

The brain-meningeal interface functions as a reservoir and entry site for brain parenchymal macrophages

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26

27 **Abstract**

28 Microglia are regarded as self-maintaining brain parenchymal macrophages without
29 contribution from adult hematopoiesis. While peripheral macrophage engraftment into the brain has
30 been reported, the biological variables governing central nervous system (CNS) macrophage niche
31 access remain unclear. We show that CNS macrophage engraftment depends on (1) the balance
32 between tissue-resident macrophage (TRM) self-renewal, (2) the temporal alignment of niche
33 opening with the availability of engraftment-competent cells and (3) proximity to the vacated niche,
34 rather than prolonged niche vacancy. Fate mapping revealed that monocytes entering the subdural
35 space undergo border-associated macrophage (BAM)-like differentiation, clonal expansion, and
36 transpial migration into the parenchyma, whereas mature BAMs directly repopulate selectively
37 vacated parenchymal niches. We further identified a parenchymal macrophage population with a
38 peripheral BAM-like transcriptional program in aged and neurodegenerative human brains,
39 challenging the dogma of microglia-exclusivity. Together, these discoveries provide a predictive
40 framework for understanding macrophage maintenance and replacement, and for developing
41 macrophage-based cellular therapies.

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Main

The CNS contains anatomically distinct macrophage niches populated by TRMs^{1–4}. Among these, microglia (MG) represent the sole macrophage population within the brain parenchyma, whereas BAMs reside at CNS interfaces, including the leptomeninges (LM), perivascular (PV) spaces (summarized as subdural BAMs; sdBAMs), the dura mater (duBAMs), and the choroid plexus (CP; cpBAMs). One of the tenets of CNS immune privilege is the restricted accessibility of the brain parenchyma to peripheral immune cells. Hence, MG and sdBAMs are maintained predominantly through local self-renewal^{5,6} without contribution from circulating monocytes under homeostasis⁷. While all CNS macrophage compartments are initially seeded by yolk sac–derived progenitors^{2,8–10}, only MG and sdBAMs maintain a predominantly embryonic ontogeny throughout adulthood, whereas cpBAMs and duBAMs undergo partial replacement by bone marrow (BM) derived monocytes, resulting in a mixed ontogeny^{2,9,11–13}.

Macrophage depletion and repopulation paradigms have become central tools to interrogate macrophage function and maintenance. However, distinct pharmacological and genetic depletion strategies produce divergent repopulation outcomes, ranging from complete restoration through residual MG self-renewal to extensive peripheral macrophage engraftment^{1,7,14–18}. A unifying framework that defines principles governing peripheral macrophage access and CNS niche population, and identifies competent cellular sources is currently lacking. Addressing this is important since macrophage replacement therapy transitions from preclinical proof-of-concept studies to clinical application¹⁹. Recent therapeutic success in patients with CSF1R-associated leukoencephalopathy (ALSP) demonstrated that MG replacement could alter disease progression in humans. However, these approaches rely on irradiation- and chemotherapy-based conditioning to create permissive niches for donor-cell engraftment^{14,20}. As the field reaches this translational inflection point, a mechanistic understanding of how CNS macrophage niches are maintained, accessed, and reconstituted will be critical for the development of safer and more effective replacement strategies.

Here, using complementary pharmacological and genetic macrophage depletion models in mice, we systematically dissected the determinants governing CNS macrophage reconstitution and defined physiological principles controlling peripheral macrophage engraftment into the CNS. We identified the coincidence of niche opening and availability of engraftment-competent cells as principal determinants governing competitive occupation of CNS macrophage niches and identified BAMs as spatially privileged reservoirs capable of directly colonizing the MG niche through transpial migration across CNS border interfaces. Finally, we provide evidence that these principles extend to humans by identifying CD163⁺GPNMB⁺ brain-engrafting peripheral-like macrophages in the aged and neurodegenerating human brain.

Monocyte contribution to BAMs is subset-specific and broadens after niche clearance.

To characterize CNS macrophage populations according to established life-cycle signatures and guide their phenotypic and genetic targeting, we re-analyzed a scRNA-seq dataset¹ of macrophages from adult mouse dura, LM/PV, CP, and brain parenchyma. Unsupervised clustering identified classical (CM; *Ccr2*, *Ly6c2*, *S100a8*) and non-classical monocytes (NCM; *Itgal*, *Ace*, *Ear2*), MG expressing canonical markers (*P2ry12*, *Tmem119*, *Sall1*), and transcriptionally distinct BAM populations (**Figure 1a-b**). BAMs segregated into MHCII^{low} and MHCII^{high} states, characterized by reciprocal expression of tissue-resident (*Mrc1*, *Lyve1*, *Folr2*, *Pf4*, *Cd163*) and antigen-presentation genes (*H2-Aa*, *H2-Ab1*, *Cd74*)^{1,3,9,21}, with an intermediate population (intBAMs) co-expressing both programs (**Figure 1a-b; Extended Data Figure 1a-b**). Consistent with established macrophage life cycle programs⁹, long-lived self-renewing TLF (*Timd4*, *Lyve1*, *Folr2*) signatures were enriched in MHCII^{low}/intBAMs, whereas monocyte-derived CCR2-associated signatures predominated in MHCII^{high} BAMs and CMs (**Extended Data Figure 1b-c**).

Based on cluster-specific signatures, we designed a high-dimensional flow cytometry (HDFC) panel and analyzed dura, CP, and brain from MG-specific *Sall1*^{GFP} reporter mice (**Figure 1b-c**). HDFC resolved MHCII^{high}, MHCII^{high}FOLR2^{high} (intBAMs), FOLR2^{high}MHCII^{low}, and double-negative BAMs, together with a brain-enriched MG population, recapitulating the scRNA-seq populations (**Figure 1a-c**). IntBAMs were most prominent in the dura (**Extended Data Figure 1d**); FOLR2^{high} BAMs co-expressed CD38 and CD206, with FOLR2 and CD38 used interchangeably throughout the study, while CCR2 was detectable in MHCII^{high} and intBAMs (**Extended Data Figure 1e**). *Sall1*-GFP expression was predominantly confined to the MG/MG-like cluster. (**Extended Data Figure 1f**). Complementary histological analysis in *Cx3cr1*^{GFP/wt} mice confirmed the anatomical distribution of these populations across CNS border compartments (**Extended Data Figure 1g**).

Monocytes contribute to BAMs across all CNS borders at steady state and after depletion.

ScRNA-seq identified *S100a8* as a highly specific marker for CM. To assess the contribution of hematopoietic stem cell (HSC)-derived monocytes to CNS macrophages, we employed adult

109 *Mrp8(S100a8)^{Cre/wt}·R26^{TdTomato}* mice to HDFC. Cre-mediated recombination labeled neutrophils
110 (~100%) and ~25% of circulating CMs, whereas MG remained unlabeled (<0.04%), confirming the
111 model's specificity. We detected low but measurable TdTomato recombination across BAM
112 populations (0–10% depending on the subset) with the most consistent monocyte contribution to
113 intBAMs in brain and dura (**Figure 1d-e**).

114 Colony-stimulating factor 1 receptor (CSF1R) signaling is required for the survival and
115 maintenance of macrophages²², and its pharmacological inhibition induces their rapid
116 depletion^{1,23,24}. To examine CSF1R-dependency and the niche recovery of CNS macrophages
117 following pharmacological depletion, we treated adult mice with the CSF1R inhibitor (CSF1Ri)
118 BLZ945 for 7d, followed by immediate HDFC analysis or a 7d recovery period after treatment
119 withdrawal. CSF1R inhibition resulted in efficient depletion of all CNS macrophage populations
120 across CNS compartments. Upon treatment discontinuation, MG and BAMs in the brain fully
121 repopulated within 7d comparable to vehicle-treated controls. In contrast, cpBAM and duBAMs
122 trended towards incomplete repopulation, suggesting a lower intrinsic self-renewal capacity than
123 MG (**Figure 1f-g**).

124 To assess the monocyte dependency of CNS macrophage niche replenishment, we compared
125 repopulation following CSF1Ri in *Ccr2^{wt}* and *Ccr2^{ko}* mice, as CCR2 is required for egress of CM
126 from the BM and their tissue recruitment²⁵. Eight days after CSF1Ri withdrawal, we observed an
127 increased fraction of CCR2-expressing BAMs across all border compartments in *CCR2^{wt}* mice
128 (**Extended Data Figure 1h-i**, see **Extended Data Figure 2a-b** for UMAPs and CCR2-KO
129 confirmation), suggesting recruitment of CCR2⁺ monocyte-derived macrophages (MDMs) into the
130 recovering niche. In contrast, MG showed no CCR2 expression or differential abundance between
131 genotypes. MHCII^{high} BAMs (including intBAMs) across brain and dura trended towards reduced cell
132 numbers at baseline (vehicle) in *CCR2^{ko}* mice. However, only BAMs in the brain displayed a
133 significant decrease in repopulation across all (except double-negative) subsets (**Extended Data**
134 **Figure 2c**). These findings indicate that BAM repopulation, including MHCII^{low} subsets, involved

135 CCR2⁺ macrophages derived from CM. While cpBAMs and duBAMs repopulated independently of
136 CCR2, access to the subdural compartment depended on CCR2.
137 To test monocyte entry into the niche-depleted brain, we fate-mapped MG in
138 *Sall1*<sup>CreERT2/wt;R26^{TdTomato} mice, exploiting the MG-restricted expression of *Sall1*^{18,26,27}, which is
139 absent from BAMs and MDMs. Following CSF1Ri treatment, repopulating macrophages uniformly
140 retained TdTomato labeling, demonstrating derivation from pre-existing MG rather than monocytes
141 **(Extended Data Figure 2d-e).**</sup>

142 Together, these data established that MG self-renew following pharmacological CSF1R-
143 mediated niche depletion without contribution from CM. In contrast, BAMs across all extra-
144 parenchymal CNS compartments, exhibited constitutive engraftment by MDMs, which was further
145 enhanced after niche depletion. While du- and cpBAMs repopulated in a CCR2-independent
146 manner, sdBAM renewal depended on CCR2. However, the brain parenchyma remained refractory
147 to MDM engraftment, possibly reflecting a combination of restricted niche access and the robust
148 self-renewal of MG.

150 **CSF1R and PU.1 control differentiation of Ly6C^{high} blood monocytes but not their** 151 **maintenance.**

152 The failure of CM to engraft the MG-depleted brain following CSF1Ri prompted us to
153 investigate whether CSF1R signaling regulates monocyte maintenance or differentiation
154 competence. Given conflicting reports regarding monocyte CSF1R dependency^{28–37}, we combined
155 pharmacological and genetic approaches to systematically dissect its role.

156 Murine monocytes encompass classical (CM; Ly6C^{high}CCR2^{high}CD43^{low}) and non-classical (NCM;
157 Ly6C^{low}CD43^{high}) subsets^{38–40}, with CMs progressively differentiating into NCMs through an
158 intermediate monocyte state (IM; Ly6C^{int}CD43^{int})^{40,41}, all of which express CSF1R⁴².

159 Time-resolved HDFC analysis of monocyte subsets during CSF1Ri (**Figure 2a**) showed rapid
160 depletion of IMs and NCMs, whereas CM numbers remained stable (**Figure 2a-c**). Following
161 treatment withdrawal, IMs recovered first, followed by NCMs, consistent with sequential CM-to-IM-

162 to-NCM reconstitution and rapid restoration of monocyte differentiation competence. To determine
163 whether CM preservation reflected true CSF1R independence rather than continuous BM
164 replenishment, we analyzed CSF1Ri-treated *Ccr2*^{ko} mice, in which Ly6C^{high} monocyte egress is
165 impaired²⁵. Despite markedly reduced baseline CM abundance, BLZ945 did not further deplete CMs
166 and its subsets⁴³, whereas IMs and NCMs remained strongly reduced (**Extended Data Figure 2f-l**)
167 arguing against compensatory replenishment as the basis for CM persistence.

168 Having confirmed *Cx3cr1* expression and efficient recombination across BM and all blood monocyte
169 subsets in *Cx3cr1*^{CreERT2/wt;R26^{TdTomato}} and *Cx3cr1*^{GFP} mice (**Extended Data Figure 3a-c**), we next
170 genetically deleted *Csf1r* using *Cx3cr1*^{CreERT2/wt;Csf1r^{fl/fl}} mice to avoid potential off-target effects of
171 BLZ945⁴⁴. Despite loss of CSF1R expression, unsupervised clustering (**Figure 2d-f; Extended**
172 **Data Figure 3d-f**) and lineage tracing (**Extended Data Figure 3g-i**) identified persistent CSF1R⁺
173 Ly6C^{high} cells as bona fide CMs, while NCMs were nearly completely depleted.

174 To distinguish persistence from replacement, we generated competitive BM chimeras with
175 TdTomato-labelled CSF1R-WT or CSF1R-KO donor cells, where preferential WT contribution would
176 indicate CSF1R-dependent cell loss. NCMs were replenished by WT but not CSF1R-KO donors,
177 whereas CMs showed comparable donor contribution, indicating selective NCM depletion but CM
178 persistence (**Figure 2g-h; Extended Data Figure 3j-k**). PU.1 deletion similarly impaired NCM
179 replenishment while sparing CMs (**Figure 2i-j; Extended Data Figure 3l-m**).

180 Collectively, CSF1R defined a differentiation checkpoint within the monocyte trajectory. Loss of
181 CSF1R signaling led to NCM depletion while preserving CMs, indicating a block in subset
182 progression rather than uniform depletion. Despite the rapid restoration of differentiation
183 competence after CSF1Ri, CM failed to outcompete the rapid MG-mediated brain repopulation.

184

185 **Impaired TRM self-renewal enables monocytes to replace all CNS macrophages, including**
186 **microglia and subdural BAMs.**

187 We next examined whether reducing TRM competitiveness was sufficient to shift the balance
188 towards monocyte engraftment to CNS macrophage niches. Permanent *Csf1r* deletion in

189 *Cx3cr1*^{CreERT2/wt};*Csf1r*^{fl/fl} mice reduced the pool of proliferative MG and resulted in the emergence of
190 CLEC12A⁺F4/80^{high} parenchymal macrophages following niche repopulation¹⁸ (**Extended Data**
191 **Figure 3n**). To establish their origin, we generated head-shielded chimeras with TdTomato-labelled
192 BM, enabling MDM tracing while avoiding irradiation-induced CNS conditioning (**Extended Data**
193 **Figure 3o**)^{7,45–47}. In control mice, donor-derived cells were restricted to CNS border compartments,
194 including sdBAMs (**Extended Data Figure 4b**), whereas following TRM depletion, TdTomato⁺
195 MDMs replaced macrophages across the dura, CP, subdural compartment and brain parenchyma
196 (**Figure 3a-d**; **Extended Data Figure 4a-b**). Brain-engrafting MDMs retained peripheral
197 macrophage markers, including F4/80, CLEC12A, MHCII and AXL (**Figure 3c**; **Extended Data**
198 **Figure 4a/c**).

199 Together, these data indicated that MDMs contributed continuously to BAM turnover, with increased
200 replacement following depletion. However, access to the parenchymal microglial niche required
201 sustained disruption of TRM occupancy.

202

203 **Exhaustion of MG self-renewal enables MDM brain engraftment.**

204 Repeated cycles of pharmacological MG depletion have been reported to delay niche refilling⁴⁸ and
205 “age” MG⁴⁹, suggesting that the MG proliferative capacity may become limited over time. Reanalysis
206 of published scRNA-seq data from this model⁴⁹ (**Extended Data Figure 4d**) revealed that
207 repopulating macrophages lacked *Sal1* and expressed peripheral macrophage-associated genes
208 despite acquiring canonical MG markers *P2ry12* and *Tmem119* (**Figure 3e**). Notably, brain-
209 engrafting TdTomato⁺ MDMs in our genetic model similarly acquired P2Y12 and TMEM119 (**Figure**
210 **3f**), indicating that these markers alone do not establish yolk-sac origin. We therefore combined
211 iterative CSF1Ri with *Flt3*^{Cre};*R26*^{TdTomato} monocyte fate mapping (**Extended Data Figure 4e**). While
212 a single depletion-repopulation (DR) cycle did not increase parenchymal MDM engraftment, three
213 cycles resulted in marked accumulation of TdTomato⁺ MDMs (**Figure 3g**; **Extended Data Figure**
214 **4f**). Circulating monocyte populations remained preserved across depletion cycles, indicating that

engraftment coincided with progressive impairment of MG self-renewal rather than altered monocyte availability or differentiation (**Extended Data Figure 4g-i**).

ScRNAseq analysis⁴⁹ identified multiple MDM transcriptional states, including MHCII^{high} (*H2-Ab1*, *Cd74*, *Ciita*, *Mgl2*), disease-associated macrophage-like (DAM-like; *Gpnmb*, *Spp1*, *Fabp5*, *Cd68*, *Lgals3*), and interferon-response (IFN repop. Mac; *Stat1*, *Stat2*, *Ifnar1*) populations. Despite this heterogeneity, all clusters shared a core transcriptional signature (*Ms4a7*, *Itga4*, *Axl*, *Clec12a*, *Itgal*, *Mrc1*, *Fxyd5*, *Pf4*, *H2-Aa*) distinct from MG and consistent with MDM origin (**Figure 3h; Extended Data Figure 4j-k**)

Competent monocytes directly exploit transient niche openings, clonally expand and enter the brain through a BAM-like state and transpl route.

We next decoupled niche opening from monocyte engraftment competence, which are simultaneously perturbed by CSF1Ri and *Cx3cr1*-driven *Csf1r* deletion. We generated head-shielded *Cx3cr1*^{CreERT2/wt};*Csf1r*^{fl/fl} chimeras reconstituted with TdTomato-labelled CSF1R-WT or CSF1R-KO BM. When niche opening coincided with the presence of engraftment-competent cells, CSF1R-WT MDMs rapidly repopulated the dura, CP, LM and brain parenchyma, whereas CSF1R-KO monocytes contributed minimally despite increased circulating CM numbers. (**Figure 4a-c; Extended Data Figure 5a**). Engrafting CSF1R-WT MDMs acquired macrophage morphology and both MHCII^{high} and MHCII^{low} states, whereas the few CSF1R-KO MDMs remained predominantly MHCII^{high}, consistent with an impaired differentiation trajectory^{40,50} (**Extended Figure Data 5b-d**). Similarly, PU.1-WT, but not PU.1-KO, monocytes robustly repopulated CNS niches despite comparable circulating CM numbers (**Figure 4d-e; Extended Data Figure 5e-h**).

In parallel, we employed a MG-specific depletion strategy to preserve CMs. *Sal1* is neither expressed in CM (**Extended Data Figure 5i-j**) or BAMs (**Figure 1c**) nor induced upon CNS entry and differentiation of brain-engrafting MDMs (**Figure 3e**). Consequently, *Sal1*^{CreERT2/wt};*Csf1r*^{fl/fl} mice enable selective MG depletion. While CSF1Ri treatment and *Cx3cr1*-driven CSF1R deletion induced

widespread depletion of CNS macrophages, *Sal1*-driven depletion did not reduce BAM numbers across CNS compartments but resulted in a significantly increased abundance of MHCII^{high} sdBAMs (depletion model differences illustrated in **Extended Data Figure 5k-l**). Comparing both genetic depletion models following a 2d repopulation period, *Sal1*^{CreERT2/wt};*Csf1*^{fl/fl} mice exhibited an increased accumulation of CLEC12A⁺F4/80⁺ peripheral macrophages in the brain compared to *Cx3cr1*^{CreERT2/wt};*Csf1*^{fl/fl} and the control condition (**Figure 4f**).

Hence, CM-sparing transient niche opening enabled direct CNS engraftment, indicating that the requirement for prolonged niche availability is model-dependent and reflects the concomitant impairment of CM.

We next examined how MDMs repopulate restrictive CNS niches. Repopulating macrophages formed discrete clusters in the LM and brain parenchyma, with early seeding events (**Figure 4c**) and columnar arrangements extending from the leptomeningeal surface into the cortex (**Figure 3d/4g**). In dual-reporter chimeras reconstituted with mixed TdTomato- and GFP-labelled BM (**Extended Data Figure 6a-b**), LM and parenchymal MDMs formed spatially segregated donor-derived clusters, demonstrating local clonal expansion (**Figure 4g-h**), whereas dura and CP showed greater donor intermixing (**Extended Data Figure 6c-d**). Light-sheet imaging further revealed spatial concordance between TdTomato⁺ leptomeningeal and parenchymal MDMs across the brain surface (**Video S1**).

