Microglia are resident inflammatory cells of the CNS and have important roles in development, homeostasis and a variety of neurologic and psychiatric diseases. Difficulties in procuring human microglia have limited their study and hampered the clinical translation of microglia-based treatments shown to be effective in animal disease models. Here we report the differentiation of human induced pluripotent stem cells (iPSC) into microglia-like cells by exposure to defined factors and co-culture with astrocytes. These iPSC-derived microglia have the phenotype, gene expression profile and functional properties of brain-isolated microglia. Murine iPSC-derived microglia generated using a similar protocol have equivalent efficacy to primary brain-isolated microglia in treatment of murine syngeneic intracranial malignant gliomas. The ability to generate human microglia facilitates the further study of this important CNS cell type and raises the possibility of their use in personalized medicine applications.
OP9 feeder layers, whereas for differentiation of INC-01 human iPSC, a feeder-free differentiation protocol was developed. Prior to differentiation to iPSC-HPC, iPSC express the stem cell markers Nanog and Tra-1-81 (Fig. 1b–d). Differentiation of iPSCs to iPSC-HPC (stage 1) resulted in the loss of Nanog and Tra-1-81 expression and gain of the hematopoietic markers CD34 and CD43 (Fig. 1e–g). Subsequent culture of iPSC-HPC on astrocyte monolayers (stage 2) supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF) and interleukin 3 (IL-3) resulted in the loss of CD34 and CD43 expression and the gain of CD11b and Iba1 expression in as early as 7 d (Fig. 1h–j). These CD34−, CD43−, CD11b+ and Iba1+ microglia-like cells continued to increase in number over the next one week. At the end of stage 2 differentiation, ~9% of the mixed astrocyte–microglial progenitor-like cells were positive for ENTPD1 (CD39), a plasma membrane protein specific to microglia (Supplementary Fig. 1). On average, $1 \times 10^6$ NCRM-5 and INC-01 iPSC resulted in $2 \times 10^6$ and $3 \times 10^6$ microglia-like cells, respectively. A fibroblast-derived iPSC (ND.1) resulted in a slightly lower yield of $8 \times 10^5$ microglia-like cells per $1 \times 10^6$ iPSC, and Inc-07s-1 (an additional feeder-free iPSC) resulted in a yield of $2 \times 10^6$ microglia-like cells per $1 \times 10^6$ iPSC.

Following stage 2 differentiation, the resulting human iPSC-MG cells were harvested and phenotypically characterized using immunocytochemistry staining. In addition to CD11b and Iba1, the human iPSC-MG expressed HLA-DR, CD45, TREM-2 and CX3CR1 but were negative for CD86, CD206, CD200R and CD200 expression (Fig. 2a). Because the phenotypes of other immune system phagocytic cells such as dendritic cells (DC) and macrophages (Mac) can resemble that of microglia, the gene expression signatures of human iPSC-MG, DC and Mac were compared. Also included in the analyses were the human iPSC lines NCRM-5 and iNC-01 iPSC resulted in $2 \times 10^6$ and $3 \times 10^6$ microglia-like cells per $1 \times 10^6$ iPSC, slightly lower yield of $8 \times 10^5$ microglia-like cells per $1 \times 10^6$ iPSC, and INC-07s-1 (an additional feeder-free iPSC) resulted in a yield of $2 \times 10^6$ microglia-like cells per $1 \times 10^6$ iPSC.
iNC-01 and NCRM-5, from which the human iPS-MG were derived, and commercially obtained primary human fetal brain-isolated microglia. We generated 53,617 gene-fragment expression values per biological triplicate sample and the 60% of data found to be noise-biased was discarded. After subjecting the data to one-factor ANOVA under Benjamini and Hochberg false discovery rate multiple-comparison correction conditions, we restricted our analysis to 18,302 gene fragments with a corrected $P < 0.05$. Using Tukey's post hoc analysis, we then quantified the number of these differentially expressed genes having both $P < 0.05$ and an absolute fold-change in expression ≥ 1.5 (14,475 genes) in pairwise comparisons of all the experimental groups. Based on correlation-based clustering analysis, the gene expression patterns of iPS-MG resembled those of human fetal microglia as well as those of DC and Mac, consistent with differentiation toward the myeloid lineage. The cells that most differed from iPS-MG in terms of gene expression were iPSCs. These gene expression relationships can be visualized using correlation-based clustering (Fig. 2b) and covariance-based principal component analysis (Fig. 2c) for Ingenuity microglia annotated genes. Recently, a unique microglial gene signature has been identified in both fetal and adult human microglia^21. This human microglial signature consists of six genes: P2RY12, GPR34, MERTK, C1QA, PROS1 and GAS6. We extracted individual gene expression data for these six microglia genes from the human microarray dataset described above and found that all six of these genes were highly expressed in the iPS-MG but not in the iPSC, Mac or DC (Supplementary Fig. 2). Quantitative reverse-transcription PCR (RT-qPCR) analyses confirmed the expression of these genes in the iPS-MG but not the iPSC, with the exception of P2RY12, which was expressed in both but to a greater degree in iPS-MG (Table 1). Expression of this distinct microglial gene signature supports the microglia-like identity of the iPS-MG and distinguished them from other myeloid cell types such as DC and Mac.

