Video Article

Processing of Bronchoalveolar Lavage Fluid and Matched Blood for Alveolar Macrophage and CD4⁺ T-cell Immunophenotyping and HIV Reservoir Assessment

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URL: <https://www.jove.com/video/59427>

DOI: [doi:10.3791/59427](https://doi.org/10.3791/59427)

Keywords: Immunology and Infection, Issue 148, Bronchoalveolar lavage (BAL), alveolar macrophages, immunophenotyping, CD4 T cells, HIV RNA, HIV reservoir, myeloid cells, antiretroviral therapy (ART), alveolar macrophages (AMs)

Date Published: 6/23/2019

Citation: Salahuddin, S., Thomson, E., Méziane, O., Farnos, O., Pagliuzza, A., Chomont, N., Olivenstein, R., Costiniuk, C., Jenabian, M.A. Processing of Bronchoalveolar Lavage Fluid and Matched Blood for Alveolar Macrophage and CD4⁺ T-cell Immunophenotyping and HIV Reservoir Assessment. *J. Vis. Exp.* (148), e59427, doi:10.3791/59427 (2019).

Abstract

Bronchoscopy is a medical procedure whereby normal saline is injected into the lungs via a bronchoscope and then suction is applied, removing bronchoalveolar lavage (BAL) fluid. The BAL fluid is rich in cells and can thus provide a 'snapshot' of the pulmonary immune milieu. CD4 T cells are the best characterized HIV reservoirs, while there is strong evidence to suggest that tissue macrophages, including alveolar macrophages (AMs), also serve as viral reservoirs. However, much is still unknown about the role of AMs in the context of HIV reservoir establishment and maintenance. Therefore, developing a protocol for processing BAL fluid to obtain cells that may be used in virological and immunological assays to characterize and evaluate the cell populations and subsets within the lung is relevant for understanding the role of the lungs as HIV reservoirs. Herein, we describe such a protocol, employing standard techniques such as simple centrifugation and flow cytometry. The CD4 T cells and AMs may then be used for subsequent applications, including immunophenotyping and HIV DNA and RNA quantification.

Video Link

The video component of this article can be found at <https://www.jove.com/video/59427/>

Introduction

One of the most significant challenges facing a cure to HIV infection is the presence of the latent HIV reservoir which causes a rebound of plasma viremia following the interruption of antiretroviral therapy (ART)^{1,2}. While the HIV reservoir during long-term ART is well documented in several tissue compartments, including secondary lymphoid organs, gut-associated lymphoid tissue (GALT), and the central nervous system (CNS), the lungs have been overlooked as an area of study since the pre-ART era³. However, the lungs play a central role in the pathogenesis of HIV. Indeed, pulmonary symptoms were among the first indicators of AIDS-related opportunistic infections⁴. Even in the modern ART era, persons with HIV are at a greater risk of developing both infectious and noninfectious pulmonary diseases than persons without HIV. For example, persons with HIV infection are at elevated risk for invasive *Streptococcal pneumoniae* infection, as well as chronic obstructive pulmonary disease (COPD)^{5,6}. Furthermore, coinfection of tuberculosis (TB) and HIV is a significant public health challenge in certain regions of the world, notably, sub-Saharan Africa, as HIV-infected individuals are 16 to 27 times more likely to have TB than persons without HIV⁷. Although some explanations for this susceptibility to pulmonary infection and chronic disease have been proposed^{8,9,10}, the precise cellular mechanisms by which individuals with suppressed HIV plasma viral load remain at higher risk for pulmonary complications have not been fully elucidated. Importantly, HIV is a very strong risk factor for pulmonary infection and chronic disease, independent of smoking status⁶.

Analysis of the immune environment of the lung is, therefore, crucial in order to understand its role in health and disease. Although noninvasive, induced sputum samples tend to contain large amounts of epithelial cells and debris with rare pulmonary lymphocytes and no AMs, limiting their role to specific applications. Conversely, large biopsies of tissue cannot be obtained in the absence of suspected disease due to associated risks of significant bleeding and pneumothorax (collapse of the lung). Furthermore, the majority of pulmonary immune cells are mainly located at the mucosal level where the lungs are continuously stimulated by antigens during breathing. To that end, bronchoscopy to obtain BAL fluid

has the advantage of providing relatively safe access to lymphocytes and AMs (see **Figure 1**). Macrophages constitute the largest proportion of cells within BAL fluid, followed by lymphocytes¹¹. It is useful, therefore, to establish a method by which BAL fluid may be processed for use in subsequent applications, such as immunophenotyping, cell culture, transcriptomics, or any further applications. The protocol for processing the BAL fluid outlined here is adapted from general procedures previously described and optimized for the various downstream assays employed. This methodology allows for the isolation of both pulmonary lymphoid and myeloid mucosal immune cells for their phenotypical and functional characterization, as well as an assessment of the HIV reservoir in adults living with HIV.

