A Fast, Easy, and Customizable Eight-Color Flow Cytometric Method for Analysis of the Cellular Content of Bronchoalveolar Lavage Fluid in the Mouse

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The cell composition of bronchoalveolar lavage fluid (BAL) is an important indicator of airway inflammation. It is commonly determined by cytocentrifuging leukocytes on slides, then staining, identifying, and counting them as eosinophils, neutrophils, macrophages, or lymphocytes according to morphological criteria under light microscopy, where it is not always easy to distinguish macrophages from lymphocytes. We describe here a one-step, easy-to-use, and easy-to-customize 8-color flow cytometric method for performing differential cell count and comparing it to morphological counts on stained cytospins. This method identifies BAL cells by a simultaneous one-step immunolabeling procedure using antibodies to identify T cells, B cells, neutrophils, eosinophils, and macrophages. Morphological analysis of flow-sorted cell subsets is used to validate this protocol. An important advantage of this basic flow cytometry protocol is the ability to customize it by the addition of antibodies to study receptor expression at leukocyte cell surfaces and identify subclasses of inflammatory cells as needed.

Keywords: flow cytometry • bronchoalveolar lavage • inflammation • asthma • macrophages

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INTRODUCTION

Numerous mouse models of airway diseases have been developed and are widely used to study the pathophysiology of such airway disorders as allergic asthma, chronic obstructive pulmonary disease, and lung fibrosis (Crestani et al., 2012; Daubeuf & Frossard, 2013; Kips et al., 2003; Mouratis, 2011; Nials & Uddin, 2008; Shin, Takeda, & Gelfand, 2009; Vodovotz et al., 2006). In these models, evaluation of the leukocytes recruited to the airways is a critical procedure for evaluating the extent of inflammation and the effect of anti-inflammatory drugs or strategies (Nials & Uddin, 2008).

Bronchoalveolar lavage (BAL), together with its analysis, is an essential technique for measuring inflammatory response and exploring immune mechanisms in the lungs (Daubeuf & Frossard, 2012; Henderson, 1994; Hunninghake, Gadek, Kawanami, Ferrans, & Crystal, 1979). BAL fluid (BALF) reflects the inflammatory cells that have infiltrated the airways in, for example, allergic asthma models, where it contains eosinophils, neutrophils, macrophages, and lymphocytes (Blé et al., 2008, 2009; Daubeuf & Frossard, 2013; Delayre-Orthez, Becker, Auwerx, Frossard, & Pons, 2008; Delayre-Orthez, Becker, De Blay, Frossard, & Pons, 2004, 2005a; Delayre-Orthez et al., 2005b; Deschamps et al., 2011; Gasparik et al., 2012; Hachet-Haas et al., 2008; Reber, Daubeuf, Pejler, Abrink, & Frossard, 2014; Zhang et al., 2009). After either May-Grünwald Giemsa or Diff-Quick staining of cytospun cells, BALF cellular composition is commonly determined by morphological criteria under optical microscopy (described in Daubeuf & Frossard, 2012). Other authors have successfully used hematology analyzers, such as the Sysmex XT-2000i or ADVIA 120 (Mathers, Evans, Bleby, & Tornow, 2007). Nonetheless, these counts cannot discriminate between lymphocyte subtypes (e.g., T and B cells) nor can they be used to study receptor expression at leukocyte cell surfaces (Van Rijt et al., 2004).

Today, however, the wide availability of multicolor flow cytometers with eight or more colors and the development of a wide range of fluorochrome-labeled antibodies enable rapid and efficient analysis of several cell types in large numbers of samples. Specifically, flow cytometry simultaneously allows analysis of large numbers of cells, compared with the much smaller number that can be counted manually by an investigator (10,000 events or more versus the traditional 200 to 400 cells counted on a cytospin in morphological analysis). In addition, flow cytometry saves time in the analysis of a large number of samples, specifically, in preparing, staining, and counting the cytospun cells. However, flow cytometry requires expertise and a large number of compatible multicolor antibodies, which is more expensive than investment in equipment required for basic manual counting. This is, however, compensated by time savings and superior results.

We describe here a rapid, easy-to-use, reliable, and customizable flow cytometry method that makes it possible to count BAL cells without complex protocols and uses stable fluorescent antibodies that can be conserved. This procedure is currently used for high-throughput screening in our French and German mouse clinics for mouse phenotyping and drug discovery research (Abboud et al., 2015; Fuchs et al., 2011; Horsch et al., 2015). It is designed to be customizable through the use of additional antibodies that can identify subclasses of inflammatory cells such as $CD4^+$ T cells, dendritic cells, and M1 and/or M2 macrophages. We give examples for this customization below.

FLOW CYTOMETRY PROCEDURE AND ANALYSIS

BASIC PROTOCOL

Mouse BAL fluid (see Daubeuf & Frossard, 2012) is centrifuged (5 min, 300 × *g*), and pelleted cells are resuspended in 500 μl PBS-EDTA. Cells are labeled with antibodies to identify and count the inflammatory cells. This protocol describes a fast, one-step, and easy-to-use protocol for this identification.

Table 1 Monoclonal Antibodies for Flow Cytometry Analysis of Leukocytes in BAL Fluid in the Mouse[a]

