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TITLE:

Flow Cytometric Analysis for Identification of the Innate and Adaptive Immune Cells of Murine Lung

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lung, immune cells, gating strategy, flow cytometry

SUMMARY:

In this study, we present an effective and reproducible protocol to isolate the immune populations of the murine respiratory system. We also provide a method for the identification of all innate and adaptive immune cells that reside in the lungs of healthy mice, using a 9-color-based flow cytometry panel.

ABSTRACT:

The respiratory tract is in direct contact with the outside environment and requires a precisely regulated immune system to provide protection while suppressing unwanted reactions to environmental antigens. Lungs host several populations of innate and adaptive immune cells that provide immune surveillance but also mediate protective immune responses. These cells, which keep the healthy pulmonary immune system in balance, also participate in several pathological conditions such as asthma, infections, autoimmune diseases, and cancer. Selective expression of surface and intracellular proteins provides unique immunophenotypic properties to the immune cells of the lung. Consequently, flow cytometry has an instrumental role in the identification of such cell populations during steady-state and pathological conditions. This paper presents a protocol that describes a consistent and reproducible method to identify the immune cells that reside in the lungs of healthy mice under steady-state conditions. However, this protocol can also

be used to identify changes in these cell populations in various disease models to help identify disease-specific changes in the lung immune landscape.

INTRODUCTION:

The murine respiratory tract contains a unique immune system responsible for fighting pathogens and maintaining immune homeostasis. The pulmonary immune system consists of cellular populations with significant heterogeneity in their phenotype, function, origin, and location. Resident alveolar macrophages (AMs), originated from fetal monocytes, reside in the alveolar lumen[1], while bone marrow-derived interstitial macrophages (IMs) reside in the lung parenchyma[2]. IMs can be further subclassified by the expression of CD206. $CD206^+$ IMs populate the peribronchial and perivascular area, while $CD206^-$ IMs are located at the alveolar interstitium[3]. A few subclassifications of IMs have been proposed recently[3-6]. Although IMs are less studied than AMs, recent evidence supports their crucial role in the regulation of the immune system of the lung[7]. In addition, CD206 is also expressed in alternatively activated AMs[8].

Pulmonary dendritic cells (DCs) are another heterogeneous group of lung immune cells with respect to their functional properties, location, and origin. Four subcategories of DCs have been described in the lung: conventional $CD103^+$ DCs (also known as cDC1), conventional $CD11b^+$ DCs (also known as cDC2), monocyte-derived DCs (MoDCs), and plasmacytoid DCs[9-13]. The first three subclasses can be defined as major histocompatibility complex (MHC) II^+CD11c^+[9,10,14,15]. Plasmacytoid DCs express MHC II and are intermediately positive for CD11c but express high levels of B220 and PDCA-1[9,13,16]. In naïve murine lungs, CD103 DCs and CD11b DCs are located in the airway interstitium, whereas plasmacytoid DCs are located in the alveolar interstitium[17].

Two major populations of monocytes reside in the lung during steady state: classical monocytes and non-classical monocytes. Classical monocytes are $Ly6C^+$ and are critical for the initial inflammatory response. In contrast, non-classical monocytes are $Ly6C^-$ and have been widely viewed as anti-inflammatory cells[3,16,18]. Recently, an additional population of $CD64^+CD16.2^+$ monocytes was described, which originate from $Ly6C^-$ monocytes and give rise to $CD206^+$ IMs[3].

Eosinophils mainly appear in the lungs during helminth infection or allergic conditions. However, there is a small number of eosinophils in the pulmonary parenchyma during steady state, known as resident eosinophils. In contrast to the resident eosinophils, inflammatory eosinophils are found in the lung interstitium and bronchoalveolar lavage (BAL). In mouse models of house dust mite (HDM), inflammatory eosinophils are recruited into the lung after antigen-mediated stimulation. It has been proposed that resident eosinophils might have a regulatory role in allergy by inhibiting T helper 2 (Th2) sensitization to HDM[19].

In contrast to the rest of pulmonary myeloid cells, neutrophils express Ly6G but not CD68 and are characterized by a signature of the $CD68^-Ly6G^+$ immunophenotype[16,20,21]. Visualization studies have shown that during steady state, the lung reserves a pool of neutrophils in the intravascular compartment and hosts a considerable number of extravascular neutrophils[22]. Similar to eosinophils, neutrophils are not found in BAL at steady state; however, several forms

of immune stimulation, such as LPS challenge, asthma, or pneumonia, drive neutrophils into the alveolar lumen, resulting in their presence in BAL[21-23].

A substantial number of $CD45^+$ cells of the lung represent natural killer (NK), T cells, and B cells and are negative for most myeloid markers[24]. In the lungs of naïve mice, these three cell types can be identified based on the expression of CD11b and MHC II[18]. Around 25% of pulmonary $CD45^+$ cells are B cells, whereas the percentage of NK cells is higher in the lung than other lymphoid and non-lymphoid tissues[24-26]. Among pulmonary T cells, a considerable fraction is $CD4^-$ $CD8^-$ and plays an important role in respiratory infections[26].

Because the lung hosts a very complex and unique immune system, several gating strategies for the identification of lung immune cells have been developed and reported[16,18,20,27]. The gating strategy described herein provides a comprehensive and reproducible way to identify up to 12 different pulmonary myeloid and non-myeloid immune populations using 9 markers. Additional markers have been used to validate the results. Furthermore, a detailed method is provided for the preparation of a single-cell suspension that minimizes cell death and allows the identification of the most complete profile of the immune cell compartment of the lung. It should be noted that the identification of non-immune cells of the lung, such as epithelial cells ($CD45^-$ $CD326^+CD31^-$), endothelial cells ($CD45^-CD326^-CD31^+$), and fibroblasts requires a different approach[28,29]. Identification of such populations is not included in the protocol and method described here.

PROTOCOL:

All studies and experiments described in this protocol were conducted under guidelines according to the Institutional Animal Care and Use Committee (IACUC) of Beth Israel Deaconess Medical Center. Six to ten weeks old C57BL/6 mice of either sex were used to develop this protocol.

1. Surgical excision and tissue preparation

1.1. Euthanize the mouse by intraperitoneally injecting 1 mL of tribromoethanol (prepared according to standard protocol; **Table of Materials**).

NOTE: CO_2 asphyxiation should be avoided in lung studies as it might cause lung injury and alter the features and properties of lung immune cells. Cervical dislocation should also be avoided as it might cause mechanical injury of the lung.

1.2. Transfer the mouse to a clean and dedicated area for surgical operation.

1.3. Stabilize the mouse dorsal side down by using needles or tape on the four extremities. Use 70% ethanol to sanitize the skin of the ventral area.

1.4. Perform an incision in the skin, from the neck to the abdomen. Carefully remove the skin from the thoracic area.

1.5. Carefully remove the sternum and ribs.

1.6. Flush the lungs by injecting 10 mL of cold PBS directly in the right ventricle, using an 18–21 G needle, until the lungs become completely white.

1.7. Carefully remove the thymus and heart without touching the lungs.

1.8. Gently detach the lungs from the surrounding tissues and transfer them to a tube with cold BSA buffer (**Table 1**).

NOTE: Effort should be made to remove all adjacent fat from the lungs before further preparing the single-cell suspension, as this could bias the readouts.

2. Preparation of single-cell suspension

2.1. Transfer the lungs to an empty Petri dish and mince them with two fine scalpels. Transfer all the pieces of the minced lung to a new 50 mL conical tube. Use 5 mL of digestion buffer to wash the plate and add it to the 50 mL tube containing the minced lung (**Table 1**).

