Author/s:
Loh, L;Koutsakos, M;Kedzierska, K;Hinks, TSC

Title:
Influenza A Virus-Infected Lung Epithelial Cell Co-Culture with Human Peripheral Blood Mononuclear Cells

Date:
2020-01-01

Citation:
Loh, L., Koutsakos, M., Kedzierska, K. & Hinks, T. S. C. (2020). Influenza A Virus-Infected Lung Epithelial Cell Co-Culture with Human Peripheral Blood Mononuclear Cells. MAIT CELLS: METHODS AND PROTOCOLS, 2098, pp.141-147. https://doi.org/10.1007/978-1-0716-0207-2_9.

Persistent Link:
https://hdl.handle.net/11343/274426

Chapter 9

Influenza A Virus-Infected Lung Epithelial Cell Co-Culture with Human Peripheral Blood Mononuclear Cells

Liyen Loh, Marios Koutsakos, Katherine Kedzierska, and Timothy S. C. Hinks

Abstract

Sensing of influenza A virus (IAV) infection by pattern recognition receptors can occur by either direct infection of lung epithelial cells or uptake of virus-infected cells by innate cells such as dendritic cells/monocytes. This triggers a series of downstream events including activation of the inflammasome, the production of cytokines, chemokines, and the upregulation of stress-induced ligands that can lead to the activation of innate cells. These cells include innate lymphocytes such as MAIT, NKT, NK, and γδ T cells. Here we describe a method used to allow activation of human innate lymphocytes in co-culture with an IAV-infected human lung epithelial cell line (A549) to measure ex vivo effector functions (TNF and IFNγ) in a mixed culture environment. We describe (1) infection of the human lung epithelial cell line, (2) co-culture with PBMC, and (3) measurement of activation using intracellular cytokine staining.

Key words Virus, MAIT cell, Flow cytometry, Tetramer, Infection, Human, Epithelial cell

1 Introduction

The innate immune response serves as the first line of defense during viral infections. Sensing of influenza A virus (IAV) infection by pattern recognition receptors (e.g., TLR and RIG-I) can occur by either direct infection of lung epithelial cells or uptake of virus-infected cells by innate cells such as dendritic cells/monocytes. This triggers a series of downstream events including activation of the inflammasome, the production of cytokines, chemokines, and the upregulation of stress-induced ligands that can lead to the activation of innate cells. These cells include innate lymphocytes such as MAIT, NKT, NK, and γδ T cells. These lymphocytes can be activated by non-classical MHC interactions, cytokine-mediated signals or both. This method allows for the activation of human innate lymphocytes in co-culture with IAV-infected human lung epithelial cells (A549) and is used to measure *ex vivo* effector functions (TNF and IFNγ) in a mixed culture environment [2]. The objective is to

Helen Kaipe and Isabelle Magalhaes (eds.), *MAIT Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 2098, https://doi.org/10.1007/978-1-0716-0207-2_9,

measure and recapitulate the events of early IAV infection in vitro, in a co-culture system with human peripheral blood mononuclear cells (PBMC) and IAV-infected human lung epithelial cells.

The method described in this chapter comprises three main steps: (1) infection of a human epithelial cell line, (2) co-culture with PBMC to activate the virus responsive cells, and (3) intracellular cytokine staining to measure the extent of functional activation.

2 Materials

2.1 Reagents and Buffers

1. Complete RPMI (cRPMI): Roswell Park Memorial Media, 10% heat-inactivated fetal calf serum (FCS), 100 U/mL Penicillin, 100 U/mL Streptomycin, and 100 μM MEM Vitamins.
2. Human lung epithelial cell line, A549 (ATCC, VA, USA).
3. PR8 virus (influenza A strain/H1N1/Puerto Rico/1934).
4. Trypsin Versene (In-house preparation).
5. Trypan Blue and Counting Chamber.
6. Brefeldin A—Golgi PLUG (BD, CA, USA).
7. Live/dead Fixable Aqua Dead Cell Stain Kit (ThermoFisher, MA, USA).
8. Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, USA).
9. 10% Lysol or 1% Virkon.
10. Fluorescence activated flow cytometry (FACS) buffer: phosphate-buffered saline (PBS), 2 mM EDTA, 0.5% bovine serum albumin (BSA). From a 500 mL bottle of PBS, add 40 mL to a 50 mL falcon containing 2.5 g BSA powder, vortex hard, then filter-sterilize back into PBS bottle using a syringe through a 0.22 um filter.
11. 1% paraformaldehyde (PFA) solution. Dilute 16% paraformaldehyde 1:16 with FACs buffer.
12. Antibodies for flow cytometry (Table 1).
13. Phosphate-buffered saline (PBS).

