



Generation of functional human microglia-like cells (hMGLs) from H9 hESCs ^(1,2)

Introduction: This method is based on protocols generated by the Blurton-Jones lab (Abud et al. 2017; McQuade et al., 2018). It generates human microglial-like cells (hMGLs) from undifferentiated H9 human embryonic stem cell line (hESCs - WA09, WiCell Research Institute) in a serum-free, feeder-free system (STEMdiff™, Stemcell Technologies). hMGLs are morphologically and transcriptionally similar to human microglia.

Before starting your experiment, make sure to go over the following materials checklist:

Materials	Catalog #	Notes
Human ESC H9 cell line	WA09	WiCell Research Institute
Hematopoietic Kit	05310	H9 hESC → Hematopoietic Progenitor Cells (HPCs); Days -1 to 12
mTeSR™ Plus	100-0276	H9 maintenance medium
ReLeSR™	100-0483	Stemcell Technologies Inc
Matrigel hESC-qualified	354277	Corning™
DMEM/F-12, HEPES	11039021	No phenol red - Gibco™
ROCK inhibitor Y-27632	72302	Stemcell Technologies Inc
6-well plates (TC-treated)	353046	Corning Falcon
12-well plates (TC-treated)	353043	Corning Falcon
15 mL Falcon conical tubes	430790	Corning™
Bambanker Freezing Media	BB05	Bulldog Bio Inc

STAGE 1: hESC Maintenance & preparation

- 1) Confirm H9 hESCs are mycoplasma-free, karyotypically normal, and at passage 30–50
- 2) Cells are maintained in mTeSR™1 or mTeSR™ Plus on Matrigel-coated plates
- 3) Prepare Matrigel-coated 6-well plates (At least 1 hour before use)
 - Dilute Matrigel 1:30 in DMEM/F-12
 - Coat wells by adding 500 µl/well
 - Incubate for 1 hour at RT
 - Aspirate excess

Day -1 (Seeding H9 hESCs)

- 1) Aspirate mTeSR™ from H9 cultures
- 2) Wash once with DPBS (Ca²⁺/Mg²⁺-free).
- 3) Add 1 ml ReLeSR™ (1 ml/well for 6-well plates) and return plate to incubator.
- 4) Incubate 3–5 min at 37°C until colonies begin to detach.
- 5) Add 1 ml mTeSR™ at a 1:1 ratio (1 ml/well for a 6-well plate)
- 6) Collect into 15 mL conical tube.
- 7) Centrifuge at 300×g for 5 min and aspirate supernatant.
- 8) Resuspend in mTeSR™ + 10 µM Y-27632 and count cells.
- 9) Seed onto Matrigel-coated 12-well plates at 5 × 10⁴ cells/cm² (approximately 1.5–2 × 10⁵ cells/well of a 12-well plate).
- 10) Incubate overnight at 37°C, 5% CO₂.



Note: Seed as small clumps, not single cells. Use a 5 min incubation and pipette gently to retain small aggregates of 3–10 cells. Aggregate size at seeding is a key determinant of HPC yield.

Plating aggregates:

- To achieve the required colony density for differentiation of cell aggregate counts, we typically plate aggregates as a gradient from 1 μ l, 2 μ l, 4 μ l, 6 μ l ...and choose desired number of colonies the next day for differentiation. A plating density of 40-80 aggregates/well (10-20 aggregates/cm²) is recommended).
 - Aggregate diameters ranging from 100-200 μ m are ideal for use with STEMdiff™ Hematopoietic Kit; however, since aggregates \geq 50 μ m in diameter are also likely to grow into colonies, count any aggregate \geq 50 μ m.
- 7) Place the plate in a 37°C incubator. Shake the plate using several quick, short, back-and-forth and side-to-side motions to distribute the cell aggregates. Do not disturb the plate for 24 hours.

STAGE 2: HPC generation (STEMdiff™ Hematopoietic Kit - Cat. #05310)

Hematopoietic Medium A (1:200 dilution- Mesoderm induction)

- Prepare fresh by adding 5 μ l Supplement A per 1 mL Basal Medium (1:200 dilution). Immediately store excess HPC medium in -20°C
- Store at 4°C for up to 1 week:

STEMdiff™ Hematopoietic Basal Medium	45 ml
STEMdiff™ Hematopoietic Supplement A	225 μ l

Hematopoietic Medium B (HPC specification):

- Prepare fresh by adding 5 μ l Supplement B per 1 mL Basal Medium (1:200 dilution)
- Store at 4°C for up to 1 week:

STEMdiff™ Hematopoietic Basal Medium	75 ml
STEMdiff™ Hematopoietic Supplement B	375 μ l

Day 0: Mesoderm induction

- 1) Confirm that 16 - 40 small colonies are attached to the cultureware (4-10 colonies/cm²). Do not proceed if cultures have insufficient or overplated colonies, as differentiation will be compromised.
- 2) Aspirate medium from wells.
- 3) Add 1 mL Medium A per well of 12-well plate.
- 4) Return to incubator and incubate at 37°C for 2 days.

