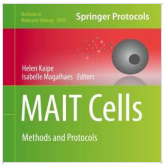

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Influenza A Virus-Infected Lung Epithelial Cell Co-Culture with Human Peripheral Blood Mononuclear Cells

Book Chapter

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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.

ATTACHMENTS

Loh2020_Protocol_InfluenzaAVirus-InfectedLungEp.pdf

DOI

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EXTERNAL LINK

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KEYWORDS

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

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GUIDELINES

Please read the full protocol before starting any steps.

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 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.

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

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MATERIALS TEXT

Citation: Liyen Loh, Marios Koutsakos, Katherine Kedzierska, Timothy S. C. Hinks (09/02/2021). Influenza A Virus-Infected Lung Epithelial Cell Co-Culture with Human Peripheral Blood Mononuclear Cells. https://dx.doi.org/10.17504/protocols.io.bmenk3de

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, shown below).
13. Phosphate-buffered saline (PBS).

Plastic:

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

Equipment:

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

Table 1 (Antibodies):

Example flow cytometric activation panel and IAV nucleoprotein expression

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Marker	Fluorophore	Laser	Clone	Dilution
Surface stain activation				
Live Dead	Aqua	Violet		1/800 (stain in PBS prior to surface stain)
MRI-5-OP-RU Tet	SA-BV421	Violet		*Titrate 1/200-1/400
CD19	APC-H7	Red	H1B19	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	1/100
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

SAFETY WARNINGS

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

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE STARTING

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 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.

IAV Infection of Human Lung Epithelial Cell Line, A549

1 **24:00:00** prior to infection, in two T75 flasks, seed 5 x 10^6 A549 cells in a total volume of **20 mL media** .

One flask for IAV infection and the second flask for uninfected control A549s.

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2 On the day of infection:

2.1

Leave one flask of A549 cells in the incubator (uninfected control).

2.2

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) **On ice** and add **174 µl** **virus** to **10 mL** **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 MOI 10:
An MOI of 10 using $1x10^6$ PBMC per well requires $1x10^7$ virus particles/well.
The volume required/well of a $1x10^9$ plaque forming units (pfu)/mL virus titer is:
$1x10^7$ pfu/$1x10^9$ pfu/mL = 0.01 mL/well or 10 µL/well.

4

Incubate flask horizontally for **01:00:00** in the **37 °C** incubator (5% CO_2).

5

Remove both T75 flasks from incubator and add **10 mL** **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** **Trypsin versene** to each flask. Gently tilt the flask to ensure that the solution coats the entire flask.

7

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Incubate for **00:05:00** in the **37 °C** incubator (5% CO_2).

8 Add **10 mL cRPMI** to T75 flasks and transfer the contents into two 50 mL falcon tubes.

9 

Centrifuge **500 x g, 25°C, 00:05:00** .

10 Aspirate supernatant.

11 Resuspend cells in **2 mL cRPMI** and perform cell counts using trypan blue estimation.

12 Adjust the volume of A549 cells so that the final concentration is $2x10^6$ cells/mL.

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

13 Thaw PBMCs in **37 °C water bath** .

14 

Gently pipette dropwise into **9 mL pre-warmed cRPMI** per cryovial.

15 

Centrifuge **500 x g, 00:05:00** .

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.

16 

Aspirate media and count cells.

17 Resuspend PBMCs at $10x10^6$ cells/mL in cRPMI.

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18 For each sample, aliquot **100 µl cells (1x10^6 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.

19

Check IAV nucleoprotein levels:

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. (Steps 25-27 followed by steps 31-38, shown here in subsequent substeps for convenience)

19.1

Spin down plate by centrifuging at **400 x g, 4°C, 00:05:00** . Discard supernatant in waste container containing 10% Lysol or 1% Virkon in class II biosafety cabinet.

19.2 15m

Stain cells with live/dead discrimination marker Aqua (1:800) final volume of 50 µL/well. Use PBS as a diluent. Incubate at **Room temperature** in the dark for **00:15:00** .

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.

19.3

Centrifuge plate at **400 x g, 4°C, 00:05:00** . Discard supernatant.

19.4 20m

Resuspend the cells in **100 µl cold cytofix/perm solution** and incubate **On ice** in the dark for **00:20:00** .

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19.5

Wash cells with **100 µl diluted (1:10 in dH2O) perm/wash buffer** . Centrifuge for **450 x g, 4°C, 00:05:00** .

19.6

Resuspend cells in **50 µl intracellular cytokine stain** , see (Table 1) below.

