

Generation and Maturation of Human Monocyte-derived DCs

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[Abstract] Dendritic cells (DC) are antigen-presenting cells, which play a critical role in the regulation of the adaptive immune response. They act as a bridge between the innate and the adaptive immune systems. An approach to study their function and potentiality is to generate DC-like cells by culturing CD14⁺ monocyte-enriched peripheral blood mononuclear cells (PBMC). In the presence of GM-CSF and IL-4, these cultures give rise to large numbers of DC-like cells. Generating human-DC from PBMC is a useful tool to study biological functions of human DC.

Materials and Reagents

1. Blood sample to obtain CD14⁺ cells
2. Histopaque -1077 (Sigma-Aldrich, catalog number: 10771)
3. DPBS (Sigma-Aldrich, catalog number: D8537)
4. autoMACS Rinsing Buffer (Miltenyi Biotec, catalog number: 130-091-222)
5. Albumin from bovine serum (Sigma-Aldrich, catalog number: A1933)
6. FITC-anti-CD14 antibody (BioLegend, catalog number: 301804)
7. Recombinant human GM-CSF (Pepro Tech, catalog number: 300-03)
8. Recombinant human IL-4 (Pepro Tech, catalog number: 200-04)
9. RPMI-1640 medium (Life Technologies, Invitrogen™, catalog number: 21875034)
10. 2-mercaptoethanol (Life Technologies, Gibco®, catalog number: 21985-023)
11. Antibiotics: penicillin-streptomycin (Life Technologies, Gibco®, catalog number: 15070)
12. Heat inactivated (at 56 °C for 30 min) qualified fetal bovine serum (Life Technologies, Invitrogen™, catalog number: 26140-087)
13. PE-anti-CD1a antibody (BioLegend, catalog number: 300106)
14. Recombinant human IL1β (Thermo Fisher Scientific, catalog number: RIL1BI)
15. Recombinant human TNFα (Thermo Fisher Scientific, catalog number: RTNFAI)
16. Rabbit Immunoglobulin G (IgG) (Sigma-Aldrich, catalog number: I5006)
17. FITC-anti-CD83 antibody (BioLegend, catalog number: 305306)
18. PE-anti-CD80 antibody (BioLegend, catalog number: 305208)

19. APC-anti-CD40 antibody (BD, catalog number: 555591)
20. Isotype-matched mAbs (BioLegend, catalog number: 400113)
21. Erylyse buffer (see Recipes)
22. Complete medium (see Recipes)
23. AutoMACS running buffer (see Recipes)
24. FACS wash buffer (see Recipes)
25. Propidium iodide (Sigma-Aldrich, catalog number: P4170) (see Recipes)

Equipment

1. Sterilized serological pipettes (2, 5, 10, 25 ml) (Corning, Costar[®], catalog numbers: 4486, 4487, 4487, 4488, 4489)
2. 50 ml conical tubes (BD, Falcon[®], catalog number: 352070)
3. Human CD14⁺ magnetic microbeads (Miltenyi Biotec, catalog number: 130-050-201)
4. Filtration unit with pore size of 0.22 μ m and polyethersulfone (PES) membrane (Thermo Fisher Scientific, catalog number: 431096)
5. 6-well tissue culture plate (BD, Falcon[®], catalog number: 353046)
6. FACS tube (Beckman Coulter, catalog number: 2523749)
7. Refrigerated centrifuge with swing out rotor
8. AutoMACS separator (Miltenyi Biotec)
9. CyAn ADP flow cytometer (Beckman Coulter)

Procedure

See Notes for a flow chart with the steps to generate DCs.

1. Take a buffy coat preparation (50 ml) from a healthy donor. Dilute heparinized blood with an equal volume of 1x PBS at room temperature (RT) and put it down slowly over Histopaque to obtain human peripheral blood mononuclear cells (PBMCs).
2. Add 20 ml of Histopaque (warmed to RT) to 50 ml conical tubes. Then slowly overlay 20 ml of the diluted blood mixture on top of the Histopaque. Centrifuge at 535 x g for 35 min at room temperature with the brake off.
3. Carefully remove the lymphocyte interface (white ring between the media and Histopaque) with a sterilized serological pipette and transfer to a new 50 ml conical tube (Figure 1).

