

A stepwise procedure for isolation of murine bone marrow and generation of dendritic cells

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Abstract Bone marrow derived dendritic cells (BMDCs) are routinely employed in cell based assays to evaluate immunomodulatory and anti-inflammatory activities. Hence, simplified, stepwise, defined and standardized methods are required for isolation of bone marrow cells from mice, propagating them in presence of growth factors and obtaining high and reproducible yields of BMDCs. Here, we describe a detailed, stepwise protocol with pictorial representation to isolate bone marrow from mouse femur and development of dendritic cells. Mouse bone marrow cells are cultured in presence of granulocyte-macrophage colony stimulating factor (GM-CSF) for 6 days to generate BMDCs.

Keywords: bone marrow, dendritic cells, primary culture

INTRODUCTION

Dendritic cells (DCs) are key immune cells which orchestrate the innate and adaptive immune responses by playing a central role in initiation and regulation of immune responses [1-3]. DCs have been widely used as target cells to investigate new drugs and molecules for immunomodulatory and anti-inflammatory properties [4-6]. DCs have emerged as one of the standard tools to study immunobiology and understand the pharmacological action of new drugs [7]. Recent developments in employing DCs in various cell-based screening platforms have demanded obtaining high yield of DCs. In vitro culture and expansion of DCs from bone marrow (BM)-derived hematopoietic precursors using GM-CSF was first described in rats [8]. It was followed by generation of large number of BMDCs from mouse and rat bone marrow [9, 10]. Subsequently, DCs were generated from human BM mononuclear cells, providing a procedure to isolate human DCs which have high clinical importance [11]. A 10-day procedure for generating BMDCs from whole murine bone marrow precursors by Lutz et al. has been described and widely used by researchers [12].

Generation of high yields of healthy BMDCs from murine bone marrow progenitors may involve complex, multistep and sophisticated procedures. The basic procedure of isolating bone marrow from mouse femur is complicated and requires expertise. There is need of a defined and simple protocol for the generation of BMDCs from mouse bone marrow. Here we describe a simplified, clear, detailed and stepwise procedure to isolate mouse bone marrow and generate BMDCs. We hope that a stepwise and easy to understand protocol along-with a comprehensive pictorial representation of various steps involved will be helpful for this purpose in a user friendly manner.

MATERIALS

Animals

Specific pathogen-free male C57BL/6 mice (20 to 25 g, 8-10 weeks

old) used in the study were obtained from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Hyderabad, India. Mice were kept in the in-house animal facility maintained at 22±3°C and 55±15% relative humidity with 12 hr light-dark cycle. They were given autoclaved pelleted feed and filtered drinking water *ad libitum*.

All experiments employing the mice were performed under the protocols approved by the Institutional Animal Ethics Committee (IAEC) of Dabur Research Foundation.

Chemicals/reagents/others

- ✓ RPMI-1640 medium (Life technologies, India)
- ✓ Fetal Bovine Serum FBS (Life technologies, India)
- ✓ Penicillin-streptomycin (Himedia, India)
- ✓ Ethanol (Sigma, USA)
- ✓ HBSS (Sigma, USA)
- ✓ Trypan blue (Sigma, USA)
- ✓ Recombinant mouse granulocyte-macrophage colony stimulating factor (rmGM-CSF), 1000 ng/ml (R&D systems, Minneapolis, USA)
- ✓ Lipopolysaccharide (LPS, from *E. coli* serotype 0127:B8)-100 ng/ml (Sigma, USA)
- ✓ FITC-conjugated CD11c (HL3) (557400, BD Biosciences PharMingen, USA)
- ✓ DMSO (Merck, India)
- ✓ Disposable syringe (1 ml Insulin) 29G × ½ (Dispovan, U-40)
- ✓ Kleenex tissues
- ✓ Petridish (Non-TC grade)
- ✓ Pipette tips
- ✓ 15 ml and 50 ml centrifuge tubes

Equipment

- ✓ Centrifuge (Heraeus)
- ✓ CO₂ Incubator (Shell labs)

- ✓ Hemocytometer (Sigma, Z37,535-7)
- ✓ Inverted microscope (Nikon Diaphot-300)

- ✓ Laminar air flow (Klenzaids)

PROCEDURE

Isolation of bone marrow – day 0

Figure 1 shows the pictorial representation of experimental steps involved in the isolation of femur bone from a C57BL/6 mouse.

