Chapter 12

Generation of Mouse Bone Marrow-Derived Macrophages

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Abstract

Isolation of resident macrophages from mouse tissues involves complex procedures for a small yield. This is inconvenient for many functional macrophage assays, which require large numbers of relatively homogeneous cells. An alternative method is the culture of bone marrow cells in vitro with appropriate growth factors, to allow the differentiation of precursor cells into large numbers of macrophages. This procedure is easy and inexpensive except for the use of M-CSF, the macrophage colony stimulating factor, and it is characterised by high yield and reproducibility. Once obtained, bone marrow-derived macrophages (BMMs) can be used for a considerable number of functional and structural assays and are commonly regarded as a model for the role of resident macrophages in the innate immune system.

Key words: Bone marrow, Macrophage, Mouse, Macrophage colony stimulating factor, CSF-1, Innate immune system

1. Introduction

Tissue-resident macrophages are an essential component of the innate immune system. They derive from blood monocytes, which migrate to the tissues and differentiate, and establish throughout the tissue to accomplish functions of defence against pathogens as well as homeostasis (1). These functions are varied and for this reason their study is important, but their sparse presence in the tissue makes isolation hard and yields insufficient for common assays. An alternative strategy to study macrophages is in vitro differentiation, a convenient way to obtain a highly pure and abundant population of cells. In vitro differentiation can be achieved from blood monocytes, but in the mouse, a much more efficient technique involves the use of bone marrow. In it, there is an abundance of precursor cells that respond to growth factors by rapidly dividing and producing millions of differentiated macrophages from a single mouse in just a few days.

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The growth factor most extensively used in this method is M-CSF, also known as colony stimulating factor 1 (CSF-1). It specifically affects the proliferation and differentiation of committed precursor cells into macrophages and it is essential for macrophage survival and function (2). The resulting population of macrophages is quite homogeneous and the procedure reproducible. For this reason, this method is widely used to test these innate immune cells on functional assays such as phagocytosis, pathogen killing, antigen presentation and cytokine production in the presence of different stimuli. Gene function can be assessed by comparing BMMs from *knock-out* mice against those from isogenic controls. Protein, DNA or RNA can be isolated and studied, and numerous other assays performed.

It is important to note that although BMMs are a widely used, invaluable model for resident macrophages, they are not like resident macrophages. An indication of this is that resident macrophages themselves are not homogeneous, for they differentiate to suit the environment of each tissue and the pathogens each tissue is likely to encounter (1).

2. Materials

1. 6–9 week mice (see Note 1).
2. RPMI 1640 tissue culture medium, supplemented with 1× GlutaMAX (Gibco) and 10% heat-inactivated Foetal Bovine Serum (FBS, see Note 2).
3. Saline solution: 0.9% sodium chloride solution for irrigation, sterile (Baxter). Keep sterile and store at 4°C.
4. Purified recombinant M-CSF (CSF-1), available from protein specialised companies. Dilute upon arrival, aliquot and store at −80°C. Before use, thaw and add to tissue culture medium. Store this supplemented medium at 4°C for up to 2 months.
5. Surgical scissors and forceps: one clean set and one sterile set.
6. 70% ethanol solution.
7. 27-gauge needles.
8. 20-ml syringe.
9. Low-lint tissues (e.g. KimWipes, Kimberly-Clark).
10. Standard bacterial Petri dishes, sterile.
11. Three 50-ml sterile tubes.

