Chapter 4

Isolation of Dendritic Cell Progenitor and Bone Marrow Progenitor Cells from Mouse

Nobuyuki Onai and Toshiaki Ohteki

Abstract

Dendritic cells (DCs) comprise two major subsets, conventional DC (cDC) and plasmacytoid DC (pDC) in the steady-state lymphoid organ. These cells have a short half-life and therefore, require continuous generation from hematopoietic stem cells and progenitor cells. Recently, we identified DC-restricted progenitors called common DC progenitors (CDPs) in the bone marrow of mouse. The CDPs can be isolated from mouse bone marrow based on the hematopoietic cytokine receptors, such as Flt3 (Fms-related tyrosine kinase 3) (CD135), c-kit (CD117), M-CSF (macrophage colony-stimulating factor) receptor (CD115), and IL-7 (interleukin-7) receptor-α (CD127). The CDPs comprise of two progenitors, $CD115^+$ CDPs and $CD115^-$ CDPs, and give rise to only DC subsets in both in vitro and in vivo. The former CDPs are the main source of cDC, while the later CDPs are the main source of pDC in vivo. Here, we provide a protocol for the isolation of dendritic cell progenitor and bone marrow progenitor cells from mouse.

Key words Conventional dendritic cells (cDCs), Plasmacytoid DCs (pDCs), Common DC progenitors (CDPs), Cytokine receptor

1 Introduction

Dendritic cells (DCs) are professional antigen-presenting cells and are essential for the induction and maintenance of immunity [1, 2]. Several subsets of DCs have been identified in the lymphoid and nonlymphoid tissues. There are two major DC subsets in the lymphoid tissue such as conventional DCs (cDCs) and plasmacytoid DCs (pDCs).

Recently, a new nomenclature has been proposed, such as pDC1 and pDC2, based on the ontogeny of the cells and their functions. cDC1 is a classical $CD8\alpha^+$ cDCs which is a dependent Batf3, while cDC2 is inclusive of $CD8\alpha^-$ cDCs and $CD4^-CD8\alpha^-$ cDCs [3].

All DC subsets have a short half-life and do not proliferate in the surrounding environment; therefore, it is essential to continuously generate them from hematopoietic stem cells via progenitors [4, 5].

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Recently, we identified DC-restricted progenitors, such as common DC progenitors, CDPs, which give rise to only DC subsets both in vitro and in vivo, and not to other cell lineages; they also process high-proliferation capacity. The CDPs comprise of two progenitors, such $CD115^+$ and $CD115^-$ CDPs. The former is a main source of cDCs, while the later is the main source of pDCs in the steady state [6, 7]. CDPs differentiate into cDC subsets via pre-cDCs [8, 9] and mature $CCR9^+$ pDCs via $CCR9^-$ pDCs [7, 10]. It has been shown that the CDPs are derived from macrophage and DC progenitors (MDPs), which give rise to monocytes, macrophages, and DC subsets [9, 11]. Recently, we found lymphoid-primed multipotent progenitor (LMPPs) directly giving rise to and MDPs in vivo and revised the load map for DC development [7]. Here, we provide a protocol for the isolation of dendritic cell progenitor and bone marrow progenitor cells from mouse.

2 Materials

2.1 Preparation of Bone Marrow Cell (BMCs)

1. C57BL/6 mice, 8–12 weeks old.
2. 70% ethanol.
3. Phosphate-buffered saline (PBS).
4. 10 ml syringes with 19 G needles.
5. Mortar and pestle.
6. Nylon meshes (150 μm pore size).
7. Histopague-1077 (Sigma-Aldrich).
8. 15 and 50 ml Falcon tubes.

2.2 Isolation of Lineage Negative Cells from BMCs

1. PE-Cy5-conjugated antibodies against lineage antigens. For DC and BM progenitors isolation: CD3ε (145-2C11); CD4 (GK1.5); CD8α (53-6.7); B220 (RA3-6B2); CD19 (MB19-1); CD11b (M1/70); CD11c (N418); I-A/I-E (M-15/114.15.2);Gr-1 (RB6-8C5); TER119 (TER119); NK1.1 (PK136). For pre-cDC isolation: CD3ε (145-2C11); CD4 (GK1.5); CD8α (53-6.7); B220 (RA3-6B2); CD19 (MB19-1); CD11b (M1/70);Gr-1 (RB6-8C5); TER119 (TER119); NK1.1 (PK136).
2. Staining buffer: PBS 1% fetal calf serum (FCS), 2 mM EDTA.
3. Anti-Cy5/Anti-Alexa Flour 647 microbeads (Miltenyi Biotec).
4. AutoMACS Pro Separator (Miltenyi Biotec).