To establish this protocol, we used the following criteria to recruit study participants¹⁵. For participants to be eligible to participate in this study, they had to be HIV-infected individuals who met the following criteria: (1) on ART for at least 3 years; (2) suppressed viral load (VL) for a minimum of 3 years; (3) CD4 T cell count of $\geq 200/\text{mm}^3$; (4) willing to undergo research spirometry and bronchoscopy. Patients with the following criteria were excluded from the study: (1) contraindication(s) to bronchoscopy; (2) high bleeding risk: coagulopathy or on warfarin or clopidogrel therapy; (3) thrombocytopenia (low platelets); (4) active pulmonary infection or another acute pulmonic process; (5) pregnant/trying to become pregnant.

Protocol

This research protocol was established directly based on the principles included in the Declaration of Helsinki and received approval from the Institutional Review Boards of the McGill University Health Centre (RI-MUHC, #15-031), the Université du Québec à Montréal (UQAM, #602) and the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CR-CHUM, #15-180).

1. Bronchoalveolar Lavage

NOTE: This section describes bronchoscopy as performed by a licensed respirologist with assistance from a respiratory therapist^{16,17}.

1. Prepare the pieces of apparatus needed for the procedure, including a bronchoscope and saline. Administer anesthetic spray to the back of the patient's throat. Avoid excessive use of topical anesthesia when possible. Apply cardiac leads to the chest in order to monitor the heart rate and rhythm and an oxygen probe to the first finger of a hand in order to monitor the oxygen saturation. Insert nasal cannula into the nostrils to provide supplemental oxygen.
2. Position the patient, preferably in the supine position. Administer sedation as follows: midazolam 0.01-0.04 mg/kg and fentanyl 50-100 μg (to facilitate patient comfort and minimize cough reflex) intravenously, in the presence of a respirologist or anesthetist.
3. Advance the flexible bronchoscope until it is wedged in the desired subsegmental bronchus. Instill saline (50-60 mL at a time) with the syringe, and then apply gentle suction (50-80 mmHg). The lavage fluid will collect in the syringe and then be transferred to a collecting container.
4. Repeat the flush to a total of 200-300 mL of lavage. Collect at least 100 mL of BAL fluid if possible.
5. Place the BAL fluid on ice.

2. Isolation of BAL Cells

NOTE: The following procedure must be carried out under sterile conditions in a biological safety cabinet, class II (BSL2) or higher.

1. **Keep the BAL samples on ice until they are processed.**
 1. Vortex the BAL in the original collection tube and transfer it to a 50 mL tube using a serological pipette. If the BAL fluid appears very turbid or contaminated by filamentous tissue, filter the fluid through a 70 μm nylon mesh filter into a new 50 mL tube.
 2. Centrifuge at 200 x g for 10 min at 4 °C. Transfer the supernatant to a new 50 mL tube. Gently break up the pellet with a pipette tip and resuspend it in 1 mL of RPMI 1640 medium.
 3. Transfer 1 mL of the supernatant to each of 10x 1.5 mL microcentrifuge tubes and the remaining supernatant to 15 mL tubes, 10 mL in each. Store all supernatant tubes at -80 °C.
2. **Process the BAL cell pellet.**
 1. Resuspend the pellet in 10 mL of RPMI 1640 for every 25 mL of the original sample. Centrifuge at 200 x g for 10 min at 4 °C. Transfer the supernatant to a new 15 mL tube (discard after ensuring there are enough cells in the pellet).
 2. Resuspend the pellet in 1 mL of RPMI 1640 + 10% fetal bovine serum (FBS) and count using trypan blue and a hemocytometer.

NOTE: If the BAL fluid is not separated by the adherence of cells before sorting, proceed to section 4.

3. Adherence of BAL Cells (Optional)

NOTE: This alternative protocol can be performed prior to or instead of cell sorting. The following procedure must be carried out under sterile conditions in a BSL2 cabinet (or higher).

1. Transfer the desired number of BAL cells for sorting to a new 15 mL tube and make up the correct volume for 1.5×10^6 macrophages/mL. Plate 2 mL of cells per well in 6-well plates and incubate for 2 h at 37 °C with 5 % CO_2 , to allow time for adherence.
2. Following incubation, carefully aspirate the media containing nonadherent cells and transfer it to a 15 mL tube. Centrifuge at 300 x g for 10 min at room temperature (RT). Remove the supernatant and resuspend at 1×10^7 cells/mL in phosphate-buffered saline (PBS) + 2 % FBS and transfer the suspension to a 5 mL round-bottomed polystyrene tube. This lymphocyte fraction is now ready to stain for cell sorting.
3. To the remaining adherent cells in the plate, add 1 mL per well of cell-disassociation solution (see the **Table of Materials**) and incubate for at least 15 min at 37 °C with 5% CO_2 , until the cells separate easily from the plate with a pipette tip.

