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TITLE:**Characterization of Human Monocyte Subsets by Whole Blood Flow Cytometry Analysis****AUTHORS AND AFFILIATIONS:**

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SUMMARY:

Here we present a protocol for characterizing monocyte subsets by whole blood flow cytometry. This includes outlining how to gate the subsets and assess their expression of surface markers and giving an example of the assessment of the expression of M1 (inflammatory) and M2 markers (anti-inflammatory).

ABSTRACT:

Monocytes are key contributors in various inflammatory disorders and alterations to these cells, including their subset proportions and functions, can have pathological significance. An ideal method for examining alterations to monocytes is whole blood flow cytometry as the

minimal handling of samples by this method limits artifactual cell activation. However, many different approaches are taken to gate the monocyte subsets leading to inconsistent identification of the subsets between studies. Here we demonstrate a method using whole blood flow cytometry to identify and characterize human monocyte subsets (classical, intermediate, and non-classical). We outline how to prepare the blood samples for flow cytometry, gate the subsets (ensure contaminating cells have been removed), and determine monocyte subset expression of surface markers – in this example M1 and M2 markers. This protocol can be extended to other studies that require a standard gating method for assessing monocyte subset proportions and monocyte subset expression of other functional markers.

INTRODUCTION:

Monocytes are a type of white blood cells which play a major role in promoting and resolving inflammation. There are three main subsets of monocytes recognized, classical (~85%), intermediate (~5%), and non-classical (~10%) monocytes, which are characterized by their level of cluster of differentiation (CD)14 and CD16 expression¹. The proportions of monocyte subsets can differ with the presence of disease, such as an increased proportion of intermediates in various inflammatory states^{2,3} including cardiovascular disease, where the level of intermediates is associated with clinical events^{4,5}. Furthermore, in disease conditions, monocytes can also undergo functional changes, with many changes detectable by a difference in surface marker expression^{6,7}. One such example is monocyte M1-skewing, an increase in markers associated with M1 macrophages, which has been observed in cardiovascular disease, diabetes, obesity, and metabolic syndrome⁷⁻¹⁰.

Despite the popularity of flow cytometry to assess monocyte subset proportion and function, there is a considerable variability in sample preparation and subset gating between studies which makes it difficult to compare findings between such studies. Importantly, there is no consensus in the demarcation of monocyte subsets, yet a standardized approach is essential given the clinical significance of changes in subset proportions in several diseases. Part of the difficulty in gating arises from the fact that monocytes differentiate from the classical through the intermediate to the non-classical subset¹¹ and as such, monocytes exist as a continuous spectrum rather than distinct populations¹². Interestingly, Zawada *et al.* showed that using either a rectangular or trapezoid gating of the intermediate subset, both resulted in a higher intermediate subset that predicted a cardiovascular endpoint¹³. This highlights that, at least for calculating proportions, the key issue is applying a consistent gating strategy between different samples (and studies), rather than attempting to definitively discriminate between subsets. While definitive gating may be more important when assessing function, the change in marker expression between subsets is incremental^{12,14}, and thus again, consistency in gating is perhaps key. As such, an objective gating method that reproducibly apportions the monocyte subsets between different samples is needed. The purpose of the method presented here is to gate monocyte subsets with a clear explanation and justification for the gating technique employed and assess the subsets for surface marker expression, thus providing a method which allows researchers to have confidence in the use of this technique when assessing different samples.

PROTOCOL:

This study has been approved by the WSLHD Human Research Ethics committee (HREC) (approval AU RED HREC/15/WMEAD/289).

1. Sample Preparation for Whole Blood Flow Cytometry

Note: As human blood is potentially infectious, the sample set-up should be performed in a biohazard hood.

1.1. Collect the blood samples from the participants into 3 mL ethylene diamine tetra acetic acid (EDTA) tubes.

1.2. Determine the white blood cell (WBC) count using a hematology analyzer or hemocytometer.

1.3. Dilute with phosphate buffered saline (PBS) (pH ~7.4) to adjust the concentration to $\sim 5 \times 10^6$ WBC/mL.

1.4. Prepare sufficient master mix for the number of tubes (*e.g.*, for 14 tubes, prepare 16 x master mix) by combining 16 x volumes of 50 μ L blood, 0.75 μ L anti CD14-V450, 0.5 μ L anti CD16-APC, and 0.625 μ L anti HLA-DR-PerCP. Vortex and pipette 51.9 μ L of mix into each tube (**Table 1**).

Note: Antibodies should be titrated to determine optimal staining concentrations for the fluorescent antibodies used.

1.5. Add surface marker (M1 and M2 or isotype control, phycoerythrin (PE) labelled) antibodies (example as per **Table 2**) and PE labelled markers for T cells (CD3), B cells (CD19), neutrophils (CD66b), and natural killer (NK) cells (CD56) (**Table 3**). Vortex and incubate for 30 min, 4 °C in the dark.

