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Corresponding Author:	Paul King Monash Medical Centre Clayton Melbourne, VIC AUSTRALIA
Corresponding Author's Institution:	Monash Medical Centre Clayton
Corresponding Author E-Mail:	paul.king@monash.edu
Order of Authors:	Lovisa Dousha
	Roleen Sharma
	Steven Lim
	James Ngui
	Ashley Buckle
	Paul King
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TITLE:

Assessing Respiratory Immune Responses to *Haemophilus influenzae*.

AUTHORS AND AFFILIATIONS:

Lovisa Dousha[1,2], Roleen Sharma[1,2], Steven Lim[2,3], James Ngui[2,4], Ashley M Buckle[5], Paul T. King[1,2]

[1]Monash Lung and Sleep, Monash Medical Centre, Melbourne, Australia
[2]Monash University Department of Medicine, Monash Medical Centre, Melbourne, Australia
[3]Flow Cytometry Science Technology Platform, Francis Crick Institute, London, United Kingdom
[4]Clinical Immunology, Monash Medical Centre, Melbourne, Australia
[5]Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Melbourne, Australia

Email Addresses of Co-authors:

Lovisa Dousha	(lovisa.dousha@monash.edu)
Steven Lim	(steve.lim@crick.ac.uk)
Roleen Sharma	(roleen.sharma@monash.edu)
James Ngui	(james.ngui@monahhealth.org)
Ashley Buckle	(ashley@ptngconsulting.com)
Paul T King	(paul.king@monash.edu)

Corresponding Author:

Paul T King	(paul.king@monash.edu)

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SUMMARY:

Haemophilus influenzae induces inflammation in the respiratory tract. This article will focus on the use of flow cytometry and confocal microscopy to define immune responses by phagocytes and lymphocytes in response to this bacterium.

ABSTRACT:

Haemophilus influenzae (Hi) is a prevalent bacterium found in a range of respiratory conditions. A variety of different assays/techniques may be used to assess the respiratory immune/inflammatory response to this bacterium. Flow cytometry and confocal microscopy are fluorescence-based technologies that allow detailed characterization of biological responses. Different forms of Hi antigen can be used, including cell wall components, killed/inactivated preparations, and live bacteria. Hi is a fastidious bacterium that requires enriched media but is generally easy to grow in standard laboratory settings. Tissue samples for stimulation with Hi may be obtained from peripheral blood, bronchoscopy, or resected lung (e.g., in patients undergoing surgery for the treatment of lung cancer). Macrophage and neutrophil function may be comprehensively assessed using flow cytometry with a variety of parameters measured, including phagocytosis, reactive oxygen species, and intracellular cytokine production.

Lymphocyte function (e.g., T cell and NK cell function) may be specifically assessed using flow cytometry, principally for intracellular cytokine production. Hi infection is a potent inducer of extracellular trap production, both by neutrophils (NETs) and macrophages (METs). Confocal microscopy is arguably the most optimal way to assess NET and MET expression, which may also be used to assess protease activity. Lung immunity to *Haemophilus influenzae* can be assessed using flow cytometry and confocal microscopy.

INTRODUCTION:

*Haemophilus influenzae (*Hi) is a normal commensal bacterium present in the pharynx of most healthy adults. Hi may have a polysaccharide capsule (types A–F, e.g., type B or HiB) or lack a capsule and be nontypeable (NTHi)[1]. Colonization of the mucosa with this bacterium begins in early childhood, and there is a turnover of different colonizing strains[2]. This bacterium is also capable of invasion of both the upper and lower respiratory tract; in this context, it may induce activation of the immune response and inflammation[3,4]. This inflammatory response may cause clinical disease and contribute to a variety of important respiratory conditions, including sinusitis, otitis media, bronchitis, cystic fibrosis, pneumonia, and chronic obstructive pulmonary disease (COPD). Most of these conditions are due to NTHi strains[2]. This article will describe methods to assess respiratory immune responses to Hi using flow cytometry and confocal microscopy.

The methods described below have been adapted from well-established techniques that have been modified to assess the inflammatory response to Hi. The selection of an appropriate antigenic form of Hi is a key part of this assessment. Antigenic preparations range from cell wall components to live bacteria. To establish and standardize assays, the use of peripheral blood samples may be very helpful initially.

Flow cytometry enables the measurement of a variety of parameters and functional assays from one sample at a cellular level. This technique has the advantage that specific cellular responses (e.g., production of reactive oxygen species (ROS) or intracellular cytokine production) can be assessed when compared to other more general methods such as enzyme-linked immunosorbent assay (ELISA) or ELISspot.

