

1 **Functional Mature Human Microglia Developed in Human iPSC Microglial Chimeric Mouse Brain**
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1 **Abstract**
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3 Microglia, the brain-resident macrophages, exhibit highly dynamic functions in neurodevelopment and
4 neurodegeneration. Human microglia possess unique features as compared to mouse microglia, but
5 our understanding of human microglial functions is largely limited by an inability to obtain human
6 microglia under homeostatic states. We developed a human pluripotent stem cell (hPSC)-based
7 microglial chimeric mouse brain model by transplanting hPSC-derived primitive macrophage precursors
8 into neonatal mouse brains. The engrafted human microglia widely disperse in the brain and replace
9 mouse microglia in corpus callosum at 6 months post-transplantation. Single-cell RNA-sequencing of
10 the microglial chimeric mouse brains reveals that xenografted hPSC-derived microglia largely retain
11 human microglial identity, as they exhibit signature gene expression patterns consistent with
12 physiological human microglia and recapitulate heterogeneity of adult human microglia. Importantly, the
13 engrafted hPSC-derived microglia exhibit dynamic response to cuprizone-induced demyelination and
14 species-specific transcriptomic differences in the expression of neurological disease-risk genes in
15 microglia. This model will serve as a novel tool to study the role of human microglia in brain
16 development and degeneration.

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1 Introduction

2

3 As the resident macrophages of the central nervous system (CNS), microglia play critical roles in
4 maintenance of CNS homeostasis and regulation of a broad range of neuronal responses ^{1, 2}. Recent
5 studies indicate that dysfunction of microglia contributes to neurodevelopmental and neurodegenerative
6 diseases, including Alzheimer's disease (AD) ³⁻⁷. Moreover, genome-wide association studies have
7 shown that many neurological disease risk genes, particularly neurodegenerative diseases, are highly
8 and sometimes exclusively expressed by microglia ⁸⁻¹⁰. These observations provide a compelling
9 incentive to investigate the role of microglia in models of abnormal brain development and
10 neurodegeneration. Most studies of microglia largely rely on rodent microglia. However, there is
11 increasing evidence that rodent microglia are not able to faithfully mirror the biology of human microglia
12 ¹¹. In particular, recent transcriptomic studies have clearly demonstrated that a number of immune
13 genes, not identified as part of the mouse microglial signature, were abundantly expressed in human
14 microglia ^{8, 12}. Moreover, a limited overlap was observed in microglial genes regulated during aging and
15 neurodegeneration between mice and humans, indicating that human and mouse microglia age
16 differently under normal and diseased conditions ^{12, 13}. These findings argue for the development of
17 species-specific research tools to investigate microglial functions in human brain development, aging,
18 and neurodegeneration.

19 Functional human brain tissue is scarcely available. In addition, given the considerable
20 sensitivity of microglia to environmental changes ⁸, the properties of available human microglia isolated
21 from surgically resected brain tissue may vary significantly, due to different disease states of the
22 patients and the multi-step procedures used for microglial purification. In order to study human
23 microglia in a relatively homeostatic state, many scientists have turned to human pluripotent stem cells
24 (hPSCs). Recent advances in stem cell technology have led to the efficient generation of microglia from
25 hPSCs ¹⁴⁻¹⁹, providing an unlimited source of human microglia to study their function. However, when
26 cultured alone or co-cultured with neurons and astrocytes in 2-dimensional (2D) or 3D
27 organoid/spheroid culture systems, these hPSC-derived microglia best resemble fetal or early postnatal
28 human microglia. This is indicated by much lower expression of key microglial molecules such as
29 TREM2, TMEM119, and P2RY12 in the hPSC-derived microglia, as compared to microglia derived
30 from adult human brain tissue ^{16, 18, 20}. Thus, even with these novel *in vitro* models, it has been
31 challenging to advance understanding of human microglial function in adult ages or in
32 neurodegeneration during aging.

33 Recent studies from us ^{21, 22} and others ²³⁻²⁵ have demonstrated that neonatally engrafted
34 human neural or macroglial (oligodendroglial and astroglial) progenitor cells can largely repopulate and
35 functionally integrate into the adult host rodent brain or spinal cord, generating widespread chimerism.
36 This human-mouse chimeric approach provides unique opportunities for studying the pathophysiology
37 of the human cells within an intact brain. In this study, we developed a hPSC microglial chimeric mouse
38 brain model, by transplanting hPSC-derived microglia into neonatal mouse brains. The engrafted
39 hPSC-derived microglia can proliferate, migrate, and widely disperse in the brain. We hypothesize that
40 the limited functional maturation of hPSC-derived microglia in *in vitro* models is primarily caused by the
41 fact that those microglia are maintained in an environment that lacks the complex cell-cell/cell-matrix
42 interactions existing in an *in vivo* brain environment ⁸. To test this hypothesis, we employed single-cell
43 RNA-sequencing and super-resolution confocal imaging to examine the identity and function of hPSC-
44 derived microglia developed for six months in the mouse brain under both homeostatic and toxin-
45 induced demyelination conditions.

1 **Results**
2

3 **Generation of hPSC microglial chimeric mouse brains**

4 Microglia originate from yolk sac erythromyeloid progenitors (EMPs) during primitive hematopoiesis.
5 EMPs further develop to primitive macrophage precursors (PMPs) that migrate into the developing neural
6 tube and become microglia with ramified processes within the CNS environment¹. We first derived PMPs
7 from hPSCs, including one human induced pluripotent stem cell (hiPSC) line and one human embryonic
8 stem cell (hESC) line, using a published protocol¹⁸. Briefly, the yolk sac embryoid bodies (YS-EBs) were
9 generated by treating the EBs with bone morphogenetic protein 4 (BMP4), vascular endothelial growth
10 factor (VEGF), and stem cell factor (SCF). Next, the YS-EBs were plated into dishes with interleukin-3
11 (IL-3) and macrophage colony-stimulating factor (M-CSF) to promote myeloid differentiation. At 2–3
12 weeks after plating, hPSC-derived PMPs emerged into the supernatant and were continuously produced
13 for more than 3 months. The cumulative yield of PMPs was around 40-fold higher than the number of
14 input hPSCs (Figure 1A), similar to the results from previous studies^{16, 18, 26}. PMPs are produced in a
15 Myb-independent manner that closely recapitulated primitive hematopoiesis^{1, 18, 27}. We confirmed the
16 identity of these hPSC-derived PMPs by staining with CD235, a marker for YS primitive hematopoietic
17 progenitors^{28, 29}, and CD43, a marker for hematopoietic progenitor-like cells^{28, 29}. As shown in Figure 1B,
18 over 95% of the hPSC-derived PMPs expressed both markers. Moreover, the human PMPs are highly
19 proliferative as indicated by Ki67 staining (95.4 ± 2.2%, n = 4) (Figure 1B). Using this method, we routinely
20 obtain ample numbers of hPSC-derived PMPs with high purity as required for cell transplantation
21 experiments.

22 We engrafted hPSC-derived PMPs into the brains of postnatal day 0 (P0) immunodeficient mice
23 that are Rag2/IL2Rγ-deficient and also express the human forms of CSF1, which facilitates the survival
24 of xenografted human myeloid cells and other leukocytes^{30, 31}. We deposited cells into the white matter
25 overlying the hippocampus and sites within the hippocampal formation (Figure 1C). In order to visualize
26 the distribution of donor-derived microglia, at 6 months post-transplantation, we stained the mouse
27 brain sections with human-specific antibody recognizing TMEM119 (hTMEM119). TMEM119 is a
28 marker that is only expressed by microglia, but not other macrophages^{14, 17, 32}. We found that the
29 donor-derived hTMEM119⁺ microglia widely disperse in the brain (Figure 1D). As early as 3 weeks
30 post-transplantation, donor-derived microglia had already migrated along corpus callosum and passed
31 through the rostral migratory stream to the olfactory bulb (Figure 1E). At 6 months post-transplantation,
32 human microglia widely dispersed in multiple brain regions, including olfactory bulb, hippocampus, and
33 cerebral cortex, and exhibited a highly ramified morphology (Figure 1F and G). Frequently, we also
34 observed clusters of human microglia in the cerebellum (Figure 1H), which might be a result from the
35 strong ability of immune cells trafficking along blood vessels and/or the choroid plexus³³. Similar to our
36 previous studies^{24, 25}, we assessed the engraftment efficiency and degree of chimerization by
37 quantifying the percentage of hTMEM119⁺ cells among total DAPI⁺ cells in the forebrain in sagittal brain
38 sections covering regions from 0.3 to 2.4 mm lateral to midline and found that about 8% of the total
39 cells were human microglia in the 6-month-old mouse brains (Figure 1D and L). As shown by individual
40 data points overlaid on the bar graphs (Figure 1L), there were variations in chimerization among
41 animals. In addition to the images showing distribution of donor-derived cells in Figure 1D, we thus also
42 included tile scan images collected from another chimeric mouse brain that represents the lower level
43 of chimerization (supplementary Figure 1A). In the developing brain, microglia are known to use white
44 matter tracts as guiding structures for migration and that they enter different brain regions³⁴. In order to
45 examine the migration pattern of our transplanted cells, we deposited PMPs into different sites, the
46 lateral ventricles of P0 mice. As early as three weeks post-transplantation, we found that the majority of
47 donor-derived cells migrated along the anterior corpus callosum, rostral migration stream, and then
48 entered the olfactory bulb. Moreover, some of those cells of migrated posteriorly along the corpus
49 callosum (supplementary Figure 1B), suggesting that engrafted cells likely used corpus callosum to
50 migrate to various brain regions. These results demonstrate that hPSC-derived PMPs survive in mouse
51 brain and that they migrate to a variety of structures.

1 To examine whether transplanted hPSC-derived PMPs efficiently differentiated to microglia in
2 the mouse brain, we double-stained brain sections for both human nuclei (hN) and hTMEM119. As
3 early as 8 weeks post-transplantation, the vast majority of hN⁺ donor-derived cells (93.2 ± 2.2%, n = 7)
4 were positive for hTMEM119 (Figure 1I and M), indicating the robust and efficient differentiation of
5 hPSC-derived PMPs into microglia. Moreover, the vast majority of the donor-derived cells expressed
6 PU.1, a transcription factor that is necessary for microglial differentiation and maintenance³⁵⁻³⁸, and
7 were positive for human specific CD45 (hCD45), which is expressed by all nucleated hematopoietic
8 cells (Figure 1J)^{39, 40}. Similarly, at 6 months post-transplantation, the vast majority of donor-derived
9 cells expressed hTMEM119 (supplementary Figure 2A) and P2RY12 (93.4 ± 3.8%, n = 7;
10 supplementary Figure 2B and C). hTMEM119 and P2RY12 was not expressed in PMP cultures
11 (supplementary Figure 2D). Moreover, we examined the distribution of human donor cells in border
12 regions, including choroid plexus, meninges, and perivascular spaces. We found that most of the
13 hTMEM119⁺/hN⁺ donor-derived cells were seen in those border regions. Furthermore, we co-stained
14 hTMEM119 with CD163, an established marker for non-microglial CNS myeloid cells^{41, 42}. In choroid
15 plexus, we found that some of the hTMEM119⁺ cells co-expressed CD163, suggesting that these
16 transplanted cells differentiated into choroid plexus macrophage (cpMΦ), but not microglia
17 (supplementary Figure 3A). In order to better visualize meninges and perivascular space, we triple-
18 stained hN and CD163 with laminin, a marker that has been commonly used to visualize vascular
19 structures in the mammalian brain⁴³. There was also a small number of hN⁺ and CD163⁺ co-expressing
20 cells in these regions, indicating that the transplanted cells differentiated into meningeal macrophage
21 (mMΦ) and perivascular macrophages (pvMΦ) (supplementary Figure 3B and C). There was a
22 possibility that some transplanted cells might remain as progenitors and maintain their hematopoietic
23 progenitor-like cell identity. As shown in supplementary Figure 3D, we found that a small population of
24 hN⁺ cells expressed CD235 in the regions close to the lateral ventricles. Overall, these results
25 demonstrate that the vast majority of engrafted hiPSC-derived PMPs differentiate into hTMEM119⁺
26 microglia, with a relatively small number giving rise to other of hTMEM119⁺ CNS myeloid cells in a brain
27 context-dependent manner or remaining as progenitors.

