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Multiplexed immunophenotyping of human antigen-presenting cells in whole blood by polychromatic flow cytometry

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Abstract

We describe two modular protocols for immunostaining and multiparameter flow cytometric analysis of major human antigen-presenting cells (dendritic cells, monocytes, B lymphocytes) in minimally manipulated whole blood. Simultaneous detection of up to eight colors is enabled by careful selection and testing of cell-subset-defining monoclonal antibodies (anchor markers) in the appropriate fluorochrome combinations, to demonstrate the quantification of surface expression levels of molecules involved in chemotaxis (e.g. CX₃CR1, CCR2), adhesion (e.g. CD11b, CD62L), antigen presentation (e.g. CD83, CD86, CD209) and immune regulation (e.g. CD101) on circulating antigen-presenting cells. Each immunostaining reaction requires as little as 50–100 μl of peripheral whole blood, no density-gradient separation, and the entire procedure from preparation of reagents to flow cytometry can be completed in <5 h.

INTRODUCTION

Antigen presentation is an innate immune function that is crucial in self/non-self discrimination and in the pathogenesis of most immune-mediated diseases. The chief immune cells endowed with this function, known also as professional antigen-presenting cells (APCs), include dendritic cells (DCs), macrophages, monocytes and B lymphocytes. DCs, macrophages and monocytes, including immature monocytes, are particularly competent in antigen capturing and presentation and in activating antigen-specific T-cell responses through major histocompatibility complex class II (MHC II) molecules 1–7. MHC II molecules are encoded in the highly genetically polymorphic human leukocyte antigen (HLA) region that produces multiple molecules required for antigen presentation, including HLA-DR, a MHC II molecule predominantly and abundantly expressed by APCs. Because APCs contribute crucially to the pathogenesis of a range of diseases, detailed characterization of their functional status would be valuable in devising strategies for diagnosis, immunologic monitoring and therapy and would furnish important insight into mechanisms of APC mobilization. More generally, the human immune system is poorly understood^{8,9}, and there is an urgent need to improve our knowledge using the latest technologies.

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AUTHOR CONTRIBUTIONS

E.F. contributed to the conception, design, performance and analysis of the protocols and wrote the manuscript. L.E. contributed to the configuration and establishment of the flow cytometer in the laboratory and to the writing of the manuscript. J.A.T. participated in the conception of the protocols and the writing of the manuscript. L.S.W. contributed to the conception, design and analysis of the protocols and the writing of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Phenotypic characterization of antigen-presenting cells has been hampered by the large number of surface markers required to identify a subpopulation or subset, and furthermore limitations of conventional three- to four-color flow cytometers are major drawbacks^{1,10,11}. Advantages of single-cell immunophenotyping of blood antigen-presenting cells by flow cytometry have been demonstrated and proven feasible¹², offering relatively high sensitivity and statistical power compared with non-flow-based technologies. Polychromatic (>6 colors) flow cytometry increases the data-capturing throughput and enables concurrent, multiplexed analysis of cell subsets and populations in the same reaction tube (e.g. calculation of percentages or ratios of cell subsets, parallel comparison of surface expression density of a molecule between two cell populations), as well as a qualitative assessment and characterization of the functional status of cells^{13,14}. In conjunction with the use of minimally manipulated leukocytes derived from erythrocyte-lysed fresh blood, instead of density-gradient-enriched mononuclear cells, immunophenotypic data can be captured with minimal introduction of artefacts or factors that affect immunostaining¹⁵.

Selection and rigorous testing of monoclonal antibodies and fluorochromes are crucial to successful polychromatic flow cytometry. Recently, Jansen *et al.* reported an eight-color immunostaining protocol for flow cytometry that uses MHC II, CD14, CD11c and CD123 with/without CD19 as cell-subset-defining markers¹⁶. Our early experience with eight-color flow cytometry using six subset-defining monoclonal antibodies concomitantly (targeting CD14, CD16, CD19, CD11c, CD123 and HLA-DR) indicated that an accurate definition of DC subsets is better achieved using the stringent Lineage cocktail 1 (Lin1)-FITC negative selection markers or antibodies (BD Biosciences) that target CD3, CD14, CD16, CD19, CD20 and CD56; these lineage markers are all undetectable on the two major DC subsets. In other words, a lack of expression of Lin1 effectively excludes T lymphocytes, CD14⁺ monocytes, CD14⁺CD16⁺ monocytes, CD16⁺ monocytes, polymorphonuclear neutrophils (PMNs), B lymphocytes and natural killer cells. Moreover, CD11c (integrin α_X) and CD123 (IL-3 receptor- α), without the exclusion of lineage markers, are not DC-specific as they are expressed by a variety of myeloid and lymphoid cells.

Therefore, to maximise the capabilities of an eight-color flow cytometer (BD LSR II with blue, red and violet lasers) while preserving the accurate identification of monocyte subsets, B lymphocytes and DC subsets in addition to retaining the capacity to analyse three to four target molecules of interest, we have devised two protocols with similar workflow, Protocols A and B (Fig. 1), that have undergone testing and ascertainment. The two immunostaining protocols are modular and can be easily performed in parallel, or independently, to define the major circulating antigen-presenting cell subsets in whole blood: CD14⁺, CD14⁺CD16⁺ and CD16⁺ monocytes; CD19⁺HLA-DR⁺ B cells; and myeloid (MDCs or preDC1) and plasmacytoid (PDCs or preDC2) DCs. Performing both protocols concurrently can reduce the elapsed time from phlebotomy to immunostaining ('arm-to-antibody' time) and minimize any potential time-dependent *in vitro* confounding effects when studying innate cellular attributes that are intrinsically volatile. The use of predeveloped, standardized fluorescent beads for normalization of fluorescence intensity data as well as for periodic fine-tuning of the flow cytometer^{17,18} can reduce non-biological and/or instrumental variation arising from day-to-day operation of the flow cytometer, especially for multicenter and/or longitudinal immunophenotyping studies.

Monocytes are progenitors for macrophages and certain DCs, and two principal subpopulations have been described: CD14⁺HLA-DR⁺ ('inflammatory') and CD16⁺HLA-DR⁺ ('resident') monocytes^{19,20}. Further characterization based on their differential expression of chemokine receptors and adhesion molecules identified inflammatory CD14⁺ monocytes as CX₃CR1^{low}CCR2⁺CD62L⁺CD11b^{high}, and resident CD16⁺ monocytes as CX₃CR1^{high}CCR2⁻CD62L⁻CD11b^{int}^{2,20}. CD14⁺ and CD16⁺ monocytes differ in size

[~18 μm or intermediate-high forward scatter channel (FSC), and ~14 μm or low-intermediate FSC, respectively], cytoplasmic granularity [relatively higher side scatter channel (SSC) and lower SSC, respectively] and in their functional capacities^{1,2,19,20} (see also Fig. 2a for FSC and SSC comparisons). Although less well understood, CD14⁺CD16⁺ monocytes are another subset, possibly in a transitional or intermediary state, that is expanded and active in inflammatory disorders^{6,21-23}. Even in the absence of an exogenous stimulus, the CD14⁺CD16⁺ subset differentiates within 12 h from CD14⁺ monocytes, and therefore we recommend proceeding with the immunostaining within 4 h of blood donation and to rotate the blood collection tubes if possible to avoid subsequent miscalculation of monocyte subset ratios and changes in the expression levels of certain surface molecules (Fig. 3).

MDCs and PDCs derive from different hematopoietic lineages and have divergent physiological functions^{7,24-29}. In conjunction with positive expression of MHC II molecules such as HLA-DR and the absence of Lin1 markers, MDCs and PDCs can be further classified as Lin1⁻HLA-DR⁺CD11c⁺ and Lin1⁻HLA-DR⁺CD123⁺, respectively. CD303 (BDCA-2) can be used alternatively to identify PDCs, whereas CD1c⁺ (BDCA-1) and CD141⁺ (BDCA-3 or thrombomodulin) are markers that identify additional MDC subsets³⁰⁻³². CD303 is a PDC-specific C-type lectin involved in antigen uptake, processing and presentation to T cells and regulates type I interferon release, a cell-defining attribute of PDCs³³.

