

Multiparametric analysis of myeloid populations by flow cytometry

Running head: Myeloid populations by flow cytometric analysis

Sara A. Mathie, Alastair L. Corbin, Hayley L. Eames, Irina A. Udalova

Corresponding author: Irina Udalova; Sara Mathie, Kennedy Institute of Rheumatology, Oxford University, Oxford, United Kingdom, irina.udalova@kennedy.ox.ac.uk; sara.mathie@kennedy.ox.ac.uk

Abstract

Flow cytometry is extensively used for the immune-profiling of leukocytes in tissue during homeostasis and inflammation. The multi-parametric power of using fluorescently conjugated antibodies for specific surface and activation markers provides a comprehensive profile of immune cells. This chapter describes the identification and characterisation of myeloid populations using flow cytometric analysis in an acute model of resolving inflammation. This model allows the examination of heterogenic populations across different systemic and tissue locations. We describe tissue processing, antibody staining, and analysis, which includes a newly described viSNE tool to generate two-dimensional clustering within myeloid populations. We also reference the use of transgenic reporter mice on specific myeloid cells that provides enhanced specificity and profiling when defining myeloid heterogeneity.

Key words; cellular heterogeneity, myeloid, monocytes, macrophages neutrophils, flow cytometry, transgenic reporter mice, viSNE

1. Introduction

Myeloid cell populations consisting of neutrophils, macrophages, monocytes, predominate many chronic inflammatory conditions. The prevalence of these cells in a range of chronic disease is well described. Macrophages are distributed throughout the body in various organs, tissue, and fluids and provide an effective first line defense [1-3]. Upon invasion with pathogen or injury, they can send signals for the recruitment of other immunologic cells. Macrophages display heterogeneity of phenotype and can adapt to their local environment [4, 5]. Different subsets of monocytes exist and can mature into macrophages in tissue [6]. Inflammatory monocytes and patrolling monocytes are distinct in their Ly6C expression, hi and lo, respectively. However, other cells express Ly6C, therefore defining monocytes subsets requires further discrimination. Permutations in the expression of the markers: CD11b, Ly6C, F4/80, CD64, CX3CR1, MHCII can separate monocyte subsets and tissue macrophages. CX3CR1 is a marker of tissue monocytes and macrophages and negatively correlates with Ly6C.

Of recent, it has been established that at homeostasis tissue macrophage populations are often represented by a mix of embryonically derived macrophages seeded during embryonic development, and capable of maintaining themselves through self-renewal [7, 8] and monocyte-derived macrophages entering the tissue in the adult animals from the blood. The proportion of embryonically derived and monocyte-derived macrophages varies between the tissues, with microglia macrophages being exclusively embryonically derived and colon macrophages being exclusively monocyte-derived after weaning [7, 9, 10]. In the tissue, monocyte adaptation to the tissue environment and transition into macrophages adds further complexity to the cell populations. This has large implications on how we study and interpret monocytes and macrophages function and phenotype, during both homeostasis and disease.

It is now emerging that this heterogeneity observed in monocytes and macrophages may be mirrored in neutrophil populations. Neutrophils display distinct phenotypes depending on location and may demonstrate heterogeneity between organs [11].

We have developed a basic flow cytometric panel to identify and phenotype myeloid subsets in blood and tissue. This panel assesses myeloid populations through recruitment from the blood, to migration and activation in the tissue. We can examine and characterize the myeloid populations consisting of monocytes, macrophage, and neutrophils which predominate this response [12, 13]. In this chapter, we utilize a 6 day air-pouch inflammatory model. This acute resolving inflammatory system mimics the synovium environment of the knee joint. [12]. The same panel is applicable and forms the basis, for the analysis of more complex models of disease in our laboratory, such as rheumatoid arthritis, obesity, and colitis [14, 15]. We can examine heterogeneity amongst myeloid subsets across different systemic and tissue compartments. This chapter describes methods for tissue processing, antibody staining and flow cytometry analysis, including viSNE, a tool that permits the mapping of high-dimensional cytometry data onto two dimensions, to examine and characterize myeloid populations in air pouch model of inflammation [16]. We also reference studies with transgenic reporter mice for myeloid markers that and can be incorporated into our flow panel and demonstrate improved cellular specificity.