Extracellular traps are expressed by neutrophils (NETs)[5–7] and by other cells such as macrophages (METs)[8]. They are increasingly recognized as a key inflammatory response, particularly in infection in the lung[9]. They may be assessed by confocal fluorescence microscopy. This technique allows definitive identification of NETs/METs and distinguishes their expression from other forms of cell death[6].

Both flow cytometry and confocal microscopy are fluorescence-based assays. Their success is dependent on optimal straining protocols of biological samples. These methods do take some time to learn and require appropriate supervising expertise. The instruments involved are also expensive both to purchase and run. The optimal setting for their use includes major universities and tertiary referral hospitals.

The methods used in this protocol are transferable for the study of other similar organisms involved in respiratory disease (e.g., *Moxarella catarrhalis* and *Streptococcus pneumoniae*). NTHi also interacts with other common respiratory bacteria[10].

PROTOCOL:

This work was approved by the human research ethics committee of Monash Health. The protocol follows the guidelines of the human research ethics committee.

1. Antigenic preparation

NOTE: Three different antigenic preparations can be used to assess the immune response to Hi. These are 1) a subcellular component (typically from the bacterial cell wall); 2) killed and inactivated bacteria; and 3) live bacteria. Determine the use of each antigenic preparation prior to the initiation of any experiments.

1.1. Subcellular components

1.1.1. Obtain subcellular components from sources, including commercial preparations, in-house developed components and/or from other investigators.

NOTE: Subcellular components usually from the bacterial cell wall may be used and include outer-membrane proteins such as P6 and lipooligosaccharide (LOS)[11,12]. These subcellular components are usually derived in specialized centers. The description of how they are made is beyond the scope of this article. Direct contact with experts in this field is recommended to obtain these components.

1.2. Killed/inactivated bacteria

1.2.1. Obtain the bacteria from the appropriate sample (e.g., sputum or bronchoscopy). Confirm the strain as *H. influenzae* using an appropriate microbiology laboratory. Perform typing of the *H. influenzae* samples to confirm they are nontypeable (by a specialist microbiology laboratory).

1.2.2. To obtain a representative antigen, use multiple NTHi strains (at least 5, each of approximately the same amount) to make a pooled antigen. Store the strains at -70 °C in glycerol broth in microcentrifuge tubes.

NOTE: In this experiment, ten distinct strains were used.

1.2.3. Thaw the strains (one microcentrifuge tube at a time) out onto chocolate agar plates and grow overnight in a 37 °C incubator with 5% CO_2. Add the bacteria to 500 μL of phosphate-buffered saline (PBS) and wash them twice (spin at 300 x *g* for 5 min). Use a MacFarland standard[10] or a spectrophotometer[13] to aliquot NTHi to a concentration of 10^8 mL (using a 5 mL container or equivalent).

1.2.4. Heat inactivate the bacteria by placing them in a water bath at a temperature of 56 °C for 10 min.

1.2.5. Sonicate the bacteria using a continuous sonication setting at a low-to-mid range speed for 30 s.

1.2.6. Aliquot the appropriate volume of samples into microcentrifuge tubes. For a sample of 10^8 mL, use aliquots of 50 µL (i.e., 20 samples per mL), freeze them at -70 °C until required[14].

1.3. Live bacteria

1.3.1. First, characterize live bacteria as mentioned in step 1.2.1. Use one well-characterized strain. Ensure that the strain is also stored in glycerol broth at -70 °C as a reserve.

1.3.2. Grow the bacteria on enriched media such as chocolate agar plates or in broth.

1.3.2.1. To use chocolate agar plates, spread the bacteria for a minimum of every 3–4 days with sterile spreaders.

1.3.2.2. Alternatively, use Brain Heart Infusion (BHI) broth enriched with factors X (hemin) and V (β-nicotinamide adenine dinucleotide) to grow the bacteria. Inoculate NTHi from overnight plates in 5–10 mL of BHI broth supplemented with both hemin and β-nicotinamide adenine dinucleotide (both 10 µg/mL) and culture overnight at 37 °C in a 5% CO_2 incubator.

NOTE: This has the advantage of reproducibility, higher colony-forming units (CFU) counts, and all bacteria being at a similar phase of log growth (on plates, bacterial viability can be greater depending on the position in the culture)[13,15].

1.3.3. Use a multiplicity of infection (MOI) of 100 bacteria to one cell to elicit a strong immune response while maintaining cellular viability. Use MacFarland Standard[10] or spectrophotometer[13] to assess the number of bacteria.

1.3.4. Ensure that the media used for live NTHi assays is free of any human serum, as this will kill the bacteria[16]. Animal serum samples (e.g., fetal calf serum) do not generally cause any problem.

