bio-protocol

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Efficient Method to Differentiate Mouse Embryonic Stem Cells into Macrophages *in vitro*

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Abstract

Macrophages are key cells in the innate immune system and play a role in a variety of diseases. However, macrophages are terminally differentiated and difficult to manipulate genetically via transfection or through CRISPR-Cas9 gene editing. To overcome this limitation, we provide a simplified protocol for the generation of mouse embryonic stem cells-derived macrophages (ESDM). Thus, genetic manipulation can be performed using embryonic stem cells, selecting for the desired changes, and finally producing macrophages to study the effects of the previous genetic manipulation. These studies can contribute to many areas of research, including atherosclerosis and inflammation. Production of ESDM has been previously achieved using embryoid body (EB) intermediates. Here, we optimized the EB method using a simplified medium, reducing the number of recombinant proteins and medium recipes required. Our EB-based differentiation protocol consists of three stages: 1) floating EB formation; 2) adherence of EBs and release of floating macrophage progenitors; and, 3) terminal differentiation of harvested macrophage progenitors. The advantages of this protocol include achieving independent floating EBs in stage 1 by using a rocker within the tissue culture incubator, as well as the exclusion of small EBs and cell clusters when harvesting macrophage progenitors via cell filtration.

Keywords: Macrophage, Terminal differentiation, Mouse embryonic stem cells, Cell filter, Embryoid bodies

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Background

Macrophages are professional phagocytes and central players in the innate immune system that can engulf and degrade many types of ligands and particles, present antigens to cells of the acquired immune system, and release inflammatory cytokines. Macrophages play key roles in multiple diseases, such as tuberculosis, sarcoidosis, and Gaucher disease. Cholesterol-laden macrophages in the arterial intima give rise to foam cells in the pathogenesis of atherosclerosis. Especially in its early stages, atherosclerosis is reversible, as foam cells can efflux excess cholesterol out of the artery via the reverse cholesterol transport pathway, or emigrate out of the early lesion. However, if the macrophage is overwhelmed, it can undergo different types of cell death, potentially leading to the accumulation of extracellular cholesterol deposits that may be harder to clear. Thus, due to the role of macrophages in the development of cardiovascular diseases, via both their cholesterol metabolism and inflammatory activity, they are an important target of vascular disease research.

Mouse models have been the most widely used models of atherosclerosis. However, mechanistic studies often require the study of macrophages *in vitro*. Furthermore, the differentiation of macrophages from cultured precursor or stem cells allows one to first perform stable gene knock-out (by CRISPR-Cas9 or other methods) or over-expression, allowing researchers to examine the effects of specific genes on macrophage structure and function (Hai *et al.*, 2018; Rojo *et al.*, 2019; Luo *et al.*, 2021; Ritchey *et al.*, 2021). Methods to derive macrophages from mouse embryonic stem (ES) cells have previously been developed through the intermediate formation of embryoid bodies (EB). This method involves the release of non-adherent macrophage progenitor cells from the EB, which can be replated and differentiated into mature ES-derived macrophages (ESDM) (Wiles, 1993; Moore *et al.*, 1998; Moore and Freeman, 1999). These EB-based macrophage differentiation methods all share the use of multiple cytokines, including macrophage colony-stimulating factor (M-CSF) and IL-3, in different time courses. Some improvements were made by using conditioned media from L cells, a murine fibroblast-like cell line derived from subcutaneous adipose tissue, as a source of M-CSF. Two groups modified the EB-based method, employing L cell-conditioned medium to substitute for M-CSF, but these protocols also use different media formulations at different stages of the differentiation (Zhuang *et al.*, 2012; Yeung *et al.*, 2015). These EB-based methods may have issues with the size and uniformity of the EBs. Additionally, there can be issues with EB adherence to the dish and merging with each other during EB formation. As the EB methods also require the harvesting of non-adherent macrophage progenitor cells, it is possible that these cells can become contaminated with small EBs, which may then result in low yield and purity of differentiated macrophages. Combined, these issues may lead to a low overall success rate, making it difficult to scale up.

In the present protocol, we optimized the EB method to create ESDM using only one media formula. We found that our method worked on both wild-type AC173 DBA/2 mouse ES cells, as well as CRISPR/Cas9 gene-edited clones of these ES cells. Some of our innovative modifications include the depletion of mouse embryonic fibroblast (MEF) feeder cells during ES cell preparation, use of a rocker in the tissue culture (TC) incubator during EB formation, and use of 30 μm filters to remove small EBs from the macrophage progenitor cells. Our simplified ESDM protocol (Figure 1) is labor-saving and economical, with high reproducibility and high yield of macrophages that are suitable for large scale use in subsequent experiments.