NOTE: Digestion buffer should be prepared immediately before use. Use 5 mg/mL of collagenase[28]. Combining 1 or 5 mg of collagenase with BSA buffer or protein-free PBS did not improve results (**Supplemental Figure S1**).

2.2. Secure the lid of the tube and digest the lung for 30 min on an orbital shaker at a speed of 150 rpm at 37 °C. Stop the reaction by adding 10 mL of cold BSA buffer.

2.3. After digestion, use an 18 G needle to mix and dissolve the lung pieces. Place a 70 µm filter strainer at the top of a new 50 mL conical tube.

NOTE: Usage of a smaller micron filter might result in the loss of major myeloid populations.

2.4. Slowly transfer the digested lung mixture directly on the strainer. Use the rubber side of a 10 mL syringe plunger to smash the remaining lung pieces on the filter. Wash the processed material on the filter with BSA buffer.

2.5. Centrifuge the single-cell suspension at 350 × *g* for 8 min at 7 °C.

2.6. Carefully discard the supernatant and resuspend the cells in 1 mL of ACK lysis buffer. Mix well using a 1 mL pipet, and incubate for 90 s at room temperature.

2.7. Add 10 mL of cold BSA buffer to stop the reaction and centrifuge at 350 × *g* for 7 min at 4 °C. Carefully discard the supernatant and resuspend the pellet in Staining Buffer to count the cells using a hemocytometer.

2.8. Resuspend the cells at a concentration of 5 × 10^6 cells/mL and use them for surface staining (see section 3).

NOTE: For this purpose, plate the cells in a 96-well round-bottom plate followed by antibody staining and washes. If a plate centrifuge is not available, use flow tubes instead of plates. With this protocol, ~15–20 × 10^6 cells per lung can be obtained from a 6–10-week-old C57BL/6 mouse of average size.

3. Surface antibody staining

3.1. Transfer 1 × 10^6 cells in 200 µL per well in a 96-well plate. Centrifuge the plate at 350 × *g* for 7 min at 4 °C. In the meantime, prepare the Fc-block solution by diluting anti-16/32 antibody (1:100) in staining buffer (**Table 1**).

3.2. Resuspend the cells in 50 µL of the pre-prepared Fc-blocking solution (**Table of Materials**) and incubate for 15–20 min at 4 °C or on ice.

3.3. Add 150 µL of staining buffer and centrifuge the plate at 350 × *g* for 5 min at 4 °C. Meanwhile, prepare the surface antibody cocktail by diluting surface antibodies (1:100; **Table 2**) in staining buffer.

NOTE: (i) Anti-16/32 antibody for Fc-blocking can be used with the surface antibodies in the same mixture. (ii) If fixable viability dye is used, add it to the surface antibody cocktail at a dilution of 1:1,000.

3.4. Resuspend the cells in 50 µL of the pre-prepared surface antibody cocktail and incubate for 30–40 min at 4 °C in the dark. Wash the cells with staining buffer twice.

NOTE: If no intracellular staining is required, resuspend the cells in 200 µL of staining buffer and proceed directly to the acquisition of data on the flow cytometer. Alternatively, cells might be fixed and stored at 4 °C for acquisition later. We recommend using the cells for flow cytometry within 24 h.

4. Cell fixation and intracellular staining

4.1. Prepare the fixation/permeabilization buffer (Fix/Perm Buffer) by mixing three parts of fixation/permeabilization diluent and 1 part of fixation/permeabilization diluent of the FoxP3/Transcription Factor Staining Buffer Set (**Table 1**).

4.2. Resuspend the cells in 50 µL of the pre-prepared Fix/Perm Buffer per well of the 96-well plate, where cells were plated as described in section 3, and incubate them for 20–25 min at 4 °C in the dark.

4.3. Dilute the 10x permeabilization buffer as 1: 10 in purified deionized water to prepare 1x permeabilization buffer.

4.4. Wash the cells once with 1x permeabilization buffer. Meanwhile, prepare the intracellular antibody cocktail by diluting intracellular antibodies (1:100) in 1 mL of permeabilization buffer.

4.5. Resuspend the cells using 50 µL of the pre-prepared surface antibody cocktail per cell of the 96-well plate and incubate for 40 min at 4 °C in the dark.

4.6. Wash the cells once with permeabilization buffer and once with staining buffer. After the final wash, resuspend the cells in 200 µL of staining buffer.

NOTE: If no flow cytometer with plate reader is available, transfer the cells into flow cytometry tubes.

4.7. Acquire a minimum of 1.5×10^6 cells per sample on the flow cytometer.

NOTE: For single colors and unstained control samples, $0.5–1 \times 10^6$ cells per sample will be sufficient. It is recommended to titer the individual antibodies used to achieve optimal staining and reduce costs. The present protocol has been optimized using Fix/Perm Buffer prepared using the FoxP3 staining buffer set. Because CD68 is a cytoplasmic and not a nuclear marker, other permeabilization solutions such as a low concentration of paraformaldehyde or cytofix/ cytoperm kits from various vendors might be sufficient.

REPRESENTATIVE RESULTS:

Gating strategy

The first step of our gating strategy is the exclusion of the debris and doublets (**Figure 1A**). Careful exclusion of doublets is critical to avoid false-positive populations (**Supplemental Figure S2**). Then, immune cells are identified using $CD45^+$, a marker for hematopoietic cells (**Figure 1B**). The live-dead stain can be added to exclude dead cells. However, this protocol results in the death of <5% of the $CD45^+$ cells (**Figure 1C**), whereas more $CD45^-$ cells are identified as dead (**Supplemental Figure S3**).

To identify monocytes and neutrophils, in contrast to previous studies that used specific antibodies for each of these populations[16,18], we prefer to use an anti-GR-1 antibody that identifies both $Ly6C^+$ and $Ly6G^+$ cells. Using the anti-GR-1 antibody together with anti-CD68 allows the separation of the lung immune cells into three clusters: $CD68^-GR\text{-}1^+$, $CD68^+$(that can be further identified as $GR\text{-}1^+$ or $GR\text{-}1^-$), and $CD68^-GR\text{-}1^{-/int}$ (**Figure 1D**). CD68 is a marker that is predominantly detected intracellularly. Surface CD68 was probed but could not be detected without fixation/permeabilization (**Supplemental Figure S4**).

Polymorphonuclear cells (neutrophils) were identified as $CD45^+CD68^-GR\text{-}1^+CD11b^+$ (**Figure 1E**). These results were verified using GR1 together with an antibody specific for Ly6G (**Figure 2**), a unique marker for polymorphonuclear neutrophils[16,18,27]. Within the $CD45^+CD68^-GR\text{-}1^{-/int}$ population, approximately 10–20% are $MHCII^-$ and $CD11b^{low}$ and represent the NK cells (**Figure** 1F). NK1.1, a unique marker for NK cells, was used to confirm this (**Figure 3**)[25,30]. The $CD45^+CD68^-GR1^{-/int}CD11b^-$ population consists of $MHCII^-$ T cells and $MHCII^+$ B cells (**Figure 1F**). Two additional antibodies were used to verify T cells and B cells—CD3 and B220, respectively (**Figure 3**).

