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High-Yield Monocyte/Macrophage Differentiation from hiPSC

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Abstract

Here, a highly efficient method for differentiating monocytes/macrophages from hiPSC is described. The process utilizes commercially-available materials to derive CD34+ progenitor cells that are apically released from a hemogenic endothelium. Subsequently, the hemogenic endothelium gives rise to highly pure (>95%), CD34-CD14+ monocytes in 19-23 days and yields more monocytes by day 35 when compared to previous methods. These monocytes are further differentiated to macrophages after 7 days. The efficient workflow and increase in monocyte output boost feasibility for high throughput studies and enables clinical-scale iPSC-derived manufacturing processes.

Guidelines

Summary

Here, a highly efficient method for differentiating monocytes is described. The process utilizes commercially-available materials to derive CD34+ progenitor cells that are apically released from a hemogenic endothelium. Subsequently, the hemogenic endothelium gives rise to highly pure (>95%), CD34-CD14+ monocytes in 19-23 days and yields more monocytes by day 35 when compared to previous methods. The efficient workflow and increase in monocyte output boost feasibility for high throughput studies and enables clinical-scale iPSC-derived manufacturing processes.

Materials

Materials:

1. mTeSRTM Plus cGMP (StemCell, Catalog #100-0276)
2. Rock inhibitor (Y-27632, Miltenyi Biotech, 130-106-538)
3. Gentle Cell Dissociation Reagent (StemCell, Catalog # 100-0485)
4. Corning® Matrigel® hESC-Qualified Matrix (Fisher Scientific, 08-774-552)
5. Falcon® 6-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate (Corning Catalog # 353046)
6. Suspension Flat Bottom 6-well Cell Culture Plate (Sarstedt, Catalog # 83.3920.500)
7. DPBS (Without Mg^{2+} or Ca^{2+}) (Gibco, Catalog # 14190144)
8. STEMdiff Hematopoietic Kit (StemCell, Catalog # 05310**)**
9. Fisherbrand™ Sterile Cell Strainers 70 µM (Fisher, 22-363-548)
10. Cell Dissociation Solution Non-enzymatic 1x (Sigma, C5914-100ML)
11. Monocyte factory medium:
    - X-VIVOTM 15 Serum-free Hematopoietic Cell Medium (Lonza, Catalog #: 04-418Q)
    - 50 mM 2-Mercaptoethanol (Thermo-Fisher, 31350010)
    - GlutaMAX Supplement 1X (Thermo-Fisher, 35050061)
    - 100 ng/ml recombinant human M-CSF (Peprotech, 300-25)
    - 25 ng/ml recombinant human IL-3 (Peprotech, 200-03)

12. Macrophage differentiation media (MDM):
    - X-VIVOTM 15 Serum-free Hematopoietic Cell Medium
    - 50 mM 2-Mercaptoethanol
    - GlutaMAX Supplement 1X
    - 100 ng/ml human recombinant M-CSF

13. LPS-EB, LPS from E. coli O111:B4 (Invivogen, tlrl-3pelps)
14. rhIFNγ (Thermo-Fisher PHC4031)

mTeSR™ Plus **STEMCELL Technologies Inc. Catalog #**100-0276

StemMACS™ Y27632 **Miltenyi Biotec Catalog #**130-106-538

Gentle Cell Dissociation Reagent **STEMCELL Technologies Inc. Catalog #**100-0485

Corning™ Matrigel™ hESC-Qualified Matrix **Fisher Scientific Catalog #**08-774-552

Falcon® 6-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate with Lid Individually Wra **Corning Catalog #**353046

Cell culture plate 6 well surface: Suspension flat base **Sarstedt Catalog #**83.3920.500

Gibco™ DPBS no calcium no magnesium **Thermo Fisher Scientific Catalog #**14190144

STEMdiff™ Hematopoietic Kit **STEMCELL Technologies Inc. Catalog #** 05310

Fisherbrand™ Sterile Cell Strainers **Fisher Scientific Catalog #**22-363-548

Cell Dissociation Solution Non-enzymatic 1x **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**C5914-100ML

Gibco™ 2-Mercaptoethanol (50 mM) **Thermo Fisher Scientific Catalog #**31350010

GlutaMAX™ Supplement **Thermo Fisher Scientific Catalog #**35050061

Recombinant Human M-CSF **peprotech Catalog #**300-25

Recombinant Human IL-3 **peprotech Catalog #**200-03

LPS-EB (LPS from E. coli O111:B4) **InvivoGen Catalog #**tlrl-3pelps

IFN-γ Recombinant Human Protein **Thermo Fisher Catalog #**PHC4031

Differentiation of iPSCs to Hematopoietic Progenitor Cells (HPCs)

1h 5m

1 Start with large cultures of pluripotent stem cells around 50–70% confluence cultured on Matrigel hESC.

2 Coat 6-well plates with fresh Matrigel (1 mg /plate) 1 h to Overnight at 37 °C . 1h

3 Prepare iPSC maintenance medium (mTeSRplus) with [M] 10 micromolar (µM) Rock inhibitor (concentration depending on your line). Add 1.5 mL of medium supplemented with rock inhibitor to each well of the Matrigel coated 6-well plate.