Antibody	Fluorochrome	Supplier	Catalog number	Concentration	Laser	Filter
General panel						
Live-Dead	DAPI	BD Bioscience	564907	10 µg/ml	Violet (405 nm)	450/50
CD45	AlexaFluor700	BioLegend	103128	1.7 µg/ml	Red (633 nm)	730/45
CD11b	APC Alexa750	BD Bioscience	557657	0.8 µg/ml	Red (633 nm)	780/60
CD11c	FITC	BD Bioscience	557400	1.5 µg/ml	Blue (488 nm)	530/30
CD3	BV605	BD Bioscience	564009	0.2 µg/ml	Violet (405 nm)	605/12
CD19	BV650	BD Bioscience	563235	0.2 µg/ml	Violet (405 nm)	655/8
I-A/I-E-(MHC-II)	PerCP-Cy5.5	BioLegend	107626	0.2 µg/ml	Blue (488 nm)	695/40
Ly-6 G (GR-1)	Pe-eFluor 610	eBioscience	61-5931	0.4 µg/ml	Blue (488 nm)	610/20
Supplementary antibodies used for results presented in Figure 3						
F4/80	BV510	BioLegend	123135	1 µg/ml	Violet (405 nm)	525/25
CD206 (MMR)	PE-CY7	BioLegend	141719	1 µg/ml	Blue (488 nm)	780/60
CD197 (CCR7)	PerCP-Cy5.5	eBioscience	45-1971	1 µg/ml	Blue (488 nm)	695/40
CXCR4 (CD184)	APC	eBioscience	17-9991	1.5 µg/ml	Red (633 nm)	660/20
CD4	APC	BD Bioscience	561091	0.5 µg/ml	Red (633 nm)	660/20

[a]The "General panel" of antibodies refers to results illustrated in Figure 1, and the "Supplementary antibodies" give results as presented in Figure 3.

Materials

Bronchoalveolar lavage (BAL) fluid from mouse
Phosphate buffered saline containing 3 mM EDTA (PBS-EDTA), kept on ice
FC Block solution (to block non-specific FcγII/IIIR antibody binding and reduce background staining; BD Bioscience, cat. no. 553142)
4′,6-diamidino-2-phenylindole (DAPI; fluorescent dye for exclusion of nonviable cells on flow cytometric analysis; BD Bioscience, cat. no. 564907)
Anti-mouse antibodies (see Table 1)
  CD45-AlexaFluor700
  CD11b-APC Alexa750
  Ly-6G (Gr-1)-PEeFluor610
  CD11c-FITC
  I-A/I-E-PerCP-Cy5.5
  CD3-BV605
  CD19-BV650

1.5-ml protein Lobind microtubes (Eppendorf, cat. no. 0030108116)
MixMate vortex (Eppendorf)
Sterile tips
Precision pipets (2.5, 20, 200, and 1000 µl)
8-color or more flow cytometer (LSR II or FACS Aria II, BD Bioscience)
FlowJo software

Blockade of the Fc-mediated binding of antibodies

1. Homogenize BAL cells by vortexing them slowly (1000 rpm, 5 sec) and place 150 µl of each BAL suspension into a separate well in a black 96-well microplate (or in separate black microtubes).
2. Add 5 µl FC Block (0.01 mg/ml in PBS-EDTA) to each well or tube.
3. Incubate 10 min at room temperature under agitation (900 rpm on a Mixmate vortex).

