

Peer Review File

Manuscript Title: iPSC-microglia promote brain organoid maturation via cholesterol transfer

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

In this paper, Park et al describe a culture system named iMicro, in which stem cell-derived brain organoids derived using an unguided approach are co-cultured with separately derived microglia. The authors claim that this co-culture system supports greater 'maturity', with the organoid affecting the development of the microglia and the microglia promoting the maturation of neural cells in early stage brain organoids. They then use this platform to explore the role of microglia in lipid transport in neural cells. Systematic studies of cell-cell interactions between human microglia and human neurons are much needed, and this work is an important effort in this direction. As described below, however, many of the conclusions are not sufficiently supported by the data.

There are a number of concerns in the field about how closely microglia/monocyte-like cells resemble microglia (or what microglia state they resemble). This is particularly important to address as in vitro maintenance of microglia has been associated with rapid changes in their gene expression, function that can confused interpretation of the data (PMID: 28521131). Without doubt the advantage of this in vitro system is the human aspect. However, it's unclear what unique aspects of human biology can be investigated with this co-culture system versus using more sophisticated, in vivo studies in animals.

Insufficient experimental details are often provided in the manuscript. Information on how many iPSC clones were included, how many differentiation batches or how many samples were used for each experiment should be included. Validation of a new platform should be done in multiple hiPSC clones. Even when these details were shown, the samples are rather modest and it's unclear what the power for detecting changes in this system is. For instance, co-cultured iMac cells are dramatically underrepresented in the single-cell data (n= 30), especially compared to the iMac alone condition. Fig. 1b, c suggests that there should be far more iMac cells in each organoid. Does this underrepresentation affect the single cell results?

Other reports that claim that microglia innately develop in organoids derived using unguided methods (presumably because non-ectodermal lineage stochastically develop). For example: PMID: 30301888. This potential confounding factor should be addressed experimentally.

One of the challenges of co-culturing different cell lineages is adapting cell culture media in this transition. It's unclear what the effect of this abrupt change is. What is the activation state of these cells? How are they affected by this transition? For instance, it's unclear how useful is the DEG analysis between iMicro and the other cell types in co-cultured organoids (Fig. 3a)? Such differences

would be trivial, as they are fundamentally different cell types. A critical comparison is between co-cultured iMicro cells and non-cultured iMac cells. Is there any evidence that NPC or neurons can affect microglia differentiation? This would be an important aspect to address.

The “maturation” claims need more evidence. Neuronal maturation involves more than neurite growth and proliferation and cannot be separated from electrophysiological maturation (especially in light of the long-term functional maturation of human stem cell-derived neurons). If the authors want to make maturation claims, they should include a detailed patch clamp electrophysiological characterization of neurons.

One of the main roles of microglia in development is to regulate synapses in neurons. This feature, especially in the context of the maturation claims of the paper, should be verified in this system.

From Fig. 1j, it seems that the overall number of iMicro is very low. How can these few cells modulate ‘maturation’ in the whole organoid? Also, why are NPC and co-NPC mixed in one cluster?

Statistical significance testing in Fig. 2a seems to require an ANOVA rather than multiple Man-Whitney t-tests.

Fig. 1f and Fig. 1g are anecdotic. At a minimum, the authors must demonstrate multiple replicates of these experiments. Ideally, the authors should provide a quantified metric to show that these cellular movements are not by chance. Moreover, these results require a control: do iMac acquire these functionalities because of co-culture with organoids?

Fig. 2l and Fig. 2m require quantification. A random seeding of cells would ensure that some number of iMicro would be in proximity to Ki67+ NPCs—it should therefore be tested if this proximity is not merely due to chance. Likewise, the representation in Fig. 2m does not prove that NPCs are phagocytosed by iMicro. Is the SOX2 signal fully enclosed in all three dimensions by the IBA1 signal? Moreover, SOX2 is a nuclear marker, and this phenomenon should be tested with other markers.

What is the evidence that the neurites in Fig. 2h are axons? This panel seems to plot “the total number of axons per cell”. Moreover, studying neuronal morphology in 3D organoids will be more informative here than after dissociation and 2D culture.

In testing whether BODIPY containing lipids are transferred from iMicro to NPC (Fig 3f, g, h), the comparison should be performed to other types of cells (i.e. non-iMac) such as iPSC, not just free BODIPY.

Just expression of PLIN2 is not sufficient to make claims about the utility of the system. Loss or gain of function studies are needed. Can the changes observed in NPC be induced by adding PLIN2+ lipid droplets?

Other issues:

- A control organoid should be shown as a comparison for Fig. 1b and Fig. 1c.
- Fig. 1m appears superfluous. This simple labeling could be incorporated into Fig. 1a.
- Fig. 2b has a y-axis label “number of cells per organoid ($\times 10^5$)”. This should perhaps be ($\times 10^5$). Fig.

2b should have a column for the total number of all cells from each organoid, to provide a stable reference point.

- The name “iMac” is already charged with meaning in the computer world. I would suggest another abbreviation for this system.

Referee #2 (Remarks to the Author):

In this study, Park et al investigate the role of human microglia in regulating neural progenitor cell (NPC) responses and maturation in early development. This was achieved using a new model of iPSC-derived organoids transplanted with primitive macrophages derived from the same iPSC line, which then mature into microglia-like cells. These organoids model very early developing brain with abundance of NPCs yet lack of mature cells like astrocytes – importantly, this is distinct from previous work using human organoids at an advanced maturation state where either human iPSC-derived microglia were transplanted in (Abud 2017 Neuron) or endogenously derived within organoids (Ormel 2018 Nat Comms). Using single cell sequencing, live imaging, proteomics, flow cytometry, and immunofluorescence, the authors make 3 major conclusions. First, transplanting in macrophages that become microglia-like cells (termed iMicro) limits the growth of organoids, suggesting an impact on limiting NPC expansion in development. Second, iMicros increase the maturation of NPCs into neurons and axon development. Third, iMicro transfer cholesterol/esters to NPCs in the organoids, and this may modify NPC metabolism and their maturation. The overall conclusion is that microglia advance brain development by providing cholesterol to NPCs to support their maturation. This is exciting as it builds on existing knowledge of lipids regulating NPC responses and neurogenesis, by now implicating human microglia as suppliers of cholesterol to support neuronal maturation. In addition, the organoid system + transplant protocol developed here is very interesting and could be of great value to the field of human brain development.

With regards to the data, I have the following suggestions:

Major suggestions:

1. The organoid + transplant system is very interesting, it would be useful to have a bit more characterization. Can the authors give quantitative info on the iMac/iMicro penetration in the organoid? Is it all the way through and can this be shown by z-stacks. If not all the way through, then is there a gradient of NPC maturation (neuron number, axon length) based on proximity to iMicros? Can the authors stain for P2RY12 and TMEM119 to complement the flow cytometry work – is expression (there or not) heterogeneous, or do all iMacs alter their expression?
2. iMac transplant causes a decrease in the size of the organoids. However it is still unclear how the microglia are regulating this change in organoid size. Can the authors quantify NPC proliferation and death in organoids alone and following iMac transplant, and quantify the frequency of iMicro phagocytosis of NPCs? Can the authors clarify why the single cell sequencing not reflect the change in numbers of the cells following transplant?
3. The single cell sequencing of the NPCs/neurons/iMicros has yielded some interesting concepts as to how the cells are responding and interacting. This would be greatly strengthened by validation at the RNA level (RNAscope) or protein level (ICC) throughout the manuscript. For instance, validating expression of a subset of genes involved in neurogenesis, axon development, proliferation,

metabolism.

4. Regarding the cholesterol transfer, it is a really interesting hypothesis that needs to be further investigated. The killer experiment would be functionally interfering with the cholesterol transfer specifically from iMicro, and eliminating the positive effects of transplant on neuronal maturation – this would make the definite link between microglia cholesterol synthesis and neuronal responses. In addition, what is the consequence of cholesterol uptake in the NPCs: do the NPCs which are BODIPY+ have longer axons? Are there more mature neurons which are BODIPY+ (after NPCs having taken it up) compared to BODIPY negative neurons? Do the BODIPY+ neurons have altered metabolism? Can the authors hypothesize how the cholesterol impacts the NPCs?
5. Overall, it would be useful to have quantifications of the data provided, for a) iMicro responses to laser injury and Abeta phagocytosis, b) iMicro proliferation, c) proximity of iMicro to proliferating NPCs/ axons, d) PLIN2+ iMicro+ cells, d) PLIN2+ LIPIDSPOT610+ IBA1+ cells in human and mouse (fig4)
6. Can the authors clarify what the n numbers indicated stand for, are these are numbers of organoids from 1 clone, or different clones from the same line, or different iPSC lines. It would be most robust to have multiple lines represented.

Minor suggestions:

1. The numbers of iMicro in the organoid appears very small from the quantification in extended data Fig3– which is not what it looks like from the high number of cells in Fig.1b. What is the absolute number of iMicro in the organoids? Is the density of iMicro similar to the density of microglia in human developing brain?
2. Since the maturation of neurons is increased with iMicro coculture, can the authors explain why no significant increase in the % of neurons is seen in organoids in Fig2b?
3. Can the authors clarify the age of the human samples used in the UMAPs in Fig.4?
4. Can the authors provide quantification with statistics of APOE from the transcriptomics and proteomics in extended Fig5d?
5. Figure 2h represents number of neurons and axon length in a strange plot, can this be re-graphed so we can see individual data points and the mean? It is difficult to tell the difference as presented, even with significance indicated.
6. Can the authors ensure that the time point of the organoid is indicated in the text and figure legends for all the single cell sequencing/proteomics.
7. The use of proteomics is really interesting, yet could have been further analysed to yield more information as we only see one of the proteins highlighted. Does the pathway analysis of proteomics show the same as the transcriptomics? Can the proteomics results also be validated by staining for several proteins of interest?
8. Can the authors reference Abud 2017 Neuron and Ormel 2018 Nat Comms in the paper- as these are organoids in which microglia are present.
9. Can the authors increase the n numbers for the d30 qPCR of Sall1 , as it is very variable.

Referee #3 (Remarks to the Author):

In this work, Park et al report the development of a novel organoid system which allows the study of the role of microglia during the early developmental steps of the human brain. These studies identified a unique role for microglia in limiting neuronal progenitor cell proliferation and the promotion of axonogenesis. Furthermore, the mechanistic investigation of these observations identified a novel pathway by which microglia exported cholesterol and its esteros to NPCs. Finally, the investigators validated their findings via the analysis of mouse and human brain samples. Taken together, these studies provide novel insights into the mechanisms that control brain development, while describing a unique system for the investigation of the brain in health and disease. Hence, these studies will certainly have a profound impact in the field and should be of interest to the broad readership of Nature. This work is performed by experts in the field, the experiments are properly controlled and described. However, it is my opinion that some issues should be addressed before this work is ready for publication.

Major comments.

1. Please compare the transcriptional profile of iMicro with that of embryonic microglia, to allow the reader to fully evaluate the possibilities and limitations offered by this new organoid system.
2. Although APOE is highly expressed in iMicro, no data are provided to support its role in lipid transport. The authors should use blocking antibodies or genetic inactivation to demonstrate the role of APOE in microglia-neuron lipid transport.
3. Provide a quantification of the data shown in Fig. 3b.
4. Quantify data shown in Fig 3e. Also, what is the difference between top and bottom panels? Please label them.
5. Quantify data in Figs. 4b and 4c.
6. Dysregulated sphingolipid metabolism plays an important role in CNS inflammation and neurodegeneration driven by glial cells (Chao et al, 179:1483–1498). Do the authors know if in addition to cholesterol and its derivatives, microglia can transfer additional lipids to neurons. This is an important point to incorporate to the discussion.

Minor comments.

7. Line 132, please use “signs” instead of “signed”.
8. Sentence in lines 275-276, please adjust as it doesn't read fine (“....ability to export lipids, among with cholesterol, for uptake...”).

Author Rebuttals to Initial Comments:

We thank the three reviewers for their careful reviews of the manuscript and the associated detailed and relevant comments they provided. In this revised manuscript, we performed the requested experimentations and analysis (summary and details below) to strengthen the characterization of microglia-sufficient brain organoids resulting from the co-culture of brain organoids with primitive-like macrophages (iMac) from the same source of iPSC. Reflecting the updated author list, the reviewers will appreciate that these additional experiments were generated in at least two separate labs at the Gustave Roussy Institute in Paris and the Singapore Immunology Network in Singapore, thus strengthening our study.

In summary, the manuscript has undergone some major modifications to show the following:

- Our microglia-sufficient **model is validated** using multiple iPSC lines (7 independent lines).
- The presence of **innate microglia** has been characterized with 4 of the 7 iPSC lines **but not in the KYOU1 line we have used for our whole study**.
- The **co-culture of brain organoids with iMac has a profound impact on the neurogenesis process** as demonstrated by further analysis and experimental studies showing specifically less neural rosettes in numbers, their reduction in size as well as a significant reduced proliferative capacity of NPC associated with increased pathways related to neuronal differentiation, neurogenesis and synaptogenesis.
- Supporting, the maturation of organoids upon macrophage addition, **patch-clamp electrophysiological analysis** suggests that the addition of iMac promotes an increase in the functional maturation of neurons.
- The **transfer of cholesterol from iMicro to NPC/neurons** is an active process mediated through the generation of APOE+ lipoprotein-like particles, and directed by the ATP-binding cassette transporter ABCA1 (also known as the cholesterol efflux regulatory protein) on iMicro, specifically shown by using Probucol, an effective inhibitor of ABCA1-mediated cholesterol efflux. In addition, using supernatant transfer experiments, we show that the release of cholesterol from iMac was enough to induce changes in organoid growth; such changes were also abrogated by the use of the ABCA1 inhibitor probucol.

Please find below our detailed responses to each reviewer's comments.

Referees' comments:

Referee #1 (Remarks to the Author):

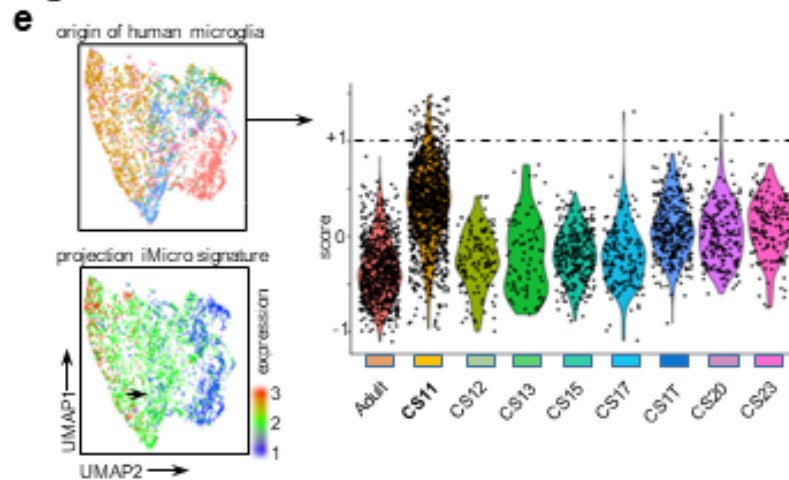
In this paper, Park et al describe a culture system named iMicro, in which stem cell-derived brain organoids derived using an unguided approach are co-cultured with separately derived microglia. The authors claim that this co-culture system supports greater 'maturity', with the organoid affecting the development of the microglia and the microglia promoting the maturation of neural cells in early-stage brain organoids. They then use this platform to explore the role of microglia in lipid transport in neural cells. Systematic studies of cell-cell interactions between human microglia and human neurons are much needed, and this work is an important effort in this direction. As described below, however, many of the conclusions are not sufficiently supported by the data.

There are a number of concerns in the field about how closely microglia/monocyte-like cells resemble microglia (or what microglia state they resemble). This is particularly important to address as in vitro maintenance of microglia has been associated with rapid changes in their gene expression, function that can confused interpretation of the data ([PMID: 28521131](https://pubmed.ncbi.nlm.nih.gov/28521131/)).

We fully agree with the reviewer that this is an important point (as highlighted in Bohlen *et al*, Neuron, 2017) and an important focus of this study.

We have shown in Figure 1 that iMac undergo significant transcriptomic and phenotypic changes that make them acquire a state closer to microglia (thus called iMicro), supported by expression of microglia-specific transcription factor (*SALL1*), regulons (*IRFs*, *CEBP*, *EGRs*, *AP-1*) and phenotypic markers such as *P2RY12* and *CX3CR1* (at the protein level). To attempt to answer to which microglia developmental state iMicro could correspond to or be closer to, we integrated our iMicro transcriptomic data with the Allen Brain atlas data to find that our iMicro have the highest similarity with CS11 human brain microglia (Fig. 4e) and E13.5 in mouse development (Fig. 4b).

Figure 4



Finally, by immunofluorescence staining, we also show that the specific PLIN2+ Lipid droplet expressing IBA-1+ iMicro we described in our human organoid model can be observed in mouse and human fetal brain (E13.5 and 15-21 weeks respectively) (Fig. 4b-d and Extended data, Fig. 9d). All these data suggest that iMac differentiate into a state closer to embryonic microglia. The figures and associated texts have been updated accordingly.

-Bohlen CJ, Bennett FC, Tucker AF, Collins HY, Mulinyawe SB, Barres BA. Diverse Requirements for Microglial Survival, Specification, and Function Revealed by Defined-Medium Cultures. *Neuron*. 2017;94(4):759-773.e8. doi: 10.1016/j.neuron.2017.04.043.

Without doubt the advantage of this in vitro system is the human aspect. However, it's unclear what unique aspects of human biology can be investigated with this co-culture system versus using more sophisticated, in vivo studies in animals.

This is an important point and we want to highlight that iPSC are easily targetable by gene editing approaches such as CRISPR and the role of any gene can thus be tested in principle in this system, in either organoid cells versus macrophages or *vice versa* or both. In addition, mounting evidences from our laboratory and others show how microglia differ between mouse, non-human primate or human not only in adult but also during the various stages of development (Kanton *et al.*, *Nature*, 2019; Geirsdottir *et al.*, *Cell*, 2019). In our microglia-sufficient brain-organoid model, we show that the neurogenesis process is drastically changed upon addition of autologous iPSC-derived macrophage/microglia, mimicking the early colonization of yolk sac macrophages in embryonic brain and their effect on NPC and neurons. Our model represents therefore an ideal model to study the

early interactions between microglia and brain cells in healthy development and in a perturbed environment as well as in genetic models of human neurodevelopmental diseases or even neurodegeneration such as Alzheimer's Disease. Thus, we believe that both approaches (human microglia-sufficient organoids vs sophisticated *in vivo* studies in animals) are valid and more importantly complementary.

-Geirsdottir L, David E, Keren-Shaul H, et al. Cross-Species Single-Cell Analysis Reveals Divergence of the Primate Microglia Program. *Cell*. 2019;179(7):1609-1622.e16.

-Kanton S, Boyle MJ, He Z, et al. Organoid single-cell genomic atlas uncovers human-specific features of brain development. *ee574(7778):418-422*.

Insufficient experimental details are often provided in the manuscript.

We apologize for the lack of experimental details in the first version of the manuscript. We have added more technical details (days, repeats, statistical analysis, name of iPSC clone, etc.) where appropriate in the text, the legends/Figures and the Method sections. This is reflected in the examples below and as highlighted:

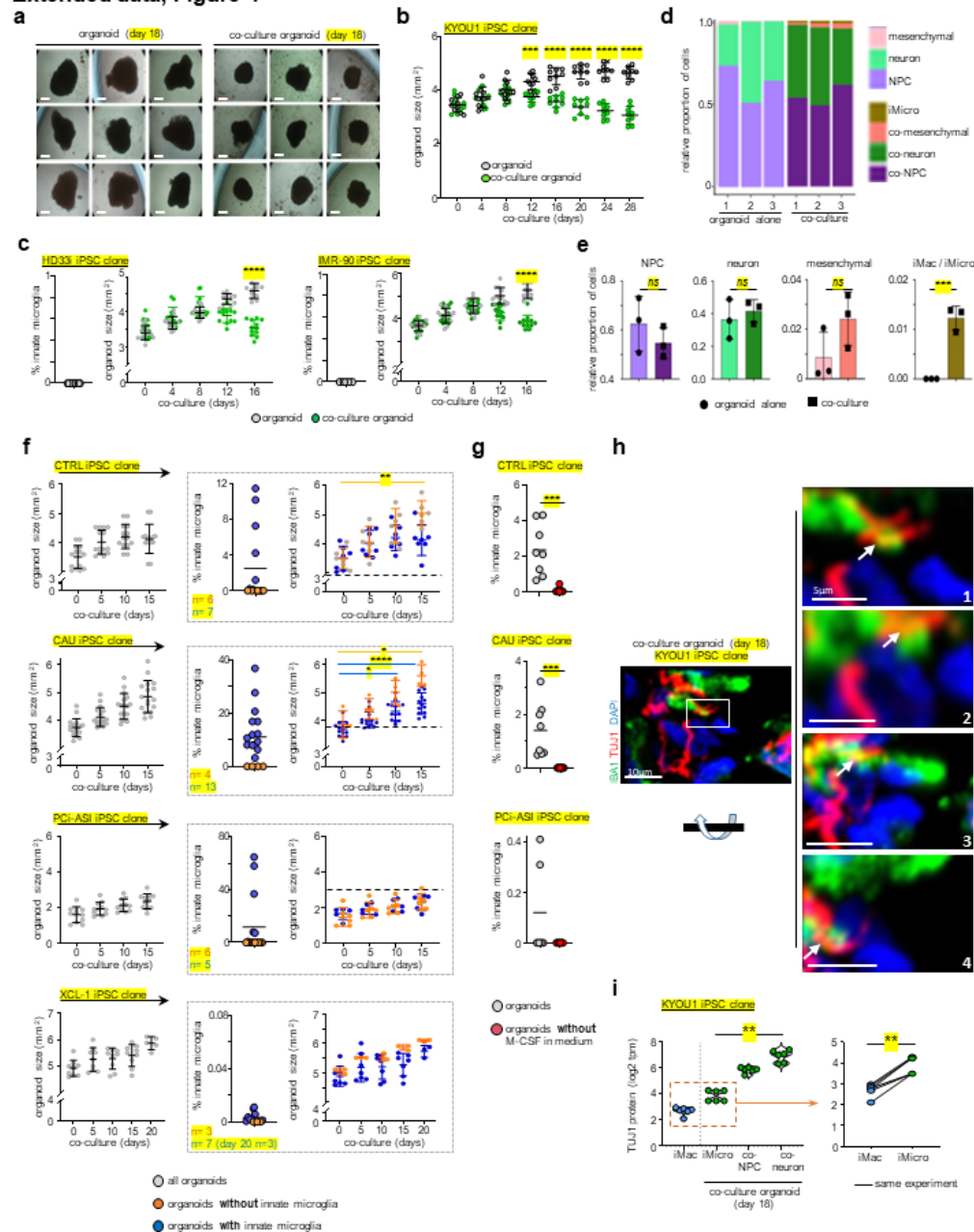
Example of Figure legends:

Figure 1. iMac become microglia-like cells in brain organoids. **a**, Schematic overview of the generation of microglia-sufficient brain organoids. iMac and brain organoid are obtained **26 days** after initial iPSC culture and constitute **day 0** iMac and organoids. Co-cultured organoids are analysed from 15 days onward (**day 15**) after co-culture of **day 0** iMac and organoids. Unless stated, the KYOU1 iPSC line is used throughout the study. **b**, Brain organoids were co-cultured with or without iMac for 18 days, cleared and imaged in 3D. **Scale bar, 300 μ m**. **Boxed area** highlighted on the right. Arrowhead, a cluster of iMac on organoid. **Scale bar, 100 μ m**. (**Representative of n=3**). **c**, Sectioning and immunofluorescence staining of brain organoid co-cultured with or without iMac for 18 days. **Scale bar, 100 μ m**. **Boxed area** highlighted on the right. **Scale bar, 20 μ m**. (**Representative of n=4**). **d**, RT-qPCR data showing the expression levels of microglia-specific markers in iMac alone (**n=3**) or iMac co-cultured with brain organoids (**n=3**) for 15 or 30 days. **Statistical analysis, One-way Anova**. **e**, Flow cytometry data showing the expression levels of microglia-specific markers in **day 18** iMac (**n=3**) and iMac co-cultured with brain organoids (**n=3**) for 18 days. **f**, tSNE plots showing the gene set score for canonical markers used to define four different cell types (neurons, NPC, iMac and mesenchymal cells) under the various organoid culture conditions obtained at **day 18** (**concatenated representation**). **g**, tSNE plots showing the projection (on a concatenated representation) of the four different cell types coming from organoids cultured in the presence (co-culture) and absence (organoid alone) of iMac for **18 days**. Co-cultured iMac were magnified and highlighted in the right panel. **h**, Heatmap showing the expression levels of regulons involved in the acquisition of microglia identity in iMac and co-cultured iMac (co-iMac). **i**, Violin plots comparing the expression levels of regulons involved in the acquisition of microglia identity between iMac and co-iMac. **Statistical analysis, Mann-Whitney test**. **j**, Representative voltage responses obtained from whole-cell patch-clamp recordings at 0 pA, rheobase and maximum action potential (AP) firing (bottom to top). **k**, Analysis of the first, second and third AP observed during maximum AP firing of organoid alone (**n=8**) and co-culture (**n=12**). **Statistical analysis, Mann-Whitney U test**. **l**, Characterization of AP firing patterns in organoid alone and co-cultured with iMac. Example traces representing firing patterns used for the characterization are shown (left panel). Distribution of AP firing patterns for neurons from control (**n=40**) and co-culture (**n=66**) organoids. **m**, Representation of intrinsic properties of neurons from each organoid alone (**n=20**) and co-culture organoids (**n=23**). **Statistical analysis performed using Mann-Whitney test**.

Induced pluripotent stem cell culture. Human iPSC KYOU1 (KYOU-DXR0109B, #ACS-1023) and IMR-90 (#CCL-186) were obtained from ATCC. CTRL iPSC line was obtained from Dr Ragnhildur Thora Karadottir. CAU iPSC was obtained from Atlantis Bioscience (#PCi-KER_KIT), HD33i from the NINDS iPSC Repository at Coriell Institute (#ND36997), PCi-ASI from Phenocell (#PCi-ASI_0.5M) and XCL-1 from XCell Science (#IP-001-1V). All iPSC lines had verified pluripotency and were mycoplasma-free. The cells were maintained on Matrigel (Corning, #354277) in mTeSR1 medium (STEMCELL Tech, 85850), which was changed every day. When iPSCs reached about 70% confluence, the cells were passaged using ReLeSR (STEMCELL Tech, #05872). Unless specified, the KYOU1 iPSC line is used throughout the manuscript to generate iMac and brain organoids.

Example of Figures:

Extended data, Figure 4



Information on how many iPSC clones were included, how many differentiation batches or how many samples were used for each experiment should be included. Validation of a new platform should be done in multiple hiPSC clones.

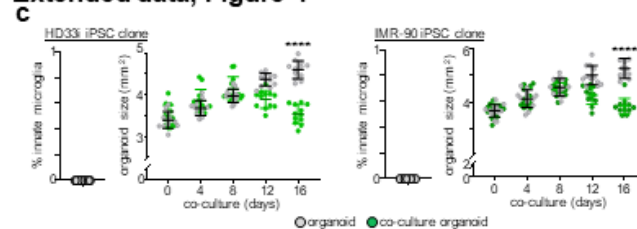
We thank the reviewer for his/her comments and we have updated throughout the manuscript and the methodology section information on the number of differentiation batches or samples used for each experiment.

About using multiple iPSC clones, we agree that it is an important point and our new platform has now been validated using multiple iPSC clones. Alongside the KYOU1 iPSC clone used as the main model, we tested 6 more iPSC clones: HD33i, IMR-90 (Extended data, Fig. 4c), and CTRL, CAU, PCi-ASI and XCL-1 clones (Extended data, Fig. 4f). In all cases, iMac can be generated with the yield being line-dependent.

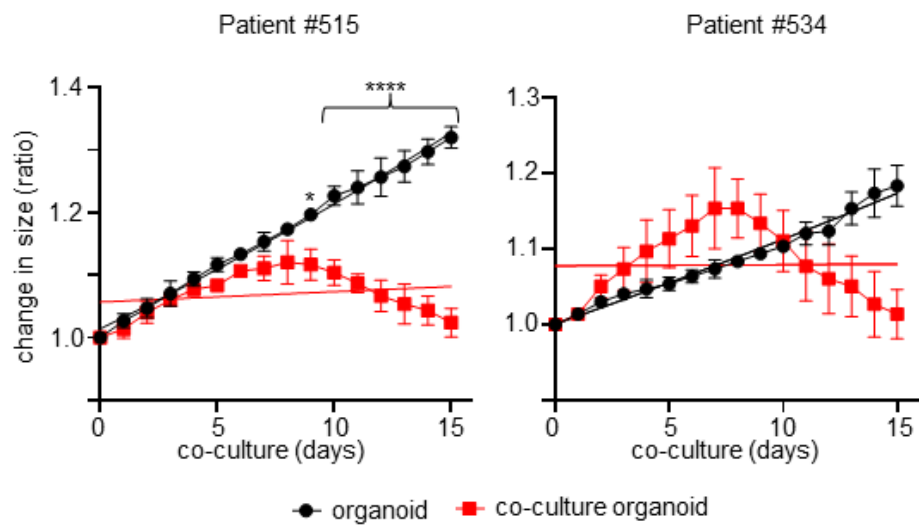
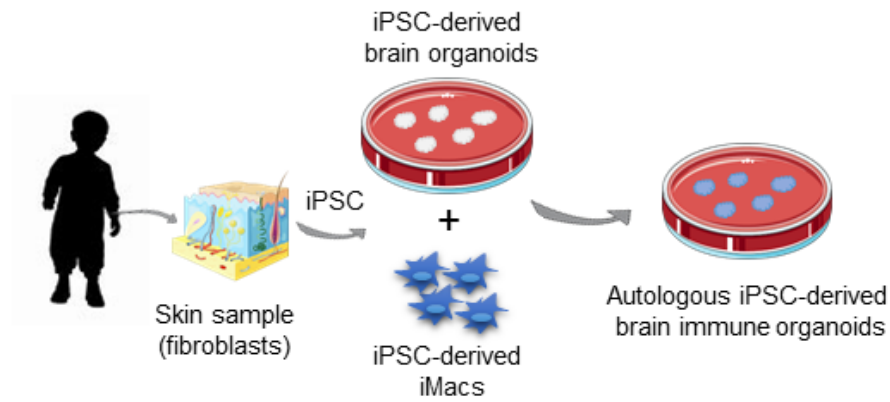
However, using these different clones, we noted the generation of innate microglia in brain organoid (Discussed also below). Importantly, the apparition of innate microglia is also clone-dependent with none detected in brain organoids generated from iPSC clones KYOU1, HD33i and IMR-90 (Extended data, Fig. 4b, c), but some detected with iPSC clones CTRL, CAU, PCi-ASI and XCL-1 (Extended data, Fig. 4f, g).

Importantly, among the clones not generating innate microglia, as observed with iPSC clone KYOU1, the addition of iMac to brain organoids generated using iPSC clones HD33i and IMR-90 (Extended data, Fig. 4c) has an effect on organoid size starting from day 12 and with significant differences reached after 16 days of co-culture, thus validating our observations in three independent iPSC lines.

Extended data, Figure 4

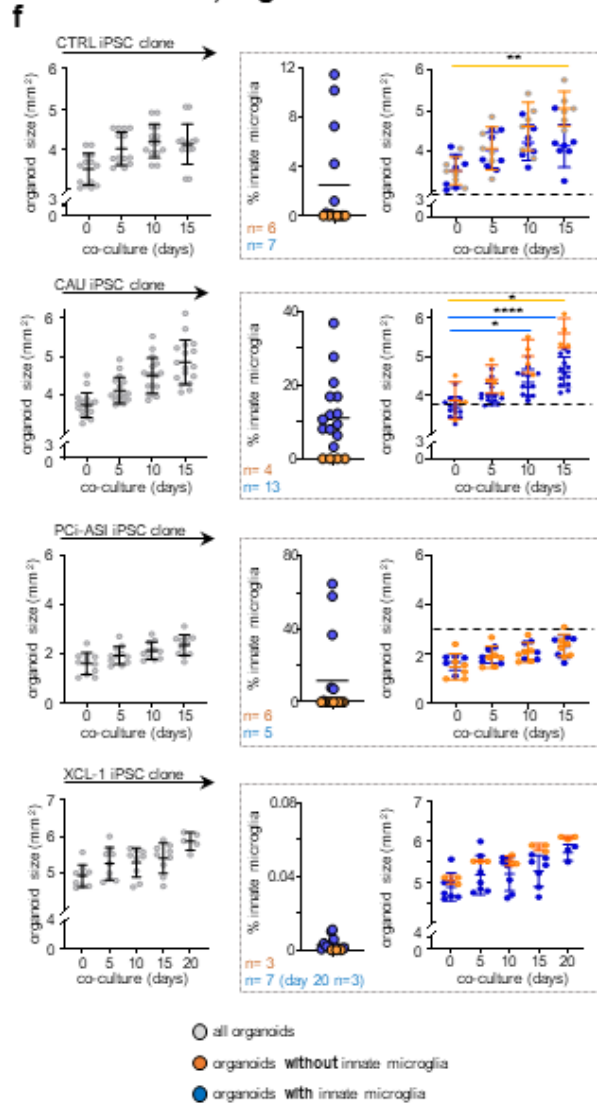


We have also validated our observations in more than 5 independent iPSC lines generated from 5 diffuse intrinsic pontine glioma (DIPG) patients (2 of which are shown below for the reviewer's discretion).



Innate microglia were observed with iPSC clones CTRL, CAU, PCi-ASI and XCL-1 and yet their proportion is heterogeneous within each iPSC clone organoid culture (Extended data, Fig 4f). Interestingly, as it was observed upon the addition of iMac to brain-organoids, the reduction in size was only observed in organoids with innate microglia whose size was above 3mm² (iPSC clones CTRL and CAU), but not below (iPSC clone PCi-ASI); while no changes in organoid size could be observed with a low % of innate microglia (iPSC clone XCL-1) (Extended data, Fig 4f).

Extended data, Figure 4

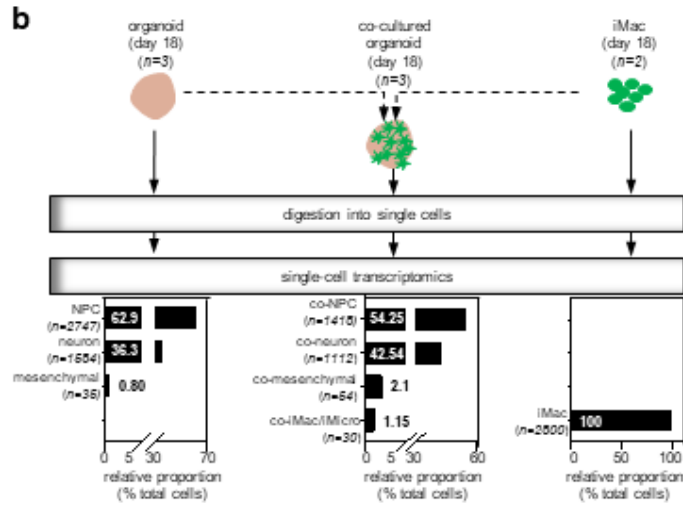


Both text (and method sections) and figures have been amended accordingly.

Even when these details were shown, the samples are rather modest and it's unclear what the power for detecting changes in this system is. For instance, co-cultured iMac cells are dramatically underrepresented in the single-cell data (n= 30), especially compared to the iMac alone condition. **Fig. 1b, c** suggests that there should be far more iMac cells in each organoid. Does this underrepresentation affect the single cell results?

We do not believe that such underrepresentation affects the single cell results. As we demonstrate throughout this study, our co-culture system enabled us to observe profound and consistent changes at the transcriptomic, proteomic, phenotypic, metabolic (lipidomic) and functional levels upon addition of iMac. We believe that the underrepresentation of co-cultured iMac/iMicro cells in the single-cell data (n=30, representing 1.15% of cells analysed by 10X technology, Extended data, Fig. 2b) may represent a technical issue inherent to such approach resulting in a low recovery of iMicro versus others cell types.

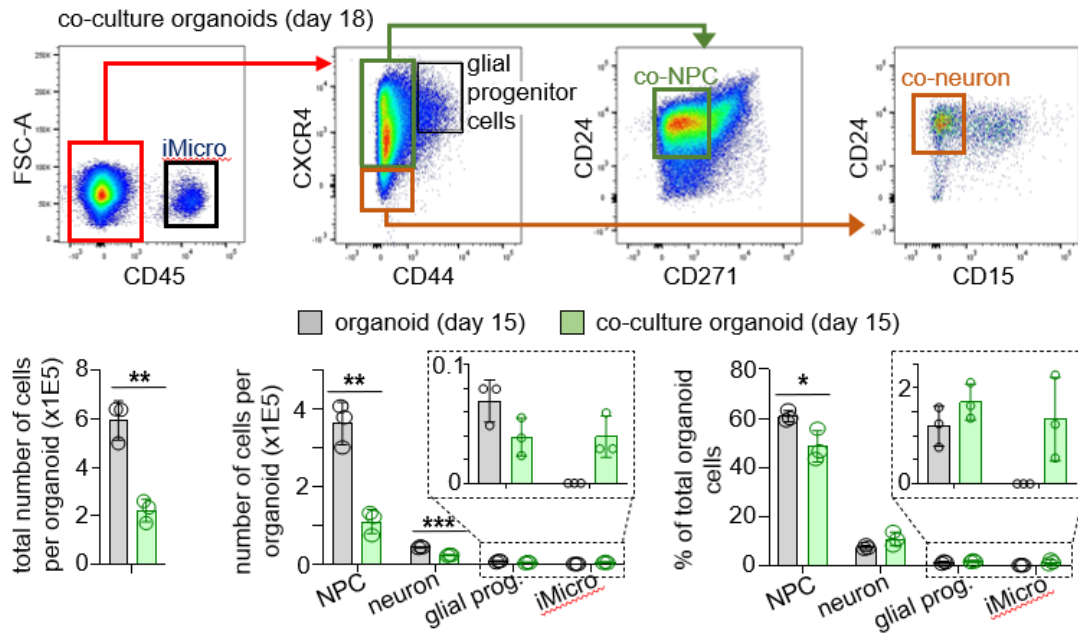
Extended data, Figure 2



Flow cytometry analysis and immunofluorescence imaging clearly demonstrate that the proportion of iMicro is higher in co-cultured brain organoids. We want to point that unless clearly stated, 150,000 iMac were added to each organoid when the co-culture is started, thus representing about 0.7-2.4% (cell count from flow cytometry data, Fig. 2b) of the co-cultured organoids while representing 1.15% of cells in the single cell data, resulting in a 5-7.5 fold reduction in expected numbers.