In healthy mice under steady-state conditions, the majority of $CD45^+$ cells detected in BAL are AMs, the main residents of the airways. Hence, BAL was performed to assess the markers that characterize AMs[20,31]. These cells are $CD45^+CD68^+Siglec\text{-}F^+CD11c^+$ but, unlike other myeloid cells, do not express high levels of CD11b (**Figure 4**). The same combination of immune markers also identifies AMs in homogenates of total lungs (**Figure 1G,H**). In addition to AMs, in the $CD45^+CD68^+Sigle\text{-}F^+$ gate (**Figure 1G**), there is a distinct cell population that is positive for CD11b but not for CD11c. This $CD45^+CD68^+Siglec\text{-}F^+CD11b^+CD11c^-$ population of leukocytes represents eosinophils (**Figure 1G**)[19,32].

In the gate with the $CD45^+CD68^+SiglecF^-$ cells (**Figure 1G**), there is a $CD11c^+MHC^+$ population that represents pulmonary DCs (**Figure 1I**). Several investigators identify pulmonary DCs as $CD11C^+MHCII^+CD24^+$[16,18]. CD24 expression was assessed to confirm the identity of this population (**Figure 5A**). The majority of the lung DCs are either $CD103^+CD11b^-$ or $CD103^-CD11b^+$ (**Figure 1J**). The $CD103^+CD11b^-$ DCs represent the $CD103^+$ conventional DCs and are identified as $CD45^+CD68^+SiglecF^-MHCII^+CD103^+CD11b^-$. In contrast, $CD103^-CD11b^+$ DCs are divided into conventional DCs and MoDCs based on CD64 expression (**Figure 1K**). Therefore, conventional $CD11b^+$ DCs are identified as $CD45^+CD68^+SiglecF^-MHCII^+CD103^-CD11b^+CD64^-$ while the MoDCs are identified as $CD45^+CD68^+SiglecF^-MHCII^+CD103^-CD11b^+CD64^+$. In contrast to conventional DCs, MoDCs are low-positive for the DC marker CD24 and positive for pan-macrophage markers, including F4/80, CD64 and MERTK (**Figure 5A**)[9-11,13,33,34].

IMs and classical and non-classical monocytes are in the $CD45^+CD68^+Siglec\text{-}F^-CD11c^{-/int}CD11b^+$ gate (**Figure 1L**) and are distinguished based on CD64 and GR-1 expression (**Figure 1M**). CD64, a pan-macrophage marker, is mainly expressed by IMs and AMs, as well as a subset of DCs. Classical monocytes are also known as $Ly6C^+$ monocytes and non-classical monocytes as $Ly6C^-$ monocytes. Both monocytes and IMs are negative for Ly6G; however, classical monocytes express Ly6C and are therefore positive for GR-1. In contrast, both interstitial macrophages and non-classical monocytes are negative for GR-1 and Ly6C[3,4,11,16,18,20,35,36]. In addition, non-classical monocytes are $CD11c^{int}$, while classical monocytes are $CD11c^-$. Although this difference in CD11c expression is generally insignificant for distinguishing the two types of monocytes at baseline, it could be critical for pathological conditions when classical monocytes accumulate. Based on the above, interstitial macrophages are defined as $CD45^+CD68^+Siglec\text{-}F^-CD11c^{-/int}CD11b^+GR\text{-}1^-CD64^+$, classical monocytes as $CD45^+CD68^+Siglec\text{-}F^-CD11c^-CD11b^+GR\text{-}1^+CD64^-$, and non-classical monocytes as $CD45^+CD68^+CD11c^{-/int}CD11b^+GR\text{-}1^-CD64^-$. Both AMs and IMs are positive for all the macrophage markers, including CD68, CD64, F4/80, and MERTK proto-oncogene. However,

unlike IMs, AMs are CX3CR1^{-}, in contrast to IMs, which could be explained by the difference in the origin of the two types of lung macrophages[4,37-39] (**Figure 5B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Gating strategy of immune cells present in the murine lung. After careful exclusion of debris, doublets, dead cells, and the non-immune cells (CD45^{-}) (**A–C**), CD45^{+} cells were separated into 3 main clusters based on the expression of CD68 and GR-1 (**D**)**.** Neutrophils belong to the CD68^{-}GR-1^{+} population (**E**) while the CD68^{-}GR-1$^{-/int}$ population consists of NK cells, B cells, and T cells (**F**). CD68^{+} cells can be further divided into Siglec F^{+} cells (**G**), which are AMs and eosinophils (**H**), and Siglec F^{-} cells (**G**), which consist of monocytes, IMs, and DCs (**I–M**). Abbreviations: SSC-A = peak area of side-scattered light; FSC-A = peak area of forward-scattered light; SSC-H = peak height of side-scattered light; L/D = live/dead staining; MHC = major histocompatibility complex; SF = Siglec F; GR-1 = GPI-linked myeloid differentiation marker (Ly-6G); NK = natural killer; IMs = interstitial macrophages; DCs = dendritic cells; AMs = alveolar macrophages.

Figure 2: **Ly6G in the lung is expressed only by CD45^{+}CD68^{-}GR-1^{+}CD11b^{+} cells identified as polymorphonuclear neutrophils.**

Figure 3: Verification of the identification of NK cells, T cells, and B cells by the gating strategy. Markers specific for NK 1.1, CD3, and B220 were used to verify the identification of NK cells, T cells, and B cells, respectively. It should be noted that NKT cells, if present, might fall in the CD3^{+} cell population within the NK cell gate, and caution is required to avoid contamination of NK with NKT cells. Abbreviations: NK = natural killer; MHC = major histocompatibility complex.

Figure 4: The majority of the immune cells obtained by bronchoalveolar lavage in naïve mice are alveolar macrophages. Abbreviations: AMs = alveolar macrophages; DCs = dendritic cells; IM = interstitial macrophage; SSC-A = peak area of side-scattered light; FSC-A = peak area of forward-scattered light; SSC-H = peak height of side-scattered light; SF = Siglec F; GR-1 = GPI-linked myeloid differentiation marker (Ly-6G).

Figure 5: Comparison of the expression of different markers in immune cells. (**A**) Comparison of CD24, CD64, MERTK, F4/80, and CD103 expression in pulmonary DCs. (**B**) Comparison of CD68, CD64, MERTK, F4/80, CD11c, Siglec F (SF), CD11b, and CX3CR1 in pulmonary macrophages and monocytes. Abbreviations: DCs = dendritic cells; MoDCs = monocyte-derived DCs; AMs = alveolar macrophages; IMs = interstitial macrophages; SF = Siglec F; MERTK = myeloid-epithelial-reproductive tyrosine kinase.

Table 1: Buffers.

Table 2: Usage of monoclonal antibodies.

Supplemental Figure S1: The best cellular dissociation is achieved by 5 mg/mL of collagenase 1 in prewarmed PBS + 0.5% BSA. In all different conditions, 0.2 mg of DNAse I was also included.

Abbreviations: BSA = bovine serum albumin; FSC-A = peak area of forward-scattered light; L/D = live/dead staining; SF = Siglec F.

Supplemental Figure S2: Inclusion of doublets might result in false-positive populations. Abbreviation: FP = false positive.

Supplemental Figure S3: More CD45$^-$ than CD45$^+$ have features consistent with dead cells.

Supplemental Figure S4: Surface staining for CD68 is not adequate to properly distinguish the individual immune cell populations of the lung.

