Journal of Visualized Experiments

Contact-free Co-culture Model for the Study of Innate Immune Cell Activation During Respiratory Virus Infection

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62115R1
Full Title:	Contact-free Co-culture Model for the Study of Innate Immune Cell Activation During Respiratory Virus Infection
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Immunology and Infection
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Singapore
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TITLE:
Contact-free Co-culture Model for the Study of Innate Immune Cell Activation During Respiratory Virus Infection

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KEYWORDS:
Co-culture, human nasal epithelial cells, innate immune cells, innate T-cells, respiratory virus, Influenza, cytokines, flow cytometry

SUMMARY:
This protocol details an investigation of the early interactions between virally infected nasal epithelial cells and innate cell activation. Individual subsets of immune cells can be distinguished

based on their activation in response to viral infections. They can then be further investigated to determine their effects on early antiviral responses.

ABSTRACT:
The early interactions between the nasal epithelial layer and the innate immune cells during viral infections remains an under-explored area. The significance of innate immunity signaling in viral infections has increased substantially as patients with respiratory infections who exhibit high innate T cell activation show a better disease outcome. Hence, dissecting these early innate immune interactions allows the elucidation of the processes that govern them and may facilitate the development of potential therapeutic targets and strategies for dampening or even preventing early progression of viral infections. This protocol details a versatile model that can be used to study early crosstalk, interactions, and activation of innate immune cells from factors secreted by virally infected airway epithelial cells. Using an H3N2 influenza virus (A/Aichi/2/1968) as the representative virus model, innate cell activation of co-cultured peripheral blood mononuclear cells (PBMCs) has been analyzed using flow cytometry to investigate the subsets of cells that are activated by the soluble factors released from the epithelium in response to the viral infection. The results demonstrate the gating strategy for differentiating the subsets of cells and reveal the clear differences between the activated populations of PBMCs and their crosstalk with the control and infected epithelium. The activated subsets can then be further analyzed to determine their functions as well as molecular changes specific to the cells. Findings from such a crosstalk investigation may uncover factors that are important for the activation of vital innate cell populations, which are beneficial in controlling and suppressing the progression of viral infection. Furthermore, these factors can be universally applied to different viral diseases, especially to newly emerging viruses, to dampen the impact of such viruses when they first circulate in naïve human populations.

INTRODUCTION:
Respiratory viruses are perhaps amongst the most widespread pathogens causing severe healthcare and economic burden. From the periodic global outbreaks of emerging epidemic strains (e.g., H1N1, H5N1, H3N2, MERS, COVID-19) to the seasonal strains of influenza every year, viruses are a constant threat to public health. Although vaccines form the main bulk of the response to these global public health challenges, it is sobering to note that these countermeasures are merely responsive[1,2]. Furthermore, a delay between the emergence of a new infectious strain and the successful development of its vaccine is inevitable[3], leading to a period when measures available to curb the spread of the virus are highly limited.

These delays are further emphasized by the costs that are inflicted upon society—economically and socially. The seasonal flu alone is responsible for approximately $8 billion in indirect costs, $3.2 billion in medical costs, and 36.3 thousand deaths in the United States of America annually[4]. This is before consideration of the research costs that are necessary to fund vaccine development. Epidemic outbreaks have even more severe effects on society, compounded by the increasing rate of globalization every year, as evidenced by the global disruptions caused by the emergence and rapid spread of severe acute respiratory syndrome coronovirus 2 (SARS-CoV-2)[5-7].

Recent studies have shown that infected patients having a greater population of activated innate T cells tend to have a better disease outcome[8-10]. Furthermore, the innate T cell population is categorized into multiple subgroups: the mucosal-associated invariant T (MAIT) cells, Vδ1 γδ T cells, Vδ2 γδ T cells, and the natural killer T (NKT) cells. These subgroups of innate T cells also exhibit heterogeneity within their populations, increasing the complexity of the interactions between the cell populations involved in the innate immune response[11]. Hence, the mechanism that activates these innate T cells and the knowledge of the specific subgroups of innate T cells may provide a different avenue of research to curtail the infectious effects of these viruses on the human host, especially during the period of vaccine development.

Epithelial cells infected by influenza produce factors that activate innate T cells rapidly[12-14]. Building upon that finding, this contact-free Air-Liquid Interface (ALI) co-culture model aims to mimic the early chemical interactions (mediated by soluble factors released by the infected epithelial layer) between the infected nasal epithelial layer and the PBMCs during early infection. The physical separation between the nasal epithelial layer (cultured on membrane inserts) and the PBMCs (in the chamber underneath) and the epithelial integrity prevent direct infection of the PBMCs by the virus, allowing a detailed study of the effects of epithelial-derived soluble factors on the PBMCs. The identified factors can therefore be further investigated for their therapeutic potential in inducing the appropriate innate T cell population that may protect against influenza infection. This paper therefore has detailed the methods of establishing a co-culture for the study of innate T-cell activation from epithelium-derived soluble factors.

PROTOCOL:

NOTE: Refer to **Table 1** for recipes of media used in this protocol.

1. Establishment of the 3T3 feeder layer

1.1. Establishment from frozen stocks

1.1.1. Thaw a cryovial of NIH/3T3 fibroblasts from frozen stocks. Add the contents of the cryovial to 2 mL of complete Dulbecco's Minimal Essential Media (DMEM) and resuspend the cells.

1.1.2. Centrifuge for 5 min at 300 × *g* and room temperature/pressure (rtp), and remove the supernatant. Resuspend the cells in complete DMEM.

1.1.3. Count the cells using trypan blue staining. Add 10 µL of trypan blue to 10 µL of the resuspended cell suspension. Mix thoroughly, and add 10 µL of the suspension to a hemocytometer to count the cells.

1.1.4. Seed cells at a density of 1×10^4 cells/cm^2 in an appropriate culture dish (e.g., T75 flask). Incubate the resulting culture flask for 3 days at 37 °C in a 5% CO_2 atmosphere.

1.2. Mitomycin C treatment

1.2.1. At 3 days, ensure confluency is 60%–80%, remove the medium, and wash the cells with 1x phosphate-buffered saline (PBS).

NOTE: It is important that the 3T3 feeder layer does not reach full confluency; otherwise, the mitomycin C treatment will not be effective.

1.2.2. Add mitomycin C-supplemented medium 3 to the flask, and incubate for 3.5 h at 37 °C in a 5% CO_2 atmosphere. Remove the mitomycin C-containing medium, and wash the cells 2x with 1x PBS.

1.3. Seeding 3T3 cells in 6-well plates

1.3.1. Add 3 mL of 1x trypsin-EDTA to the flask for 3–5 min to disassociate the cells. Collect the disassociated cells in a fresh 15 mL tube. Centrifuge the 15 mL tube for 5 min at 300 × *g*, rtp; discard the supernatant. Resuspend the cells in complete DMEM.

1.3.2. Count the cells using trypan blue staining. Seed the cells in a 6-well plate at 7.5×10^5–2.5×10^6 cells/well. Incubate the plate overnight at 37 °C in a 5% CO_2 atmosphere.

NOTE: The 3T3 feeder layer is considered ready if the cells are healthy. At this point, seed the human nasal epithelial stem/progenitor cells (hNESPCs) onto the feeder layer for expansion.

2. Establishment of human nasal epithelial cell (hNEC) culture

NOTE: Clinical samples should be obtained from patients who are free of symptoms of upper respiratory tract infection.

2.1. Processing nasal tissue into single-cell suspension

2.1.1. Wash nasal tissue in 1x Dulbecco's PBS (dPBS) (PBS without Mg^{2+} and Ca^{2+}) containing 100 µL/mL of an antibiotic-antimycotic mixture. Cut the tissue into small fragments, and treat the sample in 10 mg/mL of a neutral protease overnight at 4 °C with shaking.