NOTE: Use the methods described below to analyze standard tissue samples such as peripheral blood, bronchoscopy samples (particularly bronchoalveolar lavage (BAL)), and resected lung tissue. Incubate the samples with Hi antigen from 10 min to 24 h or more.

2. Assessment of phagocytic function by flow cytometry

NOTE: This assay requires cells in solution and is typically done in whole blood or using BAL fluid. This assay is modified from a previously published protocol based on the use of inactivated *Staphylococcus aureus* preparation and Pansorbin[17]. Inactivated whole blood and fixed *H. influenzae* is substituted for the Pansorbin[17].

2.1. Mix killed, inactivated NTHi (1.2) with propidium iodide (PI) at 100 μg/mL in phosphate-buffered saline (PBS) at a 1:1 ratio for 30 min at room temperature. Centrifuge at 450 x *g* for 5 min, and then wash in 500 μL of PBS. Spin down at 450 x *g* for 5 min and resuspend the pellet in 500 μL of PBS.

2.2. Incubate 450 μL of peripheral blood (from venepuncture of a human subject) with 50 μL of inactivated PI labeled *H. influenzae* for 20 min in a water bath at 37 °C in a 5 mL tube.

2.3. Remove the samples from the water bath and add 5 μL of dihydrorhodamine-1,2,3 (DHR). Vortex for 10 s, and then place it back in the water bath for a further 10 min.

2.4. Remove the samples from the water bath and lyse the erythrocytes (500 μL of volume as listed above in step 2.2) with 10:1 (i.e., 5 mL) of 0.8% ammonium chloride (150 mM NH_4Cl, 1 mM $NaHCO_3$, and 0.1 mM EDTA) solution.

NOTE: Alternatively, an automated system such as Q-prep may be used.

2.5 Analyze the samples on a flow cytometer within an hour. Analyze a minimum of 3,000–5,000 cells (from each subset of interest) from each sample to obtain representative results (**Figure 1**).

2.5.1. To analyze the samples for phagocytosis, determine the proportion of cells having ingested labeled bacteria (measure the proportion of cells having the fluorescent stain).

2.5.2. Quantify the ROS by the oxidation of DHR, which results in a fluorescent signal (the shift in median fluorescence is compared between baseline and stimulated samples).

NOTE: Neutrophils are more granular and have more side scatter, while macrophages are bigger and have more forward scatter.

2.5.3. Distinguish the neutrophils and macrophages morphologically from each other by their size (macrophages are larger) and granularity (neutrophils are more granular).

NOTE: Specific markers for neutrophils and macrophages are not generally used, although CD14 may be used to label blood monocytes. Flow cytometry may potentially be used to distinguish between different functional forms of monocytes and macrophages (e.g., M1 and M2 subsets)[18].

2.6. Modify the assay as appropriate (e.g., use live unlabeled NTHi to stimulate phagocytes and measure the ROS expression by DHR cleavage).

2.6.1. Isolate peripheral blood mononuclear cells by density gradient centrifugation. Layer the blood over the designated polymer for density gradient centrifugation and spin at 2300 x *g* for 30 min. Remove the mononuclear layer using a pipette, wash it twice with PBS and resuspend the cells in PBS at 10^6 cells per mL.

2.6.2. As an alternative, use BAL macrophages.

2.6.3. Suspend the monocytes/macrophages at a concentration of 10^6 cells/mL in the culture medium (RPMI). Infect them with live NTHi at an MOI of 100:1 for 1 h as described in step 1.3.

2.6.4. Add DHR to the suspension as described in step 2.3 for 10 min. Perform the analysis for ROS using flow cytometry.

3. Assessment of lymphocyte function in peripheral blood

3.1. Use whole blood or mononuclear cell preparations for flow cytometry assays[14].

3.2. Acquire samples from each subject by venepuncture. Collect 4 mL of blood from a peripheral vein in a lithium heparin tube and divide the samples into aliquots for control and antigen stimulation.

3.3. Add costimulatory antibodies (anti-CD28 and CD49d, 1 µL/mL) to both the samples. Add NTHi to the antigen sample and incubate at 37 °C and 5% CO_2 for 1 h. For the killed NTHi preparation, add 200 µL of the antigen to 2 mL of blood. For live NTHi, add live NTHi cells at an MOI of 100:1 to the white cells (as measured by hemocytometer).

3.4. Add the Golgi-blocking agent Brefeldin A (10 µL/mL) to the samples and incubate them for another 5 h.

NOTE: Blocking the Golgi apparatus prevents the cytokines from being exported outside the cell.

3.5. If whole blood is used, lyse the erythrocytes with 0.8% ammonium chloride (step 2.4) to leave only leukocytes in solution. Fix the leukocytes using 500 µL of 1%–2% paraformaldehyde for 1 h.

