Title

Stem-cell-derived human microglia transplanted in mouse brain to study human disease

Authors

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Abstract

Microglia are critically involved in complex neurological disorders with a strong genetic component, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Frontotemporal dementia (FTD). While mouse microglia can recapitulate aspects of human microglia physiology, they do not fully capture the human genetic aspects of disease and they do not reproduce all human cell states. Primary cultures of human microglia or microglia derived from human iPSC are difficult to maintain in brain relevant cell states *in vitro*. Here, we describe MIGRATE (Microglia *In vitro* Generation Refined for Advanced Transplantation Experiments), providing a combined *in vitro* differentiation and *in vivo* xenotransplantation protocol to study human microglia in the context of the mouse brain. This article details an accurate step-by-step workflow that includes *in vitro* microglia differentiation from human pluripotent stem cells (PSCs), transplantation into the mouse brain and quantitative analysis of engraftment. Compared to current differentiation and xenotransplantation protocols, we present an optimized, faster and more efficient approach that yields up to 80% chimerism. To quantitatively assess engraftment efficiency by flow cytometry, access to specialized flow cytometry is required. Alternatively, the percentage of chimerism can be estimated by standard immunohistochemical analysis. The MIGRATE protocol takes ~40 days to complete from PSCs culturing to engraftment efficiency assessment.

Introduction

Phenotype equals genotype plus environment. This relationship holds particularly true for microglia, which adapt to their environment in the brain and change their phenotype according to specific developmental, physiological or pathological circumstances. Extensive transcriptomic studies covering a wide range of animal models and human samples have shed light on the unique heterogeneity of human microglia compared to other species, including primates[1,2,3]. In fact, a large percentage of human genes involved in Alzheimer's Disease (AD) lack good one-to-one mouse orthologues[4] and human microglia express genes relevant to human neurodegenerative disease that are not expressed by other mammals[1].

Primary human microglia can be isolated in limited numbers from surgical resections or postmortem tissue. However, once removed from the brain, they undergo rapid and profound transcriptomic and phenotypic changes[5,2]. Several labs have recently developed protocols to differentiate microglia *in vitro* from pluripotent stem cells (PSCs)[6–12] (*see* Timmerman et al., 2018; Pocock & Piers, 2018; Haenseler & Rajendran, 2019 *for detailed review*[13–15]). These protocols have many strengths, including the production of high numbers of microglia-like cells, relatively easy handling and implementation, and the potential of working with specific disease-associated variants or developing genome-wide genetic screenings using CRISPR/Cas9 technology. However, similarly to the culture of primary microglia, PSC-derived microglia *in vitro* do not fully recapitulate microglia transcriptional signatures *in vivo*[4,16,17]. Thus, while human microglia derived from stem cells solve the genotype part of the equation, it is essential to provide them with yet unknown factors only present in the CNS microenvironment to obtain appropriate phenotypes[4,16,17]. The brain cellular environment is also critical to study the complex cellular interactions that drive diseases such as AD[18,19]. As such, our[4] and other groups[17,20,21] have recently developed protocols for xenotransplantation of human stem cell-derived microglia into the mouse brain.

Here, we present an optimized version of the protocol described in our original study[4]. We show that, by the introduction of a few minor technical modifications to the *in vitro* differentiation protocol, a remarkable improvement in engraftment efficiency is achieved. We name this protocol "Microglia *In vitro* Generation Refined for Advanced Transplantation Experiments", abbreviated as "MIGRATE".

Development of the Protocol

An increasing number of laboratories have developed protocols to differentiate microglia *in vitro* from PSCs in the past 5 years[6–12,22]. Several of these methods were based on earlier studies describing the generation of monocyte- or macrophage-like cells from PSCs in feeder- and serum-free culture conditions[23,24], which in turn built upon methods developed for the generation of myeloid cells[25–27].

All current protocols of microglia xenotransplantation are based on earlier studies of human macrophage xenotransplantation[28]. The first proof-of-concept of xenotransplantation of *in vitro* generated human microglia-like cells into the mouse was provided by Abud et al.[9], using the complex MITRG mice (*M-CSF*$^{h/h}$ *IL-3/GM-CSF*$^{h/h}$ *SIRPa*$^{h/h}$ *TPO*$^{h/h}$ *RAG2*$^{-/-}$ *IL2Rg*$^{-/-}$; The Jackson Laboratory, #017711). Since then, this approach has been considerably simplified and, of critical importance, now uses immunodeficient mice expressing the human variant of *CSF1* that is required for the survival of human myeloid cells, as the murine homologue does not bind the human receptor[28]. Although the MITRG mice express multiple human growth factors, Hasselmann et al. have shown no differences between this model and *hCSF1*^{KI} mice[17] (*Rag2*$^{-/-}$ *IL2rγ*$^{-/-}$ *hCSF1*^{KI} ; The Jackson Laboratory, #017708). We[4] and others[20,21] have further confirmed that humanization of CSF1 is sufficient for human microglia integration into mouse CNS. Of note, there is no evidence that *IL2ry* deficiency is needed for human cell transplantation, but this mouse is the only commercially available option. After transplantation, the PSC-derived microglia colonize large areas of the brain and recapitulate the transcriptomic signature of primary human microglia isolated from human brain obtained during neurosurgery[4,17,20,21]. These studies implemented different *in vitro* protocols for the generation of human microglia, resulting in diverse engraftment efficiencies, summarized in **Table 1**[7,12,22,24].

MIGRATE is a further refined version of one of these microglia xenotransplantation protocols, where we used microglia derived from embryonic stem cells and *Rag2*$^{-/-}$ *Il2rγ*$^{-/-}$ *hCSF1*^{KI} mice[4]. By introducing some minor modifications to the original cell culture protocol, we have succeeded in considerably improving the yield of microglial progenitors and chimera efficiency. By following the MIGRATE protocol, it is possible to mimic the *in vivo* ontogeny of human microglia.

Compared to our recently published work[4], the induction of the first stage (between Days 0-4) is now performed on embryoid bodies (EBs) generated in 96-well-plates (adapted from Van Wilgenburg et al.[24]**; Steps 8-17 of procedure, Fig. 1A-B and Extended Data Fig. 1**). The stem cells are plated in mTeSR1 media supplemented with BMP4 (for mesodermal commitment and primitive streak-like cell induction) and with VEGF and SCF (for the formation of hemangiogenic progenitors)[29]. The generation of hematopoietic cells is initiated at Day 4 by transferring the EBs to standard 6-well plates containing X-VIVO 15 media supplemented with SCF, M-CSF, IL-3, Flt-3 and TPO (adapted from Yanagimachi et al.[23]) (**Steps 17-21 of procedure, Fig. 1A-B and Extended Data Fig. 1**). For the final myeloid lineage, EBs are cultured in X-VIVO 15 media supplemented with Flt-3, M-CSF and GM-CSF, and they start producing visible progenitor cells that are ready to be harvested at Day 18 (**Steps 21-24 of procedure, Fig. 1A-B and Extended Data Fig. 1**).

Xenotransplantation of microglial progenitors into the mouse brain was performed as previously described, with a bilateral injection in newborns after 2 days of endogenous mouse microglia depletion with the CSF1R inhibitor BLZ945 (**Fig. 1A**). Compared to our formerly reported transplantation protocol[4], we increased the number of transplanted cells from 200,000 to 500,000 per mouse (see **Anticipated Results section**).

Applications of the method

The MIGRATE protocol can be used to address a variety of questions concerning normal physiology and pathology of human microglia. The technique could also be used to study *in vivo* genetic manipulation or pharmacological intervention, as well as investigate genotype-phenotype interactions with different patient-derived PSCs, which extends the use of this approach to a wide variety of conditions and experimental settings. This is of particular interest in complex neurological disorders with strong genetic components, such as AD, Parkinson's disease (PD) and Frontotemporal dementia (FTD). Cell lines carrying specific disease-related mutations or differentially loaded by polygenetic risk (as identified by single nucleotide polymorphisms (SNPs)) can be xenotransplanted into mouse models of neurological disorders to study how a human-specific genetic landscape impacts microglial biology *in vivo*. Recent work has shown that human microglia present major differences in response to AD compared to their mouse counterparts[4,17,30], as well as a unique heterogeneity and enrichment in expression of neurodegenerative disease-related genes compared to microglia from other mammals, including primates[1].

Specific applications of the protocol may include the study of various physiological and disease conditions of the brain. For instance, multiple microglial genes have been implicated in modulating AD risk[31–33]. As already indicated, a large proportion of these genes do not have good mouse orthologs or display a low similarity at the amino acid primary sequence[4]. For example, the APOE polymorphism, the major genetic risk factor for AD, does not exist in rodents, TREM2 shows low homology in protein sequence at the C-terminal domain, and there is no ortholog for CR1. Other genes, such as *CD33* and the *MS4A4* cluster, present many-to-many orthology, suggesting functional divergence[1,4]. It is clear that humanized systems are crucial to study the role of these microglial genes in AD pathogenesis, along with the possibility of exploring the contribution of polygenic risk carried by the complex genetic landscape of patient-derived cell lines.

Many other human diseases show a microglia component that can be studied with this approach. Nasu-Hakola disease, a genetically heterogeneous disease characterized by a combination of systemic bone cysts and dementia, is caused by homozygous missense mutations in TREM2 or its intracellular binding partner DAP-12 (*TYROBP*)[34] that affect osteoclasts and microglia. Also, in PD, it is now accepted that microglia-specific transcriptomic changes are associated with increased vulnerability and disease progression[35,36]. PD mutations including SCNA[37] and LRKK[38] affect microglia phenotypes and microglial-dependent mechanisms are involved in dopaminergic cell loss and α-synuclein pathology[39].

Excessive synaptic pruning by microglia contributes to AD[40] and schizophrenia[41,42]. Several of the genes involved in this process, such as those encoding components of the complement pathway, are mainly expressed in human microglia, limiting the potential of research in mouse models[2,41]. Induced microglia-like cells *in vitro* were employed to partially address these questions[42], but xenotransplantation models are likely to provide much more insight as the microglia are exposed to brain environmental cues and multicellular interactions.

A highly interesting application is the use of chimeric mice for the study of human-specific viruses with brain tropism. For example, HIV specifically infects human cells and causes encephalopathy with dementia symptoms. However, while there is ample knowledge on the impact of HIV on white blood cells in the blood circulation[43], little is known about how HIV affects the brain. Additionally, some cases of HIV-associated dementia involve the presence of macrophage-tropic virus[44]. Another example is the Zika virus, which causes a condition resembling Guillaume-Barré syndrome[45], but no mechanism has yet been established for viral entry and spread through the brain.

Comparison with other methods

The field of microglial biology and neuroimmunology is in rapid evolution. Although several methods are available for *in vitro* generation of human-stem-cell-derived microglia using a wide variety of culture conditions, media, and cytokines, there is no consensus yet on the most reliable approaches[13]. **Table 1** summarizes two main factors important for xenotransplantation experiments that vary across different protocols: yield of cells generated *in vitro*, and the percentage chimerism in the mouse brain.

Table 1. Comparison of human microglia xenotransplantation protocols

Xenotransplantation protocol	*In vitro* Protocol used	*In vitro* yield (times number of seeded PSCs)	Site of injection, number of engrafted cells and number of injection points	Graft efficiency	Timepoint and region
Mancuso et al., 2019	**Claes et al., 2018**	**0.5 times**	**From bregma: Anteroposterior, −1 mm; lateral, ±1 mm. 100k cells/injection (1 injection/side)**	**~ 9%**	**2 months (full brain)**
Hasselmann et al., 2019	McQuade et al., 2018	125 times	Lateral ventricles and overlying cortex. 62.5k cells/injection (4 injections/side)	~80%	2 months (CTX, HIP, STR)
Svoboda et al., 2019	Douvaras et al., 2017	2.2 times	Lateral ventricles. 4-500k cells/injection (1 injection/side)	~27%	4 months (full brain)
Xu et al., 2020	Van Wilgenburg et al., 2013	10 times	From bregma midline, posterior -2.0 mm, dorsoventral depth = −1.5 and −1.2 mm, midline = ±1.0 mm. 100k cells/injection (2 injections/side)	~ 8%	6 months (full brain)
Fattorelli & Martinez-Muriana et al.	**MIGRATE**	**10 times**	**From bregma: Anteroposterior, −1 mm; lateral, ±1 mm. 250k cells/injection (1 injection/side)**	**~ 60-80%**	**2.5-6 months (full brain)**

General procedure

Compared to ours[4] and others[17,20-21] previously published protocols, MIGRATE first generates EBs using 96-well-plates (adapted from Van Wilgenburg et al.[24]) to provide

reproducibility and consistency in EB formation, number and size. Consistent EB formation ensures reliable yields of microglial progenitors across multiple differentiation inductions and cell lines. When inducing hematopoiesis and microglia differentiation, MIGRATE and other methods[20,21] use similar cytokine cocktails with slightly different timing/combinations, while Hasselmann et al.[17] use a ready-to-go commercially available kit. Using commercial kits presents practical advantages, but we prefer home-made kits as all the components are fully known and therefore it is possible to adapt the protocol according to any circumstances. All available protocols engraft macrophage/microglia-like progenitors during early postnatal stages (P0-P4). However, MIGRATE requires a reduced number of injections: one per brain hemisphere (*see Table 1 for detailed comparison*).

