

## Isolation of murine peritoneal macrophages

1. Fill a 10-ml syringe with 3% Brewer thioglycollate medium. Attach 25-G needle and inject 2 ml of the solution per mouse into the peritoneal cavity. Be sure to avoid the bladder. Allow inflammatory response to proceed for 4 days, then euthanize mice by rapid cervical dislocation.
2. Soak the abdomen of each mouse with 70% alcohol and then make a small incision along the midline with sterile scissors. Retract the abdominal skin manually to expose the intact peritoneal wall.
3. Fill a 5-ml syringe with cold harvest medium. With the beveled end of a 20-G needle facing inward, insert needle through peritoneal wall along the mouse's left side (spleen side) and inject 5 ml of the cold harvest medium into each mouse.
4. Using the same syringe and needle, aspirate fluid from peritoneum. Move needle away from the viscera to cause tenting of the peritoneal wall, and withdraw peritoneal fluid slowly.
5. Remove needle from syringe and dispense peritoneal fluid into a 50-ml conical polypropylene centrifuge tube on ice.
6. Centrifuge the peritoneal exudate cells (PEC) in a refrigerated centrifuge 10 min at  $400 \times g$  ( $\sim 1000$  rpm in Eppendorf 5810R),  $4^{\circ}\text{C}$ . Discard supernatant and resuspend cell pellet in cold DMEM/F12-10 by gently tapping the bottom of the tube and pipetting up and down.
7. Count cells a hemacytometer as described in APPENDIX 3A, first diluting  $20 \mu\text{l}$  of cell suspension into  $180 \mu\text{l}$  of DMEM/F12-10 (final dilution of  $10\times$ ) and then applying  $10 \mu\text{l}$  to a hemacytometer.
8. To obtain monolayers of peritoneal macrophages, the cell concentration is adjusted to  $1.5 \times 10^6$  ( $1-3 \times 10^6$ ) total nucleated cells/ml in DMEM/F12-10 medium. The cells are allowed to adhere to the substrate by culturing them 2 hr at  $37^{\circ}\text{C}$ . Nonadherent cells are removed by gently washing three times with warm PBS. At this time, cells should be greater than 90% macrophages. Macrophages can be analyzed at this time or placed back into culture in DMEM/F12-10 medium and maintained for several days.

## Isolation of bone marrow derived macrophage

### Culture medium: L-929 conditioned medium (LCM)

L-929 is a murine fibroblast cell line that is used as a source of M-CSF. L-929 cells (ATCC no. CCL-1) are cultured in DMEM contain 10% FBS and 2% Glutamine until confluent. L-929 conditioned medium is then harvested at confluence; cells are removed by centrifugation, and supernatants are stored at  $-80^{\circ}\text{C}$  until use.

### Prepare mouse bone marrow suspension

1. Euthanize mice by rapid cervical dislocation. Using aseptic technique, peel the skin from the top of each hind leg and down over the foot. Cut off the foot along with the skin and discard. Cut off the hind legs at the hip joint with scissors, leaving the femur intact. Place femurs in a plastic dish containing sterile PBS.
2. Remove excess muscle from legs with gauze.
3. Using sharp scissors or razor blade soaked in ethanol, carefully sever leg bones proximal to each joint.
4. Attach 10-ml syringe to 25-G needle and fill with cold sterile wash medium (Dul-becco's phosphate-buffered saline without calcium and magnesium).
5. Insert needle into bone marrow cavity of femur. Flush bone cavity with 2 to 5 ml of the wash medium, until bone cavity appears white.
6. Place a cell strainer in the 50-ml conical centrifuge tube, transfer the bone marrow directly into the cell strainer. Remove the plunger from the 10 ml syringe and use the black rubber end to mash the bone marrow and release the bone marrow cells into the 50-ml conical centrifuge tube.
7. Centrifuge cells 5 min at 1,200 rpm, room temperature. Resuspend cell pellet in macrophage complete medium by tapping tube and pipetting up and down.

### Culture macrophages

8. Add cells 1 mouse per 150 mm sterile plastic petri dish in 20 ml macrophage LCC medium. Incubate in  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  incubator.
9. On day 3, add another 5 ml of macrophage LCM medium to each dish.

### Harvest macrophages

10. On day 5-7, remove and discard culture supernatants. Wash remaining adherent cells with 5 ml prewarmed Dulbecco's phosphate-buffered saline without calcium or magnesium. Discard wash, add 3 ml trypsin to each dish, and incubate 5 min at  $37^{\circ}\text{C}$ . Cells can be gently scraped to dislodge, but this should not be necessary if cultivated on non-tissue culture treated petri plates.
11. Pool dislodged cells into a 50-ml conical centrifuge tube, and add an equal volume of full DMEM medium. Centrifuge cells 5 min at 1,200 rpm, and remove supernatant.
12. Resuspend cells in 3 to 5 ml LCM medium and count cells using a hemacytometer.

*At this time the cells should be essentially 100% macrophages, with the yield being  $2-3 \times 10^7$  macrophages per mouse. Macrophages can be analyzed directly at this time or they can be replated for adherence and further experimentation. Typically, the cells are plated in LCC medium (see recipe) at  $1 \times 10^5$  cells/well in 48-well tissue culture plates,  $2 \times 10^5$  cells/well in 24-well tissue culture plates, or  $1 \times 10^6$  cells/well in 6-well tissue culture plates.*