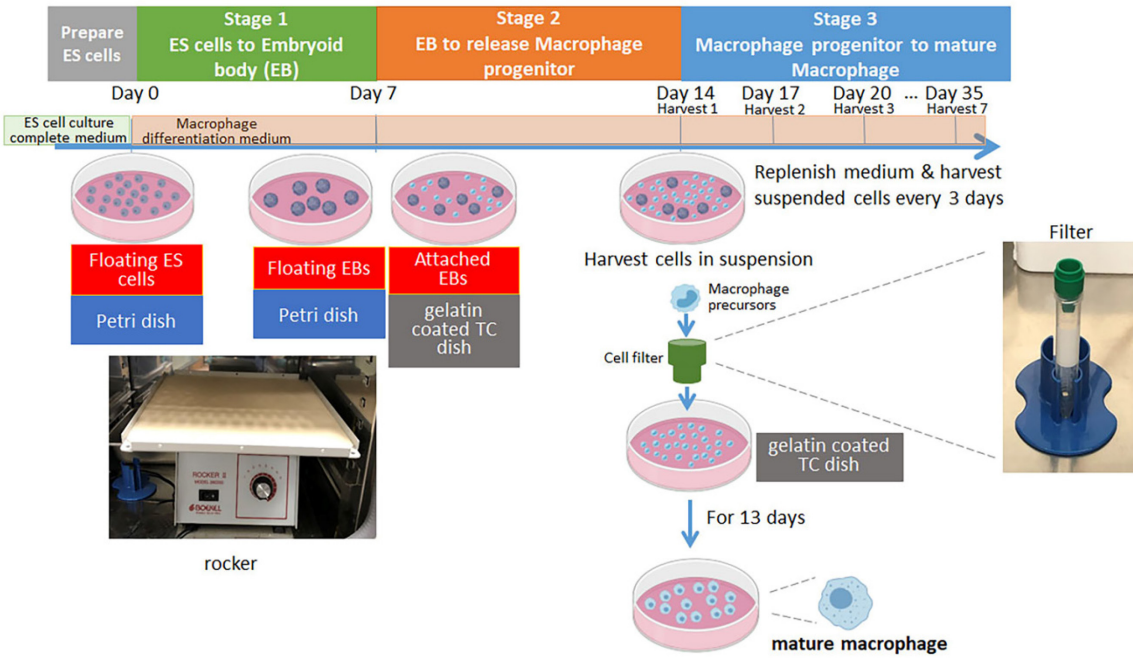

Figure 1. Schematic outline of a simplified three stage protocol for mouse embryonic stem cell derived macrophage (ESDM) differentiation.
Prepare L cell conditioned medium and feeder cells before beginning stage 1. Stage 1. Culture floating mouse ES cells in low-adherent Petri dish placed on a rocker for 7 days with macrophage differentiation medium (MDM). Stage 2. Transfer newly formed floating embryoid bodies (EBs) to gelatin-coated TC dish for attachment and growth from day 7 to day 14 in MDM. Stage 3. Harvest and filter medium with floating macrophage precursors to remove EBs, and plate filtrate in a gelatin-coated TC plate in MDM. Fully functional ESDM are obtained 13 days later.

Materials and Reagents

Materials

1. 100 mm × 15 mm not-TC-treated bacteriological Petri dish (Corning Falcon, catalog number: 351029)
2. 100 mm TC-treated cell culture dish (Corning Falcon, catalog number: 353003)
3. 6-well clear flat bottom TC-treated multiwell cell culture plate (Corning Falcon, catalog number: 353046)
4. T75 TC flask (CytoOne, catalog number: CC7682-4875)
5. T225 TC flask (Corning, catalog number: 431081)
6. 50 mL centrifuge tube, conical bottom, sterile (Corning Falcon, catalog number: 352098)
7. 15 mL centrifuge tube, conical bottom, sterile (Corning Falcon, catalog number: 352099)
8. Cryogenic vials, 1.2 mL (Corning, catalog number: 430487)
9. 30 µm monofil nylon sterile filter (Sysmex, catalog number: 04-004-2326)
10. Vacuum filtration flasks, 500 mL, 0.22 µm membrane (Cellpro, catalog number: V50022)
11. Syringe filters, 0.2 µm (Fisherbrand, catalog number: 09-720-3)
12. 1.5 mL Eppendorf tube (Sarstedt, catalog number: 72.690.301)
13. DBA/2J AC173/GrsrJ mouse embryonic stem cell (The Jackson Laboratory, catalog number: 000671C02)
14. Puromycin-resistant mouse embryonic fibroblast (MEF) feeder cells (Cell Biolabs, catalog number: CBA-312)
15. L cells, clone 929 (ATCC, catalog number: CCL-1)
16. Cell lifter (Corning, catalog number: 3008)
17. 500 mL sterile glassware bottle (Cleveland Clinic Glassware core)

