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Fully defined human pluripotent stem cell-derived microglia and tri-culture system model C3 production in Alzheimer's disease

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ABSTRACT

Aberrant inflammation in the CNS has been implicated as a major player in the pathogenesis of human neurodegenerative disease. We developed a new approach to derive microglia from human pluripotent stem cells (hPSCs) and built a defined hPSC-derived tri-culture system containing pure populations of hPSC-derived microglia, astrocytes, and neurons to dissect cellular cross-talk along the neuroinflammatory axis in vitro. We used the tri-culture system to model neuroinflammation in Alzheimer's disease with hPSCs harboring the APPSWE+/+ mutation and their isogenic control. We found that complement C3, a protein that is increased under inflammatory conditions and implicated in synaptic loss, is potentiated in tri-culture and further enhanced in APPSWE+/+ tri-cultures due to microglia initiating reciprocal signaling with astrocytes to produce excess C3. Our study defines the major cellular players contributing to increased C3 in Alzheimer's disease and presents a broadly applicable platform to study neuroinflammation in human disease.

ATTACHMENTS

c76wbiqa7.pdf

DOI

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PROTOCOL CITATION

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KEYWORDS

human pluripotent stem cell-derived microglia, tri-culture system model C3, Alzheimer's disease

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MATERIALS TEXT

Materials for Derivation of microglia from hPSCs:

E8 medium:

A	B
Activin A	7.5 ng/ml
BMP4	30 ng/ml
CHIR 99021	3 µM
ROCK inhibitor (Y-27632)	10 µM

Essential 6 medium:

A	B
Activin A	10 ng/ml
BMP4	40 ng/ml
IWP2	2µM

Essential 6 medium:

A	B
Activin A	10 ng/ml
BMP4	40 ng/ml
IWP2	2 µM
FGF2	20 ng/ml

Essential 6 medium:

A	B
VEGF	15 ng/ml
FGF2	5 ng/ml
ROCK inhibitor (Y-27632)	10 µM

Essential 6 medium:

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A	B
VEGF	15 ng/ml
FGF2	5 ng/ml
SCF	200 ng/ml
IL-6	20 ng/ml

Essential 6 medium:

A	B
SCF	100 ng/ml
IL-6	10 ng/ml
TPO	30 ng/ml
IL-3	30 ng/ml

Neurobasal medium:

A	B
B-27 supplement, L-glutamine and BDNF	
Ascorbic acid	
GDNF	
cAMP	
IL-34	100 ng/ml
M-CSF	20 ng/ml

RPMI:

A	B
FBS	10%
L-glutamine	
Penicillin–streptavidin with IL-34	100 ng/ml
M-CSF	10 ng/ml

F12 medium:

A	B
B-27 supplement, l-glutamine and IL-34	100 ng/ml
M-CSF	20 ng/ml

Materials for Cortical neuron protocol:

Essential 8 medium: ROCK inhibitor (Y-27632; [M]**10 Micromolar (µM)**)

Essential 6 medium:

A	B
LDN193189	100 nM
SB431542	10 µM
XAV939	2 µM

Materials for Astrocyte protocol:

Astrocyte induction medium:

A	B
N2, heparin-binding EGF-like growth factor	10 ng/ml
Leukemia inhibitory factor	10 ng/ml

Materials for Fluorescence-activated cell sorting analysis:

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FACS buffer:

A	B
BSA	1%
EDTA	2 mM
DNase I	30 µg/ml
Normocin in PBS	

FACS antibodies and their dilutions:

Human VEGFR2/KDR/Flk-1 PE-conjugated Antibody **R&D Systems Catalog #FAB357P**

APC Mouse Anti-Human CD235a Clone GA-R2 (HIR2) (RUO) **BD Biosciences Catalog #551336**

APC/Cyanine7 anti-human CD41 Antibody **BioLegend Catalog #303715**

PerCP-Cy™5.5 Mouse Anti-Human CD43 Clone 1G10 (RUO) **BD Biosciences Catalog #563521**

FITC Mouse Anti-Human CD45 Clone HI30 (RUO) **BD Biosciences Catalog #560976**

PE anti-human CX3CR1 Antibody **BioLegend Catalog #341604**

APC/Fire™ 750 anti-human CD11b Antibody **BioLegend Catalog #301351** , all at **5 µl** per **100 µl** test.