Note: Markers for lymphocytes, neutrophils, and NK cells are included only for the validation of the gating method.

1.6. Add 250 μ L of combined red blood cell lysis/WBC fixing solution, vortex gently immediately, and incubate for 10 min in the dark at 4 °C.

1.7. Add 250 μ L of PBS and spin cells down at 260 x g for 10 min at room temperature.

1.8. Remove supernatant, re-suspend cells in 300 μ L of 1% formaldehyde.

Note: Formaldehyde is toxic. Use nitrile gloves and use in fume hood.

1.9. Store at 4 °C protected from light until analysis is performed.

Note: Flow analysis is recommended to be done within 48 h of sample preparation.

2. Flow Cytometry

2.1. Check flow cytometer log to ensure facility staff have performed quality control checks.

Note: To ensure consistency between analyses, instrument quality control and maintaining consistent target fluorescence intensities using control beads are recommended.

2.2. Click on “New experiment”. Select bivariate (scatter) plots by clicking on the icon and use dropdown menus to select axis parameters. Ensure inclusion of a CD16/CD14 plot and a plot displaying a detector (*i.e.*, fluorescence channel) alongside time to monitor the acquisition.

2.3. Insert tube and click “Acquire”. Check the instrument voltage settings ensuring that detector signals are not off scale.

2.4. Observe cells falling in the monocyte gate of the CD14/CD16 plot. Set recording threshold to 5000 events for the classical monocyte gate and click on “Record”.

2.5. Continue to record data for remaining tubes. After data for all tubes has been recorded, export flow data as .fsc files for analysis.

Note: To ensure accuracy, single color compensation controls should be recorded. A compensation matrix can be calculated and applied to the data before analysis to account for spectral spill over^{15,16}.

3. Monocyte Gating

3.1. Open files in the analysis software. Double click tube name and select parameters from the dropdown menus to visualize the cells on a forward scatter area FSC(A)/forward scatter height FSC(H) plot. Create a doublet exclusion gate by clicking on the polygon gate tool icon and enclosing the cells as in (**Figure 1A**).

3.2. Select the gated cells (by double clicking on the gated region) and in the new display box adjust dropdown menu parameters to display the cells on an FSC(A)/side scatter SSC(A) plot. Click on the rectangular gate icon and generously select the monocyte population based on forward and side scatter properties to exclude the majority of lymphocytes, NK cells, and granulocytes (**Figure 1B**).

3.3. Select the gated cells and redisplay on a CD14/CD16 plot, selecting the parameters by using the dropdown menus. Click on the polygon gate to select monocytes based on their characteristic “ γ ” shape (**Figure 1C**).

3.4. Select the gated cells and display the monocytes on a CD16/HLA-DR plot by using the dropdown menus to select parameters. Click on the polygon gate to select the HLA-DR positive cells and exclude any remaining NK cells and neutrophils¹⁷ (**Figure 1D**).

3.5. Select the gated cells and display the HLA-DR positive cells on a CD14/HLA-DR plot using dropdown menus to select parameters. Click on the polygon gate and draw a gate to exclude the HLA-DR high/CD14 low cells (B cells express high levels of HLA-DR but not CD14) (**Figure 1E**).

Note: B cell contamination may occur and therefore should be investigated. If the non-classical population in **Figure 1C** is not distinct from the cells to its left, then contamination is likely. Step 3.5 can be skipped if B cells are not overlapping with non-classical monocytes.

3.6. Select the gated cells and use dropdown menus to display them on a CD16/CD14 plot. From plot options select “Zebra plot” which will enable monocyte subset gates to be drawn to determine subset proportions (**Figure 1F**).

Note: If zebra plot is unavailable on the analysis software, pseudo color (smooth) or contour plot may be suitable.

3.7. Click on the rectangular gate icon and select the classical monocytes by drawing an approximate rectangular gate around the CD14 high/CD16 low, classical monocyte population. Under “Display” select “Show medians” to display the median fluorescence intensity for classical monocytes. Adjust the gate such that the population has an equal distribution from the median on the left and right encompassing all the cells to the left.

3.8. Select the intermediate population by drawing a rectangular gate that encompasses the cells to the right of the classical gate. Adjust the bottom of the gate to exclude the non-classical cells by aligning the gate with the bottom of the concentric circles that are completely within the classical monocyte gate, which ensures that the intermediate subset has a CD14 expression comparable to the main classical population consistent with the current nomenclature.

3.9. Gate the non-classical subset by drawing a rectangular box down from the lower edge of the intermediate subset, selecting all the cells to the bottom of the population (**Figure 1F**).

3.10. Under “Display” select “Show gate frequencies” to determine the percentage of each monocyte subset.

4. Validation of Gating Method

4.1. To determine whether potentially contaminating cell types are effectively gated-out, first identify different cell populations with antibodies against T cells (CD3), B cells (CD19), neutrophils (CD66b), and NK cells (CD56) (**Figure 2**).

