

Chapter 3

Isolation of Human Blood DC Subtypes

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Abstract

Human blood dendritic cells (DCs) are a rare, heterogeneous cell population that comprise approximately 1% of circulating peripheral blood mononuclear cells (PBMCs). Their isolation has been confounded by their scarcity and lack of distinguishing markers and their characterisation perplexed by the recent discovery of phenotypic and functionally distinct subsets. Human blood DCs are broadly defined as leukocytes that are HLA-DR positive and lack expression of markers specific for T cell, B cell, NK cell, monocyte and granulocyte lineages. They can be subdivided into the CD11c⁻ (CD123⁺CD303⁺CD304⁺) plasmacytoid DC and CD11c⁺ myeloid DC, which can be further subdivided into three subsets based on differential expression of CD1c, CD141 and CD16. DC can be isolated from peripheral blood by using an initial density gradient centrifugation step to enrich for mononuclear cells followed by immunomagnetic depletion of cells expressing markers specific for leukocyte lineages and undesired DC subsets. Subsequent flow cytometry-based cell sorting allows the isolation of highly pure individual DC subsets that can then be used for functional studies.

Key words: Dendritic cells, blood, immunomagnetic selection, flow cytometry.

1. Introduction

Human blood DCs are a heterogeneous cell population that originate from bone marrow precursors and comprise approximately 1% of circulating peripheral blood mononuclear cells (PBMCs). Their isolation in sufficient numbers and purity to perform functional studies is challenged by their scarcity and a lack of distinguishing markers. Hence to date they have been poorly

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characterised. Blood DCs have been classically defined as leukocytes that lack markers of other leukocyte lineages CD3 (T cells), CD14 (monocytes) CD19/20 (B cells), CD56 (NK cells), CD15 (granulocytes) and CD34 (haematopoietic progenitors), and express high levels of major histocompatibility complex (MHC) class II molecules (HLA-DR) (1). Early isolation protocols enriched for a lineage negative (lin^-) DC population by density gradient centrifugation and/or immunoselection using cocktails of lineage antibodies (2), but these preparations are now known to comprise several subpopulations. CD11c broadly divides lin^- HLA-DR $^+$ blood DC into the CD11c $^-$ plasmacytoid (pDC) and CD11c $^+$ myeloid (mDC) subsets (3–5). pDCs comprise approximately 18% of the lin^- HLA-DR $^+$ population and can also be distinguished from mDC by their expression of CD123, CD303 (BDCA-2) and CD304 (BDCA-4/neuropilin-1) (6). CD11c $^+$ myeloid DC (mDC) comprises over 70% of lin^- HLA-DR $^+$ cells and can be further subdivided into three distinct subsets (6–8). The CD1c $^+$ (BDCA-1) subset represents around 19% of lin^- HLA-DR $^+$ cells and is the most extensively studied mDC subset. The CD16 $^+$ subset constitutes approximately 50% of lin^- HLA-DR $^+$ cells and has been largely overlooked due to the inclusion of CD16 in lineage antibody cocktails and their poor viability in vitro. The CD141 $^+$ (BDCA-3) subset is the least abundant, constituting around only 3% of lin^- HLA-DR $^+$ cells and is hence poorly characterised.

The discovery of these new DC surface markers and more sophisticated immunomagnetic selection, including commercially available kits for some DC subsets and flow cytometric cell sorting technologies, has now allowed the isolation of the distinct DC subpopulations and their functional analysis for the first time. The level of purity required is a crucial factor when choosing an isolation protocol. Even small percentages (<2%) of contaminating lineage cells, particularly NK or T cells, can have profound effects on DC phenotype and function and this may lead to spurious conclusions. Positive immunomagnetic selection of such rare cell populations is often confounded by the non-specific binding of dead cells to magnetic beads. Furthermore, despite the usefulness of some of the markers to distinguish individual subsets, their application for direct positive immunoselection is often precluded by direct functionality of antibody binding (e.g. CD303) or by weak expression on other DC subsets or lineage cells (e.g. CD1c is also expressed on a population of B cells, CD141 is weakly expressed by some CD1c $^+$ mDC and pDC and CD123 is weakly expressed by some mDC subsets). It is also important to note that DC viability, phenotype and function can be modulated to various extents by different isolation protocols. For this reason, when establishing an isolation protocol, DCs should be carefully phenotyped and some basic functional studies

