Video Article

Homing of Hematopoietic Cells to the Bone Marrow

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Abstract

Homing is the phenomenon whereby transplanted hematopoietic cells are able to travel to and engraft or establish residence in the bone marrow. Various chemokines and receptors are involved in the homing of hematopoietic stem cells. [1, 2]

This paper outlines the classic homing protocol used in hematopoietic stem cell studies. In general this involves isolating the cell population whose homing needs to be investigated, staining this population with a dye of interest and injecting these cells into the blood stream of a recipient animal. The recipient animal is then sacrificed at a pre-determined time after injection and the bone marrow evaluated for the percentage or absolute number of cells which are positive for the dye of interest. In one of the most common experimental schemes, the homing efficiency of hematopoietic cells from two genetically distinct animals (a wild type animal and the corresponding knock-out) is compared. This article describes the hematopoietic cell homing protocol in the framework of such an experiment.

Video Link

The video component of this article can be found at <http://www.jove.com/video/1104/>

Protocol

1. We begin the homing experiment by extracting cells to be used in the experiment. For this current experiment we are interested in finding out if the homing of whole bone marrow to the niches in the long bones of recipient animals is different for bone marrow cells extracted from WT animals such as C57BL/6J (WT) and those that are transgenic knockouts for Lysophosphatidic Receptor 1 (LPA1) (KO).
2. Before we start the experiment, we will collect the materials we need for this experiment. In our lab all mouse dissections are carried out in a laminar flow tissue culture hood especially if the cells are going to be transplanted into a recipient animal instead of being used for *ex-vivo* analysis. We start by placing a blue sheet inside the hood. Other objects needed include a pair of forceps and a sharp pair of scissors which have been autoclaved, a sterile disposable scalpel and alcohol swabs. We will need modified PBS and a sterile 6 well plate, a mortar, pestle, 50cc blue topped conical tube and a 70µM disposable filter.
3. We start by taking one WT and one KO mouse.
4. These mice are euthanized by CO₂ inhalation and then dipped in 70% isopropanol for 5 seconds. This step prevents contamination of the cells of interest with mouse fur and in our hands has not led to any compromise in yield of cells or experimental results.
5. The mice are then dissected in turn using a pair of forceps and sharp scissors. We usually start with the WT mouse as a matter of routine. A small snip is made in the ventral skin of the mouse overlying the abdomen and the skin is then stretched using the hands. The skin comes off the hind limbs easily. The femur and tibia are then extracted taking care not to dislodge the head of the femur as this contains a large amount of bone marrow. The upper limbs are detached from the animal's torso by simply applying traction to them while keeping the torso immobile. This removes the upper limbs as one piece. The humeri are then extracted from the upper limbs.
6. The removed bones are placed in modified PBS which has been added to 6 well plates. Modified PBS is made by adding 2 ml of heat inactivated Fetal Bovine Serum and 2 cc of EDTA 0.5 M pH8. This solution is then filtered through a Nalgene 0.45 µm filter and can be stored at 4 degrees Celsius for at least a month.
7. It is essential to label the wells in the plate in order to be able to identify WT and KO bones correctly.
8. The mouse carcasses are removed and disposed off.
9. A new sheet is placed in the hood and the bones are cleaned using a disposable scalpel.
10. The cleaned bones are placed one mouse at a time in the mortar. Two ml of modified PBS is added to the mortar and the bones are crushed using a pestle. It is important to first fragment the bones with 10 to and fro movements of the pestle and then pulverize them with circular rotatory movements. I usually do 50 clockwise, then 50 counterclockwise movements and so on until the material left in the mortar is white in color and the modified PBS added to the material remains white or transparent.
11. After every set of 50 circular crushing movements, we transfer the fluid from the mortar to a 70 µm filter placed on top of a 50 ml conical tube. Do not forget to add 2 ml of PBS to the mortar after every transfer to the filter. You do not want to try and pulverize dry bones or leave them dry for any length of time as this will lead to apoptosis of cells.
12. Once the material in the mortar becomes white, the material in the filter is transferred to the mortar and the crushing is again performed. This ensures that the cells in the filter are not wasted. Usually it takes one or two sets of circular rotatory movements to again make the material in the mortar white in color at which time the extraction of cells is complete.