Reagents

1. 0.05% Trypsin/0.53 mM EDTA (Caisson Labs, catalog number: T019-100GM)
2. Gelatin type A (MP, catalog number: 901771)
3. DMEM base powder, reconstituted with sterile water by Cleveland Clinic Media Preparation Core (Caisson Labs, catalog number: DMP08)
4. DMEM (1×); high glucose; w/o L-glut; w/Na pyruvate; w/o phenol red (Cleveland Clinic media preparation core, catalog number: 21-500CUST)
5. Sterile PBS (Cleveland Clinic Media Preparation Core, catalog number: 121-500)
6. Fetal bovine serum (Millipore Sigma, catalog number: F4135-500ML)
7. Penicillin-G (Sigma-Aldrich, catalog number: P3032-100MU)
8. Streptomycin (Gibco, catalog number: 11860-038)
9. MEM nonessential amino acid (Caisson Labs, catalog number: NAL03-100ml)
10. 2-Mercaptoethanol, 55 mM in Dulbecco's phosphate buffered saline (Gibco, catalog number: 21985023)
11. Dimethyl sulfoxide (DMSO) (Alfa Aesar, catalog number: 42780)
12. Formalin, buffered, 10% (Fisher Chemical, catalog number: SF100-4)
13. Recombinant mouse IL-3 protein (R&D Systems, catalog number: 403-ML-010)
14. Recombinant mouse LIF protein (Millipore Sigma, catalog number: ESG1107)
15. PD0325901, MEK inhibitor (Reprocell/Stemgent, catalog number: 04-0006-02)
16. CHIR99021, GSK-3β inhibitor (Reprocell/Stemgent, catalog number: 04-0004-02)
17. Mitomycin C (Tocris, catalog number: 3258)
18. APC-Cy7 anti-mouse CD11c (BD Pharmingen, catalog number: 561241)
19. PE Rat anti-mouse Ly-6C (BD Pharmingen, catalog number: 560592)
20. FITC rat anti-mouse Ly-6G (BD Pharmingen, catalog number: 561105)
21. PE-CF594 rat anti-CD11b (BD Horizon, catalog number: 562317)
22. Mouse FcR blocker (Stemcell Technologies, catalog number: 18720)
23. DiI (ThermoFisher, catalog number: D3911)
24. DAPI mounting medium (Vectashield catalog number: H-1200)

Solutions and media

1. 0.1% gelatin coating solution (see Recipes)
2. Basic cell culture medium (see Recipes)
3. MEF cell freezing medium (see Recipes)
4. Mouse ES cell culture basal medium (see Recipes)
5. Mouse ES cell culture complete medium (see Recipes)
6. Macrophage differentiation medium (see Recipes)
7. Macrophage maintenance medium (see Recipes)

Equipment

1. Forma direct heat CO_2 tissue culture incubator (Thermo Fisher Scientific, model: 310 Series)
2. Refrigerated benchtop centrifuge (Kendro Sorvall Legend RT, catalog number: 75004377)
3. Ultra-low freezer (Thermo Scientific, catalog number: TSX60086D)
4. Mr. Frost freezing container (Thermo Scientific, catalog number: 5100-0001)
5. Liquid nitrogen storage (Taylor Wharton, model: K Series)
6. Refrigerated microcentrifuge (Labnet Prism, catalog number: C2500-R)
7. Adjustable speed rocker (Boekel Scientific, catalog number: 260350)
8. Cell counter (Beckman Coulter, Z2)
9. Flow cytometer (BD LSR Fortessa)

10. Epifluorescent microscope (Olympus, model: IX51) with CCD camera (Q Imaging, model: EXi Aqua Fast 1394b)

Software

1. FlowJo (BD Biosciences, FlowJo™ v10.8)
2. Cell Sens Dimension imaging software (Olympus, CS-DI)

Procedure

A. L cell conditioned medium preparation (source of M-CSF)

1. Take a vial of L cells out of the liquid nitrogen tank. Plate the cells in 10 mL of basic cell culture medium in a T75 TC flask and culture in TC incubator until ~80% confluent.
2. When the culture is 80% confluent, aspirate the supernatant and rinse cells with 10 mL of PBS. Trypsinize the cells as described above. Pipet the mixture up and down, to suspend into single cells.
3. Transfer detached cells into 15 mL tube and add 8 mL of basic cell culture medium.
4. Add 25 mL of basic cell culture medium to each of five T-225 TC flasks. Then add 1 mL of detached cells to each flask. Culture in TC incubator until ~80% confluent (~5 days).
5. Harvest the supernatant in 50 mL tubes and store at -80°C. Replenish the T-225 flask with 25 mL of fresh basic cell culture medium and harvest the media after 2 days. Repeat this step until cells start to detach from the plate.
6. After accumulating 500 mL, thaw L cell conditioned medium, pool, and filter sterilize (0.2 μm). Use directly, or refreeze in 45 mL aliquots for use in macrophage differentiation medium (MDM) preparation. We routinely use one large batch of L cell conditioned medium in a particular study. However, we have not noticed significant batch differences.

B. Feeder cell preparation (inactivated mouse embryonic fibroblasts used for culturing ES cells in order to maintain their pluripotency)

1. Coat one T75 TC flask with 4 mL of 0.1% gelatin in solution. Rock the plate to cover the bottom surface with gelatin. Incubate the flask in 37°C 5% CO_2 incubator (TC incubator) for at least 1 h before use. Aspirate the 0.1% gelatin solution before use (no gel forms, gelatin protein simply adheres to the TC surface).
2. Feeder cell culture
   a. Thaw one MEF vial (1 mL) in a 37°C water bath. Move thawed cells into a 15 mL tube and add 2 mL of basic cell culture medium.
   b. Spin the cells at 500 × *g* at room temperature for 5 min. A pellet should be seen on the bottom.
   c. Discard supernatant and resuspend the pellet evenly in 10 mL of basic cell culture medium.
   d. Add resuspended MEF cells into the gelatin-coated flask. Rock the plate side to side and culture in TC incubator. Cells tend to be ~70% confluent in ~5 days.
3. Feeder cell freezing (when MEF cells are ~70% confluent)
   a. Discard the supernatant. Rinse cells with 10 mL of sterile PBS.
   b. Detach the cells with 4 mL of Trypsin-EDTA in TC incubator for 5 min. Pipette the mixture up and down to suspend into single cells.
   c. Transfer detached cells into a 15 mL tube and add 8 mL of basic cell culture medium to quench the trypsin. Spin the cells at 500 × *g* at room temperature for 5 min. A pellet should be seen on the bottom.
   d. Discard supernatant and resuspend the pellet evenly with 6 mL of MEF cell freezing medium (for

culture in one T75 TC flask). Aliquot 1 mL of cells into each 1.2 mL freezing vial. Place vials in controlled-rate freezing jar and transfer the jar to -80°C overnight. Transfer frozen cells into the liquid nitrogen tank the next day.

