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

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.

Over recent years, neuroinflammation has been increasingly implicated in the progression of various neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, as well as in aging. Microglia are thought to be key players in triggering an inflammatory state in the brain that may be beneficial initially but can precipitate or exacerbate disease pathology when prolonged. Other glial cells such as astrocytes interact with microglia and may further contribute to aberrant inflammation and neurotoxicity. However, parsing out cellular cross-talk in vivo and understanding species-specific differences in the neuroinflammatory response remain major challenges in the field. hPSC technology has the potential to overcome those challenges and to present a fully human, defined and scalable platform to study neuroinflammation. An essential requirement for such an hPSC-based model are differentiation strategies that can reproducibly generate pure populations of microglia, astrocytes and neurons in a synchronized, robust, efficient and timely manner. Protocols based on dual inhibition of SMAD signaling allow for the efficient production of highly pure neural precursors and postmitotic neurons from hPSCs. Likewise, we have recently reported a strategy to rapidly derive pure populations of astrocytes from hPSCs. In contrast, several protocols have been published for generating microglia-like cells from hPSCs; but those approaches commonly rely on embryoid body formation and are poorly defined with respect to ontogeny, require manipulations such as hypoxia for patterning, or necessitate cell sorting for purity. Importantly, current microglial differentiation protocols are not specifically optimized towards primitive hematopoiesis as defined by early induction of KDR⁺CD235A⁻ hemangioblasts. Recapitulating those early developmental steps in vitro is important as the microglial lineage entirely traces back to primitive hematopoiesis.

Results
Narrow window for primitive hematopoietic patterning of hPSCs. Here we present a new strategy to direct the differentiation of hPSCs into microglial precursors via primitive hematopoiesis (Fig. 1a). First, we patterned hPSCs towards primitive streak mesoderm by activating WNT signaling via treatment with the GSK3β inhibitor CHIR99021. We then inhibited WNT signaling by using the porcine inhibitor IWP2 and concurrently activated Nodal signaling, mimicked by exposure to activin A. Those conditions promoted the generation of KDR⁺CD235A⁻ primitive hematopoietic hemangioblasts versus KDR⁺CD235A⁺ definitive hematopoietic precursors consistent with the paradigm proposed by Sturgeon et al. Remarkably, we observed that WNT inhibition must occur within a very narrow developmental window, limited to 18–24 h after WNT activation, to efficiently generate a KDR⁺CD235A⁻ population (Fig. 1b). After optimizing cell density and small-molecule concentrations, WNT activation for 18 h followed by WNT inhibition and Nodal activation for ~2 d (2 d + 6 h) yielded ~27% KDR⁺CD235A⁻ cells by day 3 of differentiation (Fig. 1c). We next asked whether KDR⁺CD235A⁻ hemangioblasts versus the KDR⁺CD235A⁻ definitive precursors can give rise to hematopoietic cells under those conditions.
culture conditions. The KDR^+CD235A^+ hemangioblast population produced CD41^+CD235A^+CD43^+ hematopoietic cells within 3 days after isolation by cell sorting (day 6 of hPSC differentiation) in the presence of minimal hematopoietic cytokines (Fig. 1d). In contrast, the KDR^+CD235A^- population failed to produce hematopoietic cells. Those data are consistent with reports suggesting that definitive precursors give rise to hematopoietic cells only at later stages of differentiation, under hypoxic conditions, or in the presence of additional hematopoietic cytokines.

By day 7 after replating (day 10 of differentiation), the KDR^+CD235A^- fraction yielded 41% CD45^+ cells, the population that later gives rise to microglia. Other populations which arose include uncommitted CD41^+CD235A^+CD43^+ primitive erythromyeloid progenitors (EMP), CD41^+ megakaryocytes and CD235A^+ erythrocytes (only when treated with erythropoietin (Extended Data Fig. 1a)). Primitive EMPs, megakaryocytes and erythrocytes are all lineages produced during primitive hematopoiesis. The definitive KDR^+CD235A^- population still produced no hematopoietic...
cells, demonstrating all hematopoietic cells produced by day 10 of differentiation were derived from KDR\textsuperscript{+}CD235A\textsuperscript{-} hemangio blasts (Fig. 1d). Interestingly, unsorted samples containing both the KDR\textsuperscript{+}CD235A\textsuperscript{-} and KDR\textsuperscript{+}CD235A\textsuperscript{-} populations produced hematopoietic cells at nearly the same efficiency as the sorted KDR\textsuperscript{+}CD235A\textsuperscript{-} population, suggesting that KDR\textsuperscript{+}CD235A\textsuperscript{-} cells do not need to be purified for the robust production of hematopoietic cells (Fig. 1d). This finding streamlines the differentiation process by eliminating the cell sorting step and instead allows for the simple replating of the mixed population at day 3 in the presence of hematopoietic cytokines. Almost 60% of the cells at day 10 of differentiation are of hematopoietic identity, forming floating colonies in semi-suspension above a VE-CADHERIN\textsuperscript{+} hemogenic endothelium (Extended Data Fig. 1b,c).

Developmental stages of hPSC-derived microglia are similar to in vivo differentiation trajectory. We next performed single-cell RNA sequencing (scRNA-seq) at day 6 and day 10 of differentiation (in the presence of erythropoietin) to fully characterize the heterogeneity of the cells produced, and to identify the developmental trajectory of the transitioning cellular states given the paucity of data on human primitive hematopoiesis in current literature. Cells from pooled day-6 and day-10 scRNA-seq data were grouped into separate clusters using the PhenoGraph clustering algorithm\textsuperscript{1}, and a force-directed graph layout of the cells was generated to visualize the data (Fig. 2a). The more mature hematopoietic populations of erythrocytes, megakaryocytes and macrophage precursors (PMACs) were identified by the trajectory analysis tool Palantir\textsuperscript{22} as three distinct arms stemming from a common primitive EMP population, mimicking the trajectory of primitive hematopoiesis. To investigate the differentiation status of individual cells in the trajectory, we used Palantir’s ‘differentiation potential’ calculation\textsuperscript{21}, which views differentiation as steps through a Markov chain that represents by a lack of available Markov paths from a specific cell to other arms of differentiation. We found that the highest differentiation potential fell within the hemogenic endothelial clusters and the primitive EMP-like cells at the center of the map, and the lowest fell at the more mature hematopoietic clusters at the ends of the three arms (Fig. 2b). We further used Palantir to model the expression trends of key genes over pseudotime along the trajectory of each arm and found that the erythrocyte arm expressed the signature genes of embryonic hemoglobin (HBE1) and GYPΑ, the megakaryocyte arm expressed key megakaryocytic genes of ITGA2B, ITGB3 and GP1BA, and the macrophage precursor arm expressed CSF1R, PTPRC, SPI1 and CX3CR1 (ref. \textsuperscript{25}; Fig. 2c). In addition, we assessed expression of HOXA genes and CDX4 (markers of definitive hematopoiesis)\textsuperscript{24} along all three arms and found absent expression (CDX4 was undetectable) or very low expression, further indicating that our hematopoietic cells are of the primitive hematopoietic lineage (Extended Data Fig. 1d). Complementing this imputed gene trend modeling, we plotted the unimputed expression of those signature genes against pseudotime and obtained comparable results in which each arm enriched for the expression of its corresponding identity genes while showing near-absent HOXA expression (Extended Data Fig. 1e,f). Thus, we ensured that the trends as inferred by Palantir from imputed data were also observed in nonimputed data.

We compared the composition and trajectories of our differentiation to data from in vivo mouse development to verify whether we were indeed producing microglial precursors in a dish. We isolated the expression profiles of the cells along only the macrophage precursor arm and compared their gene expression to the gene signatures of primitive EMPs and pre-macrophages (megakaryocytic precursors known as PMACs) derived from in vivo profiling of mouse microglial development\textsuperscript{21} (Fig. 2d). At pseudotimes of 0.16–0.8, which correspond to the portion of the trajectory in which the early primitive EMP and primitive EMP/PMAC clusters fall (Fig. 2a,b), our cells showed higher expression of the mouse primitive EMP signature. At pseudotimes of 0.8 and above, which correspond to the mature PMAC1/2 clusters, our cells showed higher expression of the mouse PMAC signature\textsuperscript{21}. These trends were confirmed using unimputed data as well (Extended Data Fig. 2a). We note that, of the 141 genes present in the mouse EMP and PMAC signatures, 130 genes were present in our scRNA-seq dataset. A subset of these genes was separately expressed in the human primitive EMP or PMAC clusters, with some genes expressed in both (Fig. 2d), likely representing human-to-mouse species-specific differences. To demonstrate specificity, we isolated the expression profiles of cells along the megakaryocyte arm or the erythrocyte arm and compared those genes to the gene signatures of mouse primitive EMPs and PMACs. We noticed a slight increase in the expression of primitive EMP genes but no increased expression in PMAC genes, showing that the stage-specific enrichment of the primitive EMP and PMAC signatures along the macrophage trajectory is unique to this lineage (Extended Data Fig. 2b,c).

We also mapped our data onto whole-mouse gastrulation scRNA-seq data\textsuperscript{26} by using the MNN batch correction algorithm\textsuperscript{27}. We found that our PMAC cluster mapped near the mouse gastrulation...
myeloid cluster (Fig. 2e) and mapped most closely to populations that emerged at embryonic day (E) 8.5, one day before microglial precursor cells initially seed the mouse brain25 (Fig. 2f). Taken together, these data demonstrate the presence of primitive EMPs and EMP-derived PMACs in our cultures, which closely correlate to mouse myeloid cells at the time of early microglial development, indicating that our in vitro hPSC differentiation strategy follows the postulated developmental road map for microglia.

