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Cellular and Biochemical Analysis of Bronchoalveolar Lavage Fluid from Murine Lungs.

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Abstract

Bronchoalveolar lavage (BAL) is a technique used to collect the contents of the airways. The fluid recovered, called BAL fluid (BALF), serves as a dynamic tool to identify various disease pathologies ranging from asthma to infectious diseases to cancer in the lungs. A wide array of tests can be performed with BALF, including total and differential leukocyte counts (DLC), enzyme-linked immunosorbent assays (ELISA) or flow-cytometric quantitation of inflammatory mediators, such as cytokines, chemokines and adhesion molecules, and assessment of nitrate and nitrite content for estimation of nitric oxide synthase (NOS) activity. Here, we describe a detailed procedure for the collection of BALF for a variety of downstream usages, including DLC by cytological and flow-cytometry-based methods, multiplex cytokine analysis by flow cytometry, and NOS activity analysis by determining nitrate and nitrite levels.

Keywords

Differential leukocyte count; Cytokines; Nitric oxide synthase; Flow cytometry; Tracheostomy

1. Introduction

The lung is considered the most exposed organ in the body for its continuous interactions with external airborne antigens and other toxins [1]. Such constant exposures to exogenous substances at the airway mucosal surface can trigger immunological reactions in the lungs, resulting in disorders such as asthma and chronic obstructive pulmonary disease (COPD) [2, 3, 4, 5, 6, 7, 8]. When interstitial lung disorders are suspected, biopsy is not the first choice for identifying pathological and biochemical changes due to its invasive nature. Bronchoalveolar lavage (BAL) is often performed instead to examine the immune cells present in the lungs and to determine cytokine, chemokine, and adhesion molecule profiles.

BAL is a saline-based wash of the airways first established in 1970 [9, 10, 11]. In humans, it is a minimally invasive procedure to investigate lung pathophysiologies [2, 3, 12, 13, 14, 15, 16, 17] and often used to diagnose patients suffering from interstitial lung disorders [2]. The fluid recovered by BAL is known as BAL fluid (BALF) and contains a multitude of airway

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constituents, including cells, lipids, proteins, and other chemical or biological substances from the mucosal surface of the bronchial tree [18]. Most proteins present in BALF include albumin, immunoglobulin, α1-anti trypsin, transferrin, fibronectin, collagen, and collagenase. In addition, prostaglandins and a few metabolites that are either locally synthesized or reach the lungs via active transport by immunoglobulins or via passive transport by albumin are also found in BALF [4]. The cellular contents of BALF are predominantly leukocytes, with a few exceptions like erythrocytes and platelets [19, 20]. Identifying the pattern and population of differential leukocyte counts (DLC) plays a crucial role in characterizing various lung diseases [10]. In laboratory research using *in vivo* animal models for various lung disorders [21, 22, 23], BAL serves as an important and most commonly used technique to study inflammatory cell infiltration, biochemical, and molecular changes [2, 24].

Conventionally, DLC is performed by cytological staining of air-dried BALF smears with Wright-Giemsa, May- Grunwald-Giemsa, or Differential-Quik stain [25, 26]. Following staining, 200–500 cells are counted under a microscope and manually classified into neutrophils, eosinophils, macrophages, basophils, etc. However, DLC by this conventional method is prone to human errors and its diagnostic validity may be challenged for yielding false results. In this context, with the advancements of microfluidics technology and abundance of cell-specific antibodies, flow-cytometry-based DLC has become a more rapid and trustworthy tool.

In addition to DLC, BALF has been used for the detection of cytokines, chemokines, and adhesion molecules. However, independent ELISA kits were used to quantify the proteins, which is both time and cost consuming. In contrast, flow-cytometry-based multiplex assays provide a solution to minimize these challenges by facilitating the simultaneous detection of multiple proteins in a single sample. Furthermore, markers used for detecting oxidative stress in the lungs can also be measured in BALF. Oxidative stress plays a crucial role in the pathophysiology of lung disease by generating reactive oxygen species (ROS), which when combined with nitric oxide, forms potent peroxy nitryl radicals, resulting in the nitrosylation of proteins leading to lipid peroxidation [27, 28, 29, 30, 31, 32, 33].