Materials for Immunohistochemistry, live/dead assay and high-content imaging:

Primary antibodies and their dilutions:

IBA Ab **Wako Catalog #019-19741**

Monoclonal Anti-MAP2 (2a 2b) antibody produced in mouse **Sigma – Aldrich Catalog #M1406**

Purified anti-SPI1 (PU.1) Antibody **BioLegend Catalog #658002**

Anti-PSD95 antibody [6G6-1C9] - Synaptic Marker (ab2723) **Abcam Catalog #ab2723**

Purified anti-GFAP Antibody (Previously Covance catalog# PCK-591P) **BioLegend Catalog #829401**

AQP4 Antibody (C-19) **Santa Cruz Biotechnology Catalog #sc-9888**

Anti-Human/Mouse Bf1 Polyclonal **Takara Catalog #M227**

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Recombinant Anti-TBR1 antibody [EPR8138(2)] (ab183032) **Abcam Catalog #ab183032**

Anti-β-Tubulin III antibody produced in rabbit **Sigma – Aldrich Catalog #T2200**

Anti-Ctip2 antibody [25B6] (ab18465) **Abcam Catalog #ab18465**

Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb #9664 **Cell Signaling Technology Catalog #9664S**

Polyclonal Rabbit Anti-Human C1q Complement Unconjugated Ig fraction 2 mL **Agilent Technologies Catalog #A013602-1**

Secondary antibodies:

Donkey anti-Mouse IgG (H L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 488 **Thermofisher Catalog #A-21202**

Alexa Fluor® 488 AffiniPure Donkey Anti-Chicken IgY (IgG) (H L) **Jackson Immunoresearch Catalog #AB_2340375**

Donkey anti-Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 555 **Thermofisher Catalog #A-31572**

Donkey anti-Mouse IgG (H L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 555 **Thermofisher Catalog #A-31570**

AF647 Donkey anti-mouse IgG **Thermofisher Catalog #A-31571**

Alexa Fluor® 647 AffiniPure Donkey Anti-Chicken IgY (IgG) (H L) **Jackson Immunoresearch Catalog #AB_2340379**

Derivation of microglia from hPSCs 6w 2d 18h

1 Dissociate hPSCs maintained in Essential 8 medium by Accutase to obtain a single-cell suspension.

2 Plate a total of 60,000 cells per cm2 in E8 medium containing activin A (R&D 338-AC; [M]**7.5 ng/ml**), BMP4 (R&D; [M]**30 ng/ml**), CHIR 99021 (Tocris; [M]**3 Micromolar (µM)**) and ROCK inhibitor (Y-27632; [M]**10 Micromolar (µM)**) onto Matrigel-coated plates.

3 After **18:00:00** , change medium to Essential 6 medium containing activin A (**10 ng/ml**), BMP4 (**40 ng/ml**) and IWP2 (Selleck; [M]**2 Micromolar (µM)**). 18h

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4 On day 2, change medium to Essential 6 medium containing activin A (**10 ng/ml**), BMP4 (**40 ng/ml**), IWP2 (**2 Micromolar (µM)**) and FGF2 (R&D; **20 ng/ml**).

5 On day 3, dissociate cultures with Accutase and replate at 60,000 cells per cm2 in Essential 6 medium containing vascular endothelial growth factor (VEGF; R&D) (**15 ng/ml**), FGF2 (**5 ng/ml**) and ROCK inhibitor (Y-27632; **10 Micromolar (µM)**).

6 On day 4, Remove the ROCK inhibitor and change medium to Essential 6 with VEGF (**15 ng/ml**) and FGF2 (**5 ng/ml**).

7 On days 5 and 6, Feed cultures with Essential 6 medium containing VEGF (**15 ng/ml**), FGF2 (**5 ng/ml**), SCF (**200 ng/ml**) and IL-6 (**20 ng/ml**).

8 On days 7 and 9, change medium to Essential 6 with SCF (**100 ng/ml**), IL-6 (**10 ng/ml**), TPO (**30 ng/ml**) and IL-3 (**30 ng/ml**).