**Two methods to generate microglia from hPSCs.** We next established two separate methods in the functional maturation of primitive EMPs or PMACs into microglia (Fig. 3a). The first method...
...mimics the in vivo developmental trajectory during which microglial precursors seed the brain and develop into microglia within the neural environment. To recapitulate this paradigm, we harvested cells in suspension at day 10 and directly co-cultured them with postmitotic, day-30 hPSC-derived cortical neurons in the presence of interleukin (IL)-34 and macrophage colony-stimulating factor (M-CSF), cytokines important for microglial survival and maturation. Additionally, RNA-seq of the hPSC-derived cortical neurons showed that they themselves express multiple cytokines that can support microglial maturation, including IL-34, CSF1, CX3CL1, CD200 and expression of TGFB2, which has been specifically identified as a growth factor critical for maintaining a homeostatic phenotype for microglia in culture (Extended Data Fig. 3a). Approximately 50,000 cells in suspension were seeded for every 300,000 cortical neurons, yielding ~16% hematopoietic cells in co-culture at the time of plating. Remarkably, within 4 d of co-culture, adherent, ramified, IBA1+ and PU.1+ microglia-like cells emerged (Fig. 3b(i)). By 10 d of co-culture, IBA1+ cells were evenly distributed throughout the neuronal culture (Fig. 3b(ii)). These cells were also CX3CR1+ and made up more than 30% of the co-cultures as early as day 5, indicating ongoing cell proliferation (Fig. 3c).

To address whether other primitive hematopoietic lineages emerge in these co-cultures, we generated primitive hematopoietic cells expressing green fluorescent protein (GFP) from a constitutively active nuclear GFP hPSC line (MEL1 GPI:H2B-GFP; Supplementary Fig. 1) and co-cultured these cells with hPSC-derived cortical neurons. At day 6 of co-culture, we found that the majority of GFP+ cells were CD45+, indicating that they differentiated along a microglial rather than megakaryocytic or erythrocytic trajectory. Of these, 82% expressed CX3CR1, indicating that most CD45+ cells had already transitioned towards microglia (Fig. 3d). Approximately 15% of GFP+ cells were not CD45+. Half of those cells were immature CD41+CD235A+ primitive EMPs, whereas the other half were negative for these markers, possibly indicating an even earlier hematopoietic-committed stage. These data demonstrate that co-culturing day-10 primitive EMPs and PMACs with cortical neurons yields a robust population of microglial cells within 4 d, although small populations of uncommitted hematopoietic cells persist.

To derive an even more pure and synchronized population of microglia, we developed a second strategy of maturing microglia from the progenitor stage without co-culture (Fig. 3a(iii)). We took the bulk population of hematopoietic cells in suspension at day 10, followed by exposure to either serum-containing medium (RPMI + 10% serum with the addition of IL-34 and M-CSF) or by using defined, serum-free conditions (IMDM/F12 with the addition of IL-34 and M-CSF) for 7–11 d. At 4 d of culture, half of the cells had transitioned to the primitive macrophage stage, with 50–60% expressing CD11B (mature macrophage/microglial marker) and CX3CR1 (restricted to tissue-resident macrophages such as microglia). By 11 d of culture, close to 99% of the cells expressed CD11B and over 85% expressed CX3CR1 (Fig. 3e). At this stage, all cells were adherent, displayed an elongated morphology and were PU.1+ (Extended Data Fig. 4a,b). In contrast, primary human peripheral blood mononuclear cells (PBMCs), matured in parallel under the same culture conditions, expressed CD11B but largely lacked CX3CR1 expression (Fig. 3e). We found this protocol to be highly reproducible in more than ten different hPSC lines, showing high CD45+ efficiency at day 10 of differentiation and resulting in a pure population of IBA1+ primitive macrophages by day 21 (Extended Data Fig. 4c–e).

We co-cultured the resulting pure population of primitive macrophages with hPSC-derived cortical neurons to fully transition these cells to microglia. After 4 d of co-culture, the microglial cells appeared ramified and continued to be IBA1+ (Fig. 3f). When compared to matured PBMCs that were co-cultured with neurons, the microglia-like cells had lower levels of CD45 and maintained CX3CR1, whereas the PBMC-derived cells were CD45+ in the absence of CX3CR1 (Fig. 3g). Additionally, scRNA-seq on the sorted hPSC-derived microglial cells revealed that they represent a homogeneous cell population devoid of undifferentiated precursors (Extended Data Fig. 4f,g). Pairwise distances calculated between cells in the microglial sample after diffusion map embedding fell in a clean unimodal distribution, indicating transcriptional variance among these cells is centered around one rather than multiple phenotypes (Extended Data Fig. 4f). In contrast, the distribution of pairwise diffusion distances calculated between cells at day 10, before co-culture, show multiple distinct peaks indicating the heterogeneous nature of these cells (Extended Data Fig. 4g).

We next asked whether either of the two derivation methods yielded cells transcriptionally more similar to primary human microglia. We assessed the expression of signature microglial genes in the hPSC-derived microglial cells sorted from co-culture after 14 d using CD45+CX3CR1+ as compared to primary microglial cells...
cultured in vitro (cDNA from Celprogen\textsuperscript{31,32}; Fig. 3h). hPSC-derived microglial cells derived from either method expressed the signature microglial genes of \textit{TMEM119}, \textit{C1QA}, \textit{CX3CR1} and \textit{GPR34} at similar levels to the primary microglia cultured in vitro, whereas PBMCs did not express these markers. We also compared hPSC-derived microglia to acutely isolated primary adult human microglia from postmortem tissue via bulk RNA sequencing. Following unsupervised hierarchical clustering, we found that both methods yielded
microglial cells that cluster with primary human microglia obtained from postmortem cortical brain tissue (frontal and temporal, aged 60–77 years old; Fig. 3i). However, neither method fell exactly in the same sub-branch as the acutely isolated adult primary human microglia. Gene ontology (GO) pathway analysis on the genes differentially expressed between the hPSC-derived microglia and acutely isolated adult primary microglia (Extended Data Fig. 5a,b) revealed that the adult microglia were enriched in cell cycle transition and immune activation pathways, potentially because these cells were isolated from an in vivo, aged environment, whereas hPSC-derived microglia were enriched in cell differentiation and projection neuron developmental pathways, potentially due to contaminating hPSC-derived cortical neuronal cells sorted from co-culture (Extended Data Fig. 5c,d). We also performed gene-set enrichment analysis (GSEA) using seven stage-specific signatures of developing microglia to test whether the hPSC-derived microglial cells were at an earlier developmental stage than the acutely isolated adult primary microglia. Indeed, we found that the hPSC-derived microglia enriched for the postnatal microglial gene signatures, whereas the acutely isolated adult primary microglia enriched for the adult microglial gene signatures, further explaining why hPSC-derived microglia did not cluster exactly with acutely isolated adult primary microglia (Extended Data Fig. 5e–h). Finally, we also compared the expression of signature microglial genes in hPSC-derived microglia to that in the acutely isolated adult primary microglia using normalized counts from the RNA-seq data (Extended Data Fig. 3b). The majority of microglial-specific markers were expressed in the hPSC-derived microglia at similar levels to those in acutely isolated adult microglia (namely GAS6, GPR34, PROS1 and C1QA). However, TMEM119, P2RY12 and SALL1 were expressed at much lower levels in the hPSC-derived microglia than the acutely isolated adult primary microglia. We postulate that this is due to the in vitro culture of the hPSC-derived microglial cells because these genes have been shown to be downregulated during in vitro culture, and we have shown that our cells express more similar levels of these genes when compared to in vitro cultured primary microglia (Fig. 3h). We also postulate that the differences in TMEM119, P2RY12 and SALL1 expression come from the postnatal-like stage of the hPSC-derived microglia as compared to the acutely isolated adult microglia. In fact, TMEM119, P2RY12 and SALL1 are all found in the adult 1 and adult 2 panels of gene signatures (Extended Data Fig. 5g,h).

Finally, we compared the transcriptome of our hPSC-derived microglia to that of previously published microglial differentiation with publicly available RNA-seq datasets using multidimensional scaling (MDS) following the methodology used by Grubman et al. (Extended Data Fig. 6a,b). We also included the transcriptomes of the fetal and adult primary microglia used as comparisons in these studies, and we added the datasets of an additional study that specifically profiled acutely isolated adult primary microglia from human postmortem brain tissue. In our analysis, dimension 1 versus dimension 2 separated the adult primary microglia from the fetal primary microglia/hPSC-derived microglia (Extended Data Fig. 6a), whereas dimension 2 versus dimension 3 separated out the serum-cultured fetal and adult primary microglia used by Abud et al. (Extended Data Fig. 6b). We found that our hPSC-derived microglia (mg1 or mg2) clustered closely to the microglia differentiated from other protocols in both dimension 1 versus 2 and dimension 2 versus 3, and nearer to the fetal microglia as opposed to the adult microglia (Extended Data Fig. 6a). This suggests that our hPSC-derived microglia are highly similar to published differentiated microglia and are more similar to fetal primary microglia than adult.

We next evaluated functional similarities between hPSC-derived microglial cells and primary microglia. We observed that hPSC-derived microglial cells in co-culture with neurons survey their environment, retracting and extending their processes to sample the surrounding neurons akin to homeostatic microglia in vivo (Supplementary Videos 1 and 2). When challenged with the yeast antigen zymosan, the cells were also able to perform efficient phagocytosis, as compared to an astrocyte control (Extended Data Fig. 7a,b and Supplementary Video 3). Finally, another role of microglial cells is to prune synapses in the developing brain. When co-cultured with mature hPSC-derived neurons forming synapses (day 70 and older; Extended Data Fig. 7c,d), microglial cells showed inclusions containing synaptic material upon confocal imaging (Fig. 3(i)). While there were more inclusions that contained general neuronal material tagged with red fluorescent protein (RFP), the number of inclusions specifically composed of synaptic materials was 1–2% (Fig. 3(j)), resembling the basal level of synaptic uptake reported for primary microglial cells during homeostasis.