Yield of microglial progenitors

The MIGRATE protocol results in high yields of microglial progenitors, with production levels of approximately 10-times the number of seeded stem cells (11,2±4.8 - mean±SD- million cells/induction across 6 independent MIGRATE differentiations used in this study). As such, our protocol is more time- and cost-effective compared to a number of protocols previously used for xenotransplantation[7,12]. Other methods, such as Van Wilgenburg et al. and McQuade et al., provide yields similar to MIGRATE[22] or higher[24].

Engraftment efficiency

MIGRATE achieves high, long-lasting (60-80%) human chimerism shortly after transplantation into the mouse brain. We quantified the relative fraction of human engrafted cells over the total microglial content and found that at postnatal day 21 the percentage chimerism was 33±7% (**Fig. 4, see *Anticipated Results*)**, which increased up to 80% at 3-6 months of age (**Fig. 5, see *Anticipated Results***). The minor modifications introduced to MIGRATE resulted in a significantly improved performance compared to the original protocol by Mancuso et al. 2019 (~9% at 2 months). This is also the case for other published studies[20,21], based on Van Wilgenburg et al.[24] and Douvaras et al.[7] differentiation methods, which reported 8% of chimerism at 6 months[21], and 27% at 4 months, respectively[20]. Hasselmann et al.[17] reported a similar graft efficiency compared to MIGRATE (~80% human cells in specific brain regions in 2-months old animals).

Overview of the procedure

The MIGRATE protocol for xenotransplantation of human-derived microglia involves *in vitro* differentiation of human stem cells to microglia and their transplantation into new-born immunodeficient mice (**Fig. 2**). MIGRATE consists of an initial *in vitro* stage including stem cell culture (**Steps 1-6**) and induction of MIGRATE differentiation protocol (**Steps 8-24**), coordinated with *in vivo* work such as mice coupling (**Step 7**), endogenous mouse microglia depletion (**Steps 25-26**) and engraftment of human microglial progenitors (**Steps 27-33**). After human-derived microglia transplantation, the efficiency of chimerism can be assessed by flow cytometry (**Steps 34-50**) or by histochemical analysis (**Supplementary information**).

Experimental design

Stem cells culture

Stem cells need to be in culture and ready to use before starting MIGRATE protocol (**Steps 1-6**). Stem cells are thawed and seeded at a density of ~50,000 cells/cm^2 to ensure that they grow and reach the required confluence at Day 0. Typically, we start from 1 well of a 6 well-plate containing 70-80% confluent stem cells at Day 0 (**Fig. 1B**). At that moment, stem cells

are in their log phase of expansion and the recovery rates after detachment are over 1 million viable cells (we need at least 1M stem cells to start a full 96-well plate of EBs). Hence, higher or lower confluence rates may affect recovery yields and stem cell proliferative capacity, and may impact the number of wells that can be seeded in **Steps 8-13** and adequate formation of the EBs. In the MIGRATE protocol, we have standardized the number of seeded cells to 10-15k per well in a 96-well plate. Plating <10k cells will result in small EBs that will be lost during media changes. Seeding >15k cells will produce larger EBs and significantly higher amounts of dead cells without increasing microglial progenitors yields at day 18.
It is of fundamental importance to ensure the fitness of the stem cells before starting the MIGRATE protocol, especially regarding their pluripotent status. This is of particular importance if the cells are genetically modified. Common techniques include Alkaline phosphatase expression, immunocytochemistry to test for pluripotency markers staining (i.e. Oct4, Nanog, Sox2 and others), qPCR or complementary techniques (e.g. teratoma assay or bioinformatics methods). Extensive information on stem cells adequacy and fitness is described by Martí et al.[46].

Manipulation of embryoid bodies
During Days 0 to 3, we induce EB formation by seeding a cell suspension of stem cells in a low-adherent U-form 96-well plate. We change 75% of the medium every day, carefully removing dead cells. We first carefully refresh 50% of the media using a multichannel pipette. After completion of the first wash, we turn the plate by 180 degrees and repeat the procedure. This rotation ensures that dead cells are removed uniformly and EBs are less stressed. Of note, media change must be always performed on the side of the well to avoid touching the EB (**Step 14**).

On Day 4, EBs are carefully transferred from a 96-well plate to a 6-well plate. Here, EBs are individually collected using large orifice P1000 pipette tips. Alternatively, regular 1000 pipette tips can be cut to increase tip diameter, ensuring that EBs can go through. The use of standard tips or lower volume pipettes will compromise EBs viability (**Steps 15-17**).

From Day 4 on, media changes will be performed in a 6-well plate. To properly handle EBs and avoid losing them during these steps, we recommend collecting the culture medium and all the floating EBs into a 50 mL tube. To avoid any damage to the EBs, we collect them using a 25 mL serological pipette. Once transferred, EBs will sink by gravity accumulating in the bottom of the falcon and culture medium can be carefully removed. Of note, it is possible to leave some remaining media (<2 mL) in the falcon containing the EBs. Freshly prepared media can be added now to the 50 mL falcon, and EBs and media can be further transferred back to the same 6-well plate (**Steps 17-21**).

For microglial progenitors' collection at Day 18, EBs and medium containing floating precursors are passed through a sterile reversible cell strainer (37 μm) and collected into a 50 mL tube. In this step, only microglial precursors will pass through the filter whereas EBs will remain at the top of the strainer. Instead of cell reversible strainers, microglial progenitors can alternatively be collected by letting the EBs sink by gravitation to the bottom of a 50 mL tube, and collecting the supernatant. After centrifugation of collected medium at 300 rcf for 5 minutes, the pellet of microglial progenitors can be resuspended in an appropriate volume for transplantation (**Steps 22-24**).

Cytokines and growth factors preparation and maintenance
Stocks of cytokines and growth factors are prepared using a solution of sterile DPBS with 0.1% BSA (1/100). All the reagents are kept at -80 ºC up to 12 months after reconstitution. Cytokines and growth factors are highly sensitive to temperature changes and they lose

efficacy upon thawing. To avoid thawing-freezing cycles, we aliquot all the reagents in appropriate volumes for single use.

Coordination of new-born pups and microglia differentiation
Coordination of plug-checks and *in vitro* microglial progenitor differentiation is a challenging part of the protocol to ensure that new-born pups are available at the point of cell collection and transplantation. Hence, six days before starting the MIGRATE protocol, we couple the animals and confirm successful mating by plug-checking (**Step 7**). Five days after positive plug-checks, we start Day 0 of the MIGRATE protocol (**Step 8**). If mice coupling does not yield positive plugs, stem cells can be passaged and kept in culture for the upcoming week, when we repeat the procedure. Using this strict timeline, pups will be born on Day 14. CSF1R inhibitor BLZ945 is then provided at day P2 and P3, and graft microglial progenitors can be injected at P4, which is day 18 of the MIGRATE protocol (**Steps 25-33**).

Xenotransplantation of microglial progenitor cells
Proper equipment (see Materials section), ethical procedures and SOPs are needed for the xenotransplantation of microglial progenitors into immunodeficient mice. The total number of pups to be injected mostly depends on the type of experiment to be performed. We have found the success rate of human cells integration to be reproducible between different cell lines, and consistent within litters and session of transplantation. With the percentage of chimerism achievable with the MIGRATE protocol already at 2-3 months of age, we found that 0.2M to 0.8M human microglia cells can be recovered from a full brain. Nevertheless, we cannot exclude that a particular cell line that was not tested in this study may fail efficient engraftment into the mouse brain. To rule out this possibility, we suggest planning a pilot experiment to test the MIGRATE protocol with the cell line of interest and to prove its successful xenotransplantation from at least two independent inductions.

According to the specific application and the number of replicates needed for an investigation, the number of pups to inject can be estimated beforehand. We recommend to always include a few extra transplanted pups to test engraftment efficiency of a litter and to account for the potential death of some animals unrelated to the experiment.

Limitations

The main limitation of the MIGRATE protocol is that suitable microglial progenitors are limited to collection on Day 18. Similarly to any iPSCs differentiation protocols, the MIGRATE protocol results in some variability across multiple inductions, showing differences in microglial progenitor yield at Day 18 (11.2±4.8 million cells/diff). However, this inter-differentiation variability only limits the number of pups that can be grafted at the moment of collection, without affecting engraftment efficiency *per se*. Despite having shown successful engraftment of three independent cell lines (one hESCs and two different hiPSCs, *see Materials for detailed information of the cell lines*), we cannot rule out the possibility of inter-cell line variability and unsuccessful engraftment of other hiPSCs due to yet unknown factors. Therefore, we encourage researchers to run pilot experiments to confirm the suitability of the particular cell line of interest.
Cryopreservation of microglial progenitors would be ideal when performing xenotransplantation studies, but we have not succeeded in our endeavours, despite various attempts. We have tried freezing and thawing cells at Day 18 using several different dedicated freezing media (e.g. BAMBANKER®, Wako cat. no. 302-14681; CryoStor® CS10, Stemcell

technologies cat. no. 07930) but, in our hands, the viability of the microglial progenitors drops drastically upon thawing.
All available grafting protocols[4,17,20,21] are limited in their ability to evaluate the percentage of chimerism before processing the tissue. This limitation makes it difficult to control inter-animal variability. We have found, however, that reproducibility is quite good within one transplantation session and we provide a description for the evaluation of graft efficiency in three weeks old mouse brain by flow cytometry analysis.
It is important to highlight that xenotransplantation experiments include stem cell maintenance, differentiation protocol and immunodeficient animals' husbandry and, therefore, are high-cost procedures.

Materials

Biological materials

- Human embryonic stem cells (hESCs) H9 (WA09) (WiCell Research Institute, Inc., RRID:CVCL_9773)
- Human induced-pluripotent stem cells (hiPSCs) BIONi010-C-2 (Bioneer, EBiSC Cat# BIONi010-C-2, RRID:CVCL_II81)
- Human induced-pluripotent stem cells iPSC Epithelial-1 (Sigma-Aldrich, cat. no. IPSC0028-1VL) (ECACC Cat# 66540499, RRID:CVCL_EE38)
- *Rag2*$^{-/-}$ *IL2rγ*$^{-/-}$ *hCSF1*^{KI} mice obtained from Jacksons Labs (strain 017708). Mating for experiments was performed by housing mice in trios (1 male per 2 females). Higher rates of positive pregnancies are achieved when working with young mice.