9 2w 5d
On day 10, collect the cells in suspension and either (1) co-culture with cortical neurons in Neurobasal medium containing B-27 supplement, l-glutamine and BDNF, ascorbic acid, GDNF, cAMP and IL-34 (**100 ng/ml**) and M-CSF (**20 ng/ml**) for **120:00:00** for direct transition to microglia, or (2) culture in RPMI with 10% FBS, l-glutamine, and penicillin–streptavidin with IL-34 (**100 ng/ml**) and M-CSF (**10 ng/ml**) for **168:00:00** – **168:00:00** until cells were adherent and elongated to transition to primitive macrophages.

10 2w 4d
For serum-free culture, harvest cells in suspension on day 10 and culture in 75% IMDM, 25% F12 medium containing B-27 supplement, l-glutamine and IL-34 (**100 ng/ml**) and M-CSF (**20 ng/ml**) for **168:00:00** – **264:00:00** .

11 1w


Co-culture transitioned macrophages with cortical neurons with the addition of IL-34 and M-CSF for **168:00:00** for upregulation of microglial-specific markers.

12 Use induced pluripotent stem cells (iPSC) lines to test reproducibility of the microglial differentiation (DBR1, IFNAR1, IL-10RB and STAT1) are each separately derived from patient fibroblasts and are reprogrammed using a nonintegrating Sendai viral vector in the Notarangelo laboratory at the National Institutes of Health (NIH).

Cortical neuron protocol 2w 2d

13 Dissociate hPSCs with Accutase and plate at 200,000 cells per cm2 onto Matrigel-coated plates in Essential 8 medium

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with ROCK inhibitor (Y-27632; [M]**10 Micromolar (µM)**).

14 2w 2d

Treat cells with Essential 6 medium containing LDN193189 ([M]**100 Nanomolar (nM)**) and SB431542 ([M]**10 Micromolar (µM)**) for **288:00:00** , with the addition of XAV939 ([M]**2 Micromolar (µM)**) for the first **96:00:00** of differentiation.

15 Feed cultures with N2 medium with 1:1,000 B-27 supplement for an additional week to allow the development of neural progenitor cells.

16 Dissociate neural progenitor cells and replate on poly-l-ornithine/fibronectin/laminin-coated plates and maintain in neurobasal medium, BDNF, ascorbic acid, GDNF, cAMP, l-glutamine and B-27 supplement for neuronal differentiation and maturation.

Astrocyte protocol 4w 5d

17 Differentiate hPSCs into astrocytes according to the method by Tchieu et al.

18 4w 5d

Briefly, pulse cortical neural stem cells with nuclear factor IA through an inducible lentiviral construct for **120:00:00** , after which CD44+ progenitors are sorted and replated and maintained in astrocyte induction medium containing N2, heparin-binding EGF-like growth factor ([M]**10 ng/ml**) and leukemia inhibitory factor ([M]**10 ng/ml**) for a minimum of **672:00:00** .

Fluorescence-activated cell sorting analysis 50m

19 20m

Dissociate cells with Accutase for **00:20:00** and resuspend in FACS buffer containing 1% BSA, [M]**2 Milimolar (mM)** EDTA, [M]**30 µg/ml** DNase I and Normocin in PBS.

20 30m

Wash cells and incubate in FACS buffer with antibody for **00:30:00** **On ice** at **4 °C** in the dark.

21

Wash cells and resuspend in FACS buffer and strained through [M]**40 Micromolar (µM)** caps to eliminate cell clumps.

22

Perform data collection on a BD LSR II and FACSDiva v8. Gating (Supplementary Fig. 2), and complete subsequent

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analysis using FlowJo software v10.5.3.

Droplet-based single-cell RNA-sequencing library preparation and sequencing 3w 3d 0h 20m

23 Prepare four samples for single-cell sequencing at different days of the microglial differentiation: ‘day 6’ at day 6 of differentiation, ‘day 10’ at day 10 of differentiation, ‘day 10 suspension’, which only included cells in suspension at day 10 of differentiation, and ‘microglia’, which included end-stage microglial cells cultured with neurons for 14 d.

