Bronchoalveolar Lavage and Lung Tissue Digestion

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[Abstract] Bronchoalveolar lavage (BAL) is a simple but valuable and typically performed technique commonly used for studying the pathogenesis of lung diseases such as asthma and COPD. Cell counts can be combined with new methods for examining inflammatory responses, such as ELISA, Flow cytometric analysis, immunohistochemistry, quantitative polymerase chain reaction, and HPLC to assess cellular expression for inflammatory cytokines and growth factor. Here we describe a basic procedure to collect BAL fluid and digest lung tissue for assessing a number of pulmonary components.

Materials and Reagents

1. Mice
2. Avertin (Sigma-Aldrich, catalog number: T48402)
3. PBS (Sigma-Aldrich, catalog number: D8537)
4. RPMI-1640 (Sigma-Aldrich, catalog number: R8758)
5. FACS buffer (PBS/0.5% BSA)
6. Diff-Quick stain kit (Dade Behring, catalog number: B4132-1A)
7. Single Cytology Funnels (Biomedical Polymers, Inc., catalog number: BMP-CYTO-S50)
8. Superfrost slides (Thermo Fisher Scientific, catalog number: 22-034-979)
9. Liberase Blendzyme (F. Hoffmann-La Roche, catalog number: 05401119001)
10. DNase (F. Hoffmann-La Roche, catalog number: 10104159001)
11. Trypan blue (Life Technologies, Invitrogen™, catalog number: 15250-061)
12. Mouse Fc block (BD Biosciences, Pharmingen™, catalog number: 553141)
13. Antibodies for T cells ($CD3^+$)
14. Antibodies for B cells ($B220^+$)
15. Antibodies for eosinophils (Siglec-F^+CD11c^-)
16. Antibodies for alveolar macrophages (AMs, Siglec-$F^+CD11c^+CD11b^{int}F4/80^+$)
17. Antibodies for interstitial macrophages (Ims, Siglec-$F^-CD11c^-CD11b^+F4/80^+$)
18. Antibodies for neutrophils (Siglec-$F^-CD11c^-CD11b^+Ly6G^+$)
19. Antibodies for dendritic cells (DCs) (Siglec-$F^-CD11c^{hi}MHCII^{hi}$)
20. Erythrocyte lysis buffer (see Recipes)

21. Tissue digestion solution (see Recipes)

Equipment

1. 1 ml, 3 ml, and 10 ml sterile syringes
2. 21 gauge lavage tube
3. 21 gauge sterile needles
4. Cotton thread No. 40
5. 50 ml flask
6. 15 ml and 50 ml conical tubes
7. Microtubes
8. 100 Micron cell strainer (BD Biosciences, Falcon®, catalog number: 352360)
9. Hemocytometer
10. Centrifuge (Beckman Coulter, model: Allegra™ 6R)
11. Cytospin cytocentrifuge (Thermo Fisher Scientific/Shandon, model: A7830002)
12. Microscope
13. BD LSR II flow cytometer
14. 3-Way Stopcocks (Bio-Rad Laboratories, model: 732-8103)
15. Magnetic stir bar (VWR International, model: 58949-006)
16. Multi-Position Magnetic Stirrers (VWR International, model: 12621-042)

Procedure

1. Anesthetize the mouse by intraperitoneal injection of 1 ml 2.5% Avertin in PBS.
2. Using scissors to expose thoracic cage and neck. Dissect tissue from neck to expose trachea.
3. Proceed to open the diaphragm by cutting the rib cage to expose both the heart and lungs. Take caution not to pierce heart or lungs.
4. Use forceps to slide 1 inch long piece of thread underneath trachea.
5. Make a small incision in the trachea, to allow passage of 21 gauge lavage tube into trachea. The distance between the proximal end of the trachea and the tracheal incision is 2-3 mm.
   Note: Do not cut trachea all the way through.
6. Cut a 1-1.5 inch segment of 21 gauge tube, carefully pass a 21 gauge needle into the tubing. Insert tubing into trachea and tie thread into single knot around tubing in trachea.
7. Slowly inject 1 ml cold PBS with 0.1 mM EDTA into lungs using “input” 3 ml syringe via 3-way stopcocks: Watch lungs inflating and do not overinflate.

8. Collect ~1 ml BAL fluid (BALF) from lungs using “output” 3 ml syringe into microtubes on ice.
9. Repeat steps 7-8 for 3 washes per animal, each using 1 ml PBS/0.1 mM EDTA through 3-way stopcocks. Remove syringe from needle, inject recovered lavage fluid to 15 ml falcon tube on ice.
   Note: For lung tissue digestion, please go to step 22.
10. Centrifuge microtubes containing ~1 ml BAL at 1,500 rpm for 5 min with brake.
11. Pipet supernatant (BALF) from these tubes into fresh microtubes, store at -80 °C until ready to perform ELISA.
12. Pipet 500 µl PBS to resuspend cell pellet in centrifuged microtubes and add all cells back into 15 ml conical tubes.
13. Centrifuge 15 ml conical tubes at 1,500 rpm for 5 min with brake and resuspend cells with 1 ml erythrocyte lysis buffer and keep on ice for 5 min.
14. Centrifuge the cells at 1,500 rpm for 5 min.
15. Discard supernatant and resuspend BAL cells in 500 µl RPMI or FACS buffer.
16. Add 50 µl of trypan blue to 50 µl saved aliquot, mix well and count cells using a hemocytometer. Calculate and record cell concentration.
    Note: Dark blue cells are dead and should not be counted.
17. Compute volume needed for 0.5-1 x 10^5 cells for each slide.
18. Pre-wet cytospin funnels by spinning with 300 µl PBS onto glass slides (reusable) at 600 rpm for 5 min.
19. Prepare microtubes containing 0.5-1 x 10^5 cells in 300 µl total volume. Spin BAL cells onto fresh labeled glass slides at 600 rpm for 10 min.
20. Remove all slides from the cytospin apparatus, and allow to air dry at least 2 h.
21. After dry, stain slides using Diff-Quick stain kit as follows:
    25 sec in fixative solution
    15 sec in solution I
    15 sec in solution II
    Rinse the slide in distilled water.
22. Perform differential cell counts under microscope at 100x magnification using oil-immersion lens (Figure 1).