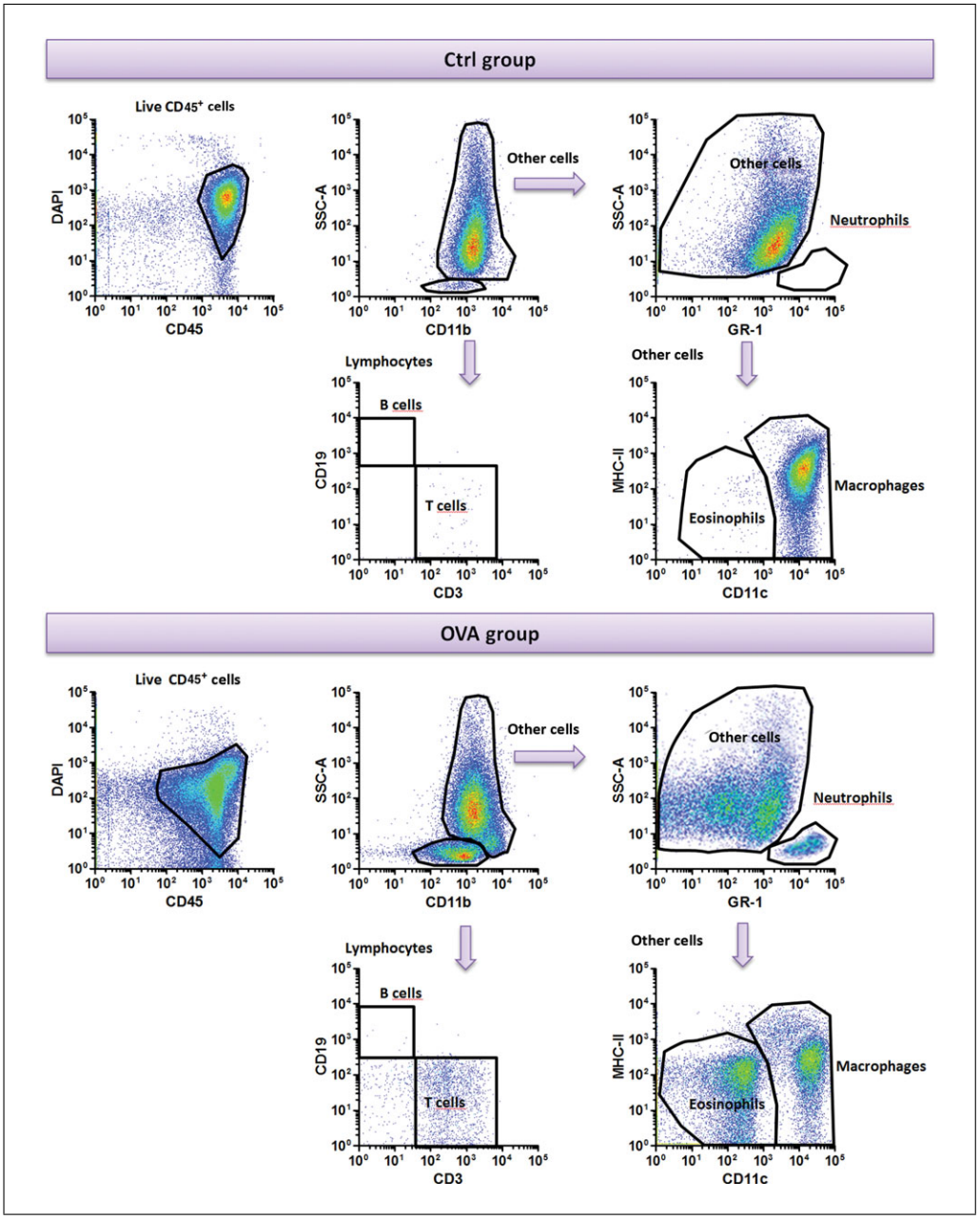


Figure 1 Analysis of labeled BALF cells by 8-color flow cytometry. FlowJo software was used to select BALF cells on a 2-D plot, in naive mice (Ctrl group) and in a model of ovalbumin-induced asthma (OVA group). First, live CD45$^+$ and DAPI$^-$ leukocytes were selected to eliminate debris, erythrocytes, and dead cells; then lymphocytes were gated on an SSC-A/CD11b plot to discriminate CD19$^+$ B cells and CD3$^+$ T cells. Other cells were analyzed with an SSC-A/GR1 plot to identify neutrophils, and an MHC-II/CD11c plot was used to separate eosinophils from macrophages in the remaining cells. This procedure identified 98.7 ± 1.4% of the live leukocytes.

Antibody solution

4. Add 1 ml PBS-EDTA to a 1.5-ml protein Lobind microtube and keep on ice.

   This is the volume required for 36 samples.

5. Add the following seven antibodies (volume of stock solution; final concentration):

   CD45-AlexaFluor700 (3.5 µl; 1.7 µg/ml)
   CD11b-APC Alexa750 (2 µl; 4 µg/ml)
   Ly-6G (Gr-1)-PEeFluor610 (1 µl; 0.2 µg/ml)

CD11c-FITC (3 µl; 1.5 µg/ml)
I-A/I-E-PerCP-Cy5.5 (1 µl; 0.2 µg/ml)
CD3-BV605 (10 µl; 0.2 µg/ml)
CD19-BV650 (10 µl; 0.2 µg/ml).

6. Homogenize antibody solution by vortexing.

Cell labeling and flow cytometry analysis

7. Place 25 µl antibody solution in each well containing BAL cells from step 2 (96-well microplate or microtubes).
8. Incubate 30 min at room temperature under agitation (900 rpm on Mixmate vortex).
9. Add 5 µl DAPI (pre-dilute, 1/500 in PBS-EDTA) to each well or microtube.
10. Acquire data on the LSR II flow cytometer.

Data analysis with FlowJo software

11. Transfer data to FlowJo software to identify and count inflammatory cells, as described below; gating conditions are shown on Figure 1.
12. Select live $CD45^+$ and $DAPI^-$ leukocytes to eliminate debris, erythrocytes, and dead cells.
13. Gate lymphocytes and "other cells" on SSC-A/CD11b plot.
14. Discriminate $CD19^+$ B cells and $CD3^+$ T cells from lymphocytes.
15. Discriminate "other cells" on SSC-A/GR1 plot to gate $GR1^+$ neutrophils and $GR1^-$ leukocytes.
16. Discriminate $GR1^-$ leukocytes on MHC-II/CD11c plot to identify and gate eosinophils from $CD11c^+$ macrophages in the remaining leukocytes.
17. Create table to report counts of identified live leukocytes as $CD19^+$ B cells, $CD3^+$ T cells, eosinophils, neutrophils, and macrophages.
18. Export results to Excel and calculate the percentage of the total identified live leukocytes represented by each leukocyte population. Use these percentages and the total number of BAL cells (see above) to obtain the number of each leukocyte population in BAL.

This procedure identifies 98.7 ± 1.4% of live leukocytes.

SUPPORT PROTOCOL

TOTAL CELL COUNTS

Mouse BAL fluid (see Daubeuf & Frossard, 2012) is centrifuged (5 min, 300 × *g*), and pelleted cells are resuspended in 500 µl PBS-EDTA. Total cells are counted either manually on a hemocytometer (e.g., Neubauer or Malassez chamber), or with a Muse cell analyzer (Merck Millipore), an ADVIA 120 hematology analyzer (Siemens), or a fluorescent image-based cell analyzer (Cellometer Auto X4 or Celigo, Nexcelom). Total cells may be counted either before or after flow cytometry analysis.

Materials

Bronchoalveolar lavage (BAL) fluid from mouse
Phosphate buffered saline containing 3 mM EDTA (PBS-EDTA), kept on ice
PBS-EDTA containing 1% BSA (PBS-BSA), kept on ice
Anti-mouse antibody, CD45-PE (Abcam, ab25603)