24 Prepare ‘Day-6’ and ‘day-10’ samples by treating cultures with Accutase for **00:20:00** to achieve a single-cell suspension. 20m

25 Prepare ‘Day-10 suspension’ by collecting and straining cells in suspension through a **40 Micromolar (µM)** filter to achieve a single-cell suspension.

26 Prepare the ‘microglia’ sample by sorting co-culture models of neurons and microglia for expression of CX3CR1.

27 Resuspend all samples were at 1,000 cells per µl in FACS buffer before sequencing.

28

Perform single-cell sequencing using 10x Genomics Chromium Single Cell 3' Library & Gel bead Kit V2 according to the manufacturer’s protocol.

29 Add an input of 8,700 cells to each 10x channel.

30

Sequence libraries on an Illumina NovaSeq device.

Single-cell RNA-sequencing data preprocessing

31 Process scRNA-seq data using the SEQC processing pipeline.

SEQC generates a cells-by-genes count matrix after read alignment, multimapping read resolution and cell barcode and unique molecular identifier correction.

32 SEQC included 3 steps:

32.1 Step 1: Remove putative empty droplets based on the cumulative distribution of molecule counts for each barcode.

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32.2 Step 2: Derive putative apoptotic cells based on >20% of molecules from the mitochondria.

32.3 Step 3: Identify low-complexity cells as cells where the detected molecules are aligned to a small subset of genes.

The number of cells per sample after SEQC processing was 5,253, 4,320, 5,555 and 4,961, and median library sizes were 19,195, 4,039, 10,126 and 16,716 molecules per cell (day 6, microglia, day 10 and day-10 suspension, respectively.

33 Normalize counts for library size by dividing each gene molecule count by the total number of molecules detected in the cell, then multiplying by 10,000 to convert the original counts to transcripts per 10,000 reads.

34 Log transform data using natural log and a pseudocount of 1.

Cell filtering

35 

For each sample, cluster cells using the PhenoGraph clustering algorithm.

36 Remove clusters of cells with low numbers of detected genes (~200) as putative empty droplets.

37 Remove clusters with high mitochondrial RNA and a low number of detected genes as putative dying cells.

38 Remove four clusters not pertaining to hematopoietic differentiation, including two early mesoderm clusters that expressed low levels of MESP1 and PDGFRA but not KDR, PECAM1 or CDH5, one cluster belonging to the cardiac lineage expressing NKX2.5 and ISL1, and one cluster belonging to mature endothelial cells.

Nearest neighbor graph construction

39 Use principal components to calculate Euclidean distances between cells.

40 Use an adaptive Gaussian kernel to convert Euclidean distances between cells' k-nearest neighbors into affinities, as described by Haghverdi et al.

By using a Gaussian kernel, affinities between cells decrease exponentially with their distance, thereby increasing affinity to nearby cells and decreasing affinity to distant cells compared to the original Euclidean distances. Moreover, by using kernels with cell-adapted widths, differences in densities across regions of the data manifold are accounted for.

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41 Use nearest neighbor graphs as a basis for force-directed graph layouts and diffusion map embeddings.

Clustering and force-directed graph layout

42 Pool data from day 6, day 10 and day-10 suspension samples for trajectory modeling.

43 Perform principal-component analysis on the data, and select the first 20 principal components for further analyses, to reduce noise due to the high degree of dropouts in scRNA-seq.

44 Calculate force-directed graph layouts using the ForceAtlas2 algorithm54, based on the 30-nearest neighbors graph of the data that is constructed as described above.

45 Perform clustering with PhenoGraph, using default parameters.

Diffusion map embedding

46 To approximate the low-dimensional data manifold representing the differentiation trajectory, construct a diffusion map embedding using an adaptive Gaussian kernel-based nearest neighbor graph (k=20; described above).

47 Construction of a diffusion map is a nonlinear method to recapitulate the low-dimensional structure underlying high-dimensional observations.

48 Select the first four diffusion components of the diffusion map for trajectory modeling.

49 Convert the diffusion distances between cells (that is, the Euclidean distances between cells in the 'diffusion map space') subsequently into pseudotime distances between individual cells as described by Haghverdi et al.