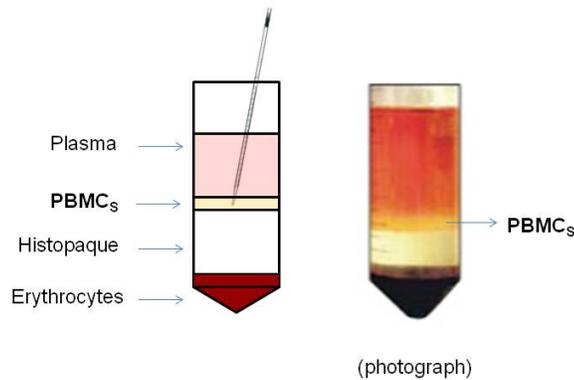


Figure 1. A schematic representation of the result of the density gradient stratification (modified by dublinlisacoolsaet.wordpress.com)

4. Fill the tube with RT PBS to 50 ml and centrifuge at $470 \times g$ for 7 min at room temperature with the brake on (acceleration: 5, deceleration: 5).
5. Put off the supernatant and re-suspend the cell pellet with 4 ml of Erylyse buffer and incubate at RT for 8 min.
6. Fill the tube with PBS to 50 ml and centrifuge at $470 \times g$ for 7 min at RT with the brake on.
7. Resuspend the pellet in 10 ml of PBS, count with dead cell exclusion the PBMCs and centrifuge cell suspension at $470 \times g$ for 10 min.
8. Isolate human CD14⁺ cells: Use human CD14⁺ magnetic microbeads according to the manufacturer's instructions:
 - a. Put off the supernatant and re-suspend the cell pellet in 80 μ l of Running Buffer per 2×10^7 total cells in a 50 ml conical tube.
 - b. Add 20 μ l of CD14 Microbeads per 2×10^7 total cells.
 - c. Mix well and incubate for 15 min in the refrigerator (2-8 °C).
 - d. Wash cells by adding 1 ml of Running Buffer per 10^7 cells and centrifuge at $470 \times g$ for 10 min. Put off the supernatant.
 - e. Re-suspend up to 10^8 cells in 500 μ l of Running Buffer.
 - f. Proceed to magnetic separation with the autoMACS separator.
9. Collect CD14⁺ cells, wash with 10 ml complete medium, resuspend the pellet in 10 ml of complete medium and count with dead cell exclusion. CD14⁺ cells should be 10-15% of PBMC (Figure 2).

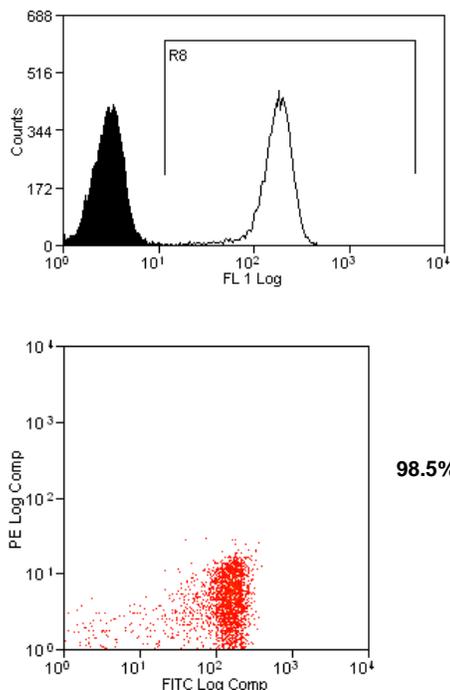


Figure 2. Analysis of CD14⁺ cells after separation at day 0. A. Histogram: the black peak is the isotype control antibody, the white peak CD14⁺ cells; B. Dot plot.

10. Centrifuge at 470 x g for 8 min at room temperature with the brake on and put off the supernatant;
11. Re-suspend at 5 x 10⁶ cells in 3 ml complete medium supplemented with 100 ng/ml of GM-CSF and 50 ng/ml of IL-4 and then culture cells in 6-well plate. Incubate cells at 37 °C and 5% CO₂ (the cytokines are dissolved in DNase/RNase free water and diluted in complete medium).
12. Day 3: Added 2 ml/well of fresh complete medium supplemented with 100 ng/ml of GM-CSF and 50 ng/ml IL-4.
13. Day 6: Monocytes are differentiating into immature dendritic cells, they are in suspension and so be careful when removing the plate from incubator. Take medium with cells out of the 6-well plate into a 50 ml tubes and centrifuge at 210 x g for 10 min at 4 °C. Put off the supernatant and re-suspend cells at 1 x 10⁶ cells/ml in complete medium. Collect 1 x 10⁵ cells into FACS tube and add 10 µl PE-anti-CD1a and 10 µl FITC-anti-CD14 antibodies for 20 min at 4 °C and then wash with FACS wash buffer at 470 x g for 5 min. Put off the supernatant and re-suspend cells in 200 µl and run samples on CyAn ADP flow cytometer. The percentage of CD1a⁺ cells should be higher than 90% as measured by flow cytometer analysis (Figure 3). Immature human derived-DC (iDC) are then ready for

experimental use. If iDC population is between 70-90% they can be used for experiments, while if they are less than 70%, restart culture.