In this chapter, we describe detailed procedures to perform the collection of BALF from mice followed by performing DLC using both conventional cytological and flow-cytometric methods. In addition, we also describe a procedure to assay multiple cytokines in BALF with flow -cytometry using a commercially available multiplex kit. Finally, we describe a method to quantify nitrate and nitrite levels in BALF as an indicator of NOS activity, which suggests the extent of ROS generation in the airways

2. Materials

2.1. BALF Collection

1. Mice: 8–12 weeks old (*see* Note 1).
2. Anesthetics: Ketamine (100 mg/kg) and xylazine (10 mg/kg) cocktail (9:1 ratio).
3. Sodium Pentobarbital: 100 mg/kg body weight for euthanasia.

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4. 18-Gauge cannula: Used for tracheostomy.
5. Surgical thread.
6. Phosphate-buffered saline (PBS) with protease and phosphatase inhibitor: 2.66 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCl, 8.06 mM $Na_2HPO_4–7H_2O$, pH ~7.4. Add protease and phosphatase inhibitor cocktail (1×).
7. 2-mL Syringe.
8. Surgical dissection tools: Pointed forceps, serrated forceps, pointed bent scissors, scalpel, and conventional small scissors.
9. Microfuge tubes: 1.5-mL and 2-mL sizes for collection and storage of BALF.
10. Ice in an ice bucket.

2.2. DLC by Cytology

1. 0.4% (w/v) Trypan blue solution (Cat# 15250061 ThermoFisher) or weigh 0.4 g of trypan blue and dissolve in 100 mL of PBS.
2. Automated or manual cell counter: Countess™ Cell Counting Chamber Slides with Countess II FL Automated Cell Counter or a hemocytometer using a microscope.
3. Cytospin (*see* Note 2).
4. Glass slides: 25 × 75 × 1.0 mm.
5. Coverslips: 22 × 22 mm.
6. Romanowsky-Giemsa (modified Giemsa) staining kit: Diff-Quik Stain Kit or commercially available equivalent with a fixative (methanol), eosinophilic xanthene dye (eosin Y), and basophilic thiazine dye (methylene blue).
7. Absolute ethanol: Histological grade. Used for dehydration of cells.
8. Xylene.
9. Mounting medium for placing cover slip.
10. Microscope.
11. Cell counter.

2.3. DLC by Flow Cytometry

1. Flow cytometer: Equipped with two lasers capable of distinguishing 575–585 nm and 660 nm.
2. Flow cytometry data analysis software.
3. 3% (w/v) Bovine serum albumin (BSA): Dissolve 3 g of BSA in 100 mL of PBS.
4. Monoclonal antibody cocktail: Combine antibodies against CD36, CD2, CD19, CD45, and CD294 in PBS at their recommended concentrations (*see* Table 1 for more details).

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5. DAPI nuclear stain solution: 100 μg/mL DAPI in PBS. Make the working concentration of DAPI by diluting a 1 mg/mL DAPI stock solution to 1:10 in PBS.

2.4. Multiplex Cytokine Assay by Flow Cytometry

1. Flow cytometer: Equipped with two lasers capable of distinguishing 575–585 nm and 660 nm.
2. Flow cytometry data analysis software.
3. Multichannel pipettors: 5–200 μL.
4. Reagent reservoirs for multichannel pipettors.
5. Mouse cytokine multiplex detection kit: BioLegend 13-plex LEGENDplex™ Inflammation Panel or equivalent (*see* Note 3).
6. Microplate vacuum manifold or centrifuge: used for washing the filter or V-bottom 96-well plate provided in the multiplex kit.
7. Wash buffer: Thaw the entire container of 20× wash buffer from the multiplex kit and bring to room temperature. Add 475 mL of deionized water to make 1× wash buffer. This solution can be stored at 2– 8 °C for upto 1 month.
8. 1.5-mL Polypropylene microfuge tubes.
9. Refrigerated centrifuge.
10. Vortex mixer.
11. Sonicator bath.
12. Aluminum foil.
13. Paper towels.
14. Plate shaker.

2.5. Nitric Oxide Synthase (NOS) Assay

1. Reaction buffer: 50 mM HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.4, 5 μM FAD (flavin adenine dinucleotide), 0.1 mM NADPH (nicotinamide adenine dinucleotide phosphate hydrogen), 0.2 U/mL nitrate reductase in 290 μL of distilled water.
2. 1 mM Potassium ferricyanide prepared in Millipore water. Prepare this solution freshly on the day of experiment.
3. Greiss reagent: Dissolve 0.2% (w/v) N-(1-Naphthyl) ethylenediamine (NED), 2% (w/v) sulphanilamide, and 5% (v/v) 95% phosphoric acid in double distilled water and stir it using a magnetic stirrer until the solution appears free of any particles.
4. UV-visible microplate spectrophotometer.