4. Gently but thoroughly scrape the adherent cells from the well surface using a pipette tip, and use 1 mL of liquid in the well to assist with the detachment. Transfer the cells to a new 15 mL tube. Wash the wells with 1 mL of PBS and add this to the same tube. Make up the content of the tube to 5 mL with PBS.
5. Centrifuge at $300 \times g$ for 10 min at RT. Remove the supernatant, resuspend at 1×10^7 cells/mL PBS + 2% FBS, and transfer the suspension to a 5 mL round-bottomed polystyrene tube. This myeloid fraction is now ready to stain for cell sorting.

4. Isolation of Peripheral Blood Mononuclear Cells

NOTE: The following procedure must be carried out under sterile conditions in a BSL2 cabinet (or higher).

1. On the same day of the bronchoscopy (generally directly before the BAL collection), obtain six tubes of venous blood from a donor in ethylenediaminetetraacetic acid (EDTA) tubes (approximately 10 mL per tube).
2. Separate the blood by centrifuging the blood tubes at $300 \times g$ for 15 min at RT. Transfer the plasma to 1.5 mL microcentrifuge tubes in 1 mL aliquots and store it at -80°C .
3. **Perform density gradient separation.**
 1. Add 2 mL of RPMI 1640 to each blood tube and mix well using a serological pipette.
 2. Transfer to 3x 50 mL tubes and make up the volume in each tube to 25 mL with RPMI 1640.
 3. Prepare another batch of 3x 50 mL tubes, each containing 20 mL of lymphocyte separation medium (LSM) (see the **Table of Materials**) at RT. Slowly and gently layer the 25 mL of diluted blood on top of the LSM for each of the three tubes, holding the tube at a 45° angle.
 4. Centrifuge at $600 \times g$ for 25 min at RT with low acceleration and no deceleration (brake off).
4. **Perform a washing of peripheral blood mononuclear cells (PBMCs).**
 1. Transfer the layer of cells at the interface of the two liquid phases in the tube to a 50 mL tube using a serological pipette; if there is more than 30 mL of volume, divide it into two tubes. Make up the volume in each tube to 50 mL with PBS.
 2. Centrifuge at $700 \times g$ for 5 min at RT and remove as much supernatant as possible.
 3. Resuspend the pellet and make up the volume to 25 mL with PBS. Centrifuge at $350 \times g$ for 10 min at RT and remove as much supernatant as possible.
 4. Repeat the wash step described in step 4.4.3.
 5. Resuspend the pellet in 5 mL of PBS + 2% FBS and count the cells.

5. Sorting Whole BAL cells and PBMCs

NOTE: The following procedure must be carried out under sterile conditions in a BSL2 (or higher).

1. Prepare sorting buffer containing PBS + 5% FBS + 25 mM HEPES (pH 7.4). Prepare 5 mL round-bottomed polystyrene tubes with 1 mL of FBS for the collection of sorted cell subsets.
2. **Perform staining.**
 1. Prepare 3x 5 mL round-bottomed polystyrene tubes, each for BAL (whole cells or lymphocyte and myeloid fractions after adherence) and PBMCs (see section 4). For each subset, prepare one tube with cells to sort and two tubes of 5×10^5 cells to use for unstained and viability stain compensation controls.
 2. Centrifuge at $350 \times g$ for 5 min at 4°C . Remove the supernatants, resuspend the cells for controls in 100 μL of PBS, and store them at 4°C until the compensation controls can be prepared as described in step 5.2.6.
 3. Prepare a 1:20 dilution of Fc receptor (FcR) blocking reagent in PBS + 5% FBS (see the **Table of Materials**-to prevent the nonspecific binding of antibody to FcR on FcR-expressing cells). Resuspend the cells to sort them at 1×10^7 cells in 250 μL of FcR-blocking mixture. Incubate them for 1 h at 4°C .
 4. After incubation, add the appropriate antibody cocktail (see **Table 1**) to the cells and incubate for 1 h at 4°C in the dark.
 5. After 1 h of staining, add 1 mL of PBS to the cells and centrifuge at $350 \times g$ for 5 min at 4°C . Remove the supernatant and resuspend cells in sorting buffer to have 1×10^7 cells in 250 μL . Filter the cells through a 70 μm filter if needed.
 6. Prepare compensation controls.
 1. Add three drops each of anti-mouse Ig, κ , and negative control compensation beads (see the **Table of Materials**) per 1 mL of PBS in a microcentrifuge tube and transfer 100 μL to each 5 mL round-bottomed polystyrene tube to be used for compensation. Prepare one tube for each fluorochrome present in the cocktail to be used.
 2. Add 1 μL of each antibody in the cocktail to a different tube containing beads. Add 1 μL of viability stain to one of the tubes of 5×10^5 cells set aside in step 5.2.1. Incubate for 20 min at 4°C in the dark.
 3. Add 1 mL of PBS to each tube and centrifuge at $350 \times g$ for 5 min at 4°C . Remove the supernatant and resuspend the pellet in 250 μL of PBS. Store at 4°C in the dark until needed.
7. Sort the cells by fluorescence-activated cell sorting (FACS) into collection tubes prepared with 1 mL of FBS and swirl gently to coat the sides of the tubes with serum.
 1. Sort BAL cells at low pressure. Gate cells first to exclude noise and include live, $\text{CD}45^+$ cells, and within this population gate out doublet cells (See **Figure 3**). Within the larger myeloid population sort $\text{CD}206$ and $\text{CD}169$ double positive cells as AMs; within the smaller lymphocyte population isolate $\text{CD}3^+$ cells and sort both $\text{CD}4$ and $\text{CD}8$ single positive populations (See **Figure 3**; gating strategy detailed in the representative results section).
 2. When sorting PBMCs, gate cells first to exclude noise and include live, $\text{CD}45^+$ cells, and within this population gate out doublet cells. Next, gate on $\text{CD}3$ cells and within the $\text{CD}3^+$ population, gate first on $\text{CD}14$ and sort single-positive monocytes, and then