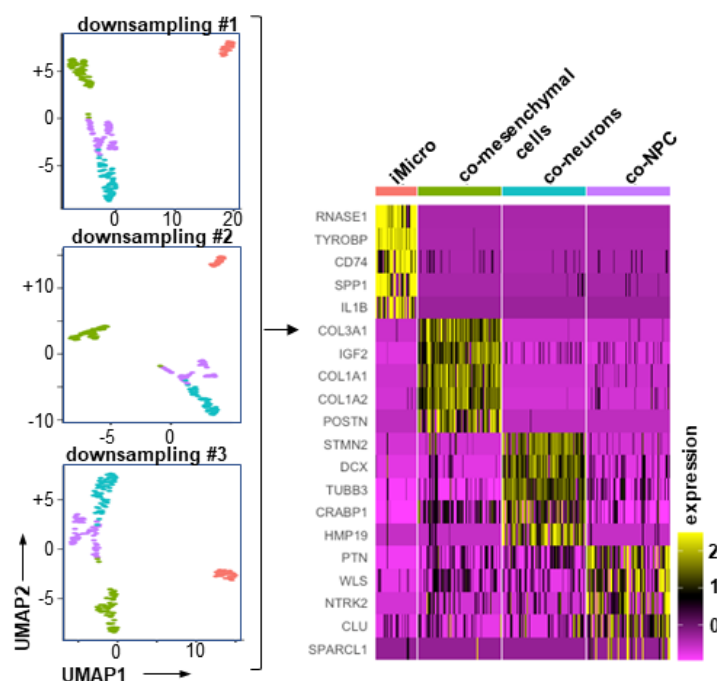
Figure 2

b



However, to confirm that the low number of iMicro does not impact the single cell result and clustering analysis, we performed down-sampling analysis. Each cell type identified by the scRNA sequencing data were randomly down-sampled so each cell type had equal number of cells. Variable gene expression analysis, UMAP, and clustering on the down-sampled cells were then performed with the Seurat Script on R and as described in the Methods section of the manuscript. From the new clusters, the gene expression was evaluated and the cell types annotated accordingly. A minimum of 3 down-sampling analysis was done all yielding to a similar heat-map analysis (Reviewer Figure below).

As such, the underrepresentation of iMicro (vs iMac alone) in our 10X scRNAseq dataset did not impact our analysis, conclusions nor clustering outcomes. **We can thus confirm that the clustering analysis presented in Fig. 1 and studied throughout the manuscript is stable despite the low number of iMicro versus iMac.**



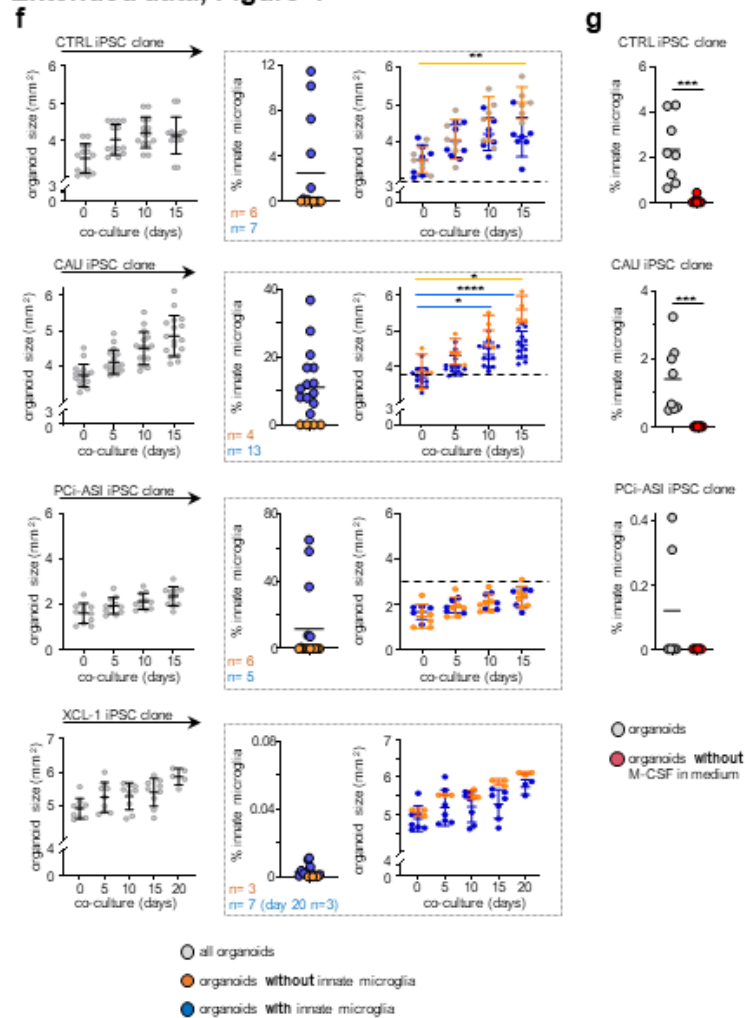
Other reports that claim that microglia innately develop in organoids derived using unguided methods (presumably because non-ectodermal lineage stochastically develop). For example: [PMID: 30301888](https://pubmed.ncbi.nlm.nih.gov/30301888/). This potential confounding factor should be addressed experimentally.

We thank the reviewer for highlighting this important point. The study by Ormel *et al.*, reported innate microglia spontaneously developing in brain organoids. Investigating innate microglia in various iPSC lines, we conclude that innate microglia is line-dependent and their apparition is also dependent on addition of M-CSF (CSF-1).

Importantly, **we did not detect the spontaneous generation of innate microglia in organoids generated from the KYOU1 iPSC clone used throughout our study** neither at the transcriptomic (Fig. 1g, Extended data, Fig. 4d, e) nor protein expression levels (Fig. 1b, c, Extended data, Fig 1, Supplementary Video. 1, 4, 5). The same was observed in brain organoids generated using iPSC lines HD33i and IMR-90 (Extended data, Fig. 4c).

The heterogeneous spontaneous detection of innate microglia was observed in brain organoids generated from iPSC clones XCL-1 (0-0.28% innate microglia), CTRL (0-12% innate microglia), CAU (0-40% innate microglia) and PCi-ASI (0-70% innate microglia) (Extended data, Fig. 4f). Importantly, the induction of innate microglia in organoids generated from the CTRL, CAU and PCi-ASI iPSC lines (not done for XCL-1 line) was dependent on M-CSF present in the culture medium as its removal abrogated their generation (Extended data, Fig. 4g).

Extended data, Figure 4



The text and figures have been amended accordingly. The reference suggested by the reviewer has also been added in the manuscript.

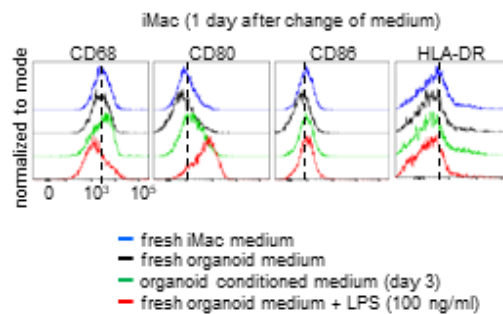
-Ormel PR, Vieira de Sá R, van Bodegraven EJ, et al. Microglia innately develop within cerebral organoids. *Nat Commun.* 2018;9(1):4167. (PMID: 30301888).

One of the challenges of co-culturing different cell lineages is adapting cell culture media in this transition. It's unclear what the effect of this abrupt change is. What is the activation state of these cells? How are they affected by this transition?

The reviewer rightfully comments on the potential impact that the change of medium could have on iMac's activation status once put in co-culture. We considered this point as indeed the formulations of media are different between the various cultures / co-cultures (Extended data, Fig. 1b).

We did not observe any significant differences in the expression of CD68, CD80, CD86 and HLA-DR activation markers by iMac when cultured in fresh organoid medium by flow cytometry analysis. As expected however, iMac incubated with organoid conditioned medium presented a slight increase of the CD80 activation marker, although not to the level observed upon activation of iMac by LPS showing a strong upregulation of CD80 (Extended data, Fig. 1b).

Extended data, Fig. 1b

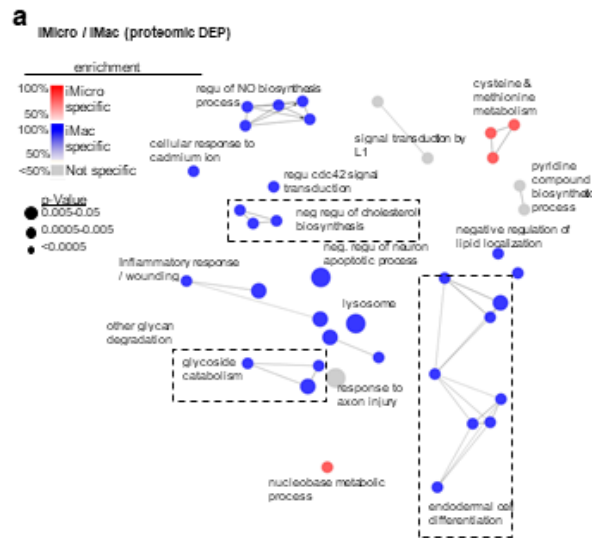


For instance, it's unclear how useful is the DEG analysis between iMicro and the other cell types in co-cultured organoids (**Fig. 3a**) (**Extended data, Fig. 8a in the updated manuscript**)? Such differences would be trivial, as they are fundamentally different cell types. A critical comparison is between co-cultured iMicro cells and non-cultured iMac cells.

We fully agree with the reviewer and have amended the analysis and figures. We performed the DEG analysis between co-cultured iMac (iMicro) and iMac (represented as a heat-map in Extended data, Fig. 3a), which clearly shows that the co-culture prompts an important remodelling of the transcriptomic landscape in iMac, especially towards a microglia-like program as shown in Fig. 1.

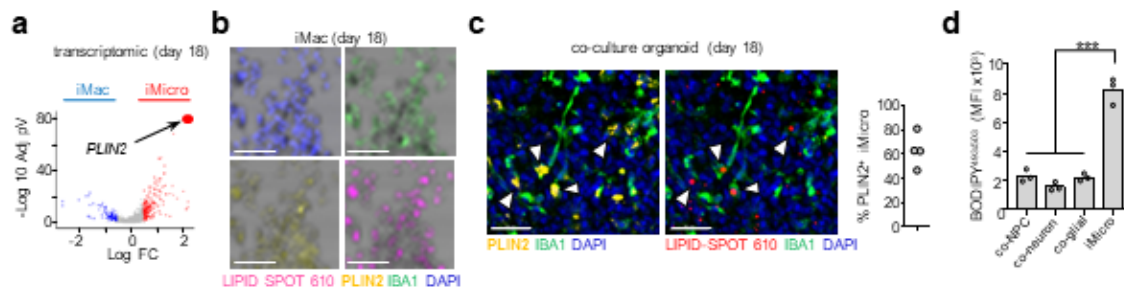
Using the proteomic data obtained by mass-spectrometry analysis on purified iMac and iMicro, we also performed a thorough comparative analysis of the differentially expressed proteins (DEP) between iMac and iMicro obtained after 18 days of co-culture (Amended in Extended data, Fig. 8a). This clearly confirms the DEG analysis showing a complete remodelling of iMac upon their co-culture with brain organoids, among which the pathways related to neurogenesis and regulation of cholesterol metabolism.

Extended data, Figure 8



For clarity, we have changed and considerably updated Fig. 3. The DEG analysis (Fig. 3a) between iMac and iMicro clearly reveals that PLIN2 was the most upregulated DEG in iMicro. By immunofluorescence analysis, we confirmed that iMac presented high levels of PLIN2+ BODIPY^{493/503} lipid droplets (Fig. 3b, Supplementary Video. 7), which were also present and preponderant in iMicro compared to other cells of co-cultured organoids (Fig. 3c, d). These observations prompted us to further investigate the transfer of cholesterol between iMicro and co-NPC (Fig. 3e-q, Extended data, Fig. 8).

Figure 3



Text and figures have been accordingly updated in the manuscript.

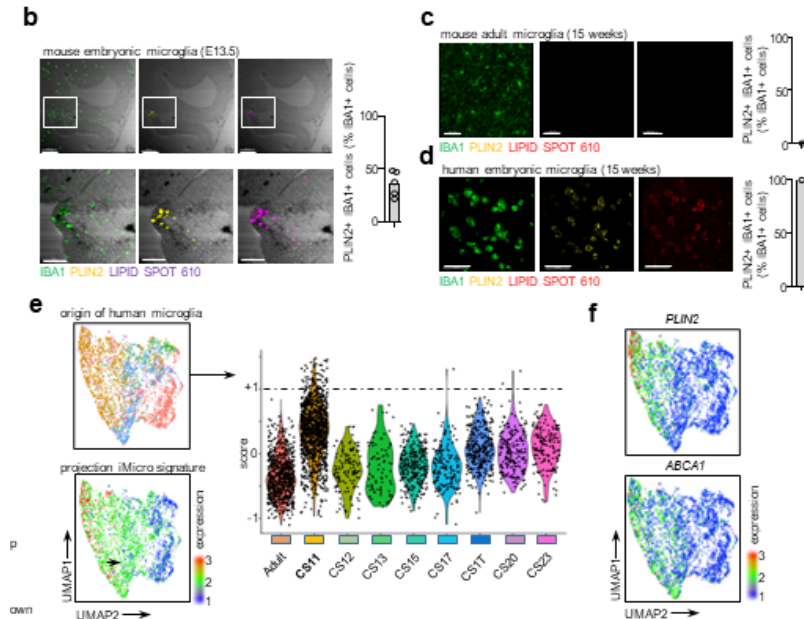
Is there any evidence that NPC or neurons can affect microglia differentiation? This would be an important aspect to address.

Macrophage to microglia differentiation is clearly resulting from a crosstalk with NPC and neurons that provide local cues supporting such process. We previously showed that iMac can differentiate to microglial-like cells *in vitro* upon co-culture with iPSC-derived neurons in a 2D system (Takata *et al*, Immunity, 2017). In the present study, we validate this finding in a 3D brain organoid model. iMac co-cultured with NPC/Neuron in a 3D brain organoid differentiate and acquire phenotypic traits resembling those of microglial cells in terms of transcriptomic profile (Fig. 1f, g and Extended data, Fig 3), regulons signature (Fig. 1h) and expression of microglial specific markers (P2RY12, CX3CR1) (Fig. 1e and Extended data, Fig. 1e). Functionally, they embed within the organoids and make contacts with

NPC and neurons (Fig. 1b, c; Extended data, Fig 1c, d; Supplementary video. 1, 4, 5), while displaying macrophage/microglia specific functions such as reaction to laser injury and endocytosis of amyloid-beta peptides (Extended data, Fig. 1g, h respectively).

More importantly, we finally demonstrate that the signature of iMicro is similar to human (Fig. 4d-f and Extended data, Fig. 9d) and mouse fetal microglia (Fig. 4b), thus validating that the PLIN2+ iMicro population we observe in our immune-sufficient brain organoid is anatomically and physiologically present during the fetal neurogenesis process.

Figure 4



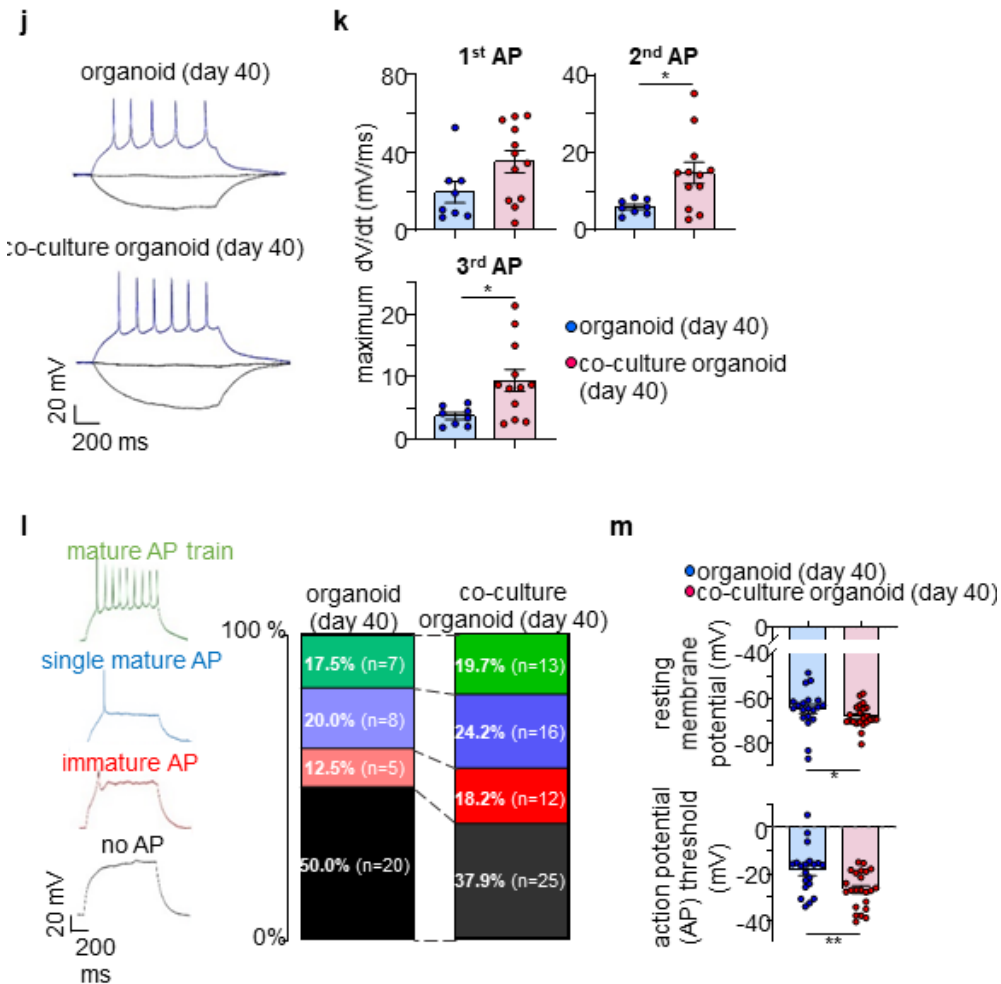
-Takata, K., et al., *Induced-Pluripotent-Stem-Cell-Derived Primitive Macrophages Provide a Platform for Modelling Tissue-Resident Macrophage Differentiation and Function*. *Immunity*, 2017. 47(1): p. 183-198 e6.

The “maturation” claims need more evidence. Neuronal maturation involves more than neurite growth and proliferation and cannot be separated from electrophysiological maturation (especially in light of the long-term functional maturation of human stem cell-derived neurons). If the authors want to make maturation claims, they should include a detailed patch clamp electrophysiological characterization of neurons. One of the main roles of microglia in development is to regulate synapses in neurons. This feature, especially in the context of the maturation claims of the paper, should be verified in this system.

We agree with the reviewer for this important comment which we have addressed by assessing how iMicro modulate the electrophysiological maturation of neurons using patch-clamp characterization analysis. Notably co-culture of brain organoids with iMac showed an increase of maximum action potential (AP) firing compared to organoids (Fig. 1j). Further analysis showed an increase of the rate of membrane potential charge (dV/dt) of the second and third AP firing of neurons during the maximum AP firing in co-cultured organoids (Fig. 1k).

To characterize deeper the electrophysiological maturation, we quantified the AP firing patterns in organoid and co-cultured organoids to show that the addition of iMac drastically reduced the amount of firing patterns devoid of any AP, while strongly increasing immature, single mature and mature AP train firing patterns (Fig. 1l). Associated with the characterisation of the intrinsic properties of neurons displaying lower AP threshold and resting membrane potentials (Fig. 1m), these results altogether demonstrate an increase maturation of neurons in brain organoids in the present of iMicro.

Figure 1



The text and figures have been amended in the revised version of the manuscript.

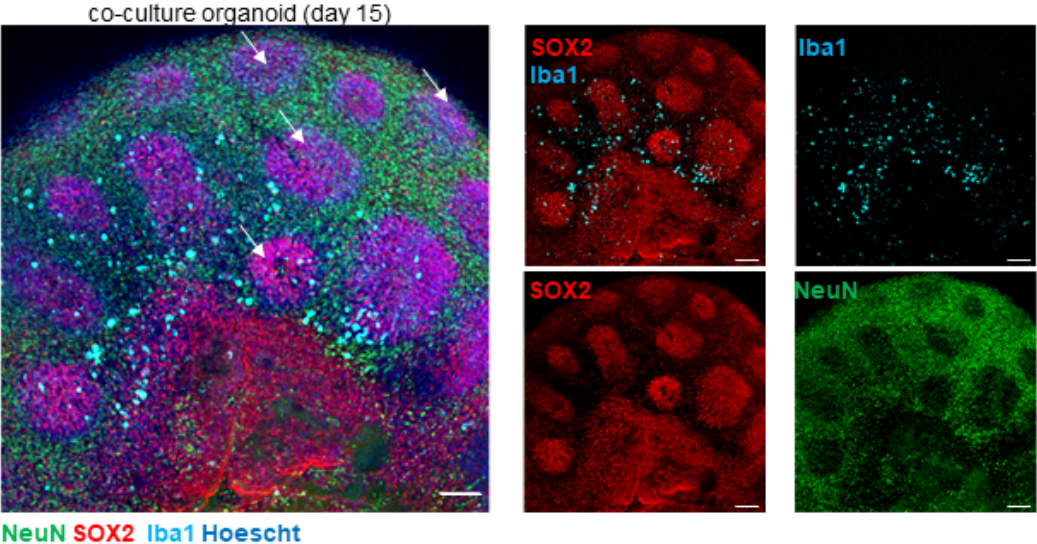
From **Fig. 1j** (**Fig. 1g in updated manuscript**), it seems that the overall number of iMicro is very low. How can these few cells modulate 'maturation' in the whole organoid? Also, why are NPC and co-NPC mixed in one cluster?

As we discussed before, the overall iMicro number recovered for single cell analysis was low (5-7.5 fold lower than expected compared to flow cytometry quantification; Extended Data, Fig. 2b and Fig. 2b respectively) but did not affect the further clustering and single cell analysis. However, we want to highlight that iMicro are embedded and distributed throughout the organoids (Fig. 1b,c; Extended data, Fig. 1c,d and Supplementary video 1, 4, 5) are in contact with NPC and neuronal cells (Fig 2.l-n,

Extended data, Fig. 4h). We hope that the reviewer will appreciate that iMicro also accumulate around crucial areas of the organoids, co-localising at the periphery where NPC structures or neural rosettes are (Extended data, Fig. 1d, Extended data, Fig. 5d and Supplementary Video. 4, 5), thus further promoting their maturation as shown in our new proteomic analysis of neural rosettes from organoids and co-culture organoids (Extended data, Fig. 5).

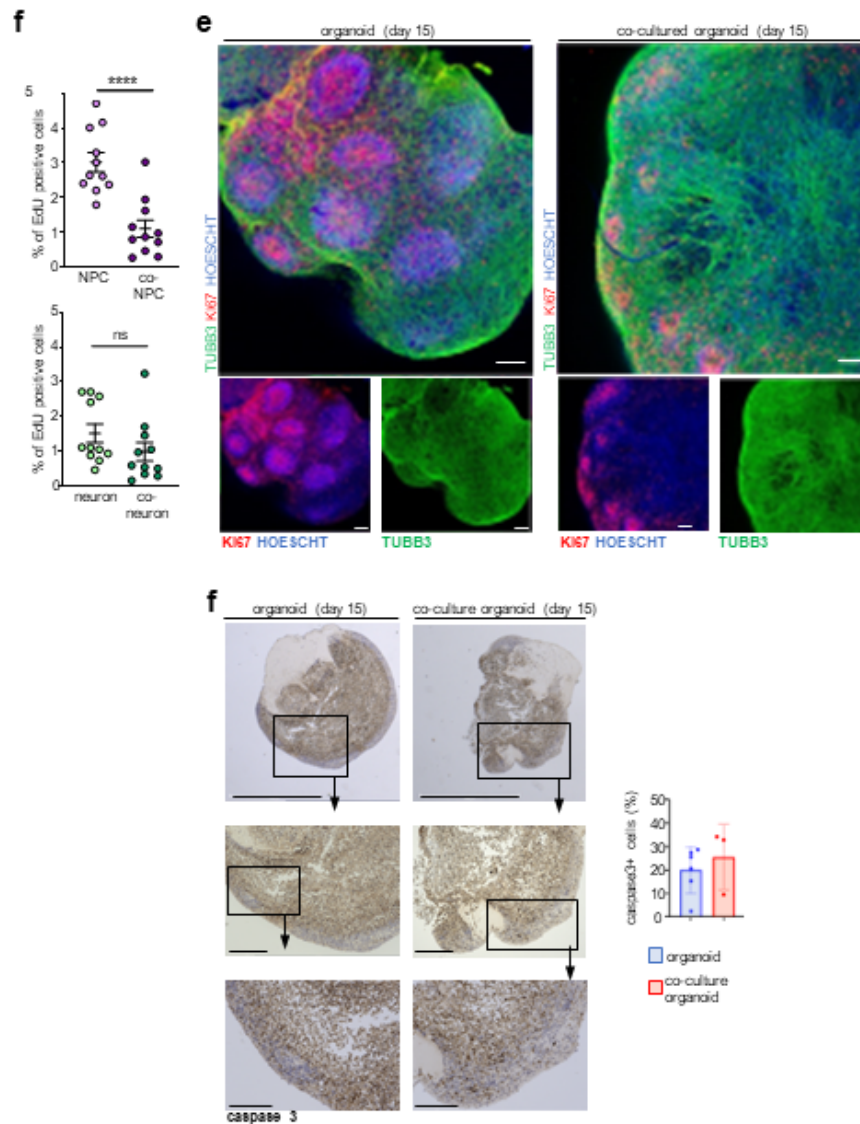
Extended data, Figure 5

d



In this latest analysis, we integrated in the new version of the manuscript, we show that iMicro situated at the vicinity of neural rosettes leads to their size reduction (Extended data, Fig. 5a, b, and Extended data, Fig. 5d), characterized by a marked reduction of the proliferative capacities (Fig. 2f; Extended data, Fig. 6c-e) of co-NPC. This was however not significantly associated with an increased apoptosis (measured by IHC to Caspase3) of SOX2+ co-NPC nor co-neurons (Extended data, Fig. 5c; Extended data, Fig. 6f). These changes are also associated with an increased neurogenesis (Fig. 2; Extended data, Fig. 5c, e).

Figure 2 Extended data, Figure 6



The manuscript has been accordingly updated.

About “Also, why are NPC and co-NPC mixed in one cluster?”, NPC and co-NPC are mixed in the same cluster as a result of the integration of the 10x single-cell datasets from “organoid alone” and “co-culture organoid” by the clustering algorithm Seurat. Integration using Seurat v4 aims to match (or ‘align’) shared cell populations across datasets. Integration first identifies cross-dataset pairs of cells that are in a matched biological state (‘anchors’) to perform comparative scRNA-seq analysis across experimental conditions.

Statistical significance testing in **Fig. 2a** seems to require an ANOVA rather than multiple Man-Whitney t-tests.

We apologise for this error and corrected the statistical analysis for Fig. 2a and similar graphs in Extended Data, Fig. 4 using one- or two-way ANOVA accordingly. As shown in Fig. 2a below the data remain statistically significant. The manuscript has been updated accordingly.

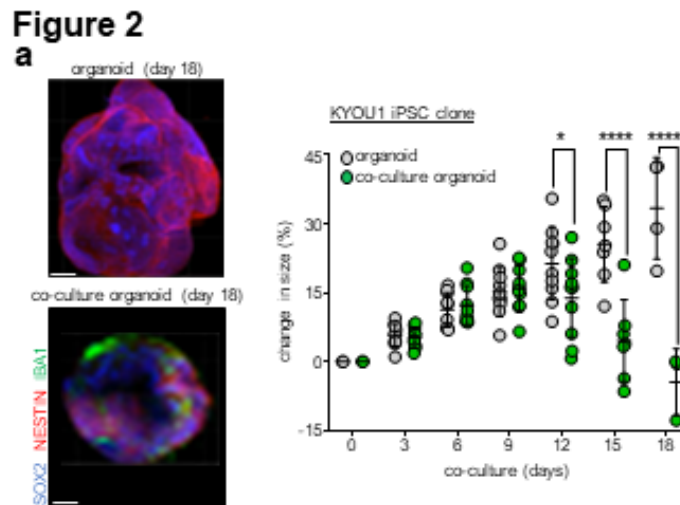
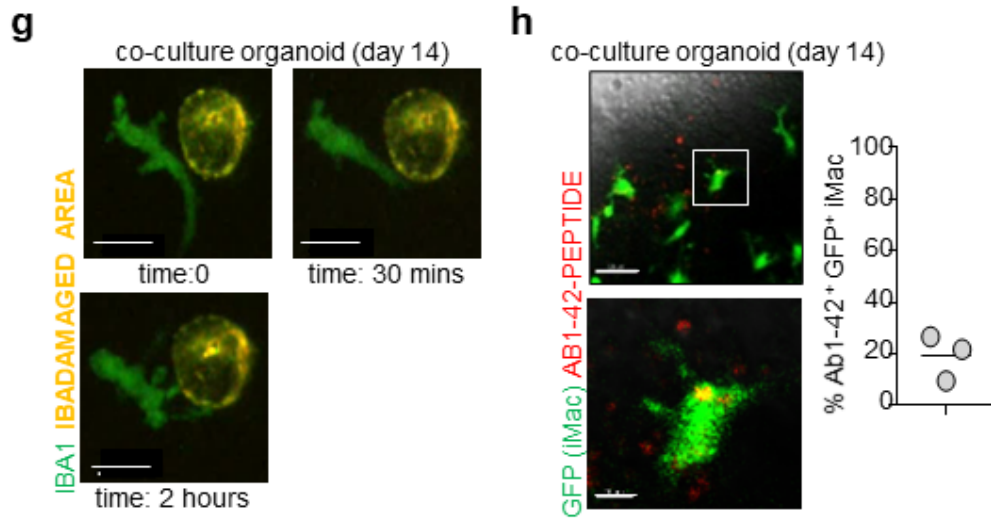


Fig. 1f and **Fig. 1g** (**Extended Data, Fig 1g and 1h in the updated manuscript**) are anecdotal. At a minimum, the authors must demonstrate multiple replicates of these experiments. Ideally, the authors should provide a quantified metric to show that these cellular movements are not by chance. Moreover, these results require a control: do iMac acquire these functionalities because of co-culture with organoids?

We fully agree with the reviewer that the data presented (Extended Data, Fig. 1g (former Fig. 1f) and Extended Data, Fig. 1h (former Fig. 1g)) are anecdotal (thus now in extended data) but were provided to illustrate that iMac are functional and perform expected functions. They demonstrate that co-cultured iMac (herein called iMicro) perform various functions characteristic of macrophage/microglia, ie. response to laser injury (Extended data, Fig. 1g, Supplementary Video. 2) and phagocytosis of Amyloid-beta peptides (Extended data, Fig 1h, Supplementary Video. 3); acquisition of functionalities which we already described in the associated 2D co-coculture (Takata *et al.*, Immunity 2017). Thus, these figures aim at showing that co-iMac/iMicro also have these functions within 3D organoids.

Extended data, Fig. 1g is showing a typical response of IBA1+ iMicro in response to laser damage of many various observations. We have provided the quantification for Extended data, Fig. 1h (n=3). Both text and figures have been amended accordingly.

Extended data, Figure 1



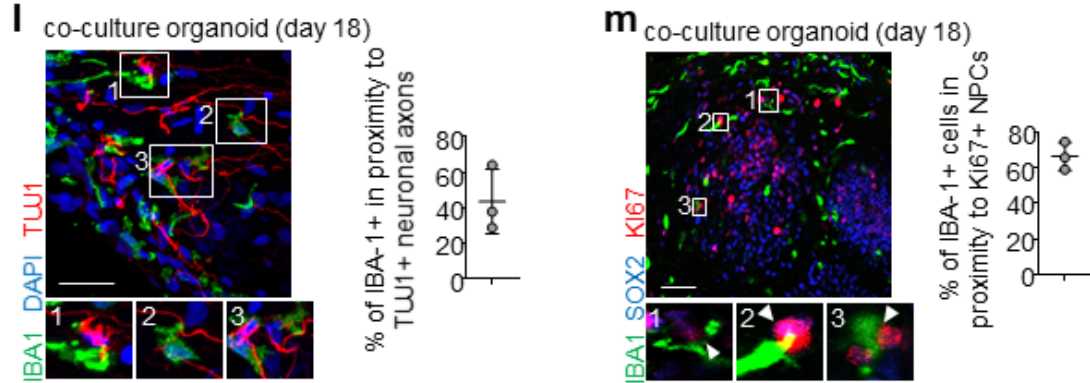
-Takata, K., et al., *Induced-Pluripotent-Stem-Cell-Derived Primitive Macrophages Provide a Platform for Modelling Tissue-Resident Macrophage Differentiation and Function*. *Immunity*, 2017. 47(1): p. 183-198 e6.

Fig. 2l and **Fig. 2m** require quantification. A random seeding of cells would ensure that some number of iMicro would be in proximity to Ki67+ NPCs—it should therefore be tested if this proximity is not merely due to chance.

Likewise, the representation in **Fig. 2m** does not prove that NPCs are phagocytosed by iMicro. Is the SOX2 signal fully enclosed in all three dimensions by the IBA1 signal? Moreover, SOX2 is a nuclear marker, and this phenomenon should be tested with other markers.

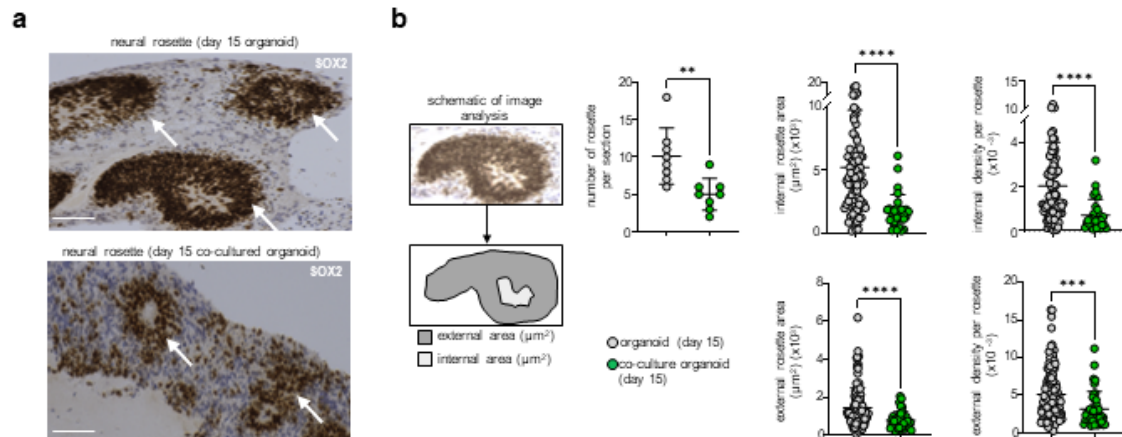
We performed quantification for Fig. 2l, m and n in the updated version of the manuscript. Measurement of the proximity was quantified using the Imaris imaging software and positivity was determined when all markers were co-localising. Between 25 and 65% of IBA1+ iMicro were in proximity to TUJ1+ neuronal axons (Fig. 2l), and 55-80% of IBA1+ iMicro were adjacent to Ki67+ SOX2+ NPCs (Fig. 2m). The iMac are added randomly to the wells at the start of the co-culture (day 0) and such observations on their distribution and embedding in brain organoids have always been observed and in different analysis by immunofluorescence (Fig. 1, 2 and Extended data, Fig. 1) and confocal microscopy (Supplementary Videos 1, 4-6).

Figure 2



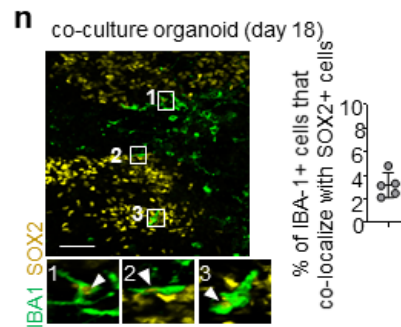
Microglia play an important role in the regulation of NPC biology in the cortex as it develops by the phagocytosis of NPC as the neurogenesis process reaches completion (Hughes *et al*, 2010, *Glia*. and Cunningham *et al*, 2013, *J Neuroscience*). This is indeed what we observed in co-culture organoids as the co-culture organoids present less and disorganised neural rosettes as proven by the reduction in the inner and outer areas and cell densities (Extended data, Fig. 5a, b).

Extended data, Figure 5



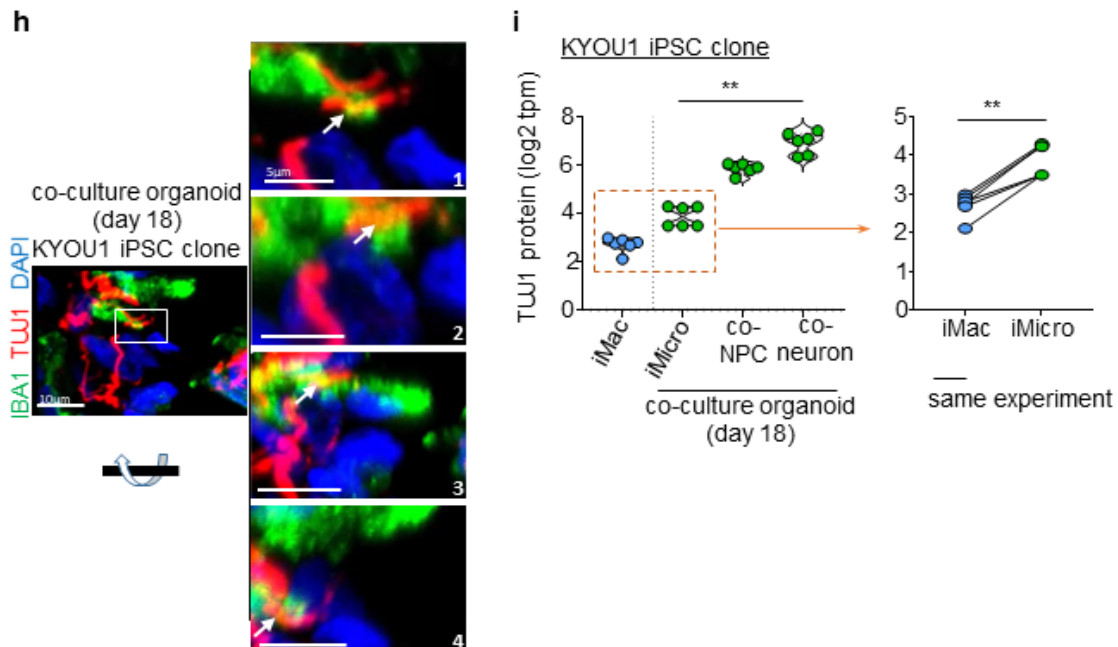
We quantified the % of IBA-1+ iMicro in proximity to SOX2+ NPC using the IMARIS imaging software in Fig. 2n. Positivity was determined when all markers (IBA1, SOX2) were co-localising. We evaluated that 2 to 5% of IBA1 iMicro showed co-localisation with SOX2+ NPC.

Figure 2



To further ascertain that neuronal cells are phagocytosed by iMicro, we evaluated the presence of the neuron/NPC beta tubulin protein TUJ1 (TUBB3) in iMicro. By immunofluorescence analysis, we could see that the TUJ1 signal was fully enclosed in 3D by iMicro (Extended data, Fig. 4h, one representative of many observations is shown); observation further confirmed by mass-spectrometry proteomic analysis of purified iMac, iMicro, co-NPC and co-neurons (Extended data, Fig. 4i).