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5. Clear 96-well plates.

3. Methods

3.1. BALF Collection

1. Euthanize mice with an overdose of sodium pentobarbital (100 mg/kg body weight) injected intraperitoneally.
2. Place the mice in supine position and make an incision at the cervical region using surgical scissors (or scalpel) and removing the skin (Fig. 1).
3. Carefully separate the tissues near the thyroid region to either side. Make a vertical incision on the external connective tissue (adventitia) to expose the trachea. Take precautions not to disturb any blood vessels to avoid bleeding (*see* Note 4).
4. Using a sharp scalpel, make a small horizontal incision on the trachea without severing (*see* Note 5). Slowly insert an 18-gauge cannula into the trachea, with the needlepoint facing toward the lungs. Secure the cannula in place by tying a surgical thread around the trachea (Fig. 1a, b).
5. Using a 5-mL syringe, slowly inject 1 mL of PBS with protease and phosphatase inhibitor into the cannula. Collect the BALF by slowly drawing the injected PBS back into the syringe (*see* Note 6). Place the collected BALF in a 2-mL microfuge tube and place it on ice until BALF samples from all mice are collected.
6. After collecting the BALF from all the experimental mice, centrifuge the BALF at 2000 × *g* for 5 min at 4 °C.
7. Transfer the supernatants into new microfuge tubes (*see* Note 7). Store them in aliquots of 100 μL at −80 °C until use (*see* Note 8).
8. Resuspend the cell pellets in 200 μL of PBS and keep at 4 °C until use (*see* Note 9).

3.2. Total and Differential Leukocyte Counts

3.2.1. DLC by Cytological Staining

1. Transfer 20 μL of the cell suspension from Subheading 3.1 step 8 into a microfuge tube and add an equal volume of trypan blue. Mix gently.
2. Place the solution on a Countess™ Cell Counting Chamber Slide and count the total number of cells using Countess II FL Automated Cell Counter. Alternatively, count the cells manually under a microscope using a hemocytometer and a cell counter.
3. Place 100 μL of the cell suspension from Subheading 3.1 step 8 and onto a glass slide. Use a Cytospin to disperse the cells uniformly onto the slide (*see* Notes 2 and 10).

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4. Leave the slide at room temperature for 30 min (*see* Note 11). Fix the air-dried cells for 30 s in the methanol fixative solution provided in the Diff-Quik Stain Kit.
5. Stain the slide with Diff-Quick Solution II for 30 s followed by counterstaining with Solution I for 30 s. Drain well between the stains.
6. Rinse the stained slide in tap water to remove excess stain and dehydrate in absolute ethanol. Place coverslip using two drops of mounting medium.
7. Perform the differential cell count using a digital light microscope at 100× magnification by oil immersion technique. Using a cell counter, count at least 200 cells per slide along a zigzag path, left to right and right to left as shown in Fig. 3.
8. Identify individual cell types based on the color and appearance as described in Table 2 and shown in Fig. 2.

3.2.2. DLC by Flow Cytometry

1. To 100 μL of the BALF cell suspension from Subheading 3.1, step 8, add 100 μL of 3% BSA in PBS and incubate for 1 h at room temperature as a blocking step. Prepare an additional cell sample as a no- stain (no antibodies) control to be used for flow cytometry (*see* Note 12).
2. Centrifuge the samples at 600 × *g* for 5 min and discard the supernatant.
3. Resuspend the cell pellet in 80 μL of PBS, add 20 μL of the antibody cocktail (Table 1), and incubate at room temperature for 1 h. Protect the cells from light (*see* Note 13).
4. Centrifuge the samples at 600 × *g* for 5 min and discard the supernatant (*see* Note 14).
5. Resuspend the pellets in PBS and centrifuge at 600 × *g* for 5 min and discard the supernatant. Repeat this step for one more time.
6. Add 100 μL of the diluted DAPI solution to the cell pellet and incubate for 10 min at room temperature.
7. Wash the cells with PBS and centrifuge the samples at 600 × *g* for 5 min and discard the supernatant to remove excess DAPI stain.
8. Resuspend the cell pellet in 100 μL of PBS and immediately perform flow cytometry.
9. Set the flow cytometer to capture at least 40,000 nucleated events.
10. Using the no-stain control sample from step 1 on an FSC vs. SSC plot, apply gating to eliminate unstained cells.
11. On an SSC vs. CD45 plot, apply gating to isolate total lymphocyte count.