2.1.2. Centrifuge for 5 min at 200 × *g*, and remove the supernatant after centrifugation. Incubate the pellet in 1–2 mL of 1x trypsin-EDTA (37 °C, 15 min), and quench by adding a volume of fetal bovine serum equal to 10% of the volume in the tube.

2.1.3. Mechanically dissociate the digested tissue into single-cell aggregates by pipetting, and then pass the resulting suspension through a 70 µm cell strainer. Resuspend the cells in complete DMEM.

NOTE: After this step, the cell suspension is a mixture of terminally differentiated cells and stem

cells that are referred to as human nasal epithelial stem/progenitor cells (hNESPCs). The objective of the subsequent steps is to select for and enrich the hNESPC population from the mixture of different cell populations.

2.2. Seeding a single-cell suspension onto the 3T3 feeder layer for selection of hNESPCs

2.2.1. Centrifuge the cell suspension from step 2.1.3 (300 × *g*, 5 min, rtp), and remove the supernatant. Resuspend the cells in 3–5 mL of medium 3.

NOTE: Medium 3 is formulated to promote the selective growth of hNEPSCs.

2.2.2. Count the cells via trypan blue staining. Seed 1×10^6 cells in 2 mL of medium 3 per well of a 6-well plate onto a ready 3T3 feeder layer. Incubate the resulting co-culture at 37 °C in a 5% CO_2 atmosphere.

NOTE: After seeding, take care not to agitate the plate as it will affect the attachment of the hNESPCs.

2.2.3. Change medium 3 (2 mL) every 2–4 days by removing the old medium entirely and replacing it with fresh medium 3.

NOTE: Medium is changed to replenish nutrients and to prevent overly acidic conditions from negatively influencing the growth of the hNESPCs. The interval between each medium change depends on how acidic (yellow) the medium becomes. Intervals should be shortened if the medium turns acidic quickly.

2.2.4. After 7–10 days, observe that hNESPCs are at a confluency suitable for transferring them onto membrane inserts. See **Figure 1** for representative morphology of hNESPCs at 3 different timepoints (2 days, 5 days, and 10 days).

2.3. Transferring hNESPCs onto membrane inserts

NOTE: Due to the mitomycin C treatment, the 3T3 feeder layer will slowly degrade over the hNESPC expansion period. This will result in a weakened adhesion to the surface of the well, allowing their dislodgement by flushing with a pipette, leaving behind only the healthy hNESPCs.

2.3.1. Remove the medium, and add 500 µL of 1x dPBS to each well. Flush the cells 3x with a micropipette to dislodge the 3T3 cells, and discard the 1x dPBS. Observe under the microscope that the round hNESPCs remain while the spindle-shaped 3T3 feeder layer is dislodged.

2.3.2. Add 500 µL of a cell disassociation reagent per well, and incubate at 37 °C in a 5% CO_2 atmosphere for 5–10 min, or until the hNESPCs have detached from the well surface.

2.3.3. Collect the hNESPC suspension, centrifuge (300 × *g*, 5 min, rtp), and remove the supernatant.

2.3.4. Resuspend the pellet in medium 3. Count the cells via trypan blue staining. Seed 3×10^4 cells in 150 µL of medium 3 per membrane insert (24-well plate) or 1×10^5 cells in 300 µL of medium 3 per membrane insert (12-well plate) (**Figure 2**). Incubate the cells at 37 °C in a 5% CO_2 atmosphere.

2.3.5. Change the medium (medium 3) of the apical and basal chambers every 2 days. Navigate a pipette tip between the supporting arms of the membrane insert to access the basal chamber, remove the old medium, and then reintroduce fresh medium by the same method. Although the apical chamber is readily accessible, take care not to disturb the growing hNESPC layer.

NOTE: Do not disturb the medium in the apical chamber for at least 2 days after seeding as the cells need time to attach to the membrane.

2.4. Differentiation of hNESPCs into hNECs

2.4.1. When hNESPCs reach 100% confluency (approximately 3–7 days from seeding on the membrane insert), start the ALI culture. Change the basal medium to differentiation medium, and remove the medium from the apical chamber. Only add medium to the basal chamber without adding it to the apical chamber when changing the medium every 2–3 days, as indicated in **Figure 3** and step 2.3.5.

NOTE: Medium is changed to differentiation medium to promote the differentiation of the hNESPCs into hNECs in ALI. The hNESPCs take approximately 3–4 weeks to reach full maturity to become hNECs in ALI, at which point the cells would have grown into a multilayer with different populations of cells in each layer. Cilia should also be observed at a magnification of 400x (cilia can be identified by their beating movement, which cause the microscope field to appear like it is vibrating). At this point, the cells are ready to be used for the co-culture experiments. Refer to **Figure 4** for the cross-section of a fully differentiated hNEC layer.

2.4.2. After obtaining mature hNECs, wash the cells in the apical chamber 3x with 1x dPBS at 2–3-day intervals for 1 week prior to the co-culture experiment. Synergize the wash step with the basal medium changes, and perform the washes as follows.

2.4.2.1. Add 50 µL (24-well)/150 µL (12-well) of 1x dPBS into the apical chamber of the membrane insert, and incubate the cells at 37 °C for 10 min. Remove the dPBS after 10 min of incubation to remove accumulated mucus and dead cells over the course of differentiation.

NOTE: Depending on the nature of the experiment, sodium bicarbonate solution can be used to wash off the mucus layer entirely. However, for viral infection in co-culture experiments, the mucus layer is retained, and only excess mucus is removed with dPBS wash. This retention is to mimic the physiological mucus layer present on the nasal epithelia that will interact with the

incoming viral infection.

3. Transepithelial electrical resistance (TEER) measurement

NOTE: Confirmation of epithelial integrity is important to ensure that an intact and healthy epithelial layer is obtained. An intact epithelial layer is determined through TEER measurement performed on 4 random wells using a voltohmmeter.

3.1. Rinse the electrode with 70% ethanol, and sterilize it with ultraviolet light for 15 min before use to ensure sterility. Rinse with 1x dPBS after sterilization.

3.2. Add 100 µL (for 24-well) or 300 µL (for 12-well) of 1x dPBS to the apical chamber of the membrane insert. Incubate the plate at 37 °C for 10 min; then, remove the dPBS.

3.3. For each well to be measured, prepare an unused well with 1 mL (for 24-well) or 3 mL (for 12-well) of prewarmed (to 37 °C) differentiation medium. Place the membrane insert in the prepared well, and add 200 µL (for 24-well) or 600 µL (for 12-well) of prewarmed differentiation medium to the apical chamber. Equilibrate at 37 °C (subject to the incubation temperature of the type of virus added, e.g., 35 °C for Influenza) for 15 min.

NOTE: Prepare a blank (empty membrane insert) in the same way for the calculation of the TEER.

3.4. Equilibrate a 15 mL tube of prewarmed differentiation medium at the same temperature for 15 min as well. Place the electrodes in the 15 mL tube, and switch on the voltohmmeter.

NOTE: At this point, the reading should be 0 resistance, as the electrodes are in the same medium. If any other reading is obtained, equilibrate the electrode in the 15 mL tube until the reading is 0.

3.5. Starting from the blank, position the electrodes in each well such that one electrode is submerged in the apical chamber medium and the other in the basal chamber medium. Record the reading only when a constant reading is obtained for a period of at least 5 min.

3.6. After each measurement, wash the electrode with PBS before the next measurement. For each well tested, take measurements for three samples at different positions of the membrane. To calculate the net TEER of each sample, subtract the background resistance, given by the blank membrane insert, from each measurement.

3.7. Calculate the total TEER reading for a well using the following equation:

$$Epithelial\ Integrity\ (\Omega cm^2) = Net\ TEER(ohms) \times Area\ of\ membrane\ (cm^2)$$

NOTE: The TEER values of hNECs for viral infection experiments should be >1000 Ωcm^2 [13,15,16].

