

1 **Microglia and macrophages alterations in the CNS during acute SIV**
2 **infection: a single-cell analysis in rhesus macaques**

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17 Running title: Microglia and macrophage responses to acute SIV infection *in vivo*

18 **ABSTRACT**

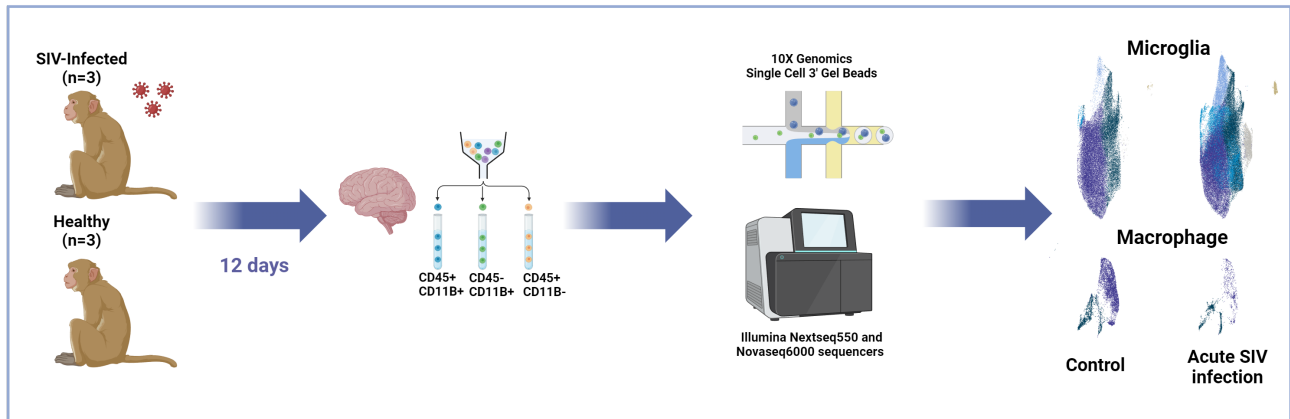
19 Human Immunodeficiency Virus (HIV) is widely acknowledged for its profound impact
20 on the immune system. Although HIV primarily affects peripheral CD4 T cells, its
21 influence on the central nervous system (CNS) cannot be overlooked. Within the brain,
22 microglia and CNS-associated macrophages (CAMs) serve as the primary targets for
23 HIV, as well as for the simian immunodeficiency virus (SIV) in nonhuman primates.
24 This infection can lead to neurological effects and the establishment of a viral
25 reservoir. Given the gaps in our understanding of how these cells respond *in vivo* to
26 acute CNS infection, we conducted single-cell RNA sequencing (scRNA-seq) on
27 myeloid cells from the brains of three rhesus macaques 12-days after SIV infection,
28 along with three uninfected controls. Our analysis revealed six distinct microglial
29 clusters including homeostatic microglia, preactivated microglia, and activated
30 microglia expressing high levels of inflammatory and disease-related molecules. In
31 response to acute SIV infection, the population of homeostatic and preactivated
32 microglia decreased, while the activated and disease-related microglia increased. All
33 microglial clusters exhibited upregulation of MHC class I molecules and interferon-
34 related genes, indicating their crucial roles in defending against SIV during the acute
35 phase. All microglia clusters also upregulated genes linked to cellular senescence.
36 Additionally, we identified two distinct CAM populations: CD14^{low}CD16^{hi} and
37 CD14^{hi}CD16^{low} CAMs. Interestingly, during acute SIV infection, the dominant CAM
38 population changed to one with an inflammatory phenotype. Notably, specific
39 upregulated genes within one microglia and one macrophage cluster were associated
40 with neurodegenerative pathways, suggesting potential links to neurocognitive
41 disorders. This research sheds light on the intricate interactions between viral

42 infection, innate immune responses, and the CNS, providing valuable insights for
43 future investigations.

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45 **Keywords:** HIV, SIV, neuroHIV, microglia, macrophages, scRNA-seq

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48 AUTHOR SUMMARY

49 HIV's entry into the central nervous system (CNS) can lead to neurological
50 dysfunction, including HIV-associated neurocognitive disorders (HAND), and the
51 establishment of a viral reservoir. While microglia and CNS-associated macrophages
52 (CAMs) are the primary targets of HIV in the CNS, their responses during acute HIV
53 infection remain poorly defined. To address this, we employed the scRNA-seq
54 technique to study microglial and CAM populations in rhesus macaques during acute
55 SIV infection. By identifying signature genes associated with different phenotypes and
56 mapping them to various biological and pathological pathways, we discovered two
57 myeloid cell clusters strongly linked to neurodegenerative disorders. Additionally,
58 other clusters were associated with inflammatory pathways, suggesting varying
59 degrees of activation among different myeloid cell populations in the brain, possibly
60 mediated by distinct signaling pathways. All microglia clusters developed signs of the
61 cellular senescence pathway. These findings shed light on the immunological and
62 pathological effects of different myeloid phenotypes in the brain during acute SIV
63 infection, providing valuable insights for future therapeutic strategies targeting this
64 critical stage and aiming to eliminate the viral reservoir.

65 INTRODUCTION

66 The human immunodeficiency virus (HIV) is an enveloped retrovirus that
67 contains two copies of a single-stranded RNA genome, which can cause acquired
68 immunodeficiency syndrome (AIDS) by significantly impairing the immune system.
69 HIV remains a global health challenge with profound implications for individuals,
70 communities, and societies. The estimated number of people with HIV (PWH) is 36.7
71 million worldwide as of 2016, and according to the latest epidemiology study in 2020,
72 PWH comprise approximately 1.2 million in the United States.⁽¹⁾ Similar to HIV in
73 genomic, structural, and virologic perspectives, the simian immunodeficiency virus
74 (SIV) also belongs to the primate retrovirus family. *In vivo*, both viruses cause
75 persistent infection. Infection of nonhuman primates (NHPs) by SIV mimics many key
76 aspects of HIV infection in humans, including immunodeficiency, opportunistic
77 infections, and CNS infection which can be associated with neurological impairments.
78 (2-5)

79 The development of HIV infection can be classified into three stages, acute HIV
80 infection, chronic HIV infection, and if untreated, eventually AIDS.⁽⁶⁾ The acute
81 infection period is defined as the stage immediately after HIV infection and before the
82 development of antibodies to HIV, during which the virus rapidly multiplies and spreads
83 throughout the body.⁽⁷⁾ Sexually-mediated HIV transmission generally starts with
84 mucosal CD4+ T cells and Langerhans cells,⁽⁸⁾ and then travels to gut-associated
85 lymphoid tissue (GALT). Intravenous infection-mediated HIV transmission leads to
86 initial infection of CD4+ T cells in lymph nodes, the spleen, and GALT.^(9, 10) Lymphoid
87 tissue and other organ macrophages can also be infected. HIV-infected cells can
88 produce large amounts of virus to infect additional target cells and can also migrate
89 and carry the virus to other tissues and organs including the central nervous system

90 (CNS). The earliest post-infection time for detecting HIV/SIV RNA in the CNS (brain
91 or cerebrospinal fluid) ranges from 4 days to 10 days.⁽¹¹⁻¹³⁾ Like the deteriorative effect
92 of HIV in the periphery, the inflammatory events and neurotoxicity elicited by the
93 HIV/SIV can damage neurons as well as other supportive cells in the brain which can
94 eventually lead to HIV-associated neurocognitive disorders (HAND). While the
95 extensive use of antiretroviral therapy (ART) has significantly reduced the occurrence
96 of dementia, the most severe type of HAND, the overall prevalence of HAND still
97 hovers around 50%.⁽¹⁴⁻¹⁷⁾

98 In CNS HIV/SIV infection, CNS-associated macrophages (CAMs) and microglia
99 are thought to play a central role in defending against the invading pathogen and
100 trigger neuroinflammation.^(18, 19) Responding to the virus and/or virally-infected cells
101 that enter the brain as the initial innate immune response, they are activated and can
102 release numerous proinflammatory cytokines including interferons, IL-6, IL-1 β , and
103 TNF- α to contribute to the control and clearance of the virus or infected cells from the
104 CNS.⁽¹¹⁾ However, macrophages and microglia can also contribute to the pathological
105 events of HIV/SIV infection. In acute SIV/HIV infection, infected blood monocytes
106 represent another cell type other than CD4+ T cell that has been proposed to seed the
107 virus in the CNS. Infection of rhesus monkeys with SIV indeed results in an increase
108 in monocytes trafficking to the brain.⁽²⁰⁾ Once trafficking monocytes enter the brain,
109 they can further differentiate to CAMs, and under experimental conditions (such as
110 depletion of CD8+ cells) can lead to rapid development of SIV encephalitis.⁽²¹⁾ On the
111 other hand, blocking of an integrin (α 4), which is highly expressed on monocytes, by
112 natalizumab was found to profoundly hinder CNS infection and ameliorate neuronal
113 injury.⁽²²⁾ However integrin(α 4) is also expressed on CD4+ T cells, thus attributing the
114 effect to monocytes is uncertain. Although microglia, which are the CNS-specific

115 resident myeloid cells, do not participate in bringing the HIV/SIV to the CNS, they are
116 actively involved in neuronal damage once they are infected and/or activated. (14, 23, 24)
117 In addition, infected CAMs and/or microglia are thought to make up a viral reservoir in
118 the brain under suppressive ART treatment, complicating efforts for an HIV cure.

119 Although the general responses of microglia and CAMs to acute SIV infection
120 has been widely studied by using bulk assays, our understanding of different microglial
121 or CAMs phenotypes have is minimal for this important period in which virus enters
122 the brain. In this study, we performed high-throughput single-cell RNA sequencing
123 (scRNA-seq) on microglia and CAMs from the brains of rhesus macaques during acute
124 SIV infection as well as in control uninfected animals to address the limitations of bulk
125 assays and investigated the different effects of acute SIV infection on varied myeloid
126 phenotypes. We identified homeostatic microglia, preactivated microglia, activated
127 microglia, and two phenotypes of CAMs in the brains. We further characterized these
128 subsets of cells by comparing their transcriptomic profiles in the uninfected and acute-
129 infected conditions. Different responses were evoked in different microglial and
130 macrophage phenotypes in acute SIV infection. Interestingly, there were two activated
131 cell clusters that were found to be closely associated with neurological disorders.
132 Finally, although we did not observe modulation of the expression of the anti-apoptotic
133 molecule BCL-2 (upregulation of BCL-2 is one of the mechanisms for promoting the
134 survival of infected cells) (25, 26) by SIV in microglia and CAMs, we found another anti-
135 apoptotic molecule, CD5L was highly expressed in infected microglia which might be
136 a novel potential pathway to elucidate the reservoir establishment in the CNS.

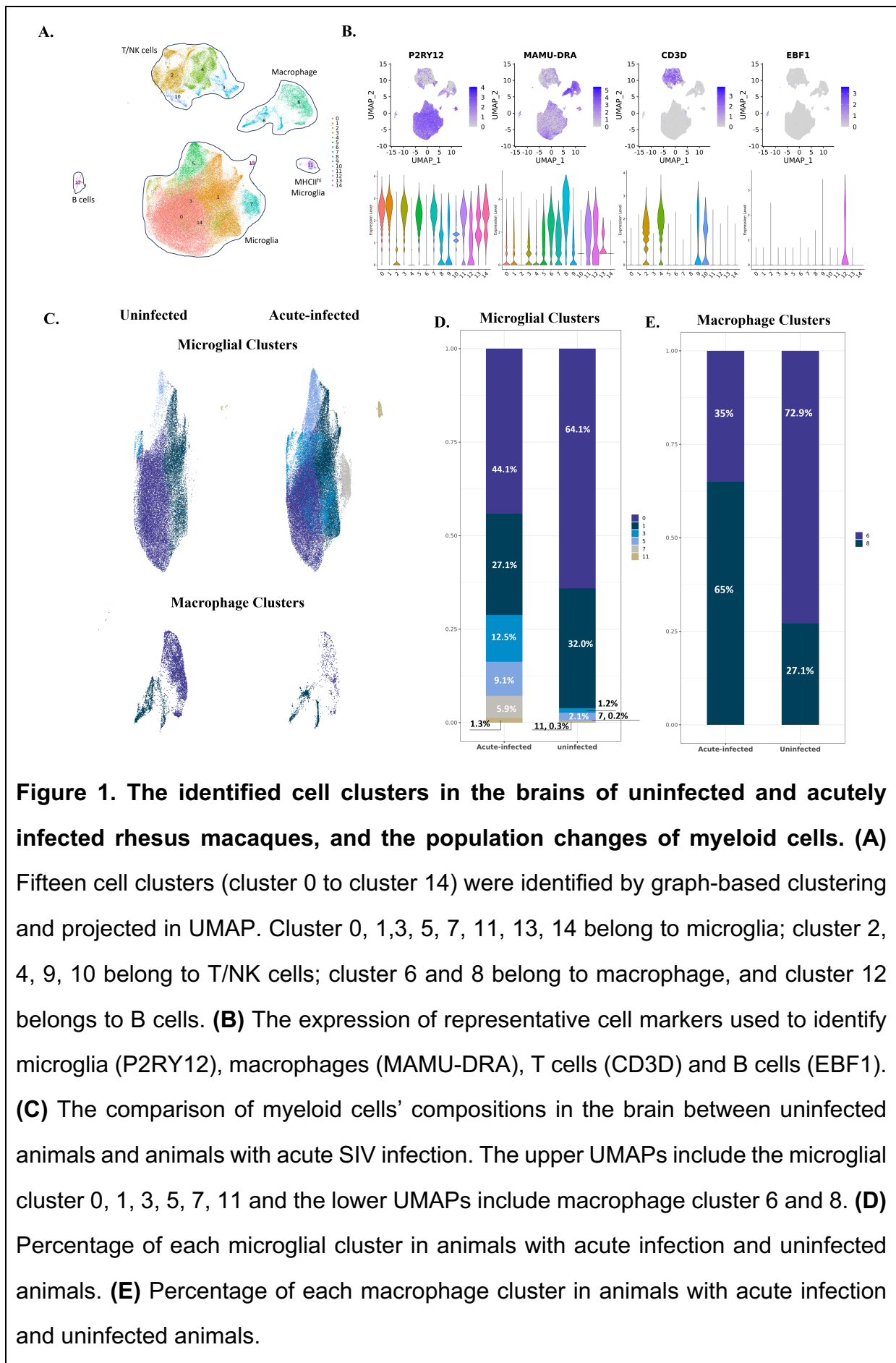
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138 RESULTS