▲ **CRITICAL** *All experiments involving live vertebrates should be performed according to national and institutional regulations, laws and guidelines. All the experiments performed in this study were performed in accordance with the local Ethical Committee of Laboratory Animals of the KU Leuven (government license LA1210591, ECD project number P177/2017) and European Union guidelines.*

▲ **CRITICAL** *The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma. Cell lines should not be listed in the misidentification and cross-contamination database provided by ICLAC.*

▲ **CRITICAL** *All experiments involving the use of human samples and human stem cells must be performed in accordance with the relevant institutional and national regulations. In all cases, cell lines generation from donated patients cells and use for research need to be in compliance with published protocols, which include the obtaining of informed consent for donation from living donors and the approval of the whole donation process by the relevant Ethical Committee.*

Reagents

- 10X PBS (Phosphate Buffered Saline), pH 7.4 (VWR, cat. no. 75801-002)
- 2-Mercaptoethanol (50 mM) (ThermoFisher, cat. no. 31350010)
  ! CAUTION *2-Mercaptoethanol may sensitize the skin. Wear gloves, goggles and lab coat while manipulation.*
- Accutase® solution (Sigma, cat. no. A6964)
- Agarose Top Vision Low Melting point agarose (Thermo Scientific, cat. no. R0801)

- Alexa 594 donkey anti mouse IgG (H+L) (ThermoFisher, cat. no. A21203, RRID: AB_2535789)
- Alexa Fluor® 488 AffiniPure Donkey Anti-Chicken IgY (IgG) (H+L) (Jackson ImmunoResearch, cat. no. 703-545-155, RRID: AB_2340375)
- Anti IBA1 Polyclonal Antibody, Rabbit polyclonal IgG, 50µg supplied at 0.5mg/ml in TBS (Wako, cat. no. 019-19741, RRID: AB_839504)
- Anti-P2RY12 antibody produced in rabbit (Merck, cat. no. HPA014518-100UL, RRID: AB_2669027)
- Antibiotic-antimycotic (100x, ThermoFisher, cat. no. 15140122)
- Antibody (APC Anti-human CD45, 1:50, BD Biosciences, cat. no. 555485, RRID: AB_398600)
- Antibody (BV421 Anti-mouse CD45, 1:500, BD Biosciences, cat. no. 563890, RRID: AB_2651151)
- Antibody (PE Pan-CD11b, 1:50, Miltenyi, cat. no. 130-109-285, RRID: AB_2654645)
- BLZ945 100MG 953769-46-5 MFCD28142668 (Selleck cat. no. S7725-100MG)
- Corning® Matrigel® matrix, hESC (VWR, cat. no. BDAA356277)
- Dako Glycergel®, Aqueous Mounting Medium, 15 mL (Agilent, cat. no. C0563)
- DMEM/F12 (ThermoFisher, cat. no.10565018)
- Dolethal (Pentobarbital Sodium, Vetoquinol, cat. no. BE-V171692)
  ! CAUTION *Pentobarbital sodium is harmful if swallowed. If administered, pentobarbital sodium may induce dizziness, sleepiness and confusion. Manipulate wearing proper personal protection equipment. Seek medical advice in case of intoxication.*
- Donkey anti-Rabbit IgG (H+L) Alexa Fluor 488 (ThermoFisher, cat. no. A-21206, RRID: AB_2535792)
- Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (ThermoFisher, cat. no. A-21202, RRID: AB_141607)
- Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (ThermoFisher, cat. no. A-21207, RRID: AB_141637)
- Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (ThermoFisher, cat. no. A-31573, RRID: AB_2536183)
- DPBS 1x (Gibco, cat. no. 14190250)
- Essential 8™ Medium (ThermoFisher, cat. no. A1517001)
- Ethanol absolute ≥99.8%, AnalaR NORMAPUR® ACS, Reag. Ph. Eur. analytical reagent, 1L (VWR, cat. no. 20821.296)
- Ethylene glycol (Sigma-Aldrich, cat. no. 324558-1L)
- Ethylenediaminetetraacetic acid disodium salt solution 0.5M (Sigma Aldrich, cat. no. E7889-100ml)
- Formaldehyde solution 4%, buffered, pH 6.9 (Sigma-Aldrich, cat. no. 1004965000)
  ! CAUTION *Formaldehyde is a toxic and carcinogenic reagent. Upon contact and inhalation, formaldehyde might sensitize the skin, cause eye damage and induce respiratory issues. Wear protective gloves/protective clothing/eye protection/face protection and manipulate inside a fume hood.*
- Gibco™ Fetal Bovine Serum (Life Technologies, cat. no. 10270106)
- GlutaMAX™ Supplement (ThermoFisher, cat. no. 35050061)
- Heparin Leo 25000 IU/5 ml (Leo Pharmaceuticals Ltd., 5 ml vial)
- HuNu antibody Anti-Nuclei Antibody, clone 235-1 (Sigma-Aldrich, cat. no. MAB1281, RRID: AB_94090)
- MACS BSA Stock Solution, 75ml (Miltenyi, cat. no. 130-091-376)
- Methyl-β-cyclodextrin (Sigma-Aldrich, cat. no. C4555-5G)

- mTeSR™1 (Stemcell technologies, cat. no. 85850)
- Normal Donkey Serum (Bio connect, cat. no. 017-000-121)
- Percoll® PLUS, pack of 250 mL (GE Healthcare, cat. no. 17-5445-02)
- Recombinant Human BMP-4 50ug (Peprotech, cat. no. 125-05)
- Recombinant Human FLT3-ligand 50ug (Peprotech, cat. no. 300-19)
- Recombinant Human GM-CSF 50ug (Peprotech, cat. no. 300-03)
- Recombinant Human IL-3 50ug (Peprotech, cat. no. 200-03)
- Recombinant Human M-CSF (CSF1) 50ug (Peprotech, cat. no. 300-25)
- Recombinant Human SCF 50ug (Peprotech, cat. no. 300-07)
- Recombinant Human TPO 50ug (Peprotech, cat. no. 300-18)
- Recombinant Human VEGF165 50ug (Peprotech, cat. no. 100-20)
- RevitaCell™ Supplement (100X) (ThermoFisher, cat. no. A2644501)
- Rockinhibitor InSolution™ Y-27632 - Calbiochem (Merck, cat. no. 688001)
- STEM121 antibody (Takara Euro, cat. no. Y40410, RRID: AB_2801314)
- TWEEN® 20 Detergent - CAS 9005-64-5 - Calbiochem (Merck, cat. no. 655205-250ML)
- Viability dye (eFluor 780, ThermoFisher, 65-0865-14)
- X-VIVO™ 15 Serum-free Hematopoietic Cell Medium (Lonza, cat. no. BE02-060F)

Equipment

- 0.3ml Insulin Syringe with 30G x 8mm Needle [Pack of 100] (BD Micro-Fine, cat. no. 324826)
- 10 mL Serological Pipet, Sterile, Individually Wrapped (Pack of 500) (Sarstedt cat. no. 86.1254.001)
- 15 mL centrifuge tubes, polypropylene, conical bottom w/ plug seal cap, sterile, natural, 500/cs (Corning, cat. no. CLS430766)
- 20ml sterile disposable graduated eccentric luer slip syringe (pack of 120) (BD Plastipak, cat. no. 300613)
- 25 mL Serological Pipet, Sterile, Individually Wrapped (Pack of 200) (Sarstedt cat. no. 86.1685.001)
- 26s gauge, small hub RN NDL, 2 in point style 2, 6PK (Filter Service cat. no. 7758-02)
- 5 mL Serological Pipet, Sterile, Individually Wrapped (Pack of 500) (Sarstedt cat. no. 86.1253.001)
- 50 mL centrifuge tubes, polypropylene, conical bottom w/ plug seal cap, rack packed, sterile, natural, 25/rack, 500/cs (Corning, cat. no. CLS430290)
- 5ml Tube Flat Bttm w/Scrw Cap PP Sterile (Sarstedt, cat. no. 60.558)
- 6-well plates: Cell Culture Multiwell Plate, 6 Well, Ps, Clear, Cellstar®, Tc, Lid With Condensation Rings, Sterile, Single Packed (Greiner Bio-one, cat. no. 657160)
- Addision forceps-1x2 teeth/straight/12cm
- Bone Forceps (FST, cat. no. 16060-11)
- Countess II Automated Cell Counter (Life Technologies)
- Countess™ Cell Counting Chamber Slides (Invitrogen, cat. no. C10228)
- Dumont #15a bone cutting forceps - carbon steel/11.5cm (FST, cat. no. 11215-01)
- Dumont #5 - Forceps (FST, cat. no. 11251-10)
- Easystrainer 70 Mm, For 50 Ml Tubes, For Tubes 227xxx/210xxx, Blue, Sterile, Single Packed (Greiner Bio-one, cat. no. 542070)
- Fine Scissors - ToughCut®/Straight/Sharp-Sharp/11.5cm (FST, cat. no. 14058-11)

- Graefe forceps (FST, cat. no. 11051-10)
- Hamilton syringe 1701 RN - 10 µl- without needle (Filter Service cat. no. 7653-01)
- Infraroodlamp 1R 11 (Fiers, cat. no. X435.1)
- MACSQuant® Tyto® Cartridges (Miltenyi, cat. no. 130-104-791)
- MACSQuant® Tyto® Cell Sorter (Miltenyi)
- Mayo Scissors - ToughCut®/Straight/Blunt-Blunt/15cm (FST, cat. no. 14110-15)
- Microplate, 96 well, U-bottom, Cell-Repellent Surface (Greiner Bio-one, cat. no. 650970)
- Nikon's stereoscopic microscope with 7.5x zoom and 115 mm working distance (Nikon, cat. no. SMZ745)
- No 23 Sterile Carbon Steel Scalpel Blade (Swann Morton, cat. no. 0210)
- Noyes Spring Scissors (FST, cat. no. 15012-12)
- RAZOR BLADE HM650V (Thermo Scientific, cat. no. 152540)
- Rectangular cover glasses (VWR, cat. no. 631-0146)
- Reversible strainers 37um LARGE 25/box (Stemcell technologies cat. no. 27250)
- SAFETY Blood Collection Set + Luer Adapter 23G x 3/4" tubing length 12" (30 cm), single-packed, sterile, not made with natural rubber latex (Greiner Bio-one, cat. no. 450096)
- SC2157R Series Short Handle Open Stock Brushes (Dinasty, cat. no. SC2157R)
- Schott KL1500 LCD (Schott Cold Light Sources, cat. no. 150200)
- Standard Pattrern forceps (FST, cat. no. 11001-12)
- SuperFrost™ Microscope Slides (Thermo Scientific, cat. no. Thermo Scientific™ AAAA000084##32E00MNZ10MH#)
- Tip, Pipette, 1000µl, Wide Bore, Clear, Racked, Sterile (Axygen, cat. no. T-1005-WB-C-R-S)
- Trypan Blue Stain (0.4%) for use with the Countess™ Automated Cell Counter (ThermoFisher, cat. no. T10282)
- Vibratome vt1000 S (Leica)

Software

- FCS express 7, version 7.04.0014
- Fiji/ImageJ, version 2.0.0-rc-69/1.52p

Reagent setup

Stem cell media

Prepare the stem cell media by taking the appropriate amount of E8 basal or flex and adding E8 supplements (1/50 dilution) and antibiotics-anti-mycotics (1/100 dilution). E8 media can be prepared in advance and stored at 4°C. We recommend keeping the media no longer than 1 month at 4°C and regularly checking for potential contaminations.

mTeSR1 + BVS

Prepare the mTeSR1 media by taking the appropriate amount of mTeSR1 and adding mTeSR1 5x supplements (final concentration 1x). mTeSR1 media can be prepared in advance and stored at 4°C. We recommend keeping the media no longer than 1 month at 4°C and regularly checking for potential contaminations. We recommend adding BVS cytokines fresh (never store them at 4°C for longer than a week). To make mTeSR + BVS media, add

50ng/mL human BMP4, 50ng/mL human VEGF and 20ng/mL human SCF to the mTeSR1 media with supplements prepared in advance.

Differentiation medium + SMIFT
Prepare the differentiation media by taking the appropriate amount of X-VIVO 15 media and adding 2mM Glutamax, 100U/mL Antibiotics-anti-mycotics and 0,055mM 2-Mercaptoethanol. Differentiation media can be prepared in advance and stored at 4°C. We recommend keeping the media no longer than 1 month at 4°C and regularly checking for potential contaminations. We recommend adding SMIFT cytokines fresh (never store them at 4°C for longer than a week). To make differentiation media + SMIFT, add 50ng/mL human SCF, 50ng/mL human M-CSF, 50ng/mL human IL3, 50ng/mL human FLT3 and 5ng/mL human TPO to the differentiation media prepared in advance.

Differentiation medium + FMG
We recommend adding FMG cytokines fresh (never store them at 4°C for longer than a week). To make differentiation media + FMG, add 50ng/mL human FLT3, 50ng/mL human M-CSF and 25ng/mL human GM-CSF to the differentiation media prepared in advance.

FACS buffer
Prepare fresh FACS buffer by taking 500ml PBS 1x (*sterile from Tissue Culture)* and adding 2mM EDTA (2ml in 500ml PBS) and 2% FCS (10ml in 500ml PBS). FACS buffer can be stored at 4°C up to 1 month.

