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Isolation and Characterization of Mononuclear Phagocytes in the Mouse Lung and Lymph Nodes

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Abstract

There is a diverse population of mononuclear phagocytes (MPs) in the lungs, comprised of macrophages, dendritic cells (DCs), and monocytes. The existence of these various cell types suggests that there is a clear division of labor and delicate balance between the MPs under steady-state and inflammatory conditions. Here we describe how to identify pulmonary MPs using flow cytometry and how to isolate them via cell sorting. In steady-state conditions, murine lungs contain a uniform population of alveolar macrophages (AMs), three distinct interstitial macrophage (IM) populations, three DC subtypes, and a small number of tissue-trafficking monocytes. During an inflammatory response, the monocyte population is more abundant and complex since it acquires either macrophage-like or DC-like features. All in all, studying how these cell types interact with each other, structural cells, and other leukocytes within the environment will be important to understanding their role in maintaining homeostasis and during the development of disease.

Keywords

Macrophage; Monocyte; Dendritic cell; Mononuclear phagocyte; Lung; Flow cytometry; Pulmonary; Interstitial

1 Introduction

The lung consists of three anatomic compartments. These include the airway lumen, the vasculature, and the space in between known as the interstitium. In the lower airways, alveolar macrophages serve as a first line of defense against invading pathogens [1]. If alveolar macrophages and incoming neutrophils are incapable of containing the pathogens, then an adaptive immune response is initiated to assist in pathogen clearance [2]. The airways and alveoli are bounded by epithelial cells, which form a protective barrier between the ambient environment and the interstitium. Underneath the epithelial barrier are dendritic cells (DCs), interstitial macrophages (IMs), and tissue monocytes [3–5].

[4]Notes

DCs link innate and adaptive immunity by acquiring, processing, and trafficking foreign and self-antigens to the draining lymph nodes (LNs), where they present peptides on MHC molecules and activate cognate T cells [6–10]. A clear division of labor exists between the two overaching DC subtypes, known as classical DC1 (cDC1) and classical DC2 (cDC2). CDC2 can be divided into two additional subtypes: an IRF4, KLF4-dependent DC2 and an IRF4, Notch2-dependent DC2 [9, 11–22]. Interstitial macrophages appear to be most densely located in the bronchovascular bundles. Within the IM population, we have recently identified three unique subtypes [23]. Similar populations exist in the stroma of other organs; however their precise functions remain unclear. Lastly, during inflammation, monocytes can differentiate and assume either macrophage-like or DC-like properties. For example, during acute lung injury monocytes can replenish tissue-resident macrophages if a niche is open, or they can differentiate into inflammatory or "recruited" macrophages (different from tissue-resident macrophages), thus contributing to wound repair and clearance of cellular debris and microbes [24–27]. Alternatively, monocytes can differentiate into a DC-like cell, migrate to the draining LNs, and induce adaptive immunity [23, 28–30].

For decades, monocytes have been viewed as precursors to tissue-resident macrophages. However, we now know that monocytes continuously traffic through nonlymphoid and lymphoid tissue without becoming bona fide long-lived, self-renewing macrophages or classical dendritic cells [31]. Most interestingly, in both mice and humans, extravascular lung and LN monocytes are as abundant as DCs in the steady state and are even more abundant during inflammation [28, 31–33]. The role of monocytes play in adaptive immunity is underappreciated, and although it has been shown that they can induce CD4 and CD8 T cell proliferation, how they preferentially activate lymphocytes and under what conditions is unclear [28]. In this chapter, we describe methods to identify and isolate murine pulmonary MPs from whole lungs and lung-draining LNs.

2 Materials

2.1 Antibody Clones Used for Staining

The following antibody clones (Table 1) are the ones most commonly used in our laboratory. In our hands they give reliable results; however similar antibodies can be purchased from eBioscience, BD Pharmingen, BioLegend, or other sources. Alternative fluorescent conjugates can also be used.

2.2 Intravenous Injection of Anti-CD45 Antibody

1. Fluorochrome-conjugated anti-CD45 antibody. Fluorochromes such as FITC or PE provide the best results in our experience. In addition, the use of the correct congenic anti-CD45, specifically anti-CD45.1 or anti-CD45.2, also provides better results than general anti-CD45.

2. 1× PBS without calcium and magnesium.