within the CD3⁺ population gate on CD4 and CD8 and sort both single positive populations (gating strategy detailed in the Representative Results section).

6. Immunophenotyping of AMs and PBMCs

NOTE: The following procedure must be carried out under sterile conditions in a BSL2 cabinet (or higher).

1. Add up to 1 million each of AMs and PBMCs to two separate 5 mL round-bottomed polystyrene tubes. Centrifuge at 300 x *g* for 5 min at 4 °C and remove the supernatant.
2. Perform FcR blocking to improve the specificity of the antibody staining. For this, resuspend the cells in 100 µL of PBS + 2% FBS and add 1.4 µL of FcR blocking reagent. Incubate for 20 min at 4 °C.
3. **Perform extracellular staining.**
 1. Following the incubation with FcR block, add the desired extracellular antibody cocktail, vortex the tubes, and incubate for 1 h at 4 °C in the dark.
 2. Wash 2x by adding 500 µL of PBS and centrifuging at 350 x *g* for 5 min at 4 °C.
4. **Prepare for fixation and permeabilization (see the Table of Materials for specific reagents used).**
 1. Prepare permeabilization solution with 1 part permeabilization buffer and 3 parts diluent buffer. Resuspend the pellet in 1 mL of permeabilization solution and incubate for 40 min at 4 °C in the dark.
 2. Prepare wash solution using 1 part wash buffer and 4 parts H₂O. Add 2 mL of wash solution to the permeabilized cells and centrifuge at 350 x *g* for 5 min at 4 °C. Remove the supernatant.
5. **Perform intracellular staining.**
 1. Resuspend the cells in 100 µL of 1x wash solution, add the desired intracellular antibodies, vortex the tubes, and incubate for at least 1 h at 4 °C in the dark.
 2. Add 2 mL of wash solution and centrifuge at 350 x *g* for 5 min at RT. Remove the supernatant and resuspend the pellet in 200 µL of PBS. Store the cells at 4 °C in the dark until needed.

7. Remainder of BAL Cells

NOTE: The following procedure must be carried out under sterile conditions in a BSL2 cabinet (or higher).

1. Cell numbers permitting, cryopreserve live cells from the BAL cell pellet (from step 2.2.2).
 1. Prepare freeze media containing 90% FBS + 10% dimethyl sulfoxide (DMSO).
 2. Centrifuge the cells at 300 x *g* for 10 min at 4 °C. Remove the supernatant and resuspend in 1.5 mL of freeze media in a cryogenic vial. Transfer the cryogenic vials to a controlled-rate freezing container (see the **Table of Materials**) and place them at -80 °C. Transfer the cells to liquid nitrogen for long-term storage once the temperature is reached.
2. **Preserve the BAL cells as dry pellets.**
 1. Transfer the remaining cells to a 1.5 mL microcentrifuge tube. Centrifuge in a counter-top centrifuge at 6,000 x *g* for 1 min. Remove as much supernatant as possible without disturbing the pellet. Store the pellet at -80 °C.

8. HIV DNA and RNA Quantification

NOTE: The following procedure must be carried out under sterile conditions in a BSL2 cabinet (or higher).