Miltenyi adult brain dissociation kit (130-107-677)
Following manufacturers' instructions, prepare enzymatic digestion cocktails and store them appropriately.

Isotonic Percoll solution (SIP)
Always prepare Isotonic Percoll solution fresh upon use. Add 9 parts percoll to 1 part of 10x PBS (without Ca2+ and Mg2+) to make SIP solution. Vortex the solution thoroughly. Proceed to preparing the working 30% working percoll solution by mixing 3ml of SIP with 7 ml of cold FACS buffer. The final volume is 10ml for each sample. Vortex the solution well and keep on ice until usage. Do not store the leftovers, but prepare fresh for every new experiment.

Procedure

▲ **CRITICAL** *Human microglial precursors are transplanted into the brain of immunodeficient mice. It is therefore essential to avoid any kind of bacterial or viral infection, including opportunistic pathogens. As such, immunodeficient mice cannot be handled, transplanted nor housed together with immunocompetent mice, and require adapted facilities.*

▲ **CRITICAL** *Cell lines should be regularly checked to ensure that they are not infected with mycoplasma.*

Stem cells thawing and culturing on Matrigel-coated plates (Days -7 to 0) ● **TIMING 7 days**

1. On **Day -7**, aliquot hMg according to manufacturers' recommendations (considering batch-to-batch variations, usually ~270-280ul/aliquot). Thaw a vial of hMg and place it on ice immediately.

2. Prepare 12.5mL of DMEM/F12 media (enough for coating two full 6-well-plates) and quickly resuspend the thawed hMg in DMEM/F12 media (~1/50 dilution). Distribute 1mL of DMEM/F12 + hMg per well, and leave for 30-60 mins at room temperature to dry.

   ■ **PAUSE POINT** *Matrigel-coated plates can be stored for a few days at 37ºC, or up to two weeks at 4ºC if covered in parafilm.*

3. Once hMg-coated plates are ready, thaw a vial of stem cells (freeze ~1 million cells/vial) by leaving the tube containing stem cells for 2 min in a water bath at 37ºC. After thawing, take cells up with a p1000 pipette and transfer them directly to a 15 mL falcon tube with 3mL of Stem cells media (see **Reagent set-up**) and spin them down at 300 rcf for 5 min.

   ■ **PAUSE POINT** *Stem cell media can be prepared and stored at 4 ºC for up to two weeks.*

   ▲ **CRITICAL STEP** *Don't leave cells for too long in the water bath or cell viability will be seriously compromised. Avoid repeated pipetting to ensure optimal cell viability after thawing.*

4. After spinning, remove the supernatant (3 mL) and resuspend the cell pellet in 3ml or 6ml of Stem cells media + RevitaCell (1/100) depending on the size of the pellet. Shake the plate (8-shaped shaking) to thoroughly disperse cells in the wells and incubate at 37ºC, 5% CO_2.

   ▲ **CRITICAL STEP** *If the pellet is very small, resuspend in 3ml and plate in one well only. Otherwise, consider dividing cells into 2 wells to avoid splitting too soon as this will stress the cells.*
   ? TROUBLESHOOTING

5. On **Day -6**, carefully aspirate the stem cell media plated on Day -7 and fully refresh 3 ml of Stem cells media without RevitaCell.

▲ **CRITICAL STEP** *Media must be fully changed as RevitaCell may induce toxicity. For stem cells maintenance, fully refresh 3 mL of Stem cells media every other day until Day 0.*

6. (OPTIONAL) *Splitting cells*. If stem cells reach confluency before Day 0, split cells into new 6-well hMg-coated plates by following these steps:

- Remove stem cell media (3 mL) and add 1 ml EDTA 0.5mM.
- Further incubate EDTA 0.5% for 5 min at room temperature.
- After incubation, carefully remove the EDTA 0.5mM and add 1 mL of Stem cells media + RevitaCell (1/100) and detach the colonies by scrapping the well with a cell scraper.
- Take 0.5 mL of Stem cells media + RevitaCell (1/100) containing the stem cells colonies and transfer them to a new well with 3 mL of Stem cells media + RevitaCell (1/100).
- Shake the plate (8-shaped shaking) to thoroughly disperse cells in the wells and incubate at 37ºC, 5% CO_2.

▲ **CRITICAL STEP** *Do not forget to fully refresh the Stem cells media + RevitaCell the day after splitting to Stem cells media only. RevitaCell can only be added up to 18-24h upon splitting/thawing according to manufacturers' instructions.*

Mice coupling and plug check (Days -6 to -5)
● **TIMING 2 days**

7. On **Day -6**, couple the appropriate number of mice needed to ensure that at least 1 female will be pregnant. On **Day -5**, plug check all coupled females and confirm that they are plug positive.

▲ **CRITICAL STEP** *If none of the females are plug positive, do not start MIGRATE protocol. Postpone the experiments for 1 week and start over.*
? TROUBLESHOOTING

MIGRATE protocol (Days 0 to 18) ● **TIMING 19 days**

8. On **Day 0**, collect cells from one 70-80% confluent well of the cultured plates by removing the Stem cell media (3 mL), adding 1mL of accutase and incubating for 5 min at 37ºC. After incubation, add 3 mL of 1x DPBS to inactivate accutase enzymatic activity.

▲ **CRITICAL STEP** *At this point, stem cells should exhibit around 70-80% confluency. Higher or lower confluence rates may affect recovery yields, stem cell proliferative capacity, and formation of the EBs.*
▲ **CRITICAL STEP** *Do not incubate longer than 5 min with accutase. Viability of the stem cells may be compromised.*
▲ **CRITICAL STEP** *Gently pipette the cells after accutase application, in order to ensure the formation of a single-cell suspension.*

9. Gently flush the well to collect all detached cells and transfer everything to a new 15 mL falcon tube. Take an aliquot to count cell suspension in a haemocytometer while centrifuging the collected cells for 5 min at 300 rcf, room temperature.

10. After spinning down, take out the supernatant (~ 4 mL) and resuspend pelleted stem cells in the required volume of mTeSR1 + BVS (see **Reagent set-up**) to get a final concentration of 10^6 cells/ml.

■ **PAUSE POINT** *mTeSR1 and 1x supplements (50 mL in total) can be prepared on Day 0 and then used for the rest of the procedure (Day 0 to 4) if kept at 4 ºC. However, cytokines must be freshly added every day.*

11. Take out 1 mL of stem cells suspension in mTeSR1 + BVS and resuspend gently in 10 mL of mTeSR1 + BVS + 10µM Rock inhibitor to get a final concentration of 10^5 cells/mL.

12. Transfer the cell suspension in mTeSR1 + BVS + 10 µM Rock inhibitor (11 mL) into a reagent reservoir. Using a 8 or 12-well multichannel pipette, plate 110 µL of stem cell suspension into each well of a 96-well U-bottom ultra-low adherence plate. After plating, spin down the 96-well plates at 100 rcf for 3min at room temperature.

▲ **CRITICAL STEP** *To avoid contamination during centrifugation, cover the plate sides with a layer of parafilm.*
▲ **CRITICAL STEP** *Gently handle the plate after centrifugation to avoid resuspension of the pelleted cells at the bottom of the U-shaped 96 well plate.*
? TROUBLESHOOTING

13. After centrifugation, check under the microscope that the stem cell suspension has clustered together into the bottom of the well (**Extended Data Fig. 1**). Gently put the 96-well plate in the incubator at 37ºC, 5% CO_2.

14. Every day from **Days 1 to 3**, change the media of the 96-well plates where EBs are growing by following these steps:

- Prepare 10 mL of fresh mTeSR1 + BVS and pour into a plastic bucket adequate for multichannel pipettes.
- To change the media, first remove 50 µl using a 8 or 12-wells multichannel pipette.
- Add 50 µl of fresh mTeSR1 + BVS.
- Repeat this procedure for a total of 75% medium change. For the second media change, rotate the plate 180º to ensure a complete removal of dead cells on both sides of the embryoid bodies.

▲ **CRITICAL STEP** *Imprecise media changes may result in loss of the embryoid bodies. To avoid such loss, carefully drip down on the side of the well while collecting the media from the well. Avoid by all means placing the tip of the pipette in the middle of the well, where the embryoid bodies are positioned.*
▲ **CRITICAL STEP** *Although it is normal to observe some evidence of cell death during these stages, dead cells and cell particles should be removed by daily media changes and there should not be high numbers of floating cells between Day1 and Day4. Elevated cell death is correlated to lack of EB growth and is an indicator of failure of the protocol, so the differentiation should be stopped.*
▲ **CRITICAL STEP** *For the MIGRATE protocol to be successful, EBs should be of spherical shape and have a diameter of ~400-500um at Day1, growing up to 700-800um at Day4 (representative pictures are provided in **Extended Data Fig.1**).*

15. On **Day 4**, collect the embryoid bodies one-by-one using a p1000 pipette and p1000 tips with large orifice (*see materials sections for specific details*). For harvesting:

- Place the p1000 tip on the centre and bottom of the well
- Carefully aspirate the media and the EB, and immediately transfer everything to a 50 mL falcon tube.
- Repeat this procedure in all the wells of the 96-well plate.
- Check under the microscope that all the embryoid bodies have been collected.

▲ **CRITICAL STEP** *Usage of regular p1000 tips may damage the EBs and impair the production of microglial progenitors. Always use p1000 tips with large orifice. If the required p1000 tips are not available, alternatively cut the tip of regular p1000 tips to make the diameter wider.*
? TROUBLESHOOTING

16. Let the EBs in the 50 mL falcon tube sink by gravity for a couple of minutes. Once they have sunk, carefully remove the medium (~ 8-9 mL of **mTeSR1 + BVS**) using a 10 mL serological tip without taking out the embryoid bodies. To avoid EB loss, some medium (<2 mL) can be left in the 50 mL falcon tube.

17. Using a 25 mL serological pipette, resuspend the EBs in 20 mL of fresh Differentiation medium + SMIFT (see **Reagent set-up**) and plate 4 mL/well containing ~20 EBs/well in a 6-well plate (no coating is applied to the plate from this step onwards). Note that only 5 out of 6 wells of a 6 well plate will be used.

■ **PAUSE POINT** *X-VIVO and supplements (antibiotics-anti-mycotics, 2-Mercaptoethanol and Glutamax) can be prepared for the upcoming media changes (~60 mL in total/6wp) and stored at 4°C. The X-VIVO media can be stored at 4°C for up to 1 month, but the media needs to be regularly checked to ensure no contamination has occurred. Cytokines must be freshly added at the moment of the media change.*

▲ **CRITICAL STEP** *To ensure an equal distribution of the EBs across the wells, EBs can be further distributed using a p1000 pipette and wider tips. Ideally, about 20 EBs should be distributed per well of a 6-well plate. Significantly higher or lower EB density is not recommended. If EBs are lost during the process, adjust to ~20 EBs/well by plating a lower number of wells.*
! CAUTION *2-Mercaptoethanol may sensitize the skin. Wear gloves, goggles and lab coat while handling.*

? TROUBLESHOOTING

18. On **Day 8**, use a 25 mL serological pipette to take up the medium of the 6-well plate containing EBs very carefully and transfer everything to a 50 mL falcon tube. Some of the bodies will be attached to the bottom of the 6-well plate; some will not. Attached EBs can be left in the wells of the 6-well plate. If floating EBs are picked up, collect them in a 50 mL falcon tube and let them sink by gravity as described in **Steps 16-17.**

19. Use a 25 mL pipette to take out the media (~ 18 mL of **Differentiation medium + SMIFT**) from the 50 mL falcon without collecting the sunken EBs.

20. Using a 25 mL serological pipette, resuspend the EBs in 20 mL of fresh **Differentiation medium + SMIFT** and plate 4 ml/well with floating EBs back in the same 6-well plate.

▲ **CRITICAL STEP** *Keep an equal distribution of the EBs across the 6-well plate, as explained in step 17.*

21. On **Day 11**, perform a media change by repeating the same procedure as in **Steps 18-20**. In this case, remove medium (~ 18 mL of **Differentiation medium + SMIFT**) and resuspend in 20 mL fresh Differentiation medium + FMG (see **Reagent set-up**). Over the following 7 days, progenitors will accumulate in the media, which is not changed until Day 18.