We reasoned that trans-pially brain-engrafting MDMs would transiently acquire a BAM-like intermediate state during their sequential transition from the blood through the LM and into the brain parenchyma. To identify markers and reporter mice suitable for tracing such a BAM-intermediate state, we leveraged the scRNAseq dataset from the 3xDR model⁴⁹. We specifically searched for markers that were (i) expressed in mature BAMs, (ii) absent from CM, and (iii) induced in repopulating leptomeningeal MDMs. We identified *Mrc1* (CD206) and *Pf4* as candidate markers (**Extended Data Figure 6e-f**). Another prototypical BAM marker, *CD163*, was excluded because it was neither transcriptionally expressed in monocyte-derived repopulating BAMs (**Extended Data Figure 6g-h**) nor detected in TdTomato⁺ repopulating leptomeningeal BAMs at d7 or d17 of

269 repopulation in *Cx3cr1*^{CreERT2/wt};*Csf1r*^{fl/fl} head-shield chimeras (Extended Data Figure 6i,
270 experimental detail in **Figure 4a/d**). A prerequisite for tracing BAM-intermediate states is the
271 absence of reporter activation in CM. Accordingly, we assessed TdTomato expression in CM
272 following 5d tamoxifen treatment and detected no recombination in *Mrc1*^{CreERT2/wt};*R26*^{TdTomato} and
273 *Lyve1*^{CreERT2/wt};*R26*^{TdTomato} and only minimal (<1.5 %) recombination in *Pf4*^{Cre/wt};*R26*^{TdTomato} mice
274 (**Extended Data Figure 7a**).

275 Next, we performed state-mapping using *Pf4*^{Cre/wt};*R26*^{TdTomato} BM transplanted into
276 *Cx3cr1*^{CreERT2/wt};*Csf1r*^{fl/fl} recipients (**Extended Data Figure 7b**). Following depletion and
277 repopulation, *Pf4*-TdTomato⁺ macrophages, were detected across all CNS compartments, including
278 the LM (**Extended Data Figure 7c/e**), CP, dura, and brain parenchyma whereas monocytes in the
279 respective tissues remained TdTomato⁻ (**Extended Data Figure 7b**; gating strategy in **Extended**
280 **Data Figure 7d**). These findings indicate that *Pf4* expression is induced by monocytes after tissue
281 entry and during their differentiation into BAMs.

282 *Mrc1* is reported to be rapidly downregulated by brain-engrafting progenitors during the
283 embryonic brain colonization^{10,51}. To capture the transient nature of the BAM-state during LM
284 transition, we next generated head-shield chimeras using *Mrc1*^{CreERT2/wt};*R26*^{TdTomato} BM cells (mixed
285 1:1 with *Cx3cr1*^{GFP/wt}) transplanted into *Cx3cr1*^{CreERT2/wt};*Csf1r*^{fl/fl} recipients. Under this model,
286 monocytes adopting a BAM-like state at the CNS borders would be identified as TdTomato⁺CD206⁺
287 cells, whereas cells subsequently entering the parenchyma would be expected to downregulate
288 CD206 while retaining permanent TdTomato labeling. HDFC analysis revealed that a substantial
289 fraction of macrophages in the dura and CP was TdTomato⁺, whereas recombination was markedly
290 lower in peripheral macrophages in the brain parenchyma. Importantly, within the TdTomato⁺
291 populations, CD206 protein expression was retained in border compartments but almost completely
292 absent in the brain consistent with local niche imprinting driving CD206 induction and repression,
293 respectively (**Figure 4i-j**).

294 Histological analysis confirmed the presence of CD206⁺TdTomato⁺ leptomeningeal macrophages,
295 whereas brain-engrafting TdTomato⁺ MDMs downregulated CD206 expression (**Figure 4k**).

Collectively, these findings demonstrated that monocytes enter at CNS interfaces, transiently acquire a BAM state and subsequently seed the underlying parenchyma via transspial migration.

BAMs are a local reservoir for brain parenchymal macrophages.

To test whether MHCII^{low} BAMs themselves could serve as a local source of parenchymal repopulation, we employed the *Sall1*^{CreERT2/wt};*Csf1r*^{fl/fl} model, which enables selective ablation of MG while preserving BAM populations. We focused on early repopulation (d7) to capture cells before full phenotypic adaptation to the brain niche⁵¹. At this stage, HDFC revealed marked accumulation of CLEC12A⁺F4/80⁺ peripheral macrophages in the brain, enriched for BAM-like MHCII^{high}, double-negative, and FOLR2^{int}CD206^{low} states consistent with an ongoing transitional loss of CD206 (**Extended Data Figure 7f-g**).

However, BAM-like repopulating cells could derive either from resident BAMs or newly recruited monocytes transitioning through a BAM intermediate. To distinguish these possibilities, we used a CT2A tumor-bearing hemisphere as an internal monocyte-rich inflammatory reference and performed scRNA-seq 7d after depletion onset. In Cre⁺ mice, homeostatic MG were lost and replaced by a distinct macrophage cluster positioned between monocyte-derived, tumor-associated macrophages and MG (**Extended Data Figure 7h-i**); subsequent analyses were restricted to the contralateral non-tumor hemisphere.

Compared with homeostatic MG, repopulating macrophages were enriched for BAM-associated genes (Pf4, Msr1, Mrc1, Cd36, Ms4a7, Clec12a, Axl) and showed reduced expression of canonical MG markers (P2ry12, Sall1, Sparc, Hexb; **Extended Data Figure 7j**). Reclustering identified a dominant BAM-like population, alongside smaller DIM-like and MG-like states, each containing proliferating cells. We validated our BAM-like annotation by projecting the gene signature onto the Macrophage-Verse⁶, a murine reference atlas for CNS macrophages across developmental and adult stages, where the signature exhibited maximal enrichment within annotated BAM populations (**Extended Data Figure 8a-b**).

323

324 **Repopulating macrophages do not derive from microglia.**

325 Dedifferentiated MG were excluded as a source of peripheral-like repopulating macrophages using
 326 MG lineage tracing in *Sal11*^{CreERT2/wt};*Csf1r*^{fl/fl};*R26*^{TdTomato} mice (**Extended Data Figure 8c-d**). We
 327 identified *Pf4* among the most upregulated genes in repopulating macrophages (**Extended Data**
 328 **Figure 7j**). While *Pf4* is a prototypical BAM marker it is also expressed in yolk sac progenitors and
 329 primitive macrophages during early CNS colonization^{52,53}. To exclude a potential CNS endogenous
 330 MG progenitor origin of *Pf4*⁺ cells, we first assessed the specificity of *Pf4* expression using
 331 *Pf4*^{Cre/wt};*R26*^{TdTomato} mice. In this model, recombination robustly labeled BAM populations across
 332 CNS interfaces and a non-negligible fraction of TdTomato⁺ cells were also detected within P2Y12⁺
 333 MG (Extended Data Figure 8e). To resolve the identity of these cells, we performed FLASHseq of
 334 TdTomato⁺ cells isolated from brain and dura. This analysis revealed two transcriptionally distinct
 335 populations: a predominantly dural BAM cluster expressing *Pf4*, *Mrc1*, and *Cd163*, and a brain-
 336 derived MG cluster expressing canonical MG genes, including *Sal11* and *P2ry12* while lacking *Pf4*
 337 expression (**Extended Data Figure 8f**). *Pf4*-TdTomato⁺ MG were further dependent on CSF1R
 338 signaling and showed active *Sal11* expression in dual *Sal11*^{GFP/wt};*Pf4*^{Cre/wt};*R26*^{TdTomato} reporter mice
 339 by HDFC and immunofluorescence (**Extended Data Figure 8g-h**).

340

341 **Repopulating macrophages do not derive from circulating or skull-derived monocytes.**

342 Having excluded a MG origin, we next tested whether repopulating macrophages could derive from
 343 CM. We generated head-shield chimeras by transplanting *UBC*^{GFP/GFP} donors in
 344 *Sal11*^{CreERT2/wt};*Csf1r*^{fl/fl} and *Sal11*^{wt/wt};*Csf1r*^{fl/fl} control mice, achieving a ~20-40% chimerism in CM
 345 (**Figure 5a; Extended Data Figure 8i**). On d7 after depletion start, GFP⁺ cells were detected within
 346 subdural and dural/CP BAM populations across both conditions but did not show increased
 347 contribution upon depletion, consistent with baseline monocyte turnover of sdBAMs (**Figure 5a-c**,
 348 see **Extended Data Figure 8j-k** for cluster identity projected on conventional plot (left) and GFP
 349 density plot per subset (right) and conventional gating/analysis).

350 Histological analysis identified only rare GFP⁺F4/80⁺ MDMs with the majority of repopulating
351 parenchymal F4/80⁺ macrophages remaining GFP⁻, while frequently expressing CD206.
352 Importantly, no differential increase in GFP⁺ macrophage abundance was observed across
353 conditions (**Figure 5d; Extended Data Figure 9a-b** for GFP channel).

354 Analogous results were obtained by parabiosis, which achieved comparable blood chimerism and
355 likewise showed no increased contribution of circulating GFP⁺ cells to repopulating parenchymal
356 macrophages; rare GFP⁺F4/80^{high} cells were largely restricted to the olfactory bulb and
357 periventricular regions (Extended Data Figure 9c-e). Although skull-derived monocytes can engraft
358 a chronically microglia-deficient niche as recently reported⁵⁴, UBC^{GFP/GFP} calvarial bone-flap
359 transplantation onto *Sal1*^{CreERT2/wt};*Csf1*^{fl/fl} mice revealed no detectable contribution of skull-derived
360 macrophages to the repopulating parenchymal macrophage pool following transient microglia-
361 selective depletion, as assessed by HDFC and immunofluorescence (**Extended Data Figure 9f**).

362

363 **CD163 identifies BAM-derived macrophages and is not re-induced in MDMs.**

364 Next, we sought to identify lineage-restricted markers distinguishing embryonic BAMs from
365 monocyte-derived MHCII^{high} BAMs. For this, we leveraged the 3xDR model, in which iterative
366 CSF1Ri progressively exhausted the self-renewal capacity of MG and promoted their replacement
367 by MDMs.

368 Differential gene expression analysis revealed that *Cd163* was selectively expressed in embryonic
369 BAMs (in control condition) but not re-induced in repopulating BAMs. In contrast, other MHCII^{low}
370 BAM-associated markers, including *FOLR2*, *CD38*, and *Lyve1*²⁶, were re-expressed in repopulating
371 BAMs, indicating that these markers reflect tissue adaptation rather than lineage origin (Extended
372 Data Figure 6f-h).

373 To validate these findings, we analyzed BAMs in the repetitive depletion/repopulation model.
374 CD163⁺ BAMs progressively declined with each successive depletion round, most prominently
375 within the subdural and CP niches, while the overall fraction of TdTomato⁺ BAMs increased,
376 consistent with gradual replacement by CD163⁻ MDMs (**Figure 5e-g**). Taken together, these data

377 demonstrate that CD163 is not induced upon tissue entry, even after several weeks of niche
378 exposure, establishing CD163 as a lineage-restricted marker of bona fide mature BAMs.
379 We stained brains of *Sal1*^{CreERT2/wt}:*Csf1r*^{fl/fl} head-shield chimeras (**Figure 5a**) for CD163 and LYVE1
380 to determine whether BAMs could be detected within the repopulated brain parenchyma. Indeed,
381 IBA1⁺CD163⁺GFP⁻ and IBA1⁺LYVE1⁺GFP⁻ macrophages were readily observed within the
382 parenchyma (**Figure 5h**; see **Extended Data Figure 9g-h** for GFP channel). Given the short time
383 they resided in this compartment, the presence of CD163⁺/Lyve1⁺ macrophages strongly supports
384 retention of a pre-existing lineage identity rather than de novo induction. Together, these data show
385 that BAMs can be a local reservoir for brain parenchymal macrophages.

386

387 **Peripheral macrophages are detectable in the aged human brain independent of** 388 **Alzheimer's pathology**

389 Given that chronic proliferative stress may progressively exhaust long-term MG self-renewal, we
390 asked whether peripheral macrophages can be detected within the aging human CNS parenchyma
391 when MG are no longer able to sustain local niche demand under physiological or
392 neurodegenerative conditions. We leveraged a scRNAseq brain atlas of aged human individuals
393 with and without Alzheimer's disease (AD) from the Religious Orders Study and Memory and Aging
394 Project (ROSMAP)⁵⁵. The dataset comprises samples from six brain regions: the entorhinal cortex
395 (EC), hippocampus (HC), anterior thalamus (TH), angular gyrus (AG), middle temporal cortex (MT),
396 and prefrontal cortex (PFC).

397 Within the human brain macrophage landscape, we identified two clusters (C6 and C12) strongly
398 enriched for the core murine MDM signature (**Figure 6a-b**). Both clusters expressed CD163; while
399 a further cluster C10 was enriched for a published human BAM signature⁵⁶, consistent with a bona
400 fide BAM identity (**Figure 7c**). In contrast, C6 was further characterized by elevated expression of
401 peripheral macrophage markers (ITGA4, LGALS3, CXCR4, FXYD5, C5AR1), together with disease-
402 associated microglia (DAM)-related genes (GPNMB, ITGAX, TREM2 and CD9) and BAM-
403 associated genes (CD163, SIGLEC1, F13A1, and MSR1) (Figure 6d). C6 was enriched in AD while

being consistently detected across conditions in all brain regions (**Figure 6e**). To localize these cells within the human brain parenchyma, we performed immunofluorescence staining on postmortem human brain sections obtained from AD patients (n=2, including one individual with trisomy 21-associated AD pathology) as well as one age-matched non-AD control. Analyses were conducted across multiple anatomical brain regions. We focused on CD163 as a lineage-associated peripheral macrophage marker, in combination with IBA1 to identify CNS-resident macrophages. Consistent with recent reports that CD163 marks human MDMs^{26,56,57}, we detected ramified CD163⁺IBA1⁺ cells within the brain parenchyma across multiple anatomical regions in both control and disease samples. Notably, CD163⁺IBA1⁺ parenchymal cells were detected in relatively AD-spared regions such as the substantia nigra and gyrus rectus of AD patients as well as in multiple regions of an aged non-AD individual, including the calcarine sulcus and medulla oblongata (**Figure 6g-h**).

To independently validate the identity of C6, we compared its transcriptional signature with the recently established human brain myeloid taxonomy⁵⁸, comprising the FreshMG and PsychAD replication cohort. C6 showed strong concordance with the GPNMB⁺ AD-associated myeloid population in both datasets, including shared upregulation of defining genes such as GPNMB, PTPRG, MITF, SAMD4A and CD163 (**Figure 6i**). Importantly, this population was detectable in neuropathology-free individuals and increased during normal aging, while showing a substantially stronger expansion with AD neuropathology. The close transcriptional correspondence of this independently identified GPNMB⁺ population with C6, supports the interpretation that these populations represent closely related macrophage states and suggests that the aging human brain macrophage landscape contains a previously underappreciated fraction of non-MG peripheral macrophages.

Discussion

The principles that determine when and how monocytes or peripheral macrophages gain access to CNS compartments remain incompletely understood. Apparently conflicting repopulation outcomes across macrophage depletion strategies^{7,15–17} have complicated interpretation of these studies and contributed to the view that prolonged niche availability dictates peripheral engraftment²⁶. We show that these models are not contradictory; rather, they represent distinct perturbations of a common biological framework centered on two key parameters and their temporal alignment: presence of engraftment-competent cells and TRM self-renewal capacity.

Broad pharmacological CSF1Ri efficiently depletes MG but also transiently impairs CM differentiation and engraftment, despite preserving their abundance. Rapidly self-renewing MG re-establish niche dominance and limit peripheral engraftment. In contrast, persistent impairment of TRM self-renewal, through either irreversible genetic disruption of CSF1R signaling or iterative depletion-repopulation cycles, exhausted yolk sac-derived TRMs (including MG, sd- and cpBAMs) and shifted the competitive repopulation toward peripheral engraftment. Importantly, selective TRM depletion demonstrated that MDM engraftment does not require prolonged niche vacancy if engraftment-competent cells are preserved during transient niche opening. Despite partial acquisition of canonical MG genes (*P2ry12*, *Tmem119*), brain-engrafting monocytes failed to fully adopt a bona fide MG identity (*Sal1*^{27,59}), potentially reflecting ontogenetically imprinted epigenetic constraints^{26,54} or insufficient time within the CNS niche⁶⁰. We show that brain-engrafting MDMs are transcriptionally heterogeneous yet share a conserved core MDM transcriptional program, which, consistent with recent reports^{54,61}, includes acquisition of a BAM-like transcriptional profile.

We extended these findings by mapping the spatiotemporal acquisition of the BAM state by CNS-infiltrating monocytes, demonstrating that monocytes progressively differentiate through a BAM identity as they transition from blood to the LM and, under permissive conditions, into the adjacent brain parenchyma. Consistent with the transplacental seeding of the macrophage-devoid embryonic brain⁵⁴ and the postnatal colonization of the perivascular space by leptomeningeal BAMs⁶², we show that a re-opened parenchymal niche in adult mice is again accessible directly through the pial

455 surface to monocyte-derived MHCII^{high} and embryonic MHCII^{low} CD206⁺/Lyve1⁺/CD163⁺ BAMs.
456 Together, these findings suggest that the LM serve not solely as a barrier, but as a macrophage-
457 brain entry interface.

458 Beyond self-renewal capacity, anatomical accessibility further determines the mode of niche
459 reconstitution. The subdural compartment and brain parenchyma constitute restrictive niches in
460 which monocyte entry is limited and Ccr2-dependent, whereas the dura and CP support continuous,
461 Ccr2-independent monocyte influx^{11,13,26,63}, closely mirroring temporal replacement dynamics in
462 human HSC-transplanted patients⁵⁶. Consequently, restricted compartments are repopulated
463 predominantly through local clonal expansion following sporadic seeding, whereas accessible
464 compartments undergo continuous replacement.

465

466 Peripheral macrophage engraftment into the brain parenchyma has been documented in aging
467 mice⁶⁰, in neurodegeneration in both mice^{64,65} and humans^{26,66}, as well as in HSC-transplanted
468 leukemia patients⁵⁶. Although definitive lineage tracing of brain-engrafting MDMs remains largely
469 restricted to mice, converging human evidence supports their presence in the parenchyma. Sex-
470 mismatched HSC transplantation studies identified Y-chromosome-positive donor-derived
471 macrophages and, by single-nucleus RNA sequencing, an enrichment of CD163-expressing
472 macrophages in transplanted brains⁵⁶, while xenotransplantation studies independently supported
473 CD163 as a marker of brain-engrafting human MDMs^{26,57}. Together with reports of CD163⁺
474 macrophage accumulation in perivascular and subpial regions during neurodegeneration⁶⁶, these
475 findings suggest that peripheral macrophages can access the human parenchyma through routes
476 consistent with transpial entry. Here, we extend these findings to physiological aging, identifying
477 CD163⁺IBA1⁺ parenchymal macrophages across multiple brain regions in aged individuals without
478 overt neuropathology. Recent studies independently support the proposed ontogeny and phenotype
479 of these cells^{58,67,68}. Somatic mutation-based lineage tracing revealed age-associated hematopoietic
480 contribution to the human microglial compartment⁶⁸. Population-scale profiling further identified an
481 age-associated increase in a transcriptionally related CD163⁺GPNMB⁺ macrophage state that

expands prominently in AD⁵⁸, while spatial proteomics independently identified parenchymal CD163⁺TMEM119⁺ BAM-like microglia in both aged control and AD brains⁶⁷. Together with our lineage-tracing data, these findings support a model in which age-associated decline in TRM self-renewal may progressively permit peripheral macrophage engraftment, with incoming cells acquiring microglial features while retaining a BAM-associated identity.

The ability to predictably repopulate even the restrictive microglial niche suggests that similar principles may govern macrophage niche occupation beyond the CNS.