4. Feeder cell inactivation (when MEF cells are ~70% confluent, approximately 6×10^6 cells)
   a. Add 1 mL of 1 mg/mL mitomycin C to 100 mL of basic cell culture medium to make a final concentration of 10 µg/mL.
   b. Retrieve MEF cells in T75 TC flask. Aspirate the medium and rinse once with 10 mL of PBS. Add 20 mL of MEF medium with 10 µg/mL mitomycin C to the flask.
   c. Incubate for at least 2 h in TC incubator.
   d. Aspirate the medium and rinse cells with 15 mL of PBS 3 times to remove mitomycin C. Trypsinize, spin, and resuspend cells in 6 mL of MEF cell freezing medium. Aliquot 1 mL of cells into each 1.2 mL freezing vial. There should be approximately 1×10^6 cells in each vial. Label the vials as inactivated MEF cells (IMEF) to avoid error. Freeze cells as described above.
5. Inactivated feeder cell plating (~7 days before the starting of differentiation)
   a. Coat each well of a new 6-well TC plate with 0.5 mL of 0.1% gelatin as described above. Aspirate the 0.1% gelatin solution before use.
   b. Thaw one IMEF vial in a 37°C water bath. Transfer thawed cells into a 15 mL tube and add 2 mL of basic cell culture medium.
   c. Spin the cells 500 × *g* at room temperature for 5 min. A pellet should be seen on the bottom.
   d. Aspirate the supernatant and resuspend cells in 12 mL of basic cell culture medium for use in one 6-well TC plate.
   e. Add resuspended IMEF cells into the gelatin-coated 6-well plate (2 mL per well). Shake the plate side to side and culture in TC incubator. The IMEF cells should appear ~50% to 70% confluent the next day (Figure 2A).

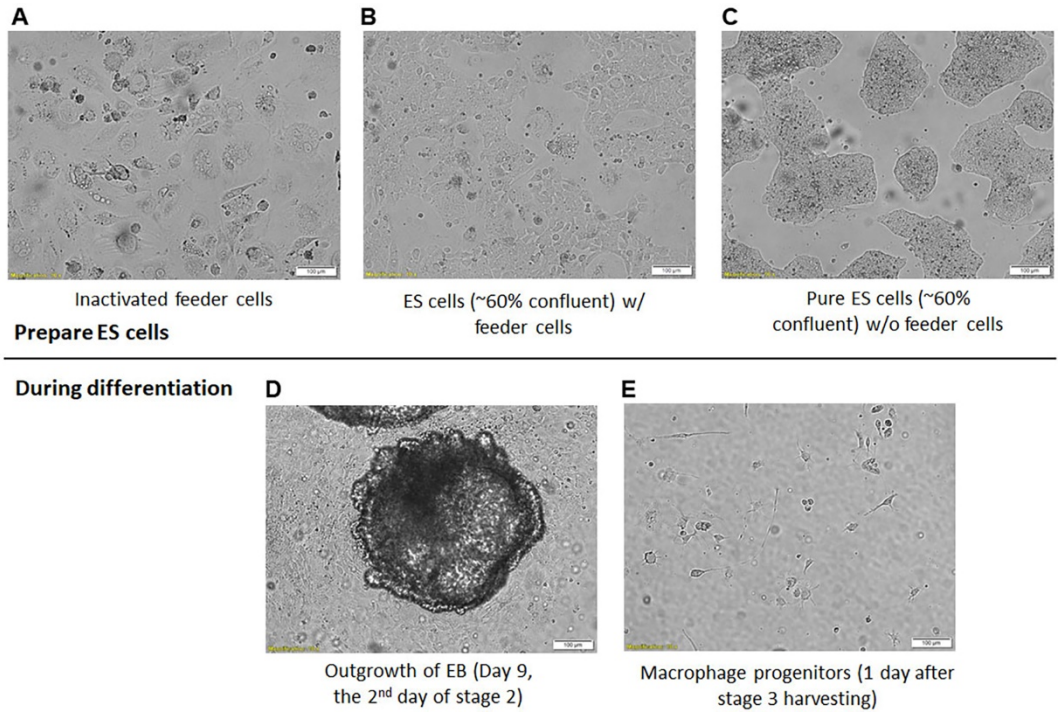

Figure 2. Appearance of cells before and during differentiation.
A. Inactivated feeder cells. **B.** ES cells plated on feeder cells. **C.** ES cells plated on gelatin coated TC plated. **D.** Outgrowth of cells from plated EB during stage 2. **E.** Suspended macrophage progenitor cells one day after replating onto a gelatin coated TC plate. 10× objective lens, scale bar = 100 µm.