22. On **Day 18**, check that microglial progenitors are visible in the supernatant, and are ready to be harvested. To collect them, take up the medium (~ 20 mL of **Differentiation medium + FMG**) and pass it through a cell strainer (37 µm; only microglial progenitors will pass through it), collecting the cell suspension in a 50 mL falcon tube.

▲ **CRITICAL STEP** *To facilitate the strainer inversion, while this is still sitting on the tube that collected microglial progenitors, place the new 50mL tube upside down on top of the strainer, then invert. The EBs should then stay inside the new tube.*

23. Take an aliquot of the harvested microglial progenitors and count in a haemocytometer. In the meantime, spin down at 300 rcf for 5 min.

24. Before xenotransplantation, remove as much of the medium as possible and resuspend the cells in 1x PBS in an appropriate volume for a final concentration of 250k cells/µL. Place the cells immediately on ice and proceed to the animal facility for grafting.

▲ **CRITICAL STEP** *The pellet usually needs to be resuspended in a small volume of PBS (10-50 µl, depending on the yield of the differentiation). Start by adding only half of the final required volume of PBS on top of the cell pellet, as this will increase once the cells are resuspended, then adjust accordingly to reach the required concentration. This will avoid overdiluting the cell suspension.*

Transplantation of human microglia into the mouse brain (Day 16 to 18)

● **TIMING variable** *Depending on the number of pups, number of cell lines grafted and expertise of the researcher, xenotransplantation of an average litter (6-10 pups) can take 30-75 minutes, including preparation of the equipment. In our experience, a single researcher is able to transplant 1-4 litters. The number of cell lines and total number of pups injected entirely depends on the experimental purposes, but it is important to have in mind that 1-2 pups per litter and cell line should be used for engraftment efficiency analysis.*

25. At post-natal day 2 (**P2**; corresponding to **Day 16** of MIGRATE protocol if mating had worked as expected and pups are born on Day 14), inject *Rag2*$^{-/-}$*IL2rγ*$^{-/-}$ *hCSF1*^{KI} newborns 48h before transplantation intraperitoneally with CSF1R inhibitor BLZ945 dissolved in 20% (2-hydroxypropyl)-β-cyclodextrin, at a dose of 200 mg/kg body weight.

▲ **CRITICAL** *An adequate animal facility with appropriate installations and SOPs for immunodeficient mice is required to perform these steps. Always handle mice under the laminar flow, keep sterile conditions as much as possible and properly clean all tools and material that enter the flow.*

▲ **CRITICAL STEP** *It might be difficult to completely dissolve the BLZ945 compound, and a precipitate may be observed after storage at 4°C. Always ensure the compound is well resuspended before injecting the mice.*

26. Repeat the injection of BLZ945 at **P3** (24h before transplantation).

27. At **P4** (after collection of **Day 18** Microglial progenitors from the *in vitro* differentiation but before taking the mice cages out from the rack), prepare all the necessary equipment for transplantation: stereomicroscope, light source, ice box, vial with cell suspension kept on ice, Hamilton syringes with needles (26s gauge), petri dishes with sterile water, 70% ethanol and sterile PBS, heating pad or lamp.

! CAUTION *Always work under the hood and keep sterile conditions during the xenotransplantation procedure. Any tool or equipment that enters the hood needs to be cleaned in advance. Always clean your gloves with 70% ethanol before handling the pups.*

28. Wash the Hamilton syringes with ethanol, sterile water and finally sterile PBS.

▲ **CRITICAL STEP** *Make sure that no air bubbles are formed in the syringe while cleaning or this may affect the actual volume of cell suspension that is injected in the pups.*

29. Take out the mice cage and anesthetize pups by hypothermia (30-60s on ice).

▲ **CRITICAL STEP** *Do not leave mice on ice longer than 60s, or this may affect recovery after transplantation.*

▲ **CRITICAL STEP** *No more than two pups should be anesthetized at the same time, since the time needed for injections must be taken into account.*

30. Place the anesthetized mice under a stereomicroscope. To facilitate transplantation, the head of the mouse can be placed on a small support (e.g. cap of an Eppendorf tube).
31. Make two small incisions with a scalpel blade at the injection sites to facilitate the insertion of the needles.

32. Bilaterally inject with Hamilton syringes 1µL of cell suspension per site at coordinates from bregma: anteroposterior, −1 mm; lateral, ±1 mm (see magnified image in **Fig. 3**).

▲ **CRITICAL STEP** *Gently resuspend the cells with the Hamilton syringe before injecting, in order to exclude the presence of any cell precipitate and avoid clogging of the needle due to cell clumps.*
? TROUBLESHOOTING

33. After the injections, allow mice to recover on a heating pad at 37 °C before transferring them back to their cage.

▲ **CRITICAL STEP** *The use of a heating pad is critical for the recovery of the anesthetized pups. A heating lamp may also be used, but a correct distance should be ensured between the injected pups and the light source, to avoid overheating.*
▲ **CRITICAL STEP** *Work with one litter at a time. We suggest dividing the litter into two groups that can be transplanted sequentially: once the first half of the pups is injected, let them recover on the heating pad/lamp light. After recovery, the injected pups can be transferred back to the cage, and the second half of the litter can be collected. This will avoid excessive manipulation of the pups and reduce the stress of the females.*
▲ **CRITICAL STEP** *It is possible to transplant different cell lines within the same litter, but this will depend entirely on the availability of equipment needed for the procedure. However, it is critical to wash thoroughly the Hamilton syringes between cell lines with 70% ethanol and sterile PBS.*

Quantification of engraftment efficiency (Day 35 (P21)) ● **TIMING 6 hours**

34. Before starting, prepare the FACS buffer, Miltenyi adult brain dissociation kit and isotonic percoll solution (3 mL per sample) according to in house and manufacturers' protocol (see **Reagent set-up**), pre-cool centrifuge to 4°C and set up the oven at 37°C.
▲ **CRITICAL STEP** *Insufficient vortexing of the SIP solutions may cause unwanted separation during myelin removal.*

Sample collection

35. At P21, euthanize grafted mice with an overdose of sodium pentobarbital.

! CAUTION *Pentobarbital sodium is harmful if swallowed. If administered, pentobarbital sodium may induce dizziness, sleepiness and confusion. Manipulate wearing proper personal protection equipment. Seek medical advice in case of intoxication.*

36. Immediately transcardially perfuse the animals with a solution of ice-cold 1x DPBS+5U of heparin.

37. Harvest the brain, dissect tissue and store it in **FACS buffer** on ice until further dissociation.

Enzymatic brain dissociation

38. Place the harvested tissue in enzymatic cocktail 1 of Miltenyi's adult brain dissociation kit and break the tissue down to small pieces using spring scissors.

39. Proceed to enzymatic dissociation of the tissue following Miltenyi's adult brain dissociation kit instructions.

40. Once dissociated, filter the cell suspension through a 70 µm strainer into a 50 mL falcon tube (*use low-adherent tubes*) and rinse through with 5ml of cold **FACS buffer**.

41. To dilute the enzymes, top up volume to 20 mL with cold **FACS buffer** and centrifuge at 300 rcf at 4°C for 15 minutes.
▲ CRITICAL STEP *In our experience, the usage of low-adherent tubes allows for a better recovery of the sample, so this is suggested for the centrifugation steps.*

Myelin removal using Percoll (30%)

42. Resuspend the pellet from tissue dissociation in 10ml of **30% Percoll solution**. Transfer the resuspended cells in 15mL low-adherence falcon tubes by using a Pasteur pipette.

43. Mix gently and centrifuge at 300 rcf at 4°C for 15 minutes. Myelin should accumulate on the top of the tube while cells pellet down.

44. Carefully clear myelin from the tube, then remove the rest of the supernatant.

Surface antibody staining for Fluorescence-activated cell sorting (FACS)

45. Prepare the antibodies mix (*see table below*) in cold **FACS buffer**.
We use the following combination of antibodies and fluorochromes (which should be adjusted depending on the FACS analyzer optical bench):

Antibody	***Catalog number***	***Fluorochrome***	***Final dilution***
Pan-CD11b	*130-109-285*	*Phycoerythrin (PE)*	*1:50*
Anti-mouse CD45	*563890*	*Brilliant-violet 421 (BV421)*	*1:500*
Anti-human CD45	*555485*	*Allophycocyanin (APC)*	*1:50*
Viability Dye eFluor 780	*65-0865-14*	*APC-Cy7*	*1:2000*

46. Resuspend the pellet into the appropriate volume needed for the staining (e.g. 200 µl for half brain; 400-500 µl for full brain). If the tube used for myelin removal is dirty, transfer the resuspended pellet into a new 15 mL falcon tube.

▲ **CRITICAL STEP** *You may also need unstained samples, as well as single staining for each antibody. If you are troubleshooting new staining, it is recommended to also run Fluorescence Minus One (FMO) controls.*

47. Incubate at 4°C for 30 minutes.

48. Add 1 mL of cold **FACS buffer** and centrifuge at 300 rcf at 4°C for 5 minutes.

49. Remove the supernatant and add the appropriate volume of buffer for FACS (e.g. 250 µL for half brain, 500 µL for full brain).

50. Run the samples in a FACS machine and analyse data to measure graft efficiency. To ensure comparable results, always run the same number of events in each sample (e.g. 200,000 events/sample).

Timing

Steps 1 to 6, Stem cells thawing and culturing: **7 days**.

- **Steps 1 to 4**, coating and stem cells thawing: 60-90 minutes.
- **Step 5**, media change: 10 minutes.
- **Step 6 (optional)**, stem cells splitting: 30 minutes.

Step 7, Mice coupling and plug check: **2 days**.

Steps 8 to 24, MIGRATE protocol: **19 days**.

- **Steps 8 to 13**, start of the differentiation (Day 0): 1 hour.
- **Step 14**, media changes (Days 1 to 3): 15-30 minutes.
- **Steps 15 to 17**, EBs transfer to 6-well plates (Day 4): 1 hour.
- **Steps 18 to 21**, media changes (Days 8 and 11): 15-30 minutes.
- **Steps 22 and 24**, microglial progenitors collection (Day 18): 1 hour.

Steps 25 to 33, Transplantation of human microglia into the mouse brain: **4 days**.

- **Steps 25 and 26**, mouse microglia depletion (P2 and P3): 1 hour.
- **Steps 27 to 33**, xenotransplantation (P4): 1-2 hours.

Steps 34 to 50, Quantification of engraftment efficiency: **6 hours**.

- **Step 34**, reagents preparation: 30 minutes.
- **Steps 35 to 37**, sample collection: 60-90 minutes.
- **Steps 38 to 41**, enzymatic brain dissociation: 60-90 minutes.
- **Steps 42 to 44**, myelin removal: 20-30 minutes.
- **Steps 45 to 47**, antibodies staining: 40-60 minutes.
- **Steps 48 to 50**, FACS: 1 hour.

Troubleshooting table

Step	Problem	Possible reason	Solution
4	Too high or too low colony density after stem cells thawing.	Cells plated in only one well when the pellet was big, or split into many wells with a small pellet.	Adjust the amount of media used to resuspend the cells. When in doubt, it's better to plate into one well and split cells soon, rather than having high amounts of cell death because of low density of plating in many wells.

7	None of the plug-checked females is pregnant: *in vitro* and *in vivo* parts are not synchronised.	Inaccurate plug-checking. Not enough number of mating couples.	Couple mice again on new Day -6, and plug-check the following day. Meanwhile, split the stem cells in order to have the correct confluency on new Day 0.
12	Stem cells count is below 1 million at Day 0.	Latest splitting was too diluted, slow growth or not healthy status of stem cells.	If stem cells are healthy and in their log phase of growth (even if the confluency is less than ideal, leading to a cell count below 1M), Day 0 of MIGRATE can be started with half or ¾ of a 96 well plate. EBs should be carefully checked day by day to ensure a correct growth.
15	On Day 4, EBs are difficult to pick up or break down by pipetting.	Poor handling, usage of normal rather than large p1000 tips.	Large orifice p1000 tips must be used. The pipette may be set on ~400-500µL, and three wells at a time may be collected. With the greater amount of media, extremely gentle pipetting should be used in the wells where EBs couldn't be picked up, avoiding bubbles.
17	Overcrowded wells of EBs impair generation of microglial progenitors.	Unequal distribution of EBs in 6 well plate stage.	Large orifice p1000 tips can be used to pick EBs and move them to less crowded wells to ensure equal distribution.
17	Lost EBs lead to low numbers of EBs/well.	EBs are lost in media changes during Day1-3, transfer during Day4 or in following media change steps.	EBs can be distributed in a smaller number of wells where density is maintained of ~20EBs/well. Yield will decrease, but microglial progenitors will be correctly generated.
32	Clogging of Hamilton syringe during injections.	Cell suspension was not well resuspended, or left for too long sitting on ice so precipitate formed.	Always ensure that microglial progenitor cells are well resuspended in PBS after collection. Resuspend again the suspension with Hamilton syringe before injections to avoid clumps.