Tri-culture system models neuroinflammatory axis between microglia and astrocytes. The ability to efficiently generate nearly pure microglia within 25 d of differentiation, with properties that are similar to their primary counterpart, set the stage to build a functional, fully hPSC-derived tri-culture platform composed of human microglia combined with similarly pure populations of human astrocytes and cortical neurons. We generated these co-cultures using a tri-culture system composed of human primary microglial cells and astrocytes. First, we adapted our primary microglial cells to survive in a fully serum-free condition using a reduced medium containing GDNF and cAMP (NB/BAGC) and optimized the ratio of cells via a tri-culture system (Methods): tri-culture, microglia and neurons, astrocytes and neurons. After 1 week of tri-culture, cell ratios stabilized; the microglial number decreased, as not all plated cells attached, and the astrocyte number increased due to proliferation, resulting in a final ratio close to 1:11:20 of microglia:astrocytes:neurons. Base medium culture conditions were optimized, in the presence of IL-34 and M-CSF, to reduce production of baseline inflammatory cytokines using complement C3 production as readout (Extended Data Fig. 8b). The condition of Neurobasal medium with brain-derived neurotrophic factor (BDNF), ascorbic acid (AA), glial cell line-derived neurotrophic factor (GDNF) and cAMP (NB/BAGC) resulted in very low baseline C3 induction with excellent neuronal survival and maintenance. After 1 week, tri-cultures showed ramified IBA1 microglial cells and many GFAP astrocyte processes interacting with MAP2 cortical neurons (Fig. 4e). The tri-cultures were largely devoid of any apoptotic cells positive for cleaved caspase-3 (CC3), confirming robust survival of all three cell types (Fig. 4f and Extended Data Fig. 8c).

To test whether the tri-culture can recapitulate a neuroinflammatory axis between microglia, astrocytes and neurons, we challenged our system with the inflammatory stimulus lipopolysaccharide (LPS) to pharmacologically model a neuroinflammatory state. We focused on the production of complement C3 as a surrogate marker of neuroinflammation because recent literature suggests that it is increased during several neuroinflammatory states including aging and neurodegenerative disorders such as Alzheimer’s disease. In Alzheimer’s disease, C3 is directly implicated in causing aberrant synaptic pruning. We first assessed C3 secretion by enzyme-linked immunosorbent assay (ELISA) under various co-culture conditions using established plating ratios (Fig. 4a; Methods): tri-culture, microglia and neurons, astrocytes and neurons, and neurons only.
At baseline, C3 was only present in cultures that contained microglia, while C3 levels were extremely low in astrocyte/neuron co-cultures and undetectable in neuron-only cultures. Interestingly, in tri-cultures, the baseline C3 levels were dramatically higher than in microglia/neuron-only cultures, suggesting a potentiation of C3 secretion via cellular cross-talk in the presence of both microglia and astrocytes (Fig. 4g). After LPS treatment, C3 was increased in all cultures containing microglia, but again greatly potentiated under tri-culture conditions (Fig. 4g). To rule out the possibility that such potentiation could simply reflect an increase in microglial numbers, we quantified IBA1+ cells by immunofluorescence (IF) using a high-content imaging microscope. IBA1+ cells were actually somewhat decreased in tri-cultures versus microglia/neuron co-cultures ruling out an increase in microglial numbers as the cause of higher C3 levels in tri-culture (Extended Data Fig. 9a). We further corroborated that LPS stimulation induces an inflammatory state beyond C3 secretion by observing increased levels of classical inflammatory cytokines by ELISA such as IL-6, TNF-α, IL-1β, IFN-γ and GM-CSF, of which IL-6 and TNF-α showed potentiation in tri-culture as well (Extended Data Fig. 9b).

To define the key cellular players contributing to C3 potentiation in tri-culture, we next generated a C3 knockout (KO) hPSC line using CRISPR–Cas9 (Extended Data Fig. 9c,d), which showed a complete lack of C3 production after differentiation to microglia (Extended Data Fig. 9e,f). We differentiated this line into C3 KO astrocytes and C3 KO microglia and generated tri-cultures that contained C3 KO astrocytes, WT microglia and WT neurons (C3KO/WT) or WT astrocytes, C3 KO microglia and WT neurons (C3KOM). The number of microglia scored by the percentage of IBA1+/DAPI (3–5%) and the number of astrocytes scored by the percentage of GFAP+/DAPI (>30%) were similar across the WT tri-culture, C3KO/WT and C3KOM cultures (Extended Data Fig. 9g).

C3KO/WT cultures at baseline and upon LPS stimulation showed reduced C3 levels as compared to WT tri-cultures but higher levels than microglia/neuron-only cultures, indicating that both astrocytic C3 and increased microglial C3 contribute to overall C3 potentiation in WT tri-culture (Fig. 4h). These data demonstrate reciprocal signaling between microglia and astrocytes in tri-culture, in which microglia activate astrocytes, and astrocytes reactivitate microglia. We further corroborated evidence for such reciprocal signaling by testing whether conditioned medium from astrocyte/neuron or microglia/neuron co-cultures can induce C3 expression in microglia or astrocytes respectively. Indeed, microglia-conditioned medium (MCM) induced C3 expression in astrocytes, indicating the ability of microglial-secreted factors to activate astrocytes, and microglia, indicating the ability of astrocyte-secreted factors to activate microglia (Extended Data Fig. 9b).

Interestingly, in C3KOM cultures at baseline or stimulated with LPS, C3 levels were very low, suggesting that microglia must express C3 to effectively induce astrocytes to secrete C3 in tri-culture (Extended Data Fig. 9i). We asked whether this is due to C3 itself acting as an inductive factor for C3 expression. We added C3 to astrocyte/neuron cultures and found that it did not induce C3 expression in astrocytes; however, adding C3 to microglia/neuron cultures did increase C3 expression (Extended Data Fig. 9i). These data indicate that C3 is itself a feed-forward mediator of C3 expression in microglia, which is normally secreted by both microglia and astrocytes in tri-culture. We next asked whether the collapse of C3 in C3KOM tri-cultures could also be due to an altered state in C3 KO microglia that is less primed to activation, such that C3 KO microglia act as a ‘resistor’ within the inflammatory loop. Indeed, when examining a panel of cytokines secreted by WT or C3 KO microglia at baseline or upon the addition of inflammatory C3, we found that C3 KO microglia were unable to induce secretion of two inflammatory cytokines, TNF-α and IL-6 (Fig. 4i). Conversely, TNF-α but not IL-6 was able to directly induce C3 expression when added to astrocyte/neuron cultures, identifying it as one of the inducers of C3 secretion by microglia upon activation (Fig. 4i).

These data characterize cellular cross-talk between microglia and astrocytes in an inflammatory loop present at baseline and exacerbated upon pharmacological induction of a neuroinflammatory state. In this loop, C3-producing microglia are the initiating cell that signal to astrocytes to produce C3, which in turn re-induce microglia to produce more C3. Among the milieu of mediators that facilitate this cross-talk, we identified C3 secreted from microglia and astrocytes as one such feed-forward inducer of C3 in microglia, as well as TNF-α secreted upon microglial activation as a C3 inducer in astrocytes (Fig. 4k). These studies demonstrate the power of the hPSC-derived tri-culture system to identify an emergent cellular property upon culture of all three cell types together (C3 potentiation), the major cellular contributions to this potentiation (astrocytic activation and microglial reactivation), as well as two key cytokine mediators that facilitate this potentiation (C3 and TNF-α).

Increased complement in tri-culture model of Alzheimer’s disease. We next applied this platform to model neuroinflammation in a disease state, Alzheimer’s disease. We used a targeted human embryonic stem cell (hESC) line homozygous for the APPsw mutation47, as well as its isogenic control (Extended Data
Fig. 10a,b). Differentiated Alzheimer’s disease- and isogenic control hPSC-derived neurons were validated by FOXG1 and MAP2 expression, as well as CTIP2 and TBR1 for cortical identity (Fig. 5a,b). The APP<sup>SWE</sup>+/+ neurons alone showed increased amyloid-β production, a hallmark of the APP<sup>SWE</sup>+/+ model of Alzheimer’s disease (Fig. 5c), with elevated levels of all three amyloid peptides (Extended Data Fig. 10c). We plated WT differentiated astrocytes (GFAP<sup>+</sup>) and WT differentiated microglia (IBA1<sup>+</sup>) in co-culture with matured APP<sup>SWE</sup>+/+ neurons (day 80) or isogenic control neurons to construct tri-cultures (Fig. 5d). After 8 d, we measured the C3 levels by ELISA in tri-cultures with APP<sup>SWE</sup>+/+ neurons compared with those containing isogenic control neurons. Interestingly, C3 levels significantly increased in APP<sup>SWE</sup>+/+ neuron-containing tri-cultures (Fig. 5e). In contrast, C3 was not
Fig. 5 | Tri-culture model of Alzheimer's disease shows increased C3 due to reciprocal signaling from microglia to astrocytes.  

**a,** IF showed that the day-80 APP<sup>SWE</sup>+/+ neurons and isogenic control neurons expressed FOXG1 (**a**) and the cortical layer markers CTIP2 and TBR1 (**b**). Scale bars, 40 µM.  

**c,** ELISA showed that day-50 APP<sup>SWE</sup>+/+ neurons secreted more total amyloid than isogenic control neurons. *n* = 3 distinct culture supernatants; two-tailed t-test, **P** = 0.0016. Error bars represent the s.d., and center line is the mean.  

**d,** IF showed APP<sup>SWE</sup>+/+ tri-cultures with day-80 APP<sup>SWE</sup>+/+ neurons (MAP2<sup>+</sup>), WT hPSC-derived microglia (IBA1<sup>+</sup>) and astrocytes (GFAP<sup>+</sup>); and isogenic control tri-cultures with day-80 isogenic control neurons (MAP2<sup>+</sup>), WT hPSC-derived microglia (IBA1<sup>+</sup>) and WT astrocytes (GFAP<sup>+</sup>). Scale bar, 100 µM.  