**Human iPS-MG demonstrate functional properties of microglia**

In addition to expressing the unique microglial gene signature shown above, microglia-like cells should also demonstrate the characteristic functional properties of microglia, such as phagocytosis of foreign particles, the production of reactive oxygen species (ROS) and secretion of inflammatory cytokines. Under baseline culture conditions, iPS-MG actively phagocytosed pHrodo red BioParticles conjugates, as evidenced by the acidification and resulting visualization of these red fluorescent particles within circular intracellular structures (Fig. 3). Following stimulation with phorbol myristate acetate (PMA), iPS-MG produce reactive oxygen species as evidenced by the oxidation and resulting green fluorescence of the CellROX green reagent indicator dye over time (Fig. 3). The mean corrected fluorescence intensities of control cells in the phagocytosis and ROS experiments were 2,383,034.40 and 4,363,224.28 relative units, respectively. After the addition of the pHrodo red BioParticles or the CellROX reagents, the mean corrected fluorescence intensities of the cells at 7 min were 18,489,482.66 and 5,104,373.38 relative units, respectively. Also, following 16 h of stimulation with LPS, the concentration of tumor necrosis factor alpha (TNFα) in iPS-MG culture media was found to be 60 ± 7.6 pg/mL. TNFα concentrations were not detectible in iPS-MG culture media before LPS stimulation or in fibroblast culture media before and after LPS stimulation (detection limits: TNFα > 15.6 pg/mL).

**Murine iPSCs differentiate into microglia-like cells via a hematopoietic progenitor-like intermediate**

To demonstrate the potential therapeutic value of iPS-MG, murine iPS-MG were differentiated from murine iPSC using a two-stage method analogous to that for human iPS-MG (Fig. 4a). Murine iPSCs generated by lentiviral reprogramming of murine embryonic fibroblasts isolated...
GFP+ cells in as early as 3 d (Fig. 4g). Subsequent culture of iPS-HPC on astrocyte monolayers supplemented with OP9 murine stromal cell layers, cells were directed to an intermediate iPS-HPC (stage 1) state with loss of Oct4 and expression of Cx3cr1Gfp+ (Fig. 4e). Following co-culture on OP9 murine stromal cell layers, cells were biweekly media changes for maintenance. GFP+ cells constituted ~4% of the other cell types listed above. Expression levels for each class of gene, analyzed by heat map examining expression levels and relationships of all genes after noise analysis between nMG, iPS-MG, adult brain-isolated microglia (aMG), DC, bone marrow derived macrophages (BMM), peritoneal macrophages (PM), mouse Cx3cr1Gfp+ iPSC iPSC and Nestin+ neural stem cells (NSC). The panels for each cell type were gated on live GFP+ cells. Red, primary antibody; blue, isotype. The figure is representative of two independent experiments (n = 2). (b) Heat map examining expression levels and relationships of all genes after noise analysis between nMG, iPS-MG, adult brain-isolated microglia (aMG), DC, bone marrow derived macrophages (BMM), peritoneal macrophages (PM), mouse Cx3cr1Gfp+ iPSC iPSC and Nestin+ neural stem cells (NSC). The panels for each cell type were gated on live GFP+ cells. Red, primary antibody; blue, isotype. The figure is representative of two independent experiments (n = 2).

Figure 5 Mouse iPS-MG display phenotypic markers and gene expression consistent with primary neonatal microglia. (a) Flow cytometric analysis of nMG and iPS-MG demonstrates comparable levels of cell-surface markers. The panels for each cell type were gated on live GFP+ cells. Red, primary antibody; blue, isotype. The figure is representative of two independent experiments (n = 2). (b) Heat map examining expression levels and relationships of all genes after noise analysis between nMG, iPS-MG, adult brain-isolated microglia (aMG), DC, bone marrow derived macrophages (BMM), peritoneal macrophages (PM), mouse Cx3cr1Gfp+ iPSC iPSC and Nestin+ neural stem cells (NSC). (c) Three-dimensional principal component (PC) analysis of the microarray data. iPS-MG total RNA was compared with that of the other cell types listed above. Expression levels for each class were averaged from three independent cell cultures or differentiation experiments (n = 3).

from Cx3cr1Gfp+ knock-in mice were used as a starting cell population. Expression of the fractalkine receptor CX3CR1 is a necessary, but not sufficient, criterion for defining murine microglia, and the microglia of Cx3cr1Gfp+/− knock-in mice express GFP under control of the Cx3cr1 promoter22. GFP expression by iPS-MG was used to facilitate their in vitro and in vivo identification.

Cx3cr1Gfp+ iPSC express Oct4 by but not CD34, CD11b or GFP (Fig. 4b–d). Following co-culture on OP9 murine stromal cell layers, cells were directed to an intermediate iPS-HPC (stage 1) state with loss of Oct4 expression and gain of CD34 expression (Fig. 4e,f). At stage 1, none of the co-cultured cells expressed either CD11b or GFP (Fig. 4g). Subsequent culture of iPS-HPC on astrocyte monolayers supplemented with GM-CSF, M-CSF and IL-3 (stage 2) resulted in the appearance of GFP+ cells in as early as 3 d (Fig. 4h–j). These GFP+ cells continued to increase in number over the following 7–14 d and required only biweekly media changes for maintenance. GFP+ cells constituted ~4% of the mixed astrocyte–microglia cell culture (Supplementary Fig. 3). Loss of CD34 expression and gain of CD11b expression occurred over this time period as well, consistent with the generation of murine iPS-MG (Fig. 4l,j). On average, for every 1 × 10^6 murine iPSC, the yield of microglia-like cells was 3 × 10^5. The iPS-MG also expressed CD39 and CD45 but not Ly6C, Ly6G, B220, Thy1.2 and NK1.1, a phenotypic profile that closely resembled that of neonatal brain-isolated microglia (nMG) (Fig. 5a)18,22. The yields following the differentiation of adult fibroblast derived iPS-C were equivalent to those of embryonic fibroblast-derived iPS.

