Chapter 23

Flow Cytometric Methods for the Assessment of Allergic Disease

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Abstract

Multiparametric flow cytometry is a powerful technique that allows the quantification and characterization of heterogeneous populations of cells. Advances in flow cytometric instrumentation, software, and reagents are occurring at a rapid pace, and flow cytometric methods are increasingly being applied to better understand cellular responses associated with a diverse array of disease conditions. This chapter provides an overview of some common applications of flow cytometry in characterizing mouse models of allergic airway disease.

Key words Allergy, Lung, Flow cytometry, FACS

1 Introduction

Allergic airway inflammation can involve multiple immune cell subsets including macrophages, dendritic cells, eosinophils, neutrophils, B lymphocytes, T lymphocytes, and natural killer cells. Accurately characterizing the cellular infiltration of the lungs is therefore an important component of investigating the immunological mechanisms responsible for the initiation and progression of allergic airway disease. This has historically been accomplished by cytocentrifugation and differential staining of cells recovered from a bronchoalveolar lavage (BAL), which allows identification of neutrophils, eosinophils, monocytes, and lymphocytes based on morphology and nuclear characteristics. However, this approach is limited in its ability to identify rare cells or accurately distinguish between biologically distinct populations of cells that do not dramatically differ in cellular morphology.

Flow cytometry employs fluorescently conjugated antibodies to identify the phenotypes of individual cells within a heterogeneous population. Multiparametric flow cytometry thereby allows extensive quantitative identification and characterization of a much

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broader range of cell populations than traditional differential staining. Recent advances in flow cytometry instrumentation, software, and reagents have greatly enhanced our ability to simultaneously assess the expression of multiple cell markers, which has led to an improved appreciation of the complexity and heterogeneity of the immune response in multiple inflammatory conditions. This chapter discusses some of the common applications of flow cytometry in assessing allergic airway disease.

1.1 Selecting Markers to Phenotypically Characterize Heterogeneous Populations of Cells

While multiparametric flow cytometric methods are increasingly being applied to mouse models of allergic disease, the field is still developing and has yet to reach consensus on the best markers and phenotyping schemes with which to characterize specific models. Thus, marker selection largely depends on the specific experimental objective. For example, an investigator may wish to simply determine the frequency of CD4+ T lymphocytes infiltrating the lungs, which will require a very different set of markers than those used to assess the activation status of lung dendritic cells.

A universal consideration in developing accurate phenotyping schemes is to examine a sufficient number of markers to accurately identify the population of interest. This is easy to accomplish when a population is identifiable by a highly specific marker, but can be challenging when attempting to discriminate between populations with shared or overlapping marker expression. As a general principle, the greater the number of markers that are simultaneously examined, the better the ability to resolve distinct subsets and accurately capture the overall heterogeneity of a population. However, increasing the number of markers requires careful validation of fluorochrome combinations and is ultimately limited by the detectors on the available instrument and the number of fluorochromes that can be simultaneously resolved based on their emission spectra.

The choice of fluorochromes that can be used in an experiment is largely determined by the instrument available to analyze the samples. For example, a BD LSRII with multiple spatially separated lasers will allow the simultaneous analysis of a much broader range of fluorochromes than a BD Calibur. It is therefore important to first determine the lasers and detectors that are available on the cytometer that is to be used for analysis before designing panels of compatible fluorochromes (*see* **Note 1**).

1.2 Fluorescence-Activated Cell Sorting

Fluorescence-activated cell sorting (FACS) is a natural extension of flow cytometric analysis and allows subsets identified in a heterogeneous mixture to be isolated and collected as highly purified viable populations. These sorted cells can then be used for a range of downstream applications, including in vitro experiments, gene expression profiling, and adoptive transfer. The protocols for preparing cells for FACS are largely identical to those used for general

flow cytometric analysis, but may need to be scaled up in order to recover adequate number of cells for downstream applications. FACS also requires more complex and expensive cytometric instrumentation than normal flow cytometric analysis, and access to cell sorters is often more limited than to analyzers. Therefore, experiments involving FACS generally warrant more careful advance planning and scheduling, and it is generally advisable to first validate flow cytometry protocols on a normal analyzer before progressing to cell sorting.

1.3 Intracellular Cytokine Detection

In addition to identifying markers expressed on the surface of cells, flow cytometry can also be used to detect intracellular markers. This technique can be used to identify phosphorylated signaling proteins or cytokine production in individual cells, thereby allowing a detailed assessment of functional responses in a heterogeneous population. Given that lung inflammation is thought to be mediated by Th2-polarized CD4+ T cells, intracellular cytokine staining has historically been applied to allergy models to determine the proportion of Th1 and Th2 cells infiltrating the lungs [1, 2]. With the advent of more complex multiparametric flow cytometry, this analysis can be expanded to also simultaneously identify Th9 and Th17 subsets, which have also recently been implicated in the pathology of airway inflammation [3, 4]. While this type of analysis can be accomplished by individually examining the expression of canonical cytokines associated with each of these Th subsets, examining them simultaneously allows the identification of cells that coexpress various combinations of cytokines, thereby providing a more accurate representation of the functional and phenotypic heterogeneity within a population.