Neutrophils can be identified and distinguished from monocytes, macrophages and DCs by virtue of their high FSC and SSC¹¹ – thus, the use of a different gating strategy (see also Fig. 2a) and immunophenotypic profile: CD16^{high}CX₃CR1⁻CD62L⁺CD11b^{high}. In line with recent findings, neutrophils differentiate from the CX₃CR1-negative lineage of myeloid progenitors that diverge from the macrophage-DC precursors early in hematopoiesis²⁹. Investigators interested in this cell population can maximize the yield of immunophenotyping data by gating on the granulocyte cluster (Fig. 2a).

Protocol A details the use of eight fluorochrome-conjugated monoclonal antibodies (or ten parameters) to characterize in whole blood CD14⁺, CD14⁺CD16⁺ and CD16⁺ monocyte and B-cell surface expression of chemokine receptors (e.g. CX₃CR1 or fractalkine receptor, CCR2), adhesion molecules (e.g. CD11b or integrin- α_M , CD62L or L-selectin) and costimulatory/accessory antigen presentation molecules (e.g. CD83, CD86, CD209 or DC-SIGN). Determination of monocyte subset ratios is also shown. Protocol B takes advantage of the Lin1 immunostaining strategy focusing on the identification of CD11c⁺ MDCs and CD123⁺ PDCs, with demonstration of the alternative and/or concomitant use of the DC markers CD1c and CD303 in combination with Lin1⁻HLA-DR⁺ to ascertain the identity of MDCs and PDCs, respectively. Specific to this protocol is the use of a violet laser-excitable fluorochrome, particularly Pacific Blue (peak excitation/emission of ~410/455 nm) or an equivalent such as eFluor 450 (peak excitation/emission of ~405/450 nm), conjugated with anti-CD123 antibody that opens up other commonly used channels (e.g. PE, Alexa Fluor 700, APC-Alexa Fluor 750) for use.

Isotype control immunoglobulins are not needed for quantitative immunophenotyping¹⁸. In a recent large-scale, gene-immunophenotyping study of nearly 200 individuals that we carried out¹⁴, we eliminated the use of the isotype controls after ascertaining beforehand that the monoclonal antibodies are specific. Isotype control immunoglobulins are liable to fluctuate over time, and the use of fluorescent microspheres is a solution to the time-dependent effects or variability observed in longitudinal studies¹⁸. We recommend only the initial use of isotype-matched control immunoglobulins to establish that a target antibody is

specific, then proceed to longitudinal immunophenotyping using fluorescent microspheres to normalize the acquired data.

When performing large-scale, quantitative immunophenotyping by polychromatic flow cytometry over a prolonged period (i.e. on the order of months), the investigator should have a precise hypothesis *a priori*, determine the power of the study and calculate the sample size required, to enable estimation of the amount of antibodies and reagents required. Apart from instrumental variation that can be remedied by using standardized fluorescent microspheres¹⁸, a batch-to-batch effect of reagents is probably another major source of variability. In a carefully planned study with a known number of immunostaining reactions, the investigator can then order an oversized batch and pool the antibodies to circumvent batch variation.

Each immunostaining reaction requires as little as 50–100 μ l of fresh whole blood without prior density-gradient separation. This method provides a cost-effective, practical approach to high-throughput polychromatic immunophenotyping of the major APC subsets in whole blood and serves as a framework upon which to incorporate additional analysis parameters, depending on the flow cytometer setup and fluorochromes used.

MATERIALS

REAGENTS

- Fresh whole blood from consenting healthy human donors ! **CAUTION** Universal precaution and work in Category 2 biological safety cabinets; where possible, use screened blood and dispose of bio-hazardous waste as per local institutional protocol. ! **CAUTION All experiments using human tissue must conform to National and Institutional regulations.**
- Citrate phosphate dextrose adenine (CPDA)-pretreated blood collection tubes (Greiner bio-one, cat. no. 455094; Sarstedt, cat. no. 01.1610.001) **▲ CRITICAL** Our testing of multiple lots of CPDA and sodium heparin tubes indicated that the former did not contain endotoxin levels above the detection limit of the assay, whereas the latter had more variable background levels of endotoxin (range <0.01 to 1.04 EU/ml, $n = 10$ total number of tubes tested from at least four lots); indicators of increased neutrophil degranulation, cell death and monocyte activation were noted with sodium heparin tubes.
- Monoclonal antibodies (see Table 1) ! **CAUTION** Sodium azide is a preservative present in most antibody preparations that is irritating to the skin and can be explosive when accumulated in plumbing; flush and dispose of with copious water. Even though azide is present, to avoid microbial contamination, open vials containing antibody in a laminar flow hood and remove aliquots with sterile pipette tips. **▲ CRITICAL** Prior to starting a longitudinal, quantitative immunophenotyping study, perform a titration analysis to determine the saturating amount or concentration of each antibody required to ensure maximal coverage of the expressed target antigen in order to attribute any variation observed to true biological differences. This can be performed by using incremental doses of a single antibody to immunostain a donor's blood samples under the same experimental conditions and determine the dose required to obtain a plateau of fluorescence intensity by constructing a line plot of antibody concentration vs mean (or median) fluorescence intensity, in conjunction with overlaid curves displayed histographically³⁴. The optimal concentration is usually the one at the beginning of a saturation plateau³⁴. For markers not requiring absolute quantitation in a study,

to reduce cost, adequate staining can be often obtained with less than a saturating amount of antibody.

- Isotype control immunoglobulins (see Table 1).
- Zenon Pacific Blue Mouse IgG_{2a} Labeling Kit (Invitrogen, cat. no. Z25156) – only if performing manual conjugation of Pacific Blue with purified anti-CD123 (Miltenyi Biotec, clone AC145, cat. no. 130-090-940) (see REAGENT SETUP for conjugation procedure); otherwise, not required if using a manufacturer-preconjugated Pacific Blue equivalent such as anti-CD123–eFluor 450 (eBioscience, clone 6H6, cat. no. 48-1239).
- BD FACS Lysing Solution, 10x (BD Biosciences, cat. no. 349202)
- BD CellFIX, 10x (BD Biosciences, cat. no. 340181)
- FluoroSpheres, 6-peak calibration beads (DakoCytomation, cat. no. K0110) or 8-peak calibration beads (DakoCytomation, cat. no. K0112)
- Sterile, endotoxin-free PBS, 1x (Sigma, cat. no. D8537)
- Sterile, endotoxin-free distilled H₂O (GIBCO Invitrogen, cat. no. 15230-089).

EQUIPMENT

- BD LSR II flow cytometer (BD Biosciences, cat. no. 33300456)
- Eppendorf Centrifuge 5417R with Fixed-Angle Rotor with Aerosol-Tight Lid
- Category 2 biological safety cabinet
- Sterile 1.5-ml or 2.0-ml polypropylene microcentrifuge tubes (Sarstedt), or 2-ml 96-well Polypropylene Deep-Well Plates (BD Falcon) in conjunction with adhesive PCR film (Thermo Scientific) for sealing during incubation. **! CAUTION** When using 96-well polypropylene plates with adhesive sealing film, avoid contact of blood and reagents with the film
- 10-ml FACS tubes
- BD FACSDiva software (BD Biosciences, <http://www.bdbiosciences.com>) for data acquisition
- FlowJo software (TreeStar Inc., <http://www.flowjo.com>) for data analysis

REAGENT SETUP

- Anti-CD123 monoclonal antibody
Either manually conjugate purified anti-CD123 monoclonal antibody (Miltenyi) with Pacific Blue (Invitrogen) as per manufacturer's instructions (see also PROCEDURE **step 3B**) or use manufacturer-preconjugated anti-CD123–eFluor 450 (eBioscience) thereby obviating the conjugation procedure detailed in **step 3Biii**. Store all antibodies at 4°C.

