Journal of Visualized Experiments

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

--Manuscript Draft--

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| **Article Type:** | Invited Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE59427R1 |
| **Full Title:** | Processing of Bronchoalveolar Lavage Fluid and Matched Blood for Alveolar Macrophage and CD4+ T-cell Immunophenotyping and HIV Reservoir Assessment |
| **Keywords:** | Bronchoalveolar lavage (BAL); Alveolar macrophages; Immunophenotyping; CD4 T-cells; HIV RNA; HIV Reservoir; Myeloid cells; antiretroviral therapy (ART); alveolar macrophages (AMs) |
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| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Open Access (US$4,200) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Montreal, Quebec, Canada |

Cover Letter

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Dr Jay Upponi
JoVE Science Editor, Immunology and Infection

January 11, 2018

**RE: JoVE59427R1**

Dear Dr Upponi,

Thank you very much for the opportunity to revise and re-submit our original research methods article entitled, "**Processing of Bronchoalveolar Lavage (BAL) Fluid and Peripheral Blood Samples from ART-treated Chronically HIV-infected Individuals to Isolate Alveolar Macrophages and CD4+ T cells for Subsequent Immunophenotyping and HIV Reservoir Assessment."** As you know, this methods paper is based on study "*HIV persistence in mucosal CD4+ T-cells within the lungs of adults receiving long-term* suppressive antiretroviral therapy" as published in the journal AIDS (AIDS 2018 Oct 23;32(16):2279-2289). We thank the reviewers for their helpful comments. Each comment was addressed on a point-by-point basis and we are confident that these revisions have definitely improved the quality of the manuscript.

No writing assistance was provided in the preparation of the manuscript. This work has not been published elsewhere nor submitted for publication simultaneously. All authors have reviewed and approved the final version of the revised manuscript.

In addition to the final manuscript being published in the journal *AIDS*, the preliminary results of this study were presented in part at both the *International AIDS Society* 2017 conference, Paris, and the *Conference on Retroviruses and Opportunistic Infections* 2018, Boston, MA.

We strongly believe our novel methods manuscript is suited to the scope and readership of *JoVE* and we look forward to your response regarding our revised manuscript.

Sincerely yours,

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**TITLE:**

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

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**KEYWORDS:**

Bronchoalveolar lavage (BAL), alveolar macrophages, immunophenotyping, CD4 T cells, HIV RNA, HIV reservoir, myeloid cells, antiretroviral therapy (ART), alveolar macrophages (AMs)

**SUMMARY:**

We describe a method for processing bronchoalveolar lavage fluid and matched peripheral blood from chronically HIV-infected individuals on antiretroviral therapy to assess pulmonary HIV reservoirs. These methods result in the acquisition of highly pure CD4 T cells and alveolar

macrophages that may subsequently be used for immunophenotyping and HIV DNA/RNA quantifications by ultrasensitive polymerase chain reaction.

**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.

**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 era[3]. However, the lungs play a central role in the pathogenesis of HIV. Indeed, pulmonary symptoms were among the first indicators of AIDS-related opportunistic infections[4]. 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 the 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 HIV[7]. Although some explanations for this susceptibility to pulmonary infection and chronic disease have been proposed[8–10], 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 status[6].

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 lymphocytes[11]. 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 participants[15]. For participants to include in the study, we looked for 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 ≥200/$mm^3$; (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 BAL as performed by a licensed respirologist with assistance from a respiratory therapist[16,17].

1.1. 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.

1.2. Position the patient, preferably in the supine position. Administer sedation as follows: midazolam 0.01–0.04 mg/kg and fentanyl 50–100 mcg (to facilitate patient comfort and minimize cough reflex) intravenously, in the presence of a respirologist or anesthetist.

1.3. 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.

1.4. Repeat the flush to a total of 200–300 mL of lavage. Collect at least 100 mL of BAL fluid if possible.

1.5. Place the BAL fluid on ice as soon as possible.

**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.

2.1. Keep the BAL samples on ice until they are processed.

2.1.1. 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.

2.1.2. 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.

2.1.3. 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.

2.2. Process the BAL cell pellet.

2.2.1. 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).

2.2.2. 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).

3.1. Transfer the desired number of BAL cells for sorting to a new 15 mL tube and make up the correct volume for 1.5 x $10^6$ 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.

3.2. 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 x $10^7$ 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.

3.3. 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.

3.4. 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.

3.5. Centrifuge at 300 x *g* for 10 min at RT. Remove the supernatant, resuspend at 1 x $10^7$ 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).

4.1. 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).

4.2. Separate the blood by centrifuging the blood tubes at 300 x *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.

4.3. Perform density gradient separation.

4.3.1. Add 2 mL of RPMI 1640 to each blood tube and mix well using a serological pipette.

4.3.2. Transfer to 3x 50 mL tubes and make up the volume in each tube to 25 mL with RPMI 1640.

4.3.3. 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.

4.3.4. Centrifuge at 600 x *g* for 25 min at RT with low acceleration and no deceleration (brake off).

4.4. Perform a washing of peripheral blood mononuclear cells (PBMCs).

4.4.1. 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.

4.4.2. Centrifuge at 700 x *g* for 5 min at RT and remove as much supernatant as possible.

4.4.3. Resuspend the pellet and make up the volume to 25 mL with PBS. Centrifuge at 350 x *g* for 10 min at RT and remove as much supernatant as possible.

4.4.4. Repeat the wash step described in step 4.4.3.

4.4.5. 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).

5.1. 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.