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12. Identify T-lymphocytes on a CD45 vs. CD2, where $CD2^+$ cells are T-lymphocytes and $CD2^-$ cells are B-lymphocytes. Alternatively, on a CD45 vs. CD19 plot, identify $CD19^+$ as B-lymphocytes and $CD19^-$ cells as T-lymphocytes.

13. To identify eosinophils and neutrophils, plot SSC on the *x*-axis and CD294 on the *y*-axis. Here, eosinophils appear toward the *y*-axis and away from the *x*-axis while neutrophils appear away from the *y*-axis and toward the *x*-axis (*see* Note 15).

14. Determine the number of each cell type in a BALF sample using a flow cytometry data analysis software.

3.3. Multiplex Cytokine Assay by Flow Cytometry

1. Completely thaw the BALF supernatant from Subheading 3.1, step 7, and keep on ice prior to performing the assay.

2. Create a template for loading the standards and samples (*see* Table 3).

3. Sonicate the bottle of pre-mixed beads from the multiplex cytokine assay kit for 1 min (*see* Note 16).

4. Reconstitute the mouse inflammation panel standard cocktail using 250 μL of the assay buffer, keep it at room temperature for 10 min, and label it as C7.

5. Prepare 1:4 dilutions serially in the following sequence: C6, C5, C4, C3, C2, and C1. Use the assay buffer alone for the 0 pg/mL standard.

6. Prior to initiating the assay, wet the wells of the 96-well filter plate with 100 μL of the wash buffer and let it sit at room temperature for 1 min.

7. Remove the wash buffer by placing the filter plate on a vacuum manifold (*see* Note 17).

8. With the filter plate on an inverted plate lid, first add 25 μL of assay buffer to all the wells, and then add 25 μL of standards prepared at steps 3 and 4, or BALF supernatants to respective standard or sample wells (*see* Note 18).

9. Briefly vortex the bead mixture for 30 s and add 25 μL to each of the standard and sample wells with the filter plate on the inverted plate lid.

10. Seal the plate with a plate sealer, wrap the plate with aluminum foil, and incubate for 2 h at room temperature on a plate shaker at 500 rpm.

11. Remove the solution as described in step 6 and add 200 μL of the wash buffer to each well on the inverted plate lid (*see* Note 19).

12. Remove the wash buffer by applying vacuum to the filter plate on the manifold and blot any residual wash buffer using a paper towel.

13. Repeat steps 11 and 12 one more time.

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14. Add 25 μL of the detection antibody solution from the kit to each well. Seal the plate with a new plate sealer, wrap it with aluminum foil, and incubate for 1 h at room temperature on a plate shaker at 500 rpm.
15. After the 1-h incubation with the detection antibody, add 25 μL of SA-PE from the kit directly to each well. Seal the plate with a new plate sealer, wrap the plate in an aluminum foil and incubate for 30 min at room temperature on a shaker at 500 rpm.
16. Wash the plate twice by repeating steps 11 and 12. Add 150 μL of the wash buffer to each well on the inverted plate lid. Using a plate shaker, shake the plate briefly to resuspend the beads. The beads are ready for flow cytometry and should be analyzed on the same day.
17. Vortex the plate for 5 s and place it on an autosampler.
18. Set the flow rate to low and set the number of beads to be acquired at 300 per sample.
19. Analyze the data using the LEGENDplex™ data analysis software provided with the kit [34] (*see* Note 20).

3.4. Nitric Oxide Synthase Activity Assay

1. Completely thaw the BALF supernatants from Subheading 3.1, step 7 and keep on ice prior to performing the assay. Prepare all the reagents freshly on the day of the assay (*see* Note 21).
2. Prepare another identical set of tubes, omitting nitrate reductase. This sample set is used for determining nitrite content alone.
3. Incubate 100 μL of the BALF supernatant samples with 400 μL of the reaction buffer at 37 °C for 30 min to convert nitrate to nitrite.
4. Add 500 μL of 2 mM potassium ferricyanide to the sample tubes to make the final concentration to 1 mM. Incubate at 25 °C for 10 min to oxidize any unreacted NADPH in the reaction buffer.
5. Add 1 mL of Griess reagent and incubate at 25 °C for 10 min. Read the absorbance at 543 nm (*see* Note 22). The linear limit of detection for the assay is 1 mM [35].