139 The constitution of the brain's myeloid cell population was altered by acute SIV 140 infection.

141 To examine the effect of acute SIV infection on brain myeloid cells, we
142 inoculated three rhesus macaques with SIV_{mac251}, and processed the brains at 12-
143 days post-inoculation to enrich resident and infiltrating immune cells for scRNA-seq.
144 Cells from three uninfected animals were similarly analyzed. In the infected animals,
145 high viral loads were found in the plasma at this stage, and productive brain infection
146 (high viral RNA/DNA ratios) was found (**Figure S1, Table S1**). Uniform Manifold
147 Approximation and Projection (UMAP) visualization of the scRNA-seq data revealed
148 that the transcriptional patterns of the cells purified from the brains of the six rhesus
149 macaques (three uninfected and three acute SIV-infected) grouped in five distinct
150 regions. Graph-based clustering revealed that two of those regions contained distinct
151 clusters, whereas in the other three existed additional subclusters of cells, totaling
152 fifteen separate clusters (**Figure 1A**).

153 After comparing the expression of different cell markers between those 15 cell
154 clusters (**Figure 1B and Table S2**), we identified seven microglial clusters with high
155 expression of microglial core genes (P2RY12, GPR34, and CX3CR1) within the main
156 microglia cluster, and one separate cluster of microglia-like cells that also expressed
157 high levels of major histocompatibility complex class II (MHC II) molecules. We also
158 found two macrophage clusters, which had high expression of MHC II and S100
159 molecules (MAMU-DRA, CD74, S100A6, and S100A4). In addition, we identified four
160 T/NK cell clusters with high expression of the genes for T cell co-receptors,
161 granzymes, and NK cell granule proteins (CD3D, GZMB, and NKG7), and one B cell
162 cluster with high expression of B cell markers (EBF1 and MS4A1). The scope of this



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Figure 1. The identified cell clusters in the brains of uninfected and acutely infected rhesus macaques, and the population changes of myeloid cells. (A) Fifteen cell clusters (cluster 0 to cluster 14) were identified by graph-based clustering and projected in UMAP. Cluster 0, 1,3, 5, 7, 11, 13, 14 belong to microglia; cluster 2, 4, 9, 10 belong to T/NK cells; cluster 6 and 8 belong to macrophage, and cluster 12 belongs to B cells. **(B)** The expression of representative cell markers used to identify microglia (P2RY12), macrophages (MAMU-DRA), T cells (CD3D) and B cells (EBF1). **(C)** The comparison of myeloid cells' compositions in the brain between uninfected animals and animals with acute SIV infection. The upper UMAPs include the microglial cluster 0, 1, 3, 5, 7, 11 and the lower UMAPs include macrophage cluster 6 and 8. **(D)** Percentage of each microglial cluster in animals with acute infection and uninfected animals. **(E)** Percentage of each macrophage cluster in animals with acute infection and uninfected animals.

177 study is to investigate the myeloid cells in response to the acute SIV infection, so we
178 only included the myeloid cells, microglia and CNS-associated macrophages (CAMs),
179 for subsequent analyses.

180 We first assessed whether we could detect cells expressing SIV transcripts.
181 Indeed, in both the microglia and CAM clusters, SIV-infected cells could be identified,
182 in total 0.15% of these myeloid cells (**Table S1**). Given the sensitivity of scRNA-seq,
183 this percentage may be artificially low due to false negatives. In the five regions of
184 brain analyzed for SIV DNA from the three acutely infected monkeys, we found an
185 average of 162 copies of the SIV proviral genome per million cells (**Figure S1, Table**
186 **S1**). If one approximates brain macrophages and microglia as comprising 10% of CNS
187 cells, this would imply that 0.16% of these cells are infected, quite similar to our finding
188 of 0.15% of these cells with SIV transcripts. Thus, at least during the acute infection
189 stage, it is likely that the level of expression from the viral genome is sufficient to be
190 recognized in scRNA-seq experiments. Given the similarity in the proportion of cells
191 with SIV DNA and SIV transcripts, it is likely that most infected cells are expressing
192 SIV transcripts, consistent with the high SIV RNA to DNA ratio (**Figure S1, Table S1**).

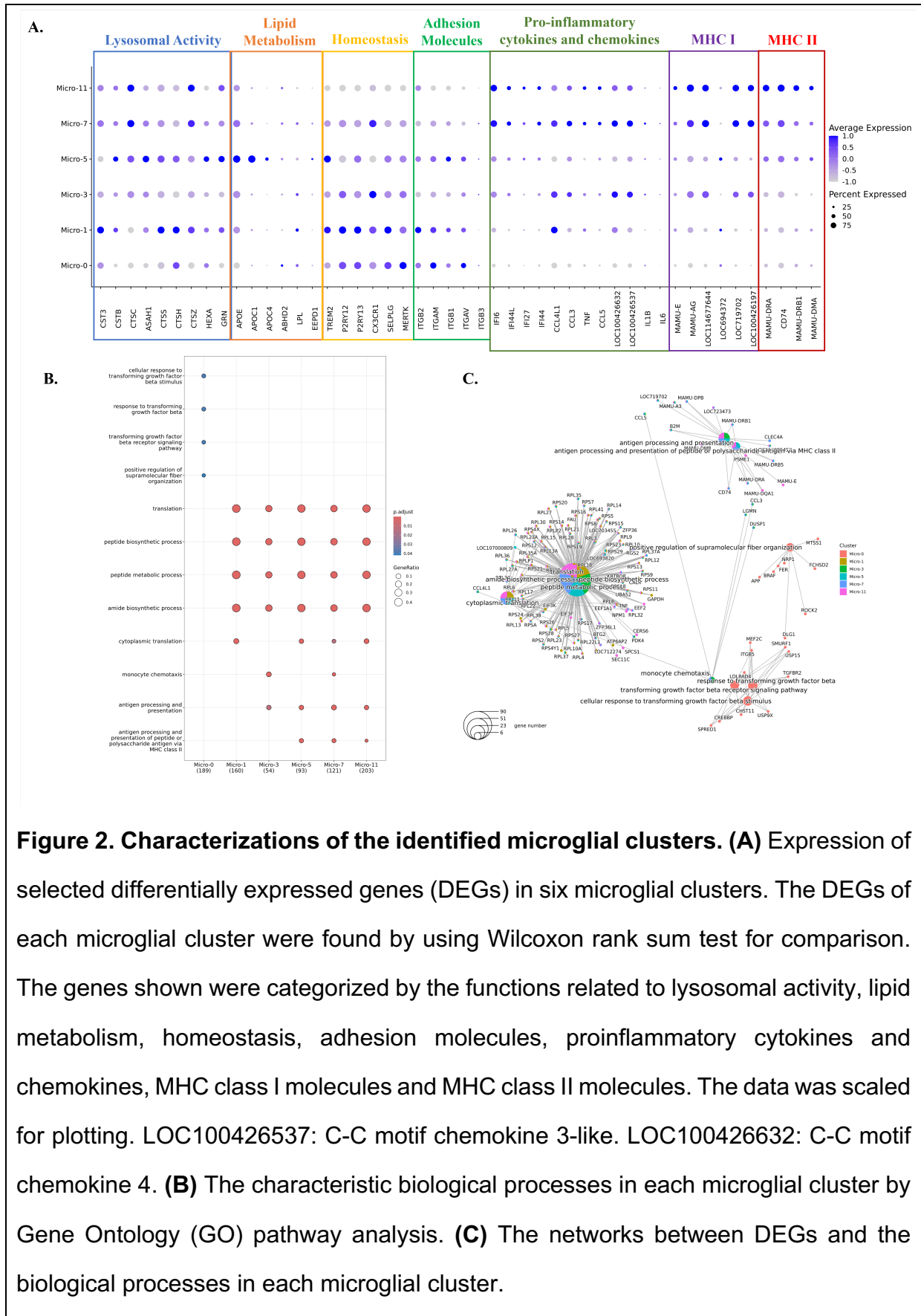
193 We next examined whether the infected myeloid cells overall exhibited
194 differences in their gene expression patterns. Other than SIV itself, only two genes
195 (LOC100426197, a class I histocompatibility gene; and LOC693820, the 40S
196 ribosomal protein S29) reached statistical significance for differential expression
197 between the infected myeloid cells and uninfected ones in the acutely infected animals
198 (**Table S3**). This is likely due to activation of even the uninfected cells in the infected
199 animals, as comparing the SIV-positive cells to all the myeloid cells in the uninfected
200 animals yielded 226 DEGs (**Table S3**). Confirming the effect of the acute infection on
201 uninfected cells, when comparing these bystander uninfected cells from the acutely

202 infected animals to those in the uninfected animals, 428 DEGs were identified (**Table**
203 **S3**). 182 DEGs were in common between the last two comparisons (**Figure S2**).

204 To better to assess how acute infection alters the myeloid populations, we then
205 separately compared the different microglia and macrophage clusters between the
206 acutely infected animals and the control uninfected animals. Cluster 13 and cluster 14
207 were excluded for their low cell population (cluster 13 has 273 cells and cluster 14 has
208 177 cells) and fewer identified markers other than microglial core genes (**Table S2**).
209 Therefore, we did downstream analyses on the six microglial clusters (0: Micro-0, 1:
210 Micro-1, 3: Micro-3, 5: Micro-5, 7: Micro-7, 11: Micro-11) and two macrophage clusters
211 (6: Macro-6 and 8: Macro-8).

212 As described briefly above, the initial identification of cell phenotypes revealed
213 that Micro-11 had a high expression of microglial core genes, but from the UMAP
214 (**Figure 1A**), it did not aggregate with other microglial clusters. In addition, the cells in
215 Micro-11 also had high expression of MHC class II molecules (**Figure 2A**). Even
216 though MHC class II molecules typically are not highly expressed on the microglia cells
217 compared with CAMs,⁽¹⁸⁾ some of the microglial cells in white matter or under
218 pathological conditions might have higher expression.⁽²⁷⁻²⁹⁾ Thus we included this
219 cluster in the analyses for microglia instead of CAM.

220 Comparing the microglial constituents between the uninfected and infected
221 animals (**Figure 1C and Figure 1D**), we found that Micro-0 and Micro-1 decreased
222 their proportions under acute SIV infection, which was compensated by the increased
223 proportions of microglial cells from other four clusters. Furthermore, this increase in
224 acute infection was more remarkable for Micro-3 and Micro-7, each increasing at least
225 10-fold, suggesting those two clusters were highly reactive to the infection. For the



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237 macrophage clusters, Macro-6 was the predominant macrophage population
238 comparing to Macro-8 in the brains of uninfected animals (1.9-fold higher), however,
239 this pattern was switched during SIV infection (**Figure 1C and Figure 1E**). There was
240 a 3.5-fold higher population of Macro-8 over Macro-6 in animals with acute SIV
241 infection, suggesting important roles of Macro-8 in reacting to early viral invasion.