We provide a new conceptual framework for macrophage replacement strategies that avoids the adverse effects of irradiation- or chemotherapy-based conditioning⁶⁹, using iterative TRM exhaustion⁵⁴ or shielded monocytes to enable greater temporal and compositional control. Our findings further reveal that CNS border compartments are not impenetrable barriers, but conditionally permissive interfaces through which macrophages can transition across anatomically connected niches. In this framework, the LM emerge as an immunologically active reservoir and conditional gateway for macrophage entry into the brain parenchyma.

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Main Figure Legends

Figure 1: Monocyte contribution to BAMs is subset-specific at steady state and broadens after niche clearance. (a) UMAP of re-analyzed scRNAseq dataset¹ displaying CNS macrophages. (b) Heatmap with mean expression of curated BAM-signature marker genes across annotated clusters shown in (a). (c) UMAPs and bivariate plots of HDFC analysis of *Sal1*^{GFP/wt} mice (n=4 *Sal1*^{GFP/wt}, n=5 *Sal1*^{wt/wt}). (d) Schematic of *Mrp8*^{Cre/wt}*R26*^{TdTomato} mice. (e) TdTomato recombination in blood and CNS macrophages. (f) Top, experimental outline of depletion/repopulation experiment. Bottom, UMAP of BAMs from brain, CP and dura pre-gated as CD45⁺CD11b⁺Ly6C⁻CD64⁺CD206⁺/Clec12a⁺ macrophages. (g) Quantification of clusters in (f) and MG (gated as CD45⁺CD11b⁺Ly6C⁻CD64⁺CD206⁻Clec12a⁻P2y12⁺CD49d⁻).

Statistical tests: (e) Two-sided Welch's t-tests (g) Pairwise unpaired Wilcoxon rank-sum tests between groups within each tissue and macrophage subset.

Figure 2: CSF1R and PU.1 control differentiation of Ly6C^{high} monocytes into downstream subsets but not for their maintenance. (a) Top, experimental scheme: *Flt3*^{Cre/wt}:*R26*^{TdTomato} mice (n = 4 mice/group). Bottom, UMAP and cluster-identity projected onto bivariate plot. (b) UMAP-based density plot of monocyte subsets. (c) Longitudinal quantification of absolute monocyte counts. (d) Left, experimental scheme of *Cx3cr1*^{CreERT2/wt}:*Csf1*^{fl/fl} (n=22) vs *Cx3cr1*^{wt/wt}:*Csf1*^{fl/fl} (n=15). Right, UMAP of CD11b⁺/singlets/live/NK1.1⁻Ly6G⁻Siglec-F⁻ cells. (e) Projection of cluster identities in (d) onto bivariate plot. (f) Absolute counts of monocytes. (g-h) Head-shielded BM chimeras with reconstitution of *Cx3cr1*^{CreERT2/wt}:*Csf1*^{fl/fl} recipients with either TdTomato⁺ CSF1R-WT or CSF1R-KO BM. (g) Experimental scheme. (h) Left, Representative FC plots of blood monocytes; middle, FC plots with cluster-based annotation (Clustering; **Extended Data Figure 3j**) of monocyte subsets showing gating of TdTomato⁺ monocytes; right, fraction of TdTomato⁺ monocytes per condition (n=9 CSF1R-WT, n=9 CSF1R-KO). (i) As in (g-h), but using TdTomato⁺ PU.1-WT or PU.1-KO donor BM (n=8 PU.1-WT, n=8 PU.1-KO). See **Extended Data Figure 3i** for clustering.

685

686 Statistical tests: (c) Two-sided unpaired t-tests with BH correction (f) Linear mixed-effects model
687 followed by estimated marginal means pairwise comparisons with BH correction. (h, j) Two-sided
688 unpaired t-tests.

689

690 **Figure 3: Impaired TRM self-renewal enables monocytes to replace all CNS macrophages,**
691 **including microglia and subdural BAMs.** (a) Experimental scheme of head-shield chimeras.

692 *Cx3cr1*^{CreERT2/wt}:*Csf1r*^{fl/fl} or *Cx3cr1*^{CreERT2/wt}:*Csf1r*^{wt/wt} mice were reconstituted with
693 *Cx3cr1*^{CreERT2/wt}:*R26*^{TdTomato} BM, followed by tamoxifen-induced recombination and 4-week
694 repopulation. TdTomato recombination was re-induced 2 days before tissue collection.

695 (b) Percentage of TdTomato⁺ macrophages as gated in (**Extended Data Figure 4a**) (n=5 depletion,
696 n=5 control, one experiment). (c) Heatmap of Z-score scaled median fluorescence intensity in
697 peripheral macrophages from depletion mice and MG from control mice as defined in (**Extended**
698 **Data Figure 4a**). (d) Representative sections of mouse brains from mice in (a-c), stained for Iba1.

699 Scale bars, 1mm, 100 μ m. (e) UMAP of the re-analyzed published 3xDR paradigm by⁴⁹ (n=3 control,
700 n=2 3xDR, experimental scheme in **Extended Data Figure 4d**) with violin plots highlighting key
701 differentially expressed genes between circled clusters. (f) Representative brain sections of head-

702 shield chimera mice from experiment in (a) stained for P2Y12 and TMEM119. Scale bar, 10 μ m. (g)

703 Experimental scheme, gating and percentage of peripheral macrophages of repetitive (1xDR, 3xDR
704 and controls) CSF1Ri experiment using *Flt3*^{Cre/wt}*R26*^{TdTomato} mice. (pre-gating in **Extended Data**
705 **Figure 4f**). n=4 1xVehicle, n=4 1xDR, n=5 3xVehicle and n=5 for 3xDR, one experiment. (h) Venn
706 diagram of shared and uniquely expressed genes among repopulating macrophages.

707 Statistical tests: (b, h) Unpaired two-sided Welch's t-test. (c) Unpaired two-sided t-tests with BH
708 correction.

709

Figure 4: Competent monocytes directly exploit transient niche opening, clonally expand and enter the brain through a BAM-like state and transpl route. (a) Experimental design of head-shielded BM chimeras reconstituted with CSF1R-WT or CSF1R-KO donor BM. Bottom, representative gating of TdTomato⁺ macrophages. (b) Absolute count of TdTomato⁺ macrophages by HDFC for brain, CP and dura. Leptomeningeal count was determined by confocal imaging shown in (c). n=9 CSF1R-WT and n=9 CSF1R-KO, one experiment. (c) Representative en face confocal imaging of the dorsal brain surface (Scale bar, 1mm, 100 μ m). (d-e) As in (a-c) but using TdTomato⁺ PU.1-WT or PU.1-KO donor BM (n=8 PU.1-WT, n=8 PU.1-KO), 7d tamoxifen treatment and tissue harvest on d17. (f) *Top Left*, experimental scheme comparing early repopulation across models, n=4/group. Mice received tamoxifen (T) from d0-4. *Top right*, quantification of absolute counts of peripheral macrophages 2d after treatment. *Bottom*, representative gating of CD45⁺CD11b⁺Ly6C⁻Ly6G⁻NK1.1⁻CD64⁺F480^{high}Clec12a^{high} peripheral macrophages. (g-h) Clonal proliferation experiments using dual-donor color chimera experiment shown in Extended Data Figure 6a-d. (g) Dot plot, representative image and monte carlo spatial permutation analysis of the spatial distribution of GFP⁺ or TdTomato⁺ Iba1⁺ repopulating MDMs in brain sections. Scale bar: 100 μ m. n=10 mice (6 Mrc1, 4 Cx3cr1). (h) As in (g) but derived from dorsal en face confocal imaging of the LM. n=8 (4 Mrc1, 4 Cx3cr1). Scale bar: 100 μ m. (i) Experimental design and representative gating of CD206-TdTomato⁺ repopulating macrophages and CD206 expression across tissues. (j) Frequency of TdTomato⁺ peripheral macrophages and CD206⁺ cells among TdTomato⁺ peripheral macrophages. (k) Representative brain section showing CD206⁺TdTomato⁺ leptomeningeal macrophages and CD206⁻TdTomato⁺ repopulating parenchymal macrophages. Scale bar: 10 μ m.

Statistical tests: (b, e, j) Unpaired two-sided Welch's t-test. (f) Pairwise two-sided Welch's t-tests.

Figure 5: BAMs are a local reservoir for brain parenchymal macrophages. (a) Experimental scheme of head-shield chimera and representative FC plot showing CD45⁺CD11b⁺Ly6C⁻CD64⁺ pre-gated macrophages used for clustering analysis in (b). (b) UMAP and GFP expression overlay of

CNS macrophages (Ly6C⁺CCR2⁺ CM were also included into the analysis). (c) Percentage of GFP⁺ cells in clusters shown in (b) n=4 Cre⁻, n=3 Cre⁺, one experiment. (d) Representative brain sections from (d) showing GFP-CD206⁺F480⁺, intraparenchymal macrophages in *Sal11*^{CreERT2/wt}*Csf1*^{fl/fl} repopulated mice (for absence of GFP see **Extended Data Figure 9a**). Scale bar: 20 μm. (e-g) FC analysis of BAMs in brain, CP and dura from experiment outlined in **Figure 3g** of repetitive (1xDR, 3xDR and controls) depletion/repopulation using *Flt3*^{Cre/wt}*R26*^{TdTomato} monocyte-reporter mice. (e) Representative FC plots of BAMs (pre-gated on F480^{high}CD206⁺ macrophages) gating for CD163⁺ BAMs. (f) Absolute counts of CD163⁺ BAMs as in (e). (g) Percentage of TdTomato⁺ BAMs. (h) Representative brain sections from (a) showing GFP-CD163⁺Iba1⁺ and Lyve1⁺Iba1⁺ intraparenchymal macrophages in *Sal11*^{CreERT2/wt}*Csf1*^{fl/fl} repopulated mice. See **Extended Data Figure 9g-h** for absence of GFP. Scale bar: 10 μm and 20 μm.

Statistical tests: (c, f, g) Unpaired two-sided Welch's t-test.

Figure 6: Peripheral macrophages are detectable in the aged human brain independent of Alzheimer's pathology. (a) UMAP of scRNAseq human brain macrophages (ROSMAP⁵⁵). (b) Enrichment analysis of murine core MDM signature onto human ROSMAP CNS macrophages. (c) Feature plot of CD163 and AddModuleScoring of published⁵⁶ human BAM signature. (d) Differentially expressed genes between C6 and MG. (e) Fractional composition of C6 macrophages across healthy and AD brains. (f) Fractional composition of C6 macrophages across brain regions. (g-h) Representative brain sections of (g) trisomy 21, (h) left, AD patient and right aged control. Scale bar : 100 μm, 10 μm. (i) Cross-dataset comparison of the ROSMAP⁵⁵ C6 transcriptional signature with the GPNMB⁺ AD-associated myeloid (ADAM) population identified in the FreshMG and PsychAD cohorts⁵⁸.

Statistical tests: (d, e, i) Two-sided Wilcoxon rank-sum tests with multiple-testing correction.

Extended Data Figure Legends

Extended Data Figure 1:

(a) Volcano plot of differentially expressed genes between MHCII^{high} and intBAMs per tissue shown in **Figure 1a**. (b) Heatmap of Z-scored mean expression of macrophage modules from⁹ in CNS macrophages from **Figure 1a**. (c) AddModuleScore of genes shown in (b). (d) Fractional composition of CNS macrophages in mice shown in **Figure 1c**. (e) Left, density plots of FOLR2, CD38, CD206 and CCR2 expression in BAMs across tissues in *Sal1*^{GFP/wt} mice shown in **Figure 1c**. Right, classification of BAM subsets based on MHCII and FOLR2/CD38 used throughout study. (f) UMAPs and fraction of GFP⁺ cells across tissues in *Sal1*^{GFP/wt} mice shown in **Figure 1c**. (g) Representative images of brain sections, LM, CP and dura whole mounts in *Cx3cr1*^{GFP/wt} mice stained for CD206 and MHCII. Scale bar: 20 μm. (h) Top, experimental scheme of CNS macrophage repopulation in *Ccr2*^{wt} and *CCR2*^{ko} mice 8d after BLZ945 treatment. Bottom, fraction of CCR2⁺ macrophages after CSF1Ri in *CCR2*^{ko} and *CCR2*^{wt} mice. (i) CCR2 density plots in *CCR2*^{wt} and *CCR2*^{ko} mice. Grey curves represent *CCR2*^{ko} cells and colored curves represent *CCR2*^{wt} BAM subsets. Dashed vertical lines indicate subset-specific CCR2 positivity thresholds defined from the 99th percentile of CCR2 expression in *CCR2*^{ko} controls for each tissue and BAM subset; shaded areas indicate CCR2-positive events.

Statistical tests: (a) Wilcoxon rank-sum test with Bonferroni correction; adj.p value < 0.05 and log2FC > 0.5. (f) two-sided Welch's t-tests with BH correction. (h) Linear model per tissue and per BAM subset (*CCR2_pos_fraction* ~ treatment) with BH correction.

Extended Data Figure 2:

(a) UMAPs of BAMs and projection of cluster identity onto bivariate plot from mice shown in **Extended Data Figure 1h-i**. (b) Density plot of CCR2, CD163, and CD9 expression of BAMs in CP and dura from mice in **Extended Data Figure 1h-i**. (c) Absolute macrophage counts in *Ccr2^{wt}* and *Ccr2^{ko}* mice from experiment in **Extended Data Figure 1h-i**. (d) Left, experimental schematic of MG lineage-tracing using *Sal1^{CreERT2/wt}:R26^{TdTomato}* mice. (e) Left, representative bivariate plot of brain macrophages. Right, quantification of TdTomato⁺ brain macrophages. (f-i) HDFC analysis of blood collected on d5 following BLZ945 or vehicle treatment in *CCR2^{wt}* and *CCR2^{ko}* mice (experiment shown in **Extended Data Figure 1h**). (f) Left, UMAP and projection of cluster identity for peripheral blood monocytes (pregated on CD11b⁺/singlets/live/Ly6G⁻/Siglec-F⁻/NK1.1⁻). Right, Expression of CD11c, MHCII, CD319, and CD177 overlayed on UMAP (FMO-corrected prior to visualization for CD319 and CD177) used to identify and exclude monocyte-derived dendritic cells. Color indicates arcsinh-transformed expression, clipped to the 1st–99th percentile for visualization. (g) Bivariate FC plot showing lack of CCR2 expression in *CCR2^{ko/ko}* compared to *CCR2^{wt/wt}* mice in vehicle treated groups. (h) UMAP displaying monocytes across treatments and genotypes. (i) Baseline comparison of absolute monocyte subset counts in vehicle-treated *CCR2^{wt}* and *CCR2^{ko}* mice. (j) Relative changes in monocyte subset abundance following BLZ945 treatment in *CCR2^{wt/wt}* and *CCR2^{ko/ko}* mice. Absolute cell counts were compared within each genotype and subset to the mean of vehicle-treated controls (set to 100%) to account for baseline differences between genotypes. n=5–6 mice per genotype and treatment group. (k) Left, bivariate FC plot of CD319 versus CD177 (FMO-corrected) expression in CM subsetted from (h) illustrating CM subset definition. Middle, feature plots show FMO-corrected CD319 and CD177 expression (color intensity indicates arcsinh-transformed expression, clipped to the 1st–99th percentile). Right, projection of CD319/CD177-defined subset identities onto UMAP embeddings of CM. (l) Relative changes in CD319/CD177-defined CM subsets following BLZ945 treatment quantified as in (i).

Extended Data Figure 3:

(a-b) Gating strategy and quantification of TdTomato-recombined monocytes in **(a)** BM and **(b)** peripheral blood monocytes in tamoxifen- and no-tamoxifen-treated (tamoxifen on days 0-4, FC on day 5) *Cx3cr1^{CreERT2/wt}:R26^{TdTomato}* mice (n = 3 mice per group). **(c)** *Cx3cr1*-GFP expression in peripheral blood monocytes in *Cx3cr1^{GFP/wt}* (n= 18) (normalized to *Cx3cr1^{wt/wt}* controls). **(d-f)** Representative FC plot of blood pre-gated on CD11b⁺/singlets/live/NK1.1-Ly6G-Siglec-F⁻ cells from mice in **Figure 2d-f**. **(e)** Heatmap showing median marker expression per cluster scaled per marker across all clusters from mice in **Figure 2d-f**. **(f)** CSF1R expression across all clusters from mice in **Figure 2d-f**. **(g)** Schematic of *Cx3cr1^{CreERT2/wt}:Csf1r^{fl/fl}:R26^{TdTomato}* mice (n=4). **(h)** TdTomato expression in CSF1R⁻ monocytes with **(i)** quantification of the TdTomato⁺ fraction among CSF1R⁺ and CSF1R⁻ populations. **(j)** Left, UMAP and projection of cluster identity onto FC plots from mice in **Figure 2g-h**. Right, ridge-plots of marker expression across monocyte subsets used for quantification shown in **(k)**. **(k)** Absolute counts of monocyte subsets across donor conditions in mice shown in **Figure 2g-h**. **(l)** Left, UMAP and projection of cluster identity onto FC plots from mice in **Figure 2i-j**. Right, ridge-plots of marker expression across monocyte subsets used for quantification shown in **(m)**. **(m)** Absolute counts of monocyte subsets across donor conditions in mice shown in **Figure 2i-j**. **(n)** Top, experimental scheme of tamoxifen-induced *Csf1r* deletion in *Cx3cr1^{CreERT2/wt}:Csf1r^{fl/fl}* mice followed by 4-week recovery. Bottom left, representative gate (pre-gated on CD45⁺/singlets/live/CD11b⁺Ly6C⁻CD64⁺) of macrophages across conditions. Right, quantification of peripherhal macrophages as defined on the left. **(o)** Left, image of head-shield chamber. Right, quantification of shielding efficiency. Points represent dosimeter per mouse at indicated location. Two independent experiments.

Statistical tests: (a, b, f, i, k, m, n, o) Two-sided unpaired Welch's t-test.

Extended Data Figure 4:

(a) Representative gating strategy of CNS macrophages in CP, dura, and brain. Ridge plots show TdTomato expression of gated populations used for quantification in **Figure 2b**. **(b-c)**

842 Representative brain sections from the experiment outlined in **Figure 3a**. **(b)** Control mice showing
843 TdTomato⁺ macrophages in the LM and perivascular space. **(c)** Repopulated mice showing MHCII,
844 AXL, and F4/80 expression in TdTomato⁺ MDMs. Scale bars: 100 μ m. **(d)** Experimental scheme of
845 the published repetitive depletion–repopulation (3xDR) paradigm by⁴⁹ (GSE207948) reanalyzed in
846 this study. Two-month-old mice underwent three cycles of 2-week PLX5622-mediated depletion and
847 repopulation prior to scRNA-seq analysis at day 130 (n=3 Control, n=2 3xDR). **(e)** FC plot displaying
848 TdTomato⁺ blood monocytes in *Flt3*^{Cre/wt}*R26*^{TdTomato} mice with quantification shown on the right.
849 **(f)** Gating strategy of brain macrophages shown in **Figure 3g**. **(g)** Peripheral blood monocyte
850 comparison across 1xDR and 3xDR depletion groups. Blood was collected on days 5, 7, 9 and 11
851 of last depletion cycle shown in **Figure 3g**. UMAP with cluster identity projected onto FC plot.
852 **(h)** UMAP-based density plots across depletion and repopulation. **(i)** Line plots of absolute monocyte
853 count across time and conditions. **(j)** Left, AddModuleScores onto 3xDR scRNAseq data from
854 **Figure 3e** with **(k)** corresponding dot plot of marker expression profiles.

855

856 Statistical tests: (i) Linear mixed-effects models ($\text{cell_count_norm} \sim \text{cycle} \times \text{Day} + (1 \mid \text{mouse_id})$)
857 with estimated marginal means pairwise comparisons between 1X and 3X cycles within BLZ945-
858 treated mice at each time point with BH correction.

859

860 **Extended Data Figure 5:**

861 **(a)** Absolute count of TdTomato⁺ monocytes at day 5 of tamoxifen treatment from experiments in
862 **Figure 4a**. **(b)** Quantification of area, perimeter and circularity of TdTomato⁺ cells detected in pia
863 whole mount images in **Figure 4c**. **(c)** representative FC plot of MHCII expression among TdTomato⁺
864 repopulating macrophages in brains in **Figure 4a**. **(d)** Left, fractional composition of MHCII⁺ and
865 MHCII⁻ cells with absolute counts displayed on the right. **(e)** Absolute count of TdTomato⁺ monocytes
866 at day 5 of tamoxifen treatment from experiments in **Figure 4d**. **(f)** Gating strategy for brain
867 macrophages (gate 7) of experiment shown **Figure 4d**. **(g)** Representative en face confocal imaging
868 of the dorsal brain surface of experiment shown in **Figure 4d** (Scale bar, 1mm, 100 μ m).

