Protocol

Bone Marrow-Derived Macrophages (BMM): Isolation and Applications

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INTRODUCTION

Bone marrow-derived macrophages (BMM) are primary macrophage cells, derived from bone marrow cells in vitro in the presence of growth factors. Macrophage colony-stimulating factor (M-CSF) is a lineage-specific growth factor that is responsible for the proliferation and differentiation of committed myeloid progenitors into cells of the macrophage/monocyte lineage. Mice lacking functional M-CSF are deficient in macrophages and osteoclasts and suffer from osteopetrosis. In this protocol, bone marrow cells are grown in culture dishes in the presence of M-CSF, which is secreted by L929 cells and is used in the form of L929-conditioned medium. Under these conditions, the bone marrow monocyte/macrophage progenitors will proliferate and differentiate into a homogenous population of mature BMMs. The efficiency of the differentiation is assessed using fluorescence-activated cell sorting (FACS) analysis of Mac-1 and 4/80 surface antigen expression. Once differentiated, the BMMs are suitable for numerous types of experimental manipulations, including morphological, gene expression, and physiological studies. For example, phagocytic cells such as macrophages have a unique ability to ingest microbes. We describe a test for the phagocytic efficiency of BMMs by exposing them to fluorescently labeled yeast zymosan bioparticles. Also, a method to deliver DNA or small interfering RNAs (siRNAs) into these hard-to-transfect cells is described. Finally, the proliferation of the BMMs is assayed using carboxyfluorescein succinimidyl ester (CFSE), a fluorescein derivative that partitions equally between daughter cells after cell division.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Antibody, anti-mouse 4/80 antigen, allophycocyanin (APC)-conjugated (eBioscience)
Antibody, anti-mouse CD16/32 (Fc block) (eBioscience)
Antibody, anti-mouse Mac-1, fluorescein isothiocyanate (FITC)-conjugated (eBioscience)
Cell line, murine, L929 (ECACC)
Celltrace CFSE Cell Proliferation Kit (Molecular Probes)
<**!**>DAPI (4′,6-diamidino-2-phenylindole) (Molecular Probes)
<**!**>Dimethyl sulfoxide containing 5 mM carboxyfluorescein succinimidyl ester (CFSE stock)

CFSE is a nonfluorescent cell-permeant. In the cytosol, CFSE is cleaved by esterases to an impermeant fluorescent molecule that can be used to track cell proliferation. Store in the dark.

Ethanol, 70%

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Fetal bovine serum (FBS; Invitrogen)
<!>FITC-labeled phalloidin (Fluka) (optional; see Step 24)
<R>Glycerol mounting medium
HEPES (Invitrogen)
<R>L929 medium
<R>Lymphocyte medium
<R>Lymphocyte medium containing 10% L929-conditioned medium (see Step 3) (BMM medium)
Mice, 8-10 wk old
Mouse Macrophage Nucleofector Kit (Amaxa)
<R>Paraformaldehyde in PBS (4% PFA)
<R>Phosphate-buffered saline (PBS), sterile (ice-cold for Step 20; prewarmed to 37°C for Step 48)
Plasmid or siRNA of interest
RPMI-1640 (Invitrogen)
<!>Triton X-100 (Sigma)
<!>Trizol (Invitrogen)
<!>Trypsin, 1X (Invitrogen)
Zymosan A BioParticles, Alexa Fluor 594-conjugated (Molecular Probes)
Zymosan A BioParticles opsonizing reagent (Molecular Probes) (optional; see Step 16)