Table 1 (Antibodies):
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)
MRI-5-OP-RU Tet	SA-BV421	Violet		*Titrate 1/200-1/400
CD19	APC-H7	Red	H1B19	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	1/100
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

19.7

30m

Incubate **On ice** in the dark for **00:30:00** .

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19.8

Wash cells with **150 µl perm/wash buffer** . Centrifuge for **450 x g, 4°C, 00:05:00** .

19.9

Repeat with a second wash with **200 µl FACs buffer** . Centrifuge for **450 x g, 4°C, 00:05:00** .

19.10 Resuspend cells in **100 µl 1% PFA** and transfer to bullet tubes.

Keep samples in the dark and at cold until acquisition on the flow cytometer.

19.11 For suggested flow cytometric gating strategy see Fig. 1. below:

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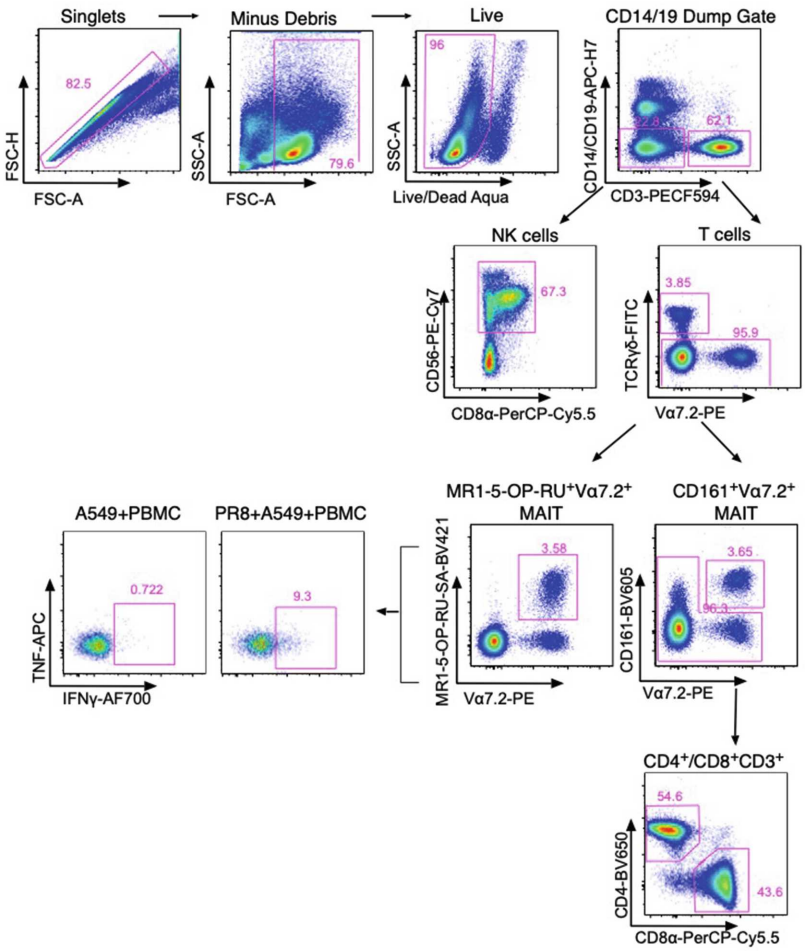

Fig. 1
Flow cytometry gating strategy for MAIT cells and other lymphocyte subsets

20 Add **100 µl** of infected and uninfected A549 cells to separate wells in the 96-well plate.

21 Add **100 µl** of uninfected A549s or IAV-infected A549s ($2x10^5$ cells) into wells containing PBMC. Leave one well with PBMC only, add **100 µl cRPMI** to this well. Place plate in the **37 °C** incubator (5% CO_2).

22

After **03:00:00** – **04:00:00** , add brefeldin A (BFA-GOLGI PLUG), 1:2000 to all wells.

23

Incubate for a further **06:00:00** in the **37 °C** incubator (**10:00:00 total co-culture**).

24

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Remove plate and continue with intracellular cytokine (ICS) staining or place in the **4 °C** covered in foil to stain the next day.

Intracellular Cytokine Staining 1h 35m

25

Spin down plate by centrifuging at **400 x g, 4°C, 00:05:00** . Discard supernatant in waste container containing 10% Lysol or 1% Virkon in class II biosafety cabinet.