2 Materials

2.1 Cell Isolation

1. Experimental mice sensitized and challenged with ovalbumin and appropriately matched control mice.
2. Equipment: Dissecting scissors; fine-tipped forceps; tracheal cannula; 30-G needles; 1 ml syringes; 10 ml syringes and surgical thread (4–0 silk); 30 mm culture dishes; 70 μm nylon cell strainers; 50 ml polypropylene conical centrifuge tubes; and a refrigerated centrifuge.
3. Stock solution of Hank's buffered salt solution (HBSS) without calcium or magnesium.
4. Stock solution of 0.5 M EDTA, pH 8.0.
5. Stock solution of RPMI 1640 medium.
6. Stock solution of penicillin–streptomycin (10,000 I.U. penicillin and 10 mg/ml streptomycin).

7. 100 % bovine serum albumin (BSA).
8. 100 % fetal bovine serum (FBS), standard grade, heat inactivated.
9. DNase I.
10. Collagenase type I.
11. BAL buffer: HBSS with 0.5 % BSA and 0.5 mM EDTA.
12. Digestion buffer (prepare on the day of the experiment): RPMI supplemented with 10 % FBS, 50 I.U./50 μg/ml of penicillin–streptomycin, 1.5 mg/ml of collagenase, and 150 μg/ml of DNase I.
13. ACK lysis buffer: 8.024 mg/l of NH_4Cl, 1.001 mg/l of $KHCO_3$, 3.722 mg/l of Na_2EDTA, pH 7.4.

2.2 Antibody Staining and Flow Cytometric Analysis

1. Equipment: 12 × 75 mm polystyrene round-bottom tubes; a refrigerated centrifuge; and a flow cytometer.
2. Fc block: Purified anti-mouse CD16/CD32 monoclonal antibody, clone 2.4G2 (*see* **Note 2**).
3. FACS buffer: HBSS with 1 % FCS and 0.3 mM EDTA.
4. FACS blocking buffer: FACS buffer with 5 μg/ml of Fc block.
5. Monoclonal antibodies (*see* **Note 3**).
6. Viability dye (e.g., LIVE/DEAD® Fixable Dead Cell Stains, *see* **Note 4**).
7. BD CompBeads (*see* **Note 5**).

2.3 Cellular Activation for Intracellular Cytokine Detection

1. Sterile round-bottom 96-well culture plate.
2. Culture medium: RPMI 1640 supplemented with 10 % FCS, 50 μg/ml of penicillin–streptomycin, and 2 mM L-glutamine.
3. Dimethyl sulfoxide (DMSO, ACS reagent grade).
4. Brefeldin A (BFA): Prepare a stock solution at a concentration of 10 mg/ml in DMSO and store at 4 °C.
5. DNase I: Prepare a stock solution at a concentration of 2 mg/ml in sterile PBS and store in small aliquots at −20 °C.
6. Ovalbumin: Prepare a stock of 50 mg/ml in sterile deionized water and store in small aliquots at −20 °C.
7. Phorbol 12-myristate 13-acetate (PMA): Prepare a stock solution at a concentration of 1 mg/ml in DMSO and store in small aliquots at −20 °C.
8. Ionomycin: Prepare a stock solution at a concentration of 1 mg/ml in DMSO and store in small aliquots at −20 °C.
9. Cell culture-grade monoclonal anti-CD28 antibody (clone 37.51).
10. Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences).

3 Methods

3.1 Preparing a Single-Cell Suspension of Airway Cells (See Note 6)

1. Obtain experimental mice that have been sensitized and challenged with ovalbumin and appropriately matched control mice.
2. Working with one mouse at a time, euthanize the mouse by CO_2 asphyxiation, but do not perform a cervical dislocation. Proceed immediately with the following steps.
3. Place the mouse in a supine position and wipe down the neck and chest area with 70 % ethanol.
4. Make a vertical incision over the upper thoracic cavity and carefully remove the skin and muscle tissue to expose the trachea.
5. Pull up the trachea with tweezers and insert a length of surgical thread behind the trachea and on top of the esophagus.
6. Make a small horizontal incision between two tracheal rings.
7. Insert a tracheal cannula into the incision and secure it to the trachea using the surgical thread.
8. Fill a 1 ml syringe with 900 ml of BAL buffer and fit it to the tracheal cannula.
9. Slowly inject the buffer to fully inflate the lungs and then slowly withdraw the fluid. Transfer the recovered buffer to a 15 ml conical tube and store on ice (*see* **Note 7**).
10. Repeat **steps 8** and **9** three more times to collect approximately 3 and 4 ml of fluid.
11. Centrifuge cells at 300 × *g* for 5 min at 4 °C.
12. Aspirate the supernatant and resuspend the pellet in 1 ml of ACK lysis buffer. Gently swirl the tube for 30–60 s to lyse any residual red blood cells.
13. Fill the tube with HBSS and centrifuge at 300 × *g* for 5 min at 4 °C.
14. Aspirate the supernatant and resuspend the cells in 1 ml of FACS buffer until ready to proceed with cell stimulation for intracellular cytokine detection (*see* Subheading 3.3) or antibody staining to identify cell surface markers (*see* Subheading 3.4).

