Video Article

Generation and Identification of GM-CSF Derived Alveolar-like Macrophages and Dendritic Cells From Mouse Bone Marrow

Yifei Dong¹, Arif A. Arif¹, Grace F. T. Poon¹, Blair Hardman¹, Manisha Dosanjh¹, Pauline Johnson¹

¹Microbiology and Immunology, University of British Columbia

Correspondence to: Pauline Johnson at pauline@mail.ubc.ca

URL: <http://www.jove.com/video/54194>

DOI: [doi:10.3791/54194](https://doi.org/10.3791/54194)

Keywords: Immunology, Issue 112, alveolar macrophage, dendritic cells, GM-CSF, mouse, bone marrow-derived, *in vitro* cell culture.

Date Published: 6/25/2016

Citation: Dong, Y., Arif, A.A., Poon, G.F., Hardman, B., Dosanjh, M., Johnson, P. Generation and Identification of GM-CSF Derived Alveolar-like Macrophages and Dendritic Cells From Mouse Bone Marrow. *J. Vis. Exp.* (112), e54194, doi:10.3791/54194 (2016).

Abstract

Macrophages and dendritic cells (DCs) are innate immune cells found in tissues and lymphoid organs that play a key role in the defense against pathogens. However, they are difficult to isolate in sufficient numbers to study them in detail, therefore, *in vitro* models have been developed. *In vitro* cultures of bone marrow-derived macrophages and dendritic cells are well-established and valuable methods for immunological studies. Here, a method for culturing and identifying both DCs and macrophages from a single culture of primary mouse bone marrow cells using the cytokine granulocyte macrophage colony-stimulating factor (GM-CSF) is described. This protocol is based on the established procedure first developed by Lutz *et al.* in 1999 for bone marrow-derived DCs. The culture is heterogeneous, and MHCII and fluoresceinated hyaluronan (FL-HA) are used to distinguish macrophages from immature and mature DCs. These GM-CSF derived macrophages provide a convenient source of *in vitro* derived macrophages that closely resemble alveolar macrophages in both phenotype and function.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54194/>

Introduction

Several *in vitro* culture methods have been described to generate bone marrow-derived macrophages (BMDMs) and bone marrow-derived DCs (BMDCs) using one or a combination of growth factors. BMDMs can be generated by culturing bone marrow cells using either macrophage colony stimulating factor (M-CSF) or GM-CSF^{1,2}. For BMDCs, the addition of FLT3 ligand to the bone marrow culture gives rise to non-adherent classical and plasmacytoid DCs (CD11c^{high}/MHCII^{high} and CD11c^{lo}, B220⁺ respectively) after 9 days in culture^{3,4}. In contrast, non-adherent cells generated after 7 to 10 days in culture with GM-CSF alone^{5,6}, GM-CSF and IL-4⁷, or GM-CSF and FLT3 ligand^{8,9} generate BMDCs more closely resembling inflammatory DCs (CD11c^{high}, MHCII^{high} CD11b⁺)¹⁰. While these *in vitro* cultures are used to generate macrophages or DCs, it is unclear if each culture gives rise to pure populations. For example, although adherent cells in the GM-CSF cultures are described to be macrophages⁵, the non-adherent cells from the same culture are used as DCs^{6,11-13}, with the presumption that they are homogeneous and any observed variability is due to different stages of development^{14,15}. Furthermore, studies have found GM-CSF to be an essential growth factor for alveolar macrophage development *in vivo*^{16,17}, and can be used *in vitro* to generate alveolar-like macrophages^{16,17,18}.

Other than adherence, the procedures for generating macrophages and DCs from GM-CSF treated bone marrow cultures are very similar suggesting heterogeneity may exist within GM-CSF bone marrow cultures. This indeed seems to be the case as two papers report the presence of BMDMs in the non-adherent fraction of BMDC cultures. In one paper, they identified a population of cells as CD11c⁺, CD11b⁺, MHCII^{mid}, MerTK⁺, and CD115⁺, which expressed a gene expression signature that most closely resembled alveolar macrophages and had a reduced ability to activate T cells¹⁹. The second paper used MHCII and FL-HA to identify an alveolar macrophage-like population (CD11c⁺, MHCII^{mid/low}, FL-HA^{high}) that was distinct from immature (CD11c⁺, MHCII^{mid}, FL-HA^{low}) and mature DCs (CD11c⁺, MHCII^{high}), both phenotypically and functionally¹⁸. These papers both illustrate that GM-CSF BMDC cultures are heterogeneous, containing both macrophage and DC populations indicating that care should be taken when interpreting data from BMDC cultures.

