Isolation of Hematopoietic Stem/Progenitor Cells From Murine Bone Marrow

Crude Bone Marrow Cell Preparation

1. Isolate bone marrow cells from mice and pass cells through 40/70μm nylon mesh.
2. Centrifuge at 1400 rpm for 10 minutes and resuspend cells in 5mL of red blood cell lysis buffer (ack lysing buffer).
3. Incubate for 5 minutes at room temperature and overlay cell suspension onto 5mL of heat-inactivated FBS.
4. Centrifuge at 1400 rpm for 10 minutes and resuspend cells in RPMI1640 (10-20 mL) and determine the cell density.
5. Centrifuge cells at 1400 rpm for 10 minutes and resuspend cells in MACS buffer at $1x10^8$ cells /mL.

Antibody Treatment

1. Add biotin-conjugated lineage marker antibodies (BD Bioscience: 2μL each antibody/10^6 cells). a. To also remove IL-7Rα positive cells from crude BM, add biotin-conjugated anti-IL-7Rα antibody (BD Bioscience: 1μL/10^6 cells), simultaneously.
2. Mix and incubate on ice for 15 min.
3. Wash cells twice with 10mL MACS buffer and centrifuge at 1400 rpm for 10 minutes
4. Resuspend cells with MACS buffer at 80μL/10^7 cells (for fewer cells, use same volume).
5. Add anti-biotin micro beads (Miltenyi Biotec) at 20μL/10^7 cells (for fewer cells, use same volume).
6. Mix and incubate cells for 15 min on ice.
7. Wash cells with 10mL cRPMI and centrifuge at 1400 rpm for 10 minutes.
8. Resuspend up to 10^8 cells in 500μL MACS buffer (for fewer cells, use same volume).

Lineage Negative Cell Collection (MACS)

MS column: up to 10^7 positive cells and up to $2x10^8$ total cells

LS column: up to 10^8 positive cells and up to $2x10^9$ total cells

1. Place the column in the magnetic field of a suitable MACS separator.

2. Prepare column by washing with proper amount of buffer (MS: 500µL; LS: 3mL) and apply cell suspension at up to 10^8 cells per 500µL buffer onto the column (MS: 500–1000µL; LS: 1–10µL).
3. Rinse with appropriate amount of buffer (MS: 3 x 50µL; LS: 3 x 3mL) and remove column from separator. a. When you want to collect positive cells, place column on a suitable collection tube, and pipette appropriate amount of buffer (MS: 1mL; LS: 5mL) onto the column and firmly flush out positive fraction using the plunger supplied with the column.
4. Centrifuge at 1400 rpm for 10 minutes.

FACSCaliber (Cell Purity Confirmation)

1. Suspend cells in FACS buffer (10^7 or fewer cells per 100µL buffer) and add 10µL of Streptavidin-APC.
2. Mix cells, and incubate for 5 minutes in the dark on ice.
3. Wash cells with 1-2mL of FACS buffer per 10^7 cells centrifuging at 4000 rpm for 5 minutes and analyze by FACS.

Isolate LKS^+ and LKS^- (CMP, GMP, and MEP) Fractions From Lineage Negative Cells

1. Incubate lineage negative cells with mouse IgG for 15 minutes on ice.
2. Stain cells with PE anti-Sca-1, FITC anti-CD16/32, and APC anti-c-kit for 30 minutes on ice.
3. Wash cells with FACS buffer and resuspend in 400µL of buffer (PBS).
4. Sort each fraction using FACSAria.

Cell Culture

1. Culture hematopoietic progenitor cells with GM-CSF (10ng/mL) for 24-72 hours in 96-well round-bottom culture plate at 200µL/well.

Cell Harvest

1. Collect floating cells from the 96-well plate
2. Harvest adherent cells from plate with 0.05% Trypsin and EDTA
    a. Wash cells with 200µL HBSS
    b. Rinse cells with 200µL EDTA (0.025%)
    c. Incubate cells with 50µL Trypsin/EDTA for 3 minutes at 37°C.
    d. Incubate cells with 50µL Trypsin/EDTA for 5 min at 37°C.
    e. Rinse with 100µL cRPMI1640
3. Centrifuge at 4000 rpm for 5 minutes.

4. Resuspend cells in sorting buffer (PBS)
5. Sort cells using FACSAria.

Hematopoietic Cell Culture Media

1. For myeloid culture: StemPro-34 SFM medium (Invitrogen) containing L-glutamine (2mM), penicillin (100U/mL), and streptomycin (100mg/mL).
2. For lymphoid culture: X-VIVO15 medium (Biowhittaker), which seemed to be optimal for lymphoid cultures, containing detoxified BSA (1%, Stem Cell Technology), 2-Mercaptoethanol ($5x10^{-5}$M), L-glutamine (2mM), penicillin (100U/mL), and streptomycin (100mg/mL).

LKS^+ Cells (Immunity 24, 801-812, 2006)

1. Sorted LKS^+ cells should be cultured at 10,000 cells/well in 96 well round bottom plate in the presence of FL (100ng/mL) and SCF (20ng/mL) with or without TLR ligands for 24-72 hours.
    a. Similar results can be obtained when 1600, 5000, or 7500 LKS^+ cells were plated per well.
    b. FL and SCF together prompt cell viability, but alone cause little differentiation.
    c. Expanded cells should express the Mac-1 and or Gr-1 myeloid markers, but not B220.

LKS^- Cells (Immunity 24, 801-812, 2006)

1. Sorted LKS^- cells should be cultured at 10,000 cells/well with medium alone, GM-CSF (20ng/mL), M-CSF, or TLR ligands for 24-72 hours.
    a. Only a subset of lineage-negative myeloid progenitors respond to GM-CSF.
    b. Virtually no viable cells are recoverable from wells with no stimulus.

CMPs, GMPs, and MEPs (Immunity 24, 801-812, 2006)

1. Stimulate sorted CMPs or GMPs with TLR ligands for 24-48 hours. a. MEPs die in culture, regardless of stimulus, and cells expressing the erythrocyte-associated TER119 marker are not recoverable.
    a. GMPs produce $F4/80^+$ cells in response to TLR ligands within 24 hours.
    b. GMPs produce $Mac\text{-}1^+F4/80^+$ and $Mac\text{-}1^+F4/80^-$ cell after culture for 72 hours.
    c. CMPs also produce $F4/80^+$ cells in response to TLR ligands, but require more time and at a lower yield.