**e,** ELISA showed that APP<sup>SWE</sup>+/+ tri-cultures secrete more C3 than isogenic control tri-cultures. *n* = 3 (distinct culture supernatants); two-way ANOVA with Bonferroni post hoc test, ****P < 0.0001. Error bars represent the s.d., and center line is the mean.  

**f,** ELISA showed that C3KOA tri-cultures with APP<sup>SWE</sup>+/+ neurons had lower C3 secretion as compared to APP<sup>SWE</sup>+/+ tri-cultures with WT astrocytes. C3KOM APP<sup>SWE</sup>+/+ tri-cultures secreted low levels of C3. *n* = 3 separate culture supernatants; two-way ANOVA with post hoc Tukey's test, ****P < 0.0001, *F* = 182.9, df = 5. Translucent bars (TRI) represent data originally presented in e. Error bars represent the s.d., and center line is the mean.  

**g,** Western blot (cropped) showed that APP<sup>SWE</sup>+/+ cultures that contain microglia had higher levels of C1QA as compared to isogenic control cultures (TRI, M/N, C3KO A and C3KOM).  

**h,** Neuroinflammatory loop schematic in an in vitro model of Alzheimer's disease where APP<sup>SWE</sup>+/+ neurons activate microglia, which in turn activate reciprocal signaling to astrocytes leading to increased C3 release as well as increased C1Q secretion and deposition.
highly produced or increased in co-cultures of APP<sup>SW</sup>+/- neuron/WT astrocyte or in APP<sup>SW</sup>+/- neuron-only cultures, indicating that microglia must be present in the cultures for robust C3 production.

To determine whether the source of the increased C3 in the APP<sup>SW</sup>+/- tri-cultures was due to astrocytes or microglia, we generated microglia and astrocytes from C3 KO hPSCs and assembled tri-cultures containing C3 KO astrocytes, WT microglia and APP<sup>SW</sup>+/- neurons or isogenic control neurons (C3KOA); or WT astrocytes, C3 KO microglia and APP<sup>SW</sup>+/- neurons or isogenic control neurons (C3KOM). Remarkably, in C3KOA Alzheimer's disease tri-cultures, we observed greatly reduced levels of C3 comparable to those of isogenic control tri-cultures, suggesting that some increased C3 in the Alzheimer's disease tri-cultures is secreted by astrocytes activated by microglia. However, C3KOA Alzheimer's disease tri-cultures still showed increased C3 levels compared to C3KOA isogenic tri-cultures, as well as microglia/neuron cultures, suggesting that some increased C3 in the tri-cultures is secreted by microglia activated by astrocytes (Fig. 5f). In C3KOM cultures, we detected only low levels of C3 production, indicating that C3-expressing microglia must be present to induce robust C3 expression in APP<sup>SW</sup>+/- tri-cultures (Fig. 5f). These data suggest that the neuroinflammatory loop is exacerbated in neurons of the tri-culture model of Alzheimer's disease, which further triggers reciprocal signaling between microglia and astrocytes leading to the emergent property of increased C3 production when all three cell types are cultured together.

Upstream of C3 is C1Q, a complement protein that complexes with a cleavage product of complement C3 and tags synaptic material for clearance<sup>6</sup>. Strikingly, we found that there was an increase in C1Q protein deposition found in APP<sup>SW</sup>+/- cultures when compared to WT cultures (Fig. 5g), but only under co-culture conditions that included microglia. C1Q has been shown to accumulate in Alzheimer's disease in vivo<sup>27</sup>, and we recapitulate this finding in an in vitro model of Alzheimer's disease. We corroborate these data with C1Q ELISA results showing increased C1Q secretion in APP<sup>SW</sup>+/- tri-cultures when compared to WT tri-cultures, and the presence of C1Q secretion again only in cultures containing microglia (Extended Data Fig. 10d). These data suggest that microglia are the main source of C1Q<sup>6</sup> in our system and that APP<sup>SW</sup>+/- tri-cultures have increased C1Q secretion and accumulation as compared to WT tri-cultures. C1Q has been identified as an inductive factor secreted from activated microglia that can activate astrocytes<sup>3</sup>, and in a previous study we have shown that, in combination with other inflammatory cytokines, C1Q can activate hPSC-derived astrocytes<sup>4</sup>. Given the higher levels of C1Q secretion in APP<sup>SW</sup>+/- tri-cultures (Extended Data Fig. 10d), we postulate that C1Q may act as an inductive factor in tri-culture models of Alzheimer's disease (Fig. 5e) and possibly other inflammatory cytokines involved in microglia–astrocyte cellular cross-talk.

Interestingly, despite increased C1Q secretion in tri-culture models of Alzheimer's disease, the deposition of C1Q was lowest under tri-culture conditions when compared to microglia/neuron, C3KOA and C3KOM cultures for both APP<sup>SW</sup>+/- and WT cultures (Fig. 5g). This seemingly contradictory finding suggests that there may be a homeostatic balance in minimizing C1Q deposition when microglia, astrocytes and neurons are cultured together, such that microglia and astrocytes can optimally phagocytose C1Q complexes and deplete C1Q accumulation (Fig. 5g). We speculate that, if the system is perturbed, by either the addition of AD neurons, the subtraction of astrocytes or the deletion of C3, homeostasis is disrupted leading to higher levels of C1Q accumulation. In C3KOM tri-cultures, there is increased C1Q deposition and secretion, suggesting an autocrine C3–C1Q negative inhibition in microglial cells during homeostasis that is disinhibited upon C3 KO (Extended Data Fig. 10e). In C3KOA tri-cultures, C1Q deposition is increased but not its secretion (Extended Data Fig. 10e), suggesting that there may be an altered state in C3 KO astrocytes that hinders their ability to clear C1Q complexes<sup>6</sup>, either directly or indirectly by an inability to signal to microglia to perform this function. It will be intriguing to use our culture system in future studies to further dissect the mechanisms responsible in both microglia and astrocytes for guiding the balance between C1Q secretion and deposition.

Based on the results from our new in vitro tri-culture system, we built a model of the cellular contributions to neuroinflammation in Alzheimer's disease focused on complement C3 (Fig. 5h). We report increased C3 levels in a tri-culture model of Alzheimer's disease versus isogenic control tri-cultures and reveal that this increase is due to astrocytic C3 induced by microglia, as well as due to microglial C3 re-induced by astrocytes. The results point to an inflammatory loop involving APP<sup>SW</sup>+/- neurons that trigger microglia to induce reciprocal signaling with astrocytes. In addition, we find increased C1Q in culture models of Alzheimer's disease in the presence of microglia, as a candidate Alzheimer's disease-specific modulator of the cellular cross-talk between microglia and astrocytes.

Discussion
These findings demonstrate that our tri-culture system enables the dissection of cellular cross-talk by genetic manipulation, and allowed us to study the mechanisms of increased complement C3 production in tri-culture upon LPS stimulation and in a model of Alzheimer's disease. Furthermore, the technology could be readily adapted to study any other disease-relevant neuroinflammatory pathway in Alzheimer’s disease or other neurodegenerative disorders. The identification of the key cellular players contributing to the neuroinflammatory axis in humans should enable the development of directed, cell-type-specific therapeutic strategies.

In fact, the hPSC-derived tri-culture system could serve as a scalable platform for the screening of compounds that specifically target the cross-talk between microglia, astrocytes and neurons as a new therapeutic modality in Alzheimer's disease or other neurodegenerative disorders.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41593-020-00796-z.

Received: 9 March 2020; Accepted: 24 December 2020; Published online: 8 February 2021

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**Methods**

**Derivation of microglia from hPSCs.** hPSCs maintained in Essential 8 medium were dissociated with Accutase to obtain a single-cell suspension. A total of 60,000 cells per cm² were plated in E8 medium containing activin A (R&D 338-AC; 7.5 μg/mL), BMP4 (30 ng/mL), PDGFRA (50 ng/mL), CHIR99021 (Gtosci; 3 μM) and ROCK inhibitor (Y-27632; 10 μM) onto Matrigel-coated plates. After 18 h, medium was changed to Essential 6 medium containing activin A (10 ng/mL), BMP4 (40 ng/mL) and IWF2 (2 μM) and FGF2 (R&D; 20 μg/mL). On day 3, cultures were dissociated with Accutase and replated 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 μM). On day 4, the ROCK inhibitor was removed and medium changed to Essential 6 with VEGF (15 ng/mL) and FGF2 (5 ng/mL). On days 5 and 6, cultures were fed with Essential 6 medium containing VEGF (15 ng/mL), FGF2 (5 ng/mL) and IL-6 (20 ng/mL). On days 7 and 9, medium was changed to Essential 6 with SCF (100 ng/mL)-, IL-6 (10 ng/mL), TPO (30 ng/mL) and IL-3 (30 ng/mL). On day 10, the cells in suspension were collected and either (1) co-cultured 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 5 d for direct transition to microglia, or (2) cultured in RPMI with 10% FBS, l-glutamine, penicillin–streptomycin with IL-34 (100 ng/mL)− and M-CSF (20 ng/mL) for 7–11 d until cells were adherent and elongated to transition to primitive macrophages.

For serum-free culture, cells in suspension on day 10 were harvested and cultured in 75% FCS medium containing B-27 supplement, l-glutamine, cytokines (VEGF, IL-6), SCF (200 ng/mL) and growing Matrigel on a microtiter plate. Cells were treated with Essential 6 medium containing activin A (10 ng/mL), FGF2 (5 ng/mL)− and IL-6 (20 ng/mL) for 7–11 d. Transitioned macrophages were then co-cultured with cortical neurons with the addition of IL-34 and M-CSF for 7 d for upregulation of microglial-specific markers. Induced pluripotent stem cells (iPSCs) lines used to test reproducibility of the microglial differentiation (DBR1, IFNARI, IL-10RB and STAT1) were each separately derived from patient fibroblasts or hPSCs, treated withactivin A (10 ng/mL)− and BMP4 (40 ng/mL)− and generating Sendai virus vector in the Notarangelo laboratory at the National Institutes of Health (NIH).