2.3 Antibody Staining and Cell Sorting for DC and BM Progenitors

1. Staining buffer. Store at 4 °C.
2. Primary antibodies: FITC-conjugated anti-CD34 (RAM34), PE-conjugated anti-CD135 (A2F10.1), APC-conjugated anti-CD117 (ACK2), Brilliant Violet 421-conjugated anti-CD127 (A7R34), and biotin-conjugated anti-CD115 (AFS-98).

3. Streptavidin-APC-Cy7.
4. Propidium iodide solution (1000×). Dissolve at 10 mg/ml in PBS and store at 4 °C in the dark (*see* **Note 1**).
5. FCS-IMDM: Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin.
6. Cell sorter: BD FACSAria III (Becton Dickinson Immunocytometry Systems).

2.4 Antibody Staining and Cell Sorting for Pre-cDC

1. Staining buffer. Store at 4 °C.
2. Primary antibodies: FITC-conjugated anti-I-A/I-E (M-15/114.15.2), PE-conjugated CD135 (A2F10.1), APC-conjugated anti-CD11c (N418), PE/Cy7-conjugated anti-CD172a (P84).
3. Propidium iodide solution (1000×).
4. FCS-IMDM.
5. Cell sorter: BD FACSAria III (Becton Dickinson Immunocytometry Systems).

3 Methods

All procedures should be performed under sterile condition.

3.1 Preparation of Bone Marrow Cells (BMCs)

1. Wet the whole body of the mouse with 70% ethanol for sterilization.
2. Remove the femurs, tibias, ilium, and backbone from five mice, and place them into ice-cold PBS (*see* Fig. 1a).
3. Remove the muscles from the femurs, tibias, ilium, and backbone using scissors and forceps, and transfer them into a new Petri dish containing PBS (*see* Fig. 1b).
4. Add 10 ml of ice-cold PBS into mortar and crush the bones (the femurs, tibias, and ilium) using pestle (*see* Fig. 1c) or add 10 ml of ice-cold PBS into dish, and flush out marrow using syringe with 19 G needle to obtain the bone marrow cell suspension from bone shaft (*see* Fig. 1d) (*see* **Note 2**).
5. Pass the cell suspension through a nylon mesh to remove debris.
6. Add 10 ml of ice-cold PBS into mortar and transfer cleaned backbone. Crush and grind the backbone using the pestle to obtain the spinal marrow (*see* **Note 3**).
7. Pass the cell suspension through a nylon mesh to remove debris.

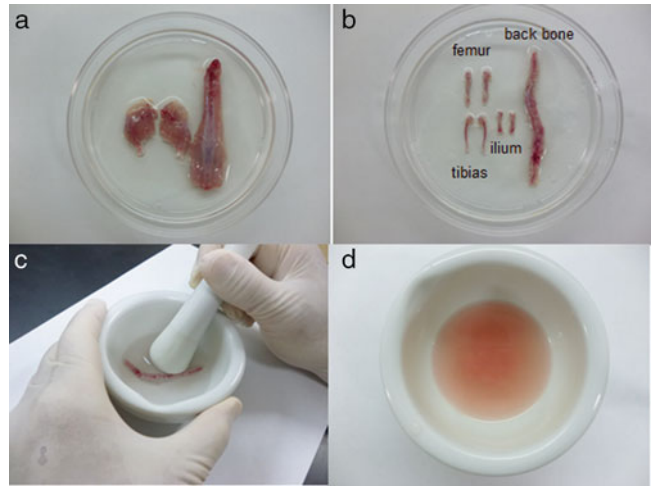

Fig. 1 Preparation of cell suspension from the femur, tibias, ilium, and the backbone. (**a**) Isolated legs and backbone from mouse. (**b**) Isolated femurs, tibias, ilium, and backbone after removal of excess muscle and fat. (**c**) Crush and grind the backbone using pestle to obtain the spinal marrow. (**d**) Cell suspension from the backbone

8. Mix bone marrow and spinal marrow cell suspensions, and centrifuge 5 min at 400 × *g* at room temperature.
9. During centrifugation, add 5 ml of room temperature histopaque-1077 into a 15 ml tube.
10. Remove the supernatant and resuspend the cells in 5 ml of PBS at room temperature.
11. Carefully overlay the 5 ml of cell suspension onto histopaque-1077.
12. Centrifuge for 30 min at 18 °C at 900 × *g* with acceleration and brakes set to "zero."
13. After centrifugation, carefully aspirate the uppermost layer. Subsequently transfer the intermediate mononuclear cell layer into a new tube.
14. Wash the cells with an excess of ice-cold PBS (5 ~ 10× volume). and centrifuge for 5 min at 4 °C at 400 × *g*.
15. Resuspend the cells in PBS, and count them.