4 Prepare 96-well plate for counting cell clusters. Add 40 µL DPBS to 3 empty wells of the 96-well plate.

5 Dissociate iPSC cultures with GCDR:

5.1 Remove iPSC medium and wash 1 mL DPBS to remove dead cells.

5.2 Treat cells with 1 mL GCDR to each well of a 6-well plate for 5-6 min.

5.3 Remove GCDR from the well.

6 Scrape the cells in 1 mL mTeSRplus + Rock inhibitor and transfer to a 15 mL Falcon. Using a 5 ml pipette, gently pipette up and down 2-3 times in order to achieve clusters of ~ 50-100 cells.

7 Pipette 5 µL of cell cluster mixture into each well of the 96-well plate (n = 3 wells) for counting.

Note

Important! It is recommended to pipett 5 µL of cells with a p20 pipette to get a more accurate representation of cell cluster size that will be plated. Use of a p10 pipettor may triturate cells too far. Additionally, 5 µL volume can be adjusted if there are too many cell clusters to easily and accurately count or cell cluster number.

8 Count all cell clusters of ~100 cells (100–200 µm diameter). Determine # of clusters per µL (total count of correct sized clusters in 3 wells/15 µL total cell cluster suspension).

Note

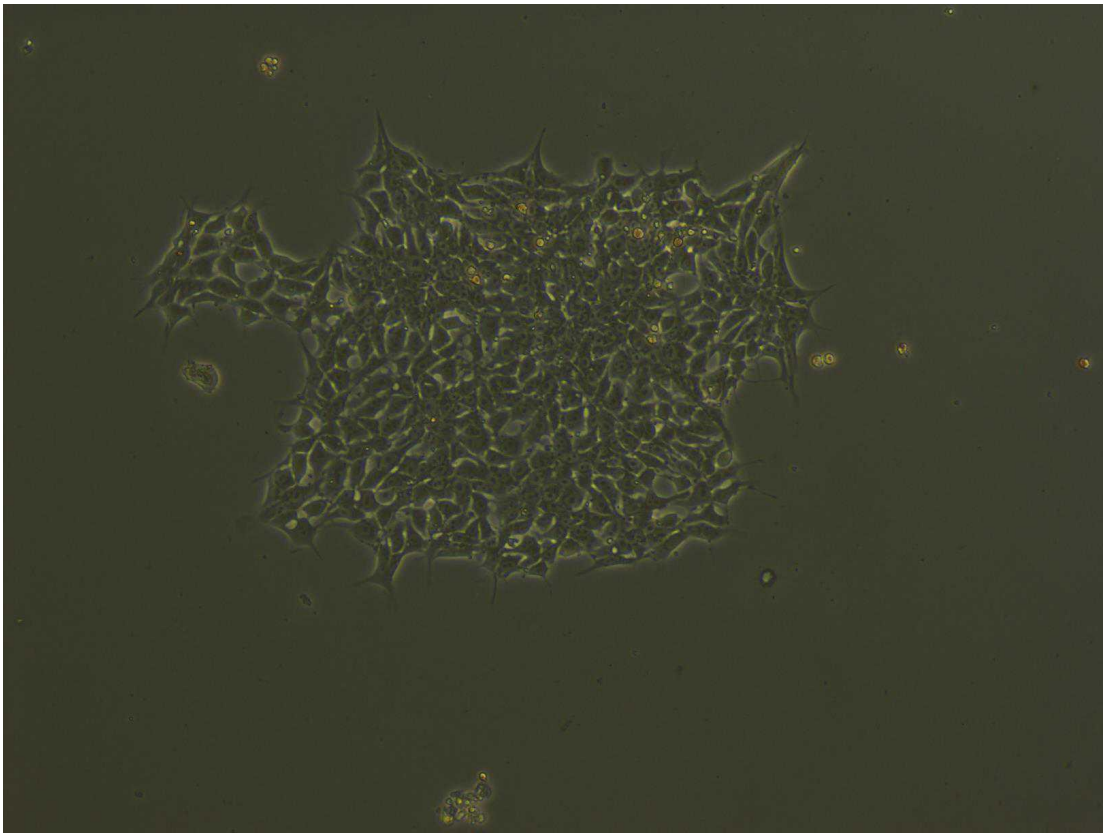
Important! Do not count clusters that are too big or too small. Clusters that are too small can be ignored as they likely will not survive passage, and the few that remain will not differentiate. However, clusters that are too large should be avoided. If there are many large clusters, triturate the cell suspension and recount. It can also be helpful to let larger clusters settle to the bottom of the tube if there are only several in suspension and trituration would create too many smaller clusters.