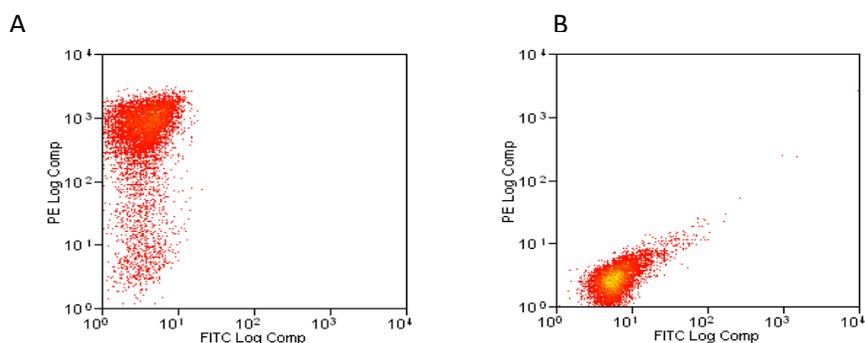


Figure 3. A: expression of CD1a on iDC at day 6; B: expression of CD14 on iDC at day 6

14. Place 2×10^6 iDCs/well in a 6-well plate and incubate for 24 h in complete medium only or supplemented with 50 ng/ml TNF- α and 50 ng/ml IL-1 β to generate mature DC (mDC).
15. Day 7: Flow cytometry procedure.
 - a. Put 6-well plate on ice for 10 min.
 - b. Harvest cells with a micropipette with a volume range from 100 μ l and 1,000 μ l and wash three times with 2 ml of refrigerated PBS. Transfer iDCs and mDCs in two different 15 ml tubes.
 - c. Centrifuge at 210 \times g for 10 min, and then put off the supernatant.
 - d. Re-suspend them in 100 μ l of PBS 1x and block non-specific sites with 5 μ l rabbit IgG for 5 min at RT.
 - e. Divide cells into FACS tube and incubate with 10 μ l fluorochrome-conjugated monoclonal antibody and isotype-matched negative controls for 20 min at 4 $^{\circ}$ C (The following mAbs were used: FITC-anti-CD83, PE-anti-CD80, APC-anti-CD40.).
 - f. Filled with 1 ml FACS wash buffer and centrifuge at 1,500 rpm for 5 min and put off the supernatant. Re-suspend cells in 200 μ l 1x PBS and analyze samples on CyAn ADP flow cytometer.
 - g. Cells were gated according to their light scattering properties to exclude cell debris and contaminating lymphocytes, and no dead cells by propidium iodide positive cells exclusion. (Figure 4).