EQUIPMENT SETUP

Flow cytometer—Our BD LSR II is equipped with three lasers: violet (405 nm excitation by Coherent VioFlame, 25 mW solid-state laser), blue (488 nm excitation by Coherent Sapphire, 20 mW solid-state laser) and red (633 nm excitation by JDS Uniphase 1344P, 17 mW HeNe laser). Bandpass filters 440/40 and 525/50 are configured with the violet laser; 530/30, 575/26, 695/40 and 780/60 with the blue laser; 660/20 and 730/45 and 780/60 with

the red laser. The specifications of long-pass dichroic mirrors used were 505 nm, 550 nm, 685 nm, 710 nm and 755 nm (see Table 1). When frequent, in-house calibration and fine-tuning of flow cytometer as detailed by Perfetto *et al.*¹⁷ is not possible, regular (quarterly) evaluation and servicing of the flow cytometer by the manufacturer's engineer should be scheduled. ! **CAUTION** Internal components of a flow cytometer pose serious health hazards and should not be tampered with by unqualified personnel.

PROCEDURE

Preparation of reagents (steps 1–2), 30 min

1 Label 2.0-ml microcentrifuge tubes. **▲ CRITICAL STEP** 1.5-ml microcentrifuge tubes can be used if reaction volumes are scaled down proportionately. However, the yield of rare cell populations might be low. When working with large numbers of staining reactions, 2-ml 96-well Polypropylene Deep-Well Plates in conjunction with adhesive sealing film are useful; avoid contact of blood with adhesive surface. We do not recommend the use of polystyrene FACS tubes for immunostaining APCs as these cells might become activated by this surface substrate. When polypropylene FACS tubes are used, exercise care when discarding the supernatant as the pellet can be difficult to visualize. Use sterile plasticware.

2 Prepare fresh 1x BD FACS Lysing Solution and 1x BD CellFIX solution by diluting 1:10 the respective 10x solutions with sterile, endotoxin-free H₂O prior to use. Keep 1x FACS Lysing Solution at 15–20°C, and CellFIX solution and 1x PBS on ice. **▲ CRITICAL STEP** Keep all solutions and antibody mixes sterile.

Staining (steps 3–11), 2 h

3 Prepare the antibody master mix for immunostaining of monocyte subsets and B lymphocytes (see **Protocol A**) and/or DC subsets (see **Protocol B**).

A. Antibody mix for monocyte subsets and B lymphocytes

- i. Add anti-human CD14–Pacific Orange, CD16–Pacific Blue, CD19–Alexa Fluor 700, and HLA–DR–PerCP (or HLA-DR–FITC) anchor antibodies to a sterile 1.5-ml microcentrifuge tube (Table 1).
- ii. Keep mix on ice and in the dark.

B. Antibody mix for DC subsets

- i. Add anti-human Lin1–FITC cocktail antibodies, HLA-DR–PerCP and CD11c–APC anchor antibodies to a sterile 1.5-ml microcentrifuge tube (Table 1); include anti-CD123–eFluor 450 in the mixture when substituting for CD123–Pacific Blue and skip **step 3Biii**.
- ii. Keep mix on ice and in the dark.
- iii. Manually conjugate purified anti-CD123 monoclonal antibody (Miltenyi) with Pacific Blue at 15–20°C using the Invitrogen Zenon Pacific Blue Mouse IgG_{2a} Labeling Kit (refer to manufacturer's instructions for details). Briefly, 1 µg (~20 µl of Miltenyi anti-human CD123, cat. no. 130-090-940) of purified anti-CD123 antibody (antibody volume not crucial if 20 µl is used per 100 µl of whole blood) is incubated for 5 min with 5 µl of Component A (consisting of Pacific Blue-labelled Fab fragment targeting Fc portion of anti-CD123 antibody), followed by incubation for 5 min with 5 µl of Component B (consisting of non-specific IgG that binds free Fab fragments). Anti-CD123–Pacific Blue is now conjugated and must be used within 30 min. Proceed immediately to **step**

4. ■ PAUSE POINT Following addition of Component A, the anti-CD123–Pacific Blue conjugate can be kept in the dark at 4°C for several weeks with the supplement of 2 mM sodium azide before proceeding to addition of Component B, according to the manufacturer’s instructions.

4 Pipet 100 µl of CPDA-treated fresh whole blood into each tube. If blood droplet is caught on the tube sidewalls, samples can be spun down gently by pressing the ‘Short’ button on the Eppendorf Centrifuge 5417R for 2 s (350 *g*).

5 Add antibodies to whole blood. For immunostaining of monocyte subsets and B lymphocytes see **Protocol A**. For immunostaining DC subsets see **Protocol B**.

A. Immunostaining monocyte subsets and B lymphocytes

- a.** Add to whole blood the appropriate volume of antibody master mix prepared above for the respective protocol. Typically, most antibodies will be added in a volume of ~10 µl so the final volume is ~180 µl; total volume to be added depends on the sum of the volume of the constituent antibodies.
- b.** Incubate in the dark for 30 min at 15–20°C.

CRITICAL STEP During this incubation you can continue with step 6.

B. Immunostaining DC subsets

- i.** Add 25 µl of the anti-CD123–Pacific Blue antibody prepared above to 100 µl of whole blood. Skip this step and proceed to **step 5Biii** if using anti-CD123–eFluor 450.
- ii.** Incubate in the dark for 10 min at 15–20°C.
▲ CRITICAL STEP During this, and the subsequent, incubation one can continue with step 6.
- iii.** Add the antibody master mix prepared in **step 3Bi**. Typically, most antibodies will be added in a volume of ~10 µl so the final volume is 180–200 µl; total volume to be added depends on the sum of the volume of the constituent antibodies.
- iv.** Incubate in the dark for 20 min at 15–20°C.

6 While Protocol A and Protocol B antibodies are incubating with blood samples, perform single-color staining for later fluorochrome spectral overlap compensation by applying the same, single antibody that was used in the master mix for each individual fluorochrome. Incubate samples in the dark for 30 min at 15–20°C. Omit this step when using standardized fluorescence-labeled microspheres for setting spectral compensation (see also PROCEDURE – Acquisition). **▲ CRITICAL STEP** Performing single-color staining for all fluorochromes each time when immunophenotyping is performed can also serve as a quality control and help to troubleshoot and gauge the quality of the antibody and the staining.

7 Following incubation of the antibody mix with blood, lyse erythrocytes by adding 2.0 ml of 1x FACS Lysing Solution per 100 µl of blood used. Incubate for 10 min at 15–20°C with inversion of tubes every 2–3 min. **▲ CRITICAL STEP** Ensure cells are well resuspended for proper lysis.

8 After incubation, centrifuge at 350 *g* for 7 min at 15–20°C.

9 Carefully pipet off and discard supernatant. Resuspend cell pellet in 1.0 ml of cold, endotoxin-free 1x PBS for each 100 μ l whole blood used and mix well.

CRITICAL STEP Perform all subsequent steps at 4°C.

10 Centrifuge at 350 *g* for 7 min at 4°C.

11 Carefully aspirate off and discard supernatant. Resuspend in 300 μ l of 1x BD CellFIX. ■ **PAUSE POINT** Fixed cells can be kept at 4°C for up to 24 h prior to analysis by flow cytometry.

? TROUBLESHOOTING

Acquisition (steps 12–16), 1 h

12 Transfer fixed samples from microcentrifuge tubes to 10-ml FACS tubes.

13 Switch on flow cytometer and allow it to warm up for 30 min. Open FACSDiva software program.

14 Calibrate PMT voltages by first creating a FSC vs SSC bivariate plot in FACSDiva to visualize cell clusters, then create one-dimensional histograms for each fluorochrome used in the experiment, and set voltages by acquiring blank and fluorescent beads (DakoCytomation FluoroSpheres) gauged on histograms according to the manufacturer's instructions. In addition, if desired, use an unstained sample of fixed cells (see Step 3 above) to establish the PMT voltages so that the majority of events (cells) contained within a gate encompassing monocyte or lymphocyte clusters (i.e. clusters that are not granulocytes, dead cells or cell debris) are in the first decade (log) of fluorescence. Benchmark against blank beads (Dako Cytomation) to monitor the autofluorescence of the target cell population on a day-to-day basis.