242 **The increased microglial populations in acute SIV infection were activated**
243 **microglia but they have different activation patterns and pathways.**

244 To better understand the factors behind the changes in microglial populations
245 during acute SIV infection, we further characterized the clusters. We initially compared
246 them regarding the expression of lysosomal proteins, lipid-metabolic proteins,
247 homeostatic molecules, integrins, pro-inflammatory cytokines, chemokines, MHC
248 class I and MHC class II molecules for their important roles in the immune surveillance
249 and maintaining homeostasis of CNS (**Figure 2A**). These included commonly reported
250 homeostatic genes of microglia include purinergic receptors (P2RY12 and P2RY13),
251 fractalkine receptor (CX3CR1), selectin P (SELPLG), triggering receptor expressed on
252 myeloid cells (TREM2), and tyrosine-protein kinase MER (MERTK). Micro-0 and
253 Micro-1 had higher expression of these genes compared with the other four microglial
254 clusters, indicating they were less likely to be associated with activation and
255 inflammation. However, compared with Micro-0, Micro-1 had enhanced lysosomal
256 activities and higher expression of some inflammatory molecules (APOE, CCL3 and
257 CCL4), suggesting it might be in a slightly more activated, or preactivated state. Micro-
258 3 and Micro-5 both had lower expression of homeostatic genes than Micro-0 and
259 Micro-1, and higher expression of genes associated with microglial activation. Micro-
260 3 highly expressed chemokines CCL3, CCL4 and MHC class I molecules, and Micro-
261 5 highly expressed lysosomal proteins, apolipoproteins (APOE, APOC1 and APOC4)

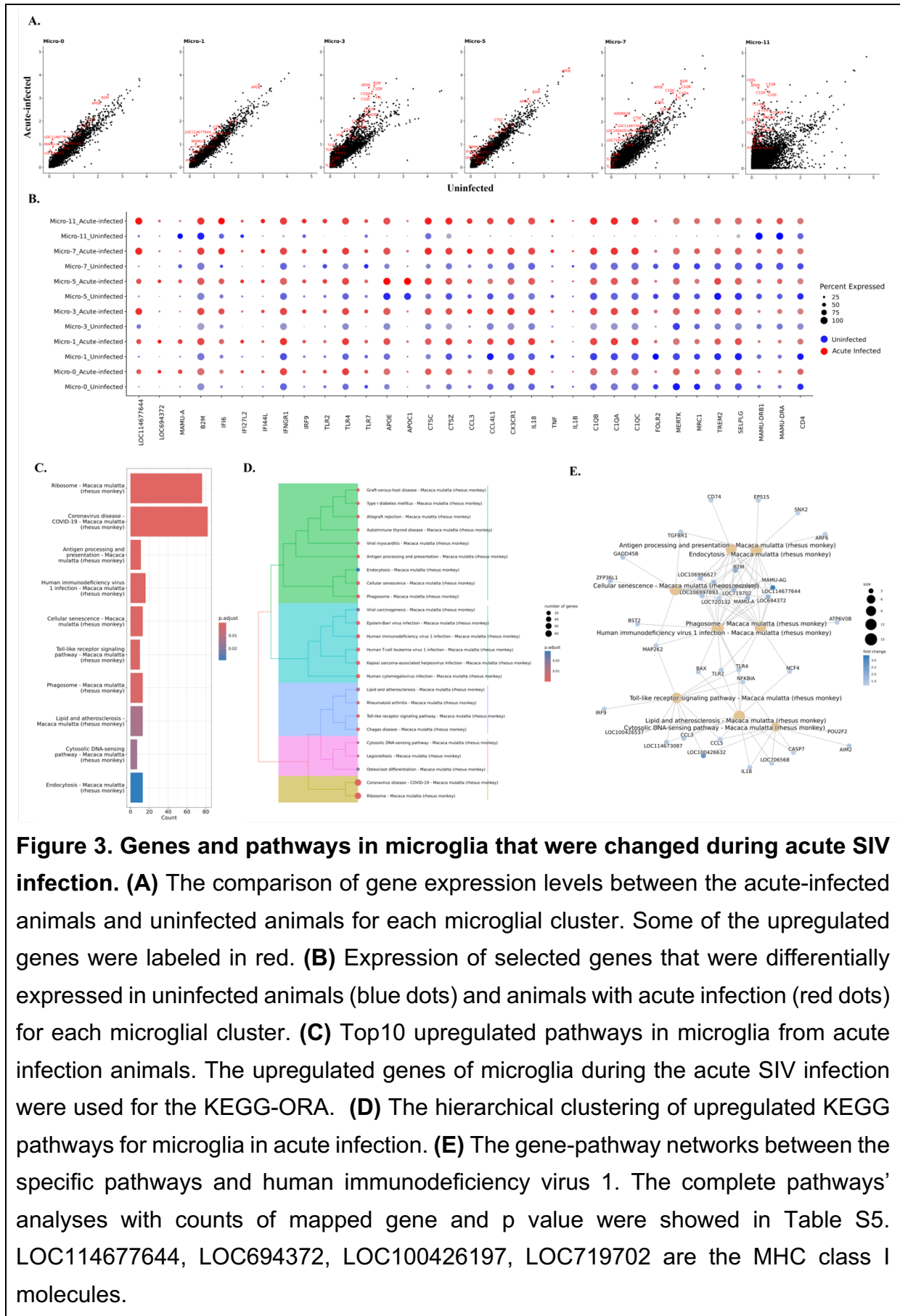
262 and MHC class II molecules, which suggested that both Micro-3 and Micro-5 might be
263 the activated, or response to infection, potentially disease-related microglia. Given that
264 more inflammatory molecules and cytokines were highly expressed, and the
265 homeostatic genes were rarely expressed in Micro-7 and Micro-11, those two
266 microglial clusters might be in a higher activated state. However, Micro-11 had higher
267 expression of MHC class II molecules with lower expression of homeostatic genes,
268 suggesting it might be the most activated microglial population. Thus, in concert with
269 our findings above the proportion of homeostatic microglia decreases, whereas the
270 proportion of activated microglia increases, during acute SIV infection.

271 We then implemented GO analyses with the DEGs in each microglial cluster to
272 assess biologic functionalities (**Figure 2B and 2C**). Micro-0 was identified with the
273 pathways associated with transforming growth factor beta (TGF β), which further
274 confirmed the homeostatic status of this microglial cluster.⁽³⁰⁾ The other five microglial
275 clusters were all active in the translation and biosynthesis, but Micro-3, Micro-5, Micro-
276 7 and Micro-11 had additional pathways related to microglial activation, such as
277 antigen-presenting ability in MHC class I or MHC class II manner and chemotactic
278 ability. However, the activated microglia clusters appeared specialized in different
279 inflammatory or activation pathways, suggesting the heterogeneity of the activated or
280 pathogenic microglial phenotypes in the brain. In summary, these results indicated that
281 Micro-0 represented homeostatic microglia, Micro-1 might be a preactivated cluster
282 with more activities in protein translation and lysosomal functions, and the other four
283 clusters were the activated microglia with upregulation of different inflammatory
284 molecules and pathways.

285 **Genes and pathways related to the MHC class I and type I interferon production**
286 **were upregulated in all microglial clusters responding to the acute SIV infection.**

287 In the characterization of the microglial clusters above, we found one
288 homeostatic cluster, one preactivated microglial cluster and four activated clusters.
289 Although the cells from uninfected animals and infected animals were aggregated
290 together for clustering, they still differ in the expression of some genes. Therefore, we
291 identified the genes that were significantly upregulated in each microglial cluster due
292 to acute SIV infection (**Table S4**). More upregulated genes with high fold-change in
293 acute SIV infection were found in activated clusters Micro-3 and Micro-11, where the
294 average expression of the genes associated with activated microglia were low in
295 uninfected animals (**Figure 3A**). Therefore, for those two clusters, it might be that only
296 the microglial cells from infected animals were truly activated microglia. For the other
297 activated or preactivated microglial clusters, the cells from uninfected animals did have
298 higher expression of the genes associated with activated microglia compared with the
299 homeostatic cluster Micro-0, and those clusters further upregulated the expression of
300 those activation genes in responses to acute SIV infection.

301 To better understand the changes in individual microglial clusters during acute
302 SIV infection, we did a comparison between uninfected and acute infection samples
303 for each microglial cluster (**Figure 3B**). We found that the genes that were related to
304 MHC class I and interferon (IFN) signaling pathways were universally upregulated in
305 microglial clusters during acute infection. It is well recognized that IFNs can block
306 HIV/SIV replication,⁽³¹⁻³³⁾ and in this study we found that the key transcription factor in
307 JAK-STAT pathway for IFN production, IRF9, was significantly upregulated in
308 microglial cells. The increased fold-change of IRF9 ranged from 1.4 (Micro-11) to 8.3
309 (Micro-3), indicating this molecule was extensively upregulated in acute SIV infection.
310 Furthermore, the genes encoding type I interferon inducible proteins (IFI genes),
311 especially IFI6, IFI27 and IFI44 were also significantly upregulated in all microglial



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326 clusters. Those IFI genes are classified into interferon-stimulated genes (ISGs), which
327 can be stimulated by the IFNs' signaling to augment the restriction of HIV/SIV
328 replication and cell entry. In addition, we also found that the IFN γ receptor (IFNGR1)
329 was slightly increased in most microglial clusters (~1.1-fold change) and highly
330 increased in Micro-11 cluster (4.5-fold change). Other upregulated genes included
331 those encoding for apolipoproteins (e.g., APOE, APOC1), lysosomal proteins (e.g.,
332 CTSC, CTSZ), complement components (e.g., C1QA, C1QB, C1QC), chemokines
333 (e.g., CCL3, CCL4, CX3CR1), and proinflammatory cytokines (e.g., IL18, TNF, IL1B).
334 Although most the aforementioned molecules were found to be upregulated in all
335 microglial clusters, Micro-1, Micro-3, Micro-7, and Micro-11 were the clusters that had
336 higher expression of those genes compared with Micro-0 and Micro-5 under acute SIV
337 infection (**Figure S4A**). It should be noted that the Micro-5 cluster was found to have
338 the highest expression of APOE and APOC in the uninfected condition as well as acute
339 infection condition compared with other clusters (**Figure 3B**). APOE and APOC are
340 also associated with neuroinflammation and neurodegeneration,⁽³⁴⁾ so their higher
341 expression in Micro-5 indicated the activated nature of this cluster.

342 Surprisingly, different from the upregulated MHC class I molecules, the MHC
343 class II molecules remained unchanged or downregulated for most microglial clusters.
344 The downregulation of MHC class II molecules was more obvious in Micro-7 and
345 Micro-11, in which the MHC class II molecules had higher expression than other
346 clusters (**Figure 2A**). The other downregulated genes were related to
347 immunoregulation and microglial homeostasis (e.g., MERTK, TREM2, MRC1, FOLR2,
348 SELPLG). This downregulation was significant in Micro-0, Micro-1, Micro-5, and Micro-
349 7, but for Micro-3 and Micro-11 these genes' expression was upregulated. When we
350 compared the expression of these immunoregulatory or homeostatic molecules

351 between different microglial clusters in acute SIV infection, we found that their
352 upregulation in Micro-3 leads this cluster to have higher expression of those genes
353 compared with other clusters (**Figure S4A**). Intriguingly, CD4 gene expression was
354 also downregulated in all microglial clusters (fold-change ranging from 1.1-1.3) and
355 had highest expression in Micro-0 during the infection. CD4 serves as an important
356 receptor for virus entry to the microglia⁽³⁵⁾ as well as the other targets of SIV/HIV
357 infection, and its downregulation might indicate the defensive strategy of activated
358 microglia in acute SIV infection. While CD4 downregulation by HIV and SIV infection
359 of cells is known to occur through viral accessory proteins targeting the CD4 protein
360 for degradation,⁽³⁶⁾ the downregulation of its transcript in all of the microglial clusters
361 in acute infection likely is a result of cellular activation.