5.2. Perform staining.

5.2.1. 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 x $10^5$ cells to use for unstained and viability stain compensation controls.

5.2.2. Centrifuge at 350 x *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.

5.2.3. 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 x $10^7$ cells in 250 µL of FcR-blocking mixture. Incubate them for 1 h at 4 °C.

5.2.4. After incubation, add the appropriate antibody cocktail (see **Table 1**) to the cells and incubate for 1 h at 4 °C in the dark.

5.2.5. After 1 h of staining, add 1 mL of PBS to the cells and centrifuge at 350 x *g* for 5 min at 4 °C. Remove the supernatant and resuspend cells in sorting buffer to have 1 x $10^7$ cells in 250 µL. Filter the cells through a 70 µm filter if needed.

5.2.6. Prepare compensation controls.

5.2.6.1. 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.

5.2.6.2. 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 x $10^5$ cells set aside in step 5.2.1. Incubate for 20 min at 4 °C in the dark.

5.2.6.3. Add 1 mL of PBS to each tube and centrifuge at 350 x *g* for 5 min at 4 °C. Remove the supernatant and resuspend the pellet n 250 µL of PBS. Store at 4 °C in the dark until needed.

5.2.7. 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.

5.2.7.1. Sort BAL cells at low pressure. Gate cells first to exclude noise and include live, CD45+ cells, and within this population gate out doublet cells (See **Figure 3**). Within the larger myeloid population sort CD206 and CD169 double positive cells as AMs; within the smaller lymphocyte population isolate CD3+ cells and sort both CD4 and CD8 single positive populations (See **Figure 3**; gating strategy detailed in the representative results section).

5.2.7.2. When sorting PBMCs, gate cells first to exclude noise and include live, CD45+ cells, and within this population gate out doublet cells. Next, isolate CD3+ cells and within this population, gate first on CD14 and sort single-positive monocytes, and then 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).

6.1. 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.

6.2. 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.

6.3. Perform extracellular staining.

6.3.1. 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.

6.3.2. Wash 2x by adding 500 µL of PBS and centrifuging at 350 x *g* for 5 min at 4 °C.

6.4. Prepare for fixation and permeabilization (see the **Table of Materials** for specific reagents used).

6.4.1. 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.

6.4.2. Prepare wash solution using 1 part wash buffer and 4 parts $H_2O$. 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.

6.5. Perform intracellular staining.

6.5.1. 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.

6.5.2. Add 2 mL of wash solution and centrifuge at 350 x *g* for 5 min at RT. Remove the supernatant and 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).

7.1. Cell numbers permitting, cryopreserve live cells from the BAL cell pellet (from step 2.2.2).

7.1.1. Prepare freeze media containing 90% FBS + 10% dimethyl sulfoxide (DMSO).

7.1.2. 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.

7.2. Preserve the BAL cells as dry pellets.

7.2.1. 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).

### 8.1. Total HIV DNA quantification

8.1.1. 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).

8.1.2. Prepare standard curve dilutions.

8.1.2.1. As above, use a DNA extraction kit to extract DNA from a pellet of 2 x $10^6$ ACH-2 cells (see the **Table of Materials**).

8.1.2.2. After elution of the DNA, perform serial 10-fold dilutions of the ACH-2 DNA to generate six dilutions, ranging from 3 x $10^5$ cells to 3 cells per 15 µL.

8.1.3. Perform a preamplification step.

8.1.3.1. In a separate room, prepare the master mix for *n* + 2 samples comprising 1x polymerase buffer, 3 mM of $MgCl_2$, 300 µM dNTPs, and 2.8 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.

8.1.3.2. 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.

8.1.3.3. 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.

8.1.3.4. 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).

8.1.4. Perform real-time PCR.

8.1.4.1. To quantify CD3 and HIV DNA, prepare two master mixes containing 1x PCR reaction master mix (see the **Table of Materials**), 1250 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.

8.1.4.2. 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) to have a total reaction volume of 20 µL.

8.1.4.3. 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.

8.1.4.4. 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/$10^6$ cells.

**8.2. HIV RNA quantification**

8.2.1. 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).

8.2.2. Well-quantified LTR-gag RNA synthesized in vitro is used as a 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 $10^5$ cells to three copies of LTR-gag RNA in 17 µL.

8.2.3. 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.

8.2.4. Perform reverse transcription (RT) and preamplification PCR.

8.2.4.1. 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.

8.2.4.2. 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.

8.2.4.3. Run the plate for 16 cycles according to the manufacturer's instructions, with an annealing temperature of 55 °C.

8.2.5. Perform real-time PCR.

8.2.5.1. 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**).

8.2.5.2. Distribute 13.6 µL of each master mix in qPCR-adapted tubes. Dilute the RT preamplification PCR products at 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.

8.2.5.3. 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 nonsmokers[20], 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+[21] cells and, then, 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 CD169[22] 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 cells[23], and the sialoadhesin receptor CD169[22].

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 any 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 and all positive cells are taken. To isolate monocytes, this population is subsequently gated on CD14 and all single-positive cells are sorted. To isolate lymphocyte subsets, CD3+ cells are gated on CD4 and CD8; CD4 single-positive and CD8 single cells are sorted.

**FIGURE AND TABLE LEGENDS:**

**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.

**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).

**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.

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

**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 blood[15]. 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 macrophages[15]. 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 symptoms[3]. However, we were able to recruit participants without any active

pulmonary symptoms or pathologies and all participants signed an ethical consent form[15]. We were able to recruit participants from our center who were participating in other studies, such as a spirometry screening study for obstructive lung disease[24], 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 studies[25]. 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 has fairly consistent numbers of lymphocytes, for example, 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 persistence[26]. 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 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 SAMHD-1[31]. 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 cells[32]. 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.