3.6. Count the cells by hemocytometer, permeabilize 10^6 cells with 100 µL of 0.1% saponin for 15 min and incubate the cells with fluorescent-labeled antibodies. Wash the cells and analyze them using a flow cytometer.

NOTE: The quantity of fluorescent-labeled antibodies will be specific for each cytokine. Follow the manufacturers' instructions. Typically, 10^6 cells in 100 µL of solution will be stained for 1 h. Most commercially obtained antibodies will be enough to perform 25–100 tests.

3.7. Determine the proportion of antigen responding cells by gating the relevant lymphocyte population (cells are gated on by CD45, then by CD3, and later by CD4 or CD8 expression) as shown in **Figure 2**. Perform background staining on non-stimulated cells for all the cytokines to be analyzed. Screen 100,000 cells to analyze each cytokine for both stimulated cells and control.

4. Assessment of lymphocyte function/inflammatory mediators in lung tissue

4.1. It is not usually possible to obtain enough lymphocytes from bronchoscopy; therefore, use the lung tissue from lobectomy samples. The optimization of lung tissue samples requires close liaison with the anatomical pathology service. Ensure the tissue has a margin of at least 3 cm from the tumor and is the cellular tissue without significant emphysema (as determined by the pathologist).

4.2. Break the lung tissue into a cellular suspension before it can be used for flow cytometry assays. Digest the lung tissue chemically (e.g., with collagenase) or disaggregate it mechanically.

NOTE: The mechanical disaggregation of tissue may be preferred as this is associated with better surface staining of cells (e.g., CD3/4 labeling)[19].

4.2.1. Obtain lobectomy samples from a pathologist (usually obtained from patients undergoing treatment of lung cancer). Slice about 20–40 g of the sample into 3–5 mm^3 sections. Place them inside a sterile 50 µL chamber before being mechanically fragmented using an appropriate disaggregator.

4.2.2. After tissue disaggregation, lyse the red blood cells with 0.8% NH_4Cl as mentioned in step 2.4.

4.2.3. Resuspend the cells in sterile RPMI (the volume will depend on the cell numbers and generally is 10–20 mL), and then filter them through a 100 µm sterile nylon mesh. Count the number of viable cells using the trypan blue exclusion method.

4.3. NTHi infection assay

4.3.1. Resuspend the lung cells to a final cell concentration of 4 x 10^6 cells/mL per tube (control and stimulated samples).

4.3.2. Use a suspension of 4 x 10^6–6 x 10^6 cells in 1 mL of RPMI for the (negative) control tube. Use the same amount for the NTHi tube (any additional sample may be used as a positive control with SEB). Infect the cells in the NTHi tube at an MOI of 100 bacteria per cell. Loosen the cap half a rotation to allow gas transfer in the tubes.

4.3.3. Place the cells in a tube rotator and incubate them at 37 °C while rotating at 12 rpm.

4.3.4. To prevent the extracellular export of cytokines, add a Golgi blocker (Brefeldin A) to the

cell suspensions 1 h after stimulation to a final concentration of 10 µg/mL. Return the cell suspensions for a further 16–22 h incubation on rotation (step 4.3.3).

4.3.5. Wash the cell suspension with 500 µL of PBS containing 1% bovine serum albumin (BSA) and 0.01% NaN3. Fix and permeabilize the cells as described in step 3.5 and step 3.6, respectively.

4.3.6. Add the antibodies for intracellular cytokine staining (the choice of antibodies is to be determined by the investigator, as listed in the NOTE in step 3.6). Stain the cell suspension for specific human lymphocyte cell-surface markers (e.g., CD45, CD3, etc.) for 1 h. Wash the cells with PBS, fix and permeabilize them as described in steps 3.5–3.6.

4.3.7. Incubate the cells with intracellular cytokine staining antibodies (e.g., IFN-γ and TNF-α or IL-13 and IL-17A) for 1 h.

NOTE: It is recommended to stain surface markers first, then intracellular as fixation can cause conformational changes in the surface proteins to which antibodies bind.

4.3.8. Wash the cells with 500 µL of PBS and resuspend in 100 µL of PBS before data acquisition on a flow cytometer.

4.4. A bead array assay may be used in conjunction to analyze the supernatant described in step 3.2 (Perform this without Brefeldin A). This allows analysis of a wide variety of different inflammatory mediators (potentially up to 40–50 mediators). Collect the supernatant in 100 µL aliquots and store at -70 °C until ready for analysis. Alternatively, one can use multiplex chemiluminescent assays[20].

4.4.1. Use multiplex bead arrays to analyze the thawed samples. Use the stored supernatant to analyze cytokine production following the manufacturer's instructions.

4.4.2. Perform the acquisition of the multiplex bead array on a flow cytometer. Use relevant software to perform the downstream data analysis.