15 Determine fluorochrome pair compensations by first acquiring samples from single fluorochrome–antibody staining. Use bi-exponential (or 'Logicle') display of *x*-axis and *y*-axis when determining amount of compensation³⁵. Set compensation now or after collection of all samples, depending on capabilities of flow cytometer and software capabilities; automatic compensation might be available on some platforms. An orderly workflow including the systematic organization of bivariate plots displaying antibody–fluorochrome pairs is essential to successful polychromatic flow cytometry (Fig. 4). For literature on flow cytometry instrument setup and fluorochrome compensation, investigators are referred to excellent reviews published by others^{10,33–39}. ▲ **CRITICAL STEP** Each target antigen can have a drastically different cell-surface distribution and biophysical properties, requiring configuration of a unique set of fluorochrome compensation values. Use an abundantly expressed surface epitope when setting compensation.

16 Begin acquisition of data.

? TROUBLESHOOTING

Analysis (steps 17), 1 h

17 After acquisition, export data in the *.fcs format and analyze using the FlowJo software (see also ANTICIPATED RESULTS and Figures; for latest information on cross-platform compatibility of FlowJo files, check the FlowJo website.). For monocytes follow option A. For B lymphocytes follow option B. For DC subsets, follow option C.

A. Monocytes

- i. Place gate around CD14⁺ and CD16⁺ monocyte clusters on a FSC (linear) vs SSC (logarithmic) bivariate scatter plot (Fig. 2a, pink enclosure), and drill down to produce a one-dimensional histogram for HLA-DR–PerCP (Fig. 2b).
- ii. Select HLA-DR⁺ cells (Fig. 2b, within pink gate), and use a quantile contour (zebra) plot to display expression of CD14–Pacific Orange vs. CD16–Pacific Blue.
- iii. Gate on discrete clusters of CD16⁺ (resident), CD14⁺CD16⁺, CD14⁺ (inflammatory) monocytes (Fig. 2c). Leave FITC, PE, APC and APC–Alexa Fluor 750 channels open for quantification of antigen expression levels displayed in a one-dimensional histogram. When HLA-DR–FITC is used, the PerCP or PerCP–Cy5.5 channel can be used for quantification, as demonstrated with CD209–PerCP–Cy5.5 (Fig. 2h).

B. B lymphocytes

- i. Place gate around the lymphocyte cluster on the FSC vs SSC plot (Fig. 2e), drill down on this population and create a CD19–Alexa Fluor 700 vs HLA-DR–PerCP (Fig. 2f). HLA-DR-expressing B cells are CD19⁺HLA-DR⁺. PE, APC, APC–Alexa Fluor 750 and FITC or PerCP/PerCP–Cy5.5 are fluorochrome channels left available for quantification of antigen expression levels, and are displayed histographically (Fig. 2g). PerCP or PerCP–Cy5.5 can be used as the quantification channel when HLA-DR–FITC instead of HLA-DR–PerCP is used (Fig. 2i).

C. DC subsets

- i. Analyze ungated cells on a Lin1–FITC vs HLA-DR–PerCP bivariate plot. DCs are Lin1[–]HLA-DR⁺ (Fig. 5b, pale-blue box).
- ii. Using a CD11c–APC vs CD123–Pacific Blue plot, calculate the MDC:PDC ratio or percentage (Fig. 5c).
- iii. To determine the expression levels of target molecules for both DC subsets, create two-dimensional scatter plots from the Lin1[–]HLA-DR⁺ cluster (Fig. 5b, pale-blue box), generating CD11c vs HLA-DR (for CD11c⁺HLA-DR⁺ MDCs) and CD123 vs HLA-DR (CD123⁺HLA-DR⁺ PDCs) plots (Fig. 5d), from which histograms displaying expression levels of molecules in the PE, APC–Alexa Fluor 750 and Alexa Fluor 700 channels can be created (Fig. 5e and 5f).

? TROUBLESHOOTING

TRUBLESHOOTING

See Table 2 for troubleshooting guidance.

ANTICIPATED RESULTS

Protocol A

The gating of monocytes on a FSC vs SSC bivariate plot is important for identifying the monocyte subsets that have different sizes and granularity^{1,2}. CD16⁺ monocytes have lower FSC and SSC, and are situated close to the lymphocyte cluster (Fig. 2a, green oval), whereas CD14⁺ monocytes, owing to their larger numbers, size (higher FSC) and higher cytoplasmic granularity (higher SSC), cluster near granulocytes (Fig. 2a, red oval). The former subset can be missed or its percentages underestimated if the gating is too conservatively placed on

only the latter subpopulation. Within the monocyte gate, HLA-DR⁺ cells (Fig. 2b) are separated based on expression of CD14 and CD16 and are displayed on a quantile contour (zebra) plot that can also be used to calculate relative ratios or percentages of CD16⁺ (Fig. 2c, green box), CD14⁺CD16⁺ (Fig. 2c, yellow box) and CD14⁺ (Fig. 2c, red box) subsets. We have found the zebra display useful in guiding the gating of monocyte subsets. Histograms showing expression profiles of the monocyte subsets are consistent with those reported previously: CD16⁺HLA-DR⁺ monocytes are CX₃CR1^{high}CCR2⁻CD11b^{low}CD62L⁻ (Fig. 2d, green tracings), and CD14⁺HLA-DR⁺ monocytes are CX₃CR1^{low}CCR2⁺CD11b^{high}CD62L⁺ (Fig. 2d, red tracings)²⁰. CD19⁺HLA-DR⁺ B cells do not express CX₃CR1 or CCR2, but the majority do express CD62L. A recent study reported that ~34% (19–44%) of B cells in peripheral blood of healthy human donors express CD11b on the cell surface, and that CD11b mediates homing of memory B cell subsets, in contrast to naïve cells⁴⁰. Accumulating evidence supports the divergent lineage and functions of CD11b⁺ and CD11b^{-low} B cells^{40–42}. Another analysis of monocyte subsets and CD19⁺HLA-DR⁺ B cells is demonstrated by CD86–PE, CD83–APC, CD209–PerCP–Cy5.5 through use of HLA-DR–FITC instead of HLA-DR–PerCP (Fig. 2). Although the expression of CD209 is negligible or undetectable on resting, circulating monocytes and HLA-DR⁺CD19⁺ B cells from a healthy blood donor (Fig. 2h and 2i), the functionality of this fluorochrome-labeled antibody is demonstrated by its increased expression on CD11c⁺HLA-DR⁺ cells in the monocyte gate following cytokine activation (Fig. 2j). We advise immunostaining within 4 h of blood donation, as changes in the monocyte subset dynamics occur with time (Fig. 3). Protocol A utilizes ten parameters or eight fluorochromes (FITC, PE, PerCP or PerCP–Cy5.5, Alexa Fluor 700, APC, APC–Alexa Fluor 750, Pacific Orange and Pacific Blue).