3. 1 mL syringe and 27-gauge needle for tail vein or retro-orbital (requires experience for consistency) injections.

4. Heat lamp and mouse restrainer.

2.3 Single-Cell Suspension of BAL, Lung, and Lung-Draining Lymph Node

1. Scissors.

2. Blunt-curve and fine-point straight-tip forceps.

3. Needles: 18-gauge and 26-gauge.

4. Syringes: 1 mL, 3 mL, and 30 mL.

5. 35 mm × 10 mm round culture dishes or 12-well tissue culture plate.

6. FACS tubes.

7. Case of glass Pasteur pipettes and a rubber bulb.

8. Buffers: 1× PBS without calcium and magnesium, 1× Hank's buffered salt solution (HBSS) without calcium and magnesium.

9. Stock solution of 0.5 M EDTA pH 8.0.

10. EDTA 100 mM, pH 8.0 stored at room temperature.

11. Hank's buffered salt solution complete buffer (HBSS complete): In a 500 mL bottle of HBSS without calcium and magnesium, add 1 mL 30% BSA and 300 μL 0.5 M EDTA.

12. For tissue digestion, make 2 mg/mL stock solution of Liberase TM (Roche) and a 10× stock solution of collagenase D in RPMI, 37.5 mL of RPMI with 1 g of collagenase D (Roche). For lung digestion, one could either use 400 μg/mL of Liberase TM or 2× collagenase D in RPMI. For LN digestion, only use 1× collagenase D and not Liberase TM.

2.4 Fluorescence-Activated Cell Sorting (FACS) Stainingfor Single-Cell Suspension of BAL, Lung, and Lung-Draining Lymph Node

1. HBSS complete and FACS tubes.

2. Centrifuge and FACS machine.

3 Method

3.1 Intravenous Injection of Anti-CD45 Antibody to Eliminate Contaminating Intravascular Leukocytes from Extravascular Leukocytes in Lung Analysis

The lungs contain a vast network of pulmonary blood vessels through which leukocytes constantly traffic. Although vascular perfusion can help eliminate some leukocytes from the lung vasculature, a high number will always remain. In order to distinguish intravascular leukocytes from the leukocytes that reside in the lung tissue, anti-CD45 antibodies can be given intravenously.

1. Dilute 5 μL of anti-CD45 antibody in 200 μL of 1× PBS.

2. Place mouse under heat lamp to dilate tail veins.

3. Inject prepared antibody solution into the tail vein 4–5 min before sacrifice.

3.2 Single-Cell Suspension of Airway Cellsfrom Bronchoalveolar Lavage (BAL)

1. Euthanize the mouse in a CO_2 chamber following standard protocols prescribed by your institution (*see* Note 1).

2. Expose the trachea by making a vertical midline incision and spread the skin.

3. Under the skin, there are two large masses of tissue. These are the submaxillary glands. Gently separate the glands from the midline with forceps (do not cut to avoid bleeding). The trachea will be easily seen and exposed.

4. Grab the outer fascial membrane covering the trachea with forceps. Carefully cut it away to expose the cartilage rings of the trachea.

5. Position the mouse upright and insert one side of a blunt forceps behind the trachea.

6. Through the largest uppermost cartilage ring, insert an 18-gauge needle, with the bevel facing outward. Attach a 3 mL syringe containing 1 mL of 1× HBSS (or PBS alone, no EDTA, if lung digestion follows). Do not insert the needle too deep into the trachea. Only insert it far enough to sufficiently cover the needle opening. After needle insertion, clamp down on the needle with the other side of the blunt forceps to hold the needle in place (*see* Note 2).

7. Lavage the lungs four times with 1 mL of 1× HBSS or PBS. Do not add more volume to the syringe (*see* Note 3).

8. Collect lavage fluid in a 5 mL FACS tube and centrifuge at $300 \times g$ for 5 min at 4 °C.

9. Remove supernatant. Rapidly tilt the FACS tubes to pour out supernatant or aspirate the supernatant using a syringe or pipette.

10. Place cells on ice, and add antibody cocktail for at least 45 min for optimal cell separation during FACS analysis (*see* Subheading 3.4).

3.3 Single-Cell Suspension of Lung and Lymph Nodes

1. To remove the lungs, place the euthanized mouse on its back, and cut open the thoracic cavity along the lower part of the rib cage. Next cut along the sides of the rib cage toward the axillary region of the mouse.