2.2 Plastic

1. T75 flasks (Corning, NY, USA).
2. 50 mL Flacon tube (Fischer Scientific, MA, USA).
3. 96-well U-bottom plate polystyrene (Greiner, Germany).

2.3 Equipment

1. Flow Cytometer, BD LSR FORTESSA, or equivalent.
2. Water Bath.

Table 1
Example flow cytometric activation panel and IAV nucleoprotein expression

Marker	Fluorophore	Laser	Clone	Dilution
Surface stain activation				
Live Dead	Aqua	Violet		1/800 (stain in PBS prior to surface stain)
MR1-5-OP-RU Tet	SA-BV421	Violet		∗Titrate 1/200–1/400
CD19	APC-H7	Red	HIB19	1/100
CD14	APC-H7	Red	MϕP9	1/100
CD8α	PerCP-Cy5.5	Blue	SK1	1/50
TCRγδ	FITC	Blue	2F11	1/50
CD4	BV650	Violet	OKT4	1/200
CD161	BV605	Violet	HP-3G10	1/50
CD3	PE-CF594	Yellow/Green	UCHT1	1/200
TCR Vα7.2	PE	Yellow/Green	3C10	1/200
CD56	PE-Cy7	Yellow/Green	NCAM16.2	1/100
Intracellular stain activation				
TNF	APC	Red	MAb11	1/50
IFNγ	AF700	Red	B27	1/150
Intracellular stain IAV-NP infection				
IAV nucleoprotein	FITC	Blue	1331	1/100

Typical flow cytometry panel compatible with a four-laser BD LSRII Fortessa flow cytometer, allowing identification of innate and adaptive lymphocyte subsets and assessment of activation measured by intracellular cytokine staining
*Batches of SA-conjugated and Tetramerized MR1-5-OP-RU will vary and require titration prior to usage

3 Methods

Personal protective equipment (PPE) should be worn at all times (gloves, lab coat, and eye protection) (*see* **Note 1**).

3.1 IAV Infection of Human Lung Epithelial Cell Line, A549

1. 24 h prior to infection, in two T75 flasks, seed 5×10^6 A549 cells in a total volume of 20 mL of media (one flask for IAV infection and the second flask for uninfected control A549s).
2. On the day of infection: leave one flask of A549 cells in the incubator (uninfected control). Wash the other flask with room

temperature PBS once, cap and gently rotate flask from side to side. Aspirate PBS with glass tissue culture pipette.

3. Thaw virus (PR8) [1] on ice and add 174 μL to 10 mL of room temperature PBS in a 50 mL falcon tube (depending on viral titer of stock) to achieve a multiplicity of infection (MOI) of ~10–30∗. Gently pipette this into the T75 containing A549 cells.

   ∗Example calculation of MO1 10:

   An MOI of 10 using 1×10^6 PBMC per well requires 1×10^7 virus particles/well. The volume required/well of a 1×10^9 plaque forming units (pfu)/mL virus titer is 1×10^7 pfu/1×10^9 pfu/mL = 0.01 mL/well or 10 μL/well.
4. Incubate flask horizontally for 1 h in the 37 °C incubator (5% CO_2).
5. Remove both T75 flasks from incubator and add 10 mL of cRPMI to the flask containing virus. Cap and gently rotate from side to side. Aspirate media from both flasks.
6. To detach A549 cells, wash flasks once with room temperature PBS, aspirate, and add 2.5 mL of Trypsin versene to each flask. Gently tilt the flask to ensure that the solution coats the entire flask.
7. Incubate for 5 min in the 37 °C incubator (5% CO_2).
8. Add 10 mL of cRPMI to T75 flasks and transfer the contents into two 50 mL falcon tubes. Centrifuge for 5 min at 500 × *g*, 25 °C. Aspirate supernatant.
9. Resuspend cells in 2 mL of cRPMI and perform cell counts using trypan blue estimation.
10. Adjust the volume of A549 cells so that the final concentration is 2×10^6 cells/mL.