869 (h) Quantification of area, perimeter and circularity of TdTomato+ cells detected in pia whole mount
870 images in **Figure 4d**. (i-j) TdTomato recombination in monocytes from (i) BM and (j) peripheral
871 blood in *Cx3cr1*^{CreERT2/wt}*R26*^{TdTomato} and *Sal1*^{CreERT2/wt}*R26*^{TdTomato} after 5d of tamoxifen treatment,
872 compared with non-tamoxifen injected controls. (k) Schematic overview on depletion model
873 differences, cross=depleted, stop sign=differentiation block. (l) Side-by-side comparison of
874 macrophage depletion across non-selective and microglia-selective depletion models in brain, CP,
875 and dura. Depletion is shown as fraction of the model-specific control mean (= dashed red line),
876 normalized to 100% (vehicle-treated mice for BLZ945; *CreERT2*^{wt/wt} *Csf1r*^{fl/fl} tamoxifen-treated mice
877 for depletion models). Analysis after 7d BLZ945 treatment or 5d following tamoxifen-induced CSF1R
878 deletion in genetic models.

879

880 Statistical tests: (a, b, e, h, i, j) unpaired two-sided Welch's t-test. (l) Two-sided unpaired Wilcoxon
881 rank-sum tests with BH correction using absolute counts.

882

883 **Extended Data Figure 6:**

884 (a) Experimental schematic of clonal proliferation experiment shown in **Figure 4g-h**. (b) Merged and
885 Iba1 channels of **Figure 4g-h**. (c) Analysis of CP and dural macrophages from mice shown in **Figure**
886 **4g-h**. Left, images of whole mounts of CP (c) and dura mater (d) stained for F480 (c) and Iba1 (d).
887 Scale bar: 100 μ m. Top right, representative dot plot depicting spatial distribution of donor-derived
888 repopulating macrophages. Bottom right, monte carlo permutation analysis as in **Figure 4g-h**. CP,
889 n=5 (1 *Mrc1*, 4 *Cx3cr1*); dura, n=5 (1 *Mrc1*, 4 *Cx3cr1*). (e) UMAP of published 3xDR scRNA-seq
890 data⁴⁹ highlighting contrasts made in (f). (f) Differentially expressed genes of control BAMs and
891 repopulating BAMs. The upper right quadrant highlights BAM-associated genes re-induced in
892 repopulating BAMs. Log2FC > 0, adj. P.Value > 0.05, pct.1 > 0.25. (g) Violin plots of canonical BAM
893 genes in clusters in (e). (h) Differential expression of control BAMs and repopulating BAMs in (e).
894 Log2FC > 0.5, p.adj. > 0.05. (i) Representative confocal images of leptomeningeal whole mounts
895 from *Cx3cr1*^{CreERT2/wt}:*R26*^{TdTomato} head-shield transplanted *Cx3cr1*^{CreERT2/wt} *Csf1r*^{fl/fl} recipient mice

shown in Figure 4a/d, stained for TdTomato, IBA1 and CD163 showing TdTomato⁺IBA1⁺CD163⁻ repopulating macrophages at day 7 and day 17 of repopulation (1 day and 10 days after tamoxifen withdrawal respectively). Scale bar : 100μm.

Extended Data Figure 7:

(a) Percentage of TdTomato⁺ cells among peripheral blood monocyte subsets from *Flt3*^{Cre/wt};*R26*^{TdTomato}, *Cx3cr1*^{CreERT2/wt};*R26*^{TdTomato}, *Pf4*^{Cre/wt};*R26*^{TdTomato}, *Mrc1*^{CreERT2/wt};*R26*^{TdTomato}, *Lyve1*^{CreERT2/wt};*R26*^{TdTomato} and *Sall1*^{CreERT2/wt};*R26*^{TdTomato} mice. For CreERT2 mouse lines, tamoxifen was administered for five consecutive days and peripheral blood was collected 1 day after tamoxifen withdrawal. (b) Experimental design and quantification of Pf4-TdTomato⁺ monocytes and MDMs across tissues. n=5 mice per group; one experiment. (c) Representative confocal whole mount imaging of leptomeningeal *Pf4*-TdTomato⁺ repopulating MDMs from mice in (b). (d) FC gating strategy for monocytes (gate A) and peripheral macrophages (gate 4) analyzed in (b) across brain, CP, and dura. (e) Representative confocal images of leptomeningeal whole mounts from *Cx3cr1*^{CreERT2/wt};*R26*^{TdTomato} head-shield transplanted *Cx3cr1*^{CreERT2/wt} *Csf1*^{fl/fl} recipient mice shown in (b), stained for TdTomato, IBA1 and CD163 showing TdTomato⁺IBA1⁺CD163⁻ repopulating macrophages at day 7 and day 17 of repopulation (1 day and 10 days after tamoxifen withdrawal respectively). Scale bar : 100μm. (f) Left, experimental scheme and representative FC plots of pre-gated (CD45⁺CD11b⁺Ly6C⁻CD64⁺) macrophages used for cluster analysis in the middle. Right, density plot of Clec12a⁺ macrophages. (g) Projection of cluster identity of Clec12a⁺ macrophages in (f) onto FOLR2-CD206 bivariate plot and absolute count of subsets across condition. (h) Experimental scheme of scRNAseq experiment. 1.5 x 10⁵ CT2A-Luc2-TdTomato cells were orthotopically injected into *Sall1*^{CreERT2};*Csf1*^{fl/fl} and *Sall1*^{wt/wt};*Csf1*^{fl/fl} mice. Seven days after tumor injection mice received tamoxifen from day 0-4 and tumor-free and tumor-bearing hemisphere were processed for scRNAseq (n = 2 Control, n = 3 Depletion, two hemispheres per mouse). (i) TSNE

922 representation of tumor-bearing and tumor-free hemispheres across conditions from (i). Dashed
923 circles indicate clusters used for contrast in volcano plot in (j).

924

925 Statistical tests: (a, b, g) Unpaired two-sided Welch's t-test.

926

927 **Extended Data Figure 8:**

928 (a) Heatmap of reclustered repopulating macrophages (including Microglia_1 cluster in **Extended**
929 **Data Figure 7i** for reference) displaying subcluster-specific gene signatures (top markers,
930 mean.AUC > 0.65, max. 20 markers per cell type). (b) AddModuleScore of BAM-like
931 cluster signature in the M-Verse⁶⁴. (c) Left, experimental scheme of MG lineage-tracing.
932 *Sal11*^{CreERT2/wt};*Csf1r*^{fl/fl};*R26*^{TdTomato} mice were treated with tamoxifen on days 0-5 and analyzed by
933 HDFC on d7. Right, representative FC plot displaying the gating (pre-gated on
934 CD45⁺CD11b⁺CD64⁺Ly6C⁻) of peripherhal macrophages and MG. (d) Left, representative TdTomato
935 gate of color-coded macrophages as defined in (c). Right, percentage of TdTomato⁺ macrophages.
936 n = 5. From one experiment.

937 (e) Representative confocal image of brain section of *Pf4*^{Cre/wt};*R26*^{TdTomato} mice stained for P2Y12
938 and CD206 showing Pf4-TdTomato⁺P2y12⁺ parenchymal macrophages. (f) UMAP of TdTomato⁺
939 macrophages sorted from dura and brain of a *Pf4*^{Cre/wt};*R26*^{TdTomato} mouse (n=1). Top, colored by
940 tissue. Bottom, annotated UMAP based on canonical MG/BAM markers shown in violin plot on the
941 right. (g) Left, experimental scheme: *Pf4*^{Cre/wt};*R26*^{TdTomato} mice were treated with BLZ945/Vehicle
942 from day 0-4 and brains analyzed by HDFC on day 8 (n = 3 Vehicle, n = 5 BLZ945, from one
943 experiment). Middle, representative gating of Pf4-TdTomato⁺ BAMs and MG used for quantification.
944 Right, absolute count of TdTomato⁺ macrophages as gated in the middle. (h) Left, representative
945 confocal image of brain sections of *Sal11*^{GFP/wt};*Pf4*^{Cre/wt};*R26*^{TdTomato} mice showing *Sal11*-GFP⁺Pf4-
946 TdTomato⁺P2y12⁺ MG. Scale bar: 100 μm and 10 μm. Right, representative FC plot of CNS
947 macrophages in brain, CP and dura showing double positive *Sal11*-GFP⁺Pf4-TdTomato⁺ MG in the
948 brain. (i) Blood chimerism of head-shield-transplanted *Sal11*^{CreERT2};*Csf1r*^{fl/fl} and *Sal11*^{wt/wt};*Csf1r*^{fl/fl}

949 mice from **Figure 5a** 4 weeks after transplantation prior to depletion. (j) Left, projection of cluster
950 identity onto MHCII-FOLR2 bivariate plot and GFP density plot from **Figure 5b**. (j) Conventional
951 analysis of **Figure 5a-b**; Left, gating strategy of peripheral macrophages (Clec12a⁺F480^{high}) and
952 MG (Clec12a⁻Cx3cr1^{high}). Right, percentage GFP⁺ macrophages from gated macrophages.

953

954 Statistical tests: (d) two-sided paired t-test, (g) two-sided Wilcoxon rank-sum test. (i, k) Unpaired
955 two-sided Welch's t-test.

956

957 **Extended Data Figure 9:**

958 (a) Confocal images displaying GFP channel from CD206⁺ macrophages in **Figure 5d**. Scale bar:
959 20 μ m. (b) Absolute count of GFP⁺IBA1⁺ macrophages in brain sections from mice in **Figure 5a**.

960 (c) Top, experimental outline of parabiosis of *Sall1*^{CreERT2}:*Csf1r*^{fl/fl} and *Sall1*^{wt/wt}:*Csf1r*^{fl/fl} mice with
961 UBC^{GFP/GFP} mice (n=3 control, n=4 depletion, from one experiment). Bottom, representative FC plot
962 showing pre-gated (CD45⁺CD11b⁺Ly6C⁻CD64⁺) macrophages and GFP⁺ cells among
963 Clec12a⁺F480^{high} peripheral macrophages used for quantification. Middle, percentage GFP⁺
964 macrophages in the brain. Right, percentage of blood chimerism in parabiotic mice. (d)
965 Representative brain sections from two different mice from (c) showing rare clusters of
966 GFP⁺Iba1⁺F480⁺ as well as more abundant GFP⁻Iba1⁺F480⁺ intraparenchymal macrophages. Scale
967 bar: 1mm, 100 μ m. (e) Percentage of GFP⁺ cells among F4/80⁺ macrophages quantified by
968 immunofluorescence from mice in (c). (f) Left, experimental scheme for skull transplantation of UBC-
969 GFP donors into *Sall1*^{CreERT2/wt}:*Csf1r*^{fl/fl} mice analyzed either 4 days (by HDFC; n = 10 depletion, n =
970 4 controls; whole brains processed) or 3 weeks (by immunofluorescence; n = 5 depletion; twenty
971 sagittal brains sections underneath transplant site were investigated per mouse) after last tamoxifen
972 treatment. Top, representative gate of GFP⁺ macrophages (pre-gated on
973 CD45⁺CD11b⁺CD64⁺F480⁺ cells). Bottom, representative confocal image of repopulated brain
974 section stained with anti-IBA1 and anti-GFP antibodies. Scale bar, 1mm and 200 μ m. (g-h) Confocal

975 images displaying GFP channel from GFP⁻ (g) CD163⁺ and (h) Lyve1⁺ macrophages in **Figure 6h**.
976 Scale bar: 10 μm.

977 Statistical tests: (b, c, e, f) Unpaired two-sided Welch's t-test.

978 **Video S1:** Light sheet microscopy of a repopulated brain hemisphere from experiment in **Figure 4d**.

979 *Cx3cr1*^{CreERT2/wt};*Csf1r*^{fl/fl} head-shield chimera were reconstituted with *Cx3cr1*^{CreERT2/wt}:*R26*^{TdTomato}
980 (PU.1-WT). Four weeks later, mice received tamoxifen for 7d and were sacrificed 10d after last
981 tamoxifen administration. Green represents TdTomato⁺ brain-engrafting MDMs.

982

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984

Methods

Animals

All animal handling, surveillance, and experimentation were performed according to the guidelines and legislation of the Swiss Federal Veterinary Office (SFVO) and the Cantonal Veterinary Office, Basel-Stadt, Switzerland, under license # 2929. Parabiosis experiments were performed at Washington University, St. Louis USA (J. Kipnis Lab). *Csf1^{fl/fl}* mice (B6.Cg-Csf1^{rtm1.2Jwp/J}) were crossed to *Sall1^{CreERT2}* kindly provided by Prof. Nishinakamura (Kumamoto University) or to *Cx3cr1^{CreERT2}* mice, received from Prof. Niess (University of Basel) generating tamoxifen-inducible *Csf1r* knockout mice. TdTomato reporter mice (B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J) were generated by crossing with the respective Cre-line, including *Sall1^{CreERT2}*, *Cx3cr1^{CreERT2}*, *Flt3^{Cre}* (kindly provided by Timm Schroeder, D-BSE Basel), *Pf4^{Cre/wt}* (kindly provided by Prof. Skoda, University of Basel), *Mrc1^{CreERT2}* (kindly provided by J. Kipnis). *Sall1^{GFP/wt}* were kindly received from Prof. Nishinakamura (Kumamoto University). Dual microglia and BAM reporter (*Sall1^{GFP/wt}* and *Pf4^{Cre/wt};R26^{TdTomato}*) were generated by crossing *Sall1^{GFP/wt}* and *Pf4^{Cre/wt};R26^{TdTomato}* mice. *Lyve1^{CreERT2/wt};R26^{TdTomato}* (Ai9) were kindly provided by J. Kipnis (Washington University, St. Louis). *Cx3cr1^{CreERT2/wt};PU.1^{fl/fl};R26^{TdTomato}* and *Cx3cr1^{CreERT2/wt};PU.1^{wt/wt};R26^{TdTomato}* controls were kindly provided by Prof. Glass (LMU, München Germany). *CCR2^{-/-}* mice were kindly provided by Dr. Cavelti-Weder (University of Basel). Mouse strain information is listed in **Supplementary Table 1**.

Tamoxifen administration

Tamoxifen was dissolved in corn oil at a concentration of 20 mg/mL by shaking overnight at room temperature (RT) in light-protected tubes. Mice received tamoxifen at a dose of approximately 75 mg/kg body weight by intraperitoneal injection once every 24 h for five consecutive d. For adult mice, a standard injection volume of 100 µL tamoxifen solution per animal was used.

BLZ945 (CSF1Ri) Treatment

BLZ945 (Novartis) was dissolved in 20% Captisol prepared in PBS and freshly prepared immediately prior to treatment. Mice were treated by oral gavage at the indicated frequency in each experiment with BLZ945 at either 200 mg/kg body weight (BW) for the dihydrochloride (HCl) form or 169 mg/kg BW for the free base form, according to the manufacturer's recommendation (Novartis). For a single depletion–repopulation cycle, mice received daily oral gavage of BLZ945 or vehicle control for 7 consecutive d, followed by a 7d repopulation phase unless otherwise indicated. For repetitive depletion–repopulation paradigms, each 7d depletion cycle was followed by either a 1-week or 3-week repopulation period, with mice sacrificed 4 weeks after completion of the final cycle.

For CSF1Ri in *Pf4^{Cre/wt};R26^{TdTomato}* mice, BLZ945 (Sotuletinib, MedChemExpress) was used. BLZ945 was dissolved by repeated vortexing and sonication in a 37 °C water bath until fully resuspended. Mice were treated with BLZ945 at a dose of 200 mg/kg body weight.

Irradiation Experiment

All irradiations were performed using a Gammacell® 40 Exactor irradiator (Best Theratronics Ltd., Canada).

The head-protection chamber was designed (by Paul Jordi, Jordi Strahlentechnik, Münchenstein, Basel) with staggered lead wall offsets to reduce scatter and edge-associated radiation leakage. All chamber walls (side, roof, and bottom) consisted of 3 cm lead shielding. Because irradiation was delivered from both top and bottom sources, the roof and floor were reinforced with an additional 1.5 cm lead layer, resulting in a total shielding thickness of 4.5 cm in these regions.

Validation of the head-shielding efficiency was measured using individually packed thermoluminescent dosimeters (TLDs; detection range 0.1–10 Gy). TLDs were positioned on the exposed back and beneath the lead-protected head within the irradiation chamber to quantify radiation exposure under head-protected conditions relative to total body irradiation. An additional

non-irradiated TLD was included as a baseline control. A total irradiation dose of 6.11 Gy was applied.

Bone marrow chimeras

Bone marrow chimeras were generated using a head-protected irradiation protocol. Recipient mice were anesthetized with ketamine/xylazine and placed into the irradiation chamber with the head shielded using a custom 3–4.5 cm lead barrier, as illustrated in Fig. S3B. Mice received two doses of 6 Gy total body irradiation separated by an interval of at least 4 h.

During the interval between irradiation cycles, bone marrow cells were isolated from donor mice under sterile conditions. Femurs and tibiae were collected and flushed with RPMI medium. Cell suspensions were filtered through a 70 μ m filter and subjected to 1min ACK lysis. Cells were subsequently washed, resuspended in sterile PBS, and counted prior to transplantation. Recipient mice received 2×10^6 sex-matched whole bone marrow cells by intravenous tail vein injection immediately following the second irradiation cycle.

For the assessment of clonal proliferation, we transplanted mixtures (1:1 ratio) of either *Cx3cr1*^{CreERT2/wt}:*R26*^{TdTomato} and *Cx3cr1*^{GFP/wt} or *Mrc1*^{CreERT2}:*R26*^{TdTomato} and *Cx3cr1*^{GFP/wt} BM into head-shielded *Cx3cr1*^{CreERT2/wt}:*Csf1r*^{fl/fl} recipient mice.

Parabiosis

Parabiosis experiments were performed between *UBC*^{GFP} mice and age- and sex-matched (female) *Sall1*^{CreERT2/wt}:*Csf1r*^{fl/fl} or *Sall1*^{wt/wt}:*Csf1r*^{fl/fl} mice as previously described⁵⁴. Briefly, mice were anesthetized with a ketamine/xylazine (100mg/kg, 10 mg/kg) mixture and the corresponding lateral sides were shaved and disinfected using alternating betadine and alcohol washes. A skin incision extending from the olecranon to the knee joint was generated, and subcutaneous fascia was carefully separated to create a free skin margin. Corresponding knee and elbow joints of parabiont pairs were connected using absorbable 5-0 Vicryl sutures, followed by approximation and closure of the dermis while excluding epidermal layers at the junction site. Animals received postoperative

analgesia with buprenorphine (Buprenorphine SR; 1.0 mg/kg) and antibiotic supplementation in the drinking water.

067

068 **Calvarium bone-flap transplantation**

069 ***Sall1*^{Cret/wt}:*Csf1r*^{fl/fl}**

070 7–10-week-old mice were anesthetized with a ketamine/xylazine mixture (80 mg/kg ketamine, 16
071 mg/kg Xylazine) and maintained at 37°C on a heating pad throughout the surgical procedure. Hair
072 over the head was removed using depilatory cream, and the surgical site was thoroughly disinfected
073 with Betadine. A midline skin incision was made to expose the parietal and interparietal bones. A
074 cranial window of approximately 4 × 6 mm was generated using an electric drill. During drilling, the
075 surgical area was carefully monitored and kept moist as needed. The cranial flap was then gently
076 removed by carefully detaching the underlying dura from the bone, while preserving the integrity of
077 the dura mater, brain parenchyma, and sinus structures.

078 Following craniotomy, a sex- and age-mismatched fluorescent donor mouse, typically UBC-GFP,
079 was euthanized. A donor calvarial flap of comparable size and from the corresponding skull region
080 was prepared. The donor dura was removed from the bone flap, and the flap was trimmed as needed
081 to match the recipient craniotomy site. The donor bone flap was then positioned over the cranial
082 window and fixed in place using dental cement. Care was taken to prevent cement leakage between
083 the fracture margins to allow proper bone apposition and healing. After the cement had hardened
084 and the transplanted flap was stably immobilized, the skin was re-approximated and sutured.