Extended data, Figure 4



The figures, texts and method sections have been updated accordingly.

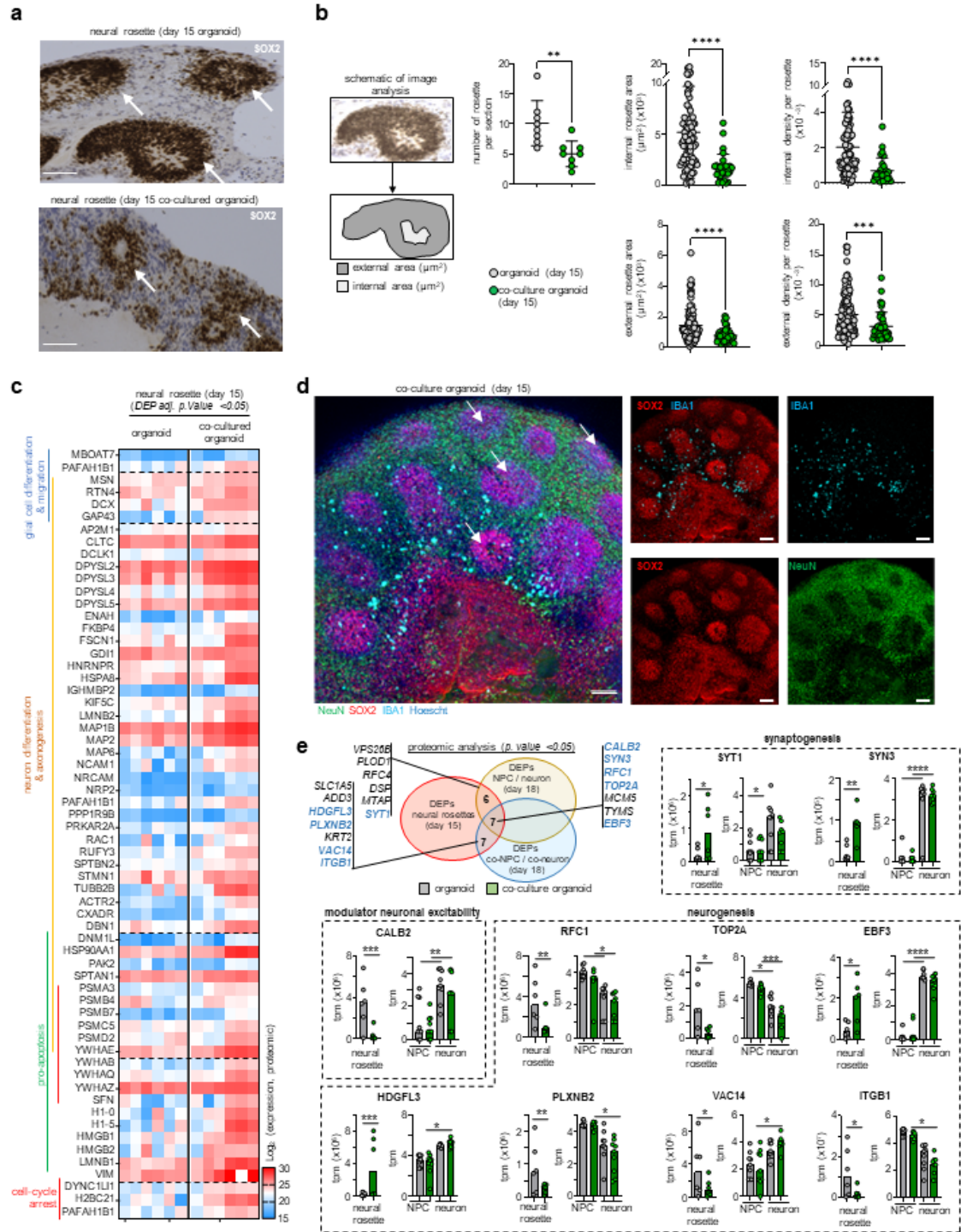
-Hughes, M.M., Field, R.H., Perry, et al., Microglia in the degenerating brain are capable of phagocytosis of beads and of apoptotic cells, but do not efficiently remove PrPsc, even upon LPS stimulation. *Glia*. 2010. 58(16):2017-2030. PMID: 23467340.

-Cunningham, C.L., Martínez-Cerdeño, V., Noctor, S.C. Microglia regulate the number of neural precursor cells in the developing cerebral cortex. *J Neurosci*. 2013;33(10):4216-4233. PMID: 20878768.

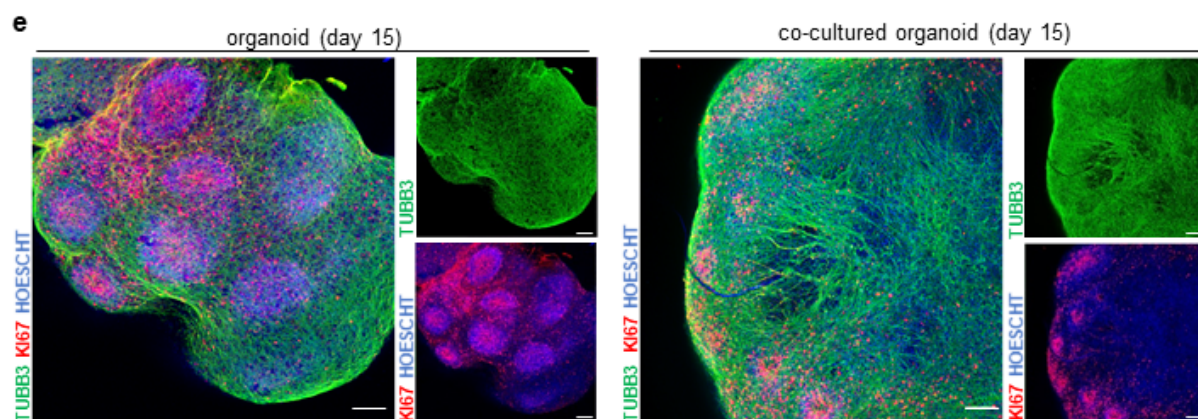
What is the evidence that the neurites in **Fig. 2h (Fig. 2i in the updated manuscript)** are axons? This panel seems to plot “the total number of axons per cell”. Moreover, studying neuronal morphology in 3D organoids will be more informative here than after dissociation and 2D culture.

We thank the reviewer for this comment and indeed, we have measured the number and length of neurites and have amended the labels accordingly (Fig. 2i (former Fig. 2h)). Analysis of transcriptomic data (Fig. 2c, d, e and Extended data, Fig. 6a, b) clearly showed an increase in neurogenesis and axon development in the presence of iMicro. Similarly, our new analysis by immunochemistry and immunofluorescence revealed that neural rosettes were fewer and less organized in co-cultured organoids (Extended data, Fig. 5a, b, d and Extended data, Fig. 6e respectively). This was confirmed at the proteomic level as excised rosettes displayed a clear profile associated with upregulation of neuron differentiation and axonogenesis pathways (Extended data, Fig. 5c, e).

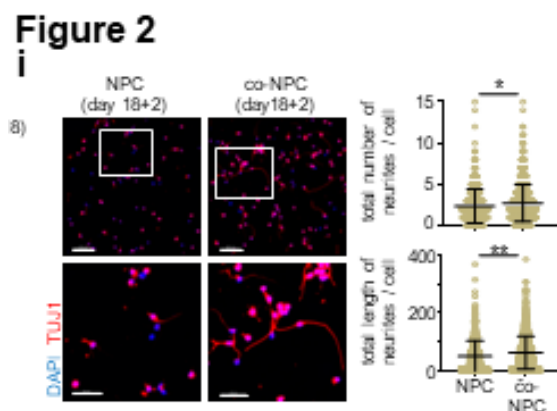
Extended data, Figure 5



Extended data, Figure 6



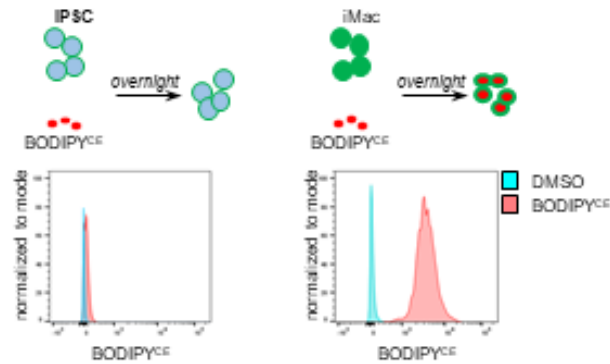
We indeed observe that neuronal cells co-cultured organoids showed longer neurites than in organoids (Fig. 1c; Extended data, Fig. 1d), and as proven after FACS sorting and ensuing 2D culture (Fig. 2i, previously Fig. 2h).



Studying neuronal morphology in 3D organoids was beyond our expertise.

In testing whether BODIPY containing lipids are transferred from iMicro to NPC (**Fig 3f, g, h**), the comparison should be performed to other types of cells (i.e. non-iMac) such as iPSC, not just free BODIPY.

We addressed this issue using the KYOU1 iPSC cell line. We could confirm that iPSC were unable to uptake the BODIPY^{CE} fluorescent probe and thus integrate it into lipid droplet for further transfer into NPCs. This experiment was done in triplicate for each condition and is shown below. We can add it in the manuscript upon reviewer's recommendation.



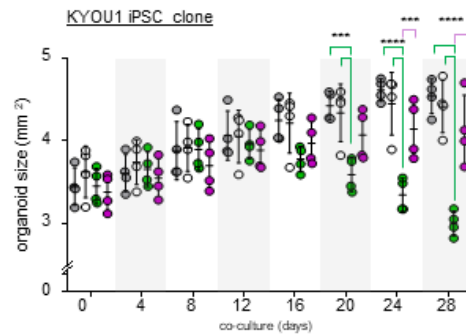
Just expression of PLIN2 is not sufficient to make claims about the utility of the system. Loss or gain of function studies are needed. Can the changes observed in NPC be induced by adding PLIN2+ lipid droplets?

We fully agree with this important point and have performed additional studies to support our claims. PLIN2 is the most important DEG overexpressed in iMicro compared to iMac (Fig. 3a). This high expression of PLIN2 in iMac (Fig. 3b) and iMicro (Fig. 3c) directed us to the biology of lipid droplets and the downstream transfer of cholesterol, both of which have been largely described in macrophages. Indeed, we now show that the size reduction of organoids upon the addition of iMac can be partially recapitulated by transfer experiments of iMac supernatant to organoids alone, reaching significance at day 24 and a trend from day 20 (Fig. 3j).

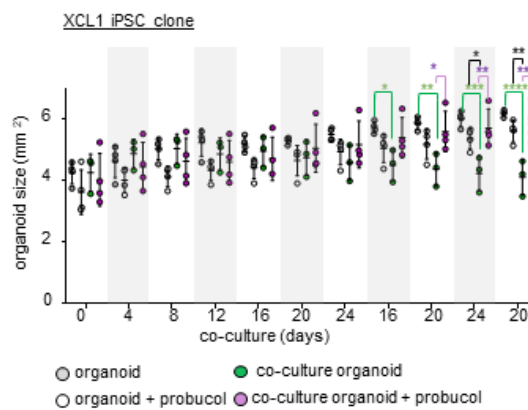
We further show that this phenomenon is driven by the release of cholesterol from iMac/iMicro into the microenvironment via the ATP-binding-cassette receptor and cholesterol transporter ABCA1 highly expressed by iMac/iMicro (Fig. 3n, Extended data, Fig. 8i). Indeed, as already described in the literature (Chung *et al.*, 2010, JBC; Smith *et al.*, 2004, J Lipid Res.; Chroni *et al.*, 2004, Biochemistry; Kypreos *et al.*, 2008, Biochemistry), blocking of ABCA1 using probucol promoted the accumulation of neutral-lipids (and cholesterol species) into iMac (Fig. 3o) and impaired the subsequent esterification of cholesterol into cholesterol esters following its release. (Fig. 3p, Extended data, Fig. 8j) that will integrate lipoprotein-like particles. This is mirrored in our lipidomic analysis on culture medium from iMac treated with probucol as we observed reduced levels of cholesteryl-esters and increased levels of triglycerides and phospholipids (Fig. 3p and Extended data, Fig. 8j). Consequently, the reduction of size induced by the addition of iMac was abrogated upon the addition of probucol to co-culture organoids generated from the KYOU1 iPSC clone (Fig. 3q) and the XCL-1 iPSC clone (Added below and which can be added to the manuscript at reviewer's suggestion).

Figure 3

p



Extra Data

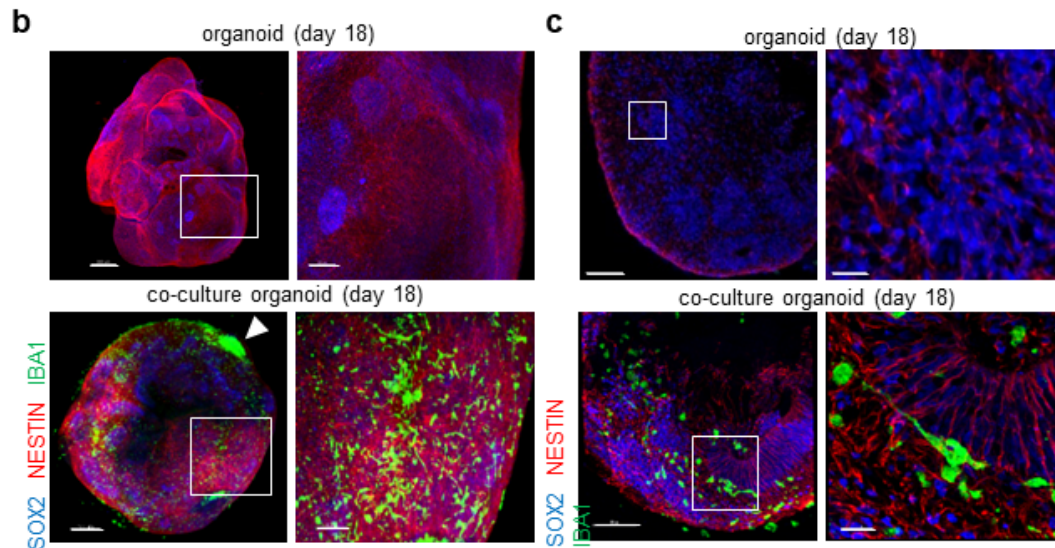


- Chung S, Timmins JM, Duong M, et al. Targeted deletion of hepatocyte ABCA1 leads to very low density lipoprotein triglyceride overproduction and low density lipoprotein hypercatabolism. *J Biol Chem.* 2010;285(16):12197-12209. doi:10.1074/jbc.M109.096933.
- Smith JD, Le Goff W, Settle M, et al. ABCA1 mediates concurrent cholesterol and phospholipid efflux to apolipoprotein A-I. *J Lipid Res.* 2004;45(4):635-644. doi:10.1194/jlr.M300336-JLR200.
- Chroni A, Liu T, Fitzgerald ML, Freeman MW, Zannis VI. Cross-linking and lipid efflux properties of apoA-I mutants suggest direct association between apoA-I helices and ABCA1. *Biochemistry.* 2004;43(7):2126-2139. doi:10.1021/bi035813p.
- Kypreos KE. ABCA1 promotes the de novo biogenesis of apolipoprotein CIII-containing HDL particles in vivo and modulates the severity of apolipoprotein CIII-induced hypertriglyceridemia. *Biochemistry.* 2008;47(39):10491-10502. doi:10.1021/bi801249c.

Other issues:

- A control organoid should be shown as a comparison for **Fig. 1b** and **Fig. 1c**.
We thank the reviewer for this important comment and we added immunostaining of control organoids at low (Fig. 1b) and high resolution (Fig. 1c).

Figure 1



- **Fig. 1m** appears superfluous. This simple labelling could be incorporated into **Fig. 1a**.

We agree and we have incorporated Fig. 1m into Extended Data, Fig. 1a; a more detailed representation of Fig. 1a.

- **Fig. 2b** has a y-axis label “number of cells per organoid (x1E-5)”. This should perhaps be (x1E5). **Fig. 2b** should have a column for the total number of all cells from each organoid, to provide a stable reference point.

We thank the reviewer for spotting these errors which we have rectified. We also added the total number of cells and iMicro for each organoid (n=3 for organoids; n=3 for co-culture organoids) (Fig. 2b). Both text and figures have been amended.

- The name “iMac” is already charged with meaning in the computer world. I would suggest another abbreviation for this system.

We appreciate the comment put forward by the reviewer. Such terminology of iMac and iMicro has however first been published by our group in “Takata *et al*, Immunity, 2017” and we would like to keep it as we believe it is an acronym that can quickly suggest an iPSC origin in the mind of the readers.

-Takata, K., et al., *Induced-Pluripotent-Stem-Cell-Derived Primitive Macrophages Provide a Platform for Modelling Tissue-Resident Macrophage Differentiation and Function*. Immunity, 2017. 47(1): p. 183-198 e6. (PMID: 28723550).

Referee #2 (Remarks to the Author):

In this study, Park et al investigate the role of human microglia in regulating neural progenitor cell (NPC) responses and maturation in early development. This was achieved using a new model of iPSC-derived organoids transplanted with primitive macrophages derived from the same iPSC line, which then mature into microglia-like cells. These organoids model very early developing brain with abundance of NPCs yet lack of mature cells like astrocytes – importantly, this is distinct from previous work using human organoids at an advanced maturation state where either human iPSC-derived microglia were transplanted in (Abud 2017 Neuron) or endogenously derived within organoids (Ormel 2018 Nat Comms). Using single cell sequencing, live imaging, proteomics, flow cytometry, and immunofluorescence, the authors make 3 major conclusions. First, transplanting in macrophages that become microglia-like cells (termed iMicro) limits the growth of organoids, suggesting an impact on limiting NPC expansion in development. Second, iMicros increase the maturation of NPCs into neurons and axon development. Third, iMicro transfer cholesterol/esters to NPCs in the organoids, and this may modify NPC metabolism and their maturation. The overall conclusion is that microglia advance brain development by providing cholesterol to NPCs to support their maturation. This is exciting as it builds on existing knowledge of lipids regulating NPC responses and neurogenesis, by now implicating human microglia as suppliers of cholesterol to support neuronal maturation. In addition, the organoid system + transplant protocol developed here is very interesting and could be of great value to the field of human brain development.

With regards to the data, I have the following suggestions:

Major suggestions:

1. The organoid + transplant system is very interesting; it would be useful to have a bit more characterization. Can the authors give quantitative info on the iMac/iMicro penetration in the organoid? Is it all the way through and can this be shown by z-stacks. If not all the way through, then is there a gradient of NPC maturation (neuron number, axon length) based on proximity to iMicros?

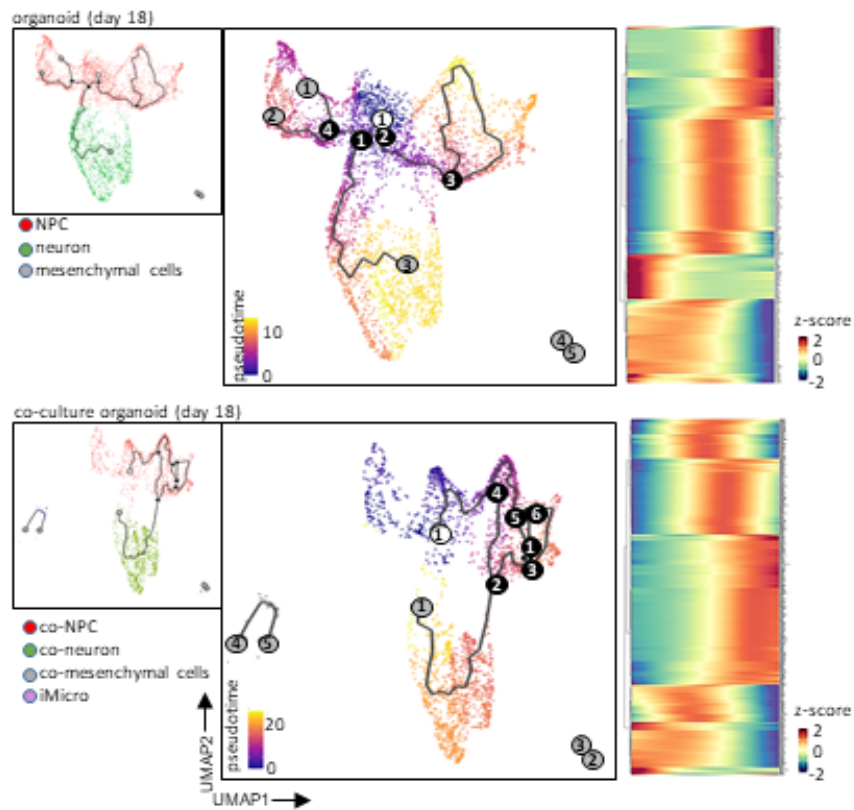
We thank the reviewer for this important suggestion. It is clear from the various immunofluorescence and supplementary videos that iMac/iMicro penetrate the organoid from the periphery and migrate deeply into the core of the organoid (Fig. 1b, c; Extended Fig. 1c, d; Supplementary Video. 1, 4, 5), and we show that during this journey, they interact with both co-NPC and co-neurons, promoting neurogenesis as demonstrated by our extensive transcriptomic, proteomic and associated functional analysis (Fig. 1, 2 and associated Extended data, Fig. 1-6).

Neural rosettes enriched in NPC/co-NPC were fewer, smaller, less organised and surrounded by iMicro in co-culture organoids compared to organoids alone (Extended data, Fig. 5a, b, d; Extended data, Fig. 6e and Supplementary Video 7). In the presence of iMicro, neural rosettes presented lower proliferative activity (Staining with Ki67 and EdU, Fig. 2f, Extended data, Fig. 6e and Supplementary Video. 6), less co-NPC, and displayed higher markers of neurogenesis (Fig. 2g, h and Extended data, Fig. 5c, e).

It was therefore difficult to exactly quantify the penetration of iMac/iMacro throughout co-culture organoids, and to measure a gradient of NPC maturation based on their proximity to iMicro. Since there was not a clear heterogeneity of NPC in co-culture organoid, this suggested that the whole NPC population was reprogrammed. To evaluate this, we performed a heatmap of differentiation with Monocle 3 (Trapnell *et al*, Nat Biotechnol. 2014) using our 10X transcriptomic data and which permitted to predict the cell trajectory of differentiation. While we are currently investigating this in

a follow-up study, cell-trajectory analysis are shown below, and suggest that iMicro re-shaped the gradient of NPC development in organoid alone.

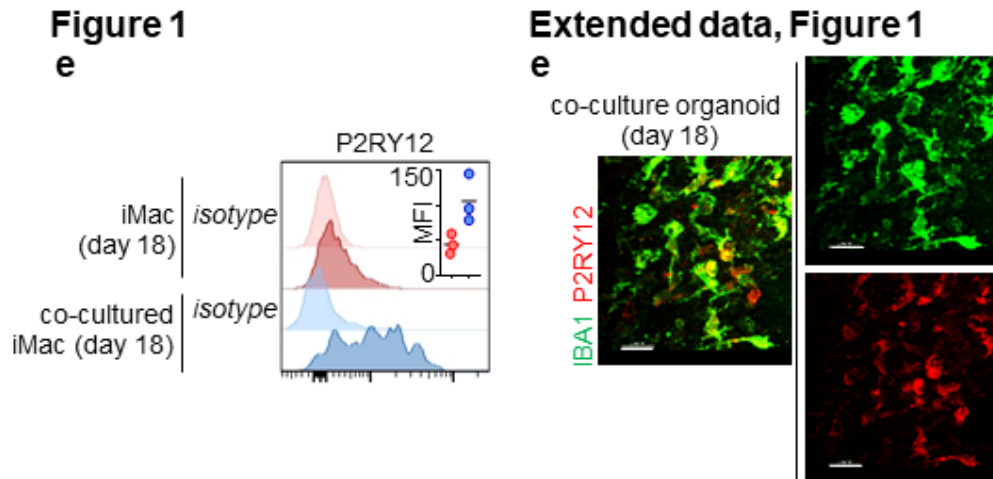
Trajectory analysis



-Trapnell C, Cacchiarelli D, Grimsby J, et al. The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat Biotechnol.* 2014;32(4):381-386. doi:10.1038/nbt.2859.

Can the authors stain for P2RY12 and TMEM119 to complement the flow cytometry work – is expression (there or not) heterogeneous, or do all iMacs alter their expression?

As the reviewer suggested, we added the immunofluorescent staining of co-cultured organoids for P2RY12 (Extended data, Fig. 1e) which complement the flow cytometry staining (Fig. 1e). We did not detected heterogeneous expression at late stage of co-culture (data not shown).



Antibody for TMEM119 (polyclonal rabbit anti-human TMEM 119) did not work and did not yield to a staining by immunofluorescence, in accordance with the low expression detected by flow cytometry. The reliability of this marker as a specific marker for microglia has however recently been called into question. Indeed, its expression has been shown not to be exclusively nor homogeneously expressed by microglia (Su *et al*, Transl Vsi Sci Technol, 2019 and Vankriekelsvenne *et al*, Glia, 2022).

-Vankriekelsvenne E, Chrzanowski U, Manzhula K, et al. Transmembrane protein 119 is neither a specific nor a reliable marker for microglia. *Glia*. 2022;70(6):1170-1190. PMID: 35246882.

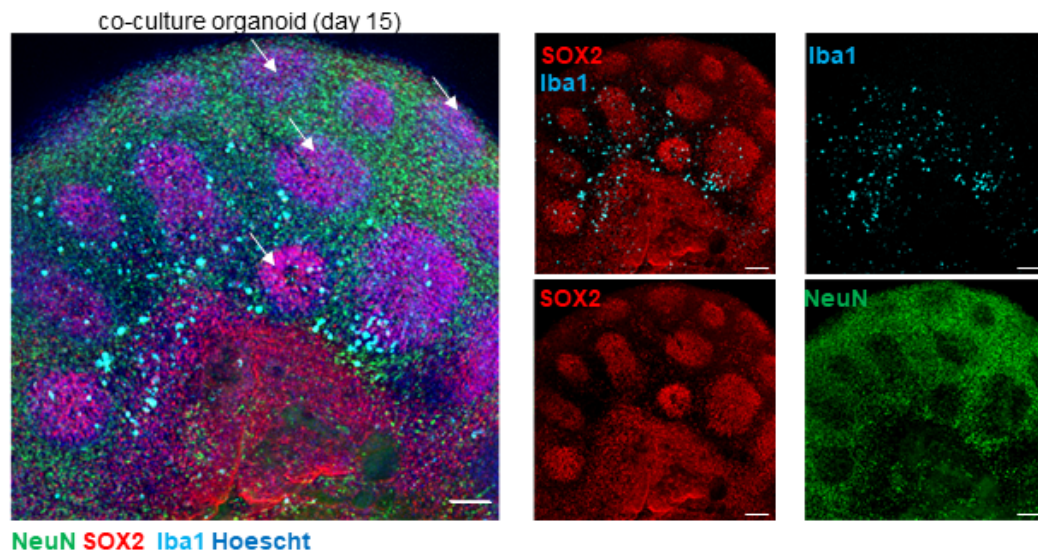
Su N, März S, Plagemann T, et al. Occurrence of Transmembrane Protein 119 in the Retina is Not Restricted to the Microglia: An Immunohistochemical Study. *Transl Vis Sci Technol*. 2019;8(6):29. PMID: 31853425.

2. iMac transplant causes a decrease in the size of the organoids. However, it is still unclear how the microglia are regulating this change in organoid size. Can the authors quantify NPC proliferation and death in organoids alone and following iMac transplant, and quantify the frequency of iMicro phagocytosis of NPCs?

We thank the reviewer for this comment and provide now more data quantifying more precisely NPC proliferation reduction upon co-culture. We show that upon the initiation of the co-culture, iMicro slowly penetrate and embed in the organoid structure from the periphery, making connection with neurons and NPC (Fig. 2l, m, n) and accumulating not only at the periphery (Fig. 1b, c; Extended data, Fig. 1c, d and Supplementary Video. 4), but also at the vicinity of neural rosettes essentially composed of co-NPC (Extended data, Fig. 1c, d, Extended data, Fig. 5d, Supplementary Video. 7).

Extended data, Figure 5

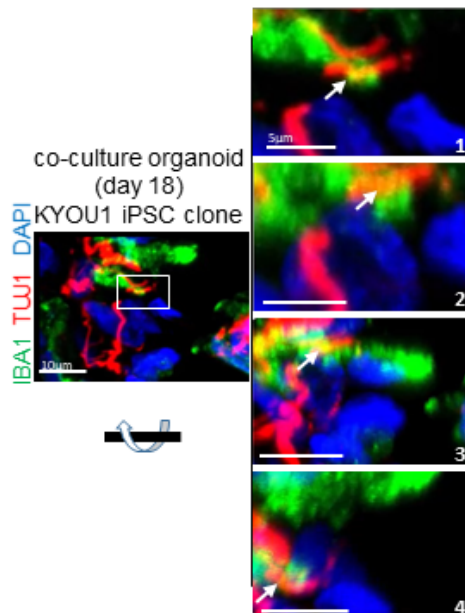
d



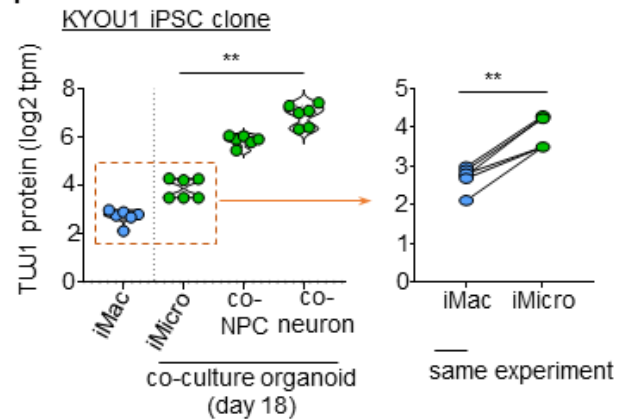
The distribution of iMicro was associated with observation of phagocytosis of co-NPC (Fig. 2n) as 2-5% of IBA1+ iMicro co-localised with SOX2+ co-NPC. Quantifying precisely this phenomenon of phagocytosis in 3D organoids was unfortunately beyond our expertise and the scope of the study. We however pushed further our analysis using z-projection of immunofluorescence staining where we could provide evidence that the TUJ1 signal was embedded in IBA1+ iMicro (Extended Fig. 4h). Using our proteomic analysis on purified cells, we also demonstrated that IBA1+ iMicro showed positivity for the neuronal-specific TUJ1 protein marker (Extended data, Fig. 4i).

Extended data, Figure 4

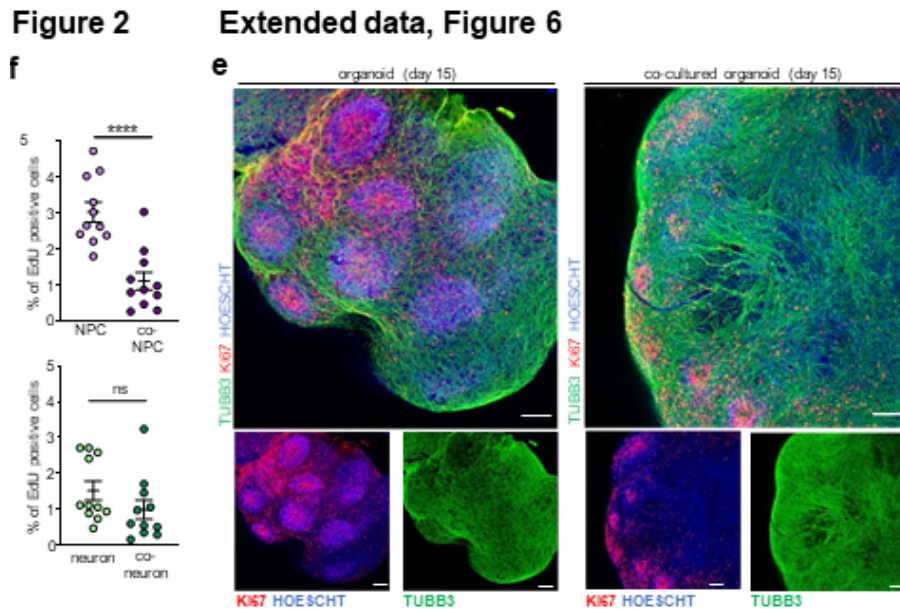
h



i



Co-NPC clearly exhibit significantly less proliferation by EdU staining in co-culture organoids compared to organoids alone (Fig. 2f). Neural rosettes are also significantly smaller and less organised in co-culture organoids compared to organoid alone at day 15 (Extended data, Fig. 5a, b and Extended data, Fig. 6e). This is also confirmed by the proteomic analysis by mass-spectrometry of the rosettes from co-culture organoids vs organoid alone, showing a heightened profile of proteins involved in neuronal differentiation and axonogenesis, pro-apoptosis / cell-cycle arrest (Extended data, Fig. 5c, e).



All these data have been added to the new version of the manuscript.

-Hughes, M.M., Field, R.H., Perry, et al., Microglia in the degenerating brain are capable of phagocytosis of beads and of apoptotic cells, but do not efficiently remove PrPSc, even upon LPS stimulation. *Glia*. 2010. 58(16):2017-2030. PMID: 23467340.

-Cunningham, C.L., Martínez-Cerdeño, V., Noctor, S.C. Microglia regulate the number of neural precursor cells in the developing cerebral cortex. *J Neurosci*. 2013;33(10):4216-4233. PMID: 20878768.

Can the authors clarify why the single cell sequencing not reflect the change in numbers of the cells following transplant?

We welcome the reviewer's comment in relation to Fig. 2b and Extended data, Fig 3d (Extended data, Fig. 2d in the update manuscript). Addition of iMac to brain organoid leads to a significant reduction in the number of co-NPC and co-neurons (Fig. 2b). This observation is also reflected in the atrophy of NPC-rich neural rosettes of co-culture organoids (Extended data, Fig. 5a, b; Extended data, Fig. 6e) and the strong reduction in the proliferative activity of co-NPC by EdU incorporation and SOX2 staining (Fig. 2f and Extended data, Fig. 6e respectively). This significant reduction in the proliferative capacity of co-NPC was however not associated with an increased death of neuronal cells (Caspase 3 staining, Extended data, Fig. 6f).

Extended data, Figure 5

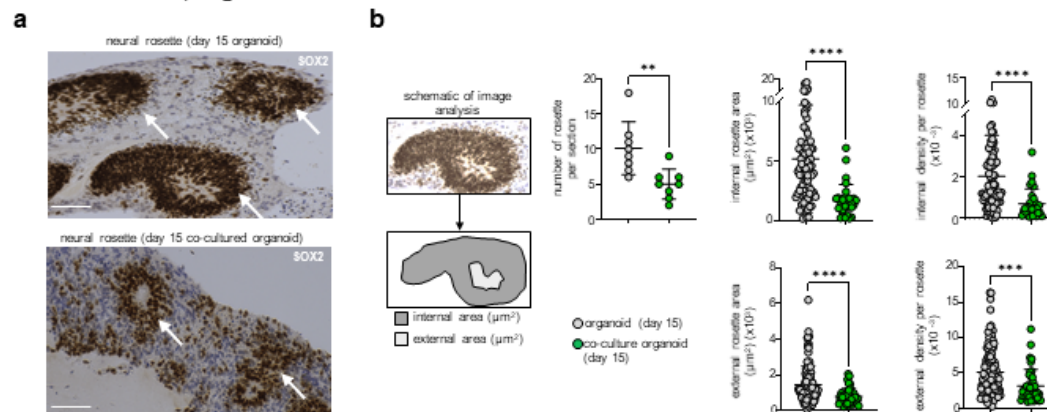
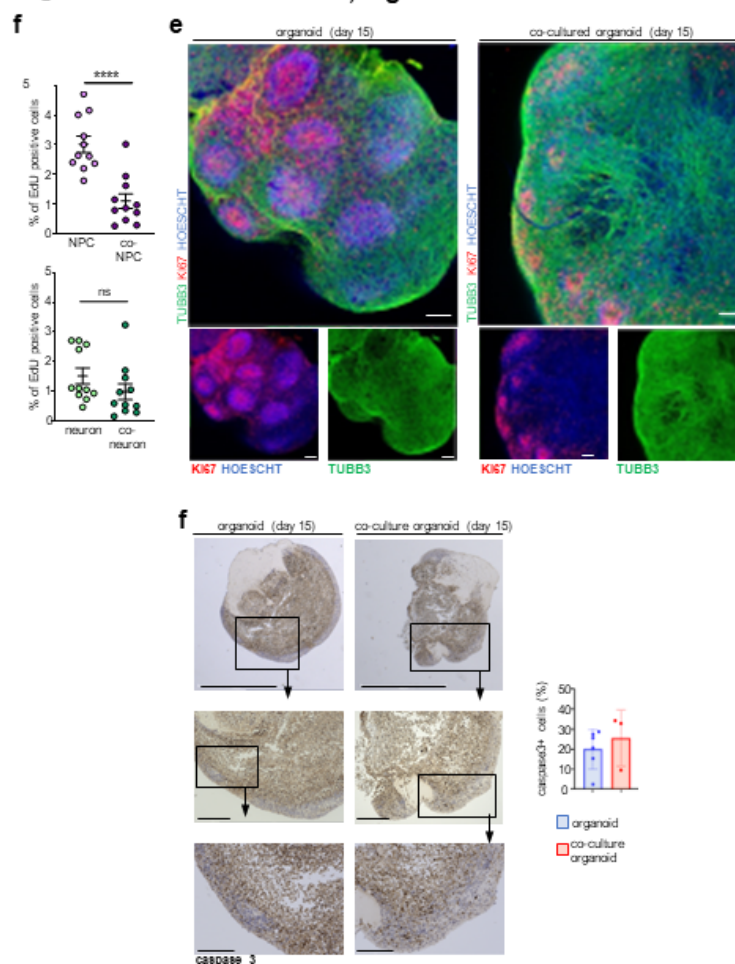


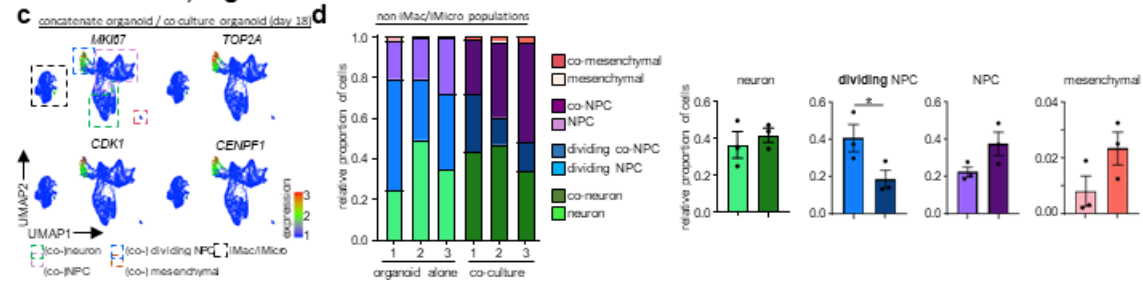
Figure 2

Extended data, Figure 6



We agree that this reduction in total co-NPC is not necessarily translated in the analysis of the single cell sequencing data (Extended data, Fig. 4c, d) at first glance. However, the subdivision of cell populations between dividing and non-dividing cells based on the expression of *MKI67*, *TPO2A*, *CDK1* and *CENPF1* shows a significant decrease of dividing NPC in co-culture organoids in the single cell data (Extended data, Fig. 6c, d).