The gene expression signatures of murine iPSC, iPS-MG, nMG, adult brain-isolated microglia, bone marrow-derived macrophages, peritoneal macrophages and DC were compared. Also included in the analyses were Nestin+ neural stem cells NSC, given previous reports of differentiation of embryonic stem (ES) cells into microglia using a neuronal differentiation protocol23. We generated 35,556 gene-fragment expression values per biological replicate sample and the 20% of data found to be noise-biased was discarded. After subjecting the data to one-factor ANOVA under Benjamini and Hochberg false discovery rate multiple-comparison correction conditions, we restricted our analysis to 26,842 gene fragments with a corrected P < 0.05. Using Tukey’s post hoc analysis, we then quantified the number of these genes having both P < 0.05 and an absolute fold-change in expression ≥ 1.5 in pairwise comparisons of all the experimental groups, resulting in 20,232 genes. The results of this a posteriori analysis indicated that the gene expression patterns of iPS-MG most closely resembled those of nMG. The next-closest resemblance was to those of adult brain-isolated microglia. The cells that most differed from iPS-MG in terms of gene expression patterns were iPSCs and neural stem cells. These gene expression relationships can be visualized using correlation-based clustering (Fig. 5b) and covariance-based principal component analyses (Fig. 5c).

Murine iPSC-MG demonstrate in vitro and in vivo functional properties of native microglia

In addition to their phenotypic and gene expression resemblance to nMG, murine iPSC-MG also demonstrated the in vitro functional properties of nMG. Following stimulation with PMA, both cell types actively phagocytosed pHrodo E. coli BioParticles conjugates as evidenced by the acidification and resulting visualization of red fluorescent particles within circular intracellular structures (Fig. 6a). The mean cell fluorescence intensities of nMG and iPS-MG after the addition of the pHrodo E. coli BioParticles were qualitatively similar, at 140,166.72 and 152,096.58 relative units respectively. Under baseline culture conditions, both nMG and iPS-MG generated ROS, as evidenced by the oxidation and resulting red fluorescence of the CellROX Deep Red reagent indicator dye within cells (Fig. 6b). The mean cell fluorescence intensities after the addition of the CellROX reagent were 338,876.45 relative units for nMG and 235,213.10 relative units for iPS-MG. Control fibroblasts neither phagocytosed foreign particles nor produced ROS under the same conditions (Fig. 6a,b).

Migration to sites of CNS pathology such as brain tumors is an in vivo functional hallmark of microglia24. The ability of murine iPSC-MG to migrate to and infiltrate experimental gliomas in mice was therefore assessed. One week following the intracranial implantation of syngeneic, luciferase-expressing malignant glioma cells, iPS-MG were injected into the cerebral hemisphere contralateral to the tumor site. Brains were immunohistochemically analyzed 1 week later, and luciferase-expressing tumor cells were found in areas of high nuclear density (Fig. 6c,g). There was an expected abundance of infiltrating Iba1+ microglia in these areas (Fig. 6d,h), and while many of them were GFP+ and represented native microglia (Fig. 6e,i), many were also Iba1+ GFP+ and represented the iPS-MG cells previously injected into the contralateral hemisphere (Fig. 6f,j). These results demonstrate the ability of iPS-MG to engraft within an unconditioned brain and migrate from one hemisphere to the other in response to a tumor.
Murine iPS-MG increase the survival of intracranial malignant glioma-bearing mice

The ability of native microglia to phagocytose tumor cell debris, load antigens onto major histocompatibility class II (MHC II) molecules and display costimulatory molecules such as B7-1 and B7-2 suggest their ability to present tumor antigens in vivo. Additionally, microglia are capable of directly killing tumor cells by producing nitric oxide, ROS and TNF-α (ref. 4). As a potential clinical application of iPS-MG, we assessed the ability of intracranially injected nMG or iPS-MG, in combination with subcutaneously injected DC (ref. 25), to prolong the survival of malignant glioma-bearing mice. C57BL/6 mice were intracranially implanted with syngeneic GL261 malignant glioma tumor cells expressing luciferase to facilitate the in vivo imaging of tumor size and growth. One week following implantation, animals were imaged to confirm tumor engraftment and randomized into five groups. Each control or treatment group was then injected intracranially (IC) and subcutaneously (SC) with different combinations of vehicle control (phosphate buffered saline; PBS) or cells (DC, nMG or iPS-MG) pulsed in vitro with tumor cell lysates, as shown in Supplementary Table 1.

The median survival of the untreated control group (Group A: IC and SC injection of PBS) was 40 d, while that of the SC DC treated group (Group B) and IC nMG treated group (Group C) were 41 d ($P = 0.859$, log-rank test) and 46 d ($P = 0.086$, log-rank test), respectively. The combination IC nMG + SC DC treated group (Group D) had a median survival of 205.5 d ($P = 0.008$, log-rank test, degrees of freedom (df) = 1, $\chi^2 = 6.983$) while the median survival of the IC iPS-MG + SC DC (Group E) group was undefined, as more than 50% of the animals were alive at the end of the 1-year observation period ($P = 0.001$, log-rank test, df = 1, $\chi^2 = 10.22$). P-values were calculated in comparison to Group A (Fig. 7 and Supplementary Table 1). Treatment of animals with cells pulsed in vitro with murine embryonic fibroblast lysates before injection did not prolong survival and demonstrated the antigen specificity of the antitumor treatments (data not shown).

DISCUSSION

Experimental mouse models of human diseases in which microglia have been genetically modified or ablated have been especially useful in demonstrating the beneficial and detrimental effects of microglia on the pathogenesis of a variety of brain and spinal cord disorders. A major obstacle to further laboratory study and clinical translation of these findings has been the lack of an abundant source of normal and disease-specific human microglia. One partial solution to overcoming this obstacle has been the differentiation of ES cells into microglia-like cells using a modified neuronal differentiation protocol. ES cells can be readily expanded to large numbers before differentiation. ES cell-derived microglia are capable of engrafting in the unconditioned CNS (refs. 23, 28–30). While these protocols represent a significant advance in the study of microglia, they require several weeks to complete and do not mirror the normal microglial developmental process. In contrast to neurons and macroglia, microglia are of mesodermal rather than neuroectodermal origin and arise from embryonic yolk sac myeloid precursors that travel to the brain and spinal cord before differentiating into mature, functional cells within the CNS (refs. 31–35). An analogous protocol for the differentiation of human ES cells has not been reported, and even if one were to be developed, the translation of ES cell-derived microglia to the clinical therapeutic setting would likely be constrained by the unavailability of patient-specific ES cells as a starting population, as well as histocompatibility issues and ethical concerns. To address these shortcomings, a protocol for the differentiation of human iPSC into microglia-like cells was developed. Human iPSC can be readily generated from normal and disease-specific individuals and can also be expanded to large numbers in vitro. Additionally, a protocol using a myeloid rather than neural-cell intermediate state was purposely designed to be more consistent with the in vivo developmental lineage of microglia.