085 Postoperative care included analgesic treatment without antibiotic administration. No postoperative
086 infections were observed. Following surgery, mice were housed individually during the recovery
087 period.

088 For GFP+ cell detection by HDFC, whole brains were processed and acquired. For
089 immunofluorescence analysis, at least twenty 40-µm-thick sagittal brain sections within the
090 transplantation area were analyzed per mouse.

091

092 **Tumor cell injection for scRNAseq**

093 7–15-week-old mice were anesthetized and maintained under anesthesia using isoflurane
094 throughout the surgical procedure. Animals were mounted onto a stereotactic frame (Neurostar,
095 Germany), and the scalp was disinfected using povidone-iodide solution prior to a midline incision.
096 A burr hole was manually drilled 2 mm lateral to the cranial midline and 1 mm posterior to the bregma
097 suture. Intracranial injections were performed using the StereoDrive v1 software (Neurostar,
098 Germany) and a 10 µL Hamilton syringe (#80300, Hamilton, USA). The syringe was lowered to a
099 depth of 3.0 mm below the dura surface and subsequently retracted by 0.5 mm to create a small
100 reservoir. A total of 1.5×10^5 CT2A-Luc2-TdTomato tumor cells suspended in 4 µL were injected at
101 a rate of 1 µL/min. Following injection, the needle was left in place for at least 2 min and then slowly
102 retracted in 50 µm incremental steps. The incision was subsequently sutured.

103 Tumor engraftment was confirmed on day 7 following intracranial implantation by bioluminescence
104 imaging. Mice received an intraperitoneal injection of D-luciferin (150 mg/kg^{-1}), and imaging was
105 performed 10 min after injection using the NEWTON 7.0 instrument (Vilber, France) and
106 bioluminescence counts measured using NEWTON v7 Software (Vilber, France) imaging system
107 and software (Vilber, France).

108

109 **Mouse tissue collection and processing**

110 **CNS tissue**

111 For all mouse cohorts, animals were deeply anesthetized by intraperitoneal injection of
112 ketamine/xylazine (80 mg/kg ketamine [Ketanarkon, Streuli Tiergesundheit, Switzerland] and
113 16 mg/kg xylazine [Rompun, Elanco, USA]) and transcardially perfused with PBS prior to tissue
114 collection. Following perfusion, the dura mater was carefully peeled from the cranium, the fourth
115 ventricle was removed, and brains were immediately processed for downstream applications. Brain
116 hemispheres were separated, with one hemisphere immersion-fixed in 4% paraformaldehyde (PFA)
117 for histological and immunofluorescence analyses, while the contralateral hemisphere was used for

flow cytometric analyses following dissection of the lateral ventricle choroid plexus. Brain tissue was subsequently minced and maintained in ice-cold PBS until processing.

All tissues were enzymatically digested at 37 °C for 30 min using 1 mg/mL collagenase type IV (#LS004188, Worthington Biochemical Corporation, USA) and 250 U/mL DNase I (#10104159001, Roche, Switzerland) in digestion buffer consisting of HBSS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ (#14205-050, Gibco, USA), 1% MEM non-essential amino acids (NEAA), 1 mM sodium pyruvate, 44 mM sodium bicarbonate (#25080-060, Gibco, USA), 25 mM HEPES (#H0887, Gibco, USA), 1% GlutaMAX-I, and 1% antibiotic-antimycotic (#15240062, Gibco, USA). Following digestion, tissues were mechanically dissociated by repeated trituration using a 1000 μL pipette tip. Brain-derived cell suspensions were subsequently filtered through a 70 μm cell strainer and subjected to myelin and debris removal using debris removal solution (#130-109-398, Miltenyi Biotec, Germany) according to the manufacturer's instructions. Cells were washed with PBS and maintained on ice until further downstream applications.

Dura and choroid plexus samples were processed using the identical enzymatic digestion protocol; however, no myelin/debris removal step was performed. Following enzymatic dissociation, cell suspensions were filtered through a 35 μm mesh cell strainer cap (Falcon®, #352235) prior to downstream flow cytometric analyses.

Blood

Mice were gently restrained and tail disinfected with 70% ethanol. Peripheral blood was collected from the lateral tail vein by puncture and immediate collection of $2 \times 10 \mu\text{L}$ blood into a 96-well plate containing 100 μL PBS supplemented with 1 mM EDTA and 1:100 mouse FcBlock (TruStain FcX (#101320, BioLegend, USA)).

Spectral flow cytometry and FACS

145

146 **Staining and acquisition**

147 All samples were acquired on a Cytex Aurora 5-Laser Spectral Analyzer (Cytex Biosciences, USA)
148 using standard daily quality-controlled Cytex-Assay-Settings unless otherwise stated.

149

150 **CNS tissues**

151 Single cell suspensions were resuspended at equal volumes in PBS and distributed in 96-well plate
152 (round bottom). All centrifugation and incubation steps were performed at $300 \times g$ at 4°C , protected
153 from light, unless otherwise stated. Viability staining was performed by incubating cells with a
154 Zombie Viability dye (Zombie Aqua or NIR Viability kit) for 15 min. Subsequently, Fc-block was
155 performed by incubating cells in a 1:50 dilution of mouse TruStain FcX (#101320, BioLegend, USA)
156 for 15 min. Antibody mastermixes (full-stains and FMOs) were freshly prepared on the day of staining
157 in Brilliant Stain Buffer (#00-4409-75, ThermoFisher Scientific, USA). Cell surface markers were
158 stained with surface antibody mastermixes for 20 min and then washed three times in autoMACS
159 Running Buffer. Antibodies used for flow cytometry are listed in **Supplementary Table 2**.
160 Fixation/permeabilization step was performed by incubating the cells for 20 min at RT using a Cyto-
161 Fast Fix/Perm Buffer set (#426803, BioLegend, USA). Finally, the samples were washed twice,
162 resuspended in a final volume of $200\ \mu\text{L}$ of autoMACS Running Buffer, and acquired. Absolute
163 counts were quantified by normalizing cell count by acquisition volume.

164

165 **Blood**

166 All centrifugation and incubation steps were performed at $400 \times g$ at 4°C , protected from light, unless
167 otherwise stated. For longitudinal studies of blood over multiple days, reagents (antibody
168 mastermixes, FMO mastermixes, viability dyes and FcBlock-EDTA mix) was prepared in advance.
169 To correct for potential batch effects, blood of a C57BL/6 mouse was collected transcardially into
170 1mM EDTA supplemented with 1:100 FcBlock and frozen at -80°C prior to experiment start. For
171 each timepoint, 1 vial of blood was thawed and stained side by side with other samples and used to

assess whether batch normalization is required. After blood collection as described in “Mouse tissue collection and processing”, blood was centrifuged and resuspended in Zombie Viability dye (Zombie Aqua or NIR Viability kit) for 15 min. Subsequently, cell surface markers were stained with surface antibody mastermixes for 20 min and washed three times in autoMACS Running Buffer. Then, blood was fixed/lysed by adding 150 μ L of BD FACS Lysing Solution (BD Biosciences, #349202) for 10 min at RT. Cells were centrifuged at $600 \times g$ at 4 °C, washed twice and resuspended in 200 μ L autoMACS running buffer (20 μ L initial whole blood resuspended in 200 μ L acquisition volume) and subsequently acquired on a Cytex Aurora 5-Laser Spectral Analyzer (Cytex Biosciences, USA). Absolute counts were quantified by normalizing cell count by acquisition volume.

Flow cytometry analysis

For conventional flow cytometry analysis, frequencies/counts, acquisition volume/sample and median fluorescence intensities (MFI) of gated populations were exported along corresponding metadata and analyzed in R.

FlowSOM analysis

Data was unmixed using the SpectroFlo software (Cytex) and then manually pre-gated on CD45⁺/CD11b⁺, live, single cells and then cells of interest (i.e CD45⁺CD11b⁺Ly6C⁻CD64⁺ macrophages) using FlowJo v10 Software. The analysis was subsequently performed in R v4.3.2. Data was transformed by arc-sinh transformation using variance stabilizing cofactors for each channel (estParamFlowVS and transFlowVS functions from the FlowVS package). Manual corrections of cofactors were applied when necessary. Clustering was performed using FlowSOM and ConsensusClusterPlus using the wrapper function cluster from the CATALYST package. The resulting clusters were manually annotated and merged based on marker expression. Clustering identity was compared to conventional gating by projection of cluster identity onto bivariate plots. For visualization and statistical quantification, single cell object was transformed into a dataframe and analysed as indicated in respective figure legends.

199

200 **Fluorescence-activated cell sorting**

201 FLASHseq of TdTomato+ macrophages

202 Samples and collection plates were maintained at 4 °C throughout the tissue preparation sorting
203 procedure, and PBS was used as the sorting and sample preparation buffer in all steps. Tissue was
204 prepared as described in “Mouse tissue collection and processing”. Viability staining was performed
205 using Zombie NIR at 1:1000 dilution in PBS for 20 min. No FcBlock was performed as no surface
206 staining was required.

207 Pf4-TdTomato⁺ live singlet cells from brain and dura preparations were single-cell sorted on a BD
208 FACSDiscover S8 Cell Sorter using a 100 µm nozzle into lysis buffer-prefilled Eppendorf twin.tec®
209 384-well PCR plates (Cat. No. 0030128508, Eppendorf) mounted on a cooled plate holder, with one
210 cell deposited per well. Plate was immediately sealed and centrifuged at 700 × g at 4 °C and
211 transferred into -80 °C. scRNA-sequencing libraries were generated according to the FLASH-seq
212 protocol (v4).

213

214 **10X scRNAseq of tumor engrafted mice**

215 Samples and collection tubes were maintained at 4 °C throughout the tissue preparation, sorting
216 procedure, and PBS was used as the sorting and sample preparation buffer in all steps

217 Mice were deeply anesthetized and perfused as described in “Mouse tissue collection and
218 processing”. Brain was split into tumor-bearing and tumor-free hemisphere by mid-sagittal cut and
219 processed as described in “Mouse tissue collection and processing”. ACK lysis was performed once
220 for 5 min. Cells were Fc-blocked and stained with anti-CD45 and anti-CD11b for 30 min at 4 °C.
221 Cells were washed and Hashtag-antibodies added followed by 20 min incubation. Samples were
222 washed and then pooled before the final wash and subsequent resuspension in PBS and 0.02%
223 BSA. DAPI was added for dead cell exclusion.

224

225 **Immunofluorescence**

226 ***Mouse samples***

227 Mice were transcardially perfused with PBS. Brains were subsequently dissected and post-fixed
228 overnight in 4% paraformaldehyde (PFA) at 4 °C, followed by thorough washing in PBS. Dura mater
229 and choroid plexus samples were fixed in 4% PFA for 10 min at room temperature prior to washing
230 in PBS. Brain tissue was sectioned using a vibratome (40 µm sagittal sections and 27 µm coronal
231 sections) and processed as free-floating sections. Choroid plexus and isolated pia preparations
232 were similarly stained free-floating. Dura samples were carefully dissected under a
233 stereomicroscope and stained on glass slides. All samples were blocked and permeabilized in PBS
234 containing 3% bovine serum albumin (BSA) and 0.5% Triton X-100, followed by incubation with
235 primary antibodies overnight at 4 °C. After washing, appropriate fluorescent secondary antibodies
236 (**Supplementary Table 2**) were applied for 1 h at room temperature, followed by additional washing
237 steps prior to mounting.

238 **Human samples**

239 Human tissue samples (**Supplementary Table 3**) obtained from healthy and Alzheimer donors were
240 formalin-fixed and paraffin-embedded (FFPE). Tissue blocks were sectioned at 5 µm using a Microm
241 HM 355S microtome (Thermo scientific) and mounted onto glass slides (Epredia).
242 Immunofluorescence was performed with the Ventana Discovery Ultra (Roche Diagnostics Suisse
243 SA) automated stainer. In brief, tissue sections were deparaffinized and rehydrated. Antigens were
244 retrieved by heat in Cell Conditioning buffer 1 (CC1, ref. 950-124, Ventana) at 98°C for 40 minutes.
245 Primary antibody against IBA1 (goat monoclonal, 1:500; Novus Biologicals) and CD163 (rabbit
246 monoclonal, 1:150; Abcam) was manually applied and incubated for 1 hour at 37°C. After washing,
247 secondary antibodies donkey anti-goat Cy3 (Jackson ImmunoResearch) and donkey anti-rabbit
248 Alexa Fluor 647 (Jackson ImmunoResearch) were applied and incubated for 1 hour at 37°C. Nuclear
249 counterstaining was performed using DAPI, incubated at RT for 30 min. All the secondary antibodies
250 were used at a concentration of 1:1000. After staining, slides were washed three times with 1×PBS
251 and mounted using ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific).

252

253 **Microscopy**

254 **Stained slides**

255 Fluorescence imaging was performed using inverted spinning-disk confocal microscopy systems
256 (Nikon Eclipse Ti and Nikon Ti2; Nikon). High-resolution imaging was acquired using a 40× air
257 objective, while lower magnifications (4×, 10×, and 20×) were used for overview and large-area
258 imaging.

259 Images were acquired using standard filter sets appropriate for the respective fluorophores. Z-stacks
260 and tiled acquisitions were performed where indicated.

261 For quantitative analyses, imaging settings (including laser power, exposure time, and detector gain)
262 were kept constant within each experiment for samples stained and imaged under identical
263 conditions.

264

265 **Whole leptomeninges microscopy**

266 Whole-pia imaging was performed on intact brains using spinning-disk confocal microscopy (Nikon
267 Ti2) with a 4× objective. Z-stack tile scans were acquired across the entire pial surface of the dorsal
268 cortex in ice-cold PBS prior to downstream processing.

269 Due to the autofluorescence of the leptomeninges, signal detection was effectively restricted to the
270 surface, with minimal contribution from underlying cortical tissue, as verified by three-dimensional
271 reconstructions.

272 Equivalent acquisitions were performed on fixed hemispheres or whole brains for structural
273 comparison.

274 **Tissue clearing and lightsheet microscopy**

275 Brains were cleared using EZ Clear with refractive index matching prior to imaging. Cleared brain
276 hemispheres were immersed in oil and imaged using a benchtop mesoSPIM system equipped with
277 a motorized XY stage and controlled via mesoSPIM-control software. Imaging was performed using
278 a 5×/0.14 NA air objective (working distance 3.4 mm) and a Photometrics IRIS 14 camera.

279 tdTomato fluorescence was excited at 561 nm and detected using a 595/50 bandpass emission filter.
280 Dual-sided illumination was used to reduce shadowing artifacts and improve signal uniformity.

281 Tile scans and z-stacks were acquired to cover entire hemispheres. Image stitching was performed
282 in Fiji, and three-dimensional visualization was carried out using Imaris.

283

284 **ScRNAseq data analysis**

285 **BAM-Atlas**

286 Raw count matrices for four wild-type samples from Van Hove et al. (GSM3687213–GSM3687216;
287 dura mater, choroid plexus, enriched subduralmeninges, and whole brain) were obtained from NCBI

288 GEO. Cells were filtered by MAD-based outlier detection ($\text{nmads} = 4$) on detected gene count, total
289 UMI count, and mitochondrial read fraction, followed by PCA based outlier removal on QC metrics
290 (scatter). Genes with average count $< 10^{-3}$ were excluded. Non-myeloid populations (dendritic cell
291 subsets, B cells, T cells, NK cells, ILC, neutrophils, and doublets) identified at Louvain resolution
292 0.6 were removed, retaining microglia, macrophages, and monocytes.

293 The myeloid subset was normalised using SCTransform, regressing out dissociation stress score,
294 cell cycle scores (S and G2/M phase), and ribosomal RNA fraction. Datasets were integrated across
295 tissues using Seurat canonical correlation analysis (CCA; `SelectIntegrationFeatures`, `nfeatures =`
296 `3,000`; `PrepSCTIntegration`; `FindIntegrationAnchors`; `IntegrateData`). PCA was run on the integrated
297 assay; UMAP (`dims = 1:15`, `n.neighbors = 20`) and tSNE (`dims = 1:15`) were computed on the
298 integrated space. Louvain clustering was performed at resolutions 0.1–1.2; resolution 0.8 was used
299 for annotation. Clusters were manually assigned to cell types based on canonical marker
300 expression, and contaminating populations (B cells, mast cells, endothelial, stromal, CD45^- , and
301 unassigned cells) were removed to produce the final annotated object. Differentially expressed
302 genes between BAM subpopulations were identified using `FindMarkers` (Seurat, SCT assay, `min.pct`
303 `= 0.25`, `logfc.threshold = 0.4`).

304

305 **Tumor-bearing and tumor-free hemisphere 10X scRNAseq**

306 CD45^+ cells were sorted from CT-2A glioma-bearing mouse brains across four sample groups
307 (Csf1r-Cre^{+-} × tumour contralateral hemisphere) and sequenced on a NovaSeq SP lane (800M
308 reads). Reads were aligned to mm10Ensembl 102 using STARsolo. Cells were filtered, 600 UMIs
309 and 450 detected genes; doublets were removed in two steps using HTO classification and
310 `scDbtFinder`. Batch effects across sample groups were corrected using `FastMNN` (`batchelor`; `k = 20`,
311 `d = 50`). Louvain clustering was performed on the MNN-corrected space at `k = 10`; all visualisations
312 use `UMAP.MNN` (`n_neighbors = 15`) and `TSNE.MNN` embeddings. Cell-type annotation combined
313 `SingleR` (ImmGenData; Philip Schmassmann dataset ⁷⁰) with manual marker-based assignment.
314 Cluster 1 (MG-like cells) was re-clustered independently; subclusters were characterised using

scoreMarkers (scrn), and gene sets defined by markers with mean AUC >0.65 were projected onto the M-Verse multi-dataset integration using AddModuleScore (Seurat). For Figure 6D, pseudobulk differential expression was computed in MG-like and Microglia_1 clusters using limma on aggregated counts (≥ 20 cells per pseudobulk), with the contrast tumor-free hemisphere Cre+ versus tumor-free hemisphere Cre-.

FLASHseq

Cells sorted from the brain and dura mater of Pf4^{Cre/wt};R26^{tdTomato} wild-type mice, and from the brains of Cx3cr1^{GFP/wt} and Cx3cr1CreERT2/wt;R26^{tdTomato} mice, as described above, were processed using the FLASHseq protocol (768 single-cell libraries). FLASH-seq libraries were sequenced on an Illumina NextSeq 500/550 platform to generate 36-bp single-end reads. A custom reference genome was assembled from mm39 supplemented with eGFP, eYFP, CreERT2, and TdTomato transgene sequences and indexed with Ensembl 110 annotation. Reads were aligned using STAR and gene-level counts were quantified with featureCounts within Snakemake workflows, executed on the SciCore HPC cluster. The resulting count matrix was imported into Seurat (min.cells = 3, min.features = 300) and filtered (detected features > 300 and < 7,500, UMI count > 300, mitochondrial fraction < 8%). Data were log-normalised (scale factor = 10,000), highly variable features identified (vst, nfeatures = 2,000), and UMAP computed on the first 25 PCs. Cells were classified as BAMs (UMAP_2 > 8) or microglia (UMAP_2 $\leq$ 8) based on UMAP coordinate position. Differential marker genes between BAMs and microglia were identified using scoreMarkers (scrn), performed on the Pf4-Cre $\times$ Ai14 wild-type subset.