**Cortical neuron protocol.** hPSCs were dissociated with Accutase and plated at 200,000 cells per cm² onto Matrigel-coated plates in Essential 8 medium with ROCK inhibitor (Y-27632; 10 μM). Cells were treated with Essential 6 medium containing LDN193189 (100 nM) and SB31542 (10 μM) for 12 d, with the addition of XAV939 (2 μM) for the first 4 d of differentiation. Cultures were fed with N2 medium with 1:1000 B-27 supplement for an additional week to allow neuronal maturation. On day 6, medium was changed to Essential 6 with SCF (100 ng/mL)-, IL-6 (10 ng/mL)-, TPO (30 ng/mL)- and IL-3 (30 ng/mL). On day 10 of differentiation, and 'day-10' samples were prepared by harvesting the cells in suspension.

**Microglial differentiation: 'day 6' at day 6 of differentiation, 'day 10' at day 10 of differentiation, and '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. 'Day-6' and 'day-10' samples were prepared by treating cultures with Accutase for 20 min to achieve a single-cell suspension. 'Day-10 suspension' was prepared by collecting and suspending cells in suspension through a 40-μm cell strainer. Microglial cells in differentiation were prepared by sorting co-culture models of neurons and microglia for expression of CX3CR1. All samples were resuspended at 1,000 cells per μl in FACS buffer before sequencing. Single-cell sequencing was performed using 10x Genomics Chromium Single Cell 3’ Library & Gel bead Kit V2 according to the manufacturer's protocol.

An input of 8,700 cells was added to each 10x channel. Libraries were sequenced on an Illumina NovaSeq device.

**Single-cell RNA-sequencing data preprocessing.** scRNA-seq data were processed using the SEQC processing pipeline1. SEQC generates a cells-by-genes count matrix after read alignment, mapping unmapped read sequence and cell barcode and unique molecular identifier correction. SEQC included an initial filtering step removing (1)putative empty droplets based on the cumulative distribution of molecules counts for each barcode, (2)putative apoptotic cells based on 20% of molecules derived from the mitochondria, and (3) low-complexity cells identified 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). Counts were normalized 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. Data were then log transformed using natural log and a pseudocount of 1.

**Cell filtering.** For each sample, cells were clustered using the PhenoGraph clustering algorithm13. Clusters of cells with low numbers of detected genes (~200) were removed as putative empty droplets. Clusters with high mitochondrial RNA and a low number of detected genes were removed as putative dying cells. Four clusters not pertaining to hematopoietic differentiation were removed, including two early mesoderm clusters that expressed low levels of MESP1 and PDGFRα but not KDR, PECAM1 or CDH5, one cluster belonging to the cardiac lineage expressing NKG2 and ISL1, and one cluster belonging to mature endothelial cells.

**Nearest neighbor graph construction.** Principal components were used to calculate Euclidean distances between cells. An adaptive Gaussian kernel was used to test nearest Euclidean distances between cells' k-nearest neighbors into affinities, as described by Haghverdi et al.8. 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. Nearest neighbor graphs were used as a basis for force-directed graph layout and diffusion map embeddings.

**Clustering and force-directed graph layout.** Data from day 6, day 10 and day-10 suspension samples were pooled for trajectory modeling. Principal-component analysis was performed on the data, and the first 20 principal components were selected for further analyses, to reduce noise due to the high degree of dropouts in scRNA-seq1. Force-directed graph layouts were calculated using the ForceAtlas2 algorithm14, based on the 30-nearest neighbors graph of the data that was constructed as described above. Clustering was performed with PhenoGraph, using default parameters13.

**Diffusion map embedding.** To approximate the low-dimensional data manifold representing the differentiation trajectory, a diffusion map embedding was constructed using an adaptive Gaussian kernel-based nearest neighbor graph (k = 20; described above)1. Construction of a diffusion map is a nonlinear method to recapitulate the low-dimensional structure underlying high-dimensional observations. The first four diffusion components of the diffusion map were selected for trajectory modeling. The diffusion distances between cells (that is, the Euclidean distances between cells in the 'diffusion map space') were subsequently converted into pseudotime distances between individual cells as described by Haghverdi et al.8. 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 cells15.

**Trajectory characterization.** To further characterize the trajectory, we used Palantir22. Palantir is a tool that, using pseudotime distances, identifies trajectory end points ('terminal cells') and, 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'). As the analysis was performed on the data, and the first 20 principal components were used, to further characterize the trajectory, we used Palantir22. Palantir is a tool that, using pseudotime distances, identifies trajectory end points ('terminal cells') and, 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'). As the input approximate start cell of the Palantir trajectory, a random cell from the CDH5-high, KDR-high and PECAM1-high hemogenic endothelium clusters was used. The number of neighbors was set to k = 20, and the number of diffusion components was set to 2. For all other parameters, default settings were used.

**Calculation of gene trends over pseudotime.** To recover expression trends of individual genes over pseudotime, we first imputed our processed count matrix using MAGIC. MAGIC is a method to deme the cell count matrix and fill in zeros due to dropouts. MAGIC included differentiating cells and, moreover, measures entropy in cell phenotypes to measure their plasticity ('differentiation potential') and commitment to specific cell fates ('branch probability'). As the input approximate start cell of the Palantir trajectory, a random cell from the CDH5-high, KDR-high and PECAM1-high hemogenic endothelium clusters was used. The number of neighbors was set to k = 20, and the number of diffusion components was set to 2. For all other parameters, default settings were used.
pseudotime (Supplementary Fig. 2b) were calculated 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 were included.

**Diffusion distance distributions.** To investigate whether cells in the microglia sample were still in the process of differentiating, we inspected the distribution of pairwise diffusion distances among the cells. In accordance with single-cell studies of cell differentiation\(^{19,20}\), 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.

A multiscaled diffusion map embedding (described earlier) of the cells was used 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 compared to the nonembodied data. Distances were calculated 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.** For comparison to previously published mouse signatures, we used published PMACs\(^{25}\). RNA was extracted from human microglia from patients aged 55–77 years (T2200; 1:1,000), CTIP2 (Abcam, ab18465; 1:200), CC3 (Cell Signaling Tech, 9664S; 1:500), TBR1 (Abcam, ab183032; 1:200), TUJ1 (Sigma-Aldrich, T2200; 1:500), PU.1 (BioLegend, 658002; 1:100), PSD95 (Abcam, ab2723; 1:200), GFAP (BioLegend, 658002; 1:100), DAPI (Life Technologies, I369; 1:500), TBR1 (Abcam, ab183032; 1:200), TU1 (Sigma-Aldrich, T2200; 1:1,000), CTP2P (Abcam, ab18465; 1:200), C3 Cell (Signaling Technology, 9664S; 1:200) and C1Q (Dako, A013602-1; 1:1,000). Secondary antibodies included donkey anti-mouse Alexa Fluor 488 (Thermo Fisher, A-21202), donkey anti-chick Alexa 488 (Jackson ImmunoResearch, AB_2340375), donkey anti-rabbit Alexa Fluor 488 (Thermo Fisher, A-31572), donkey anti-mouse Alexa Fluor 555 (Thermo Fisher, A-31570), donkey anti-mouse Alexa Fluor 647 (Thermo Fisher, A-31571) and donkey anti-chick Alexa Fluor 647 (Jackson ImmunoResearch, AB_2340379) at 1:500.

**Engulfment of synaptic proteins imaging.** Microglia were co-cultured with day-70+ neurons on culture dishes (Ibidi) for up to 30 d and stained with PSD95 and IBA1 (100 d in total). Cultures were imaged 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. Data were processed and analyzed with Imaris v9.2.2: a surface volume mask was generated in the IBA1 channel, within which another mask was 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.** For the phagocytosis assay, microglial cells or astrocyte controls were incubated with Zymosan A bioparticles conjugated with Alexa Fluor 488 for 5 h in an Olympus VivaView fluorescence incubator microscope. For the surveying assay, microglial cells were infected with a lentiviral construct expressing GFP and were co-cultured with day-30 cortical neurons for 7 d, then incubated in an Olympus VivaView fluorescence incubator microscope for 16 h. Time-lapse imaging was completed at 2 min per frame.

**RNA sequencing.** Approximately 50,000–100,000 hPSC-derived microglia from three different hPSC lines, H1, H9 and the WT iPSC line SA241-1, were sorted from neurological co-cultures by expression of CX3CR1. RNA was extracted using the Zymo RNeasy Micro Kit. RNA was converted to cDNA using the SuperScript IV First-Strand Synthesis System from Invitrogen. cDNA was amplified using gene-specific primers before sequencing on an Illumina NextSeq 500 platform. Reads were then processed using HTSeq to compute a raw expression count matrix, which was then processed using DESeq from R/BioConductor to analyze differential expression between samples.

**Primary human microglia.** Brain tissue was provided by the Netherlands Brain Bank (NBB). Informed consent for brain autopsy, the use of tissue and use of clinical information was 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). Brain tissue was collected from one male and three females without a history of a neurological or psychiatric disorder in the age range of 60–77 years. Microglia were isolated from the medial frontal gyrus and temporal superior gyrus as described before\(^{21}\). In short, 2–10 g of tissue was collected within 13 h after death and stored in Hibernate medium on ice, and the isolation procedure was started within 2–24 h after autopsy. A single-cell suspension was generated by dissociating the tissue mechanically through a metal sieve, followed by an enzymatic digestion using collagenase type I (1,700 U ml\(^{-1}\); Worthington) and DNase I (200 µg ml\(^{-1}\); Roche). Microglia were further purified using a Percoll gradient and positive selection using CD11b-conjugated magnetic microbeads (Miltenyi Biotec). Microglia were lysed in RLT buffer (Qiagen), and RNA was extracted according to the protocol provided by the RNeasy Mini Kit (Qiagen).

