Chapter 15

Evaluation of Activation and Inflammatory Activity of Myeloid Cells During Pathogenic Human Coronavirus Infection

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Abstract

Innate immune cells play a vital role in mounting an effective host response to a variety of pathogen challenges. Myeloid cells such as neutrophils and monocyte-macrophages are major innate leukocytes that orchestrate protective immunity to viral lung infections. However, a dysregulated cytokine response can promote excessive infiltration and robust pro-inflammatory activity of neutrophils and monocyte-macrophages, leading to fatal disease. Following virus infection, the beneficial or deleterious role of infiltrating neutrophils and monocyte-macrophages is determined largely by their ability to secrete inflammatory cytokines and chemokines. A majority of studies use the total number of infiltrating cells and their activation status as measures to demonstrate their role during an infection. Consequently, the ability of neutrophils and Inflammatory Monocyte Macrophages (IMMs) to secrete inflammatory cytokines and chemokines, and its correlation with the disease severity, is not well defined. In this chapter, we report useful markers to identify lung infiltrating innate immune cells and define their activation status. We also describe a simple method to measure intracellular cytokine production to evaluate the inflammatory activity of neutrophils and IMMs in a mouse model of human coronavirus infection.

Key words Coronavirus, Neutrophils, Inflammatory monocyte-macrophages, Lungs, Cytokines and chemokines

1 Introduction

Myeloid cells such as neutrophils and monocyte-macrophages are key immune cells that make up a large proportion of tissue infiltrating innate leukocytes following a pathogen challenge. Both neutrophils and inflammatory monocytes-macrophages (IMMs) are rapidly recruited to the site of infection and play crucial roles in the host defense against viral lung infections [1, 2]. The antiviral functions of neutrophils and monocyte-macrophages are facilitated following the recognition of pathogen-associated molecular patterns (PAMPs) by the cell surface and endosomal toll-like receptors (TLRs) and intracellular RIG-I like (RLRs) and Nod-like receptors

Rahul Vijay (ed.), *MERS Coronavirus: Methods and Protocols*, Methods in Molecular Biology, vol. 2099,
https://doi.org/10.1007/978-1-0716-0211-9_15,

(NLRs). Detection of viral PAMPs (viral proteins and nucleic acids) by these sensors leads to the activation of a cascade of signaling events resulting in the production of antiviral molecules like interferons (IFNs), interferon-stimulated genes (ISGs), and inflammatory cytokines and chemokines [3–5]. IMMs and neutrophils also participate in the phagocytosis of virus-infected cells and orchestrate effective adaptive T cell responses, both of which are essential for effective virus clearance [6].

In addition to host protective function of myeloid cells during viral lung infections, several recent studies demonstrate their role in mediating cytokine storm and thus exacerbating the host immune response to virus infections [2, 7]. The deleterious functions of neutrophils and IMMs are linked to dysregulated type I IFN (IFN-I) responses, particularly during high pathogenic virus infections [8, 9]. For example, while a controlled neutrophil response is protective during influenza A virus infection, an excessive neutrophil accumulation is detrimental [10, 11]. Similarly, an exaggerated monocyte-macrophage response resulting from delayed IFN-I signaling is detrimental during human coronavirus infections [8]. IMMs and neutrophils also express increased levels of death receptors such as DR5 and FAS, and the interaction of these receptors with their ligands TRAIL and FASL, respectively, promotes airway epithelial and lung microvascular endothelial cell death [9, 12, 13]. Additionally, excessive inflammatory cytokines and chemokines produced by IMMs and neutrophils impair antiviral T cell responses, leading to ineffective virus clearance and reduced survival [8].