1. **Total HIV DNA quantification**
 1. To avoid the inhibition of polymerase chain reaction (PCR) with BAL lysate debris, use a DNA extraction kit (see the **Table of Materials**) to extract DNA from a sample of BAL cells according to the manufacturer's instructions. Use 15 µL of this DNA combined with a master mix in the preamplification step described below (step 8.1.3).
 2. Prepare standard curve dilutions.
 1. As above, use a DNA extraction kit to extract DNA from a pellet of 2 x 10⁶ ACH-2 cells (see the **Table of Materials**).
 2. After elution of the DNA, perform serial 10-fold dilutions of the ACH-2 DNA to generate six dilutions, ranging from 3 x 10⁵ cells to 3 cells per 15 µL.
 3. Perform a preamplification step.
 1. In a separate room, prepare the master mix for *n* + 2 samples comprising 1x polymerase buffer, 3 mM of MgCl₂, 300 µM dNTPs, and 2.5 U of Taq DNA polymerase (see the **Table of Materials**) and 300 nM of each of the four primers (see step 8.1.3.2). Perform all measures in triplicate wells.
 2. Use primers hCD3OUT5', hCD3OUT3', ULF1, and UR1 to generate amplified DNA from both human CD3 and HIV (see the sequences in **Table 2**). Note that both genes are preamplified in the same tube. Mix gently and spin down the tube to ensure complete mixing.
 3. Distribute 35 µL of master mix per well in a 96-well PCR plate and add 15 µL of standard or sample DNA. The total reaction volume is 50 µL.

4. Perform the preamplification (denaturation at 95 °C for 8 min, followed by 12 cycles of 95 °C for 1 min, 55 °C for 40 s, 72 °C for 1 min, and elongation at 72 °C for 15 min).
4. Perform real-time PCR.
 1. To quantify CD3 and HIV DNA, prepare two master mixes containing 1x PCR reaction master mix (see the **Table of Materials**), 1,250 nM appropriate primers, and 100 nM probe. Use primers HCD3IN5' and HCD3IN3' and probe CD3 FamZen to quantify human CD3 in one reaction, and primers UR2 and LambdaT and probe UHIV FamZen to quantify HIV DNA in another reaction (see the sequences in **Table 2**). Distribute 13.6 µL of each mix in qPCR-adapted tubes.
 2. Dilute the preamplification PCR product at 1:10 in sterile water, DNase, RNase, and protease free. Add 6.4 µL of each diluted sample to 13.6 µL of qPCR mix in qPCR-adapted tubes for a total reaction volume of 20 µL.
 3. Perform the real-time PCR using the following program: denaturation at 95 °C for 4 min and 40 cycles of 95 °C for 3 s and 60 °C for 10 s with single acquisition.
 4. Extrapolate the number of HIV copies and number cell equivalents in each reaction tube from the standard curves. Calculate the number of HIV DNA copies/10⁶ cells.

2. HIV RNA quantification

1. Extract RNA from a sample of BAL cells, using an RNA extraction kit (see the **Table of Materials**) according to the manufacturer's instructions. Use 17 µL of this RNA in the reverse transcription and preamplification step described below (step 8.2.4).
2. LTR-gag RNA synthesized in vitro and precisely quantified is used as standard; it is spiked into healthy donor RNA extract for GUSB normalization. Prepare six serial 10-fold dilutions of this standard, corresponding to 3 x 10⁵ cells to three copies of LTR-gag RNA in 17 µL.
3. Distribute 17 µL of each standard dilution and each sample in a 96-well PCR plate and treat the samples with DNase (see the **Table of Materials**) for 10 min at 25 °C to remove contaminant genomic DNA. Stop the reaction by adding 2 µL of 25 mM EDTA and incubate the samples for 10 min at 65 °C.
4. Perform reverse transcription (RT) and preamplification PCR.
 1. Perform this step using a one-step RT-PCR kit (see the **Table of Materials**) according to the manufacturer's instructions. Use primers GUSB forward 1, GUSB reverse 1, UR1, and ULF1 to generate amplified cDNA from both human GUSB as housekeeping gene and LTR-gag HIV RNA (see the sequences in **Table 2**). The GUSB values will be used to normalize the HIV values.
 2. Distribute 31 µL of master mix per well in the same 96-well PCR plate containing the DNase-treated standards and samples and mix well. The total reaction volume is 50 µL.
 3. Run the plate for 16 cycles according to the manufacturer's instructions, with an annealing temperature of 55 °C.
5. Perform real-time PCR.
 1. Prepare two master mixes containing 1x PCR reaction master mix (as above in step 8.1.4.1), 1250 nM appropriate primers, and 100 nM probe. Use primers GUSB forward 2, GUSB reverse 2, and probe GUSB-HEX to quantify GUSB cDNA in one reaction; use primers UR2, LambdaT, and probe UHIV FamZen to quantify HIV cDNA in another reaction (see the sequences in **Table 2**).
 2. Distribute 13.6 µL of each master mix in qPCR-adapted tubes. Dilute the RT preamplification PCR products 1:10 in sterile water, DNase, RNase, and protease free, and add 6.4 µL of each diluted sample or standard to the appropriate PCR mix. The total reaction volume is 20 µL.
 3. Perform the real-time PCR using the following program: denaturation at 95 °C for 4 min and 40 cycles of 95 °C for 3 s and 60 °C for 10 s with single acquisition (select the green channel for FamZen and yellow for HEX).