NOTE: This bead array assay may also be performed on supernatant from peripheral blood and/or BAL samples.

5. Assessment of lung proteolysis by confocal microscopy

NOTE: Fluorescent confocal microscopy is complimentary to flow cytometry and can be used to assess protease and ROS inflammatory responses. Extracellular traps such as NETs and METs are composed of extracellular chromatin (DNA) with other inflammatory mediators, particularly proteases such as neutrophil elastase (NE) and matrix metalloproteinases (MMP). They can be assessed in BAL and lung tissue using confocal microscopy, and this has been described previously by Sharma, R. et al.[21].

5.1. Assess direct protease expression in lung tissue (as described in step 4.2.1) using confocal microscopy and *in situ* zymography[15,17].

5.1.1. Use either fresh or frozen unfixed lung tissue. Mount the sections cut to a thickness of 4 µm on superfrost/adhesion slides. Pre-warm the sections in 1x reaction buffer for 5 min.

5.1.2. Add fluorescein-labeled gelatin substrate (30 µg/mL per section) directly on individual slides to measure proteolysis via the action of metalloproteinases.

5.1.3. Place the slides in a horizontal position and incubate in a light-protected, humidified chamber at 37 °C for 1 h.

5.1.4. Rinse the sections in 1x reaction buffer before being mounted.

5.1.5. As a negative control, add only the reaction buffer without fluorescent gelatin to the sections.

5.1.6. Use a confocal microscope to assay the lysis of the substrate by examination. Use ImageJ to determine the area of the lung with evidence of proteolysis. For this, measure the lung area that has staining above the background of the quenched sample. As another control, use the lung tissue with no fluorescent gelatin added[15].

NOTE: Perform this on a minimum of 10 high power fields of view (FOV) for each sample.

5.2. Measure the extracellular ROS expression in lung tissue by immunoreactivity for 3-nitrotyrosine (a toxic oxidative stress product)[13].

REPRESENTATIVE RESULTS:

The representative results show how inflammatory immune responses to NTHi can be assessed/quantitated by flow cytometry and confocal microscopy. A key part of the interpretation of the results is the comparison in fluorescence between control and stimulated samples. A number of preliminary experiments are usually required to optimize the staining of samples. How many different colors can be examined simultaneously will depend on the number of channels available on the flow cytometer/confocal microscope. Results are shown for the assessment of 1) ROS production, 2) intracellular cytokine staining of human lung tissue, and 3) *in situ* zymography to measure lung proteolysis.

Figure 1 shows the representation of ROS production by monocytes. The measurement of ROS is by the oxidation of DHR123 to produce fluorescence. Cells are gated on, and their median fluorescence is assessed by flow cytometry. The median fluorescence of the stimulated sample is compared to the control.

Figure 2 shows the intracellular production of cytokines by lymphocytes derived from human lung tissue. The lung tissue needs to be broken down into single-cell suspension before flow

cytometry assays can be performed to assess inflammatory mediator production, e.g., cytokine production by lymphocytes. Lung tissue can be broken down mechanically or digested chemically, e.g., by collagenase. The mechanical breakdown methods may produce superior results, particularly in terms of retaining cell surface staining (e.g., CD3 and CD4). Filtering of the samples is important to exclude debris that may interfere with the analysis.

Figure 3 shows the expression of protease activity as measured by *in situ* zymography. Unfixed tissue is used to assess protease activity. These samples are typically frozen at -70 °C until analysis. The area of the tissue that has protease fluorescence is measured, and results are compared between control and stimulated samples.

FIGURE AND TABLE LEGENDS:

Figure 1: ROS production by monocytes. (**A**) The panel shows the gating strategy for peripheral blood mononuclear cells (PBMC), with forward scatter and side scatter being used to define the phagocyte population. In panel (**B**), the phagocyte population is further defined by CD14 expression to label the monocytes. This monocyte population is analyzed for DHR fluorescence in control (**C**) and stimulated samples (**D**).

Figure 2: Cytokine production in lung tissue. Cells are first analyzed for their expression of the leukocyte marker CD45 (**A**) using flow cytometry. This population is then analyzed further for CD3 expression (**B**) and CD4/CD8 expression (**C**). CD3/CD4+ cells are assessed for intracellular cytokine production in control (**D**) and NTHi-stimulated samples (**E**).

Figure 3: Lung *in situ* zymography. Panel (**A**) shows the expression of chromatin/DAPI in sections of lung tissue. Panel (**B**) shows fluorescent staining, indicating the presence of MMP activity. Panel (**C**) shows the merged image indicating that the MMP activity is also co-localized with the expression of chromatin.