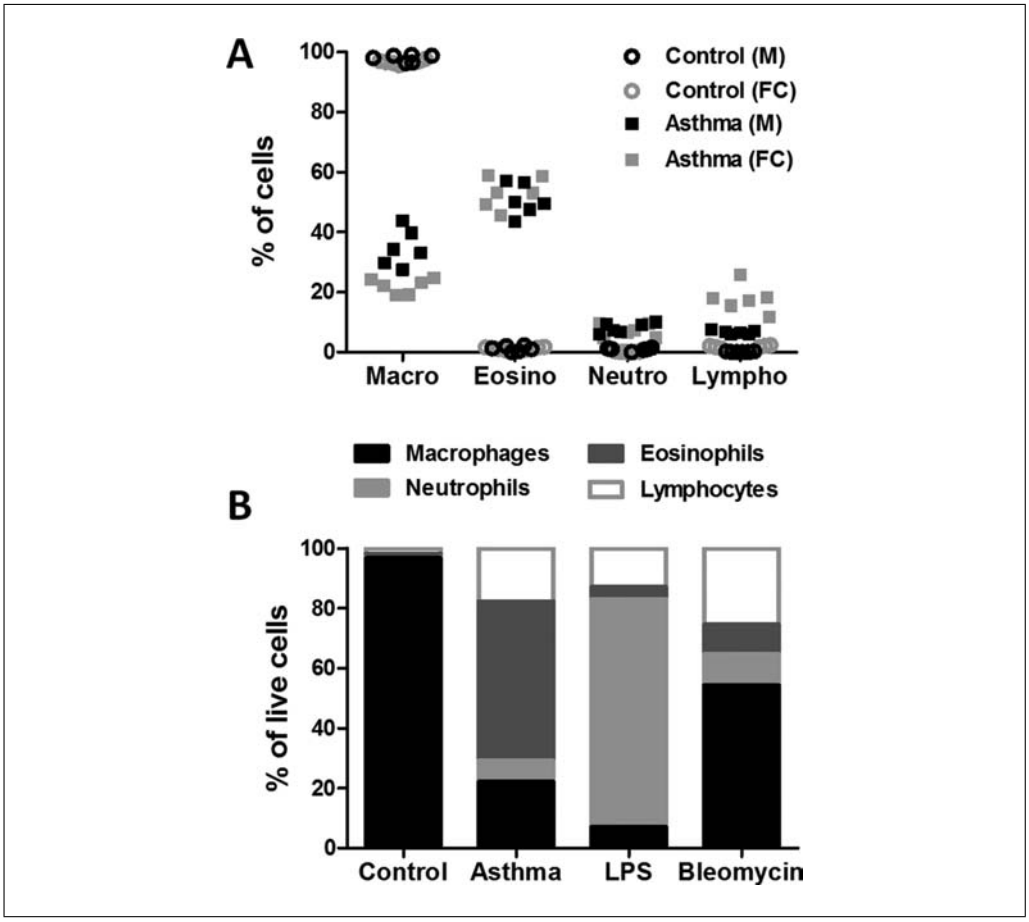


Figure 2 Cell population in BALF in airway disease. (**A**) Comparison between manual morphological counting (M) and flow cytometry (FC) analysis in naive mice (Control) and in a model of ovalbumin-induced asthma (Asthma). Data show that flow cytometry is useful for differentiating macrophages from lymphocytes (see Commentary, Background Information for details). Manual counting and flow cytometry produce similar results for eosinophils and neutrophils. (**B**) Illustration of the usefulness of the flow cytometry analysis procedure in three models of airway diseases: A model of ovalbumin-induced asthma, an acute inflammatory model induced by intranasal administration of *Escherichia coli* lipopolysaccharide (LPS, 200 μg/kg) with BALF 24 hr later and a model of pulmonary fibrosis induced by bleomycin (one intranasal administration, 1 mg/kg) with BALF 14 days later. Macro, Macrophages; Eosino, Eosinophils; Neutro, Neutrophils; Lympho, Lymphocytes; LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid.

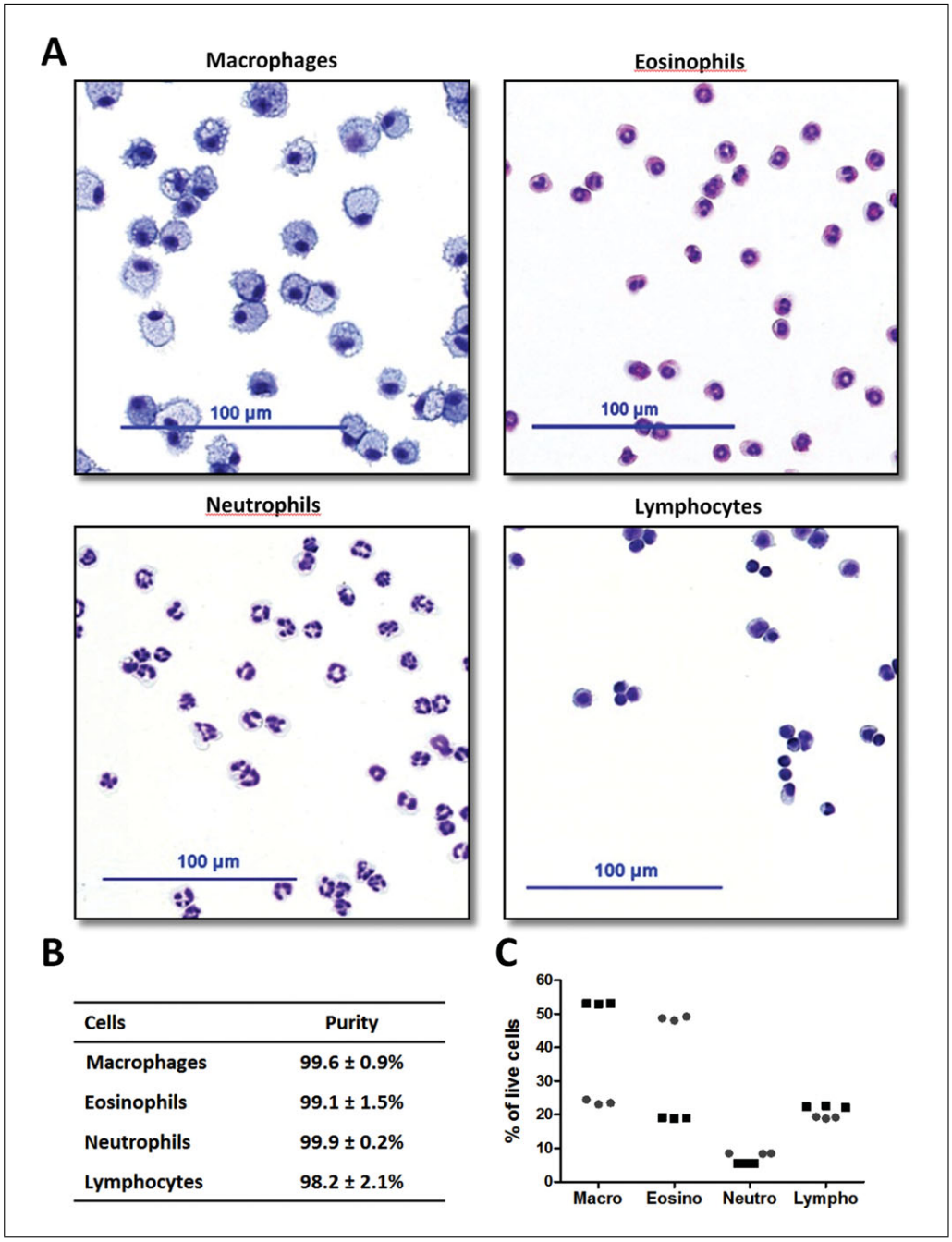
Conventional bright field microscope
Hemocytometer (e.g., Neubauer, Malassez)
Muse cytometer (Millipore)
Precision pipets (2.5, 20, 200, and 1000 μl)
Sterile tips
1.5-ml microtube

Total cell count with a manual hemocytometer

1a. Place 5 μl cell suspension on a hemocytometer.