While distances in standard diffusion maps are related to a random Markov walk of length 1 along the edges of the 'affinity graph', diffusion distances in multiscale space generalize over random walks of all lengths, thereby better capturing phenotypic similarities and differences between cells.

Trajectory characterization

50 Use Palantir to further characterize the trajectory.

Palantir is a tool that, using pseudotime distances, identifies trajectory end points ('terminal cells') in data of differentiating cells and, moreover, measures entropy in cell phenotypes to measure their plasticity ('differentiation potential') and commitment to specific cell fates ('branch probability').

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51 As the input approximate start cell of the Palantir trajectory, use a random cell from the CDH5-high, KDR-high and PECAM1-high hemogenic endothelium clusters.

The number of neighbors was set to k=20, and the number of diffusion components was set to 4. For all other parameters, default settings were used.

Calculation of gene trends over pseudotime

52 To recover expression trends of individual genes over pseudotime, impute our processed count matrix using MAGIC.

MAGIC is a method to denoise the cell count matrix and fill in zeros due to dropouts, by sharing information across similar cells via data diffusion. MAGIC is run with the number of neighbors set to k=40, the random walk length t=6, and further settings as default.

53 Based on the imputed count matrix, calculate gene trends using generalized additive models as described by Setty et al.

54 Calculate Spearman correlations between unimputed gene counts and pseudotime (Supplementary Fig. 2b) per branch.

For each branch, only those cells that had a branch probability equal to or higher than the branch probability at the start of the trajectory are included.

Diffusion distance distributions

55 To investigate whether cells in the microglia sample were still in the process of differentiating, inspect the distribution of pairwise diffusion distances among the cells.

In accordance with single-cell studies of cell differentiation, we assumed that differentiation happens asynchronously: different cells are assumed to be at different stages of differentiation at one point in time. Therefore, the transcriptomes of differentiating cells are expected to lie on an elongated manifold. In contrast, cells with a homogeneous phenotype are thought to be centered around one (multidimensional) mode, thus lying on an approximately sphere-shaped ‘manifold’. The distribution of pairwise distances between cells, calculated over multiple dimensions, is informative as to the shape of the manifold: if this distribution is unimodal, it suggests that the cells lie on a
spherical manifold.

56 Use a multiscaled diffusion map embedding (described earlier) of the cells to calculate pairwise distances between the cells.

Multiscaled diffusion maps are thought to capture the shape of the manifold well, while reducing levels of noise as

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compared to the nonembedded data.

57 Calculate distances for different numbers (4–9) of diffusion components, to show robustness of the results to the number of components selected.

Comparison to mouse gene signatures

58 For comparison to previously published mouse signatures of EMPs and PMACs25, translate signature mouse gene names that had a one-to-one mouse–human ortholog (as defined by Ensembl BioMarts59) to human gene names.

All other genes were excluded from the analysis. We further excluded genes for which no transcripts were detected in our data. Heat maps showing pseudotime expression trends of signature genes (Figs. 2d and 3i) are based on MAGIC56 imputed expression values.

59 Normalize imputed gene expression for each gene to range between 0 and 1.

60 Include all cells that had a minimum myeloid branch probability of 0.1 in the heat map.

61 Order cells by pseudotime, and cluster genes using centroid clustering.

62 For the macrophage gene signature, append cells from the microglia sample to the trajectory cells and given an artificial pseudotime of 1.1.

Integration into single-cell mouse embryogenesis atlas

63 For integration of trajectory data into a recently published single-cell transcriptomics atlas of mouse gastrulation and early organogenesis, use data of all cells of the hematoendothelial lineage (15,875 cells).

Only genes with a one-to-one mouse–human ortholog (as described earlier) are included in the analysis. To further restrict organism-related bias, the gene set is limited to genes that were highly variable in the reference mouse data.

64 Define highly variable genes as described by Satija et al.

The final number of genes included was 1,356.

65

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To perform further batch correction, use a fast implementation of mutual nearest neighbors batch correction: fastMNN (https://rdrr.io/github/LTLA/batchelor/man/fastMNN.html) performs batch correction on the principal-component matrix instead of the gene expression matrix.