2. Pull back the sternum with blunt forceps, and locate the largest mediastinal lung-draining lymph node (LLN) under the right side of the heart below the thymus. A small blood vessel perpendicular to the trachea and superior vena cava indicates

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[1] We use CO_2 as our preferred method of euthanasia due to its low cost and quick application. However, alternative methods of euthanasia, such as lethal injection of pentobarbital, can also be used. Cervical dislocation should be avoided since it can cause bleeding into the lungs. Euthanasia methods should be approved by your institutional animal care and use committee (IACUC).

[2] If the needle appears to be unable to suction delivered fluid, rotate the tip a bit, or slightly expose needle opening to the air to release lack of suction.

[3] Never place more than 1 mL of HBSS or 1× PBS into the airways. Excess fluid forced into the airways will cause lung injury and bleeding will occur. Fluid extracted from airways of naïve mice should not contain red blood cells. If this happens, there are mainly two reasons: (1) The bronchoalveolar lavage was not extracted immediately after mouse euthanasia, or (2) lavage fluid is being injected into the mouse too aggressively. Also, do not add EDTA to BAL fluid since EDTA will inhibit lung digestion.

the location of the large LLN, which is slightly below this small blood vessel. There are also two very small lymph nodes slightly above the blood vessel, which are easily seen in an inflamed mouse but not in a steady-state mouse. Since the left side LLNs are very difficult to find, even during inflammation, only extract the one, large LLN on the right side for consistency and proper data analyses.

3. Place the LLN in a 35 mm culture dish containing 1 mL collagenase D for digestion.

4. Keep on ice until all samples have been collected.

5. Expose the heart. Nick the left atrium, and insert a 27-gauge needle with a 30 mL PBS-filled syringe into the right ventricle, and perfuse the lungs until they turn white. Note that even when the lung is white, there are still many intravascular leukocytes present. This highlights why intravenous injection of anti-CD45, which stains vascular leukocytes, is important (*see* Subheading 3.1), especially for extravascular lymphocyte, monocyte, and granulocyte analysis.

6. Remove lobes individually, and place them in a 35 mm culture dish containing 1 mL of Liberase TM or collagenase D for digestion. Keep on ice until all samples have been collected.

7. Lung digestion: Place lungs on a glass microscope slide, and cut them into very tiny pieces with scissors. Place the minced lungs back into the digestion buffer.

8. Lymph node digestion. Tease each sample apart with two 26-gauge needles attached to 1 mL syringes. To tease, hold down the lymph node with one needle while breaking open the lymph node with the other needle. When teasing is done correctly, concentrated cells bursting from the lymph node are easily observed in the media.

9. Place minced and teased cells in an incubator for 30 min at 37 °C.

10. Following incubation, add 100 μL of 100 mM EDTA to inhibit further tissue digestion.

11. Place culture dishes on ice, and homogenize the cell suspension by repeated pipetting with a glass Pasteur pipette and rubber bulb. Filter cells through 100 μm nylon filter (*see* Note 4), and collect cells into a 5 mL FACS tube. Wash the dish with HBSS complete to collect remaining cells. Filter the wash into the same FACS tube using the 100 μm nylon filter.

12. Centrifuge cells at $300 \times g$ for 5 min at 4 °C.

13. Decant or aspirate supernatant, leaving behind up to 200 μL volume with cells. Make sure not to double-tilt the FACS tube while pouring off the supernatant since this can lead to loss of cells.

[4.]Do not use any filter smaller than 70 μm, because DCs and macrophages may not easily pass through a filter that is too fine. For example, a 40 μm nylon filter may result in reduced recovery of DCs and macrophages for FACs analysis. Lung cells and sometimes lymph nodes should be refiltered through a 70 μm or 100 μm nylon filter to remove clumped cells that could clog the FACS machine.

3.4 Stain Single-Cell Suspension of Airway, Lung, and Lymph Nodes for FACS

1. Place cells on ice and make an antibody master mix for FACS staining. Use approximately 1 μL of antibody per sample in a final volume of 100 μL of HBSS complete for all antibodies except the MHC II (IA/IE) antibody, which is very strong. For the MHC II antibody use 0.3 μL for each lung and LLN sample and 1 μL for each blood sample. As an example, if there are five samples that require the same stain, add 5 μL of each antibody into 500 μL HBSS complete, and then add 100 μL of the antibody mix to each sample. Stain cells with antibodies for at least 45 min up to 1.5 h for optimal cell separation during FACS analysis.