4. Isolation of peripheral blood monocytes and NK cells

NOTE: Blood samples should be obtained from healthy volunteers and used on the same day of isolation.

4.1. Collect 30–40 mL of whole blood from each donor in 10 mL blood collection tubes.

4.2. Isolate PBMCs using density gradient centrifugation, and obtain PBMCs from the buffy coat after the following steps (see also the **Table of Materials**).

4.2.1. Thoroughly mix ~10 mL of blood from the blood collection tube in a vacutainer before diluting with an equal volume of balanced salt solution (PBS).

4.2.2. Add 15 mL of density gradient medium to the base of a 50 mL tube.

NOTE: The ratio of density gradient medium to diluted blood should be 2:3–3.5.

4.2.3. Layer 35 mL of diluted blood on the density gradient medium with a pipette gun (with the dispense setting set to the lowest possible) by tilting the tube by 45° and allowing the diluted blood to fall dropwise onto the inner wall of the tube so that the layer of density gradient medium is not disturbed.

4.2.4. Centrifuge at 800 × *g*, 18–20 °C for 30 min (brake: off). Carefully remove and discard the upper plasma layer without disturbing the lower layer.

4.2.5. Transfer the buffy coat to a new tube without mixing the red blood cell layer, gradient density medium layer, or the buffy coat. Once the buffy coat has been collected in a new 50 mL tube, top up the volume to 50 mL with 1x PBS.

4.2.6. Centrifuge at 400 × *g*, 18–20 °C, 8 min (brake: off), and remove the supernatant with a pipette gun. Resuspend the cell pellet with 50 mL of 1x PBS, and centrifuge at 120 × *g*, 18–20 °C, 10 min to remove platelets.

4.2.7. Discard the supernatant by pipetting, without disturbing the cell pellet.

NOTE: As the cell pellet might be loose, it is inadvisable to discard the supernatant by pouring.

4.2.8. Resuspend the cell pellet with complete RPMI (Roswell Park Memorial Institute) medium. Perform a cell count by adding 10 µL of 3% acetic acid with methylene blue to 10 µL of the resuspended cell suspension. Mix thoroughly, and add 10 µL of the mixture to a hemocytometer to count the cells.

4.3. Dilute PBMCs with complete RPMI medium to a density of 2×10^6/mL (for 24-well) or 4×10^6/mL (for 12-well).

5. hNEC Viral infection and transition to hNEC:PBMC co-culture

NOTE: H3N2 (A/Aichi/2/1968) is used as the representative strain of infection in this protocol. Multiplicity of infection (MOI) of 0.1 is used as the representative MOI in this protocol.

5.1. Day 0 (Infection of hNECs)

NOTE: One well from the hNECs grown from the same donor is used to obtain a representative cell count for every well used in the experiment.

5.1.1. In the representative well, add 150 µL of 1x trypsin-EDTA to the apical chamber of the membrane insert and 350 µL of 1x trypsin-EDTA to the basal chamber, and incubate at 37 °C for 10 min, or until the cells detach from the membrane.

5.1.2. Flush the cells on the membrane by pipetting up and down, and collect the suspension in a 1.5 mL centrifuge tube. Add 200 µL of complete DMEM to quench trypsin activity. Count cells via trypan blue staining to obtain the cell count per well.

5.1.3. Calculate the required MOI of the virus based on the cell count per well, and dilute the virus stock accordingly with complete RPMI on ice.

NOTE: MOI 0.1 of 1.26×10^6 hNECs per well = 1.26×10^5 H3N2 viral particles per well in 30 µL (for 24-well)/100 µL (for 12-well)

5.1.4. For the remaining wells for the infection experiment, add 50 µL (for 24-well)/150 µL (for 12-well) of 1x dPBS into the apical chambers of the membrane inserts, incubate at 37 °C for 10 min, and remove the 1x dPBS.

5.1.5. Change the basal medium in the membrane inserts to complete RPMI medium by transferring the inserts to a new plate with complete RPMI added to the wells (350 µL (for 24-well)/700 µL (for 12-well)).

NOTE: When hNESPCs have fully differentiated to hNECs, they are tolerant/permissive to different media for up to 72 h, with no changes to morphology or organization when the medium is switched to RPMI[12]. RPMI is used to support the growth and maintenance of the PBMC population.

5.1.6. Add the prepared 30 µL (for 24-well)/100 µL (for 12-well) of the virus inoculum into the apical chamber of the membrane insert, incubate at 35 °C in a 5% CO_2 atmosphere for 1 h, and remove the viral inoculum from the apical chamber.

5.1.7. Change the basal medium of membrane insert to fresh complete RPMI medium and incubate for at 35 °C in a 5% CO_2 atmosphere for 24 h.

5.2. Day 1 (establishment of hNECs + PBMCs co-culture)

5.2.1. Seed the required number of PBMCs in 150 µL (for 24-well)/300 µL (for 12-well) of complete RPMI medium by directly adding the PBMC suspension into the basal chamber of each well of infected hNECs from Day 0 (1.5×10^6 for 24-well plates and 3×10^6 for 12-well plates). Incubate at 37 °C for 24/48 h.

NOTE: The final volume in the basal chamber after the establishment of the co-culture is 500 µL (for 24-well)/1000 µL (for 12-well).

5.3. Days 2–3 (harvesting of PBMCs 48/72 h post-viral infection)

5.3.1. Collection of apical supernatants (48/72 h post-viral infection)

5.3.1.1. Add 50 µL (for 24-well)/150 µL (for 12-well) of 1x dPBS to each apical chamber, and incubate at 37 °C for 10 min. Collect the 1x dPBS in 1.5 mL tubes. Aliquot 25 µL (for 24-well)/50 µL (for 12-well) of the supernatant for plaque assay in a new 1.5 mL tube, and immediately freeze both the stock and the aliquots at -80 °C.

5.3.2. Collection of cellular RNA from hNECs (48/72 h post-viral infection)

5.3.2.1. Transfer the membrane insert to a clean well, add 300 µL (for 24-well)/600 µL (for 12-well) of RNA lysis buffer to the apical chamber, and incubate at rtp for 5 min. Collect the supernatant in a 1.5 mL centrifuge tube, and store it at -80 °C until RNA extraction for molecular analysis.

5.3.3. Harvesting PBMCs (48/72 h post-viral infection)

5.3.3.1. With the broad base of a sterile pipette tip, gently scrape the surface of the well to dislodge the activated PBMCs that may be adherent to the well surface. Collect the basal medium containing PBMCs in a 2 mL centrifuge tube.

5.3.3.2. Flush the wells 2x with 300 µL of 1x dPBS-E, and collect the wash in the same 2 mL tube. Centrifuge the 2 mL tube (500 × *g*, 5 min, rtp), and collect the supernatant in a fresh 2 mL tube without disturbing cell pellet. Store the supernatant at -80 °C for cytokine and chemokine analysis; resuspend the cell pellet in 200 µL of 1x dPBS.

6. **Flow cytometry**

NOTE: This section of the protocol is continued directly from the previous section using the PBMC cell suspension from step 5.3.3.2. Ensure minimal light exposure during the following steps in this section. A sample panel of surface staining markers can be found in **Table 2**.

6.1. Surface staining

6.1.1. Transfer the resuspended PBMC cell pellet into a 96-V bottom well plate. Centrifuge the plate at 300 × *g* for 3 min, and remove the supernatant.

6.1.2. Incubate all the PBMCs (except for the "unstained") with 50 µL of a viability stain for 15 min at rtp. Top up to 200 µL with 150 µL of Magnetic-Activated Cell Sorting (MACS) buffer. Centrifuge the plate at 300 × *g* for 3 min, and discard the supernatant.

6.1.3. Prepare a panel of surface staining antibodies of interest with appropriate dilution ratios and a final volume of 50 µL per reaction (top-up with MACS buffer to obtain the final volume). Perform surface staining by adding 50 µL of the prepared antibody mix to the cells using a multi-channel pipette. Incubate at 4 °C for 15 min in the dark.