C. Mouse ES cell culture for differentiation

1. Thaw one vial of ES cells (approximately 1.5×10^6 cells) in 37°C water bath. Transfer thawed cells into 15 mL tube and add 3 mL of mouse ES cell culture basal medium. Spin 500 × *g* at room temperature for 5 min.
2. Aspirate supernatant. Resuspend the pellet in 12 mL mouse ES cell culture complete medium.
3. Retrieve the IMEF cells coated 6-well plate from incubator. Aspirate the basic cell culture medium.
4. Add 2 mL of the resuspended ES cells into each well. Rock plate side to side. Culture in TC incubator. After 3 days, cells should be ~70% confluent (Figure 2B, approximately 1.5×10^6 cells per well).

D. Differentiation of mouse ES cell into embryoid bodies (EBs) (stage 1, day 0)

1. Removal of IMEF cells (important: small amount of IMEF cells can disrupt the differentiation)
   a. Trypsinize (0.5 mL per well) one well of the 6-well plate of ES cell/IMEF cell co-culture and resuspend into 12 mL of mouse ES cell culture complete medium. Plate 2 mL into each well of a gelatin-coated 6-well TC plate (no feeder cells, 6-fold dilution). ES reach ~60 to 70% confluency in five days in the absence of IMEF feeder cells (Figure 2C).
   b. Retrieve ES cells in 6-well plate from incubator when ~70% confluent. Aspirate the medium, rinse with PBS once (2 mL per well), and trypsinize (0.5 mL/well) for 5 min in TC incubator. Spin cells down and aspirate supernatant.
   c. Resuspend cell pellet in 12 mL of mouse ES cell culture basal medium and plate on another gelatin-coated 6-well TC plate, at 2 mL/well. Incubate ES cells with residual feeder cells in TC incubator for 30 min.
   d. Harvest the supernatant to obtain pure ES cells (residual IMEF cells adhere on the bottom). Spin and resuspend the pellet in 5 mL of PBS.
2. Count the cells with cell counter. Calculate the volume of the suspension needed to obtain 6×10^5 cells for each differentiation.
3. Add 6×10^5 ES cells into a 15 mL tube, pellet the cells, and resuspend in 10 mL of MDM.
4. Transfer the cell suspension to a 100 mm Petri dish (sterile not-TC treated bacteriological), which is designated as day 0 of the differentiation. Place dish on horizontal rocker, set at ~9 s per cycle, which is inside the TC incubator. The rocker prevents possible attachment and merging of EBs. Small EBs floating in medium should be visible after two days (Figure 3).
5. Add an additional 10 mL of MDM in the Petri dish on day 4, and keep cells on rocker. Manage the rocker's angle of tilt to avoid spilling.

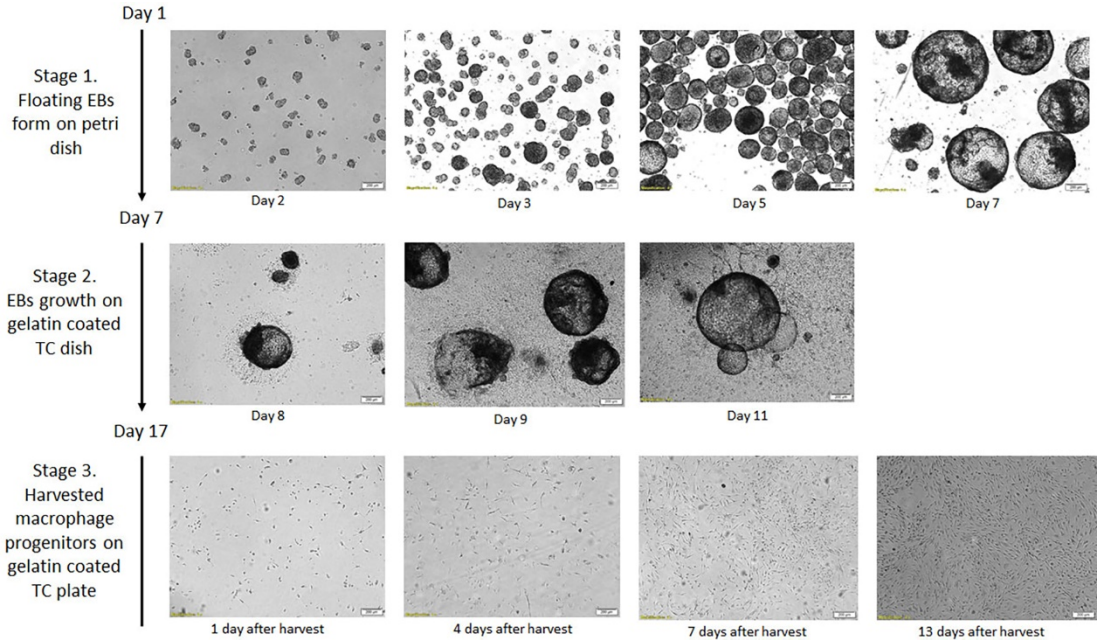

Figure 3. Light microscope bright field images during ESDM differentiation.
Stages 1-3 at different days are shown (4× objective lens, scale bar = 200 μm). Stage 1. Multiple single ES cells aggregate as early E**B**s. The rocker prevents E**B**s from merging with each other or attaching to the Petri dish, ensuring independent development of large spherical E**B**s. Stage 2. E**B**s transferred to gelatin-coated TC dish spread cells out eccentrically until confluent. Stage 3. Macrophage progenitors released by the EBs are harvested and plated on a new gelatin-coated TC plate. The attached macrophage progenitors divide and differentiate.