3.2 Preparing Single-Cell Suspensions of Total Lung and Lung-Draining Lymph Nodes (*See* Note 6)

1. Obtain experimental mice that have been sensitized and challenged with ovalbumin and appropriately matched control mice (*see* **Note 8**).
2. Euthanize a single mouse by CO_2 asphyxiation, but do not perform a cervical dislocation.
3. Place the mouse in a supine position and wipe down the neck and chest area with 70 % ethanol.
4. Make a vertical incision over the thoracic cavity and carefully remove the skin and muscle tissue. Retract the rib cage to expose the lungs and heart.

5. Carefully remove the mediastinal lymph nodes found below the thymus under the right side of the heart. These lymph nodes can be challenging to find in a naïve mouse, but are generally enlarged in inflamed mice making them easier to identify. Place the lymph nodes into 1 ml of digestion buffer in a 35 mm culture dish and store on ice until the lungs have been isolated.
6. Fill a 10 ml syringe with HBSS and fit it with a 30 G needle. Insert the needle into the right ventricle and maneuver it towards the pulmonary artery. Make a small incision in the left atrium. Very gently perfuse the HBSS to clear cells from the pulmonary circulation. The lungs should turn white during the perfusion step. An additional 10 ml of HBSS can be perfused if adequate perfusion is not achieved on the first pass.
7. Dissect the lung lobes and place them in 5 ml of digestion buffer in a 35-mm culture dish.
8. Use two 30 G needles to break open the lymph nodes. Use a scalpel or a scissors to mince the lung into small pieces. Incubate the dishes at 37 °C for 30 min to allow enzymatic digestion of the tissue.
9. Transfer the lung and lymph node suspensions to separate 50 ml conical tubes passing them through a 70 μm nylon cell strainer. Use the plunger of a syringe to gently mash the residual tissue on the filter. Wash the filter two times with 10 ml of FACS buffer and keep the cell suspensions on ice until the tissues have been harvested from all the animals.
10. Repeat **steps 2–11** for each individual mouse.
11. Centrifuge the cells at 300 × *g* for 5 min at 4 °C.
12. Aspirate the supernatant and resuspend the pellets in 1 ml of ACK lysis buffer. Swirl the tubes for 30–60 s to lyse any residual red blood cells. Add 15 ml of HBSS and centrifuge the cells at 300 × *g* for 5 min at 4 °C.
13. Aspirate the supernatant and resuspend the cells in 1 ml of FACS buffer until ready to proceed with cell stimulation for intracellular cytokine detection (*see* Subheading 3.3) or antibody staining to identify cell surface markers (*see* Subheading 3.4).

3.3 Ex Vivo Restimulation to Induce Cytokine Production (See Note 9)

1. Count the cells and resuspend them in culture medium at a density of 0.2–2 × 10^6 cells/100 μl (*see* **Note 10**).
2. Label a 96-well U-bottom plate to identify samples and stimulation conditions. Pipet 100 μl of the cell suspensions into each of the appropriate wells.
3. Prepare an appropriate volume of 2× restimulation media using culture media supplemented with the components indicated in Table 1 (*see* **Note 11**).

Table 1
Composition of restimulation media

DNase I	40 μg/ml (1:50 dilution of stock)
Brefeldin A	20 μg/ml (1:500 dilution of stock)
(1) Ovalbumin or	1 mg/ml (1:50 dilution of stock)
(2) PMA and	100 ng/ml (1:10,000 dilution of stock)
Ionomycin or	1 μg/ml (1:1,000 dilution of stock)
(3) DMSO	1:1,000 dilution

The components listed in the above table should be added to the culture media for ex vivo cell restimulation to induce cytokine production

4. Add 100 μl of the appropriate 2× restimulation medium to the 100 μl of cells in the wells and mix well.
5. Incubate for 5 h in an incubator at 37 °C with 5 % CO_2.
6. After incubation, proceed to centrifugation of the plate at 300 × *g* for 4 min at room temperature.
7. Rapidly invert the plate to discard the supernatant into a waste container and gently blot the plate onto a paper towel.
8. Resuspend the cells in 200 ml of FACS buffer and proceed to **step 2** of the antibody staining protocol (Subheading 3.4) (*see* **Note 12**).

3.4 Antibody Staining to Phenotype Heterogeneous Cell Populations

1. Divide the cells into an appropriate number of wells on a 96-well round-bottomed plate (*see* **Note 12**) based on the number of available cells and the number of separate staining panels that are to be used.
2. Centrifuge the plate at 300 × *g* for 4 min at 4 °C.
3. Rapidly invert the plate to discard the supernatant into a waste container and gently blot the plate onto a paper towel.
4. Resuspend cells in 100 μl of FACS blocking buffer and leave on ice for at least 10 min.
5. Prepare antibody master mixes in FACS blocking buffer for each staining panel. The specific combination of antibodies in the master mix will depend on the experimental objectives and the available cytometer (*see* **Note 4**). Master mixes should allow for a final staining volume of 100 μl/sample and should include each of the antibodies at a concentration predetermined by titration. This will typically be in the range of 0.2–2 μl/100 μl of buffer.
6. Add 100 μl of FACS buffer to each of the wells.

