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TITLE:

Co-Culturing Microglia and Cortical Neurons Differentiated from Human Induced Pluripotent Stem Cells

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SUMMARY:

This protocol describes a methodology to differentiate microglia from human iPSCs and maintain them in co-culture with iPSC-derived cortical neurons in order to study mechanistic underpinnings of neuroimmune interactions using human neurons and microglia.

ABSTRACT:

The ability to generate microglia from human induced pluripotent stem cells (iPSCs) provides new tools and avenues for investigating the role of microglia in health and disease. Furthermore, iPSC-derived microglia can be maintained in co-culture with iPSC-derived cortical neurons, which enable investigations of microglia-neuron interactions that are hypothesized to be dysregulated in a number of neuropsychiatric disorders. Human iPSCs were differentiated to generate microglia using an adapted version of a protocol developed by the Fossati group, and the iPSC-derived microglia were validated with marker analysis and real-time PCR. Human microglia generated using this protocol were positive for the markers CD11C, IBA1, P2RY12, and TMEM119, and expressed the microglial-related genes AIF1, CX3CR1, ITGAM, ITGAX, P2RY12, and TMEM119. Human iPSC-derived cortical neurons that had been differentiated for 30 days were plated with microglia and maintained in co-culture until day 60, when experiments were undertaken. The density of dendritic spines in cortical neurons in co-culture with microglia was quantified under baseline conditions and in the presence of pro-inflammatory cytokines. In order to examine how microglia modulate neuronal function, calcium imaging experiments of the cortical neurons were undertaken using the calcium indicator Fluo-4 AM. Live calcium activity of cortical neurons was

obtained using a confocal microscope, and fluorescence intensity was quantified using ImageJ. This report describes how co-culturing human iPSC-derived microglia and cortical neurons provide new approaches to interrogate the effects of microglia on cortical neurons.

INTRODUCTION:

In the human brain, microglia are the primary innate immune cells[1]. Brain development is regulated by microglia via two routes: release of diffusible factors and phagocytosis[1]. Microglia-derived diffusible factors help support myelination, neurogenesis, synaptic formation, maturation, cell death, and cell survival[1]. Microglia also phagocytize various elements in brain synapses, axons and in both living and dead cells[2-8]. Receptors on microglia recognize tags such as calreticulin, ATP, and sialic acid and regulate cellular phagocytosis[9,10]. In the hippocampus, microglia maintain the homeostasis of neurogenesis through its phagocytic role[11].

Synaptic phagocytosis in the dorsolateral geniculate nucleus (dLGN) of the rodent brain has been shown to be regulated by microglia[1]. In rodents, it has been shown that there are two periods during the development when intense microglial synaptic phagocytosis is observed. The first period occurs during initial synapse formation and the second period occurs when connections are being fine-tuned and pruned[12]. Other factors that are involved in synaptic pruning are inflammatory proteins and the Class I major histocompatibility complex (MHC1, H2-K^b and D^b)[13,14]. It has been suggested that C1q (complement component 1q) on the microglia colocalizes with MHC1, which triggers synaptic pruning[15]. Furthermore, mouse studies show that interleukin-33 (IL-33) secreted by astrocytes regulates synapse homeostasis in the thalamus and the spinal cord through its effects on microglia, though this has yet to be investigated in humans[13]. Microglia secrete a variety of cytokines that help maintain neuronal health, such as tumor necrosis factor α (TNFα), IL-1β, IL-6, IL-10 and interferon-γ (IFN-γ) and these cytokines can modulate dendritic spine and synapse formation[16-18]. There are significant gaps in our knowledge of neuron-microglia interactions during human brain development. Most of our knowledge comes from studies from rodent models, while there is a paucity of information on the temporal and mechanistic aspects of synaptic pruning in the human cortex. Microglia support neuronal survival in the neo-cortex, and other cell types contribute as well[1]. It is not clear how microglia contribute to this preservation and what the interplay between microglia and the other cell types are. Microglia release several cytokines that affect neuronal and synaptic development but the mechanistic basis of their effects of these cytokines in neurons are largely unknown[19,20]. In order to develop a more complete understanding of the function of microglia in the human brain, it is critical to explore its interactions with different cell types found in the human brain. This report describes a method to co-culture human iPSC-derived neurons and microglia generated from the same individual. Establishing this methodology will enable well-defined investigations to interrogate the nature of microglia-neuronal interactions and to develop robust *in vitro* cellular models to study neuroimmune dysfunction in the context of different neurodevelopmental and neuropsychiatric disorders.

The role of microglia in schizophrenia

Synaptic pruning is a major neurodevelopmental process that takes place in the adolescent brain[21,22]. Multiple lines of evidence suggest that synaptic pruning during this critical period is

abnormal in schizophrenia (SCZ)[23-26]. SCZ is a chronic, debilitating psychiatric disorder characterized by hallucinations, delusions, disordered thought processes and cognitive deficits[23,24]. Microglia, the resident macrophages in the brain, play a central role in synaptic pruning[25,26]. Postmortem and positron emission tomography (PET) studies show evidence for dysfunctional microglial activity in SCZ[25-32]. Postmortem SCZ brains show well-replicated but subtle differences in the brain – pyramidal neurons in the cortical layer III show decreased dendritic spine density and fewer synapses[33-35]. Synaptic pruning is a process by which superfluous excitatory synaptic connections are eliminated by microglia during adolescence, when SCZ patients usually have their first psychotic break[22,36]. Postmortem studies show an association between SCZ and microglial activation, with increased density of microglia in SCZ brains, as well as increased expression of proinflammatory genes[27]. In addition, PET studies of human brains using radioligands for microglial activation show increased levels of activated microglia in the cortex[25-28]. Recent genome-wide association studies (GWAS) show that the strongest genetic association for SCZ resides in the major histocompatibility complex (MHC) locus, and this association results from alleles of the complement component 4 (C4) genes that are involved in mediating postnatal synaptic pruning in rodents[37]. This association has provided additional support for the hypothesis that aberrant pruning by microglia may result in the decreased dendritic spine density seen in SCZ postmortem brains. Investigations of microglial involvement in synaptic pruning in SCZ have so far been limited to indirect studies with PET imaging or inferences from investigations of postmortem brains.