3. Methods

1. Warm up tissue culture medium and tissue culture medium supplemented with rM-CSF in water bath, at 37°C.
2. Sacrifice mouse by approved method and spray lower body thoroughly with 70% ethanol solution. Pull both back legs apart until cracks are heard, indicative of femurs being disjoined from the hips.
3. With clean scissors and forceps, cut skin around one of the back legs. Pull skin down towards paw and remove.
4. Pierce leg with scissors and tear muscle alongside bone by opening scissors. Do not cut through muscle as there is danger of cutting through bone. Repeat this on both sides of femur and tibia until both bones are roughly clean.
5. Cut ligaments between femur and hip. Cut bone below the ankle joint. Place femur and tibia in a tube of ice-cold, sterile saline solution.
6. Repeat the process with the other back leg.
7. From this stage all steps must be done in sterile conditions and with sterile equipment. Wipe femur and tibia by rubbing with low-lint tissues (these need not be sterile) to remove attached tissue. Place the bones in a dish containing a small amount of 70% ethanol.
8. Prepare a syringe full of 37°C medium (RPMI 1640, supplemented with GlutaMAX and 10% FBS), with a 27-gauge needle and a sterile 50-ml tube.
9. Separate tibia from femur by bending slightly at the knee joint. Discard the knee. Hold femur with sterile forceps and cut the top end with sterile scissors (see Note 3). Insert the needle through the cut end and flush bone marrow with medium into a sterile tube. While flushing, move the needle up and down while scraping the inside of the bone. Do this until the bone appears clear. This should use approximately 5 ml per bone. Discard bone.
10. Hold tibia with sterile forceps and cut the top and bottom ends with sterile scissors. Insert the needle through the knee end and repeat the process. Repeat with remaining bones.
11. Centrifuge the cell suspension at $150 \times g$, 5 min. Discard the supernatant and replace with RPMI, supplemented with GlutaMAX, 10% FBS and recombinant M-CSF (see Notes 4 and 5). Pipette up and down several times to disaggregate bone marrow (see Note 6).
12. Label Petri dishes at a ratio of two per bone. Pipette cell suspension into dish and complete volume to 10 ml per plate

with supplemented medium with M-CSF. Incubate cells at 37°C, 5% CO_2 for 5 days (see Note 7).

13. On day 5, wash cells twice with 5 ml of saline solution at room temperature (see Note 8). Scrape the cells off the dish with a cell scraper, transfer to a sterile tube and count. There should be about 5×10^6 per dish (10^7 per bone). Centrifuge at $150 \times g$ for 5 min and resuspend in the desired volume of medium with M-CSF. The cells are ready to be assayed (see Note 9). Cells can be washed and fresh medium added; however, it is recommended that they are used within 2 days.

4. Notes

1. Differences have been noted between BMMs from male and female mice (3). To prevent variation, using sex-matched, as well as age-matched, mice is recommended.
2. It is important for the FBS to be low in endotoxin, and less than 2 EU/ml is recommended. Endotoxin is a strong macrophage activator and its presence could alter the results. Other components of FBS could also activate or inhibit activation of BMMs (4), so it is convenient to test a few batches for activation of unstimulated macrophages.
3. The femur does not need to be cut at the knee end. Removing the knee is sufficient to open the bone at that end.
4. To assess the optimal concentration of rM-CSF, titration and testing should be done prior to experiment. A concentration range from 1 ng/ml to 1 μg/ml is recommended. After 5 days, the concentration that produces yields close to 10^7 BMMs per bone should be chosen.
5. A more affordable option to rM-CSF is the use of L929 cell-conditioned medium. However, the concentration of M-CSF will vary between batches and L cells will also produce varying amounts of GM-CSF, affecting the consistency of results (5).
6. Some fractions of bone marrow might not easily disaggregate and clots might appear. These large fractions should not interfere with the growth of BMMs and will easily wash on day 5.
7. Bone marrow-derived macrophages show a foamy appearance in culture. This is due to the fact that they do not only use M-CSF, but they also internalise it and degrade it. M-CSF internalisation causes cytoplasmic vacuolation, which is evident under the microscope (6).
8. The main criterion for separation of macrophages from other cells in this model is that macrophages adhere to regular dishes

not treated for tissue culture, while other cells do not. However, different types of plastic result in different rates of adhesion. It is important to make sure that macrophages are not lifting off the dish during washes.

9. Assays must be done in medium with M-CSF, because macrophages need M-CSF not only for growth and differentiation but also for survival (2).

References

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