Equipment

Cell strainer, nylon, 70-µm (BD Biosciences)
Centrifuge, benchtop
Coverslips
Cytocentrifuge
Dishes, tissue culture, 10- or 15-cm (TPP)
Filter, 0.45-µm (Millipore)
Flask, tissue culture, 75-cm^2 (TPP)
<!>Flow cytometer, equipped with a 488-nm argon laser (e.g., FACSCalibur; BD Biosciences)
Forceps
Hemacytometer
Hood, laminar flow
Incubator, humidified, preset to 37°C, pre-equilibrated with 5% CO_2
Microcentrifuge
Microscope, fluorescence
Mortar and pestle (optional; see Step 8)
Nail polish, clear (optional; see Step 27)
Needles, 25-gauge (optional; see Step 8)
Nucleofector II (AAD-1001; Amaxa)
Pipettes
Plates, cell culture, 12- or 24-well
Policeman, rubber or a flattened 1000-µL pipette tip
Scissors, surgical
Slides, microscope
Syringes, 5-mL (optional; see Step 8)
Tubes, 15- and 50-mL
Tubes, microcentrifuge, 1.5-mL
Vortexer

METHOD

Preparation of L929-Conditioned Medium

1. Plate 4.7×10^5 L929 cells in a 75-cm^2 flask containing 55 mL of L929 medium.
2. Grow cells in a humidified incubator with 5% CO_2 at 37°C for 7 d.

3. Collect the supernatant. Filter through a 0.45-µm filter. Store 50-mL aliquots frozen at -20°C (L929-conditioned medium).

Bone Marrow Isolation and BMM Differentiation (Fig. 1)

Sterile techniques are required during and after isolation of bone marrow cells. Rinse all utensils carefully with ethanol. Harvest bone marrow in a laminar flow hood.

4. Sacrifice mice by cervical dislocation.
5. Sterilize the abdomen and hind legs with 70% ethanol.
6. Make an incision in the midline of the abdomen. Clip outward to expose the hind legs.
7. Use scissors to remove all muscle tissue from the bones. Cut the bones at both ends to free them.
8. Crush the bones in a mortar with 5 mL of lymphocyte medium supplemented with 20 mM HEPES. Alternatively, separate the femur and tibia by cutting at the knee joint. Flush the bones with lymphocyte medium using a 5-mL syringe and a 25-gauge needle.
9. Pipet the bone marrow cells up and down to bring the cells into single-cell suspension.