Extended data, Figure 6

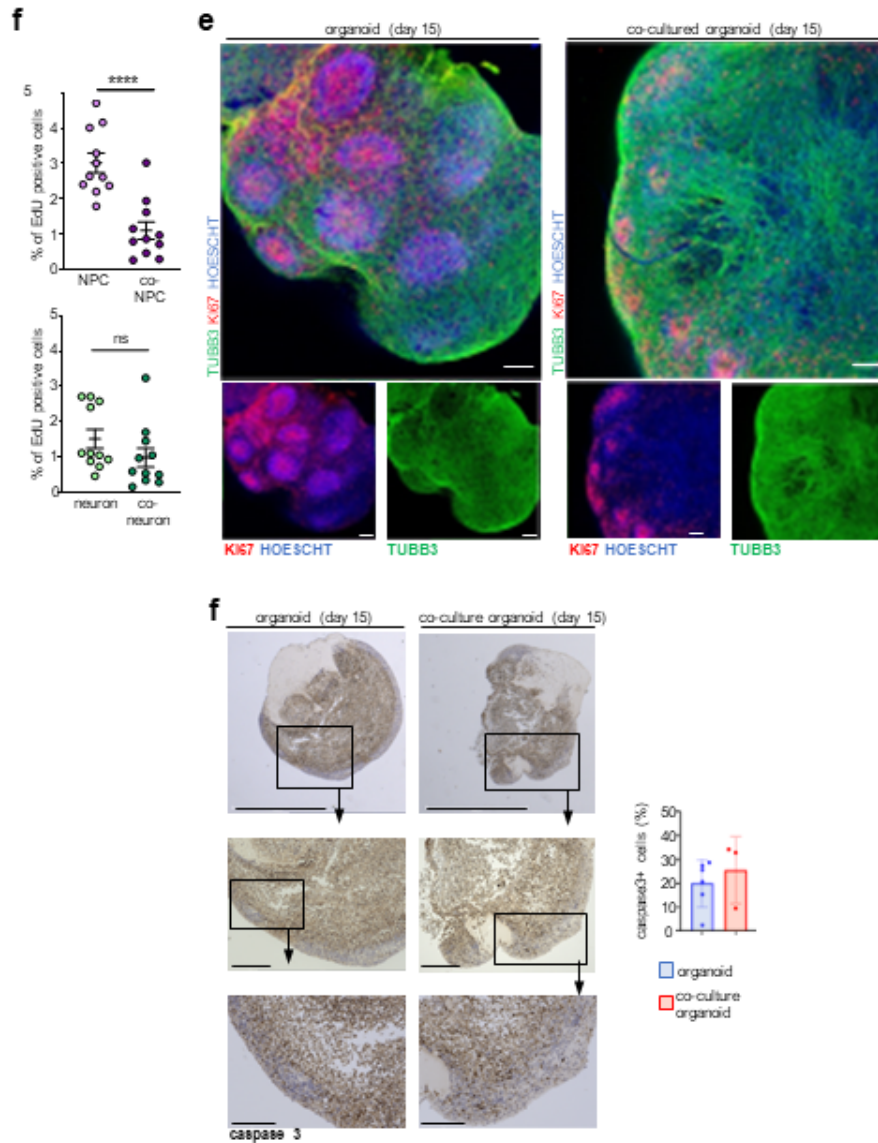


The manuscript has been updated throughout with these data and observations.

3. The single cell sequencing of the NPCs/neurons/iMicros has yielded some interesting concepts as to how the cells are responding and interacting. This would be greatly strengthened by validation at the RNA level (RNAscope) or protein level (ICC) throughout the manuscript. For instance, validating expression of a subset of genes involved in neurogenesis, axon development, proliferation, metabolism.

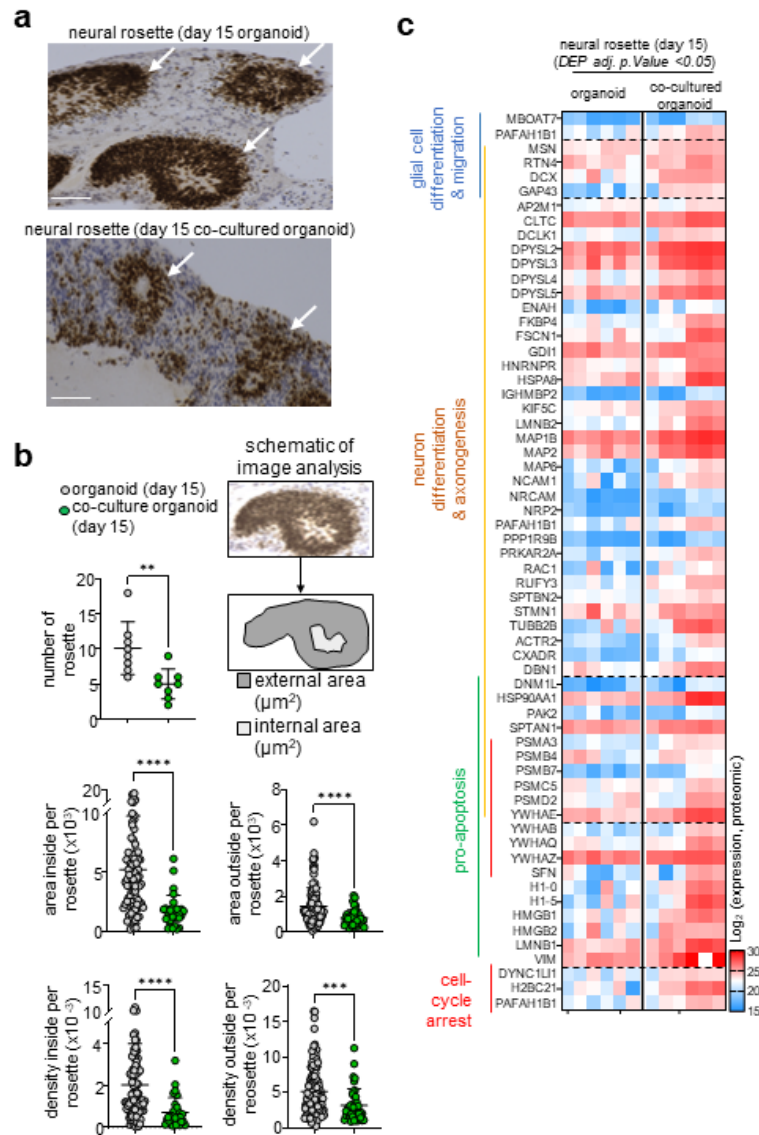
Following the reviewers' comments, we decided to further validate some of the important findings of single cell sequencing data such as the decreased expression of genes involved in **proliferation**. The reduction of the size of brain organoids upon the addition of iMac (Fig. 2a, Extended data, Fig. 4a-e) was associated with a decreased number co-NPC compared to NPC (Fig. 2b) as shown by EdU and Ki67 staining (Fig. 2f and Extended data, Fig. 6e respectively), while we could not detect a significant increase in cell death as revealed by caspase3 IHC analysis (Extended data, Fig. 6f).

Figure 2 **Extended data, Figure 6**



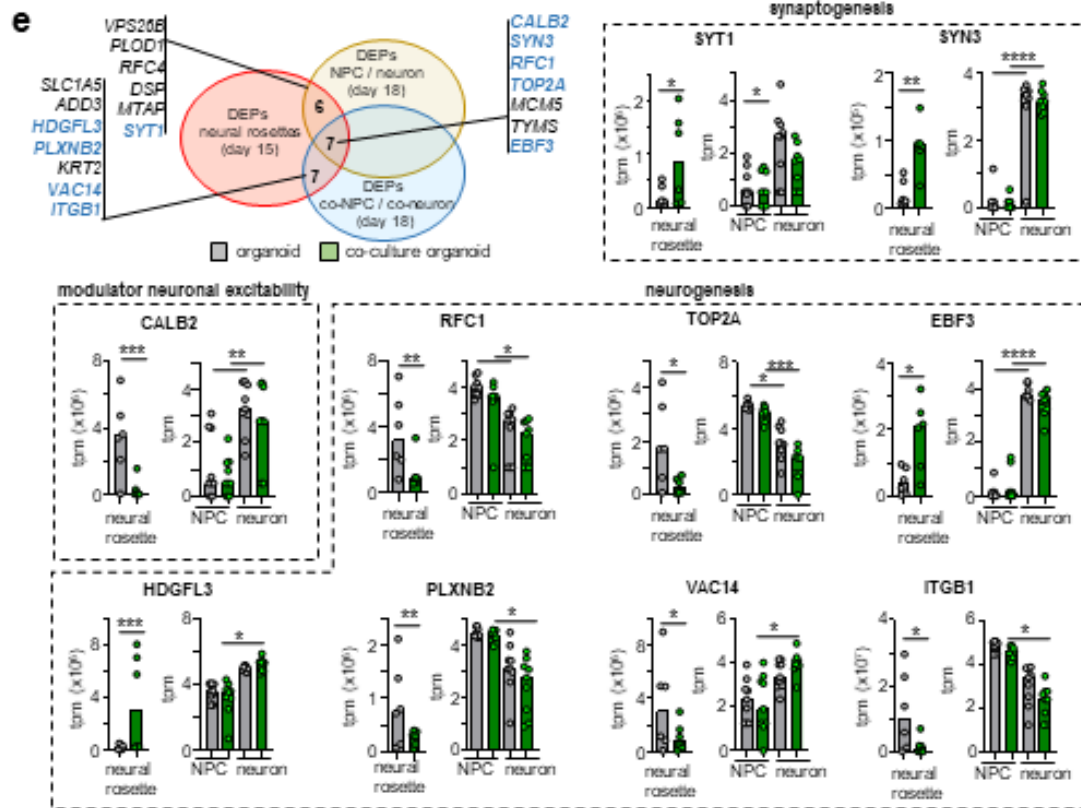
Interestingly, the SOX2 staining was mainly restricted to neural-rosettes bordered by IBA1+ iMicro (Extended data, Fig. 5d). This was associated with a significant reduction in their size and organization in co-culture organoids compared to organoids alone (Extended data, Fig. 5a, b and Extended data, Fig. 6e). We thus extended our analysis of **neural rosettes** from both organoids and co-culture organoids (day 15). Organoid sections were stained with Haematoxylin before neural rosettes were excised with a laser micro-dissector, processed and acquired by mass-spectrometry for proteomic analysis. Enriched pathway analysis using the differentially expressed proteins (DEP) revealed that neural rosettes from co-cultured organoids were significantly associated with pathways related with pro-apoptosis and cell-cycle arrest, glial cell differentiation and migration, as well as **neuronal differentiation and associated axonogenesis** which we investigated further (Extended data, Fig. 5c).

Extended data, Figure 5



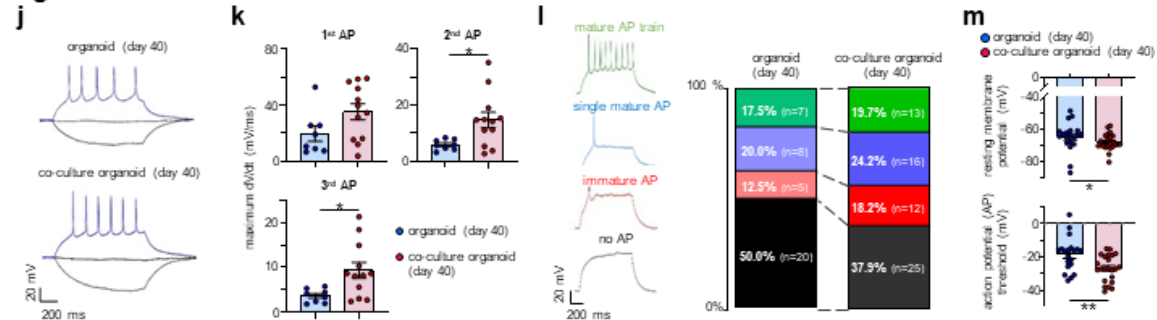
Ensuing proteomic profiles of NPC-enriched neural rosettes was analysed and compared with the bulk proteomic analysis from purified NPC, co-NPC, neuron and co-neuron. The comparison between DEP from the two proteomic analysis highlighted common signatures associated in co-cultured organoids with **synaptogenesis** (increased expression of *SYT1* and *SYN3* in co-cultured organoids), modulator of **neuronal excitability** (reduced expression of *CALB2* in co-culture organoids) as well as multiple markers associated with the **neurogenesis process** (expression of *iRFC1*, *TOP2A*, *EBF3*, *HDGFL3*, *PLXNB2*, *VAC14* and *ITGB1*) (Extended data, Fig. 5e). These findings were supported by morphological studies where co-NPC displayed more and longer neurites than NPC (as shown by the analysis of *TUJ1* stained NPC and co-NPC, Fig. 2i), and the various staining characterising neurogenesis and axon development throughout the study (*NESTIN*, *SOX2*, *TUJ1*, *TUBB3* or *NeuN*) (Fig. 1b, c; Extended data, Fig. 1c, d; Extended data, Fig. 5d and Extended data, Fig. 6e).

Extended data, Figure 5



We further validated the proteomic markers suggesting an increased neurogenesis, axonogenesis and synaptogenesis of NPC and neurons in co-culture organoids functionally by **electrophysiological whole-cell patch recording** techniques. Indeed, we could show that the addition of iMac promotes an increase in action potential firing (AP, Fig. 1j) and especially of the rheobase of the second (2nd AP) and third (3rd AP) AP (Fig. 1k) in co-culture organoids. Co-cultured organoids also presented an AP firing pattern enriched in immature AP, single mature AP and mature AP trains compared to brain organoids alone (Fig. 1l), while neurons showed improved intrinsic properties such as the significant reduction of their AP threshold and resting membrane potential (Fig. 1m).

Figure 1

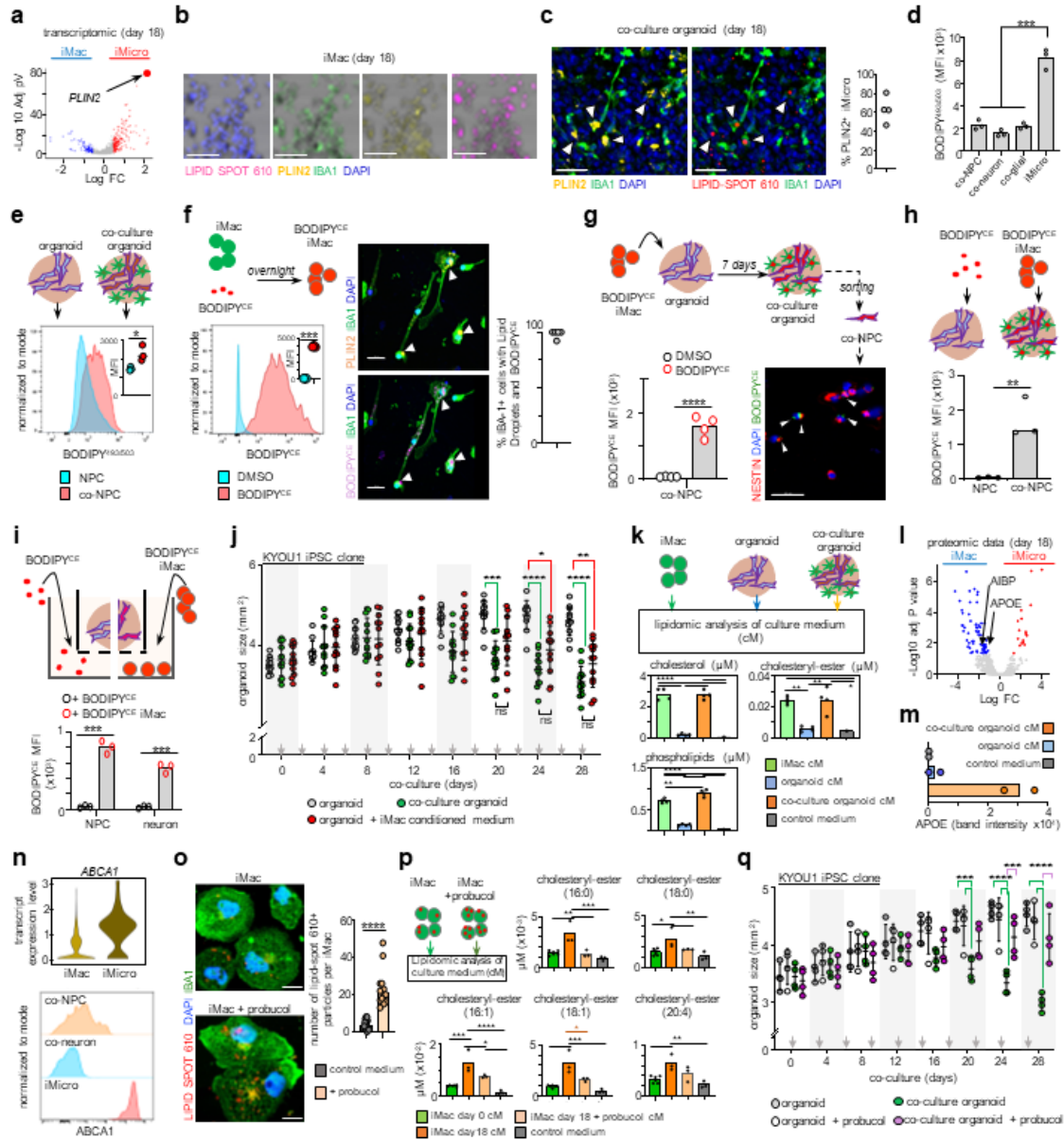


We provided in depth characterization of the generation of iMicro from iMac in co-cultured organoids both phenotypically and functionally. Compared to iMac, iMicro express proteins markers specific of

microglia such as P2RY12, CX3CR1 by both flow cytometry (Fig. 1e) and immunofluorescence analysis (for P2RY12, Extended data, Fig. 1e). Embedded in co-cultured organoid from the surface to the core, iMicro surround neural rosettes and interact with NPC and neurons as shown by immunofluorescence staining (Fig. 1b, c; Fig. 2m, n; Extended data, Fig. 1c, d and Extended data, Fig. 5d). This microglial phenotype of iMicro was also translated functionally by flow cytometry, immunofluorescence or proteomic analysis as they display functional features commonly associated with microglia such as response to laser injury (Extended data, Fig. 1g, Supplementary Video. 2), co-NPC/co-neuron phagocytosis (Extended data, Fig. 1h, Extended data, Fig. 4h, i).

We finally validate pathways linked the storage and production of APOE+ lipoprotein-like particles by flow cytometry, immunofluorescence, proteomic and lipidomic analysis by mass-spectrometry analysis (Fig. 3 and Extended data, Fig 8). In this respect, iMicro storing lipids in PLIN2+ lipid droplets (Fig. 3a-e) transfer cholesterol, cholesterol-esters and phospholipids to co-NPC (Fig. 3f-k; Extended data, Fig. 8d-f). Such mechanisms involving the proteins APOE and AIBP expressed by iMicro (Fig. 3l, m and Extended data, Fig. 8g, h) required the efflux of cholesterol from iMac/iMicro by ABCA1 (Fig. 3n-q, Extended data, Fig. 8i). Fig. 3 is further explained in the next question below.

Figure 3

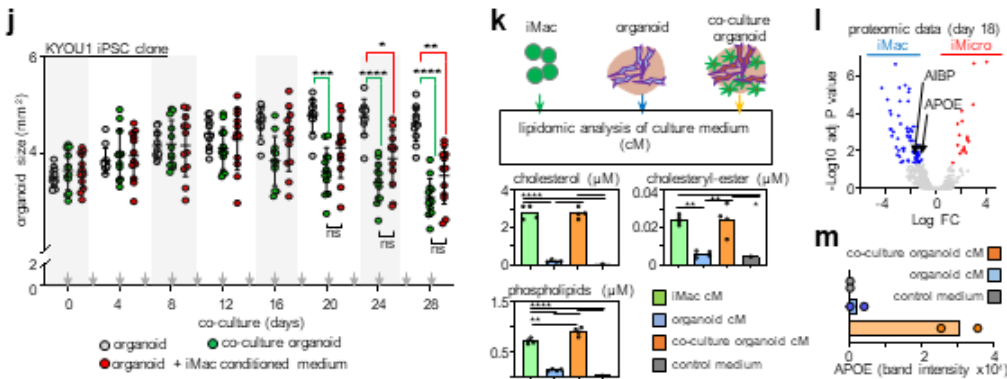


Altogether, we have extensively validated our single cell data of organoid and co-cultured organoids (at day 18 of co-culture) both phenotypically and functionally at the protein and lipidomic level throughout the manuscript.

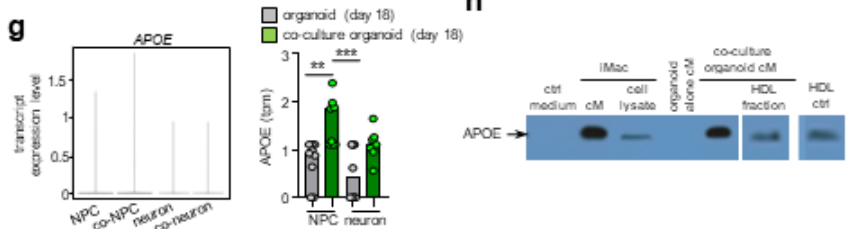
4. Regarding the cholesterol transfer, it is a really interesting hypothesis that needs to be further investigated. The killer experiment would be functionally interfering with the cholesterol transfer specifically from iMicro, and eliminating the positive effects of transplant on neuronal maturation – this would make the definite link between microglia cholesterol synthesis and neuronal responses.

As suggested by the reviewer and as we describe subsequently in Fig. 3 and associated Extended data, Fig. 8, we show that ABCA1 transporter is required to mediate such cholesterol transfer leading to the production of cholesteryl-esters and their eventual integration into APOE+ lipoprotein-like particles (LLP). Indeed, we show that the size reduction of organoids upon the addition of iMac can be partially recapitulated by transfer experiments of iMac supernatant (Fig. 3j, Extended data, Fig. 8d, e) to organoids alone. Further analysis of our proteomic data revealed that APOE (a major protein composing lipoprotein-like particles) and AIBP (Apolipoprotein 1 Binding protein) were highly expressed by iMac (Fig. 3l). This signature and a lipid profile of the supernatant of both iMac and co-culture organoids rich in cholesterol and its esters and phospholipids (Fig. 3k and Extended data, Fig. 8f) suggested that the cholesterol and its esters were transported to neuronal cells via lipoprotein-like particles; especially as co-NPC expressed high levels of APOE at the protein level while devoid of its transcript, thus supporting its microglial origin (Extended data, Fig. 8g). Indeed, not detected in the conditioned medium (cM) of organoids alone by western blot analysis, APOE was highly expressed at protein level in the cM of co-culture organoids; expression restricted to the fraction of cM enriched in High Density lipoprotein (HDL) (Fig. 3m and Extended Data, Fig. 8h). Altogether, this suggested that the APOE+ LLP transporting cholesterol and its esters of microglial origin to co-NPC resembled HDL.

Figure 3

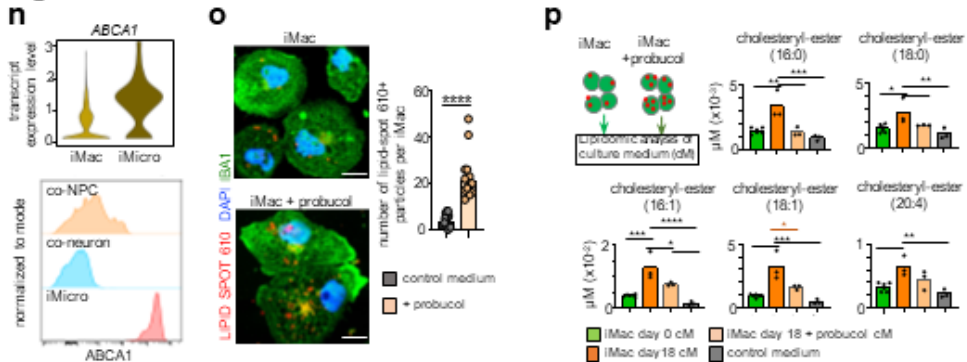


Extended data, Figure 8

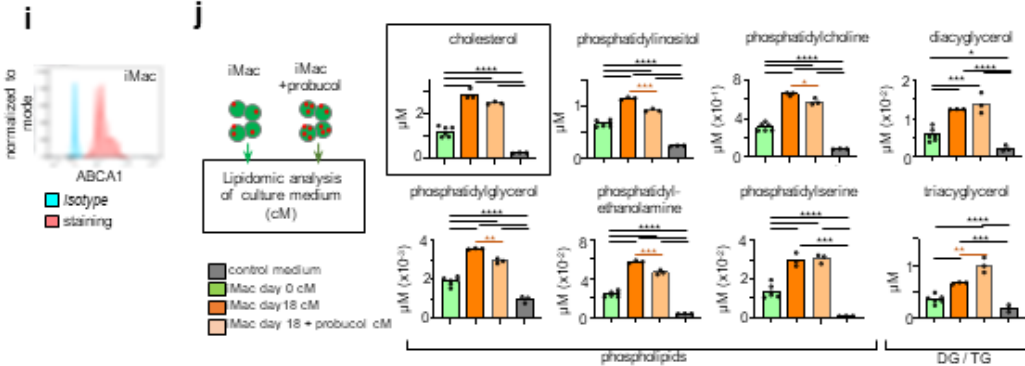


The expression of AIBP, a protein facilitating the efflux of cholesterol by the the ATP-binding-cassette receptor and cholesterol transporter ABCA1 for integration into APOE/ApoA1 lipoprotein-like particles further prompted us to explore the function of ABCA1 in our system, especially as it was highly expressed in iMac and iMicro at the transcript and protein levels but not expressed by co-NPC and co-neurons (Fig. 3n, Extended Data, Fig. 8i). As supported by previous reports (Chung *et al.*, JBC, 2010; Smith *et al.*, J Lipid Res, 2004; Chroni *et al.*, Biochemistry 2004; Kypreos *et al.*, Biochemistry 2008), the inhibition of ABCA1 with probucol led to the accumulation of lipid droplets in iMac (Fig. 3o) and prevented the release of cholesterol and its further esterification and integration into APOE+ lipoprotein-like particles as demonstrated by the lipidomic of the cultured treated iMac showing a reduction in the level of phospholipids and an increase in triglycerides (Fig. 3p; Extended data, Fig. 8j).

Figure 3



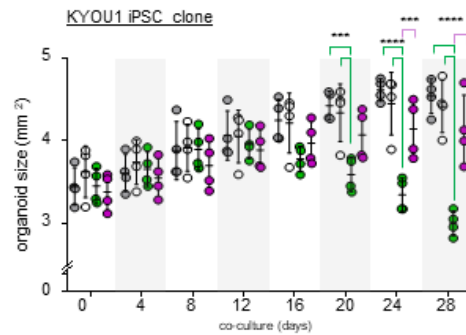
Extended data, Figure 8



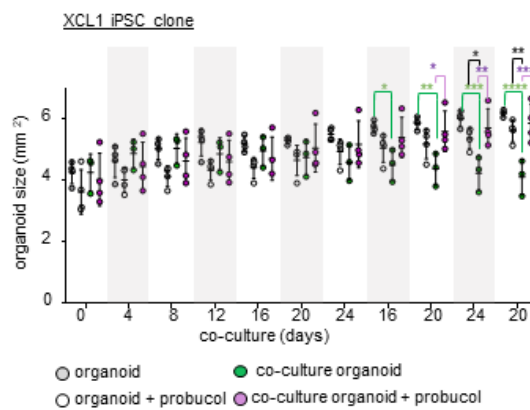
We further show that this phenomenon driven by the release of cholesterol from iMac/iMicro into the microenvironment for the generation of cholesteryl-esters and its integration into lipoprotein-like particles, was associated with the size reduction of brain organoids upon co-culture with iMac. Indeed, blocking of ABCA1 using probucol abrogated the reduction of size in co-cultured organoids generated from the KYOU1 (Fig. 3p) and the XCL-1 human iPSC clones (Shown below, and which can be included in the manuscript upon recommendation by the reviewers).

Figure 3

q



Extra Data



Altogether, we are describing how the AIBP/ABCA1 system present in iMac/iMicro promote the active release of cholesterol and its subsequent esterification into cholesteroyl-esters. APOE+ lipoprotein-like particles purified in HDL fractions would then permit the transfer of these cholesteroyl-esters to co-NPC. We further functionally interfere with this process by blocking the ABCA1 transporter and show that it does significantly abrogate the reduction of size in co-cultured organoids generated from two distinct iPSC lines.

-Chung S, Timmins JM, Duong M, et al. Targeted deletion of hepatocyte ABCA1 leads to very low density lipoprotein triglyceride overproduction and low density lipoprotein hypercatabolism. *J Biol Chem.* 2010;285(16):12197-12209. doi:10.1074/jbc.M109.096933.

-Smith JD, Le Goff W, Settle M, et al. ABCA1 mediates concurrent cholesterol and phospholipid efflux to apolipoprotein A-I. *J Lipid Res.* 2004;45(4):635-644. doi:10.1194/jlr.M300336-JLR200.

-Chroni A, Liu T, Fitzgerald ML, Freeman MW, Zannis VI. Cross-linking and lipid efflux properties of apoA-I mutants suggest direct association between apoA-I helices and ABCA1. *Biochemistry.* 2004;43(7):2126-2139. doi:10.1021/bi035813p.

-Kypreos KE. ABCA1 promotes the de novo biogenesis of apolipoprotein CIII-containing HDL particles in vivo and modulates the severity of apolipoprotein CIII-induced hypertriglyceridemia. *Biochemistry.* 2008;47(39):10491-10502. doi:10.1021/bi801249c.

In addition, what is the consequence of cholesterol uptake in the NPCs: do the NPCs which are BODIPY+ have longer axons? Are there more mature neurons which are BODIPY+ (after NPCs having taken it up) compared to BODIPY negative neurons? Do the BODIPY+ neurons have altered metabolism?

We thank the reviewer for his/her sharp observation on the presence of BODIPY+ and BODIPY- co-NPC and co-neurons which we are currently investigating in a follow up study. We believe this is beyond the scope of study as difficult to quantify and technically challenging to perform, while using our current data we can only make speculative suggestions. We nevertheless further analysed our 10X RNAseq data and explored the DEGs between PLIN2+ and PLIN2- co-NPC and co-neurons.

Indeed, the BODIPY^{493/503} staining co-localise with the expression of PLIN2 protein; a hallmark of lipid droplets.

Can the authors hypothesize how the cholesterol impacts the NPCs?

We believe that the identification of the exact molecular mechanisms mediating such effect are beyond the scope of the study and will be address in further studies.

5. Overall, it would be useful to have quantifications of the data provided, for a) iMicro responses to laser injury and Abeta phagocytosis, b) iMicro proliferation, c) proximity of iMicro to proliferating NPCs/ axons, d) PLIN2+ iMicro+ cells, d) PLIN2+ LIPIDSPOT610+ IBA1+ cells in human and mouse (**fig 4**)

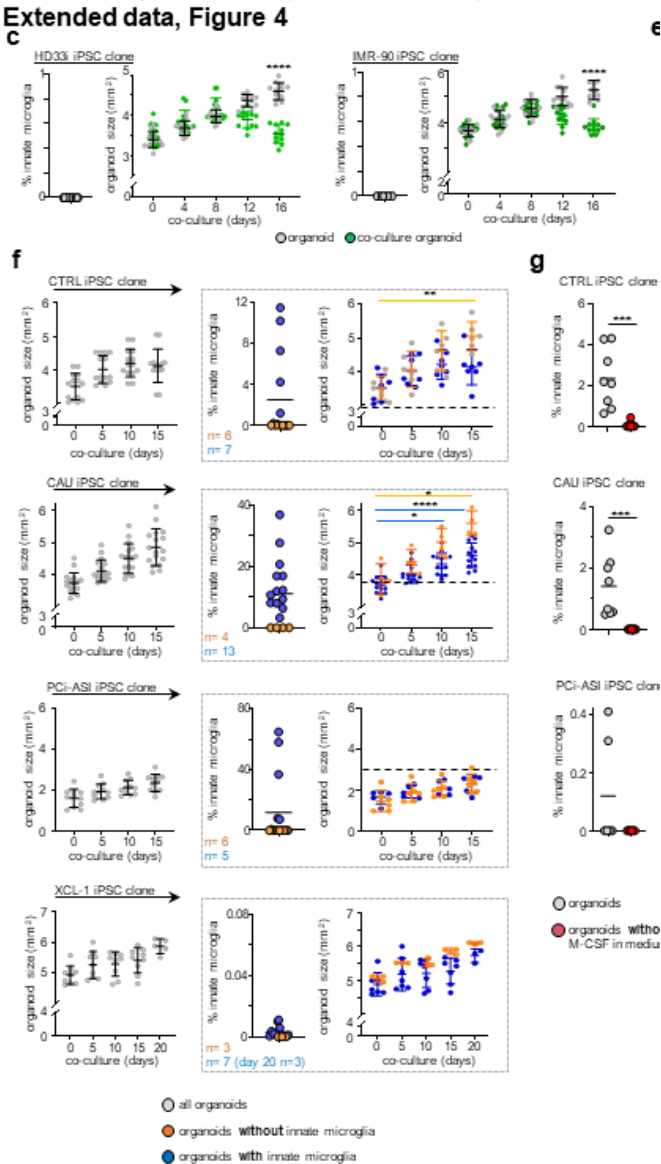
We thank the reviewer for their comment and as we have repeats for each of these experiments, we provide quantifications performed using the Imaris Imaging software whenever possible. We have performed the quantification for iMicro responses to amyloid beta peptide (Extended data, Fig. 1h), iMicro proliferation (Extended data, Fig. 1e) and their proximity to proliferating NPC and their neurites (Fig. 2l, m, n). We quantified the proportion of PLIN2+ iMicro cells (Fig. 3c) in co-culture organoids but we did not add this quantification for Fig. 3b, since 100% of iMac are Lipid-Spot-610+ PLIN2+ (detail added in the figure legend).

We quantified the proportion of Lipid-Spot-610+ PLIN2+ IBA1+ iMicro from the immunofluorescence staining of mouse and human brain microglia (Fig. 4b-d, Extended Data, Fig. 9d). In addition, extended data, Fig. 1g is a representation of several observations on the response of iMicro response to laser injury. The text, figures and method sections have been updated.

6. Can the authors clarify what the n numbers indicated stand for, are these are numbers of organoids from 1 clone, or different clones from the same line, or different iPSC lines. It would be most robust to have multiple lines represented.

We apologize for this lack of precision and have ensured that the “n” number is clearly explained throughout the text and notably in figures, figure legends and the method section where appropriate. The manuscript is based on the extensive description of our new immune-sufficient brain organoid model generated from the KYOU1 human iPSC clone. As such the “n” number represent either the number of organoids or pool of brain organoids and co-culture organoids generated from this iPSC clone.

Where we have used other human iPSC clones either in the main manuscript or in this response to Reviewer's comments, we have clearly indicated which human iPSC clone has been used. Notably, other human iPSC clones (HD33i, IMR-90, CTRL, CAU, PCi-ASI and XCL-1) have been used and associated results are presented in Extended data, Fig. 4c, f, g. In this figure, each points represent an individual organoid/co-culture organoid. The updated manuscript reflects all these changes.



Minor suggestions:

1. The numbers of iMicro in the organoid appears very small from the quantification in **extended data Fig3– (Extended Data, Fig. 4 in the updated manuscript)** which is not what it looks like from the high number of cells in **Fig.1b**. What is the absolute number of iMicro in the organoids? Is the density of iMicro similar to the density of microglia in human developing brain?

As we highlighted beforehand, there is indeed a discrepancy in the numbers of iMicro obtained from the 10X RNAseq Data (Extended data, Fig. 2b), the Flow Cytometry analysis (Fig. 2b) or the

Immunofluorescence data (Fig. 1b, Extended Data, Fig. 1c, Extended Data, Fig. 5d, Extended Data, Fig. 6e) compared to the initial number of iMac added per organoid at day 0 of coculture (150,000 unless otherwise stated). These differences mainly owe to technical variability between techniques and likely to a poor recovery of iMicro during the 10x single cell processing. Overall, the proportion of iMicro obtained by Immunofluorescence imaging and Flow cytometry analysis (0.7-2.4%) was higher compared to numbers obtained by single-cell RNAseq (1.15%) resulting in a 5-7.5-fold difference.