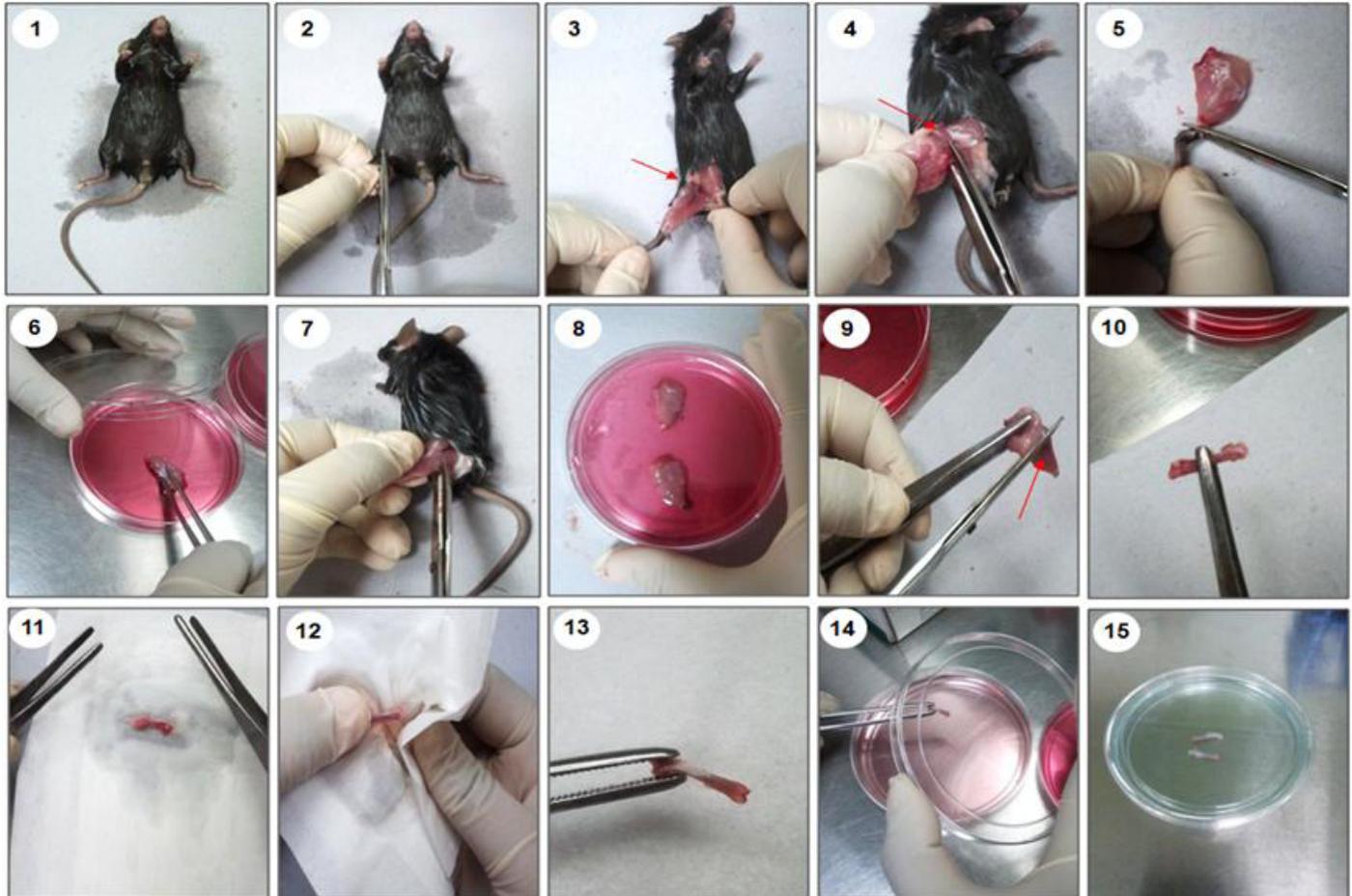


Figure 1. Isolation of femur bone from C57BL/6 mice. (1) An euthanized mouse is sprayed with 70% ethanol, (2) Incision is made in skin of the hind leg, (3) Skin is pulled backwards to expose the muscles, (4) The hind leg is cut at the pelvic joint, (5) Lower portion of hind leg is removed, (6) The leg is transferred to medium, (7-8) Excision of other leg, (9) Knee joint is cut to remove tibia, (10) A femur bone after removing surrounding muscles, (11-14) Cleaning the femur with 70% ethanol, (15) Femur kept in 70% ethanol for disinfection.