26 15m

Stain cells with live/dead discrimination marker Aqua (1:800) final volume of 50 µL/well. Use PBS as a diluent. Incubate at **Room temperature** in the dark for **00:15:00** .

> 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.

27

Centrifuge plate at **400 x g, 4°C, 00:05:00** . Discard supernatant.

28

Add **50 µl surface phenotype stain** (Table 1 below) to each well.

Table 1 (Antibodies):
Example flow cytometric activation panel and IAV nucleoprotein expression

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Marker	Fluorophore	Laser	Clone	Dilution
Surface stain activation				
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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

29 30m

Incubate for **00:30:00** **On ice** , in the dark.

30

Wash cells once with **150 µl FACs buffer** . Centrifuge for **1500 rpm, 4°C, 00:05:00** . Flick off supernatant in discard container in biohazard cabinet.

31 20m

Resuspend the cells in **100 µl cold cytofix/perm solution** and incubate **On ice** in the dark for **00:20:00** .

32

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Wash cells with **100 µl diluted (1:10 in dH2O) perm/wash buffer** . Centrifuge for **450 x g, 4°C, 00:05:00** .

33

Resuspend cells in **50 µl intracellular cytokine stain** , see (Table 1) below.

Table 1 (Antibodies):
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)
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IFNγ	AF700	Red	B27	1/150
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IAV nucleoprotein	FITC	Blue	1331	1/100

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*Batches of SA-conjugated and Tetramerized MR1-5-OP-RU will vary and require titration prior to usage

34 30m

Incubate **On ice** in the dark for **00:30:00** .

35

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Wash cells with **150 µl perm/wash buffer** . Centrifuge for **450 x g, 4°C, 00:05:00** .

36

Repeat with a second wash with **200 µl FACs buffer** . Centrifuge for **450 x g, 4°C, 00:05:00** .

37 Resuspend cells in **100 µl 1% PFA** and transfer to bullet tubes.

Keep samples in the dark and at cold until acquisition on the flow cytometer.

38 For suggested flow cytometric gating strategy see Fig. 1. below:

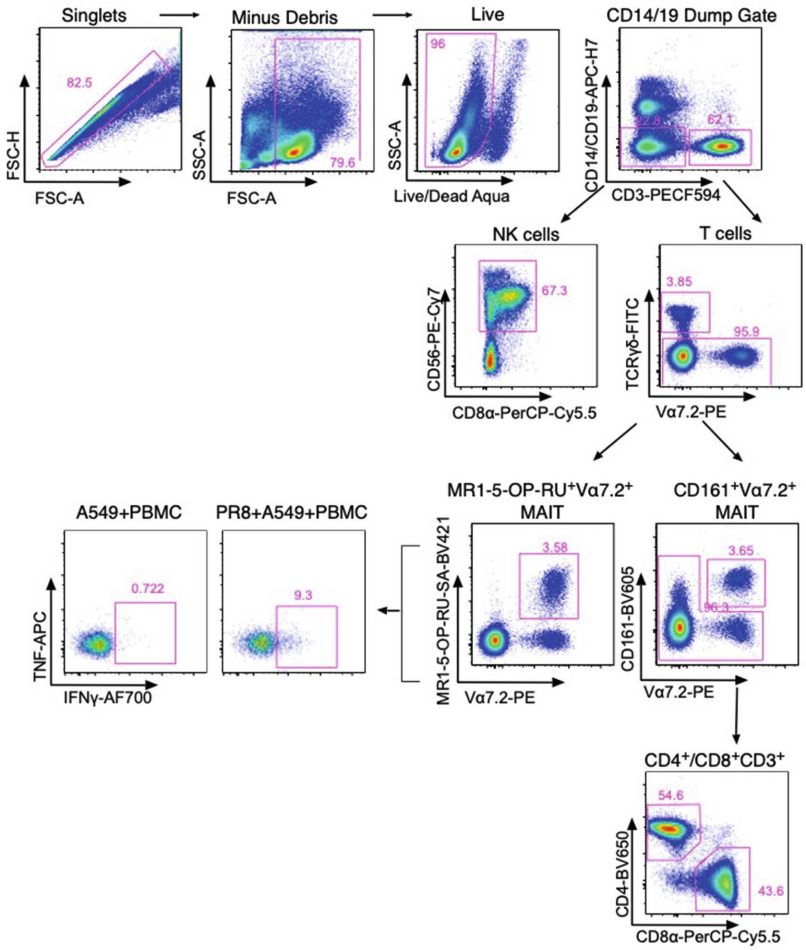

Fig. 1
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