Materials

All RPMI supplemented with L-glutamate. Dubecco's cell culture phosphate buffered saline (PBS) buffer used is free from magnesium and calcium. FACS buffer: Dubecco's cell culture PBS with 1% FCS and 0.01% NaN₃. ACK (Ammonium-Chloride-Potassium) lysing buffer used for red blood cell lysis. Falcon tubes are all conical bottom.

2.1 Blood preparation

1. Ethylenediaminetetraacetic acid (EDTA) 0.5mM.

2. 1mL luer slip syringe with 27 gauge needle.
3. 1.5mL Eppendorf tube.
4. ACK red blood cell lysis buffer.
5. FACS buffer.

2.2 Air pouch lavage and dissection of air pouch membrane (Note 1)

1. Curved forceps.
2. Fine scissors.
3. Sterile cold PBS.
4. 5 mL luer slip syringes.
5. 15 mL Falcon tube.
6. 70 microM cell strainer.
7. DNase1 grade II, from bovine pancreas.
8. Liberase™ TL Research Grade, low Thermolysin concentration.
9. RPMI medium.
10. FACS buffer.

2.3 Flow cytometric analysis

1. FACS buffer.
2. 15 mL Falcon tube.
3. 96 Well Clear Round Bottom TC-Treated Microplate.
4. 5 mL FACS round bottom polystyrene test tube.
5. Purified rat anti-mouse CD16/CD32 (Mouse BD Fc-Block™) (**Note 2**)
6. Anti-mouse fluorescently labelled antibodies: CD45, CD11b, Ly6G, SiglecF, Ly6C, F4/80, MHCII, CD11c, CD206, CD62L, live/dead stain, (**Note 3**)

7. LIVE/DEAD™ Fixable Far Red Dead Cell Stain Kit, Thermofisher Scientific
8. Anti-rat/hamster calibration bead set (**Note 4**).
9. Brilliant violet stain buffer.
10. Cytofix™ fixation buffer.
11. Flow cytometer with 4 lasers: Blue 488 nm, Yellow-Green 561 nm, Red 633 nm and Violet 405 nm.

3. Methods

3.1 Blood preparation

1. Coat syringe and needle for blood collection with EDTA. Insert syringe into 200uL EDTA and push up and down. Dispense EDTA into Eppendorf tube.
2. Collect blood, remove the needle and dispense into Eppendorf tube containing EDTA. Invert and keep on ice or at 4°C (**Note 5**).
3. Pipette 10mL ACK buffer to 15 mL Falcon tube, then with 1mL pipette transfer blood + EDTA mix from Eppendorf slowly into ACK buffer, leave for 10 mins.
4. Spin 500g, 5 mins with the brake on.
5. To wash tip off supernatant, add 10mL PBS and spin as step 4. Check cell pellet is free from red blood cells (RBC) (**Note 6**).
6. Discard supernatant and resuspend cell pellet in 1mL FACS buffer.
7. Count cell suspension and adjust the concentration to $3-5 \times 10^6$ cells/mL.

3.2 Air pouch lavage and dissection of air pouch membrane

3.2.1 Air pouch lavage

1. Inject 3mL cold PBS into dorsal air pouch ensuring needle passes through skin and into pouch space (**Note 7**).
2. Place mouse on the side, gently massage pouch.

3. Carefully hold skin around pouch and snip a small incision into the epidermis to create a “window” and reveal intact membrane.
4. Gently snip into the membrane.
5. Carefully pinching membrane, use 5mL syringe (without needle) to lavage out injected PBS (**Note 8**).
6. Dispense into chilled 15mL Falcon tube (**Note 9**).
7. Spin Falcon tube at 500g, 5mins, 4°C with the brake on (**Note 10**).
8. Resuspend cell pellet in 1 mL FACS buffer.
9. Count cell suspension and adjust concentration to $3-5 \times 10^6$ cells/mL.