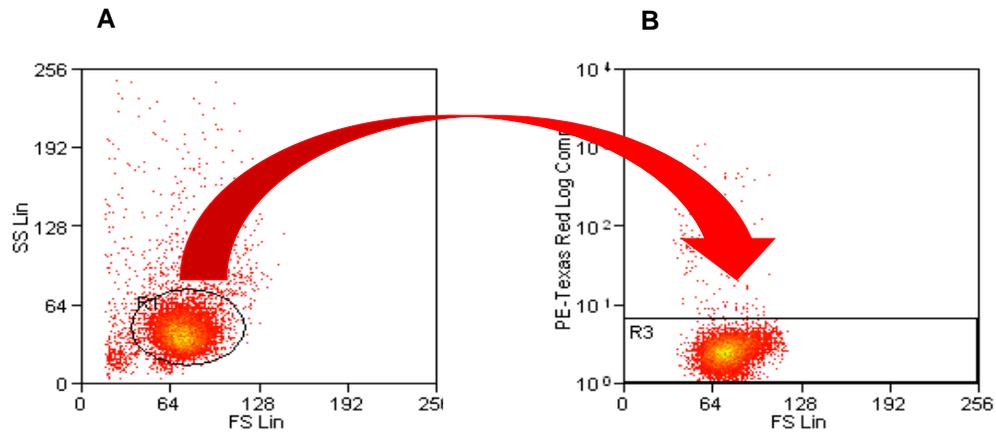
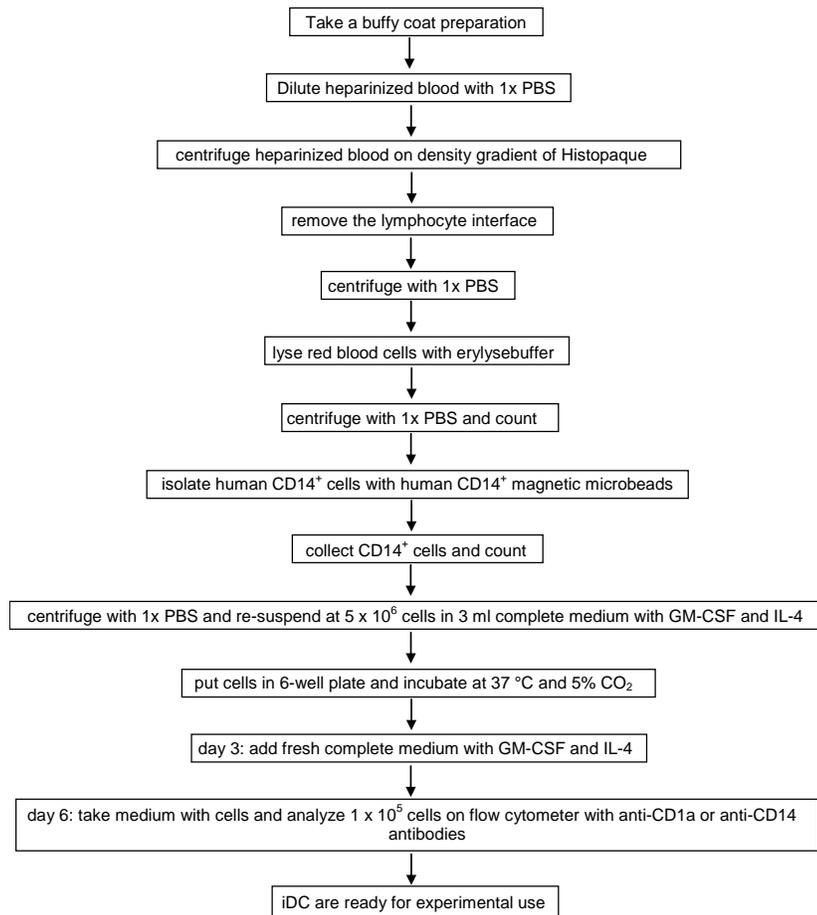


Figure 4. A: Dot plot of FS and SS parameter to identify the iDCs; B: gate to exclude the dead cells. We analyzed the cells on gate R1 and gate R3.

Notes

1. A flow chart with the steps to generate DCs.



Recipes

1. Erylyse buffer
 - 500 ml Milliq water
 - 0.83% NH₄Cl
 - 0.168% Na₂CO₃
 - 1 mM EDTA (pH 7.3)
 - Sterile filtration
2. Complete medium
 - RPML medium
 - 100 U of penicillin
 - 0.1 mg/ml streptomycin
 - 50 μM 2-mercaptoethanol
 - 10% heat-inactivated qualified fetal bovine serum
3. Auto MACS running buffer
 - 1 L auto MACS Rinsing buffer
 - 5 g BSA sterilized by passing through 0.22 μM filter
4. FACS wash buffer
 - 1x PBS
 - 0.2% BSA
 - 0.1% NaN₃
5. Propidium iodide
 - 1x PBS
 - 2% FBS
 - 0.1% NaN₃
 - 2 mg propidium iodide

Acknowledgments

This protocol was adapted from previous work by Spadaro *et al.* (2008).

References

1. Spadaro, M., Caorsi, C., Ceruti, P., Varadhachary, A., Forni, G., Pericle, F. and Giovarelli, M. (2008). [Lactoferrin, a major defense protein of innate immunity, is a novel maturation factor for human dendritic cells.](#) *FASEB J* 22(8): 2747-2757.

2. Spadaro, M., Montone, M., Arigoni, M., Cantarella, D., Forni, G., Pericle, F., Pascolo, S., Calogero, R. A. and Cavallo, F. (2014). [Recombinant human lactoferrin induces human and mouse dendritic cell maturation via Toll-like receptors 2 and 4](#). *FASEB J* 28(1): 416-429.