A majority of the studies demonstrating the beneficial or detrimental effects of neutrophils and IMMs during viral lung infections enumerate percentages and total number and define activation status of the lung infiltrating myeloid cells using surface markers [14]. We recently showed spontaneous production of several inflammatory cytokines and chemokines by neutrophils and IMMs, which correlated with severe lung pathology and reduced survival in CoV infections [8]. Thus, the identification of specific inflammatory cytokines and chemokines produced by these cells will allow us to define their pro-inflammatory status and design strategies to control inflammatory responses. In this study, we describe useful markers to identify innate immune cells infiltrating into the lung and describe a simple method to evaluate inflammatory cytokine and chemokine production by neutrophils and monocyte-macrophages during pathogenic human coronavirus infections.

2 Materials

2.1 Infection

1. Sterile, endotoxin-free, pharmaceutical grade physiological saline, 1× phosphate-buffered saline, or 1× Dulbecco's Modified Minimum Essential Medium.
2. Isoflurane, isoflurane vaporizer and induction chamber or cocktail of xylazine (12.5 mg/kg) + ketamine (85 mg/kg).
3. 1 mL syringe for anesthetic administration.
4. Sterile 200 μL pipette tips and single channel 200 μL pipette.
5. Human coronavirus aliquots.

2.2 Harvesting Lungs

1. Anesthetic—ketamine (85 mg/kg) + xylazine (12.5 mg/kg).
2. 1 mL syringe for anesthetic administration and 10 mL syringe for lung perfusion.
3. Surgical equipment (scissors, forceps, 22G 5/7 needles).
4. 1× PBS or pharmaceutical grade physiological saline and 22G 5/7 needles
5. Square (6–12 inch) styrofoam or cardboard, absorbent pads.

2.3 Digestion and Processing of Lung Tissue

1. Scissors and forceps.
2. A 12-well plate for mincing lung tissue.
3. Lung digestion DNAse I/Collagenase D buffer.
4. 15 mL and 50 mL conical tubes.
5. Tube rotator.
6. Six-well plates for homogenizing lung tissue.
7. 3 mL syringe plunger, plastic Pasteur pipettes (3 mL).
8. RPMI 10% FBS media.
9. Benchtop lab centrifuge with rotors and cups to hold 15 mL or 50 mL conical.

2.4 Incubation and/or Stimulation of Lung Cells for Intracellular Cytokine Staining

1. 96-well plates, 200 μL multichannel pipette, 200 μL single channel pipette
2. RPMI 10% FBS, and Golgi-plug.
3. TLR ligands: Poly I:C, LPS, and R837.

2.5 FACS Staining and Acquisition

1. 96-Well plates.
2. FACS buffer (PBS+ 2–5% FBS+0.01% sodium azide).
3. Cytofix/Cytoperm buffer.
4. Perm/Wash Buffer.
5. Antibodies: anti-mouse CD45 PE-Cy7 (Clone: 30-F11), anti-mouse CD11b e450 Cat (Clone: M1/70), anti-mouse CD11c

PE (Clone: N418), anti-mouse IA/IE PerCp-Cy5.5 (clone: M5/114.15.2), anti-mouse Ly6C percp-cy5.5 (Clone: HK1.4), anti-mouse Ly6G FITC (Clone:1A8,), anti-mouse TNF APC (Clone: MP6-XT22), anti-mouse IL-6 APC (Clone:MP5-20F3), anti-mouse iNOS APC (Clone: CXNFT), anti-mouse IL-1β APC (Clone: NJTEN3), anti-mouse CD80 APC (Clone: 16-10A1), anti-mouse CD86 APC (Clone:GL-1\), anti-mouse CD69 APC (Clone: H1.2F3), anti-mouse PDCA-1 PE/APC (Clone:JF05-1C2.4.1), and anti-mouse CD16/32 (clone: 2.4G2).

6. Flow Cytometer (capable of detecting six or more fluorophores).

3 Methods

3.1 Mice Infection

1. Thaw a virus aliquot on ice just before infection, avoiding repeated freeze-thaw.
2. Dilute MERS-CoV and SARS-CoV in DMEM to achieve the required dose and keep virus on ice throughout the infection time (*see* **Note 1**).
3. Under xylazine/ketamine anesthesia (confirmed by pedal reflex), slowly deliver 40–50 μL of a well-mixed virus inoculum directly into the nostrils using a 200 μL pipette (*see* **Note 2**).
4. Following virus delivery, mouse should be placed on its dorsal side in a cage with bedding for the remaining virus inoculum to be inhaled.
5. Monitor mice every 10 min until complete recovery.