66 Use the first 20 principal components of the pooled data for batch correction.

67 Perform batch correction among samples from within the same time point, after which batch correction is performed between time points.

> The sample order used for fastMNN is: mouse data, late to early, then the human day-10 and day-10 suspension samples (pooled), and finally the day-6 sample.

68 Calculate force-directed graph layout as described earlier.

69 Construct a graph of the clusters of mouse and human data using PAGA.

70 Use only graph edges with a weight of 0.2 or higher for the force-directed layout of the graph.

Data analysis platform

71 Use the SCANPY platform (v1.4) for data analysis.

Immunohistochemistry, live/dead assay and high-content imaging 2h 35m

72 50m

Fix cells in 4% paraformaldehyde for **00:10:00** at **Room temperature** , permeabilize with 0.1% Triton for **00:05:00** , wash with 0.2% Tween-20 in PBS for **00:05:00** and block with 5% donkey serum in 0.2% Tween-20 in PBS for **00:30:00** .

73 30m

Dilute primary antibodies in blocking solution and incubate with the sample **Overnight** at **4 °C**

74 45m

Dilute secondary antibodies (Alexa Fluor 488, 555 and 647) in blocking solution and incubate with the sample at **Room temperature** for **00:45:00** .

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75 Use DAPI stain to identify cell nuclei.

76 30m

Perform the live/dead assay with CC3, with the control of hPSC-derived cortical neurons incubated with 70% methanol for **00:30:00** .

Use ImageExpress Micro Confocal High-Content Imaging System to quantify microglial cell numbers in culture.

77 Take nine fields at ×5 magnification to scan an entire 96-well culture well.

Engulfment of synaptic proteins imaging 18w 4d

78 18w 4d

Co-culture microglia with day-70+ neurons on culture dishes (Ibidi) for up to **720:00:00** and stain with PSD95 and IBA1 (**2400:00:00** in total).

79

Image cultures on the Leica SP8 confocal microscope equipped with white-light laser technology and standard argon lasers (458, 476, 488, 496 and 514 nm) at ×40 magnification.

80 Process and analyze data with Imaris v9.2: a surface volume mask is generated in the IBA1 channel, within which another mask is generated for the PSD95 channel to determine the volume of PSD95 inclusions/volume of IBA1 in a given z-stack.

Phagocytosis assay and surveying assay 1w 0d 21h

81 5h

For the phagocytosis assay, incubate microglial cells or astrocyte controls with Zymosan A bioparticles conjugated with Alexa Fluor 488 for **05:00:00** in an Olympus VivaView fluorescence incubator microscope.

82 1w 0d 16h

For the surveying assay, infect microglial cells with a lentiviral construct expressing GFP and co-culture with day-50 cortical neurons for **168:00:00** , then incubate in an Olympus VivaView fluorescence incubator microscope for **16:00:00** .

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83 Compile time-lapse imaging at **00:02:00** per frame. 2m

RNA sequencing

84 Sort approximately 50,000-100,000 hPSC-derived microglia from three different hPSC lines, H1, H9 and the WT iPSC line SA241-1, from neuronal co-cultures by expression of CX3CR1.

85 Extract RNA using the Zymo RNA Micro Kit.

86 Obtain RNA from primary human microglia after sorting postmortem tissue from the frontal and temporal lobes from patients aged 60-77 years.

87 Submit all samples to the Memorial Sloan Kettering Cancer Center (MSKCC) Integrated Genomics Core for paired-end SMARTER sequencing and 30-40 million reads.

88 

Perform analysis via a standard pipeline through the MSKCC Bioinformatics core: FASTQ files were mapped using the rnaSTAR aligner.

89 Process Output SAM files using PICARD tools.

90 Process the mapped reads using HTSeq to compute a raw expression count matrix, which is then processed using DESeq from R/BioConductor to analyze differential expression between samples.

Primary human microglia 1d 15h

91 Provide brain tissue by the Netherlands Brain Bank (NBB).

> Informed consent for brain autopsy, the use of tissue and use of clinical information is obtained premortem. The procedures of the NBB are in accordance with all national laws and the procedures have been approved by the ethics committee of the VU University Medical Center (Amsterdam, Netherlands).