6.1.4. Wash by adding 150 µL of MACS buffer. Centrifuge the plate at 300 × *g* for 3 min, and discard the supernatant. If intracellular staining is not required, proceed directly to the 15 min incubation in step 6.3.

6.2. Intracellular staining (If required)

6.2.1. Add 100 µL of a fixation and permeabilization solution to each well. Incubate at 4 °C for 20 min in the dark. Top up the wells with 100 µL of 1x MACS buffer.

6.2.2. Centrifuge (500 × *g*, 3 min, 25 °C), and remove the supernatant. Repeat the wash by adding 200 µL of 1x Permeabilization Wash buffer. Centrifuge (500 × *g*, 3 min, 25 °C), and remove the supernatant.

6.2.3. Prepare the dilutions for the antibody panel of interest to achieve a final volume of 50 µL using 1x Permeabilization Wash buffer. Incubate on ice for 30 min in the dark. Add 200 µL of 1x Permeabilization Wash buffer.

6.2.4. Centrifuge the cells (500 × *g*, 3 min, 4 °C), and remove the supernatant. Resuspend the cells in 100 µL of fluorescence-activated cell sorting buffer.

6.3. Pipette 200 µL of a lysing solution into each well. Incubate for 15 min at rtp. Centrifuge the plate at 300 × *g* for 3 min and discard the supernatant.

6.4. Resuspend the cell pellets in 200 µL of MACS buffer. Perform flow cytometry in accordance to the settings outlined previously[13], or store at 4 °C in the dark.

7. Determination of cytokine and chemokine levels

7.1. Process 25 µL of the basal chamber supernatant using an immunology multiplex assay according to the manufacturer's protocol[18].

7.2. Calculate the concentration of each analyte using multiplex manager software utilizing a curve-fitting algorithm (5 parameters) for the standard curve.

8. Assessment of viral contamination

8.1. Extract viral RNA using an extraction kit on 4 sets of solutions: the stock H3N2 solution, the MOI 0.1 dilution for infection, the 10x serial dilutions of the MOI 0.1, and the basal medium from the samples (both 48 and 72 h post-viral infection)[12].

8.2. Select non-structural gene (NS1) and matrix (M1) as targets for polymerase chain reaction (PCR) (see the **Table of Materials** for primers used in the representative experiment).

8.3. Perform reverse-transcription-PCR (RT-PCR) (42 °C, 60 min) to convert the viral RNA to cDNA before performing quantitative PCR (qPCR) to measure NS1 and M1 levels as a proxy for viral presence, and correlate the levels to the standard curve obtained from the RT-qPCR performed on the 10x serial dilution of the MOI 0.1 aliquot. Refer to **Table 3** for the composition of the reaction mixes, and use the following conditions: preincubation at 95 °C for 10 min, followed by a 3-step amplification for 40 cycles: 95 °C for 10 s, 60 °C for 10 s, 72 °C for 30 s; melting curve: 95 °C for 10 s, 65°C for 60 s, and 97°C for 1 s.

9. Plaque assay

9.1. Day 0: seeding MDCK (Madin Darby Canine Kidney) in 24-well plates

9.1.1. Remove the medium from a T75 Flask of confluent MDCK cells. Wash 2x with 1x PBS to remove all traces of serum. Trypsinize the MDCK cells by adding 10 mL of trypsin and incubating at 37 °C in a 5% CO_2 atmosphere for 20–30 min until the cells detach.

NOTE: Do not tap the flask as this might result in clumping.

9.1.2. Pipette the cell suspension into a 15 mL tube containing 2 mL of complete Eagle's Minimal Essential Medium (EMEM). Centrifuge (300 × *g*, 5 min, rtp), and remove the supernatant.

9.1.3. Resuspend the cells in 6 mL of complete EMEM. Count the cells by trypan blue staining.

9.1.4. Dilute the MDCK cell suspension to a concentration of 1×10^5 cells/mL, and seed 1 mL of the diluted suspension in each well of a sterile 24-well plate. Incubate at 37 °C in a 5% CO_2 atmosphere for 24 h to obtain a confluent monolayer of MDCK cells in each well.

9.2. Day 1: infection of MDCK cells

9.2.1. Prepare the infection medium. Remove the medium from the MDCK monolayer in the 24-well plate, and wash 2x with 1x dPBS. For the second wash, leave the PBS in the wells while preparing serial dilutions of the virus.

9.2.2. Thaw the virus samples on ice, and serially dilute them in a 24-well plate to achieve serial dilutions from 10^{-1} to 10^{-6}.

NOTE: As an example, fill each well in a 24-well plate with 270 µL of infection medium.

9.2.3. Add 30 µL of the virus sample to the first well in the row. With a new pipette tip, mix well and transfer to the next well in the row to dilute the sample by 10x. Proceed until 10^{-6} dilution has been achieved; perform steps 9.2.1–9.2.3 for all virus samples.

9.2.4. Remove PBS from the MDCK plate, and infect in duplicate with 100 µL of the prepared viral dilutions. For control wells, add 100 µL of the infection medium (without the viral sample). Incubate at 35 °C in a 5% CO_2 atmosphere for 1 h, shaking the plate to eliminate dry spots every 15 min.

9.2.5. Remove the viral inoculum, and add 1 mL of liquid overlay for each well. Incubate at 35 °C in a 5% CO_2 atmosphere for 72 h.

9.3. Day 4 (72 h post-infection): plaque visualization

9.3.1. Remove the liquid overlay, and fix the cells with 4% formaldehyde in 1x PBS for 1 h. Remove the formaldehyde solution, and wash once with 1x PBS or distilled water.

9.3.2. Stain the fixed cells by adding 1% crystal violet solution for 15 min. Remove the crystal violet dye, and wash the cells with running water. Dry the plate at rtp.

9.3.3. Once dry, count plaques, and calculate the viral titer according to the following formula:

$$Number\ of\ Plaques \times Dilution\ Factor = Number\ of\ Plaque - Forming\ Units\ in\ 100\ \mu L$$

REPRESENTATIVE RESULTS:

Although conventional T cells form the main repertoire of adaptive immune response against viral infection to facilitate viral clearance, the innate T cell population works across a broader spectrum to suppress the viral load for effective clearance at a later stage. Therefore, this protocol specifically creates a robust condition to study innate T cells, their activation, and their functional population following influenza infection, without needing epithelial and immune cell samples from the same donor. This protocol can also be applied to other viruses, although it may be limited to viruses with apical release, i.e., no virus should enter the basal layer to come into contact with the PBMC compartment.

Based on the representative results in **Figure 1**, this protocol can help to obtain hNESPC populations grown from a primary cell suspension in a 3T3 feeder layer. **Figure 1** provides a sample of the expected progression of the hNESPCs as they grow on the 3T3 feeder layer. These cells will be used for differentiation in the ALI culture to obtain multilayered hNECs, complete with functional ciliated and goblet cells (**Figure 4**). Using the hNECs, innate T-cell activation can be investigated using flow cytometry. The results shown in **Figure 5** show the detection of MAIT cell, γδ-T cell, and NK cell populations, which were significantly increased in co-culture involving hNECs infected with influenza virus. This setup can then be applied to other strains of the influenza virus to tease out the universal population across strains, as well as other viruses and their ability to activate innate T cell populations. In addition, the detection panel can also be customized according to the innate immune cell population of interest to observe their respective activation under co-culture conditions with infected epithelial cells.

FIGURE AND TABLE LEGENDS:

Figure 1: hNESPCs grown on a 3T3 feeder layer 2/5/10 days from seeding. Day 2: Note the islets of hNESPCs (an example is demarcated with a white arrow) that should be observed 2 days after seeding on the 3T3 feeder layer. Day 5: The islets observed on Day 2 should now be larger (examples of islands of hNESPCs are demarcated by green circles), and the 3T3 layer should be observed to be degenerating. Day 10: The hNECPSs should be dominating the entire plate with little or no 3T3 cells visible. Scale bars for Day 2 and Day 5 = 50 µm based on a magnification of 200x, scale bar for Day 10 = 100 µm based on a magnification of 100x. Abbreviation: hNESPCs = human nasal epithelium stem/progenitor cells.