1. Euthanize a C57BL/6 mouse (male, 20 to 25 g, 8–10 weeks old) by CO₂ asphyxiation. Subsequent experimental steps will be conducted in a laminar air flow.
2. Position the mouse on its dorsal back on a clean blotting sheet and thoroughly spray all the external areas with 70% ethanol for disinfection. This step minimizes the possibility of contaminating cell preparations with fur or external particles.
3. Make an incision in each hind leg using blunt-end sterile scissors. Firmly grasp the skin and gently pull downwards to expose the muscles.
4. Cut the hind leg just above the pelvic/hip joint using sharp and sterile dissecting scissors, ensuring that the epiphysis remains intact without exposing off its contents to outside.
5. After removal of the hind leg, carefully hold it from the lower side. Using sterile scissors make an incision just above the claws to remove the lower portion of the hind leg.
6. Transfer the hind leg to RPMI-1640 medium in a sterile petridish and keep for 5 min to loosen the muscle tissue.
7. Cut the hind leg at just below the knee-joint through ligaments to remove off the tibia, ensuring that the epiphysis remains intact.
8. Dissect the femur from surrounding muscles and remove excess tissue using sterile forceps and scissors, keeping the ends of the bone intact.

9. Remove any extra leftover muscle/tissue on the femur by using lint-free tissue paper (Kleenex) and gently clean the bones using 70% ethanol.
10. Soak the intact bones in 70% ethanol in a sterile petridish for 2 min for disinfection. Repeat the same procedure with another femur.
11. Transfer the bones to HBSS in a sterile petridish for rinsing off ethanol.
12. After rinsing, transfer the bones to culture medium RPMI-1640 in a sterile petridish.
13. Trim both ends of femurs carefully using sterile, sharp scissors to expose the interior marrow shaft.
14. Flush the contents of marrow with 2 ml of HBSS using a 1-ml insulin syringe with a 29G \times $\frac{1}{2}$ needle. Collect the contents into a sterile 50-ml centrifuge tube. (The bones should appear white once all the marrow has been expelled out completely).

Preparation of BM cell suspension – day 0

Figure 2 shows the pictorial representation of experimental steps involved in the preparation of bonemarrow suspension.

