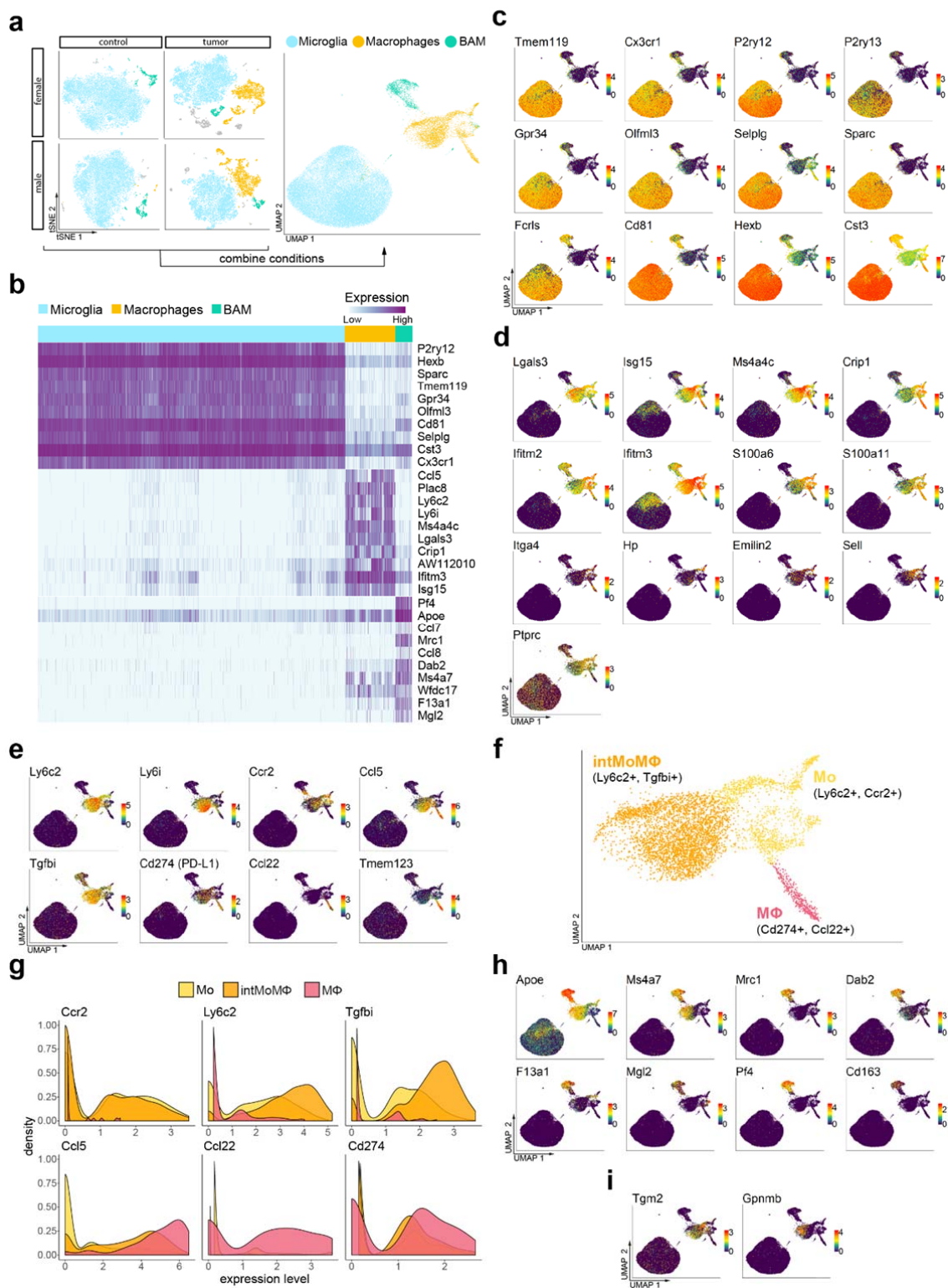
Figure 1



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Figure 2