Protocol B

This protocol is a modification of the four-color staining reported by Willmann *et al.*¹² and others. Because four fluorochrome channels (typically, Lin1–FITC, HLA-DR–PerCP, CD11c–APC and CD123–PE) are required for separating MDCs and PDCs, this limitation precludes the use of antibodies of interest that are commonly available in PE and APC. Using a three-laser flow cytometer (BD LSR II) and a violet-laser-excitable fluorochrome (e.g. anti-CD123 conjugated with Pacific Blue), we were able to maximize immunostaining combinations and attain seven-color multiplexed analysis of two major blood DC subsets; there exist future opportunities for robust eight-color analyses with the increased availability of monoclonal antibodies coupled with new fluorochromes taking advantage of the violet laser such as eFluor 605 and eFluor 650 monoclonal antibodies (eBioscience). In this protocol, Lin1 and HLA-DR are indispensable for identifying DCs (Fig. 5a and 5b) that can be subclassified into MDCs (Lin1⁻HLA-DR⁺CD11c⁺) and PDCs (Lin1⁻HLA-DR⁺CD123⁺) (Fig. 5d). CD1c and CD303 are alternative markers that can identify Lin1⁻HLA-DR⁺ MDC and PDC, respectively, (Fig. 6a and 6b) but should not be regarded as equivalent to CD11c and CD123 in view of the tremendous DC heterogeneity^{26,27,30–32,44}. MDC:PDC ratios or percentages (Fig. 5c) have been reported to alter in disease states; however, gating strategies and the use of DC-defining markers can vary between studies^{45,46}. The CD11c–CD123 bivariate plot of Lin1⁻HLA-DR⁺ cells (Fig. 5c and Fig. 5a, top row) is a common strategy used to simultaneously display ratios between the two DC subsets^{44,45}. For quantification of expression levels (i.e. in mean or median fluorescence intensity units), we find that the CD11c⁺HLA-DR⁺ and CD123⁺HLA-DR⁺ plots (Fig. 5d) generated from Lin1⁻ cells (Fig. 5b, pink box) are preferable as they provide a clear visualization of DC clusters (Fig. 5d, orange and purple boxes) and other cells or events; this is also the method demonstrated in the product sheet provided by the manufacturer of the Lin1 mixture antibodies (BD Biosciences). Protocol B has the capacity for at least nine parameters or seven fluorochromes (FITC, PE, PerCP, APC, APC–Alexa

Fluor 750, Alexa Fluor 700, Pacific Blue). Using CD101 expression profiling of circulating DCs as an example (Fig. 5f), we have concurrently identified positive CD101 surface expression on MDCs but non-expression on PDCs as a novel strategy for differentiating the two DC subsets, findings that might have implications also in their differential immunomodulatory functions^{31,47-50,52}.

Surface markers useful for the identification of circulating APC subsets are summarized in Table 3. As the complexity of polychromatic flow cytometry increases, multidimensional analyses of the data by computational modelling might one day replace manual gating and increase the throughput and objectivity of analyses⁵³. Comprehensive information on flow cytometer setup, fluorochromes, spectral overlap compensations, fluorescent bead normalization for longitudinal immunophenotyping studies, operational pitfalls and other useful technical information are detailed elsewhere^{10,18,34-39}. Further links to major manufactures of flow cytometers, accessories and fluorochrome-conjugated antibodies can be found at the end of an article by Perfetto *et al.*¹¹.

Acknowledgments

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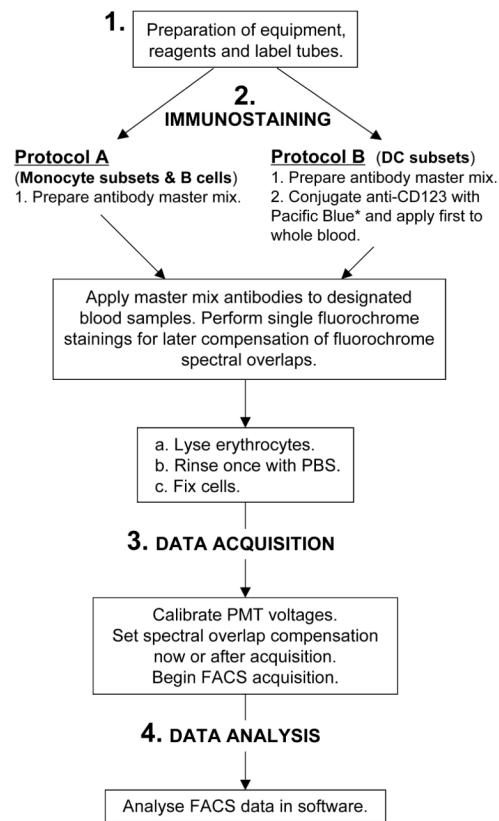


Figure 1. Diagram illustrating the workflow for multiplexed immunophenotypic analysis of the major circulating APC subsets by flow cytometry. *Manufacturer-preconjugated anti-CD123–eFluor 450 can substitute for anti-CD123–Pacific Blue and obviate the need for manual conjugation.

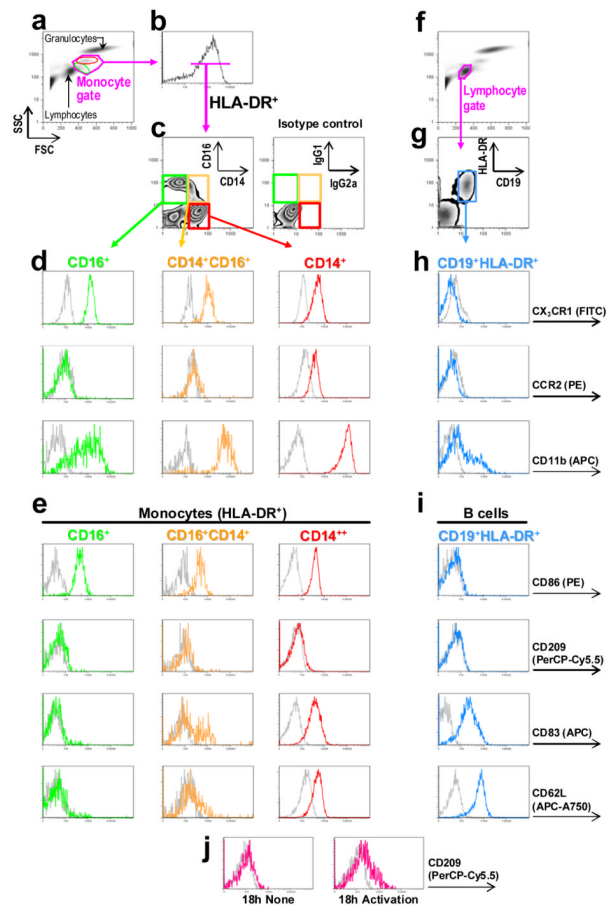


Figure 2.

Flow cytometric analysis of monocyte subsets and CD19⁺HLA-DR⁺ B lymphocytes in whole blood. (a) CD16⁺ ‘resident’ and CD14⁺ ‘inflammatory’ monocytes have preponderant localizations on a FSC (linear) vs SSC (logarithmic) bivariate plot (green and red ovals, respectively), owing to their different intrinsic biophysical properties. Cells within the monocyte gate (pink enclosure) are selected for (b) expression of HLA-DR–PerCP and further separated based on (c) expression of CD14–Pacific Orange and CD16–Pacific Blue, and displayed on a quantile contour (zebra) plot. Negative controls, nonspecific mouse IgG_{2a} and IgG₁, matching the respective fluorochrome and immunoglobulin isotypes of CD14 and CD16, respectively, show no significant positivity. (d) CD16⁺HLA-DR⁺ (green), CD14⁺CD16⁺HLA-DR⁺ (yellow) and CD14⁺HLA-DR⁺ (red) monocyte subsets are analyzed further for their expression of CX₃CR1–FITC, CCR2–PE and CD11b–APC, and (e) CD86–PE, CD209–PerCP–Cy5.5, CD83–APC and CD62L–APC–Alexa Fluor 750, as shown in one-dimensional histograms. B lymphocytes are analyzed from (f) cells localized to the lymphocyte gate that (g) express CD19 and HLA-DR. Histograms illustrate B-cell expression profiles of (h) CX₃CR1, CCR2 and CD11b, and (i) CD86, CD209, CD83 and CD62L in parallel with monocytes co-immunostained in the same reactions. (j) Functionality of the anti-human CD209 antibody in PerCP–Cy5.5 is demonstrated in a separate reaction by activation of peripheral whole blood with IL-1 β (10 ng/ml), TNF- α (50 ng/ml), IFN- γ (1000 U/ml; Peprotech) and ultra-pure lipopolysaccharide (100 ng/ml; InvivoGen); cells in the monocyte gate expressing CD11c and HLA-DR are shown.