performed (e.g. allogeneic mixed lymphocyte reaction) as described elsewhere (7). The identification of novel DC activation markers, CMRF-44 and CMRF-56, has led to the development of positive immunomagnetic selection protocols that are clinically applicable (9–11). These protocols rely on the partial activation of DCs after overnight culture and predominantly consist of the CD1c⁺ mDC subset along with some B cells and monocytes. Such preparations are potent antigen-presenting cells for the induction of T-cell responses and are also suitable for vaccination, but their heterogeneous nature and partial activation make them unsuitable for studying DC subset biology.

The following protocol is designed for the isolation of highly pure (>99%) individual human blood DC subsets that is essential for their functional characterisation. Mononuclear cells (MNCs) are first isolated by density gradient centrifugation (**Section 3.1**) followed by an initial enrichment of the desired DC subset by depletion of lin⁺ cells, residual red blood cells (using CD235a) and, where applicable, other DC subsets, by immunomagnetic selection (**Section 3.2**). Positive selection by fluorescence-activated cell sorting (FACS, **Section 3.3**) then allows for the complete removal of residual lin⁺ cells and enables precision in selecting the desired DC subpopulation based on the intensity of expression of the applicable marker. As expected for such rare cell populations, yields are low but the use of leukapheresis products allows for the isolation of multiple subsets from an individual donor and repeating the isolation procedure results in sufficient yields of even the rarest subsets (e.g. CD141⁺ DC) for most functional analyses.

2. Materials

1. Fresh whole blood or buffy coats obtained by venipuncture or leukapheresis products (*see Note 1*).
2. Ficoll–Hypaque (Pharmacia).
3. 1X Phosphate-buffered saline (PBS) pH 7.2 (Invitrogen).
4. Complete medium: RPMI-1640 (Invitrogen) supplemented with 10% human AB serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (Invitrogen).
5. Running buffer: PBS, supplemented with 0.5% bovine serum albumin (BSA) (Invitrogen) and 2 mM ethylene diamine tetra-acetic acid (EDTA) (Merck). Keep buffer cold (4–8°C).

- 6. Goat anti-mouse IgG microbeads (Miltenyi Biotec).
- 7. Purified and fluorescent monoclonal antibodies (mAbs) listed in **Tables 3.1–3.3** (*see* **Notes 2** and **3**).
- 8. Mouse serum (10% in PBS) (Sigma).
- 9. 30 µm Pre-separation filter (Miltenyi Biotec).
- 10. autoMACS™ Separator.
- 11. FACSaria™ or similar FACS instrument capable of a minimum of four-colour sorting.

Table 3.1
Unconjugated primary mouse IgG antibodies used for cell depletion (Section 3.2)

mAb	Clone, supplier	Per 1×10 ⁸ cells (µg)	CD1c ⁺ mDC	CD141 ⁺ mDC	CD16 ⁺ mDC	pDC
CD3	OKT3, ATCC	2	Yes	Yes	Yes	Yes
CD14	RMO52, Beckman Coulter	2	Yes	Yes	Yes	Yes
CD19	J3-119, Beckman Coulter	2	Yes	Yes	Yes	Yes
CD20	B9E9, Beckman Coulter	2	Yes	Yes	Yes	Yes
CD34	My10, BD Biosciences	2	Yes	Yes	Yes	Yes
CD56	N901, Beckman Coulter	2	Yes	Yes	Yes	Yes
CD235a	GA-R2, BD Biosciences	2	Yes	Yes	Yes	Yes
CD11c	BU15, Beckman Coulter	2	No	No	No	Yes
CD1c ^a	AD58E7, Miltenyi Biotec	2	No	Yes	Yes	Yes
CD141	AD5-14H12, Miltenyi Biotec	2	Yes	No	Yes	Yes
CD16	3G8, Beckman Coulter	2	Yes	Yes	No	Yes
CD304	AD5-17F6, Miltenyi Biotec	2	Yes	Yes	Yes	No