7. Centrifuge at 300 × *g* for 4 min at 4 °C.
8. Rapidly invert the plate to discard the supernatant into a waste container and gently blot the plate onto a paper towel.
9. Add 100 μl of the appropriate antibody master mix to each well.
10. Incubate for 30 min at 4 °C in the dark.
11. Add 100 μl of FACS buffer to each of the wells.
12. Centrifuge at 300 × *g* for 4 min at 4 °C.
13. Rapidly invert the plate to discard the supernatant into a waste container and gently blot the plate onto a paper towel.
14. Resuspend the cells in 200 μl of FACS buffer.
15. Centrifuge at 300 × *g* for 4 min at 4 °C.
16. Rapidly invert the plate to discard the supernatant into a waste container and gently blot the plate onto a paper towel.
17. If staining for intracellular cytokines, proceed to Subheading 3.5. Alternatively, if the analysis only involves cell surface markers, then resuspend the samples in 250 μl of FACS buffer and transfer them to tubes that are compatible with the available cytometer (e.g., 12 × 75 mm polystyrene round-bottom tubes). Store the samples at 4 °C in the dark until analysis (*see* **Note 13**).
18. Use CompBeads to prepare an unstained and single-stained compensation controls individually stained with each of the antibodies used in the master mix (*see* **Notes 5** and **14**).

3.5 Antibody Staining to Identify Intracellular Cytokines

1. Resuspend the cells in 100 μl of Cytofix–Cytoperm buffer and mix well (*see* **Note 15**).
2. Incubate for 20 min at room temperature in the dark (*see* **Note 16**).
3. Add 100 μl of 1× perm/wash buffer to each of the wells.
4. Centrifuge at 300 × *g* for 4 min at 4 °C.
5. Rapidly invert the plate to discard the supernatant into a waste container and gently blot the plate onto a paper towel.
6. Resuspend the cells in 200 μl of 1× perm/wash buffer.
7. Prepare an intracellular staining antibody master mix in 1× perm/wash buffer allowing for a final staining volume of 100 μl/sample. The specific combination of antibodies in the master mix will depend on the experimental objectives and the available cytometer (*see* **Note 17**).
8. Centrifuge at 300 × *g* for 4 min at 4 °C.
9. Rapidly invert the plate to discard the supernatant into a waste container and gently blot the plate onto a paper towel.
10. Resuspend the cells in 100 μl of the appropriate antibody master mix.

11. Incubate for 20 min at 4 °C in the dark.
12. Add 100 μl of 1× perm/wash buffer to each of the wells.
13. Centrifuge at 300 × *g* for 4 min at 4 °C.
14. Rapidly invert the plate to discard the supernatant into a waste container and gently blot the plate onto a paper towel.
15. Resuspend the cells in 200 μl of 1× perm/wash buffer.
16. Centrifuge at 300 × *g* for 4 min at 4 °C.
17. Rapidly invert the plate to discard the supernatant into a waste container and gently blot the plate onto a paper towel.
18. Resuspend the cells in 250 μl of FACS buffer, transfer them to tubes that are compatible with the available cytometer (e.g., 12 × 75 mm polystyrene round-bottom tubes), and store at 4 °C in the dark until analysis (*see* **Note 13**).

3.6 Flow Cytometric Acquisition

1. Turn on the cytometer and allow sufficient time for the lasers to warm up.
2. Select appropriate lasers and parameters to match the fluorochromes used in the experiment.
3. Load a tube of unstained cells onto the cytometer and begin to acquire events.
4. Adjust the PMT voltages so that the majority of the cells of interest are in the lower left quadrant of a linear FSS and SSC plot and within the first two logarithmic decades for each of the fluorescence parameters (*see* **Note 18**).
5. Briefly acquire an experimental sample and adjust PMT voltages to ensure that none of the fluorescence parameters are off the scale.
6. Acquire the single-color and unstained CompBeads and calculate fluorescence compensation (*see* **Note 14**).
7. Create histograms and cytograms and draw gates to allow the identification of the target populations of interest.
8. Begin acquisition of experimental samples and aim to collect a sufficient number of events to allow statistically justified conclusions to be drawn (*see* **Note 19**).
9. For data analysis and anticipated results, *see* **Notes 20–22**.

4 Notes

1. Having identified the fluorochromes that can be detected using a given instrument, the choice of specific fluorochrome combinations is largely dependent on the markers that are to be assessed. Ideal combinations will include fluorochromes

with maximal fluorescence intensities that have minimal spectral overlap with each other. Identifying appropriate combinations of fluorochromes with these characteristics becomes increasingly challenging with increasing number of markers. Generally, the brightest fluorochromes, such as phycoerythrin (PE) and allophycocyanin (APC), are best reserved for markers that are only expressed on rare cells or at a low density on the cells. Conversely, dimmer fluorochromes, such as Alexa Fluor 700, can be used with markers that are highly expressed on distinct populations. There are a number of useful Web-based tools that have been designed to facilitate the design of complementary fluorochrome panels, including the BD Biosciences Fluorescence Spectrum Viewer, Flourish from Treestar, and CytoGenie from Woodside Logic.