Extended data, Figure 2

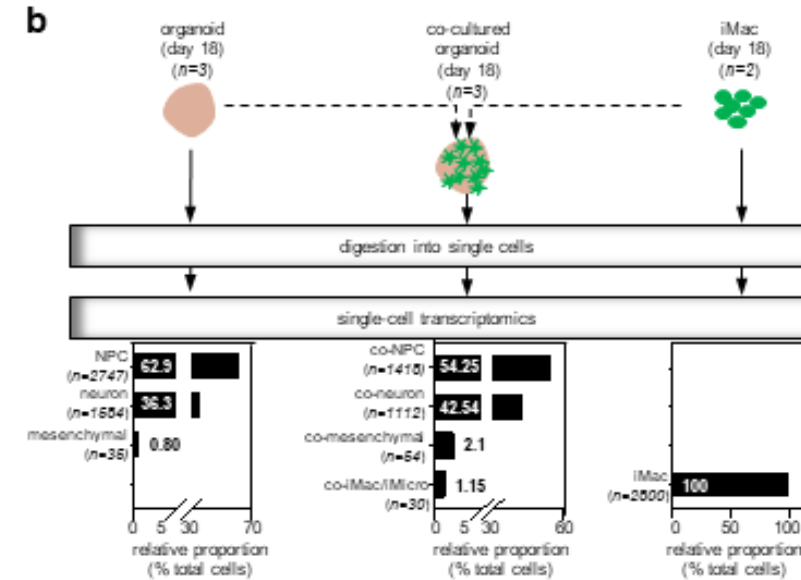
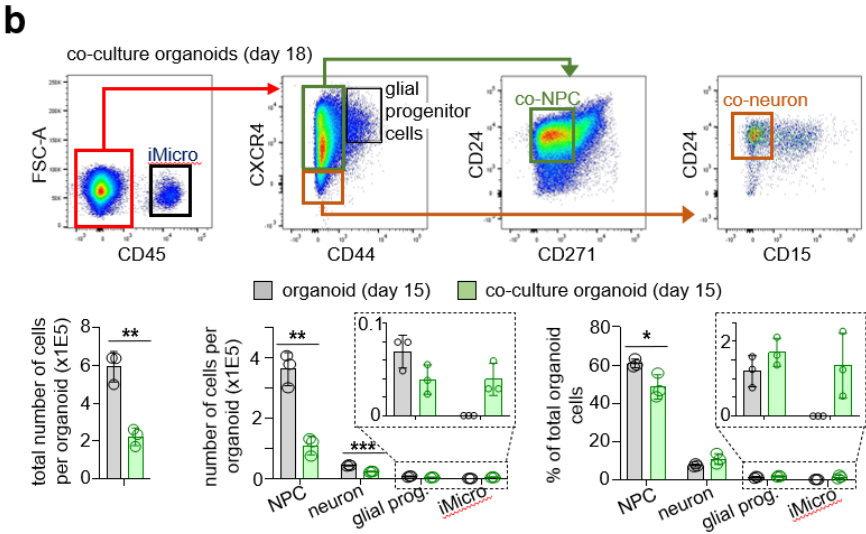
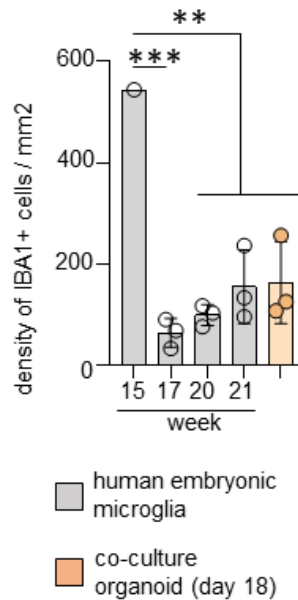


Figure 2



As to “Is the density of iMicro similar to the density of microglia in human developing brain?”, we quantified the density of IBA1+ cells in human embryonic microglia week 15-21 (Fig. 4d; Extended data, Fig. 9d) and in our organoid sections (Extended data, Fig. 1c and other available organoid sections, (n=3, 3 different subsections each) using the Imaris software (Statistical analysis, one-way Anova test). For clarity, the calculation of the density of IBA1+ cells in organoid did not consider the surface of co-culture organoids, but rather its core (Figure below). We could observe that the density

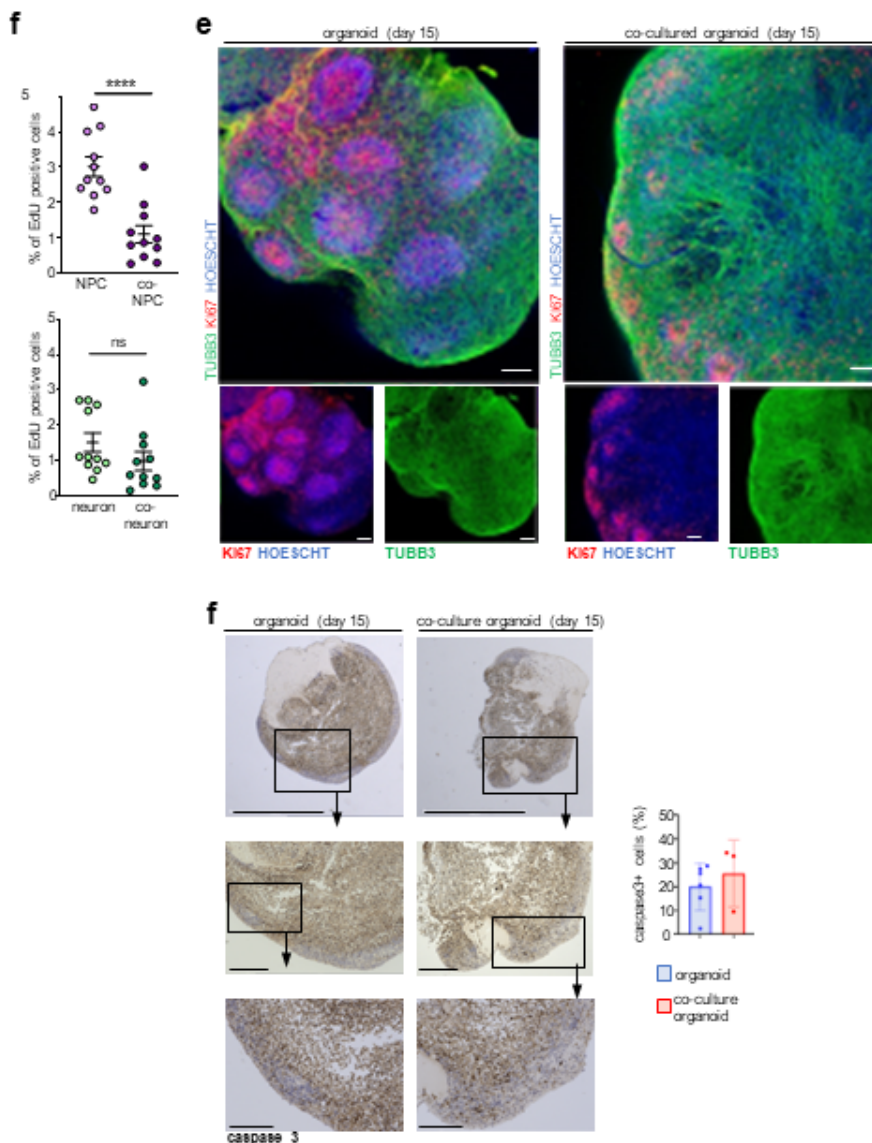
of IBA1+ iMicro in day 18 co-culture organoids resembled the density of human embryonic microglia at week 17, 20 and 21.



2. Since the maturation of neurons is increased with iMicro coculture, can the authors explain why no significant increase in the % of neurons is seen in organoids in **Fig2b**?

While the maturation of neurons is increased in co-culture organoids compared to organoids alone, we clearly demonstrate that the neural rosettes are less structured and smaller upon the addition of iMicro (Extended data, Fig. 5a, b, Extended data, Fig. 6e), while the mass-spectrometry proteomic profile shows a strong signature of apoptosis / cell-cycle arrest alongside an increased in the neurogenesis profile in neural rosettes from co-culture organoids (Extended data, Fig. 5c). This observation mirrored the reduced proliferation of co-NPC in co-culture organoids as demonstrated by EdU incorporation assay (Fig. 2f) and Ki67 staining by immunofluorescence (Extended data, Fig. 6e). Thus, it is likely that less neurons are produced.

Figure 2 Extended data, Figure 6

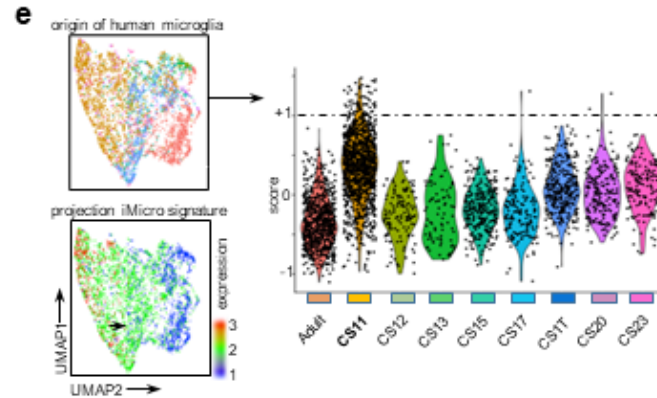


We also observed that this reduced proliferative capacity of co-NPC and co-neurons was not associated with higher levels of apoptosis orchestrated in part by iMicro at the vicinity of neural rosettes as demonstrated by our proteomic analysis (Extended data, Fig. 5c) and immunofluorescence staining using caspase 3 (Extended data, Fig. 6f). Altogether, orchestrated by iMicro, we show that NPC in our co-culture brain organoids privilege reduced proliferation and improved neurogenesis over increased apoptosis. These new data support the observation Fig. 2b and the manuscript has been accordingly updated

3. Can the authors clarify the age of the human samples used in the UMAPs in Fig.4?

We compared the profile of iMicro with that of the embryonic microglia from the Allen human brain atlas. We found that the iMicro in co-culture organoid at Day 18 may resemble that of CS11 human embryonic microglia (Fig. 4e).

Figure 4



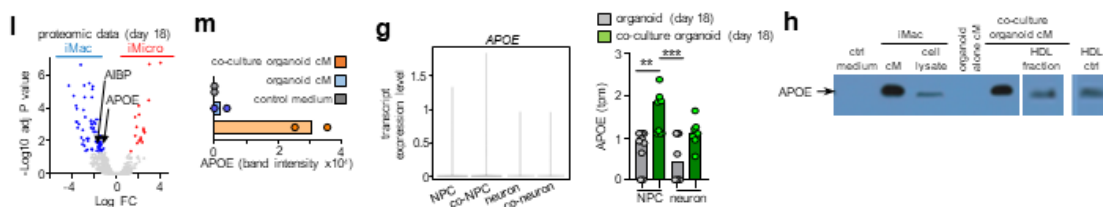
By immunofluorescence imaging, we confirmed the observations made with regards to the expression of PLIN2+ Lipid droplets by IBA1+ microglial cells in 15-, 17, 20- and 21-week-old human embryos (Fig. 4d, Extended data, Fig. 9d). The Figures and texts of the new version have been amended accordingly.

4. Can the authors provide quantification with statistics of APOE from the transcriptomics and proteomics in extended **Fig5d (Fig. 3l in the updated version of the manuscript)**?

In the revised version of the manuscript, we have extended the analysis linked with the expression of APOE. We show that the addition of iMac culture medium was sufficient to allow for cholesteryl-esters to be transferred from iMac to NPC (Fig. 3i), and was associated with a reduction of the size of brain organoids throughout the culture (Fig. 3j, Extended data, Fig. 8d, e). The lipidic composition of the culture medium from iMac and co-culture organoids (rich in cholesterol, cholesteryl-esters and phospholipids) suggested that cholesterol-esters were transferred to NPC via an APOE+ lipoprotein-like particle (LLP) (Fig. 3k, Extended data, Fig. 8f). Indeed, the DEP analysis of iMac vs. iMicro unravelled that APOE and AIBP (Apolipoprotein A1 binding protein favouring the efflux of cholesterol to APOE LLP) were strongly expressed by iMac at the start of the co-culture (day 0), thus further supporting our hypothesis (Fig. 3l with associated APOE quantification and statistical analysis). Interestingly, devoid of any RNA transcripts for *APOE*, co-NPC presented significant levels of APOE proteins and suggested that it originated from iMac (Extended data, Fig. 8g). APOE was further detected only in the culture medium of day 18 iMac and co-culture organoids while absent in the culture medium of organoid alone. Processing the culture medium of co-culture organoids by density gradient separation and ensuing western-blot analysis, we could confirm that the expression of APOE was restricted to the HDL fraction. Altogether these results strongly suggest that iMicro transfer cholesterol and its esters to co-NPC via APOE+ LPL resembling HDL. The text and figures have been amended accordingly.

Figure 3

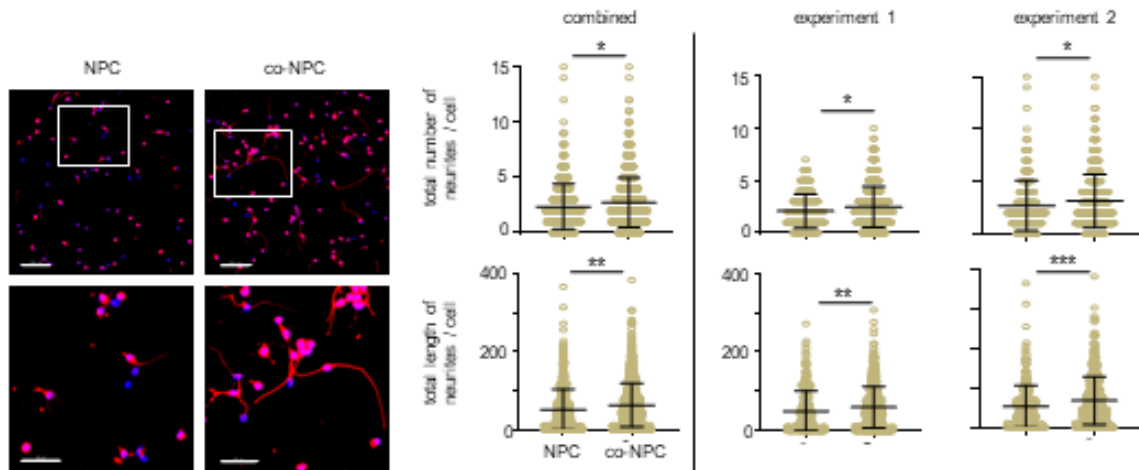
Extended data, Figure 8



5. **Figure 2h (Fig. 2i in the updated version of the manuscript)** represents number of neurons and axon

length in a strange plot, can this be re-graphed so we can see individual data points and the mean? It is difficult to tell the difference as presented, even with significance indicated.

The data plotted fig. 2i (former Fig. 2h) was re-plotted using scattered dots with Mean and standard deviation. The two independent experiments shown are separately displayed for convenience. We amended the labelling of the axis as recommended by one reviewer to neurites, which better reflects the measurements done while exact numbers of measurements are details in the Method section. The final manuscript reflects these changes.



6. Can the authors ensure that the time point of the organoid is indicated in the text and figure legends for all the single cell sequencing/proteomics.

We apologize for this lack of clarity and have amended throughout the text and figures the time point of the organoid or co-culture organoids used when necessary, and especially for all single cell sequencing, proteomic and lipidomic analysis.

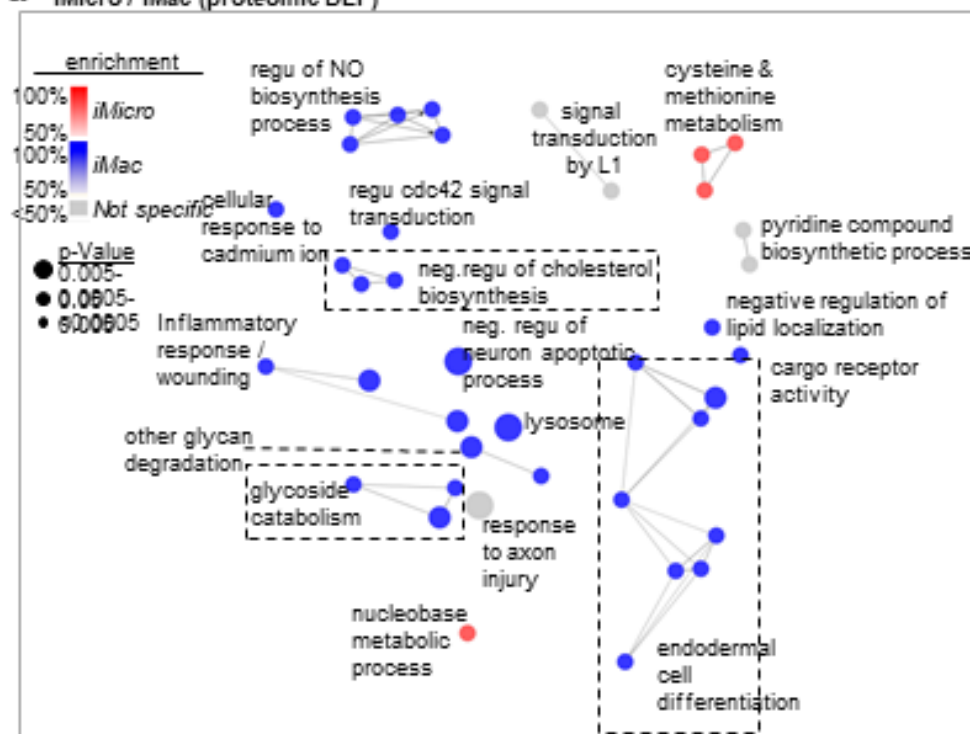
7. The use of proteomics is really interesting, yet could have been further analysed to yield more information as we only see one of the proteins highlighted. Does the pathway analysis of proteomics show the same as the transcriptomics? Can the proteomics results also be validated by staining for several proteins of interest?

We have performed pathway analysis using the differentially expressed proteins (DEP) from the proteomic data obtained by mass-spectrometry analysis for iMac/iMicro, NPC/co-NPC and neuron/co-neuron (Extended data, Fig. 8a, b, c respectively). As expected, the pathways characterised using the DEPs do not cover completely the analysis performed with transcriptomic data. Nonetheless, the analysis of iMac/iMicro DEP and DEG highlight similar pathways associated with cholesterol and lipid biosynthesis, inflammatory responses, and neurogenesis processes (such as control of the regulation of neuronal apoptosis and neuronal differentiations).

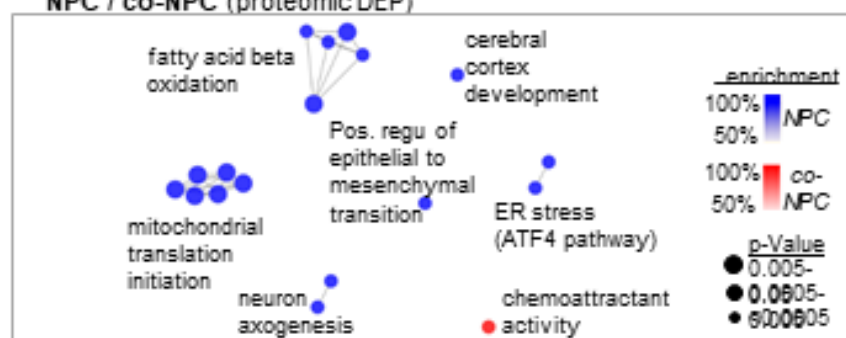
DEG and DEP of NPC/co-NPC show common pathways such as neuro- and axonogenesis, and metabolic pathways linked with fatty acid oxidation and mitochondrial biogenesis. The DEP proteomic analysis related to neuron/co-neuron highlighted pathways not unravelled by transcriptomic analysis such as neuronal activities (dopamine metabolic process), leukotriene metabolism/production or amyloid-beta formation.

Extended data, Figure 8

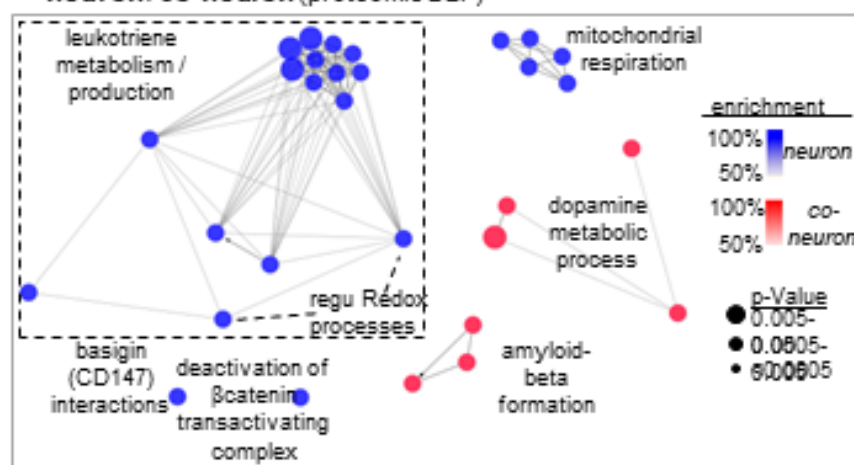
a iMicro / iMac (proteomic DEP)



b NPC / co-NPC (proteomic DEP)



c neuron / co-neuron (proteomic DEP)



We further validated these pathways and associated markers by flow cytometry analysis and immunofluorescence throughout the manuscript and as exposed in details throughout this document in response to the various reviewer's comments. For markers of **iMac/iMicro related pathways**, we investigated the following:

- Markers of **microglia phenotype** of iMicro with the staining for microglia specific markers such as P2RY12 and CX3XR1 (Fig. 1e, Extended data, Fig. 1f) as well as morphological analysis (Fig. 1c; Fig. 3f; Extended data, Fig. 1d-h; Supplementary Video, 1, 8).
- Pathways of **regulation of cholesterol biosynthesis and storage** are described extensively throughout the manuscript with staining for microglial markers such as lipid droplets (PLIN2+, Lipid Spot 610 / BODIPY^{493/503}+) (Fig. 3b, c, f, o and Supplementary Video. 8 We also validate functionally the mechanisms of storage of cholesterol by iMac/iMicro into lipid droplets using specific lipid probes (BODIPY^{493/503} and BODIPY^{CE}) (Fig. 3). We also validated these observations in human and mouse fetal brain sections (Fig. 4 b-d, Extended data, Fig. 9d).
- Pathways related to **cholesterol transfer/efflux and cargo**. We characterized the cholesterol efflux from iMac/iMicro to NPC/co-NPC respectively using successive pulse-chase experiments using lipid probes and protein expression (Fig. 3e-i). We further show that this process is mediated by ABCA1 alongside AIBP (Fig. 3n, Extended data, Fig. 8i) and that ABCA1 blockade abrogated the observed size reduction of co-culture brain organoids normally observed with time. Finally, using a combination of flow-cytometry, western blot and mass-spectrometry lipidomic analysis, we suggest that the transport of microglial-derived cholesterol and its ester is mediated via APOE+ lipoprotein-like particle detected in the same fractions as purified HDL using ultracentrifugation gradient density isolation of culture media of iMac and co-culture organoids. (Fig. 3k-m, o, p and Extended data, Fig. 8f-h, j
- **Inflammatory response / response injury pathways** with response to laser damage (Extended data, Fig. 1g and Supplementary Video. 2), uptake of Amyloid beta peptides (Extended data, Fig. 1h and Supplementary Video. 3) and to a certain extent, albeit also associated with the neurogenesis process, the proximity of iMicro to co-NPC and the associated phagocytic processes (Fig. 2l-n and Extended data, Fig. 4h, i).

With regards to the validation of **NPC/co-NPC and neuron/co-neuron associated pathways**:

- We addressed **neurogenesis-associated pathways** (cerebral cortex development, neuron axogenesis, dopamine metabolic process) via extensive morphological analysis using markers of NPC and Neurons (SOX2, TUJ1, TUBB3, NeuN, Nestin) (Extended data, Fig. 1c, d; Supplementary Video. 1, 4, 5) and the study on the length and number of neurites (Fig. 2i).
- **Proliferation** was assessed by staining for EdU (Fig. 2f) and Ki67 staining (Extended data, Fig. 6e, Supplementary Video. 6) . Apoptosis was measured by the expression of Caspase 3 (Extended data, Fig. 6f).
- **Neural rosette analysis** by micro-dissection and ensuing mass-spectrometry proteomic analysis. The histological relationship with iMicro also allowed for the overall understanding of the impact of iMicro in neurogenesis in co-culture organoids (Extended data, Fig. 5a, b, d; Extended Fig. 6e; Supplementary Video. 1, 7).

Altogether we validate numerous pathways revealed by mass-spectrometry proteomic analysis throughout the manuscript as reflected in its updated version.

8. Can the authors reference Abud 2017 Neuron and Ormel 2018 Nat Comms in the paper- as these are organoids in which microglia are present.

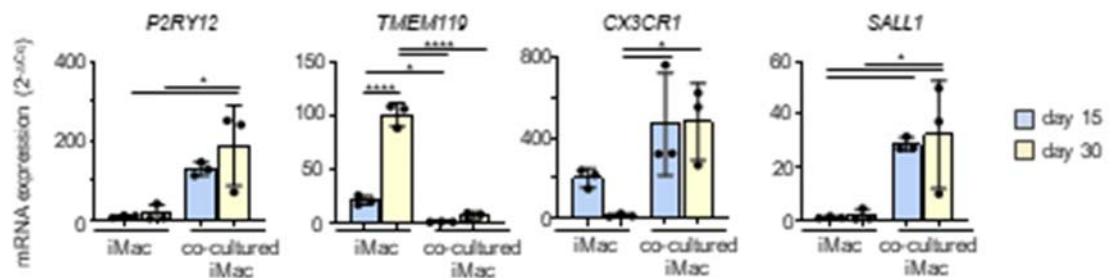
We have added the reference Abud *et al.*, 2017 Neuron and Ormel *et al.*, 2018 Nat Comms in the reference and in support of the experiments on innate microglia presented in Extended data, Fig. c, f, g.

-Abud EM, Ramirez RN, Martinez ES, et al. iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron*. 2017;94(2):278-293.e9. doi:10.1016/j.neuron.2017.03.042.

-Ormel PR, Vieira de Sá R, van Bodegraven EJ, et al. Microglia innately develop within cerebral organoids. *Nat Commun*. 2018;9(1):4167. Published 2018 Oct 9. doi:10.1038/s41467-018-06684-2.

9. Can the authors increase the n numbers for the d30 qPCR of Sall1, as it is very variable.

We have increased the repeated the qPCR cycles for SALL1 and also increased the number of cycles from 40 (*P2RY12*, *TMEM110*, *CX3CR1*) to 50 cycles (*SALL1*) on day 18 iMac and co-cultured iMac. We still observe at d30 *SALL1* mRNA expression and significantly overexpressed compared to its expression in iMac at day 15 and day 30. The updated manuscript reflects these changes (Fig. 1d).



Referee #3 (Remarks to the Author):

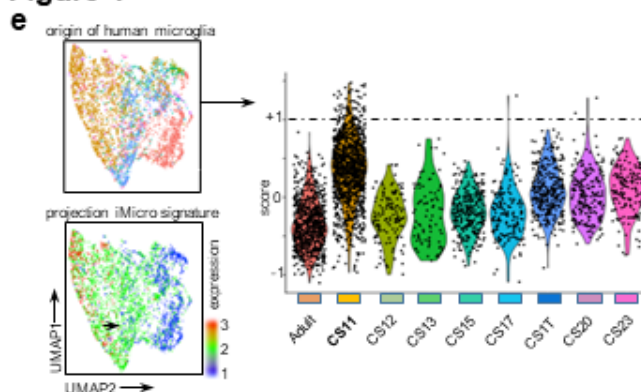
In this work, Park et al report the development of a novel organoid system which allows the study of the role of microglia during the early developmental steps of the human brain. These studies identified a unique role for microglia in limiting neuronal progenitor cell proliferation and the promotion of axonogenesis. Furthermore, the mechanistic investigation of these observations identified a novel pathway by which microglia exported cholesterol and its esters to NPCs. Finally, the investigators validated their findings via the analysis of mouse and human brain samples. Taken together, these studies provide novel insights into the mechanisms that control brain development, while describing a unique system for the investigation of the brain in health and disease. Hence, these studies will certainly have a profound impact in the field and should be of interest to the broad readership of Nature. This work is performed by experts in the field, the experiments are properly controlled and described. However, it is my opinion that some issues should be addressed before this work is ready for publication.

Major comments.

1. Please compare the transcriptional profile of iMicro with that of embryonic microglia, to allow the reader to fully evaluate the possibilities and limitations offered by this new organoid system.

We thank the reviewer for his/her comment. We integrated the transcriptional profile of iMicro with that of embryonic microglia from the Allen human Brain atlas and found that they resemble microglia from CS11 human foetal development (Fig. 4e).

Figure 4

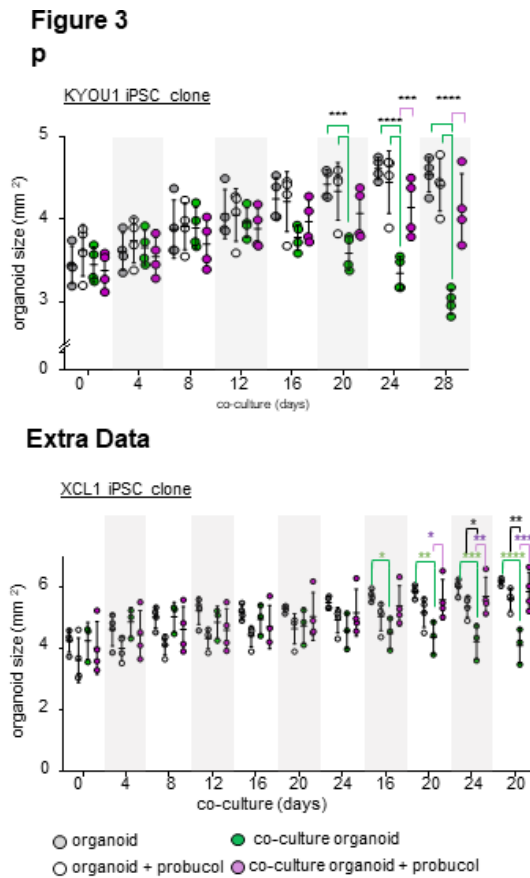


2. Although APOE is highly expressed in iMicro, no data are provided to support its role in lipid transport. The authors should use blocking antibodies or genetic inactivation to demonstrate the role of APOE in microglia-neuron lipid transport.

We thank the reviewer for his/her comments and have extensively explored this area.. Since it has been shown that the lack of APOE does not prevent the formation of HDL, although their size and lipid composition is altered (Morton *et al*, JCI, 2018), we decided to validate our hypothesis by targeting the initial steps of the formations of LPL (ie. efflux of cholesterol prior to its esterification and integration into ApoA1+ particles before APOE+ LPL/HDL are formed) rather the transport cargo itself.

Strong of the finding that ABCA1 and AIBP are the highest DEPs in iMac vs. iMicro from our proteomic data, we indeed focussed on the delivery of cholesterol / cholesteryl-esters based on published

literature showing that astrocytes provide cholesterol and its esters to neuronal cells via APOE+ HDL through interaction with ABCA1 (Ito *et al.*, Brain Res. 2014; Ioannou *et al.*, Cell, 2019), and performed inhibition experiments. We show in Fig. 3 that iMac/iMicro use the ABCA1 transporter to mediate cholesterol efflux for its esterification and integration into APOE+ apolipoprotein-like particles isolating in HDL fractions. Blocking of ABCA1 using probucol significantly reduced the levels of cholesteryl-esters generated and resulted also in the reduction of size in co-culture organoids being abrogated (Fig. 3n-q, Extended data, Fig. 8g-j).



-Morton AM, Koch M, Mendivil CO, et al. Apolipoproteins E and CIII interact to regulate HDL metabolism and coronary heart disease risk. *JCI Insight*. 2018;3(4):e98045. Published 2018 Feb 22. doi:10.1172/jci.insight.98045

-Ioannou MS, Jackson J, Sheu SH, et al. Neuron-Astrocyte Metabolic Coupling Protects against Activity-Induced Fatty Acid Toxicity. *Cell*. 2019;177(6):1522-1535.e14. doi:10.1016/j.cell.2019.04.001.

-Ito J, Nagayasu Y, Miura Y, Yokoyama S, Michikawa M. Astrocyte's endogenous apoE generates HDL-like lipoproteins using previously synthesized cholesterol through interaction with ABCA1. *Brain Res*. 2014;1570:1-12. doi:10.1016/j.brainres.2014.04.037.

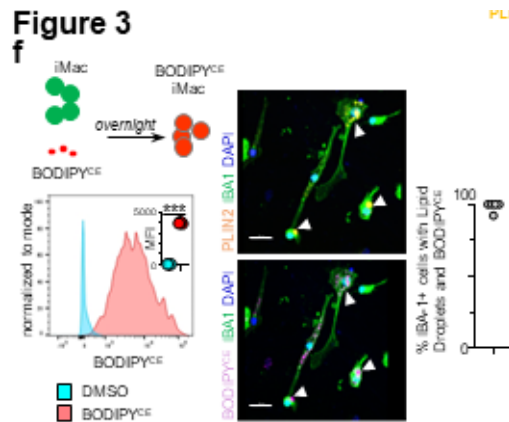
3. Provide a quantification of the data shown in **Fig. 3b** (**Fig. 3c in the updated manuscript**).

We have quantified the data shown in Fig. 3c (formerly Fig. 3b) as the % of PLIN2+ iMicro compared to the total population of iMicro. All the iMac at day 18 (100%) presented PLIN2+ Lipid-Spot 610+ lipid droplets. Text and figure have been amended accordingly.

C

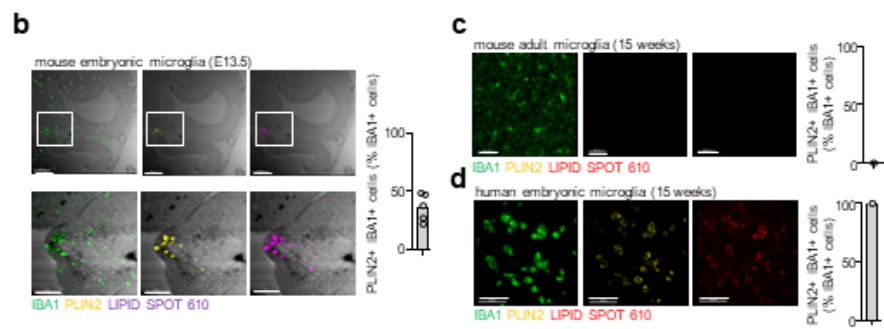
Figure 3: PLIN2+ macrophages are lipid-laden. The figure consists of two panels of immunofluorescence images and a bar graph. The left panel shows PLIN2 (green), IBA1 (yellow), and DAPI (blue) staining. The right panel shows LIPID-SPOT 610 (red), IBA1 (green), and DAPI (blue) staining. Both panels show macrophages with lipid droplets. The bar graph on the right shows the percentage of PLIN2+ macrophages (% PLIN2+ iMacro) for each condition. The y-axis ranges from 0 to 100. The x-axis has two categories: PLIN2+ IBA1+ DAPI and LIPID-SPOT 610 IBA1+ DAPI. The first category has a mean value of approximately 60, and the second category has a mean value of approximately 65. Individual data points are shown as open circles.

We apologize for the omission in Fig. 3f (Former Fig. 3e) and have added the labels for the top and bottom panels alongside a quantification made using the Imaris imaging software of the % of IBA1+ cells presenting both PLIN2 expression and BODIPY^{CE}. The final manuscript reflects these changes.

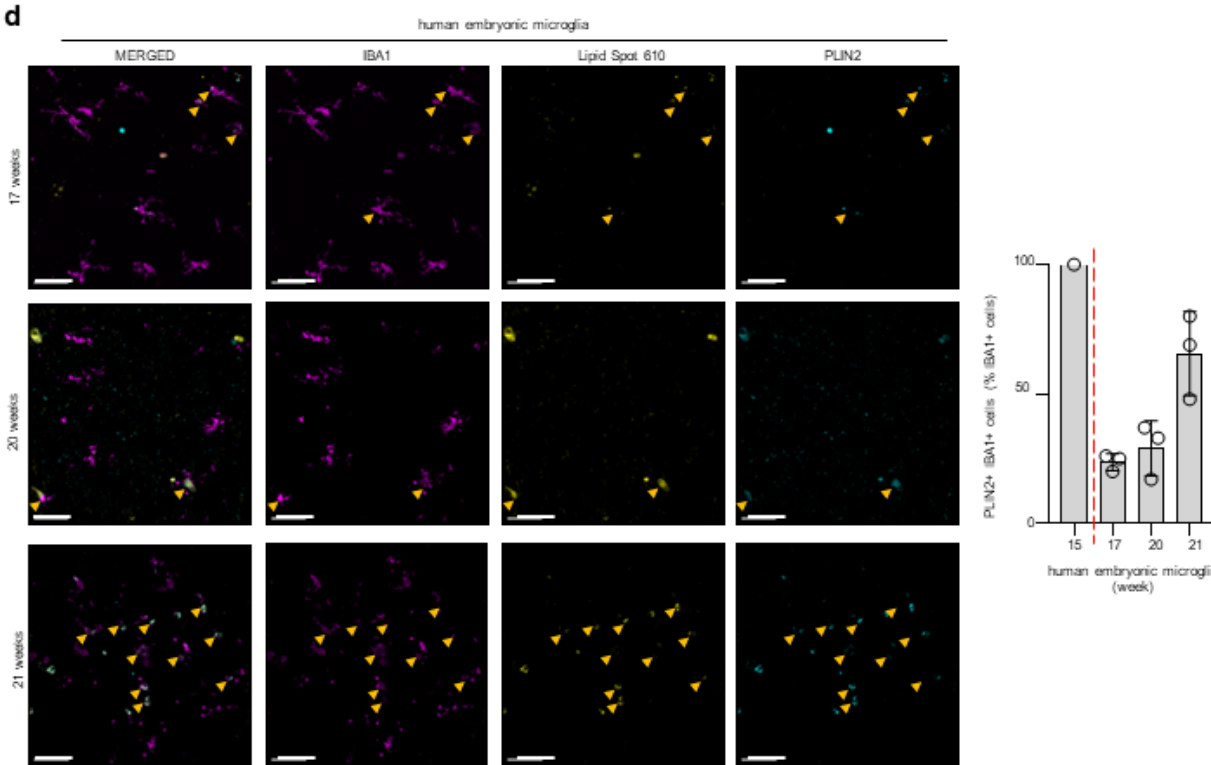


We have quantified data in Fig 4b-d and Extended data, Fig. 9d using the Imaris Imaging software to detect colocalization of IBA1, PLIN2 and Lipid Spot 610. In mouse embryonic microglia (E13.5), 25-50% of IBA+ cells contained lipid droplets while none were detected in 15-week-old mouse adult microglia (Fig. 4b, c respectively). Quantification on human embryonic microglia showed that the density of iMicro in day 18 co-culture organoid was similar to the density of human embryonic microglia at 17, 20 and 21 weeks (Fig. 4d, Extended data, Fig. 9d and Reviewer's figure below). The present updated manuscript reflects these changes.

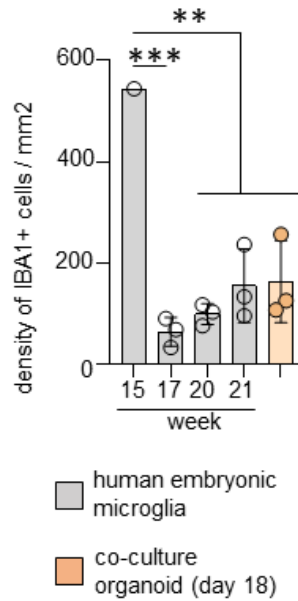
Figure 4



Extended data, Figure 9



Reviewer's figure



6. Dysregulated sphingolipid metabolism plays an important role in CNS inflammation and neurodegeneration driven by glial cells (Chao et al, 179:1483–1498). Do the authors know if in addition to cholesterol and its derivatives, microglia can transfer additional lipids to neurons. This is an important point to incorporate to the discussion.

Due to size constraint in the manuscript, we did not discuss this important point, that however we believe is beyond the scope of the study, as our model focuses on early stages of development.

Minor comments.

7. Line 132, please use “signs” instead of “signed”.

The text has been amended accordingly

8. Sentence in lines 275-276, please adjust as it doesn’t read fine (“....ability to export lipids, among with cholesterol, for uptake...”).

We have amended the text accordingly.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

Despite efforts to address the issues raised in peer-review, many technical and interpretation limitations remain. The flow and presentation of the data is still confusing, and overall, the main claims of the manuscript are not supported by the data in my opinion.

Experiments require detailed information on how many cells, organoids, differentiations, and cell lines were used. Despite efforts in this direction in the revision, insufficient details are provided about the cell lines that were used in each experiment (including their validation).

Many interpretations are not consistent with the data presented. For example, the authors state that “our model is validated in 7 independent lines”; however, co-culture experiments seem to have been performed in only 4/7 of iPSC lines. Most data come from experiments in one iPSC line. Claims are sometime made from experiments without statistical significance or controls (Ext Fig 1 g, and h; Ext Fig 6f). Quantification is sometime limited; for instance, in Figure 4b, only a small area was quantified. The PLIN2+ IBA1+/IBA1+ ratio may not reach ~40% if the cells were quantified in the upper images showed in the same figure.

To conclude that iMicro, but not iMac, resembles human microglia functional assays as well as morphological reconstruction are needed (not just transcriptomic analysis). Also, for Ext Fig 1b: day 3 may be too early to conclude that the organoid medium has no effect on the iMicro differentiation. qPCR analysis of microglia markers (like the ones used in Fig 1d: P2RY12, TMEM119, CX3CR1, SALL1) at day 15 and day 30 is necessary.