Day 2: Half-medium change with medium A

- 1) Carefully aspirate 500 μ l of Medium A from each well (aspirate from the side to avoid disturbing the cell layer).
- 2) Add 1 or 1.5 ml of fresh Medium A per well for 12-well or 6-well plate respectively.
- 3) Incubate at 37°C for 24 hours.

Morphology check: By Day 2, cells should appear as a flat, cobblestone-like monolayer with early signs of elongation at colony edges — early mesoderm morphology.

Day 3: Switch to medium B

- 1) Aspirate all Medium A from each well.
- 2) Add 1 mL Medium B per well.



Note: Medium B drives further differentiation from mesoderm toward hematopoietic progenitors via hemogenic endothelium specification Inflammation and Regeneration +1.

Days 5, 7, and 10: Half-medium changes with medium B

- 1) On each of Days 5, 7, and 10: aspirate 500 μ L spent medium; add 500 μ L fresh Medium B.
 - 2) Day 7 onward, small round cells will begin appearing in suspension above the adherent layer — these are emerging HPCs undergoing endothelial-to-hematopoietic transition.
- Do not disturb the adherent layer during media changes. Tilt the plate and aspirate from the corner.

Day 12: Harvesting HPCs

- 1) Pipette the culture medium up and down 5–10 \times vigorously to dislodge loosely adherent HPCs from the surface.
- 2) Collect all suspension cells into a 15 mL conical tube.
- 3) Wash the well once with 1 mL DMEM/F-12; add to the same tube.
- 4) Centrifuge 300 \times g, 5 min; aspirate supernatant.
- 5) Resuspend pellet in desired medium; count viable cells (Trypan Blue exclusion or automated counter).

Cell quality check:

- Morphology: round, non-adherent cells in suspension
- Flow cytometry: 25–65% CD34⁺CD45⁺ (average ~43%); CD43⁺
- Viability: >85%
- Typical yield: 5×10^5 – 2×10^6 cells per well of a 12-well plate

Cryopreservation: Freeze cells in Bambanker medium at a concentration of 0.5 – 1×10^6 cells/ml. **Note:** Freezing HPCs will significantly reduce viability. Either use them immediately to culture hMGL or use them for xenotransplant.

STAGE 3: Microglia differentiation & Maturation

Media: During this stage, we use a microglia differentiation medium of the following composition:

Basal medium	Brand	Cat. #	Stock	Final conc	500 mL	Aliquot	
1-Thioglycerol (Monothioglycerol)	Sigma™	A8960-5G		400 μ M	0.02	Progenitor Microglia Media Aliquot (PMMA) 50ml/tube	
B27 Supplement	Gibco™	17504044	50X-10ml	2X	20		
N2 Supplement	Gibco™	17502048	100X-5ml	0.5X	2.5		
Non-essential Amino Acids (NEAA)	Gibco™	11140050	100X	1X	5		
GlutaMax Supplement	Gibco™	35050061	100X	1X	5		
Human Insulin	Sigma™	I2643-50MG	50 mg/ml	5 μ g/ml	0.05		
Insulin-Transferrin-Selenium (100 \times)	Gibco™	51500056	100X-10ml	2X	10		
DMEM/F-12, HEPES, no phenol red	Gibco™	11039021			457		
Cytokines							
Recombinant Human M-CSF (HEK293E)	Biologend	574808	25 μ g/ml	25 ng/ml	500 μ l		
Recombinant Human IL-34 (CHO)	Biologend	577906	100 μ g/ml	100 ng/ml	500 μ l		
Recombinant Human TGF- β 1 (CHO)	Biologend	781804	50 μ g/ml	50 ng/ml	500 μ l		

Maturation

Human Fractalkine (CX3CL1)	PeproTech	300-31	100 μ g/ml	100 ng/ml	25 μ l (2.5 μ l/50 ml PMMA)
Human CD200 (HEK293E)	BioLegend	770006	100 μ g/ml	100 ng/ml	25 μ l (2.5 μ l/50 ml PMMA)



- **Differentiation medium:** Before use, warm the PMMA to RT (do not heat to 37°C).
- **Prepare tissue culture plates:** Coat 6-well plates with Matrigel (dilute 1:30 in cold DMEM/F-12; 250 µL/well; 1 hour at RT; aspirate excess)

Day 0: Seeding HPCs

- 1) Resuspend Day 12 HPCs in RT PMMA
- 2) Seed at 1.1×10^5 cells/well in 6-well plates in 2 mL of PMMA per well.
- 3) Incubate at 37°C, 5% CO₂.