2. Clone 2.4G2 is a rat IgG2b κ antibody and therefore cannot be used with staining panels involving an anti-rat secondary antibody. In this situation, the 2.4G2 Fc blocking antibody can be substituted with 10 % normal mouse serum.
3. It is generally most convenient if all the antibodies used in a panel are directly conjugated to fluorochromes that are compatible with the detectors on the available cytometer. If directly conjugated antibodies are not available, an indirect labeling protocol can be employed using a biotin-conjugated primary antibody followed by streptavidin-conjugated fluorochrome, or an unlabeled primary antibody and a fluorochrome-conjugated anti-isotype secondary antibody. Indirect labeling protocols can result in signal amplification, allowing greater sensitivity than direct labeling; however, they introduce additional complexity to experiments because of the need to identify compatible combinations of primary and secondary antibodies, and because of the potential of secondary antibody cross-reactivity and high background staining. Addressing these issues becomes increasingly more challenging when attempting to combine larger number of antibodies in a panel. All of the antibodies used in a panel should be titrated to determine optimal staining concentrations.
4. As discussed earlier, the specific choice of markers and fluorochromes will depend on the specific experimental objectives. Table 2 presents an example of two phenotyping schemes that could potentially be used with a 4-laser cytometer to accurately identify several major immune subsets in the lung that have been reported to be involved in allergic airway disease [5–8]. These schemes can be modified to focus on particular populations of interest, and the choice of complementary fluorochrome combinations can be optimized to accommodate the capabilities of the available cytometer. Note that these phenotyping schemes include a viability dye (e.g., 7AAD, DAPI,

Table 2
Phenotyping schemes for use with a 4-laser cytometer

A. Antibody panel	Identifiable cell populations and key defining markers
AmCyan anti-CD45 (30-F11)	*NK cells*: $CD3^-$, $NK1.1^+$
Pacific Blue anti-CD3 (17A2)	*NKT cells*: $CD3^+$, $NK1.1^+$
PE-Cy7 anti-NK1.1 (PK136)	*γδ T cells*: $CD3^+$, $TCR\gamma\delta^+$
FITC anti-TCRγδ (UC7-13D5)	*CD4+ αβ T cells*: $CD3^+$, $TCR\gamma\delta^-$, $NK1.1^-$, $CD4^+$, $CD8^{low}$
APC-Cy7 anti-CD4 (RM4-5)	Naïve subset: $CD44^{low}$, $CD62L^{hi}$
Pac. Blue anti-CD8 (53-6.7)	Effector/effector memory subset: $CD44^{hi}$, $CD62L^{low}$
PE anti-CD62L (MEL-14)	Central memory subset: $CD44^{hi}$, $CD62L^{hi}$
APC anti-CD44 (IM7)	*$CD8^+$ αβ T cells*: $CD3^+$, $TCR\gamma\delta^-$, $NK1.1^-$, $CD4^{low}$, $CD8^+$
DAPI (viability stain)	Naïve subset: $CD44^{low}$, $CD62L^{hi}$ Effector/effector memory subset: $CD44^{hi}$, $CD62L^{low}$ Central memory subset: $CD44^{hi}$, $CD62L^{hi}$
B. Antibody panel	**Identifiable cell populations and key defining markers (*see* Fig. 1)**
AmCyan anti-CD45 (30-F11)	*Alveolar macrophages*: $Autofluorescence^{hi}$, $F4/80^{hi}$, $CD11c^{hi}$, $MHCII^{int}$, $CD11b^{low}$
FITC anti-F4/80 (BM8)	*Interstitial macrophages*: $Autofluorescence^{hi}$, $F4/80^{hi}$, $CD11c^{low}$, $MHCII^{low}$, $CD11b^{hi}$
APC-Cy7 anti-Gr-1 (RB6-8C5)	*Inflammatory dendritic cells*: $CD11c^{hi}$, $CD11b^{hi}$, $Gr\text{-}1^{hi}$
Pac. Blue anti-MHCII (M5/114.15.2)	*$CD11b^{hi}$ dendritic cells*: $CD11c^{hi}$, $MHCII^{hi}$, $CD11b^{hi}$, $CD103^{low}$
PE-Cy7 anti-CD11c (N418)	*$CD103^{hi}$ dendritic cells*: $CD11c^{hi}$, $MHCII^{hi}$, $CD11b^{low}$, $CD103^{hi}$
APC-Cy7 anti-CD11b (M1/70)	*Plasmacytoid dendritic cells*: $CD11c^{med}$, $MHCII^{med}$, $mPDCA1^{hi}$
APC anti-CD103 (M290)	*Neutrophils*: $MHCII^{low}$, $CD11c^{low}$, $CD11b^{hi}$, $Ly6G^{hi}$, $F4/80^{low}$
PE anti-mPDCA1	*Eosinophils*: SSC^{hi}, $MHCII^{low}$, $CD11c^{low}$, $CD11b^{hi}$, $F4/80^{int}$
DAPI (viability stain)	*Monocytes*: SSC^{int}, $MHCII^{low}$, $CD11c^{low}$, $CD11b^{hi}$, $F4/80^{int}$, $Gr\text{-}1^{hi}$ & $Gr\text{-}1^{low}$ subsets