28 We next assessed the proliferation of engrafted cells by staining the proliferative marker Ki67.
29 As shown in Figure 1K and N, at 3 weeks post-transplantation, about 17% (16.9 ± 5.7%, n = 8) of hN⁺
30 transplanted cells expressed Ki67, indicating that these cells were capable of proliferating in the mouse
31 brain. At 6 months post-transplantation, the percentage of proliferating cells dramatically decreased and
32 less than 2% (1.7 ± 0.8%, n = 7) of total engrafted cells were Ki67 positive. These Ki67⁺ proliferating
33 human cells mainly localized in the subventricular zone, the walls along lateral ventricles, corpus
34 callosum, and olfactory bulb (Figure 1K and supplementary Figure 3E). We also examined the
35 proliferation of mouse host microglia at different brain regions at 3 weeks post-transplantation. We only
36 found a very small number of Ki67⁺ mouse microglia in the subventricular zone (supplementary Figure
37 3F), which is consistent with a previous report⁴⁴. Taken together, these findings demonstrate that
38 engrafted hPSC-derived PMPs differentiate to microglia, generating a mouse brain with a high degree of
39 human microglial chimerism in the forebrain.

40
41 **Human PSC-derived microglia undergo morphological maturation and are functional in the mouse**
42 **brain**

43 Compared with three weeks post-transplantation, hPSCs-derived microglia appeared to exhibit more
44 complex processes at 6 months post-transplantation (Figure 1E and F). Moreover, even at the same
45 stage, hPSC-derived microglia in the cerebral cortex seemed to exhibit much more complex
46 morphology, compared with the hPSCs-derived microglia in the corpus callosum and cerebellum
47 (Figure 1G and H and hTMEM119 and Iba1 staining in supplementary Figure 2A and 4, respectively).
48 In the corpus callosum, hPSC-derived microglia had fewer branches that aligned with axons; and in the
49 cerebral cortex, the microglia exhibited more complex and ramified processes (supplementary Figure
50 2A and 4), similar to observations from previous studies^{34, 45}. This prompted us to further examine the
51 morphological and functional changes of the hPSC-derived microglia along with the development of the

1 mouse brain, particularly in cerebral cortex. Previous studies have shown that there are no changes in
2 microglial number, cytokine levels, and gene expression profiles between wild type and *Rag2*^{-/-} mice⁴⁶.
3 Building upon that, we also compared the differences between xenografted hPSC-derived microglia vs.
4 host mouse microglia. We double-stained the brain sections with human and mouse specific TMEM119
5 (hTMEM119 and mTMEM119, respectively) antibodies to distinguish hPSC-derived microglia and
6 mouse host microglia (Figure 2 A and B). As shown in Figure 2A, in 6 month old mice, both hPSCs-
7 derived microglia and mouse microglia were seen in the cerebral cortex and hippocampus. Notably, we
8 observed that mouse microglia mainly resided in distal regions in the cerebral cortex and hippocampus.
9 In particular, in the corpus callosum, mouse microglia were rarely seen, and the vast majority of
10 microglia were hPSC-derived microglia (Figure 2C), indicating that hPSC-derived microglia replaced
11 the host mouse microglia. In the cerebral cortex, hTMEM119⁺ hPSC-derived microglia exhibited much
12 more complex processes at 8 weeks and 6 months post-transplantation than those cells at 3 weeks
13 post-transplantation, as indicated by the increased number of endpoints (Figure 2D). The total length of
14 processes of hPSC-derived microglia also significantly increased from week 3 to week 8 and month 6
15 (Figure 2E), suggesting the gradual maturation of hPSC-derived microglia in mouse brain. We further
16 examined the morphological differences between hPSC-derived microglia vs. mouse microglia at the
17 same time points after transplantation. In the cerebral cortex, at 3 weeks post transplantation,
18 compared with hPSC-derived microglia, mouse microglia showed a significantly higher number of
19 endpoints and a slight trend of longer processes (Figure 2D and E). However, at 8 weeks post-
20 transplantation, there was no significant difference in endpoint number and process length between
21 hPSC-derived microglia and mouse microglia (Figure 2D and E). Interestingly, at 6 months post-
22 transplantation, hPSC-derived microglia exhibited a significantly higher number of endpoints and longer
23 process length than mouse microglia. Since microglial morphology is inextricably linked to their
24 phagocytic functions^{47, 48}, we examined the expression of CD68, a marker for the phagolysosome^{49, 50}.
25 In the cerebral cortex, CD68 was expressed in some of the hPSC-derived microglia at 3 weeks post-
26 transplantation and its expression dramatically decreased from 8 weeks to 6 months post-
27 transplantation (Figure 2F and G). We observed some hTMEM119⁻/CD68⁺ cells at 3 weeks and nearly
28 no hTMEM119⁻/CD68⁺ cells at 6 months post-transplantation (Figure 2G), suggesting that nearly no
29 host mouse microglia expressed CD68 at 6 months post-transplantation. Taken together, hPSC-derived
30 microglia show variable morphologies in a spatiotemporal manner and morphologically differ from the
31 host mouse microglia.

32 Microglia have been shown to shape synapse formation by pruning synapses and to maintain
33 oligodendroglial homeostasis by phagocytizing oligodendroglial cells⁵¹⁻⁵³. We therefore investigated
34 whether hPSCs-derived microglia are functional in the mouse brain. To examine synaptic pruning
35 function, we employed a super-resolution imaging technique to visualize synapse engulfment by
36 hPSCs-derived microglia. We triple-stained hTMEM119 with both a post-synaptic marker PSD95 and a
37 pre-synaptic marker synapsin I. The 3D reconstruction images show that PSD95⁺ and synapsin I⁺
38 puncta are colocalized within hTMEM119⁺ processes, indicating that these synaptic proteins are
39 phagocytosed by hPSCs-derived microglia at eight weeks post-transplantation in grey matter (Figure
40 3A). In addition, we also validated the specificity of PSD95 puncta staining by incubating brain sections
41 with the PSD95 antibody together with a PSD95 peptide. We barely detected any PSD95⁺ puncta
42 signal after the incubation in the presence of PSD95 peptide (supplementary Figure 5A). We also triple-
43 staining hTMEM119 and PSD95 with CD68. As shown in Figure 3B and supplementary Figures 5B, 5C,
44 PSD95⁺ puncta are localized within CD68⁺ phagolysosomes in hTMEM119⁺ hPSCs-derived microglia,
45 further indicating their synaptic pruning function. Of note, this engulfment of synaptic materials was
46 observed from 3 weeks to 6 months post-transplantation, with a peak at 8 weeks post-transplantation
47 (Figure 3C). Very few mouse microglia were found to engulf synaptic proteins at 8 weeks post-
48 transplantation (supplementary Figure 6A). To examine the function of phagocytosing oligodendroglia,
49 we double-stained hCD45 with PDGFR α , a marker for oligodendroglial progenitor cell. We observed
50 that hCD45⁺ human microglia were able to engulf PDGFR α ⁺ oligodendroglia at 3 weeks post-
51 transplantation in the corpus callosum (Figure 3D). We also double stained hCD45 with the

1 oligodendroglial marker OLIG2 and similarly found that hPSCs-derived microglia in white matter
2 engulfed OLIG2⁺ oligodendroglia at 3 weeks post-transplantation (supplementary Figure 6B). In
3 addition, we detected that a small population of mouse microglia engulfed OLIG2⁺ oligodendroglia in
4 the corpus callosum at 3 weeks post-transplantation (supplementary Figure 6C).

5 Microglia, together with endothelial cells, pericytes and astrocytes, form the functional blood–
6 brain barrier^{54–56}. We double-stained brain sections with hCD45 and laminin. We found that hPSC-
7 derived microglia clustered around and were closely affiliated with laminin⁺ blood vessels in both grey
8 matter and white matter across different brain regions including the olfactory bulb (Figure 3E and
9 supplementary Figure 6D). In addition, we also found that mouse microglia similarly had close contact
10 with blood vessels in the cerebral cortex, corpus callosum, and olfactory bulb (supplementary Figure
11 6E). Altogether, these results demonstrate hPSCs-derived are functional in the mouse brain under
12 homeostatic conditions. The human microglia and host mouse microglia exhibited similar microglial
13 functions, including synaptic pruning, phagocytosis of oligodendroglia, and having contact with blood
14 vessels.

16 **Single-cell RNA-sequencing of hiPSC microglial chimeric mouse brain identifies a gene
17 expression signature consistent with adult human microglia**

18 Homeostatic human microglia at adult stages are difficult to obtain, because microglia are highly
19 sensitive to environmental changes and microglia derived from adult human brain tissue-derived are
20 usually purified through multi-step procedures that can change their biological properties significantly⁸.
21 In addition, microglia derived from hPSCs using all current differentiation protocols largely resemble
22 fetal or early postnatal human microglia^{16, 18, 20}. We hypothesize that hPSC microglial chimeric mice
23 may provide a unique opportunity to study biological properties of adult human microglia, because the
24 engrafted hPSC-derived microglia are likely to exhibit an expedited maturation process promoted by the
25 maturing environment in the mouse brain. To test this hypothesis, we examined transcriptomic profiles
26 of hiPSC-derived microglia, developed in the *in vivo* homeostatic mouse environment, using single-cell
27 RNA-sequencing (scRNA-seq). We collected brain regions where engrafted hiPSCs-derived microglia
28 preferentially dispersed, including the cerebral cortex, hippocampus, corpus callosum, and olfactory
29 bulb, from 6-month-old chimeric mouse brain for scRNA-seq. A previous study has demonstrated that
30 within hours during which microglia are isolated from the brain environment and transferred to culture
31 conditions, microglia undergo significant changes in gene expression⁸. To capture observed
32 expression patterns as close to the “*in vivo*” patterns as possible, we chose to omit a FACS sorting step
33 since it would have added substantial processing time. Owing to the wide distribution and high
34 abundance of hiPSC-derived microglia in those brain regions, we were able to capture ample numbers
35 of hiPSC-derived microglia for scRNA-seq even without enrichment by FACS sorting. After brain tissue
36 dissociation with papain and centrifugation to remove debris and myelin, single cell suspensions were
37 directly subjected to droplet-based 10X Genomic RNA-seq isolation (Fig. 4A). Using stringent criteria,
38 29,974 cells passed the quality control evaluation (with about 10,000–15,000 reads/cell) from 4 animals
39 for downstream analysis (supplementary Figure 7A).