The claims about changes in organoid size in the presence of iMac are insufficiently supported by the data. For instance, Ext Fig. 6f only shows a trend for increased CAS3 in co-cultured organoids. Critically, less than half (3/7) of the iPSC lines showed organoid size reduction; therefore, the robustness of iMicro induction protocol remains unclear. As mentioned above, samples sizes (lines, independent differentiations, organoids) are small and it's unclear what the power would be to detect such an effect.

The claims about maturation in brain organoids require more data and controls: changes in cell identity, soma size, spine density, neurite complexity, electrophysiological parameters (sEPSC frequency and amplitude, resting membrane potential, and max firing rate) and transcriptomic (by mapping onto available datasets) are key to making some definitive claims.

The conclusions about cholesterol transport actually are still not supported experimentally.

The analysis reveals to 5 different regulons; the authors claim these are related to microglial identity. However, these TFs are promiscuous and further testing of their specific involvement is needed.

The authors believe that their model can be used to model neurodegenerative conditions such as TREM2+ AD; however, they use early-stage cultures and evidence so far still points to a rather immature state.

Supplementary Fig. 8h lacks a loading control for total protein as well as a quantification.

It's unclear how candidates were selected in Figure 2g with the mass spec experiment.

Referee #2 (Remarks to the Author):

In this revision, the authors put forward the novel hypothesis that in human developing brain, microglia limit neural progenitor cell proliferation thereby promoting neuronal maturation and preventing over growth, using a new combination of iPSC derived organoids and microglia-like cells from the same donor. The authors have performed a significant amount of new experiments to address my issues, including further characterizing the cell responses in the organoids, and functionally intervening with cholesterol export to support their hypothesis that iMicro lipid transfer influences NPC responses. Furthermore, the added characterization of electrophysiological properties of the organoids in co-culture, along with comparison to human 'in vivo' microglia responses, has also strengthened the paper. The paper is well written and easy to follow. The methodology is robust and valid. The authors have now included additional sources of iPSC and interestingly shown that some innately generate microglia, and these have smaller organoids, which further supports the hypothesis.

With regards to the authors' comments and new data, I have the following comments:

1. I appreciate the authors having characterized the NPC responses in the organoid following iMac transplant, discovering the impact on proliferation rather than death, and looking at iMicro phagocytosis of NPCs. With regards to the latter, the co-localization of IBA1 with TUJ1 for a readout of phagocytosis would be greatly strengthened by a 3D rendering to show that the NPC components are inside the iMicro, as this is a standard approach when investigating microglia phagocytosis. Otherwise this could represent processes from either cell type on top of each other. I see that the authors have used the Imaris software in this study to address another question, and this software is most commonly used to do this.
2. I understand the authors' comment that understanding the mechanism by which cholesterol influences the NPCs could be beyond the scope of this study. It would be useful to have clarification however on why further assessment of BODIPY-CE transferred from iMicro to co-NPCs is considered by the authors as being beyond the scope of this study. Showing that BODIPY-CE+ NPCs have reduced proliferation may be straightforward given that the authors showed BODIPY-CE transfer to NPCs, and assessed proliferation/maturation. This would greatly strengthen the hypothesis that the cholesterol transfer regulates NPC proliferation/maturation.
3. The probucol experiment is very important in showing that blocking ABCA1 eliminates the impact of iMac transplant into the organoids. Showing that this is associated with a reduction in BODIPY-CE uptake by NPCs, and an impact on NPC proliferation, would tie together the experiments very nicely and strengthen the central hypothesis of the paper.

Minor issues:

1. I would recommend not saying that co-NPCs 'express' high levels of APOE at the protein level, but rather say it was detected, as they are not likely making it themselves so the term express is not appropriate.
2. The summary mentions that NPC acquired exported cholesterol which participated in metabolic reprogramming that accompanied NPC differentiation. I think this wording is a bit strong considering that functional validation of metabolic reprogramming in the NPCs would be needed to make this statement, so I suggest softening this.
3. I appreciate that the authors have opted for functional validation of NPC proliferation and iMicro cholesterol export, over expression validation of specific genes. There are still however quite a lot of graphs highlighting changes in particular genes that haven't been validated using another method – this is fine but it would be useful if in the text, at least where a list of specific genes are highlighted as being of particular interest, that we would have an acknowledgement the importance of validation for future work.

Referee #3 (Remarks to the Author):

I congratulate the authors for their extensive revision of this manuscript. The authors have addressed all my concerns.

Author Rebuttals to First Revision:

Dear Editor and Reviewers,

We first want to thank all reviewers for their careful consideration of the manuscript and their critical comments. In this latest version of the manuscript, we performed multiple additional experiments and analysis to further strengthen our claim with regards to the impact of the addition of primitive like macrophages (iMac) on brain organoids from the same source of iPSC, the subsequent size reduction observed in co-culture organoids, as well as the underlying mechanism. We hope that the reviewers will appreciate once more that these additional experiments required the expertise of other research labs including at the Institute for Molecular and Cell Biology (Singapore), the Yoon Loo Lin School of Medicine (Singapore), the Duke-NUS Medical School (Singapore) and the Gustave Roussy Institute in Paris, France, highlighting the reproducibility of our main observation.

In summary:

- We used **11 iPSC lines to validate our microglia-sufficient model** at different levels. The impact of the addition iMac on the size of our brain organoids is **directly validated in 8 of these iPSC lines (8/11)**
- **Innate microglia** has been investigated in **7 out of the 11 iPSC lines** and found to be present in organoids generated from **4 of these 7 iPSC lines, but not in the KYOU1 line we have mainly used in this study**. Additionally, we validated that **the presence of innate microglia was affecting the size of organoids in 2 out of these 4 iPSC lines**.
- The **co-culture of brain organoids with iMac has a profound impact on the neurogenesis process** as demonstrated by further analysis and experimental studies specifically showing reduced numbers and sizes of neural rosettes as well as a significantly reduced proliferative capacity of NPC associated with increased pathways related to neuronal differentiation, neurogenesis and synaptogenesis.
- Supporting, the maturation of organoids upon macrophage addition, **patch-clamp electrophysiological analysis** suggests that the addition of iMac promotes an increase in the functional maturation of neurons.
- The **transfer of cholesterol from iMicro to NPC/neurons** is an active process mediated through the generation of APOE+ lipoprotein-like particles isolating with HDL, and directed by the ATP-binding cassette transporter ABCA1 (also known as the cholesterol efflux regulatory protein) on iMicro, specifically shown by using Probucol, an effective inhibitor of ABCA1-mediated cholesterol efflux. In addition, using supernatant transfer experiments, we show that the release of cholesterol from iMac was enough to induce changes in organoid growth associated with a reduced proliferative activity of co-NPC; such changes were also abrogated by the use of the ABCA1 inhibitor probucol.

More specifically, we addressed the reviewer's comments below in detail.

Referees' comments:

Referee #1 (Remarks to the Author):

Despite efforts to address the issues raised in peer-review, many technical and interpretation limitations remain. The flow and presentation of the data is still confusing, and overall, the main claims of the manuscript are not supported by the data in my opinion.

We regret to see that the reviewer finds our manuscript hard to read and confusing, especially as it has not been an issue raised by Referee #3, while Referee #2 stresses on the fact that **"the paper is well written and easy to follow"**. We have nonetheless carefully read the manuscript, sought comments from independent collaborators and further reorganize Figures and Supplementary Figures to increase its fluidity.

For clarity, we have also reviewed the methodology and added technical details when they were lacking. Where appropriate, we have also paid particular attention to clearly mention the number of organoids, experiments, batches, timeline, name of iPSC clone when KYOU1 is not used, statistical analysis, etc. for every figure panel showed. This is mainly reflected in the figures and their legends and we have highlighted an example below:

Extended data, Figure 5. a, Images of organoids cultured in the absence (organoid alone, n=9) or presence (co-culture organoid, n=9) of iMac for 18 days. Scale bar, 500 μ m. **b,** Organoid size was measured over time in the absence and presence of iMac. Statistical analysis, 2-way ANOVA (n=9 for each condition). **c,** Evolution of the size of organoids generated from the XCL-1 iPSC clone over time from day 0 to day 20 in the absence and presence of iMac. Statistical analysis, 2-way ANOVA (n=3 for each condition, representative of at least 2 separate experiments). **d,** Size of brain organoids generated from iPSC clones HD33i and IMR90 devoid of innate microglia in the presence or absence of autologous iMac from day 0 to day 16, (n=12 each condition for each iPSC line). Proportion (%) of “innate” microglia in each organoid at day 16 (n=8, n=12 respectively). Statistical analysis, 2-way ANOVA. **e,** Size of brain organoids generated from iPSC clones eCTRL, CAU, PCi-ASI and XCL-1 from day 0 and up to day 15 (day 20 for XCL-1 iPSC clone) (n=14, 17, 11 and 9 respectively). Boxed plots represent the proportion (%) of innate microglia in each organoid at day 16, and the change of sizes between organoids with and without innate microglia (number of each organoid displayed on the graph). Statistical analysis, 2-way ANOVA. **f,** Impact of the presence of M-CSF in the culture medium on the proportion of innate microglia in brain organoids generated from CTRL, CAU and PCi-ASI iPSC clones (n= 8, 6 and 8 respectively). Statistical analysis, Mann Whitney test. **g,** Impact of the presence (n=16) and absence (n=16) of M-CSF in the organoid culture medium on the size of organoids from CTRL and CAU iPSC clones grown for up to 15 days. Statistical analysis, 2-way ANOVA. **h,** Evolution of the size of organoids generated from the iPSC of 4 distinct patients (pediatric brain cancer) over 15 days with and without iMac (n=3 for each condition). Statistical analysis, 2-way ANOVA. **i,** Proportion of four different cell types in organoids cultured in the absence (organoid alone 1, 2 and 3) or presence of iMicro (co-culture 1, 2 and 3) for 18 days. Relative cell proportions were calculated from the single cell RNA-seq data. **j,** Bar graphs showing the relative proportion of each cell type in organoids alone (n=3) and co-cultured organoids (n=3). The relative proportions were calculated from the single-cell RNA-seq data. Statistical analysis, Mann Whitney test. **k,** 3D encasing of TUJ1 (TUBB3) signal by IBA1⁺ iMicro in co-culture organoid (day 16) Scale bar, 10 μ m. Panels on the right represent four different angles. Scale bar, 5 μ m. (One representation of multiple similar observations). **l,** Mass-spectrometry analysis of TUJ1 protein describing the presence of TUJ1 protein in iMac, iMicro, co-NPC and co-neuron. Statistical analysis, left plot: one-way ANOVA; right plot: Mann-Whitney test). Three independent samples (2 co-cultured organoids and 2 organoids) from the same experiment were sorted for proteomic analysis and each sample was run in triplicate.

Our main claim is that the addition of iMac prompts a reduction of the size of brain organoids. We subsequently describe throughout the study how this size reduction occurs with the KYOU1 iPSC clone taken as reference.

We however added further evidence below of the extent to which we went to validate our co-culture model using 3 additional commercially available iPSC lines (4 lines in total: XCL-1, HD33i, IMR-90, on top of KYOU1) and iPSC lines derived now from 4 patient’s skin fibroblasts. Indirectly, we show that the innate microglia had a profound impact on the size reduction of organoids generated from 2 out of the 4 clones in which innate microglia had been found (iPSC clones CTRL and CAU).

Experiments require detailed information on how many cells, organoids, differentiations, and cell lines were used. Despite efforts in this direction in the revision, insufficient details are provided about the cell lines that were used in each experiment (including their validation).

We are sincerely surprised by this comment as we have indicated such information in the material and method section (Page 24-36 of the manuscript) and Referee#2 highlighted “The methodology is robust and valid”. Still, we carefully reviewed all the method sections, figures and legends (as highlighted in previous comment) and added any details we may have inadvertently omitted. We also specified that unless otherwise stated, the KYOU-1 iPSC line is used throughout the manuscript for consistency (Page 24 of the manuscript). **Nevertheless, hoping to clarify this point, we provide a table (see below) describing all these parameters for every experiment performed.**

Human iPSC line	Cerebral organoids (Total number)	Cerebral organoids (Number of batches)	Presence of innate microglia	Cerebral co-culture organoids (Number of batches)	Organoids with innate microglia cultured in +/- M-CSF (Number)
KYOU1	199	25	No	49	--
XCL-1	101	15	No	27	6
IMR-90	36	6	No	3	--
HD33i	32	5	No	3	--
CTRL	34	6	Yes	--	Yes
CAU	38	7	Yes	--	Yes
Pci-ASI	23	4	Yes	--	--

Note: Cerebral organoids were generated as per Lancaster et al, 2014 with starting 9,000 iPSC per well for each organoid. For co-culture experiments, 150,000 iMacs (day 26) were seeded per well along with one cerebral organoid (Day 26); the culture was maintained for a further 18 days unless stated otherwise

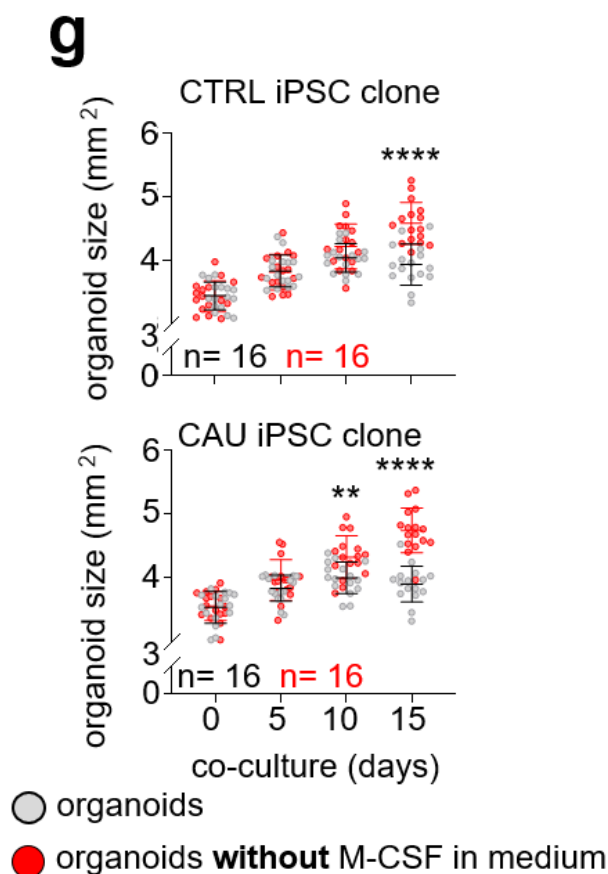
Many interpretations are not consistent with the data presented. For example, the authors state that “our model is validated in 7 independent lines”; however, co-culture experiments seem to have been performed in only 4/7 of iPSC lines. Most data come from experiments in one iPSC line.

To clarify this point, we tested **7 independent lines** but **co-cultures were performed in 4/7 of iPSC lines (KYOU1, XCL-1, HD33i, IMR-90)** as 3 of these lines generate high levels of innate microglia (and hence it is not possible to perform co-culture experiments to formally address the effect of added iMac).

Moreover, as highlighted by Referee#2, “The authors have now included additional sources of iPSC and interestingly shown that some innately generate microglia, and these have smaller organoids, which further supports the hypothesis”. Indeed, we have previously shown that the size reduction can also be observed in two other organoid cultures containing innate microglia (**CTRL** and **CAU** iPSC lines, Extended data, Fig. 4f, g), thus further strengthening our findings. This observation was not easily observed with the **Pci-ASI** organoids as not only they are the smallest in size we could observe, but they also contain very low amount of innate microglia (up to 0.4% of total cells).

However, with **CTRL** and **CAU** iPSC lines (see below), when we did not provide CSF-1/M-CSF (needed for generation of innate microglia as shown in Extended data, Fig. 5f), absence of innate microglia lead to an increase in the size of associated brain organoids compared to CSF-1 supplemented conditions, further supporting the role of microglia in promoting such size reduction (Extended data, Fig. 5g, Statistical analysis, 2-way Anova).

Extended data, Figure 5



In the previous set of revision, we also added data using XCL-1 which was left to the appreciation of the reviewers to be included in the manuscript (Page 41 of the response to reviewers). **We thus added the XCL-1 data to the manuscript for clarity (Extended data, Fig. 5c; Extended data, Fig. 9f and Extended data, Fig. 10h).** We have also shown that the addition of iMac also led to a size reduction of co-culture organoids generated using **HD33i** and **IMR-90** iPSC lines (Extended data, Fig. 5d). Therefore, the impact of the presence of iMac in our model is verified in the commercially available iPSC lines **KYOU1, XCL-1, HD33i and IMR-90 as well as indirectly in CTRL and CAU.**

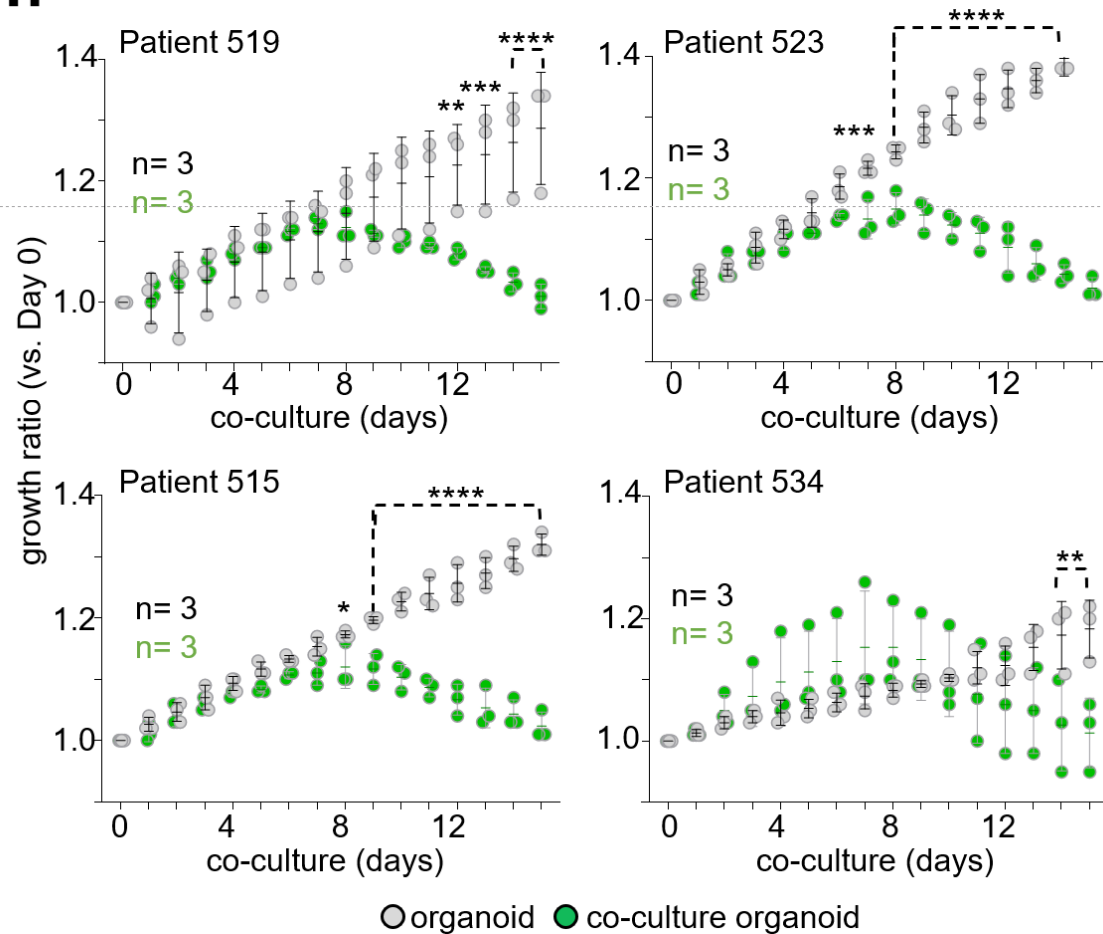
Finally, in the previous response to the reviewers, we provided unpublished data showing co-culture experiments with iPSC lines generated from 2 patients (pediatric brain cancer). Since the revision, we have also generated the following data showing similar trend from 2 more patients (Table below). All these data have been added to this latest version of the manuscript (Extended data, Fig. 5h).

	Patient-derived iPSC	Total Number of Cerebral Organoids	Number of Batches of Cerebral Organoids Made	Presence of Innate Microglia Detected	Number of Batches of Co-culture Cerebral Organoids with iPSC-derived Microglia
First revision	#515	48	8	No	3
	#534	58	10	No	3
New iPSC	#519	18	3	No	3
	#523	18	3	No	3

Note: Cerebral organoids were generated as per Lancaster *et al.*, 2014 with starting nine thousand iPSC per well for each organoid. For co-culture experiment, 150,000 iMac (Day 26) were seeded per well along with one cerebral organoid (Day 26) and the culture was maintained for further 15 days unless stated otherwise.

Extended data, Figure 5

h



Statistical analysis, 2-way ANOVA.

Thus, to summarize, we have validated co-culture experiments:

- (1) Directly with KYOU1, XCL-1, HD33i and IMR-90 iPSC lines (in the manuscript).
- (2) Directly with iPSC lines generated from 4 different patients (in the manuscript).

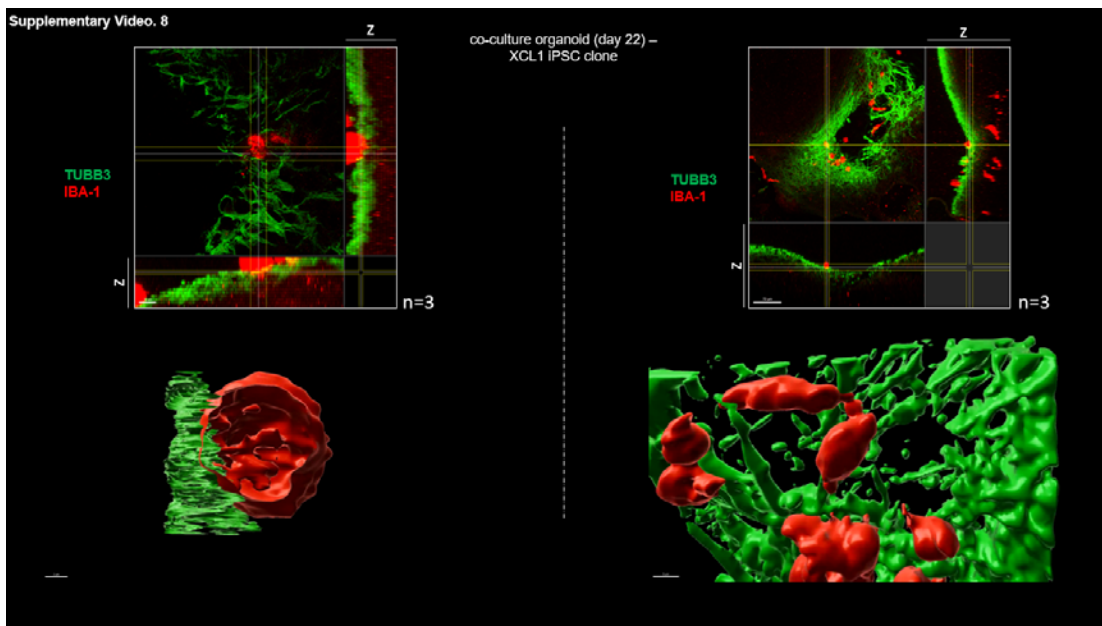
(3) Indirectly with CTRL and CAU iPSC lines (with and without innate microglia) (added in the manuscript).

Furthermore

Claims are sometime made from experiments without statistical significance or controls (Ext Fig 1 g, and h; Ext Fig 6f).

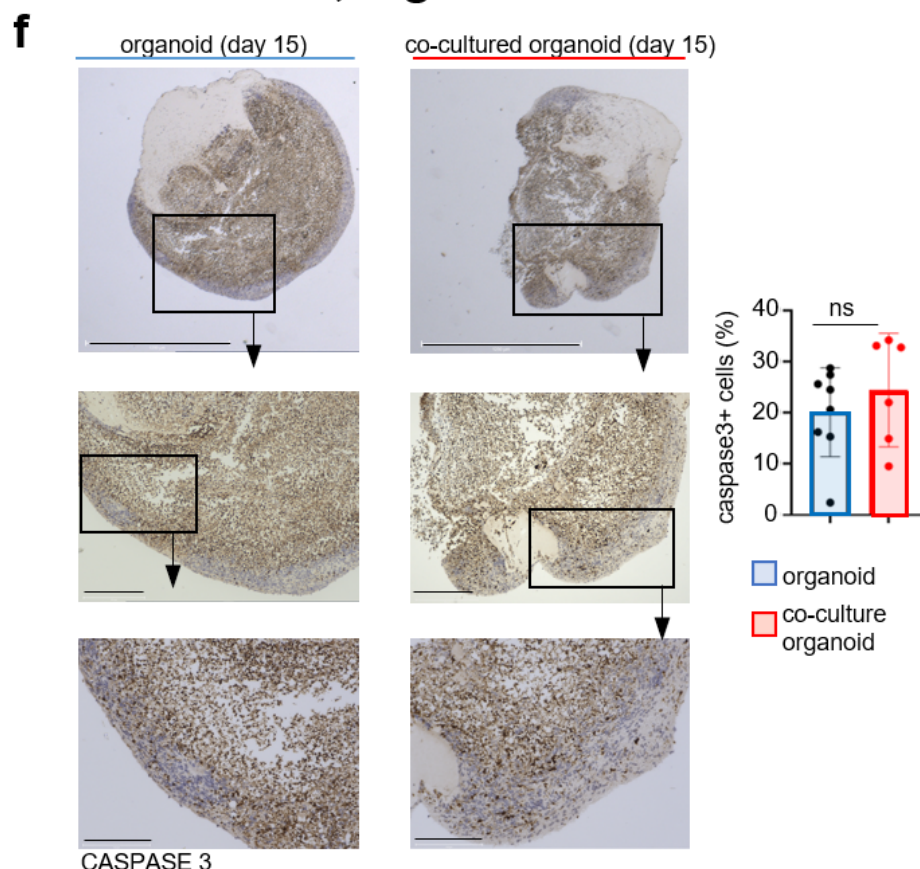
We thank the reviewer for this comment and would like to clarify on these points:

-In Extended Data Fig. 1h, i (former Extended Data Fig. 1g, h), we depicted some intrinsic features (phagocytosis and migration to damaged cells) of iMicro in the 3D environment of co-culture organoids. These observations are expected microglia properties and have been repeated independently (n=3 in both cases). We are not sure how we can quantify this and for which purpose. Using isolated iMac as controls that are not in a 3D environment is also not a perfect control in our interpretation. We however provided further evidence of the ability of iMicro to phagocytose TUJ1(TUBB3)⁺ neuronal cells, another expected property of microglia associated with neurogenesis processes, in the by 3D rendering (n=6, Supplementary Video. 8), thus supporting our initial findings in immunofluorescence staining and proteomic mass-spectrometry analysis (Extended Data, Fig. 5k, l).



-As for Extended data Fig. 6f, we have increased the number of replicates from n=3 to n=6 and clearly shown the replicates in the amended manuscript. We showed that there is no statistical (ns) difference in the level of caspase3⁺ cells between organoid and co-culture organoids; hence statistical data is seemingly absent.

Extended data, Figure 6

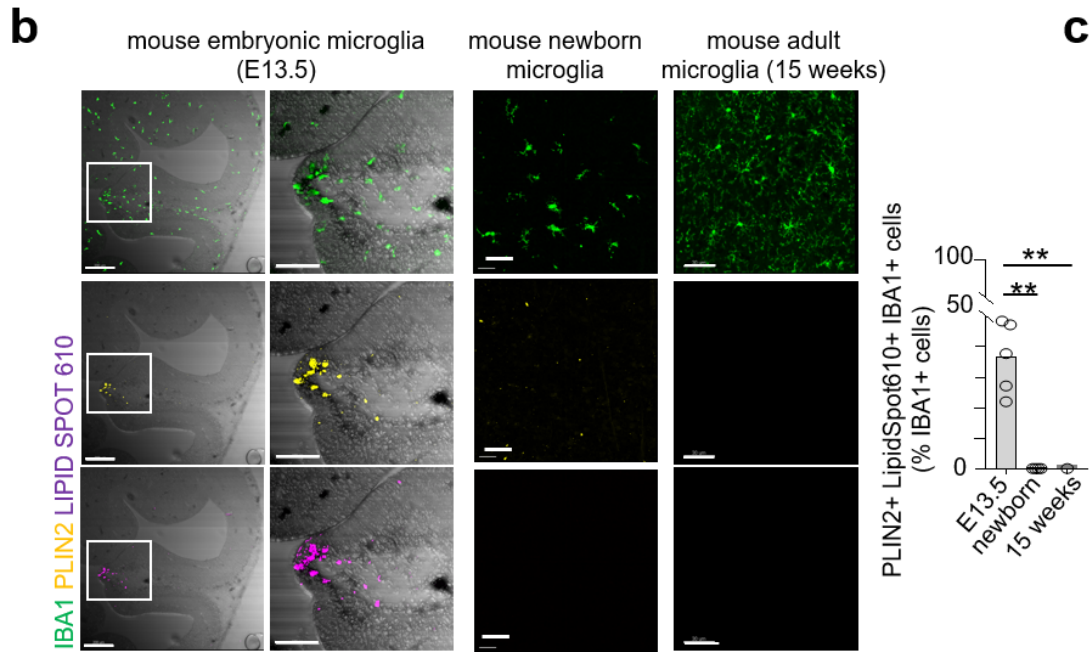


We have amended the manuscript as we have quantified more organoids and provided updated quantifications for clarity. Additionally, we nonetheless added “ns” to this graph standing for “not significant”. We apologize for this omission.

Quantification is sometime limited; for instance, in Figure 4b, only a small area was quantified. The PLIN2+ IBA1+/IBA1+ ratio may not reach ~40% if the cells were quantified in the upper images showed in the same figure.

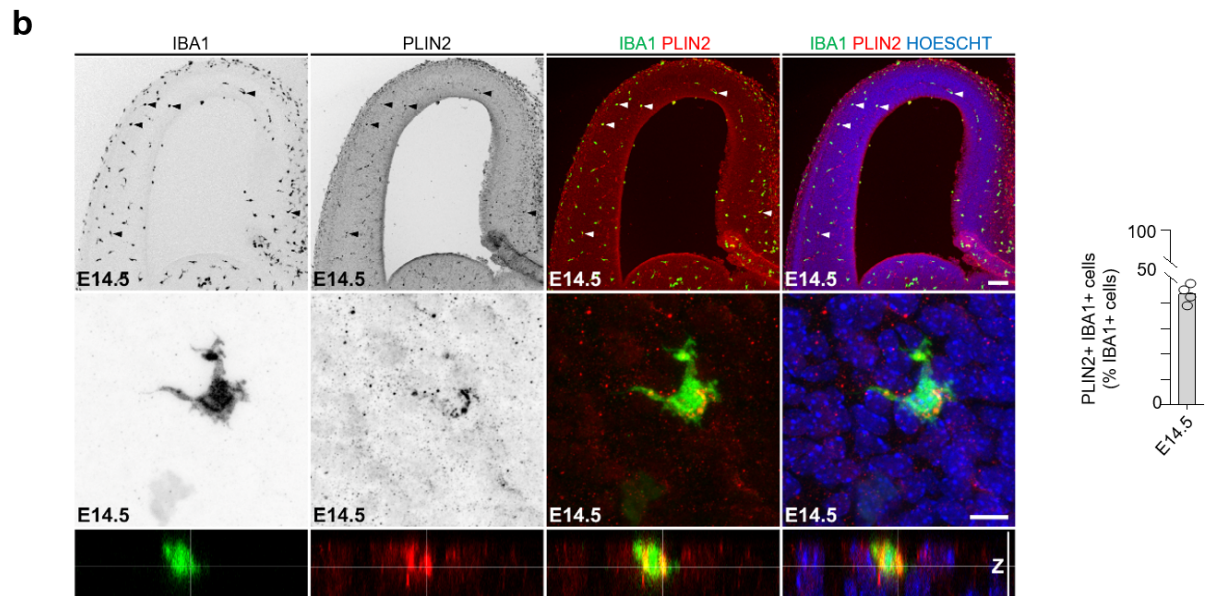
We understand the reviewer’s concern and have formatted former Fig. 4b and Fig. 4c into **Fig. 4b** in this final manuscript. We performed a new quantification based on the whole area observed while still keeping the boxed area for information. Similarly, we added the quantification of IBA1⁺LipidSpot610⁺PLIN2⁺ cells in mouse newborn microglia (n=4) and have increased the numbers of replicate for 15 weeks mouse adult microglia (n=5). This allowed us to perform better quantification and relevant statistical analysis of PLIN2⁺ LipidSpot 610⁺IBA1⁺/IBA1⁺ ratio between mouse embryonic (n=5), newborn (n=4) and adult microglia (n=5). Statistical analysis using one-way ANOVA showed that while newborn and adult microglial cells do not present lipid droplets (determined as IBA1⁺LipidSpot610⁺PLIN2⁺ cells), 20-50% IBA1⁺ microglial cells in E13.5 mouse embryonic microglia presented lipid droplets.

Figure 4



To further clarify this point, we performed another independent staining of E14.5 mouse fetal microglia (n=4) (Extended data, Fig. 11b). We could clearly detect IBA1⁺PLIN2⁺ microglia which accounted for 35-50% of all IBA1⁺ cells, within the 20-50% range observed in **Fig. 4b**.

Extended data, Figure 11

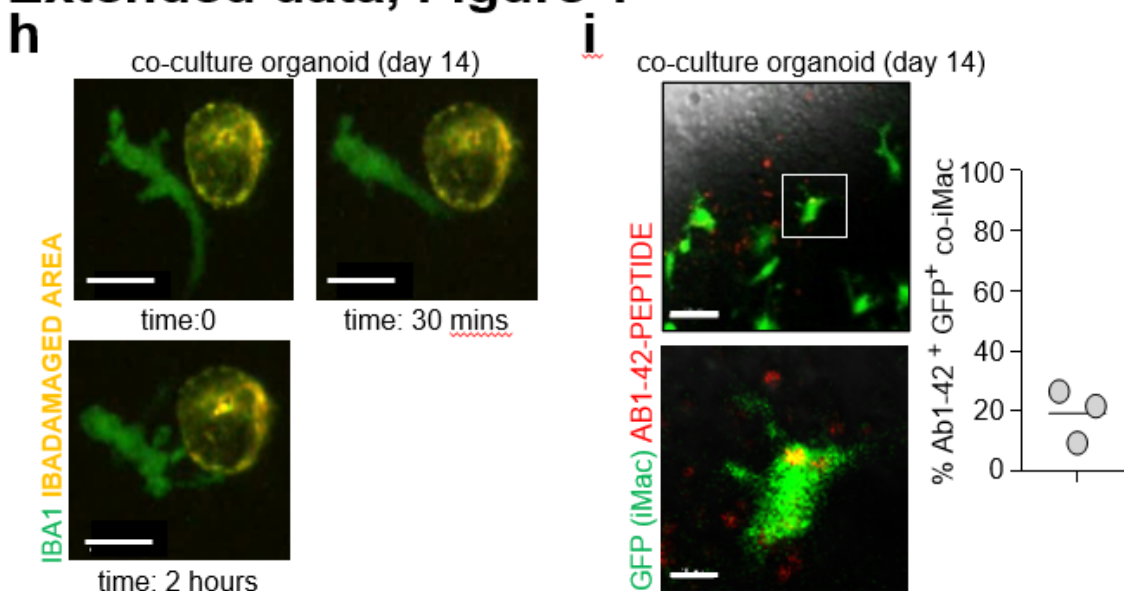


To conclude that iMicro, but not iMac, resembles human microglia functional assays as well as morphological reconstruction are needed (not just transcriptomic analysis).

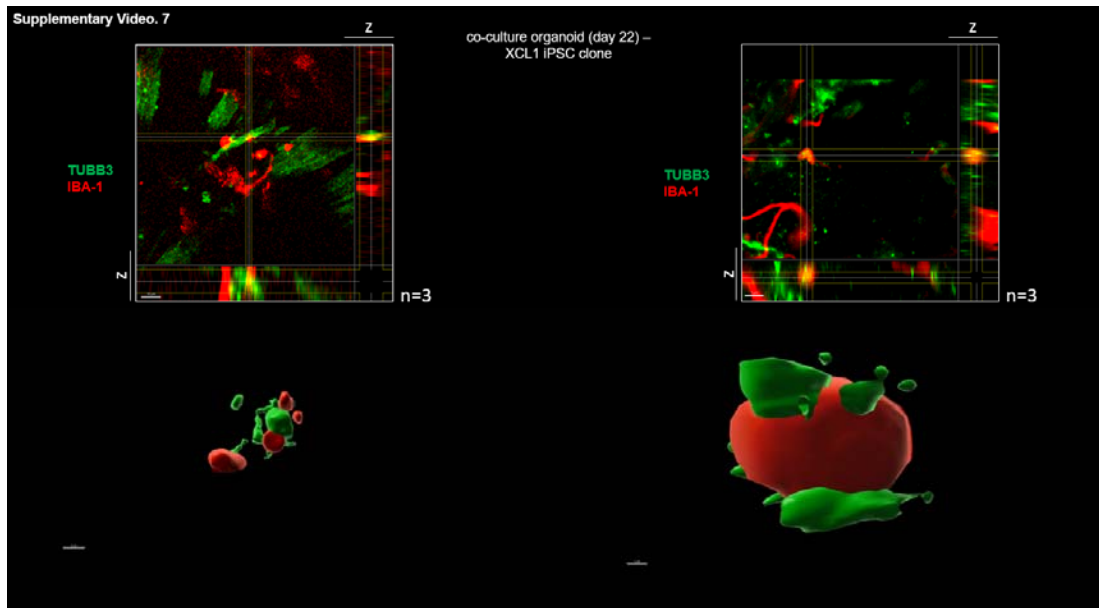
We agree with the reviewer and we have gone beyond the single transcriptomic analysis to show that iMicro resemble human microglia. Indeed, we validated our transcriptomic analysis using proteomic analysis by mass-spectrometry, flow cytometry and confocal/immunofluorescence staining associated with 3D rendering when specified. We indeed show that IBA1⁺ iMicro presenting an amoebic morphology (Fig. 1c, Extended Data, Fig. 1e, Supplementary Video. 7, 8) are present at the surface of organoids and penetrate the organoid where they interact with SOX2⁺ and NESTIN⁺ neuronal cells (Fig. 1b, c; Extended Data, Fig. 1d, e) and surround neural rosettes enriched in SOX2⁺ NPC (Extended Data, Fig. 8c). These iMicro express canonical markers of microglia as demonstrated by flow cytometry analysis for P3RY12 and CX3CR1 (Fig. 1e).

Functionally, we performed several assays commonly used to functionally describe microglia, that were moved to supplementary data such as laser injury model (Extended Data, Fig. 1h and Supplementary Video. 2), and phagocytosis assay with uptake of Amyloid beta peptides (Extended Data, Fig. 1i and Supplementary Video. 3).