DISCUSSION:

The methods listed here use fluorescence-based flow cytometry and confocal microscopy techniques that can be used in conjunction to obtain detailed information about the inflammatory lung response to Hi.

Establishing the appropriate antigenic formulation of Hi to be used is critical, and it is advisable to have specific input from a microbiologist in this regard. Live Hi induces a stronger response, while killed Hi preparations and Hi components are more standardized and are easier to store. PI will only label dead bacteria[22]; other dyes such as carboxyfluorescein succinimidylester (CFSE) could be used to label live bacteria for phagocytosis assays[23]. A series of preliminary experiments should be undertaken to optimize the appropriate antigen. For using live NTHi, an MOI of 100:1 is optimal; a lower MOI may not induce a clear immune response, while a higher MOI may be toxic to the cells. However, a dose-response curve may give useful information and may be very valuable, particularly with the initial optimization of the technique[24]. As a positive control, a commercially obtained form of inactivated *Staphylococcus aureus* antigen may be used, which is

also labeled with PI as above for the ROS/phagocytosis assay. For the T cell assays, stimulation with *Staphylococcal* superantigen E (SEB) may be used as a positive control[19,25].

The protocols for obtaining BAL and/or lung tissue samples need to be clearly established. The BAL samples can be quite variable between different operators. A hand-held syringe for the right middle lobe lavage produces good results[26]. The obtaining of the lung tissue sample from lobectomy samples requires the establishment of a collaboration with a pathologist. The lung tissue sample should have some margin from the tumor (ideally at least 3–4 cm). Bigger samples (e.g., at least 25–50 g) will yield more cells, as well as samples that are more proximal without obvious emphysema. The mechanical disaggregation of the lung tissue is time-consuming and will generally take at least 2–3 h for each sample[19,27].

Preliminary experiments should be done to optimize the staining of different fluorophores for both flow cytometry and confocal microscopy. Areas to concentrate on include identifying the best staining panel, staining/concentration, and treatment of cells/tissue to maximize viability[28]. The use of lung tissue may be associated with more tissue debris than other fluid samples such as blood or BAL, and this may have some effect on the differentiation of different cellular populations by flow cytometry. The appropriate choice of antibodies needs to be determined and optimized in preliminary experiments. For surface labeling of lymphocytes, anti-CD3 and CD4 can be used for T helper cells, anti-CD3 and CD8 for cytotoxic T cells, and anti-CD3 and CD56 for NK cells. The choice of intracellular cytokines to be studied depends on the mediators of interest and the number of parameters/colors that can be analyzed on the flow cytometer[29]. A specific challenge of working with macrophages in lung tissue is their high level of autofluorescence[30]; this problem can be dealt with by comparing stimulated cells with background control and the addition of specific markers for inflammation such as proteases and histones.

A limitation of these techniques is the requirement for appropriately trained and skilled staff to perform the experiments. Well-established flow cytometry and microscopy facilities are also required. The use of human tissue samples is associated with significant variability particularly when using lung tissue; this may require a series of preliminary experiments to optimize assays (especially with issues of background staining).

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DISCLOSURES:

The authors have nothing to disclose.