^a *See* **Note 4**

Table 3.2
‘Exclusion’ fluorescent-conjugated antibodies for FACS sorting (Section 3.3)

mAb	Clone, supplier	Volume per test (µl)	CD1c ⁺ mDC	CD141 ⁺ mDC	CD16 ⁺ mDC	pDC
Sheep anti-mouse- PE or FITC	Chemicon	2	Yes – –PE	Yes – FITC	Yes – FITC	Yes – FITC
CD15-PE or FITC ^a	MMA, BD Biosciences	5	Yes – PE	Yes – FITC	Yes – FITC	Yes – FITC

^a *See* **Note 5**

Table 3.3
'Selection' fluorescent-conjugated antibodies for FACS sorting (Section 3.3)

mAb	Clone, supplier	Volume per test (μ l)	CD1c ⁺ mDC	CD141 ⁺ mDC	CD16 ⁺ mDC	pDC
CD1b/c-FITC	B-B5, Diaclone	5	Yes	Yes ^a	No	No
CD11c-APC	S-HCL-3, BD Biosciences	5	No	No	Yes	No
CD16-PE	B73.1, BD Biosciences	5	No	No	Yes	No
CD141-APC	AD5-14H12, Miltenyi Biotec	10	Yes ^b	Yes	No	No
CD123-PE	9F5, BD Biosciences	5	No	No	No	Yes
CD304-APC ^c	AD5-17F6, Miltenyi Biotec	10	No	No	No	Yes

^a See Note 6

^b See Note 7

^c See Note 8

3. Methods

3.1. Isolation of MNCs by Density Gradient Centrifugation

1. Aliquot 20 ml whole blood into 50-ml conical tubes and add an equal volume of room temperature (RT) PBS. For leukapheresis products aliquot 10 ml and add 30 ml RT PBS (*see* Note 9). Mix well.
2. Using a 10-ml pipette or syringe and cannula slowly layer 10 ml Ficoll–Hypaque underneath the blood/PBS mixture.
3. Centrifuge at $500 \times g$ for 20 min at RT without braking.
4. Collect the mononuclear cell layer from the interphase and carefully transfer into fresh 50-ml conical tubes up to a volume of 20 ml.
5. Fill the 50-ml tubes with cold running buffer up to a 50-ml volume.
6. Centrifuge at $300 \times g$ for 10 min at 4°C to remove platelets.
7. Pipette off the supernatant and gently resuspend the cell pellets in a small volume of running buffer.
8. Pool cells into a single 50-ml tube and wash with cold running buffer.
9. Centrifuge at $500 \times g$ for 5 min at 4°C.
10. Resuspend the cell pellet in 50 ml running buffer and proceed with experiment.
11. Determine cell yield and viability using a haemocytometer and Trypan Blue exclusion.

3.2. Enrichment of lin^- Cells by Immunomagnetic Depletion

1. Resuspend MNCs in a 50-ml tube at 10^8 cells per ml in cold running buffer with a maximum of 5×10^8 MNCs per separation run (*see* **Notes 3** and **10**).
2. Add 2 μg of each primary mouse IgG antibody per 10^8 total cells as indicated in **Table 3.1** for the required DC subpopulation. Mix well and incubate for 20 min at $4-8^\circ\text{C}$ with occasional mixing (*see* **Notes 10** and **11**).
3. Wash the cells once with 10–20X labelling volume of running buffer at $500 \times g$ for 5 min at 4°C to remove unbound primary antibody.
4. Pipette off supernatant completely and resuspend the cell pellet in 900 μl running buffer per 10^8 total cells.
5. Add 100 μl goat anti-mouse IgG microbeads per 10^8 total cells. Mix well and incubate for 15 min at $4-8^\circ\text{C}$, with occasional mixing.
6. Wash cells with 10–20X labelling volume of running buffer and centrifuge at $500 \times g$ for 5 min at 4°C . Pipette off supernatant completely.
7. Resuspend the cell pellet in 0.5 ml cold running buffer per 10^8 total cells.
8. Prepare a pre-separation filter by applying 1 ml running buffer and discarding the flow-through. Pass cells through filter to remove cell aggregates.
9. Prepare and prime the autoMACSTM separation device according to the manufacturer's instructions (*see* **Note 12**).
10. Place tube containing the magnetically labelled cells in the autoMACSTM separator. Choose 'Depl025' program.
11. Collect negative fraction (outlet port 'neg1'). This fraction contains the enriched lin^- DC population. (If using an LD column collect flow-through.)