92 Collect brain tissues from one male and three female donors without a history of a neurological or psychiatric disorder in the age range of 60–77 years.

93 Isolate microglia from the medial frontal gyrus and temporal superior gyrus as described before.

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94 In short, collect **2 g** - **10 g** of tissue within **13:00:00** after death and store in Hibernate medium **On ice** (1d 15h), and start the isolation procedure within **02:00:00** - **24:00:00** after autopsy.

95 Generate a single-cell suspension by dissociating the tissue mechanically through a metal sieve, followed by an enzymatic digestion using collagenase type I (**3000 U/ml** ; Worthington) and DNase I (**200 µg/ml** ; Roche).

96 Purify microglia using a Percoll gradient and positive selection using CD11b-conjugated magnetic microbeads (Miltenyi Biotec).

97 Lyse Microglia in RLT buffer (QIAGEN), and extract RNA according to the protocol provided by the RNeasy Mini Kit (QIAGEN)

Gene ontology analysis and gene-set enrichment analysis

98

Perform GO analysis using http://geneontology.org.

The analysis type is PANTHER overrepresentation test (released 04 April 2020), the reference list was Homo Sapiens (all genes in database), the annotation dataset was GO biological process complete, and the test type is Fisher with false discovery rate correction.

99

Perform GSEA using the GSEA software (v4.0.3) from the Broad Institute, using the clusters defined in Matcovitch-Natan et al. defining microglial developmental stages.

Comparison with publicly available microglial datasets

100

Integrate publicly available datasets from the following studies according to the methodology by Grubman et al. before MDS.

The following datasets were included: Muffat et al. (GSE85839, 8 samples), Douvaras et al. (GSE97744, 14 samples), Abud et al. (GSE89189, 12 samples), Ormel et al. (GSE102335, 9 samples) and Galatro et al. (GSE99074, 38 samples).

101 Integrate a total of 17 samples from our study (13 hPSC-derived microglia and 4 adult primary microglia).

102 Briefly, convert count matrices to log2 reads per kilobase million, and remove the differences between batches using the removeBatchEffect from limma, specifying each dataset as a batch, as well as specifying common groups as design

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(adultmg, hPSC-microglia and fetal microglia).

103 Perform MDS using the plotMDS function from limma.

Tri-culture system, co-culture ratios and LPS assay 18w 4d 1h 5m

104 Differentiate cortical neurons from hPSCs and replate at 200,000 cells per cm2 on plates coated with poly-l-ornithine/fibronectin/laminin and allow to mature for **1200:00:00** - **1680:00:00** in NB/BAGC. 17w 1d

105 Dissociate astrocytes that differentiated from hPSCs with Accutase for **00:20:00** - **00:30:00** and then plate on top of the neurons at 25,000 cells per cm2 and are allowed to settle for **96:00:00** in NB/BAGC. 4d 0h 50m

106 Dissociate microglia that differentiated from hPSCs with Accutase for **00:10:00** and plate on top of the astrocyte/neuron culture at 50,000 cells per cm2 in NB/BAGC with IL-34 (**100 ng/ml**) and M-CSF (**20 ng/ml**). 10m

107

Change medium every other day with fresh addition of IL-34 and M-CSF.

> Other co-cultures included microglia and neurons (50,000 microglia per cm2 and 200,000 neurons per cm2), astrocytes and neurons (25,000 astrocytes per cm2 and 200,000 neurons per cm2) and neurons only (200,000 neurons per cm2).

108 After culture for a minimum of **168:00:00** , add LPS at **1 µg/ml** for **72:00:00** . 1w 3d

109 5m

Collect culture medium and spun down at **2000 rpm** for **00:05:00** .

110 Freeze the supernatant at **-80 °C** until further analysis.

Cytokine ELISA

111 Following the manufacturer's protocols, analyze culture supernatants for C3 and C1Q using the Millipore Luminex Multiplex Kit on the FlexMap 3D system.

112 Send supernatants from the ±LPS assay, as well as the C3+/- assay on WT and C3 KO microglia, to Eve Technologies for multiplexed analysis of 14 inflammatory cytokines using the human high-sensitivity T cell discovery array 14-plex.