40 We performed dimensionality reduction and clustering using a principal component analysis
41 (PCA)-based approach. Using t-distributed stochastic neighbor embedding (t-SNE) to visualize cell
42 clustering, we identified 11 clusters, including a cluster of xenografted hiPSC-derived microglia, which
43 we named Xeno MG (Fig. 4B). This clustering pattern was consistently and independently obtained in
44 all four animals, indicating the reproducibility of the sequencing and clustering procedures
45 (supplementary Figure 7B). We defined each cluster based on the expression of enriched genes (Table
46 S1) that could be recognized as markers for specific cell types or are reported to be abundantly
47 expressed in specific cell types (Figure 4C and supplementary Figure 7C). The clusters included 10
48 mouse cell types: astrocytes (*SCL6A11*⁵⁷, *NTSR2*⁵⁸), oligodendrocytes (*CLDN11*⁵⁹, *CNP*⁶⁰),
49 oligodendrocyte progenitor cells (OPC; *PDGFRα*, *OLIG2*⁶¹), excitatory neurons (*SYT1*⁶², *SNAP25*⁶³),
50 neuronal precursors (*SOX11*,⁶⁴ *STMN2*,⁶⁵), vascular cells (*MYL9*⁶⁶, *MGP*⁶⁷), choroid cells
51 (*LCN2*⁶⁸, *1500015O10Rik*⁶⁹), endothelial cells (*ITM2A*⁷⁰, *FLT1*⁷¹), GABAergic neuron (*NPY*⁷², *NR2F2*⁷³)

1 and mouse microglia (*P2RY12*, *C1QA*, and *CX3CR1*). The only human cell cluster, labeled Xeno MG,
2 similarly expressed the microglial markers *P2RY12*, *C1QA*, and *CX3CR1*, and accounted for about 7%
3 of total cells in our selected dissected regions (supplementary Figure 7D). Of note, a cross-correlation
4 analysis of clustered cell types showed that Xeno MG had a highest correlation coefficient value (0.776)
5 with mouse microglia, consistent with a microglial identity of the engrafted human cells (supplementary
6 Figure 7E). Furthermore, the expression of a set of canonical microglial genes (*C1QA*, *CX3CR*,
7 *TREM2*, *CSF1R*, and *P2RY12*) was only detected in Xeno MG and mouse microglia clusters (Figure
8 4D). Moreover, we performed bulk RNA-seq to analyze the pre-engraftment hiPSC-derived PMPs. We
9 compared transcriptomic profiles between PMPs and Xeno MG from 6 months old chimeric mice.
10 Notably, as shown in Figure 4E, compared with PMPs, Xeno MG highly expressed microglial identity
11 markers, such as *TMEM119*, *P2RY12*, *SALL1*, and *OLFML3*, which were barely detected in PMPs. On
12 the other hand, the expression of markers for hematopoietic progenitor cells, such as *CD59*, *CD44*, and
13 *CD38*, was low in Xeno MG but much higher in PMPs. Furthermore, we compared the transcriptomic
14 profile of Xeno MG with a published dataset generated from human brain tissue-derived human
15 microglia¹². A significant correlation was observed between Xeno MG and the published dataset¹²
16 (supplementary Figure 8A), further confirming the human microglial identity of the engrafted human
17 cells. As shown in Figures 1 and 2, the highly ramified morphology of hiPSC-derived microglia suggest
18 that they stay at a homeostatic state. We examined expression of several pro-inflammatory cytokines to
19 assess the impact of the tissue preparation procedures on the microglial state. Consistently, we found
20 very minor expression of acute pro-inflammatory cytokines such as IL-1 β , IL-1 α and TNF- α ^{74 75, 76}
21 (supplementary Figure 8B). In contrast, the pro-inflammatory cytokine, IL-6 and an anti-inflammatory
22 cytokine, IL-10, were nearly undetectable, and expression of these pro-inflammatory cytokines is often
23 correlated with a longer-lasting inflammatory response^{75, 76}. This observation suggests that only a very
24 mild inflammatory reaction was likely triggered in the Xeno MG during sample preparation, similar to
25 previous reports^{53, 74}. These results demonstrate that Xeno MG developed in the mouse brain largely
26 retain their human microglial identity and exhibit a gene expression pattern characteristic of
27 homeostatic human microglia.

28 Next, to further evaluate identity and maturation of Xeno MG in chimeric mouse brains, we
29 compared the global expression patterns of 21 genes including the 11 microglia-specific genes and 7
30 HPC-specific genes shown in Figure 4E, as well as 3 NPC genes (*NES*, *DCX*, and *SOX2*) between our
31 Xeno MG, hiPSC-derived PMPs and published datasets of hiPSC-derived microglia cultured under 2-
32 dimensional (2D) conditions (iPS MG)^{17, 77}, hiPSC-derived microglia developed in 3D cerebral
33 organoids (oMG)⁷⁸, hiPSC-derived microglia developed in mouse brain (xMG) as reported in a recent
34 study^{12, 78}. brain-tissue derived adult human microglia, including adult ex vivo microglia (Adult MG
35 ExVivo) from Gosselin et al., 2017^{17, 77} (age from 13-17 years) and Galatro et al., 2017(age from 34-102
36 years)^{17, 77}, in vitro microglia (MG InVitro) from Gosselin et al., 2017^{17, 77}, as well as blood/liver
37 macrophages^{17, 77}. As shown in Figure 4F, a principal component analysis (PCA) demonstrated that
38 Xeno MG, together with xMG, were markedly distinct from blood/liver macrophages, PMPs, and the
39 hiPSC-derived MG cultured under 2D conditions or developed in organoids. The human microglia
40 cultured *in vitro* were separate from the other two clusters, which might suggest the significant impact of
41 culture conditions on gene expression in those microglia as previously reported⁸. Our Xeno MG
42 clusters intermingled with a cluster of adult MG, suggesting their resemblance to adult human microglia.
43 Recent unbiased hierarchical clustering analyses revealed four major subclasses of adult human
44 microglia derived from human brain tissue⁷⁹. To determine if Xeno MG also exhibited similar
45 heterogeneity in chimeric mouse brain, we examined the expression of the most differentially regulated
46 genes identified from the different subclasses of adult human microglia⁷⁹. Gene expression analysis
47 revealed that *CD74*, *SPP1*, *C3*, and *CST3*, which were highly expressed in all subclasses in adult
48 human microglia, had a similarly uniform pattern of expression among most Xeno MG cells. Moreover,
49 a chemokine gene *CCL4*, the zinc finger transcription factors *EGR1*, *EGR2* and *EGR3*, *CD83*, and
50 *MCL1*, which are each characteristically expressed in individual subclasses of human microglia,
51 similarly had upregulated expression in distinct subpopulations of Xeno MG (Figure 4G and

1 supplementary Figure 8C). Taken together, these results demonstrate that Xeno MG developed in the
2 mouse brain highly resemble mature human microglia and faithfully recapitulate heterogeneity of adult
3 human microglia.
4

5 **Transcriptomic profiling analysis reveals differences between co-resident Xeno MG and mouse**
6 **microglia**

7 Previous studies reported differences in transcriptomic profiles between human and mouse microglia^{8, 12}. In the chimeric mouse brain, as xenografted hiPSC-derived microglia and host mouse microglia
8 developed in the same brain environment, this model may provide a unique opportunity to directly
9 examine the differences between human and mouse microglia. Xeno MG and host mouse microglia
10 clusters obtained from 4 independent samples of 6-month-old chimeric mouse brains were used for the
11 following comparison (Figure 5). We first compared the average levels of microglial gene transcripts in
12 Xeno MG with orthologous gene transcripts in host mouse microglia. Consistent with previous findings
13^{8, 12}, the comparison between Xeno MG and mouse microglial transcriptomes demonstrated similar
14 gene expression patterns overall ($r^2 = 0.553$; $p < 2.2 \times 10^{-16}$), and the majority of orthologous genes
15 pairs (14,488 of 15,058; 96.2%) were expressed within a twofold range (black dots, Figure 5B). Using a
16 cut-off of 2-fold difference and an FDR of 0.05, we identified that 91 gene transcripts were preferentially
17 expressed in human microglia, whereas 84 gene transcripts were preferentially expressed in mouse
18 microglia (supplementary Figure 8D and Table S2). Importantly, previously-reported signature genes
19 expressed in human microglia⁸, including *SPPI*, *A2M*, and *C3*, and signature genes expressed in
20 mouse microglia, including *HEXB*, *SPARC*, and *SERINC3*, were all differentially expressed in our
21 sequencing data (Figure 5B and C), indicating the high fidelity of our samples in resembling previously-
22 identified human vs. mouse microglial gene expression profiles. To explore the function of genes that
23 were highly expressed human microglia, we further performed Gene Ontology (GO) term analysis.
24 Many significantly enriched terms were associated with the innate immune activity of microglia, such as
25 “immune system response,” “cellular response to chemical stimulus,” and “regulation of cytokine
26 production.” This finding of enriched innate immunity-related gene in our Xeno MG could be either
27 reflect the nature of human microglia as reported previously^{12, 80}, or the result from differential
28 responses to critical signals from murine molecules, such as fractalkine. These results suggest that,
29 compared to the host mouse microglia, Xeno MG and mouse microglia exhibit overall similar patterns of
30 transcriptomic profile, but numerous species-specific differentially expressed genes were also
31 observed.
32

33 Previous studies have shown that several disease risk genes, such as genes associated with
34 AD, Parkinson’s disease (PD), multiple sclerosis (MS), and schizophrenia (SCZ), are preferentially
35 expressed in microglia^{8, 80, 81}. Moreover, relative expression of these genes in human and mouse
36 microglia are also different⁸. Therefore, we examined the expression of disease risk genes in Xeno MG
37 and mouse microglia from our chimeric mouse brain preparation. Expression of disease risk genes, as
38 reported in a recent study⁸, had a highly similar differential expression pattern in co-resident mouse
39 and human microglia (Figure 5D, E and supplementary Figure 8F-I). Specifically, with respect to MS,
40 we found that out of 32 MS genes, 29 genes, including *ZFP36L1*, *RPL5*, and *NDFIP1* were more
41 abundantly expressed in Xeno MG than in mouse microglia (Figure 5D and E). Similarly, out of 14 AD
42 genes, 10 genes including *Apoc1*, *Sorl2*, and *Mpzl1*, were more abundantly expressed in Xeno MG
43 than in mouse microglia (supplementary Figure 8F and I). Out of the 20 PD genes listed in a previous
44 report⁸, 18 genes, such as *Vps13c*, *Snca*, *Fgf20*, *Mnrrn1*, and *Lrrk2*, had the same trend of differential
45 expression with greater expression in Xeno MG than in mouse microglia (supplementary Figure 8H and
46 I). We also found that some of the disease genes were preferentially expressed in mouse microglia,
47 such as *Syt11* and *Gba* in PD. Altogether, these observations demonstrate that our hiPSC microglial
48 chimeric mouse brain can faithfully model disease-relevant transcriptomic differences between human
49 and mouse microglia, and this new model will serve as a new tool for modeling human neurological
50 disorders that involve dysfunction of microglia.
51

1 **Human PSC-derived microglia are dynamic in response to cuprizone-induced demyelination**

2 To explore whether Xeno MG are functionally dynamic in response to insult, we fed 3 months old
3 chimeric mice with cuprizone-containing diet to induce demyelination. The cuprizone model is one of
4 the most frequently used models to study the pathophysiology of myelin loss in multiple sclerosis⁸². It is
5 appropriate to use our hiPSC microglial chimeric mouse brain to examine the dynamics of human
6 microglia under a demyelination condition, considering our observation that a large number of Xeno MG
7 reside in the corpus callosum at 3 to 4 months post-transplantation and Xeno MG were found nearly
8 exclusively in the corpus callosum at 6 months post-transplantation (Figure 2C). After 4 weeks of
9 cuprizone treatment, we found that myelin structure, indicated by MBP staining in the corpus callosum,
10 was disrupted and became fragmented in our chimeric mice (Figures 6A), in contrast to the intact and
11 continuous MBP⁺ myelin structure in chimeric mice fed with control diet (supplementary Figure 9). As
12 shown in the super-resolution images in Figure 6B and C, engulfment of MBP⁺ myelin debris by both
13 Xeno MG and mouse microglia were clearly seen in the corpus callosum. Notably, more myelin debris
14 was found inside of mouse microglia, compared with Xeno MG (Figure 6D). In addition, we also
15 examined the expression of CD74 and SPP1, which is known to be upregulated in multiple sclerosis⁸³.
16 Without cuprizone treatment, variations in CD74 expression among animals were observed in Xeno MG
17 and on average, about 20% of Xeno MG expressed CD74 (Figure 6E and F). Nearly no Xeno MG
18 expressed SPP1 in the corpus callosum (Figure 6G and H). With cuprizone treatment, many of the
19 Xeno MG expressed CD74 or SPP1, recapitulating the upregulated expression of CD74 and SPP1 in
20 MS (Figure 6E-H). In mice, CD74 and SPP1 were shown to characterize white matter-associated
21 subsets of microglia that appear during development⁵³. In our study, there was a discrepancy between
22 the CD74 and SPP1 transcript levels and their protein levels. This might suggest an involvement of a
23 sophisticated control of mRNA translation that was observed in regulation of other genes critical for
24 brain development^{84, 85}. Altogether, these results demonstrate that human Xeno MG are dynamic in
25 response to insult in mouse brain.