Generating human microglia in the laboratory

Cultured primary mouse microglia have been frequently used in studying microglia, though there are several indications that rodent microglia may not be representative of human microglial anatomy and gene expression (**Table 1**)[38]. Several studies have also differentiated microglia directly from blood monocytes through transdifferentiation[39-42]. Blood monocyte-derived microglia-like cells exhibit major differences from human microglia in gene and protein expression profile pro-inflammatory responses, and they appear to be more macrophage-like in their biology[43]. Recent methodological advances now enable the generation of microglia from human iPSCs, which provide opportunities to study live microglia that more accurately resemble the biology of microglia found in the human brain (**Table 2**). These iPSC-derived microglial cells have been shown to recapitulate the phenotype, gene expression profiles, and functional properties of primary human microglia[44-48]. This paper provides a method to co-culture human iPSC-derived neurons and microglia generated from the same individual in order to develop personalized *in vitro* models of neuron-microglia interactions. For this *in vitro* co-culture model, a microglial differentiation protocol from the Fossati group was adapted (**Table 3**) and combined with an adapted version of a cortical neuronal generation protocol from the Livesey group (**Table 4**)[49,50].

PROTOCOL:

The human iPSCs used in this study were reprogrammed from fibroblasts that had been obtained through informed consent from healthy control subjects, with approval from the institutional review board (IRB). The reprogramming and characterization of iPSCs used in this study (ML15, ML27, ML40, ML56, ML141, ML 250, ML292) were described in a prior study[51].

1. Maintenance of iPSCs

1.1 Prepare a 1:50 dilution of LDEV-free reduced growth factor basement membrane matrix in DMEM/F12 without phenol red and pre-coat a 6-well plate with 1 mL of the diluted solution for at least 2 h at 37 °C prior to thawing cell stocks.

1.2 Thaw cryopreserved iPSC stocks in a 37 °C water bath for 2 min. Add the cells to a 15 mL centrifuge tube containing 5 mL of DMEM/F12. Spin the cells down at 300 x *g* for 5 min.

1.3 Remove the coating solution from the pre-coated LDEV-free reduced growth factor basement membrane matrix plate and add 1 mL of stem cell medium (SCM) with 10 µM Rock inhibitor (Y-27632).

1.4 Resuspend the cell pellet in SCM with 10 µM Y-27632 and add to the pre-coated plate for a final volume of 2 mL of SCM plus Y-27632. Maintain iPSC cell cultures in this medium for 24 h in a 37 °C incubator.

1.5 Replace the medium with 2 mL of fresh SCM without Y-27632 24 h after thawing.

1.6 After iPSCs reach 80-90% confluence, passage them onto 75 mL flasks coated with LDEV-free reduced growth factor basement membrane matrix.

1.6.1 Passage cells by first rinsing cells with HBSS and remove after letting sit for 30 s. Add 1 mL of non-enzymatic cell dissociation reagent and incubate for 4 min at 37 °C. Prepare the plate with SCM containing 10 µM Y-27632.

1.6.2 Aspirate non-enzymatic cell dissociation reagent and add 1 mL of SCM + 10 µM Y-27632 to each well. Gently scrape the well with a cell lifter and obtain cells with a 1000 µL pipette.

1.6.3 Deposit cells onto LDEV-free reduced growth factor basement membrane matrix-coated 75 mm flask in a total volume of 8 mL of SCM + Y-27632.

2. Microglia differentiation

NOTE: A schematic outlining the microglia differentiation protocol is depicted in **Figure 1A**. Media were warmed to room temperature before use.

2.1 Day 0: Perform a complete medium change with SCM medium supplemented with 80 ng/mL BMP-4. Perform daily medium changes with this same medium during Days 1-3, without any washing between medium changes.

2.2 Day 4: Prepare Day 4-5 medium: Hematopoietic medium (HM), supplemented with 25 ng/mL FGF, 100 ng/mL SCF, and 80 ng/mL VEGF. Remove the medium and replace with Day 4-5 medium containing 5 µM Y-27632.

NOTE: Cells begin to float at this point – about half the cells are floating, and half are adherent.

2.3 Day 6: Prepare Day 6-13 medium: HM supplemented with 50 ng/mL SCF, 50 ng/mL IL-3, 5 ng/mL TPO, 50 ng/mL m-CSF, and 50 ng/mL Flt3-L. Collect the supernatant, add to a 15-mL conical tube and spin down for 8 min at 300 x *g*. Resuspend the pellet in a flask with 8 mL of Day 6-13 medium supplemented with 5 µM Y-27632.

2.4 Day 10: Add 8 mL of Day 6-13 medium on top of the existing medium.

2.5 Day 14: Prepare Day 14+ medium: HM supplemented with 50 ng/mL m-CSF, 50 ng/mL Flt3-L, 50 ng/mL GM-CSF. Collect the supernatant, add to a 50 mL conical tube and spin down for 8 min at 300 x *g*. Resuspend the pellet in 8 mL of Day 14+ medium containing 5 µM Y-27632 and continue to culture in this medium.

2.6 Day 18: Add 8 mL of Day14+ medium.

2.7 Day 22: Add 8 mL of fresh Day14+ medium without removing the existing medium.

2.8 Day 25: Move cells to step 2.9 or continue to maintain in Day14+ medium until day 50 of differentiation.

2.9 After Day 25, collect the supernatant in a 50-mL conical tube and spin down for 8 min at 300 x *g*.

2.9.1 Prepare adherent medium: RPMI supplemented with 1% of 200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl solution, 25 ng/mL GM-SCF and 100 ng/mL IL-34.

2.9.2 Resuspend the pellet in the adherent medium containing 5 µM Y-27632.

2.9.3 Plate cells at a density of 50,000 cells per cm^2 on 24-well plates for different experiments: LDEV-free reduced growth factor basement membrane matrix-coated plates for microglial monoculture, 10 µg/mL poly-L-ornithine and 10 µg/mL laminin coated glass imaging plates for imaging experiments.

2.9.4 Dilute poly-L-ornithine and laminin in DPBS and add 250 µL/well for a 24 well imaging plate.

NOTE: Cells in culture are now adherent in nature (**Figure 1C**).

2.10 Maintain cells in culture for at least 14 days, with bi-weekly medium changes. After day 14 post-adherence, cells have reached maturation and may be used for experiments.

3. Cortical neuron differentiation

NOTE: A schematic outlining the cortical neuron differentiation protocol is depicted in **Figure 1G.**

3.1 Day 0:

3.1.1 Once iPSCs are confluent on LDEV-free reduced growth factor basement membrane matrix-coated plates, switch from SCM to a 50/50 mix of N2/B27 medium supplemented with 10 µM SB431542, 1 µM dorsomorphin, 100 nM LDN193189.

NOTE: N2 medium consists of basal medium supplemented with 1% N-2 supplement, 1% 200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl solution, 1% pen/strep. B27 medium consists of DMEM/F12 supplemented with 2% B-27 supplement, 1% 200 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl solution, 1% pen/strep.