3.2 Harvesting and Digestion of Lungs

1. On days 4–5 post-infection, under complete xylazine/ketamine or isoflurane anesthesia (confirmed by pedal reflex), cut open the abdominal cavity to expose the diaphragm. Make an incision through the diaphragm with scissors, remove diaphragm, and then remove the rib cage to completely expose the heart and lungs (*see* **Note 3**).
2. Fill a 10 mL syringe with ice-cold sterile PBS and attach a 25 G × 5/8 needle. Insert the needle into the right ventricle of the heart and slowly inject 5 mL of DPBS into the heart. In the meantime, use forceps to break the left atria to allow blood to drain from circulation. Inject remaining 5 mL of DPBS until lungs turn pale.
3. Separate the heart and then remove the lung from the thoracic cavity. Separate and discard any remaining connective tissue associated with the lungs (*see* **Note 4**).
4. Place the lungs into the well of a 12-well tissue culture plate filled with 2.5 mL of DPBS on ice.

5. Rinse the lungs with DPBS and transfer it into another well without DPBS. Mince the lungs into very fine pieces using scissors.
6. Transfer minced lungs with a 2.5 mL plastic transfer pipette to a 15 mL conical tube containing 5 mL of digestion buffer.
7. Place tubes on a rocker and gently rock at room temperature for 30 min (*see* **Note 5**).
8. Place a 70 μm cell strainer in a 60 × 15 mm tissue culture dish or in a well of a 6-well plate.
9. Transfer lung tissue in digestion buffer on to the cell strainer using a 2.5 mL transfer pipette. Gently press and dissociate tissue through a strainer with the flat end of a 3 mL syringe plunger. Process tissues until there is only connective tissue remaining on the strainer and rinse the strainer with complete RPMI 1640 medium.
10. Spin down lung cells in 15 mL conical tube for 5 min at 300 × *g* at 4 °C in a bucket tabletop centrifuge.
11. Discard off supernatant and resuspend the cells in 1 mL of ACK buffer for 1 min to lyse the remaining red blood cells. Neutralize the ACK buffer with 10 mL of ice-cold DPBS or 10 mL of 5% RPMI medium.
12. Spin down the cells for 5 min at 300 × *g* at 4 °C and resuspend the cells in 5 mL of ice-cold buffer.

3.3 Cell Surface Staining for Innate Immune Cells (See Note 6)

1. Spin down lung cells for 5 min at 300 × *g* at 4 °C.
2. Dilute 0.2 μg of CD16/32 antibodies in 100 μL FACS buffer and resuspend cells in FACS buffer containing CD16/32 antibodies in a 96-well plate.
3. Gently mix the cells and antibodies.
4. Incubate the cells in the dark for 15 min at 4 °C.
5. Wash the cells twice with 150 μL of FACS buffer at 300 × *g* for 5 min at 4 °C.
6. Resuspend the cells in 100 μL FACS buffer containing the antibody cocktail.
7. Incubate the cells in the dark for 20 min at 4 °C.
8. Spin down lung cells for 5 min at 300 × *g* at 4 °C and discard the FACS buffer.
9. Wash the cells twice with 150 μL of FACS buffer at 300 × *g* for 5 min at 4 °C.
10. Resuspend the cells in 200 μL of FACS buffer and acquire using a flow cytometer.
11. Figure 1 provides an example of IMM activation marker expression in CoV infected lungs.