Our results above demonstrate the differentiation of both human and mouse iPSC into cells with not only the phenotype and consensus gene expression signature but also the in vitro and in vivo functional properties of primary brain-isolated microglia. In the correlation-based clustering analysis, the gene expression patterns of the murine iPS-MG most closely resembled those of neonatal microglia and next closely those of adult microglia. In contrast, in the human analysis, the human iPSC-MG did not cluster as tightly with the commercially obtained human fetal microglia. Extraction of the individual gene expression data for the six consensus human microglia genes (P2RY12, GPR34, MERTK, CIQA, PROS1 and GAS6) from the microarray dataset did, however, demonstrate the high expression of all six genes in the iPS-MG but not in the iPSC, Mac, DC or commercially obtained human fetal microglia. While there are robust protocols for the isolation of murine microglia, similar protocols and source tissue for the isolation...
of authentic human microglia are currently lacking. This underscores the need for protocols such as ours.

Very recently, another protocol for the differentiation of human iPSC and ES cells into microglia-like cells was reported. Similarly to ours, in the protocol developed by Muffat et al., cells are differentiated through a myeloid rather than neural intermediate, and the final yields of microglia-like cells from a given number of starting iPSC are also similar. The major advantage of the other method appears to be the use of defined factors rather than co-culture with astrocytes to induce final differentiation. The use of astrocytes derived from the same starting iPSC as that used to make the microglia-like cells would, however, avoid the introduction of any allogeneic cells into the differentiation process, an important consideration for potential clinical uses. An apparent advantage of our astrocyte co-culture protocol compared to the defined-factor protocol is the use of starting iPSC propagated under serum- and feeder-free conditions rather than grown on a feeder layer of murine embryonic fibroblasts. The avoidance of any xenogeneic cells in the differentiation process may facilitate the future clinical use of human microglia-like cells. An additional difference in the two protocols is the time required for the differentiation of the iPSC into microglia-like cells. Whereas our mouse and human protocols require 2 and 4 weeks, respectively, the defined-factor protocol requires 8 weeks for both species. Finally, the microglia-like behavior of the cells generated using the defined-factor protocol was demonstrated using an elegant in vitro 3D organotypic neuroglial environment. In contrast, we demonstrated the engraftment, migration and potential clinical use of our microglia-like cells in an in vivo model that did not require myeloablative conditioning of the host animal with lethal irradiation.

The potential applications of normal and disease-specific human iPSC-MG include their use in disease modeling, drug discovery studies and toxicity screening assays. Genetic mutations of microglia-expressed genes have been associated with the pathogenesis and prognosis of a variety of human diseases. Microglia derived from the iPSC of patients with such diseases may allow direct study of the roles of those gene products in disease development and progression. One such example is superoxide dismutase-1 (SOD1), a mutation of which is implicated in amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder affecting adult motor neurons. Over 100 different SOD1 mutations have been identified, and the role of these mutations in human motor neuron and microglial function could be modeled using normal and disease-specific iPSC-MG. iPSC-MG could also be used in high-throughput screens of SOD1-active drugs and microglia-specific toxicity assays. Another potential application of human iPSC-MG is their use in personalized medicine applications. The murine iPSC-MG generated here were able to engraft within an unconditioned brain and migrate to an area of pathology. Furthermore, the murine iPSC-MG, in combination with DC, were an effective cell therapy for intracranial malignant glioma tumors. iPSC-MG could also potentially be used for the treatment of diseases such as ALS or as a delivery vector for gene-based therapies of diseases such as multiple sclerosis or Parkinson’s disease.

### METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

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### AUTHOR CONTRIBUTIONS

J.K.P conceived the project; H.P, M.J.S., D.M.I., Y.C., D.B.M. and J.K.P designed the experiments; H.P, M.J.S., D.M.I., Y.C., A.B.S., G.K. and M.A.B. performed the experiments; H.P, M.J.S., D.B.M. and J.K.P analyzed the data; K.R.J. performed microarray data analysis; A.G.E., D.M., C.L.S. and S.G. provided technical support; H.I.M. provided conceptual advice; and H.P, M.J.S., D.B.M and J.K.P wrote the manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Creation of Cx3cr1Gfp/+ mice iPS cell and human iPS cell lines. Fibroblasts isolated from embryonic day 13 Cx3cr1Gfp/+ knock-in mice were reprogrammed to the iPS state using the Dox-inducible polycistronic m4EFA lentivirus kit (ST000043; Stemgent) according to the manufacturer's instructions. Confirmation of the iPS state was obtained using alkaline phosphatase staining of CD34+ cells using episomal plasmids (Lonza contract) made available to the NIH Center for Regenerative Medicine and currently available through RUCDR Infinite Biologics. The iNC-01 cell line was obtained from Dr. H.L. Malech's laboratory of Host Defenses and Research Technologies Branch, NIAID, NIH. The iNC-01 cells were reprogrammed from human peripheral blood hematopoietic stem/progenitor cells (CD34+) that had been mobilized into the donor's peripheral blood by G-CSF treatment. The reprogramming method involved transduction with integrating STEMCCA-loxP lentivirus expressing human Oct4/KSO2c/myc from a single transcript, followed by Cre-mediated excision of the vector. Authenticity and characterization of the cell line as iPS cells arising from the healthy donor included TRA-1-60 and alkaline phosphatase staining for pluripotency markers, three-germ layer characterization by teratoma formation in immunodeficient NSG mice followed by H&E staining and histology characterization, three-germ layer characterization by embryoid body differentiation and immunofluorescence microscopy after staining for smooth muscle actin (mesoderm), α-fetoprotein (endoderm) and class III b-tubulin (ectoderm). Additional characterization included DNA fingerprinting to confirm that the origin of the cells matched the donor blood sample by DNA microsatellite analysis at seven loci in comparison with PBMCs derived from that patient using the AmpFISTR COiler kit (Applied Biosystems) and genome-wide DNA methylation analysis at >485,000 CpG methylation sites in comparison with embryonic stem cells, other iPS lines and primary hematopoietic cells using Infinium HumanMethylation450 BeadChip (Illumina). The iNC-01 iPS line was further characterized molecularly for additional pluripotency markers by global proteome analysis using electrospray ionization mass spectrometry (ESI-MS) and MALDI-TOF/TOF mass spectrometry in comparison with embryonic stem cells, other iPS lines and somatic cells and by expression analysis of 754 microRNAs using the TaqMan Human MicroRNA Array A+B v3.0.