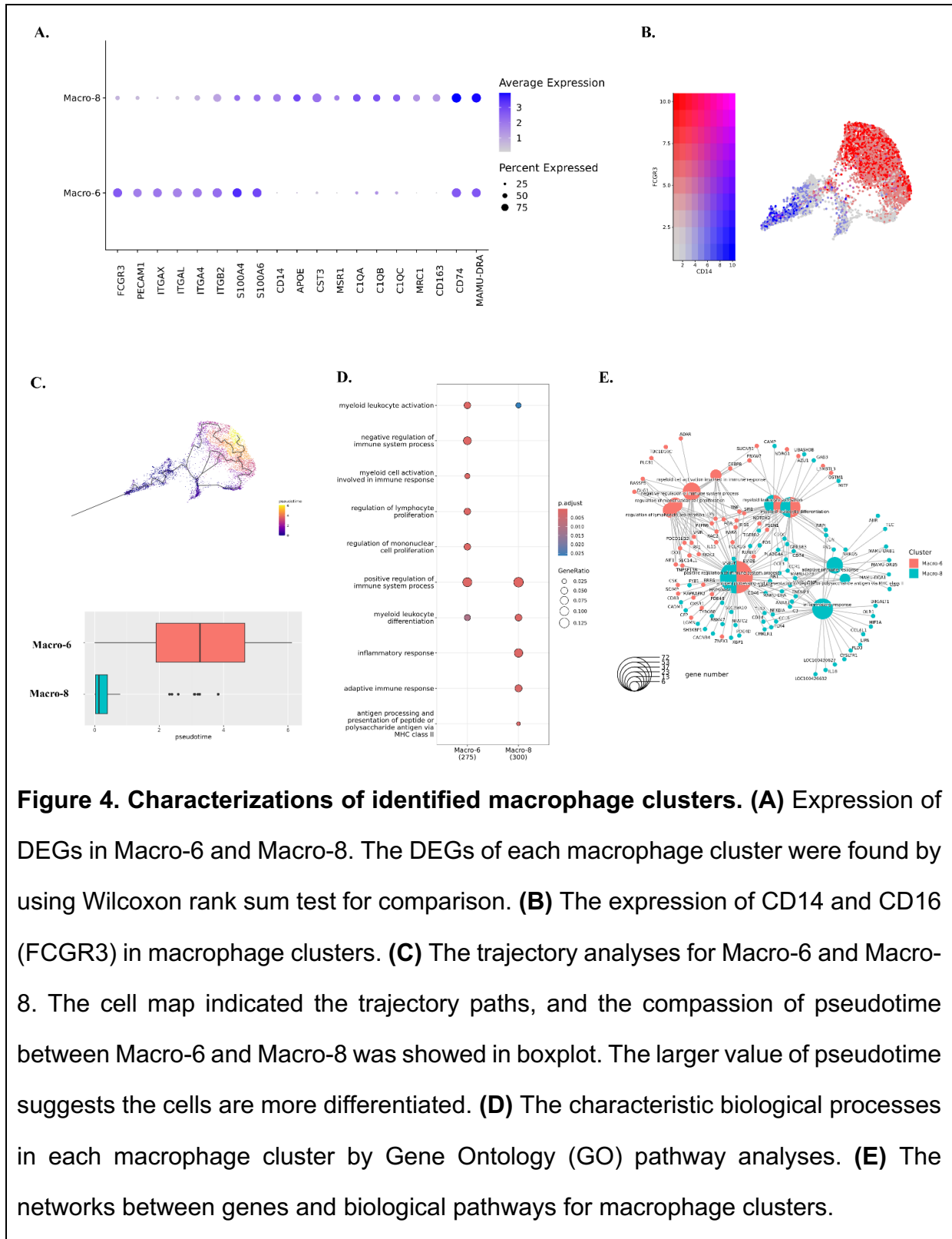
362 The genes that were significantly upregulated in microglial cells were then
363 further associated with various pathways by using the Kyoto Encyclopedia of Genes
364 and Genomes (KEGG) databases (**Figure 3C-3E**). All six clusters upregulated
365 pathways related to antigen presentation and processing as well as cellular
366 senescence. Micro-0 was found to upregulate the least number of pathological
367 pathways compared with other microglial clusters (**Table S5**), which suggests that the
368 homeostatic status in Micro-0 was least altered by acute SIV infection. The other five
369 microglial clusters were associated with more disease-related pathways under acute
370 infection (**Figure S3**). In general, these pathways could be categorized into five
371 classes (**Figure 3D**), including pathways related to antigen processing and
372 presentation, viral infections, inflammation, cytosolic DNA sensing, and ribosomal
373 activities. In the category of viral infections, we found the HIV-1 infection pathway, with
374 sixteen enriched genes. Most genes upregulated in the HIV-1 infection pathway

375 encoded MHC class I molecules, which connected the endocytosis, phagocytosis, and
376 antigen presentation pathways to HIV-1 infection (**Figure 3E**).

377 The MEK2 protein kinase (MAP2K2) molecule, involved in mitogen-activated
378 protein kinase (MAPK) pathway, also serves as the key molecule connecting cellular
379 senescence and toll-like receptor (TLR) signaling with HIV-1 infection. MAP2K2 can
380 trigger inflammation by phosphorylating the downstream kinases ERK1/2
381 (Extracellular Signal-Regulated Kinases 1 and 2) to translocate the transcription factor
382 NF- κ B and AP-1 to the nucleus for the expression of the genes encoding cytokines.
383 The TLRs utilize those pathways to induce the production of proinflammatory
384 cytokines. By comparing different TLRs, we found that TLR4, which can induce the
385 expression of type I IFNs, was upregulated in all microglial clusters (**Figure 3C**) during
386 acute SIV infection. TLR2 signaling, also acting through NF- κ B and AP-1 to produce
387 proinflammatory molecules (such as TNF- α , IL-1 β , and IL-6), was upregulated in most
388 microglial clusters. TLR7 which, as opposed to the cell-surface location of TLR4 and
389 TLR2 is located on the endosomal compartment of the cells, and can specifically
390 recognize single strand RNA (ssRNA) of HIV/SIV for the production of type I IFNs ⁽³⁷⁻
391 ³⁹⁾, was found to be upregulated particularly in Micro-3 (2.8-fold change). While the
392 TLRs were extensively upregulated in microglial cells, Micro-7 and Micro-11 had the
393 highest expression of TLR2, Micro-1 had the highest expression of TLR4, and Micro-
394 3 and Micro-7 had the highest expression of TLR7 during acute SIV infection (**Figure**
395 **S4A**), indicating different microglial clusters might favor different TLR signaling
396 pathways to produce proinflammatory cytokines. In summary, all of the upregulated
397 pathways in acute SIV infection pointed to the pathways related to interferons and
398 TLR-induced inflammatory cytokine production, highlighting the critical roles of them
399 in microglia defense against acute SIV infection.

400 **The predominant CAM cluster phenotype in acute SIV infection was**
401 **CD14^{hi}CD16^{low}**

402 In the characterization of the predicted phenotypes for those two macrophage
403 clusters, we found that Macro-6 had lower expression of the inflammatory molecules
404 which were highly expressed in Macro-8 (e.g., APOE, CST3, MSR1). Instead, it had
405 higher expression of the cell adhesion molecules (e.g., PECAM1 and integrins)
406 **(Figure 4A and Table S2)**. In addition, Macro-6 was found to have high expression of
407 CD16 (FCGR3) but low expression of CD14, and Macro-8 had high expression of
408 CD14 but low expression of CD16 **(Figure 4B)**. In human blood, CD14^{hi}CD16^{low} cells
409 are described as inflammatory classical monocytes/ macrophages which are trafficked
410 to sites of inflammation and/or infection, and CD14^{low}CD16^{hi} cells are the patrolling
411 non-classical monocytes/macrophages, which adhere and crawl along the luminal
412 surface of endothelial cells.⁽⁴⁰⁾ In this study, we found that acute SIV infection shifted
413 the dominant CAM phenotype from CD14^{low}CD16^{hi} (Macro-6) to CD14^{hi}CD16^{low}
414 (Macro-8). Although Macro-8 appears to represent the classical inflammatory
415 phenotype, it also had higher expression of the markers for anti-inflammatory M2-like
416 macrophages (e.g., MRC1/CD206, CD163). Regarding differentiation, it was reported
417 that the CD14^{hi}CD16^{low} phenotype can be directly differentiated from precursor cells
418 in bone marrow, and it is the obligatory precursor intermediate for CD14^{low}CD16^{hi}
419 phenotype in the blood.⁽⁴¹⁾ Intriguingly, through trajectory analyses we also found that
420 the CD14^{low}CD16^{hi} CAMs (Macro-6) had a larger value of pseudotime compared to
421 the CD14^{hi}CD16^{low} CAMs (Macro-8) **(Figure 4C)**, suggesting CD14^{hi}CD16^{low} cells
422 might also be the precursor intermediate for CD14^{low}CD16^{hi} cells in CNS.



423

424 **Figure 4. Characterizations of identified macrophage clusters. (A)** Expression of

425 DEGs in Macro-6 and Macro-8. The DEGs of each macrophage cluster were found by

426 using Wilcoxon rank sum test for comparison. **(B)** The expression of CD14 and CD16

427 (FCGR3) in macrophage clusters. **(C)** The trajectory analyses for Macro-6 and Macro-8.

428 The cell map indicated the trajectory paths, and the comparison of pseudotime

429 between Macro-6 and Macro-8 was showed in boxplot. The larger value of pseudotime

430 suggests the cells are more differentiated. **(D)** The characteristic biological processes

431 in each macrophage cluster by Gene Ontology (GO) pathway analyses. **(E)** The

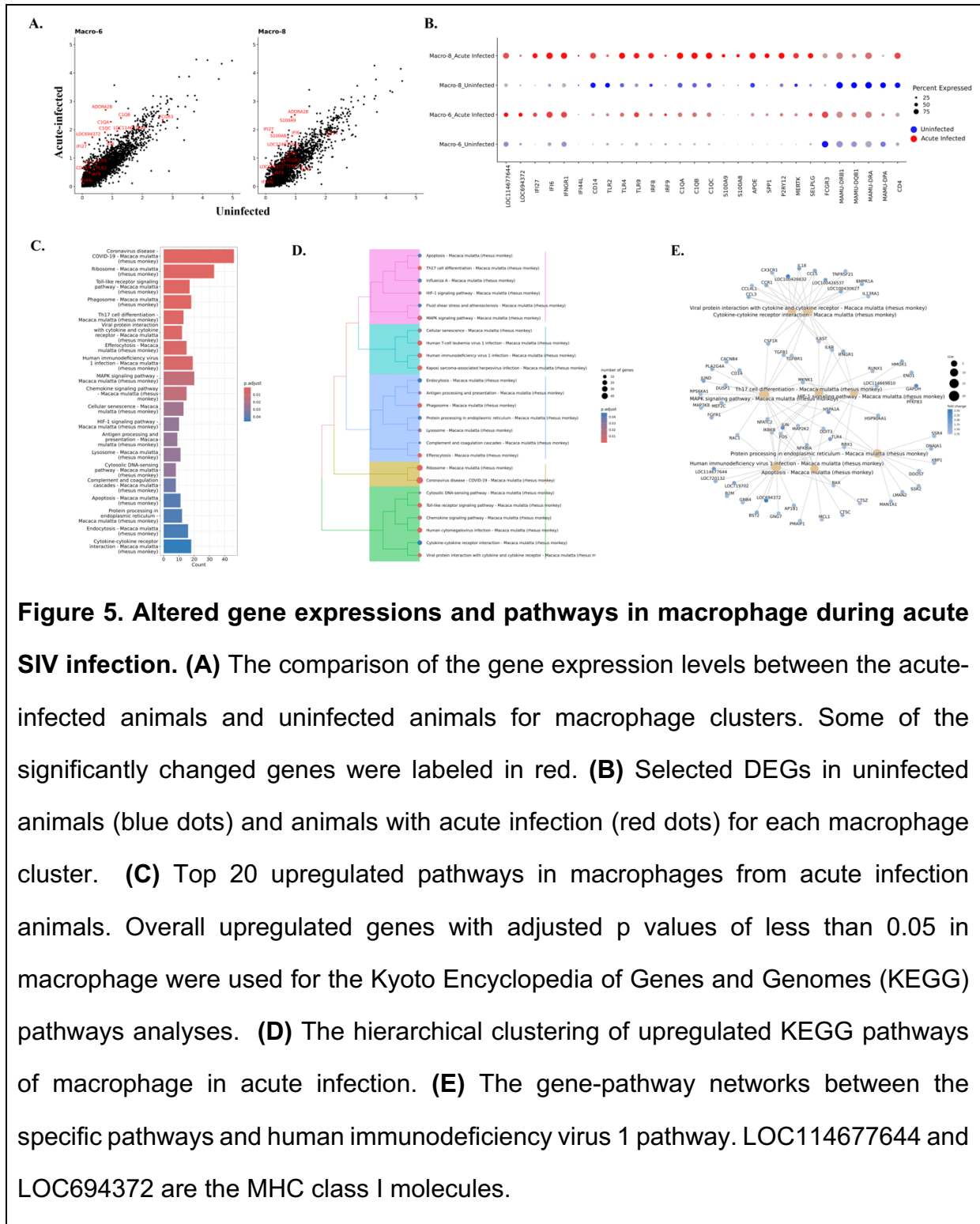
432 networks between genes and biological pathways for macrophage clusters.

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434 The GO results indicated that cells in both the Macro-6 and Macro-8 clusters
435 participated in myeloid leukocyte activation and differentiation, which suggested these
436 two phenotypes might originate or differentiate from infiltrating monocytes.
437 According to the opposite biological functions of CD14^{hi}CD16^{low} and CD14^{low}CD16^{hi}
438 monocytes in the periphery, the Macro-6 cluster had both positive and negative
439 regulations of the immune processes, however, the Macro-8 cluster lacked the
440 negative regulation of the immune responses and thus had the potential to trigger
441 inflammation (**Figure 4D**). Furthermore, those two CAM clusters might also have the
442 potential for interactions with infiltrating lymphocytes and adaptive immune responses,
443 functions that were not found in the microglia clusters. For example, Macro-6 was
444 predicted to regulate lymphocyte proliferation by secreting IL-15, which is an important
445 stimulator for T/NK cells' proliferation and activation. Macro-8 highly expressed MHC
446 class II as well as other molecules, which can present the antigens for triggering
447 adaptive immune responses (**Figure 4E**). In summary, we identified both
448 CD14^{hi}CD16^{low} and CD14^{low}CD16^{hi} macrophage phenotypes in the brains of rhesus
449 macaque, and the CD14^{hi}CD16^{low} cells (Macro-8 cluster) became the dominant
450 phenotype in acute SIV infection, whereas CD14^{low}CD16^{hi} cells (Macro-6 cluster)
451 predominated in the uninfected condition.