E. Differentiate large EBs to macrophage progenitors on day 7 (stage 2)

1. On day 7, coat 100 mm TC dish with 4 mL of 0.1% gelatin for 1 h in TC incubator. Aspirate the gelatin solution before use.
2. Retrieve the stage 1 Petri dish of large EBbs from the rocker (Figure 3). There should be >100 large spherical EBs floating in the medium.
3. Transfer all floating EBs together with the original medium into the gelatin-coated 100 mm TC dish gently with a 10 mL pipette, to avoid dissociating the EBs.
4. Gently rock the dish side to side to evenly spread the EBs. Put the stage 2 dish into TC incubator without the use of the rocker, which is not used in any subsequent steps. Culture from day 7 to day 14.
5. The EBs will attach to the gelatin-coated dish on day 8. Aspirate the media and refeed with 10 mL of MDM on days 8 and 12. Outgrowth of adherent cells from the plated EB occurs during this phase (Figures 2D and 3).
6. On day 14, the dish bottom should be fully occupied by growing EBs leaving no room for newly released macrophage progenitors to attach, promoting the macrophage progenitors to float in the medium.

F. Harvest macrophage progenitors on day 14, and every three days thereafter, to differentiate into functional ESDM (stage 3)

1. Retrieve stage 2 TC dish from TC incubator. Floating macrophage progenitors should be seen in the medium.
2. Place a 30 μm sterile filter on top of a 15 mL tube. Gently collect the media containing floating macrophage progenitors with a 10 mL pipette and apply the media slowly into the filter for gravity filtration, to remove

small EBs and cell clusters. Transfer the filtered macrophage progenitors to a new gelatin-coated 6-well TC plate, at a volume of 3 mL in each well, to start the stage 3 cultures. Refeed the 100 mm TC dish at stage 2 with 10 mL of MDM and return it to TC incubator.

3. The stage 3 macrophage progenitors in the 6-well plate should be attached by the next day (Figures 2E and 3). Aspirate medium, wash gently once with PBS, and refeed with 2 mL of MDM. The attached stage 3 macrophage progenitors keep dividing during early stages of maturation. Replenish the MDM medium every five days. Success of ESDM differentiation can be simply screened seven days after stage 3 plating (assessed by acetylated LDL uptake, described below), when ~40% of the cells display a macrophage phenotype.
4. Continue to harvest stage 2 macrophage progenitors every three days as described above. The harvesting can be sustained for up to seven rounds (~21 days after the first harvesting). The only difference we observed between different harvestings is the yield. The first two harvestings give lower yields and the later harvestings yield a larger number of macrophage progenitors.
5. Feed mature ESDM (>13 days of stage 3 plating) with 3 mL of macrophage maintenance medium per well every five days. Mature ESDM remain healthy for up to 15 days after full maturation. Mature ESDM can also be detached using trypsin and a sterile cell lifter for replating. Ideally, the comparison of different ESDMs should be conducted within the same harvesting. However, we found no significant variation on comparing results between different harvestings.

Data analysis

This section describes the assessment of macrophage function and identity of the ESDM.

A. DiI stained acetylated LDL (Ac-LDL) uptake (assay for macrophage function)

1. Follow procedure for AcLDL preparation, dialysis, and DiI labeling as previously described (Robinet *et al.*, 2013). Briefly, add 1 µL of DiI stock (10 mg/mL in DMSO) to 700 µL of PBS in a microfuge tube and vortex well. Add 350 µL of DiI/PBS to 350 µL of AcLDL (range usually 2-4 mg protein/mL), and pipet up and down to mix (do not vortex). Rock in 37°C incubator for 25-30 min and spin 10,000 × *g* at 4°C for 2 min. Filter sterilize the supernatant with a 0.22 µm filter membrane, and dilute Dil-AcLDL to the concentration of 0.75 mg protein/mL.
2. Add 500 µL of Dil-AcLDL to 7.5 mL of MDM (final concentration: 50 µg/mL DiI AcLDL). Gently mix by pipetting (do not vortex).
3. Add 1.5 mL of Dil-AcLDL solution to each well of stage 3 ESDM in a 6-well TC plate, and incubate in TC incubator for 30 min.
4. Aspirate media, rinse cells once with PBS, and add PBS to prepare cells for flow cytometry. Detach cells with a cell lifter and pass through a 30 µm cell filter to remove cell clumps. We previously determined DiI AcLDL uptake via flow cytometry on days 3, 5, 7, 9, 11, and 13 after plating stage 3, and DiI+ ESDM represented 1.3%, 2.4%, 41%, 68%, 87%, and 96% of the cells (Figures 4A and 4B), respectively, as previously described (Hai *et al.*, 2018).
5. To observe DiI by microscopy, fix Dil-AcLDL loaded adherent ESDM in 10% phosphate buffered formalin at room temperature for 10 min. Rinse cells in PBS, and treat with DAPI solution (50 µL of DAPI mounting medium in 1 mL of PBS) for 10 min to stain nuclei. Rinse in PBS and observe cells by epifluorescence microscopy using the rhodamine channel for DiI, and the UV channel for DAPI. Results are shown in Figure 4C.