3xDR

Raw count matrices for five samples (GSM7070553–GSM7070557; three control, two 3xDR) were obtained from NCBI GEO (GSE226286). Cells were filtered (detected features ≥ 200 and $\leq 4,000$; mitochondrial fraction $\leq 5\%$) and doublets removed using scDbtFinder (per-sample, with cluster-guided detection). After doublet exclusion, dimensionality reduction was recomputed on singlets (11

PCs, n.neighbors = 26). Macrophage clusters were identified at Louvain resolution 0.6, extracted, and independently re-clustered (12 PCs, n.neighbors = 9). Final cell-type annotations were assigned at resolution 0.4. Differential expression was performed using a pseudobulk strategy: counts were aggregated per mouse (make_pseudobulk()), and edgeR quasi-likelihood F-tests (filterByExpr, calcNormFactors, estimateDisp, glmQLFit, glmQLFTest) were applied. Significant genes were defined at FDR < 0.05 and $|\log_2FC| > 0.5$. The primary comparisons shown in the figures were: BAMs 3xDR versus BAMs Control (Figure 5 volcano), and each repopulating subtype individually versus MG Control (IFN repop. Mac, MHCII^{high} repop. Mac, DAM^{like} repop. Mac; Venn, top 200 upregulated genes per subtype). To assess BAM identity re-expression, BAMs (Control) and repop_BAMs (3xDR) were each compared against the same background comprising all remaining non-BAM cell populations using Wilcoxon rank-sum tests (FindMarkers, logfc.threshold = 0, min.pct = 0.1); genes were classified as shared upregulated, shared downregulated, BAM-specific, or repop_BAM-specific based on adjusted p-value < 0.05 and $\log_2FC$ directionality, and restricted to genes detected in > 25% of repop_BAM cells. Module scores for MHCII-high, DAM, and homeostatic MG signatures were computed using AddModuleScore (Seurat) and visualised as feature plots and dotplots.

358

359 **ROSMAP**

Single-nucleus RNA-sequencing data from the Religious Orders Study and Memory and Aging Project (ROSMAP) multiregion cohort were obtained from the Synapse data repository (syn52383412). Gene expression matrices were downloaded for six brain regions: angular gyrus (AG; syn52408594), entorhinal cortex (EC; syn52408588), hippocampus (HC; syn52408592), midtemporal cortex (MT; syn52408595), prefrontal cortex (PFC; syn52408599), and thalamus (TH; syn52408586). Cell type annotations were derived from the original study (Mathys, Boix et al., Nature 2024). From each regional dataset, nuclei annotated as CNS-associated macrophages (CAMs) or microglia (Mic; including P2RY12+, MKI67+, DUSP1+, and TPT1+ subtypes) were

368 extracted using Seurat v5, yielding a combined dataset of 104,181 nuclei (CAMs: 4,194; Mic:
369 99,987) across all six regions.

370 The six regional subsets were merged into a single Seurat object with region-specific cell barcode
371 prefixes. Highly variable features were identified using FindVariableFeatures (nfeatures = 2,000),
372 followed by data scaling and principal component analysis (PCA) on the variable feature set. UMAP
373 dimensionality reduction and shared nearest-neighbour (SNN) graph construction were performed
374 on the top 20 principal components with 15 neighbours (RunUMAP: dims = 1:20, n.neighbors = 15;
375 FindNeighbors: dims = 1:20). Unsupervised Louvain clustering was applied across a resolution
376 range of 0.1–0.9 in steps of 0.1, and cluster stability was evaluated using clustree v0.5. Resolution
377 0.6 was selected as it provided biologically coherent clusters without over-fragmentation.

378 Neuropathological Alzheimer's disease status was derived from the pathAD metadata field,
379 dichotomised as AD or non-AD.

380 To quantify enrichment of our murine peripheral macrophage gene signature (n = 96 genes;
381 intersection of three independent murine repopulating-macrophage gene sets; see analysis in 3xDR
382 above, converted to human orthologues) across clusters, we applied fast gene set enrichment
383 analysis (fgsea v1.32.4). The gene signature comprised the intersection of three independent
384 peripheral macrophage repopulation gene sets (top 200 genes each), retained only if present in the
385 expression matrix. For each cluster, all expressed genes were ranked by their mean expression z-
386 score (computed gene-wise from AverageExpression across clusters), such that genes most
387 specifically expressed in a given cluster rank highest. fgsea was run with scoreType = "pos" to test
388 exclusively for positive enrichment with nPermSimple = 10,000 permutations and a random seed of
389 42 for reproducibility. Normalised Enrichment Scores (NES) and nominal p-values were computed
390 per cluster; multiple testing correction was applied globally across all cluster tests using the
391 Benjamini-Hochberg (BH) procedure. Clusters with adjusted p-value < 0.05 were considered
392 significantly enriched. Results are presented as a lollipop plot (NES on x-axis, point size proportional
393 to $-\log_{10}(\text{padj})$, line style indicating significance).

To identify human BAMs, we curated a BAM gene signature from the study by⁵⁶. Specifically, marker genes reported for BAM clusters 19 and 26 were combined into a single non-redundant gene set. This curated signature was used to compute per-cell module scores using Seurat's AddModuleScore function.

To identify transcriptional features specific to candidate clusters, one-versus-rest differential expression was performed between each candidate cluster and a MG background using the Wilcoxon rank-sum test (FindMarkers, Seurat v5; min.pct = 0.10, log₂FC threshold = 0.25, Benjamini-Hochberg correction). C6 was compared against all clusters excluding C10 and C12, and C12 against all clusters excluding C6 and C10. Results are displayed as volcano plots with genes significant at p_{adj} < 0.05 and |log₂FC| ≥ 0.5. Top 10 up- and downregulated genes and a custom set of differentially expressed genes is displayed.

To characterize the regional distribution of C6, the proportion of nuclei from each brain region was computed within C6 separately for AD and non-AD donors, using all nuclei in the cluster per diagnostic group as the denominator. Results are displayed as stacked proportional bar charts. To directly contrast the extremes of the clinicopathological spectrum, C6 donor proportions were compared between neuropathologically and clinically unaffected donors (healthy; no AD pathology, no dementia) and donors fulfilling both neuropathological and clinical AD criteria (path+/clin+), using a two-sided Wilcoxon rank-sum test with BH correction applied within this subset comparison.

FreshMG and PsychAD

Pre-processed myeloid cell data from the FreshMG and PsychAD atlases were obtained as Seurat objects through the AD Knowledge Portal (syn52795302 and syn52795303). FreshMG comprises CD45+/CD11b+ FACS-sorted cells from fresh-frozen post-mortem prefrontal cortex (PFC) of 137 donors profiled by single-cell RNA sequencing (Lee, Roussos et al., Nature Genetics 2026). PsychAD comprises 1,470 frozen dorsolateral prefrontal cortex (DLPFC) samples profiled by single-nucleus RNA sequencing; it was provided as a SingleCellExperiment object and converted to Seurat v5. In both datasets, subtype labels carrying a class prefix (e.g., ADAM_GPNMB, PVM_CD163)

were stripped to their terminal identifier. Disease-associated microglia were identified as the GPNMB cluster; perivascular macrophages (PVM) as the CD163 cluster. Differentially expressed genes were identified for GPNMB cells versus all remaining myeloid subtypes, excluding CD163/PVM, using a Wilcoxon rank-sum test in both atlases. To assess transcriptional concordance with ROSMAP cluster C6, $\log_2$ fold-change values from each atlas were correlated with ROSMAP C6 DEGs across all genes detected in both matrices, using Spearman's rank correlation. Concordantly upregulated genes were defined as those with $\log_{FC} > 0$ and $p_{adj} < 0.05$ in both ROSMAP C6 and the respective atlas comparison.

All analyses were performed in R (version 4.5.1). Key packages used include: Seurat (≥ 5.0), SingleCellExperiment, scran, scater, scDbfFinder, edgeR, limma, batchelor (FastMNN), ComplexHeatmap, and the tidyverse suite. Snakemake pipelines were executed on the SciCore HPC cluster at the University of Basel

Histology

Quantification of cells

Cell detection and quantification were performed in QuPath using custom Groovy scripts. Cells were identified using the built-in watershed-based detection algorithm on the relevant marker channel (e.g., Iba1). Detected cells were classified into marker-defined populations based on fluorescence intensity thresholds in additional channels. Classification thresholds were defined empirically and kept constant within each dataset. Detection parameters were adjusted where necessary to account for differences in cell morphology between depleted and non-depleted conditions. For selected analyses, fluorescence intensity and morphological features were extracted.

Separation of leptomeninges and parenchymal/perivascular

Leptomeningeal and parenchymal/perivascular cells were distinguished based on spatial proximity to the tissue boundary. The tissue surface was defined from autofluorescence signal, and the distance of each detected cell to this boundary was calculated. Cells located within 5 μm of the

surface were classified as leptomeningeal, while all others were assigned to parenchymal/perivascular compartments.

Monte Carlo spatial analysis

Spatial organisation of labelled cells was analysed in R using a Monte Carlo label-permutation approach based on QuPath-derived centroid coordinates. Analyses were performed separately for leptomeninges, dura, choroid plexus, and brain regions. For each region, neighbour densities for same-class cell pairs were calculated in concentric annuli up to 250 μm using 5 μm radial bins.

To generate the null distribution, cell-class labels were randomly permuted within each region while keeping cell coordinates fixed, thereby preserving tissue geometry and overall cell density. This was repeated 1,000 times. Observed neighbour-density curves were then compared with the permutation-derived null distribution using global simulation envelopes. Spatial deviations between observed and randomised distributions were additionally summarized over the 20–80 μm range.

Dot maps

Spatial distributions of labelled cells were visualized as dot maps based on centroid coordinates extracted from QuPath. Cells were plotted by class for individual regions, with coordinates displayed in a two-dimensional plane and axes scaled equally. Representative regions or all regions were visualized depending on the analysis.

Cell lines

The murine malignant astrocytoma cell line CT-2A was kindly provided by Prof. Thomas Seyfried (Boston College). CT-2A cells were cultured as adherent monolayers in high-glucose DMEM without glutamine (Gibco) supplemented with 10% fetal bovine serum (PAN-Biotech), 1% GlutaMAX

(Gibco), and 1% penicillin–streptomycin (Sigma-Aldrich). Cells were maintained at 37 °C and 5% CO₂ and passaged at ~80% confluency. All cell lines were routinely tested and confirmed negative for mycoplasma contamination using a MycoAlert detection kit (#LT07-318, Lonza). CT-2A Luc2-tdTomato was produced by lentiviral transduction to express luciferase 2 (Luc2) and the tdTomato reporter protein as previously reported⁷⁰.

Mouse genotyping

Genotyping primer list can be found in **Supplementary Table 4**.

Quantification and statistical analysis

Data are presented as mean ± standard error of the mean (SEM) unless otherwise indicated. The exact number of biological replicates (n = mice), number of independent experiments and statistical tests used are specified in the corresponding figure legends. Statistical significance was defined as $p < 0.05$ (* $p < 0.05$, $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant). All statistical analyses were performed using R software (v4.3.2). Given the typical sample sizes used in experimental biology studies, formal assessment of normality was not systematically performed. Parametric or rank-based non-parametric statistical tests were selected as appropriate for the experimental design and data distribution. Comparisons between two groups were performed using two-tailed unpaired or paired t tests, or rank-based non-parametric tests where appropriate. Multiple testing correction was applied where appropriate. All graphical illustrations were created with BioRender.com

Data availability

All single-cell RNA-seq datasets generated in this study will be deposited in the Gene Expression Omnibus (GEO) and made publicly accessible under the corresponding GEO accession code upon publication. Additional processed data and code required to reproduce the analyses is available from the lead contact upon request.

SnRNA-seq profiling data are available from Synapse in coordination with the ROSMAP project. Data are accessible under accession codes syn52293442 (as part of the MIT ROSMAP Single-Nucleus Multiomics Study; Synapse: syn52293417). The data are available under controlled use conditions set by human privacy regulations. To access the data, a data use agreement is needed. This registration is in place solely to ensure anonymity of the ROSMAP study participants. A data use agreement can be agreed with either Rush University Medical Center (RUMC) or with SAGE, who maintains Synapse, and can be downloaded from their websites (<https://www.radc.rush.edu/>; <https://adknowledgeportal.synapse.org/>).

Code availability

No specific proprietary code or software was developed for this manuscript.

Materials availability

This study did not generate new unique reagents.

Acknowledgments

Calculations were performed at sciCORE (<http://scicore.unibas.ch/>) scientific computing center at the University of Basel. We thank Prof. Alfred Zippelius, Prof. Jan Niess and Prof. Ryuichi Nishinakamura for providing genetic mouse lines; the genomics, proteomics, flow cytometry histology, microscopy and animal core facilities of the University of Basel, Switzerland for technical and logistical support. We thank Alexandar Tzankov for providing human brain tissue samples. We thank Diego Calabrese and the members of the DBM Histology Core Facility for their support and staining service. We thank the microscopy facilities at the Department of Biomedicine (DBM), particularly Michael Abanto, and the Imaging Core Facility (IMCF, Biozentrum), especially Kai Schleicher, for their technical support with microscopy and imaging workflows. We also thank Marc Thommen for advice and support regarding tissue clearing protocols. We are extremely grateful to Paul Jordi from Jordi Strahlentechnik (Münchenstein, Basel) for the design and construction of the head-shielding chamber. We further thank Roman Menz (University Hospital Basel) and Thibaut Klein (Department of Biomedicine, Basel) for their support in planning the irradiation experiments. We thank the study participants and staff of the Rush Alzheimer's Disease Center, the data contributors, and the investigators of the original study. The dataset was originally generated and described by Mathys, H., Boix, C.A., Akay, L.A. et al. Single-cell multiregion dissection of Alzheimer's disease. *Nature* 632, 858–868 (2024). <https://doi.org/10.1038/s41586-024-07606-7>.

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536

537 **Author Contributions**

538 Conceptualization, D.K, R.Gl., G.H.; methodology, D.K, G.H, P.T.W.; investigation, D.K., P.T.W.,
539 S.H., G.D.B, S.D., S.Z., M.F.R., T.S., H.M., Z.B., V.S., F.G., ; formal analysis, D.K, P.T.W, S.H., P.Z.,
540 ; writing-original draft, D.K; writing-review & editing, D.K., P.T.W., G.D.B., A.C.D., M.F.R., and G.H.;
541 funding acquisition, D.K. and G.H.; supervision, G.H; data curation, D.K, M.F.R, G.D.B., S.Z., R.G.,
542 S.D., A.G., M.M.; project administration, M.F.R., G.D.B. ; Resources, G.H., M.F.R., A.G., M.D., A.Z.,
543 R.G., L.M., J.K., R.Guz.

544

545 **Competing Interests**

546 G.H. has equity in, and is a co-founder of Incephalo Inc., G.H. is a co-founder of GlioCART GmbH,
547 G.H. has received consultancy honoraria from Servier Inc., Novartis and BB Braun Aeskulap.

548

549 **Supplemental Information**

550 Extended Data Figures 1-9.

551 S1 Video, related to Figure 4.

552 Supplementary_Table_1_mouse_strains_and_other_reagents

553 Supplementary_Table_2_Antibody list for flow cytometry

554 Supplementary_Table_3_HumanSamples_IF_ROSEMAP_Metadata

555 Supplementary_Table_4_Primer List

556

557

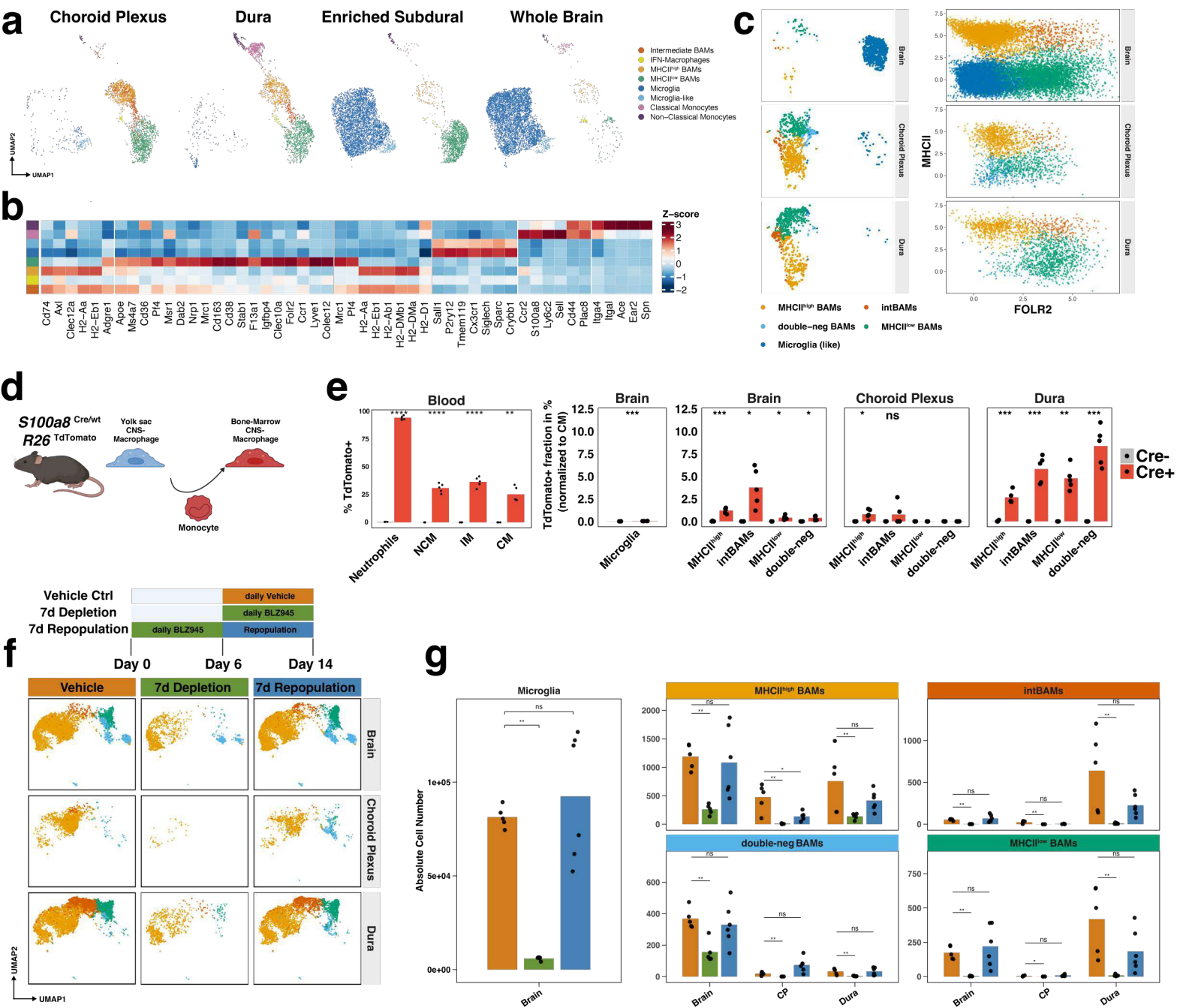
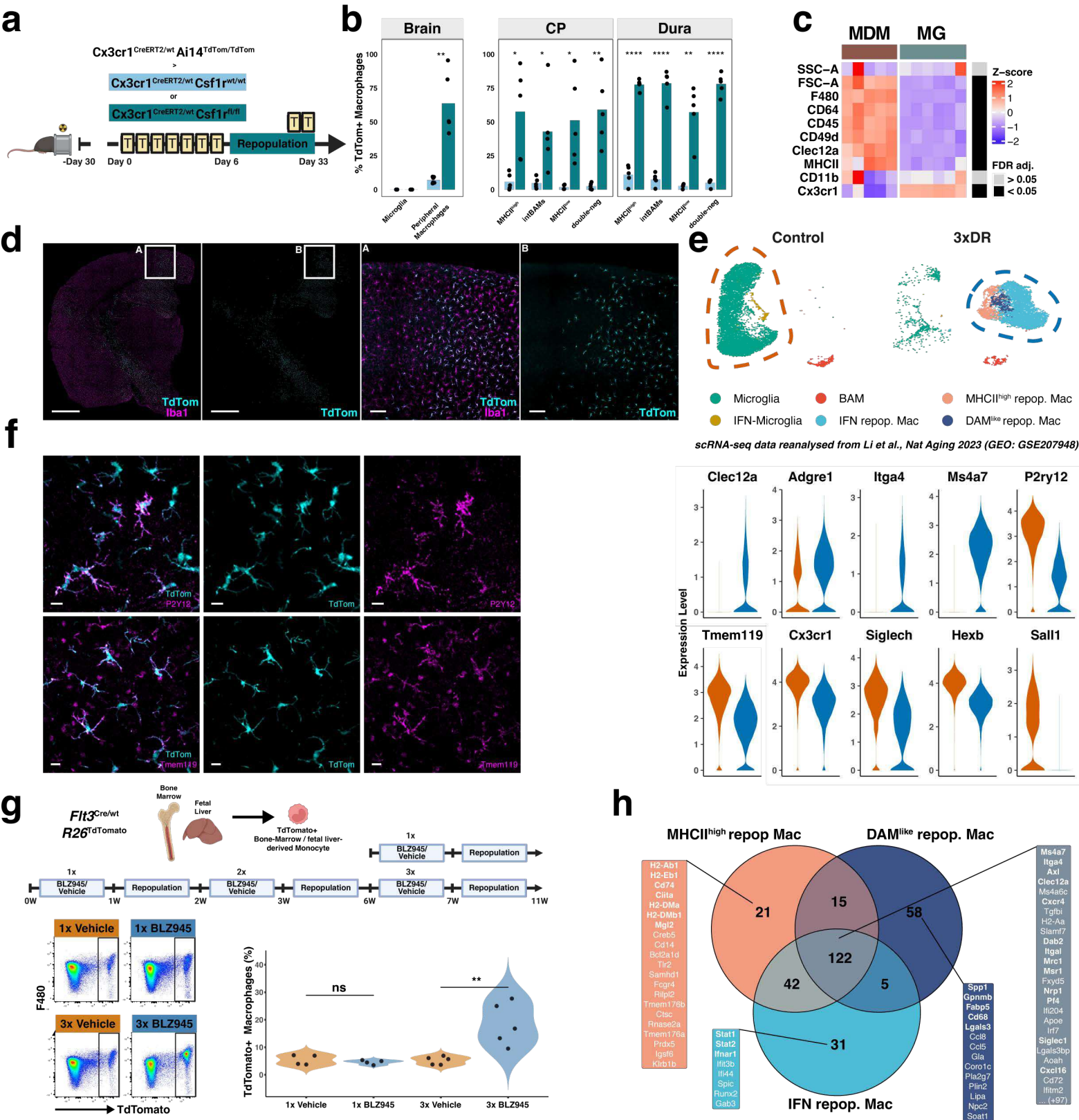


Figure 1: Monocyte contribution to BAMS is subset-specific at steady state and broadens after niche clearance.