26
27

1 Discussion

2

3 Humanized mouse models, in which the immune system is reconstituted with cells of human origin,
4 have been well-established and provide powerful tools for studying cancer, inflammatory and infectious
5 disease, and human hematopoiesis ⁸⁶. In this study, by engrafting neonatal mice with hPSC-derived
6 PMPs, we demonstrate the generation of chimeric mouse brains in which hPSC-derived microglia
7 widely disperse. We propose that the following three reasons may account for the generation of human
8 microglial chimeric mouse brain. First, as compared to other types of neural cells, microglial cells are
9 unique in that they turn over remarkably quickly, allowing the vast majority of the population to be
10 renewed several times during a lifetime ⁸⁷⁻⁸⁹. Previous studies have shown that neonatally transplanted
11 human macroglial or neural progenitor cells can outcompete and largely replace the host mouse brain
12 cells ^{21, 90, 91}. In this study, we also observe that the hPSC-derived PMPs are highly proliferative prior to
13 transplantation and transplanted cells divide for at least 6 months in the mouse host brain. Therefore,
14 the nature of high turnover rate of microglia and the competitive advantage of engrafted human cells
15 over endogenous mouse cells may result in a large number of human donor-derived microglia and
16 brain regions being repopulated by hPSC-derived microglia in the mouse brain at 6 months. Second,
17 during early brain development, microglial cells use blood vessels and white matter tracts as guiding
18 structures for migration and enter all brain regions ³⁴. Thus, transplantation of hPSC-derived PMPs to
19 the anterior anlagen of the corpus callosum of the neonatal mouse brain in this study may facilitate
20 migration of donor cell-derived microglia, resulting in wide dispersion of hPSC-derived microglia into
21 different brain regions. In addition, in support of this concept, we also observe that in the mouse brain,
22 hPSC-derived microglia are concentrated around and have close contact with blood vessels in both
23 grey matter and white matter. Lastly, although several previous studies also transplanted hPSC-derived
24 microglia into mouse brains, the generation of chimeric mice with a high degree of human microglial
25 brain chimerism has not been reported ^{16, 92}. We propose that this might be because of the age of the
26 host animals used for cell transplantation. Previous studies used adult animals for cell transplantation
27 ^{16, 92}. In our study, we transplanted hPSC-derived PMPs into the mouse brain at the earliest postnatal
28 age, P0, as in general the neonatal brain is more receptive for the transplanted cells and more
29 conducive for their survival and growth ^{21, 24, 91}. This is also supported by a recent study in which
30 neonatal animals were used for cell transplantation ⁴². Moreover, in contrast to those studies that
31 mainly examined donor-derived microglia 2 months after transplantation, we characterized the donor-
32 derived microglia up to 6 months post-transplant, which allowed the donor cells to develop for a longer
33 term in the mouse brain. Although there were variations in chimerization among animals, this human
34 microglia chimera model is highly reproducible according to scRNA-seq analysis using four chimeric
35 mouse brains. We calculated the numbers of detected mouse/human microglia in each mouse brain
36 sample and found that human microglia were consistently detected in each sample (legend to
37 supplementary Figure 7). In addition, the high reproducibility of generating such a hiPSC microglial
38 chimeric mouse brain model was also corroborated by two other recent reports ^{31, 93}.

39 Remarkably, we find that xenografted hiPSC-derived microglia developed in the mouse brain retain
40 a human microglial identity. Importantly, xenografted hiPSC-derived microglia showed expression
41 patterns of microglial maturity resembling adult human microglia derived from human brain tissue.
42 Therefore, establishment of such a hiPSC microglial chimeric mouse model provides novel
43 opportunities for understanding the biology of human microglia. First, this proof-of-concept study paves
44 the path to interrogating the species differences between human vs. mouse microglia at molecular,
45 functional, and behavioral levels using this hiPSC microglia chimeric mouse brain model. It has been
46 increasingly recognized that as compared to mouse microglia, human microglia possess unique
47 features under conditions of development, aging and disease ^{8, 11-13}. In our model, human and mouse
48 microglia develop in the same brain, but we have observed that human microglia are morphologically
49 distinct from their mouse counterparts and also exhibit signature gene expression profiles characteristic
50 of human microglia isolated from brain. Microglia are intimately involved in processes of neuronal
51 development, such as neurogenesis, synaptogenesis, and synaptic pruning ⁹⁴⁻⁹⁶. Building upon the

1 differential expression profiles, our model will be useful to investigate how human and mouse microglia
2 function differently in shaping neuronal development. Similar to a recent report ³¹, our engrafted PMPs
3 give to a small population of CNS macrophages. There are also potentially mouse-derived CNS
4 macrophages in the brain tissue that we collected for single-cell RNA-seq, but likely to represent a
5 small fraction of the total cluster. Therefore, within microglial clusters from each species, results may
6 include some similar but distinguishable cell types. However, the primary gene expression differences
7 are likely to be driven by the majority microglia. This was also supported by the observation that
8 previously-reported signature genes expressed in human vs. mouse microglia ⁸ were differentially
9 expressed in our results. Nevertheless, we take a conservative interpretation and did not claim to
10 identify any novel gene expression differences between human vs. mouse microglia based on the
11 current RNA-seq data. Second, a previous study reports that engrafted human astrocytes modulate
12 other CNS cell types in the mouse brain, particularly enhancing neuronal synaptic plasticity ²⁴. Thus, in
13 future studies, our hiPSC microglial chimeric mouse model will provide fascinating opportunities to
14 understand how the inclusion of human microglia in the developing brain ultimately impacts neuronal
15 development, synaptic plasticity, as well as the behavioral performance of the animals. Third, several
16 transcriptomic studies ^{12, 20} have clearly demonstrated that microglial genes are differently regulated
17 during aging and neurodegeneration between mice and humans, indicating the importance of
18 developing a human microglia model to study human microglial function across different development
19 stages, particularly adult microglia for studying aging-related and neurodegenerative disorders. Our
20 single-cell RNA-seq data suggests that Xeno MG resemble to adult human microglia. Moreover,
21 functional analyses demonstrate that Xeno MG are dynamic in response to cuprizone-induced
22 demyelination and recapitulate the upregulated expression of CD74 and SPP1 seen in MS patients⁸³,
23 providing proof of concept that our chimeric mouse model has the ability to exhibit functional changes
24 of human microglia under disease conditions. Combined with human iPSC technologies, such as the
25 availability of edited, isogenic cells with or without disease-related genes, the hiPSC microglia chimeric
26 mouse model will be informative in teasing out the roles of human microglia in neurodevelopmental
27 disorders, neurodegenerative disorders, as well as brain infections by viruses such as Zika virus and
28 HIV-1.

29 Similar to reports of hiPSC macroglial or neuronal chimeric mouse brain models ^{21, 24, 91}, in the
30 current hiPSC microglial chimeric mouse model, the endogenous mouse counterpart cells are still
31 present. In contrast to macroglial cells and neurons, microglial cells can be acutely depleted (up to 99%
32 depletion) in the entire brain without significantly affecting the viability of animals, by pharmacologically
33 inhibiting signaling pathways that are important for the survival and development of microglia, such as
34 colony-stimulating factor 1 (CSF1) signaling ⁹⁷ or by genetically coupling suicide genes under the
35 control of promoters of microglia-specific genes ^{1, 98}. In future studies, it will be interesting to explore the
36 possibility of creating humanized mouse brains containing solely hiPSC-derived microglia, by depleting
37 endogenous mouse microglia using pharmacological or genetic approaches in neonatal mouse brains
38 prior to engraftment of hiPSC-derived microglia. In addition, co-transplantation of human PSC-derived
39 PMPs and neural or macroglial progenitors may generate chimeric mouse brain containing human
40 microglia, neurons, and macroglial cells. Currently, in this proof-of-concept hiPSC microglial chimeric
41 mouse brain model, there is a lack of peripheral adaptive immune system in the host due to a *Rag2*^{-/-}
42 mutation. To circumvent this limitation, hiPSC-derived PMPs can be transplanted into animals in which
43 the immune system is humanized by the same hiPSC-derived hematopoietic stem cells ^{36, 99, 100}. This
44 will further allow the generation of animals with isogenic adaptive immune system and brain innate
45 immune system derived from the same human individuals. Combined with recently developed hiPSC
46 cerebral organoid models that contain microglia ^{16, 78}, chimeric mouse brain models may help further
47 our understanding of the complex interactions between human microglia and human neurons and
48 macroglial cells under normal and disease conditions.

1 **METHOD DETAILS**
2

3 **Generation, culture, and quality control of hPSC lines.**

4 One healthy control female hiPSC line and female H9 ESC line were used this study. The hiPSC line
5 were generated from healthy adult-derived fibroblasts using the “Yamanaka” reprogramming factors, as
6 reported in our previous study ¹⁰¹. The hiPSC line has been fully characterized by performing
7 karyotyping, teratoma assay, DNA fingerprinting STR (short tandem repeat) analysis, gene expression
8 profiling, and Pluritest (www.PluriTest.org), a robust open-access bioinformatic assay of pluripotency in
9 human cells based on their gene expression profiles ¹⁰², as described in our previous study ¹⁰¹. The
10 hPSCs were maintained under feeder-free condition and cultured on dishes coated with hESC-qualified
11 Matrigel (Corning) in mTeSR1 media (STEMCELL Technologies). Human PSCs from passage number
12 15 to 30 were used. The hPSCs were passaged approximately once per week with ReLeSR media
13 (STEMCELL Technologies). All hPSC studies were approved by the Embryonic Stem Cell Research
14 Oversight (ESCRO) committee at Rutgers University.
15

16 **PMP generation and culture**

17 PMP were generated from hiPSCs and H9 hESCs, using a published protocol ¹⁸. Briefly, the yolk sac
18 embryoid bodies (YS-EBs) were generated by treating the EBs with bone morphogenetic protein 4
19 (BMP4, 50 ng/ml, Peprotech; to induce mesoderm), vascular endothelial growth factor (VEGF, 50
20 ng/ml, Peprotech; endothelial precursors), and stem cell factor (SCF, 20 ng/ml, Miltenyi Biotech;
21 hematopoietic precursors). Next, the YS-EBs were plated into dishes with interleukin-3 (IL-3, 25 ng/ml,
22 Peprotech) and macrophage colony-stimulating factor (M-CSF, 100 ng/ml, Invitrogen) to promote
23 myeloid differentiation. At 2–3 weeks after plating, human hPMPs emerged into the supernatant and
24 were continuously produced for more than 3 months. The cumulative yield of PMPs was around 40-fold
25 higher than the number of input hPSCs (Figure 1A), similar to the previous studies ^{16, 18}. PMPs were
26 produced in a Myb-independent manner and closely recapitulating primitive hematopoiesis ^{118, 27}.
27

28 **Animals and Cell transplantation**

29 PMP were collected from supernatant and suspended as single cells at a final concentration of 100,000
30 cells per μ l in PBS. The cells were then injected into the brains of P0 $Rag2^{-/-}hCSF1$ immunodeficient
31 mice (*C;129S4-Rag2^{tm1.1Flv} Csf1tm1^{(CSF1)Flv} II2rg^{tm1.1Flv}/J*, The Jackson Laboratory). The precise
32 transplantation sites were bilateral from the midline = \pm 1.0 mm, posterior bregma = -2.0 mm, and
33 dorsoventral depth = -1.5 and -1.2 mm (Figure 1C). The mouse pups were anesthetized by placing
34 them on ice for 5 minutes. Once cryo-anesthetized, the pups were placed on a digital stereotaxic device
35 (David KOPF Instruments), equipped with a neonatal mouse adaptor (Stoelting). The pups were then
36 injected with 0.5 μ l of cells into each site (total 4 sites) by directly inserting Hamilton needles through
37 the skull into the target sites. The pups were weaned at 3 weeks and were kept up to 6 months before
38 they were tested for the engraftment of human cells. All animal work was performed without gender
39 bias with approval of the Institutional Animal Care and Use Committee (IACUC).
40