DISCUSSION:

Identification of pulmonary immune cells can be challenging because of the multiple immune cell types residing in the lung and their unique immunophenotypic characteristics compared to their counterparts residing in other tissues. In several pathologic conditions, cells with distinct phenotypic features appear in the lungs. For example, bleomycin-induced lung injury results in the recruitment of circulating monocyte-derived macrophages in the alveolar space, where they can remain for as long as one year and even persist after bleomycin-induced fibrosis. In contrast to tissue-resident AMs, the circulating monocyte-derived macrophages are Siglec F^{low}CD11b$^+$. Targeted depletion of the monocyte-derived macrophages results in the amelioration of bleomycin-mediated pulmonary fibrosis[16,40]. Cells with similar features are recruited to the lungs during influenza infection and provide prolonged protection against streptococcal pneumonia[15].

Based on CD11c and MHC II expression, IMs have been further subcategorized into IM1, IM2, and IM3. IM1 are immunophenotypically defined as CD11c$^-$MHC II$^-$; IM2 are defined as CD11c$^-$MHC II$^+$; and IM3 are defined as MHC II$^+$CD11c$^+$. It has been proposed that IM1, IM2, and IM3 represent the physiologic stages of monocyte to macrophage transition, rather than distinct macrophages categories, with IM3 representing the monocytic compartment[41]. As mentioned above, MoDCs are low-positive for the DC marker CD24 and positive for pan-macrophage markers, including F4/80, CD64, and MERTK[9-11,13,33,34]. However, several studies identify CD64$^+$MERKT$^+$ cells as pulmonary macrophages[4,10]. Both IM3 and MoDCs have been defined as CD64$^+$MERKT$^+$MHCII$^+$CD11C$^+$, suggesting that these two populations most likely represent the same cell type. Consistent with this hypothesis, the gating strategy presented here does not identify a distinct population representing IM3 cells, in addition to MoDCs.

Critical steps of the protocol described here include: 1) the removal of all adjacent fat from the lungs before preparing the single-cell suspension, as this could bias the readouts; 2) permeabilization before staining with the anti-CD68 antibody. A limitation of the present protocol is that it cannot identify non-immune cells of the lung, such as epithelial (CD45$^-$ CD326$^+$CD31$^-$), endothelial cells (CD45$^-$CD326$^-$CD31$^+$), and fibroblasts. Identification of such populations requires a different approach[28,29]. In addition, the protocol requires staining for CD68, an intracellular marker, which might pose a limitation if the investigator is not experienced in intracellular staining. The significance of the present protocol and gating strategy with respect to existing methods is that this strategy provides a streamlined approach that uses a lower

number of markers while allowing reproducible identification of all the immune populations of the lung.

Furthermore, a detailed method is provided for the preparation of a single-cell suspension that minimizes cell death and allows the identification of a complete profile of the immune cell compartment of the lung. Although the protocol outlined here describes the characterization and identification of lung immune populations under steady-state conditions, future applications may include the assessment of these populations in various disease models where it can help identify disease-specific changes in the lung immune landscape. In conclusion, this article presents a simple and reproducible protocol for lung single-cell preparation and a 9-color-based flow cytometry panel for the identification of 12 different immune cell populations.

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DISCLOSURES:

V.A.B. has patents on the PD-1 pathway licensed by Bristol-Myers Squibb, Roche, Merck, EMD-Serono, Boehringer Ingelheim, AstraZeneca, Novartis, and Dako. The authors declare no other competing financial interests.

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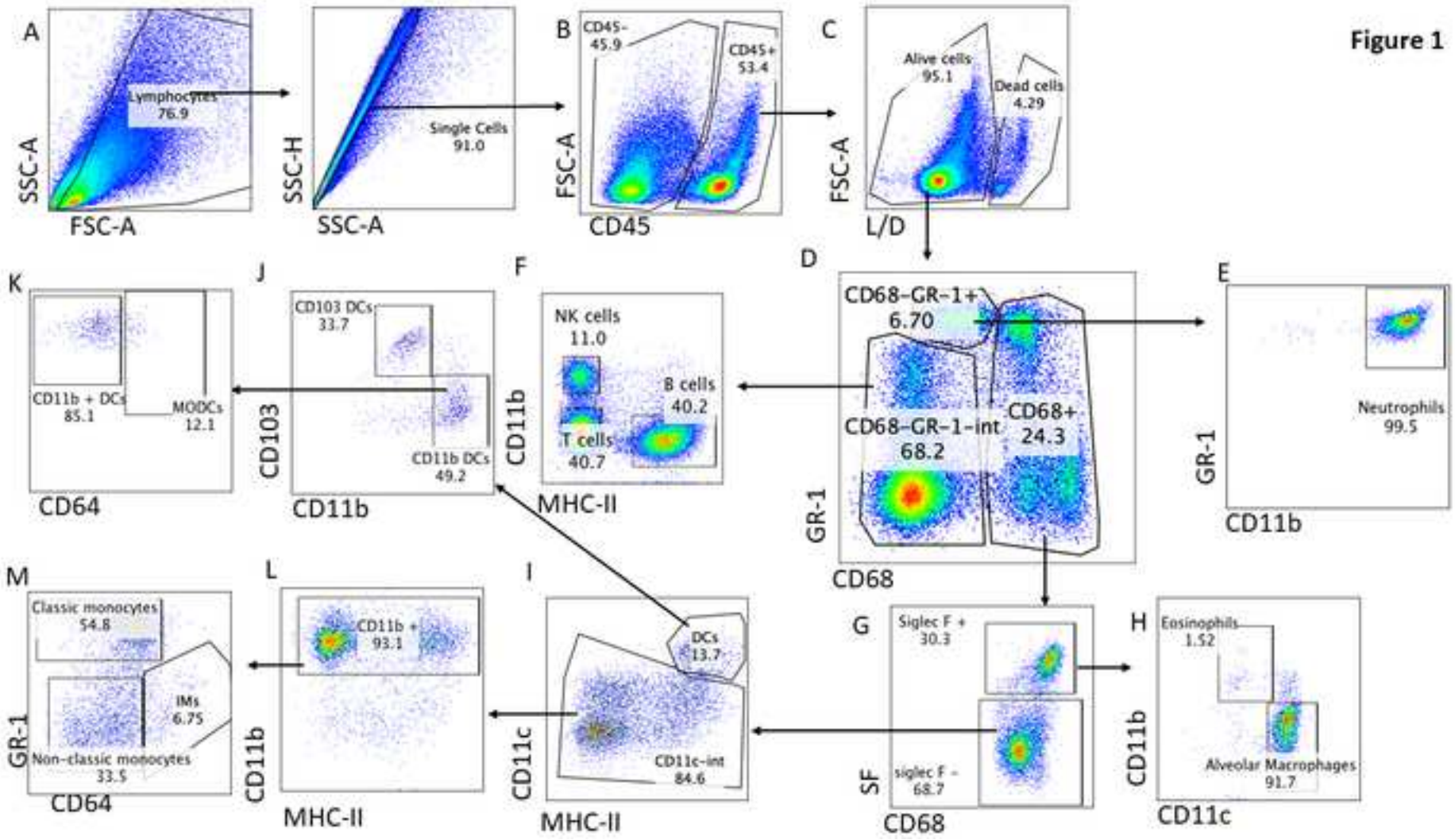

