Chapter 5

Activation of the Non-canonical Inflammasome in Mouse and Human Cells

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Abstract

The non-canonical inflammasome is a signaling platform that allows for the detection of cytoplasmic lipopolysaccharides (LPS) in immune and non-immune cells. Upon detection of LPS, this inflammasome activates the signaling proteases caspase-4 and -5 (in humans) and caspase-11 (in mice). Inflammatory caspases activation leads to caspase self-processing and the cleavage of the pore-forming protein Gasdermin D (GSDMD). GSDMD N-terminal fragments oligomerize and form pores at the plasma membranes, leading to an inflammatory form of cell death called pyroptosis. Here, we describe a simple method to activate the non-canonical inflammasome in myeloid and epithelial cells and to measure its activity using cell death assay and immunoblotting.

Key words Non-canonical inflammasome, Caspase, Caspase-11, Caspase-4, Gasdermin D, Pyroptosis, Lactate dehydrogenase, Lipopolysaccharides, Macrophages, Monocytes, Epithelial cells

1 Introduction

The inflammasome [1, 2] is an immune signaling platform that activates a family of proteases called the inflammatory caspases [3]. Canonical inflammasomes are activated by cytosolic sensors that recognize a wide range of pathogen-derived and self-derived molecules, which generally indicate the loss of cellular homeostasis [3, 4]. Canonical inflammasomes activate the effector enzyme caspase-1, which cleaves the proinflammatory cytokines Interleukin (IL)-1β and IL-18 into their bioactive forms that are then secreted [5, 6]. Caspase-1 also cleaves the pore-forming protein Gasdermin D (GSDMD). The cleaved N-terminus of GSDMD oligomerizes to create large pores in the plasma membrane, leading to cell swelling and an inflammatory form of lytic cell death called pyroptosis [7].

Recently, a novel inflammasome that recognizes the presence of cytosolic bacterial lipopolysaccharide (LPS) from Gram-negative bacteria was identified: the non-canonical inflammasome (NCI)

Ali A. Abdul-Sater (ed.), *The Inflammasome: Methods and Protocols*, Methods in Molecular Biology, vol. 2459, https://doi.org/10.1007/978-1-0716-2144-8_5,

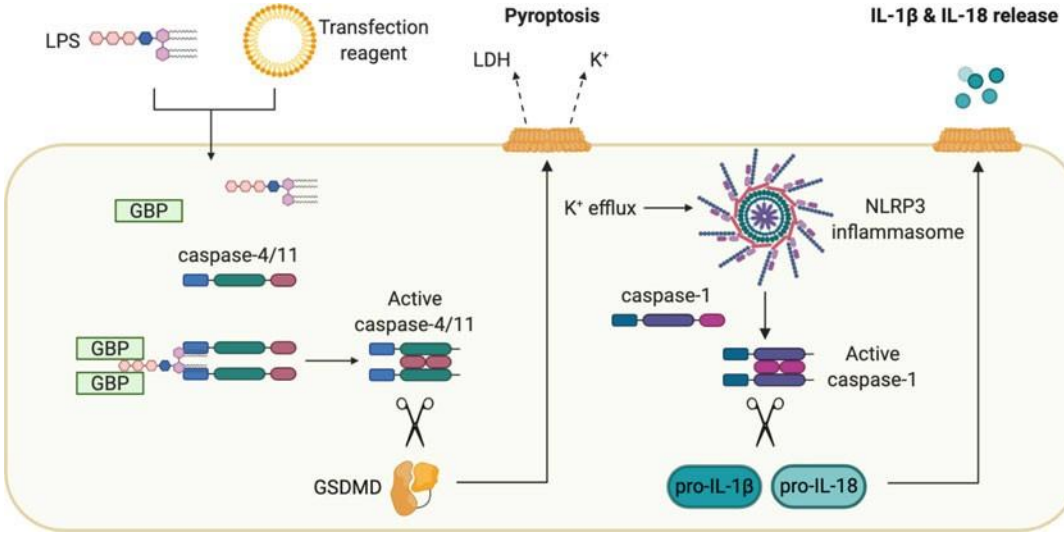

Fig. 1 The non-canonical inflammasome pathway. Cytosolic LPS is sensed by GBPs and caspase-4/11. Active caspase-4/11 cleaves GSDMD causing membrane pore formation. GSDMD pores allow the efflux of cellular contents such as LDH and potassium ions ultimately causing pyroptotic cell death. Potassium efflux from the cell also triggers NLRP3 activation and formation of the inflammasome which activates caspase-1. Caspase-1 cleaves pro-IL-1β and pro-IL-18 to their bioactive forms that are secreted