41 Both hiPSC- and hESC-derived PMPs were transplanted into mouse brains. Both engrafted
42 hiPSC- and hESC-derived microglia were analyzed, including characterization of their marker
43 expression (Figure 1), morphological changes along brain development (Figure 2), and their phagocytic
44 functions under homeostatic condition (Figures 3), as well as toxin-induced demyelination condition
45 (Figure 6). For the quantification, we pooled the data collected from both hiPSC- and hESC-derived
46 microglia. In the single-cell RNA-sequencing experiment, we only used the animals received
47 transplantation of hiPSC-derived microglia. For cuprizone-induced demyelination, three months old
48 chimeric mice were fed with cuprizone diet (Sigma-Aldrich, 0.2%) or control diet for four weeks as
49 previously report ¹⁰³.
50

Sample preparation and library construction for Single-cell RNA sequencing

1 Six months old chimeric mice that received transplantation of microglia derived from the hiPSC line
2 were used for single-cell RNA-sequencing experiments. The mice were perfused with oxygenated
3 solution (2.5 mM KCl, 87 mM NaCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 75 mM sucrose, 20 mM
4 glucose, 2 mM MgSO₄, and 1 mM CaCl₂) as reported ¹⁰⁴ and the brain was quickly extracted and kept in
5 the same cold solution for vibratome (VT1200, Leica) sectioning (500μm thickness) and dissection. The
6 brain regions were isolated from where engrafted hiPSCs-derived microglia largely dispersed, including
7 the cerebral cortex, hippocampus, corpus callosum, and olfactory bulb. The selected regions were
8 chopped with Spring Scissors (WPI) into fine pieces for further dissociation into single cells, based on
9 10x Genomic Sample Preparation Domstratd Protocol (Dissociation of Mouse Embryonic Neural
10 Tissue) with modifications. Briefly, the pieces were collected and dissociated with the Papain (1mg/ml,
11 Sigma) and DNAase I (100 unit/ml, Roche) in Hibernate solution (Gibco) in 37°C for 20 minutes.
12 Tissues were washed and triturated with wide-bore tips in cold Hibernate solution until no visible
13 chunks. The samples were spun down in 200 rcf for 2 minutes in 4°C and filtered through 30 μm cell
14 strainer to obtain single cells for cell counting and library preparation. To generate libraries, 20,000 cells
15 were loaded for each sample. Chromium™ Single Cell 3' Library and Gel Bead Kit v2, 4 rxns,
16 Chromium™ i7 Multiplex Kit, 96 rxns, and Chromium™ Single Cell A Chip Kit, 16 rxns are used from
17 10x Genomic single cell gene expression kit. cDNA libraries were generated following the manufacturer
18 instructions.
19

20 **Bulk and single-cell RNA sequencing**

21 We performed bulk RNA-sequencing analysis of hiPSC-derived PMPs as previously reported ²². Briefly,
22 total RNA was prepared with RNAeasy kit (QIAGEN) and libraries were constructed using 600 ng of
23 total RNA from each sample and the TruSeqV2 kit from Illumina (Illumina, San Diego, CA) following
24 manufacturers suggested protocol. The libraries were subjected to 75 bp paired read sequencing using
25 a NextSeq500 Illumina sequencer to generate approximately 30 to 36 million paired-reads per sample.
26 Fastq files were generated using the Bc12Fastq software, version 1.8.4. The genome sequence was
27 then indexed using the rsem-prepare-reference command. Each fastq file was trimmed using the fqtrim
28 program, and then aligned to the human genome using the rsem-calculate-expression (version 1.2.31)
29 command to calculate FPKM (fragments per kilobase of transcript per million mapped reads) values. In
30 order to analyze the transcripts, FPKM > 1 was set as cutoff to filter transcripts.
31

32 For single-cell RNA sequencing, we have used Chromium™ i7 Multiplex Kit, 96 rxns,
33 Chromium™ Single Cell 3' Library and Gel Bead Kit v2 and Chromium™ Single Cell A Chip Kit for
34 capture and library preparation. Single cell RNA sequencing was performed by RUCDR® Infinite
35 Biologics at Rutgers by using a 10X Genomics single cell gene expression profiling kit. The libraries
36 were analyzed on Agilent 4200 TapeStation System using High Sensitivity D1000 ScreenTape Assay
37 (Cat #: 5067-5584) and quantified using KAPA qPCR (Cat # KK4835). Libraries and then normalized to
38 10nM before being pooled together. The pooled library was then clustered and sequenced on Illumina
39 HiSeq 2500 in Rapid Run Mode, using the following parameters: 36bp forward read, 100bp reverse
40 read, and 8bp index read. For each individual library, the sequencing data from 4 unique indexes were
combined before further analysis.
41

42 Sequencing reads were aligned with pooled mouse (mm10) and human (hg19) reference
43 genomes and the barcodes were interpreted using Cellranger software (10X Genomics, v. 3.0.0). The
44 resulting matrices of gene counts x barcodes were coded by individual sample identifier and loaded into
45 Seurat (v. 3.0.0) software ¹⁰⁵⁻¹⁰⁷ in R/Bioconductor ¹⁰⁸. An initial analysis revealed a distinct cluster of
46 human-expressing cells. To compare expression across species, a strategy was employed similar to
47 one used previously ¹⁰⁹. A table of 17,629 unique matching genes was prepared, starting with a
48 human-mouse gene homology list obtained from Jackson Labs
49 (<http://www.informatics.jax.org/downloads/reports/index.html#marker>), and hand-curating to remove
50 duplicates. The homologous genes list is deposited in a public data archive at
51 <https://github.com/rhart604/mousify/blob/master/geneTrans.txt>. Sample/barcode identifiers for the
human-specific data were isolated and matching gene symbols were converted from human to mouse.
52

1 Sample/barcode identifiers not matching this cluster were assumed to be mouse, and these were
2 trimmed to retain only mouse gene symbols matching the homology list. Complete details of homology
3 gene translation are described elsewhere¹¹⁰.

4 To model both human and mouse results in a comparable system, we assigned individual
5 sequencing reads to the optimal species using the alignment score in the bam file, and then separated
6 the original fastq files into individual sets specific by species. These were re-processed with separated
7 mouse or human reference genome indices and then recombined after translating homologous genes.
8 In the end, only 147 out of 19,154 barcodes, or 0.76%, included sequences optimally aligning with both
9 species, likely caused by the creation of droplets containing cells from more than one species, so these
10 were eliminated from further consideration. The entire procedure, along with comparisons to alternative
11 strategies, including all required R and Python code, is described elsewhere¹¹⁰.

12 For analysis of human microglial sub-clusters, extracted human sample/barcode were restricted
13 to human gene symbol results and re-analyzed with Seurat. Gene ontology analysis used the g:Profiler
14 website (<https://biit.cs.ut.ee/gprofiler/gost>).

15 For comparisons among sources of human microglia, raw RNAseq reads from the human-
16 specific cluster were pooled by sample and aligned with reference human genome (hg38) using
17 HISAT2¹¹². Raw sequencing reads from other publications were downloaded from GEO (series
18 accessions GSE99074¹², GSE97744¹⁷, GSE102335⁷⁸, and GSE133434⁴²). After similar HISAT2
19 alignment, all count summaries were imported into a DESeq2¹¹³ data model and a variance
20 stabilization transformation was applied prior to principal components analysis.

21 **Immunostaining and cell counting**

22 Mouse brains fixed with 4% paraformaldehyde were put in 20% and 30% sucrose for dehydration. After
23 dehydration, brain tissues were blocked with OCT and frozen by solution of dry ice and pure alcohol.
24 The frozen tissues were cryo-sectioned with 30 μ m thickness for immunofluorescence staining^{114, 115}.
25 The tissues were blocked with blocking solution (5% goat or donkey serum in PBS with Triton X-100) in
26 room temperature (RT) for 1 hr. The Triton X-100 concentration was 0.8% for brain tissue. The primary
27 antibodies were diluted in the same blocking solution and incubated with tissues in 4 °C overnight. The
28 primary antibodies were listed in supplementary Table S3. Then, the sections were washed with PBS
29 and incubated with secondary antibodies for 1 hr in RT. After washing with PBS, the slides were
30 mounted with the anti-fade Fluoromount-G medium containing 1, 40,6-diamidino-2-phenylindole
31 dihydrochloride (DAPI) (Southern Biotechnology). For PSD95 peptide blocking experiment, PSD95
32 antibody (2.5 μ g/ml) was incubated with five times PSD95 blocking peptide (12.5 μ g/ml, Synaptic
33 System) or equivalent amount of PBS at RT for 30 minutes before applying secondary antibodies to the
34 brain tissues as suggested by the manufacturer instructions.

35 Images were captured with a Zeiss 710 confocal microscope. Large scale images in Figure 1D,
36 supplementary Figure 1A and 1B were obtained by confocal tile scan and automatically stitched using
37 10% overlap between tiles by Zen software (Zeiss). For 3D reconstructive images in Figure 3E, Figure
38 6A and Supplementary Figure 6B, 6D and 6E were processed by Zen software (Zeiss). To visualize
39 synaptic puncta pruning, myelin debris engulfment, super-resolution images in Figure 3A, 3B, 3D,
40 Figure 6B, 4C, Supplementary Figure 5 and Supplementary Figure 6A, 6C were acquired by Zeiss
41 Airyscan super-resolution microscope at 63X with 0.2 mm z-steps. Cell number and microglia process
42 length and endpoints were counted with ImageJ software, following our previous report²² and the
43 published protocol¹¹⁶. At least five consecutive sections of each brain region were chosen. The number
44 of positive cells from each section was counted after a Z projection and at least 7 mice in each group
45 were counted. The number of synaptic puncta or myelin debris were calculated from 7 mice in each
46 group, minimal 10 cells per animal. Engraftment efficiency and degree of chimerization were assessed
47 by quantifying the percentage of hTMEM119⁺ cells among total DAPI⁺ cells in sagittal brain sections, as
48 reported in the previous studies^{24, 25}. The cell counting was performed on every fifteenth sagittal brain
49 section with a distance of 300 μ m, covering brain regions from 0.3 to 2.4 mm lateral to the midline
50 (seven to eight sections from each mouse brain were used).

1

2 **Data analysis**

3 All data represent mean \pm s.e.m. For the data presented as bar-and-data overlap plots. When only
4 two independent groups were compared, significance was determined by two-tailed unpaired t-test with
5 Welch's correction. When three or more groups were compared, one-way ANOVA with Bonferroni post
6 hoc test or two-way ANOVA was used. A P value less than 0.05 was considered significant. The
7 analyses were done in GraphPad Prism v.5.