2. The identification of DCs, macrophages, and monocytes in the lung is outlined in Figs. 1 and 2.

3. Identification of migratory DCs and monocytes in the LLN is outlined in Fig. 3.

Migratory dendritic cells are gated using CD11c versus MHC II (Fig. 3): CD103^{+} DCs and CD11b^{+} DCs (alternatively, one could use XCR1 in place of CD103 and SIRPα in place of CD11b). Note: CD11b DCs can be divided further into two sub-populations using CD24 and Mgl2/CD301 (not shown, [22]).

Acknowledgment

Abbreviations

AM	Alveolar macrophage
BAL	Bronchoalveolar lavage
DC	Dendritic cell
IM	Interstitial macrophage
IN	Intranasal
IT	Intratracheal
LLN	Mediastinal lung-draining lymph node
MP	Mononuclear phagocyte

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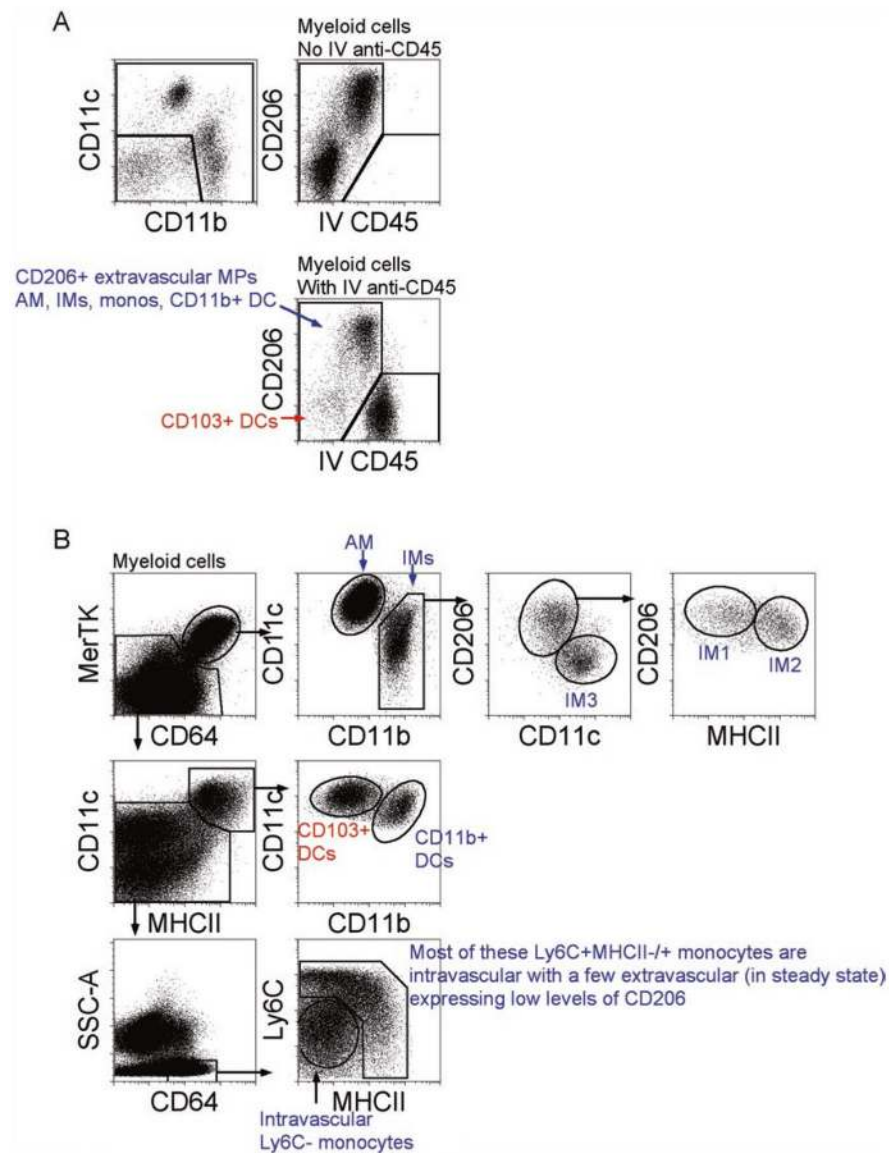
Fig. 1.
Identification of mononuclear phagocytes (MPs) in steady-state mouse lung using flow cytometry. (**a**) The use of an IV injection of anti-CD45 antibody to differentiate intravascular leukocytes from pulmonary extravascular mononuclear phagocytes. Note that intravascular leukocytes stain positively with the anti-CD45 antibody. Mononuclear phagocytes from the lung tissue and airspaces can be highly autofluorescent, so a second stain, such as CD206, is helpful. Note that Fig. 2 illustrates in detail how to identify all the CD206+ MPs and CD206−CD103+ DC. (**b**) Gating strategy for pulmonary macrophages. Macrophages