Bone Marrow

Materials and Reagents–

RPMI 1640 medium or PBS (Phosphate buffered saline: 0.9%), or Isoton
Squeeze bottle with 70% ETOH
Scissors and forceps
Cutting board or paper towels
60 x 15 mm Petri dishes
3 ml syringes
22 gauge needles
15 ml conical centrifuge tube
RBC lysis buffer

Protocol: Bone Marrow

1. Kill mouse either by cervical dislocation or CO_2 inhalation. Place the mouse on its back on cutting board and soak it with ethanol to reduce the possibility of hair becoming airborne.
2. Make a long transverse cut through the skin in the middle of the abdominal area. Reflect skin from the hindquarters and the hind legs.
3. Separate the legs from the body at the hip joint, remove the feet. Place the legs in a Petri dish containing medium.
4. Remove all muscle tissue from the femur and tibia, separate femur and tibia and cut off the epiphysis on both ends.
5. Puncture the bone end with a needle and flush out the bone marrow with 3 ml of media.
6. Remove the large debris and cell clumps by layering the cell suspension over 3 ml of heat-inactivated FBS for 10 minutes on ice. Wash the suspension by centrifugation at 300 x g for 10 minutes at 4ºC and keep in medium with at least 5% FBS.

Protocol: Spleen, Thymus and Lymph node cells

1. Locate the specific organs (LN under armpits or hind legs, Thymus lies over heart, spleen lies in the left quadrant of the abdomen).
2. Remove organs by using forceps to raise organ and scissors to carefully cut membranes from organ.
3. Place organs in small, 25mm petri dish with sterile screen. Add 1 ml of RPMI media or PBS.
4. Using the plunger portion of a 1 ml syringe, push the organ through the screen until all the cells are in a single cell suspension (do NOT rub plunger over tissue and screen since this will rupture the cells).
5. Remove the cell suspensions using the syringe, and place into a sterile capped tube.

Comments:

1. For tissue culture studies, do the whole isolation in a sterile hood and use only sterile instruments, media and dishes and screens. Do not use the same scissors you used for cutting the skin for muscle removal unless you flame them with alcohol 1st.
2. Keep cells on ice.
3. Typical yield of cells is ~3-7 x 10^7 per mouse for bone marrow, ~2 x 10^8 spleen cells, ~7 x 10^7 Thymus cells, and ~3 x 10^7 lymph node cells.

Peritoneal Cells

Materials and Reagents:

RPMI 1640 medium or PBS
Squeeze bottle with 70% ethanol
Scissors and forceps
Cutting board or paper towels
5 ml syringes
22 gauge and 23 gauge needles
Thioglycolate medium
15 ml conical centrifuge tubes

Protocol:

1. 3 days prior to the experiment, inject the mouse i.p. with 3 ml of sterile Thioglycolate.
2. Kill mouse by cervical dislocation or CO_2 inhalation. Place the mouse on its back on cutting board and soak it with ethanol to reduce the possibility of hair becoming airborne.
3. Make a small cut through layer of skin with hair and pull the hair and skin back to expose the underlying muscle. The abdomen should be bloated with fluid and cells.
4. Grab the sides of the peritoneal cavity and massage the cavity to help release adherent cells into the cavity of the mouse.
5. Hold the abdominal muscle with a forceps and make a very small incision into the muscle. Hold the muscle up so that the fluid in the abdominal cavity does not leak out.
6. Insert a sterile pasteur pipette into the incision you made in the muscle layer and withdraw the fluid from the peritoneal cavity and place into a test tube.

Comments:

1. The average yield is 2×10^7 cells per mouse. If the cells are harvested at 24 hours the primary cell type is a neutrophil. If harvested at 48-72 hours the primary cell type is an “activated” macrophage. The activated macrophage has the respiratory burst induced and is better able to phagocytize and kill ingested bacteria.
2. The yield is dependent upon the mouse strain.

Isolation of B lymphocytes

1. By cytotoxic elimination of T cells

Materials and Reagents:

Spleen cell suspension
Antibodies of known specificity and cytotoxic titer
- anti-Thy-1 (mouse cells)
- anti-CD3 (human cells)
- Anti-CD4 (will eliminate T helper cells)
- Anti-CD8 (will eliminate T cyotoxic cells)

Rabbit complement
15 ml conical centrifuge tubes

Protocol:

1. Dilute spleen cell suspension to 10^7 cells/ml. Add 1 ml of antibodies and incubate for 30 minutes.
2. Wash the cell suspension in medium at 250 x g for 10 minutes at room temperature.
3. Add 1 ml of diluted rabbit complement and incubate for 30 minutes at 37°C.
4. Wash the cell suspension 3 X in medium at 250 x g for 10 minutes at room temperature.

Comments:

1. Complement activated lysis of T cells will give a relatively pure population of B lymphocytes. To determine how pure the remaining cells are you can bind a polyclonal antibody to mouse Ig (all classes), and then label with a fluroscein dye and count the number of cells that stain positively and negatively. This will give you the relative percentage of purity of the B cell population.