3.2 Isolation of Lineage-Negative Cells from BMCs

1. Centrifuge cell suspension at 400 × *g* for 5 min at 4 °C, and aspirate the supernatant.
2. Add to the cells the appropriate PE-Cy5-conjugated antibody cocktail against lineage antigens, mix well.
3. Incubate for 30 min at 4 °C in the dark.

4. Wash the cells with ice-cold staining buffer in excess (5 ~ 10× of volume), centrifuge at 400 × *g* for 5 min at 4 °C, and aspirate the supernatant.
5. Resuspend the cell in staining buffer, and add appropriate volume of anti-Cy5/Anti-Alexa Flour 647 microbeads according to manufacturer's instructions.
6. Incubate for 15 min at 4 °C in the dark.
7. Wash the cells with ice-cold staining buffer in excess, centrifuge at 400 × *g* for 5 min at 4 °C, and aspirate the supernatant.
8. Resuspend the cells in staining buffer. Proceed with magnetic separation to obtain lineage-negative cell fraction using AutoMACSPro Separator according to manufacturer's instructions.
9. Proceed to Subheading 3.3 or 3.4.

3.3 Antibody Staining and Cell Sorting for DC and BM Progenitors

1. Centrifuge the lineage-negative cell suspension at 400 × *g* for 5 min at 4 °C, and aspirate the supernatant.
2. Add primary antibody mix to the cell suspension, mix well.
3. Incubate for 30 min at 4 °C in the dark.
4. Wash the cells with ice-cold staining buffer in excess and centrifuge for 5 min at 400 × *g*, and aspirate the supernatant.
5. Add the streptavidin to the cells, mix well.
6. Incubate for 30 min at 4 °C in the dark.
7. Wash the cells with ice-cold staining buffer in excess, centrifuge for 5 min at 400 × *g*, and aspirate the supernatant.
8. Resuspend the cells in staining buffer containing Propidium iodide (final concentration 10 μg/ml) to stain and exclude dead cells.
9. Prepare tubes containing 1 ml of FCS-IMDM for collecting the sorted target cells.
10. Sort $CD115^+$ CDPs as $lin^-CD117^{int}CD135^+CD115^+CD127^-$, $CD115^-$ CDPs as $lin^-CD117^{int}CD135^+CD115^-CD127^-$, MDPs as $in^-CD117^+CD135^+CD115^+Sca\text{-}1^-$, and LMPPs as $in^-CD117^+CD135^+CD34^+Sca\text{-}1^+$ by using a cell sorter (*see* Fig. 2) (*see* **Note 4**).

3.4 Antibody Staining and Cell Sorting for Pre-cDC

1. Centrifuge the lineage-negative cell suspension at 400 × *g* for 5 min at 4 °C, and aspirate the supernatant.
2. Add the primary antibody mix to the cell suspension, mix well.
3. Incubate for 30 min at 4 °C in the dark.
4. Wash the cells with ice-cold staining buffer in excess and centrifuge for 5 min at 400 × *g*, and aspirate the supernatant.

