Protocol for Preparation of Bone Marrow Macrophages (BMMs)

(with L-cell media and CSF-1)

Cindy Rohde, July 21, 2004

modified by AWL, 7/29/06, 9/7/06

1. Prepare BMM media (α-MEM, 15%FBS, Pen/Strep/Glut and 50 μM βME).
2. (Day 0) Euthanize 3 mice by CO_2 asphyxiation and confirm death through onset of rigor mortis or cervical dislocation.
3. Pin mouse to styrofoam lid and liberally apply 70% EtOH on the lower portion of the mouse, including the tail.
4. Using sterile technique, excise femurs and tibiae from mouse by cutting at the proximal end of the femur (hip joint) and the distal end of the tibia (ankle joint) with a pair of dissecting scissors. (Be careful not to set your mouse on fire!)
5. Remove adherent tissue as completely as possible from bones (will contaminate marrow prep) and separate bones using a sterile 10" scalpel blade. (Rinse bones/tissue 1-3x with 70% EtOH during tissue removal.)
6. Transfer bones to 35 mm dish on ice.
7. Move into T.C. hood.
8. Fill 5 mL syringe with ice cold BMM media

   5 ml per 2 femurs and 5 mL per 4-5 tibia
9. Using a 25G needle, hold bone with forceps, insert needle into intact end of bone and wash out bone marrow with 1.5 mL BMM media/femur and 0.5 mL BMM media/tibia into a 50 mL conical tube. Invert bone and wash out marrow from open end of bone with another 1 mL/femur and 0.5 mL/tibia. (Total volumes: 2.5 mL/femur and 1-2 mL/tibia)

10. Resuspend cells to make single cell suspension by bringing up the suspension with a 21 G needle attached to a 10 ml syringe and dispelling the suspension.
11. Transfer cells to a 70 μm filter set on top of a new 50 mL conical tube.
12. Allow gravity to filter cells into conical.
13. Count cells by making a 1:5 dilution with PBS. (Conc. $1.36x10^6$ cells/mL; approx. $3.4x10^7$ cells); if desired, dilute cells 1:5 into counting media (BMM media + 2% acetic acid to lyse RBCs)
14. Seed cells @ ~$1x10^7$ cells/mL in BMM media into T25 or T75 flasks.
15. Add 12 ng/mL CSF-1 and 15 ng/mL IL-3 to each flask

    (N.B. may want to decrease IL-3 to 5 ng/mL)
16. Return cells to incubator overnight.
17. (Day 1) Skip the Ficoll step if mice < 4 weeks of age. Ficoll non-adherent cells. Use 15 ml conical tubes. If volume of cells is greater than 10 ml, first spin down and resuspend in 10 ml of media. Use 5 mL cells/3 mL Ficoll. Spin @ 1800 rpm for 30 min. Make sure Ficoll and centrifuge temperature is 20-25 deg.
18. Transfer cells at interface to a fresh 50 mL conical (pool cells) and wash cells with 10 mL BMM wash media.
19. [Optional] Resuspend cells at same volume as first day in 12 ng/mL CSF-1 and 5 ng/mL of rIL-3 and grow for an additional 24 hrs
20. Remove nonadherent cells and wash x 2 with 10 mL of BMM wash media.
21. Resuspend cells in 10 mL BMM media containing 30% L-cell media and count cells diluted 1:5 with PBS. (Conc. $2x10^6$ cells/mL; approx. $1x10^7$ cells)

22. Seed cells @ $8x10^5$ cells/plate ($2x10^6$ cells/mL) into 10 non-TC treated 100 mm dishes in 8 mL BMM media containing 30% L-cell media.
23. Add 20 ng/mL CSF-1 to each plate and return plates to incubator.
24. (Day 3) Two days later, do half media change: replace 50% of the media with fresh BMM media containing 30% L-cell media and 20 ng/mL CSF-1 to each plate. (Be sure to collect floating cells and return cells to plates.)
25. (Day 5) Check cells; if not doing well, add additional CSF-1
26. (Day 6) Two days later, do a complete change of the medium: add fresh BMM media containing 30% L-cell media and 20 ng/mL CSF-1 to each plate. (Be sure to collect floating cells and return cells to plates.)
27. (Day 7) Next day, remove media, carefully wash cells 3x with 10 mL HBSS and starve cells for 16-18 hrs in BMM media alone (no CSF-1).
28. After 16-18 hrs of starvation, remove media and carefully wash cells 2x with 8 mL HBSS and 1x with 5 mL α-MEM (at this time, cells are rounded and attach only loosely to surface). Any longer period of starvation will result in significant cell loss.
29. Add 2 mL α-MEM to each plate and return plates to incubator for 5 min.
30. Stimulate cells with 10 nM CSF-1 for 0 or 1 min.
31. After stimulation, place plates on ice and aspirate media.
32. Carefully wash cells 3x with 5 mL HBSS/0.2 mM Na_3VO_4.
33. Lyse cells with 1 mL 1xLB containing 40 μg/mL aprotinin/leupeptin, 1/1000 DTT/pepstatin, 1/100 PMSF/benzamidine and 2 mM Na_3VO_4.
34. Incubate cells on ice in LB for 15 min.
35. Scrape cells from plate and transfer to an microfuge tube.

36. Vortex vigorously and incubate cells on ice for another 10 min.

37. Spin down cell debris @ 14K rpm for 15 min in cold box.

38. Transfer lysates to fresh microfuge tube and determine protein concentrations.

Results:

BMMs differentiated well and were completely confluent at time of stimulation/lysis.

Materials and Reagents:

To dissect femur and tibia:

10" scalpel blade
scalpel blade holder
dissecting scissors
forceps
styrofoam lid and diaper
beaker with EtOH

For cells:

T25 flasks
Seed cells in non-TC-treated dishes (60 mm or 100 mm) for cell growth;
Seed cells in standard TC dishes for stimulation experiments.

BMM Growth Media:

α-MEM (Earle's salts), with glutamine, with ribonucleosides and deoxyribonucleosides
15% FBS
1x Pen/strep/glut
50 μM β-ME

BMM Wash Media

As above except use 2% FBS (can use regular FBS).

Growth factors

1. rh-CSF-1 stock is 10 μM (800 μg/ml)

dilute to 1:10 (80 μg/ml) in BMM media

12 ng/ml is 1:6667 dilution ➔ 1.5 μl per 10 ml

20 ng/ml is 1:4000 dilution ➔ 2.5 μl per 10 ml

2. rm-IL-3 stock is 0.1 μg/μl (100 μg/ml)

15 ng/ml is 1:6667 dilution ➔ 1.5 μl per 10 ml

5 ng/ml ➔ 0.5 μl per 10 ml