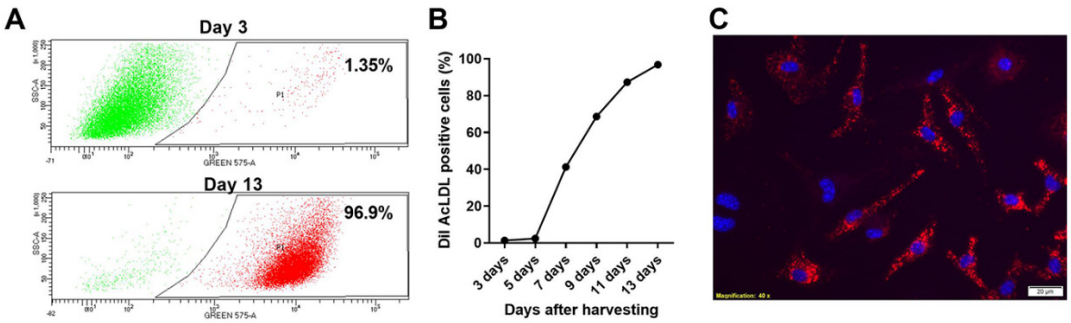

Figure 4. Stage 3 ESDM tested for DiI AcLDL uptake.
A-B. Flow cytometry for DiI-AcLDL positive ESDM at different days after harvesting macrophage progenitors. In **A,** the Y-axis is side scatter, and the X-axis is DiI fluorescence intensity (green dots are DiI negative, and red are DiI positive ESDM). **C.** DiI-AcLDL loaded ESDM (13 days after harvesting) were stained with DAPI and viewed by fluorescence microscopy (40× objective, 20 μm scale bar) using rhodamine and UV filter sets.

B. Macrophage cell markers

1. Retrieve fully differentiated (>13 days after stage 3 plating) ESDM, wash once with PBS, and detach from plates with a cell lifter. Resuspend macrophages in DMEM and pass through a 30 μm sterile filter to remove cell clumps.
2. Mix thoroughly by pipetting, centrifuge at 500 × *g* for 5 min, and aspirate the supernatant. Resuspend cell pellets with 600 μL of phenol red-free DMEM. Distribute 100 μL of cell suspension into microfuge tubes. Add 1 μL of mouse FcR blocker to each tube and incubate for 15 min. Then add 5 μL of antibodies CD11c-Apc-Cy7, Ly6C-PE, and CD11b-PE-CF594, or 2 μL of Ly6G-FITC individually to each tube (final concentration = 0.01 μg/μL for each antibody). Use one tube of cells as an unstained negative control.
3. Incubate for 30 min to 1 h in the dark. Spin cells at 500 × *g* for 5 min and aspirate the supernatant. Wash cells in 300 μL of PBS, spin down, resuspend cells in 200 μL of PBS containing 2% FBS, and run flow cytometry on the Fortessa instrument, using channels appropriate for the four labeled antibodies. Analyze data using FlowJo software. The results are shown in Figure 5. The differentiated cells were CD11b positive, CD11c dimorphic, Ly6C low, and Ly6G negative.