are double positive for MerTK and CD64 (Fig. 2). The macrophage-negative gate (MerTK−, CD64−) is used to identify DCs (MHCII^{high}, CD11c^{high}). Cells from the DC-negative gate can be further analyzed to identify extravascular monocytes, which are few and would require the use of IV anti-CD45 to identify them (not shown here) [31]
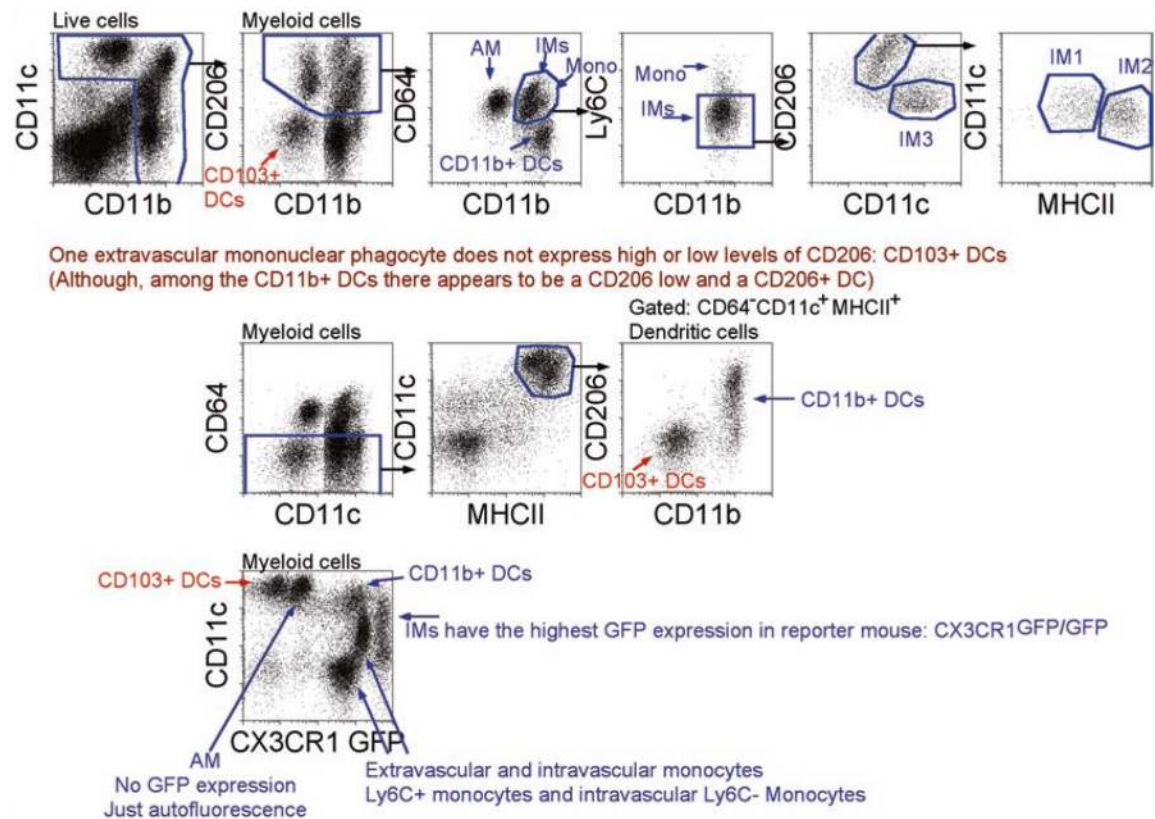
Fig. 2.
Additional ways to identify mononuclear phagocytes (MPs) in steady-state mouse lung. Live cells are selected using FSC and SSC, followed by exclusion of doublet cell and dead cells (not shown). Myeloid cells are gated using CD11c versus CD11b, illustrated in the top left graph. Most extravascular MPs in the lung can be identified using CD206 (in both mice and humans [30]) with the exception of CD103$^+$ DCs, which are CD206 negative. Outlined above is the gating strategy to identify all the pulmonary MPs. In addition, in the lung, IMs have very high CX$_3$CR1 expression as reported in the CX$_3$CR1 GFP reporter mice [24]. Intravascular Ly6C$^-$ monocytes display higher GFP expression in CX$_3$CR1^{gfp} mice than intra- or extravascular Ly6C$^+$ monocytes
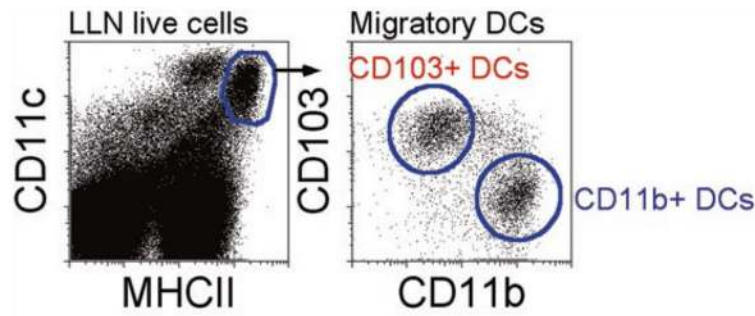
In steady state, there are a few monocyes in the LLN.
However during inflammation there are many more LN monocytes.
Use CD64 versus CD11b to identify Ly6C+ MHCII+ monocytes (make sure to exclude B cells using B220).