4. Notes

1. Mice should be housed under constant temperature and a 12-h light/dark cycle with food and water provided *ad libitum*. All procedures must be conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approval by the Institutional Animal Care and use Committee at your institution. Always use personal protective equipment while handling mice or biological samples.

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2. If a cytospin is not available, BALF cells may be spread onto the slide by smearing a droplet of cell suspension across the slide with another glass slide held at a 30–45° angle.

3. Some multiplex kits offer a choice of either a filter plate or V-bottom plate for the 96-well sample plate. Here we describe a procedure using a filter plate.

4. To avoid damaging blood vessels, carefully remove the skin layer and use two blunt forceps to pull apart the thyroid tissue to expose the trachea.

5. While performing tracheostomy, be extremely cautious not to disturb any blood vessels near the tracheal incision as it will contaminate the BAL samples and the cellular analysis will be compromised.

6. It is almost impossible to extract the whole amount of BAL (1 mL) as there will be a 20% loss, which is expected. Massaging the thorax region may facilitate maximum recovery of the injected PBS. BALF appears as a slightly cloudy solution with clearly visible particulate matter.

7. After centrifuging the BALF sample at 600 × *g* for 5 min, take precaution while separating the supernatant from the cells. Leave the last 50 μL of the supernatant to prevent collecting any cells.

8. The supernatant from the BALF samples should be stored at −80 °C until analysis. Avoid repeated freeze–thaw cycles to prevent any degradation of cytokines. It is highly recommended to store the sample in equally divided aliquots of 100 μL each.

9. Keep the resuspended cells from BALF samples on ice and process immediately on the same day for DLC to avoid cell loss due to lysis.

8. During the cytospin procedure, proper assembling of the slide, filter paper, and the solution-holding accessory is vital. Make sure the openings of all three components align to prevent loss of cells during the centrifuging step. Carefully dismantle the cytospin components to prevent smudging of the smear.

9. Do not let the smear dry for more than 30 min.

10. The cell suspension for flow analysis should always be placed in a dark container and the tubes need to be wrapped in aluminum foil to prevent bleaching of fluorescent molecules.

11. When DLC is performed using flow cytometry, wash the cells thoroughly after the incubation with primary antibodies to avoid any artifacts, which compromise the integrity of data.

12. Thereafter, the bottle of beads should be vortexed for 30 s just prior to adding to samples.

13. Eosinophils are less granular and hence appear toward the *y*-axis, whereas neutrophils appear away due to dense granules. In terms of CD-294 staining, eosinophils stain positive for CD294 and appear away from the *x*-axis, whereas neutrophils stain negative for CD294 and hence appear closer to the *x*-axis. If the plot

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is separated into 4 quadrants, eosinophils appear on the top left quadrant and neutrophils appear on the bottom right quadrant.

14. The vacuum pressure should always be set to 10 mmHg to prevent any damage to the filter located at the bottom of the plate.

15. Centrifuge the samples and perform the assay with the supernatants to prevent any particulate matters from clogging the bottom of the plate during vacuum application. Perform a protein assay to quantify the protein concentrations of the supernatants and use the same amount of proteins for all samples when performing the cytokine or NOS assays.

16. Do not touch the bottom of the well with the pipette tip as you can damage the filter; instead, introduce the samples or any solutions along the sides of the wells.

17. If the filter becomes clogged at the bottom, use a pipette to pipette up and down the contents of the well. Clear the bottom of the clogged well with a clean wipe and apply vacuum.

18. If the data obtained is not within the standard range, adjust the dilution of the samples and repeat the assay.

19. For consistency of the reaction condition, assay all the samples to be compared at the same time.

20. If the sample values are too diluted, use a concentrating centrifuge tube like Vivaspin 6 Centrifugal Concentrator and repeat the assay.

21. For consistency of the reaction condition, assay all the samples to be compared at the same time.

22. If the sample values are too diluted, use a concentrating centrifuge tube like Vivaspin 6 Centrifugal Concentrator and repeat the assay.