Cell culture. Cx3cr1Gfp/+ iPS cells were maintained on a mitotically inactivated C57BL/6-background MEF feeder layer (Globalstem) using Knockout DMEM with 10% ES-qualified FBS, nonessential amino acids, sodium pyruvate, 2-mercaptoethanol, penicillin/streptomycin (all from Gibco) and leukemia inhibitory factor (LIIF; Millipore). iPS cells were passaged upon reaching confluency. Normal mMG were isolated from postnatal day 2 brains using the Neural Tissue Dissociation Kit and CD11b MicroBeads (Miltenyi) according to the manufacturer's instructions and maintained in animal astrocyte media (Lonza) supplemented with 20% FBS and penicillin/streptomycin on 0.1% gelatin-coated tissue culture treated dishes (Sigma). Media was changed twice weekly, and OP9 cells were passaged upon reaching confluency. Normal mMG were isolated from postnatal day 2 brains using the Neural Tissue Dissociation Kit and CD11b MicroBeads (Miltenyi) according to the manufacturer's instructions and cultured in DMEM (Life Technologies) with 10% FBS, Glutamax, nonessential amino acids, sodium pyruvate, 2-mercaptoethanol (Gibco) and sodium bicarbonate (Sigma). Microglia were plated on six-well temperature-responsive tissue culture plates (Upcell) at 2 × 10⁶ cells per well in 2 mL of media. Adult murine microglia were purified from mixed glial cultures. Dendritic cells and bone marrow-derived macrophages were isolated following culture of whole bone marrow with recombinant murine IL-4 (RND Systems) and GM-CSF (Peprotech)-containing media or M-CSF (Peprotech)-containing media, respectively. Peritoneal macrophages were harvested by saline lavage. Neural stem cells were purchased from Millipore. Firefly luciferase-expressing GL261 cells (National Cancer Institute) were cultured in Neurobasal media (Gibco), supplemented with N2 and B27 additives (Gibco), Glutamax and penicillin/streptomycin. NCRM-5 and INC-01 human iPS cells were maintained as feeder-free on matrigel (BD)-coated six-well plates and split every 3 d. The cells were split 1:6 or 1:12 using EDTA (0.5 mM in Ca²⁺- and Mg²⁺-free PBS with 1.8 mg/mL NaCl). Essential 8 (Invitrogen) medium was used for growth and maintenance of NCRM-5 human iPS and were maintained in a humidified 37 °C incubator with 5% CO₂ and 21% O₂ (ref. 39). mTET3 (StemCell Technologies) media was used for INC-01 cells. The cell lines were regularly tested for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (Lonza).

Microglial differentiation

Human iPS cell lines were harvested from a well of a six-well plate using EDTA according to Chen et al. and resuspended in OP9 differentiation medium (ODM-MEM with 20% FBS and 100 µM MTG). All steps were cultured in a humidified 37 °C incubator with 5% CO₂ and 21% O₂. On day 0, iPS aggregates were added to a confluent OP9 (ATCC) plate. On day 1, medium was removed, and 20 mL of fresh ODM was added to the plate. On days 4 and 6, half of the medium was replaced with fresh medium. On day 9, cells were harvested using 5 mL of collagenase solution (1 mg/mL) by incubating it for 30 min at 37 °C (Life Technologies). Next, collagenase was aspirated, and 5 mL of trypsin/EDTA solution (0.05%/0.5 mM) was added and incubated for 15 min at 37 °C. Cell suspension was collected, and MACS sort of CD34⁺/CD43⁺ cells was carried out according to Miltenyi manufacturer's directions. Subsequently, the pellet from the previous step was resuspended using myeloid progenitor expansion medium (ODM with 200 ng/mL GM-CSF (Bender)) at a concentration of ~5–10 × 10⁶ cells/mL. Phema-coated T75 flasks were washed with 20 mL PBS and 10–20 mL of CD34⁺ cell suspension was added and incubated for 2 d. On day 11, expanded CD34⁺ cells were centrifuged and resuspended in astrocyte differentiation medium (ADM-IMDM with 10% FBS, 20 ng/mL GM-CSF, 20 ng/mL M-CSF (Peprotech) and 20 ng/mL IL3 (Peprotech)). Expanded CD34⁺ cells were plated onto confluent 10-cm plates of human astrocytes at a density of 1–2 × 10⁶ cells/10 mL/plate. On day 15, 10 mL of ADM was added. On days 18–25, media was aspirated and plates were harvested using 5 mL of trypsin/EDTA and incubated for 5–10 min. Subsequently, cell suspensions harvested from the plates were washed and MACS sorted for CD11b⁺ cells according to the manufacturer's directions. The CD11b⁺ cells were collected and resuspended using ADM; they were used for further characterization, expansion or in experiments as required.