9 Using cluster #/µL calculations, plate 20–40 clusters per well. The goal is to produce 10–20 surviving clusters of ~100 cells each per well the next day. Swirl plate in incubator to evenly distribute the clusters throughout the plate.

Note

Important! After counting, flick or invert cell suspension tube as clusters of cells will have settled to the bottom. To guarantee success on preliminary runs, it can be helpful to plate several wells of various densities from 20 to 40 clusters per well. If your cell line is particularly robust, you can plate lower densities such as 10–20 clusters per well. With quick-growing cell lines, you will also want to err toward lower densities as these colonies will be larger than slow-growing iPSC lines.

10 24 h after plating, count colonies and choose wells to begin with. Do not blindly start differentiations in all wells. Choose only wells with 10–20 undifferentiated iPSC clusters of ~100 cells each.



Representative morphology of KOLF iPSC line at day 0. Due to ROCK inhibitor, colonies might look with more loose packing.

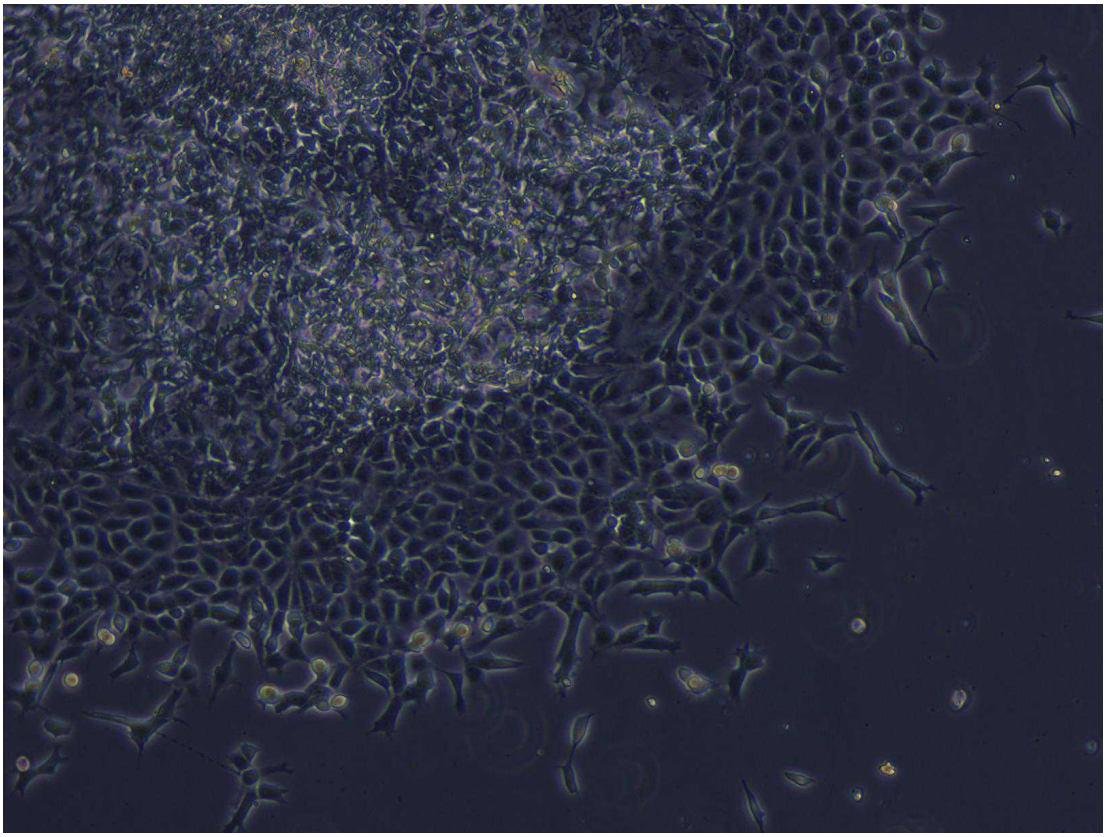
Note

Critical step: Smaller clusters may be ignored and these wells may still be used (if all clusters are small but density is good, cells can be cultured in mTeSR plus 1–2 days prior to starting experimentation). Wells that have 2 or more large clusters of iPSCs cannot be used as these larger clusters will interfere with proper differentiation. Wells with more than 30 clusters cannot be used as these will result in significantly decreased yield. Wells with less than 10 clusters may be used, but final yield will be diminished.