Note: Here T cells and neutrophils do not sit close to the “ γ ” shape of monocytes and are gated out in **Figure 1C**.

4.2. Confirm that NK cells are removed in step 3.4 (**Figure 1D**), and B cells are removed in step 3.5 (**Figure 1E**) as shown in **Figure 3**. If NK cells or B cells are not gated-out, re-adjust the gates.

5. Phenotypic Monocyte Marker Expression

5.1. Select cells from each monocyte subset. Alter dropdown parameters to create a histogram for each monocyte subset (**Figure 1F**) displaying each marker and its matching isotype (**Figure 4**).

5.2. Calculate the degree of expression of each marker (median or geometric mean) compared to the respective isotype control.

REPRESENTATIVE RESULTS:

The monocyte gating strategy and flow cytometry analysis used here (**Figure 1**) successfully gated the monocyte subsets and revealed their relative proportions. The proportions (for this sample) were calculated as 88.1% classicals, 4.33% intermediates, and 7.49% non-classicals. These subset gates were not contaminated with B cells, T cells, neutrophils or NK cells, which was confirmed with markers CD19, CD3, CD56, and CD66b, respectively. By assessing the relative position of other populations, it is clear that the T cells and neutrophils fall well outside the monocyte “ γ ” shape on a CD16/CD14 plot (**Figure 2A** and **2D**). However, both the NK cells and B cell populations overlapped with the non-classical monocyte population (**Figure 2B** and **2C**). The steps of the gating strategy (**Figure 1D** and **1E**) were confirmed to exclude the NK cells (**Figure 3A**) and B cells (**Figure 3B**). Although the B cell population included a small portion of non-classical monocytes, the amount was negligible.

Having gated the subsets, the degree to which they expressed different surface markers, M1 (CD64, CD86, and CD120b) and M2 (CD163, CD11b, and CD93) was assessed. The markers showed positive expression compared to their corresponding isotype controls, as seen by the shift of the histograms (**Figure 4**). The median of the markers was greater than that of the isotype controls.

Application of this gating strategy to a blood sample, which was split into four tubes, stained and analyzed separately, yields comparable results between tubes (**Table 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative monocyte gating strategy in whole human blood. **(A)** FSC(A) vs. FSC(H) plot: Gating the cells that have an equal area and height, thus removing clumps (greater FSC(A) relative to FSC(H)) and debris (very low FSC) K= 1000. **(B)** FSC(A) vs. SSC(A) plot: Broad selection of monocytes based on their SSC/FSC properties. **(C)** CD16 vs. CD14 plot: Gating to select monocytes based on their characteristic “ γ ” shape. **(D)** CD16 vs. HLA-DR plot: Gating to select HLA-DR positive cells and remove NK cells. **(E)** CD14 vs. HLA-DR: Gating to exclude the B

cells (HLA-DR high/CD14 low) from the monocytes. **(F)** Selected monocytes redisplayed on CD16 vs. CD14 plot to gate the monocyte subsets. For A-F color represents cell density with blue and green indicating low density, red and orange indicating high density, and orange indicating mid-range density.

Figure 2: Validation of gating strategy by identification of potentially contaminating cells. Potentially contaminating cells are identified by markers **(A)** T cells (CD3), **(B)** B cells (CD19), **(C)** NK cells (CD56), and **(D)** neutrophils (CD66b). The left side panels show identification of each population after gating as per **Figure 1A** and **1B**, with color representing cell density from high (red) to low (blue). The right side panels show each cell population (blue) superimposed on the final monocyte CD16/CD14 plot to reveal proximity of these populations to monocytes (red).

Figure 3: Confirmation that gating steps remove contaminating cells. Heat maps showing the degree of expression from high (red) to low (blue) of **(A)** CD56 and **(B)** CD19. Gating steps successfully exclude cells with high CD56 (NK cells) and high CD19 (B cells).

Figure 4: Monocyte expression of M1 (CD120b) and M2 (CD93) markers. Smoothed histograms of monocyte M1 and M2 marker expression (blue) showing clear shift from the isotype (red) for **(A)** classicals, **(B)** intermediates, and **(C)** non-classicals.

Table 1: Antibodies for whole blood flow and master mix.

Table 2: M1/M2-PE Fluorochrome labeled monoclonal antibodies for whole blood flow.

Table 3: PE Fluorochrome labeled monoclonal antibodies for lymphocytes (T cells, B cells), neutrophils, and NK cells.

Table 4: Monocyte subset proportions from one blood sample stained and analyzed separately.