452 **CAMs might be more activated in triggering immune responses under acute SIV**
453 **infection than microglia.**

454 Consistent with the genes that were upregulated in all microglial clusters, most
455 molecules related to MHC class I and IFN production were also significantly
456 upregulated in both macrophage clusters (**Figure 5A and 5B**). However, unlike
457 microglial clusters, the IFI44L gene had extremely low expression in macrophage
458 clusters. The changes of MHC class II molecules in macrophage clusters are different



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Figure 5. Altered gene expressions and pathways in macrophage during acute SIV infection. (A) The comparison of the gene expression levels between the acute-infected animals and uninfected animals for macrophage clusters. Some of the significantly changed genes were labeled in red. **(B)** Selected DEGs in uninfected animals (blue dots) and animals with acute infection (red dots) for each macrophage cluster. **(C)** Top 20 upregulated pathways in macrophages from acute infection animals. Overall upregulated genes with adjusted p values of less than 0.05 in macrophage were used for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analyses. **(D)** The hierarchical clustering of upregulated KEGG pathways of macrophage in acute infection. **(E)** The gene-pathway networks between the specific pathways and human immunodeficiency virus 1 pathway. LOC114677644 and LOC694372 are the MHC class I molecules.

472 between Macro-6 and Macro-8. The expression of MHC class II molecules in Macro-
473 6 remained unchanged except for the downregulation of MAMU-DPA, however, the
474 expression of those genes in Macro-8 was extensively downregulated in acute SIV
475 infection, which is consistent with the changes of activated microglial clusters.
476 Intriguingly, we also found that the CAMs significantly upregulated the core genes for
477 homeostatic microglia during the acute SIV infection (**Figure 5B**). Given the myeloid
478 lineage of microglia and CAMs, the similar behaviors in response to acute SIV infection
479 might be expected (e.g., APOE and SPP1), but it was surprisingly that the homeostatic
480 genes for microglia (e.g., P2RY12, GPR34) and other genes characterizing microglia
481 (MERTK, SELPLG) that were downregulated or remained unchanged in microglial
482 clusters during acute SIV infection were increased in CAMs, especially Macro-6. We
483 found that that P2RY12 expression was upregulated 4-fold, GPR34 4.5-fold, SELPLG
484 2.4-fold, and MERTK 3.7-fold in the Macro-6 cluster. Although Macro-6 highly
485 upregulated those molecules, the expressions of microglial homeostatic core genes
486 were still higher in Macro-8 during acute infection (**Figure S4B**). Furthermore, while
487 Macro-6 was characterized as CD14^{low}CD16^{hi} macrophages, and Macro-8 was
488 characterized as classic CD14^{hi}CD16^{low} macrophages, once they were in acute
489 infection condition, the CD14^{hi}CD16^{low} cells upregulated CD16 (1.5-fold), whereas the
490 CD14^{low}CD16^{hi} cells upregulated CD14 (3.3-fold). This again highlights the plasticity
491 of myeloid cells.

492 The elevation of CD14 in Macro-6 was also accompanied by the increased
493 expression of TLR4, which is the coreceptor for CD14 in inducing pro-inflammatory
494 signaling. In addition, more genes related to the inflammatory pathways (e.g., TLR2,
495 TLR9, IRF8, IRF9, C1QA, C1QB, C1QC) were found to be increased in both
496 macrophage clusters (**Figure 5B**). The fold-change of most inflammatory molecules

497 was higher in Macro-6 (e.g., C1QA, C1QB, and C1QC had a 2-fold change in Macro-
498 6 but only ~1.2-fold change in Macro-8) but the overall expression level was still higher
499 in Macro-8 compared to Macro-6 (**Figure S4B**). Macro-8 also significantly upregulated
500 more molecules related to innate defense, such as S100A8 (fold-change: 2.3) and
501 S100A9 (fold-change: 2.9), which was not observed in Macro-6. In summary, these
502 results suggest that the immunosuppressed phenotype of Macro-6 might differentiate
503 toward inflammatory phenotype, and the pre-activated phenotype of Macro-8
504 upregulated more inflammatory molecules and signaling pathways during the acute
505 infection.

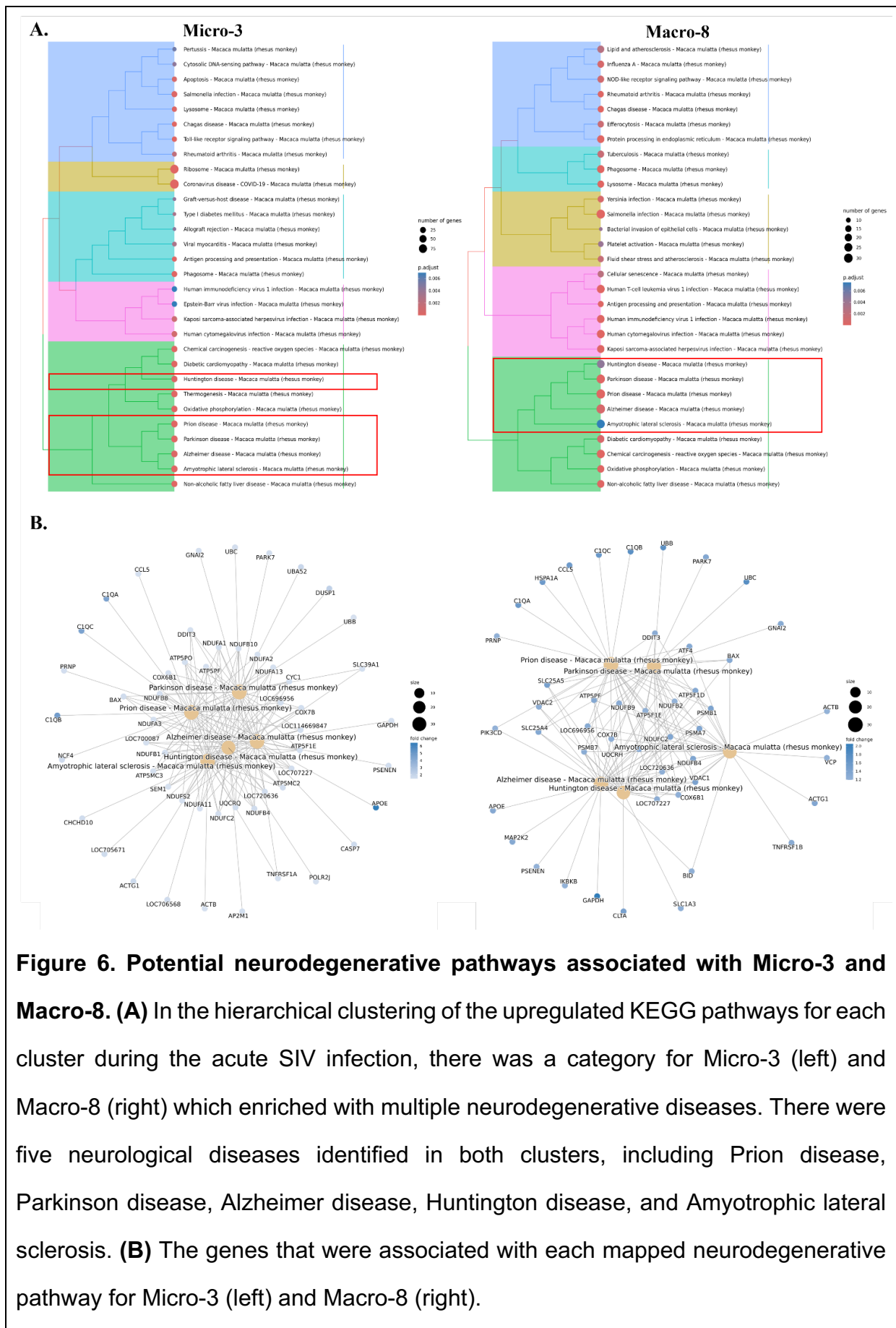
506 The KEGG gene enrichment analyses for macrophages showed more
507 upregulated inflammatory pathways compared to microglia (**Figure S5 and Table S5**).
508 Overall, the macrophages not only enhanced the pathways that were found to be
509 upregulated in microglia, but also augmented other inflammation-related pathways, for
510 example, the interaction between viral proteins and cytokine/chemokine receptors and
511 Th17 cell differentiation (**Figure 5C and 5D**). In the characterization of the CAMs by
512 their featured pathways, we found that these macrophages might have more
513 interactions with the T cells than microglial cells. When we further examined the genes
514 that were upregulated during acute SIV infection in the KEGG pathways, we found
515 that they were enriched in the ability to induce Th17 differentiation during acute SIV
516 infection. The genes that are mapped to this pathway included IL-6 and TGFB (**Figure**
517 **5E**), which were reported as key cytokines secreted by macrophages to trigger
518 differentiation of Th17 cells.^(42, 43) Given the pleiotropic functions of those two
519 cytokines, their increase might not be necessarily correlated with Th17 differentiation,
520 but their increased expression in perivascular macrophages responding to acute SIV
521 infection has been confirmed in rhesus macaque.⁽⁴⁴⁾ Even though more inflammatory

522 or immunological pathways were upregulated in macrophages compared to microglia,
523 the key pathway that connects them with HIV-1 infection might also involve the NF- κ B
524 signaling. In the gene-pathway connections (**Figure 5E**), multiple molecules and
525 kinases for NF- κ B signaling (e.g., IKBKB, JUN, FOS, MAP2K2, NFKBIA, TLR4)
526 connected the HIV-1 infection and other inflammatory or anti-viral pathways.

527 **The genes that were upregulated in Micro-3 and Macro-8 clusters in response to**
528 **acute SIV infection are associated with multiple neurological diseases.**

529 The microglial and macrophage clusters that were widely activated, with increased
530 expression of proinflammatory cytokines and chemokines in acute SIV infection, might
531 induce toxicities not only for the virus but also for neurons and other cells in the CNS.
532 Here, we found that one microglial cluster and one macrophage cluster may be
533 strongly related to neurological disorders such as HAND. The upregulated genes
534 analyzed with KEGG gene enrichment analyses for each cluster revealed that there
535 was a category of pathways including those leading to multiple neurocognitive
536 disorders in both the Micro-3 and Macro-8 clusters (**Figure 6A**). Prion disease,
537 Parkinson disease, Alzheimer disease, Huntington disease, and amyotrophic lateral
538 sclerosis were in the list of this category. In Micro-3, all of those five
539 neurodegeneration-associated pathways have extremely low adjusted p-value
540 (<0.002); in Macro-8 the Huntington disease and amyotrophic lateral sclerosis had a
541 slightly higher adjust p-value (~ 0.006). The mapped genes in those pathways were
542 related to mitochondrial functions, and we found that the fold-change of the associated
543 genes in Macro-8 was higher than the Micro-3 (**Figure 6B**).

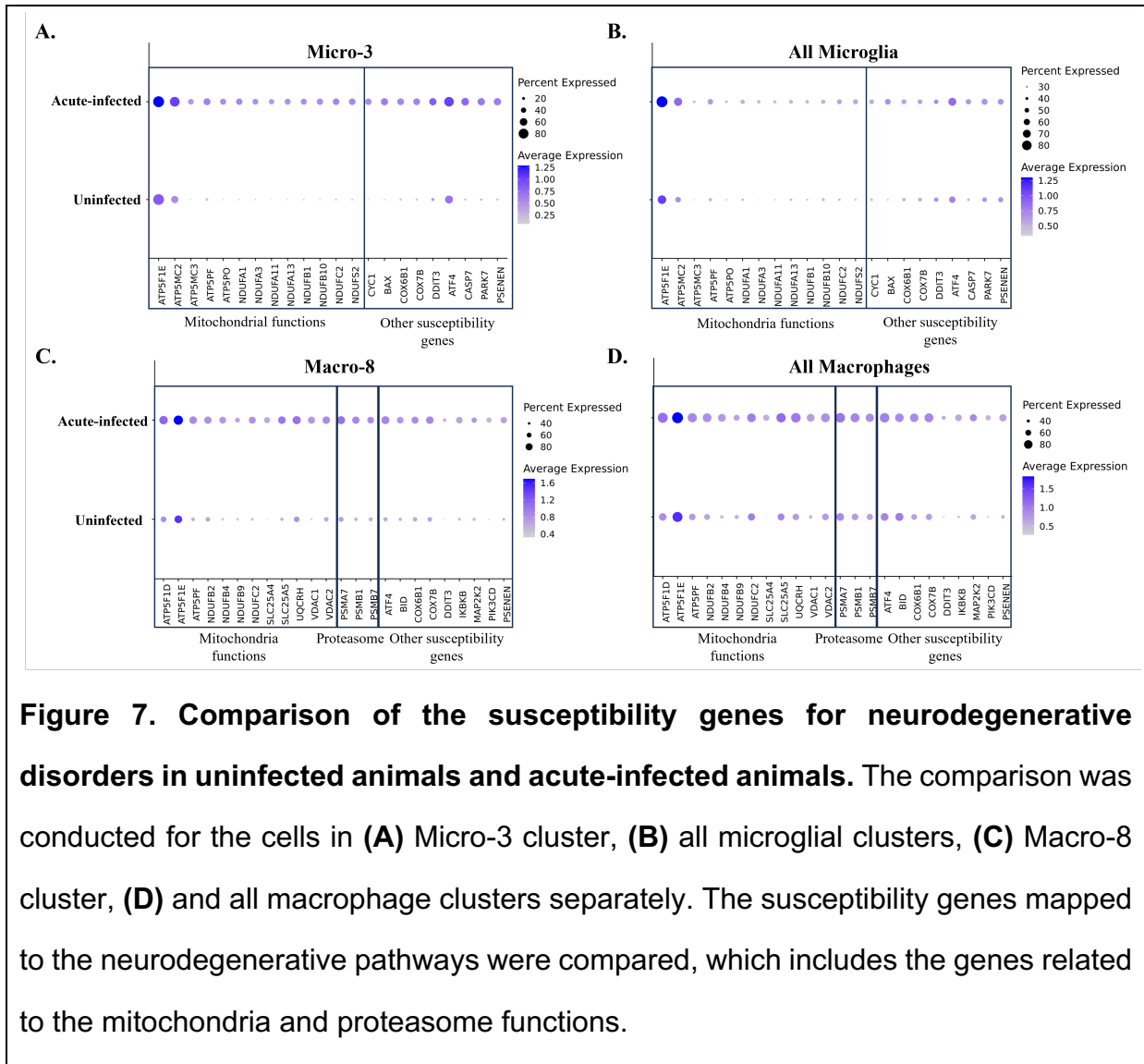
544 To further confirm the specificity of neurodegenerative molecules in Micro-3 and
545 Macro-8 during the acute SIV infection, we then compared the expression changes for



555 a number of these genes in all microglia and macrophages under uninfected
556 conditions and infected conditions. Consistent with the above, we found that those
557 genes were significantly upregulated in Micro-3 and Macro-8 in acute SIV infection
558 **(Figure 7A and Figure 7C)**. However, the expression of these genes barely changed
559 when we compared them in all microglial cells **(Figure 7B)**. Although genes that are
560 related to the mitochondria functions seemed to be upregulated when all macrophages
561 in infected and uninfected conditions were compared **(Figure 7D)**, the overall
562 changing fold was not as prominent as in the Macro-8 cluster. All of these results
563 suggested that the Micro-3 and Macro-8 clusters might be strongly associated with the
564 induction of neurological disturbances when activated in acute SIV infection.