Figure 1

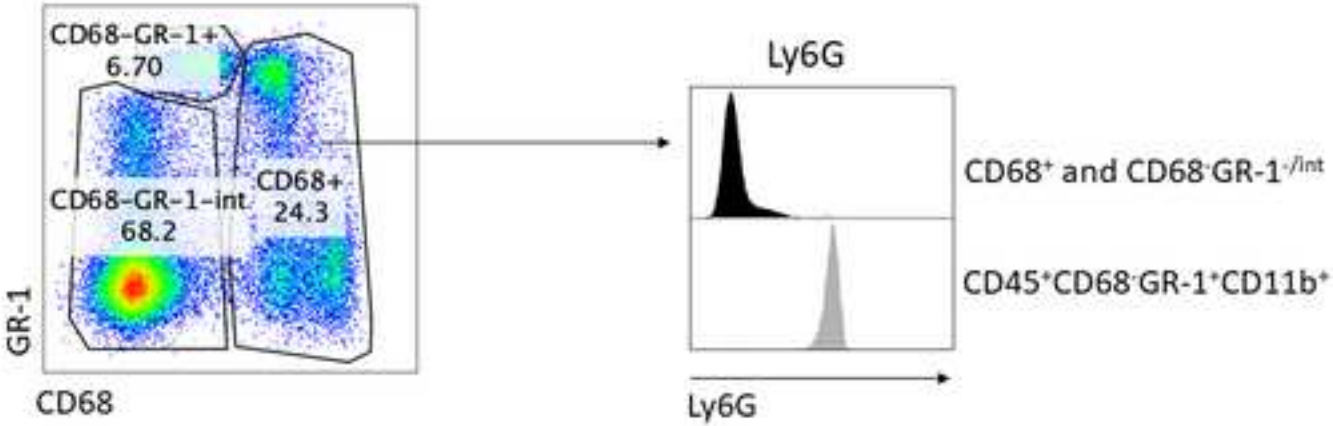

Figure 2

Figure_3

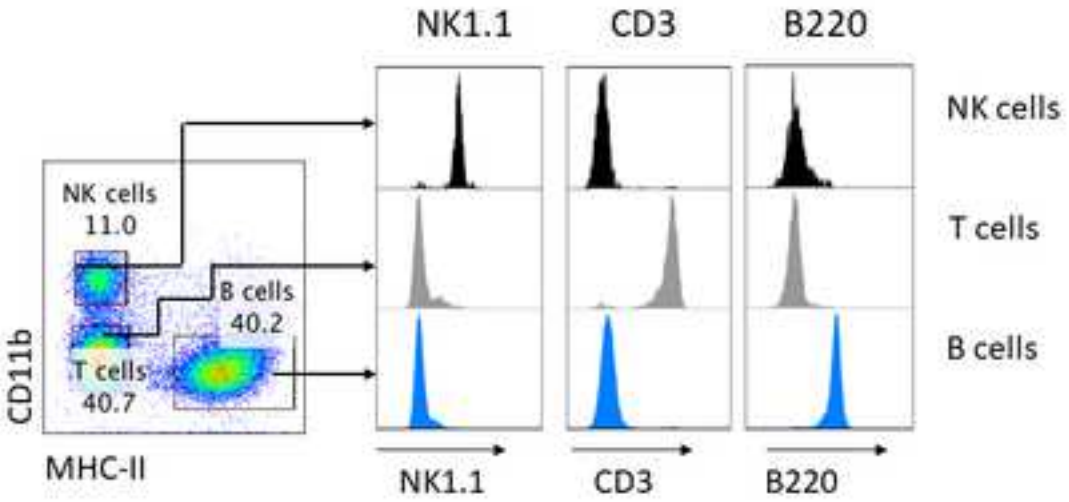

Figure 3

Figure 4

Click here to access/download;Figure;Figure_4.tif

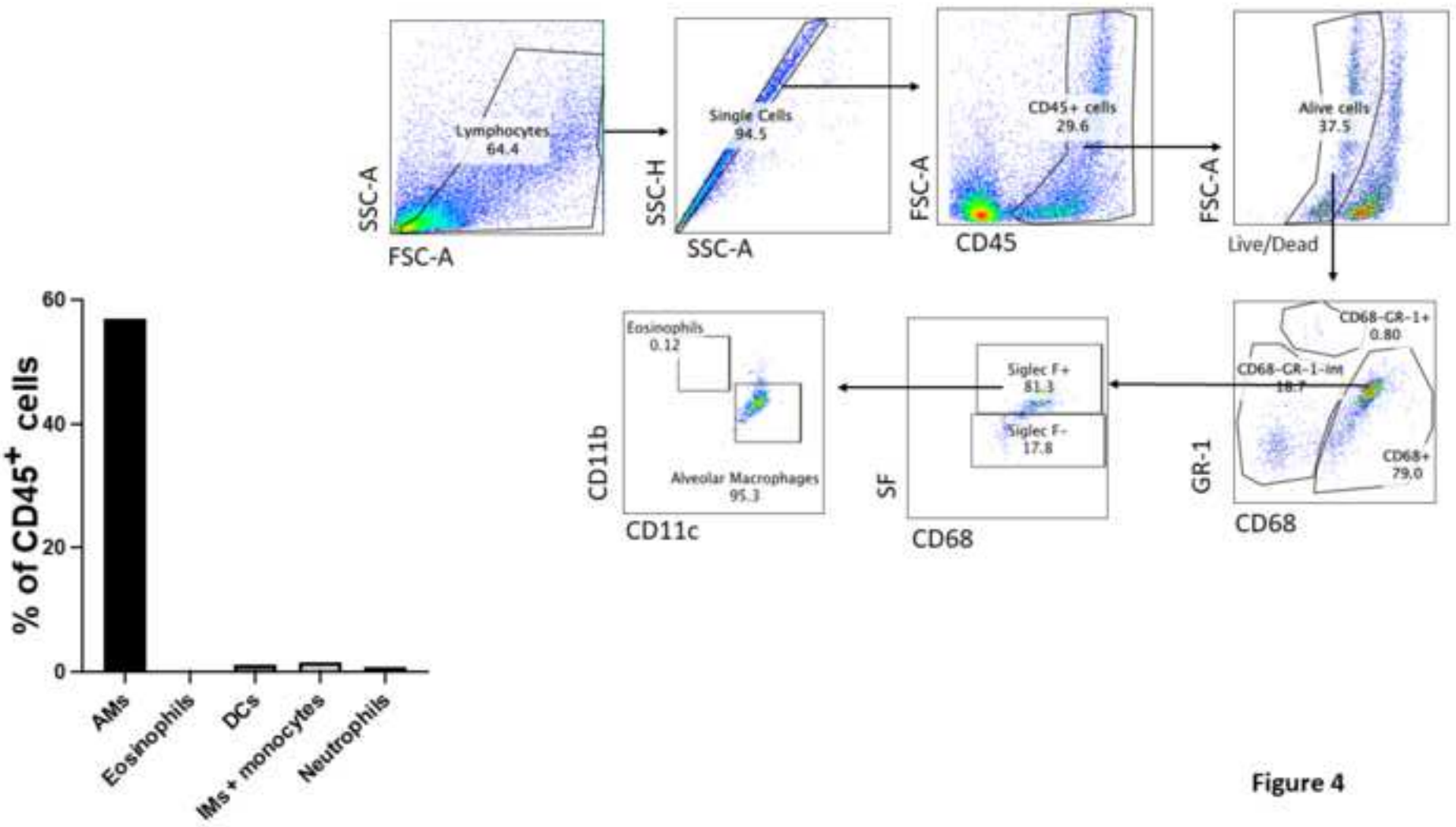

Figure 4

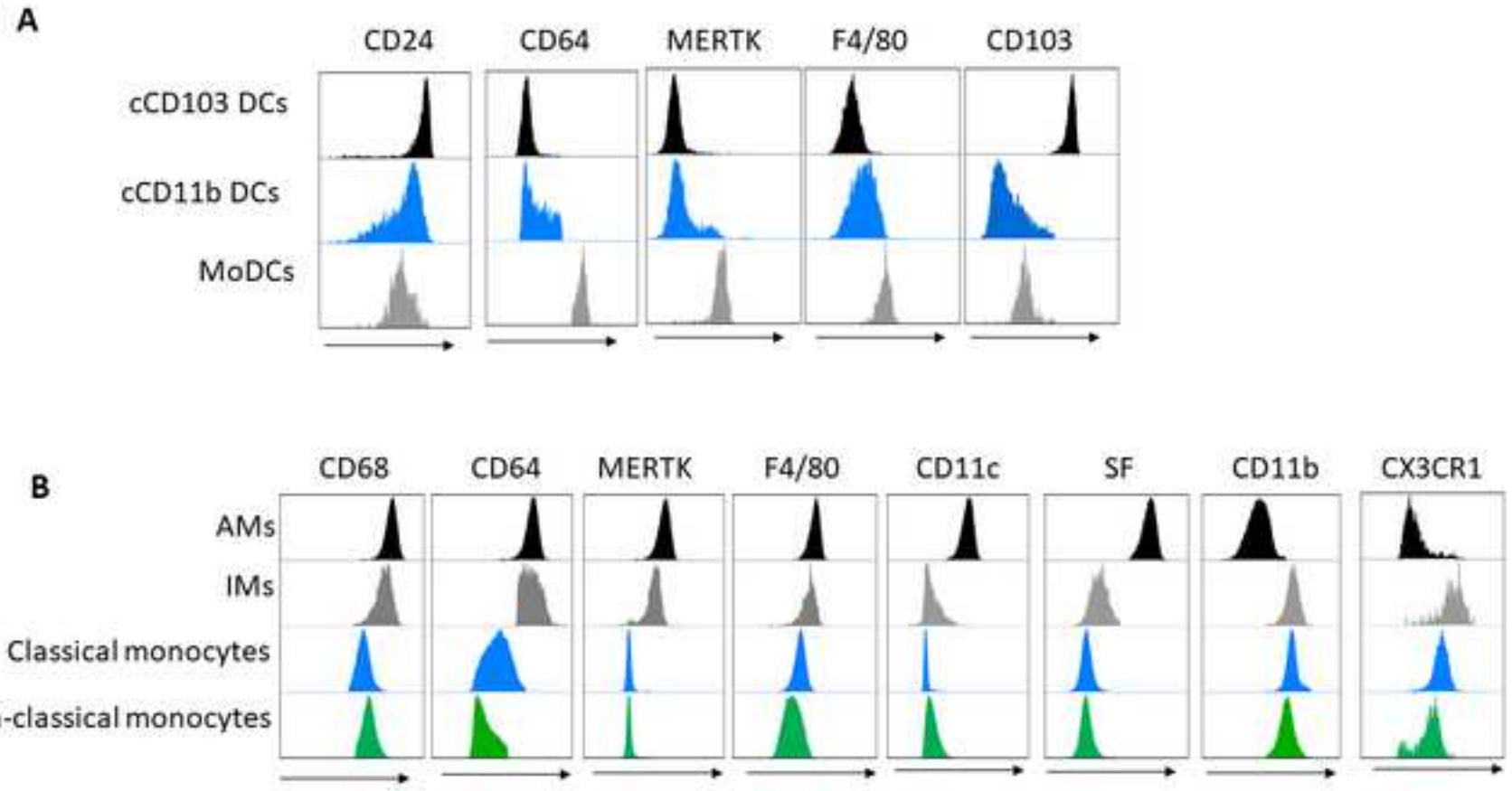

Figure 5

Table 1

BSA buffer	PBS + 0.5% Bovine serum albumin
Digestion buffer	Prewarmed (37 °C) BSA buffer + 5 mg/mL collagenase type 1 + 0.2 mg/mL DNase I
Staining buffer	PBS + 2.5% FBS
Fix/Perm Buffer	Three parts of fixation/permeabilization diluent and 1 part of fixation/permeabilization diluent of the Foxp3/Transcription Factor Staining Buffer Set
Permeabilization buffer	10x permeabilization buffer from the Foxp3/Transcription Factor staining Buffer Set diluted 10 times in purified deionized water

Table 2

Antigen	Clone	Fluorochrome	Dilution
CD45	30-F11	APC/CY7	0.111111111
Gr-1	RB6-8C5	BV421	0.111111111
CD68	FA-11	PerCPCy5.5	0.111111111
CD11b	M1/70	PECy7	0.111111111
Siglec F	S17007L	FITC	0.111111111
CD11c	N418	BV650 or BV510	0.111111111
CD64	X54-5/7.1	PE/Dazzle 594	0.111111111
MHC-II	M5/114.15.2	AF700	0.111111111
CD103	2.00E+07	PE / FITC	0.111111111
Live/Dead Fixable Far Read Dead Cell Stain Kit		FarRed (APC) or Aqua (BV510)	0.736111111
CD3	17A2	PE	0.111111111
B220	RA3-6B2	AF488	0.111111111
NK1.1	PK136	FITC	0.111111111
CD24	30-F1	PE	0.111111111
MERTK	2B10C42	PE	0.111111111
F4/80	BM8	BV605	0.111111111
CX3CR1	SA011F11	PE	0.111111111
FcBlock (CD16/32)	93		0.111111111

Surface/intracellular

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Beth Israel Deaconess Medical Center

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Vassiliki A. Boussiotis, M.D., Ph.D.
Professor of Medicine
Harvard Medical School

Vidhya Iyer Ph.D.
Review Editor
JoVE

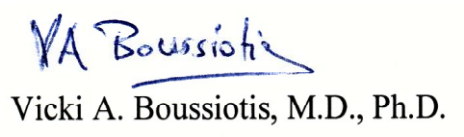
August 4, 2021

Dear Dr. Iyer,

We are pleased to submit our revised manuscript titled "Flow cytometric analysis for identification of the innate and adaptive immune cells of murine lung" (MS# 62985).

In this revision, all the comments of the editors and the reviewers have been taken into consideration and the manuscript has been amended accordingly. All the changes and additions gave been highlighted in the text and have been outlined in our point-by-point response to the reviewers' comments.

Sincerely,



Vicki A. Boussiotis, M.D., Ph.D.

Response to Reviews