**Gene ontology analysis and gene-set enrichment analysis.** GO analysis was performed using http://geneontology.org. The analysis type was 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 was Fisher with False discovery rate correction. GSEA was performed using the GSEA software (v4.0.3) from the Broad Institute, using the clusters defined in Matcovitch-Natan et al.\(^{13}\) defining microglial developmental stages.

**Comparison with publicly available microglial datasets.** Publicly available datasets from the following studies were integrated according to the methodology by Grubman et al.\(^{16}\) before MDS. The following datasets were included: Muffat et al.\(^{17}\) (GSE85839, 8 samples), Douvaras et al.\(^{18}\) (GSE97744, 14 samples), Abud et al.\(^{19}\) (GSE89189, 12 samples), Ormel et al.\(^{20}\) (GSE102335, 9 samples) and Galatro et al.\(^{21}\) (GSE99074, 38 samples). A total of 17 samples were integrated from our study (13 hPSC-derived microglia and 4 adult primary microglia). Briefly, count matrices were converted to log, reads per kilobase million, and the differences between batches were removed using the removeBatchEffect function from limma, specifying each dataset as a batch, as well as specifying common groups as design (adult, hPSC-microglia and fetal microglia). MDS was performed using the plotMDS function from limma.

**Tri-culture system, co-culture ratios and LPS assay.** Cortical neurons were differentiated from hPSCs and replated at 200,000 cells per cm\(^2\) on plates coated with poly–l-ornithine/lifibronecin/laminin and allowed to mature for 50–70 d in NB/BAGC. Astrocytes that differentiated from hPSCs were dissociated with
Accurate for 20–30 min and then plated on top of the neurons at 25,000 cells per cm² and were allowed to settle for 4d in NB/BAGC. Microglia that differentiated from hPSCs were then dissociated with Accutase for 10min and plated on top of the astrocyte/neuron culture at 50,000 cells per cm² in NB/BAGC with IL-34 (100ng ml⁻¹) and M-CSF (20ng ml⁻¹). Medium was changed 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). After culture for a minimum of 7d, LPS was added at 1μg ml⁻¹ for 72h. Culture medium was collected and spun down at 2,000r.p.m. for 5min, and the supernatant was frozen at −80°C until further analysis.

**Cell line engineering.** CRISPR-Cas9 knockout of C3. The PX458 vector containing the guide 5'TCTGCACTATCCAGGTA 3' was nucleofected into H1 hESCs. Cells were sorted on the basis of GFP expression and cultured as single-cell clones in 8 medium with the cloneR supplement. Clones were picked onto replicate plates, and genomic DNA was extracted using Bradley Lysin Buffer and Proteinase K treatment. A 450-bp PCR product was amplified around the guide RNA cut site, and the clones were screened for indels by Sanger sequencing. Clones with indels were subsequently picked and expanded, karyotyped and differentiated into microglia for further validation by ELISA for a lack of C3 protein secretion.

**Ad lines.** APP/PS1ΔE9 and WT iCa9 lines were generated using the guide RNA and oligonucleotides from Paquet et al. in an h9 background.

**Primers.** Primers used for qPCR included: C1QA (Qiagen QT0997745), C3 (F 5' AAAAGGGAGGCGAAGGATGTC 3', R 5' GATGCTGGCTGCTGGTCTCA 3'), C3CR1 (F 5' TGGGCGCTTACCATGATG 3', R 5' GCCAATGGAAGGATGAGCAGG 3'), TEM19119 (F 5' CTCTTGTGATGATTGAGGTC 3', R 5' GACAGATGAGGATGACCCG 3'), P2R12 (F 5' AAGACGCTCAGACTTAC 3', R 5' GGGCAGATGATGATGAGG 3'), GPR30 (F 5' GAGAACAGAATGTCATGAC 3', R 5' GGTTGCTGCAAGTCTGTG 3').

**Statistics and reproducibility.** All data presented in this study are representative of at least three independent experiments, including IP panels, ELISA results and FACS data. Western blots and gel electrophoresis were repeated at least twice. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications. Cells were randomly assigned into experimental culture groups (TRI, M/N, A/N and N) after accounting for their respective genotypes (APP/PS1ΔE9, WT and C3KO). Data collection and analysis were not performed blind to the conditions of the experiments because all quantified data were collected in an automated fashion (ELISA, FACS and ImageExpress) such that bias would not be introduced. Data distribution was assumed to be normal, but this was not formally tested. Data are presented as the mean ± s.d. All statistical analyses were performed using GraphPad Prism v8.0.1: one-way ANOVA with Tukey’s post hoc or Student’s t-test 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 were considered significant with P < 0.05 as indicated in figure legends. Samples were 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). For RNA samples from three postmortem human brain tissue samples were used for RNA-seq experiments; donors were aged 77 (male), 60 (female) and 84 (female) years.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All RNA-seq data, bulk and scRNA-seq, have been deposited to the Gene Expression Omnibus under GSE139549 and GSE19552. Publicly available RNA-seq datasets used include GSE85839 (ref. 1), GSE97744 (ref. 2), GSE89198 (ref. 3), GSE102335 (ref. 4) and GSE90727 (ref. 5). Other data and reagents in this study are available from the corresponding author upon reasonable request.

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**Acknowledgements**

We are grateful to the members of the Studer laboratory for their helpful discussions and support for this project. Additionally, we thank the MSKCC flow cytometry core for cell sorting; A. Viale at Integrated Genomics Operation Core (MSKCC) for the RNA-seq studies; P. Zumbo and D. Betel at the Weill Cornell Medical College (WCMC) Applied Bioinformatics Core for MDS analysis; R. Ralph at the automated optical microscopy service with the Microscopy and Image Analysis Core (WCMC) for high-content imaging data analysis; V. Bokyo at the Molecular Cytology Core (MSKCC) for help with confocal imaging and quantification; and the Molecular Cytogenetics Core (MSKCC) for karyotyping analysis. We also thank the team of the NBRI for providing us with postmortem brain tissue and the Notarangelo laboratory at NIH for reprogramming the iPSC lines used to test reproducibility of the microglial differentiation (DBRI, IFNAR1, IL-10RB and STAT1). S.R.G. was supported by the Ruth L. Kirschstein Individual Predoctoral NRSA for MD/PhD Fellowship (1F30MH15166-91) and by a Medical Scientist Training Program grant from the NIH National Institute of General Medical Sciences (award no. T32GM007739) to the Weill Cornell/Rockefeller/Sloan Kettering Tri-Institutional MD-PhD Program. J.T. was supported by a Tri-I Starr Stem Cell Scholar fellowship. N.S. was supported by a Glenn/AFAR Postdoctoral Fellowship; R.M.W. was supported by an F32 Ruth L. Kirshstein Postdoctoral Fellowship (MH116590); and G.G. was supported by an EMBO long-term postdoctoral fellowship and a NYSTEM postdoctoral fellowship. The work was supported in part by R21 NS084334, R01AG156298 to L.S. and the core grant P30CA08748.

**Author contributions**

S.R.G.: conception, study design, data analysis and interpretation, writing of manuscript, development and execution of differentiation of microglia from hPSCs and development of tri-culture system. L. Nikkema: scRNA-seq bioinformatic analysis and interpretation. J.T.: differentiation of astrocytes from hPSCs. N.S.: generation of isogenic APP/PS1ΔE9/hPSC lines. R.M.W.: generation of GPI-H2B-GFP iPSC line. O.H.: generation of microglia from multiple iPSC lines for reproducibility validation. G.G.: development of paradigm for cortical neuronal differentiation. M.S. and L.W.: human primary microglial isolation and RNA preparation. L.S.: sample processing for scRNA-seq.
M.S.: development of Palantir. D.P.: conception and design of scRNA-seq data analysis and interpretation. P.Z. and D.B.: comparison of RNA-seq data to publicly available microglial datasets. L. Studer.: conception, study design, data analysis and interpretation, and writing of manuscript.

**Competing interests**

L.S. holds equity and is a scientific co-founder and paid consultant of BlueRock Therapeutics. S.R.G. and L.S. are listed as inventors of a related patent application filed by the Memorial Sloan Kettering Cancer Center.

**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s41593-020-00796-z.
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41593-020-00796-z.
Correspondence and requests for materials should be addressed to L.S.