Representative Results

In most nonsmokers, BAL fluid is received in a sterile container and is a slightly turbid yellow-orange-colored liquid. The fluid may be pinker in color if the donor underwent endobronchial biopsies during the bronchoscopy and some bleeding occurred. The fluid may be darker in color if the donor is a smoker. After centrifugation, the BAL supernatant will be almost clear and slightly orange, while the cell pellet can range in color from off-white to very dark brown, depending on the condition of the sample and whether the donor was a smoker or not.

When counting the whole BAL sample, different cell types can be visualized, including larger, round macrophages around 17 µm in diameter and smaller round lymphocytes around 7.3 µm in diameter^{18,19} (see **Figure 2**). Macrophages are enlarged in smokers by about 40%¹⁸. The distinction between the cell types allows for counting the macrophages and lymphocytes separately. There may also be some debris visible in the field, especially in samples from smokers. Macrophages are the most abundant cell type in the BAL, accounting for approximately 85% of cells in nonsmokers²⁰, and they are enriched in smokers so they may seem almost exclusive.

The BAL cells have a tendency to aggregate, so they must be mixed well during all manipulations. The pellet may appear dark even after several wash steps. If filamentous debris is evident in the fraction after staining for cell sorting, pass the cells through a 70 µm filter before running them through the cell sorter.

The sorting of BAL cells must be done at a low pressure to ensure droplet sizes large enough to accommodate the macrophages. The cells are first gated to include all CD45⁺ cells, and then based on viability to ensure all dead cells are excluded (see **Figure 3**). Singlet cells are then chosen and within this, two populations are gated based on size and morphology, namely larger myeloid cells and smaller lymphocytes (see **Figure 3**). Within the larger cells, cells are gated on CD206^{22,23} and CD169²² and the double-positive cells are sorted as AMs, while within the smaller cells, CD3⁺ cells are chosen and gated on CD4 and CD8; CD4 single-positive and CD8 single-positive cells are sorted (see **Figure 3**). The markers used were chosen based on previously described phenotypes of AMs, such as the mannose receptor CD206, found on phagocytic cells²³, and the sialoadhesin receptor CD169²².

When sorting the PBMCs, cells are first gated on forward and side scatter which should show a homogeneous lymphocyte population, all of which are taken, excluding noise close to the zero-axis (data not shown). The population is gated on viability and CD45, and live CD45⁺ cells are used. This population is then gated on CD3; to isolate monocytes, the CD3⁺ population is subsequently gated on CD14 and all single positive cells are sorted. To isolate lymphocyte subsets, the CD3⁺ cells are gated on CD4 and CD8 and both single positive and cell populations are sorted.

Figure 1: Protocol overview. A schematic showing the workflow of the protocol, including potential downstream uses of the generated samples. PBMC = peripheral blood mononuclear cells; BAL = bronchoalveolar lavage; LSM = lymphocyte separation medium. [Please click here to view a larger version of this figure.](#)

Figure 2: Microscope field view of whole BAL fluid. Microscope images from (A) a nonsmoker and (B) a smoker with visible lymphocytes (L), macrophages (M), and red blood cells (RBC). Magnification is 1,000x (10x ocular and 100x lens with oil immersion). [Please click here to view a larger version of this figure.](#)

Figure 3: Representative gating strategy for the cell sorting of whole BAL cells. Gating strategy used to sort alveolar macrophages (AM), CD4, and CD8 T cells from whole BAL cell samples. [Please click here to view a larger version of this figure.](#)

Sample	Antibody	Fluorochrome	Clone	Volume per test (μL)
BAL and PBMC	Live/Dead	APC-H7	-	1
	CD45	PE-Cy7	HI30	5
	CD3	Alexa700	UCHT1	2
	CD4	PE-cy5	RPA-T4	4
	CD8	BV605	SK1	3
BAL only	CD206	PE	19.2	10
	CD169	BB515	7-239	5
PBMC only	CD14	BV786	M5E2	5

Table 1: Flow panel for the sorting of whole BAL cells and isolated PBMCs.