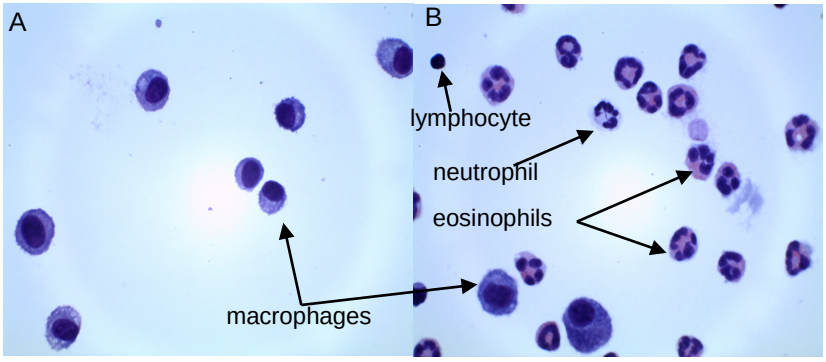

Figure 1. Photograph of cytospun BAL cells stained with Diff-Quick. (A) Control BAL; (B) BAL from asthmatic mice.

23. The following protocol is for lung tissue digestion. Immediately after lavage, perfuse the lung vascular bed using a 10 ml syringe filled with 5 ml PBS. Make a small incision in the left ventricle and connect a 21 G needle and insert needle into the right ventricle. Accurate perfusion will result in a color change to white.
24. Transfer lung lobes to a petri dish and chop it to small digestible pieces using a razor blade.
25. Transfer grounded lung tissue into a 50 ml flask containing 20 ml/lung of tissue digestion solution and magnetic stir bar. Incubate, stirring at regular speed, at 37 °C for 30-45 min.
    Note: This can be performed in 37 °C incubator.
26. Disperse the suspension by repeated aspiration through a 10 ml syringe, transfer to a 50 ml conical tube and centrifuge for 5 min at 1,500 rpm at 4 °C.
27. Lyse remaining erythrocytes by suspension in erythrocyte lysis buffer for 2 min at room temperature. Wash cells with 10 ml cold PBS/0.5% BSA and centrifuge for 5 min at 1,500 rpm at 4 °C.
28. Wash cells twice with 10 ml cold PBS/0.5% BSA, and filter through a 100-µm cell strainer.
29. Resuspend 1 millions cells in 50 µl of 1:200 Fc block in FACS buffer and incubate for 10 min on ice.
30. Wash the cells with 1 ml of PBS/0.5% BSA and spin down the cells for 5 min at 4 °C.
31. Discard the supernatant and stain cells with antibodies (1:100 in FACS buffer) and incubate for 30 min on ice.
    Note: All the fluorochrome-conjugated mAbs were purchased from eBioscience or Biolegend.
32. Wash the cells with 1 ml of PBS/0.5% BSA and spin down the cells for 5 min at 4 °C.
33. Resuspend the cells in 500 µl PBS/0.5% BSA and analyze the cells using BD LSR II flow cytometer (Figure 2).

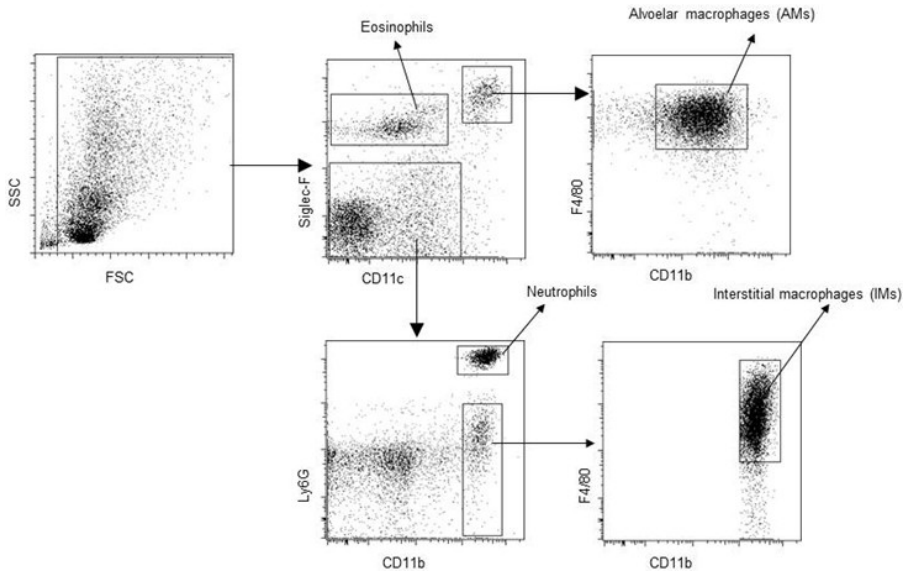

Figure 2. Gating strategy for lung digested cells. This strategy also applies to BAL cells.

Recipes

1. Erythrocyte lysis buffer
   NH_4Cl 16.4 g
   $KHCO_3$ 2 g
   EDTA 0.5 M 400 µl
   2 L ddH_2O
   Titrate with HCl to pH 7.2-7.4
2. Tissue digestion solution
   Serum-free RPMI 1640
   0.13 mg/ml Liberase Blendzyme
   20 U/ml DNase

Acknowledgments

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References

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