DISCUSSION:

Whole blood flow cytometry is an ideal approach to study monocytes as the cells are examined in conditions close to their physiological microenvironment providing an insight into their roles in infection and inflammatory conditions. Furthermore, the use of fresh (*i.e.*, unprocessed) blood samples minimizes the alterations or cell transformations that can occur due to storage or handling^{18,19}, such as those known to occur with freeze-thawed monocytes²⁰. Prompt sample preparation is recommended as some markers are upregulated if samples are kept at room temperature prior to processing¹⁹. The optimal concentrations of M1 and M2 markers were determined by titration, and this should be done for any new antibody to limit non-specific binding whilst ensuring that the degree of shift is due to antigen expression and not restricted by lack of antibody. The removal of red blood cells and fixation of white blood cells with lysis solution is an important step in this protocol as the presence of red blood cells can interfere with flow cytometry^{21,22}. Note that while some lysis solutions are compatible with no-wash staining, clearer populations are evident in our hands when a wash step is used.

Correct setup of the flow cytometer is also critical when comparing expression of monocyte markers. We recommend that researchers maintain consistent target fluorescence intensities of control beads and perform quality control on the instrument to be used to provide consistent results across different samples run on different days. In addition to this, isotype controls are used to assist in interpreting any non-specific background signal generated by non-specific antibody binding. Monocytes have high levels of Fc receptors¹¹ and therefore are prone to non-specific binding. Of note, the level of non-specific binding differs for the different subsets, and thus the use of an isotype control becomes important when comparing the degree of marker expression between subsets.

Another important criterion to be considered is the gating steps employed. Some studies suggest that it is crucial to draw a tight gate around the monocyte population in FSC(A)/SSC(A) plot to get rid of most of the non-monocytic CD16 positive cells²³⁻²⁵, but this may lead to loss of some monocytes as non-monocyte cells can overlap with monocytes on FSC/SSC plots²⁶. Rather, to exclude any other blood cells that may contaminate the monocytes, the inclusion of a third monocyte marker in addition to CD14 and CD16, is essential^{26,27}. For this reason, HLA-DR is often used and is ideal as it is not expressed by NK cells or neutrophils^{17,28}. Although lymphocytes (B cells and T cells) may express HLA-DR, they differ from monocytes in respect to CD14 expression. While HLA-DR is an ideal third marker, CD86 has also been recommended^{5,27,29} but was not used here as it is also an M1 marker and thus its degree of expression on monocyte subsets was assessed.

Validation of the gating strategy used is of crucial importance. While NK cells are known to overlap with non-classicals if they are not gated out²⁸, we notice that B cells can also overlap with the non-classicals (**Figure 2B**); whether this is the case in other studies may depend on the fluorochrome choice, instrument configuration, detector sensitivity, or even the disease condition being examined. Here, the overlapping B cells showed high expression of HLA-DR and were not gated out by selection of HLA-DR positive cells in **Figure 1D**. Rather, to remove B cells we used an additional plot of CD14/ HLA-DR, where the B cells separate out from non-classicals due to their higher HLA-DR and low CD14 expression.

There are also many different ways in which the gates for the monocytes themselves have been drawn in the literature; these include quadrants (the subsets are separated by quadrant markers), and rectangular or trapezoid boxes¹³ (with separate boxes drawn for each subset) which furthermore differ in their placement delineating where one subset ends and another starts. These differences likely reflect the fact that monocytes exist as a continuum of cells, differentiating from classical to non-classical, rather than as clearly distinct populations. However, because variations in techniques for identifying the subsets themselves can lead to differences in the calculated monocyte subset proportions, it becomes important that the gating method is reasonably objective, rather than subjective, as this will make the method more robust and reproducible. Some studies use an isotype control for CD16 to determine the border between the classical and intermediate subsets³⁰. On the other hand, to define the separation between intermediates and non-classicals, it has been proposed that the cut-off line may be vertical or oblique, with the choice up to the investigators, the proviso being it should

be reproducible, but a rectangular gate has been recommended to facilitate comparisons between studies^{13,30,31}. Here, increased objectiveness was obtained by plotting the data on a zebra plot and applying objective visual rules, because zebra plots provide an additional visualization cue by mixing color gradient to each equal probability bin over a traditional contour plot. The right border of the classical subset was drawn such that the population was evenly distributed around the population median. The division between the intermediates and non-classicals were also standardized by having the base of the intermediates align with the bottom of the concentric circles within the classical population (*i.e.*, the intermediate population clearly expresses high levels of CD14, as per the standard nomenclature¹).

While some studies have suggested the use of additional markers, such as C-C chemokine receptor type 2 (CCR2) or 6-sulfo LacNAc (SLAN) for obtaining successful enumeration of monocytes and to reveal their clinical significance^{32,33}, in our hands the level of expression of many monocyte functional markers varies widely between individuals¹⁴. Such variation may limit the usefulness of these markers to define subsets based on their expression. Automated computational approaches have also been used to visualize and cluster monocyte subsets including visual interactive stochastic neighbor embedding (viSNE), t-distributed stochastic neighbor embedding (tSNE), or Spanning-tree Progression Analysis of Density-normalized Events (SPADE)^{34,35}, which can provide visual representation of the cells based on the set of multiple markers used. While this has been shown to increase the accuracy of the gating strategy in monocyte subset classification, it is recognized that a drawback is the number of antibodies (and corresponding fluorophore channels) required. Its usefulness will depend on the questions being asked; the extra complexity may not be warranted, for example, in enumeration studies.