3.2.2 Dissection and digestion of air pouch membrane

1. To collect membrane, hold visible membrane near incision with forceps, snip carefully under the epidermis to separate the membrane from overlying soft tissue. Collect in pre-weighed 15mL Falcon tube containing 1mL of serum-free RPMI. (**Note 11**).
2. Add 1mL of RPMI containing 10 μ g DNase and 2.5 μ g Liberase, place in water bath or shaker for 1hr @ 37°C.
3. After 1 hr incubation, pour membrane digest mix through a 70 microM sieve into a 50mL Falcon tube (**Note 12**).
4. With the flat end of a 1 mL syringe plunger gently massage membrane to release any leukocytes through sieve. Wash through with RPMI.
5. Transfer into 15 mL Falcon tube and spin 500g, 5 mins, 4°C.
6. Discard supernatant, check no RBC contamination. Add 10 mL PBS, and spin as step 5.
7. Resuspend cell pellet in FACS buffer.
8. Count cell suspension and adjust concentration to $3-5 \times 10^6$ cells/mL.

3.3 Flow antibody staining

1. Plate 100µL cell suspension prepared from blood, membrane and lavage and diluted in FACS buffer in well of the round bottom 96-well plate.
2. Add 100µL cold FACS buffer to each well and centrifuge 4 mins at 500g, 4°C with the brake on. Discard supernatant.
3. Dilute Fc-block in FACS buffer 1:100 and add 30µL per well. Incubate for 15 mins at 4°C.
4. Without washing, add 30µl antibodies diluted in FACS buffer. Vortex plate gently and incubate for 20 mins at 4°C in the dark. (**Note 13**).
5. To wash, add 150µL FACS buffer, spin as in step 3. Discard supernatant and repeat step 5.
6. After 2nd wash discard supernatant and fix with 50µL Cytofix™ for 20 mins, resuspend cells gently.
7. Wash x 2 in FACS buffer as step 5, resuspend in 150µL FACS buffer.
8. Create a single-colour compensation tube for each fluorochrome: In a 5 mL FACS tube, add 300µL FACS buffer +10µL negative bead and 10µL anti-Rat/hamster Ig bead + 4µL of single fluorochrome antibody. Vortex and incubate in the dark for at least 15 mins (**Note 14**).

3.4 Flow cytometric analysis – Spectral compensation and gating

1. Using unstained samples, set forward and side scatter, so that myeloid populations are clearly identifiable, central on the plot, Figure 1B.
2. Use unstained sample to set voltage values to define the negative gate. Using a spare stained sample, verify positive events are distinguished from negative gate for each fluorochrome. Adjust voltage values so that the maximal fluorescent intensity is not off the scale.
3. Run each tube containing compensation beads separately on the flow cytometer. Gate on singlet population based on forward and side scatter, Figure 1C. Collect and save 5000 events. Follow BD FACS Diva software instructions to calculate spectral compensation.

4. Start running samples. Collect around 50,000 events from sample wells. The gating strategy for selecting CD11b⁺ leukocytes is depicted in Figure 2 (**Note 15**).
5. We have also analyzed cells taken from the air pouch model carried out in transgenic reporter mice with Ly6G^{tdTom} and Cx3CR1^{GFP}, incorporated into this basic panel. (**Note 16**).
6. Analysis carried out using FlowJo (Treestar) software program. FSC files can be run through viSNE (Cytobank) software (**Note 17**).

4. Notes

1. Mice are given a subcutaneous injection of 3 mL air on Day 0, on Day 3 the pouch is topped up with injection of 3 mL of air. On day 6, the pouch was injected with 1 mg zymosan. Blood, membrane, and exudate are taken at 4 hours after challenge.
2. This antibody reacts specifically with extracellular domains of the mouse Fc γ III and Fc γ II receptor to block non-specific binding of antigens. We find this antibody gives clean and consistent blocking compared to whole serum alternatives.
3. Our panel consists of antibodies conjugated with fluorochromes selected for optimal detection of each antigen: We matched bright fluorochromes with low-density antigen and vice versa. To minimize spillover we spread multiple markers on the same cells type over different laser lines. Fixable live dead marker Far-Red dye in APC-Cy7 (Thermofisher), allows for intracellular detection of cytokines and transcription factors. Table of antibody panel, Figure 1A. For gating strategies for we also create fluorescent-minus-one (FMO) controls which we use to confirm gating of these myeloid cell types. This is the full panel of antibodies except one. FMO controls are used to determine data spread effects induced by high spectral compensation values and prevent overestimation of populations.
4. In our protocol we use BD™ CompBead calibration beads for compensation. This saves on precious samples, especially for membrane where we have a low cell yield.