3.1.2 Change the medium with the above supplements added daily for 7 days.

3.2 Day 7:

3.2.1 Pre-coat plates in LDEV-free reduced growth factor basement membrane matrix for at least 2 h.

3.2.2 Passage cells 1:1 onto pre-coated plates. Rinse cells with 1 mL/well HBSS and remove after letting it sit for 30 s. Add 1 mL/well of cell detachment medium (e.g., Accutase) and incubate for 4-5 min at 37 °C. While incubating, prepare 15 mL conical tubes with 5 mL of DMEM.

3.2.3 Gently pipette enzymatic dissociation agent to remove cells from the plate with a P1000 pipettor. Collect enzymatic dissociation agent and cell mixture in the 15 mL conical tube containing 5 mL DMEM.

3.2.4 Centrifuge the tubes for 5 min at 300 x *g*. Resuspend the pellet in 1 mL of N2/B27 medium containing 10 µM Y-27632. Continue daily feedings with N2/B27 medium without any supplements.

3.3 Day 12: Passage cells 1:1 using methods described in 3.2.2. Continue daily feedings with N2/B27 medium.

3.4 Day 15/16: Passage cells 1:2 using methods described in 3.2.2. Continue daily feedings with N2/B27 medium.

3.5 Day 18/19: Passage cells 1:3 using methods described in 3.2.2. Continue daily feedings with N2/B27 medium until day 25.

3.6 Day 25: Feed cells with N2/B27 medium supplemented with 10 μM of DAPT.

3.7 Day 28: Feed cells with fresh, untreated N2/B27 medium.

3.8 Day 30: Passage cells using methods described in 3.2 onto the microglia culture plates and maintain in BrainPhys medium supplemented with 1% B-27 supplement.

4. Microglia/neuron co-cultures

4.1 Plate Day 30 cortical neurons on top of microglial cultures at a density of 50,000 cells per cm^2. Supplement medium with laminin 1 μg/mL to improve cell adherence.

4.2 Maintain cultures in a mix of 50% adherent medium and 50% NM. Perform half-medium change bi-weekly until experimentation.

4.3 Perform experiments after neurons reach day 60.

5. Interferon-γ treatment

5.1 Prepare fresh medium supplemented with 100 ng/mL IFN-γ. Add the medium and let it incubate for 24 h. Remove the medium and proceed to experimentation.

6. Immunocytochemistry

6.1 Fix cells in the culture dish with 100 μL of 4% paraformaldehyde at room temperature for 20 min.

6.2 Rinse cells in 1 mL of PBS thrice for 5 min each.

6.3 Add 1 mL of PBST (PBS + 0.1% Triton X) for 10 min.

6.4 Add blocking buffer: 1 mL of PBS plus 5% goat serum for 1 h.

6.5 Add primary antibody diluted in 100 μL of PBS + 1% goat serum - overnight at 4 °C. Optimize all primary antibodies accordingly.

6.6 Rinse cells in 1 mL of PBS thrice for 5 min each.

6.7 Add secondary antibody, diluted in 100 μL of PBS plus 1% goat serum - for 1 h at room temperature.

7. Spine analysis

7.1 Obtain images on a confocal microscope at 60x magnification.

7.2 Use ImageJ function NeuronJ[52] to analyze images.

7.3 Obtain measurements for neurite length, spine length, and spine count through NeuronJ.

8. Calcium imaging

8.1 Prepare fresh medium with 3 µM Fluo-4AM dye. Incubate co-cultures in this medium for 30 min at 37 °C. Then rinse the cells with PBS, add live-cell imaging solution to the cells, and proceed to the imaging.

8.2 Using a confocal microscope equipped for live-cell imaging, obtain time-lapse images at 40x for 2 min. Activity can be recorded at baseline, with exposure to 15 mM glutamate, or in the setting of depolarization with 5 mM potassium chloride.

8.3 Using ImageJ, measure fluorescence intensity over time for individual cell bodies. Using the selection tool, select individual region of interest (ROI) surrounding each cell body. Open the **ROI Manager** and press **Add** to select. Continue to add new selections to the ROI manager.

8.4 Use the **Set Measurements** tool to measure **Mean Gray Area**. When the number of desired cell bodies has been added to the ROI manager, select them all and then use the **Multi-Measure** tool. This will provide a readout of the mean gray area for each over the time course of the video file. The exported data will give mean fluorescence intensity for the region of interest for each frame.

8.5 Determine fluorescence intensity ratio, F/F_0, where F is the fluorescence intensity at a given time and F_0 is the initial fluorescence intensity. F/F_0 can be graphed over time to examine spontaneous activity in neurons or examined at the maximum fluorescent intensity in the setting of stimulation.

REPRESENTATIVE RESULTS:

Protocol Validation

The iPSC-derived microglia were generated from seven iPSC lines over three different rounds of differentiation. Control iPSC lines ML27, ML56, ML292, and ML364 and schizophrenia iPSC lines ML40, ML141, and ML250 were utilized. Characterization of these iPSC lines have been described previously[51]. These iPSC-derived microglia were validated using ICC and qPCR. Microglia generated from the adapted protocol exhibited typical ramified microglial morphology (**Figure 1C**), and expressed microglial markers CD11c, TMEM119, and IBA1, as examined by immunocytochemistry (**Figure 1D,E**). Cells with nuclei expressing microglial markers CD11c, P2RY12, and IBA1 were quantified. CD11c, P2RY12, IBA1 and TMEM119 were present in 63%,

60%, 65%, and 44% of the cells respectively, which is consistent with data described in the original differentiation protocol paper[49]. These experiments were performed with iPSC lines ML27, ML40, ML141, and ML250. Expression of specific genes was examined using qPCR to confirm the expression of microglial genes AIF1, CX3CR1, ITGAM, ITGAX, P2RY12, and TMEM119 (**Figure 1F**). This data was obtained from iPSC-derived microglia from two lines and normalized to an iPSC line. The SYBR Green real-time PCR protocol was used.

Dendritic Spines

Cortical neurons and microglia in co-culture were visualized using confocal microscopy (**Figure 2A**). Dendritic spines were quantified in the co-cultured cortical neurons (**Figure 2B**). Co-cultures were analyzed to determine the proportion of microglia and cortical neurons by marker analysis using P2RY12 and MAP2 to identify microglia and neurons respectively. In these co-cultures, 32.5% of the cells were positive for P2RY12 and 37.7% of the cells were positive for MAP2. Cortical neurons co-cultured with microglia treated with IFN-γ exhibited no significant differences in spine count, spine length, and neurite length when compared to cortical neurons co-cultured with untreated microglia (**Figure 2D**). This data was collected from the four control iPSC lines ML27, ML56, ML292, ML364, with two separate wells per experimental condition and ten images obtained from each well.