This protocol describes how to isolate bone marrow, culture bone marrow cells in GM-CSF, and identify the alveolar macrophage-like population from the immature and mature DCs in the bone marrow culture by flow cytometry using FL-HA binding and MHCII expression. This procedure is based on the established procedure of Lutz *et al.*⁶ and is able to generate 5 - 10 x 10⁶ non-adherent cells on day 7 of a 10 ml culture. The culture is usable from days 7 to 10 and yields a heterogeneous population of macrophages, immature and mature DCs, as well as some progenitors at day 7. This provides a simple method to grow and isolate *in vitro* alveolar-like macrophages in large quantities.

Protocol

Mice were euthanized in accordance with the Canadian Council on Animal Care guidelines for ethical animal research by procedures approved by the University of British Columbia Animal Care Committee.

1. Acquiring a Single Cell Bone Marrow Suspension from Mouse Femur and Tibia

1. Switch on a biological safety cabinet (BSC) 15 min prior to start of procedure to purge cabinet air and allow for air flow stabilization, then clean/spray BSC surface with 70% ethanol. Keep everything as sterile as possible for the entirety of the protocol. Do not cut the bones under non-sterile conditions.
2. Prepare dissection tools by soaking in 70% ethanol (1 pair of dissection scissors, 2 pairs of forceps), Hank's Balanced Salt Solution with 5% fetal calf serum (HBSS-FCS), sterile Petri dishes (100 mm x 15 mm), 1 ml syringes, 26½ gauge needles, conical collection tubes (15 ml or 50 ml), and paper towels in the BSC.
3. Euthanize the mouse using protocols approved by the institution's animal research ethics committee. Bring into the BSC, place on top of one or two paper towels and spray the mouse with 70% ethanol.
4. In the BSC, open a sterile Petri dish aseptically, aliquot 10 ml of HBSS-FCS to the base and 1 - 2 ml to the lid.
5. Place the mouse on its stomach with the back facing up and lift the skin around the thoracic sections with fingers of the non-dominant hand. Use the scissors to make an incision where the skin is lifted.
6. Hold on to both ends of the cut, one end with each hand and carefully pull apart the skin around the mouse from the back to the stomach, continue pulling until two ends of the incision meet at the stomach.
7. Flip the mouse to stomach facing up, and with one hand pull the lower half of the skin down towards the leg, keep pulling back the skin until the legs are exposed.
8. Hold the foot of the mouse up and cut the Achilles tendons above the ankle joint and the ligaments connecting the muscles to foot at the lower end of the tibia with scissors. This loosens the foot from the leg bones.
9. While holding the leg up by the foot, cut the muscles and ligaments with scissors all around the knee joint at the lower end of the femur to sever the thigh muscles from the lower leg. Use forceps to gently pull loosened muscles away from the fibular and femoral surfaces. Take care not to sever the femoral artery to avoid heavy bleeding.
10. With one hand holding on to the foot, press down open scissors at the top end of the femur at the hip joint, rotate the leg and pull gently to separate the femur from the hip joint while using the scissors to make the final cut to free the leg from the body.
11. From this point onwards, only hold the tissue with sterile forceps. Hold on to the leg at one end and pull off the foot on the other.
NOTE: This should not require much force, as connecting ligaments are cut in step 1.9. Alternatively, cut the tibia just above the ankle where the bone is fused.
12. Hold the leg by the distal end of the femur with one set of forceps and proximal end of the tibia and fibula with another, carefully bend the tibia and fibula in the opposite direction of the knee joint to separate them from the femur and patella.
13. Use forceps to pull down the remaining ligaments and muscles still attached to the lower leg bones. Here, remove the fibula along with the connective tissues with forceps as it is too small to be flushed for bone marrow cells. Place the cleaned tibia on the inverted Petri dish lid.
14. Hold the lower end of the femur with one set of forceps and the patella with another, bend the patella and surrounding tissues in the opposite direction of the joint for removal. Then clean off remaining connective tissues from the femur; pulling them away with forceps, and place it on the inverted Petri dish lid. Discard the patella.
15. Repeat 1.7 - 1.13 with the other leg if needed. Transfer the bones to 1.6 ml microcentrifuge tubes containing 1 ml sterile HBSS-FCS for transportation if required.
16. Place bones on the inverted Petri dish lid, with 1 - 2 ml of HBSS-FCS, use forceps to clean any residual tissue still attached to the tibia and femur, then transfer the bones to the base of Petri dish containing 10 ml of HBSS-FCS. Alternatively, clean the bones and remove attached muscle tissue using a 70% ethanol soaked tissue.
17. Cut each of the bones in half with scissors. Partially shield the Petri dish with a sterile lid while cutting to ensure the bones stay in the dish. Alternatively, cut the end of each bone to allow flushing in the next step.
18. Attach the 26½ G needle on to the 1 ml syringe, and draw up 1 ml HBSS-FCS from the Petri dish. Insert the tip of the needle into the end of a bone piece and flush out the bone marrow cells (soft red tissue inside the bones). Insert the needle into the head of the bone and the open end, until all the red colored marrow is removed. Observe the bones as white in color.
19. Pass any visible clumps of bone marrow through the syringe a few times to form a single cell suspension.
20. Discard the flushed bones. Use a 10 ml pipette to mix the cell suspension well and transfer to the collection tube. Rinse the Petri dish once with 5 ml of HBSS-FCS to collect residual cells in the dish. Place cells on ice.
NOTE: The bone marrow cell suspension is still viable if stored for 2 - 3 hr at 4 °C.