13. The solution in the conical tube is now centrifuged (please remember to keep the cells from the WT and KO mouse separate. Hence you will have two different tubes (one with WT cells, the other with KO cells). Centrifuge at 1200 rpm for 10 minutes.
14. Aspirate off the supernatant using separate aspirator pipettes for each tube. Add 2 cc of ACK lysis buffer to each pellet. This lysis buffer should be sterile. Incubate on ice for 5 minutes.
15. Add 8 cc of modified PBS to each tube. Take 10 ul from each tube and pipette into two separate wells in a 96 well plate. Place the tubes in the centrifuge and spin them at 1200 rpm for 10 minutes.
16. While the tubes are spinning, add 30 ul of trypan blue dye and 60 ul of modified PBS to each well of the 96 well plate that carries the cells of interest.
17. Add 10 ul of this mixture (please mix well by pipetting up and down) to a hemocytometer and count the cells in the four outer squares.
18. The cell count in each sample per ml is the number of cells thus counted divided by 4 and multiplied by 100 000.
19. Once the centrifugation is complete, remove the tubes from the centrifuge and resuspend the cells in PBS without calcium, magnesium or L glutamine at a concentration of 20 million cells per ml. You will likely have about 5 ml of volume for the WT and 5 ml of volume for the KO animal. From this step onward we will be using PBS, not modified PBS for the protocol.
20. To each tube add Dil, a cell staining dye, to achieve a final concentration of 5uM. Vortex immediately for 10 seconds. Incubate at 37 degrees for 10 minutes and avoid exposure to light.
21. Fill the tubes with PBS and spin for 10 minutes at 1200 rpm.
22. After centrifugation, aspirate the supernatant and resuspend the cells in PBS at 40 million cells per ml.
23. Load 500 ul into 1 cc syringes with a 27G needle (5 syringes for the WT mouse and 5 syringes for the KO mice). It may be prudent to prepare 6 syringes for each cohort. Having one extra can help as tail vein injections which we are going to do next can be tricky.
24. Cover the syringes with foil in order to keep the Dil from disintegrating.
25. Inject 500 ul of the cell mixture into each recipient animal; (5 for each cohort).
26. These animals need to be irradiated with 9 Gy of irradiation 4-24 hours prior to injection.
27. 48 hours after injection, harvest bone marrow from the hind limbs of each recipient animal.
28. Process as described above for the donors. Resuspend the cells from each animal in 1 cc of PBS. Obtain a cell count using a hemacytometer.
29. Read on the LSR II flow cytometer from BD or an equivalent instrument. The result can be expressed as the percentage or absolute number of cells in the analyzed bone marrow which are Dil positive.

Discussion

Homing is the process whereby bone marrow cells including stem cells, progenitor cells and differentiated cells, their way into the bone marrow after being injected into the blood stream or bone marrow cavity of mice. As is obvious from the definition, a scientist may chose to study the homing of hematopoietic stem cells, progenitor cells or differentiated cells identified by immunophenotyping and isolated by cell sorting. Typically scientists inject bone marrow cells depleted for lineage markers and hence enriched for progenitor and stem cells.

The dose of the cells of interest injected is a variable in this experiment. The number of cells can vary from 500 000 per recipient mouse to 20 million per recipient mouse. The more cells one can inject the easier the detection in the bone marrow. Cell number availability may limit the number injected. Sorting 20 million LKS SLAM cells is extremely difficult perhaps even impractical with the current technologies in most laboratory settings, in which case one might decide to use a number of cells towards the lower end of the range given above.

The host animal in which homing is being studied can also be varied. This recipient animal may be a WT C57BL/6J mouse which has been lethally irradiated with 9 Grays of radiation as we did in this experiment, a WT C57BL/6J mouse or a W/Wv mouse which has not been irradiated. Whether the mouse is irradiated or not depends on the question the experimenter is trying to answer. If one is interested in studying homing in a setting where a large amount of cytokines is being released and vascular leak is being induced, an irradiated WT mouse is an appropriate choice. A smaller number of cells (500 000) may be injected into a WT mouse which has not been irradiated so that the stem cells can home to a small number of unoccupied niches without the above confounders.

W/Wv mice are deficient in c-KIT receptor function and can engraft stem cells without any preconditioning. This allows us to study the homing of stem cells in the absence of vascular injury or cytokine release caused by radiation. The logistic problem with the use of these mice is that it is difficult to obtain adequate numbers of W/Wv mice to serve as recipients due to breeding colony size concerns.

In brief, although we describe a standard homing protocol, the variations used depend upon the question to be answered and must be determined during the design of the experiment.

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