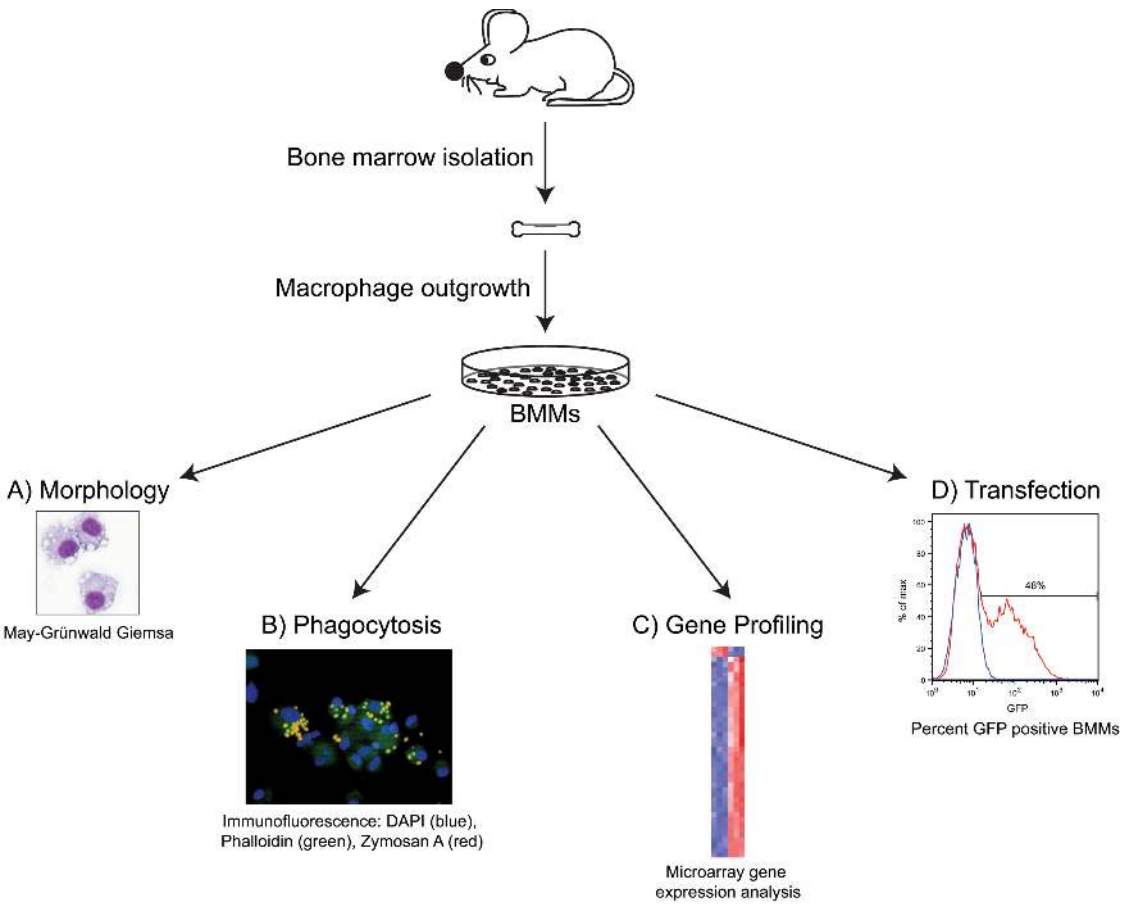

FIGURE 1. Flow chart of isolation and examples of applications for BMMs. Cells are isolated from bone marrow and cultured in vitro. BMMs are suitable for numerous applications including, but not limited to, the examples shown here. (*A*) Morphological examination of cytospins using histological stains (e.g., May-Grünwald-Giemsa staining to visualize nuclei and granules). (*B*) Assays for phagocytic capacity. Fluorescence microscopy is used to visualize DAPI-stained nuclei (blue), phalloidin-FITC-labeled cytoskeletons (green), and phagocytosed Alexa Fluor 594-conjugated zymosan A bioparticles (red). The zymosan A bioparticles appear yellow as they colocalize with the green phalloidin marker. (*C*) Gene expression analyses. Because of their homogeneity, BMMs are an excellent primary cell source. (*D*) Transfection studies. Transfection of BMMs using an Amaxa Nucleofector system results in 40%-50% transfection efficiency. BMMs were transfected with 1 µg of green fluorescent protein (GFP)-expressing plasmid and were assayed 24 h later for GFP expression (red) compared to mock transfected controls (blue). (For color figure, see doi: 10.1101/pdb.prot5080 online at www.cshprotocols.org.)

10. Pass the cells through a cell strainer. Wash the strainer with another 5 mL of lymphocyte medium.

11. Count the bone marrow cells using a hemacytometer. Adjust the concentration to 2×10^6 cells/mL in BMM medium.

12. Plate the cells as needed: e.g., 25 mL in a 15-cm culture dish (for protein or nucleic acid extraction), 12 mL in a 10-cm culture dish, 1 mL per well for 24-well or 2 mL per well for 12-well plates (for phagocytosis assays).

13. Differentiate cells in a humidified incubator with 5% CO_2 at 37°C. Wash cells twice with PBS every 2-3 d, and add fresh BMM medium.

    Macrophage progenitors adhere to the cell dish and are not washed away. Macrophages are fully differentiated at day 6. Grow cells to be used for phagocytosis assays for 7 d.

14. Process BMMs as required for assays for phagocytic activity (Steps 15-28), nucleic acid or protein extraction (Steps 29-30), or harvesting of intact cells (Steps 31-36) for subsequent experimentation.

Phagocytosis Assay (Fig. 1B)

All steps that include fluorescent dyes should be performed in the dark.