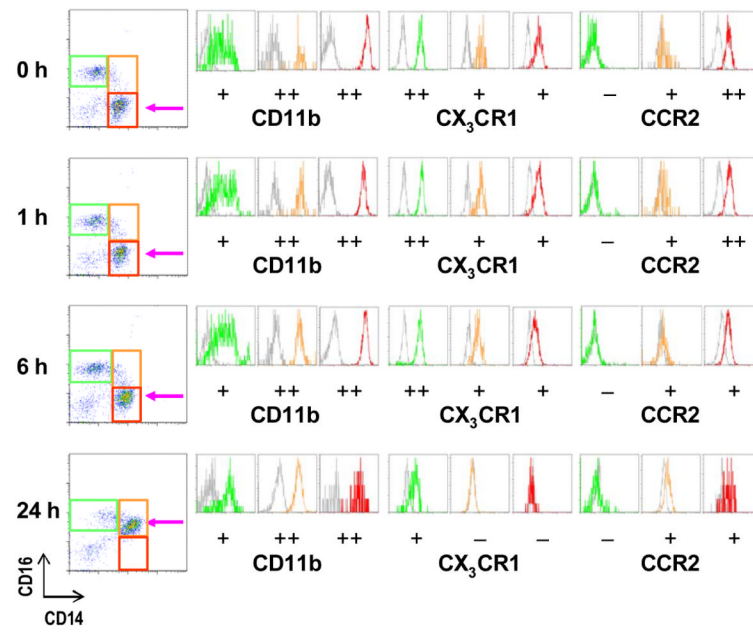


Figure 3.

A time-course experiment showing the natural differentiation or transition of CD14⁺ monocytes into CD14⁺CD16⁺ cells (pink arrow at 24 h) in citrated whole blood even in the absence of any stimulus. Note that CX₃CR1 expression levels on all monocyte subsets decrease over time, whereas CCR2 is expressed only on CD14⁺ and at a low level on CD14⁺CD16⁺ monocytes but is also downregulated with time. FSC, forward scatter channel. SSC, side scatter channel. '-', negative expression. '+', low-to-intermediate level of expression. '++', high level of expression.

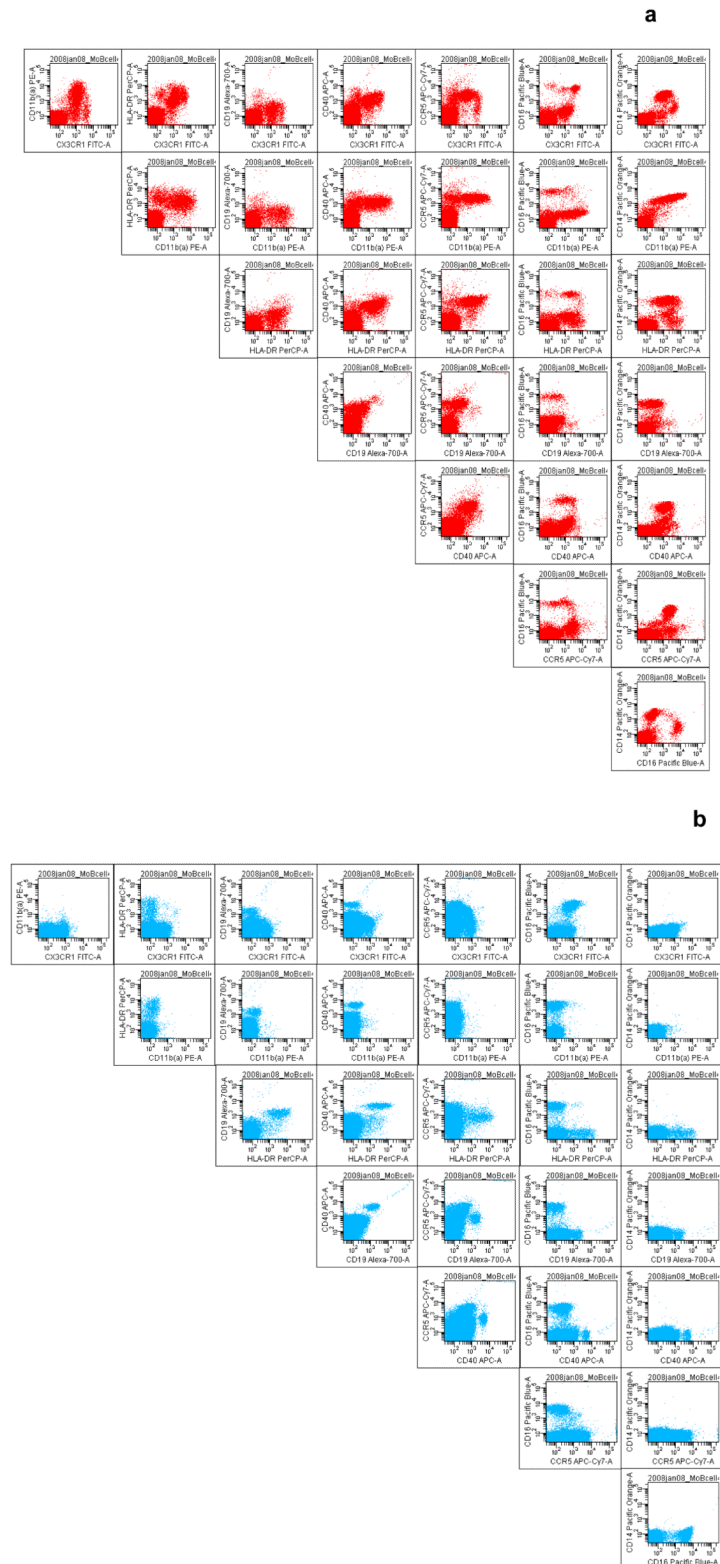


Figure 4. The systematic display of antibody–fluorochrome pairs created in the Worksheet window of the BD FACSDiva software is useful in organizing the large number of bivariate plots, for

visualizing the correct spectral overlap compensation, quality control and for discovering novel cell subsets. This mode of display is also useful for visualizing single-color staining and for ascertaining fluorochrome ‘spillover’. Acquisition events or cells captured in the **(a)** monocyte gate and **(b)** lymphocyte gate are shown. Antibodies demonstrated include those targeting CX₃CR1–FITC, activated CD11b (CD11b(a))–PE, HLA-DR–PerCP, CD19–Alexa Fluor 700, CD40–APC, CCR5–APC-Cy7, CD16–Pacific Blue, and CD14–Pacific Orange (see Table 1 for details).

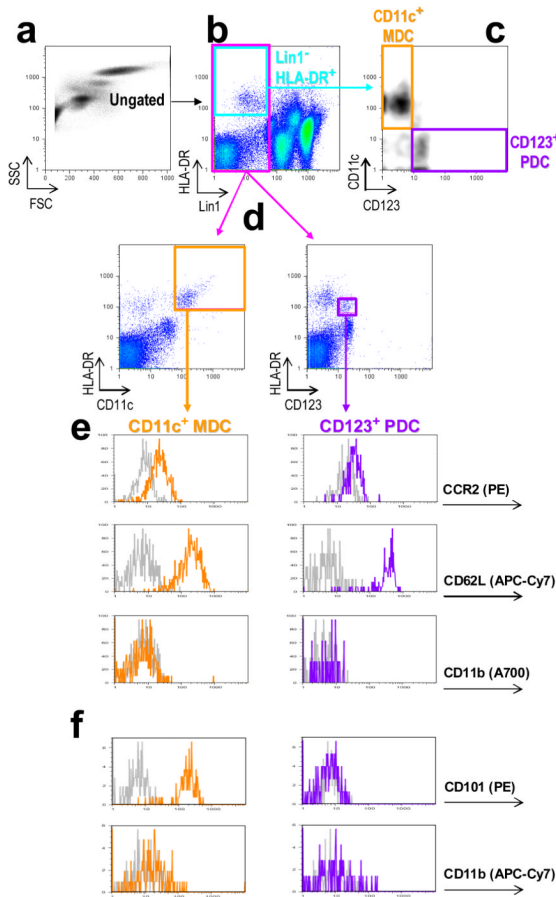


Figure 5.