Target	Step	Primer Name	Primer Sequence
HIV Total DNA or HIV LTR-Gag RNA	Pre-amplification PCR	UR1	5'-CCA TCT CTC TCC TTC TAG C-3'
		ULF1	5'-ATG CCA CGT AAG CGA AAC TCT GGG TCT CTC TGG TTA GAC-3'
	Real-time PCR	UR2	5'-CTG AGG GAT CTC TAG TTA CC-3'
		LambdaT	5'-ATG CCA CGT AAG CGA AAC T-3'
		UHIV FamZen:	5'-/56-FAM/CA CTC AAG G/ZEN/C AAG CTT TAT TGA GGC/3IABkFQ/-3'
CD3 DNA	Pre-amplification PCR	HCD3 out 5'	5'-ACT GAC ATG GAA CAG GGG AAG-3'
		HCD3 out 3'	5'-CCA GCT CTG AAG TAG GGA ACA TAT-3'
	Real-time PCR	HCD3 in 5'	5'-GGC TAT CAT TCT TCT TCA AGG T-3'
		HCD 3 in 3'	5'-CCT CTC TTC AGC CAT TTA AGT A-3'
		CD3 FamZen:	5'-/56-FAM/AG CAG AGA A/ZEN/C AGT TAA GAG CCT CCA T/3IABkFQ/-3'
GUSB RNA	Pre-amplification PCR	GUSB Forward 1:	5'-ACC TAG AAT CTG CTG GCT ACT A-3'
		GUSB Reverse 1:	5'- GTT CAA ACA GAT CAC ATC CAC ATA C-3'
	Real-time PCR	GUSB Forward 2:	5'-TGC TGG CTA CTA CTT GAA GAT G-3'
		GUSB Reverse 2:	5'- CCT TGT CTG CTG CAT AGT TAG A-3'
		GUSB-HEX:	5'-/5HEX/TCGCTCACA/ZEN/CCAAATCCTTGACC/3IABkFQ/-3'

Table 2: Primer and probe sequences for HIV DNA and RNA quantification.

Discussion

Herein we described a method for processing BAL fluid to obtain CD4 T cells and AMs, alongside matched PBMCs, which can be studied to investigate the HIV reservoir within the lungs. We recently reported on HIV DNA quantification in CD4 T cells from matched peripheral blood and BAL samples, and our group demonstrated that HIV is 13 times more abundant in pulmonary CD4 T cells than in those from peripheral blood¹⁵.

However, the levels of HIV DNA in AMs are donor-dependent and so, thus far, there has not been a consistent correlation between HIV DNA levels in the lymphocytes compared to macrophages¹⁵. The access to these primary macrophage cell subsets, however, will be a vital tool to interrogating this question and gaining a better understanding of the viral load in the lung in the context of the HIV reservoir.

In the pre-ART era and in several other studies utilizing BAL fluid, participants underwent bronchoscopy in order to diagnose a suspected pathology or obtain a microbiological diagnosis for respiratory symptoms³. However, we were able to recruit participants without any active pulmonary symptoms or pathologies and all participants signed an ethical consent form¹⁵. We were able to recruit participants from our center who were participating in other studies, such as a spirometry screening study for obstructive lung disease²⁴, as well as those undergoing other research procedures, such as leukapheresis and colonoscopy. Previous research amongst people living with HIV demonstrated that altruism is a key factor motivating participation in research studies²⁵. Like with many human specimens, we noted a great deal of person-to-person variability. There was no way to "predict" from which participants we would obtain BAL with good versus poor cell yields. Unlike peripheral blood, which yields fairly consistent numbers of lymphocytes, the cell numbers in BAL fluid are very variable. Injecting a greater volume of normal saline into the lungs (with the hopes of obtaining a greater return of BAL fluid) is not always possible as larger volumes of normal saline are often associated with more coughing and a higher risk of fever postbronchoscopy. We noticed that using a smaller (rather than larger) diameter bronchoscope enabled the respirologist to reach deeper into the bronchi and obtain fluid containing greater quantities of cells. A consistent finding was that tobacco smokers had much larger proportions of AMs than lymphocytes within their BAL fluid, which is expected as AMs engulf debris and particulate matter. Furthermore, we observed that BAL fluid from smokers contained debris which may block the equipment used, such as PCR machines and flow cytometers. Similar issues may be observed in areas of high pollution or individuals exposed more frequently to poor air quality.

With regard to their role in the establishment of HIV reservoirs and viral persistence, the purity of CD4 T cells and AMs is a key consideration. For this reason, we opted to use fluorescence-activated cell sorting (FACS) to obtain highly pure cell populations. It is also possible that the collected BAL fluid may be contaminated with blood as some minor bleeding is expected during a bronchoscopy; the presence of naive B cells would indicate this, and cells can be washed in a red blood cell lysis buffer to circumvent this problem. Another challenge with studying BAL fluid relates to quantifying inflammatory markers and cytokines, which are important for understanding HIV persistence²⁶. As the instilled saline dilutes the BAL fluid, levels of inflammatory mediators and cytokines may be difficult to measure. Although a urea correction factor has been proposed to account for dilution, there is relatively little literature describing its use^{27,28}.