15. Incubate BMMs grown in BMM medium for 7 d in 12- or 24-well plates (from Step 13) for an additional 24 h in lymphocyte medium (i.e., BMM medium without L929-conditioned medium).

    This starves the cells for M-CSF.

16. Add 5 µL of fluorescently labeled zymosan A bioparticles and 5 µL of opsonizing reagent to a 1.5-mL reaction tube containing 500 µL PBS. Incubate at 37°C for 1 h.

    Opsonization is optional but increases phagocytosis.

17. Wash the bioparticles twice, 1 mL of PBS each wash, by centrifugation at 1250*g* for 15 min. Count the number of particles.

18. Wash the BMMs twice with RPMI-1640. Count an aliquot of the BMMs from a well. Calculate the number of bioparticles required to add for a multiplicity of infection (MOI) of 10.

    Alternatively, test a range of MOIs, e.g., 5-25.

19. Briefly vortex the opsonized bioparticles. Centrifuge the particles onto the BMMs at 450*g* for 2 min. Incubate the samples in a humidified 37°C, 5% CO_2 incubator for 1 h.

    Remember to include a negative control.

20. Stop phagocytosis by adding ice-cold PBS. Wash the BMMs four times with cold PBS.

21. Scrape the cells with a rubber policeman (or a flattened 1000-µL pipette tip). Centrifuge the cells onto microscope slides using a cytocentrifuge at 500*g* for 5 min.

    Alternatively, cells can be fixed directly in wells. Perform all subsequent steps at room temperature in the dark.

22. Fix cells in freshly prepared 4% PFA for 20 min. Wash twice with PBS.

23. Prepare PBS containing 1% FBS and 0.5% Triton X-100. Permeabilize the cells with this solution for 15 min. Prepare PBS containing 1% FBS (i.e., no Triton). Wash the cells three times with this solution.

24. (Optional) Prepare 1 µg/mL FITC-labeled phalloidin in PBS containing 10% FBS. Stain the cells with this solution for 30 min.

    Phalloidin stains the actin cytoskeleton.

25. Prepare 1 µg/mL DAPI in PBS containing 1% FBS. Remove the medium from the cells and add the DAPI. Incubate for 10 min.

26. Wash the cells twice in PBS containing 1% FBS. Wash in H_2O. Air-dry briefly.

27. Mount coverslips with a few drops of glycerol mounting medium.

    Optionally, seal the coverslips with clear nail polish.

28. Analyze the cells using a fluorescence microscope.

    The phagocytic index is the average number of particles per 100 macrophages.

BMM Extraction

29. Add 4 mL Trizol directly to a 15-cm dish of BMMs. Resuspend the cells by pipetting. Transfer to tubes.

30. Extract RNA, DNA or protein according to the needs of the subsequent experiment.

Harvesting BMMs

The volumes used here are for cultures in a 15-cm dish. Adjust accordingly for other dish sizes.

31. Wash cells with PBS. Add 4 mL of room-temperature trypsin. Swirl the dish briefly to mix. Incubate the samples for 5 min in the incubator at 37°C.

32. Replace the trypsin with 4 mL of fresh trypsin. Incubate for 20-25 min at 37°C with occasional swirling.

    Macrophages are very sticky.

    See Troubleshooting.

33. Add 2 mL of additional trypsin. Resuspend the cells completely by pipetting.

34. Add 6 mL of lymphocyte medium. Resuspend the cells by pipetting. Transfer the cells to a 15-mL tube.

35. Centrifuge at 200*g* for 10 min. Resuspend the pellet in fresh lymphocyte medium.

36. Process cells as required for FACS (Steps 37-40), transfection (Steps 41-47), or cell proliferation (Steps 48-51).

Phenotypic Characterization of BMMs by FACS

All steps are performed at 4°C in the dark.

37. Resuspend 5×10^5 BMMs in 200 µL of lymphocyte medium supplemented with 20 mM HEPES. Add Fc block antibody (diluted 1:400). Incubate for 10 min.