2. Plating and Culturing of Bone Marrow Cells (BMCs)

1. Prepare the following reagents and supplies.
 1. Prepare red blood cell (RBC) lysis buffer by making 0.84% ammonium chloride in a final solution of 2 mM Tris pH 7.2, then sterilize by filtering through a 0.2 micron filter.
 2. Prepare BMC media by adding 10% FCS, 20 mM HEPES, 1x nonessential amino acid, 55 µM 2-mercaptoethanol, 50 U/ml penicillin and streptomycin, 1 mM sodium pyruvate, and 2 mM L-glutamine to RPMI medium 1640.
 3. Obtain commercially available recombinant GM-CSF or use GM-CSF containing supernatant from AG8.653 myeloma cells transfected with the GM-CSF gene²⁰. Determine the concentration of GM-CSF in the supernatant by ELISA and add the equivalent of 20 ng/ml to the BMC media.
2. Spin down the bone marrow cells at 314 x g for 5 min. The pellet is red, due to RBCs. In the BSC, aspirate the supernatant using a sterile glass Pasteur pipette attached to vacuum suction, loosen the pellet by tapping, then add 10 ml of RBC lysis buffer. Vortex to resuspend the cells and incubate in RBC lysis buffer at room temperature for 5 min.
3. Add 10 ml of HBSS-FCS then spin cells at 314 x g for 5 min and aspirate the supernatant. Observe the cell pellet as white color. Resuspend cells in desired volume of BMC media.
4. Count the resuspended-cells using a hemocytometer after staining dead cells with 0.4% trypan blue.
NOTE: If bone marrow from one leg (single tibia and femur) is used, resuspend in 5 ml of BMC media, take 10 µl of cell suspension and dilute it with 70 µl of 0.4% trypan blue, mix well and transfer 10 µl of the 1/8 cell dilution to the hemocytometer chamber. Count the viable cells in

four quadrants: the average of the four quadrant counts $\times$ dilution factor $\times 10^4$ = number of cells per ml. A femur and a tibia from one leg will generally yield approximately $2 - 4 \times 10^7$ cells after RBC lysis.

5. Estimate the number of 10 ml dishes of bone marrow cultures needed for downstream experiments based on the number of total cells needed for experiments at the end of the culture.

NOTE: Each dish requires 2×10^6 bone marrow cells at initial seeding and yields $5 - 10 \times 10^6$ cells at day 7.

1. Aliquot the total number of cells for plating into a new tube, spin down at $314 \times g$ for 5 min, aspirate the supernatant, and resuspend cells at 4×10^6 cells/ml in BMC media.
6. Prepare a new sterile 100 mm $\times$ 15 mm Petri dish with 9.5 ml of BMC media containing 200 ng of GM-CSF (recombinant or from GM-CSF containing supernatant). Add 500 μ l of the 4×10^6 cells/ml bone marrow cells to the center of the Petri dish for the final concentration of 2×10^5 cells/ml in 10 ml.
NOTE: It is important to use a sterile Petri dish not a tissue culture dish. Also, when adding the cells, ensure cells remain concentrated at the center and minimize disturbance to the dishes after seeding and during media changes. Incubate cells at 37°C and 5% CO_2 . This is day 0 in culture.
7. On day 3, add 10 ml of fresh BMC media containing 200 ng of GM-CSF to each dish of cells. Take care to minimize disturbance of the cells concentrated in suspension. Total volume of cell culture is now 20 ml.
8. Observe cells under the light microscope at 100X magnification. Observe numerous non-adherent cells (round) and a few adherent cells (flat). Cells in groups of three or four resembling petals of a flower can be seen, indicating proliferation.
9. On day 6 perform a half media change. Carefully remove 10 ml of media into a sterile 50 ml conical tube, spin down at $314 \times g$ for 5 min, aspirate and discard the supernatant.
10. Resuspend the cell pellet in 10 ml of fresh BMC media containing 20 ng/ml of GM-CSF, gently vortex and add to the original cell culture dish for a total volume of 20 ml. Observe cells under the microscope.
NOTE: Day 7 culture should be at 70% or greater in confluency with adherent flat cells and dense clouds of round non-adherent cells.