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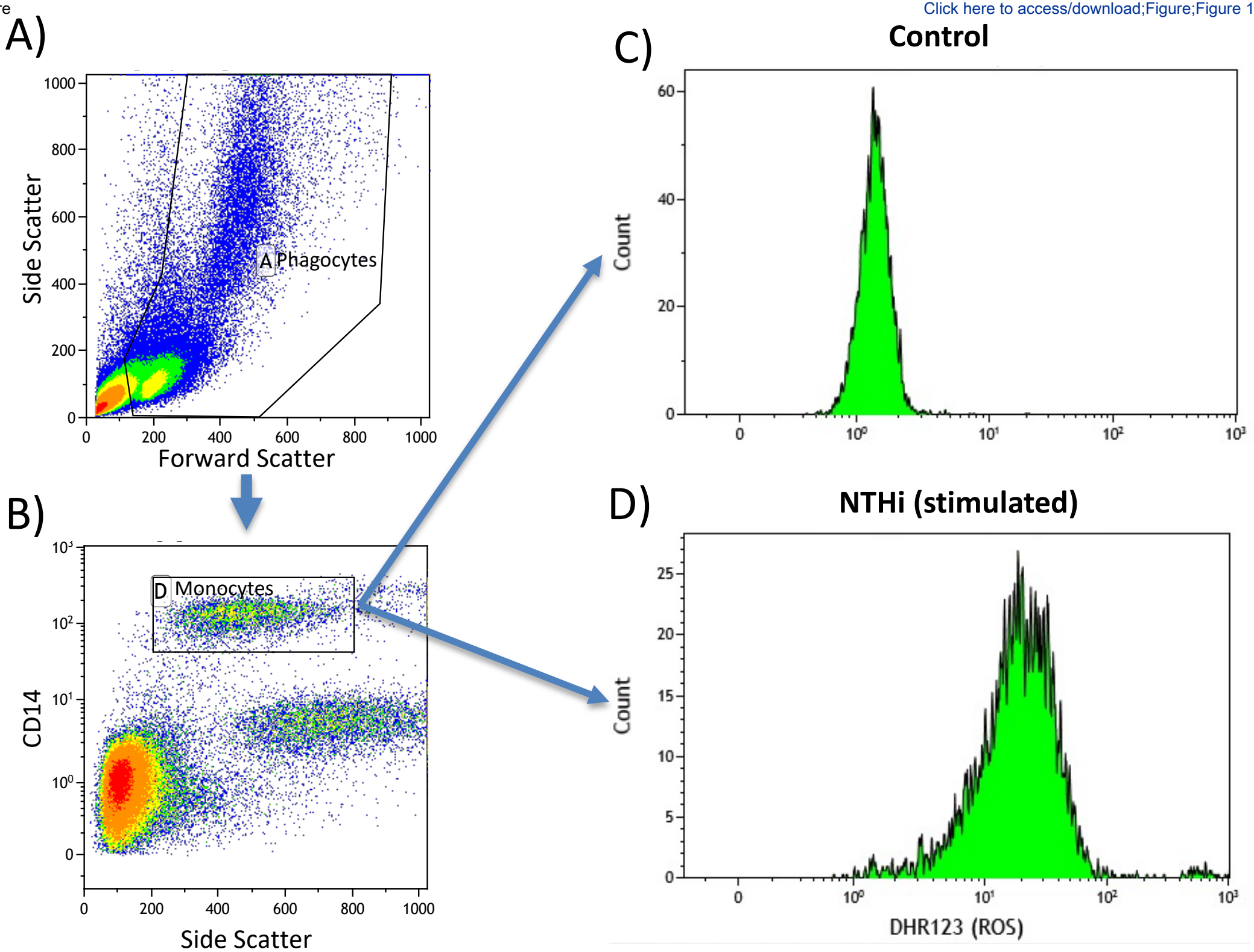
Figure



Figure

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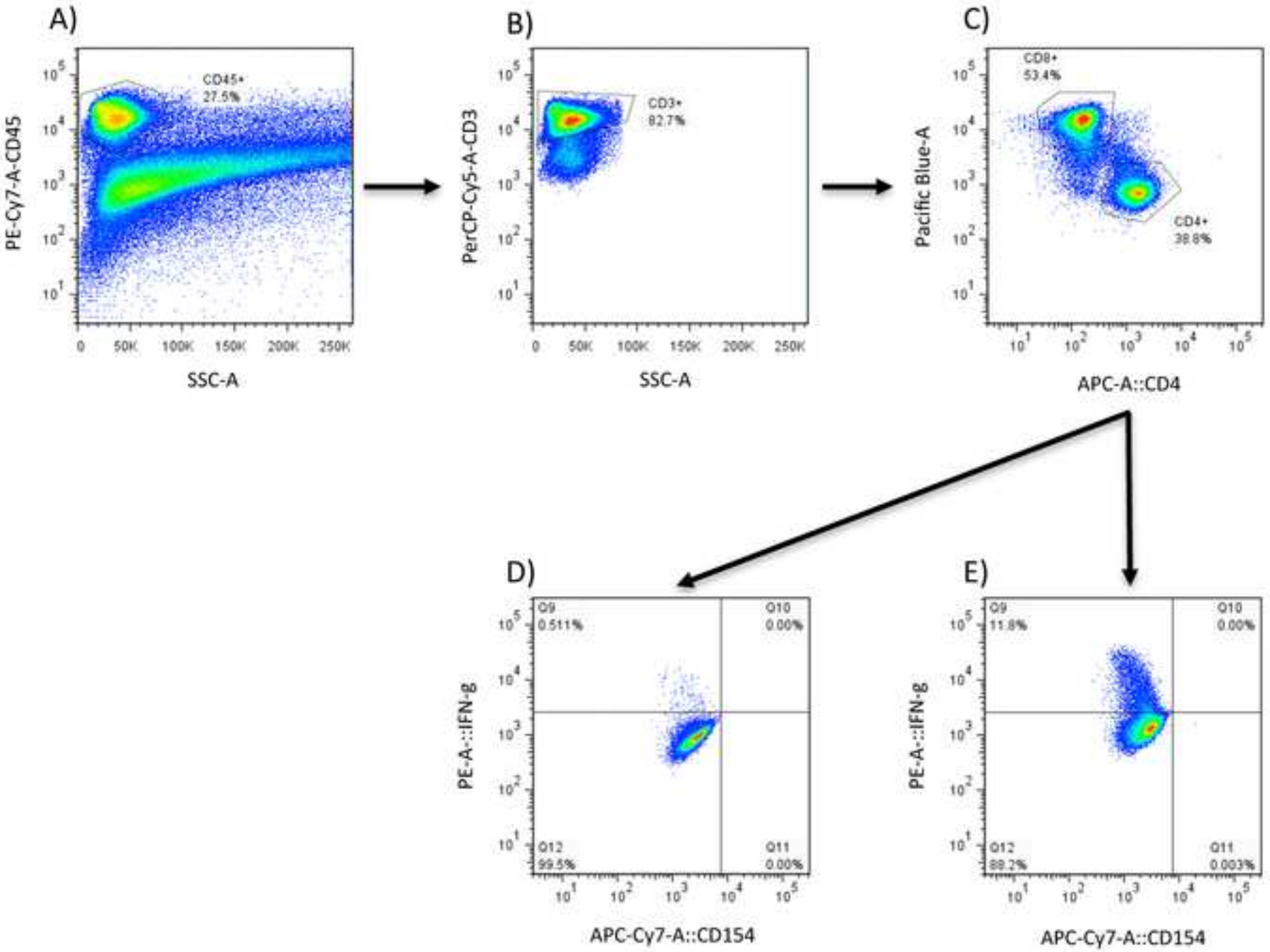