Calcium Imaging

Neurons co-cultured with microglia were stained with calcium fluorescence indicator in order to examine differences in neuronal firing with stimulation from glutamate with and without IFN-γ induced microglial activation (**Figure 2E**). Cortical neurons co-cultured with microglia treated with IFN-γ showed significant reduction in fluorescence intensity after stimulation with glutamate compared to cortical neurons co-cultured with untreated microglia (**Figure 2F**). This data was collected for three healthy control iPSC lines ML27, ML56, and ML292, with two wells per experimental condition and five images obtained from each well.

Supplementary Figure 1 further validates antibodies and staining protocol.

FIGURE AND TABLE LEGENDS:

Figure 1: Differentiation and validation of iPSC-derived microglia. (**A**) Schematic depiction of microglial differentiation from iPSCs through microglial maturity and co-culture with cortical neurons. (**B**) Representative image of microglial progenitor cells after re-plating following day 25 of differentiation at 10x magnification. (**C**) Representative image of microglia in monoculture at day 14 at 10x magnification. (**D**) Immunocytochemistry staining of CD11c, P2RY12, IBA1, and TMEM119 to confirm expression of microglial markers, shown at 20x magnification. (**E**) Percentage of cells positively stained for microglial markers CD11c, P2RY12, IBA1, and TMEM119. (**F**) qPCR validation showing microglia exhibiting microglial-signature genes including AIF1, CX3CR1, ITGAM, ITGAX, P2RY12, and TMEM119. (**G**) Schematic depicting cortical neuron differentiation from iPSCs. (**H**) Immunocytochemistry staining of CTIP2, CUX1, SATB2, and MAP2 to confirm generation of cortical neurons, shown at 63X magnification.

Figure 2. Functional changes in microglia and neuron co-cultures with and without interferon-gamma treatment. (**A**) Representative image of microglia and cortical neuron co-culture, with neurons stained for MAP2, pre-synaptic marker bassoon and post-synaptic marker homer, and microglia stained for TMEM119. (**B**) Representative image of dendritic spines in microglia/neuron co-cultures, with and without IFN-γ treatment. Scale bar = 50 μm. (**C**) Percentage of cells positive for P2RY12 or MAP2 in the co-cultures (mean + SEM). (**D**) Cortical neurons co-cultured with microglia treated with IFN-γ showed no significant differences when compared to untreated microglia in terms of spine count (Mann-Whitney test, $P > 0.05$, mean + SEM), spine length (Mann-Whitney test, $P > 0.05$, mean + SEM), or neurite length (unpaired t test, $P > 0.05$, mean + SEM). (**E**) Representative images of cortical neurons co-cultured with microglia in the presence of the calcium indicator Fluo-4AM, before and after glutamate stimulation and with and without IFN-γ treatment. (**F**) Cortical neurons co-cultured in the presence of microglia treated with IFN-γ had significantly lower fluorescence intensity with glutamate stimulation when compared to neurons co-cultured with untreated microglia (Mann-Whitney test, $P = 0.0003$, mean + SEM).

Table 1: Overview of current protocols for differentiation of iPSCs to microglial cells.

Table 2: Overview of media used for microglial differentiation, listed with the concentration of cytokines used.

Table 3: Brief overview of microglial differentiation protocol from Douvaras et al.[49] and adaptations made in this study.

Table 4: Brief overview of neuronal differentiation protocol from Shi et al.[50] and adaptations made in this study.

Supplementary Figure 1: (**A**) Control iPSCs stained Microglia specific antibodies CD11c, P2RY12 and (**B**) TMEM119 and Iba1 to ensure these antibodies do not stain non-microglia cells. Control iPSC line did not exhibit any positive staining for these markers. (**C**) Co-cultures analyzed by cell type visible by specific line. Co-cultures were stained with a neuronal marker MAP2 or microglial marker P2RY12.

Supplementary Table 1: List of primary antibodies used in this protocol and their optimal dilutions.

Supplementary Table 2: List of primers used for RT PCR experiments with forward and reverse sequences.

DISCUSSION

The development of differentiation methods along different trajectories for pluripotent stem cells have opened many avenues for the investigation of brain function and disease processes[53-55]. Initial studies had focused on the development of specific neuronal cell types hypothesized to be important in specific brain disorders[56,57]. Recently, brain organoids have also provided new ways to study disease biology using patient-specific three-dimensional models[51,58]. The two-

dimensional and three-dimensional cellular models provide specific advantages when trying to tackle different scientific questions[59]. While the early studies focused on cells along the neuronal lineage, recent developments now enable generation of other cell types in the brain, i.e. microglial cells and brain microvascular endothelial cells[60,61]. While studying the development of these cell types have provided valuable information and knowledge, it is important understand the interplay between these cell types to fully understand the role of neuroimmune and neurovascular interactions on brain function and development. This paper provides a detailed protocol on generating and co-culturing cortical neurons and microglia derived from the same iPSC line in order to develop personalized *in vitro* co-culture models to study the effects of different cell types on neuronal biology. By optimizing a method to efficiently differentiate microglia from iPSCs, we are now poised to examine disease-specific phenotypes using microglia and cortical neurons differentiated from iPSCs from disease subjects. Furthermore, this experimental approach allows for cross-culturing of microglia and neurons from control and disease subjects that can be leveraged to delineate specific contributions of microglia and neurons in disease-related phenotypes.

A number of rodent studies have examined co-cultures of microglia and neurons. A tri-culture with astrocytes, microglia and primary neurons reported significant improvement in neuronal health and reduced caspase 3/7 activity in the co-cultures[62]. An additional co-culture protocol for primary cerebellar granule neurons and primary cortical microglia from mice revealed that the use of co-culture methodology helps prevent the negative effect of toxicants on neuronal function and survival by mediating the release of cytokines[63]. These findings suggest that it is important to study co-cultures of different cell types to accurately depict relevant neurobiology and highlight the need for these experiments to be undertaken using human cells to understand the role neuro-immune interactions in psychiatric disease biology.

Only one previous study has investigated co-cultures of human iPSC-derived microglia or precursor macrophages with cortical neurons[48]. Co-cultures of embryonic MYB-independent iPSC-derived macrophages with iPSC-derived cortical neurons found that co-culture with microglia led to downregulation in pathogen-response pathways, upregulation in homeostatic function pathways, and promoted an anti-inflammatory and pro-remodeling cytokine response than corresponding monocultures, further suggesting the important crosstalk between microglia with neurons that can be recapitulated in *in vitro* co-culture models[48]. This study used iPSC-derived neurons to help mature MYB-independent iPSC-derived macrophages into microglial cells whereas the current protocol utilizes iPSC-derived microglia that had been differentiated to maturity separately. Given the significant interplay between these cell types, using a methodology where the maturation of microglia is dependent on the health of the neurons may present challenges and confounding factors when studying disease-related phenotypes.