Figure 2: Well diagrams for membrane inserts in 24-well and 12-well plates. Note the medium volume to be used for each compartment. hNESPCs are seeded in the apical chambers of the membrane inserts. Abbreviation: hNESPCs = human nasal epithelium stem/progenitor cells.

Figure 3: Well diagrams for membrane inserts in 24-well and 12-well plates for ALI co-culture establishment. Note the medium volume to be used for each compartment. Note the differences in medium volume to be used for the different intervals (2 days/3 days) between medium changes. Abbreviation: ALI = air-liquid interface.

Figure 4: β4-Tubulin and MUC5AC co-stain of an hNEC layer. β4-Tubulin is stained in green, while MUC5AC is stained in red. The nuclei are stained in blue with DAPI. MUC5AC indicates the presence of mucus-producing goblet cells, while β4-tubulin indicates the presence of cilia on ciliated cells. Scale bar = 20 µm based on 600x magnification. Abbreviations: hNEC = human nasal epithelial cell; MUC5AC = mucin 5AC; DAPI = 4',6-diamidino-2-phenylindole.

Figure 5: Representative results of PBMCs incubated with or without nasal epithelium or influenza-infected epithelium for 24 h. Activation of MAIT, Vδ1 T cells, Vδ2 T cells, and NK cells was determined by cell-type-specific markers including Vα 7.2 TCR, Vδ1 TCR, Vδ2 TCR, CD56, and CD69 staining. The values above the gates indicate the percentage of CD69-positive cells. Abbreviations: PBMCs = peripheral blood mononuclear cells; Epith = nasal epithelium; FLU-Epith

= influenza-infected epithelium; MAIT = mucosal-associated invariant T cells; NK = natural killer; TCR = T-cell receptor; CD = cluster of differentiation.

Table 1: Recipe for media used.

Table 2: Sample surface staining markers.

Table 3: Recipe for reaction mixes of reverse-transcription polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR).

DISCUSSION:

Innate immune responses against viruses are an under-investigated field of study in antiviral management. The airway epithelial cells and innate immune cells work in concert to suppress viral replication during an infection, besides serving as a determinant of overactive adaptive response if the viral load is not kept in check[12,13,17]. However, the development of a relevant human model for the study of epithelial-innate immune crosstalk to investigate the activation of innate immune cells to confer an appropriate antiviral response remains a challenge. Hence, this ALI co-culture model represents a versatile technique that can be used to assess a whole host of interactions between the nasal epithelial layer and the immune cells. As this model combines in-vitro-differentiated hNECs, PBMC activation analysis via flow cytometry, and viral infection, many of the crucial steps have been clearly demarcated to ensure the success of this protocol. In addition, further modification can also be done to the part of the airway involved where in-vitro-differentiated cells from both the upper and lower airways can be used, adding another layer of versatility to the protocol.

However, when working with epithelial-immune cell crosstalk in viral infection, it is critical that the viruses do not interact directly with the PBMCs to identify early local epithelial-derived soluble factors released by the hNECs. Therefore, this model is more suitable for examining viruses with polarized viral release, wherein viruses only bud out from the apical surface into the apical chamber, e.g., influenza viruses[12] and SARS-CoV-2 virus[19]. In addition, to prevent leakage of apical-release viruses into the basal chamber compromising the experiment, an intact epithelial layer of sufficient thickness is vital. Therefore, it is important that TEER measurement and viral RNA quantification be performed to ensure that the results are free of viral leakages into the basal chamber[13,16,20]. A TEER reading of >1000 implies an intact multilayer of cells suitable for viruses with polarized release; the basal media should be free of any viral RNA contamination[13,15,16]. However, the utility of the model for bidirectional release viruses, such as rhinoviruses, remains to be explored[16]. Such viruses are not limited to releasing their progeny in a polarized manner and may bidirectionally release new viruses into both apical and basal regions of the epithelium. Further optimization is required before this model can be applied to viruses with non-polarized release.

As this protocol involves working with human samples from different individuals, no two samples of hNECs will exhibit the same properties and responses[12,16]. For example, the viscosity of the mucus produced by the terminally differentiated hNEC layer could differ greatly. The speed of

cilia development may also be different. It is important, therefore, to exercise some level of flexibility when adhering to the guidelines laid out in this protocol. While it is certainly possible to utilize epithelial cell lines, this would remove the complexity (mucus, interactions between different cell types) of the interactions between the different cell types of the epithelial layer, which would be ideal for investigation of how epithelial crosstalk influences the responses of the PBMCs. A primary cell line is necessary to mimic the physiology of the nasal tissues, where the cells are multilayered, and the different cell types are localized to their own individual niche, although variability might be an issue. Variability in this respect can be overcome by utilizing single-cell RNA sequencing to differentiate and separate the heterogenous population of cells.

The types of interactions that can be assessed are indeed limited by the origin of the PBMCs and the hNECs. When the PBMCs and hNECs are obtained from different donors, ensuring epithelial integrity and separation is crucial. When PBMCs come into contact with hNECs, allogenic immune reactions could occur. Hence, the only interactions that are relevant are interactions mediated by soluble factors that can pass through the membrane inserts, as has been described in the protocol above. However, when both populations of cells originate from the same individual, this model has an added layer of utility as conventional immune cell reactions between the hNECs and the PBMC population can now be assessed, including T-cell-mediated cytotoxicity and antibody-mediated responses. In addition, epigenetic studies can also be performed to examine how modifications to the genome may affect cytokine gene/protein expression/secretion.

Furthermore, different cell populations can be added to the basal chamber to further investigate a specific population. This can be performed by isolating the cell populations of interest (T cells, NK cells, monocytes) and introducing them to the basal chamber instead of the PBMCs. However, this model cannot be used to investigate cellular interactions that require direct contact owing to the separation of the two cell populations by the membrane. As such, the investigation of adaptive immune responses may be limited by this detail. In conclusion, this ALI co-culture model offers a versatile starting point for the in vitro investigation of the crosstalk between nasal tissues and immune cells. The protocol described in this manuscript attempts to provide a guideline that will be helpful even if the populations/conditions are altered.

ACKNOWLEDGMENTS:
We would like to thank the research staff in NUS Department of Otolaryngology and Department of Microbiology and Immunology for their help with the hNEC culture- and viral-culture-related work. We would also like to thank the surgeons and surgical team in National University Hospital, Department of Otolaryngology, for their assistance in providing the cell and blood samples required for the study.

FUNDING:
This study was funded by National Medical Research Council, Singapore No. NMRC/CIRG/1458/2016 (to De Yun Wang) and MOH-OFYIRG19may-0007 (to Kai Sen Tan). Kai Sen Tan is a recipient of fellowship support from European Allergy and Clinical Immunology (EAACI) Research Fellowship 2019.

DISCLOSURES:
All authors declare no conflict of interest.

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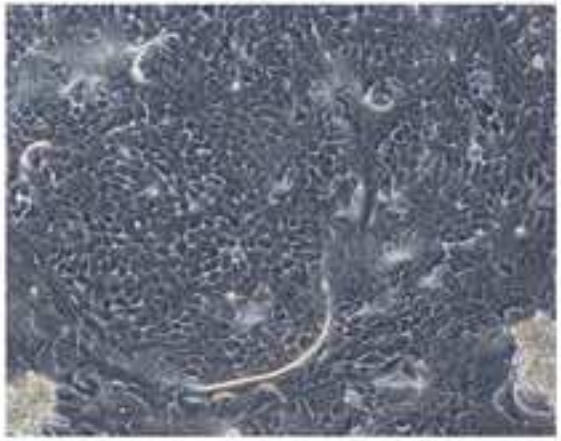
Figure 1.