**ACKNOWLEDGMENTS:**

The authors would like to acknowledge their funders: the Canadian Institutes of Health Research (CIHR) (grant #153082 to C.C., M.A.J., and N.C.); the *Réseau SIDA et maladies infectieuses du Fonds de recherche du Québec-Santé* (FRQ-S) who granted funding to C.C. and M.A.J.; the McGill University Faculty of Medicine who granted funding to C.C. This study was also supported in part by The Canadian HIV Cure Enterprise (CanCURE) Team Grant HIG-133050 to M.A.J., C.C., and N.C. from CIHR in partnership with the Canadian Association for AIDS Research (CANFAR) and the International AIDS Society (IAS). M.A.J. holds the CIHR Canada Research Chair tier 2 in Immunovirology and C.C. and N.C. hold an FRQ-S Junior 1 and Junior 2 research salary award, respectively. E.T. 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, including the respiratory therapists, and Dr. Marianna Orlova for the provision of the microscopy photos. Most importantly, the authors wish to thank the many volunteers without whom this research would not be possible.

**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1



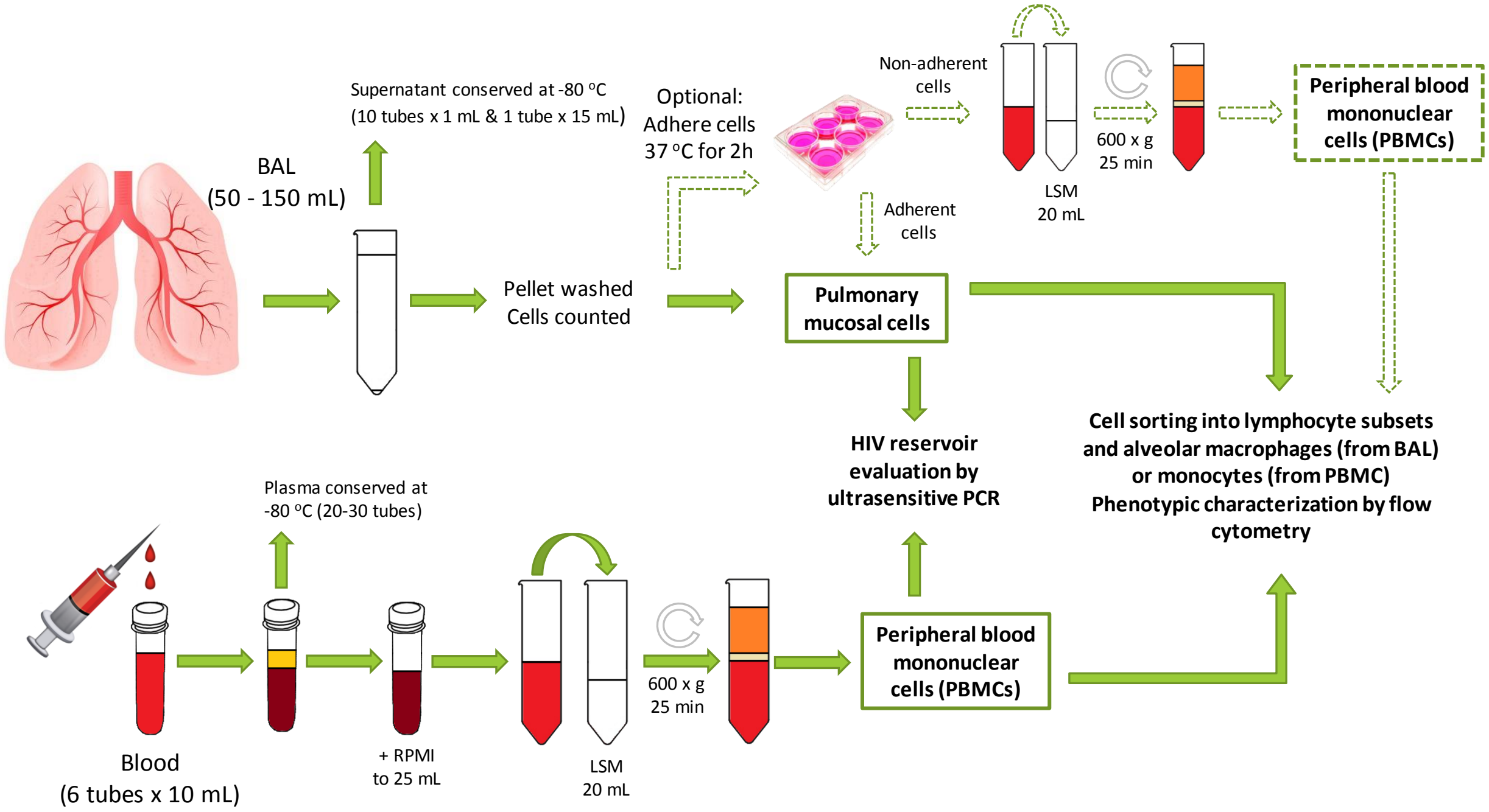

Figure 2



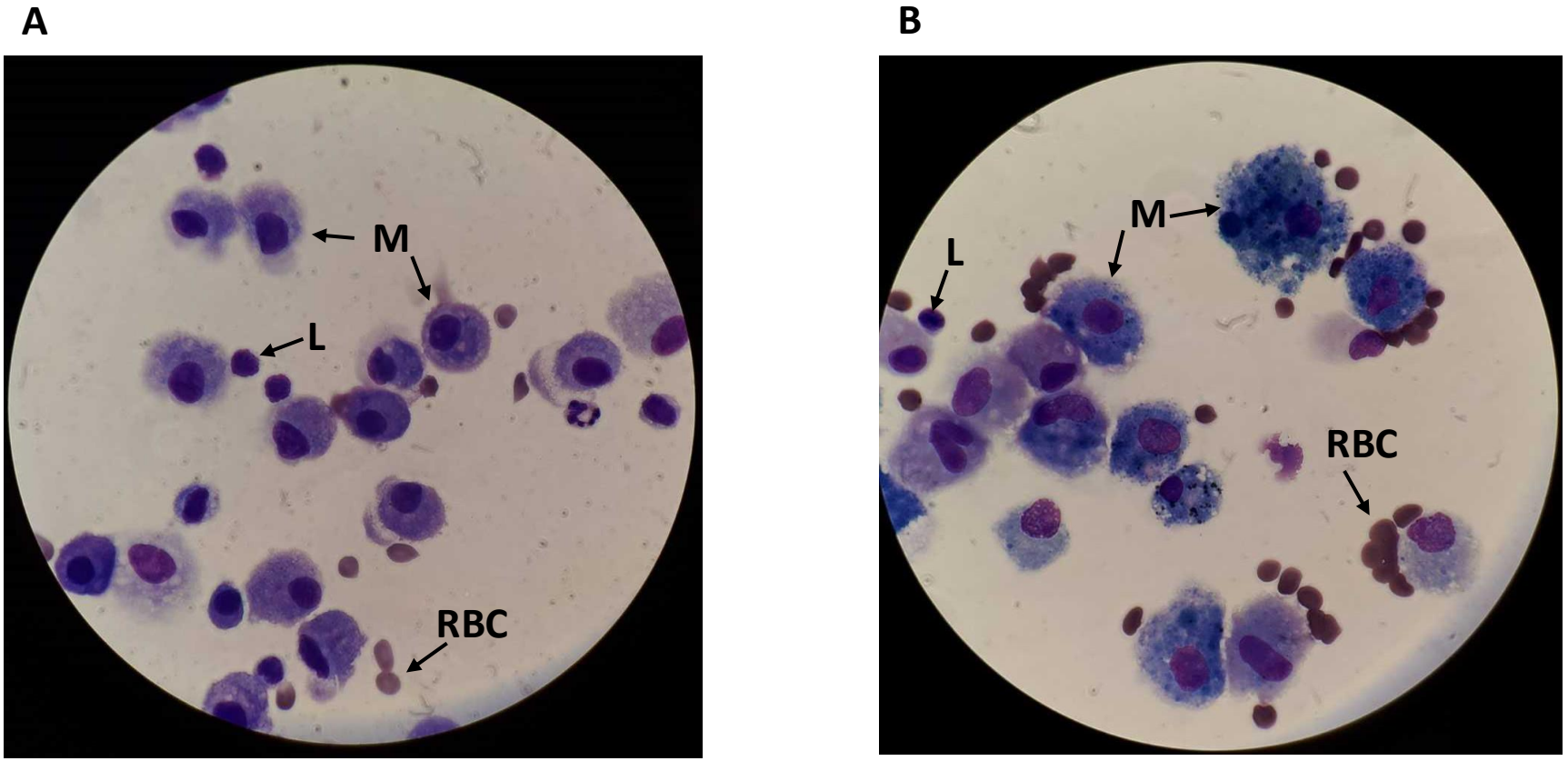

Figure 3