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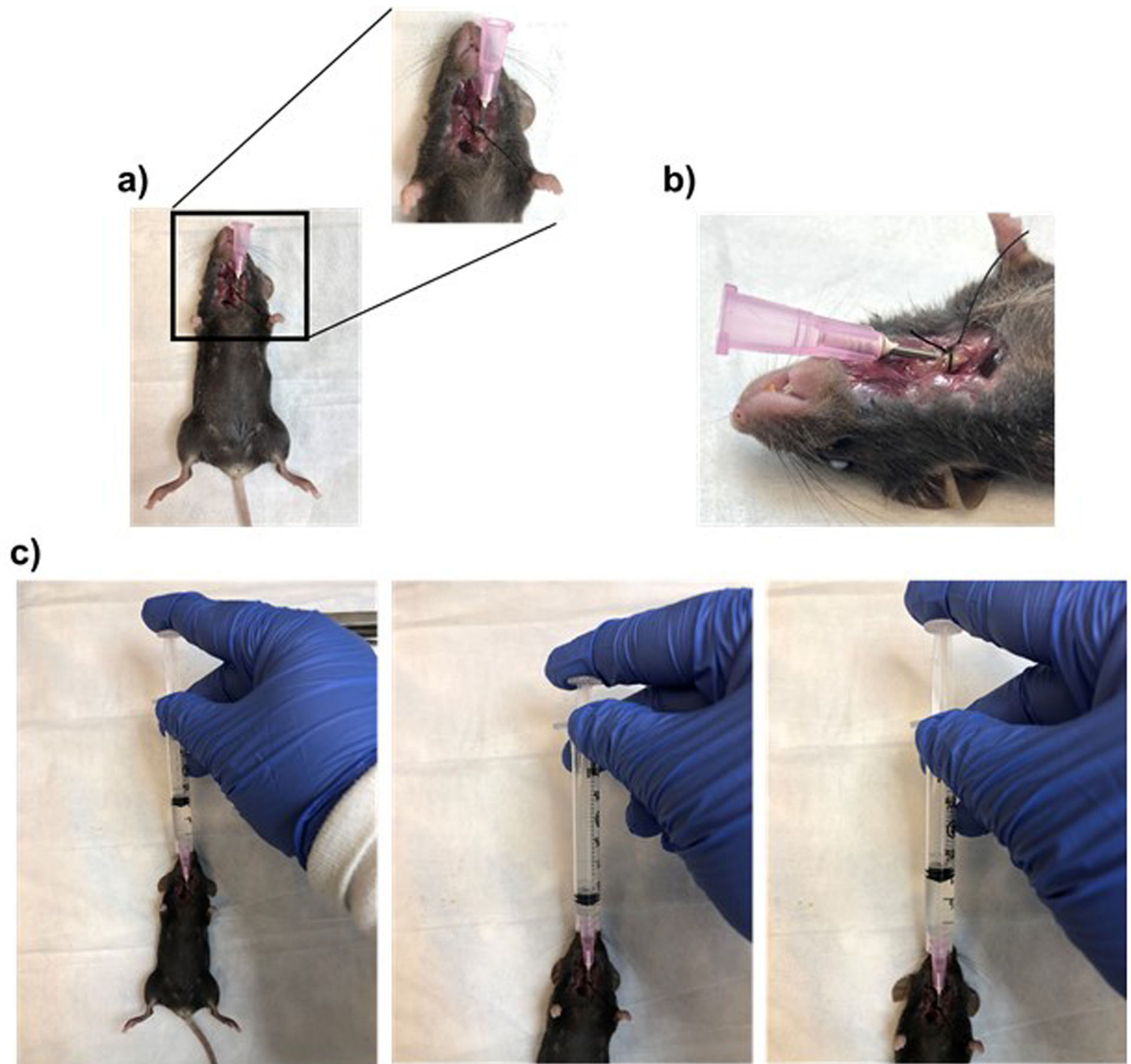

Fig. 1.
Collection of bronchoalveolar lavage (BAL). (a) Image showing tracheostomized mice in supine position. Inset shows magnified area of trachostomy. (b) Lateral view of tracheostomized mice. (c) From left to right: images showing the collection of BAL from tracheostomized mice using syringe connected to the canula