11 Prepare Medium A from StemDiff Hematopoietic kit by diluting Supplement A 1:200 in basal medium. You will need 3 mL Medium A per well for the entire differentiation.

12 Remove mTeSRplus and add 2 mL Medium A per well (Day 0).

13 48 h later, supplement cultures with additional 1 mL Medium A per well (Day 2).



14 48 h later, remove all medium A and add 2 mL Medium B (1:200 dilution of supplement B into basal medium) per well (Day 4).

15 Repeat addition of 1 mL medium B every 48 h three times (Day 6, 8, 10).

Note

Important! Do not remove medium B during the differentiation process ever, the differentiating cells are secreting crucial cytokines into the medium and removal of these will hinder the differentiation process. Over the course of differentiation, the phenol red in the medium will begin to turn yellow—this is expected. By day 6–7 you should be able to see round, phase-bright cell clusters forming within the endothelial cell layers. If these do not occur, the differentiation is not likely to be successful. Common issues are that clusters were originally plated at too high a density or too close to each other.

16 On Day 10, collect round, floating, phase-bright cells by agitating the plate gently and collecting all medium with a serological pipette.

Note

Shaking plate too vigorously or washing/rinsing the well may result in lift-off of immature or non-CD43+ cells leading to contaminated cultures. Be gentle when collecting cells.

16.1 Centrifuge 300 x g, 00:05:00 . To continue collecting cells on day 12, replace 2 mL supernatant (conditioned medium) per well (add to 1 mL fresh medium). 5m

16.2 At this point cells may be analyzed for HPC markers by flow cytometry or ICC (cells should always be >95% CD43+).

Differentiation of HPC to monocytes 5m

17 On Day 12, CD34+ cells are harvested from the plate leaving behind a hemogenic endothelium.

Note

The CD34+ cells can be cryopreserved at 10^6 cells/mL in Cryostor CS10 (STEMCELL Technologies) according to the manufacturer's protocol and the remaining hemogenic endothelium is cultured in 2 mL of monocyte factory medium. The hemogenic endothelium begins producing monocytes over the next 8-9 days and for 30+ weeks.

18 On Day 19 monocytes are harvested by gently rinsing monocytes off each well before passing through a 70 µm nylon filter and centrifuging at 300 x g, Room temperature, 00:05:00 . 5m

19 CD45+, CD14+, CD11b+ cells are checked by flow cytometry. We usually obtain >85% CD14+ cells.

20 The medium in each harvested well is immediately replaced with 2 mL of fresh monocyte factory medium.

Note

iPSC-derived monocytes are used immediately.

Differentiation of iPSC-derived monocytes into macrophages

5m

21 Freshly harvested monocytes are plated at 2.5-3x10^5 per well of a 6-well plate ultra-low attachment plate in 2 mL of macrophage differentiation media (MDM) Day 0 (**Day 19 from beginning**).

22 On Day 4, 1 mL of media is removed and each well is supplemented with 2 mL of MDM.

23 Between days 7 and 10 (**Day 26-29**), monocyte-derived macrophages are gently dissociated from the plate using Cell Dissociation Buffer, enzyme-free, pelleted at 300 x g, Room temperature, 00:05:00, resuspended in fresh MDM medium, and plated for experiments. CD11b+, CD68+ cells are checked by flow cytometry.

5m

Macrophage activation

24 iPSC-derived MDM are plated at 5x10^5 cells per well in a 6 well plate in 2 mL of MDM medium without M-CSF.

25 Cells are left untreated or activated with 500 ng/mL of LPS, 20 ng/mL of rhIFNγ, or both LPS and rhIFNγ.

26 After 24 hours of activation, supernatants are harvested and assayed for human IL-6, TNF, and IL-1β via enzyme linked immunosorbent assay (ELISA).

Table 1. iPSC lines used.

	A	B	C
	Line	**Reprogramming method**	**Cell source**
	KOLF	CRISPR Cas9 ARID2	Fibroblast male 55-59 yrs
	AIW002-02	CytoTune™-iPS 2.0 Sendai Reprogramming Kit to generate iPS (non integrative system)	PBMC male 37 yrs
	A18945	episomal reprogramming to generate iPSc	Female CD34 + cord blood usin g a three-plasmid < 1 yr

Protocol references

This protocol was adapted from

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