Anticipated results

The MIGRATE protocol enables human-derived microglial progenitors to xenograft in mouse brains, generating a suitable model to study physiological and pathological roles of human microglia *in vivo*.

We tested MIGRATE across three genotypically different ESC/iPSC cell lines: H9 (ESC), BIONi010-C-2 (*C-2*, iPSC) and Sigma-Aldrich iPSC Epithelial-1 (*Sigma*, iPSC). We found similar engraftment efficiency across cell lines and mice, with an average chimerism of 33±7% at 3 weeks after xenotransplantation (**Fig. 4A-B and Supplementary Fig. 1**). Transplanted microglial progenitors were generated from two independent *in vitro* inductions for every cell line in analysis. All the xenotransplantation experiments were successful across all the cell lines. All tested mice from all litters displayed successful integration of human cells and were included in the engraftment efficiency analysis.

Starting from broadly available stem cell lines (e.g. the human embryonic stem cell line H9 at Wicell), application of MIGRATE differentiation is expected to fully cover major brain regions (**Fig. 5C-D**). After optimization and validation experiments, we identified cells harvested at Day 18 as ideal for xenotransplantation purposes, yielding percentages of chimerism of up to 80% in mice of 3-6 months of age (**Fig. 5C-D**).

To ensure that transplanted litters have an ideal chimerism, human microglia transplantation can be quantified by FACS 3-4 weeks after grafting. Additional verification of human microglia distribution can be provided by immunohistochemistry using human microglia markers such as hCD11b or hP2RY12 (**Fig. 5A-B**). In our experience, successful microglia xenografts reach around 30-40% at P21 (**Fig. 4** and **Fig. 5A-B**). Lower microglia chimerism can eventually occur, but it is recommended to test an additional littermate to exclude the possibility of technical issues during the FACS protocol and/or interindividual variability. Depending on the concrete experimental questions, working with a low percentage of chimerism is possible but we have seen that reduced amounts of human microglia do not completely distribute across the brain, clustering in specific regions like striatum (in proximity of the injection sites) and frontal cortex.

As an example of application of our humanized system, we have previously described that the injection of oligomeric amyloid-β (oAβ) in human-microglia chimeras induced a differential response between human and mouse cells *in vivo*[4]. Further details on the experimental outcome and transcriptomic analysis can be found in the original publication in *Nature Neuroscience* linked to this protocol.

Authors contributions statements

N.F., A.M-M., B.D.S. and R.M. conceived the study. N.F., A.M-M. and R.M. performed all the experiments and wrote the manuscript. I.G. and L.W. assisted with microglia xenotransplantations and engraftment efficiency analysis. All authors read and approved the final manuscript for publication.

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Competing interests

The authors declare no competing interests. B.D.S. receives grants from different companies that support his research and is a consultant for several companies, but nothing is directly related to the current publication.

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Figure Legends

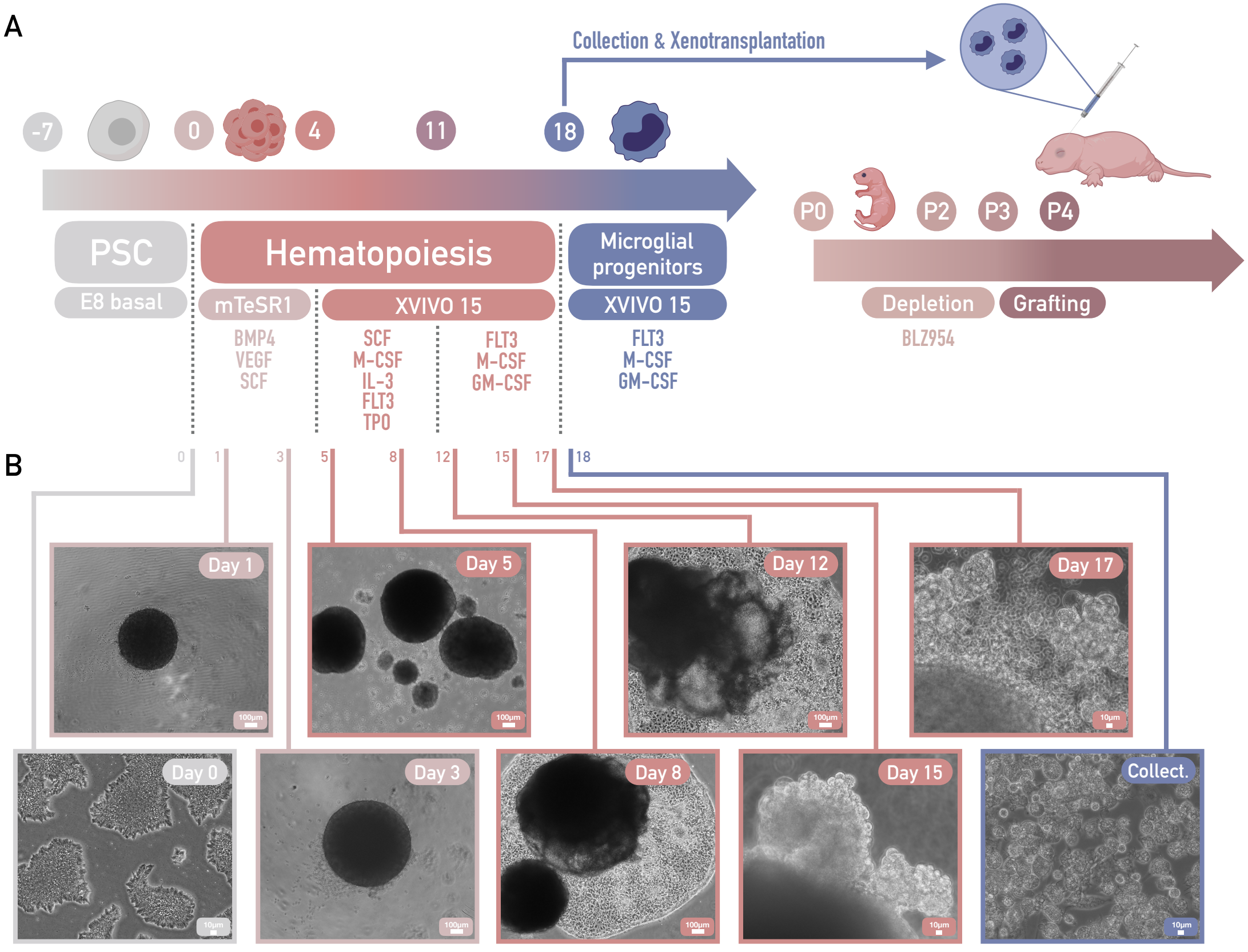
Fig. 1 | MIGRATE protocol schematic. (A) Schematic of the MIGRATE protocol and xenotransplantation into the mouse brain. The three main stages of the differentiation are indicated (PSC, Hematopoiesis, Microglial progenitors), along with the culturing media and cocktails of cytokines to be used at every step. Microglial progenitors can be harvested at Day 18 and transplanted into newborns following depletion of endogenous microglia. **(B)** Representative pictures of key steps of the differentiation protocol (H9 cells, length of scale bars is indicated in the picture).

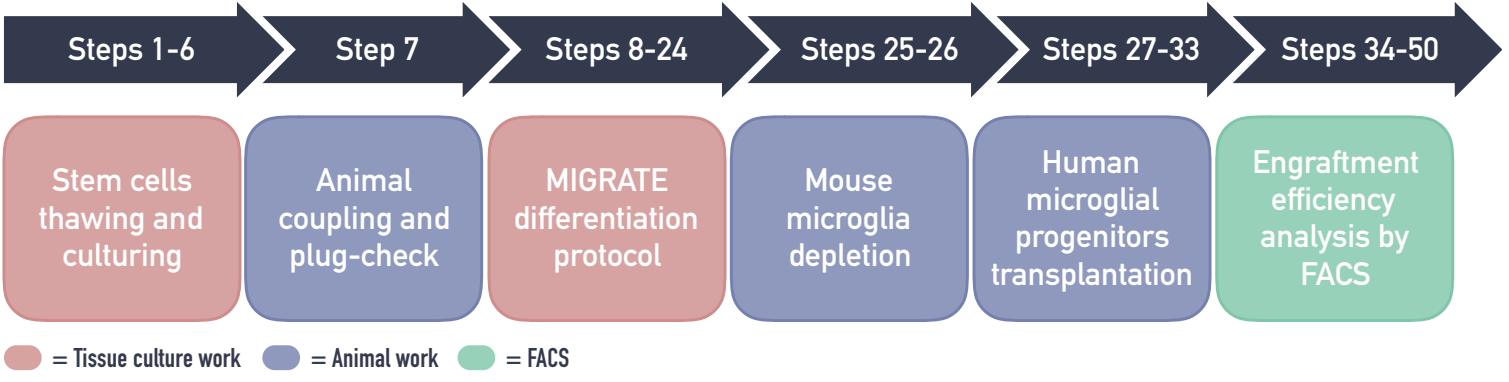
Fig. 2 | Schematic to show an overview of the MIGRATE protocol. Tissue culture work is shown in pink, animal work in blue and FACS analysis in green. Steps refer to ***Procedure*** workflow.

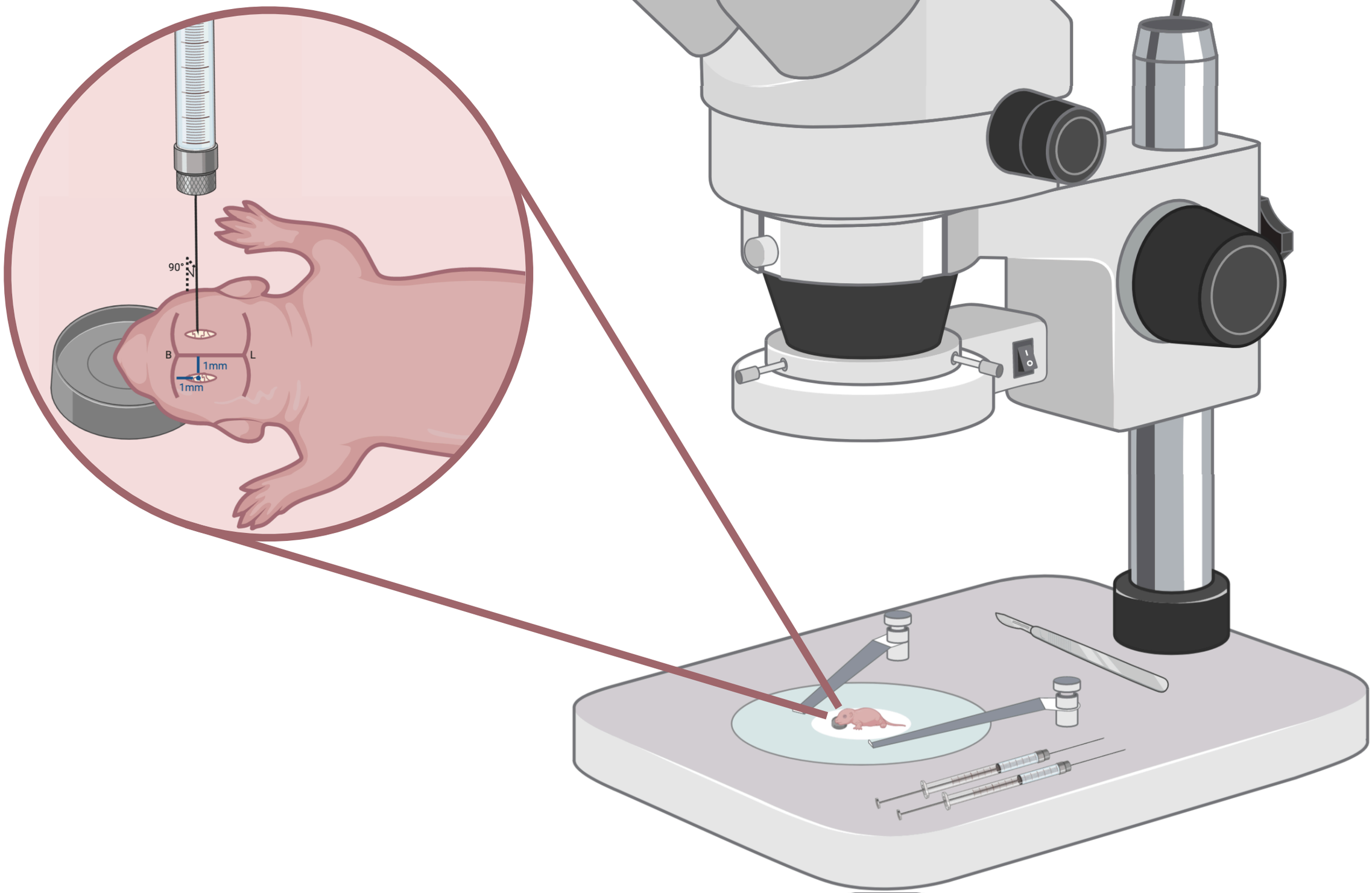
Fig. 3 | Xenotransplantation schematic. Schematic illustration of the xenotransplantation procedure, including equipment and injection coordinates. At P4, mice are anesthetized by hypothermia (not shown) and placed under a stereomicroscope. A scalpel blade is used to make small incisions at the injection sites to ensure correct insertion of the needles. Pups are injected bilaterally with 1 µl of cell suspension at coordinates from bregma (B): anteroposterior, −1 mm; lateral, ±1 mm (*zoom in*, in blue); depth, 2 mm. The Hamilton syringe is held perpendicular (90°) to the pup's head. After the injections, mice were allowed to recover on a warming pad at 37 °C and then transferred back to their cage (not shown). Abbreviations: B, bregma; L, lambda.

