Transduction of BM cells and in vitro differentiation to neutrophils draft

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A. Harvesting Retrovirus

Note; Phoenix Eco packaging cells (see ATCC or web sites of Nolan lab, Stanford Univ. http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html)

MIEG3 retrovirus vectors (Williams DA, at el. *Blood*. 2000; 96:1646-54)[1]

Lipofectamine reagent (GIBCO BRL)

sodium butyrate (Sigma)

1. Prepare Phoenix cells on T75 flask to be 80% confluent in 10mL of DMEM (high glucose) with 10% FCS.
2. Add plasmids (MIEG3, MIEG3HR1 or MIEG3FR2) to the Phoenix cells (8μg DNA to each flask) using Lipofectamine reagent as the way the company recommends.
3. 18 hours after introducing plasmid DNA to Phoenix Eco cells, change the medium to DMEM containing 10mM sodium butyrate[2] and 10% FCS. 8 hours after, wash the cells twice with warmed PBS gently, and

add 10mL of fresh alpha MEM with 20% FCS for BM cells. Incubate at 32 or 37˚C.

4. Two to four days after introducing plasmid DNA to Phoenix Eco cells, collect the supernatant every 24 hours, filter with 0.45μm filter, and store in -80˚C freezer until they are used for the transduction. We usually titer on 3T3 cells. Use freshly thawed sup for transduction. To thaw sup, put the tubes of virus sup at RT for 10min, then incubate them at 37˚C until the sup is thawed completely.

B. Transduction mouse bone marrow progenitor cells with retrovirus and differentiation to Neutrophils in vitro.

Note; 5-FU(Adrucil®, Phamecia)

cytokines (murine SCF, MGDF, G-CSF, IL-3)

CH296 (Takara Shuzo, Retronectin®)

Cell Dissociation Buffer (GIBCO BRL)

Fetal bovine serum (Hyclone Lab. Inc. “defined”)

1. Inject 5-FU to mice (3mg/mouse) intrapetritoneally (On Day 48-72 hours before BM harvest).
2. On Day0, harvest bone marrow cells (BM cells) from femur and tibia using 5mL syringes and 22G needles to flush BM with 3ml/mouse of alpha MEM without serum. Total of 1 to 3 x10^7 cells are usually

obtained from one mouse. Wash BM cells with PBS (1300rpm, 10min) and incubate the pellet in RBC lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, and 0.1mM EDTA) for 3 minutes on ice. After spinning down, wash BM cells with PBS (1300rpm, 10min), get count the cell counts, spin down again (1300rpm, 10min), and then resuspend the cells (5-10 x 10^6 cells/mL) in alpha MEM containing 20% FCS and cytokine cocktail (100ng/mL murine SCF, 100ng/mL MGDF, and 100ng/mL G-CSF) for the prestimulation. Incubate the BM cells for 2days for the prestimulation at 37˚C and 5% CO2.

3. On day1, coat non-tissue culture coated 6well plate with CH296 in PBS (4µg/cm^2, that is 40µg/2mL/well) at 4˚C, O/N for the retrovirus infection. The Next day (day2) decant supernatant, block the wells with 2% BSA in PBS for 30minutes at RT, and wash once with 5mL of PBS/well gently.
4. As described below, BM cell are infected on Day 2, 3, and 4 with retrovirus supernatant total of 3 times (once in 24 hours) Use the medium without retrovirus as the mock trandsduction[3,4].

   <u>On the day2</u>, suspend the BM cells (5x10^6 cells in 4mL) in the retrovirus sup plus cytokines (100ng/mL murine SCF, 100ng/mL MGDF, and 100ng/mL G-CSF) and incubate on the CH296 coated 6 well plates.

On day 3, remove supernatant, spin down(1300rpm, 10min) the BM cells. Add half volume of 4ml virus sup (2ml) so as not to make plates dry. Resusupend the cells in the other half of retrovirus sup (2ml) with cytokines (finally to be 100ng/mL murine SCF, 100ng/mL MGDF, 100ng/mL G-CSF in 4ml sup), and add to the well from which the cells were taken.

On day 4 when infection is done in the same way as day3, but change the cytopkines to 0.3ng/mL G-CSF and 50 IU/ml IL-3 for start of differentiation.

On day 5, take both suspension cells and adherent cells using Cell Dissociation Buffer and resuspend in the fresh alpha MEM medium containing G-CSF, IL-3 and 20% FCS.

5. On day 8 sort the cells for GFP+ cells by FACStar and continue to culture the sorted cells in alpha MEM containing 20% serum and cytopkines (0.3ng/mL G-CSF and 50u/mL IL-3). 2 x 10^6 sorted cells / each genotype are needed to get 2-4 x 10^7 cells on Day 16. Every other day (Day10, 12, 14) after sorting, get cell counts and add the same media with fresh cytokines (0.3ng/mL G-CSF and 50 IU/ml IL-3) to adjust cell concentration to be 5 x 10^5/ml. You may need to split the expanded cells to another flasks.

Typically, cell number expands 50-200 fold from the input cells on Day 2 through Day14-16 if no sorting.

6. On day12-14 count the suspension cells and you can do the assays. Use suspension cells but not adherent cells. Most of the cells (more than 85%) are PMN.