3. Collecting and Preparing BMDC and BMDMs for Flow Cytometry

1. Keep all buffers at 4°C .
2. Harvest the cells from day 7 to day 10. To harvest the cells, first transfer 10 ml of the culture supernatant to a 50 ml conical collection tube, then tilt the dish vertically by approximately 30 degrees and wash the dish by pipetting the remaining 10 ml of culture from the top of the dish. Repeat this wash 5 times, each time rotating the dish by one third.
3. Transfer the remaining 10 ml of cells to the same collection tube and discard the plate and adherent cells.
NOTE: The washes will remove non-adherent and loosely adherent cells; the flat adherent cells are resistant to the washes. Typically, between $5 - 10 \times 10^5$ non-adherent cells are expected from one plate at day 7 and $2 - 5 \times 10^6$ cells at day 10, although Lutz *et al.* reported 10×10^6 cells at day 10. The adherent cells are not normally taken. However, if these cells need to be compared to the non-adherent cells, they can be removed as follows. Add 5 ml of 0.7 mM EDTA in 1x PBS, pH 7.4 to the dish after the non-adherent cells are removed, incubate at 37°C for 5 min, pipette up and down to remove the adherent cells and collect in a separate tube.
4. Spin down the collected cells at $314 \times g$ for 5 min and aspirate the supernatant. Resuspend in 5 ml sterile BMC media at 4°C , vortex gently to mix, and count using trypan blue and hemocytometer.
NOTE: Each dish will yield $5 - 10 \times 10^6$ non-adherent cells from a day 7 culture. From now on, perform all steps at 4°C .
5. For flow cytometric analysis, transfer 2×10^5 cells for each sample into 5 ml polystyrene round bottom tubes, add 300 μ l of FACS buffer at 4°C (1x phosphate buffered saline, 2 mM EDTA and 2% bovine serum albumin) to each sample.
6. Spin cells down at $314 \times g$ for 5 min and aspirate the supernatant. Observe a visible white, opaque pellet at the bottom of the tube.
7. Resuspend cells in 100 μ l of unlabeled Fc receptor Ab to block Fc receptor binding (Use 1/10 supernatant from 2.4G2 producing myeloma cell line) and incubate for 20 min at 4°C . Then add 300 μ l of FACS buffer to each sample, gently vortex, then spin cells down at $314 \times g$ for 5 min and aspirate the supernatant.
8. Resuspend cells in 100 μ l of CD11c-PeCy7 at 0.2 μ g/ml, Gr1-Pacific Blue at 0.25 μ g/ml, MHCII-APC at 0.05 μ g/ml, and FL-HA at approximately 2 μ g/ml to identify macrophages and DCs.
NOTE: FL-HA was prepared in house using fluorescein and rooster comb hyaluronic acid sodium salt as previously described²¹.
9. Incubate for 20 min at 4°C to allow binding to the cell surface. Add 300 μ l of FACS buffer to each sample, gently vortex, then spin cells down at $314 \times g$ for 5 mins at 4°C and aspirate the supernatant. **NOTE:** If biotinylated antibodies are used in 3.8, repeat 3.8 - 9 with fluorescent-streptavidin.
10. Wash cells with another 300 μ l of FACS buffer, gently vortex to mix and spin down the cells at $314 \times g$ for 5 min. Aspirate the supernatant and resuspend the cells in 200 μ l of FACS buffer containing 0.1 - 0.2 μ g per ml of propidium iodide or DAPI to label nonviable cells. Cells are now ready for flow cytometric analysis²².
NOTE: See Results and Figure 3 for details of the populations and how the cells were gated.