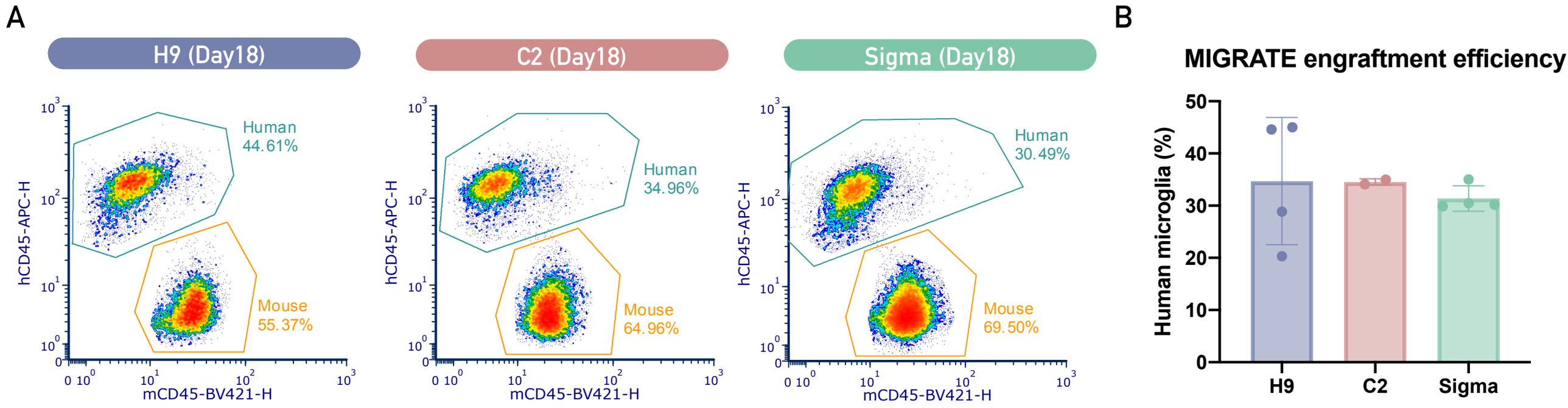
Fig. 4 | *In vivo* engraftment efficiency is reproducible across different ESC/iPSC cell lines when using MIGRATE protocol. FACS analysis to show *in vivo* engraftment efficiency of microglial progenitors derived from different ESC/iPSC cell lines and inductions at 3 weeks after xenotransplantation. **(A)** Representative FACS plots of H9-, C2- and Sigma-derived microglial progenitors harvested and transplanted at Day18. **(B)** Summary of all the replicates analysed for the *in vivo* engraftment efficiency of H9-, C2- and Sigma-derived microglial progenitors. Each line was harvested at Day 18 from at least 2 independent inductions and transplanted in P4 neonates.

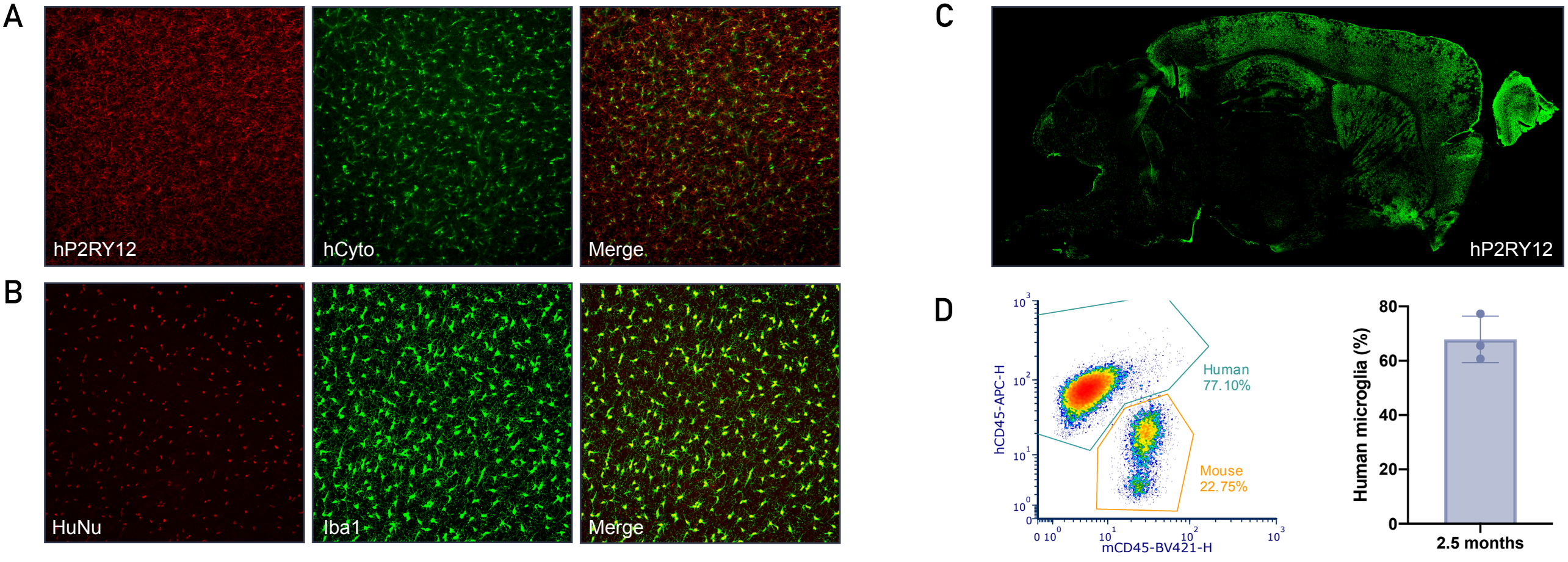
Fig. 5 | MIGRATE microglial progenitors xenotransplantation reach high chimerism
(A and B) Representative images of engrafted human microglia in cortical sections at p21 showing human microglia identity by hCyto+/hP2RY12+ staining **(A)** and high levels of endogenous microglia replacement by HuNu+/Iba1+ staining **(B)**. **(C)** Sagittal full brain section scan showing an overview distribution of human engrafted microglia (hP2RY12+, in green) 6 months after xenotransplantation. **(D)** Representative FACS plot and summary of 3 replicates (2 independent inductions) of H9 engrafted microglia 2.5 months after transplantation.


A
Collection & Xenotransplantation
-7
0
4
11
18
P0
P2
P3
P4
PSC
E8 basal
Hematopoiesis
mTeSR1
BMP4
VEGF
SCF
XVIVO 15
SCF
M-CSF
IL-3
FLT3
TPO
FLT3
M-CSF
GM-CSF
Microglial progenitors
XVIVO 15
FLT3
M-CSF
GM-CSF
Depletion
BLZ954
Grafting
B
0
1
3
5
8
12
15
17
18
Day 1
Day 5
Day 12
Day 17
Day 0
Day 3
Day 8
Day 15
Collect.
100µm
10µm

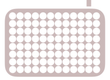

Steps 1-6
Step 7
Steps 8-24
Steps 25-26
Steps 27-33
Steps 34-50
Stem cells thawing and culturing
Animal coupling and plug-check
MIGRATE differentiation protocol
Mouse microglia depletion
Human microglial progenitors transplantation
Engraftment efficiency analysis by FACS
= Tissue culture work
= Animal work
= FACS


90°
B
L
1mm
1mm

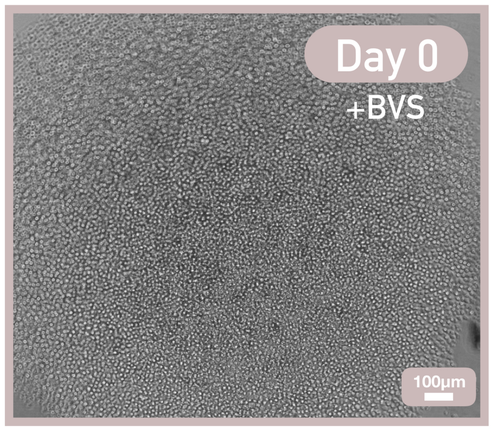

A
H9 (Day18)
C2 (Day18)
Sigma (Day18)
Human
44.61%
Mouse
55.37%
Human
34.96%
Mouse
64.96%
Human
30.49%
Mouse
69.50%
hCD45-APC-H
mCD45-BV421-H
B
MIGRATE engraftment efficiency
Human microglia (%)
50
40
30
20
10
0
H9
C2
Sigma

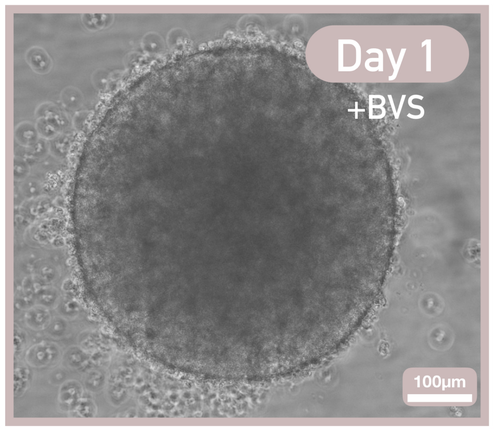

A
hP2RY12
hCyto
Merge
B
HuNu
Iba1
Merge
C
hP2RY12
D
hCD45-APC-H
mCD45-BV421-H
Human
77.10%
Mouse
22.75%
Human microglia (%)
2.5 months