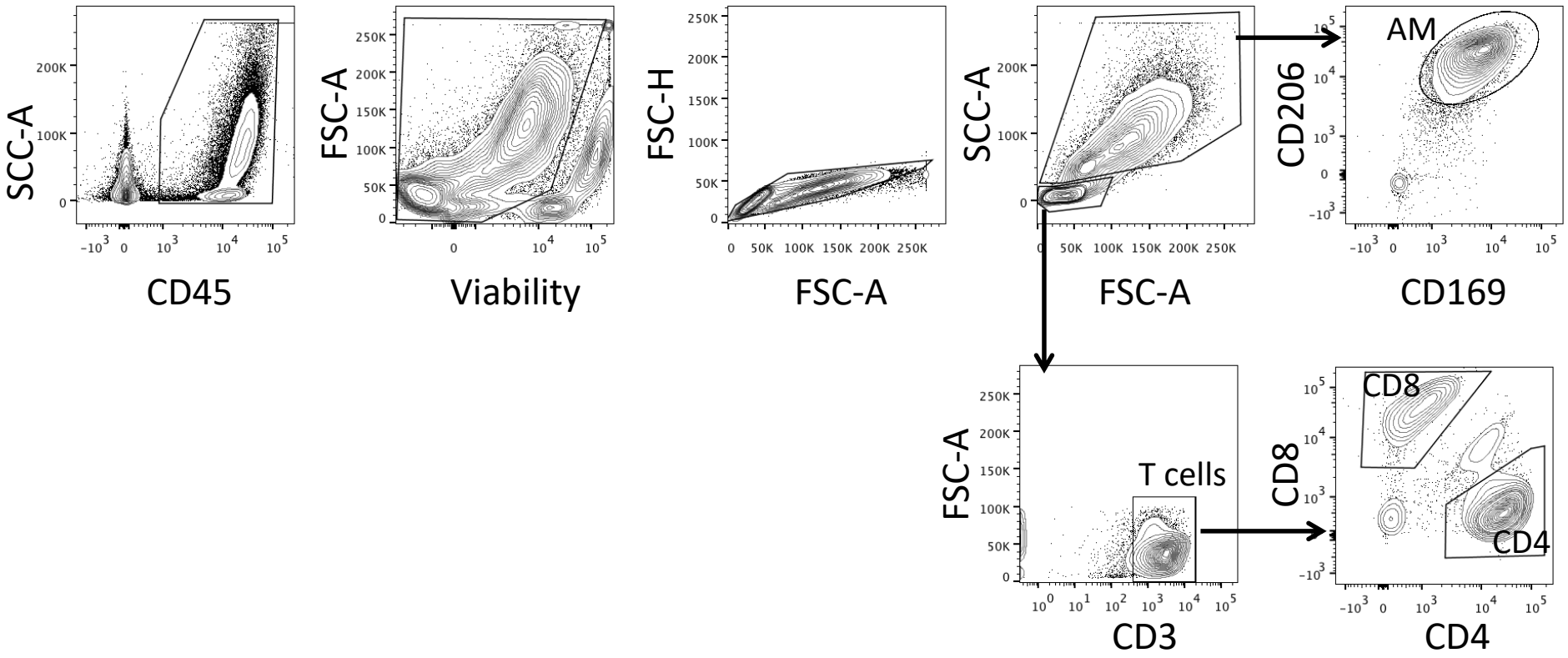

**Table 1: Panel for cell sorting of bronchoalveolar lavage (BAL) cells and is**

| 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 |

**olated peripheral blood mononuclear cells (PBMC)**

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

| Target | Step | Primer Name | |
|---|---|---|---|
| HIV Total DNA or | Pre-amplification PCR | UR1 | 5'-CCA TC |
| HIV LTR-Gag RNA | | ULF1 | 5'-ATG CC |
| | Real-time PCR | UR2 | 5'-CTG AG |
| | | LambdaT | 5'-ATG CC |
| | | UHIV FamZen: | 5'-/56-FA |
| CD3 DNA | Pre-amplification PCR | HCD3 out 5' | 5'-ACT GA |
| | | HCD3 out 3' | 5'-CCA GC |
| | Real-time PCR | HCD3 in 5' | 5'-GGC TA |
| | | HCD 3 in 3' | 5'-CCT CT |
| | | CD3 FamZen: | 5'-/56-FA |
| GUSB RNA | Pre-amplification PCR | GUSB Forward 1: | 5'-ACC TA |
| | | GUSB Reverse 1: | 5'- GTT CA |
| | Real-time PCR | GUSB Forward 2: | 5'-TGC TG |
| | | GUSB Reverse 2: | 5'- CCT TC |
| | | GUSB-HEX: | 5'-/5HEX/ |

| Primer Sequence |
| --- |
| T CTC TCC TTC TAG C-3' |
| A CGT AAG CGA AAC TCT GGG TCT CTC TGG TTA GAC-3' |
| G GAT CTC TAG TTA CC-3' |
| A CGT AAG CGA AAC T-3' |
| M/CA CTC AAG G/ZEN/C AAG CTT TAT TGA GGC/3IABkFQ/-3' |
| C ATG GAA CAG GGG AAG-3' |
| T CTG AAG TAG GGA ACA TAT-3' |