Feeder-free differentiation of human iPS-microglia on astrocytes

INC-01 human iPS cells were cultured feeder-free in mTET3R1 media (Stemcell Technologies). Human iPS cells were passaged when 70% confluent, using PBS/EDTA/NaCl to loosen the colonies from the plate, and replated onto a matrigel-coated plate at ratios of 1:5 to 1:8. The cultures were further incubated for 2 d at 37 °C, 5% CO₂, 5% O₂. Medium was changed to fresh mTET3R1 on day 1. On Day 0, Stemdiff APEL medium (Stemcell Technologies) was warmed to room temperature (20–25 °C, 3 mL per well of a six-well plate). Media A was prepared by adding the desired growth factors just before use. Media A consisted of Stemdiff APEL medium (Stemcell Technologies), 30 mg/mL LVEGF (Peprotech), 30 mg/mL hBMIP4 (Peprotech), 40 ng/mL hSCF (Peprotech) and 50 ng/mL hActivin A (Invitrogen). mTET3R1 medium was aspirated from iPS culture wells and replaced with freshly prepared Media A. The cultures were further incubated for 4 d at 37 °C, 5% CO₂, 5% O₂. No further media changes were carried out during this time. On days 4, 7 and 10, media A was removed and replaced with media B and incubated for 3 d at 37 °C, 5% CO₂, 5% O₂. Media B was prepared by adding the growth factors to Stemdiff APEL medium (3 mL per well), 300 ng/mL hSCF (Peprotech), 300 ng/mL hIL3 (Peprotech), 10 ng/mL hIL-3 (Peprotech), 10 ng/mL hIL-6 (Peprotech), 50 ng/mL hG-CSF (Peprotech) and 25 ng/mL hBMIP4 (Peprotech). Cells were harvested on day 15 to analyze for hematopoietic differentiation using 1 mL of Accutase (Innovative Cell Technologies) per well. Cells from 3 wells of a six-well plate culture were analyzed for CD34, CD45 and CD43 expression by
FACS, and cells from the other three wells were plated onto 10–cm dish of human astrocyte in 10 mL of media C (500 mL IMDM (Gibco), 10% defined FBS (Gibco), 5 mL Penicillin/Streptomycin (Gibco), 20 ng/mL of hIL3 (Peprotech), 20 ng/mL of hGMCFSF (Peprotech) and 20 ng/mL of hM-CSF (Peprotech). The astrocyte–hematopoietic progenitor co-culture plates were further incubated at 37 °C, 5% CO2, for 1–2 weeks for differentiation to microglia. CD39+ microglia was isolated using MACS (Milteny). Mouse iPSC–microglia differentiation on OP9 feeder layers. Cs3cr1- or- ipSC were differentiated into microglia using a two-stage protocol. In the first stage, a modified protocol for the hematopoietic differentiation of iPSCs, Cs3cr1- or- iPSC colonies were dissociated into a single-cell suspension using 1 mg/mL collagenase (Invitrogen) and Accutase (Stemcell Technologies) and plated at a density of 1.5 × 10^5 cells per 15-cm tissue culture dish of confluent OP9 stromal cells. This stage 1 culture was maintained in α-MEM with 10% defined FBS (Hyclone), 2-mercaptoethanol and penicillin/streptomycin for 7 d. On day 4, the media was fully replaced. After 7 d of stage 1 culture, cells were harvested by dissociation with trypsin (Gibco) and transfected onto a confluent astrocyte monolayer for stage 2 culture. Stage 2 cultures were maintained in DMEM/F12 media (Gibco) with 10% defined FBS, 2 mM 2-mercaptoethanol, 2 mM Glutamax (Gibco), nonessential amino acids (Gibco), 20 ng/mL recombinant murine GM-CSF (Peprotech), 20 ng/mL recombinant murine M-CSF (Peprotech), 20 ng/mL recombinant murine IL-3 (Peprotech) and penicillin/streptomycin. Media in stage 2 cultures was replaced twice weekly. Purification of iPSC-MG from stage 2 cultures was carried out using anti-CD11b or anti-CD39 magnetic beads (Milteny) according to manufacturer’s instructions followed by optional FACS sorting of iPSC-MG by gating on GFP+ cells.

Immunofluorescence. iPSC cultures, stage 1 cultures and Stage 2 cultures were established in eight-well Permanox-coated chamber slides (Thermo Fisher). Cells were fixed with 4% paraformaldehyde, blocked with Background Buster (Innovex Biosciences) and incubated with primary antibodies overnight at 4 °C. Cells were washed with PBS and incubated with the appropriate secondary antibodies for 1 h at room temperature. For the human iPSC, rabbit anti-human Nanog (Stemgent, Cat # 09-0020) and mouse anti-human Tra-1-81 (1:100; Stemgent, Cat # 09-0011) were used as primary antibodies and anti-rabbit Alexa Fluor 488 and anti-guinea pig antibodies as secondary antibodies (Molecular Probes). For stage 1, differentiated cells were stained with rabbit anti-human CD34 (Abcam, Cat # ab81289) and APC-conjugated-mouse anti-human CD43 (BD, Cat # ab81289, Clone # 560198), and the signal for CD34 was detected using anti-rabbit Alexa Fluor 488 (Invitrogen, Cat # A-11008). For stage 2, microglia-like cells were detected using the mouse anti-human CD11b (Abcam, Cat # ab91145) and rabbit anti-human iHBA (Wako, Cat # 019-19741). Secondary antibodies used in this case were anti-mouse Alexa Fluor 546 and anti-rabbit Alexa Fluor 488 for mouse cells, the following primary/secondary antibody combinations were used: rabbit anti-mouse Oct4 polyclonal (Stemgent, Cat # 09-0023) and anti-rabbit Alexa Fluor 546 antibody (Invitrogen, Cat # A10040); rat mAb CD34 mAb (MEC14.7; Abcam, Cat # ab8158)/anti-rat Alexa Fluor 647 (Invitrogen, Cat # A-21247) antibody; anti-mouse CD11b mAb directly conjugated to Alexa Fluor 488 (BD Biosciences, Cat # 557686, clone M1/70) secondary not needed. All primary antibodies were used at a 1:100 dilution in PBS with 0.1% BSA, while secondary antibodies were used at a 1:200 dilution in the same buffer. Following staining, the slides were mounted using Vectashield mounting medium with DAPI (Vector Labs) and imaged using the Zeiss LSM 510 confocal microscope. All isotype control primary mAb-stained cells lacked fluorescence labeling. The all antibodies used are commercially validated antibodies.