8

9 **Data availability**

1 The accession number of the single-cell RNA sequencing data reported in this study is GEO:
2 GSE129178.**Acknowledgements**

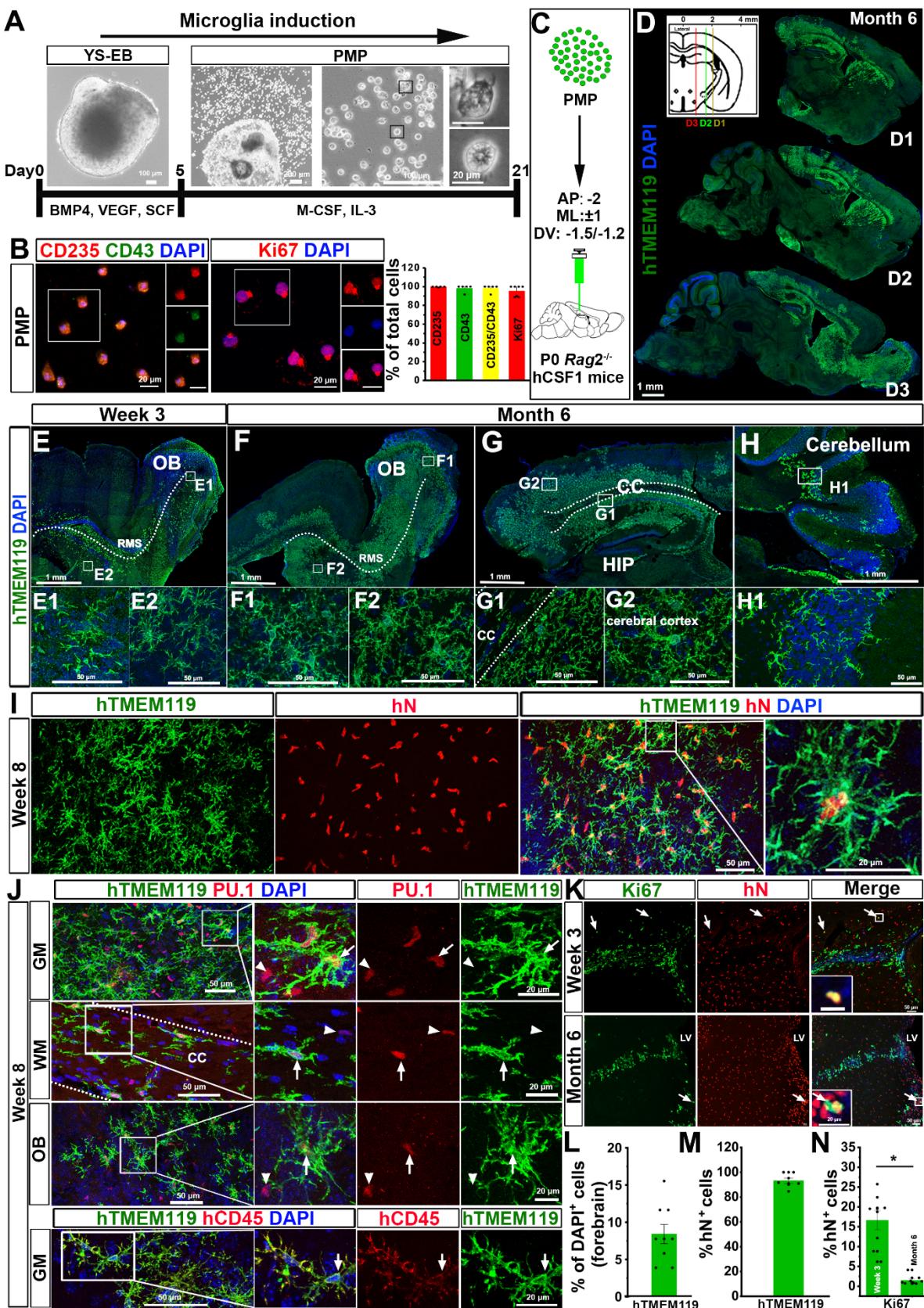
3 This work was in part supported by grants from the NIH (R21HD091512 and R01NS102382 to P.J.)
4 R.P.H. was supported by R01ES026057, R01AA023797, and U10AA008401. X.L. was supported by
5 the Jiangsu Government Scholarship for Overseas Studies from China. We thank Dr. Cheryl Dreyfus
6 from Rutgers Robert Wood Johnson Medical School for the suggestions on cuprizone model, Dr.
7 Noriko Kane-Goldsmith from Rutgers University for assistance with Zeiss Airyscan super-
8 resolution imaging, and Dr. Brian Daniels from Rutgers University for critical reading of the manuscript.
9 We also thank Ms. Maharaib Syed from Rutgers University for the assistance with
10 immunohistochemistry.

11
12 **Author Contributions**

13 P.J. and R.X. designed experiments and interpreted data; R.X. carried out most of experiments with
14 technical assistance from A.B., X.L., A.P., and K.K.; R.P.H. performed the gene expression analysis,
15 interpreted the data, and provided critical suggestions to the overall research direction; P.J. directed the
16 project and wrote the manuscript together with R.X. and input from all co-authors.

17
18 **Competing Financial Interests**
19 The authors declare no competing financial interests.
20

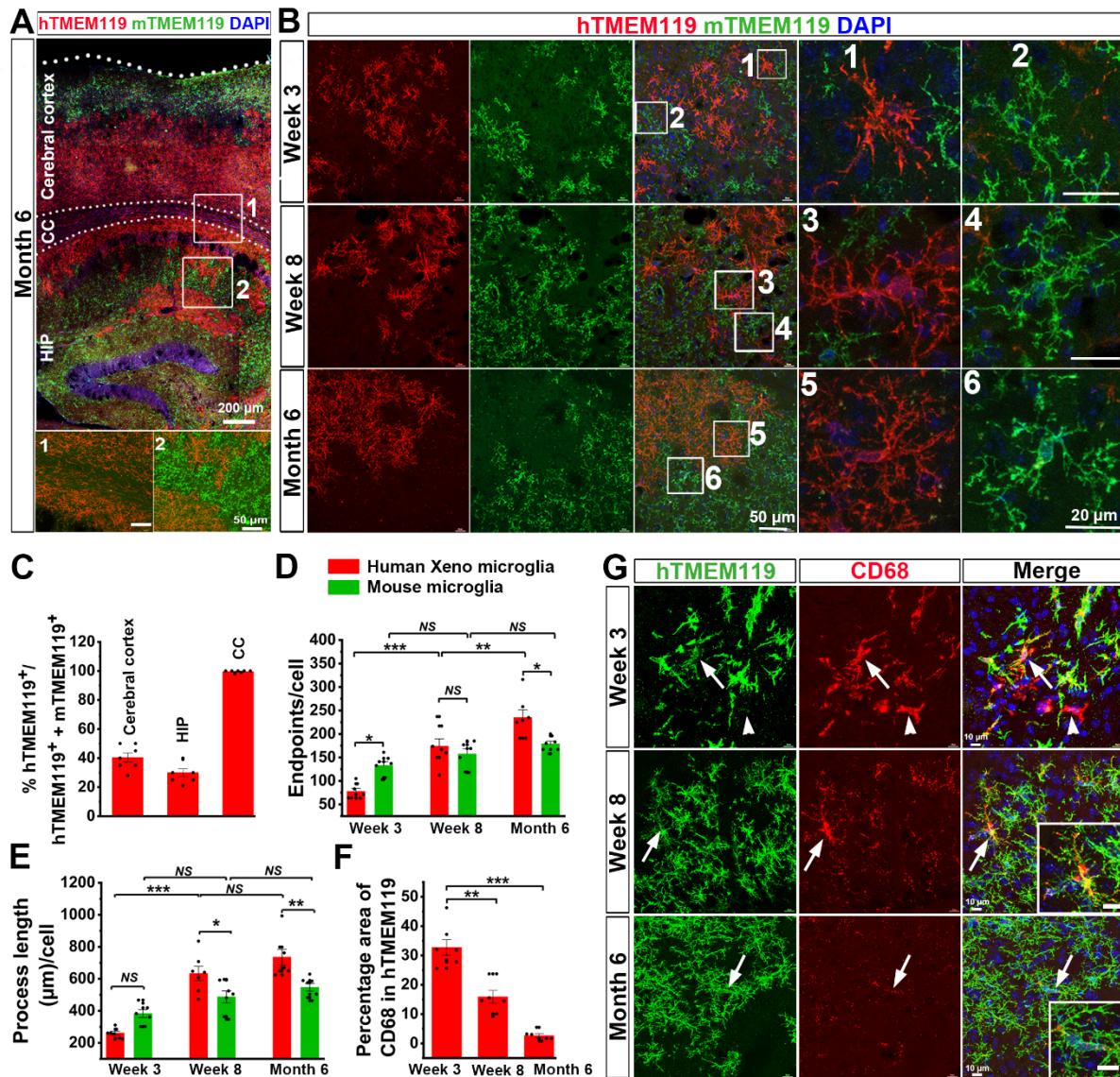
Xu et al., Figure 1



1 **Figure 1. Generation of hPSC microglial chimeric mouse brains.**

2 (A) A schematic procedure for generating primitive macrophage precursor (PMP) from hiPSCs or
3 hESCs-derived yolk sac embryoid bodies (YS-EB). Insets: representative bright-field images at different
4 stages. Scale bars represent 100 μ m, 200 μ m, and 20 μ m as indicated in the images
5 (B) Representative images and quantification of CD235 $^+$, CD43 $^+$, CD235 $^+$ /CD43 $^+$, and Ki67 $^+$ cells in
6 PMP. Quantification of pooled data from one hiPSC line and one hESC line. The experiments are
7 repeated for at least 5 times (n = 5-7) and for each experiment, the two stem cell lines are used. Data
8 are presented as mean \pm s.e.m. Scale bars: 20 μ m in the original and enlarged images.
9 (C) A schematic diagram showing that hPSC-derived PMP are engrafted into the brains of P0 *rag2* $^{-/-}$
10 hCSF1 mice.
11 (D) Representative images from sagittal brain sections showing the wide distribution of xenografted
12 hPSC-derived microglia at six months post-transplantation. Anti-human-specific TMEM119
13 (hTMEM119) selectively labels xenografted hPSC-derived microglia. Scale bar: 1 mm.
14 (E-H) Representative images from sagittal brain sections showing the distribution of hTMEM119 $^+$
15 xenografted hPSC-derived microglia at 3 weeks and 6 months post-transplantation in different brain
16 regions. OB, olfactory bulb; RMS, rostral migratory stream; CC, corpus callosum; HIP, Hippocampus.
17 Scale bars: 1 mm or 50 μ m in the original or enlarged images, respectively.
18 (I) Representative images of hTMEM119 $^+$ cells among the total donor-derived hN $^+$ cells in grey matter
19 at 8 weeks post-transplantation. Scale bars, 50 μ m or 20 μ m in the original or enlarged images,
20 respectively.
21 (J) Representative images of PU.1- and human-specific CD45 (hCD45)-expressing cells in the donor-
22 derived hTMEM119 $^+$ cells in different brain regions at 8 weeks post-transplantation. Scale bars, 50 μ m
23 or 20 μ m in the original or enlarged images, respectively.
24 (K) Representative images of Ki67 $^+$ cells among the total donor-derived hN $^+$ cells at 3 weeks and 6
25 months post-transplantation. Scale bars, 50 μ m or 20 μ m in the original or enlarged images,
26 respectively.
27 (L) Quantification of the percentage of hTMEM119 $^+$ cells in total DAPI $^+$ cells in the forebrain at 6
28 months post-transplantation (n = 9 mice). The data are pooled from the mice received transplantation of
29 microglia derived from both hESCs and hiPSCs. Data are presented as mean \pm s.e.m.
30 (M) Quantification of the percentage of hTMEM119 $^+$ cells in total hN $^+$ cells (n = 7 mice). Data are
31 presented as mean \pm s.e.m.
32 (N) Quantification of Ki67 $^+$ cells among the total donor-derived hN $^+$ cells at 3 weeks or 6 months post-
33 transplantation (n = 8 mice for each time point). The data are pooled from the mice that received
34 transplantation of microglia derived from both hESCs and hiPSCs. Student's *t* test. **P < 0.01. Data are
35 presented as mean \pm s.e.m.

Xu et al., Figure 2



1 **Figure 2. Human PSC-derived microglia undergo morphological maturation.**

2 (A) Representative images of hTMEM119⁺ hPSC-derived microglia and mTMEM119⁺ mouse microglia
3 in the cerebral cortex, corpus callosum (CC) and hippocampus (HIP) in 6 months old mice. Scale bars
4 represent 200 μ m and 50 μ m in the original and enlarged images, respectively.