3.3. Positive Selection of DC Subpopulations by FACS Sorting

1. Centrifuge negative fraction at $500 \times g$ for 5 min at 4°C . Pipette off supernatant completely.
2. Label cell pellet with appropriate fluorescent sheep anti-mouse Ig and CD15 antibody for desired DC population (**Table 3.2**).
3. Mix well and incubate for 15 min at $4-8^\circ\text{C}$, with occasional mixing.
4. Wash the cells once with running buffer and centrifuge at $500 \times g$ for 5 min at 4°C . Pipette off supernatant completely.
5. Resuspend the cell pellet in 50 μl 10% mouse serum.
6. Repeat Steps 3 and 4.

7. Label the cell pellet with appropriate ‘selection’ antibody mixture for identification of desired DC population (**Table 3.3**).
8. Repeat Steps 3 and 4.
9. Resuspend the cell pellet in running buffer (10^7 – 10^8 cells/ml).
10. Sort desired DC population by flow cytometry FACSaria™ or similar device according to the criteria in **Fig. 3.1** (*see Note 13*).

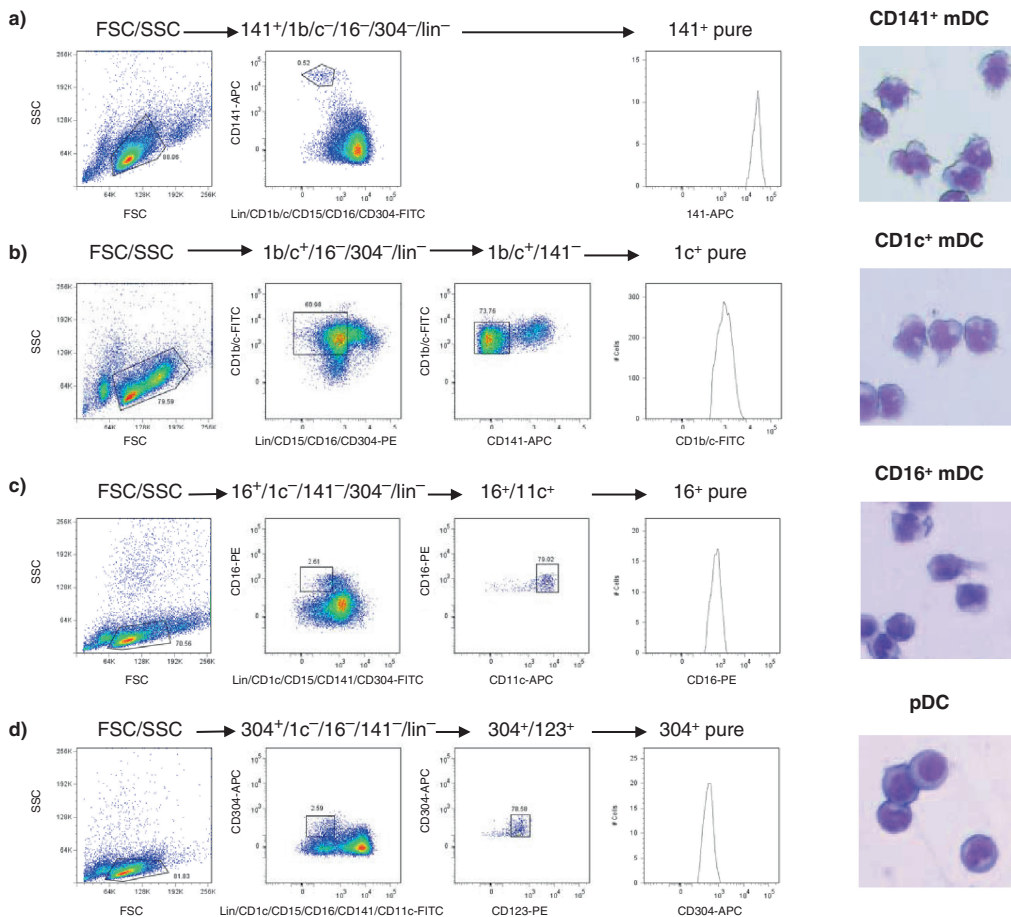


Fig. 3.1. Gating criteria for FACS sorting and morphology of human lin⁻ DC subpopulations. For all populations cells are first gated by FSC and SSC to remove dead cells and debris (left of marked region) and cell aggregates (right and top of marked region). **(a)** Isolation of the CD141⁺ DC population. As CD141 is weakly expressed on some CD1c and PDC, this population is sorted as lin⁻, CD1c⁻ and CD304-FITC negative, and CD141-APC bright. **(b)** The CD1c⁺ DC population is sorted as lin⁻, CD16⁻ and CD304-PE negative, CD141-APC negative and CD1b/c-FITC positive. **(c)** The CD16⁺ DC population is sorted as lin⁻, CD1c⁻, CD141⁻ and CD304-FITC negative, CD11c-APC bright and CD16-PE positive. **(d)** PDCs are sorted as lin⁻, CD1c⁻, CD141⁻ and CD11c-FITC negative, CD304-APC positive and CD123-PE positive.

11. Collect cells into complete medium for phenotypic or functional analysis. Expected yields per 5×10^8 starting MNCs are $5\text{--}8 \times 10^5$ CD1c⁺ mDC, $0.5\text{--}1.0 \times 10^5$ CD141⁺ mDC, $0.5\text{--}3.5 \times 10^5$ CD16⁺ mDC and $3\text{--}5 \times 10^5$ pDC (*see Note 14*).

4. Notes

1. Acquisition of blood products requires approval from an appropriate ethics committee and written informed consent from the donor. When working with human blood appropriate biosafety practices (PC2 facilities) must be followed. Ideally DCs should be freshly isolated, however, good cell yields and viabilities can be obtained from whole anti-coagulated blood or leukapheresis products left sealed in their collection bag at RT for up to 20 h.