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Figure 3

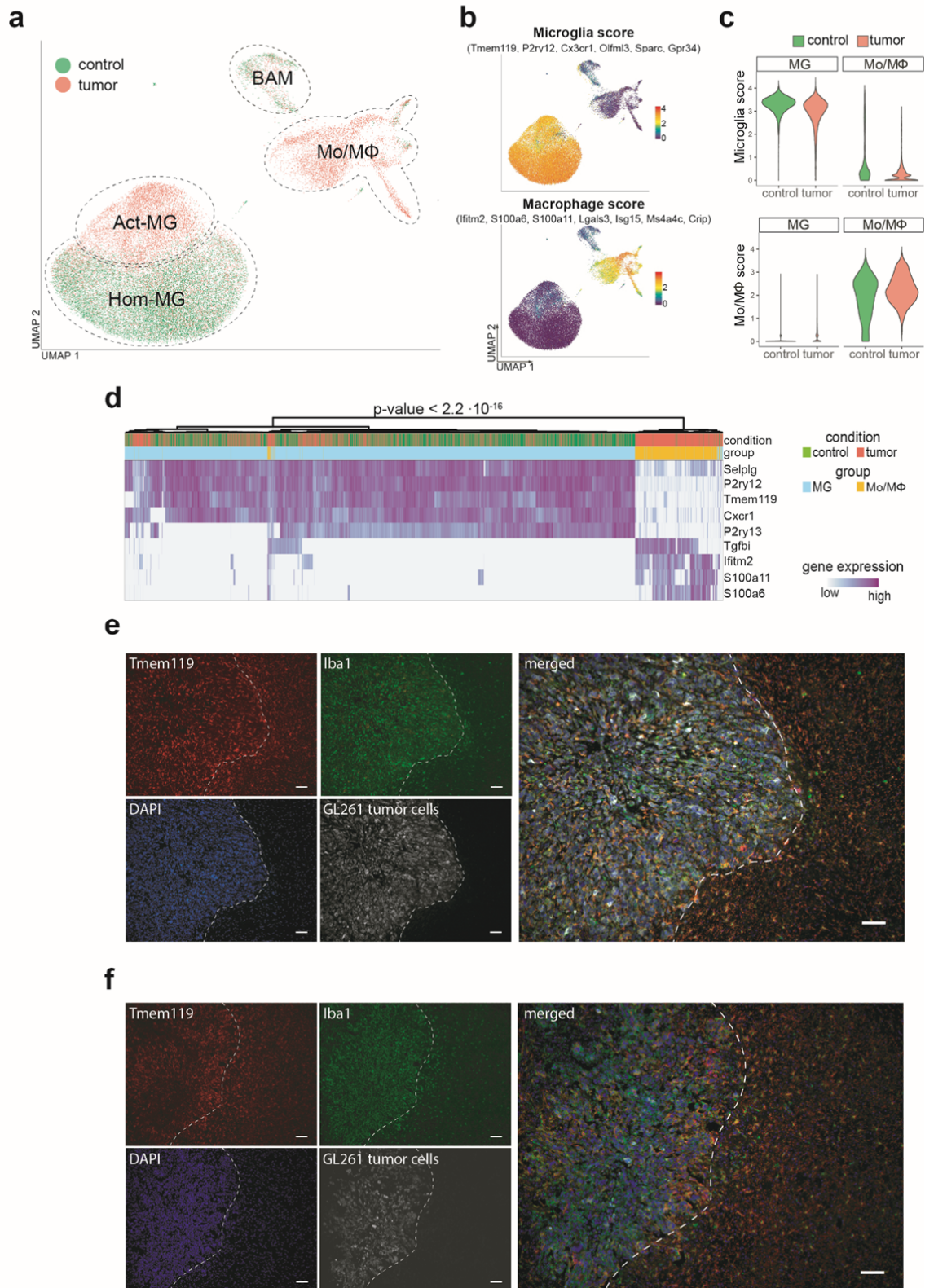