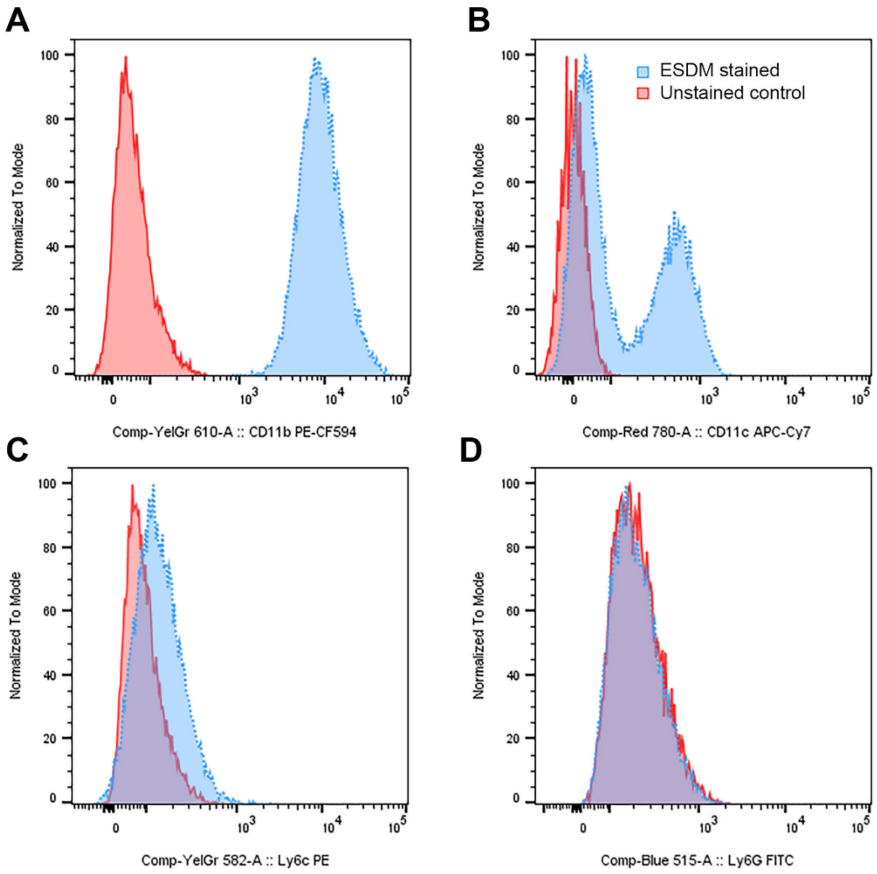

Figure 5. Flow cytometry for cell marker proteins.
The following markers were used: macrophage specific CD11b (**A**), CD11c (**B**), the monocyte maker Ly6C (**C**), and the neutrophil specific marker Ly6G (**D**). The cells were first gated by forward and side scatter to select only single cells. The red traces are for unstained ESDM, and the blue traces for stained ESDM.

C. Other macrophage functional tests

1. ESDM are competent for AcLDL mediated accumulation of cellular free and esterified cholesterol measured biochemically, as previously described (Hai *et al.*, 2018).
2. ESDM are competent for inflammasome activation and IL-1β release into the media, assessed by IL-1β ELISA, as previously described (Ritchey *et al.*, 2021).

Recipes

1. 0.1% gelatin coating solution (Table 1)

a. Add gelatin powder into a 500 mL bottle, then add sterile water as indicated below. Do not overfill the bottle, as contents will overflow during sterilization.
b. Gelatin powder does not dissolve until sterilized. Autoclave with loosely covered lid. The solution should be clear after sterilization.
c. Let the solution cool. Tighten lid and store at room temperature. Use within 6 months.

Table 1. 0.1% gelatin coating solution

Composition	Volume (for 400 mL stock)
sterile water	400 mL
gelatin powder	0.4 g

2. Basic cell culture medium (for MEF cell and L cell culture) (Table 2)

a. Mix all ingredients below in a sterile environment.
b. Mix well and store at 4°C for up to 4 months.

Table 2. Basic cell culture medium

Composition	Volume (for 500 mL)	Final concentration
DMEM/high glucose medium	445 mL	
FBS	50 mL	10%
Penicillin/Streptomycin (10,000 units/mL)	5 mL	100 units/mL

3. MEF cell freezing medium (Table 3)

a. Mix all ingredients below in a sterile environment.
b. Mix well and store at 4°C. Use within 1 month.

Table 3. MEF cell freezing medium

Composition	Volume (for 10 mL)	Final concentration
basic cell culture medium	8 mL	80%
DMSO	1 mL	10%
FBS	1 mL	10%

4. Mouse ES cell culture basal medium (Table 4)

a. Mix all ingredients below in a sterile environment.
b. Sterilize the mixture by passing through 0.22 μm filter.
c. Store at 4°C and use within 3 months.

Table 4. Mouse ES cell culture basal medium

Composition	Volume (for 500 mL)	Final concentration
DMEM/high glucose medium	405 mL	
FBS	75 mL	15%
Penicillin/Streptomycin (10,000 units/mL)	5 mL	100 units/mL
MEM nonessential amino acid (100×)	5 mL	1×
2-Mercaptoethanol (55 mM)	900 μL	0.1 mM
Recombinant Mouse LIF Protein (10^7 units/mL)	50 μL	10^3 units/mL

5. Mouse ES cell culture complete medium (Table 5)

a. Mix all ingredients below in a sterile environment.
b. Store at 4°C and use within 1 month.

Table 5. Mouse ES cell culture complete medium

Composition	Volume (for 50 mL)	Final concentration
mouse ES cell culture basal medium	50 mL	
PD0325901 (10 mM)	5 µL	1 µM
CHIR99021 (30 mM)	5 µL	3 µM

6. Macrophage differentiation medium (MDM) (Table 6)

a. Mix all ingredients below in a sterile environment.
b. Sterilize the mixture by passing through 0.22 µm filter.
c. Store at 4°C and use within 3 months.

Table 6. Macrophage differentiation medium

Composition	Volume (for 500 mL)	Final concentration
DMEM/high glucose medium	340 mL	
FBS	75 mL	15%
Penicillin/Streptomycin (10,000 units/mL)	5 mL	100 units/mL
MEM nonessential amino acid (100×)	5 mL	1×
2-Mercaptoethanol (55 mM)	900 µL	0.1 mM
IL-3 (100 µg/mL in sterile PBS w/ 0.1% BSA)	15 µL	3 ng/mL
L cell conditioned medium	75 mL	15%

7. Macrophage maintenance medium (Table 7)

a. Mix all ingredients below in a sterile environment.
b. Sterilize the mixture by passing through 0.22 µm filter.
c. Store at 4°C and use within 3 months.

Table 7. Macrophage maintenance medium

Composition	Volume (for 500 mL)	Final concentration
DMEM/high glucose medium	340 mL	
FBS	75 mL	15%
Penicillin/Streptomycin (10,000 units/mL)	5 mL	100 units/mL
MEM nonessential amino acid (100×)	5 mL	1×
2-Mercaptoethanol (55 mM)	900 µL	0.1 mM
L Cell conditioned medium	75 mL	15%

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Competing interests

The authors declare that they have no competing interests.

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