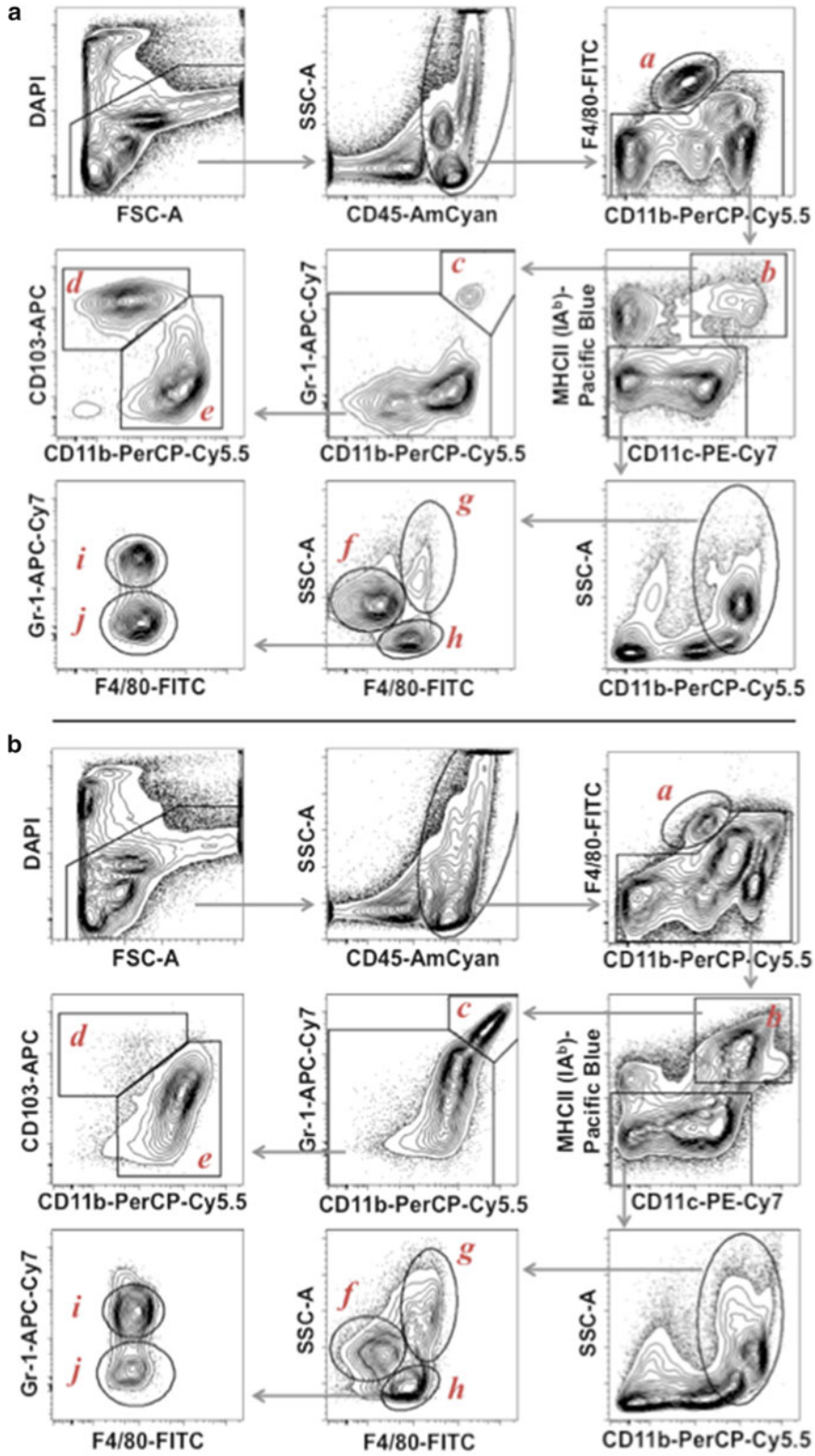
The above table lists antibody panels that can accurately identify several major immune subsets in the lung that have been reported to be involved in allergic airway disease

or LIVE/DEAD® Fixable Dead Cell Stain, Invitrogen) to facilitate the exclusion of dead cells, which are often autofluorescent and exhibit nonspecific antibody binding. Certain cell types, such as alveolar macrophages, are also highly autofluorescent and it can be difficult to distinguish this from 488 nm excited fluorochromes, which can present a challenge during data analysis. One useful strategy to deal with this is to keep the 530/50 "FITC" channel open to specifically allow for the

detection of cellular autofluorescence, which can be very useful in distinguishing highly autofluorescent macrophages from other cell types [9]. Another strategy, employed in Table 2 and in Fig. 1, is to use FITC-conjugated F4/80, a marker expressed by alveolar macrophages, allowing alveolar macrophages to be identified by any positive signal in the FITC channel.

5. Multiparametric phenotyping schemes generally use combinations of fluorochromes that exhibit some degree of spectral overlap. To address this issue, fluorescence compensation using single-color-stained controls is employed to more accurately represent the true signal in a given detector by subtracting spillover from other detectors. Ideally, compensation should be performed using suspensions of cells or antibody capture beads (e.g., BD CompBeads) individually stained with the same antibodies used in the experimental panel. It should be noted that antibody capture beads provide a number of advantages over cells and are generally preferable for more accurate and consistent compensation. A limitation of antibody capture beads is that they can only capture specific antibody isotypes, and cells must be used in instances where the antibody isotype does not match the beads. In situations where only limited numbers of cells are available, as is often the case with BAL preparations, it can sometimes be challenging to reserve adequate number of cells for single-color compensation controls. However, it should be noted that while the cells used for compensation do need to express the antigen of interest, they do not need to be from the same source as the experimental sample (i.e., splenocytes containing CD4+ T cells can be used to compensate BAL T cell samples stained with an anti-CD4 antibody).
6. The first step in any flow cytometric protocol is to generate a single-cell suspension from the tissue of interest. In the case of allergic airway disease models, inflating the lungs with a physiological saline solution and collecting the resulting BAL fluid offers a way to specifically isolate cells that have infiltrated into the airway space. As a complementary approach, digestion of