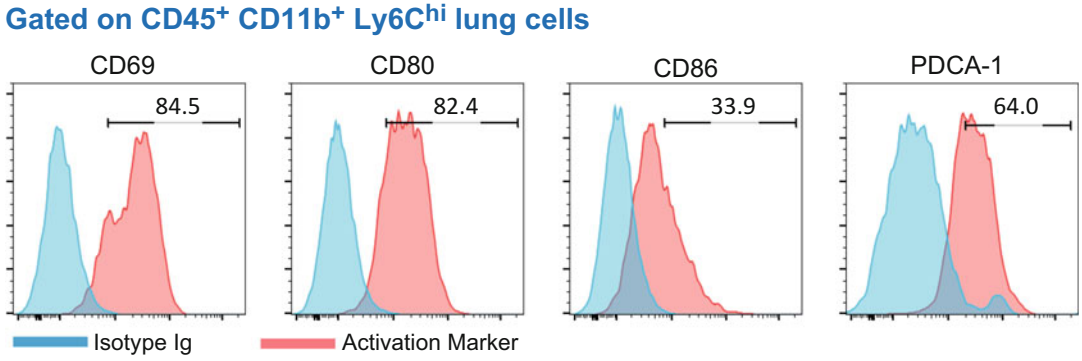

Fig. 1 Ideal markers to determine activation status of IMMs. Lung cells harvested from SARS-CoV-infected BALB/c mice (3 dpi) were surface stained for IMMs and activation markers as described in the methods section

3.4 Incubation and or Stimulation of Lung Cells for Intracellular Cytokine Staining (See Note 7)

1. Count live cells using a hemocytometer by staining with trypan blue.
2. Spin down lung cells for 5 min at 300 × *g* at 4 °C and resuspend the cells in RPMI 10% FBS at one million cells per 100 μL/well.
3. Dispense 100 μL of cells into a well of 96-well plates and add additional 100 μL RPMI 10% FBS media with or without Golgi-plug (1 μg/mL) with or without a TLR agonist (LPS 10–100 ng/mL, R837 and Poly I:C 100 ng to 1 μg/mL).
4. Incubate cells for 6–7 h at 37 °C in CO_2 incubator.
5. After incubation, wash cells twice with RPMI 10% FBS media.

3.5 Cell Surface and Intracellular Cytokine Staining for FACS (See Note 8)

1. Spin down lung cells for 5 min at 300 × *g* at 4 °C.
2. Dilute 0.2 μg of CD16 antibodies in 100 μL FACS buffer and resuspend cells in FACS buffer containing CD16/32 antibodies in a 96-well plate.
3. Gently mix the cells and antibodies.
4. Incubate the cells in the dark for 15 min at 4 °C.
5. Wash the cells twice with 150 μL of FACS buffer at 300 × *g* for 5 min at 4 °C.
6. Resuspend the cells in 100 μL FACS buffer containing 0.25 μg of cell surface identification and activation marker antibodies (*see* Table 1).
7. Incubate the cells in the dark for 20 min at 4 °C.
8. Spin down lung cells for 5 min at 300 × *g* at 4 °C and discard the FACS buffer.
9. Wash the cells twice with 150 μL of FACS buffer at 300 × *g* for 5 min at 4 °C.

Table 1
Cell surface markers to identify lung resident and lung infiltrating innate immune cells

No.	Innate immune cell	FACS markers
1	Alveolar macrophage	$CD45^+CD11c^+SiglecF^+$or $CD45^+CD11c^+F4/80^+$
2	Neutrophils	$CD45^+Ly6C^{int}Ly6G^+$or $CD45^+CD11b^+Gr1^+$
3	Monocytes	$CD45^+CD11b^+Ly6C^{hi}CCR2^+$
4	Macrophages	$CD45^+CD11b^+F4/80^+$
5	Dendritic cells	$CD45^+CD11b^-CD11c^+$MHC-II^+
6	Natural killer cells	$CD45^+CD3^-NKP46^+$/$CD45^+CD3^-$NK1.1 (B6)/$CD45^+CD3^-DX5^+$(BALB/c)
7	Eosinophils	$CD45^+CD11b^+CD11c^-SiglecF^+$