| T CAT TCT TCT TCA AGG T-3' |
| C TTC AGC CAT TTA AGT A-3' |
| M/AG CAG AGA A/ZEN/C AGT TAA GAG CCT CCA T/3IABkFQ/-3' |
| G AAT CTG CTG GCT ACT A-3’ |
| A ACA GAT CAC ATC CAC ATA C-3’ |
| G CTA CTA CTT GAA GAT G-3’ |
| GT CTG CTG CAT AGT TAG A-3' |
| TCGCTCACA/ZEN/CCAAATCCTTGGACC/3IABkFQ/-3' |

| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| 70 µm Sterile Cell Strainer | Fisher Scientific | 22363548 |
| ACH-2 Cells | NIH | 349 |
| BD FACSAria | BD Biosciences | N/A |
| BD LSRFortessa X-20 | BD Biosciences | N/A |
| Bronchoscope | Olympus | BF-1TH190 |
| Cell Disassociation Solution | Sigma | C5914 |
| CD169 BB515 | BD Biosciences | 565353 |
| CD14 BV786 | BD Biosciences | 563698 |
| CD206 PE | BD Biosciences | 555954 |
| CD3 Alexa700 | BD Biosciences | 557943 |

| | | |
|---|---|---|
| CD4 PE-Cy5 | BD Biosciences | 555348 |
| CD45 PE Cy-7 | BD Biosciences | 557748 |
| CD8 BV605 | BD Biosciences | 564116 |
| CompBead Plus | BD | 560497 |
| DNase I | Invitrogen | 18068015 |
| dNTP Set 100 mM | Invitrogen | 10297-018 |
| Dimethyl Sulfoxide (DMSO) | Sigma | D8418 |
| EDTA | Invitrogen | AM9912 |
| FBS | Wisent Bioproducts | 080-150 |
| FcR Blocking Reagent, Human | Miltenyi | 130-059-901 |
| FlowJo v10 | FlowJo LLC | N/A |