Fig. 1 Example of a gating strategy to identify major lung myeloid populations. Whole lungs from a naïve (**a**) and a challenged (**b**) C57BL/6 mouse were digested, processed, and stained with an antibody panel similar to that described in Table 2B. Viable hematopoietic cells are identified as being DAPI^{low} and CD45^{positive}. Lung macrophages (*a*) are identifiable as a distinct F4/80^{high}, CD11b^{intermediate} population. The alveolar macrophages within this population are highly autofluorescent and are CD11c^{high} and MHCII^{intermediate} (not shown). Within the F4/80^{low} population, dendritic cells (*b*) can be identified as being CD11c^{hi} and MHCII^{hi}. These dendritic cells can be further subdivided into Gr-1^{hi}, CD11b^{hi} inflammatory monocyte-derived DCs (*c*), CD103^{+} DCs (*d*), and CD11b^{+} myeloid DCs (*e*). Differences in F4/80 expression and granularity, as assessed by SSC, can be used to identify the MHCII^{low}, CD11b^{hi} cells as neutrophils (*f*), eosinophils (*g*), and monocytes (*h*). The monocyte population can be further subdivided into inflammatory Gr-1^{hi} monocytes (*i*) and resident Gr-1^{low} monocytes (*j*). Data kindly provided by Dr. Daigo Hashimoto


a
DAPI
FSC-A
SSC-A
CD45-AmCyan
F4/80-FITC
CD11b-PerCP-Cy5.5
a
CD103-APC
CD11b-PerCP-Cy5.5
d
e
Gr-1-APC-Cy7
CD11b-PerCP-Cy5.5
c
MHCII (IA^b)-Pacific Blue
CD11c-PE-Cy7
b
Gr-1-APC-Cy7
F4/80-FITC
i
j
SSC-A
F4/80-FITC
g
f
h
SSC-A
CD11b-PerCP-Cy5.5
b
DAPI
FSC-A
SSC-A
CD45-AmCyan
F4/80-FITC
CD11b-PerCP-Cy5.5
a
CD103-APC
CD11b-PerCP-Cy5.5
d
e
Gr-1-APC-Cy7
CD11b-PerCP-Cy5.5
c
MHCII (IA^b)-Pacific Blue
CD11c-PE-Cy7
b
Gr-1-APC-Cy7
F4/80-FITC
i
j
SSC-A
F4/80-FITC
g
f
h
SSC-A
CD11b-PerCP-Cy5.5

total lung tissue allows an isolation of all the cells in both the alveolar and interstitial spaces. Depending on the experimental objective, it can also be informative to examine cells in the mediastinal lung-draining lymph nodes.

7. Do not inflate the lungs with more than 1 ml of buffer because forced overinflation can result in damage to the lung.
8. The protocols for the isolation of cells from the BAL and digested lung can be combined, in that the pre-lavaged lungs can be dissected and digested. In this case, the pulmonary circulation should be perfused prior to performing the BAL. It should also be noted that the BAL is unlikely to entirely remove all cells from the airways, so the suspension of cells from the lavaged lung will likely contain a mixture of interstitial cells and partially depleted alveolar cells.
9. The detection of intracellular cytokines generally requires restimulating the cells ex vivo in the presence of an inhibitor of protein transport. This section can be skipped if the experimental analysis is only focused on the characterization of cell surface markers.
10. Using higher number of cells will facilitate the final analysis, but this will be limited by the cell yields from the tissues.
11. Ex vivo restimulation can be performed using the same antigen that was initially used to sensitize and challenge the mice (in this case, ovalbumin), which allows an assessment of the antigen-specific cytokine response. The cells can also be stimulated with an antigen-nonspecific agonist, such as PMA and ionomycin, which provides a more generalized assessment of the overall cytokine response. When stimulating the cells with a specific antigen, it is useful to also include a nonspecific stimulation condition as a positive control. It is also important to include an unstimulated condition as a negative control.
12. The subsequent antibody staining steps can be performed directly in the same 96-well plate that was used for stimulation. Alternatively, the cells can be transferred to 12 × 75 mm polystyrene round-bottom tubes for antibody staining. It is generally more convenient to use 96-well plates when processing larger number of samples (>10) because rapidly inverting the plate to discard the supernatant and adding the buffers with a multichannel pipette are faster than aspirating and resuspending the cells in individual tubes.
13. Samples should be analyzed by flow cytometry as soon as possible to avoid potential artifacts that may result from prolonged storage. This is particularly important in the case of unfixed cells where cell death is a significant concern. Formaldehyde-fixed cells that are kept in the dark at 4 °C can generally be stored for longer periods of time but should generally be analyzed within 72 h to minimize loss of fluorescence signal.