Analysis of two major DC subsets in whole blood. (a) Ungated cells that are (b) Lin1⁻HLA-DR⁺ (within pale-blue box) are analysed on a (c) bivariate density plot showing CD11c vs CD123 expression levels to allow for identification of MDCs (within orange box) and PDCs (within purple box), respectively, and for determination of DC subsets ratio or percentages. To profile expression levels of specific surface antigens on DC subsets, (b) Lin1⁻ cells (within pink box) are gated on and further analysed for (d) CD11c⁺HLA-DR⁺ and CD123⁺HLA-DR⁺ expression, corresponding to MDCs (orange) and PDCs (purple), respectively. Histograms show (e) expression levels of CCR2-PE, CD62L-APC-Cy7 and CD11b-Alexa Fluor 700, and (f) of CD101-PE and CD11b-APC-Cy7.

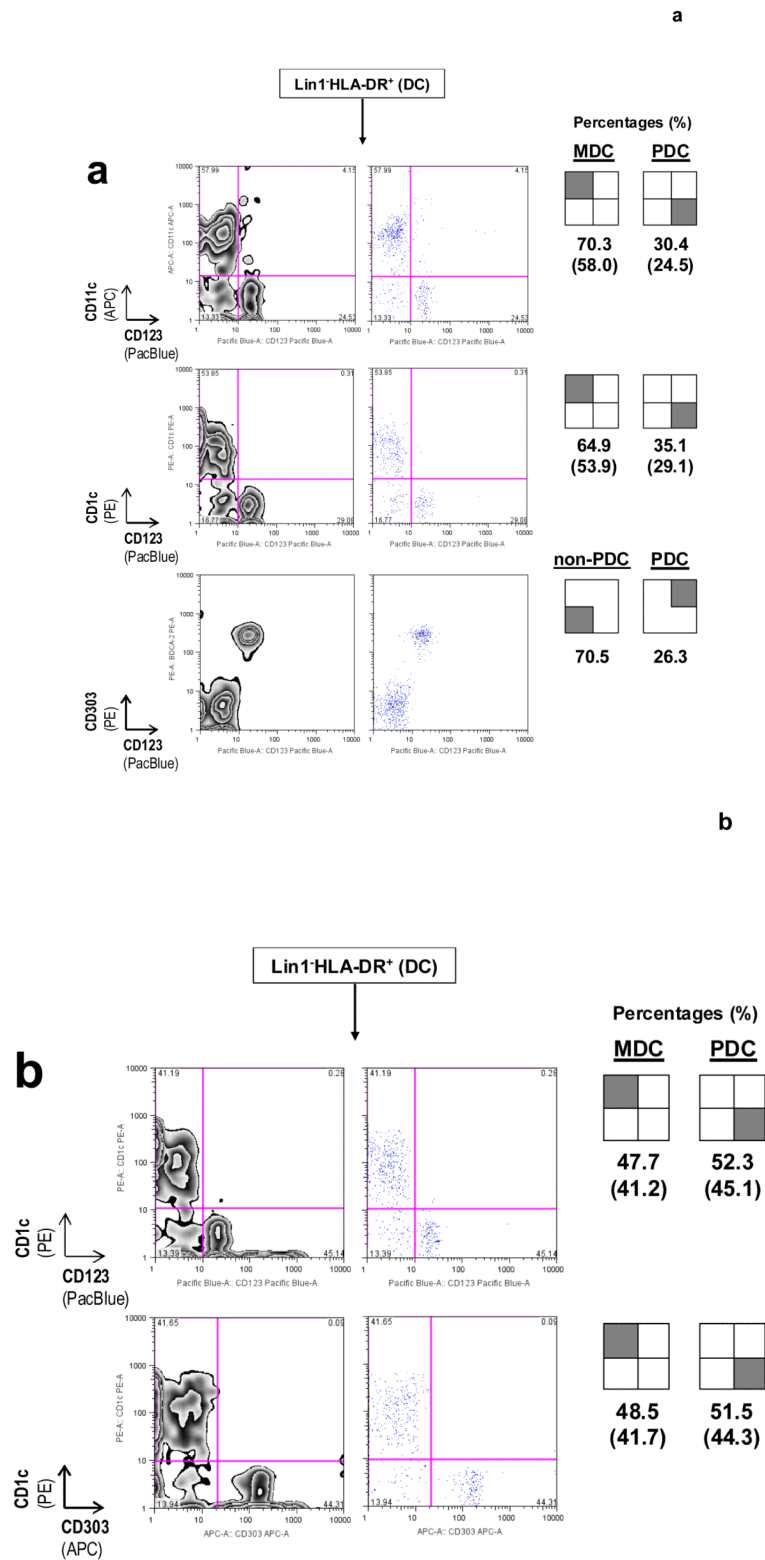


Figure 6. DC subset-defining markers for identifying MDCs and PDCs as described in Protocol B. (a) CD11c and CD123, in conjunction with Lin1⁻HLA-DR⁺, are surface markers that are

commonly used to identify MDCs and PDCs, respectively. CD1c⁺ (or BDCA-1⁺) MDCs might represent a major subset of CD11c⁺ MDCs, as reported previously³⁰⁻³². Co-staining for CD123 and CD303 (BDCA-2) on Lin1⁻HLA-DR⁺ cells simultaneously identifies PDCs in the same staining reaction⁴³; numbers in brackets represent raw percentages for non-PDCs (70.5%) and Lin1⁻HLA-DR⁺CD123⁺CD303⁺CD11c⁻ PDCs (26.3%). Concordance between Lin1⁻HLA-DR⁺CD123⁺ and Lin1⁻HLA-DR⁺CD303⁺ cells is typically in the range 0.971 to 0.989 (mean 0.981, $n = 6$). **(b)** Immunostaining from another blood donor illustrates a variation in the MDC:PDC ratio or percentages. CD1c and CD303 can be used as alternative markers to identify specific Lin1⁻HLA-DR⁺ MDC and PDC subsets, respectively, and in determining subset ratios or percentages. Numbers inside brackets represent percentages calculated from all four quadrants, whereas percentages outside brackets are normalized for MDCs and PDCs only. Data are representative of at least three independent donors.