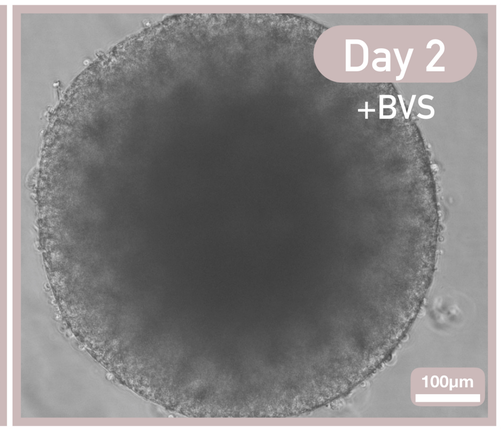
96wp

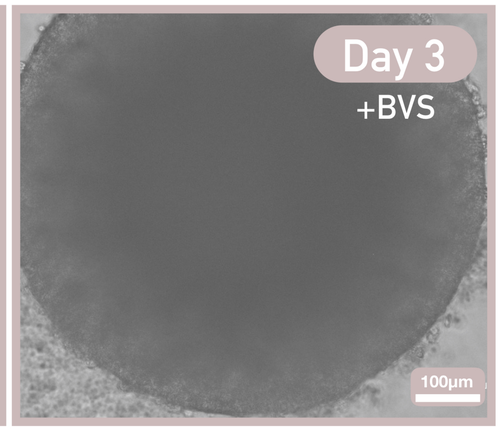


6wp


Day 0
+BVS
100μm


Day 1
+BVS
100μm


Day 2
+BVS
100μm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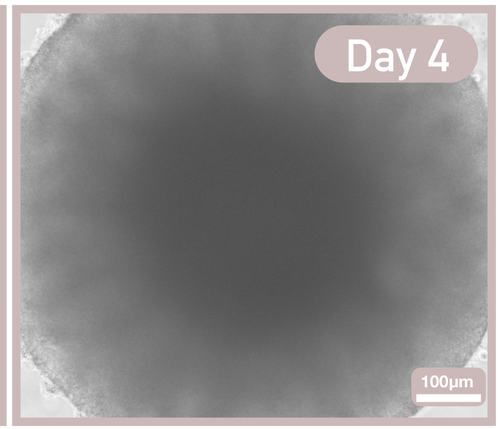
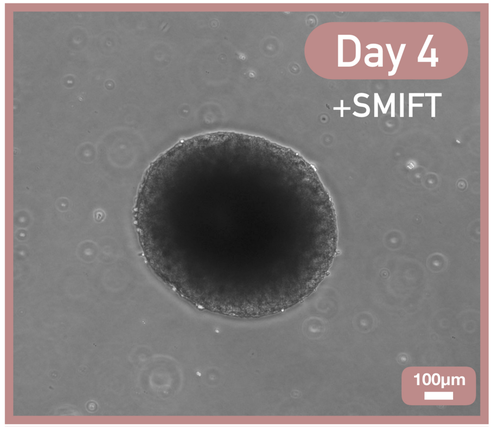
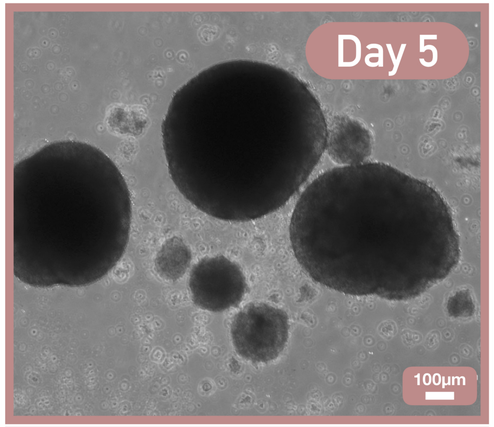
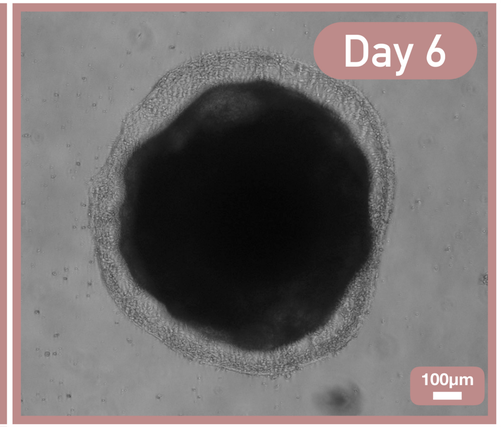
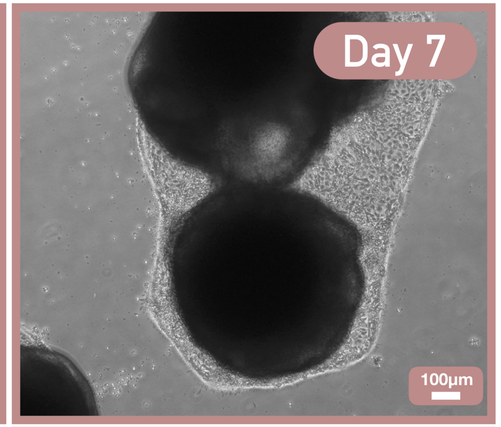
+BVS
100μm

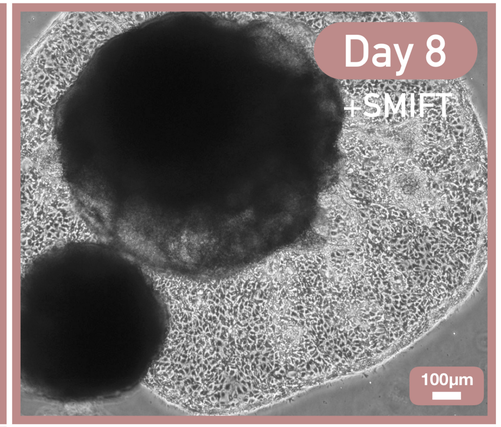

Day 4
100μm

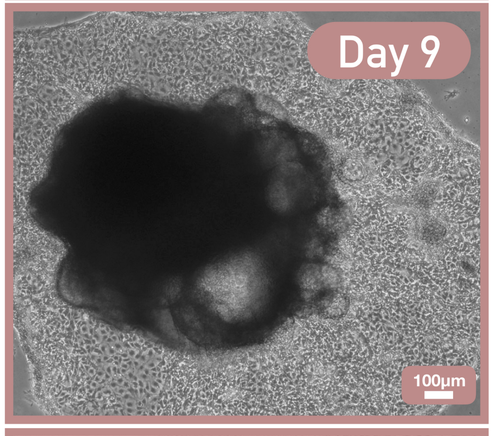

Day 4
+SMIFT
100μm

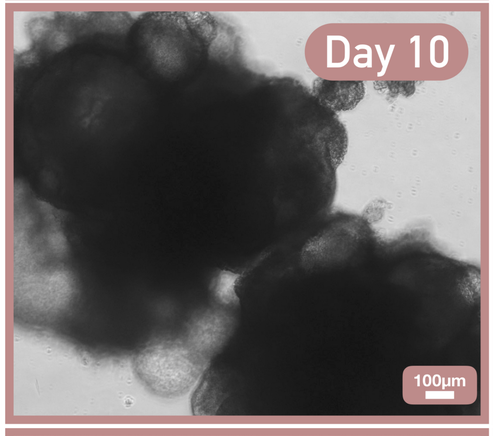

Day 5
100μm

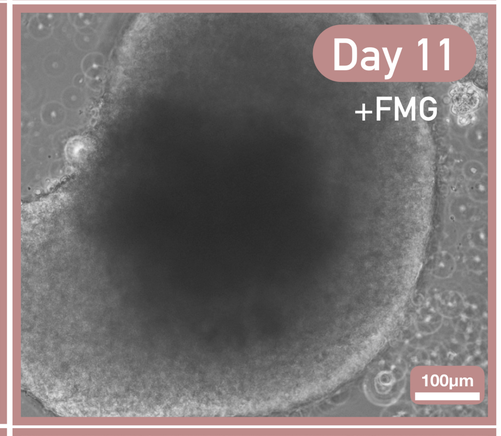

Day 6
100μm

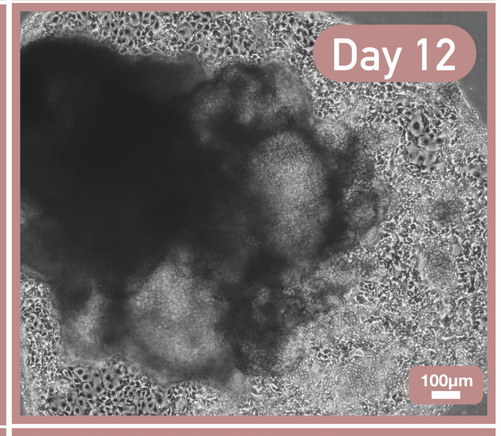

Day 7
100μm

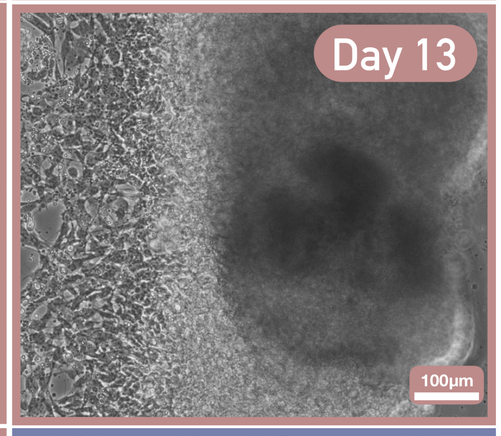

Day 8
+SMIFT
100μm

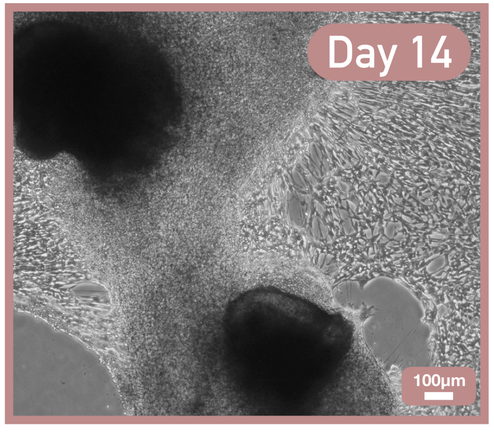

Day 9
100μm

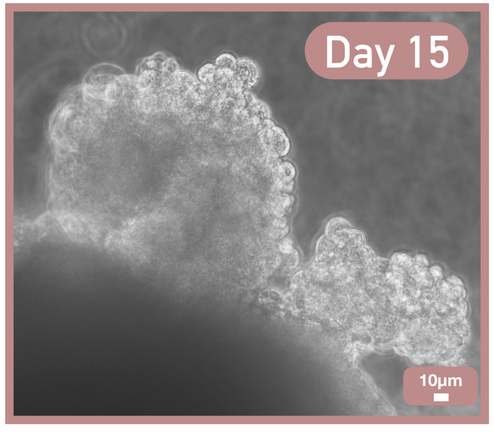

Day 10
100μm

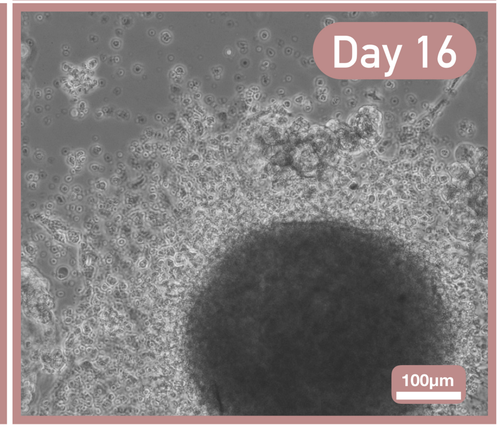

Day 11
+FMG
100μm

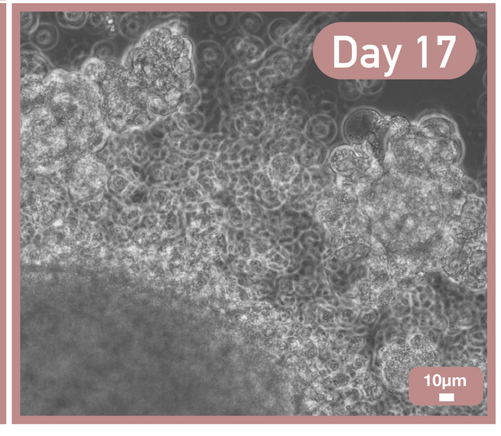

Day 12
100μm

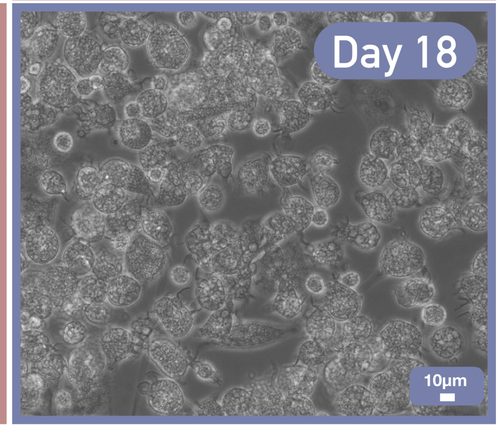

Day 13
100μm


Day 14
100μm


Day 15
10μm


Day 16
100μm


Day 17
10μm


Day 18
10μm