FACS analyses. CD11b+ sorted cells were collected from the astrocyte feeder cells using MACs (Milteny). DAPI was used to determine LIVE/DEAD cells. Next, the cells were washed and stained with primary antibodies Ms anti-human anti-CD11b (1:200) (Abcam, Cat # ab91145) and HLA-DR- (1:200; Abcam, Cat # CR3/43) (CR3/43) ab7856, rabbit pAB anti-CD45 (1:200; Abcam, ab10558), mouse mAb anti-CD86 (1:200; Abcam, Cat # BU63) ab23356, rabbit anti-human Iba1 (1:100; Wako, Cat # 019-19741), CD80 (1:200; Abcam), rabbit pAB CXCR4 (1:200; Abcam, Cat # ab8021) and goat pAb TREM-2 (1:200; Abcam, Cat # ab85851) antibodies, along with their corresponding isotype controls for 1 h at 4 °C. After incubation, the cells were washed and stained with corresponding secondary antibodies tagged with the following Alexa Fluor 488 or Alexa Fluor 546 as appropriate for 45 min on ice, and subsequently the signals were analyzed using Moflo Astrios Cell Sorter (Beckman Coulter, Inc.). For mouse iPSC-MG, CD11b+ sorted cells were collected from the astrocyte feeder cells using MACs (Milteny). Subsequently, the cells were incubated with an Fc receptor block consisting of Rat anti-mouse CD16/32 (1:100; BD Pharmingen, Cat # 553142) and mouse IgG (1:50; Jackson ImmunoResearch) for 15 min at 4 °C. Staining was carried out for the dead cells using LIVE/DEAD Fixable Blue Dead Cell Stain Kit for UV excitation (Molecular Probes) for 30 min at 4 °C. Next, the cells were washed and stained with fluorochrome-conjugated rat anti-mouse Ly6C-APC-Cy7 (1:400; Biolegend, Cat # 128026, clone # HK1.2), rat anti-mouse Ly6G-PE (1:200; BD Pharmingen, Cat # 551461, clone # IA8), rat anti-mouse CD45-PerCP-Cy5.5 (1:50; BD, Cat # 550994, clone # 30-F11), rat anti-mouse CD39-PE-Cy7 (1:200; ebioscience, Cat # 25-0391-80, clone # 24DMS1), rat anti-mouse Thy1.2-Alexa Fluor 700 (1:1,200; Biolegend, Cat # 105320, clone # 30-H12), anti-mouse NK1.1-PE (1:200) mAb (ebioscience, Cat # 12-5941-83, clone # PK136) and rat anti-mouse B220-APC (1:1,000; BD Pharmingen, Cat # 553092, clone # RA3-6B2) antibodies and their corresponding isotype controls for 1 h at 4 °C. All antibodies used are commercially validated. After incubation, the cells were washed twice and fixed with 1% paraformaldehyde. The signals were analyzed using an LSR II flow-cytometer (BD).

Microarray hybridization. For human iPSC-MG, total RNA was isolated from NCRM-5 and INC-01 human iPSC lines, iPSC-MG and human fetal microglia (Clonexpress) using miRNeasy kit (Qiagen). RNA from human dendritic and bone marrow macrophages (ALLcells) was also used. Isolated total RNA was amplified with the WT Expression Kit (Ambion) and labeled with the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix). Labeled cRNA were hybridized to the Affymetrix GeneChip Human Gene 2.0 ST Array (Affymetrix, Inc.) in a blinded, interleaved fashion. The Affymetrix Gene Chip Scanner 3000 was used to scan each array and gene-probe intensities were generated using the Affymetrix AGCC software (Affymetrix, Inc.). RNA was obtained from murine nMG, adult microglia, Cs3cr1- or- iPSC, iPSC-MG, peritoneal macrophages, bone marrow-derived macrophages, bone marrow-derived dendritic cells and neural stem cells. RNA was extracted from cells using the miRNeasy mini kit (Qiagen), amplified with the WT Expression Kit (Ambion) and labeled with the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix). Labeled cRNA were hybridized to the Affymetrix GeneChip Mouse Gene 1.0 ST Array (Affymetrix, Inc.) in a blinded, interleaved fashion. The Affymetrix Gene Chip Scanner 3000 was used to scan each array, and gene probe intensities were generated using the Affymetrix AGCC software (Affymetrix, Inc.).