Figure 2: CSF1R and PU.1 control differentiation of Ly6Chigh monocytes into downstream subsets but not for their maintenance.



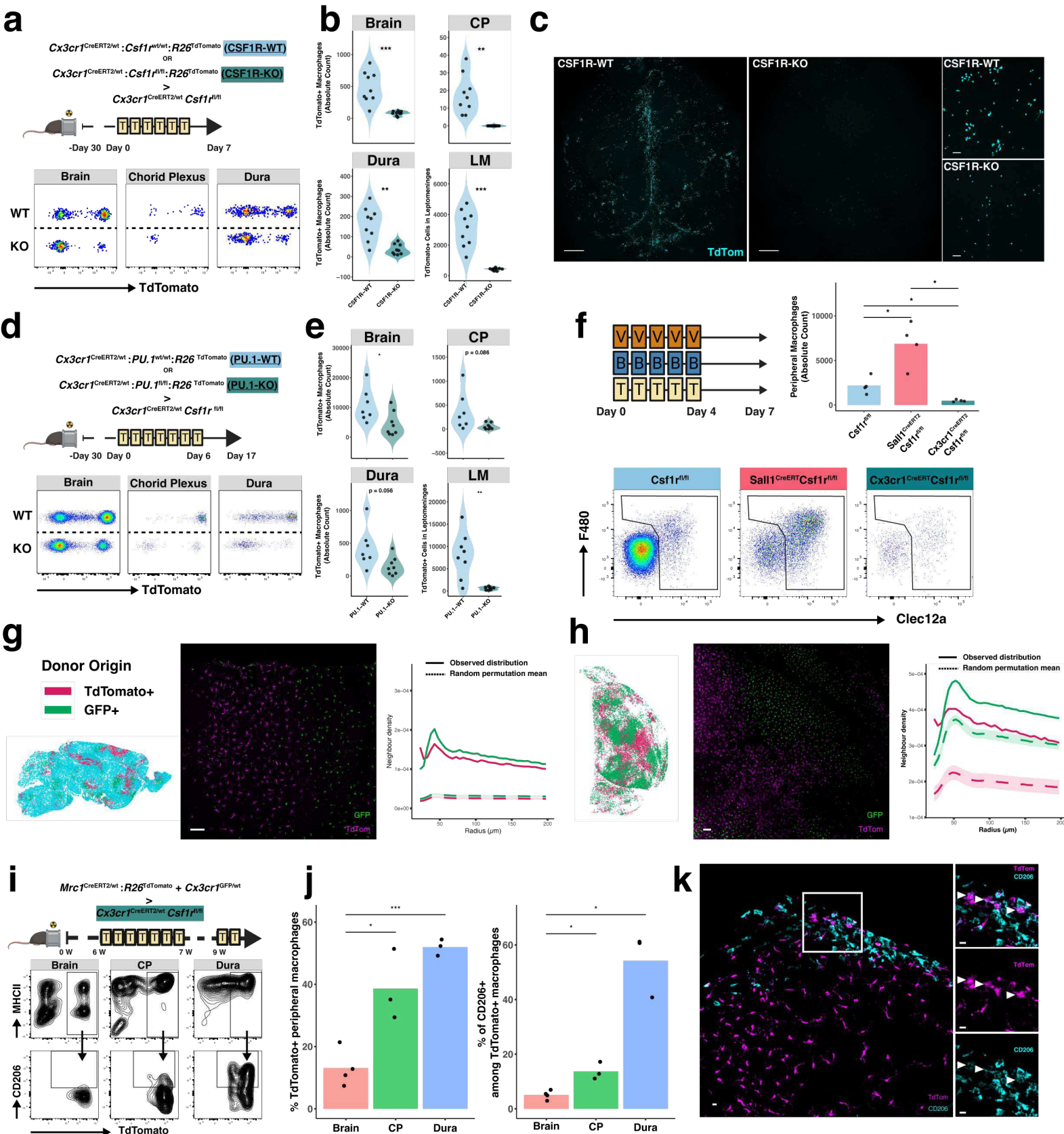


Figure 4: Competent monocytes directly exploit transient niche opening, clonally expand and enter the brain through a BAM-like state and transpl route.

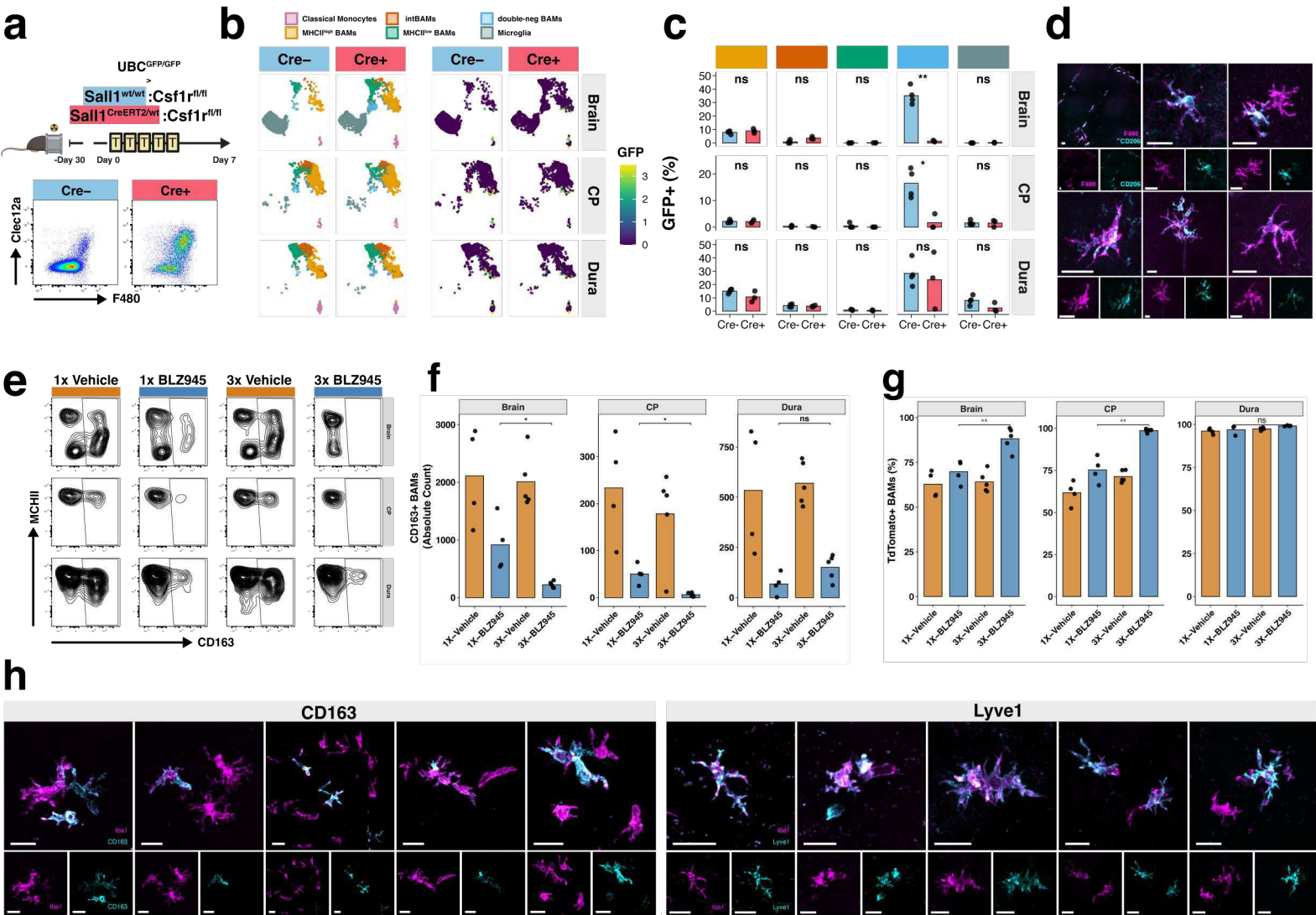
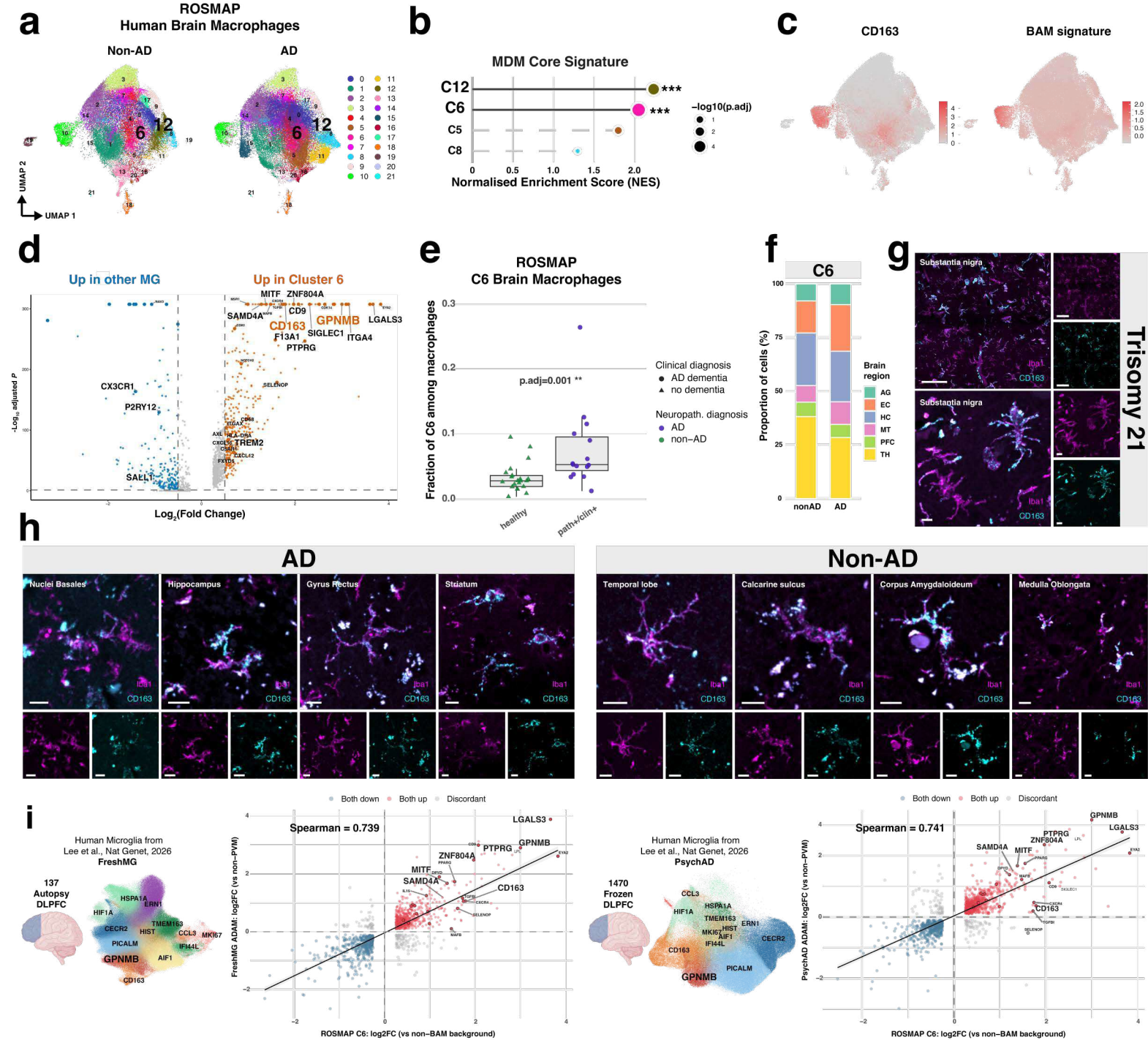
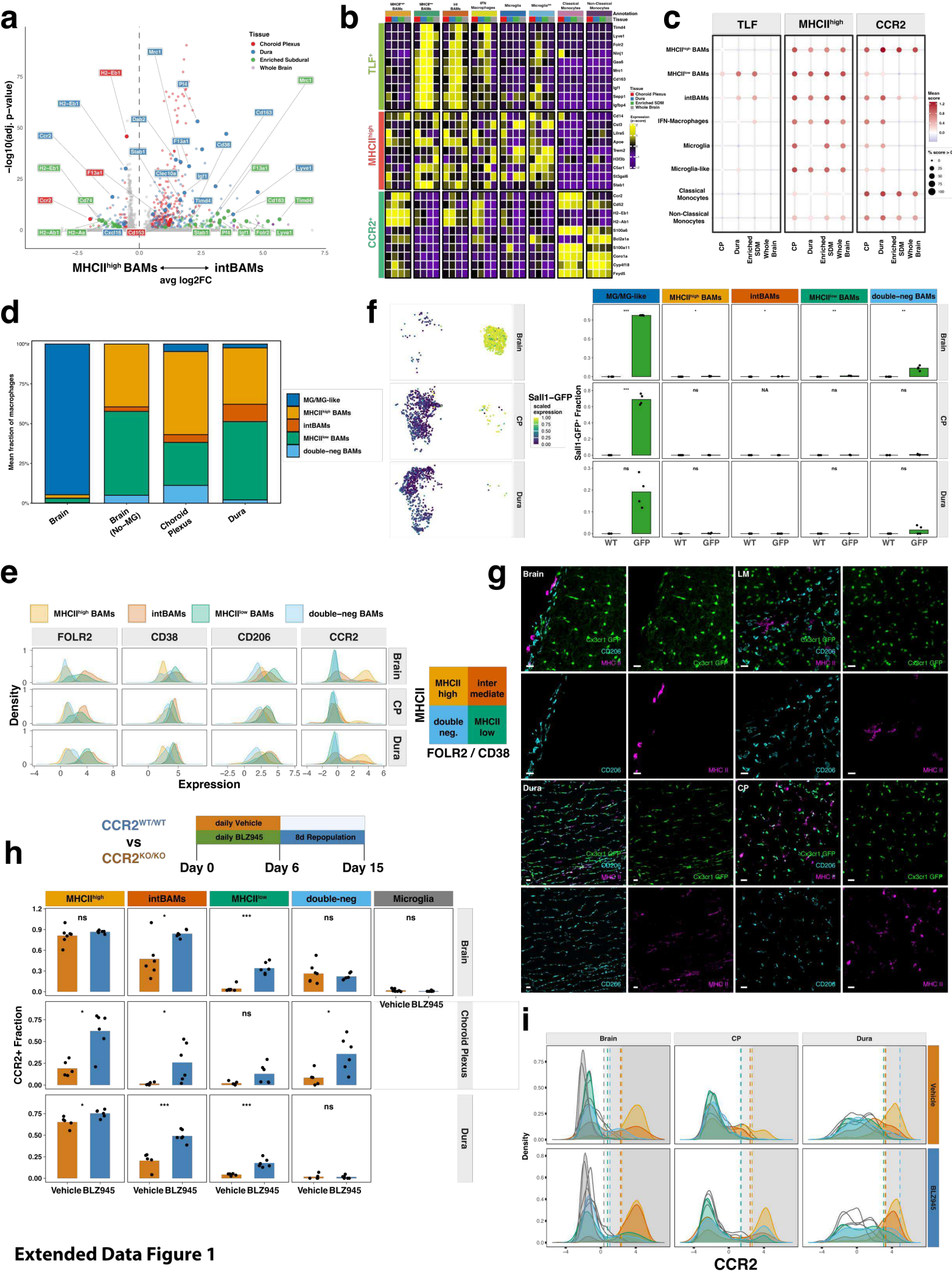
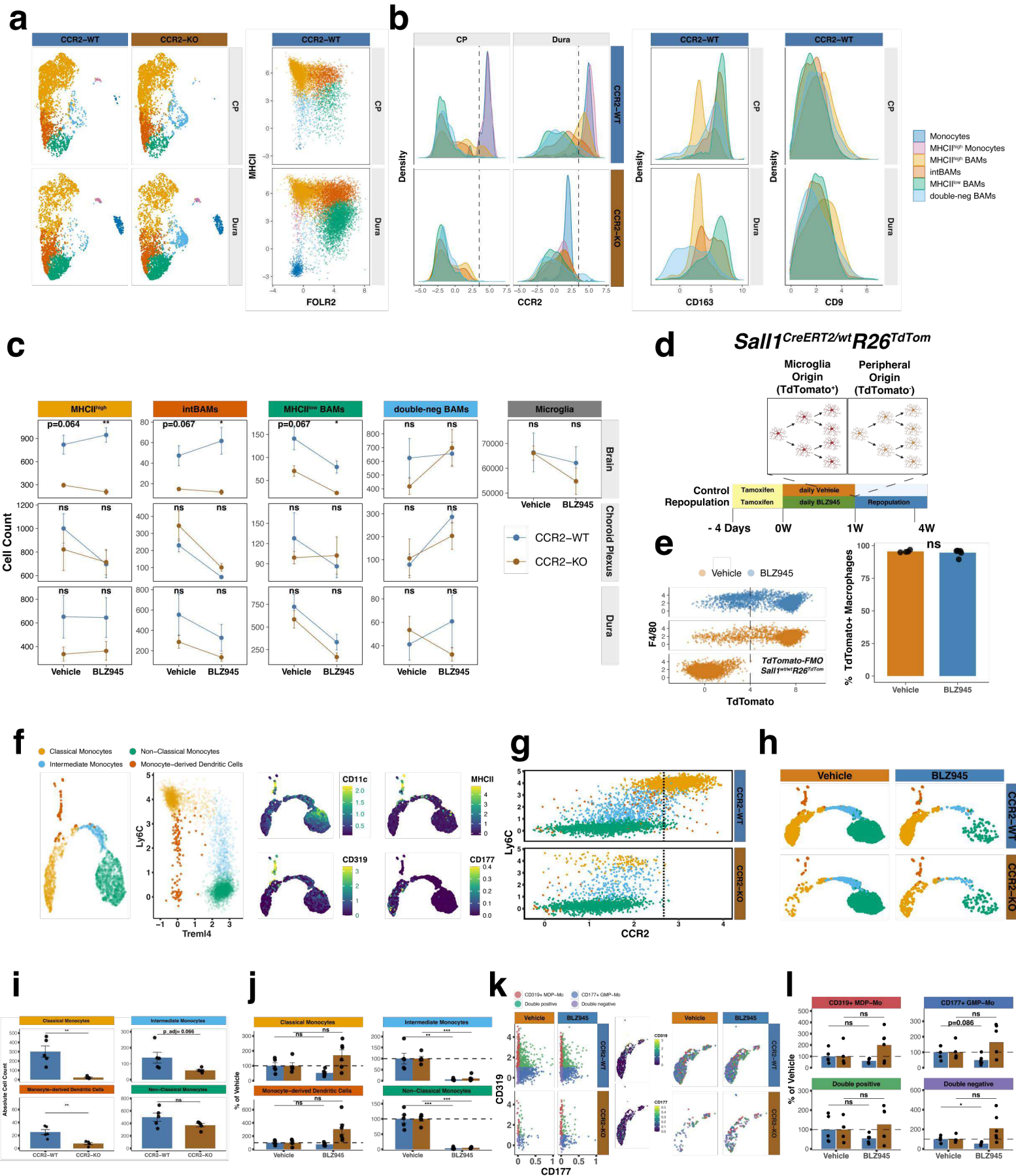


Figure 5: BAMS are a local reservoir for brain parenchymal macrophages.