Monocytes gated with our technique show proportions in line with the literature and the expression of surface markers by the three subsets can be readily determined. Overall, the technique and methodology explained here provides a standardized and straightforward method of enumerating monocyte subset proportions and assessing surface marker expression, which can be extended to include other markers as well, thereby validating their functional roles in various other conditions.

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DISCLOSURES:

The authors have nothing to disclose.

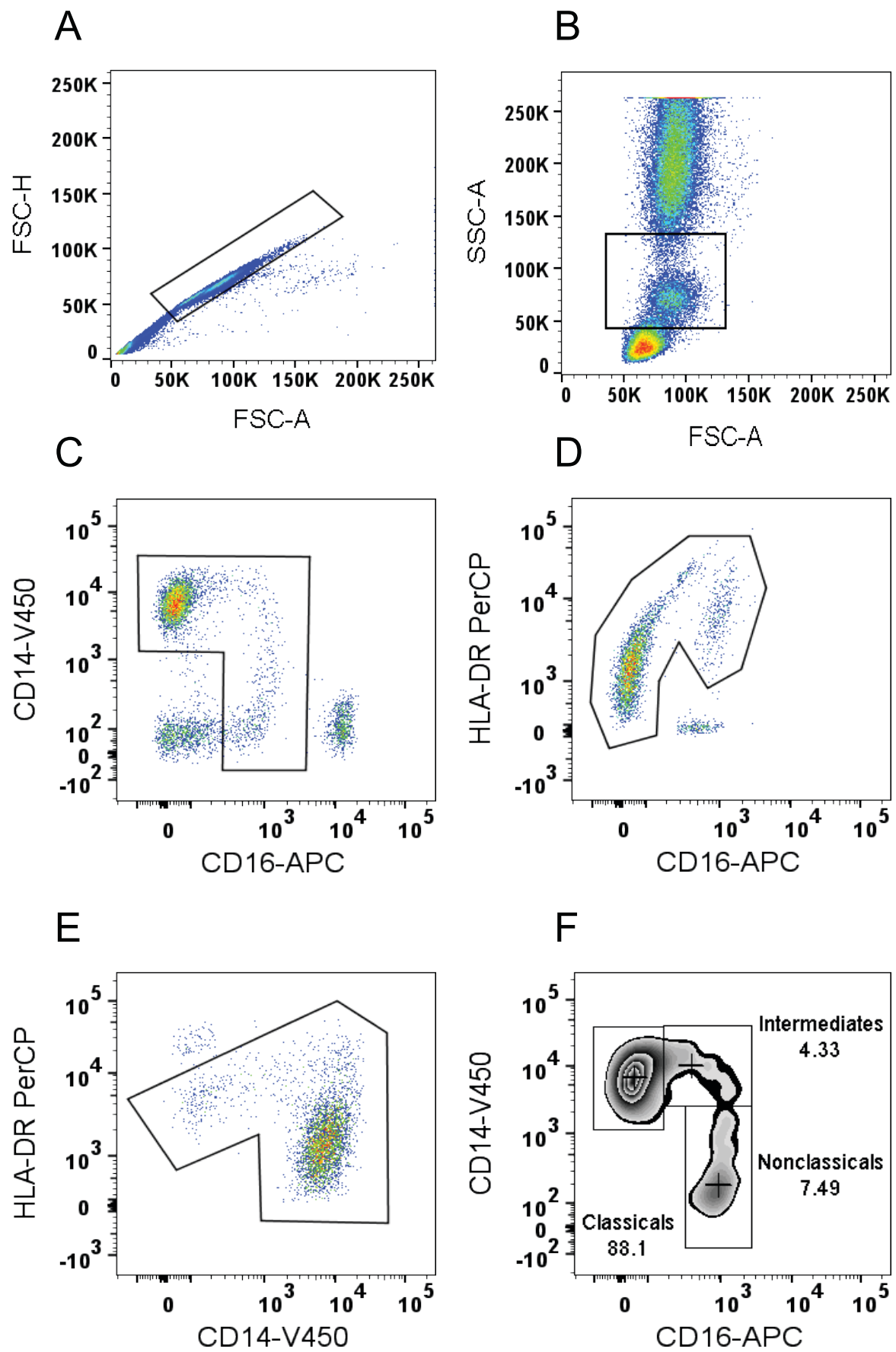
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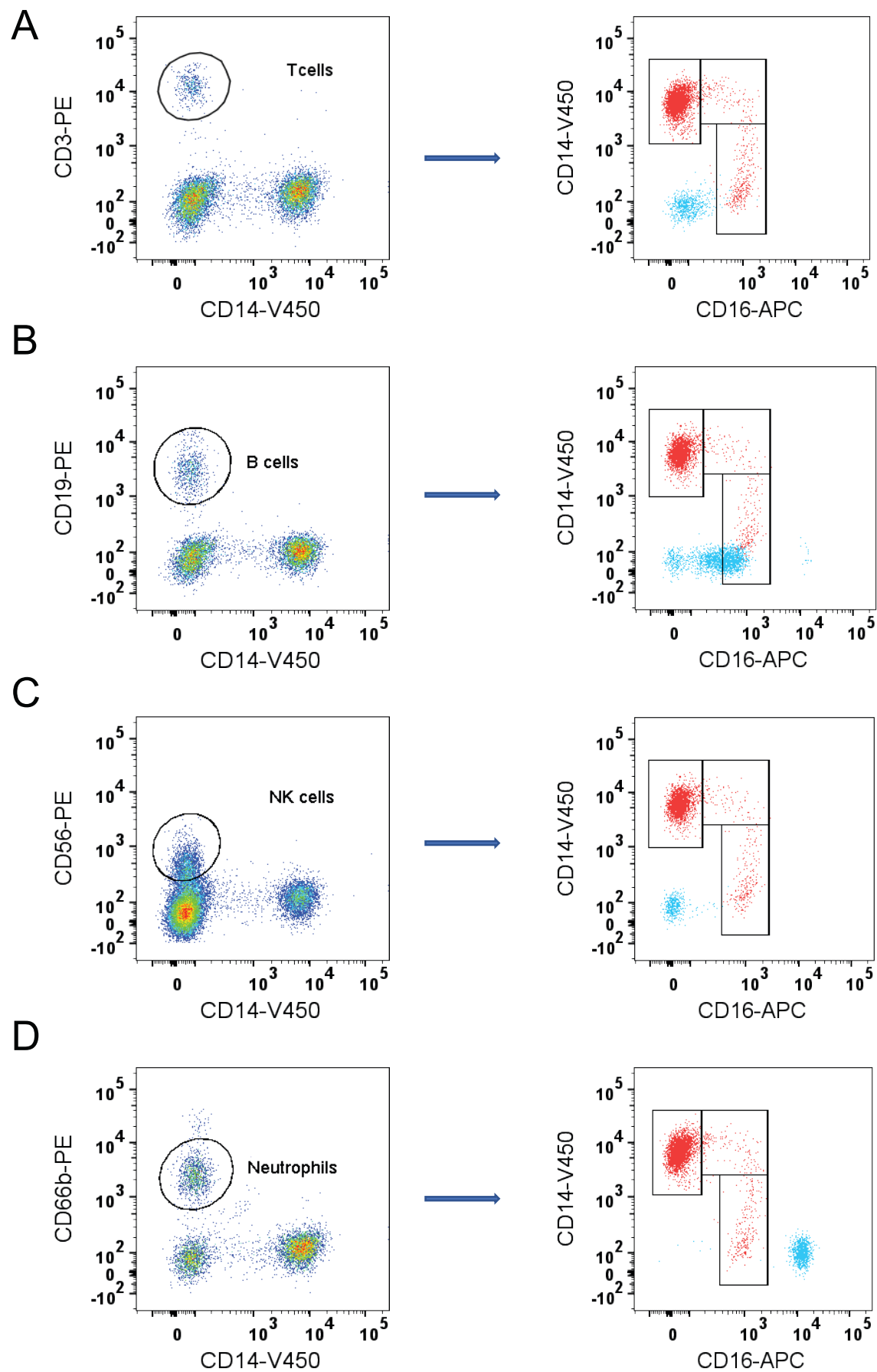
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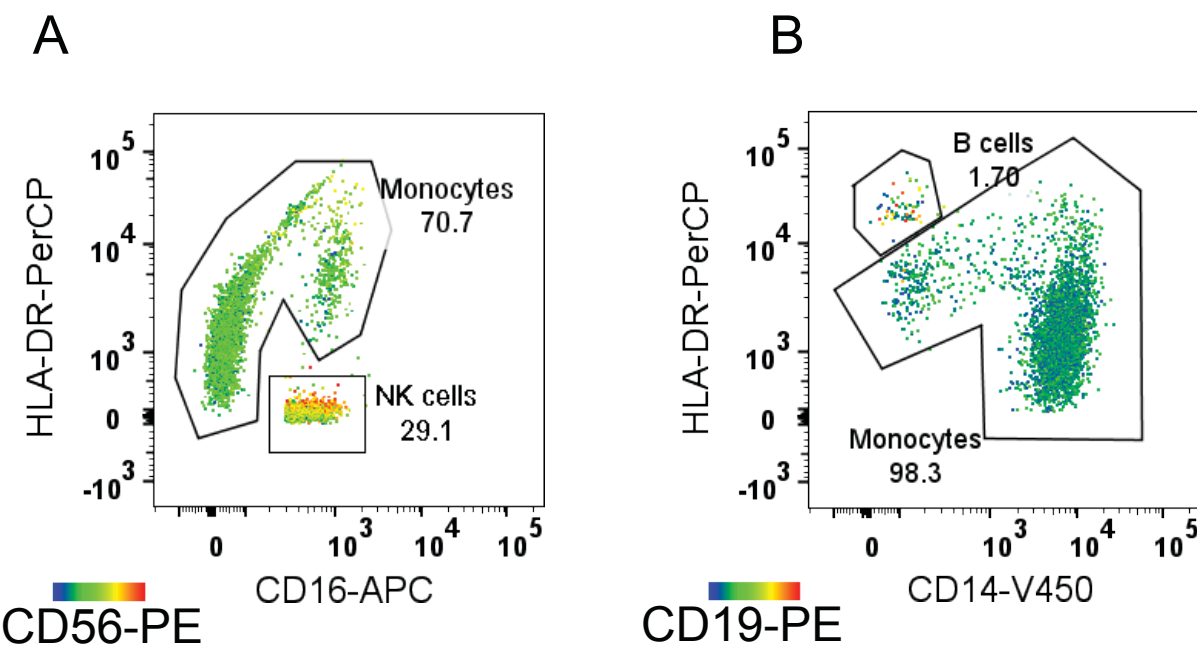
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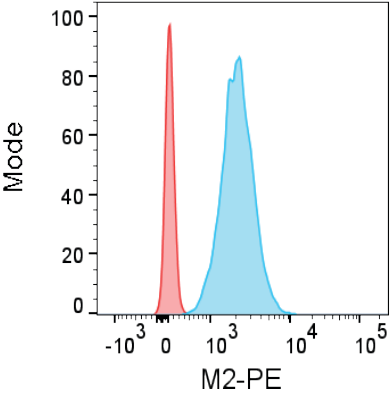
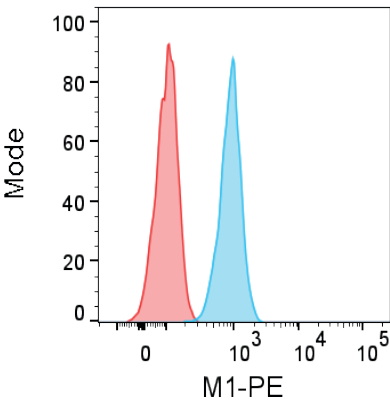