Representative Results

A flowchart summarizing the major steps of this method is shown in **Figure 1**. The density and morphology of the bone marrow culture at different times of culture are shown in **Figure 2**. At day 1, the cells are small and sparse but by day 3, there are more cells, some are larger and a few have begun to adhere. By day 6 there is a definite adherent and non-adherent fraction (**Figure 2A**). The culture can be harvested from day 7 - 10 with a higher percentage of CD11c⁺ cells present at day 10. **Figure 2B** shows the day 7 culture before harvest, as well as the separated adherent and non-adherent cell fractions. The adherent fraction consists of the cells left after the light washes that remove the non-adherent fraction. The non-adherent fraction is greatly enriched for cells with a dendritic morphology, which can be seen more clearly in the digitally magnified images in the insets in **Figure 2B**. Once the non-adherent fraction is removed (day 7 or day 10), the cells are analyzed by flow cytometry after labeling with fluorescent antibodies against key cell surface markers, as described above. **Figure 3** shows representative flow cytometry plots of the non-adherent fraction of the culture at day 7 and day 10.

For the identification of both macrophages and DCs, gate on CD11c⁺ and Gr1⁻ cells after first gating on size and live cells (**Figure 3A and B**). In day 7 cultures, the CD11c⁺ Gr1⁻ population in the non-adherent cell fraction is typically 60 to 70% of total live cells, and this is increased to 90% or more on day 10 (**Figure 3B**). The CD11c⁺ Gr1⁻ population at day 7 and day 10 can be divided into three main subsets using MHCII and FL-HA binding: MHCII^{mid/low} FL-HA binding^{high} macrophages (P1), MHCII^{mid} FL-HA binding^{low} (P2) cells containing immature DCs, and MHCII^{high} FL-HA binding^{low} mature DCs (P3) (**Figure 3C** and reference¹⁸). The FL-HA^{low}, MHCII^{low} population (P0) observed at day 7 has been shown to contain progenitors for the P1-P3 cells¹⁸. This population is not evident at day 10 implying that further maturation of the culture has occurred. This is also supported by the greater percentage of CD11c cells in the culture as well as slightly higher percentages of the P2 and P3 DC populations at day 10. **Figure 3D** shows MerTK, considered to be a macrophage marker, is highly expressed on both macrophage and immature DC populations (P1 and P2), but is expressed to a lower extent on mature DCs (P3 cells). Notably, analysis of the adherent fraction reveals the presence of the P0, P1 and P2 populations, but not the P3 mature DC population (data not shown). Thus macrophages are present in the adherent and non-adherent fractions but only mature DCs are present in the non-adherent fraction.

Figure 1: A Flow Chart Indicating the Key Steps of the Method. Primary cells are harvested from the bone marrow, plated and maintained in culture for 7 - 10 days when they are harvested for use in further experiments, purified or used in FACS analysis. [Please click here to view a larger version of this figure.](#)

Figure 2: Microscopic Examination of GM-CSF Derived Bone Marrow Cells. **A)** Images of the bone marrow culture at day 1, 3, and 6 showing the increase in cell number, changes in morphology, and emergence of distinct adherent and non-adherent fractions. **B)** Left panel shows the day 7 bone marrow culture and the typical cell density. The middle panel shows the day 7 adherent cells after harvest of the non-adherent fraction and the right panel shows the non-adherent fraction harvested at day 7. Insets are digitally magnified images of cells in this fraction with dendritic morphology. The white scale bar represents 50 μ m. [Please click here to view a larger version of this figure.](#)

Figure 3: Representative Phenotype of the GM-CSF Culture at Day 7 and Day 10. Non-adherent cells from the culture are labeled with CD11c, Gr1, MHCII, FL-HA, and MerTK at day 7 or day 10 and analyzed for their phenotype by flow cytometry. **A)** Cells were first gated on size then live cells. **B)** Shows a plot of the gated cells with CD11c and Gr1 (the neutrophil/monocyte marker) and identifies the CD11c⁺Gr1⁺ population containing the macrophage and dendritic cells. Gating on this population, **C)** shows the flow cytometry plot of MHCII and FL-HA binding, which shows the separation of the alveolar-like macrophages (P1) from immature DCs (P2) and mature DCs (P3), as described in Poon *et al.*¹⁸. **D)** Histograms comparing MerTK expression between the P1 (macrophages), P2 (immature DCs), and P3 populations (mature DCs) at day 7 and day 10. [Please click here to view a larger version of this figure.](#)