14. Accurate compensation controls require single-stained samples for each of the fluorochromes that are being used, and these single-stained samples must be at least as bright as the stained sample. It is also critical to include an unstained sample with identical autofluorescence properties to the single-stained samples. These compensation controls should be acquired on the cytometer on each day of testing using the same photomultiplier tube voltages that are used for the experimental samples. While compensation has historically been performed manually, this process can become very laborious when dealing with large number of fluorescent parameters and can be a significant source of variability. Multiple software platforms that are used for the acquisition and analysis of flow cytometry data now include algorithms for automatic compensation, and the use of these methods is generally preferable to manual compensation. It should also be noted that a number of analysis programs allow compensation to be performed post acquisition. There are a number of resources that provide a more thorough discussion of compensation [10].
15. This section of the protocol uses the BD Cytofix/Cytoperm Fixation/Permeabilization kit and largely follows the manufacturer's instructions. It can be modified to accommodate similar formaldehyde-based fixation and saponin-based permeabilization buffers provided by other manufacturers.
16. Prolonged incubation in formaldehyde-based fixation buffers can result in loss of fluorescent signals and dimmer staining of certain intracellular antigens. Fixed samples can safely be stored for several hours to days prior to proceeding with the permeabilization step. However, it is recommended that after 30 min, the formaldehyde buffer be washed off and the cells resuspended in FACs buffer for prolonged storage.
17. As is the case with cell surface markers, the specific choice of intracellular markers and fluorochromes in a panel will depend on the specific experimental objectives. Table 3 presents an example of an 8-color phenotyping scheme that could be used to subdivide the CD4+ T cell compartment into Th1, Th2, Th9, and Th17 subsets. Depending on the experimental objectives and the capabilities of the available cytometer, this phenotyping scheme could easily be modified to examine alternative combinations of cytokines, for example, simultaneously examining IL-2 and TNFα production by CD4+ and CD8+ T cells.
18. On some instruments, Cytometer Setup and Tracking (CS&T) can be used to automatically determine PMT voltages in the optimal linear response range. In these situations, the CS&T-assigned voltage settings should typically not require drastic adjustments unless experimental samples are off of the scale.

Table 3
8-color phenotyping scheme used to subdivide the CD4+ T cell compartment

Antibody panel	Identifiable cell populations and key defining markers
Surface stain	
AmCyan anti-CD45	*CD4+ αβ T cells*: $CD45^{hi}$, $TCR\beta^{hi}$, $CD4^{hi}$, $CD8^{low}$
Pac. Blue anti-TCRβ (H57-597)	*Th1*: $IFN\gamma^{+}$
PerCP-Cy5.5 anti-CD4 (RM4-5)	*Th2*: $IL\text{-}4^{+}$/$IL\text{-}5^{+}$
APC-Cy7 anti-CD8 (53-6.7)	*Th9*: $IL\text{-}9^{+}$
UV LIVE/DEAD stain	*Th17*: $IL\text{-}17A^{+}$
Intracellular stain	
PE-Cy7 anti-IFNγ (XMG1.2)	Note that subsets of CD4+ cells may coexpress combinations of these markers and may not be clearly definable within a single subset
PE anti-IL-4 (11B11) or PE IL-5 (TRFK5)	
FITC anti-IL-17A (TC11-18H10)	
APC anti-IL-9 (RM9A4)	

The above table lists an antibody panel that can accurately subdivide the CD4+ T cell compartment into Th1, Th2, Th9, and Th17 subsets

19. The total number of events collected will depend on background staining levels and the frequency of the population of interest. When dealing with a population that represents a fairly high frequency of the total sample (<10 %), collecting 5,000 events typically results in a coefficient of variation (CV) on the order of 5 %, which is generally acceptable in the field. When dealing with a rarer population, the total number of events collected must be increased. For example, if the population of interest represents only 0.1 % of the total sample, approximately 500,000 total events would need to be collected to ensure a 5 % CV. When acquiring several samples in which the frequency of the population of interest varies, it is generally good practice to gate on the population of interest and set a stopping gate to collect a consistent number of events within this population [11].
20. The acquisition of flow cytometry data results in the generation of a listmode file that can be exported for subsequent analysis. There are a number of free and commercially available programs available for data analysis. While there are significant differences in the interfaces of these programs, the fundamental principles of analysis remain the same. For the applications discussed in this chapter, the analysis largely focuses on determining the frequency of particular subpopulations within the sample, e.g., the percentage of macrophages among lung hematopoietic cells, or the percentage of IFNγ-producing cells among CD4 T cells. This is generally accomplished by sequentially gating populations until the population of interest is adequately identified. Gates are typically drawn based on whether a population is positive or negative for a marker of interest.

This practice is generally straightforward for brightly expressed markers that result in distinct positive and negative populations, but can be more challenging for dim markers or those with a broad distribution. For these challenging markers, additional staining controls should be implemented (*see* **Note 21**).

21. Fluorescently labeled isotype control antibodies have historically been used to help determine background staining levels and thereby identify positively stained cells. While this can be helpful in identifying nonspecific Fc receptor-mediated antibody binding, when conducting multiparameter experiments, it is generally more accurate to employ a "fluorescence-minus-one" (FMO) control [10]. FMO controls are samples stained with all the antibodies in the panel except for the antibody of interest, providing an accurate measure of background staining that can be used to set a threshold for positivity for a given marker.
22. Gating strategies will vary based on the specific markers used in an experiment, and multiple strategies can typically be used to ultimately identify the population of interest. In complex multidimensional experiments it is often wise to attempt to visualize populations using several alternative gating strategies to determine the best way to identify sample heterogeneity. Figure 1 provides an example of a gating strategy used to identify major myeloid populations in total digested lung that was stained using a panel similar to that presented in Table 2B.

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