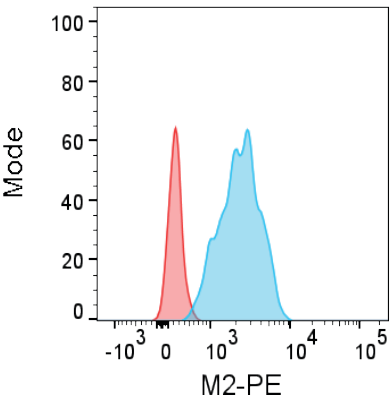
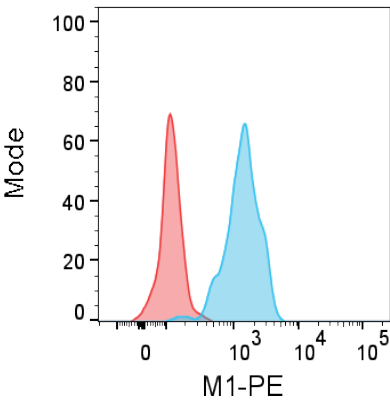
M1 Marker (CD120b-PE)

M2 Marker (CD93-PE)

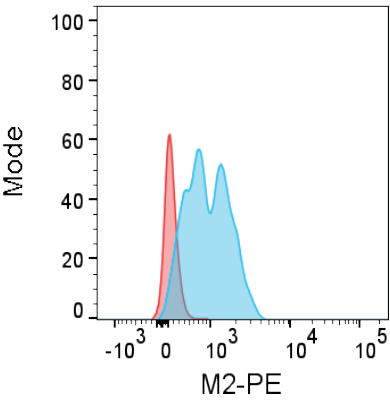
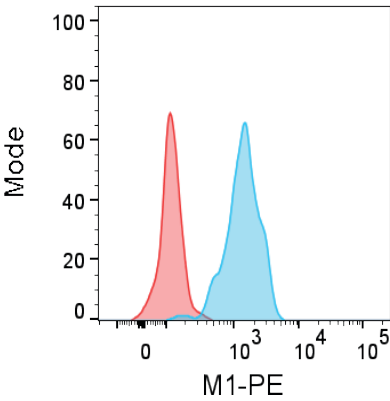
(A) Classical



(B) Intermediate



(C) Non-classical



Antibody	Volume (50 µL blood)	Volume (16x) 800 µL blood
CD14 - V450	0.75 µL	12 µL
CD16 - APC	0.5 µL	8 µL
HLA-DR-PerCP	0.625 µL	10 µL