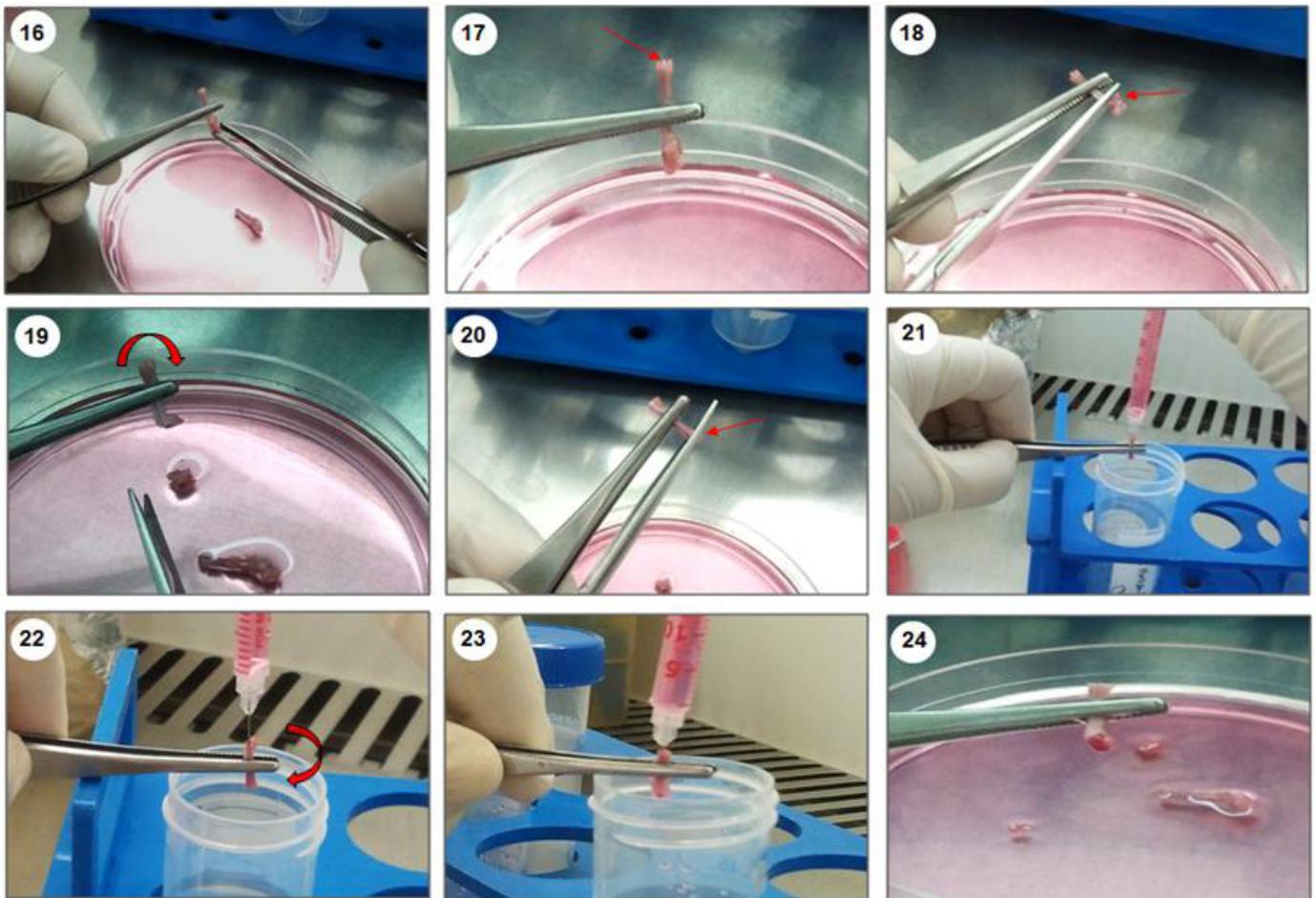


Figure 2. Preparation of bone marrow suspension. (16) Femur is first rinsed with HBSS and then transferred to medium, (17-20) Both ends of femur are cut, (21) Bone marrow is flushed by 29G \times $\frac{1}{2}$ needle, (22-23) Flushing the bone marrow from other end by inverting the femur, (24) A clean femur appears white from which marrow has been collected.

15. Dilute the BM cell suspension collected above with HBSS to a final volume of 20 ml. Disintegrate any clusters within the bone marrow suspension by vigorous pipeting.
16. Centrifuge the cell suspension at 250 g \times 8 min.
17. Remove the supernatant and resuspend cell pellet in 20 ml of HBSS. Centrifuge the cell suspension at 250 g \times 8 min (wash 1).
18. Again wash the cell pellet in HBSS as mentioned above (wash 2).
19. After 2 washes, resuspend the cell pellet from each femur gently in 20 ml of HBSS to prepare a homogeneous suspension. Take out an aliquot of cell-suspension for cell count.
20. Count the total number of cells using a hemocytometer and trypan blue staining method for cell viability.
21. Centrifuge the cell suspension at 250 g \times 8 min.

22. According to the cell count obtained, resuspended the cell pellet in culture medium (RPMI-1640 + 10% FBS + 20 mM penicillin/streptomycin) to achieve a final cell density of 10×10^6 cells/ml.

Seeding of cells (day 0)

Figure 3 shows the pictorial representation of experimental steps involved in the seeding of bone marrow precursor cells for BMDC cultures.