The total cell count should be performed within 24 hr of BAL (see Daubeuf & Frossard, 2012).

2a. Count cells and calculate total cell concentration according to the instructions for the hemocytometer you are using. Calculate total number of cells as a function of the volume of lavage fluid (500 μl).



Cells	Purity
Macrophages	99.6 ± 0.9%
Eosinophils	99.1 ± 1.5%
Neutrophils	99.9 ± 0.2%
Lymphocytes	98.2 ± 2.1%

Figure 3 Quality control of purity of the cells from the one-step flow cytometry procedure. (**A**) The flow cytometry procedure and cell selection presented on Figure 1 used a FACSARIA II cell sorter (BD Bioscience). Sorted cells were cytospun and stained with Diff-Quick (see Daubeuf & Frossard, 2012). (**B**) Sorted macrophages, eosinophils, neutrophils, and lymphocytes were identified and counted on a cytospin to determine the purity and efficiency of the flow cytometry procedure. (**C**) Intra-individual reproducibility of the flow cytometry procedure on BALF cells is represented for a duplicate experiment in a murine asthma model (gray dots and black squares). Macro, Macrophages; Eosino, Eosinophils; Neutro, Neutrophils; Lympho, Lymphocytes; BALF, bronchoalveolar lavage fluid.

Total cell count with Muse cell analyzer (Merck Millipore)

1b. Homogenize BAL cells by vortexing the suspension slowly (1000 rpm, 5 sec) and distribute 50 µl of each BAL suspension in separate 1.5-ml microtubes.

The total cell count should be performed within 24 hr of BAL.

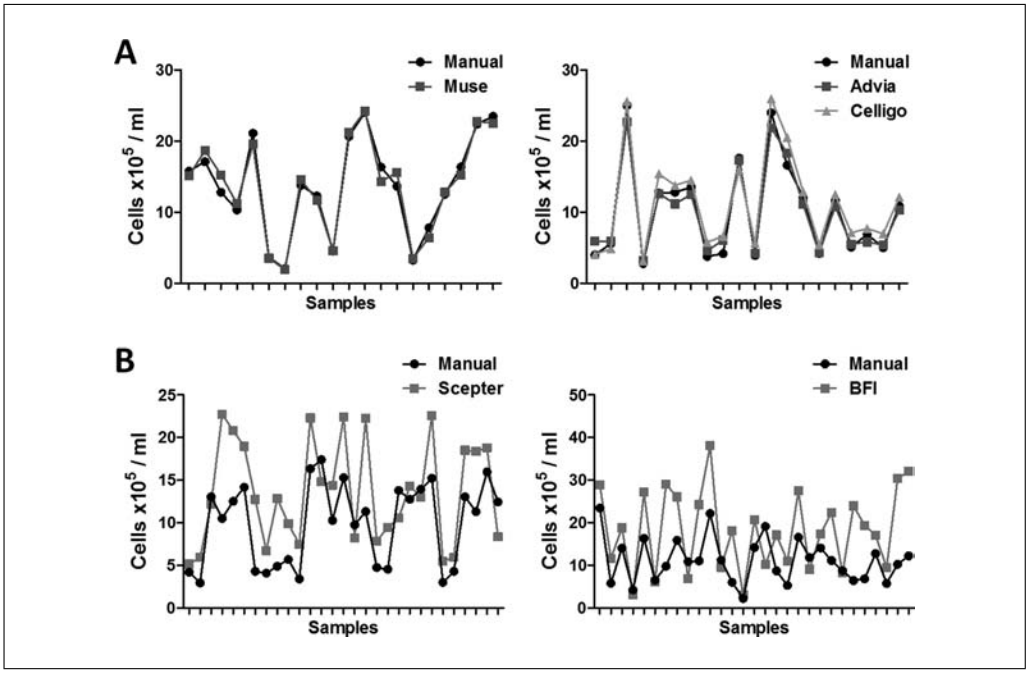


Figure 4 Total cell counting can be replaced by a few automated cell counters. (**A**) Total cells were counted with Muse, ADVIA 120, and Celigo S and compared to manual counting (on a hemocytometer). (**B**) Comparison performed on the same samples for total cell counts obtained with the manual method and either an automated cell counter with bright-field images on counting slides (BFI) or an impedance-based particle detection counter (Scepter).

2b. Add 50 µl CD45-PE antibody solution (0.5 µg/ml in PBS-BSA) to each 1.5-ml microtube.

3b. Add 1 ml PBS-EDTA to each tube.

4b. Incubate 20 min at room temperature.

5b. Acquire data on the Muse cell analyzer.

COMMENTARY

Background Information

Performing bronchoalveolar lavage (BAL) is an essential approach for studying airway inflammation in animal models of airway diseases, because it allows the identification and counting of the inflammatory leukocytes recruited to the respiratory tract. The development of flow cytometry offered (Van Rijt et al., 2004) and, as described in this article, continues to offer new perspectives for differential cell counting, compared to standard manual morphological counts (described in the procedure by Daubeuf & Frossard., 2012). Manual counting is a reproducible technique but limited to morphological characteristics and is tedious, time-consuming, and therefore expensive. It takes longer to obtain results than with flow cytometry. Moreover, Figure 2A shows that manual counts using morphological criteria under optical microscopy produce a higher number of macrophages (black square) than flow cytometry techniques do (gray square), and vice versa for lymphocytes. This result reveals that manual counts frequently confuse lymphocytes with macrophages and that our simple one-step flow cytometry technique is useful for distinguishing macrophages from lymphocytes attracted to the airway lumen in a murine model of ovalbumin-induced asthma. Figure 2B illustrates the usefulness of the procedure to discriminate leukocytes in BAL, regardless of the proportions of each leukocyte, in three models of airway diseases: A model of asthma, an acute inflammatory model induced by lipopolysaccharide (LPS), and a model of pulmonary fibrosis.