Figure 2. Onai *et al.*

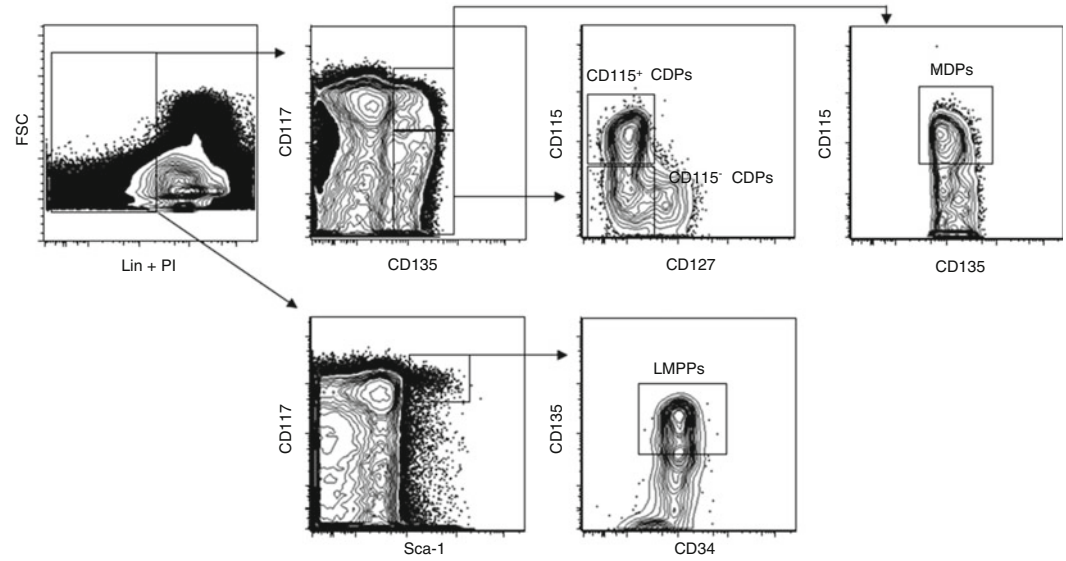

Fig. 2 Isolation of DC progenitors and BM progenitors from BM Lin$^-$ cells were divided by CD117, CD135, and Sca-1 expression. Lin$^-$CD117$^+$CD135$^+$ cells and lin$^-$CD117^{int}CD135$^+$ cells were further divided by CD115 and CD127 expression. Lin$^-$CD117$^+$Sca-1$^+$ cells were divided by CD34 and CD135 expression. CD115$^+$ CDPs, CD115$^-$ CDPsMDPs, and LMPPs were defined as lin$^-$ CD117^{int}CD135$^+$CD115$^+$CD127$^-$, lin$^-$CD117^{int}CD135$^+$CD115$^-$CD127$^-$, lin$^-$CD117$^+$CD135$^+$CD115$^+$Sca-1$^-$, and lin$^-$CD117$^+$Sca-1$^+$CD135$^+$CD34$^+$, respectively

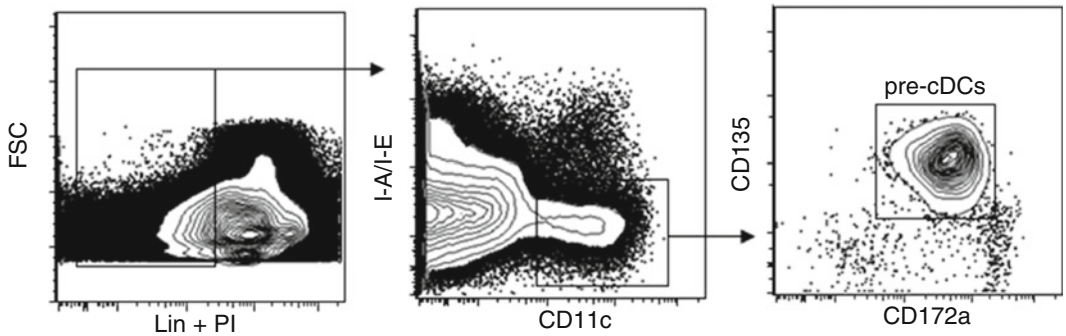

Fig. 3 Isolation of pre-cDC from BM. Lin$^-$ cells were divided by CD11c, I-A/I-E, CD135, and CD172a. pre-cDCs were defined as lin$^-$CD11c$^+$I-A/I-E$^-$CD135$^+$CD172a^{int} cells

5. Resuspend the cells in staining buffer containing Propidium iodide (final concentration 10 μg/ml) to stain and exclude dead cells.
6. Prepare tubes containing 1 ml of FCS-IMDM for collecting the sorted target cells.
7. Sort pre-cDCs as lin$^-$CD11c$^+$I-A/I-E$^-$CD135$^+$CD172a^{int} cells (*see* Fig. 3).

4 Notes

1. Propidium iodide is light sensitive.
2. In this protocol, we introduced bone marrow cell preparation from the femurs, tibias, ilium, and backbone using mortar and pestle for crushing and grinding the bones. Using this method, the total number of bone marrow cells from the mice is increased. There is no functional difference between the progenitors isolated from the femurs, tibias, ilium, and backbone.
3. Remove and discard the white funiculus that will be extracted as well during the crushing.
4. Sorted $CD115^+$ CDPs, $CD115^-$ CDPs, MDPs, and LMPPs are cultured in FCS-IMDM supplemented with Flt3-ligand (10 ng/ml). The progenies derived from these progenitors are analyzed on day 8 after culture.

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