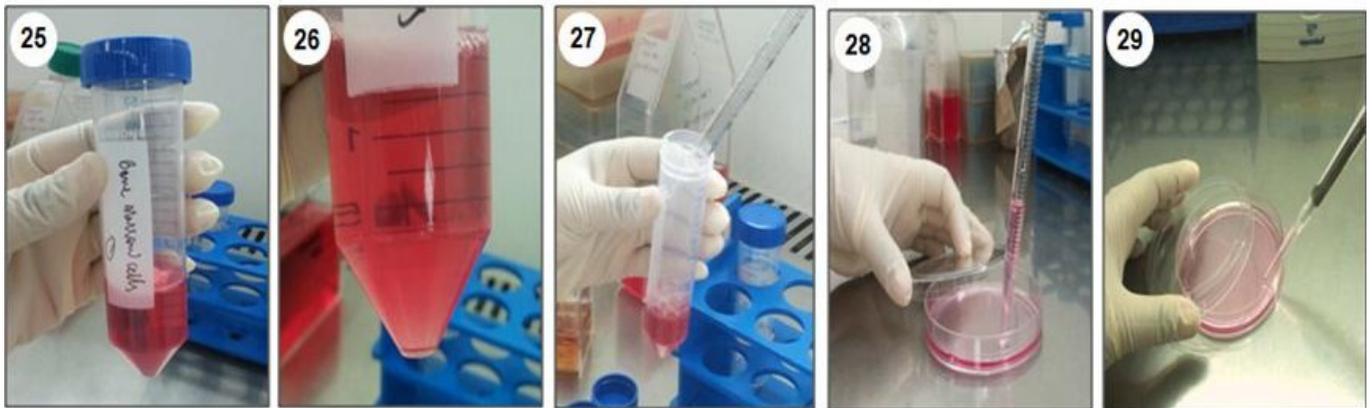


Figure 3. Seeding of bone marrow precursor cells. (25-26) Pellet of bone marrow cells, (27) Supernatant is removed, (28) Growth medium with rmGM-CSF is added to a petridish, (29) Bone marrow cells are seeded into medium.

23. Add 9.6 ml of culture medium (RPMI-1640 + 10% FBS + 20 mM penicillin/streptomycin) into sterile, pre-labeled 90-mm petridishes (non-tissue culture grade).
24. Add 0.2 ml of cell suspension (10×10^6 cells/ml) into each petridish to achieve the final cell density of 2×10^6 cells/petridish.
25. Add 0.2 ml of rmGM-CSF from rmGM-CSF stocks (1000 ng/ml) into above petridishes so that final concentration of rmGM-CSF in 10 ml is 20 ng/ml.
26. Gently swirl the petridishes to ensure uniform mixing of contents.
27. Incubate the cells at 37°C, 5% CO₂ and 95% humidity in CO₂ incubator for 3 days.

Replenishment of culture with fresh culture medium (day 3)

28. Add 10 ml of fresh culture medium with 20 ng/ml rmGM-CSF into each petridish with BMDC cultures. The total volume in each petridish is now 20 ml.
29. Gently rotate the petridishes for proper mixing of contents.
30. Incubate the cultures at 37°C, 5% CO₂ and 95% humidity in a CO₂ incubator further for another 3 days.

Harvesting of BMDCs (day 6)

31. Harvest primary BMDCs from each petridish by collecting non-adherent cells by gently pipeting them with culture medium. Loosely-adherent BMDCs get easily dislodged into suspension by this process while the adherent macrophages remain attached to the petridish.
32. Collect the cell suspension from each petridish into sterile 50-ml centrifuge tubes. Discard the adherent cells which contain macrophages.
33. Transfer the cell suspensions to polypropylene tubes for FACS analysis.
34. For treatments in biological assays, centrifuge the cell suspension at $250 \text{ g} \times 8 \text{ min}$.
35. Resuspend the cell pellet in culture medium (RPMI-1640 + 10% FBS + 20 mM penicillin/streptomycin) to make a homogeneous suspension.
36. Count the cells using a hemocytometer and trypan blue staining method for cell viability.
37. Adjust the cell density as per required number for biological assay.

Flow cytometry analysis of BMDCs

38. Harvest day-6 immature BMDCs from 90-mm petridishes.
39. Count the cells and collect 1×10^6 cells into each sterile centrifuge tubes.
40. Centrifuge the cells at $250 \text{ g} \times 8 \text{ min}$.
41. Remove the supernatant and resuspend the cell pellet in 5 ml of PBS.
42. Centrifuge the cells at $250 \text{ g} \times 8 \text{ min}$ (wash 1).
43. Remove the supernatant and repeat the wash once (wash 2).
44. Incubate the cells with FITC-labeled CD11c antibody and isotype control in 100 μl of PBS, at 4°C in dark.
45. After staining for 30 minutes, add 0.9 ml of PBS into each tube.
46. Repeat the wash steps twice by centrifuging the cells at $250 \text{ g} \times 8 \text{ min}$.

47. Remove the supernatant and resuspend the cell pellet in 0.3 ml of buffer (PBS + 5% FBS).
48. Transfer the cell suspensions to polypropylene tubes for FACS analysis.

ANTICIPATED RESULTS

Cell yield

1. On day 0, the expected bone marrow yield is $10 \pm 1.654 \times 10^6$ (per femur) (n = 25).
2. On day 6, the expected BMDC yield is $2 \pm 0.661 \times 10^6$ (per 90-mm dish) (n=25).

Morphology of BMDCs

Figure 4 shows the typical morphological features of BMDCs in culture.