We would like to thank the editor and the reviewers for their thoughtful suggestions for the improvement of our manuscript. All the comments of the reviewer and the editor have been taken into consideration and the manuscript has been revised accordingly. Below please find a point-by-point response to the reviewer's comments.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
2. Please revise your title to better describe the protocol in the paper and the video. Maybe something like "Flow cytometric analysis for the identification of the innate and adaptive immune cells of murine lung."
3. Please provide an email address for each author.
4. Please keep the word count of the summary between 10 and 50 words.
5. Please keep the word count of the abstract between 150 and 300 words.
6. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.
7. Use numbered steps in your protocol 1., 1.1., 1.1.1., 1.1.1.1.; no letters, bullets, dashes.
8. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
10. 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. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
11. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.
12. Please remove the embedded Tables from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls file. The table legend or caption (title and description) should appear in the Figure and Table Legends section after the

Representative Results in the manuscript text.
13. Please consider providing solution composition as Tables in separate .xls files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.
14. Please include a title and a description of each figure and/or table in its legend. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).
15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
16. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.
17. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.
18. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.
19. Please sort the Materials Table alphabetically by the name of the material.
20. Files uploaded to the "Supplemental Files (as requested by JoVE)" section of your Editorial Manager account are only for JoVE's internal use and will NOT be published with your article. If you would like your files to be available for download with your article, then please move them to the "Supplemental Code Files" section of your Editorial Manager account.