We note that our experience confirms the high stability of all the antibodies described in this protocol; they can be used for several years.

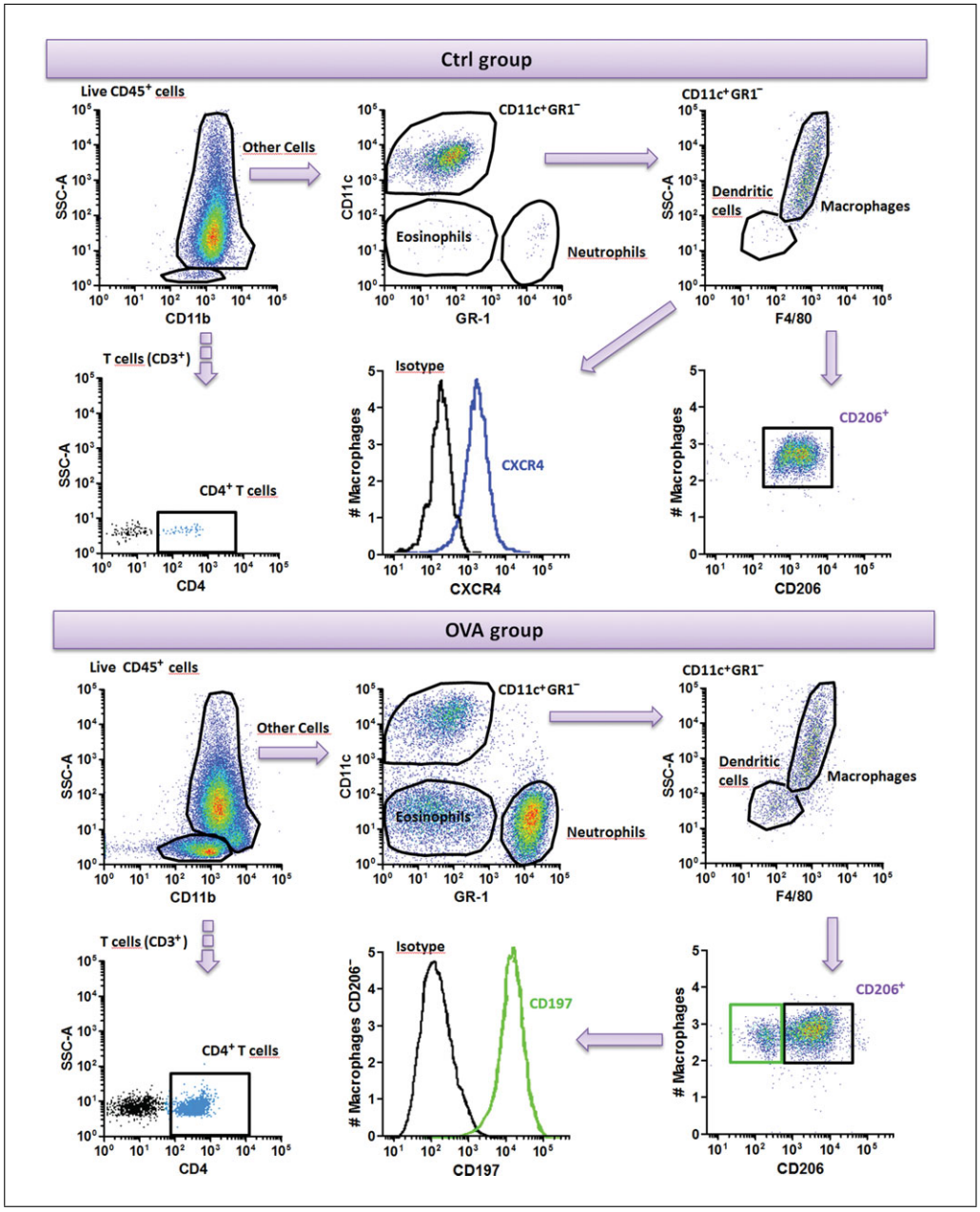


Figure 5 Analysis of labeled BALF cells by multicolor flow cytometry, examples of customization. FlowJo software was used to select BALF cells, on 2-D or histogram plot, in naive mice (Ctrl group) and in a model of ovalbumin-induced asthma (OVA group). First, live CD45$^+$ DAPI$^-$ leukocytes were selected to eliminate debris, erythrocytes, and dead cells; then lymphocytes were gated on a SSC-A/CD11b plot to discriminate first CD3$^+$ T cells and then CD4$^+$ T cells. Other cells were analyzed with CD11c/GR1 plot to identify neutrophils and eosinophils from CD11c plus GR1$^-$ cells (macrophages) in the remaining cells. Addition of other markers can discriminate dendritic cells (F4/80$^-$) from macrophages (F4/80$^+$) or identify receptor expression at the cell surface, as shown here for CD206, CD197, or CXCR4 on macrophages. BALF, bronchoalveolar lavage fluid.

Critical Parameters

To ensure BAL cell viability and stability, and the quality of labeling, the following standard steps necessary for manual counting were avoided: Hypotonic shock to lyse red cells, repeated centrifugation, and washing. In addition, the quality of the tubes and microplate is important: Low-adsorption microtubes resist surface coating with the antibodies (in PBS-EDTA without BSA or serum), and the microplates must be able to prevent macrophage adhesion. We use black U-96-well plates from Eppendorf (cat. no. 0030 601.807) and find they successfully prevent macrophage adhesion and protect samples from light. The microplate is sealed with

Table 2 Time Requirements for Processing 36 or 60 Samples

Step	Flow cytometry	Manual
BAL cell concentration	20 min	20 min
Hemolysis		60 min
Total cell count (Muse versus Manual)	60 min	180 min
Antibody labeling	60 min	
Flow cytometry (LSR II)	60 min	
Analysis (FlowJo)	100 min	
Cytospin		90 min
Diff-Quick staining		60 min
Differential cell counting		420 min
Total for 36 samples	300 min (5 hr)	830 min (>13 hr)
Total for 60 samples	480 min (8 hr)	1500 min (25 hr)

opaque aluminum storage foil (Eppendorf, cat. no. 0030 127.889) to protect samples from light completely during antibody labeling. In addition, the presence of EDTA in endotoxin-free PBS is essential to limit macrophage activation and cell adhesion and to guarantee the quality, purity, and reproducibility of the flow cytometry procedure and analysis (Fig. 3).