Microarray data analysis. Gene fragment data summarization and normalization were performed separately for human and mouse samples using the Partek Genomic Suite v2 (http://www.partek.com/) with the ‘RMA’ option selected under default settings. Subsequent analyses of these data were also performed separately for mouse and human, while the analyses performed were the same. Data quality was assessed via Tukey box plot, PCA scatter plot and correlation-based heat map using functions in R (https://cran.r-project.org/). LOWESS (locally weighted scatterplot smoothing) modeling of the data (CV is approximately mean expression) was used to characterize noise for the system and discard noise-biased data. Differential expression was tested for via ANOVA under BH correction condition followed by Tukey HSD post hoc test. Gene fragments found to have a corrected P < 0.05 by ANOVA and a post hoc P < 0.05 were deemed to have significant differential expression between the corresponding cell types if the absolute difference of means was ≥ 1.5×. Visualization of cell-type relationships post testing was accomplished using the heat.map.2() functions in R and the 3D-PCA option in Partek.

Quantitative real-time PCR (RT-qPCR). Total RNA was isolated from human iPSC and human iPSC-MG using miRNeasy kit (Qiagen). Subsequently, 1 μg of RNA was treated with amplification grade DNAese I (Life Technologies) to remove any contaminating genomic DNA. We used 250 ng of total RNA for cDNA synthesis using iScript cDNA Synthesis Kit (Bio-Rad). All Q-PCRs were performed in 20 μL volumes using SYBR Green PCR master mix (Applied Biosystems) in a 96-well optic tray using a CFX96 Real-Time PCR machine (Bio-Rad). The reactions were conducted in duplicate, and samples without
reverse transcriptase were used as nontemplate controls. Primers specific for the six microglial genes were obtained from Integrated DNA Technologies. q-PCR reactions were run with an initial denaturation temperature of 95 °C for 10 min, followed by 40 cycles of three-step amplification (denaturation at 95 °C for 30 s, -60 °C (dependent on the annealing temperature of the primer) annealing for 20s and extension at 72 °C for 20 s). The optimal annealing temperature for each gene-specific primer was determined before the experimental runs. The housekeeping gene ACTB was used as a reference.

**Phagocytosis assay.** We cultured 5 × 10⁴ mouse iPSC-MG, nMG or MEFs for 48 h on four-well CC2-treated glass chamber slides (Thermo Fisher). Cells were stimulated for 15 min with 100 nM phorbol myristate acetate (PMA) diluted in dye-free MEM media (Gibco) before the incubation of cells with pHrodo Red E. coli BioParticles conjugate (Life Technologies) for 1.5 h at 37 °C. Hoechst 33342 (Thermo Scientific) was used to stain the nuclei. Human iPSC microglia were treated similarly, but they were not activated with PMA before incubation with the BioParticles. Live-cell imaging was carried out using an LSM 510 confocal microscope using excitation/emission wavelengths of 560/585 nm (Zeiss). Nuclei were visualized with 20 µM Hoesch 33342. Corrected total cell fluorescence intensity for the confocal images was calculated using Image J.

**Reactive oxygen species assay.** We cultured 5 × 10⁴ mouse iPSC-MG, nMG or MEFs were cultured as above for the phagocytosis assay. Cells were then treated with 20 µM CellROX Deep Red reagent (Life Technologies) diluted in dye-free MEM media (Gibco) before the incubation of cells with PMA and 20 µM CellROX Green reagent (Life Technologies) dye. Nuclei were stained with Hoechst 33342 dye. Live-cell imaging was performed at 640/645 nm using the Zeiss LSM 510 confocal microscope. Analysis of the confocal images for corrected total fluorescence intensity was carried out using Image J.

**Mouse iPSC-MG engraftment studies.** Animals were anesthetized and placed in a stereotaxic frame (Stoelting). Coordinates used for stereotactic injection were 3 mm right lateral and 1 mm posterior to bregma, with an injection depth of 3.5 mm. Male C57BL/6NCr 6-week-old mice were injected with 3 µL of a 5 × 10⁴ cells/µL suspension of luciferase-expressing GL261 cells in dPBS, resulting in the implantation of 1.5 × 10⁵ cells/animal. One week later, animals were injected 3 mm left lateral and 1 mm posterior to bregma, with an injection depth of 3.5 mm, with 3 µL of an 8.33 × 10⁴ cells/µL suspension of mouse iPSC-MG in dPBS, resulting in the implantation of 2.5 × 10⁵ cells/animal. One day later, animals were killed for analysis and perfused with 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde at 4 °C for 4 h, cryoprotected with 10% sucrose solutions and cut into 8-µm-thick tissue sections using a cryostat. The sections were stained with anti-goat-anti-luciferase (1:1,000; Fitzgerald, Cat # 70C-CR202GGAF), chicken anti-GFP (1:200; Abcam, Cat # ab13970) and rabbit anti-Iba1 (1:200; Wako, Cat # 019-19741) primary antibodies, followed by donkey anti-goat Alexa Fluor 647 (Invitrogen, 1:100), donkey anti-chicken Alexa Fluor 488 (Jackson, 1:100) and donkey anti-rabbit Alexa Fluor 555 (Invitrogen, 1:100) secondary antibodies, respectively. Images were taken using a Zeiss LSM 510 confocal microscope. Mice were housed four animals per cage and monitored regularly for signs of distress and clinical deterioration. The animals were on a 12-h light and dark cycle. All animal studies were approved by the NINDS/NIDCD Institutional Animal Care and Use Committee.

**Statistical analysis.** Differential expression for the microarray experiments was tested for via ANOVA with BH correction followed by Tukey HSD post hoc tests. Gene fragments found to have a corrected P < 0.05 by ANOVA and a post hoc P < 0.05 were deemed to have significant differential expression between the corresponding cell types if the absolute difference of means was ≥1.5×. Statistical analysis for the survival curves in the animal treatment study was carried out using the log-rank test (Mantel-Cox test). P < 0.05 was considered statistically significant. A Supplementary Methods Checklist is available.

**Data availability.** Data for both the mouse and human samples are available for download from the NCBI Gene Expression Omnibus at SuperSeries GSE78116. Additional data that support the findings of this study are available from the corresponding authors upon reasonable request.

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