| | | |
|---|---|---|
| HLA-DR BV650 | BD Biosciences | 564231 |
| HyClone HEPES solution | Fisher Scientific | SH3023701 |
| Live/Dead APC-H7 | Invitrogen | L34975 |
| Lymphocyte Separation Medium (LSM) | Wisent Bioproducts | 350-000-CL |
| Mr. Frosty Freezing Container | ThermoFisher | 5100-0001 |
| OneComp eBeads | Invitrogen | 01-1111-41 |
| PBS 1X | Wisent Bioproducts | 311-010-CL |
| PCR Tubes Corbett Rotor-Gene | Axygen | PCR-0104-C |
| PerfeCTa qPCR ToughMix | Quantabio | 95112 |

| | | |
|---|---|---|
| QiaAmp DNA Mini Kit | Qiagen | 51304 |
| RNeasy Mini Kit | Qiagen | 74104 |
| Rotor-Gene Q | Qiagen | 9001550 |
| RPMI 1640 1X | Wisent Bioproducts | 350-000-CL |
| Sterile Water | Wisent Bioproducts | 809-115-CL |
| Superscript™ III One-Step RT-PCR System | Invitrogen | 12574018 |
| *Taq* DNA Polymerase | Invitrogen | 18038-042 |

| | | |
|---|---|---|
| Transcription Factor Buffer Set | BD Biosciences | 562725 |
| Trypan Blue | Sigma | T8154 |

| Comments/Description |
| --- |
| Nylon mesh filters with 70 µm pores to remove impurities from BAL sample before sorting |
| HIV-1 latent T cell clone with one integrated proviral copy which do not express CD4 |
| Cell sorter (configured to detect 16 colours simultaneously) |
| Flow cytometer (configured to detect 14 colours simultaneously) |
| EEIII HD therapeutic bronchoscope; channel width 2.8 mm; outer diameter 6.0 mm |
| Non-enzymatic formulation for gently dislodging adherent cell types from plastic or glass surfaces. |
| Sialic acid-binding molecule antibody used for flow cytometry |
| Endotoxin receptor antibody used for flow cytometry |
| Mannose receptor antibody used for flow cytometry |
| T cell co-receptor antibody used for flow cytometry |

T cell co-receptor antibody used for flow cytometry

Receptor-linked protein tyrosine phosphatase antibody used for flow cytometry

T cell co-receptor antibody used for flow cytometry

Anti-mouse Ig, κ and negative control polystyrene microparticles used to optimize fluorescence compensation in flow cytometry

Digests single- and double-stranded DNA to oligodexyribonuleotides containing a 5' phosphate to remove contamination from RNA

Consists of four deoxynucleotides (dATP, dCTP, dGTP, dTTP) for use in PCR

Apolar, protic solvent used to make media for cryopreserving live cells

Used to stop Dnase I enzyme activity

Premium fetal bovine serum to supplement media

Binds to Fc receptor on the cell surface to prevent non-specific binding of flow antibodies

Flow cytometry analysis software used for all analyses

MHC class II cell surface receptor antibody used for flow cytometry
Buffer providing maintenance of physiological pH

Viability marker used for flow cytometry

Polysucrose for isolation of PBMC from whole blood

Freezing container ensuring rate of cooling very close to -1°C/minute, the optimal rate for cell preservation
Anti-mouse, rat and hamster antibodies for compensation of PBMC samples
Phosphate buffered saline for cell washing and staining
4-strip PCR tubes with 0.1 mL capacity for use with Corbett Rotor-Gene

2X concentrated ready-to-use reaction cocktail for PCR amplification of DNA templates

Kit for isolation of genomic, mitochondrial, bacterial, parasite or viral DNA. Includes QIAamp Mini Spin Columns, QIAGEN Proteinase K, Reagents, Buffers, Collection Tubes

Kit for purification of up to 100 µg total RNA from cells, tissues, and yeast. Includes RNeasy Mini Spin Columns, Collection Tubes, RNase-free Reagents and Buffers
Real-time PCR cycler
Cell culture media

DNase, RNase & protease free

RT-PCR kit which performs both cDNA synthesis and PCR amplification in a single tube. Includes SuperScript III RT/Platinum Taq Mix, 2X Reaction Mix (containing 0.4 mM of each dNTP, 3.2 mM $MgSO_4$), magnesium sulfate

Thermostable enzyme that synthesizes DNA from single-stranded templates in the presence of dNTPs and a primer. Includes Taq DNA Polymerase, 10X PCR buffer, magnesium chloride

Buffers for intracellular staining for flow cytometry. Includes fixation/permeabilization buffer, diluent buffer, perm/wash buffer

Viability dye to count cells using haemacytometer

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**Processing of Bronchoalveolar Lavage (BAL) Fluid and Peripheral Blood Samples from ART-treated Chronically HIV-infected Individuals to Isolate Alveolar Macrophages and CD4+ T-cells for Subsequent Immunophenotyping and HIV Reservoir Assessment**

Author(s):

Syim Salahuddin, Elaine Thomson, Oussama Meziane, Omar Farnos, Amelie Pagliuzza, Nicolas Chomont, Ron Olivenstein, Cecilia Costiniuk, Mohammad-Ali Jenabian

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# Reply to editorial and referee comments

## Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

**Rebuttal:** as recommended the paper has been fully proofread by two native English-speaking scientists.

*2. Please remove the brackets enclosing the reference numbers cited in the manuscript text.*

**Rebuttal:** these changes have been made as instructed.

*3. Please rephrase the Introduction to include a clear statement of the overall goal of this method.*