A: In response to the editorial comments we have:

1) *Modified the title*
2) *Provided email addressed for all authors*
3) *Shortened the summary length*
4) *Reformatted the reference presentation in the text into a superscript number.*
5) *Renumbered the protocol steps to keep only numbers and not letters as indicators of sections and subsections.*
6) *Revised the text to remove any personal pronouns*
7) *Revised the protocol description so that is all in imperative tense.*
8) *Removed embedded tables and provided them as separate excel files.*

9) *Added acknowledgments and conflict of interest section.*

Reviewer #1:

Manuscript Summary:

The authors provide a detailed method for obtaining a single cell suspension from lung tissue and identifying immune cells in this organ during homeostasis. For the detection of these different immune cells they propose a small flow cytometry staining panel that makes optimal use of the selective surface and intracellular proteins expressed on the different immune cell populations. Overall, this manuscript has been very well written and I am convinced that this will help researchers in there panel design in order to investigate lung immune cells. The main weakness is the fact that the proposed gating strategies and flow cytometry panel are very useful in steady state, however no remarks are made on how these populations behave in infection/disease. As most scientist will not only look at steady state lungs, but rather manipulate this environment somehow, it would be interesting/add significantly to the manuscript if this was mentioned/discussed in the manuscript.

Major Concerns:

-

Minor Concerns:

line 54: CD206 expression is also observed on alternatively activated alveolar macrophages. This should be mentioned in the introduction, to avoid confusion and clarity.

A: We made this clarification in the introduction lined 79-80 and added a relevant citation (ref#8).

line 61: Conventional CD103 DCs and conventional CD11b DCs are also known as cDC1 and cDC2, respectively. It would be beneficial to include this nomenclature for clarity.

A: We added this information (lines 84-85).

line 78: inflammatory eosinophils are also found in the lung interstitium, please amend.

A: We made this clarification (line 101).

line 80: lung-resident eosinophils have been suggested to have a regulatory role in allergy, in my opinion this has not been fully proven. Could the authors revise this sentence to be less strong.

A: We revised this point as requested (line 103).

line 131: It would be helpful to include a note to emphasize the need to remove all the adjacent fat from the lungs before further preparing the single cell suspension, as this could bias the read outs.

A: We emphasized this point (lines 156-157).

line 175: Please add a note in which the scientists are recommended to titrate the individual antibodies for optimal staining and to reduce costs.

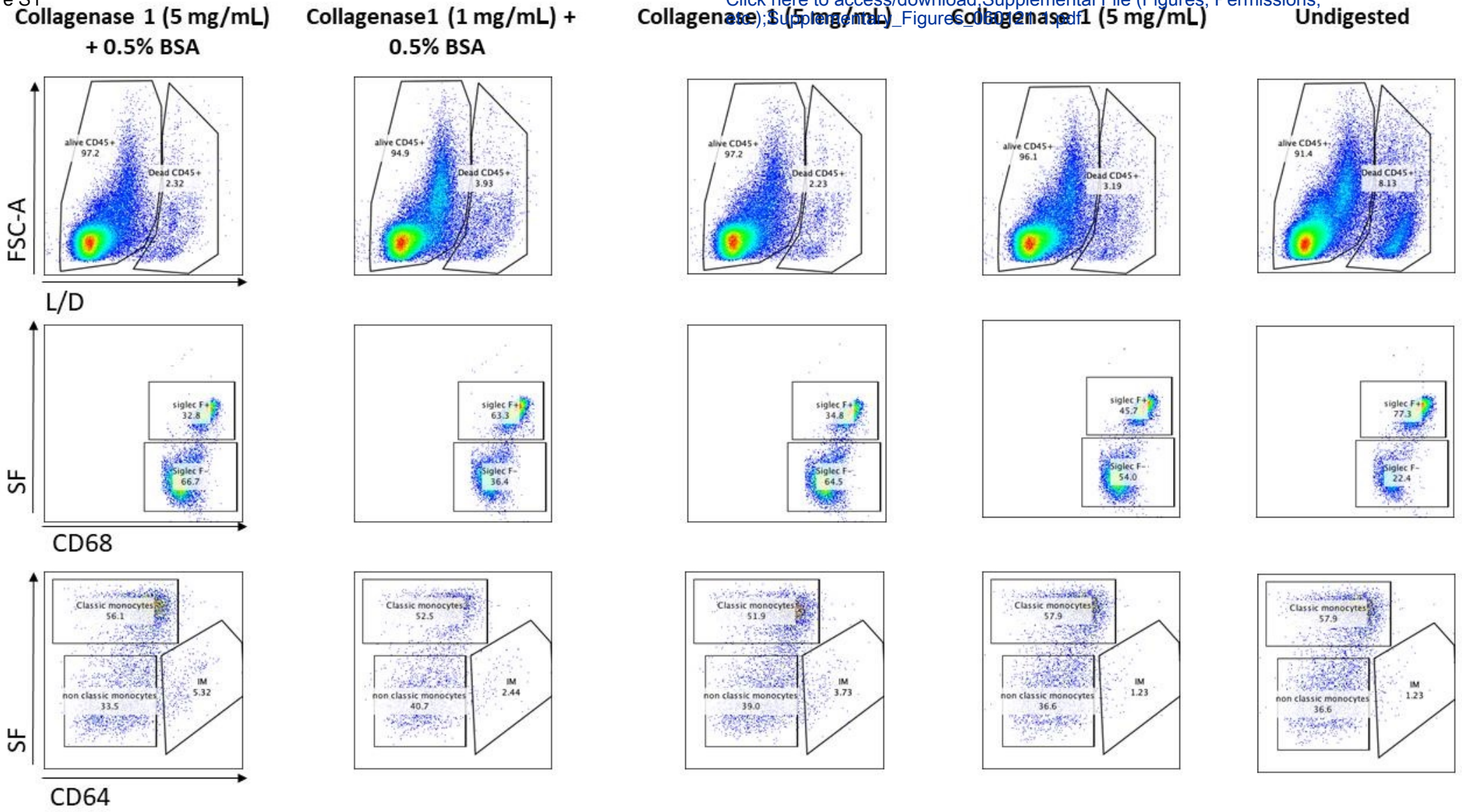
A: We added this point (lines 233-234).

line 197: To me it is not clear why the FoxP3 staining kit (that is necessary for intranuclear

staining) has to be used, whereas the CD68 is located in the cytoplasm. If so it would be of note to add a sentence providing additional possibilities to stain intracellularly (e.g. low concentration PFA, or cytofix/cytoperm kit from eBioscience (or other vendors)).
A: We added this clarification (lines 235-238)
Figure 3: Within this gating strategy could the authors provide clarity on where the NKT cells would fall? It seems as if these cell would be the little blob in CD3+ cells within the NK cell gate. Although very little, the possible contamination of the NK cell (or other gate) with NKT cells should be addressed in the manuscript.
A: We added this clarification in the Figure legend of Figure 3.