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Conditioned medium assays 1w 1d

113 Harvest medium from hPSC-derived microglia–neuron co-cultures after **48:00:00** to represent MCM. 2d

114 2d

Add MCM to astrocyte-neuron co-cultures for **48:00:00** before the cells are harvested for C3 expression analysis by qPCR.

115 Harvest medium from hPSC-derived astrocyte–neuron co-cultures after **48:00:00** to ACM. 2d

116 2d

Add ACM to microglia-neuron co-cultures for **48:00:00** before the cells are harvested for C3 expression analysis by qPCR.

Cytokine addition assays 4d

117 2d

Add human C3 (Millipore; [M]**1 µg/ml**) to microglia-neuron or astrocyte-neuron co-cultures for **48:00:00** before cells are harvested for C3 expression analysis.

118 2d

Add human TNF-α (R&D; [M]**100 ng/ml**) and IL-6 (R&D; [M]**100 ng/ml**) to astrocyte-neuron co-cultures for **48:00:00** before cells are harvested for C3 expression analysis.

Cell line engineering

119 CRISPR–Cas9 knockout of C3. The PX458 vector65 containing the guide 5' TCTGCACTATCCAGGTA 3' is nucleofected into H1 hESCs.

120 Sort cells on the basis of GFP expression and culture as single-cell clones in E8 medium with the cloneR supplement.

121 Pick clones onto replicate plates, and extract genomic DNA using Bradley Lysis Buffer and Proteinase K treatment.

122

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Amplify a 450-bp PCR product around the guide RNA cut site, and screen the clones for indels by Sanger sequencing.

123 Pick and expand, karyotype and differentiate clones with indels subsequently into microglia for further validation by ELISA for a lack of C3 protein secretion.

124 Generate AD lines. APPSWE+/+ and WT iCas9 lines using the guide RNA and oligonucleotides from Paquet et al. in an H9 background.

Primers

125

Primers used for qPCR included: C1QA (QIAGEN QT00997745), C3 (F 5' AAAAGGGGCGCAACAAGTTC 3', R 5' GATGCCTTCCGGGTTCTCAA 3'), CX3CR1 (F 5' TGGGGCCTTCACCATGGAT 3', R 5' GCCAATGGCAAAGATGACGGAG 3'), TMEM119 (F 5' CTTCCTGGATGGGATAGTGGAC 3', R 5' GCACAGACGATGAACATCAGC 3'), P2RY12 (F 5' AAGAGCACTCAAGACTTTAC 3', R 5' GGGTTTGAATGTATCCAGTAAG 3') and GPR34 (F 5' GAAG ACAATGAGAAGTCATACC 3', R 5' TGTTGCTGAGAAGTTTTGTG 3').

Statistics and reproducibility

126

All data presented in this study are representative of at least three independent experiments, including IF panels, ELISA results and FACS data. Western blots and gel electrophoresis are repeated at least twice. No statistical methods are used to predetermine sample sizes, but our
sample sizes are similar to those reported in previous publications. Cells are randomly assigned into experimental culture groups (TRI, M/N, A/N and N) after accounting for their respective genotypes (APPSWE+/+, WT and C3KO). Data collection and analysis are not performed blind to the conditions of the experiments because all quantified data are collected in an automated fashion (ELISA, FACS and ImageExpress) such that bias would not be introduced. Data distribution is assumed to be normal, but this was not formally tested. Data are presented as the mean±s.d. All statistical analyses are performed using GraphPad Prism v.8.0.1: one-way ANOVA with Tukey's post hoc or Sidak's tests to compare multiple groups, two-way ANOVA with Tukey's or Bonferroni post hoc tests to compare multiple groups with two independent variables, and an unpaired
two-tailed Student's t-test to compare two groups. Statistical differences are considered significant with $P<0.05$ as indicated in figure legends. Samples are excluded if data collection failed, for example due to machine clogs during FACS or ELISA collection (one sample in Fig. 4g) or after the outlier test (two samples in Extended Data Fig. 9b). Four RNA samples from three postmortem human brain tissue samples are used for RNA-seq experiments; donors were aged 77 (male), 60
(female) and 84 (female) years.

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