Figure

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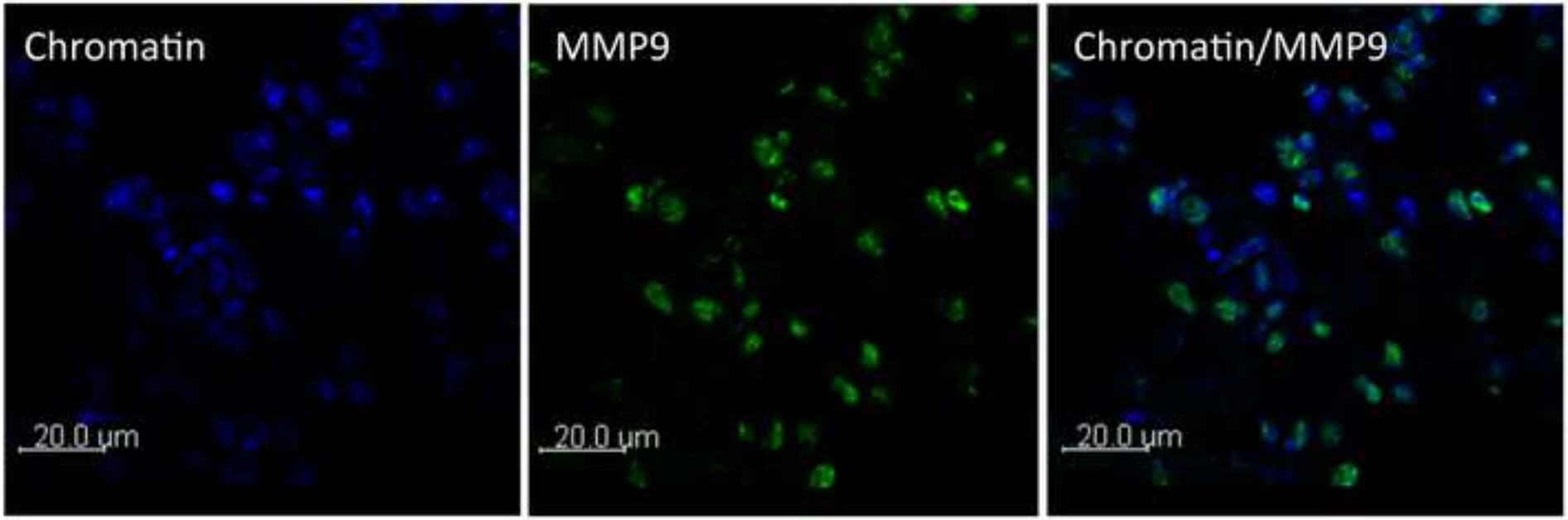

Response to Editorial Comments

1. Please employ professional copyediting services as many grammatical errors in the manuscript significantly affect the comprehension of the manuscript. Please reduce the use of personal pronouns (e.g., "we", "you", "our", etc.)

This has been reviewed and the personal pronouns have been removed.

2. The protocol section has been formatted to imperative tense. Please check and address the comments included within the attached manuscript file.
The comments have been addressed in the text.

3. 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.
This has been added at the beginning of the protocol (line 94).

4. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.
The table is in the requested format.