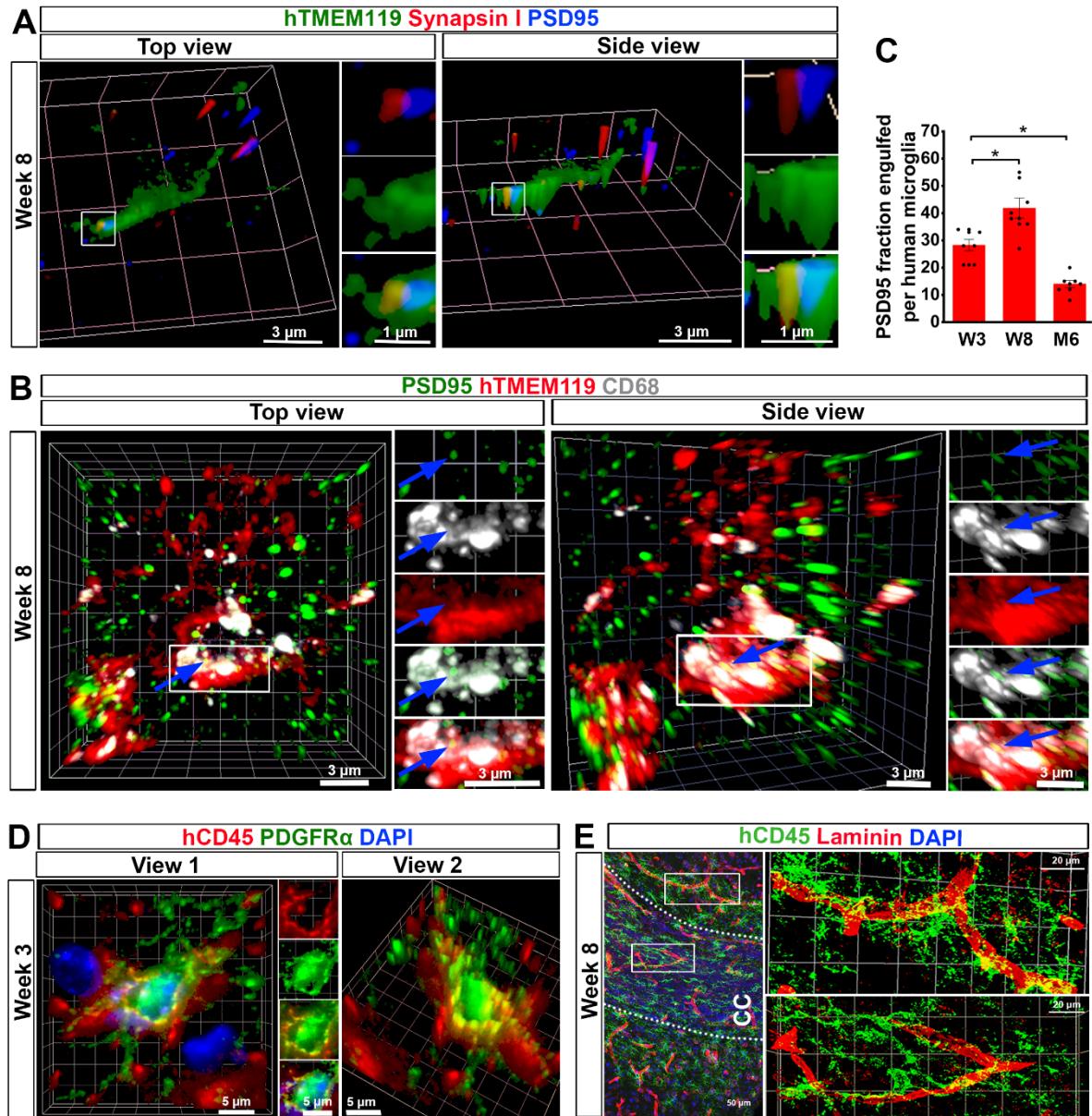
5 (B) Representative images of hTMEM119⁺ hPSC-derived microglia and mTMEM119⁺ mouse microglia
6 in the cerebral cortex in 3 weeks, 8 weeks, and 6 months old mice. Scale bars represent 50 μ m and 20
7 μ m in the original and enlarged images, respectively.

8 (C) Quantification of the percentage of hTMEM119⁺ cells in total microglia (hTMEM119⁺ plus
9 mTMEM119⁺) in the cerebral cortex, hippocampus (HIP), corpus callosum (CC) in 6 months old
10 chimeric mice (n = 7 mice for each time point). The data are pooled from the mice received
11 transplantation of microglia derived from both hESCs and hiPSCs. Data are presented as mean \pm
12 s.e.m.

13 (D and E) Quantification of endpoint numbers and total process length of mouse and hPSC-derived
14 microglia based on mTMEM119 or hTMEM119 staining respectively from grey matter at the three time
15 points (n = 7 mice for each time point). The data are pooled from the mice received transplantation of
16 microglia derived from both hESCs and hiPSCs. Two-way ANOVA is used to compare the endpoints
17 and process length between human and mouse microglia and one-way ANOVA is used for the
18 comparison within mouse or human microglia. *P < 0.05, ***P < 0.001, NS, no significance. Data are
19 presented as mean \pm s.e.m.

20 (F) Quantification of the percentage of CD68⁺ area in hTMEM119⁺ area from cerebral cortex in 3
21 weeks, 8 weeks and 6 months old chimeric mice (n = 7 mice for each time point). The data are pooled
22 from the mice received transplantation of microglia derived from both hESCs and hiPSCs. One-way
23 ANOVA test, **P < 0.01, ***P < 0.001. Data are presented as mean \pm s.e.m.

24 (G) Representative images of CD68- and hTMEM119-expressing cells in the cerebral cortex from 3
25 weeks, 8 weeks and 6 months old mice. Scale bars, 10 μ m or 2 μ m in the original or enlarged images,
26 respectively.



1 **Figure 3. Human PSC-derived microglia are functional in the mouse brain.**

2 (A) Representative 3D reconstruction of super-resolution images showing hTMEM119⁺ donor-derived
3 microglia engulf synaptic proteins, synapsin I and PSD95 in grey matter at 8 weeks post-
4 transplantation. Scale bars, 3 and 1 μ m in the original or enlarged images, respectively.

5 (B) Representative 3D reconstruction of super-resolution images showing colocalization of hTMEM119,
6 PSD95, and CD68 staining in grey matter at 8 weeks post-transplantation. Scale bars, 3 in the original
7 and enlarged images.

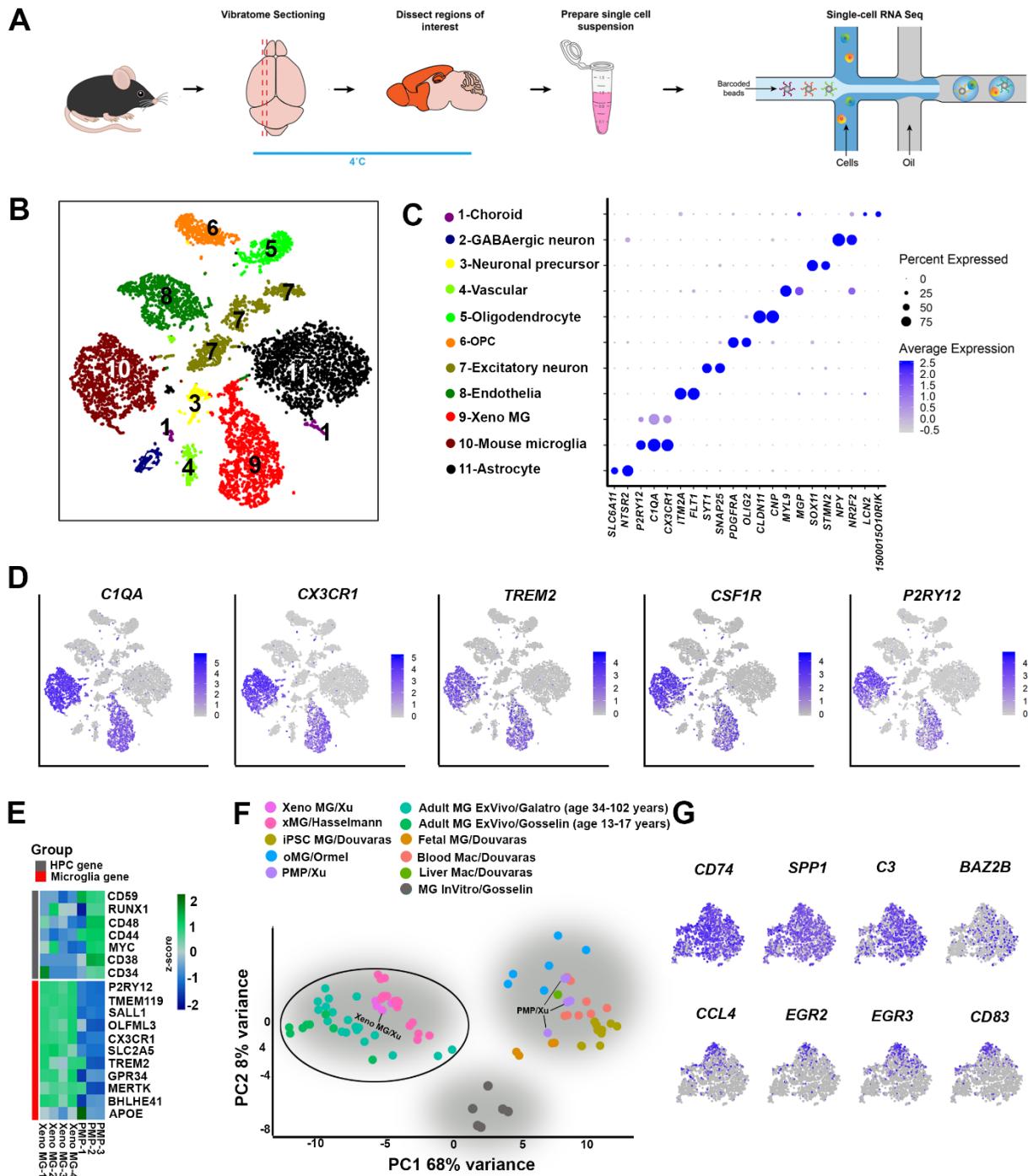
8 (C) Quantification of PSD95⁺ fraction engulfed per microglia from cerebral cortex at the three time
9 points (n = 7 mice for each time point). The data are pooled from the mice received transplantation of
10 microglia derived from both hESCs and hiPSCs One-way ANOVA test, *P < 0.05. Data are presented
11 as mean \pm s.e.m.

12 (D) Representative 3D reconstruction of super-resolution images showing that hCD45⁺ hPSC-derived
13 microglia phagocytize PDGFR α ⁺ oligodendroglial cells in the corpus callosum at 3 weeks post-
14 transplantation. Scale bars, 5 μ m.

15 (E) Representative images showing the interaction between laminin⁺ blood vessels and hCD45⁺ human
16 microglia in grey matter and white matter at 8 weeks post-transplantation. Scale bars, 50 and 20 μ m in
17 the original or enlarged images, respectively.

18

Xu et al., Figure 4



1 **Figure 4. Single-cell RNA-seq analysis of hiPSC microglial chimeric mouse brain.**

2 (A) A schematic diagram showing the experimental design. Microglia were isolated from the highlighted
3 brain regions at 6 months post-transplantation and handled at 4°C to reduce ex vivo activation. The
4 single-cell suspension was loaded into a 10X Genomics Chromium system for single-cell RNA-
5 sequencing.

6 (B) tSNE plot of 11 cell types as identified by characteristic cell-specific gene expression, following
7 translation of human gene symbols to mouse symbols as described in Methods. Arrow indicates the
8 human xenograft microglia (Xeno MG).

9 (C) Dot plot showing two representative cell-specific genes for each cell cluster. As indicated by the
10 legend, the diameter of the dot indicates the percent of cells within a cluster expressing the gene
11 (percent expression). The color saturation indicates the average expression over the cluster (average
12 expression; log normalized counts). The cluster numbers, colors of clusters in panel B, and selected
13 cell identities are shown at left.