Discussion

In this manuscript, we provide a method for generating GM-CSF derived macrophages and DCs from a single mouse bone marrow culture that is adapted from Lutz *et al.*⁶. MHCII expression and FL-HA binding distinguishes between immature DCs and macrophages in this culture (see **Figure 3C**), which previously has been difficult. This, together with another report by Helft *et al.*¹⁹, demonstrates heterogeneity within GM-CSF induced BMDC cultures that were previously thought to be only DCs. Helft *et al.*¹⁹ used quite different culture conditions to generate BMDCs yet also found a significant macrophage population. GM-CSF induced macrophages resemble alveolar macrophages and GM-CSF induced DCs resemble inflammatory DCs, whereas FLT3 ligand supplemented BMDC cultures produce classical and plasmacytoid DCs^{3,4}. Different DC and macrophage populations are also observed *in vivo* under homeostatic and inflammatory conditions, and there is much discussion in the field as to what defines a DC and a macrophage¹⁰. This is particularly true when it comes to monocyte derived inflammatory DCs and this is also relevant in this BMC. It is thus important to use several phenotypic markers, functional data, and possibly gene expression data to characterize a particular population. It is also possible that these *in vitro* DC and macrophage populations may be further sub-divided as more functional and phenotypic markers are discovered.

In this method, the markers CD11c, Gr1, MHCII, and FL-HA are used to distinguish macrophages from immature and mature dendritic cells in the non-adherent fraction of the BMC culture. This is different to Helft *et al.*¹⁹, who used the markers CD11c, CD11b, CD115, CD135, MerTK, and MHCII to distinguish a macrophage population from mature DCs. MerTK expression levels could distinguish between macrophages (P1) and mature DCs (P3), but not immature DCs (P2) (**Figure 3D**). Thus FL-HA and MHCII are useful markers to discriminate macrophages from both immature and mature DC populations. These macrophages are also functionally distinct from DCs in this BMC culture and this is demonstrated in more detail elsewhere¹⁸. Briefly, after LPS stimulation the BMC macrophages produce different cytokines than mature DCs and do not activate naïve T cells¹⁸. These macrophages also closely resemble alveolar macrophages, both phenotypically and functionally as both bind FL-HA and express CD11c, MerTK, CD200R, CD206 and F4/80 and after LPS stimulation produce TNF- α yet are unable to activate naïve T cells¹⁸. However, there are also differences: the BMC macrophages are CD11b⁺ and have lower levels of Siglec F compared to *ex-vivo* alveolar macrophages, suggesting these cells are similar but not identical to alveolar macrophages.

There are several important steps to ensure the success of this protocol. It is important to maintain sterility from exposing the bone marrow onwards. When moving in and out of the BSC, spray gloved hands and objects with 70% ethanol. Although it is helpful to rinse tools in 70% ethanol to sterilize, ethanol is toxic to bone marrow cells, so air-dry the tools and ensure the ethanol has evaporated before bringing bones or cells in contact with them. In addition, avoid contacting mouse tissues or cells with non-sterile equipment or hands during the bone marrow harvest. Although penicillin and streptomycin supplementation is provided in the media, careless handling of the cells may lead to yeast or fungal contamination. In addition, since immune cells with phagocytic activity are generated in this culture, minor contamination may not be detected by simply observing the cell culture microscopically, thus it is important to identify the signs of contamination. For example, cell numbers may be reduced and the expression of cell activation markers such as CD40 and CD86 may be increased when analyzed by flow cytometry. Media color may also turn yellow reflecting a pH change due to contamination. When contamination occurs, the BMC culture is unusable. To maintain experimental consistency, it is important to generate a single cell suspension from the bone marrow by vortexing and obtain an accurate cell count to set up the BMC cultures. Also, if any connective tissue from the bone ends up in the single cell suspension, filter through a sterile 70 micron filter to remove it. Typical cell yields for one plate of the non-adherent fraction at day 7 are between $5 - 10 \times 10^6$ cells. Cell numbers are usually a good indication that the protocol is working well. With this procedure we usually obtain between $2 - 5 \times 10^6$ cells on day 10, whereas Lutz reports 10×10^6 cells. We perform RBC lysis on the initial bone marrow cells whereas the protocol by Lutz *et al.* does not and so this step may be optional. Variations in the percent of CD11c cells as well as numbers and the proportions of macrophages and DCs can arise from different sources or batches of FCS and so it is critical to test new batches of FCS on BMC cultures for consistent experimental results. GM-CSF concentration was varied from 5 ng/ml to 40 ng/ml and this did not significantly affect the cell yield. Cell density may also influence the maturation of the culture. For example, Helft *et al.* seeded 1×10^7 cells in 4 ml media which produced a greater percent of cells with high MHCII expression¹⁹. The time point when cells are harvested can also affect the percentage of populations present in the non-adherent fraction. Cells