565 **Apoptotic resistance was not initiated during acute SIV infection in myeloid cells**
566 **in the brain.**

567 HIV was reported to upregulate the expression anti-apoptotic molecules and
568 downregulate pro-apoptotic molecules to enable the survival of infected host cells.^{(25,}
569 ^{26, 45, 46)} Those anti-apoptotic molecules (e.g., BCL2, BFL1, BCL-XL, MCL-1) and pro-
570 apoptotic molecules (e.g., BAX, BIM, BAK1, BAD) are typically found in BCL-2 family.
571 While microglia and macrophages are typically thought to be long-lived, the
572 mechanism of survival following HIV infection has not been extensively studied. To
573 examine if HIV infection of myeloid cells in the brain could lead to the establishment
574 of the virus reservoir by blocking apoptosis, we first compared the expression of anti-
575 apoptotic and pro-apoptotic molecules between macrophages and microglia in acute-
576 infected animals and uninfected animals **(Figure 8A)**. However, we did not observe
577 remarkable changes in the RNAs encoded by these molecules in macrophages and
578 microglia during acute infection. We did observe the anti-apoptotic molecule BCL2
579 was downregulated, and the pro-apoptotic molecule BAX was upregulated, suggesting



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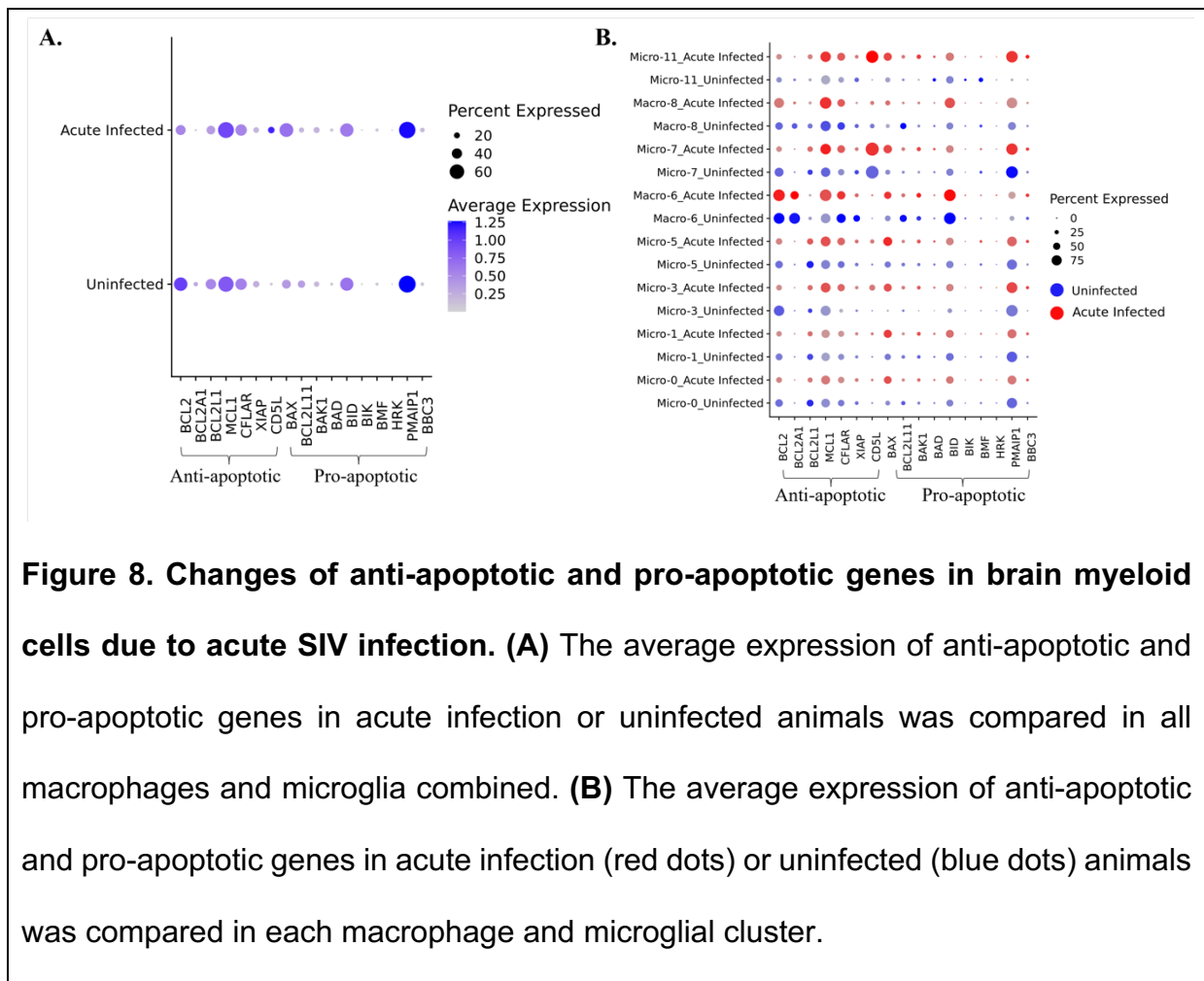
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Figure 7. Comparison of the susceptibility genes for neurodegenerative disorders in uninfected animals and acute-infected animals. The comparison was conducted for the cells in **(A)** Micro-3 cluster, **(B)** all microglial clusters, **(C)** Macro-8 cluster, **(D)** and all macrophage clusters separately. The susceptibility genes mapped to the neurodegenerative pathways were compared, which includes the genes related to the mitochondria and proteasome functions.

588 that the macrophages and microglia are prone to undergo apoptosis *in vivo* upon SIV
589 infection. Then we further investigated the change of these molecules in individual
590 myeloid cell cluster (**Figure 8B**). The BCL2 gene was downregulated in most clusters,
591 and the cluster which did not downregulate BCL2 (Macro-6) was found to maintain its
592 expression without change. On the other hand, the pro-apoptotic gene, BAX was
593 widely upregulated in most cell clusters. For other anti-apoptotic gene expressions,
594 we found that MCL1 was upregulated, except in homeostatic Micro-0 and Micro-1,
595 CFLIP (CFLAR) was upregulated in Micro-3 and Micro-11, and others remained
596 unchanged or slightly downregulated in infection. The pro-apoptotic molecules were
597 generally upregulated, for example, BAK1 was upregulated in Micro-3, Micro-7, and
598 Micro-11, BID was upregulated in Micro-3 and Macro-8, and PMAIP1 was upregulated
599 in Micro-11. However, a known pro-apoptotic molecule for microglia and macrophage,
600 BIM (BCL2L11), was found to be downregulated in Macro-6 and Macro-8.
601 Interestingly, we found that another anti-apoptotic molecule CD5L, which is not
602 included in BCL-2 family, was highly upregulated in Micro-11.
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612 **DISCUSSION**

613 Microglia are the major innate immune cells in the brain and serve multiple
614 functions. They are active in protecting the brain from invading pathogens, clearing
615 damaged synapses as well as dying cells, and promoting neuron development. While
616 not as extensively characterized as neurons, they are indeed heterogeneous, and with
617 the advent of scRNA-seq our studies and those of others have identified a number of
618 classes of microglia.⁽⁴⁷⁻⁵⁰⁾ To maintain a steady state while gaining some immunogenic
619 properties, microglia express several homeostatic genes. Downregulation and/or
620 mutations of these homeostatic genes can lead to uncontrolled neuroinflammation,
621 neurotoxicity and multiple neurocognitive diseases.⁽⁵¹⁻⁵⁵⁾ In this study, we found
622 multiple populations of microglia which changed their proportions during acute SIV
623 infection. During acute SIV infection overall activation was observed, with a lower
624 expression of homeostatic genes and a decreased proportion of cells with a
625 homeostatic phenotype. In addition, we found the major homeostatic microglial cluster,
626 Micro-0, was enriched in the TGF β signaling pathway, which was not observed for the
627 other microglial clusters. Based on others' findings, the silencing of TGF β signaling in
628 microglia resulted in loss of microglial ramification and the upregulation of
629 inflammatory markers without any external stimulus relative to wild-type microglia.^{(30,}
630 ⁵⁶⁾ Considering the pleiotropic functions of TGF β signaling in microglial cells,⁽⁵⁷⁻⁵⁹⁾ the
631 more active TGF β pathway in Micro-0 suggested this homeostatic microglial cluster
632 might be more active in stimulating microglial differentiation and regulating the
633 activation of other microglial clusters.

634 Although the proportion of cells in Micro-0 remarkably decreased during acute
635 SIV infection, this was still the dominant microglial population (consisting of 44.1% of
636 microglial cells in acute infection, compared to 64.1% in uninfected animals) in the

637 brain, suggesting that microglial activation was still under some control during the
638 acute infection stage. In addition, Micro-0 did not show prominent signs of activation
639 during this stage, which could be attributed to their high activity in TGF β signaling. The
640 Micro-1 cluster, with high expression of microglial core genes but lack of TGF β
641 signaling pathway enrichment, was much more inducible in terms of gene expression
642 during acute SIV infection than the homeostatic Micro-0 cluster. More pathogenic and
643 inflammatory pathways were upregulated for Micro-1 during acute SIV infection
644 **(Figure S3)**, highlighting the importance of TGF β signaling pathway in regulating
645 microglial activation.

646 Micro-1, similar to Micro-0, also decreased its proportion in acute infection,
647 comprising 27.1% of the myeloid cells versus 32% in the uninfected animals. These
648 decreases were accompanied by prominent changes in the proportions of other
649 microglial populations in acute infection, as Micro-3, -5, -7, and -11 all increased their
650 representation. For CAMs the dominant cluster changed from Macro-6 (65% in
651 uninfected animals) to Macro-8 (72.9% in acute SIV infection). All these were
652 characterized by increased signs of cellular activation.

653 In uninfected brains, the activated microglial clusters constituted a low but
654 noticeable presence (<5% proportion). Those activated microglia cells have been
655 reported to exist in the healthy brain at different anatomic locations and are involved
656 in diverse neurological events compared with homeostatic microglia.⁽⁶⁰⁻⁶²⁾ From
657 scRNA-seq experiments, it is difficult to determine the biological meaning of such low
658 proportion cell clusters. Therefore, to better interpret the changes in gene expression
659 during the acute SIV infection, we did the comparisons in multiple ways.

660 We found that MHC class I genes and genes related to interferon production
661 were significantly upregulated in both microglial and macrophage clusters during acute

662 SIV infection. However, the expression levels were different between individual
663 clusters, suggesting discrepancies of responses for each cluster. For virus infection,
664 MHC class I molecules are key to the host defense for their ability to present virus
665 proteins in the infected cells to the cytotoxic T/NK cells in MHC class I manner.⁽⁶³⁾ The
666 significant upregulation of MHC class I molecules in all myeloid cell clusters also
667 indicated that the reaction to initial SIV infection might not be limited to the low
668 proportion of microglia and macrophages infected with SIV, and that the bystander
669 cells also showed reactivity to the infection. This was further supported by the
670 transcriptional changes in these bystander cells (**Table S3**).