There are three critical steps in this protocol that should be followed to ensure success. First, spinning cells for medium change for the floating microglial cultures should be kept to a minimum, which is why it is outlined that media should be added on top of the existing media during this stage instead of exchanging media. This step helps prevent loss and death of differentiating microglial cells. Second, when plating cortical neurons onto microglial cultures, it

is crucial to add laminin to the medium in order to ensure that neurons will adhere to the plate. Neurons have a tendency to lift off of the plate and adding laminin helps prevent this. Third, for calcium imaging experiments, ensure that the confocal microscope used is equipped with an incubation chamber for live-cell imaging. This allows the cells to remain healthy throughout the experiment and prevents variability in data due to the timing of when the imaging data was collected.

This protocol adapted an aspect of the Fossati group protocol[49] that had led to significant loss and death of differentiating microglial cells. Rather than collecting the supernatant and floating cell mixture and pelleting the cells every four days as originally described, fresh media was added on top to prevent cell loss and death in the centrifugation process. Cells were grown in 75mm flasks rather than in 6-well plates in order to maximize volume of media that could be added during this stage.

This protocol also removed a sorting step described in the Fossatti group protocol[49]. The sorting step resulted in a much lower cellular yield and high cell death in our hands. Hence, floating cells at day 25+ were plated without using this sorting step. Cells differentiated using this modified approach had ramified morphology characteristic of microglial cells, expressed microglial genes and showed robust staining of microglial markers. The percentage of cells expressing microglial markers is similar to the data in the original protocol[49], suggesting that the purity of these cells is not significantly affected by removal of this sorting step.

Though the specific protocols for generating co-culturing iPSC-derived microglia and cortical neurons involved adapting from two well-established protocols, the microglia generated with this adapted approach have not been as extensively characterized as the original protocol. The downside of this protocol relates to length and cost of differentiation. The differentiation process takes at least 40 days and requires the use of a number of expensive reagents, especially the cytokines. Also, in this report, IFN-γ was used as an activator of microglia, but it should also be noted that this pro-inflammatory cytokine can elicit direct changes in cortical neurons as well[64] and there is a need to undertake further studies to delineate the effect of cytokines on microglia and neurons.

These experiments provide proof-of-concept approaches to examine the effect of microglia on neuronal biology, which sets the stage for interesting explorations of various facets of neuro-immune interactions in the context of disease biology using microglia and neurons generated from patients with specific neurodevelopmental and neuropsychiatric disorders. The ability to cross-culture microglia and neurons from healthy subjects and disease subjects provide interesting avenues to dissect the specific roles of these cell types in the manifestation of disease-related phenotypes. Furthermore, co-culture models can be expanded to include astrocytes, oligodendrocytes and endothelial cells in order to develop novel *in vitro* models that reflect the different niches found in the brain.

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DISCLOSURES:

The authors have nothing to disclose.

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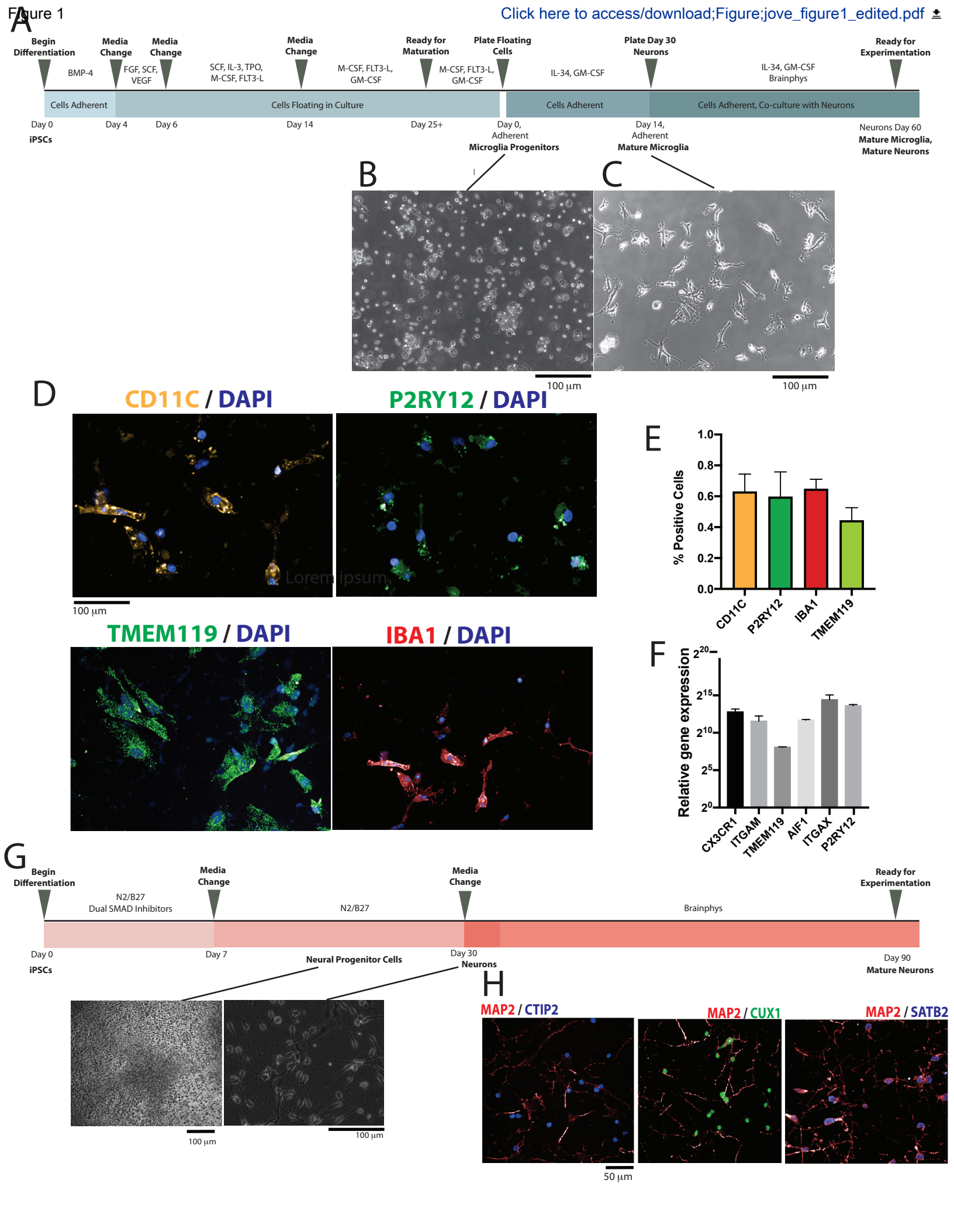
Figure 1