Tube	PE Antibodies	Code	Isotype Match	Stock Concentration
1	IgG1	BD (555749)	NA	1.0 µg/20 µL
2	CD163	BD (556018)	BD IgG1	0.125 µg/20 µL
3	CD64	BD (558592)	BD IgG1	0.06 µg/20µL
4	IgG1	BD (555749)	NA	1.0 µg/20 µL
5	CD86	BD (555658)	BD IgG1	1.0 µg/20 µL
6	CD11b	BD (561001)	BD IgG1	1.0 µg/20 µL
7	IgG2a	R &D (IC003P)	NA	25 µg/mL
8	CD120b (TNFRII)	R &D (FAB226P)	R &D IgG2a	25 µg/mL
9	IgG1	BL (400112)	NA	0.2 mg/mL
10	CD93	BL (336108)	BL IgG1	50 µg/mL

Working Volume

0.625 µL

5 µL

10 µL

1.25 µL

1.25 µL

1.25 µL

5 µL

5 µL

2.5 µL

1.25 µL

Tube	Antibody	Working Volume
11	CD3	5 µL
12	CD19	5 µL
13	CD66b	1.25 µL
14	CD56	5 µL

Tube	% Classical	% Intermediate	% Non-classical
1	84.3	5.11	10.1
2	84.2	5.05	10.5
3	84.3	5.03	10.7
4	81.6	5.03	12.1

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
BD vacutainer blood collection set	Becton Dickinson	367286	
BD vacutainer K2E 5.5 mg plus blood collection tubes -3.0 ml	Becton Dickinson	7128959	
Phosphatate Buffered Saline (PBS)	Lonza	17-516F	
5ml polystyrene round bottom FACS tube (12 x 75 mm style)	Invitro technologies	352054	
V450 Mouse Anti-Human CD14 Antibody [MφP9]	BD Biosciences	560349	
APC Mouse monoclonal Anti-CD16 Antibody [3G8]	Abcam Australia	ab140477	
Per CP Anti-Human HLA-DR Antibody [L243]	BioLegend	307628	
PE Mouse IgG1 κ Isotype Control [MOPC-21]	BD Biosciences	555749	Lot # 4283901
PE Mouse Anti-Human CD163 Antibody [GHI/61]	BD Biosciences	556018	Lot # 2335626
PE Mouse Anti-Human CD64 Antibody [10.1]	BD Biosciences	558592	Lot # 36768
PE Mouse Anti-Human CD86 Antibody [2331 (FUN-1)]	BD Biosciences	555658	Lot # 3109766
PE Mouse Anti-Human CD11b/Mac-1 Antibody [ICRF44]	BD Biosciences	561001	Lot # 3228959
PE Mouse IgG2A Isotype control [20102]	R&D Systems	IC003P	Lot # 1212031
PE Human TNF RII/TNFRSF1B Antibody [22235]	R&D Systems	FAB226P	Lot # 612051
PE Mouse IgG1, κ Isotype Control [MOPC-21]	BioLegend	400112	Lot # B220359
PE Anti-human CD93 Antibody [VIMD2]	BioLegend	336107	Lot # B143544
PE Mouse Anti-Human CD3 Antibody [SK7]	BD Biosciences	347347	Lot # 3010929
PE Mouse Anti-Human CD19 Antibody [HIB19]	BD Biosciences	561741	Lot # 2307721
PE Mouse Anti-Human CD56 Antibody [B159]	BD Biosciences	561903	Lot # 3011796
PE Anti-human CD66b Antibody [G10F5]	BioLegend	305105	Lot # B154037
OptiLyse C	Beckman Coulter	A11895	
Formaldehyde solution 37%	Sigma	F1635	
BD FACSCanto II Flow Cytometer	BD Biosciences		
Automatic haematology analyser, XT-1800i	Sysmex		
Centrifuge GS-6R	Beckman		
Flowjo software v10.0.7	Tree Star Inc		



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Xiaoyan Cao, Ph.D.
Review Associate
[JoVE](#)

Dear Xiaoyan,

Thank you for providing editorial feedback on our manuscript [JoVE5794 “Characterisation of human monocyte subsets by whole blood flow cytometry analysis”].

Please find below point-by-point responses to each individual Editorial comment (**bolded**), including where revision to the manuscript has been made.

Editorial comments:

1. Tables 2 and 3: Please move the lot numbers of antibodies to Table of Materials.

Lot number columns have been removed. Lot numbers have been added to comments column in Table of Materials

2. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please refer to specific comments in the attached manuscript.

We had initially avoided explicitly stating steps in case they varied between programs. We have added more detail which should translate well, if not perfectly, across different software.

3. Please also address specific comments marked in the attached manuscript.

These have been added as tracked changes on the manuscript to show responses.

We look forward to hearing from you regarding our submission. While we feel we have addressed the comments, we would be glad to respond to any further comments you may have.

Kind regards,

Helen Williams