Troubleshooting

In the absence of any hemolysis step, manual total cell counting with a hemocytometer (e.g., Neubauer, Malassez) can be difficult when the number of erythrocytes is high. In this situation, we have found that only a few instruments can count leukocytes accurately in BAL samples containing debris and erythrocytes. Three instruments have worked successfully for us, as described in Figure 4A: The Muse cytometer (Merck Millipore), the ADVIA 120 hematology analyzer (Siemens), or the Celigo S Imaging Cell cytometer (Nexcelom) with propidium iodide as the fluorescent dye nuclear staining (Fig. 4A). Conventional cell counters using bright-field images or impedance-based particle detection do not appear to enable discrimination between heterogeneous samples with debris and erythrocytes, and cannot be used for BALF analysis (Fig. 4B).

Anticipated Results

This fast and easy-to-use protocol is designed to efficiently and reproducibly identify the inflammatory cells recovered in BAL, i.e., macrophages, eosinophils, neutrophils, and T and B cells. It is easily customizable to identify additional cell subsets by the addition of supplementary antibodies specific to these subclasses of cells in the "antibody solution" as exemplified on Figure 5.

In these customized protocols, antibodies directed against the MHC class II molecules may be advantageously replaced by another fluorescent antibody directed against another marker. For instance, eosinophils, neutrophils, and macrophages in BALF can be efficiently counted in one step by using anti-CD11c (macrophage) and anti-GR1 (neutrophils) antibodies, eosinophils being the unlabeled cells. As Figure 5 shows, F4/80 antibody (F4/80-BV510; BioLegend; 1 µg/ml) added to the antibody solution makes it possible to differentiate between the CD11c plus F4/80$^+$ macrophage population from the CD11c plus F4/80$^-$ dendritic cells. Similarly, macrophage phenotypes may be studied by their expression of CD206 (M2 macrophages, CD206-PE/CY7; BioLegend; 1 µg/ml), CD197 (M1 macrophages, CD197-PerCP-Cy5.5, eBioscience; cat. no. 45-1971-82, 1 µg/ml), or CXCR4 (CXCR4-APC; eBioscience; cat. no. 17-9991-80, 1.5 µg/ml; Fig. 5).

In another alternative protocol, addition of anti-CD4 antibodies (CD4-APC; BD Bioscience; cat. no. 561091, 0.5 µg/ml) allows one to differentiate CD4$^+$ T cells within the CD3$^+$ T cell population (Fig. 5).

Time Considerations

Table 2 summarizes the timeline associated with the procedures. Note that several steps are very time consuming. The time does not increase linearly with the number of samples, so we present the time necessary for 36 samples. We compare the time required for flow

cytometry and manual differential count (see Daubeuf & Frossard, 2012).

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Literature Cited

Abboud, D., Daubeuf, F., Do, Q. T., Utard, V., Villa, P., Haiech, J., ... Frossard, N. (2015). A strategy to discover decoy chemokine ligands with an anti-inflammatory activity. *Scientific Reports*, *5*, 14746. doi: 10.1038/srep14746.

Andreasen, C. B. (2003). Bronchoalveolar lavage. *Veterinary Clinics of North America: Small Animal Practice*, *33*, 69–88. doi: 10.1016/S0195-5616(02)00056-6.

Blé, F.-X., Cannet, C., Zurbruegg, S., Karmouty-Quintana, H., Frossard, N., Trifilieff, A., & Beckmann, N. (2008). Allergen-induced lung inflammation in actively sensitized mice assessed by MRI. *Radiology*, *248*(3): 834–843. doi: 10.1148/radiol.2482071452.

Blé, F.-X., Cannet, C., Zurbruegg, S., Gérard, C., Frossard, N., Beckmann, N., & Trifilieff, A. (2009). Activation of the lung S1P1 receptor reduces allergen-induced plasma leakage in mouse. *British Journal of Pharmacology*, *158*, 1295–1301. doi: 10.1111/j.1476-5381.2009.00391.x.

Crestani, B., Marchand-Adam, S., Quesnel, C., Plantier, L., Borensztajn, K., Marchal, J., ... Dehoux, M. (2012). Hepatocyte growth factor and lung fibrosis. *Proceedings of the American Thoracic Society*, *9*, 158–163. doi: 10.1513/pats.201202-018AW.

Daubeuf, F., & Frossard, N. (2012). Performing bronchoalveolar lavage in the mouse. *Current Protocols in Mouse Biology*, *2*, 167–175. doi: 10.1002/9780470942390.mo110201.

Daubeuf, F., & Frossard, N. (2013). Acute ovalbumin asthma model in the mouse. *Current Protocols in Mouse Biology*, *3*, 31–37. doi: 10.1002/9780470942390.mo120202.

Delayre-Orthez, C., Becker, J., Auwerx, J., Frossard, N., & Pons, F. (2008). Suppression of allergen-induced airway inflammation and immune response by the peroxisome proliferator-activated receptor-alpha agonist fenofibrate. *European Journal of Pharmacology*, *581*, 177–184. doi: 10.1016/j.ejphar.2007.11.040.

Delayre-Orthez, C., Becker, J., De Blay, F., Frossard, N., & Pons, F. (2004). Dose-dependent effects of endotoxins on allergen sensitization and challenge in the mouse. *Clinical and Experimental Allergy*, *34*, 1789–1795. doi: 10.1111/j.1365-2222.2004.02082.x.