2. These antibody combinations are based on the isolation of a single DC subset per experiment and are designed for maximum purity of individual subsets. By using only CD3, CD14, CD19, CD20, CD34, CD56 and CD235a in the initial depletion step (**Section 3.2**) it is possible to then sort multiple DC subsets simultaneously by flow cytometry (**Section 3.3**) by excluding residual lineage cells (using PE-SAM and CD15-PE)) and staining with CD11c in combination with CD1c, CD141, CD16, CD123 or CD304, depending on the instrument's sorting capacity and fluorescent antibody combinations available. This practice is not recommended where high purities and maximum yields of individual subsets are required. Cell depletion kits and Ab-conjugated magnetic beads that use similar immunomagnetic separation technologies are also available (e.g. StemCell Technologies, Dynal®) and can alternatively be used for **Section 3.2**.
3. Antibody concentrations are optimal for starting populations of $\leq 5 \times 10^8$ MNCs. Do not exceed this cell number as it reduces binding efficiencies and increases sorting time, resulting in a reduction in the final yield, purity and cellular viability. When isolations from larger starting populations are required repeat the procedure using a maximum of 5×10^8 MNCs in each run.
4. Do not use CD1b/c antibody in this step.
5. The CD15 antibody (clone MMA) is mouse IgM and cannot be used in the initial depletion step (**Section 3.2**) as it will not bind goat anti-mouse IgG microbeads.

6. CD1c is not highly expressed on the cell surface. The addition of CD1b/c-FITC is recommended for the CD141⁺ mDC isolation procedure to absolutely exclude the CD141^{dim} CD1c⁺ cells.
7. Include CD141-APC into the CD1c⁺ mDC isolation procedure to ensure exclusion of CD1c/CD141 double positive cells.
8. CD303 (BDCA-2) also defines the pDC population but should not be used to isolate them as it is internalised upon receptor engagement.
9. Ficoll–Hypaque and PBS must be at RT before proceeding. For smaller blood or leukapheresis products density gradient centrifugation can be performed in 15-ml tubes provided the volumes are scaled down accordingly to maintain ratios of 1:2 whole blood:PBS, 1:4 leukapheresis:PBS and 1:5 Ficoll–Hypaque: blood product/PBS mixture.
10. Work fast, keep cells cold and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labelling.
11. Use a standard refrigerator rather than ice for 4–8°C incubations.
12. Separations (Steps 9–11) can alternatively be performed manually using a VarioMACSTM Separator with LD or D columns (Miltenyi Biotec) or similar manual magnetic bead separation device according to the manufacturer's instructions.
13. Keep sorting times to less than 50 min to maintain cell viability.
14. These figures are donor dependent and are highly variable.

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