3. On day 0, bone marrow progenitor cells seeded into culture petridishes show spherical morphology (Fig. 4A). Cells are small in size but with a defined cell-membrane and good health.
4. On day 3, BMDCs demonstrate spherical morphology with very early stages of “dendrites” formation on their cell surface. Initiation of colony formation can be seen at various sites with aggregation of cells together (Fig. 4B). Conversion of cells into adhered macrophages is also seen (elongated cells).
5. Immature BMDCs are obtained on day 6 of the culture in the presence of rmGM-CSF. At this stage, differentiation of cells into adherent macrophages is observed. Simultaneously, large sized colonies of BMDCs are formed at various sites (Fig. 4C). Large numbers of semi-adherent and floating BMDCs are observed.
6. Treatment of immature BMDCs on day 6 of culture with maturation stimulus LPS (100 ng/ml) for 24 h results in the maturation of BMDCs as evident by an increased population of cells with branched and extended morphology and adhered macrophages (Fig. 4D).

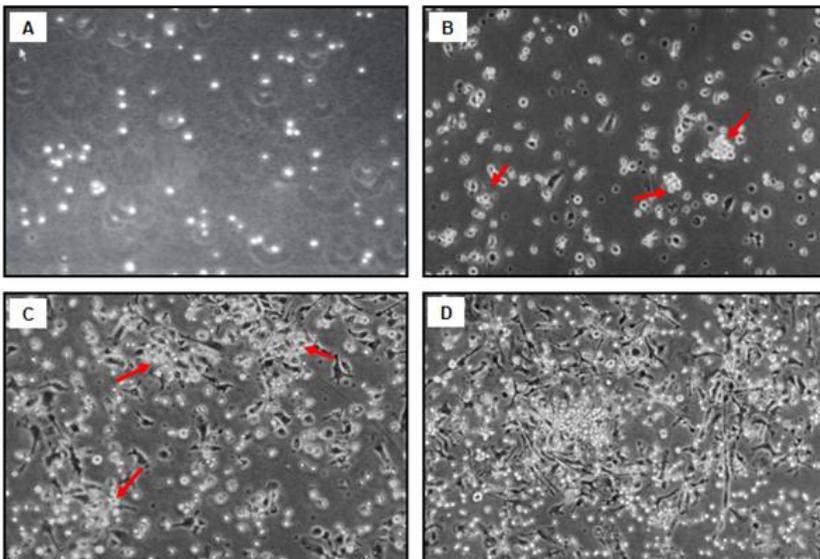


Figure 4. Morphology of murine BMDCs at different days of culture. Bone marrow cells are cultured in growth medium RPMI-1640 with 10% FBS containing 20 ng/ml rmGM-CSF for 6 days. Fresh medium is supplemented at day-3. **A.** Bone marrow precursors at day-0, **B.** day-3 BMDCs, **C.** day-6 immature BMDCs-untreated, and **D.** day-6 BMDCs treated with maturation stimulus LPS (100 ng/ml) for 24 h. The red arrows indicate the sites of colony formation at day-3 and day-6 (Magnification – 100×).

Purity of BMDCs

Surface expression of CD11c (a murine DC-specific marker) was analyzed in BMDCs by flow cytometry. Using this method, 80-85%

of the BMDCs expressing CD11c were obtained in different cultures. A representative histogram plot for CD11c expression by BMDCs is shown in Figure 5.

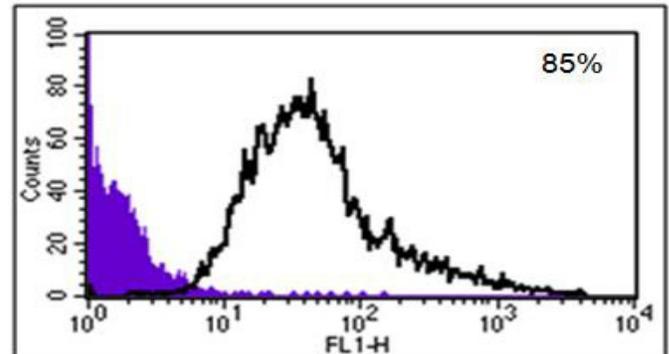


Figure 5. Representative histogram plot for CD11c expression on BMDCs. Day-6 BMDCs (1×10^6) were stained with FITC-labeled anti-CD11c and analyzed by flow cytometry. Isotype control was used as background control (purple-filled area). The percentage of CD11c positive cells is indicated in histogram.

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