671 The expression of type I interferons, especially IFN β ^(64, 65) has been found to be
672 widely upregulated in acute SIV infection. In response to the production of IFN β , the
673 downstream ISGs that are induced by type I IFNs to amplify the antiviral effects have
674 been found to be upregulated in blood and lymph nodes during the acute SIV/HIV
675 infection in rhesus macaque and human.^(66, 67) Our recent and prior studies of
676 microglial responses to acute and chronic SIV infection in the brain^(50, 68) also
677 highlighted that numerous ISGs were significantly upregulated in the myeloid cells of
678 the brain and might contribute to the HIV/SIV associated brain damages. Therefore,
679 the high expression of ISGs starting at an early stage of SIV infection can extend to
680 chronic infection in the brain's myeloid cell populations.

681 We note that one of the important pathways for type I IFN production is TLR
682 signaling, and we found that TLR2 and TLR4 were widely upregulated in microglial
683 and CAM clusters. TLR7, which can specifically bind with ssRNA from SIV, was
684 upregulated in Micro-3, Micro-5, and Micro-11, and TLR9 which can bind with the CpG
685 motif in DNA was widely upregulated in CAM clusters. All of these TLRs were reported
686 to have the ability to recognize and bind with HIV,⁽⁶⁹⁻⁷¹⁾ although TLR7 might be the

687 primary target.⁽³⁷⁾ The presence of TLR4, TLR7, and TLR9 in endosomal
688 compartments endows them the ability to recognize viral nucleic acids in the cells, and
689 indeed their signaling pathways are related to the production of IFNs.⁽⁷²⁻⁷⁴⁾ TLR
690 signaling can trigger the transcription of numerous inflammatory cytokines (e.g., TNF-
691 α , IL-6, IL-1 β , etc), which were also found upregulated in microglial or macrophage
692 clusters under acute SIV infection. All of those cytokines can serve as initiator or the
693 products for the pathways associated with NF- κ B and AP-1 signaling, which also has
694 been found to be modulated by HIV/SIV for their establishment and reactivation from
695 latency.⁽⁷⁵⁻⁷⁹⁾

696 CNS-associated macrophages, found in the interface between the parenchyma
697 and the circulation, represent an important myeloid cell population in the brain distinct
698 from microglia. Previously, all CAMs were thought to be derived from the monocytes
699 in the circulation, but recent findings showed that CAMs are highly heterogenous.
700 Some phenotypes might originate from the yolk sac and can be self-replenishable like
701 microglia, which are different from the CAMs differentiating from the circulating
702 monocytes.^(80, 81) From the transcriptional perspective, the yolk sac-derived CAMs also
703 share more similarities with microglia, and they are hard to separate based on their
704 transcriptomic profiles.⁽⁸⁰⁾ Given the distance between CAM clusters and the microglial
705 clusters on the UMAP and the low expression of microglial core genes in CAM clusters,
706 Macro-6 and Macro-8 were more likely to be derived from the circulating monocytes.
707 Like the two main phenotypes in the periphery, Macro-6 had a CD14^{low}CD16^{hi}
708 phenotype with immunosuppressive properties and Macro-8 had a CD14^{hi}CD16^{low}
709 phenotype with proinflammatory properties. During acute SIV infection, Macro-6
710 upregulated more inflammatory molecules which were already highly expressed in
711 Macro-8, indicating the Macro-6 could be activated and polarized toward the

712 inflammatory CD14^{hi}CD16^{low} phenotype. This observation is consistent with a report
713 about the transcriptomic convergency of peripheral CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺
714 population under the SIV infection.⁽⁸²⁾

715 Intriguingly, macrophages also increased the expression of microglial core
716 genes during the acute SIV infection. In a different system, it was found that the
717 monocyte-derived macrophages in the retina adopted microglia-like gene expression
718 during the degeneration processes,⁽⁸³⁾ which was similar to what we found for the
719 CAMs in acute SIV infection. However, the reasons why CAMs share more similarities
720 with microglia in response to stress or neurodegenerative diseases are still unknown.
721 Although the CAM clusters synchronized the changes for most immunoreactive
722 molecules, other immunoreactive molecules might change differently. For example,
723 most MHC class II molecules that were not changed or slightly upregulated in Macro-
724 6 were downregulated in Macro-8. The repression of MHC class II molecules in
725 professional antigen-presenting cells (APCs) was also observed during HIV infection
726 in humans, and serves as one of the immunodeficiency mechanisms of CD4⁺ T cells
727 in AIDS.⁽⁸⁴⁻⁸⁷⁾ Therefore, the unchanged MHC class II in Macro-6 suggested that this
728 cluster might be less likely to be affected by SIV-induced immunodeficiency at least in
729 acute infection. They may still maintain the ability to trigger the activation and
730 differentiation of CD4⁺ T cells for host defense against SIV virus.

731 The activation of microglia and CAMs in the brain is essential for protecting
732 against viral infection, however, the pro-inflammatory cytokines and cytotoxic
733 molecules secreted by them might also lead to the damages for neurons as well as
734 other supportive apparatus in CNS. Therefore, overreactive microglia and especially
735 monocyte-derived CAMs were thought to play a central role in HAND before the era
736 of efficacious antiretroviral therapy.^(24, 88) By enriching the upregulated genes in each

737 microglial or macrophage cluster into the various pathways, we found that Micro-3 and
738 Macro-8 clusters were linked with numerous neurological diseases, including
739 Alzheimer's, Huntington's, and Parkinson's diseases during acute SIV infection. These
740 changes CAMs and microglia also have potential in the pathogenesis of HAND by
741 various disease mechanisms associated with neurodegeneration.⁽²⁴⁾ When we further
742 identified the upregulated genes that were linked with those neurological disorders,
743 we found that, the genes related to mitochondrial respiration were significantly
744 upregulated in both Micro-3 and Macro-8. *In vitro*, HIV infection of macrophages has
745 been shown to alter their mitochondrial energetic profiles, with the specific changed
746 dependent on the stage of infection.⁽⁸⁹⁾

747 Given the unmet need for the validated biomarkers of HAND in clinical
748 settings,⁽⁹⁰⁾ some studies have sought to identify various proinflammatory cytokines
749 and molecules associated with protein misfolding as biomarkers for HAND,^(91, 92) and
750 other studies explored the specific myeloid cell phenotypes for serving as
751 biomarkers.^(88, 93) Both HIV itself and the antiretroviral drugs used to treat HIV can
752 affect mitochondria, and mitochondrial pathways are key suspects in the resulting
753 neuropathogenesis of HIV and HAND.⁽⁹⁴⁾

754 Although only Micro-3 and Macro-8 showed enrichment in multiple neurological
755 disorder pathways, all the myeloid cell clusters were associated with the cellular
756 senescence pathway, which might be another potential factor triggering or dampening
757 the neurological disturbances.^(95, 96) The potential effect of cell aging caused by acute
758 SIV infection might not only happen in microglia or macrophages but extend to
759 neurons as well as other brain cells. Indeed, while very few myeloid cells were directly
760 infected, the bystander effects of infection were widely manifested. Furthermore, the
761 inflammatory microglia and CAMs resulting from virus infection are also likely to trigger

762 or speed up cerebral aging.⁽⁹⁷⁾ Indeed epigenetic studies have found that HIV infection
763 leads to an advancement of predicted biological age in blood cells as well as in the
764 brain, and is associated with reduction in brain gray matter and cortical thickness.<sup>(98-
765 101)</sup>

766 Both HAND and the establishment of HIV/SIV reservoir in CNS can occur
767 irrespective of antiretroviral therapy.^(102, 103) Macrophages and microglia cells are
768 believed to serve as major HIV reservoir in the CNS given that they are prime targets
769 for the virus and their known longevity.⁽¹⁴⁾ Many pathways could be altered by HIV
770 infection allowing for the long-term survival of the infected macrophages and microglia,
771 and the anti-apoptotic or pro-apoptotic molecules in BCL-2 families were found to
772 regulate the survival of macrophages and/or microglia in HIV infection.^(25, 26, 104, 105)
773 However, we did not observe a wide and significant upregulation of anti-apoptotic
774 molecules or downregulation of pro-apoptotic molecules in myeloid cell populations
775 identified in this study. The cluster that upregulated anti-apoptotic genes also
776 upregulated pro-apoptotic genes, so it is difficult to identify a specific cluster that might
777 facilitate the establishment of a SIV, and by analogy HIV, reservoir. From the average
778 expression of the anti-apoptotic and pro-apoptotic molecules in BCL-2 families, the
779 overall trend for the myeloid cells of the brain under acute SIV appears to be the
780 enhancement of pro-apoptosis and suppression of anti-apoptosis, and the means in
781 which reservoir cells survive long-term in unknown.

782 Still there might be other molecules in myeloid cells that can also regulate the
783 apoptotic pathways. For example, we found upregulation of CD5L, which is also known
784 as apoptosis inhibitor of macrophage (AIM). CD5L can support the survival of
785 macrophages when the cells are challenged with infections or other dangers.⁽¹⁰⁶⁾
786 Although anti-apoptosis⁽¹⁰⁷⁾ is the most well-recognized function for this molecule, little

787 is known about the intracellular mechanisms underlying CD5L regulation of apoptosis.
788 Whether this molecule could be modulated during the SIV/HIV for modulating the virus
789 reservoir in macrophages or microglia needs further investigations.

790 Limitations to our study include the number of animals examined, study of a
791 single time point during the acute infection period, the lower sensitivity of scRNA-seq,
792 the lack of spatial assessment within the brain of where these populations of cells are
793 present, and potential differences between SIV infection of rhesus monkeys and HIV
794 infection of people. However, studies of the brain in people are largely limited to post-
795 mortem studies and lack control over conditions. Yet many considerations limit the
796 number of nonhuman primates used for terminal experimental studies. The deposition
797 of sequence data and metadata in publicly accessible databases from our studies and
798 others' enables the building of larger analyses with more subjects. These data can be
799 useful in meta-analyses across models and disease states.⁽¹⁰⁸⁾ The ability to purify
800 microglia and macrophages from the brains and the ability to examine thousands of
801 cells from each animal is an advantage enabling the identification and study of less
802 prevalent populations. The continued development of spatial transcriptomics,
803 sequencing methods and other technological and analytic advances will also help
804 alleviate these limitations.

805 In conclusion, by performing scRNA-seq to assess the brain myeloid cells in
806 rhesus macaques, we identified six microglial clusters and two macrophage clusters.
807 In response to the acute SIV infection of a small proportion of cells, all myeloid clusters
808 upregulated the genes related to MHC class I molecules and IFN signaling, which also
809 served as the key connections for other cellular responses to the HIV/SIV infection.
810 The activated microglial and macrophage clusters with more upregulated inflammatory
811 cytokines increased their proportions and the homeostatic or immunosuppressive

812 myeloid clusters decreased their proportions during acute SIV infection. Among the
813 activated clusters, both a microglial cluster and a macrophage cluster were identified
814 that exhibited dysregulation of genes associated with pathways linked to
815 neurodegenerative disorders. Changes in all of the microglial clusters may contribute
816 to worsening neurological health due to their enhanced ability to produce inflammatory
817 molecules and involvement in cellular senescence.

818

819

820 **MATERIALS AND METHODS**

821 **Animals**

822 The six male adult rhesus macaques used in this study were purchased from
823 PrimGen (Hines, IL) and New Iberia (LA), and tested negative for the indicated viral
824 pathogens: SIV, SRV, STLV1, Herpes B-virus, and measles; and bacterial pathogens:
825 salmonella, shigella, campylobacter, yersinia, and vibrio. Macaques were housed in
826 compliance with the Animal Welfare Act and the Guide for the Care and Use of
827 Laboratory Animals in the NHP facilities of the Department of Comparative Medicine,
828 University of Nebraska Medical Center (UNMC). The primate facility at UNMC has
829 been accredited by the American Association for Accreditation of Laboratory Animal
830 Care international. The UNMC Institutional Animal Care and Use Committee (IACUC)
831 reviewed and approved this study under protocols 19-145-12-FC and 16-073-07-FC.
832 Animals were maintained in a temperature-controlled ($23 \pm 2^\circ \text{C}$) indoor climate with
833 a 12-h light/dark cycle. They were fed Teklad Global 25% protein primate diet (Envigo,
834 Madison, WI) supplemented with fresh fruit, vegetables, and water *ad libitum*. The
835 monkeys were observed twice daily for health status by the animal care and veterinary
836 personnel. Three of the six animals were intravenously inoculated with a stock of

837 SIV_{mac}251 to establish acute SIV infection (93T, 94T, and 95T). The other three
838 macaques were uninfected (92T, 104T, and 111T) and used as control. Virus stocks
839 were provided by the Virus Characterization, Isolation and Production Core at Tulane
840 National Primate Research Center.