Nylon Wool Purification of B and/or T Lymphocytes

Materials and Reagents:

Spleen cell suspension
RPMI 1640 medium with 5% FBS
50 ml conical centrifuge tubes
3 way stopcock
Nylon wool
10, 30 or 60 ml disposable syringes
HCl (1%)
Glass beaker

Protocol:

1. Boil the nylon wool in 1% HCl in a glass beaker for 20 minutes. Wait until the flid is cold and then pour it off. Wash the nylon wool repeatedly to remove all HCl and check the pH. Let the nylon wool dry at room temperature, and weigh in appropriate aliquots (2 grams of nylon wool is suitable for separation of ~ 3×10^8 cells in 4 ml).
2. Prepare the nylon wool column by packing a syringe of appropriate size with nylon wool (i.e., 2 grams of nylon wool packed into a 10 or 30 ml syringe).
3. Equilibrate the column by running 10x the loading volume (amount that will fill up the syringe with nylon wool) of warm (37ºC) medium. Remove all trapped air bubbles by tapping on the column sides. Compact the nylon wool with a pipette.
4. Close the 3-way stopcock and add an extra 2 to 3 ml of warm medium. Incubate the column for 30 minutes at 37ºC.
5. Drain the column completely and add the prewarmed cell suspension (approximately 7.5×10^7 cells/ml).
6. Drain the column completely and add the prewarmed cell suspension (~ 7.5×10^7 cells/ml).
7. Incubate the column for another 30 minutes at 37ºC.
8. Fill the column with warm medium, open the stopcock and collect the first 15 to 20 ml of nonadherent cells.
9. Wash the cell suspension twice (2X) in an appropriate medium at 250 x g for 10 minutes at 4ºC.

General Protocol for Cytokine ELISA

Materials and Reagents:

Enhanced protein-binding plates
Multichannel pipettete (50-200 ul)
Parafilm
Refrigerator
Incubator (37º)
Multiwell Spectrophotometer (Plate Reader)
Capture and detection anti-cytokine antibodies (detection antibody is biotinylated)
Avadin (or Strepavidin)-alkaline phosphatase (or peroxidase) conjugate
Substrate:

a) p-nitrophenylphosphate tablets for alkaline phosphatase assay;
b) 2,2'-Azino-bis (3-ethylbenzthiazolline-6-sulfonic acid) for peroxidase assay

Coating buffer: 0.1 M $NaHCO_3$, pH 8.2
Blocking Buffer: Phosphate-buffered saline containing 10% FBS
Dilution Buffer: PBS-10% FBS
Washing Buffer: PBS containing Tween 20 (0.05%)
Substrate Solution:

a) for alkaline phosphatase, dissolve p-nitrophenyl phosphate tablets to a final concentration of 1 mg/ml in 20 mM diethanolamine-HCl buffer, pH 9.5 containing 1 mM $MgCl_2$
b)for peroxidase, dissolve ATBS to a final concentration of 0.3 mg/ml in 0.1 citric acid, pH 4.35; then add 10 ul of 30% H_2O_2 per 10 ml of solution

Stopping Solution:

a) 10 N NaOh for alkaline phosphatase;
b) 16% SDS solution in 40% N,N-dimethylformamide (SDS/DMF) for peroxidase

1. Coating Plates with Capture Antibody
    1. Dilute purified anti-cytokine antibody to 1 to 2 ug/ml in coating buffer
    2. Using enhanced protein-binding plates, add 100 ul of the diluted antibody per well
    3. Seal the plate with parafilm and incubate for 6 hours at room temperature of overnight at 4ºC.

2. Blocking the Plates
    1. Discard the antibody solution and wash the wells twice with PBS.
    2. Add200 ul/well of blocking buffer.
    3. Seal the plate with parafilm and incubate for a minimum of 2 hours at 37ºC or overnight at 4ºC.
    4. Plates can be used immediately after completion of the blocking step, or stored at 4ºC without removing the blocking solution. If stored for later use, it is recommended to place the sealed plates inside plastic bags to minimize evaporation.

3. Samples/Standards
    1. Determine the number of wells needed. It is advisable to assay each sample at least in duplicate or triplicate. Include extra wells for blanks and recombinant cytokine standards (6-7 different concentrations in the 10-2,000 pg/ml range)
    2. Add samples and standards (50 ul/well). Cover an's 'd incubate for at least 2 hours at 37oC or overnight at 4oC.
    3. Wash 4 times with PBS-0.05% Tween 20.

4. Secondary (Detection) Antibody:
    1. Dilute the biotinylated secondary antibody to 1 to 2 ug/ml in dilution buffer.
    2. Add 60 ul of diluted antibody per well, cover, and incubate for at least one hour at 37oC.
    3. Wash 6 x with PBS-0.05% Tween 20.

5. Avidin-Enzyme Conjugate:
    1. Dilute the avidin-alkaline phosphatase (or avidin-peroxidase) conjugate to the manufacturer's recommended dilution (usually 1-2 ug/ml) using dilution buffer.
    2. Add 100 ul per well, cover, and incubate for at least 30 minutes at 37ºC.
    3. Wash 6 x with PBS-Tween 20.

6. Substrate:
    1. Prepare the appropriate substrate solution (p-nitrophenyl phosphate for alkaline phosphatase, ATBS-H2O2 for peroxidase) at least 5 minutes before use. Substrate solution must be at room temperature.
    2. Add 100 ul per well. Allow to develop at room temperature for 10 to 60 minutes.
    3. Stop the reaction by addition of stop reagent. (25 ul/well of 10 N NaOH for alkaline-phosphatase reactins, or 100 ul/well of SDS/DMF solution for peroxidase reactions).
    4. Read at OD 405 nm in plate reader.