5. Blood is ready to process after a minimum of 30 mins.
6. If red blood cell lysis is required a second time, repeat from step 3 and incubate for 5 mins in ACK buffer.
7. Mice are terminated following CO₂ asphyxiation and exsanguination.
8. Using round ended forceps hold the skin and soft tissue to prevent any loss of PBS wash. Carefully insert the 5mL syringe into incision in membrane and lavage.
9. Take a note of the volume retrieved; this can vary between mice and used to normalize cells/mL.
10. The supernatant can be kept at -80°C for analysis of cellular mediators, such as cytokines, chemokines, and lipids.
11. Following lavage, pouch will be deflated, grab exposed membrane, and with scissors snip around. As it becomes detached from epidermis more membrane should be made available, gently continue snipping until collected. Record weight using pre-weighed tubes, between 50-75µg is expected membrane retrieved. The amount of membrane tissue retrieved may vary. Membrane weight used to normalize cells count reported as cells/mg tissue.
12. Tip contents of membrane into sieve, gently push membrane with end 1 mL syringe, rinse through with RPMI media, transfer into 15 mL Falcon tube to spin. Approximately 5x10⁵ cells are expected back from the membrane, 15 mL Falcon tube allows more compact pellet for a small number of cells.
13. When using more than one BD Horizon bright violet dye in a panel, there may be fluorescent interference. Using BD Horizon™ Brilliant Stain buffer 1:10 in FACS buffer will prevent any staining artefacts when using these antibodies conjugated with brilliant violet dyes.
14. For each single control to set spectralcompensation use the conjugated antibody used for the panel. To preserve antibody volume is effective at 1µL.

15. Singlets (R1) are selected based on the linear correlation between FSC-A vs. FSC-H. From this population events negative for live/dead marker are selected (R2). These live leukocytes are then gated for CD45⁺ (R3) and CD11b⁺ (R4) expression. FMO control samples are a useful reference at this stage. Gating CD11b⁺ selected events against Ly6G and SiglecF separate leukocytes into three broad populations: neutrophils (R5), eosinophils (R6) and monocytes and macrophage containing gate (R7) (Figure 2B). From gate R5, we calculate the mean fluorescent intensity expression of CD62L to determine activation status of neutrophils. CD62L selectin is highly expressed on blood neutrophils. Levels are down-regulated upon activation and expression of CD62L is decreased on neutrophil populations found in membrane and exudate. Drilling down from gate R7 mono/macrophages gate to Ly6C vs. MHCII there are 3 distinct populations: inflammatory monocytes, Ly6C^{hi} MHCII⁻; macrophages Ly6C⁻ MHCII⁺; a gate containing Ly6C⁻ patrolling lymphocytes (Figure 3B). From the R7 gate, the Ly6C⁻ MHCII⁺ population predominantly express F4/80 in tissue and exudate compartments. (Figure 3C). More in-depth analysis of monocyte and macrophage populations can be determined using CD64, CD206, CD11c depending on the tissue or disease of interest: CD64 is expressed on F4/80⁺ macrophage populations from R7. However, in more complex tissue settings, particularly in the gut, CD64-antigen is very useful at delineating resident from recently replenished and differentiated macrophages. CD206 is considered a classic marker of M2 macrophages, however, it is expressed on most tissue macrophages and may be best used for expression rather than for specifically identifying M2 macrophages population. CD11c may be used to define dendritic cell subsets from R7 gate (gating not shown) [15].
16. The development of transgenic reporter mice expression fluorescence protein on a range of myeloid specific markers is providing a popular approach to analyzing and sorting cells from tissue, using fluorescently acquired cell sorting (FACS) [5, 7]. Transgenic (tg) fluorescent

Ly6G reporter gene mice give a clear strategy for identifying and isolating neutrophils with minimum handling and activation from antibodies [11]. Transgenic fluorescent CX3CR1 reporter gene mice allow for immune-phenotyping of monocytes and macrophages. CX3CR1 is highly expressed on macrophages and expression is low inflammatory monocytes [7]. This panel can be applied to cells that express *Ly6GtdTom* and *CX3CR1gfp* by swapping CD62L and CD206 to APC.