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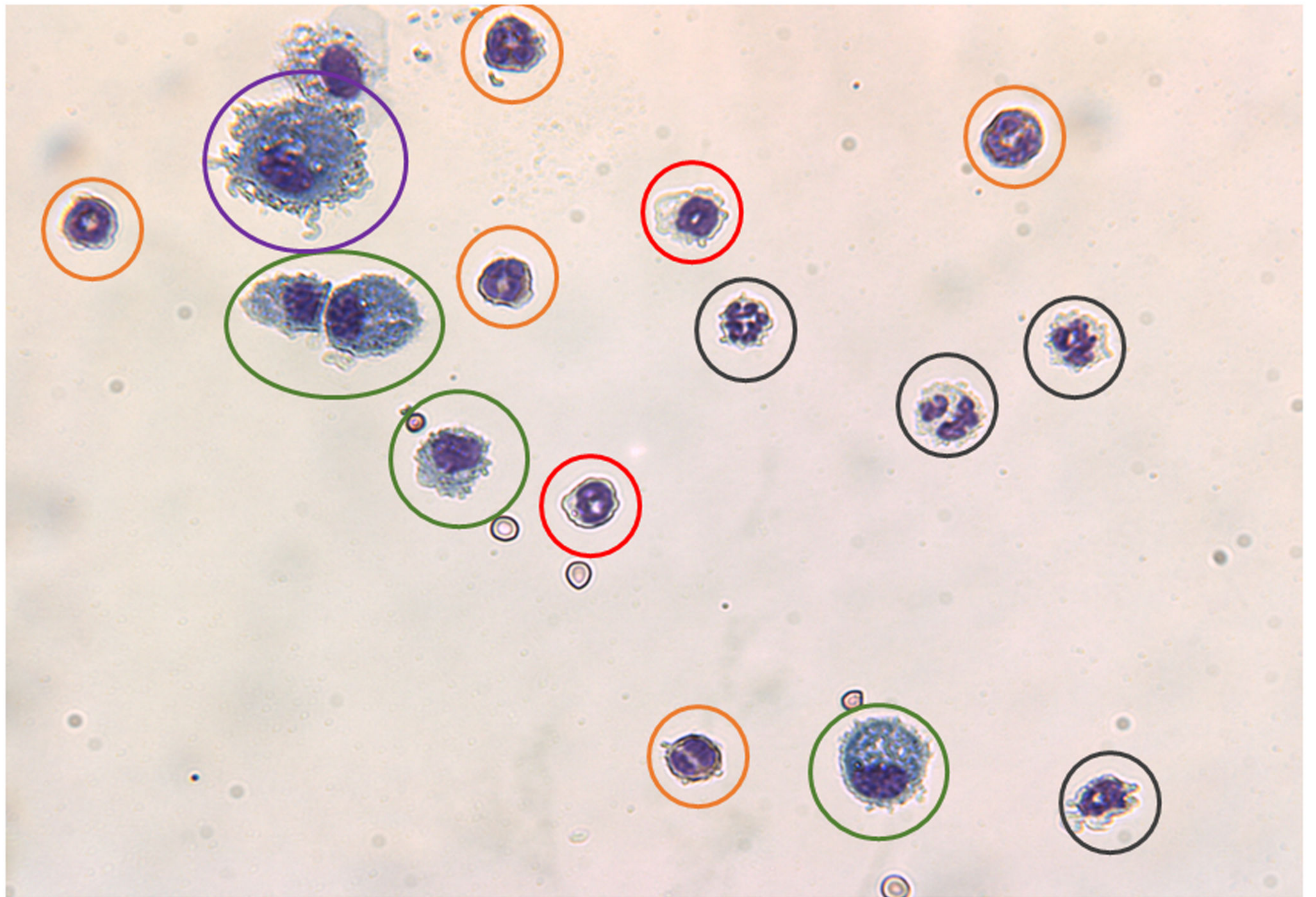

Fig. 2.

Figure showing major leukocytes found in BAL fluid. (**a**) Neutrophil (black circles); (**b**) eosinophil (red circles); (**c**) basophil (orange circles); (**d**) monocyte (green circles), and (**e**) macrophage (purple circles)