AMs are highly autofluorescent, which poses a problem during cell sorting and flow cytometry phenotypic analysis. In particular, the effect is more pronounced in smokers whose AMs may be completely black in color, significantly affecting their autofluorescence. When excited by a standard blue 488 nm laser, the AM autofluorescence is at its peak at approximately 540 nm, which overlaps with the fluorescence spectra of commonly used conjugates such as FITC and PE^{29,30}. It is worth noting that two separate lasers can be used to excite FITC and PE (e.g., PE by the yellow/green and FITC by the blue 488 laser). To overcome the inherent autofluorescence with FITC, we used unstained AMs to determine the autofluorescence background. In addition, the use of fluorescence minus one (FMO) controls can be very useful to combat these technical issues. Larger beads (e.g., 7.5 µm) can be used, which are closer in size for compensating macrophage populations, compared to smaller beads (e.g., 3.0 µm), which can be used for compensating lymphocyte populations. An even more suitable approach would be to use a small fraction of cells as the single-stain controls, using a known, highly expressed marker on the subset, such as HLA-DR or CD45, conjugated to each of the desired fluorochromes, which would allow for a much more accurate compensation than can be achieved with beads. In the case of smokers' samples, this tactic is particularly useful as the macrophages are much larger and more autofluorescent. In addition, from the preparation step, the whole BAL sample could be cultured in a plate before sorting as described in section 3 of the protocol, to allow a separation of the populations by adherence. In this way, the adherent macrophages can be isolated from other nonadherent cells such as lymphocytes. Compensation is far less challenging if the lymphocyte and AM populations are separated rather than examined together; however, relying on adherence will result in a loss of macrophages, which is an important consideration when cell numbers are already limiting. Also, an adherence step could result in the unwanted activation of adherent monocytes, which may affect downstream results generated using these cells. The value of efficiently sorting cells into purer populations must be weighed against the restriction of having fewer such cells for subsequent experiments.

Other models, most notably murine models, have been used to study macrophage immunological characteristics and biology. While these models are extremely useful and allow great insight into a cell type that is difficult to manipulate, they have limitations. Many of the cell surface markers vary between mice and humans such that the immunophenotype of human AMs is not completely understood. However, this model system requires the pooling of several mice for assays due to the low cell numbers available from each animal. In addition, the necessity to pool specimens precludes considerations of genetic predisposition and sex. Recently, it has been shown that sex plays a role in the infectivity of macrophages by HIV-1 due to the disparate expression of the restriction factor SAMHD1³¹. Nonhuman primates (NHP) represent the closest model to humans and facilitated the study of simian immunodeficiency virus (SIV) infection and its effect on the immune system, providing insight into the role of tissue-resident macrophages compared to monocyte-derived macrophages. In rhesus macaques, it has also been shown that lung macrophage isolates from BAL harbor a replication-competent virus; a viral outgrowth assay (VOA) was used to analyze the behavior of SIV in tissue-resident cells³². Such a finding is of significant research value but must still be validated in humans before it can be applied, and the high cost of using NHPs precludes the use of large sample populations. In addition, human AMs will be useful for many other applications such as *in vitro* viral/microbial infection assays and in studies of other pathogens such as Tuberculosis/HIV coinfection.

Disclosures

The authors have nothing to disclose.

Acknowledgments

The authors would like to acknowledge their funders: the Canadian Institutes of Health Research (CIHR) (grant #153082 to CC, MAJ, NC); the Réseau SIDA et maladies infectieuses du Fonds de recherche du Québec-Santé (FRQ-S) who granted funding to CC and MAJ and the McGill University Faculty of Medicine who granted funding to CC. This study was also supported in part the Canadian Institutes of Health Research (CIHR)-funded Canadian HIV Cure Enterprise (CanCURE) Team Grant HB2 – 164064 to MAJ, CC and NC. MAJ holds the CIHR Canada

Research Chair tier 2 in Immunovirology and CC and NC hold an FRQ-S Junior 1 and Junior 2 research salary award, respectively. ET holds an RI-MUHC Studentship MSc award.

In addition, the authors would like to acknowledge Josée Girouard and all clinical staff involved in coordinating and obtaining the samples, as well as the respiratory therapists; Ekaterina Iourtchenko, Hélène Pagé-Veille, and Marie-Hélène Lacombe at the RI-MUHC Immunophenotyping platform; and Dr. Marianna Orlova for provision of the microscopy photos. Most importantly, the authors wish to thank the many volunteers without whom this research would not be possible.

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