Peer review information Nature Neuroscience thanks Hansang Cho and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Characterization of primitive hematopoiesis up to Day 10 of differentiation. a, FACS analysis shows that addition of erythropoietin (EPO) from Day 6 to Day 10 of differentiation causes the emergence of CD235A+ erythrocytes at Day 10 of differentiation, as well as a reduction in the percentage of Macro (macrophage precursor) fated cells. b, Brightfield images over days 4–10 of the differentiation show that round hematopoietic cells progressively proliferate in semi-suspension through day 10, black arrows point to hematopoietic cells. Scale bar = 100 µM (Day 4, 10) and 50 µM (Day 7). c, IF shows VE-cadherin+ hemogenic endothelium at Day 10 of differentiation. Scale bar = 100 µM. d, Imputed gene expression trends over pseudotime as calculated by Palantir of HOXA genes in the 3 differentiation trajectory arms show nearly absent HOXA1–7 expression, and minimal HOXA9 and 10 expression at low pseudotimes (~1 log fold lower than genes in Fig. 2C). PMAC (PMAC arm, green), MK (megakaryocytic arm, pink), and ERY (erythrocyte arm, orange). e, Unimputed gene expression trends of HOXA1-10 genes show similar trends to imputed trends shown in A, nearly absent HOXA1-7 expression and minimal HOXA9-10 expression only at low pseudotimes in all differentiation trajectory arms. PMAC (PMAC arm, green), MK (megakaryocytic arm, pink), and ERY (erythrocyte arm, orange). f, Unimputed gene expression trends of key signature genes over pseudotime as calculated by Palantir show similar trends to imputed gene trends shown in Fig. 2C. Separate differentiation arms increasingly express their corresponding identity markers over pseudotime: ERY (GYPA, HBEG), MK (ITGA2B, ITGB3, GPIBA), and PMAC (CX3CR1, CSF1R, SPI1, PTPRC). MY (PMAC arm, green), MK (megakaryocytic arm, pink), and ERY (erythrocyte arm, orange). n = 6743 and error bars = SD for (A)–(C).
Extended Data Fig. 2 | Heatmaps of EMP and PMAC signatures generated with unimputed data along PMAC, ERY, and MK arms. a. Heatmaps showing unimputed counts of mouse yolk sac EMP PMAC signature genes along the PMAC arm ordered by pseudotime. There is increased expression of the EMP and PMAC signature genes over pseudotime along the PMAC trajectory, corresponding to the same increase in expression shown in Fig. 2D with imputed data. b. i) Heatmap of gene expression data from cells along the erythrocyte trajectory (ERY) ordered by pseudotime compared to mouse yolk sac EMP and PMAC gene signatures shows increased expression of EMP genes at pseudotimes corresponding to the in vitro human EMP/ERY clusters, but no increase in expression of the PMAC signature genes at any pseudotime. ii) Likewise, heatmap of imputed gene expression data from cells along the megakaryocytic trajectory (MK) ordered by pseudotime compared to mouse yolk sac EMP and PMAC gene signatures shows increased expression of EMP genes at pseudotimes corresponding to the human EMP/MK clusters, but no increase in expression of the PMAC signature at any pseudotime. c. Heatmaps generated from unimputed data show the same trends of increased expression of EMP signature genes at pseudotimes when EMP/ERY and EMP/MK clusters emerge, but no increased expression of the PMAC signature genes. Gene counts were individually scaled to range from 0 to 1 for all heatmaps.
Extended Data Fig. 3 | Normalized counts of genes expressed by hPSC-derived microglia and hPSC-derived neurons in co-culture. a, hPSC-derived neurons co-cultured with microglia express signature genes important for microglial maturation (IL-34, CSF1, CX3CL1, CD200, TGFβ2, TGFβ3), whereas hPSC-derived microglia express low levels or do not express these genes. Neurons = hPSC-derived neurons, micro = hPSC-derived microglia from method ii of differentiation. n = 4 samples for neurons and n = 6 for microglia. Error bars = SD, center = mean. b, hPSC-derived microglia co-cultured with hPSC-derived neurons express a large panel of microglial genes at nearly the same levels as adult acutely isolated primary microglia, except TMEM119, P2RY12, and SALL1. Primary = adult acutely isolated microglia, n = 4, macro = method ii, matured alone then co-cultured, n = 6, round = method i, direct co-culture, n = 6. Error bars = SD, center = mean.
Extended Data Fig. 4 | See next page for caption.
**Extended Data Fig. 4 | Characterization and reproducibility of maturing primitive EMPs/PMACs to homogenous hPSC-derived microglia without co-culture.**

a, Brightfield image of differentiating primitive EMPs/PMACs shows that by 11 days of culture in IL-34 and M-CSF, the cells are adherent on TC-treated plastic and display an elongated morphology. b, IF shows that all cells at day 11 uniformly express the myeloid transcription factor PU.1. Scale bar = 50µM for (a) and (b). c, FACS analysis of day 10 differentiation cultures show a 37–51% induction of CD43 + CD45 + macrophage precursors that is reproducible across 4 different hPSC lines. d, After 11 days of culture in IL-34 and M-CSF, a pure population of IBA1 + cells is reproducible in 5 hPSC lines (including 4 iPSC lines). Scale bar = 100 µM. e, Each iPSC line used to test reproducibility of the microglia differentiation was derived from patient fibroblasts using a nonintegrating Sendai viral vector. f, Pairwise diffusion distances calculated between cells in the microglial sample after diffusion map embedding fall in a unimodal distribution. Pairwise distances calculated using different numbers of diffusion components are shown, with consistent results. g, In contrast, pairwise diffusion distances between cells in the heterogenous Day 10 sample have multiple peaks across different numbers of diffusion components.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | GO pathway analysis and GSEA on differentially expressed genes between hPSC-derived microglia and acutely isolated adult primary microglia. 

a, Heatmap of top 100 differentially expressed genes between hPSC-derived microglia derived from method ii and acutely isolated adult primary microglia using DESEQ. 
b, Table of top 100 differentially expressed genes between hPSC-derived microglia (method ii) and adult primary microglia using DESEQ. 
c, GO pathway analysis identifies neuronal developmental pathways as enriched in hPSC-derived microglia (method ii). 
d, GO pathways analysis identifies immune activation pathways as enriched in acutely isolated adult primary microglia. 

e-f, GSEA on 7 embryonic to adult microglial gene signatures (yolk sac, embryonic 1 and 2, postnatal 1 and 2, adult 1 and 2) reveals that hPSC-derived microglia (method ii) enrich for postnatal 1 and 2 signatures with NES = −1.46 and FDR = 0.14; NES = −1.52, FDR = 0.16. 

G-H) GSEA reveals that adult primary microglia enrich for adult 1 and 2 signatures with NES = −1.47, FDR = 0.09. Heatmaps show top 20 ranked genes from each gene signature in primary vs. hPSC-microglia (method ii).
Extended Data Fig. 6 | Transcriptomic comparison of hPSC-derived microglia to microglia from previously published differentiation protocols. a, MDS analysis using published datasets from 4 different microglial protocols and 1 study profiling acutely isolated adult primary microglia from postmortem human brain tissue reveals that hPSC-derived microglia from both method i (mg1) and method ii (mg2) cluster near the microglia differentiated from published protocols as well as near fetal microglia. acutely isolated adult primary microglia sequenced in our study, mg1 = hPSC-derived microglia from method i, mg2 = hPSC-derived microglia from method ii of our study. adultmg_adultmg_ormel, Galatro et al35, Abud et al11, fetal_mg_adultmg_muffat = fetal microglia from Abud et al11, Douvaras et al12, Muffat et al10, mg_abud, douv, muffat, ormel) = hPSC-microglia from Abud et al11, Douvaras et al12, Muffat et al10, and Ormel et al35. Dimension 1 vs. 2 separates the adult primary microglia from the fetal microglia/hPSC-derived microglia. b, Dimension 2 vs. 3 separates out the adult primary microglia and fetal microglia cultured in serum used in Abud et al11. Our hPSC-derived microglia (mg1, mg2) cluster near the other differentiated microglia (mg_ormel, mg_douv, mg_muffat) and fetal microglia (fetalmg_douv, fetalmg_douv+serum, fetalmg_muffat+serum).
Extended Data Fig. 7 | Microglia perform efficient phagocytosis of zymosan-coated beads and mature hPSC-derived neurons form synapses in vitro.

a, Fluorescent microscopy shows that microglial cells contain zymosan-conjugated fluorescent beads as inclusions within 4 hours of incubation. Scale bar = 70 µM. b, Astrocyte control does not contain fluorescent bead inclusions after 4 hr of incubation. Scale bar = 280 µM. c, IF shows that hPSC-derived cortical neurons at day 70 express a punctate distribution of the pre-synaptic SYNI and post-synaptic HOMER1. Putative synapses are stained where both SYNI and HOMER1 are side-by-side (white arrow). d, IF shows that day 70 hPSC-derived cortical neurons also express the post-synaptic marker PSD95 in a punctate distribution. Scale bar = 50 µM (c) and (d).
Extended Data Fig. 8 | Optimization of tri-culture ratio and culture media to maximize cell survival and minimize cell activation at baseline. 

**A.** IF shows that tri-cultures with increased numbers of astrocytes plated (50 K) show fewer microglial cells (IBA1+) attached at Day 7 than with tri-cultures containing fewer (25 K) astrocytes. Scale bar = 200 µM.

**B.** Media formulations show the lowest secretion of C3 in the tri-culture by ELISA. Addition of DMEM base, F12 supplement, low glucose, high glutamine, and high pyruvate all increase C3 levels in baseline tri-cultures. n = 2 technical replicates of cell culture supernatant.