Day 2

Day 5



Day 10

Figure 2.

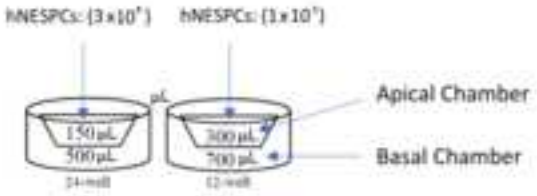


Figure 3.

Figure 4.

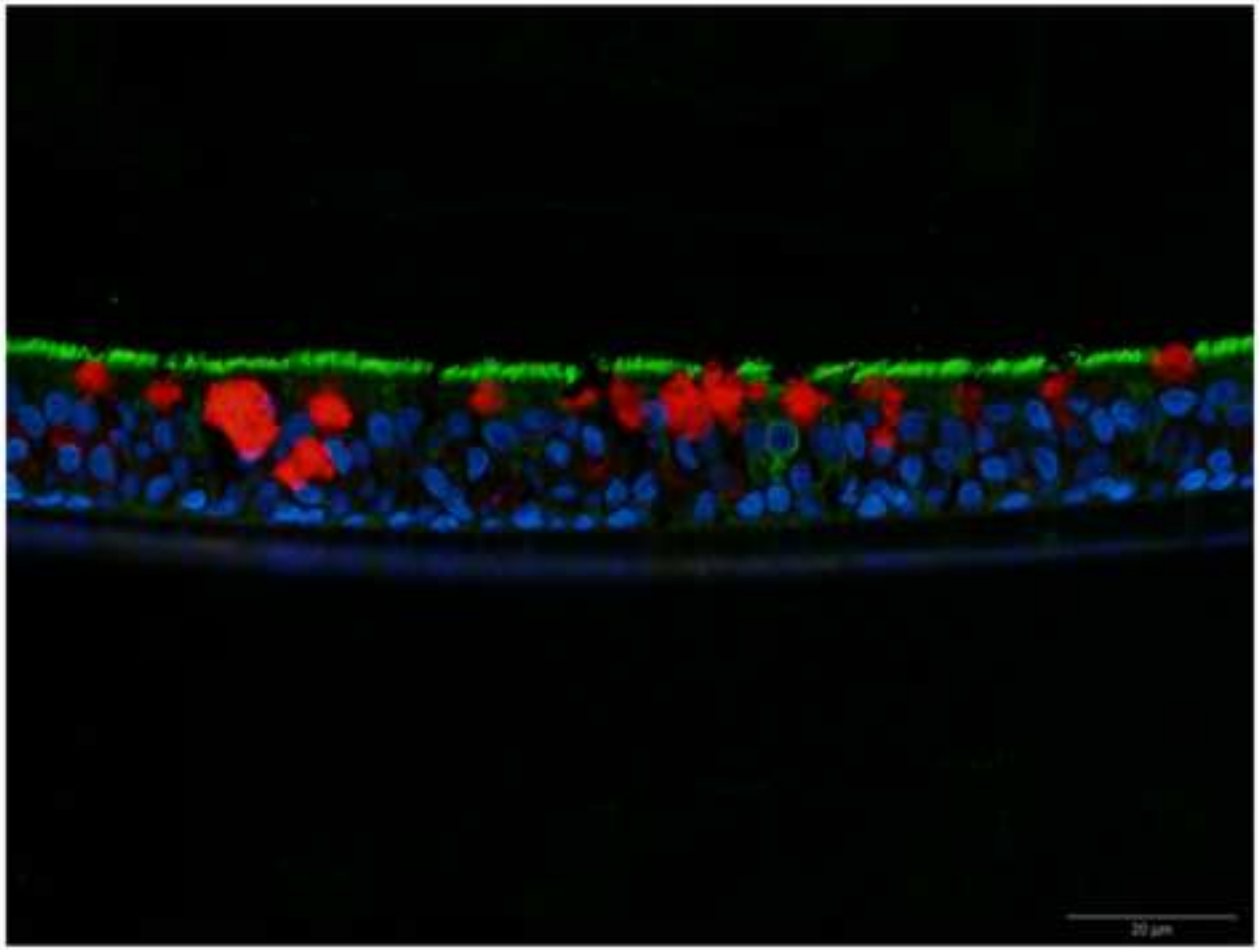


Figure 5.

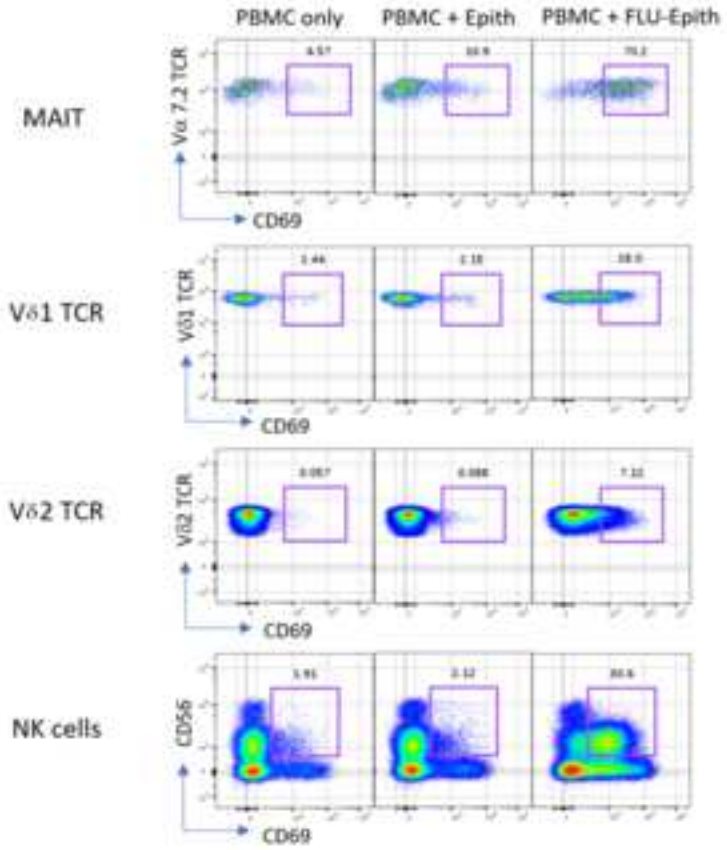


Table 1

Click here to access/download;Table;1. JoVE_Table 1.xlsx

Medium	Recipe	Composition
Medium 3	DMEM/Nutrient Mixture F-12	500 mL
	Human Epithelial Growth Factor	5 ng/mL
	Insulin	2.5 µg/mL
	Cholera Toxin	0.1 nM
	Hydrocortisone	0.5 µg/mL
	3,3',5-triiodo-l-thyronine	2 nM
	N-2 supplement	5 mL
	Antibiotic-Antimycotic	5 mL
Differentiation Media	PneumaCult-ALI Basal Medium	441 mL
	PneumaCult-ALI 10x Supplement	50 mL
	Hydrocortisone Solution (200x)	2.5 mL
	0.2% (2 mg/mL; 1000 IU/mL) Heparin Sodium Salt in Phosphate-Buffered Saline	1 mL
	Antibiotic-Antimycotic (100x)	5 mL
	PneumaCult-ALI Maintenance Supplement (100x)	500 µL
Complete Dulbecco's Minimal Essential Medium (DMEM)	DMEM/High Glucose	450 mL
	Heat-inactivated Fetal Bovine Serum	50 mL
	Antibiotic-Antimycotic (100x)	5 mL
Complete Roswell Park Memorial Institute (RPMI) Medium	RPMI 1640 (w L-Glutamine)	445 mL
	Heat-inactivated Fetal Bovine Serum	50 mL
	Antibiotic-Antimycotic (100x)	5 mL
Complete Eagle's Minimal Essential Medium (EMEM)	EMEM (w L-Glutamine)	450 mL
	Heat-inactivated Fetal Bovine Serum	50 mL
Infection Medium	EMEM (w L-Glutamine)	4 mL
	TPCK Trypsin (500 µg/mL)	8 µL
Magnetic-Activated Cell Sorting Buffer	1x PBS	498 mL
	0.5 M EDTA	2 mL
	BSA (Tissue Culture Grade)	2.5 g
Mitomycin C-containing Medium 3	Medium 3	10 mL
	Mitomycin C	500 µL