Days 2, 4, 6: Fresh medium additions

- 1) At each time-point, add 1 ml of fresh PMMA medium per well and return plate to incubator.

Day 7 (1st Cell expansion)

1. Gently collect all the medium from wells and place in a 15 ml centrifuge tube
2. Add 2 ml of fresh RT PMMA medium per well
3. Spin down medium (300g x 5min) and discard supernatant,
4. Resuspend cell pellet in 2 ml of RT PMMA
5. Plate cells onto a new plate.

Days 8, 10 and 12: Fresh medium additions

- 1) At each time-point, add 1 ml of fresh PMMA medium per well and return plate to incubator.

Day 14 (2nd Cell expansion):

- 1) Remove the old medium while leaving approximately 1 ml in the well.
- 2) Collect the removed medium, centrifuge to pellet the cells, and discard the supernatant.
- 3) Resuspend the cell pellet in 1ml RT PMMA, add it to the same well and return plate to incubator.

Days 16, 18, 20, 22: Half-medium changes

Remove 1 ml of old medium and add 1 ml fresh PMMA (follow the step on D12 above)

Morphology checkpoints:

Days 0–4: Cells adhere; begin to spread with short processes

Days 4–12: Cells develop elongated, bipolar morphology; small processes visible

Days 12–24: Cells should be adherent with increasingly ramified morphology; branching processes; small cell bodies characteristic of microglia precursors.

- No large, flat macrophage-like cells (these indicate contamination or over-activation)
- If >20% of cells appear as large, flat, amoeboid cells, check for mycoplasma and verify medium preparation.

Day 24: Maturation medium

- 1) Prepare PMMA maturation by adding 2.5 µl of CX3CL1 and CD200 to 50 mL of PMMA
- 2) Aspirate medium from well and spin down.
- 3) Replace with 1 ml of maturation medium (PMMA + CD200 + CX3CL1) into each well.
- 4) Discard supernatant, resuspend cell pellet in maturation medium and cells back into the same well.



Expected expansion: The maturation phase produces an average 4-fold expansion of cells, so 1×10^5 seeded cells will yield $\sim 4 \times 10^5$ mature hMGLs per well by Day 36.

Days 26 and 28: Harvest mature hMGLs

Continue adding 1 mL of maturation medium (PMMA + CD200 + CX3CL1) to each well.

From Day 26 onwards, hMGLs can be harvested and used for experiments.

- 1) Collect all supernatant and wash the well once with DMEM/F-12. Combine the wash with the collected supernatant in a 50 mL tube.
- 2) Centrifuge at $500 \times g$ for 5 minutes.
- 3) Resuspend the cell pellet in the desired medium for downstream applications or freeze cells in Bambanker medium at a concentration of $0.5 - 1 \times 10^6$ cells/mL.

Characteristics of mature hMGLs

Marker	Expected Result	Significance
CD45 ⁺ / CD11b ⁺	>80%	Core myeloid/microglial identity
TREM2 ⁺	>50%	Microglial phagocytic receptor (AD risk gene)
P2RY12 ⁺	Positive	Homeostatic microglia marker
CX3CR1 ⁺	Positive	Fractalkine receptor; microglial surveillance
IBA1 (AIF1) ⁺	Positive	Pan-microglial marker
TMEM119 ⁺	Positive	Microglia-specific homeostatic marker
CD14 ⁺	Low-moderate	Myeloid marker

Functional validation

Phagocytosis

- 1) Incubate hMGLs with pH-sensitive bioindicator particles (pHrodo Red E. coli bioparticles; 5 $\mu\text{g}/\text{mL}$) for up to 72 hours with live-cell imaging.
- 2) As particles are phagocytosed, cells turn red and concentrate particles intracellularly.
- 3) Expected: robust phagocytic activity visible by 12–24 hours; >70% of cells phagocytic.

Inflammatory activation (LPS Stimulation)

- 1) Stimulate with LPS (100 ng/mL) for 24 hours.
- 2) Measure supernatant cytokines by ELISA.
- 3) Expected cytokine release: TNF- α , IL-6, IL-1 β , IFN- γ , GM-CSF, IL-8, IL-12p70, IL-2 (pro-inflammatory); IL-10 (anti-inflammatory) bioRxiv +1.

Morphology assessment

- 1) Image by phase contrast or IBA1 immunofluorescence.
- 2) Homeostatic hMGLs: small cell bodies, multiple long ramified processes.
- 3) Upon injury or LPS stimulation: cells retract processes and adopt amoeboid morphology.



References

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Stem Cell Technologies protocol

<https://www.stemcell.com/products/stemdiff-hematopoietic-kit.html>

https://cdn.stemcell.com/media/files/brochure/BR27027-STEMdiff_Hematopoietic_Kit.pdf