3.2 Co-Culture (Start During the 1 h Incubation with Virus)

1. Thaw PBMCs in 37 °C water bath and gently pipette dropwise into 9 mL of pre-warmed cRPMI per cryovial and centrifuge at 500 × *g* for 5 min (*see* **Note 2**).
2. Aspirate media and count cells. Resuspend PBMCs at 10×10^6 cells/mL in cRPMI. For each sample aliquot 100 μL of cells (1×10^6 PBMC) into three wells of a 96-well U-bottom plate. These wells will correspond to Media Control, uninfected A549 + PBMC, and IAV-infected A549 + PBMC, respectively.

   To check IAV nucleoprotein levels, *see* **Note 3**. Add 100 μL of infected and uninfected A549 cells to separate wells in the 96-well plate.
3. Add 100 μL of uninfected A549s or IAV-infected A549s (2×10^5 cells) into wells containing PBMC. Leave one well

with PBMC only, add 100 uL of cRPMI to this well. Place this plate in the 37 °C incubator (5% CO_2).

4. After 3–4 h, add brefeldin A (BFA-GOLGI PLUG), 1:2000 to all wells and incubate for a further 6 h in the 37 °C incubator (total co-culture 10 h).
5. Remove plate and continue with intracellular cytokine (ICS) staining or place in the 4 °C covered in foil to stain the next day.

3.3 Intracellular Cytokine Staining

1. Spin down plate by centrifuging at 400 × *g* for 5 min at 4 °C. Discard supernatant in waste container containing 10% Lysol or 1% Virkon in class II biosafety cabinet.
2. Stain cells with live/dead discrimination marker Aqua (1:800) final volume of 50 μL/well. Use PBS as a diluent (*see* **Note 4**). Incubate at room temperature in the dark for 15 min.
3. Centrifuge plate at 400 × *g* for 5 min at 4 °C. Discard supernatant.
4. Add 50 μL of surface phenotype stain (Table 1) to each well. Incubate for 30 min on ice, in the dark.
5. Wash cells once with 150 μL of FACs buffer. Centrifuge for 5 min at 1500 rpm, 4 °C. Flick off supernatant in discard container in biohazard cabinet.
6. Resuspend the cells in 100 μL of cold cytofix/perm solution and incubate on ice in the dark for 20 min.
7. Wash cells with 100 μL of diluted (1:10 in dH_2O) perm/wash buffer. Centrifuge for 5 min at 450 × *g*, 4 °C.
8. Resuspend cells in 50 μL of intracellular cytokine stain, see (Table 1) below. Incubate on ice in the dark for 30 min.
9. Wash cells with 150 μL of perm/wash buffer. Centrifuge for 5 min at 450 × *g*, 4 °C.
10. Repeat with a second wash with 200 μL of FACs buffer. Centrifuge for 5 min at 450 × *g*, 4 °C.
11. Resuspend cells in 100 μL of 1% PFA and transfer to bullet tubes. Keep samples in the dark and at cold until acquisition on the flow cytometer. For suggested flow cytometric gating strategy *see* Fig. 1.

4 Notes

1. Biological Hazards—Human PBMC samples are classified as non-infectious. Influenza A virus—PR8-strain (H1N1) is a lab-adapted strain of IAV virus. Work should be risk assessed, and we recommend controls which include but are not restricted to the following: Lab coat, safety glasses, and gloves