are typically collected between days 7 - 10²⁵⁻²⁷, but some studies collect BMCs at day 6^{19,23,24}. If more cells are needed, culture BMCs in larger 150 mm x 15 mm Petri dishes in 40 ml at the same density. In this case a half-volume media change is done on both day 3 and day 6 (i.e., 20 ml of media removed, cells spun down and replaced with fresh 20 ml media supplemented with 20 ng/ml rGM-CSF). These plates typically generate 3 - 6 x 10⁷ cells per dish at day 7. FL-HA is an important reagent, together with MHCII, to distinguish macrophages from immature and mature DCs in the BMC culture. Thus once it is made, titrate the FL-HA for use on cells known to constitutively bind HA such as alveolar macrophages¹⁸ or BW5147 T cells and confirm its specificity using either a HA-blocking CD44 antibody (KM-81, commercially available), unlabeled HA or a CD44 deficient cell. CD11c⁺ Gr1⁺ cells in the culture can function as an internal control for non HA-binding cells, and a fluorescence minus one control (FMO) or CD44 deficient BMC cultures are highly recommended for accurate setting of the FL-HA binding gate.

Although experiments can be performed using a heterogeneous culture, it is advisable to enrich for the DC or macrophage population using either fluorescence activated cell sorting or antibody coated magnetic beads¹⁸ based on the expression of CD11c, Gr1, MHCII, and HA binding. Possible experiments include stimulation with Toll-like receptor agonists such as LPS to measure activation and cytokine production, T cell activation assays, or additional characterization by flow cytometry. For example, after 8 or 24 hr of stimulation, flow cytometry can be used to measure intracellular staining for cytokines and/or surface labeling of co-stimulatory molecules such as CD40 and CD86, as described in¹⁸. Intracellular cytokine labeling procedures require pretreatment of the cells with Brefeldin A, which prevents secretion and allows the cytokine to build up inside the cell. For T cell activation assays involving the loading of an antigen such as the OVA peptide by an antigen presenting cell, purified macrophages based on MHCII and HA binding will not activate T cells while immature and mature DCs will¹⁸. It should be noted that the sorting procedure alone may activate the dendritic cells to some extent, making it important to include negative controls in all experiments.

As with any *in vitro* method, there are advantages and disadvantages compared to using *in vivo* or *ex-vivo* cells. The disadvantage is that *in vitro* derived cells may not exactly mimic *in vivo* cells but they have the advantage that they can be generated in sufficient numbers to allow functional and biochemical analysis, something that is often not feasible with *ex-vivo* cells. This method of macrophage and DC identification will allow future research to obtain more homogenous cell populations from a culture with previously underappreciated heterogeneity. As purified macrophages from this GM-CSF culture closely resemble alveolar macrophages from the lung¹⁸, it provides a convenient source of alveolar-like macrophages for *in vitro* analysis. For example, these HA binding macrophages could be used to screen for drugs that modify alveolar macrophage function and to investigate mechanisms of *Mycobacterium tuberculosis* infection and resistance. Given the recent discovery that GM-CSF and M-CSF bone marrow-derived macrophage transplantation can alleviate pulmonary proteinosis in mice^{28,29}, this method to identify macrophages from GM-CSF cultures may help increase the efficacy of this proposed therapy.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgements

This work was funded by the Canadian Institutes of Health Research (CIHR) (Grant MOP-119503) and the Natural Sciences and Engineering Council of Canada (NSERC). NSERC also supported summer studentships to Y.D. and A.A. YD is supported by the University of British Columbia (UBC) with a 4-year fellowship award, A.A. is supported by CIHR with a graduate student Master's award (CGS-M). We thank Calvin Roskelley for assistance with the microscope used to generate the images in **Figure 2**. We also acknowledge support from the UBC Animal and Flow Cytometry Facilities.