841 **Viral loads**

842 To determine the viral load in plasma, the blood of infected animals (93T, 94T
843 and 95T) was collected at 7-day and 12-day post-inoculation of SIV. The EDTA-
844 anticoagulated plasma was separated from blood by centrifugation. Specimens of
845 brain and lymphoid organs were taken for determination of viral load in tissues. Plasma
846 SIV RNA levels were determined using a gag-targeted quantitative real time/digital
847 RT-PCR format assay, essentially as previously described, with six replicate reactions
848 analyzed per extracted sample for assay threshold of 15 SIV RNA copies/ml.⁽¹⁰⁹⁾
849 Quantitative assessment of SIV DNA and RNA in tissues was performed using gag-
850 targeted nested quantitative hybrid real-time/digital RT-PCR and PCR assays, as
851 previously described.^(109, 110) SIV RNA or DNA copy numbers were normalized based
852 on quantitation of a single copy rhesus genomic DNA sequence from the CCR5 locus
853 from the same specimen to allow normalization of SIV RNA or DNA copy numbers per
854 10⁶ diploid genome cell equivalents, as described.⁽¹¹¹⁾ Ten replicate reactions were
855 performed with aliquots of extracted DNA or RNA from each sample, with two
856 additional spiked internal control reactions performed with each sample to assess
857 potential reaction inhibition. The viral load in plasma, lymphatic tissues and brain are
858 shown in **Figure S1** and **Table S1**.

859 **Isolation of myeloid cells in the brain**

860 Twelve days after viral inoculation, necropsy was performed on deeply
861 anesthetized (ketamine plus xylazine) animals, following intracardial perfusion with

862 sterile PBS containing 1 U/ml heparin. Brains were harvested and approximately half
863 of the brain was then taken for microglia/macrophage isolation.
864 Microglia/macrophage-enriched cellular isolation was performed using our previously
865 described procedure.⁽¹¹²⁾ Briefly, the brain was minced and homogenized in cold
866 Hank's Balanced Salt Solution (HBSS, Invitrogen, Carlsbad, CA). After being
867 centrifuged, the brain tissue was then digested at 37° C in HBSS containing 28 U/ml
868 DNase I and 8 U/ml papain for 30 minutes. After digestion, the enzymes were
869 inactivated by addition of 2.5% FBS, and the cells centrifuged and resuspended in
870 cold HBSS. The cell suspension was mixed with 90% Percoll (GE HealthCare,
871 Pittsburg, PA) and centrifuged at 4° C for 15 minutes at 550 x g. The
872 microglia/macrophage pellet at the bottom was resuspended in HBSS and passed
873 through a 40 µm strainer to remove cell clumps and/or aggregates. Cells were again
874 pelleted by centrifugation and resuspended in RBC lysis buffer for 3 minutes to
875 eliminate any contaminating red blood cells. A final wash was performed before the
876 resulting cells were quantified on both a hemocytometer and Coulter Counter Z1. The
877 isolated cells were then sorted for scRNA-seq. The cells in samples 92T, 93T, 94T,
878 95T were sorted and sequenced right after the isolation, but the cells in samples 104T
879 and 111T were cryopreserved before the cell sorting and scRNA-seq. The methods
880 for cryopreservation followed our previous study, which was found to maintain the vast
881 majority of the transcriptomic features of fresh isolated microglia/macrophages.⁽¹¹²⁾
882 Specifically, after isolation as above, the cells were centrifuged at 4° C at 550 x g for
883 5 minutes and the supernatant was removed. The pellet was dissociated by tapping
884 and then resuspended by the dropwise addition of a solution of 4° C 10% DMSO in
885 FBS at a concentration of 10⁶ cells per milliliter. Cells were transferred to

886 cryopreservation tubes and slowly controlled freezing at -80° C. After 24 hours,
887 cryotubes were transferred to liquid nitrogen for long-term storage.

888 **Single cell preparation and RNA sequencing**

889 For fresh brain isolates, cells were washed in PBS, and first stained with UV-
890 blue live/dead. Cells were then washed, resuspended in MACS buffer with 0.1% BSA,
891 and counted. The cells were then stained with non-human primate CD11b microbeads
892 and CD45 microbeads (Miltenyi, San Diego, CA, USA). Four hundred million cells
893 were reconstituted in 320 μ L of MACS buffer and reacted with 180 μ L of CD11b and
894 40 μ L of CD45 microbeads at 4° C for 15 minutes. After incubation, cells were washed,
895 resuspended in 1 ml of MACS buffer, and loaded on MACS Separator LS columns.
896 The double enriched fractions were collected and counted, and then stained with
897 antibody cocktails including BV711-labeled anti-CD20 antibody, BV421-labeled anti-
898 CD3 antibody, BV605-labeled anti-CD11b antibody and PE-labeled anti-CD45
899 antibody (Biolegend, San Diego, CA) for 45 minutes at 4° C. Cells were washed with
900 e-bioscience flow cytometry staining buffer and sorted on Aria2 flow cytometer (BD
901 Biosciences, San Jose, CA, USA). The selection of cells was based on the size,
902 singlet, live and the expression of CD20, CD3, CD45, and CD11b. The CD20 positive
903 cells were all excluded, and the CD20 negative cells that were positive for either CD45
904 or CD11b or both CD45 and CD11b were collected for scRNA-seq library preparations.

905 Samples of cryopreserved cell isolates, stored in liquid nitrogen described
906 above, were rapidly thawed in a 37° C water-bath. The cell recovery procedures were
907 well described in our previous publications.⁽¹¹²⁾ After the recovery, cells were washed
908 and counted by Coulter Counter Z1. Once cell concentration was known, cells were
909 transferred to ice-cold PBS, and followed the aforementioned procedures for CD45
910 and CD11b enrichment and FACS sorting.

911 Post-sorting, isolates were concentrated to approximately 1000 cells per μL ,
912 and assessed by trypan blue for viability and concentration. Based on 10 \times Genomics
913 parameters targeting 8000 cells, the ideal volume of cells was loaded onto the 10 \times
914 Genomics (Pleasanton, CA, USA) Chromium GEM Chip and placed into the
915 Chromium Controller for cell capturing and library preparation. This occurs through
916 microfluidics and combining with Single Cell 3' Gel Beads containing unique barcoded
917 primers with a unique molecular identifier (UMI), followed by lysis of cells and
918 barcoded reverse transcription of RNA, amplification of barcoded cDNA,
919 fragmentation of cDNA to 200 bp, 5' adapter attachment, and sample indexing as the
920 manufacturer instructed with version 3 reagent kits. The prepared libraries were then
921 sequenced using Illumina (San Diego, CA, USA) Nextseq550 and Novaseq6000
922 sequencers. The sequences have been deposited in NCBI GEO (accession number
923 GSE253835).

924 **Bioinformatics**

925 Sequenced samples were processed using the 10 \times Genomics Cell Ranger
926 pipelines (7.1.0). Specifically, the scRNA data was demultiplexed and aligned to the
927 customized Mmul10 rhesus macaque reference genome (NCBI RefSeq assembly)
928 which was combined with a chromosome representing the SIV genome. For this,
929 overlapping PCR products derived from reverse-transcribed RNA isolated from
930 PBMCs of infected animals 93T and 94T were sequenced using Sanger chemistry and
931 sequences combined to yield a consensus SIV genome. This sequence was deposited
932 in NCBI GenBank, accession number PP236443. After filtering as well as counting the
933 UMI and cell barcode by the Cell Ranger count pipeline, the sequenced samples from
934 the same animals were aggregated together to generate a single file containing feature
935 barcode matrices for the downstream analyses. The counting summary statistics

936 generated by 10x Genomics for each sample are shown in **Table S6**. The downstream
937 analyses were then implemented with R (version: 4.3).

938 **Cell clustering and differentially expressed genes (DEGs)**

939 To cluster the cell and find DEGs for each cell cluster, the feature barcode
940 matrices were analyzed with Seurat R package⁽¹¹³⁾ (version: 4.3.0). We removed
941 sparsely expressed genes and low-quality cells and kept genes which had expression
942 in at least 10 cells and the cells with UMI count from 400 to 20,000, gene count from
943 400 to 10,000, and mitochondrial percentage less than 15%. The final cell counts are
944 shown in **Table S6**. Then the scRNA-seq datasets from six animals were merged to
945 generate a single Seurat Object for further analyses. The normalization, scaling and
946 finding variable genes were performed by SCTransform v2 (SCT).⁽¹¹⁴⁾ After
947 normalization, we performed principal component analyses (PCAs) with a default
948 setting of 50 principal components (PCs) for reducing dimensionality. To minimize the
949 batch effect and integrate the datasets, we implemented harmony⁽¹¹⁵⁾ (version: 1.0)
950 before clustering. The integrated dataset was then subjected to graph-based
951 clustering which used the first 30 PCs and 0.2 as resolution. We selected the top 30
952 PCs which explain approximately 80% of the total variation. The Uniform Manifold
953 Approximation and Projection (UMAP) was used as a non-linear dimensional reduction
954 method to further visualize the cell clusters. The settings for running UMAP mostly
955 followed the default except for defining the dimensionalities using the first 30 batch
956 effect corrected PCs.

957 We then characterized each cluster in two ways. First, we examined the
958 expression of general cell markers for microglia (P2RY12, GPR34, and CX3CR1),
959 CNS-associated macrophages (MHC class II), T/NK cells (CD3D, GZMB, and NKG7),
960 B cells (EBF1 and MS4A1), and endothelial cells (RGS5, CLDN5, and ATP1A2) in

961 each cluster. Next, we found the DEGs for each cell cluster by performing the Wilcoxon
962 rank sum test embedded in FindAllMarkers function of Seurat to the data that was
963 normalized by SCT. The positive markers with 0.25-fold change (log-scale) on average
964 that were detected in a minimum of 25% of cells in either of two populations were
965 calculated as biomarkers for each cluster (**Table S2**). The DEGs that were found for
966 the same macrophage/microglia cluster but under different infection conditions were
967 also identified by using the above test method and criteria (**Table S4**). When we used
968 the same way to find DEGs between infected cells and uninfected cells (Figure S2 and
969 Table S3), to avoid losing the signal for SIV, we performed FindAllMarkers function on
970 log-normalized data, which was obtained by applying LogNormalize function in Seurat
971 with scale factor as 10000. The average gene expression for the
972 macrophages/microglia in the same cluster was calculated for the uninfected cells and
973 acute-infected cells separately, and then the values were added 1 and converted to
974 \log_{10} value for plotting. Given our goal in this study to analyze the myeloid cells in the
975 brain, we then further subsetted the microglial and macrophage clusters and reran the
976 FindAllMarkers function with the aforementioned settings within them separately. The
977 DEGs that were found in subclusters of microglia or macrophages were further used
978 in Gene Ontology (GO) analyses for further characterization.

979 ***Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)***
980 ***Over-representation analyses (ORA)***

981 Over-representation analysis (ORA) is a widely used approach to determine
982 whether known biological functions or processes are enriched in an experimentally
983 derived gene list (e.g., DEGs), and the p-value in this analysis is calculated by
984 hypergeometric distribution.⁽¹¹⁶⁾ The GO-ORA and KEGG-ORA were all implemented
985 with the clusterProfiler R package⁽¹¹⁷⁾ (version: 4.0.2). The GO analysis uses Entrez

986 Gene identifiers instead of the common gene symbol. Therefore, we converted the
987 common gene symbol to their Entrez Gene identifiers by genome wide annotation
988 package for rhesus macaque (version: 3.18). This package mapped the gene symbol
989 to Entrez Gene identifiers based on NCBI databases (updated on Sep-11, 2023). The
990 featured pathways of each microglial or macrophage cluster were then identified using
991 GO-ORA based on the DEGs, and the biological process (BP) was chosen as
992 subontology for analysis. The KEGG-ORA used the DEGs that were found between
993 different conditions as input to find the upregulated pathways for microglial or
994 macrophage clusters in response to acute SIV infection. The gene annotation of
995 rhesus macaque for KEGG analyses was found in KEGG database (Mmul10, RefSeq).
996 For both GO-ORA and KEGG-ORA, the cutoff for p-value was set to <0.05. The results
997 were visualized by dot plots, bar plots, tree plots, and gene-concept network, which
998 were plotted using enrichplot package (version 1.22.0). All GO-ORA and KEGG-ORA
999 pathways detected with p-value less than 0.05 were summarized in **Table S5**. In the
1000 table, the analysis results provided geneRatio and BgRatio, which are the ratio of input
1001 genes annotated in a term and the ratio of all genes that are annotated in this term
1002 respectively.

1003 ***Trajectory analysis***

1004 The monocle3 R package^(52, 118, 119) (version: 1.2.7) was used to estimate
1005 lineage differentiation within the macrophage clusters. We extracted the macrophage
1006 clusters from the merged Seurat Object and further constructed single-cell trajectories.
1007 The trajectory graph was inferred and fitted to the cell clusters generated by Seurat.
1008 The Macro-8 cluster was defined as the root node based on the prior knowledge⁽⁴¹⁾ for
1009 ordering all macrophages in their pseudotime. For visualization, the UMAP
1010 embeddings from Seurat Object was used, the nodes and branches were delineated

1011 based on the trajectory analysis, and the cells were colored by their pseudotime. To
1012 better compare the pseudotime of cells in Macro-6 and Macro-8, they were further
1013 illustrated in box plot using ggplot2 (version: 3.4.4)

1014 **Statistics**

1015 Alpha less than 0.05 was considered as a significant difference in all
1016 comparisons. The DEGs were found using non-parametric Wilcox rank sum test, and
1017 p-values were adjusted based on Bonferroni correction. In ORA analyses, the
1018 enrichment p-value is calculated using hypergeometric distribution, and p-value was
1019 adjusted in GO-ORA analysis to compare multiple microglial clusters.

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1021

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