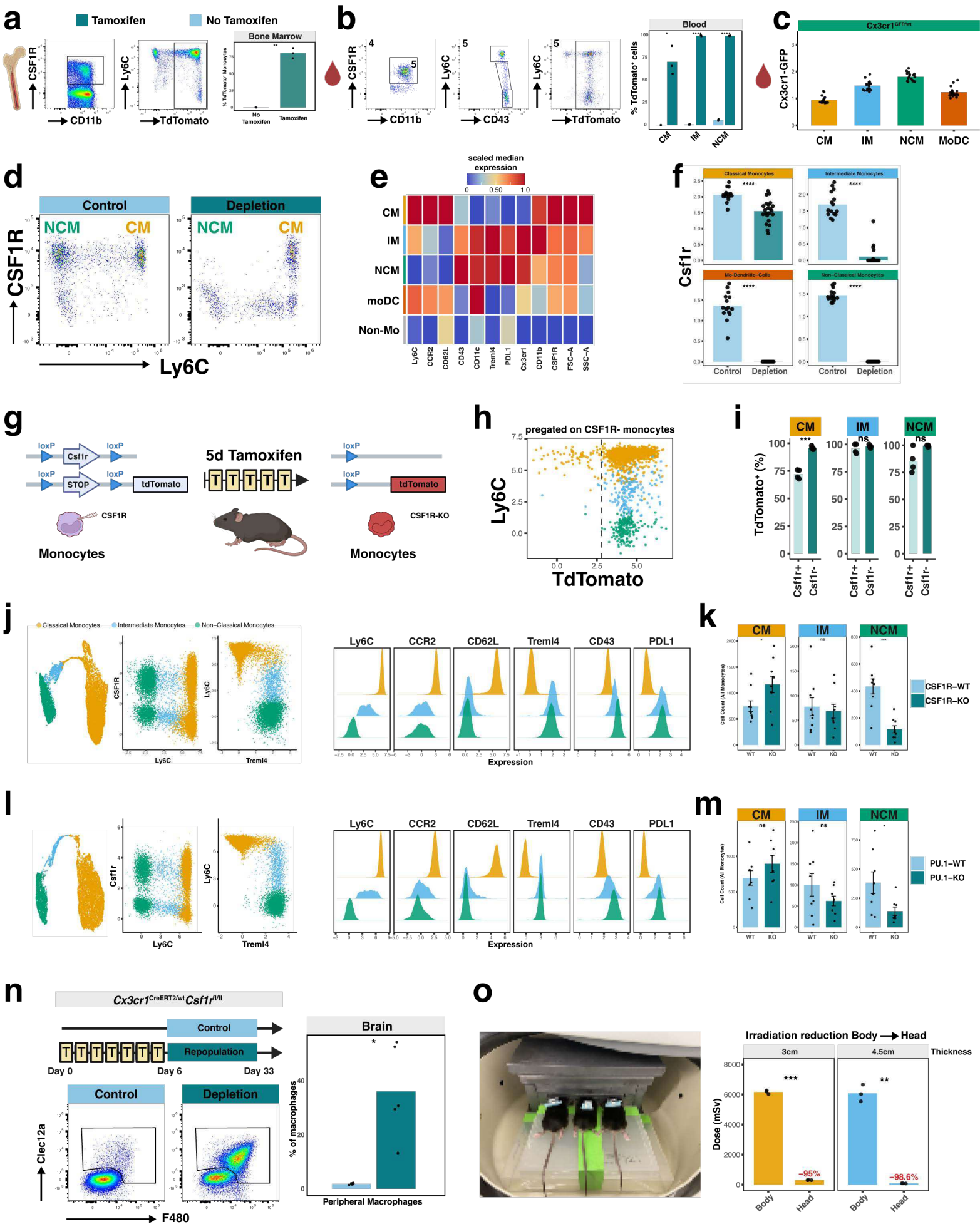




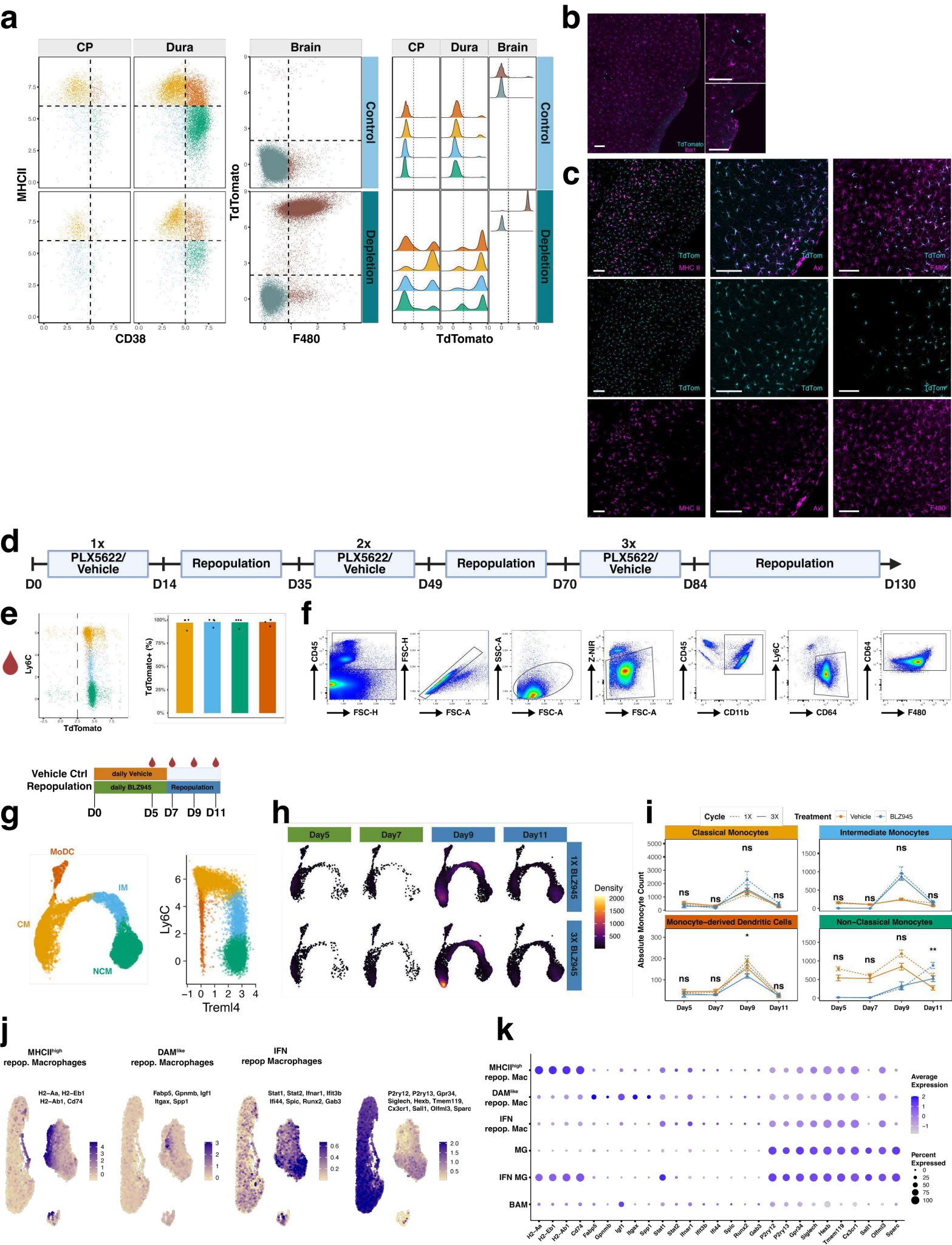
Extended Data Figure 1



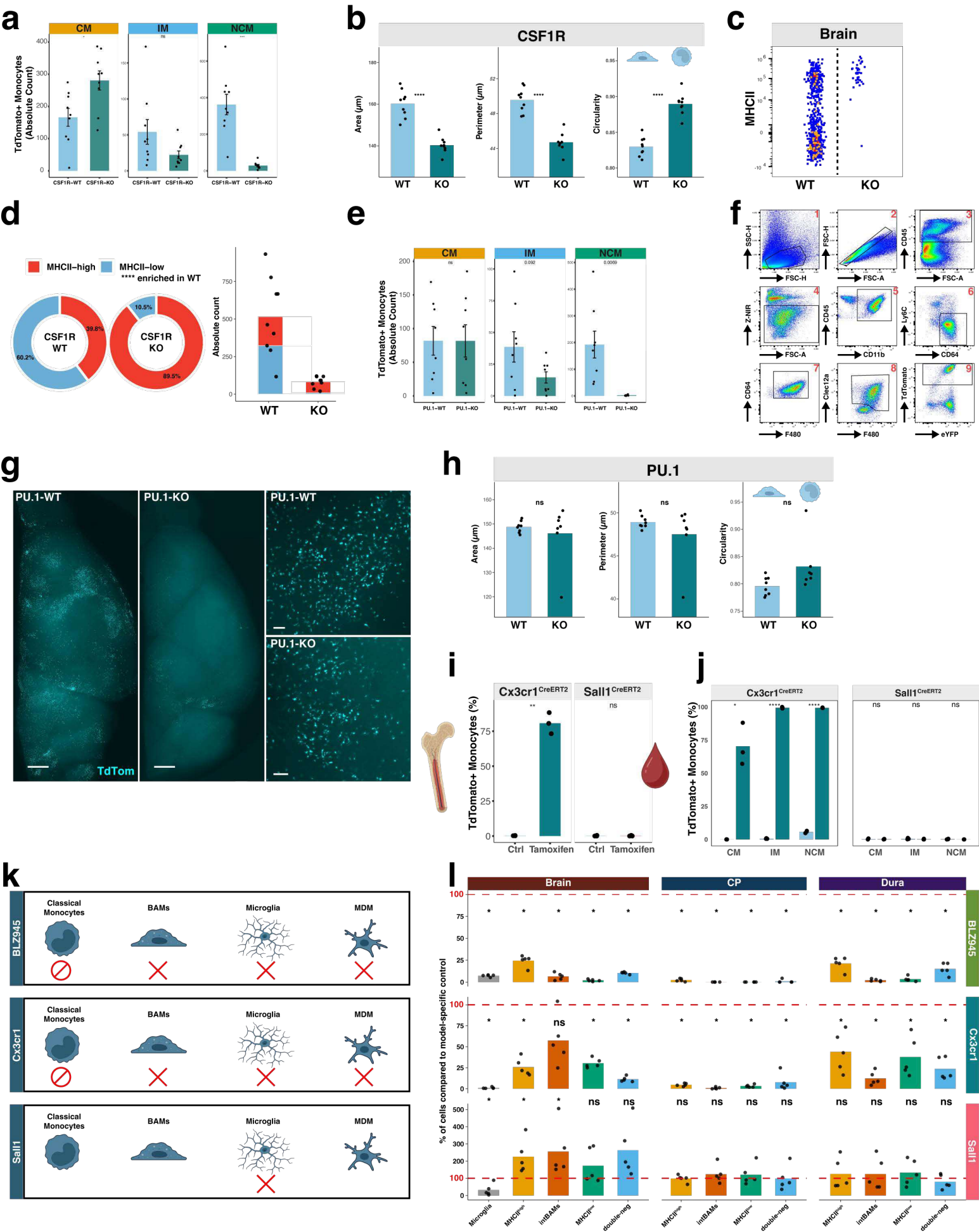
Extended Data Figure 2



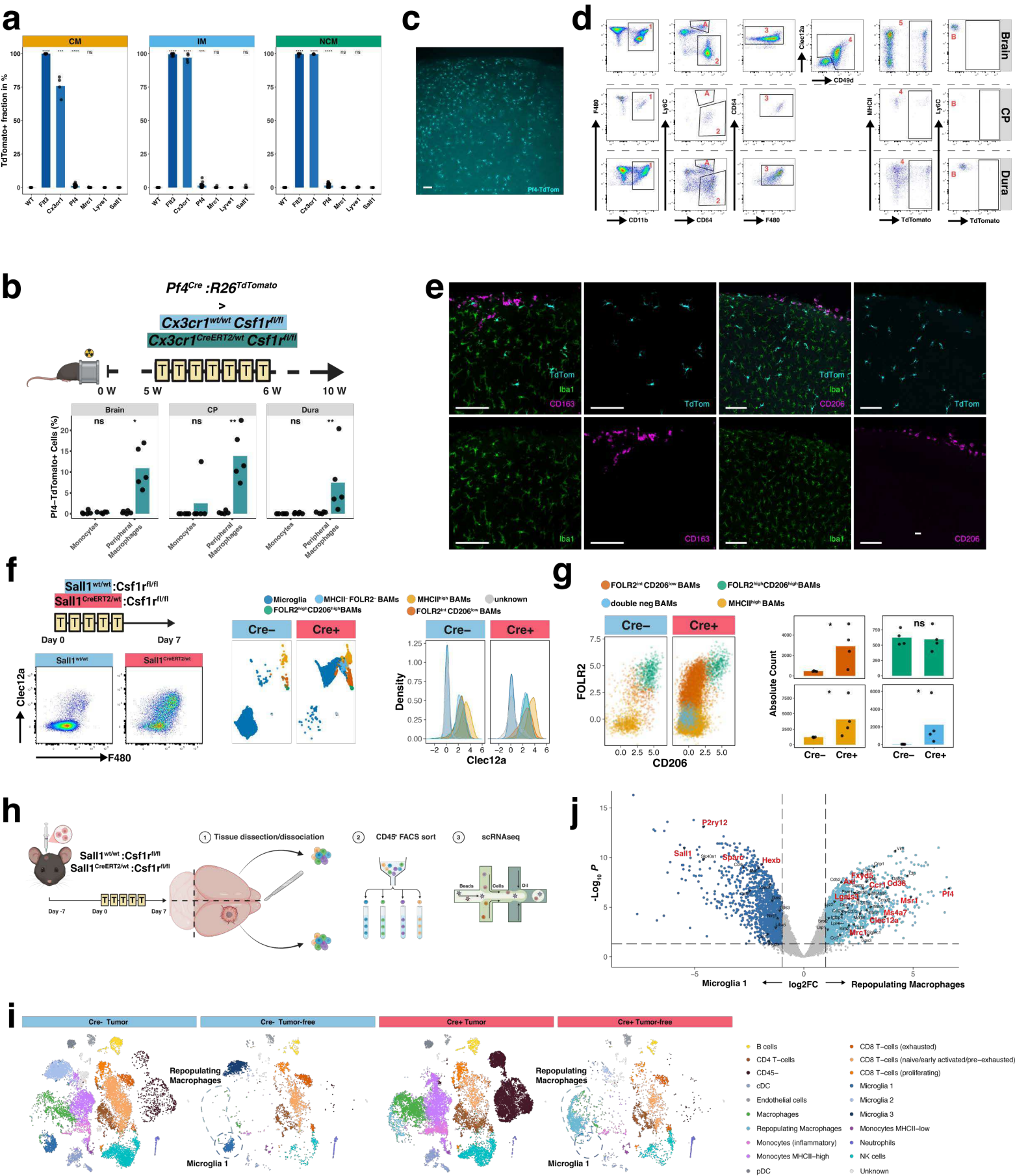
Extended Data Figure 3



Extended Data Figure 4

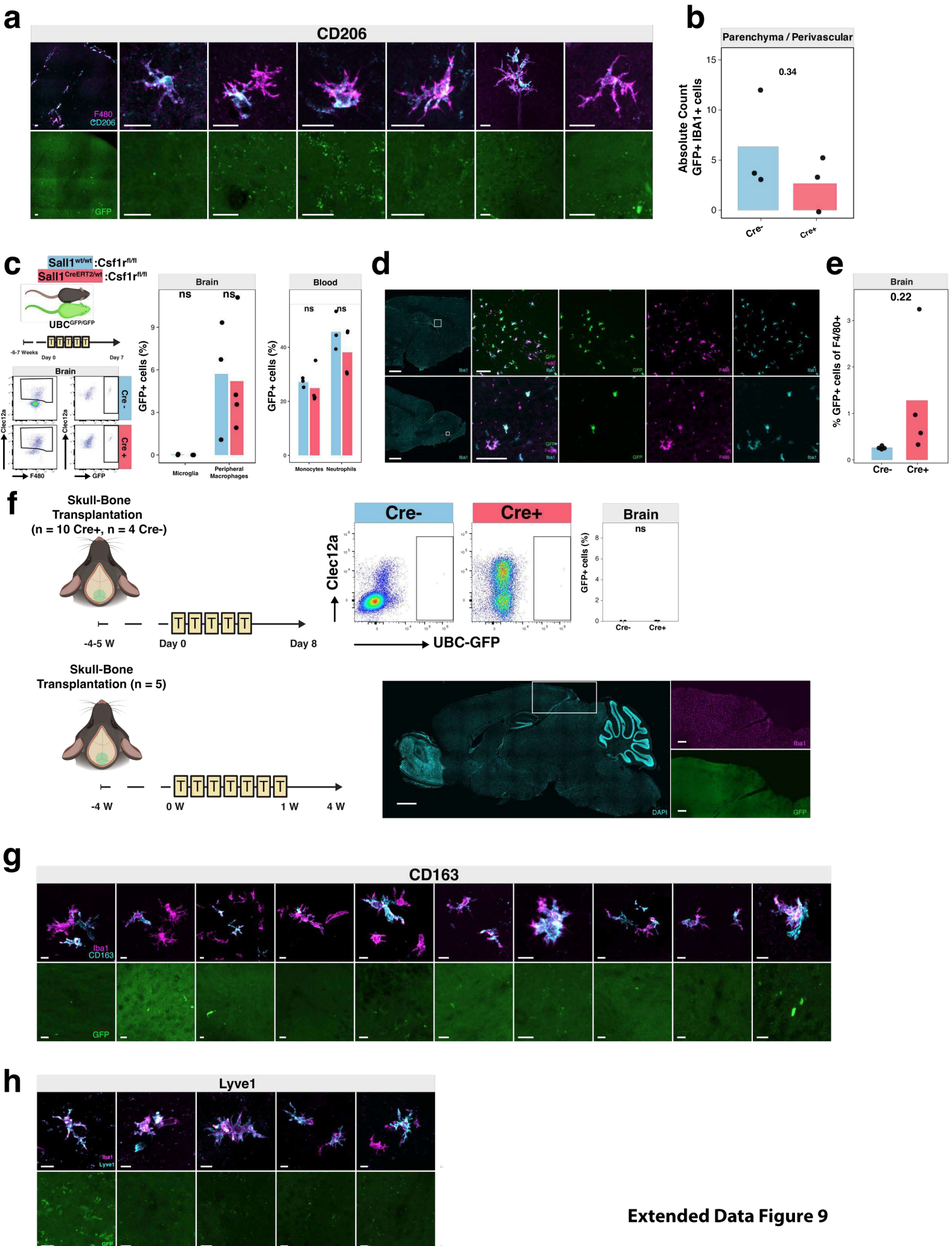


Extended Data Figure 5



Extended Data Figure 7





Extended Data Figure 9

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1mousestrainsreagents.docx](#)
- [TableS2AntibodiesFC.docx](#)
- [TableS3HumanSamples.xlsx](#)
- [TableS4primerlist.xlsx](#)
- [VideoS1.mp4](#)