Comments

10 µL/mL

Only to be added right before use

Final TPCK Trypsin Concentration of 1 µg/mL

Mitomycin C (10 µg/mL)

Table 2

Cell surface marker	Fluorophore
Vδ1 T-cell receptor (TCR)	Fluorescein isothiocyanate (FITC)
Vδ2 TCR	Peridinin-cholorphyll-protein (PerCP)
CD3	V500
CD8	Allophycocyanin-Cyanine 7 dye (APC-Cy7)
CD14	Phycoerythrin (PE)-CF594
CD56	Phycoerythrin (PE)-Cyanine 7 (Cy7)
CD69	Brilliant Violet 421 (BV421)
CD83	Allophycocyanin (APC)
CD161	Brilliant Violet 605 (BV605)
Vα 7.2	Phycoerythrin (PE)
CD38	Brilliant UltraViolet 395 (BUV395)

Table 3

qPCR Reaction Mix	qPCR Master Mix	5 µL
	Nuclease-free Water	3 µL
	Forward Primer (1 mM)	0.5 µL
	Reverse Primer (1 mM)	0.5 µL
	cDNA (12.5 ng/µL)	1 µL
	Total Reaction Volume	10 µL
RT-PCR Reaction Mix	RT-PCR 5x Buffer	2.5 µL
	Random Primers (500 ng/µL)	0.2 µL
	RNase Inhibitor	0.625 µL
	dNTP Mix	2.5 µL
	Reverse Transcriptase	0.5 µL
	RNA (200 ng/µL)	1 µL
	Nuclease-Free Water	12.675 µL
	Total Reaction Volume	20 µL

Table of Materials

Click here to access/download;Table of Materials;1. JoVE_Materials Table.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description	Generic Name Used in Manuscript
0.5% Trypsin-EDTA	Gibco	15400-054		
0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0, RNase-free	Thermofisher	AM9260G		0.5M EDTA
1.5 mL SafeLock Tubes	Eppendorf	0030120086		1.5mL Centrifuge Tube
10 mL K3EDTA Vacutainer Tubes	BD	366643		10mL Blood Collection Tubes
10x dPBS	Gibco	14200-075		
10x PBS	Vivantis	PC0711		
12-well Plate	Corning	3513		
12-well Transwell Insert	Corning	3460		membrane insert
1x FACS Lysing Solution	BD	349202		
2.0 mL SafeLock Tubes	Eppendorf	0030120094		2 mL centrifuge tube
24-well Plate	Corning	3524		
24-well Transwell Insert	Corning	3470		
3% Acetic Acid with Methylene Blue	STEMCELL Technologies	07060		
3,3',5-triiodo-l-thyronine	Sigma	T-074		
37% Formaldehyde Solution w 15% Methanol as Stabilizer in H_2O	Sigma	533998		
5810R Centrifuge	Eppendorf	5811000320		
5 mL polypropylene tubes (flow tubes)	BD	352058		
70 µm Cell Strainer	Corning	431751		
A-4-62 Rotor	Eppendorf	5810709008		

Accutase	Gibco	A1110501	Cell Dissociation Reagent
Antibiotic-Antimycotic	Gibco	15240-062	
Avicel CL-611	FMC Biopolymer	NA	Liquid Overlay
Bio-Plex Manager 6.2 Standard Software	Bio-Rad Laboratories, Inc	171STND01	Multiplex Manager Software
Butterfly Needle 21 G	BD	367287	
Cholera Toxin	Sigma	C8052	
Crystal Violet	Merck	C6158	
Cytofix/Cytoperm Solution	BD	554722	Fixation and Permeabilization Solution
Dispase II	Sigma	D4693	Neutral Protease
DMEM/High Glucose	GE Healthcare Life Sciences	SH30243.01	
DMEM/Nutrient Mixture F-12	Gibco-Invitrogen	11320033	
dNTP Mix	Promega	U1515	dNTP Mix
EMEM (w L-Glutamine)	ATCC	30-2003	
EVOM voltohmmeter device	WPI, Sarasota, FL, USA	300523	
FACS Lysing Solution	BD	349202	1x Lysing Solution
Falcon tube 15 mL	CellStar	188271	15 mL tube
Falcon tube 50 mL	CellStar	227261	50 mL Tube
Fast Start Essential DNA Probes Master	Roche	6402682001	qPCR Master Mix

Ficoll Paque Premium	Research Instruments	17544203	Density Gradient Media
H3N2 (A/Aichi/2/1968)	ATCC	VR547	
H3N2 M1 Forward Primer Sequence	Sigma	5'- ATGGTTCTGGCCAGCACTAC-3'	
H3N2 M1 Reverse Primer Sequence	Sigma	5'- ATCTGCACCCCCATTCGTTT-3'	
H3N2 NS1 Forward Primer Sequence	Sigma	5'- ACCCGTGTTGGAAAGCAGAT-3'	
H3N2 NS1 Reverse Primer Sequence	Sigma	5'- CCTCTTCGGTGAAAGCCCTT-3'	
Heat Inactivated Fetal Bovine Serum	Gibco	10500-064	
hNESPCs	Human Donors	NA	
Human Epithelial Growth Factor	Gibco-Invitrogen	PHG0314	
Hydrocortisone	STEMCELL Techonologies	7925	Collected from nasal biopsies during septal deviation surgeries
Insulin	Sigma	I3536	
Lightcycler 96	Roche	5815916001	qPCR Instrument
Live/DEAD Blue Cell Stain Kit *for UV Excitation	Thermofisher	L23105	Viability Stain
MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel II - Premixed 23 Plex	Merck Pte Ltd	HCP2MAG-62K-PX23	Immunology Multiplex Assay
Mitomycin C	Sigma	M4287	
M-MLV 5x Buffer	Promega	M1705	RT-PCR 5x Buffer
M-MLV Reverse Transcriptase	Promega	M1706	Reverse Transcriptase
N-2 supplement	Gibco-Invitrogen	17502-048	

NIH/3T3	ATCC	CRL1658	
Perm/Wash Buffer	BD	554723	Permeabilization Wash Buffer
PneumaCult-ALI 10x Supplement	STEMCELL Techonologies	5001	
PneumaCult-ALI Basal Medium	STEMCELL Techonologies	5001	
PneumaCult-ALI Maintenance Supplement (100x)	STEMCELL Techonologies	5001	
Random Primers	Promega	C1181	Random Primers
Recombinant Rnasin Rnase Inhibitor	Promega	N2511	RNase Inhibitor
RNA Lysis Buffer	Qiagen	Part of 52904	
RPMI 1640 (w L-Glutamine)	ATCC	30-2001	
STX2 electrodes	WPI, Sarasota, FL, USA	STX2	Electrode
T25 Flask	Corning	430639	
T75 Flask	Corning	430641U	
TPCK Trypsin	Sigma	T1426	
Trypan Blue	Hyclone	SV30084.01	
Viral RNA Extraction Kit	Qiagen	52904	Viral RNA Extraction Kit
V-Shaped 96-well Plate	Corning	3894	

Dear Editors and Reviwers,

We thank the editors and reviewers for the constructive suggestion that helped improved the manuscript. We have made the requested changes as suggested by the editors and reviewers listed in the point by point rebuttal (in red) below.

Editorial Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

- We have proofread the document and added in the necessary information to strengthen the manuscript and have defined all terms that were used.