10. Add 100 μL of Cytofix/Cytoperm buffer and incubate cells in the dark for 25 min at 4 °C.
11. Add another 100 μL of 1× perm buffer (diluted to 1× with ddH_2O), spin down the cells (400 × *g* for 5 min at 4 °C), and discard the buffer.
12. Add 100 μL of 1× perm buffer containing anti-cytokine antibodies. See Table 1 for markers and concentrations.
13. Incubate the cells in the dark for 25–30 min at 4 °C.
14. After incubation add another 100 μL of 1× perm buffer (diluted to 1× with ddH_2O), spin down (400 × *g* for 5 min at 4 °C) the cells, and discard the buffer.
15. Wash the cells twice with 200 μL of 1× perm buffer at 400 × *g* for 5 min at 4 °C.
16. Wash the cells once with 200 μL of FACS buffer at 300 × *g* for 5 min at 4 °C (*see* **Note 9**).
17. Resuspend cells in 200 μL of FACS buffer for FACS acquisition.
18. Acquire FACS data using a flow cytometer and analyze data using FlowJo software (*see* **Note 10**).
19. Figure 2 demonstrates intracellular inflammatory cytokine production by lung IMMs on day 1 and 3 post-SARS-CoV infection.
20. Figure 3 demonstrates intracellular cytokine production by IMMs and neutrophils following brief TLR stimulation.

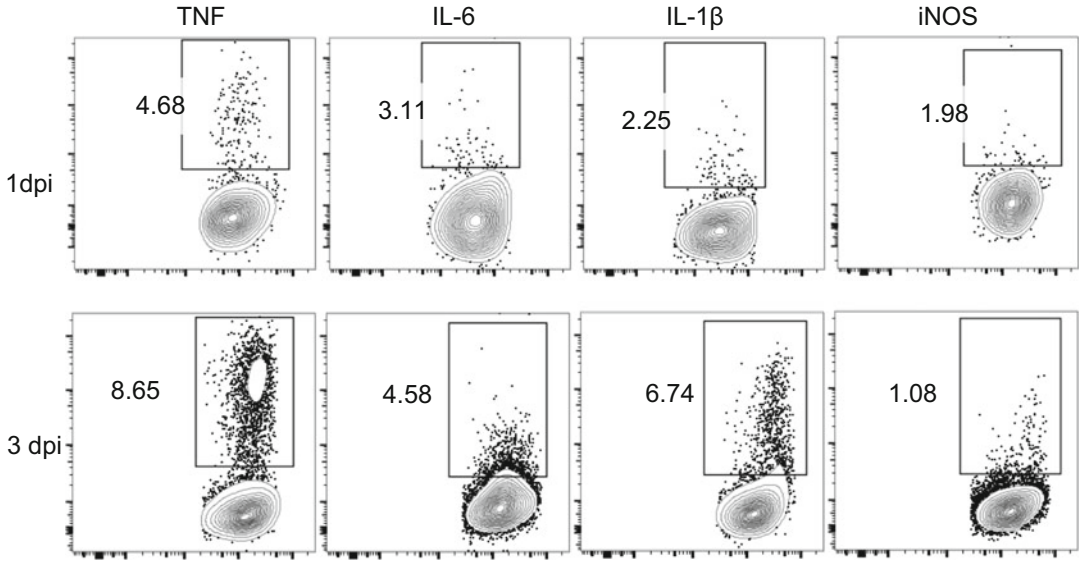

Fig. 2 Spontaneous cytokine production by IMMs. Lung cells isolated from SARS-CoV-infected BALB/c mice (1–3 dpi) were incubated for 7-h in the presence of Golgi-plug. Cells were then surface stained for IMMs ($CD45^{+}CD11b^{+}Ly6C^{hi}$) and then for intracellular cytokines TNF, IL-6, IL-1β, and iNOS