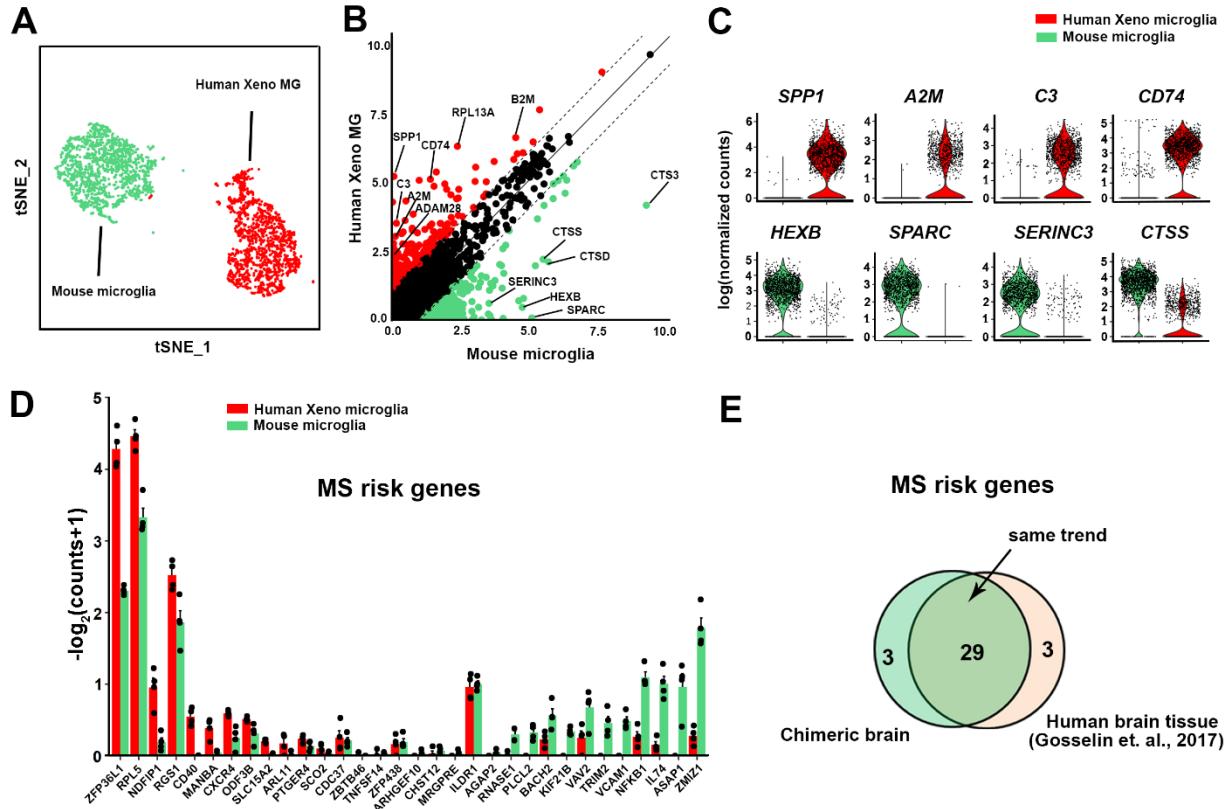
14 (D) tSNE plots with dots (representing individual barcodes/cells) colored by expression of canonical
15 microglial genes, based on expression level determined in Seurat (log normalized counts).

16 (E) A heatmap showing expression of signature genes of hematopoietic progenitor cells (HPC) and
17 microglia in PMPs and Xeno MG, showing individual replicates (n=3 for PMP, n=4 averaged human
18 clusters for Xeno MG). Color indicates the expression level normalized for each gene using a Z-score.

19 (F) Principal component analysis (PCA) of our Xeno MG, hiPSC-derived PMPs, and individual cell
20 RNA-seq expression data from publicly-available datasets, including hiPSC-derived microglia cultured
21 under 2-dimensional (2D) conditions (iPS MG)^{17, 77}, hiPSC-derived microglia developed in 3D cerebral
22 organoids (oMG)⁷⁸, hiPSC-derived microglia developed for 2-7.8 months in mouse brain (xMG)⁴².
23 brain-tissue derived adult human microglia, including adult ex vivo microglia (Adult MG ExVivo) from
24 Gosselin et al., 2017⁸ (age from 13-17 years) and Galatro et al., 2017 (age from 34-102 years)¹², in
25 vitro microglia (MG InVitro) from Gosselin et al., 2017⁸, as well as blood/liver macrophages^{17, 77}.
26 (G) t-SNE plots of selected human cells showing the expression of *CD74*, *SPP1*, *C3*, *BAZ2B*, *CCL4*,
27 *EGR2*, *EGR3*, and *CD83* gene transcripts. *CD74*, *SPP1*, *C3* and *BAZ2B* appear to be uniformly
28 expressed in all cells, but *CCL4*, *EGR2*, *EGR3*, and *CD83* are enriched in distinct subsets of in Xeno
29 MG.

30

Xu et al., Figure 5



1 **Figure 5. Transcriptomic profiling analysis of clusters of Xeno MG and mouse microglia.**

2 (A) tSNE plot highlighting only the clusters of human Xeno MG and mouse host microglia.

3 (B) Scatter plot showing mean mRNA expression levels of human and mouse genes with unique
4 orthologs from Xeno MG and mouse microglia clusters, highlighting the differentially expressed genes
5 (DEGs; at least two-fold different) in human Xeno MG (red) or mouse microglia (green) from 6 months
6 old chimeric mouse brain. Significantly different DEGs (less than 5% false discovery rate [FDR] and at
7 least two-fold different) are listed in Table S2.

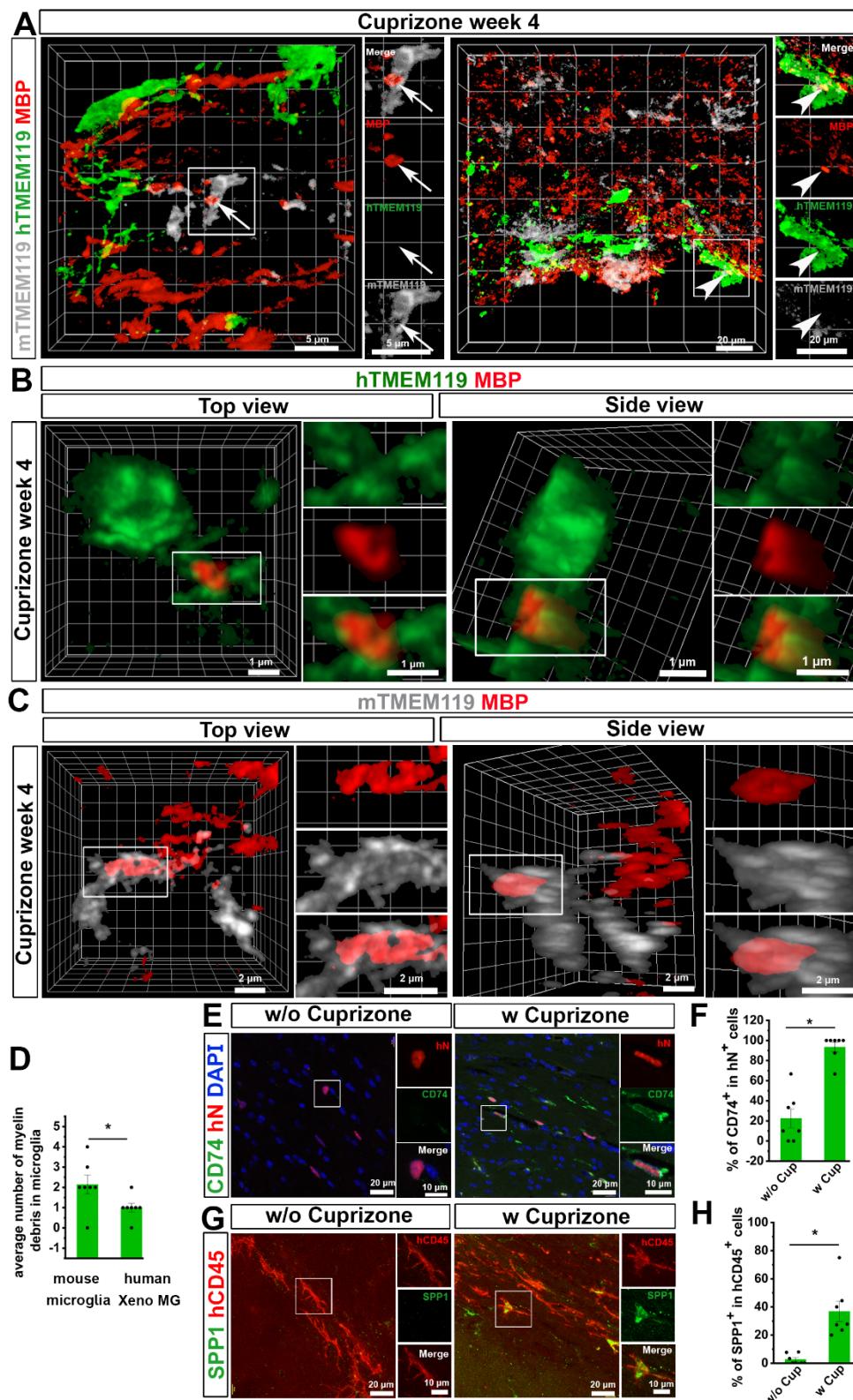
8 (C) Violin plots summarizing expression differences in individual cells within the human Xeno MG and
9 mouse microglia clusters. Dots indicate expression levels (as log normalized counts) of individual cells
10 and the violin shape summarizes the distribution of expression in the population.

11 (D) Bar plots showing the average expression (mean \pm SEM, n = 4 samples) of Multiple sclerosis (MS)-
12 associated genes in Xeno MG and mouse microglia. These genes were reported to be differentially
13 expressed between human and mouse microglia as in Gosselin et al., 2017⁸.

14 (E) Venn diagrams showing that majority of the MS associated genes that were reported to be
15 differentially expressed between human and mouse microglia are recapitulated in our chimeric mouse
16 model (29 of 32).

17

Xu et al., Figure 6



1 **Figure 6. Responses of hPSC-derived microglia to cuprizone-induced demyelination.**

2 (A) Representative 3D reconstruction images showing hTMEM119⁺ donor-derived microglia and
3 mTMEM119⁺ mouse host microglia interact with MBP⁺ myelin debris in the corpus callosum after 4
4 weeks of cuprizone-diet. Scale bars, 5 or 20 μ m in the original or enlarged images as indicated.
5 (B and C) Representative 3D reconstruction of super-resolution images showing hTMEM119⁺ donor-
6 derived microglia (B) and mTMEM119⁺ mouse host microglia (C) engulf MBP⁺ myelin debris in the
7 corpus callosum after 4 weeks of cuprizone-diet. Scale bars, 1 μ m in the original or enlarged images.
8 (D) Quantification of average number of myelin debris in mouse host microglia and human Xeno MG (n
9 = 7 mice). The data are pooled from the mice that received transplantation of microglia derived from
10 both hESCs and hiPSCs. Student's *t* test. **P* < 0.05. Data are presented as mean \pm s.e.m.
11 (E) Representative images showing CD74⁺ and hN⁺ cells after 4 weeks of diet with (w) or without (w/o)
12 cuprizone. Scale bars, 20 and 10 μ m in the original or enlarged images, respectively.
13 (F) Quantification of the percentage of CD74⁺ cells in total hN⁺ cells after 4 weeks of diet with (w) or
14 without (w/o) cuprizone (n = 7 mice). The data are pooled from the mice that received transplantation of
15 microglia derived from both hESCs and hiPSCs. Student's *t* test. **P* < 0.05. Data are presented as
16 mean \pm s.e.m.
17 (G) Representative images showing SPP1⁺ and hN⁺ cells after 4 weeks of diet with (w) or without (w/o)
18 cuprizone. Scale bars, 20 and 10 μ m in the original or enlarged images, respectively.
19 (F) Quantification of the percentage of SPP1⁺ cells in total hN⁺ cells after 4 weeks of diet with (w) or
20 without (w/o) cuprizone (n = 7 mice). The data are pooled from the mice that received transplantation of
21 microglia derived from both hESCs and hiPSCs. Student's *t* test. **P* < 0.05. Data are presented as
22 mean \pm s.e.m.

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34