References

1. Fleetwood, A. J., Lawrence, T., Hamilton, J. A., & Cook, A. D. Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. *J Immunol.* **178** (8), 5245-5252 (2007).
2. Lari, R. *et al.* Macrophage lineage phenotypes and osteoclastogenesis—complexity in the control by GM-CSF and TGF-beta. *Bone.* **40** (2), 323-336 (2007).
3. Brasel, K., De Smedt, T., Smith, J. L., & Maliszewski, C. R. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood.* **96** (9), 3029-3039 (2000).
4. Angelov, G. S., Tomkowiak, M., Marcais, A., Leverrier, Y., & Marvel, J. Flt3 ligand-generated murine plasmacytoid and conventional dendritic cells differ in their capacity to prime naive CD8 T cells and to generate memory cells *in vivo*. *J Immunol.* **175** (1), 189-195 (2005).
5. Inaba, K. *et al.* Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med.* **176** (6), 1693-1702 (1992).
6. Lutz, M. B. *et al.* An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods.* **223** (1), 77-92 (1999).
7. Labeur, M. S. *et al.* Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J Immunol.* **162** (1), 168-175 (1999).
8. Berthier, R., Martinon-Ego, C., Laharie, A. M., & Marche, P. N. A two-step culture method starting with early growth factors permits enhanced production of functional dendritic cells from murine splenocytes. *J Immunol Methods.* **239** (1-2), 95-107 (2000).
9. Brasel, K. *et al.* Flt3 ligand synergizes with granulocyte-macrophage colony-stimulating factor or granulocyte colony-stimulating factor to mobilize hematopoietic progenitor cells into the peripheral blood of mice. *Blood.* **90** (9), 3781-3788 (1997).
10. Segura, E., & Amigorena, S. Inflammatory dendritic cells in mice and humans. *Trends Immunol.* **34** (9), 440-445 (2013).
11. West, M. A. *et al.* Enhanced dendritic cell antigen capture via toll-like receptor-induced actin remodeling. *Science.* **305** (5687), 1153-1157 (2004).
12. Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., & Stockinger, B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity.* **24** (2), 179-189 (2006).

13. Goodridge, H. S. *et al.* Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. *Nature*. **472** (7344), 471-475 (2011).
14. Shalek, A. K. *et al.* Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. *Nature*. **510** (7505), 363-369 (2014).
15. Vander Lugt, B. *et al.* Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation. *Nat Immunol*. **15** (2), 161-167 (2014).
16. Shibata, Y. *et al.* GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. *Immunity*. **15** (4), 557-567 (2001).
17. Lacey, D. C. *et al.* Defining GM-CSF- and Macrophage-CSF Dependent Macrophage Responses by In Vitro Models. *J Immunol*. **188** (11), 5752-5765 (2012).
18. Poon, G. F. *et al.* Hyaluronan Binding Identifies a Functionally Distinct Alveolar Macrophage-like Population in Bone Marrow-Derived Dendritic Cell Cultures. *J Immunol*. **195** (2), 632-642 (2015).
19. Helft, J. *et al.* GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells. *Immunity*. **42** (6), 1197-1211 (2015).
20. Stockinger, B., Zal, T., Zal, A., & Gray, D. B cells solicit their own help from T cells. *J. Exp. Med.* **183** (3), 891-899 (1996).
21. de Belder, A. N., & Wik, K. O. Preparation and properties of fluorescein-labelled hyaluronate. *Carbohydr Res.* **44** (2), 251-257 (1975).
22. Stewart, C.C., & Stewart, S.J. Immunophenotyping. In: *Current Protocols in Cytometry*. Wiley, J., & Sons, Inc., eds., Chapter 6, Unit 6.2, (2001).
23. Dearman, R. J., Cumberbatch, M., Maxwell, G., Basketter, D. A., & Kimber, I. Toll-like receptor ligand activation of murine bone marrow-derived dendritic cells. *Immunology*. **126** (4), 475-484 (2009).
24. Abdi, K., Singh, N. J., & Matzinger, P. Lipopolysaccharide-Activated Dendritic Cells: 'Exhausted' or Alert and Waiting? *J Immunol*. **188** (12), 5981-5989 (2012).
25. Contreras, I. *et al.* Impact of *Leishmania mexicana* Infection on Dendritic Cell Signaling and Functions. *PLoS Negl Trop Dis*. **8** (9), (2014).
26. Feng, T., Cong, Y. Z., Qin, H. W., Benveniste, E. N., & Elson, C. O. Generation of Mucosal Dendritic Cells from Bone Marrow Reveals a Critical Role of Retinoic Acid. *J Immunol*. **185** (10), 5915-5925 (2010).
27. Grauer, O. *et al.* Analysis of maturation states of rat bone marrow-derived dendritic cells using an improved culture technique. *Histochem Cell Biol*. **117** (4), 351-362 (2002).
28. Suzuki, T. *et al.* Pulmonary macrophage transplantation therapy. *Nature*. **514** (7523), 450-454 (2014).
29. Happle, C. *et al.* Pulmonary transplantation of macrophage progenitors as effective and long-lasting therapy for hereditary pulmonary alveolar proteinosis. *Sci Transl Med*. **6** (250), (2014).