Fig. 3.

Identification of mononuclear phagocytes (MPs) in steady-state mouse lung-draining LN (LLN). Live cells are gated using FSC, SSC, doublet cell, and DAPI exclusions. Migratory dendritic cells are gated using CD11c versus MHCII: CD103$^+$ DCs and CD11b$^+$ DCs (alternatively, one could use XCR1 in place of CD103 and SIRPα in place of CD11b). CD11b$^+$ DCs can be further divided into two subpopulations using CD24 and Mgl$_2$/CD301 (not shown [22]). Monocyte gating using CD64 and/or Ly6C versus CD11b was previously shown in reference (keep in mind that neutrophils express intermediate levels of Ly6C, express slightly more CD11b and have a higher SSC than LN monocytes) [31]. Lung-derived migratory DCs have higher MHC II expression levels than resident LN DCs and B cells

Table 1

Antibody clones used to stain pulmonary mononuclear phagocytes

Antigen	Clone	Company	Conjugate
CD11c	N418	eBioscience	PE-Cy7
CD11b	M1/70	eBioscience	eF450
MHCII	M5/114.15.2	eBioscience	APC-Cy7
CD64	X54–5/7.1	BD Biosciences	AF647
MerTK	Polyclonal	R&D Systems	Unconjugated/biotin
CD206	C06822	Biolegend	PE
Ly6C	HK1.4	Biolegend	BV510
P4B0	CLA3–1	AbDSerotec	FITC
Lyve-1	ALY7	eBioscience	eF660
CD 169	SER-4	eBioscience	PE
CD36	No.72–1	eBioscience	PE
CCR2	475301	R&D Systems	PE
FcER1	42430	eBioscience	FITC
CD14	Sa2–8	eBioscience	PerCP-Cy5.5
CD45	30-F11	eBioscience	FITC
CD45.1	A20	Biolegend	PE-Cy7
CD45.2	104	Biolegend	FITC
Siglec F	E50–2440	BD Biosciences	PE/BV421
CD 103	2E7	eBioscience	PE/eF450
BrdU	Bu20A	eBioscience	APC
CD43	S7	BD Biosciences	BV421
CD24	M1/69	eBioscience	eF450
CD115	AF598	Biolegend	PE
Ly6G	1A8	Biolegend	eF450
B220	RA3–6B2	eBioscience	eF450
CD3	17A2	eBioscience	eF450
NK1.1	PK136	eBioscience	eF450
Streptavidin		eBioscience	PerCP-Cy5.5