Table 1

Monoclonal antibodies used in Protocols A and B

Lineage Cocktail 1 (Lin1) antibodies target CD3, CD14, CD16, CD19, CD20 and CD56

Protocol A - Monocyte subsets & B lymphocytes							
Target antigen	Fluorochrome	Antibody clone	Antibody host species & isotype	Manufacturer	Product catalog number*	Laser used	Bandpass filters (nm) / Long-pass dichroic mirror (nm)
CCR2	PE	48607-211	mouse IgG _{2b}	R&D Systems	FAB151P	blue	575/26 / 550
CD11b	APC	ICRF44	mouse IgG ₁	BioLegend	301310	red	660/20 / 505
CD14 ⁺	Pacific Orange	TuK4	mouse IgG _{2a}	Invitrogen	MHCD1430	violet	585/42 / 710
CD16 ⁺	Pacific Blue	3G8	mouse IgG ₁	BioLegend	302021	violet	450/50 / 755
CD19 ⁺	Alexa Fluor 700	S125-C1	mouse IgG ₁	Invitrogen	MHCD1929	red	730/45 / 550
CD62L	APC-Alexa Fluor 750	DREG-56	mouse IgG ₁	Invitrogen	MHCD62L	red	780/60 / 685
CD83	APC	HB15e	mouse IgG ₁	BD Biosciences	551073	red	660/20 / 505
CD86	PE	FUN-1	mouse IgG ₁	BD Biosciences	555658	blue	575/26 / 685
CD209	PerCP-Cy5.5	DCN46	mouse IgG _{2b}	BD Biosciences	558263	red	695/40 / 505
CX ₃ CR1	FITC	2A9-1	rat IgG _{2b}	MBL International	D070-4	blue	530/30 / 505
HLA-DR	FITC	L243	mouse IgG _{2a}	BD Biosciences	307604	blue	530/30 / 685
HLA-DR ⁺	PerCP	L243	mouse IgG _{2a}	BD Biosciences	347364	red	695/40 / 505
BD Multicolor CD83/CD86/CD209	APC/PE/PerCP-Cy5.5	HB15e/FUN-1/DCN46	mouse IgG ₁ /IgG ₁ /IgG _{2b}	BD Biosciences	334098	(see above)	
Protocol B - DC subsets							
Target antigen	Fluorochrome	Antibody clone	Antibody host species & isotype	Manufacturer	Product catalog number*	Laser used	Bandpass filters (nm) / Long-pass dichroic mirror (nm)
CCR2	PE	(see above)					
CD1c	PE	AD5-8E7	mouse IgG _{2a}	Miltenyi Biotec	130-090-508	blue	575/26 / 550
CD11b	Alexa Fluor 700	VIM12	mouse IgG ₁	Invitrogen	CD11b29	red	730/45 / 710
CD11b	APC-Cy7	ICRF44	mouse IgG ₁	BD Biosciences	557754	red	780/60 / 755
CD11c ⁺	APC	S-HCL-3	mouse IgG _{2b}	BD Biosciences	333144	red	660/20 / 755
CD62L	APC-Cy7	DREG-56	mouse IgG ₁	eBioscience	10-0629	red	780/60 / 755
CD101	PE	BB27	mouse IgG ₁	Serotec	MCA2236PE	blue	575/26 / 550
CD123 ⁺	pure**	AC145	mouse IgG _{2a}	Miltenyi Biotec	130-090-940	violet (for Pacific Blue)	450/50 / 550

Protocol B - DC subsets								
Target antigen	Fluorochrome	Antibody clone	Antibody host species & isotype	Manufacturer	Product catalog number*	Laser used	Bandpass filters (nm)	Long-pass dichroic mirror (nm)
CD123 ^{††}	eFluor 450	6H6	mouse IgG ₁	eBioscience	48-1239	violet	450/50	
CD303	APC	AC144	mouse IgG ₁	Miltenyi Biotec	130-090-905	red	660/20	
HLA-DR [‡]	PerCP	(see above)						
Lineage Cocktail 1 (Lin1) ^{‡§}	FITC	multiple	mouse IgG ₁ /IgG _{2b}	BD Biosciences	340546	blue	530/30	505

* Catalog numbers can vary in the USA, the European Union and other countries.

** For conjugation with Pacific Blue using the Invitrogen Zenon Pacific Blue Mouse IgG_{2a} Labeling Kit.

[‡] Anchor markers used in Protocols A and B.

^{††} CD123-eFluor 450 can be a substitute for CD123-Pacific Blue.

Table 2

Troubleshooting table

Step	Problem	Possible reason	Solution
3, 14, 15, 17	Fluorochrome 'spillover' (i.e. non-specific fluorescence detected in a neighboring channel, e.g. false-positive events in the PE channel attributable to FITC fluorescence)	<ol style="list-style-type: none"> i. Improper selection of fluorochrome, or inadequate understanding of antigen and epitope distribution. ii. Improper instrument setup and fluorochrome compensation. iii. Use of tandem dyes (e.g. PE-Cy5). 	<p>Check fluorescence spectrum viewer (http://bdbiosciences.com/spectra or http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-Spectra-Viewer.html). Examine single-color staining profiles and perform 'fluorescence minus one' or FMO36 control stainings to identify the culprit fluorochrome. Knowing the distribution of antigens and epitopes targeted by antibodies, and having a proper understanding of fluorochromes, their selection and instrument setup and operation are crucial for polychromatic flow cytometry^{10,34-39}. Refer also to instrument user's guide provided by the flow cytometer manufacturer.</p>
3B, 17	Dull staining of manually conjugated Pacific Blue antibody (e.g. anti-CD123) using the Invitrogen Zenon labeling kit	<ol style="list-style-type: none"> i. Suboptimal antibody: conjugate ratio. 	<p>Optimize amount of Component A used, and titrate antibody levels. Component B (blocking immunoglobulin) must also be adjusted stoichiometrically (refer to manufacturer's instructions). Alternatively, a Pacific Blue equivalent such as eFluor 450 can be used.</p>
7, 11, 17	Lack of consistency in phenotype readout.	<ol style="list-style-type: none"> i. Donor variation or instability of phenotype. ii. Lack of a consistent time interval between cell fixation and flow cytometric acquisition. iii. Use of non-standardized, non-fresh reagents. 	<p>Allow samples to stabilize in fixative for at least 1 h after immunostaining. Analyze at a fixed time interval (e.g. 16 h after cell fixation). Avoid analysing later than 24 h as fluorescence intensity or tandem dyes might begin to degrade. Use freshly prepared standardized reagents (e.g. BD FACS lysing solution, CellFIX).</p>
7, 17	Odd or variable FSC vs SSC bivariate profiles or large amounts of debris, including dead cells.	<ol style="list-style-type: none"> i. Prolonged incubation (>15 min) with FACS Lysing Solution can alter cellular biophysical properties or surface epitopes. 	<p>Stop incubation promptly at 10 min, and invert tubes less vigorously.</p>
9, 11, 17	Low cell yield.	<ol style="list-style-type: none"> i. Discarding supernatant without care. ii. Rarity of cell population. 	<p>Pipet off supernatant carefully if using microcentrifuge tubes, or pour away supernatant gently when using FACS tubes. Consider scaling up blood volumes and staining reagents.</p>

Step	Problem	Possible reason	Solution
14, 17	<p>Progressive shifting of acquisition events on FSC vs SSC bivariate plot over days and sometimes during a data collection session. This can</p>	<ul style="list-style-type: none"> i. Obstruction of internal tubings that supply or drain flow cell. ii. Fluidics or pressure problems in tubing circuit. 	<p>Ferromagnetic valves are often used to regulate pressures and buffer or waste flow within certain flow cytometers. Use of magnetic bead-based cell sorting and subsequent analysis on such flow cytometers can cause valves to malfunction, leading to increased internal pressure within the flow cell. Discontinue the use of</p>

Table 3

Physical properties and differential expression profile of surface phenotypes on CD16⁺, CD14⁺CD16⁺ and CD14⁺ monocytes (Mo), CD19⁺HLA-DR⁺ B cells, CD11c⁺MDCs, CD123⁺PDCs and CD16^{hi}CX3CR1⁻ neutrophils

Parameters	B lymphocytes				CD16 ^{hi} Neutrophils		
	CD16 ⁺ Mo	CD16 ⁺ CD14 ⁺ Mo	CD14 ⁺⁺ Mo	CD19 ⁺ HLA-DR ⁺			
FSC	low-int	low-int to high-int	high-int	low	low to int	low	high
SSC	int	int	int	low	int	low to int	high
HLA-DR	pos	pos	pos	pos	pos	pos	neg [*]
CX ₃ CR1	high	int	low	neg	n.d.	neg ^{**}	neg
CCR2	neg	int	high	neg	high	int	neg
CD11b	int	int	high	neg to int	low or neg	neg	pos
CD62L	low or neg	low	pos	int	high	high	pos
CD101	pos [†]	pos [†]	pos [†]	pos [†]	pos	neg	pos [†]

FSC, forward scatter; SSC, side scatter.

^{*} Reported positive in specific diseases⁵².

^{**} Reported negative by Geissmann *et al.*²⁰

[†] We (data not shown) and others⁵³ have demonstrated the surface expression of CD101 on these cells by flow cytometry.