Delayre-Orthez, C., Becker, J., de Blay, F., Frossard, N., & Pons, F. (2005a). Exposure to endotoxins during sensitization prevents further endotoxin-induced exacerbation of airway inflammation in a mouse model of allergic asthma. *International Archives of Allergy and Immunology*, *138*, 298–304. doi: 10.1159/000088867.

Delayre-Orthez, C., Becker, J., Guenon, I., Lagente, V., Auwerx, J., Frossard, N., & Pons, F. (2005b). PPARalpha downregulates airway inflammation induced by lipopolysaccharide in the mouse. *Respiratory Research*, *6*, 91. doi: 10.1186/1465-9921-6-91.

Deschamps, K., Cromlish, W., Weicker, S., Lamontagne, S., Huszar, S. L., Gauthier, J. Y., ... Tan, C. M. (2011). Genetic and pharmacological evaluation of Cathepsin S in a mouse model of asthma. *American Journal of Respiratory Cell and Molecular Biology*, *45*, 81–87. doi: 10.1165/rcmb.2009-0392OC.

Fuchs, H., Gailus-Durner, V., Adler, T., Aguilar-Pimentel, J. A., Becker, L., Calzada-Wack, J., ... Hrabě de Angelis, M. (2011). Mouse phenotyping. *Methods*, *53*, 120–135. doi: 10.1016/j.ymeth.2010.08.006.

Gasparik, V., Daubeuf, F., Hachet-Haas, M., Rohmer, F., Gizzi, P., Haiech, J., ... Frossard, N. (2012). Prodrugs of a CXC Chemokine-12 (CXCL12) neutraligand prevent inflammatory reactions in an asthma model in vivo. *ACS Medicinal Chemistry Letters*, *3*, 10–14. doi: 10.1021/ml200017d.

Gregory, L. G., & Lloyd, C. M. (2011). Orchestrating house dust mite-associated allergy in the lung. *Trends in Immunology*, *32*, 402–411. doi: 10.1016/j.it.2011.06.006.

Hachet-Haas, M., Balabanian, K., Rohmer, F., Pons, F., Franchet, C., Lecat, S., ... Galzi J.-L. (2008). Small neutralizing molecules to inhibit actions of the chemokine CXCL12. *The Journal of Biological Chemistry*, *283*, 23189–23199. doi: 10.1074/jbc.M803947200.

Henderson, A. J. W. (1994). Bronchoalveolar lavage. *Archives of Disease in Childhood*, *70*, 167–169. doi: 10.1136/adc.70.3.167.

Horsch, M., Aguilar-Pimentel, J. A., Bonisch, C., Come, C., Kolster-Fog, C., Jensen, K. T., ... Beckers, J. (2015). Cox4i2, Ifit2, and Prdm11 mutant mice: Effective selection of genes predisposing to an altered airway inflammatory response from a large compendium of mutant mouse lines. *PloS One*, *10*, e0134503. doi: 10.1371/journal.pone.0134503.

Hunninghake, G. W., Gadek, J. E., Kawanami, O., Ferrans, V. J., & Crystal, R. G. (1979). Inflammatory and immune processes in the human lung in health and disease: Evaluation by bronchoalveolar lavage. *The American Journal of Pathology*, *97*, 149–206.

Kips, J. C., Anderson, G. P., Fredberg, J. J., Herz, U., Inman, M. D., Jordana, M., ... Chung, K. F. (2003). Murine models of asthma. *The European Respiratory Journal*, *22*, 374–382. doi: 10.1183/09031936.03.00026403.

Kumar, R. K., Herbert, C., & Foster, P. S. (2008). The "classical" ovalbumin challenge model of asthma in mice. *Current Drug Targets*, *9*, 485–494. doi: 10.2174/138945008784533561.

Mathers, R. A., Evans, G. O., Bleby, J., & Tornow, T. (2007). Total and differential leucocyte counts in rat and mouse bronchoalveolar lavage fluids using the Sysmex XT-2000iV. *Comparative Clinical Pathology*, *16*, 29–39. doi: 10.1007/s00580-006-0655-x.

Mouratis, M. A. (2011). Modeling pulmonary fibrosis with bleomycin. *Current Opinion in Pulmonary Medicine*, *17*, 355–361. doi: 10.1097/MCP.0b013e328349ac2b.

Nials, A. T., & Uddin, S. (2008). Mouse models of allergic asthma: Acute and chronic allergen challenge. *Disease Models & Mechanisms*, *1*, 213–220. doi: 10.1242/dmm.000323.

Reber, L. L., Daubeuf, F., Pejler, G., Abrink, M., & Frossard, N. (2014). Mast cells contribute to bleomycin-induced lung inflammation and injury in mice through a chymase/mast cell protease 4-dependent mechanism. *Journal of Immunology*, *192*, 1847–1854. doi: 10.4049/jimmunol.1300875.

Shin, Y. S., Takeda, K., & Gelfand, E. W. (2009). Understanding asthma using animal models. *Allergy, Asthma & Immunology Research*, *1*, 10–18. doi: 10.4168/aair.2009.1.1.10.

Van Rijt, L. S., Kuipers, H., Vos, N., Hijdra, D., Hoogsteden, H. C., & Lambrecht, B. N. (2004). A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. *Journal of Immunological Methods*, *288*, 111–221. doi: 10.1016/j.jim.2004.03.004.

Vodovotz, Y., Chow, C. C., Bartels, J., Lagoa, C., Prince, J. M., Levy, R. M., ... Fink, M. P. (2006). In silico models of acute inflammation in animals. *Shock*, *26*, 235–244. doi: 10.1097/01.shk.0000225413.13866.fo.

Zhang, Z., Hener, P., Frossard, N., Kato, S., Metzger, D., Li, M., & Chambon, P. (2009). Thymic stromal lymphopoietin overproduced by keratinocytes in mouse skin aggravates experimental asthma. *Proceedings of the National Academy of Sciences of the U.S.A.*, *106*(5), 1536–1541. doi: 10.1073/pnas.0812668106.