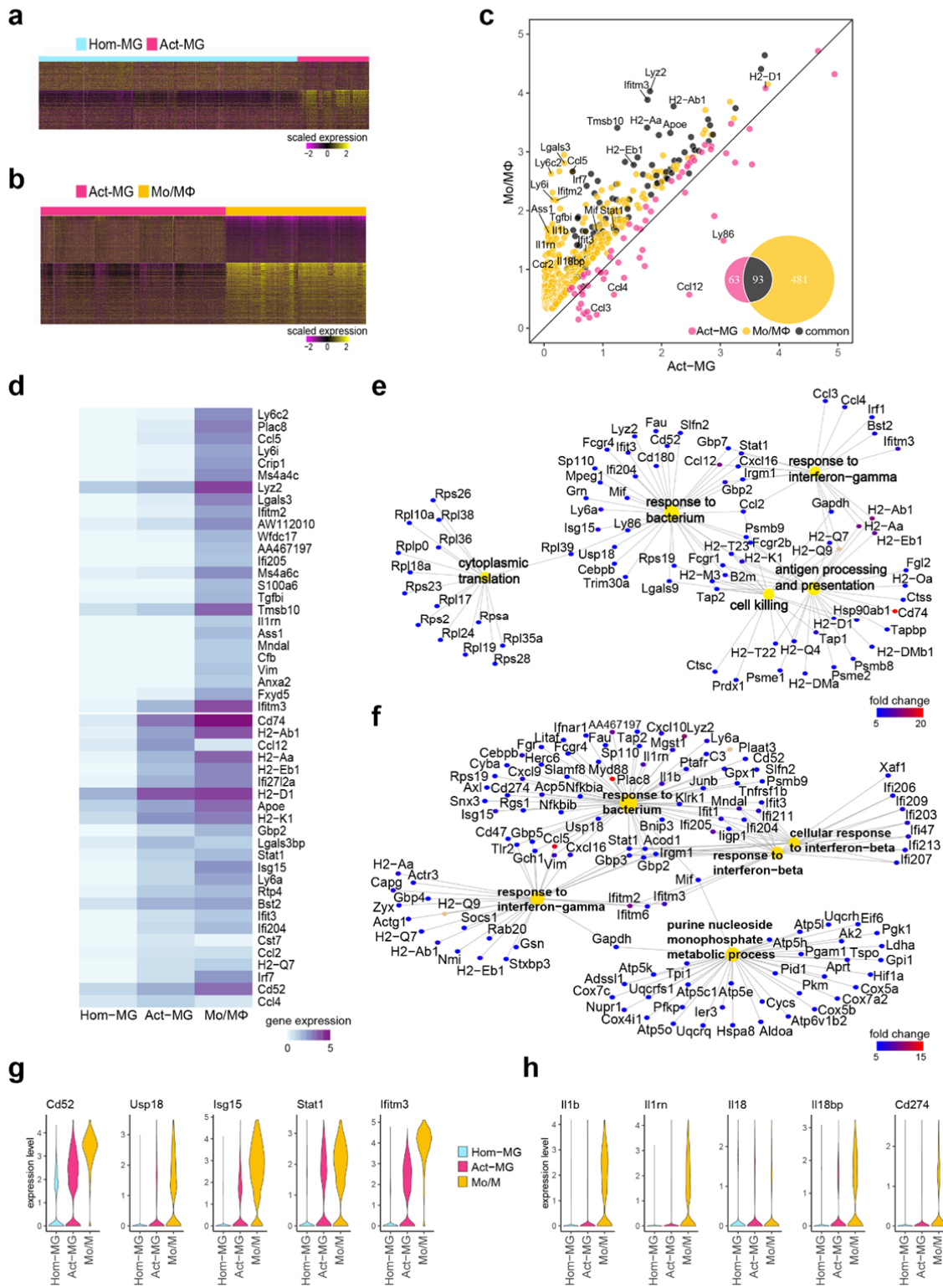
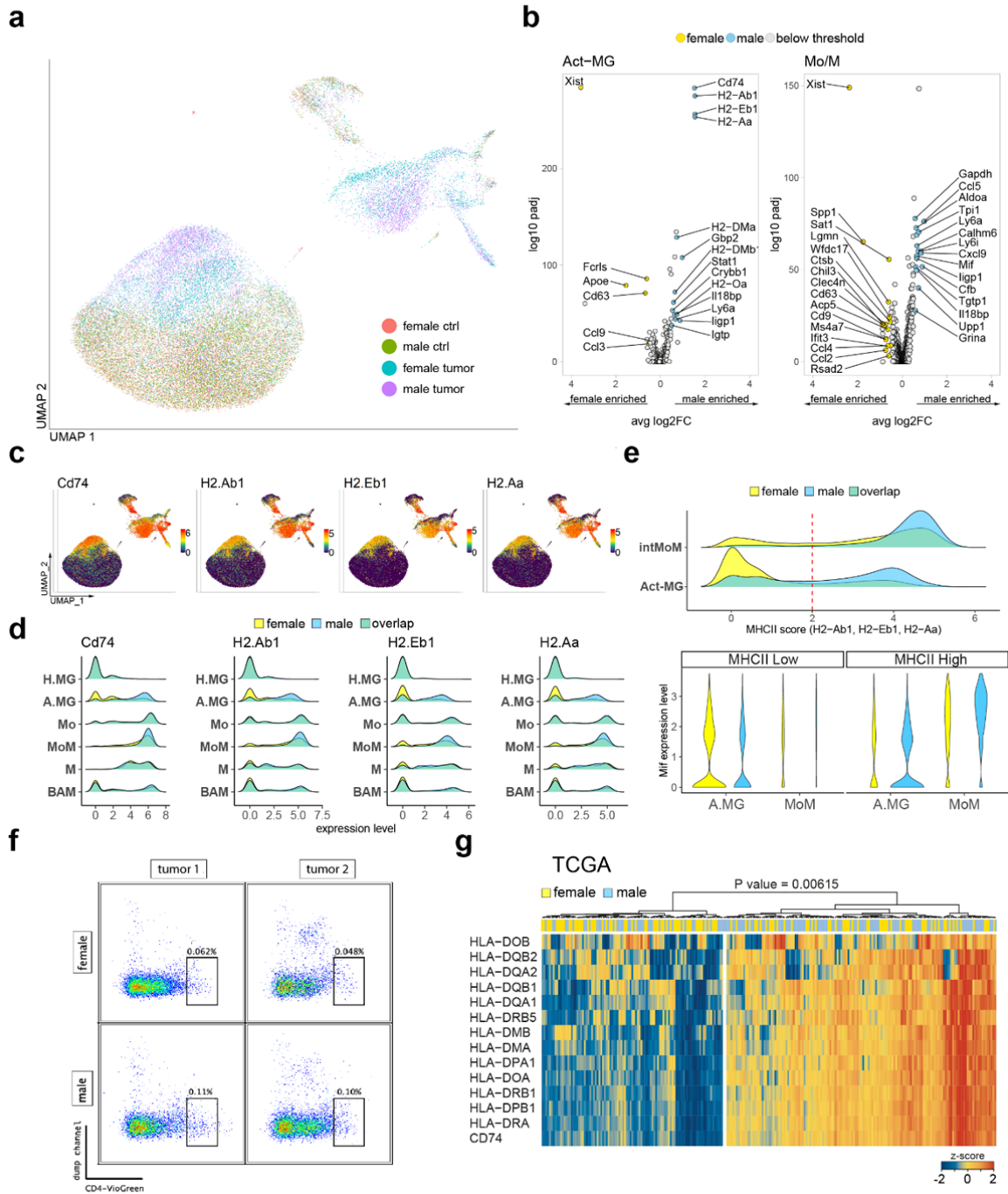


Figure 4



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Figure 5



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