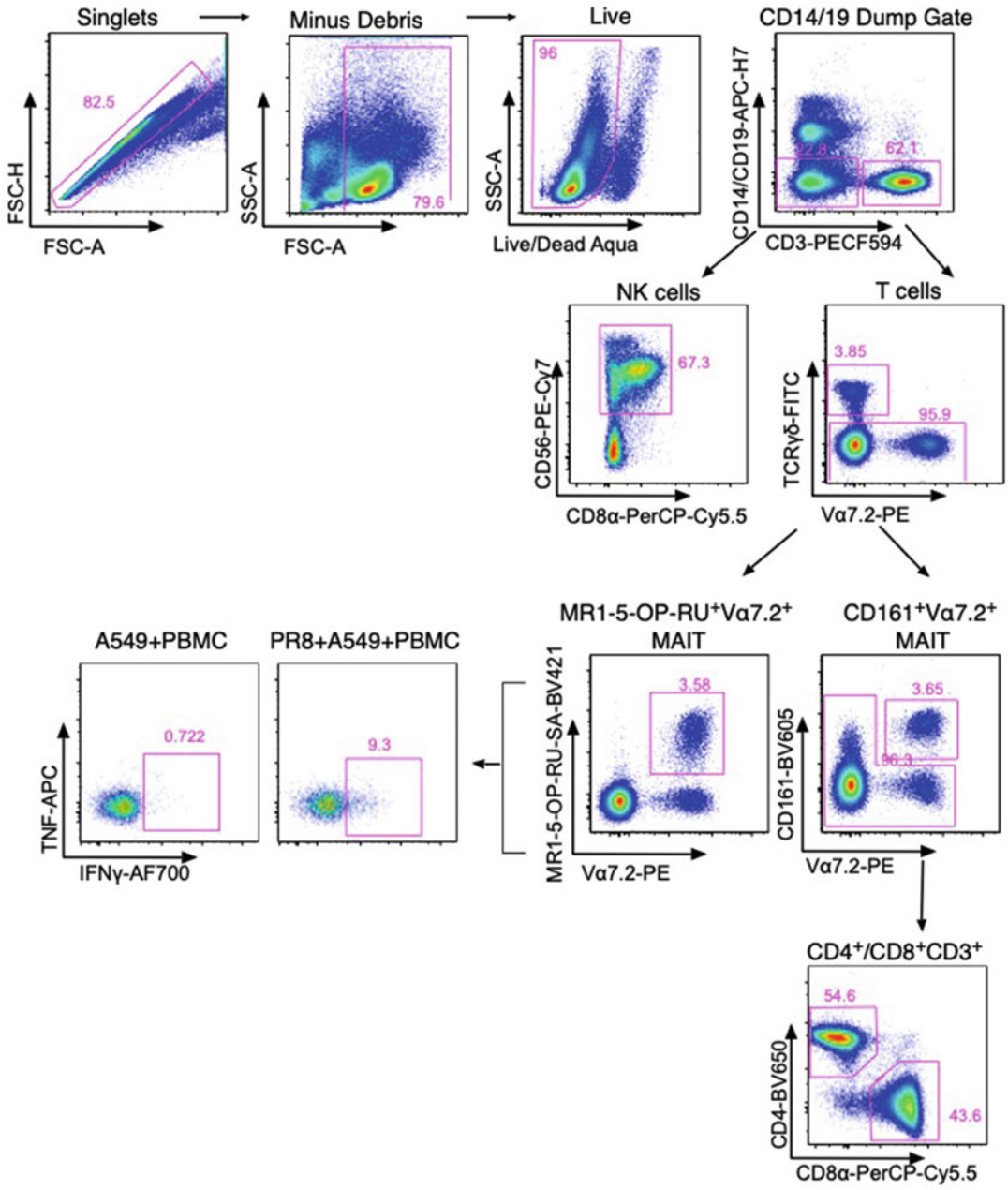

Fig. 1 Flow cytometry gating strategy for MAIT cells and other lymphocyte subsets

should be worn when performing this protocol. Work with human PBMCs and virus in a Class II biohazard cabinet. Use filter tips when working with virus. Decontaminate all pipette tips that have been used for human and virus work in 10% lysol or 1% Virkon when working in the biohazard cabinet. After use, the biohazard hood should be decontaminated by wiping down with 70% ethanol and by UV sterilization for 15 min

before any further use. All waste and its container must be disposed as hazardous waste.

2. MAIT cell responses after in vitro influenza co-culture are highly variable between donors. Freshly processed PBMCs may aid in the detection of IFNγ cytokine responses after influenza co-culture.
3. To determine if influenza virus infection of lung epithelial cells is successful after 10 h of culture, intracellular cytokine staining for influenza A virus nucleoprotein is determined by flow cytometry. Follow **steps 1–3** and **6–11** of Subheading 3.3 Intracellular cytokine staining.
4. Fixable viability dyes react with exposed amine groups within permeable cells. Therefore, to prevent wasteful reaction with proteins in cytometry buffers, it is recommended to resuspend cells in protein-free media for the viability staining step.

Acknowledgments

This work was funded by grants to TSCH from the Wellcome Trust (104553/z/14/z, 211050/Z/18/z) and K.K from the National Health and Medical Research Council (NHMRC) Program Grant (1071916). K.K. is an NHMRC Senior Research Fellow (1102792). The content represents only the authors' views and not those of the European Commission.

References

1. Koutsakos M, Illing PT, Nguyen THO et al (2019) Human CD8(+) T cell cross-reactivity across influenza A, B and C viruses. Nat Immunol
2. Loh L, Wang Z, Sant S et al (2016) Human mucosal-associated invariant T cells contribute to antiviral influenza immunity via IL-18-dependent activation. Proc Natl Acad Sci U S A 113:10,133–10,138