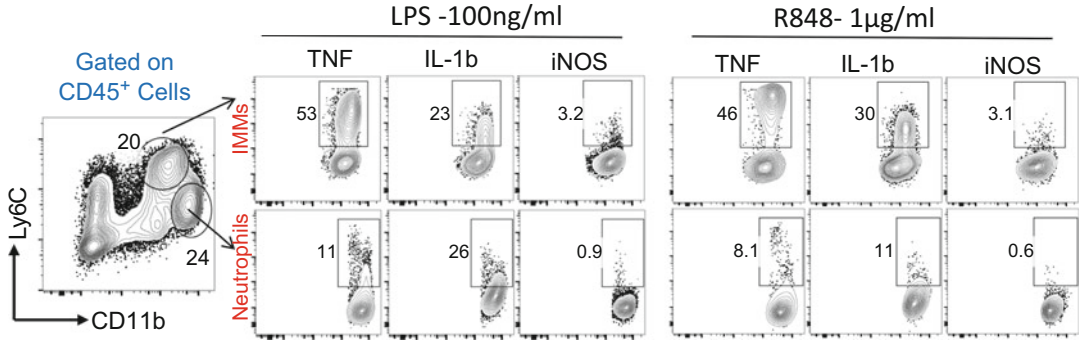

Fig. 3 Staining for intracellular cytokines in TLR-stimulated IMMs and neutrophils: Total lung cells isolated from SARS-CoV-infected BALB/c mice (3 dpi) were stimulated with LPS (TLR4 ligand, 100 ng/mL) or R848 (TLR7 ligand, 1 μg/mL) for 4—h in the presence of Golgi-plug. IMMs ($CD11b^{hi}Ly6c^{hi}$) and neutrophils ($CD11b^{hi}Ly6C^{int}$) were stained for intracellular TNF, IL-1β, and iNOS production

4 Notes

1. Both MERS-CoV and SARS-CoV should be aliquoted in ice-cold DMEM and the media should be kept on ice throughout the period of infection.
2. Ketamine/xylazine anesthesia gives uniform infection compared to isoflurane. It is essential to make sure that the animals

are completely anesthetized. Following anesthesia mice should be placed on their back on thick bedding to avoid hypothermia.

3. During MERS-CoV and SARS-CoV infection, neutrophil accumulation peaks between day 1 and 3 post-infection and monocytes between day 2 and 4 post-infection. As a result, days 2–4 are ideal time points to assess the activation and pro-inflammatory activity of myeloid cells.
4. Soon after harvesting, lungs should be placed in PBS on ice until further processed. Post-homogenization, the lung tissue can be digested at room temperature from 30 to 45 min.
5. After Collagenase/DNAse digestion, the lung tissue in 15 mL conical should be kept on ice until further processed. Following tissue homogenization, all washing and cell surface staining should be carried out on the ice or at 4 °C.
6. For efficient staining, antibodies should be diluted in 100 μL of FACS buffer and added to assigned well of 96-well round-bottom plate or tube. Both plate and tubes should be placed on flat surface of vortex machine (with very low speed) to ensure that cell pellet is broken and antibody solution is uniformly distributed.
7. The concentration of LPS for stimulation should be 10–100 ng/mL. Other TLR agonists could be used at 1 μg/mL concentration for optimum results. When adding TLR agonists and Golgi-plug, it is essential to dilute these reagents in RPMI-10 and add 100 μL (of 2× concentration) to 100 μL of cell suspension in the 96-well round-bottom plate. Alternatively, a 200 μL of RPMI-10 media with TLR agonists and Golgi-plug (both at 1× concentration) can be added to the wells.
8. Intracellular cytokine staining (ICS) should be carried out using anti-mouse antibodies conjugated with APC or PE or PerCPcy5.5 dyes for better results and use other dyes accordingly for cell surface staining. For ICS staining, cells should be incubated for 25–30 min for better results. Keep cells on ice throughout cell surface and intracellular staining.
9. After surface staining, the cells should be treated with cytofix for 15 min followed by washing and resuspension in FACS buffer. There is no need to add cytofix to ICS-stained cells as these cells were already treated with Cytofix/Cytoperm buffer immediately before ICS staining.
10. For best results, cells should be acquired in flow cytometer within 1–2 days post-staining.

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