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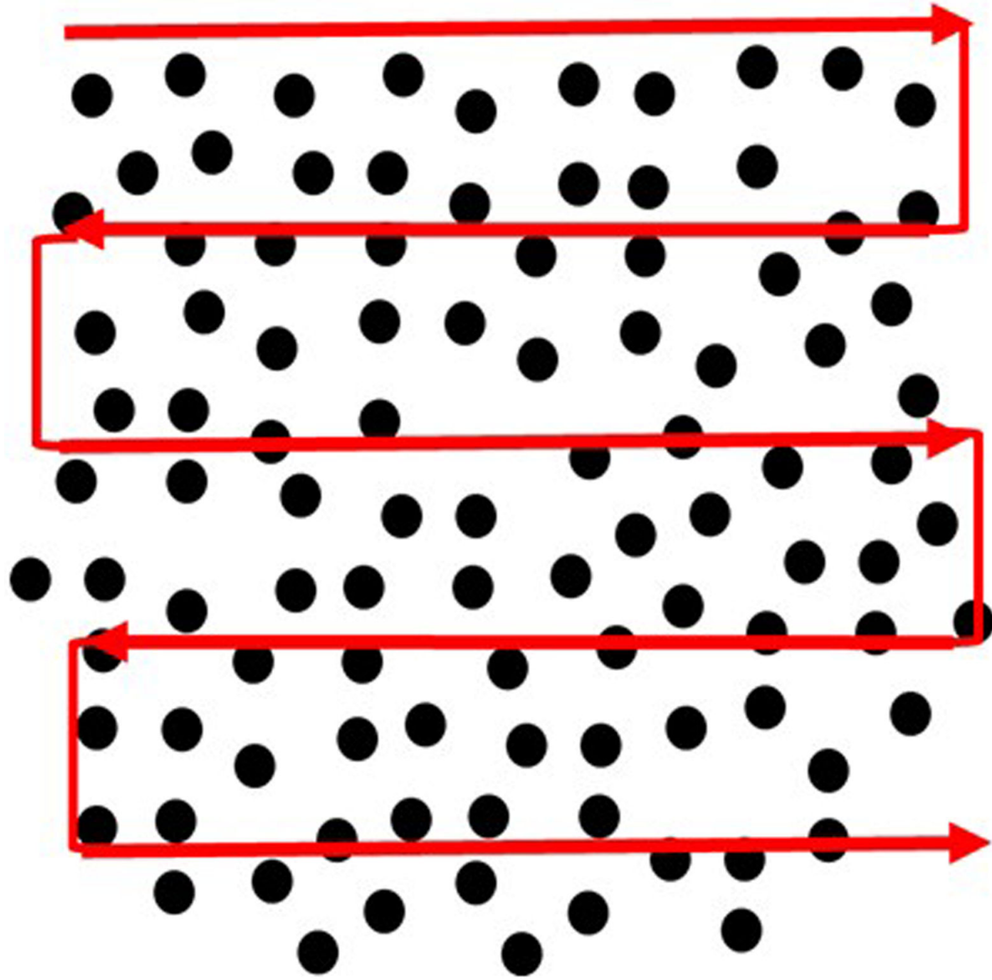

Fig. 3.
Representative image showing the pattern of counting cells on a stained BAL smear

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Table 1:

Antibodies (clones) and their working concentrations for identifying different leukocytes in murine BALF.

Antibody Target	Clone Name	Conjugation	Cell Type	Working Concentration
CD36	SMΦ	Alexa Fluor® 488	Monocytes/macrophages	0.4 μg/mL final concentration
CD2	3B6	PE	T lymphocytes	0.4 μg/mL final concentration
CD19	B-1	Alexa Fluor® 594	B lymphocytes	0.4 μg/mL final concentration
CD45	2D-1	Alexa Fluor® 680	Lymphocytes	0.4 μg/mL final concentration
CD294 (CRTH2)	No3m1scz	Alexa Fluor® 647	Granulocytes	0.4 μg/mL final concentration

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Table 2:

Morphological description of different leukocytes in BALF stained with Diffquik.

Cell Type	Color and Appearance
Neutrophils	Have a dark blue multi-lobed nucleus and pale pink cytoplasm with purple granules.
Eosinophils	Have a blue bi-lobed nucleus and cytoplasmic granules varying from red to reddish orange.
Basophils	Have a purple to dark blue nucleus and black or dark purple granules.
Monocytes	Have purple nucleus with sky blue cytoplasm.
Macrophages	Have purple nucleus with sky blue cytoplasm as monocytes but larger than other leukocytes.

Table 3:

Sample template for flowcytometric analysis of cytokines in using BioLegend 13-plex LEGENDplex™ Inflammation Panel. C0-C7 are standards and S1-S40 are samples.

	A	B	C	D	E	F	G	H	I	J	K	L
1	C0	C4	S1	S5	S9	S13	S17	S21	S25	S29	S33	S37
2	C0	C4	S1	S5	S9	S13	S17	S21	S25	S29	S33	S37
3	C1	C5	S2	S6	S10	S14	S18	S22	S26	S30	S34	S38
4	C1	C5	S2	S6	S10	S14	S18	S22	S26	S30	S34	S38
5	C2	C6	S3	S7	S11	S15	S19	S23	S27	S31	S35	S39
6	C2	C6	S3	S7	S11	S15	S19	S23	S27	S31	S35	S39
7	C3	C7	S4	S8	S12	S16	S20	S24	S28	S32	S36	S40
8	C3	C7	S4	S8	S12	S16	S20	S24	S28	S32	S36	S40

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