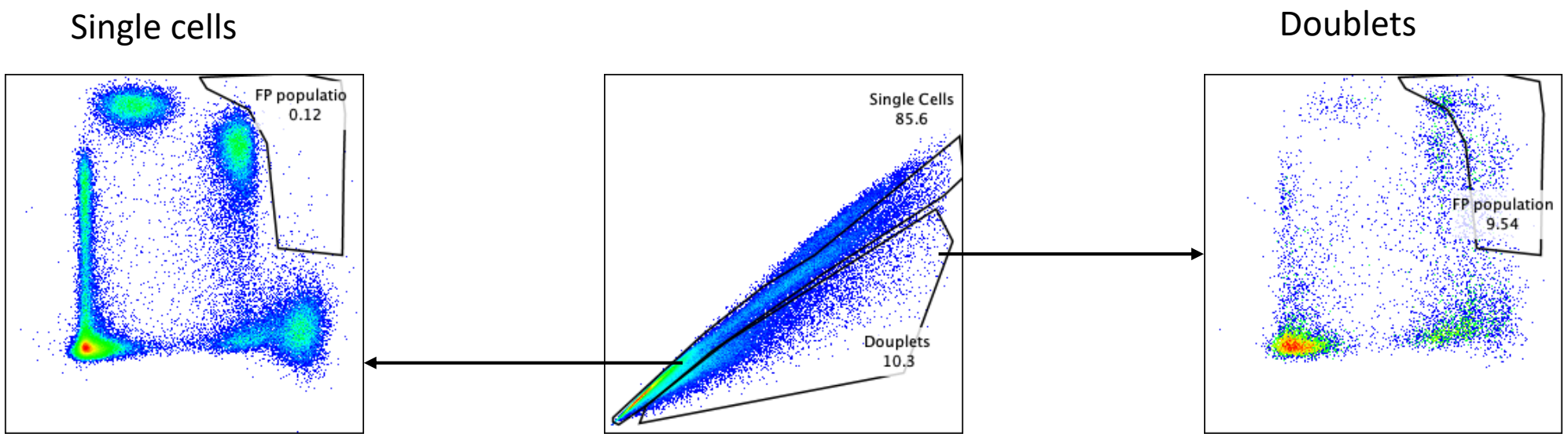
Reviewer #2:
Manuscript Summary:
Thank you for the opportunity to review this manuscript. It is an excellent piece of work and a valid contribution to the field. The methods are clearly described and easy to follow. The manuscript is concise and well written.

Major Concerns:
Nil
Minor Concerns:
For the gating fiures (Figures 1,2,3,4) it is hard to read the labels on plots (i.e the label of each cell population). These should be made clearer.
A: We increased the font size in all figures so that the labels in the plots are clearer to read.

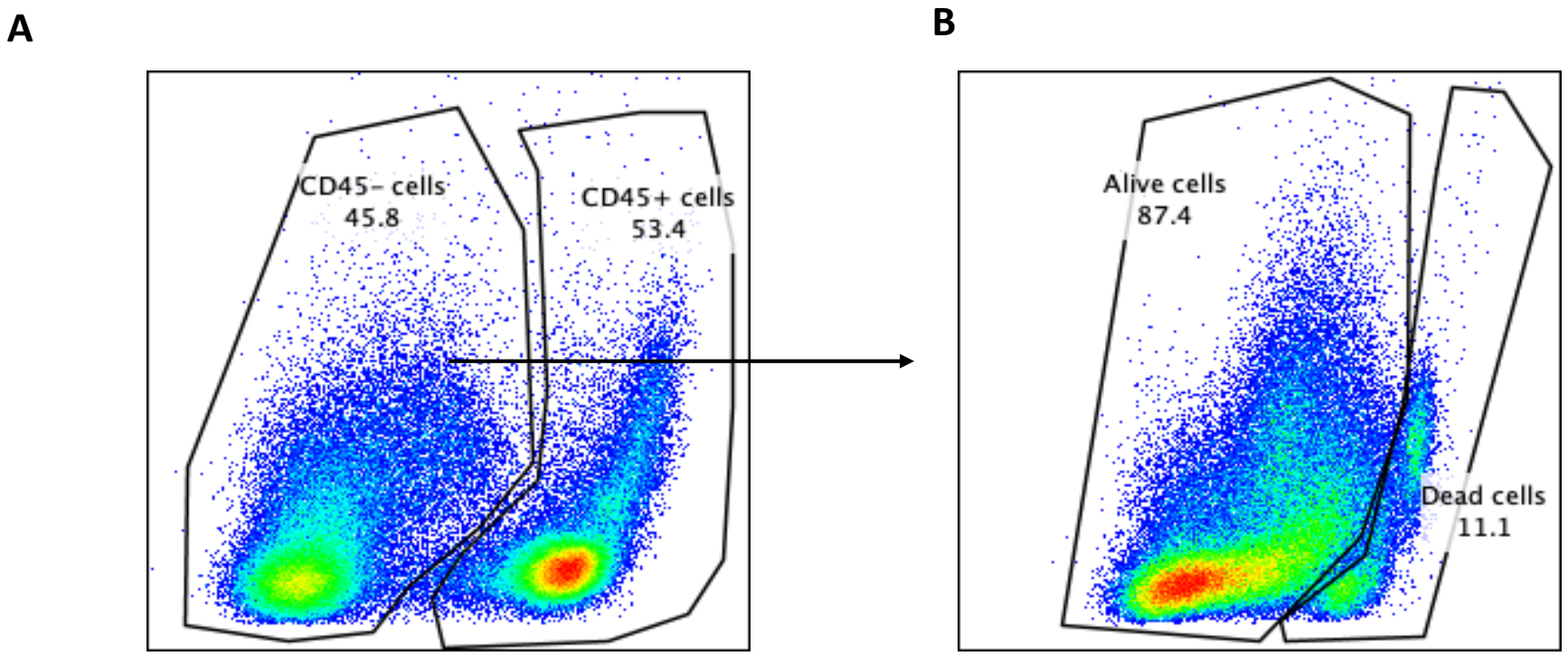


Supplemental Figure S1

Supplemental Figure S2

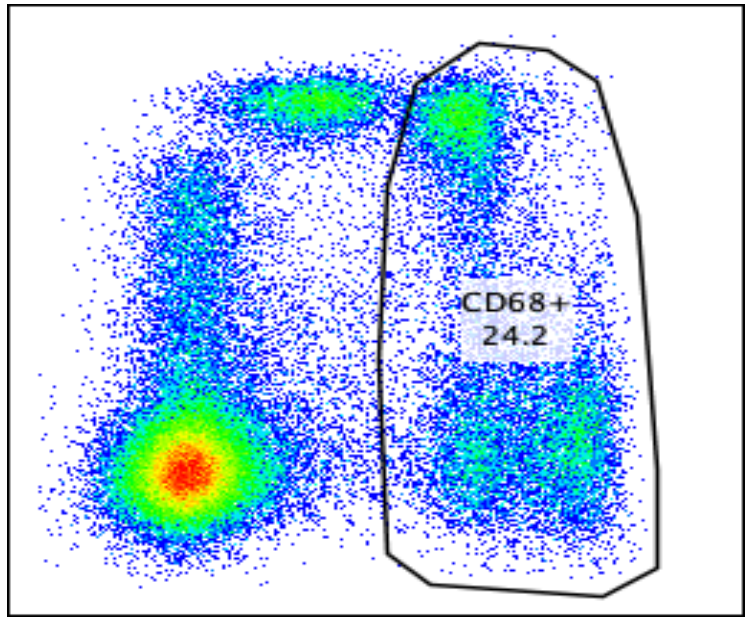


Supplemental Figure S3



Supplemental Figure S4

Intracellular CD68



Surface CD68