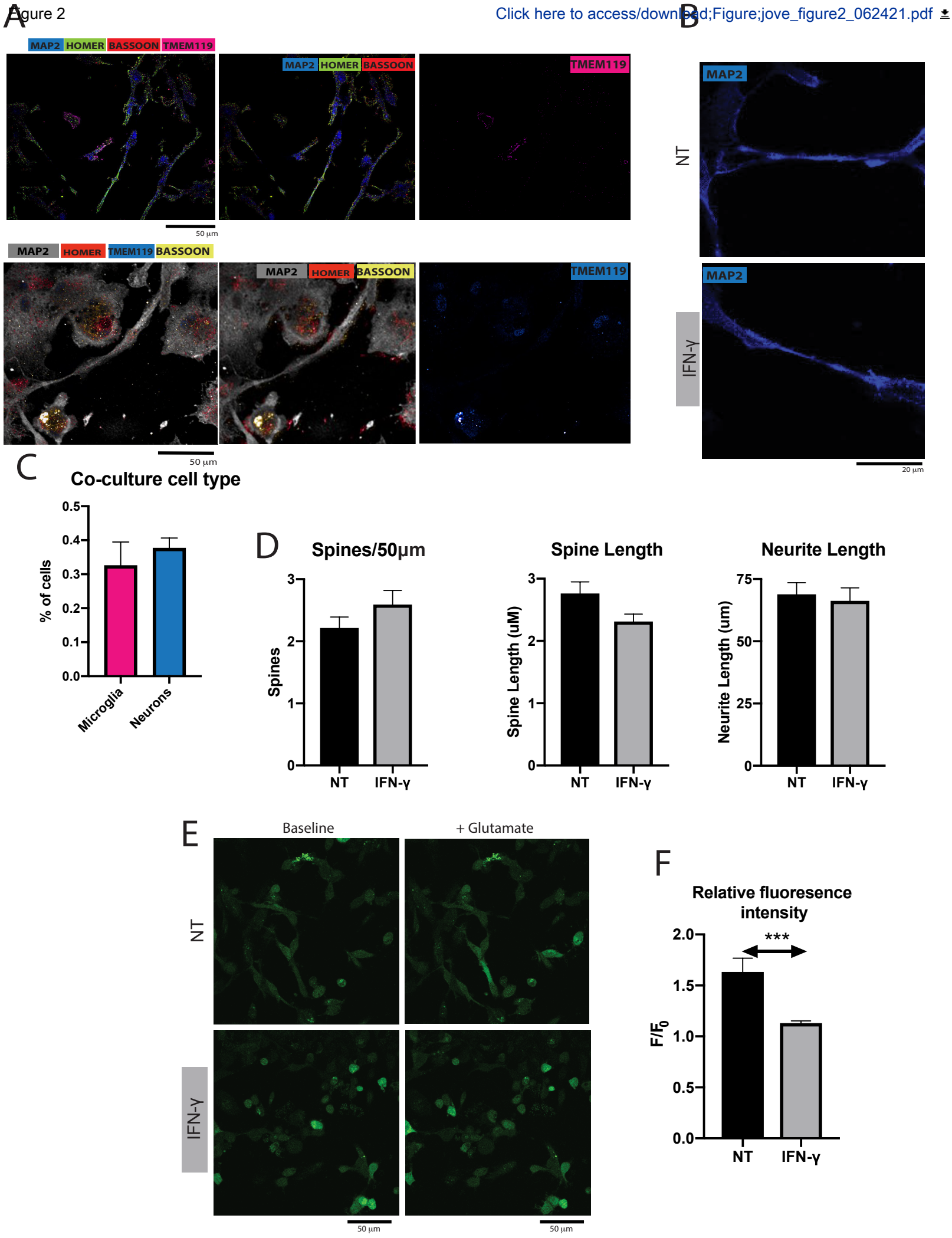


Table 1

Protocol	Supplements used	Sorting stage	Length of Differentiation
Abud et al.	FGF, BMP4, Activin A, LiCl, FGF, VEGF, TPO, SCF, IL-3, IL-6, M-CSF, IL-34, TGFb1, insulin, CD200, CX3CL1	At day 10, isolate CD34+ cells	38 days
Douvaras et al.	BMP4, FGF, SCF, VEGF, IL-3, TPO, M-CSF, Flt-3L, GM-CSF, IL-34	At day 25, for CD14+/ CX3CR1+ cells	40 days
Haenseler et al.	BMP4, SCF, VEGF, M-CSF, IL-3, GM-CSF	None	42 days

Mcquade et al.	IL-34, TGF-β1, M-CSF, CLxCL1, CD200	None	38 days
Muffat et al.	M-CSF, IL-34	None	74 days

Protein markers expiressed	Characterization
CD45, CX3CR1, ITGB5, MERTK, PROS1, TGFbR1, P2RY12, TREM2	Cytospin/Giemsa staining, transcriptomic profiling, RNA seq, flow cytometry, RT-QPCR, cell type analysis, flow cytometry, motility assay, inflamation response assay, phagocytosis assay, transplantation
CD11b, CD11c, CX3CR1, IBA1, P2RY12, TMEM119	ICC, RT-QPCR, cell type analysis, flow cytometry, RNA seq, calcium assay, motility assay
CD11b, CD14, CD45, IBA1, MERTK	ICC, RT-QPCR, cell type analysis, flow cytometry, motility assay, inflamation response assay

P2RY12, TMEM119	ICC, RNA seq, phagocytosis assay, transplantation assay
CD11B, IBA1, P2RY12 TMEM119	ICC, RNA seq, flow cytometry, cell size comparison Endotoxin response, motility assay

Table 2

Day	Medium	Cytokines	Concentration
0-3	Stem Cell Medium	BMP-4	80ng/mL
4-5	Hematopoietic Medium	FGF	25ng/mL
		SCF	100ng/mL
		VEGF	80ng/mL
6-13	Hematopoietic Medium	SCF	50ng/mL
		IL-3	50ng/mL
		TPO	5ng/mL
		M-SCF	50ng/mL
		Flt3-Ligand	50ng/mL
14-25+	Hematopoietic Medium	M-SCF	50ng/mL
		Flt3-Ligand	50ng/mL
		GM-SCF	50ng/mL
Adherent	RPMI 1640	GM-SCF	25ng/mL
		IL-34	100ng/mL

Table 3

Protocol	Day 0-3 Medium	Feeding method	Sorting
Douvaras et al.	Custom medium; medium without Lithium Chloride, GABA, Pipecolic Acid, bFGF and TGFβ1 supplemented with 80ng/mL BMP4	Every four days, cells pelleted and resuspended in fresh medium	Isolation of CD14+ or CD14+CX3CR1+ progenitors via FACS sorting
This protocol	iPSC medium supplemented with 80ng/mL BMP4	Every four days fresh medium added on top of existing medium	None