38. Add FITC-conjugated anti-Mac-1 and APC-conjugated anti-4/80 antibodies (both diluted 1:400) to the cells. Incubate for 30 min in the dark.

39. Centrifuge the cells at 300*g* for 5 min. Wash once by centrifugation with 200 µL fresh lymphocyte medium. Resuspend in 300 µL of fresh lymphocyte medium.

40. Analyze the cells by FACS.

    Macrophages are double-positive for Mac-1 and 4/80.

BMM Transfection (Fig. 1D)

BMMs are transfected using Amaxa's Nucleofector device and a Mouse Macrophage Nucleofector kit.

41. Equilibrate the Mouse Macrophage Nucleofector Solution to room temperature.

42. Prepare lymphocyte medium containing 20% FBS. Fill the appropriate number of wells in 12-well plates with 1.5 mL of this medium. Preincubate the plates in a humidified incubator with 5% CO_2 at 37°C.

43. Count harvested BMMs (from Step 36). Centrifuge at 200*g* for 10 min. Resuspend the cells in Nucleofector Solution to a concentration of 1×10^6 cells/100 µL (minimum 100 µL).

44. Add 1-2 µg of plasmid DNA or siRNA to a 1.5-mL tube. Add 100 µL of the BMM/Nucleofector mix to the plasmid or siRNA. Mix gently. Transfer to a certified Amaxa cuvette.

    Make sure that the solution covers the bottom. Avoid air bubbles.

45. Process the sample immediately using the Nucleofector II device, program Y-001**.

46. Add 500 µL of the plated medium (from Step 42) to the cuvette. Transfer the diluted cell suspension back into the prepared 12-well plate.

47. Incubate the cells in a humidified incubator with 5% CO_2 at 37°C for 24-48 h.
The cells can now be used for subsequent experiments.
See Troubleshooting.

Cell Proliferation Assay

48. Dilute the CFSE stock to 5 µM with PBS prewarmed to 37°C.

49. Remove the medium from the BMMs (from Step 36). Add the CFSE. Incubate the cells in a humidified incubator with 5% CO_2 at 37°C for 15 min.
Remember to include a negative control.

50. Wash the cells. Replace with fresh BMM medium.

51. Track cell division by FACS analysis of CFSE on FL-1 using a FACSCalibur. Calculate the number of cell divisions using the FlowJo Proliferation function.
CFSE has substantial fluorescein emission into FL-2, which requires extensive compensation.
See Troubleshooting.

TROUBLESHOOTING

Problem: BMMs are difficult to trypsinize.
[Step 32]
Solution: Flush the cells carefully with trypsin after incubation for 20-25 min. Use prewarmed trypsin.

Problem: BMM transfection fails.
[Step 47]
Solution: Using <100 µL Nucleofector solution will cause a failure in Amaxa Nucleofector transfection. Make sure the transfection solution covers the bottom of the cuvette, and avoid air bubbles.

Problem: CFSE emission is too bright for FACS analysis.
[Step 51]
Solution: Use a lower concentration of CFSE. Normally, CFSE emission is too bright the same day or even the day after. Wait two or more days before analyzing.

DISCUSSION

BMMs are an excellent model to study various mechanisms in a primary cell culture. Compared to many other primary cells, the BMMs are homogenous, have a proliferative capacity, are transfectable, and have a lifespan longer than a week. In fact, BMMs can be grown up to three weeks without noticeable cell death or altered morphology. BMMs can be used as a primary cell culture system to study gene function in vitro (e.g., ablating gene expression in transgenic mice). Altered proliferation, function, and gene expression can all be analyzed using BMMs (Weischenfeldt et al. 2008). Additionally, macrophages are specialized cells that carry out numerous tasks in the immune system such as phagocytosis, antigen presentation, cytokine production, and migration. The BMMs represent a tractable system to assay these functions in cell culture (Kanters et al. 2003; Doyle et al. 2004; Cho et al. 2007).

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