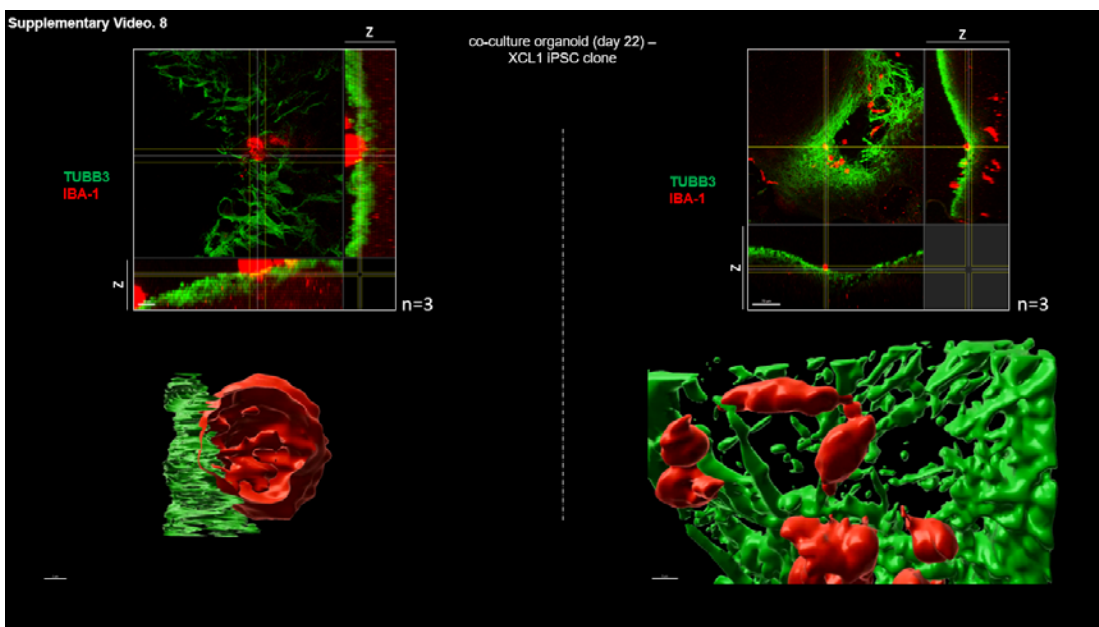
Extended data, Figure 1



We have also considerably expanded the data on the phagocytosis of co-neurons/co- NPC which represents an integrant part played by microglia during the neurogenesis process and as suggested by Reviewer#2. Using 3D reconstitution, we confirmed that iMicro were in close contact with neuronal cells (n=6, Supplementary Video. 7) thus confirming previous observation showing that iMicro present an amoebic morphology and interact with dividing SOX2⁺ NPC (Fig. 2l, m) and TUJ1⁺ neurons (Fig. 2n) .

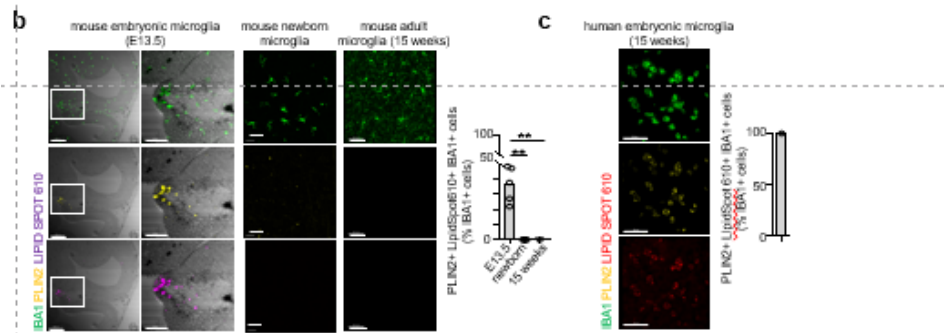


We also confirmed the ability of iMicro to engulf neuronal cells we observed using immunofluorescence (Expanded data, Fig. 5k) and mass spectrometry analysis (Extended data, Fig. 5l). Indeed, by 3D reconstitution (n=6, Supplementary Video. 8), we clearly see traces of TUBB3⁺ neurons within IBA1⁺ iMicro presenting an amoeboid morphology.

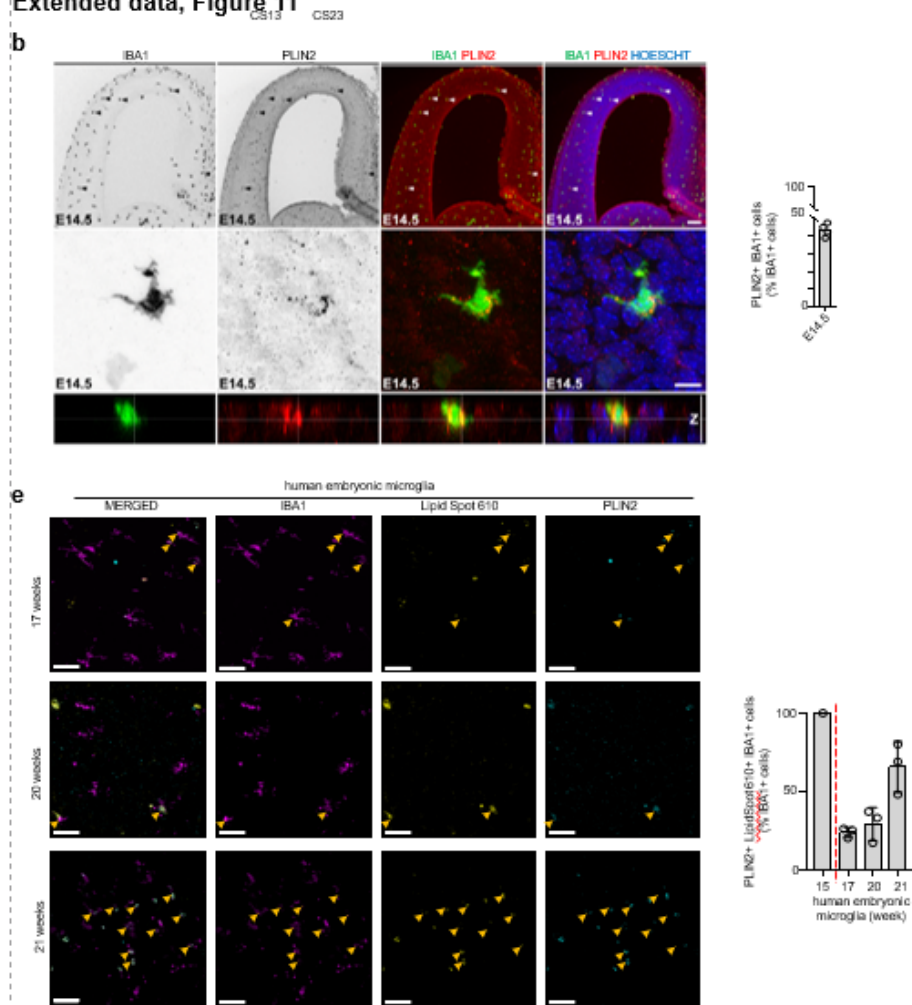


We finally demonstrated a mechanism by which iMicro promote the maturation of neurons associated with size reduction of our organoids upon co-culture based on the presence of PLIN2⁺LipidSpot610⁺ Lipid droplets and their ability to provide co-NPC with cholesterol and its esters via Lipoprotein like Particles resembling HDL (Fig. 3, Extended Data, Fig. 9, 10). **More importantly, we validated these observations as we identified fetal IBA1⁺PLIN2⁺LipidSpot610⁺ microglia in fetal murine and human brain, but not in newborn and adult microglia by immunofluorescence and immunohistochemistry (Fig. 4b, c and Extended Data, Fig. 11b, e).**

Figure 4



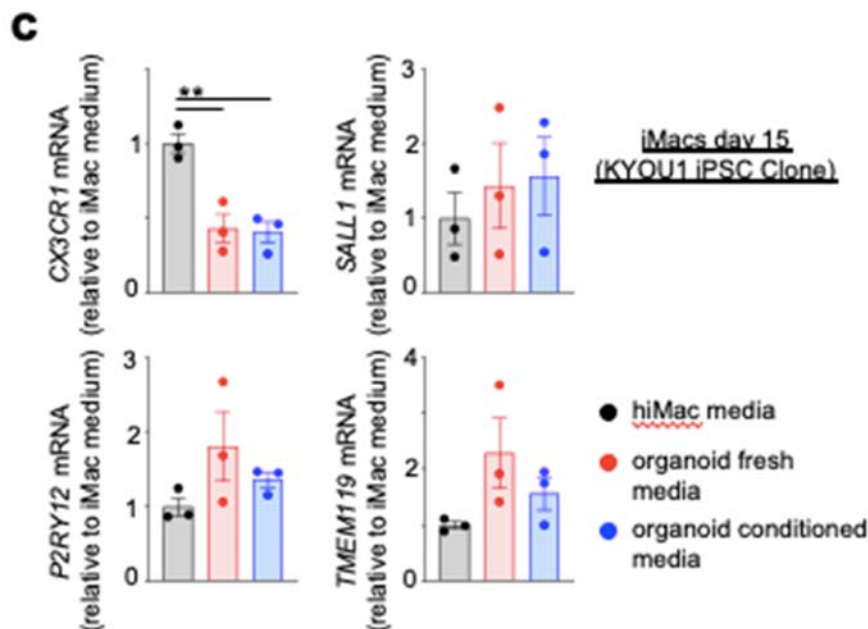
Extended data, Figure 11



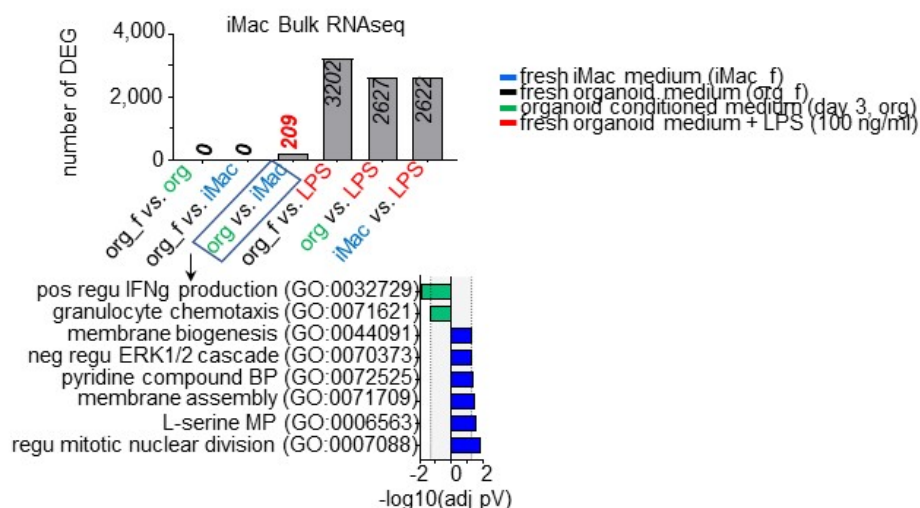
Altogether, these data confirm that iMac added to brain organoids (co-culture organoids) differentiate into iMicro resembling microglia phenotypically, morphologically and functionally, while we confirm our observations in murine and human fetal microglia.

Also, for Ext Fig 1b: day 3 may be too early to conclude that the organoid medium has no effect on the iMac differentiation. qPCR analysis of microglia markers (like the ones used in Fig 1d: P2RY12, TMEM119, CX3CR1, SALL1) at day 15 and day 30 is necessary.

We thank the reviewer for this comment and have provided a qPCR analysis of microglia markers (P2RY12, TMEM119, CX3CR1, SALL1) at day 15 (n=3, Extended Data Fig. 1c). We observed no significant changes in the expression of *SALL1*, *P2RY12* or *TMEM19* while a reduction of *CX3CR1* expression by iMac was observed in iMac cultured for 15 days in organoid fresh medium and organoid conditioned medium.



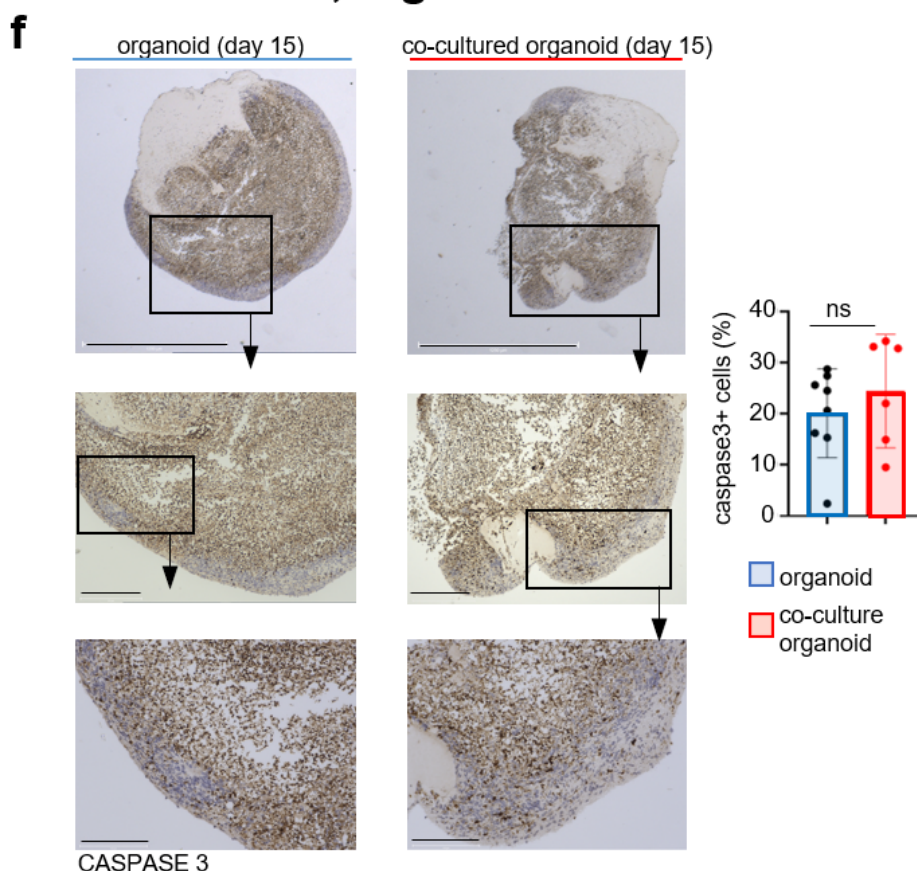
In addition, we performed bulk RNA-seq analysis of iMac cultured with organoid, iMac medium and conditioned medium from organoid and co-culture organoids for 3 days (which we add here at the reviewer's discretion). Our analysis presented below suggests that the culture of iMac in fresh organoid medium and organoid medium does not induce the expression of microglial specific genes, compared to a positive control of iMac stimulated with LPS (model of microglia activation).



The claims about changes in organoid size in the presence of iMac are insufficiently supported by the data. For instance, Ext Fig. 6f only shows a trend for increased CAS3 in co-cultured organoids.

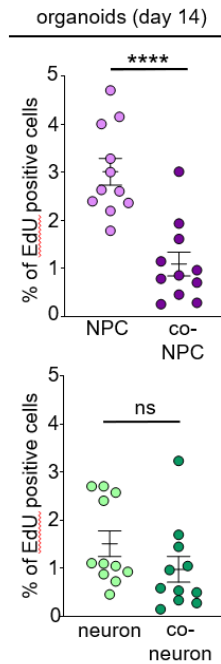
On this point, we want to clarify that while there seemed to be a trend, it is not statistically different. We refer to earlier question on statistical analysis also raised by the same reviewer in this **Referee's comments**. We added "ns" to this graph standing for "not significant". **We also have quantified more slides/areas (n=6) and provide updated quantifications (see below) confirming no statistically different counts.**

Extended data, Figure 6



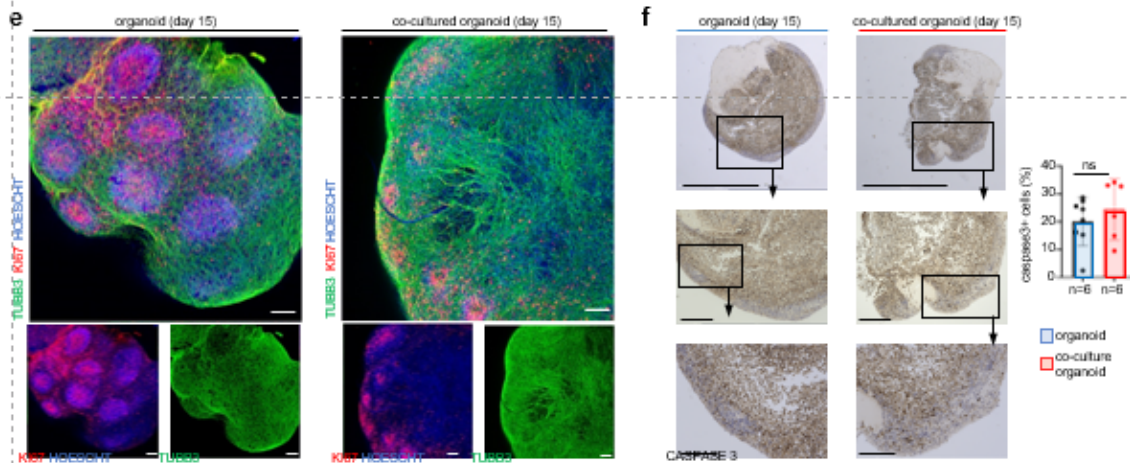
We want to clarify that such data were not used to support our claim on reduction in organoid size. Rather, this led us to conclude that the reduction in the size of brain organoids upon the addition of iMac was preferentially the results of reduced proliferation associated with increased differentiation of co-NPC to co-neurons; rather than an increase in neuronal cell death. Indeed, we confirmed this initial finding observed from the analysis of our transcriptomic data (Fig. 2c, d, e and Extended data, Fig. 6a, b, c, d) by EdU staining using flow cytometry (Fig. 2f).

Figure 2
f

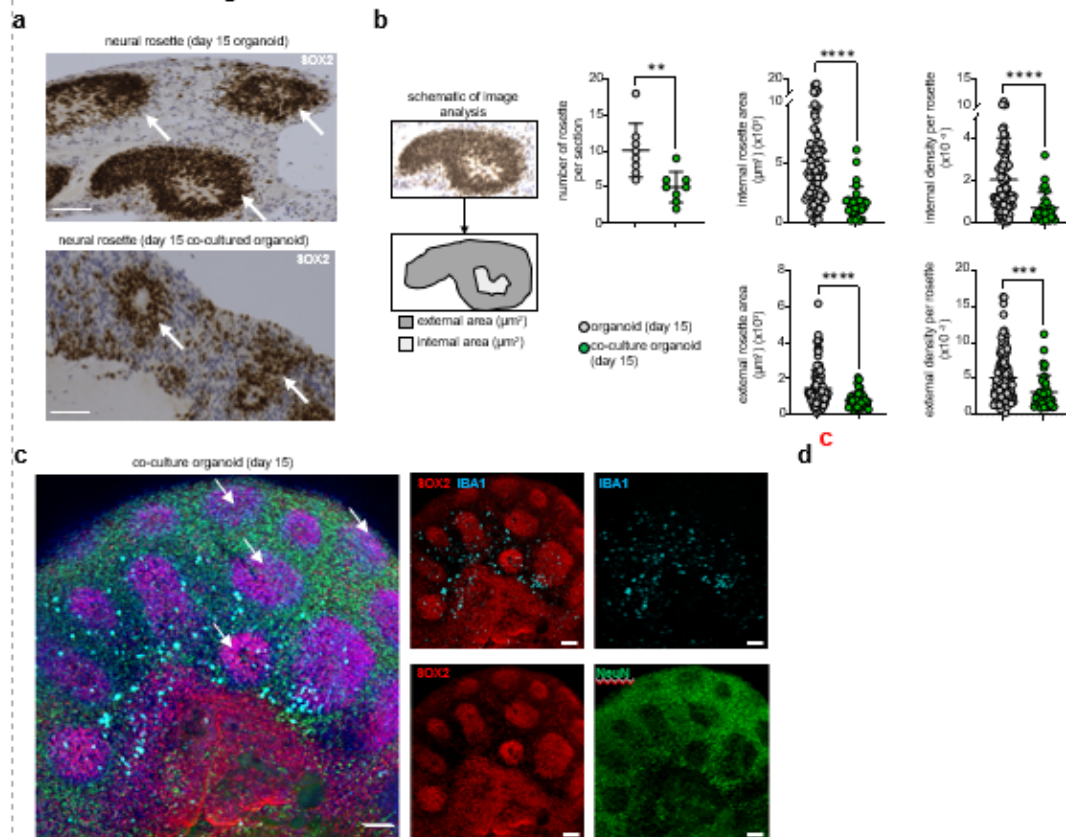


This finding was further supported by our staining for Ki67 showing a significant reduction in the expression of Ki67 in co-culture organoids compared to organoids alone (Extended Data, Fig. 6e). We could further confirm this observation as co-culture organoids presented less, small and more disorganised neural rosettes enriched in NPC compared to organoids alone (Extended Data, Fig. 8a, b, c, d).

Extended data, Figure 6



Extended data, Figure 8



Altogether, these data strongly support our claim that the changes in organoid size in the presence of iMac is the result of reduced proliferative activity of NPC alongside their maturation into neurons, rather than an increase in neuronal death.

Critically, less than half (3/7) of the iPSC lines showed organoid size reduction; therefore, the robustness of iMicro induction protocol remains unclear. As mentioned above, samples sizes (lines, independent differentiations, organoids) are small and it's unclear what the power would be to detect such an effect.

As the other referees agree and especially Referee#2: "The authors have now included additional sources of iPSC and interestingly shown that some innately generate microglia, and these have smaller organoids, which further supports the hypothesis".

To better support this, we added some data in the current manuscript, data which we previously showed in the last Response to Reviewer for their consideration (ie. Data using the XCL-1 iPSC clone as well as iPSC clones derived from 4 patients).

As such, in this manuscript, we have used 11 iPSC lines in total among which 7 from commercial sources and 4 which were generated in the lab. We show here that the addition of iMac induces a reduction in size of brain organoids in 8 out of the 11 iPSC clones used, namely **KYOU1** (our reference iPSC clone throughout this manuscript, Fig. 2a, Fig. 3j, r and Extended Data, Fig. 5a, b), **XCL-1** (Extended Data, Fig. 5c, Extended data, Fig. 9f and Extended Data, Fig. 10h), **HD33i** and **IMR-90** (Extended Data, Fig. 5d) and **four iPSC clones** generated in the lab from paediatric brain cancer patients (Extended Data, Fig. 5h).

We also showed that the presence of innate microglia in organoids generated from **CAU** and **CTRL** iPSC clones was associated with smaller organoids from about 10-15 days of co-culture respectively (Extended Data, Fig. 5g).

With regards to samples sizes, we clearly specified that unless otherwise stated, the KYOU1 iPSC clone was used throughout the study. We have also extensively revised the manuscript and the figures, adding information on the iPSC clone used, the number of organoids/repeats performed, the day from the start of co-culture the experiment was performed, as well as statistical test used.

We want to highlight that other manuscripts on brain organoids including the two seminal ones by Lancaster *et al.*, in Nature (Reference 7, 3 ESC and iPSC) and Nature Protocol (Reference 8) used only H9 human ES cells, SC101A-1 iPS cells and 1 patient derived iPS line in the former study to describe their findings. (Lancaster, M.A., et al., *Cerebral organoids model human brain development and microcephaly*. Nature, 2013. **501**(7467): p. 373-9; Lancaster, M.A. and J.A. Knoblich, *Generation of cerebral organoids from human pluripotent stem cells*. Nat Protoc, 2014. **9**(10): p. 2329-40).

The claims about maturation in brain organoids require more data and controls: changes in cell identify, soma size, spine density, neurite complexity, electrophysiological parameters (sEPSC frequency and amplitude, resting membrane potential, and max firing rate) and transcriptomic (by mapping onto available datasets) are key to making some definitive claims.

The claims about maturation was initially based on transcriptomic (Fig. 2c, d, e; and Extended Data, Fig. 6a, b) and proteomic data (Fig. 2g, h; and Extended Data, Fig. 5c, d) showing an upregulation of pathways associated with axon development and neurogenesis in the presence of iMicro and was ultimately supported by electrophysiological experiments (Fig. 1j-m). **On this point raised by the reviewer, we want to clarify that some electrophysiological parameters including the resting membrane potential, action potential threshold were already included in the revised version of the manuscript (Fig. 1m).** The firing pattern was also added in the revised version (Fig. 1l) and we added the raw data including the maximum firing rate as a supplementary table (Supplementary Table. 3). Both datasets support more mature neurons cocultured with iMac compared to organoid alone.

Regarding additional parameters including those mentioned by the reviewer, we can split neuronal development in two distinct stages involving (1) cell differentiation and maturation of general neuronal

properties and followed by (2) later stage synaptogenesis, synaptic maturation, and neuronal network formation.

During the first stage, we found no strong differences in soma-size-increase linked with cell maturation (Marchetto et al., 2010; Wegiel et al., 2014). Indeed, we took advantage of the fact that a cell's capacitance is directly proportional to its size (Koch & Poggio, 1985), and found no significant differences between the capacitance of neurons from co-culture organoids and those from control organoids (Control: 23.7 ± 1.93 pF, iMicro-cocultured: 23.6 ± 2.01 pF, Mann-Whitney U test $p = 0.899$). Nonetheless, these capacitance values don't perfectly reflect neuronal surface areas covered by neuronal branches (axons and dendrites) due to spatial clamping limitation (Bar-Yehuda & Korngreen, 2008). Transcriptomic and proteomic changes observed between the control and co-culture organoids revealed an increase in *GAP43* and *HEFM* gene expression, which are involved in axonal development and neurogenesis (Fig. 2c, d, e and Extended Data Fig. 6a, b), along with a heightened level of neuronal axon filament protein PRPH expression, and significantly longer neurites in co-NPC than NPC (Fig. 2e, g and h). Hence, iMicro likely impacts the early maturation of NPC or neurogenesis.

Furthermore, neurons in co-culture organoids present better functional outcomes, suggesting improved neuronal maturation. Specifically, the resting membrane potential of iMicro-cocultured neurons is more negative (Fig. 1m; Control: -64.8 ± 2.01 mV, Coculture: -68.1 ± 1.06 mV, Mann-Whitney U test $p = 0.034$), the action potential threshold is lower (Fig. 1m; Control: -18.5 ± 2.24 mV, Coculture: -26.7 ± 1.65 mV, Mann-Whitney test) showing any form of action potential firing (immature, mature single or mature train firing).

In addition, an increase and strengthened action potential firing dynamics is another correlate of age-dependent neuronal behaviour (Nikitin et al., 2017). An examination of the maximum dV/dt of the first three action potentials in a continuous mature action potential train showed a trend of increase in the first action potential (Control: 19.5 ± 5.49 mV/ms, Coculture: 35.2 ± 5.68 mV/ms, Mann-Whitney U test $p = 0.079$), and a significant increase in the second (Control: 5.93 ± 0.65 mV/ms, Coculture: 14.7 ± 2.76 mV/ms, Student t test $p = 0.020$) and third action potential (Control: 3.66 ± 0.53 mV/ms, Coculture: 9.40 ± 1.76 mV/ms, Student t test $p = 0.019$) maximum amplitude change. Taken together, these observations are not only reminiscent of central nervous system neurons that exhibit progressive hyperpolarisation (Perez-García et al., 2021) and a decrease in threshold to trigger action potential firing and trains, but also of an increase in action potential firing behaviour in iPSC-derived human neurons associated with their maturation (Fink et al., 2017).

Alternatively, we could compare the complexity of neuronal arborization (increase branching with time), spine density, or synaptic function (increase synaptic strength and frequency of events with maturation) to examine maturation variations and differences. Although there have been previous reports on axonal projection and synapse formation (Birey et al., 2017; Xiang et al., 2019), we believe that the development of synapses in human brain organoids is limited and not applicable for our study. Human brain synaptogenesis follows a precise, spatiotemporally controlled process involving morphogen-induced cell migration, cellular projection, contact-induced synapse generation, and pruning (Faust et al., 2021; Paolicelli et al., 2011; Shen & Cowan, 2010). However, current organoid generation protocols do not provide the quintessential morphogen gradients and structural axis required for effective synaptogenesis and microglia-dependent synaptic development (Wang et al., 2021). Hence, human organoids currently lack the natural programmed capacity for synaptogenesis, resulting in promiscuous synapse generation in organoids of all ages. Also, due to the vast disagreement on the timing of *in vivo* organogenesis and organoid culture (Hofer & Lutolf, 2021), organoid models do not recapitulate satisfactorily later stage neuronal changes. The high batch-to-batch variability of morphological and synaptic parameters further renders their analysis unreliable at immature stages.

Since neurogenesis and the maturation of intrinsic neuronal functional capabilities precedes synaptogenesis and synaptic function, and our electrophysiological data presented an increased general intrinsic neuronal property, we argue that there is an increase in the initial maturation of neurons in the presence of iMac/iMicro. However, alternate models will be required to examine the changes in neuronal communication and connectivity, if any, more commonly associated with macrophage or microglia function (Paolicelli et al., 2011).

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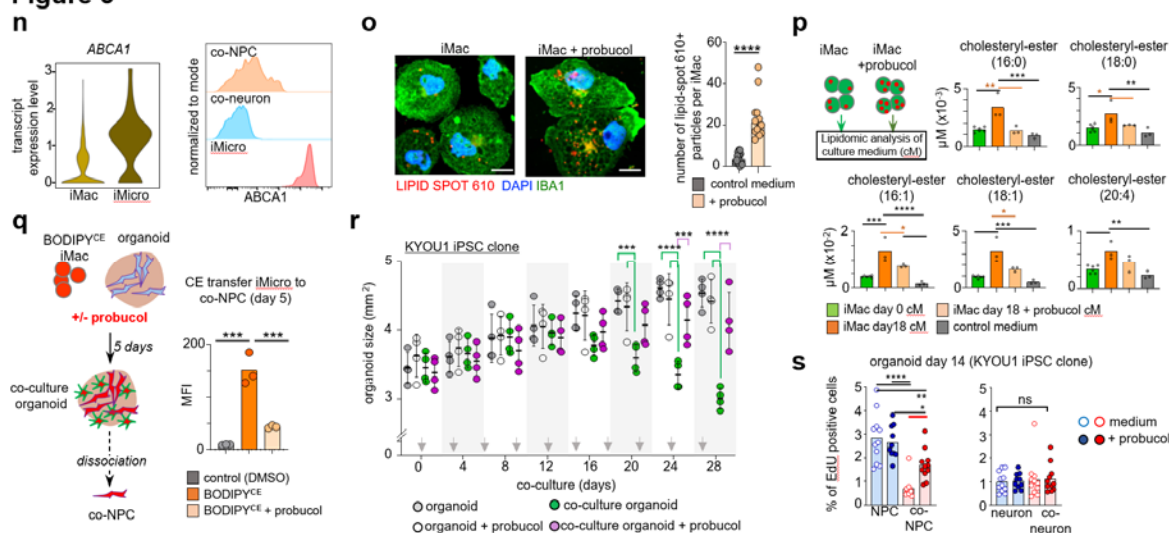
Wegiel, J., Flory, M., Kuchna, I., Nowicki, K., Ma, S. Y., Imaki, H., Wegiel, J., Cohen, I. L., London, E., Brown, W. T., & Wisniewski, T. (2014). Brain-region-specific alterations of the trajectories of neuronal volume growth throughout the lifespan in autism. *Acta Neuropathologica Communications*, 2, 28. <https://doi.org/10.1186/2051-5960-2-28>

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The conclusions about cholesterol transport actually are still not supported experimentally.

We respectfully disagree with the reviewer's comment which is in contrast with the comments from Referee#3 in the first review that considerably led us to improve this manuscript. Referee#3 is satisfied with our corrections; while we will be able to answer the few questions from Reviewer 2 which will ultimately greatly improve the manuscript. We particularly show that blockade of the ABCA1 transporter expressed by iMac / iMicro (Fig. 3n, Extended Data, Fig. 10f) leads to an increase in the amount of lipid droplets (Fig. 3o) and the ensuing reduced export of cholesterol in APOE⁺ lipoprotein like particles resembling HDL (Fig. 3p and Extended data, Fig. 10g). Blockade of ABCA1 by probucol abrogated the uptake of cholesterol by co-NPC from iMicro (Fig. 3q and Extended data, Fig. 10d, e), prevented their decrease in proliferative activity (Fig. 3s); observation associated with the absence of size reduction in probucol-treated co-culture organoids (Fig. 3r, Extended Data, Fig. 10h).

Figure 3



We ultimately show using two iPSC lines (XCL-1 and KYOU1), that the cholesterol provided by iMicro is delivered to co-NPC and is associated with increased neurogenesis and decreased size of co-culture brain organoids (Fig. 3r and Extended Data, Fig. 10h). All the data on size reduction induced by iMicro using XCL-1 iPSC line are now added in the main manuscript (Extended Data, Fig. 5c; Extended Data, Fig. 9f and Extended data, Fig. 10h).

The analysis reveals to 5 different regulons; the authors claim these are related to microglial identity. However, these TFs are promiscuous and further testing of their specific involvement is needed.

While this is an interesting point, we believe that this is out of scope of the paper. These TFs are recognized as signature of microglia identity in the literature; and we further provide staining analysis by immunofluorescence and flow cytometry analyses of protein biomarkers (SALL1 for example) specific of microglia.

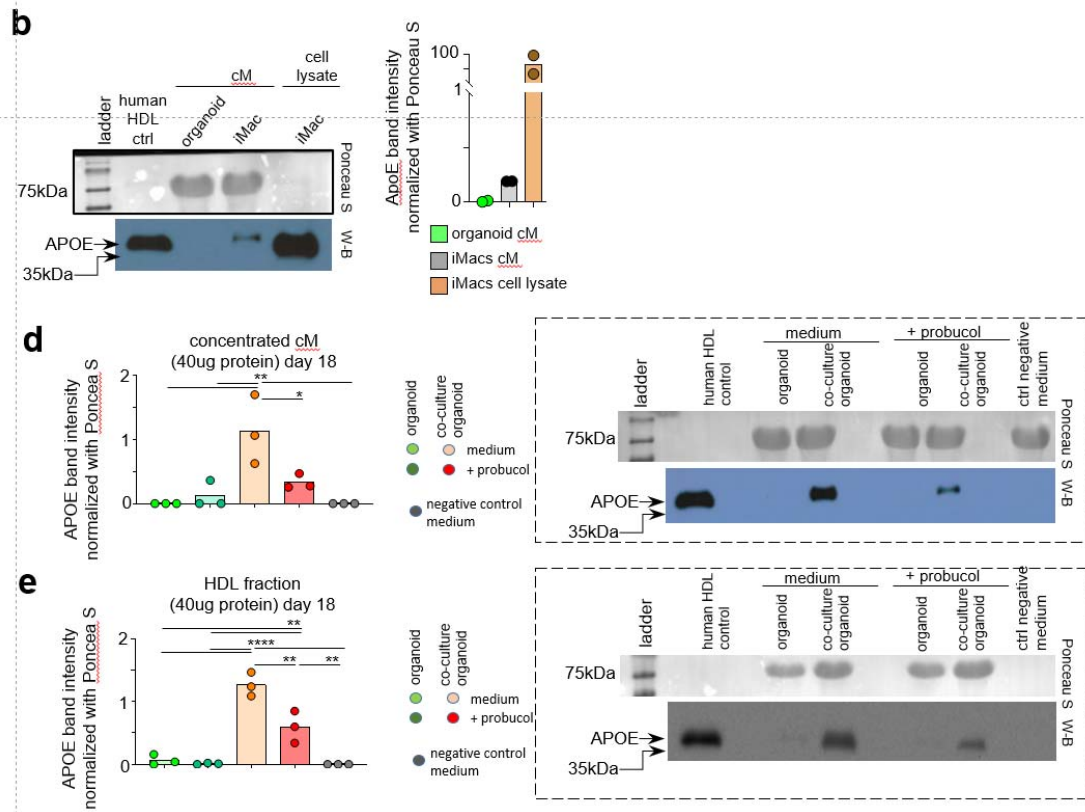
The authors believe that their model can be used to model neurodegenerative conditions such as TREM2+ AD; however, they use early-stage cultures and evidence so far still points to a rather immature state.

We are currently working on this to show the high relevance of our model in the study of neurodegenerative disease. AD has been linked with genetic polymorphism affecting microglia specific genes such as TREM2 (TREM2 R47H). We consider that such polymorphism may affect early neurogenesis process even if clinical manifestation only occurs later in life. Consistently, our preliminary data using TREM2 R47H iMac added in WT organoids show that mutated iMicro affects NPC gene expression. **However, we can remove this point of discussion if needed.**

Supplementary Fig. 8h lacks a loading control for total protein as well as a quantification.

We fully agree with the reviewer and we provided loading control as well as an extra-analysis and quantification for both APOE in iMac, the various culture supernatants and the HDL lipoprotein like particle fraction where the APOE signal was detected. Data were quantified to the protein signal on PonceauS staining equivalent to 40ug protein loaded per lane (Extended Data, Fig. 10 b, d, e). Corresponding figure, legends, methodology and text were amended accordingly.

Extended data, Figure 10



It's unclear how candidates were selected in Figure 2g with the mass spec experiment.

We agree with the reviewer that this information was missing and we have clarified this point in the method section as well as clearly specify the bioinformatic script used in each section.

Referee #2 (Remarks to the Author):

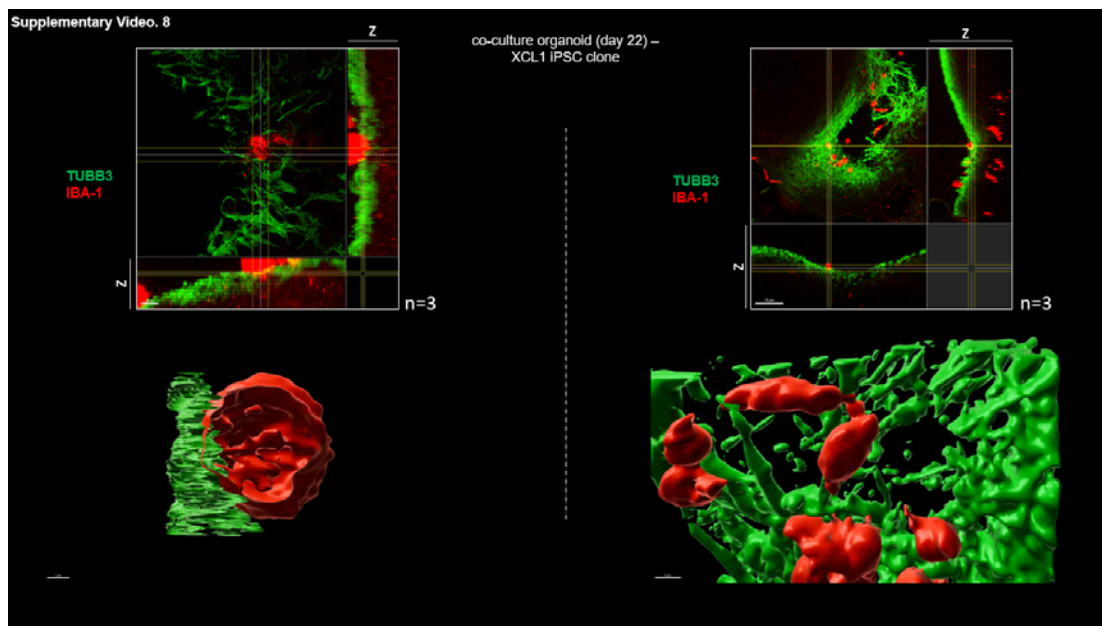
In this revision, the authors put forward the novel hypothesis that in human developing brain, microglia limit neural progenitor cell proliferation thereby promoting neuronal maturation and preventing over growth, using a new combination of iPSC derived organoids and microglia-like cells from the same donor. The authors have performed a **significant amount** of new experiments to address my issues, including further characterizing the cell responses in the organoids, and functionally intervening with cholesterol export to support their hypothesis that iMicro lipid transfer influences NPC responses. **Furthermore, the added characterization of electrophysiological properties of the organoids in co-culture, along with comparison to human 'in vivo' microglia responses, has also strengthened the paper. The paper is well written and easy to follow. The methodology is robust and valid.** The authors have now included additional sources of iPSC and interestingly shown that some innately generate microglia, and these have smaller organoids, which further supports the hypothesis.