Supplements

Rock inhibitor (10µM) used after centrifugation

Table 4 Click here to access/download;Table;microglia jove table 4.xlsx

Protocol	Neural Maintenance Medium	Neural Induction Medium	Supplements
Shi et al. 201250	N2/B27 medium. N2 medium: Basal medium with 1% N-2 supplement, 1% 200mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl solution, 1% pen/strep, 5µg/mL insulin, 1mM ;-glutamine, 100µM non-essential amino acids, 100µM 2-mercaptoethanol. B27 medium: DMEM/F12 supplemented with 2% B-27 supplement, 200mM L-glutamine, 1% pen/strep.	N2/B27 medium supplemented with 10µM SB431542 and 100ng/mL noggin OR 10µM SB431542 and 1µM dorsomorphin.	20ng/mL FGF2 upon appearance of rosettes
This protocol	N2/B27 medium. N2 medium: Basal medium with 1% N-2 supplement, 1% 200mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl solution, 1% pen/strep. B27 medium: DMEM/F12 supplemented with 2% B-27 supplement, 1% 200mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl solution, 1% pen/strep.	N2/B27 medium supplemented with 10µM SB431542, 1µM dorsomorphin, 100nM LDN193189.	

Notable Differences

Use of Insulin, NEAA, 2-mercaptoethanol

Use of 200mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl solution in B27 medium, use of 100nM LDN193189 in Neural Induction Medium

Table of Materials

Reviewer #4:
Manuscript Summary:
In this manuscript, the authors provide protocols for the iPSC differentiation into microglia-like cells, cortical neurons as well as the procedures of co-culture of the two cell types. Especially the new iPSC to microglia differentiation technique is highly interesting for microglia research. Although a ladder of different microglia differentiation protocols has emerged in the past 5 years it is clear that several improvements may improve the yield, robustness and functionality of the cells. The benefit of the presented protocol is the simplification of the Douvaras protocol what might be interesting for scientists to save resources. In addition, a robust protocol for microglia-like cell & iPSC-derived neuron co-culture will help scientists to unravel mechanisms of cellular interactions between both cell types.
However some points should be addressed:

Minor Concerns:
1. Protocol:
3.3 & 3.4.: The enzymatic dissociation reagent should be specified.
3.4.: Do you use the 15ml falcon with 5ml medium addressed in 3.3 in this step?
3.5.-3.10.: Reference 3.2 is not about passaging.
3.10.: The ingredients of the neuronal medium (NM) should be named.
→ We have made changes in the manuscript to address all these comments.

2. Figures
Figure 1 is missing in the most recent submission file and should be included again.
For better reproducibility, it would be extremely useful to see additional light microscopy images depicting all critical (morphological) transformation steps from iPSC to the mature microglia-like cells in Fig. 1A-C (i.e. d0, d4, d6, d14, d0 adherent, d14 adherent, d60). Analogous this would be useful for neuronal differentiation (Fig. 1G-H).
Fig. 2A is the only picture showing the co-culture of the iPSC-derived neurons and microglia. Unfortunately, the staining quality is very low and provides poor insight into the morphology and interactions of both cell types. This should be revised or other proof of concepts of the functionality of the co-culture of microglia-like cells and iPSC-derived neurons should be included into the manuscript.
The unit of length in Fig. 2D should be changed from "um" to "µm".
→ In figure 1, we have added light microscopy images of the neural progenitor cells and the differentiated neurons. We had taken light microscopy images of the microglia-like cells at only specific stages and unfortunately do not have the serial images from different days.
→ In figure 2, we have included two sets of images that give a clearer representation of the co-culture of microglia-like cells and the iPSC derived neurons. We have included images with both the neuronal and microglial stainings present, along with side-by-side images showing just the neuronal or the microglial stainings.

Reviewer #5:
Manuscript Summary:

This manuscript describes a protocol for co-culturing human iPSC-derived microglia and cortical neurons, which will provide a useful experimental system to understand the effects of microglia on cortical neurons.

Major Concerns:
None.

Minor Concerns:
With regards to, instead of with regard to has been frequently used, which needs to be corrected.
→ **We have not used "with regards to" in the current version.**

Reviewer #6:
Manuscript Summary:
This is a very well presented study, which describes an important protocol for the generation of iPSC-derived microglia and a neuron-microglia co-culture system. I am satisfied with the interpretation of the protocol and the representative results. However, there are a few minor points related to immunohistochemistry and figure preparation, which should be addressed before publication.

Minor Concerns:

1. *What was the dilution of the primary antibodies used during immunohistochemistry? Please add to Table of Materials.*

→ **We have added this information in the Table of Materials.**

2. *Please add information about the secondary antibodies (manufacturer, catalog number, dilution) to Table of Materials.*

→ **We have added this information in the Table of Materials.**

3. *Please do not indicate scale bars in every pictures if the magnification is the same (Fig. 1B, H, Fig. 2D).*