17. FSC files saved from FACS can be run through viSNE to give a two-dimensional picture and depict cellular clustering of markers. By using all markers simultaneously, viSNE achieves a more accurate grouping of myeloid subsets. We show example files of murine colon taken from flow cytometry studies in our laboratory. Heatmaps depict levels of expression of MHC II, Ly6C, CD11c, and F4/80 and display cell clusters and changes in marker expression levels between healthy and disease monocytes/macrophages (Figure 4). viSNE is available for download at: <http://www.c2b2.columbia.edu/danapeerlab/html/index.html>

Acknowledgements

This work is supported by Arthritis Research United Kingdom and Nova Nordisk Foundation.

5. References

1. Wynn TA, Chawla A, Pollard JW (2013) Macrophage biology in development, homeostasis and disease. *Nature* 496 (7446): 445-455.
2. Franken L, Schiwon M, Kurts C (2016) Macrophages: sentinels and regulators of the immune system. *Cell Microbiol* 18(4): 475-487.
3. Byrne AJ, Mathie SA, Gregory LG, Lloyd CM (2015) Pulmonary macrophages: key players in the innate defence of the airways. *Thorax*, 70(12): 1189-1196.
4. Udalova IA, Mantovani A, Feldmann M (2016) Macrophage heterogeneity in the context of rheumatoid arthritis. *Nat Rev Rheumatol* 12(8): p. 472-485.
5. Kierdorf K., Prinz M, Geissmann F, Gomez perdiguero E (2015) Development and function of tissue resident macrophages in mice. *Semin Immunol* 27(6): 369-378.

6. Geissmann F, Jung S, Littman DR (2003) Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19(1): 71-82.
7. Yona S, Kim KW, Wolf Y, Mildner A et al. (2013) Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 38(1): 79-91.
8. Hashimoto D, Chow A, Noizat C et al. (2013) Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* 38(4): 792-804.
9. Bain CC, Bravo-Blas A, Scott CL et al. (2014) Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Immunity* 39(5): 929-937.
10. Sieweke MH and Allen JE (2013) Beyond stem cells: self-renewal of differentiated macrophages. *Science* 342(6161): 1242974.
11. Kolaczkowska E. and Kubes P (2013) Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 13(3): 159-175.
12. Edwards JCW, Sedgwick AD, Willoughby DA (1981) The formation of a structure with the features of synovial lining by subcutaneous injection of air: An in vivo tissue culture system. *J Pathol* 134(2): 147-156.
13. Pessler F, Mayer CT, Jung SM et al. (2008) Identification of novel monosodium urate crystal regulated mRNAs by transcript profiling of dissected murine air pouch membranes. *Arthritis Res Ther* 10(3): p. R64. doi: 10.1186/ar2435.
14. Dalmas E, Toubal A, Alzaid E et al. (2015) Irf5 deficiency in macrophages promotes beneficial adipose tissue expansion and insulin sensitivity during obesity. *Cell* 161(6): 610-618.
15. Weiss M, Byrne AJ, Blazek K et al. (2015) IRF5 controls both acute and chronic inflammation. *Proc Natl Acad Sci U S A* 112(35): 11001-11006.
16. Amir el-AD, Davis KL, Tadmor MD et al. (2013) viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nature Biotechnol* 31(6): 545-552.

Figure 1: Compensation and analysis set up. **A.** List of fluorescently labeled antibodies for the FACS panel described in this chapter. **B.** Forward (FSC-A) and side scatter (SSC-A) for blood, membrane and exudate samples. **C.** Singlet selection for compensation bead set.

Figure 2: Flow cytometric analysis of the air pouch model. **A.** Gating strategy for CD11b⁺ leukocytes in the blood, tissue, and exudate. **B.** Gating of major myeloid populations in the blood, membrane tissue and exudate: neutrophils, eosinophils, and monocyte/macrophage populations

Figure 3: Monocyte and macrophage sub-populations. **A.** Gating strategy for major myeloid cells. **B.** CD11b⁺ Ly6G⁻ SiglecF⁻ monocytes/macrophage populations gated for Ly6C and MHCII: Inflammatory monocytes depicted Ly6C^{hi} and MHCII⁻; patrolling lymphocytes containing gate Ly6C⁻ MHCII⁻; Ly6C⁻ MHCII⁺ macrophages. **C.** F4/80 expression on Ly6C⁻ MHCII⁺ populations.

Figure 4: viSNE heatmaps of monocyte macrophage populations. Heat map depicting the level of expression and clustering within gate R7, CD45⁺CD11b⁺Ly6G⁻SiglecF⁻ population in the normal and inflamed murine colon.