**Rebuttal:** we provided a clear sentence at the end of the introduction to address the editorial comment.

*4. Please use the micro symbol µ instead of u and abbreviate liters to L to avoid confusion.*

**Rebuttal:** these changes have been made as instructed.

*5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Eppendorf, Sigma, Miltenyi, CompBeads, BD FACSAria™, QIAamp, Qiagen, Invitrogen, Perfecta ToughMix (Quantabio), FlowJo®, etc.*

**Rebuttal:** these changes have been made throughout that manuscript as instructed.

*6. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.*

**Rebuttal:** these changes have been made as instructed.

*7. Please revise the protocol text to avoid the use of any personal pronouns (*e.g.*, "we", "you", "our" etc.).*

**Rebuttal:** these changes have been made as instructed.

*8. Please revise the protocol to contain only action items that direct the reader to do something (*e.g.*, "Do this," "Ensure that," etc.). The actions should be described in the*

*imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.*

**Rebuttal:** these changes have been made as instructed.

*9. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.*

**Rebuttal:** We carefully reviewed all experimental details to address this comment throughout the paper.

*10. section 1.2: What are the inclusion and exclusion criteria for recruiting patients? Please also describe how to administer sedation.*

**Rebuttal:** we have added a paragraph after the ethics statement at the beginning of the protocol section (page 4) to clearly describe both inclusion and exclusion criteria used to set up this protocol. Also, as requested, we added information about the administration of sedatives (section 1.2, page 4).

*11. sections 2.1, 5.2.2: Please provide the composition of OCT embedding medium and LSM. If they are purchased, please include their information in the Table of Materials and reference the Table of Materials in the protocol steps where they are used.*

**Rebuttal:** the section referring to the OCT embedding medium has now been removed as per a reviewer recommendation and the reagent is not referred to elsewhere in the protocol. The LSM is referenced in the materials table as 'lymphocyte separation medium'.

*12. section 5.1.1: When, how and what volume of blood is collected? It is unclear. Please specify.*

**Rebuttal:** this information is now described at the beginning of section 4 of the protocol.

*13. section 8.1.2: Please specify from which step the cells are obtained.*

**Rebuttal:** cells referred in this section are indeed the whole BAL cells, the resuspended cell pellet from the initial centrifugation steps outlined in section 2. The source of the cells is now noted in section 7.1.2.

*14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*

**Rebuttal:** these instructions have now been addressed.

*15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.*

**Rebuttal**: these instructions have now been addressed.

*16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.*

**Rebuttal:** these instructions have now been addressed.

*17. Please remove the titles and Figure Legends from the uploaded figures. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.*

**Rebuttal:** these changes have been made as instructed.

*18. Figure 1: Please use SI abbreviations for all units (L, mL, µL) and include a space between all numerical values and their corresponding units (15 mL, 37 °C, etc.).*
**Rebuttal:** these changes have been made as instructed.

*19. Figure 2: Please combine all panels of one figure into a single image file. Please label the two images in the second page and describe them in the figure legend.*

**Rebuttal:** these changes have been made as instructed; the second pair of images has been removed for clarity.

*20. Figure 3: There are two figure 3 files and also two figures in each pdf file. Please only upload the final version of the Figure 3.*

**Rebuttal:** these changes have been made as instructed.

*21. Table 1: Please indicate the unit for the "Volume per test".*

**Rebuttal:** these changes have been made as instructed.

*22. Please revise the Table of Materials to include the name, company, and catalog number of all relevant supplies, reagents, equipment and software in separate columns in an xls/xlsx file. Please sort the items in alphabetical order according to the name of material/equipment.*

**Rebuttal:** these changes have been made as instructed.

23. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

**Rebuttal:** As requested we carefully reviewed the discussion section to be able to address all aspects as listed above.

*24. References: Please do not abbreviate journal titles.*

**Rebuttal:** we updated the reference list using the JOVE citation style with EndNote. However, EndNote keeps some of the abbreviation as per official journal titles which sometimes contain abbreviations.

**Reviewer #1:**

Manuscript Summary:
Salahuddin and colleagues present a well-written manuscript that clearly and logically describes their protocol for obtaining and characterizing peripheral (blood) and airway (by broncho-alveolar lavage) immune cells in individuals living with HIV.

Minor Concerns:

*1. Endobronchial biopsies can be done in research settings as the authors describe, but they are more commonly employed in studying the airway epithelium in diseases such as asthma, cystic fibrosis, etc. It is my experience that most research bronchoscopies in individuals living with HIV involve BAL and not biopsies. In this context, I suggest that the authors can retain the brief description of obtaining endobronchial biopsies (lines 148-170) if they wish (personally I would delete it), but if so then they should clearly explain that these biopsies are not used to study/compare peripheral vs. BAL immune cells. In fact, they do not have any description in their manuscript of how to analyze these cells. Again, if they do retain this part of the protocol, then they should articulate how these samples, which are dominated by airway epithelial cells, may be used in studies of people with HIV. In addition, they should cite published manuscripts in which investigators performed such biopsies and analyzed the airway epithelium in the context of HIV. I will defer to the authors but this 'component' of the protocol either needs to be discussed in an appropriate context or removed (and I favor removal).*

**Rebuttal:** As recommended by the reviewer, the biopsy section (originally section 2) has now been removed from the manuscript. Accordingly, the introduction, protocol body, representative results and Figure 1 have been appropriately updated to reflect the removal.