**C.** Control staining for CC3. IF of hPSC-derived neurons killed by 70% methanol incubation for 30 minutes shows bright CC3+ whereas IF of fixed cells without the methanol treatment does not. Scale bar = 100 µM.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Characterization of C3 KO hPSC line and C3 KO-derived microglia and activation properties of conditioned medium and C3 addition to microglia and astrocytes. a, Cell scoring by ImageExpress microscopy shows higher %IBA1+/DAPI in microglia/neuron (M/N) cultures vs. tri-cultures at day 7. n = 4 distinct cell culture wells. Error bars = SD, center = mean. b, Cytokine ELISA panel shows the secretion of inflammatory cytokines upon LPS stimulation only in cultures containing microglia, including IL-10, IL-6, TNFa, GM-CSF, IL1B, and IFNy. n = 3 cell culture supernatants. Error bars = SD, center = mean. c, Sanger sequencing shows that the C3 KO hPSC line has a 7 bp deletion (red) near the targeted PAM site (blue), guide is marked in green. d, IF shows that the C3 KO hPSC line expresses the pluripotency markers of SOX2, NANOG, and OCT4. Scale bar = 100 µM. e, i) FACS analysis shows that microglia differentiated from the C3 KO line are a pure population expressing CD11B+ and CX3CR1+. iii) IF shows that these cells are all IBA1+ and PU.1+. Scale bar = 100 µM. f, ELISA shows that C3 KO microglia (densely cultured alone) do not secrete C3 protein as compared to WT microglia. n = 2 cell culture supernatants. g, Cell scoring by ImageExpress shows similar numbers of IBA1+ microglia and GFAP+ astrocytes between the different tri-cultures (TRI, C3KOM, C3KO). n = 4 distinct cell culture wells. Error bars = SD, center = mean. h, 48hrs of astrocyte-conditioned medium (ACM) treatment induces C3 expression in hPSC-derived microglia, normalized to untreated microglia. **p = 0.0037, two-tailed t-test, n = 3. 48 hrs of microglia-conditioned medium (MCM) treatment induces C3 in astrocytes, normalized to untreated astrocytes, n = 3 independent experiments. *p = 0.0122, two-tailed t-test. Error bars = SD, center = mean. i, C3 (1ug/mL) addition to microglia induces C3 expression after 48hr, normalized to untreated microglia. *p = 0.0217, two-tailed t-test, n = 3 independent experiments. C3 addition to astrocytes does not induce C3 expression after 48hr of treatment, n = 3. Error bars = SD, center = mean.
Extended Data Fig. 10 | Characterization of the APP\textsuperscript{SWE}+/+ and WT isogenic lines. a, Sanger sequencing shows that the APP\textsuperscript{SWE}+/+ line is homozygous for the GA \textgreater{} TC mutation as compared to the isogenic wildtype line. b, IF shows that both lines express the pluripotency markers of SOX2, NANOG, and OCT4. Scale bar = 100 µM. c, Quantification of amyloid peptides 38, 40, and 42 in neuronal cultures shows that APPSWE+/+ neurons have higher levels of all peptides as compared to isogenic WT neurons. n = 3 cell culture supernatants. d, Increased C1Q secretion by ELISA in APPSWE+/+ tri-cultures, ****p < 0.0001, two-way ANOVA with Sidak’s post hoc test, n = 3 cell culture supernatants, error bars = SD, center = mean. e, Increased C1Q secretion in C3KOM but not C3KO tri-cultures. n = 3 cell culture supernatants, error bars = SD, center = mean.
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Software and code

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**Data collection**

| imaris v 9.2, GSEA v 4.03, GraphPad Prism v8.0.1, FACSDiva v8, FlowJo 10.5.3 were used. |

**Data analysis**

Statistical tests were performed using GraphPad Prism for all bar graphs. For bulk RNA-sequencing data, FASTQ files were mapped using the rnaSTAR aligner. Output SAM files are processed using PICARD tools. The mapped reads were then processed using HTSeq (v 0.11.0) to compute a raw expression count matrix, which was then processed using DESeq from R/BioConductor to analyze differential expression between samples. For scRNA-seq analysis, the Scanpy platform (v1.4) was used for data analysis. Data were processed using the SEQC pipeline, followed by clustering using the Phenograph algorithm. Force-directed graph layouts were calculated using the ForceAtlas2 algorithm. Trajectory analysis was done using Palantir.

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All RNA-sequencing data, bulk and scRNA-seq, have been deposited to GEO under GSE139549 and GSE139552. Publicly available RNA-seq datasets used include GSE8583913, GSE9774415, GSE8918914, GSE102333540, and GSE9907442. GO analysis was performed using geneontology.org. The analysis type was PANTHER overrepresentation test, released 04/07/2020, the reference list was Homo Sapiens (all genes in database), the annotation data set was GO biological process complete.
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Life sciences study design

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| Sample size | No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications (Qi et al, Tchieu et al). Qi, Y. et al. Combined small-molecule inhibition accelerates the derivation of functional cortical neurons from human pluripotent stem cells. Nat Biotechnol 35, 154-163, doi:10.1038/nbt.3777 (2017). Tchieu, J. et al. NFIA is a gliogenic switch enabling rapid derivation of functional human astrocytes from pluripotent stem cells. Nat Biotechnol 37, 267-275, doi:10.1038/s41587-019-0035-0 (2019). |
| Data exclusions | Samples were excluded if data collection failed (machine clogs during FACS or ELISA collection – 1 sample in 4g), or after the outlier test – 2 samples in S9b. |
| Replication | All data presented in this study is representative of at least 3 independent experiments, including immunofluorescence panels, ELISA results, and FACS data. Western blots and gel electrophoresis were repeated at least twice. |
| Randomization | Cells were randomly assigned into experimental culture groups (TRI, M/N, A/N, N) after accounting for their respective genotypes (APPswe+/+, WT, C3KO). |
| Blinding | Data collection and analysis were not performed blind to the conditions of the experiments because all quantified data were collected in an automated fashion (ELISA, FACS, ImageExpress) such that bias would not be introduced. |

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| Antibodies | x |
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| Palaeontology | x |
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| Human research participants | x |
| Clinical data | x |

| Methods | n/a |
| ChiP-seq | x |
| Flow cytometry | x |
| MRI-based neuroimaging | x |

Antibodies

Antibodies used

- KDR-PE – R&D, FAB357P, Clone 89106
- CD235A-APC – BD Biosciences, 551336, Clone GA-R2
- CD41-Apc/Cy7 – Biolegend, 303715, Clone HP8
- CD43-PerCP/Cy5.5 – BD Biosciences, 563521, Clone 1G10
- CD45-FITC – BD Biosciences, 560976, Clone HI3O
- CX3CR1-PE – Biolegend, 341604, 2A9-1
- IBA1 – Wako, 019-19741, polyclonal
- Map2 – Sigma-Aldrich, M1406, clone AP-20
- PU.1- Biolegend, 658002, Clone 7C6B05
- Cd11b-APC/Cy7 – Biolegend, 301351, ICRF44
- PSD95- Abcam, ab2723, Clone 666-1C9
- GFAP – Biolegend, 829401, polyclonal
- AQP4 – Santa Cruz Biotech, sc-9888, polyclonal
- FOXG1 – Takara, M227, polyclonal
Validation

KDR-PE – Human, Flow Cytometry, see manufacturer’s references
CD235A-APC – Human, Flow Cytometry, see manufacturer’s references
CD41-Apc/Cy7 – Human and other primates, Flow Cytometry, see manufacturer’s references
CD43-PerCP/Cy5.5 - Human, Flow Cytometry, see manufacturer’s references
CD45-FITC – Human, Flow Cytometry, see manufacturer’s references
CX3CR1-PE – Human and other primates, Flow Cytometry, see manufacturer’s references
IBA1 – Human, Mouse, Rat, Immunocytochemistry, see manufacturer’s references
MAP2 – Human, Mouse, Rat, Immunocytochemistry, see manufacturer’s references
PU1-Human, Flow Cytometry, see manufacturer’s references
Cd11b-APC/Cy7 – Human, Flow Cytometry, see manufacturer’s references
PSD95- Human, Mouse, Rat, Immunocytochemistry, see manufacturer’s references
GFAP – Human, Mouse, Rat, Immunocytochemistry, see manufacturer’s references
AQP4 – Human, Mouse, Rat, Immunocytochemistry, see manufacturer’s references
FOXG1 – Human, Mouse, Rat, Immunocytochemistry, see manufacturer’s references
TBR1 – Human, Mouse, Rat, Immunocytochemistry, see manufacturer’s references
TUJ1 – Human, Mouse, Rat, Immunocytochemistry, see manufacturer’s references
CTIP2 – Human, Mouse, Rat, Immunocytochemistry, see manufacturer’s references
C1Q – Human, Mouse, Rat, Immunoblot, Immunocytochemistry, see manufacturer’s references
Donkey anti-mouse Alexa 488 - ThermoFisher, A-21202, polyclonal
Donkey anti-chick Alexa 488 - Chick, Immunocytochemistry, see manufacturer’s references.
Donkey anti-rabbit Alexa 555 - Mouse, Immunocytochemistry, see manufacturer’s references.
Donkey anti-mouse Alexa 647 - Mouse, Immunocytochemistry, see manufacturer’s references.
Donkey anti-chick Alexa 647 - Chick, Immunocytochemistry, see manufacturer’s references.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) WA01 (H1) and WA09 (H9) cells were from the WiCell Stemcell Bank. CRISPR edited cell lines (H1-C3KO, H9-iCas9, MEL1 GPI-H2B-GFP) were generated in the Studer lab. iPSC lines (DBR1, IFNAR1, IL10RB, STAT1) were reprogrammed in the Notarangelo lab at the NIH.

Authentication All CRISPR-edited cell lines were validated using sanger sequencing and karyotyped. Additionally, C3KO lines were validated for loss of C3 protein by ELISA.

Mycoplasma contamination All cell lines tested negative for mycoplasma contamination.

Commonly misidentified lines (See ICLAC register) No commonly misidentified cell lines were used in this study.

Human research participants

Policy information about studies involving human research participants

Population characteristics Brain tissue was provided by the Netherlands Brain Bank (NBB). Informed consent for brain autopsy, the use of tissue, and use of clinical information was obtained pre-mortem. Brain tissues were collected from one male and three female donors without a history of a neurological or psychiatric disorder with an age range between 60 and 77 years.

Recruitment Brain tissues were requested from donors without a history of neurological or psychiatric disorders over the age of 60.

Ethics oversight 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).

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Cells were dissociated with Accutase for 20min and resuspended in FACS buffer containing 1% BSA, 2mM EDTA, 30ug/mL DNase I and Normocin in PBS. Cells were washed and incubated in FACS buffer with antibody for 30 minutes on ice at 4 degrees in the dark. Cells were washed and resuspended in FACS buffer and strained through 40µM caps to eliminate cell clumps.

Instrument
BD LSR II

Software
FACS DIVA (BD) was used to collect data, and Flowjo LLC was used for analysis.

Cell population abundance
50,000 - 100,000 microglial cells were sorted from co-cultures for RNA-sequencing analysis. Post-sort analysis was done to ensure purity of the sorted sample.

Gating strategy
FSC/SSC gating was set so that all cells were visible on the plot and away from the axes. Single cells were next gated based on FSC-W vs. FSC-H and SSC-W vs. SSC-H. DAPI-negative cells were next selected. Gating was then set on an unstained control and positive populations within samples were determined based upon this gating.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.