With regards to the authors' comments and new data, I have the following comments:

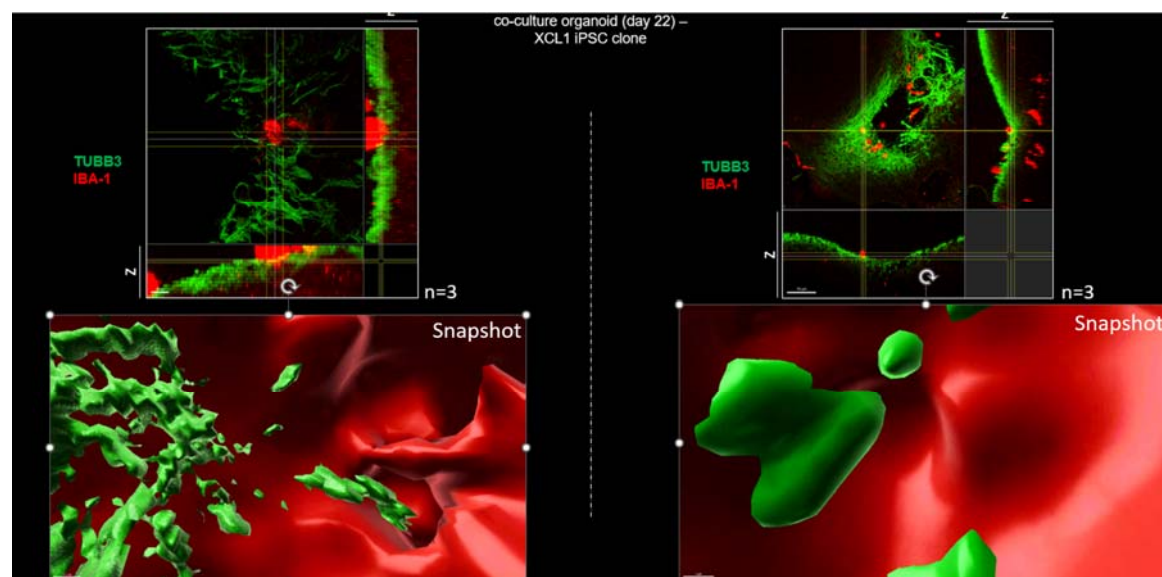
1. I appreciate the authors having characterized the NPC responses in the organoid following iMac transplant, discovering the impact on proliferation rather than death, and looking at iMicro phagocytosis of NPCs. With regards to the latter, the co-localization of IBA1 with TUJ1 for a readout of phagocytosis

would be greatly strengthened by a 3D rendering to show that the NPC components are inside the iMicro, as this is a standard approach when investigating microglia phagocytosis. Otherwise this could represent processes from either cell type on top of each other. I see that the authors have used the Imaris software in this study to address another question, and this software is most commonly used to do this.

We thank the reviewer for this comment and have performed supplementary analysis to clarify this point. By 3D rendering using the Imaris imaging software, we could demonstrate that the neuronal components were inside iMicro (Supplementary Video. 8). This confirms our initial findings on the phagocytosis of neuronal cells by iMicro in co-culture organoids by immunofluorescence and mass-spectrometry analysis (Extended Data, Fig. 5k, l respectively).



For convenience, we have attached a specific snapshot for each of these 3D rendering videos zoomed from within iMicro below.



These snapshots from 3D rendering clearly show that neuronal components (TUJ1/TUBB3) are inside iMicro, thus supporting the notion that iMicro phagocytose NPC.

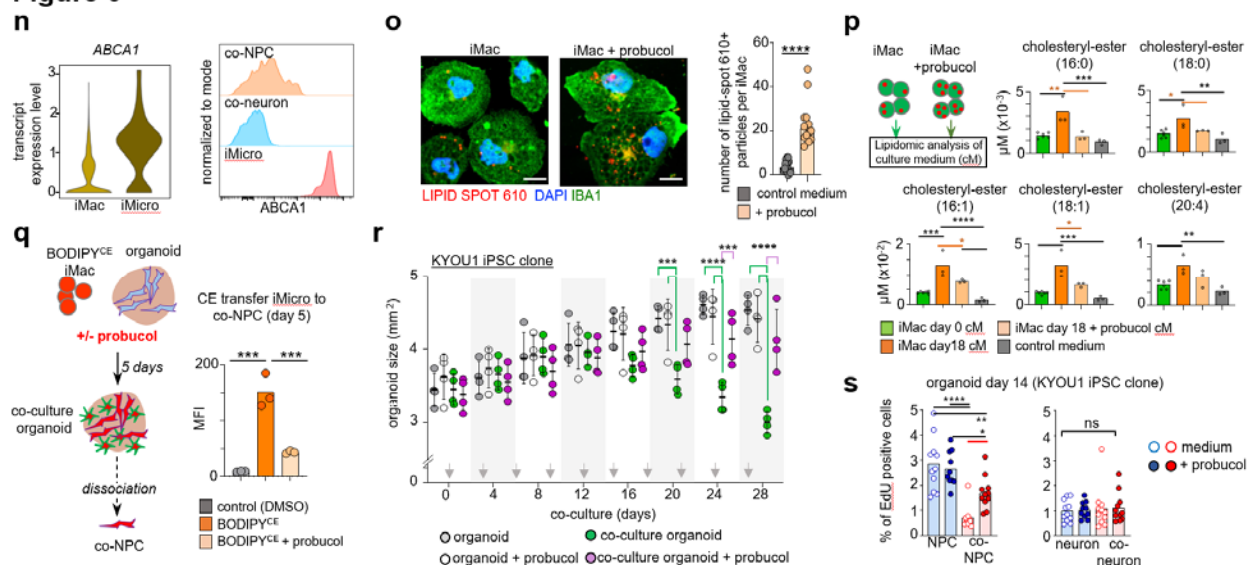
2. I understand the authors' comment that understanding the mechanism by which cholesterol influences the NPCs could be beyond the scope of this study. It would be useful to have clarification however on why further assessment of BODIPY-CE transferred from iMicro to co-NPCs is considered by the authors as being beyond the scope of this study. Showing that BODIPY-CE+ NPCs have reduced proliferation may be straightforward given that the authors showed BODIPY-CE transfer to NPCs, and assessed proliferation/maturation. This would greatly strengthen the hypothesis that the cholesterol transfer regulates NPC proliferation/maturation.

We thank the reviewer for this comment which is a question we still intend to address as part of other functional studies for follow-up studies. However, we were limited here by the low number of BODIPY-CE+ as well as that the EdU staining protocol which affected BODIPY fluorescence. We have rather focussed our attention on the critical point below.

3. The probucol experiment is very important in showing that blocking ABCA1 eliminates the impact of iMac transplant into the organoids. Showing that this is associated with a reduction in BODIPY-CE uptake by NPCs, and an impact on NPC proliferation, would tie together the experiments very nicely and strengthen the central hypothesis of the paper.

We fully agree with this comment which we addressed and strengthen the paper. We previously showed that the inhibition of ABCA1 using probucol lead to an accumulation of cholesterol and its esters in iMac and a reduction of their release in the supernatant in lipoprotein like particles (LPLs) resembling HDLs (Fig. 3n, o, p and Extended data, Fig. 10b, f, g). **We performed additional experiments to show that the blocking of ABCA1 in organoid co-culture strongly reduced the levels of ApoE within the purified HDL fraction of culture supernatant (n=3, Extended data, Fig. 10d, e) and subsequently the levels of BODIPY-CE taken up by NPC in co-culture organoids (n=3, Fig. 3q). We could finally associate the ability of probucol to abrogate the reduction of size we observed in co-culture organoids (Fig. 3r and Extended data, Fig. 10h) with a significantly diminished impact on the proliferative activity of co-NPC as shown by a higher EdU incorporation (Fig. 3s).**

Figure 3



Altogether, these results strongly support the main aim of the paper mechanistically as we show that the size-reduction in co-culture organoids is mediated in part by the incorporation of cholesterol and its esters produced by iMac/iMicro into LPL resembling HDL for their subsequent uptake by co-NPC leading to their maturation and reduced proliferation.

Minor issues:

1. I would recommend not saying that co-NPCs ‘express’ high levels of APOE at the protein level, but rather say it was detected, as they are not likely making it themselves so the term express is not appropriate.

We agree with the reviewer especially as this is clearly demonstrated in Extended data, Fig. 10c. We amended the text to “especially as high levels of APOE proteins were detected in co-NPC not expressing its transcript (Extended Data Fig. 10c).” (Line 352-354).

2. The summary mentions that NPC acquired exported cholesterol which participated in metabolic reprogramming that accompanied NPC differentiation. I think this wording is a bit strong considering that functional validation of metabolic reprogramming in the NPCs would be needed to make this statement, so I suggest softening this.

We agree with the Reviewer’s comment and have softened this statement: “Mechanistically, iMicro contained high levels of PLIN2+ lipid droplets that exported cholesterol and its esters which were taken up by NPC in the organoids.” (Line 68-70)

3. I appreciate that the authors have opted for functional validation of NPC proliferation and iMicro cholesterol export, over expression validation of specific genes. There are still however quite a lot of graphs highlighting changes in particular genes that haven’t been validated using another method – this is fine but it would be useful if in the text, at least where a list of specific genes are highlighted as being of particular interest, that we would have an acknowledgement the importance of validation for future work.

We thank the reviewer for this comment and rather than a list of genes, we have provided statements inviting for further research which we are currently being undertaken in our labs such as: “Although the mechanism is still elusive and under study, the transported cholesterol and its esters would then be utilized

by neuronal cells and be involved in the improved neurogenesis we observed in the presence of iMicro.”
(line 451-453)

Referee #3 (Remarks to the Author):

I congratulate the authors for their extensive revision of this manuscript. The authors have addressed all my concerns.

We thank the reviewer for his/her positive evaluation of the manuscript.

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

I appreciate the efforts made by the authors to improve the manuscript. However, the new data and interpretations is not addressing the issues raised. The main claims of the manuscript are not supported by the data. There is not enough transparency on what data was used and what levels of variability it covers. For instance, in looking at Ext. Fig. 5: the data is “representative of at least 2 separate experiments”; why is not all the data shown? Exd Fig 5h shows relative values while Exd Fig 5b,c,d showed absolute value for the same experimental design. For the new patch clamp recordings, how many organoids are used? Why is the patching now performed at another time point? The quality of the PLIN2 staining is problematic. Why PLIN2+ cells are aggregated in Fig4b, but PLIN2+ cells are widespread in Ext Fig 11?

Functional data to demonstrate that iMicro resemble human microglia more than iMac is lacking. This is a central point of the manuscript, and yet often this comparison is missing. The utility of the experimental approach proposed here is not demonstrated.

Referee #2 (Remarks to the Author):

In this 2nd revision, the authors put forward their novel hypothesis that in human developing brain, microglia limit neural progenitor cell proliferation thereby promoting neuronal maturation and preventing over growth, using a new combination of iPSC derived organoids and microglia-like cells from the same donor.

The authors have addressed my previous concerns by providing new analyses and experiments. For instance, they have provided videos and 3D views to convincingly demonstrate neuronal traces within microglia. They have also shown that inhibition of the cholesterol exporter ABCA1 using a drug leads to reduced cholesterol export to NPCs, and influences organoid size and NPC proliferation. All minor concerns have also been addressed with editorial changes.

Referee #3 (Remarks to the Author):

It is my opinion that the authors have made an outstanding effort at addressing the remaining comments raised by the Reviewers. The use of additional iPSC lines, and orthogonal validation of iMicro by single-cell and bulk transcriptomics, mass spectrometry, flow cytometry, IF and functional assays strengthen the reported findings. In addition, ABCA1 blockade experiments support the role of cholesterol uptake in co-NPC proliferation and size reduction in co-culture organoids. The manuscript now reads as a clear and well-written story, which highlights the relevance of these studies as well as future research directions.

Author Rebuttals to Second Revision:

We thank again the three reviewers for their careful consideration of the manuscript and their latest critical and encouraging comments. In this latest version of the manuscript, we believe that we add further evidence strengthening our claim with regards to the impact of the addition of primitive like macrophages (iMac) on 3-dimensional (3D) brain organoids from the same source of iPSC, as well as the mechanism driving this observation. We also add further evidence that iMac in our 3D organoids express more microglial markers than in the 2D co-cultures our group previously published (Takata et al, Immunity 2017).

In summary:

- The iMac in our 3D brain-organoids express significant higher levels of microglial markers compared to iMac described in our previously published observation in 2D co-cultures with cortical neurons. This strengthens our message that **the iMac in our 3D co-culture brain-organoid resemble more microglia observed in human and mouse brains.**
- Finally, we have clarified other issues raised by referee #1 (see below).

Referees' comments:

Referee #1 (Remarks to the Author):

I appreciate the efforts made by the authors to improve the manuscript. However, the new data and interpretations is not addressing the issues raised. The main claims of the manuscript are not supported by the data. There is not enough transparency on what data was used and what levels of variability it covers.

We are sincerely surprised by this comment having highlighted both in the final manuscript and the previous responses to Reviewers the data used, the day of co-culture the experiments were done, the number of organoids and independent experiments performed, as well as emphasizing that we consistently reproduced the similar observations constituting the main claim of our study as well as the number of organoids produced for each general observations for each human iPSC clone used (number of cerebral organoids, number of batches, number of co-culture organoids, etc.). For clarity, we have added a **Supplementary Table 4** in the manuscript.

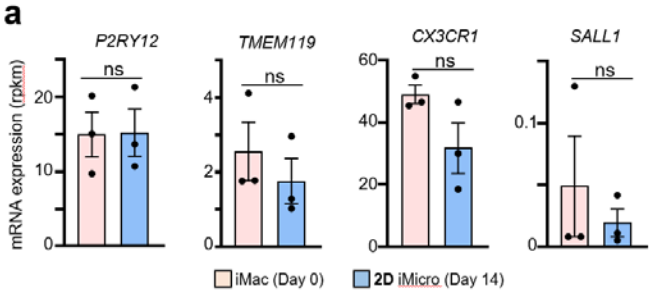
Supplementary Table

Human iPSC line	Cerebral organoids (Total number)	Cerebral organoids (Number of batches)	Presence of innate microglia	Cerebral co-culture organoids (Number of batches)	Organoids with innate microglia cultured in +/- M-CSF (Number)
KYOU1	199	25	No	49	--
XCL-1	101	15	No	27	6
IMR-90	36	6	No	3	--
HD33i	32	5	No	3	--
CTRL	34	6	Yes	--	Yes
CAU	38	7	Yes	--	Yes
Pci-ASI	23	4	Yes	--	--

Note: Cerebral organoids were generated as per Lancaster et al, 2014 with starting 9,000 iPSC per well for each organoid. For co-culture experiments, 150,000 iMacs (day 26) were seeded per well along with one cerebral organoid (Day 26); the culture was maintained for a further 18 days unless stated otherwise

Altogether, and as highlighted by Referee 2 and 3, we provided strong phenotypic, functional and mechanistic evidence using multiple iPSC lines that the addition of iMacs was reducing the size of 3D brain organoids. We extensively showed that our 3D co-cultured iMac (iMicro) closely resembled microglial cells phenotypically and functionally, and to a much stronger extent than their counterpart generated in 2D co-culture with cortical neurons who did not exhibit a significant change in the expression of key microglial genes such as *P2RY12*, *TREM119*, *CX3CR1* and most importantly the key microglial transcription factor *SALL1* (Extended data, Fig. 2a).

Extended data, Figure 2

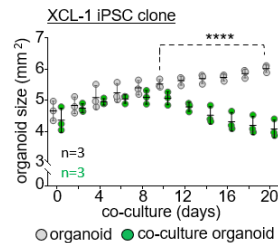


For instance, in looking at **Ext. Fig. 5**: the data is “representative of at least 2 separate experiments”; why is not all the data shown?

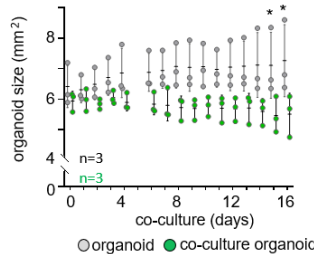
We thank the reviewer for this comment related to **Extended data Fig. 5c**. The other experiments showed a similar response and statistical significance. However, due to variability between sets of data since there is a batch dependent variability (a well-known issue in organoid biology), pooling the 2 datasets together was abrogating the statistical significance observed. Thus, we decided to show the data per batch comparing the different groups for each batch between each other.

Extended data, Figure 5

c

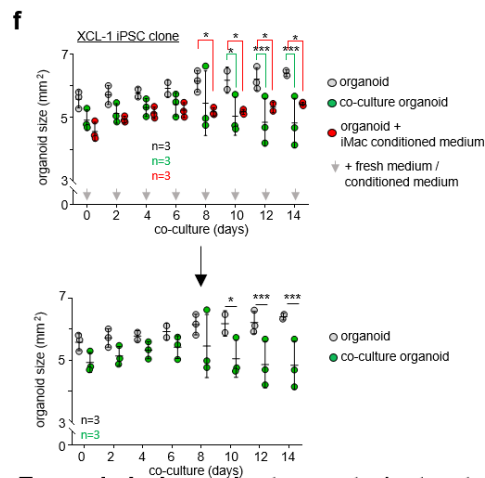


Independent repeat

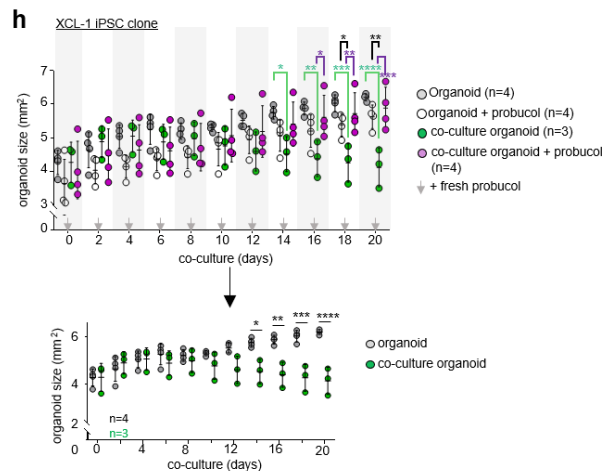


We have also demonstrated similar trend in other independent experiments described in **Extended data, Fig. 9f** and **Extended data, Fig. 10h**. We have extracted the data of interest to this comment ie. Size of organoid and co-culture organoid through time. We have additionally added the extracted data of repeats of each of these two figures for the reviewer’s consideration.

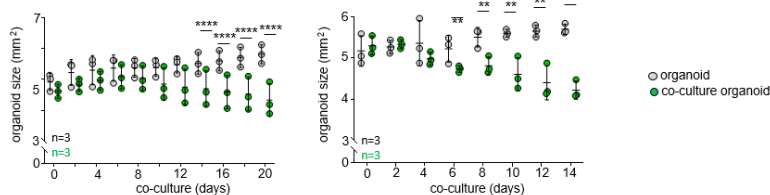
Extended data, Figure 9



Extended data, Figure 10



Example Independent repeats (extracted data of organoid and co-culture organoids)

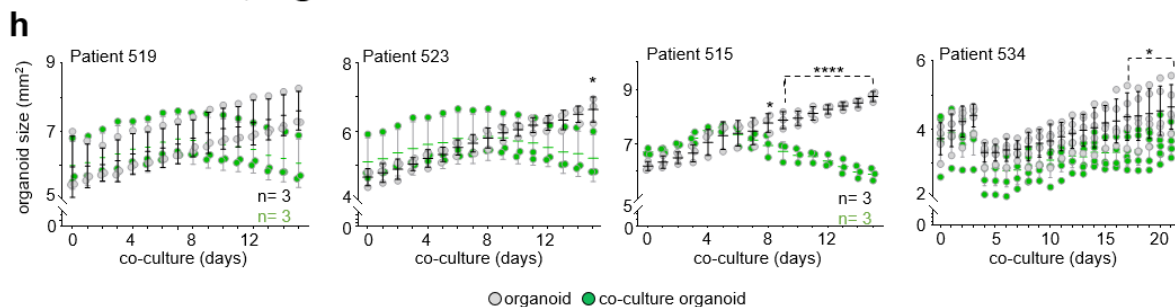


In all cases, we show that the addition of iMac to brain-organoids generated from the XCL-1 iPSC line was leading to a decrease in size. These data generated independently at the Institut Gustave Roussy in Paris and at the Singapore Immunology Network in Singapore, further demonstrate the robustness of our findings.

Exd Fig. 5h shows relative values while **Exd Fig 5b,c,d** showed absolute value for the same experimental design.

The inconsistency noted by the reviewer is due to the fact that the organoids were very heterogeneous in terms of size&shape thus explaining the variability at each timepoints among organoids when using the absolute values. As a consequence and to alleviate this heterogeneity (expected from human patients), we plotted the relative values (ratio versus. Day 0) compared to the absolute value for our organoids derived from patient's iPSC. In an effort of clarity, we have however amended the manuscript to show the absolute value in **Extended data, Fig. 5h**, while we have moved the relative value to **Supplementary Fig. 2**.

Extended data, Figure 5



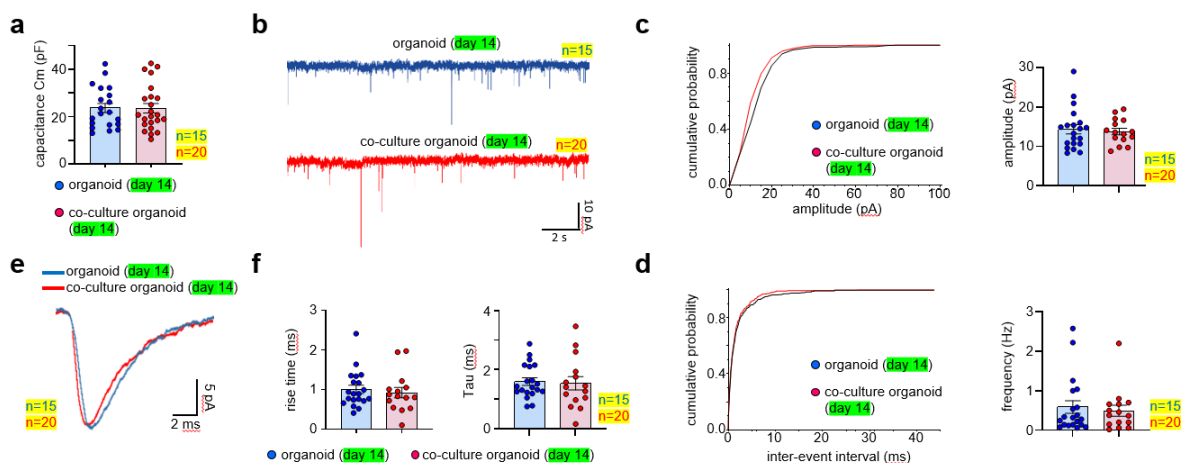
In all cases, we clearly observe that the addition of iMac leads to a reduction of size of our co-cultured organoids over-time which otherwise enlarge with time. The manuscript has been amended accordingly.

For the new patch clamp recordings, how many organoids are used? Why is the patching now performed at another time point?

We want to highlight that the number of organoids was described in **Extended data Fig. 4** with $n=15$ for organoids and $n=20$ for co-culture organoids. For clarity, we however added this information to each panel (number of organoids highlighted in yellow).

We apologize for the time point confusion since the day we consider as day 0 (initiation of co-culture) was modified upon suggestion by two reviewers and not correctly clarified in the previous revision. The second patch clamp (**Extended data Fig. 4**) was performed at day 14 (14 days after the start of co-culture) while the initial patch clamp was performed at day 15 (**Fig. 1j, k, l, m**). We have amended this in **Extended data Fig. 4** (modifications highlighted in green). Therefore, both sets of patch-clamp data are performed at relatively similar time-points.

Extended data, Figure 4



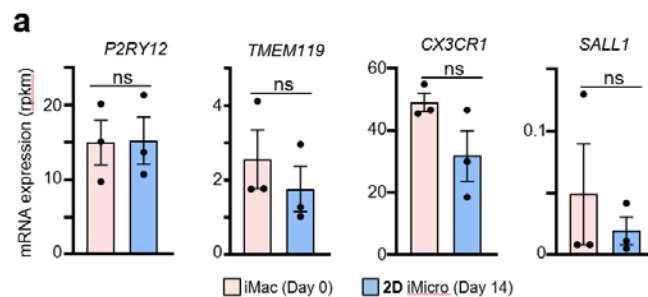
The quality of the PLIN2 staining is problematic. Why PLIN2+ cells are aggregated in **Fig4b**, but PLIN2+ cells are widespread in **Ext Fig 11**?

With regards to the PLIN2 staining, the difference results from the fact that the regions of the brain observed are different and performed in two different laboratories, thus further strengthening our observations. Staining in **Fig. 4b** is showing the *Roof Plate* while staining in **Extended data, Fig. 11d** is showing the *neocortex*, both showing the presence of lipid droplet laden embryonic microglia in two different regions of the brain. The differences in proportions and shape in these two different locations represent interesting observations beyond the scope of this study. We have amended the text accordingly to specify the regions of the brain observed.

Functional data to demonstrate that iMicro resemble human microglia more than iMac is lacking. This is a central point of the manuscript, and yet often this comparison is missing. The utility of the experimental approach proposed here is not demonstrated.

We thank the reviewer for this comment which we addressed using unpublished data. We previously showed that iPSC can generate iMac which differentiate into functional tissue resident macrophage in a 2D co-culture model of cortical neurons and resembling microglia (2D iMicro). Using unpublished RNAseq data of such 2D iMicro (Day 14 of coculture), we show that at the transcriptomic level 2D iMac do not express significantly higher levels of microglial markers (*P2RY12*, *TMEM119*, *CX3CR1* and *SALL1*), now reported in **Extended data, Fig. 2a** in sharp contrast to what was observed in our current model of 3D co-culture brain-organoid (**Fig. 1d**). **This strengthens our message that iMac in our 3D co-culture brain-organoid resemble more microglia observed in human and mouse brains.** The manuscript has been amended accordingly.

Extended data, Figure 2



Takata K, Kozaki T, Lee CZW, et al. Induced-Pluripotent-Stem-Cell-Derived Primitive Macrophages Provide a Platform for Modeling Tissue-Resident Macrophage Differentiation and Function. *Immunity*. 2020;52(2):417-418. doi: 10.1016/j.immuni.2020.01.004

Referee #2 (Remarks to the Author):

In this 2nd revision, the authors put forward their novel hypothesis that in human developing brain, microglia limit neural progenitor cell proliferation thereby promoting neuronal maturation and preventing over growth, using a new combination of iPSC derived organoids and microglia-like cells from the same donor.

The authors have addressed my previous concerns by providing new analyses and experiments. For instance, they have provided videos and 3D views to convincingly demonstrate neuronal traces within microglia. They have also shown that inhibition of the cholesterol exporter ABCA1 using a drug leads to reduced cholesterol export to NPCs, and influences organoid size and NPC proliferation. All minor concerns have also been addressed with editorial changes.

We thank the reviewer for his/her positive comments, and agree that the additional experiments previously suggested have greatly improved the manuscript. We are grateful for reviewer recommendations and support.

Referee #3 (Remarks to the Author):

It is my opinion that the authors have made an outstanding effort at addressing the remaining comments raised by the Reviewers. The use of additional iPSC lines, and orthogonal validation of iMicro by single-cell and bulk transcriptomics, mass spectrometry, flow cytometry, IF and functional assays strengthen the reported findings. In addition, ABCA1 blockade experiments support the role of cholesterol uptake in co-NPC proliferation and size reduction in co-culture organoids. The manuscript now reads as a clear and well-written story, which highlights the relevance of these studies as well as future research directions.

We thank the reviewer for his/her positive evaluation of the latest manuscript. We are grateful for reviewer recommendations and support.

Reviewer Reports on the Third Revision:

Referees' comments:

Referee #3 (Remarks to the Author):

The authors have extensively address all the comments raised by the Reviewers.

Referee #4 (Remarks to the Author):

Park et al use neuroimmune organoids to study cellular interactions between developing neural progenitors and microglia. The authors discover a novel phenomenon that lipid transfer from microglia influences organoid size. Further characterization reveals uptake of a cholesterol-ester by PLIN2+ lipid droplets and export, possibly through APOE lipoprotein-like structures in the form of high-density lipoproteins, and dependent on the cholesterol transporter ABCA1. Interestingly, PLIN2 appears restricted to developing microglia in vivo, suggesting a potentially transient role for this process.

Focusing on the organoid characterization, the models used are adequate for these studies. Variation between lines and batches is common in the field and the authors report this transparently. Analysis of organoid regionalization is limited. It is unclear to me if the organoids are claimed to be patterned towards cortex as set up in the introduction. It would help to understand what fraction of cells express regional markers such as FOXG1, EMX2, NKX2.1, GBX2, etc. The regionalization, and possible variation between transplants and controls, likely does not influence the results as Day 26 transplants are probably too late to change the general patterning (though could potentially affect subregionalization, like cortical hem vs cortex). Thus the claims about cellular interactions between microglia and NPCs are likely to be valid due to internal controls of transplant vs non-transplant in each batch, despite more characterization being desired.

In terms of presentation, I would recommend against using the term mini-brain. The study also provides limited contextualization and discussion of other papers in the field. For example, Ref 29 is included but only for patch clamp, and Ref 30 is included for innate microglia development rather than for transplanting iMgl. Other relevant studies are not mentioned:
<https://pubmed.ncbi.nlm.nih.gov/34536354/>, <https://pubmed.ncbi.nlm.nih.gov/34297942/>,
<https://pubmed.ncbi.nlm.nih.gov/37172564/>

Finally, there are probably are more sophisticated ways of measuring fidelity to in vivo microglia than qPCR (and analysis of bulk gene expression) for a few markers for comparison of 2D vs 3D iMicro/iMac, but again does not undercut the results, and the transient PLIN2 finding and comparison with developing microglia is quite interesting.

Author Rebuttals to Third Revision:

We want to thank Reviewer 3 and Reviewer 4 for their careful consideration of the manuscript and their last critical and encouraging comments. In this last version of the manuscript, we have provided the changes suggested by Reviewer 4. We do believe that we have finally made a strong claim with regards to the impact the addition of primitive like macrophages (iMac) has on a 3-dimensional brain organoid from the same source of iPSC which reflects more appropriately brain formation as our findings could be mirrored in mouse and human foetal brains. We have also described in depth the mechanisms driving the neuronal maturation by iMac.

Referees' comments:

Referee #3 (Remarks to the Author):

The authors have extensively addressed all the comments raised by the Reviewers.

We thank the reviewer for his comments which have greatly improved the manuscript, and for his/her supportive comment.

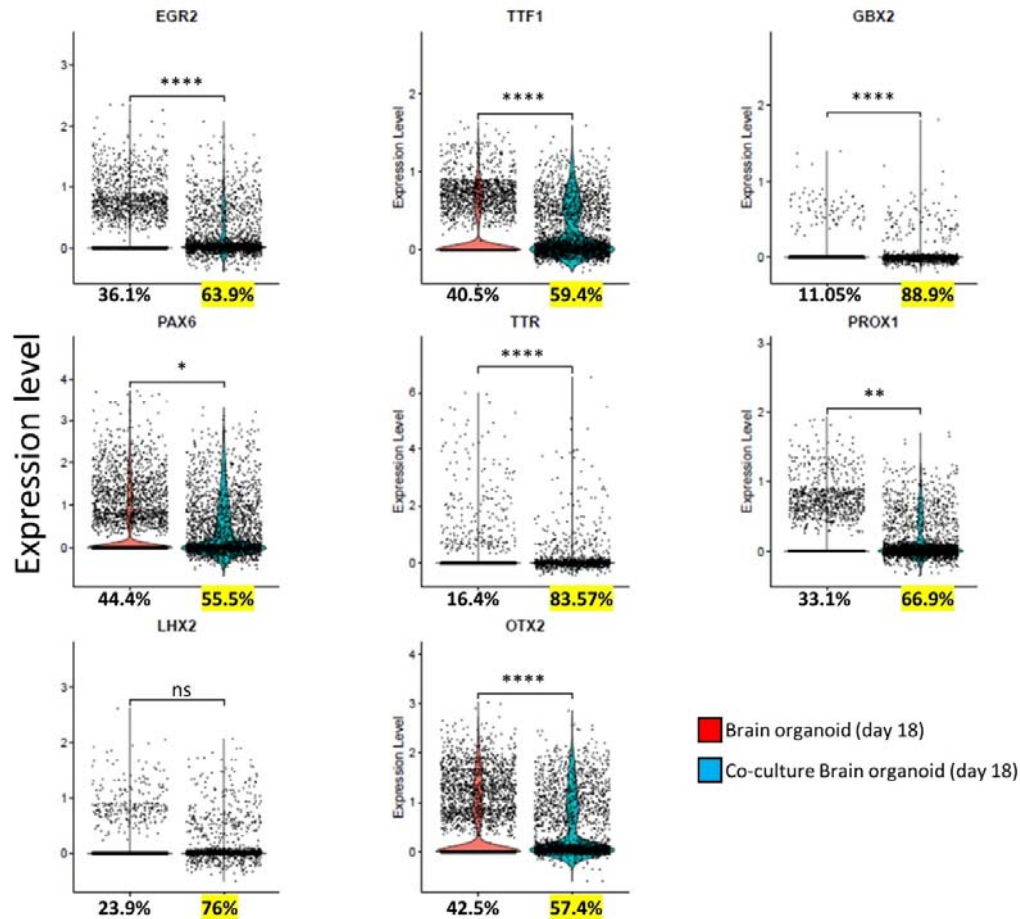
Referee #4 (Remarks to the Author):

Park et al use neuroimmune organoids to study cellular interactions between developing neural progenitors and microglia. The authors discover a novel phenomenon that lipid transfer from microglia influences organoid size. Further characterization reveals uptake of a cholesterol-ester by PLIN2+ lipid droplets and export, possibly through APOE lipoprotein-like structures in the form of high-density lipoproteins, and dependent on the cholesterol transporter ABCA1. Interestingly, PLIN2 appears restricted to developing microglia in vivo, suggesting a potentially transient role for this process.

Focusing on the organoid characterization, the models used are adequate for these studies. Variation between lines and batches is common in the field and the authors report this transparently. Analysis of organoid regionalization is limited. It is unclear to me if the organoids are claimed to be patterned towards cortex as set up in the introduction. It would help to understand what fraction of cells express regional markers such as FOXG1, EMX2, NKX2.1, GBX2, etc. The regionalization, and possible variation between transplants and controls, likely does not influence the results as Day 26 transplants are probably too late to change the general patterning (though could potentially affect subregionalization, like cortical hem vs cortex). Thus, the claims about cellular interactions between microglia and NPCs are likely to be valid due to internal controls of transplant vs non-transplant in each batch, despite more characterization being desired.

We thank the reviewer for this very interesting comment. Using our RNAseq data, we can observe that while the average expression levels of markers of brain regions such as EGR2 (Hindbrain), TTF1 (ventral forebrain), GBX2 (thalamus), PAX6 (dorsal forebrain), TTR (Choroid plexus), PROX1 (Hippocampus), LHX2 (Cortical and OTX2 (Forebrain/midbrain) in neuronal cells is lower in co-culture organoids than in organoids alone whereas the proportion of cells expressing those markers is significantly increased in co-culture organoids (Figure 1, Wilcoxon Test, proportion of cells expressing the marker is at bottom of violin plots).

Figure 1



While it is therefore likely that subregionalization is modified by the addition of iMac to brain organoids, these data suggest that at day 18 post-culture, the addition of iMac leads to a better imprinted regional identity of neuronal cells, a topic that require future studies.

In terms of presentation, I would recommend against using the term mini-brain.

We thank the reviewer for this suggestion and have replaced the term “mini-brain” with “brain organoid”.

The study also provides limited contextualization and discussion of other papers in the field. For example, Ref 29 is included but only for patch clamp, and Ref 30 is included for innate microglia development rather than for transplanting iMgl. Other relevant studies are not mentioned: <https://pubmed.ncbi.nlm.nih.gov/34536354/>, <https://pubmed.ncbi.nlm.nih.gov/34297942/>, <https://pubmed.ncbi.nlm.nih.gov/37172564/>

We thank the reviewer for this comment and we acknowledge that these papers related to the incorporation of iPSC-derived microglia into brain organoids shall be added to our manuscript. Nonetheless, we believe that this the current study provides the mechanistic behind our improved model as we show that iMac co-cultured with brain organoids further promote axogenesis and neurogenesis via the transfer of cholesterol and its esters.

We also believe that our manuscript is the first to show not only this lipid cross-talk, but also that our co-cultured brain organoid model is the first model suitable for studying embryonic microglia and associated pathologies. We have added the reference to the final version of the manuscript.

Popova, Galina et al. "Human microglia states are conserved across experimental models and regulate neural stem cell responses in chimeric organoids." *Cell stem cell* vol. 28,12 (2021): 2153-2166.e6. doi:10.1016/j.stem.2021.08.015

Xu, R., Boreland, A. J., Li, X., Erickson, C., Jin, M., Atkins, C., Pang, Z. P., Daniels, B. P., & Jiang, P. (2021). Developing human pluripotent stem cell-based cerebral organoids with a controllable microglia ratio for modeling brain development and pathology. *Stem cell reports*, 16(8), 1923–1937. <https://doi.org/10.1016/j.stemcr.2021.06.011>

Schafer, S. T., Mansour, A. A., Schlachetzki, J. C. M., Pena, M., Ghassemzadeh, S., Mitchell, L., Mar, A., Quang, D., Stumpf, S., Ortiz, I. S., Lana, A. J., Baek, C., Zaghal, R., Glass, C. K., Nimmerjahn, A., & Gage, F. H. (2023). An in vivo neuroimmune organoid model to study human microglia phenotypes. *Cell*, 186(10), 2111–2126.e20. <https://doi.org/10.1016/j.cell.2023.04.022>

Finally, there are probably are more sophisticated ways of measuring fidelity to in vivo microglia than qPCR (and analysis of bulk gene expression) for a few markers for comparison of 2D vs 3D iMicro/iMac, but again does not undercut the results, and the transient PLIN2 finding and comparison with developing microglia is quite interesting.

We thank the reviewer for this comment. We agree that there are more sophisticated ways of comparing 2D and 3D iMicro/iMac. Although of interest, this comparison was not the precise scope of the study where we focussed on the improved neurogenesis driven by iMicro resembling microglial cells both phenotypically and functionally. Finally, disposing of a 3D model, we are no longer focussing our attention on 2D iMac/iMicro.