*2. The manuscript would benefit from even a brief discussion in the Introduction as to how the authors actually developed this protocol. Specifically, it is a general technique that many investigators use and it would be appropriate to state whether or not this protocol was adapted from and/or modified from one or more published protocols by other investigators. As with the vast majority of protocols, one assumes that the authors*

*did not create this completely 'in a vacuum' at McGill but rather modified/adapted from one or even multiple sources. In fact, to the extent they can articulate that this protocol includes techniques that have been validated and are in use by other clinical investigators working in HIV and the lung will increase the likelihood that colleagues will adopt their protocol.*

**Rebuttal:** To acknowledge the reviewer comment, within the last paragraph of the introduction section, we stated that this protocol has been adapted from other published general protocols (page 3) to suit the needs of the researchers in terms of downstream assays, and we added appropriate references (*e.g.* Brenchley *et al.*, *Mucosal Immunology*, 2007; Jambo *et al.*, *Mucosal Immunology*, 2014; Mwandumba *et al.*, *The Journal of Immunology*, 2004; and Gordon *et al.*, *Vaccine*, 2008).

**Reviewer #2:**

Manuscript Summary:
In this manuscript by Salahuddin *et al.*, the authors present a method for processing bronchoalveolar lavage (BAL) fluid to evaluate alveolar macrophages and CD4+ T cells for subsequent immunophenotyping. They collect BAL fluid from chronically HIV-infected individuals and use PCR to quantify HIV DNA and RNA to investigate the pulmonary HIV reservoir. While this manuscript provides a clear rationale for this technique and is well written, we have major concerns with the methodologic rigor and level of discussion presented. Overall, the manuscript is not well referenced and would benefit from a more nuanced and thorough discussion of BAL collection techniques and the use of flow cytometry to identify immune cell populations. There is also a significant error in Figure 2.

Major Concerns:

*1. The authors should explain the rationale behind their choice of volume of BAL return ("over 100cc") and the variability of instilled saline (200-300cc) suggested in their manuscript.*
*In fact, there is a large body of literature describing the importance of standardized BAL collection, particularly for human subjects research. This is an important consideration when quantifying immune cell recruitment into the airways and measuring protein levels in supernatants, as the authors propose.*

**Rebuttal:** 200-300 ml of saline are instilled to ensure a return of approximately 100 ml in optimal conditions, as already described in several descriptions of BAL procedures (Brenchley *et al.*, *Mucosal Immunology*, 2007; Jafari *et al.*, *American Journal of Respiratory and Critical Care Medicine*, 2009; and Gordon *et al.*, *Vaccine*, 2008). Furthermore, having performed over a thousand bronchoscopies at the MUHC for both research and medical interventions, our observations suggest that a flexible volume of 200-300 mL of saline is typically needed to ensure the return of at least 100 mL during the BAL procedure. Having a return of at least 100 mL is helpful to ensure that sufficient cells are obtained for analysis. This is also discussed in the second paragraph of the discussion section.

Of note, the initial volume of saline cannot be exactly determined due to the donor-dependent variabilities in participant capacity to tolerate the procedure. If needed, for assessments other than isolation of immune cells (*e.g.* cytokine quantification), a urea correction factor may be used as already mentioned in the 3rd paragraph of the discussion.

*2. The immunophenotyping presented in this manuscript represents a basic approach and would benefit from a more comprehensive discussion of potential flow cytometry based strategies. In particular, the authors do not engage in a detailed discussion of autofluorescence, which can be a particular problem when isolating alveolar macrophages. The authors could discuss, for example, the wavelengths known to detect the most autofluorescence. Fluorophores with shorter wavelengths (e.g. FITC and PE) are generally avoided, however the authors use a PE-conjugated antibody to stain for alveolar macrophages. The authors could also reference the use of FMO (fluorescence minus one) and dump gating to assist in identification of the populations of interest.*

**Rebuttal:** the reviewer's comment about the phenotypical markers as described in the protocol is true. Indeed, in this method paper, our aim is not to propose a comprehensive phenotypical characterization of alveolar macrophages as this could largely vary according to different study topics. We therefore deliberately propose the most widely accepted markers for the AM population.
To acknowledge the reviewer's comment on the autofluorescence of AMs and the combination of fluorochromes (*e.g.* combination of FITC and PE as well as the usage of FMO as raised by the reviewer) we have now added a paragraph in the discussion section to address the challenges and potential alternatives in FACS-sorting or immune-phenotyping.

*3. We are concerned about significant errors noted in this manuscript. In particular, Figure 2 mistakenly labels red blood cells as lymphocytes.*

**Rebuttal:** We acknowledge the referee's comment as indeed, the figure was mislabeled. This is now corrected.

Minor Concerns:

*1. The authors should address the potential for monocyte activation using the adherence technique for monocyte isolation.*

**Rebuttal:** this is now mentioned as a limitation of this alternative technique in the 3rd paragraph of the discussion.

*2. Figure 3 is shown multiple times, and there does not appear to be a figure for the gating strategy for peripheral blood samples.*

**Rebuttal:** Figure 3 has been modified; the gating strategy for selecting the AM population is shown using markers as outlined in the references now included in the discussion. The PBMC sorting strategy is not included as the markers used are classical cell surface markers and the authors felt that the description in the discussion was sufficient to explain the gating strategy used.

*3. The manuscript does not appear to be fully edited, and various sections of the manuscript are highlighted for unclear reasons.*

**Rebuttal:** Sections of the manuscript were highlighted as per instructions from JoVE editors to indicate which parts of the procedure will be filmed.