2. Please revise the following lines to avoid overlap with previously published work: 307-312

- We noted the overlap and have rephrase these 5 lines.

3. Please shorten your title to “Contact-free Co-culture Model for the Study of Innate Immune Cell Activation During Respiratory Virus Infection”

- We have changed the title to the one suggested.

4. Please provide an email address for each author.

- We have provided the email addresses as requested.

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.
For example: Accutase; STX2 “chopstick” electrode; Vacutainer tubes; Falcon tube; Eppendorf; BD Cytofix/Cytoperm; Luminex; Milliplex MAP kits …panel; Bio-Plex manager 6.0 software; Qiagen; Avicel overlay etc

- We have changed the writing to use generic terms to describe the materials used, and correspondingly reference them in the table of materials

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. 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. Please add more specific details (e.g., button clicks for software actions, numerical

values for settings, etc) 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.

- We have added in the relevant details and references which we believed have further strengthen the details of the protocol.

7. 4.2.1: How much blood is to be taken in the well?

- We apologize for the confusion. No blood is taken from the well. We have edited the sentence to eliminate confusion as the volume was mentioned in the step before.

8. 8.3: What were the qRT-PCR conditions? What did the reaction mixture contain? What were primer sequences? If this has been published, please cite that/those reference(s).

- We have included the details in the manuscript at the corresponding section.

9. Please remove the embedded figures from the manuscript. All figures should be uploaded separately to your Editorial Manager account. The legends should appear only in the Figure and Table Legends section after the Representative Results.

- We have relocated the necessary parts of the manuscript to their respective section.

10. Please remove the embedded Tables from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. The table legend or caption (title and description) should appear in the Figure and Table Legends section after the Representative Results in the manuscript text.

- We have relocated the necessary parts of the manuscript to their respective section.

11. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

- We have formatted the manuscript as per the guidelines provided.

12. In representative results, line 377, what is figure X?

- We apologize for the mistake. The figure is now appropriately referenced.

13. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol (what readers should pay attention to)
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

- We have rewritten the discussion to include the changes requested.

14. Please do not abbreviate journal names in the reference list.

- We have edited the references accordingly.

15. Please sort the Materials Table alphabetically by the name of the material.

- We have sorted the material name accordingly.

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes a protocol for coculture of virally infected nasal epithelial cells and PBMC to investigate the activation of "innate" immune responses. The protocol is of interest because the use of ALI culture protocols is quite tricky and very difficult to transfer between labs. In addition, this culture setup may be used to investigate different immune cell subsets innate as well as adaptive, which is indicated by the authors towards the end of the discussion section.

Major Concerns:

1. The main focus of the manuscript should be establishment of the coculture system wheraes PBMC isolation and FACS staining procedures could be reduced and replaced by propor references.

- We thank the reviewer for the comment. We have now added in more detail and note the steps to pay attention to in the coculture section, and shortened the other sections accordingly.

2. When going into details with the coculture part of the protocol several reagents and culture media should be specified more clearly.

- We apologize for the missing details. We have now added in all details accordingly in the respective sections

3. In addition, how to harvest cells and supernatants from inserts and transwells is confusing and it has to be explained what is added to which compartment and when and where the cells and supernatants are harvested - and also why medium is changed and how.

- We noted the missing details and have now specifically state the compartment of the transwells for each step, especially in the coculture sections. All the changes are marked with tracked changes

4. The concept of investigating innate T-cell activation could be introduced in more detail to make the readouts used in the result examples more relevant to the reader.

- We have now added additional information on innate T-cell and the significance of studying them in the third paragraph of the introduction section.

5. The heterogeneity of culture systems based on primary epithelial cells and PBMC is mentioned in the discussion, but some data on the variability and how this can be circumvented would be extremely helpful. Maybe epithelial cell lines could be used if the main purpose is to investigate how soluble factors influence immune cells in the PBMC.

- We thank the reviewer for the suggestion. We have added into the discussion the following to further circumvent such variability.

"While it is certainly possible to utilize epithelial cell lines, this would remove the complexity (mucus, interactions between different cell types etc.) of the interactions between the different cell types of the epithelial layer, which would be ideal for investigation of how the epithelial crosstalk influences the response of the PBMCs. We note that a primary cell line is necessary to mimic the physiology of the nasal tissues, where the cells are multi-layered and the different cell types are localized to their own individual niche though variability might be an issue. Variability in this respect can be overcome by utilizing single cell RNA sequencing to differentiate and separate the heterogenous population of cells."

Minor Concerns:
1. Many abbreviations are used without explanation, please include a list.

- We apologize for the mistake. We have now clearly written the complete name the first time the abbreviation is used. We have also added a list of abbreviations as table S1.

2. The question about leakyness of the culture system and the virus getting access to the PBMC should be explained in more detail and maybe some data on appropriate controls could be included.

- We have added in the discussion the following statement to explain the significance of leakiness and ways to circumvent them. In addition, we also added examples on virus suitable for the model and those that are not due to the nature of their bi-directional progeny release:

"However, when working with epithelial-immune cell cross talk in viral infection, it is critical that the viruses do not interact directly with the PBMCs in order to identify early local epithelial derived soluble factors released by the hNECs. Therefore, this model is more suitably built for examining viruses with polarized viral release, where viruses only bud out from the apical surface into the apical chamber, such as influenza viruses12 and SARS-CoV-2 virus18. In addition, to prevent leakage of apical release viruses into the basal chamber compromising the experiment, an intact epithelial layer of sufficient thickness is vital. Therefore it is important that the TEER measurement and the viral RNA quantification be performed to ensure that the results are free of viral leakages into the basal chamber. A TEER reading of >1000 implies an intact multilayer of cells suitable for viruses with polarized release; while the basal media should be free of any viral RNA contamination. On the other hand, the utility of the model to bidirectional release viruses such as Rhinoviruses remains to be explored16. Such viruses were not limited to releasing their progeny in a polarized manner and may bidirectionally release new viruses into both apical and basal regions of the epithelium. More optimization are required to be conducted before this model could be applied to viruses with non-polarized release."

3. The manuscript should be looked throug to ensure that terms and wording is used consistently to avoid confusion.

- We have further proofread the manuscript to improve the consistency.

Reviewer #2:
Major Concerns:

1. Describe how long does it take to obtain differentiated cultures. Should provide H and E section of the cultures to demonstrate the differentiation of the cultures.

- We have added in the protocol section the time to obtain differentiated culture. We have also added cross section of the transwells (Figure 4) to show how the differentiated culture look like.

2. The method says use medium 3 for culturing nasal epithelial cells, but does not give the composition or the source of the medium.

- We apologize for the missing details. The details have now been added into the manuscript in the materials table.

3. Presence of mucus interfere with TER measurement using chopstick electrode. The surface of the cultures should be washed with sodium bicarbonate in order to remove

mucus. Use of endohm instead of chopstix electrode will give a better estimation of TER. The nasal epithelial culture medium base is usually LHC and DMEM.

- We have added a note in the protocol section 2.4.3 where washing with sodium bicarbonate is added as an option depending on the nature of the experiment. However, for the case of viral infection, the mucus layer should be part of the physiological interaction with the incoming virus. Therefore, in our protocol we only state to wash off excess mucus/dead cells a week before TEER measurement and experiments begins. Additionally, we have also added endohm as an option for TEER measurement in section 3 of the protocol. However, as we do not require an accurate TEER measurement but a range to ensure cell layer is intact, the chopstick electrodes should suffice in our protocol. We have added the TEER range required for viral infection experiment after 3.1.9 and in the discussion of our manuscript.

4. How does this affect when changed to RPMI during co-culture?

- When hNESPCs have fully differentiated in hNECs, they are tolerant/permissive to different media for up to 72 hours. There are no changes to morphology, organization etc. when the media is switched to RPMI. We have added this as a note after 5.1.7 with an accompanying reference.