→ **We have corrected this.**

4. *What was the reason of using different magnification in Fig. 1D?*

→ **These images were taken in a different microscope.**

5. *The scale bar is rarely visible in Fig. D. at IBA1/DAPI staining.*

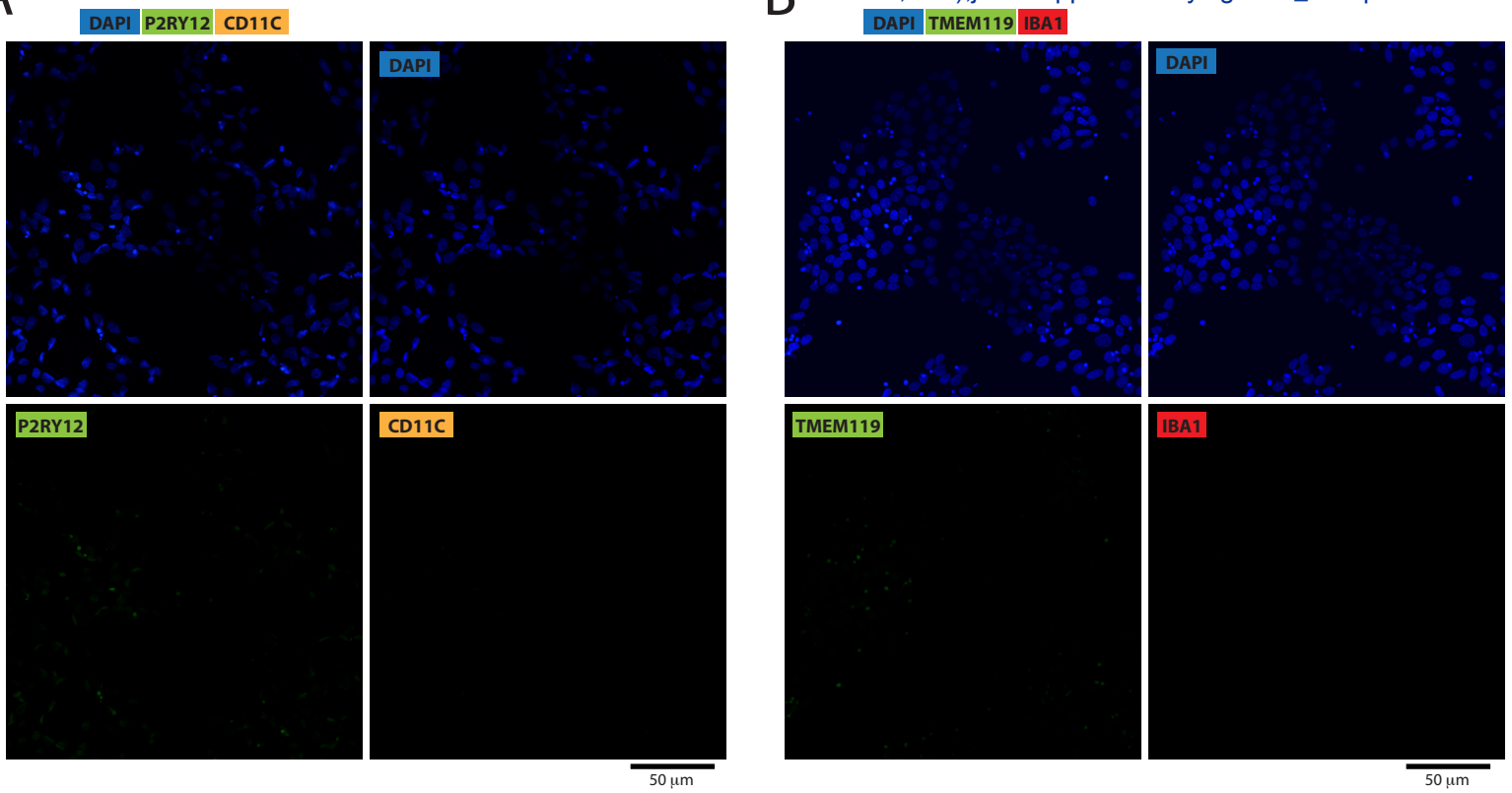
→ **We have adjusted the image so that the scale bar is clear.**

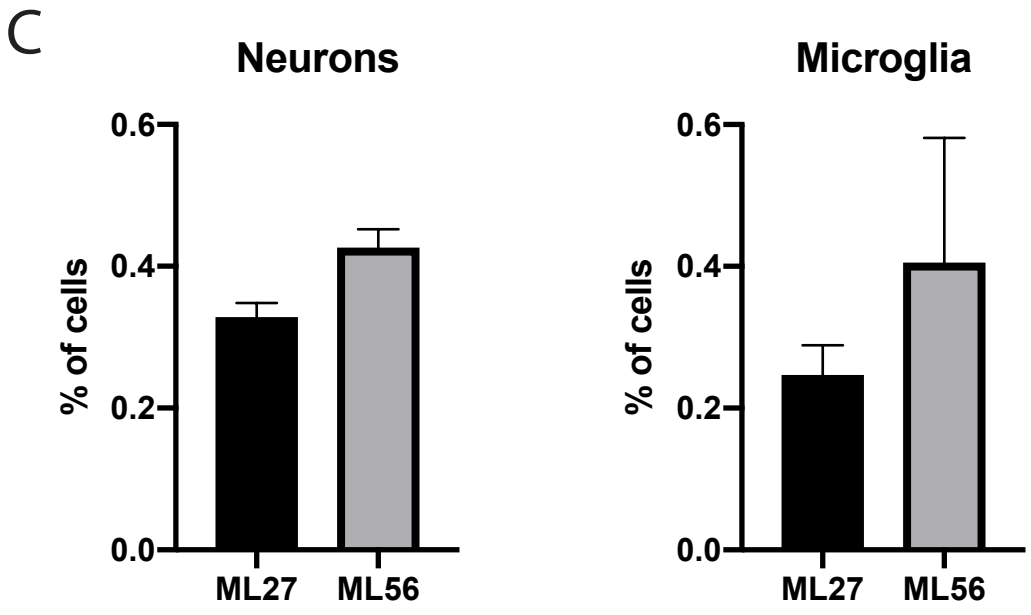
6. *Size and style of sclale bars are very different. Please standardize it!*

→ **We have standardized the size and style of the scale bars.**

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);jove supplementary figure 1_final.pdf





Supplementary Figure 1. A. Control iPSCs stained Microglia specific antibodies CD11c, P2RY12 and B. TMEM119 and Iba1 to ensure these antibodies do not stain non-microglia cells. Control iPSC line did not exhibit any positive staining for these markers. C. Co-cultures analyzed by cell type visible by specific line. Co-cultures were stained with a neuronal marker MAP2 or microglial marker P2RY12.

Primary antibody	Type	Dilution	Source	Catalogue #
Bassoon	Mouse monoclonal	1/300	Synaptic Systems	141 011
CD11C	Mouse monoclonal	1/200	Biolegend	337207
Ctip2	Rat monoclonal	1/300	Abcam	ab18465
CUTL1/CUX1	Mouse monoclonal	1/200	Abnova	H00001523-M01
Homer	Chicken polyclonal	1/500	Synaptic Systems	160 006
IBA1	Goat polyclonal	1/500	Abcam	ab5076
MAP2	Guinea pig polyclonal	1/1000	Synaptic Systems	188 004
P2RY12	Rabbit polyclonal	1/200	Biolegend	848002
TMEM119	Rabbit polyclonal	1/1000	Abcam	ab185333

Supplementary table 1. List of primary antibodies used in this protocol and their optimal dilutions.

Primer	Forward Sequence (5'-3')	Reverse sequence (5'-3')
AIF1	TCAACAAGCAATTCCTAGAC	TTTTATCTCTGCTGTTCCTG
CX3CR1	AAATACCCCATCATTCATGC	TTGTTCCAAACGTTTCTAGG
ITGAM	GGGGTCTCCACTAAATATCTC	CTGACCTGATATTGATGCTG
ITGAX	GCCTGGATTATAAGGATGTC	TTGAAAAGCTAATCCAACCC
TMEM119	GTCCACAATATTCGTCAGTC	CTGGTGCATTATATCTCAGC
P2RY12	AAGAGCACTCAAGACTTTAC	GGGTTTGAATGTATCCAGTAAG
RPL32	GTGCAACAAATCTTACTGTG	CTGCCTACTCATTTTCTTCAC

Supplementary table 2. List of primers used for RT PCR experiments with forward and reverse seq

ıuences.