[8–11]. The NCI is assembled upon the detection of LPS by members of the guanylate-binding protein (GBP) family, which allows the recruitment of inflammatory caspases to the surface of cytosolic Gram-negative bacteria or to outer membrane vesicles secreted by these bacteria. The expression of GBPs is typically induced by TLR ligands or interferon-γ [12–15]. The NCI activates caspase-11 in mice and caspase-4 and caspase-5 in human cells. The activation of these caspases is often associated with self-processing [16–18]. Caspases-4/-5/-11 cleave the pore-forming protein GSDMD, to induce pyroptotic death of infected cells. GSDMD pores also drive potassium efflux, which indirectly activates the canonical NLRP3 inflammasome and NLRP3-dependent secretion of IL-1β and IL-18 (Fig. 1) [19, 20]. NCI activity can be inhibited using the inflammatory caspase inhibitor VX-765 [21], whereas cytokine secretion can be inhibited using the NLRP3-specific inhibitor MCC950 [22]. Much of what we know about NCI is derived from studies of macrophages and epithelial cells, but the NCI can have specialized functions in other cell types such as neutrophils [23]. In this chapter, we will describe how to activate the NCI in primary mouse macrophages and human monocytic and epithelial cell lines. We will also detail how to monitor NCI activation by using immunoblotting to assess caspase activation and by measuring cell death (LDH release). GSDMD cleavage can also be used to monitor NCI activation, and a protocol to measure this is

described in this volume (Kamalaya and Boucher, Chapter 4). The whole protocol should be executed within 2 weeks for mouse cells and within 1 week for human cell lines.

2 Materials

2.1 Generation of Primary Mouse Macrophages

1. Mouse (C57BL/6).
2. Recombinant human macrophage colony-stimulating factor 1 (hM-CSF) (see Note 1).
3. Low-endotoxin fetal calf serum (see Note 2).
4. RPMI 1640, endotoxin tested.
5. DMEM.
6. Penicillin/Streptomycin-Glutamine (100× stock; 10,000 U/mL Penicillin, 10 mg/mL Streptomycin, 29.2 mg/mL L-Glutamine in 10 mM citrate buffer).
7. Complete mouse bone marrow–derived macrophage media (cBMM media): RPMI 1640, 10% heat-inactivated fetal calf serum, 1% Penicillin/Streptomycin-Glutamine, and 100 ng/mL hM-CSF freshly added to media.
8. Red blood cell lysis buffer (home-made 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA in H_2O, final pH adjusted to 7.2 or commercial).
9. DPBS.
10. Ethanol (70%, in distilled water).
11. TC-coated flat bottom 96-well plate.
12. Square 100-mm petri dishes, Sterilin.
13. 70-μm pore size cell strainers or mesh.
14. 18-G blunt needles.
15. 21-G needles.
16. 27-G needles.
17. 10-mL sterile pipettes.
18. 15-mL sterile conical tubes.
19. Cell counter or hemocytometer.

2.2 Human Cell Line Culture

1. HeLa cells (ATCC CCL2).
2. THP-1 cells (ATCC TIB-202).
3. Phorbol 12-myristate 13-acetate (resuspended in DMSO at 10 μg/mL).
4. Low-endotoxin fetal calf serum (see Note 2).
5. RPMI 1640, endotoxin tested.

6. DMEM.
7. Penicillin/Streptomycin-Glutamine (100× stock); 10,000 U/mL Penicillin, 10 mg/mL Streptomycin, 29.2 mg/mL L-Glutamine in 10 mM citrate buffer).
8. Complete THP-1 media: RPMI 1640, 10% heat-inactivated fetal calf serum, 1% Penicillin/Streptomycin-Glutamine.
9. Complete HeLa media: DMEM, 10% heat-inactivated fetal calf serum, 1% Penicillin/Streptomycin-Glutamine.
10. Trypsin/EDTA solution.
11. T75 Tissue culture Flask.
12. TC-coated flat bottom 96-well plate.
13. 10-mL sterile pipettes.
14. 15-mL sterile conical tubes.

2.3 Non-canonical Inflammasome Activation

1. Opti-MEM media.
2. Pam3CSK4 (in ultrapure water at 1 mg/mL).
3. Ultrapure EK-LPS (prepared in ultrapure water, 5 mg/mL) (see Note 3).
4. Human Interferon-γ (see Note 4).
5. FuGENE HD transfection reagent.
6. Lipofectamine LTX transfection reagent.
7. VX765 (prepared at 10 mM, in DMSO).
8. MCC950/CP-456,773 sodium salt (prepared at 10 mM in water).

2.4 Non-canonical Inflammasome Measurement

1. Western blot running buffer 10× (30 g Tris Base, 144 g Glycine, 1% SDS).
2. MeOH.
3. Chloroform.
4. CytoTox 96® Non-Radioactive Cytotoxicity Assay, LDH assay.
5. Human IL-1β ELISA kit.
6. Mouse IL-1β ELISA kit.
7. Mouse Caspase-1 p20 (Casper-1) antibody (Adipogen, Cat AG-20B-0042-C100).
8. Human Caspase-1 antibody clone D7F10 (Cell Signaling Technology, Cat 3866S).
9. Mouse Caspase-11 antibody (Abcam, Cat EPR18628).
10. Human Caspase-4 antibody clone 4B9 (Santa Cruz Biotechnology, Cat sc56056).
11. Human/mouse Tubulin antibody (Sigma-Aldrich, Cat T5168).

12. 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels.
13. NuPAGE LDS loading buffer (4x).
14. Dithiothreitol (prepared at 1 M in distilled water).
15. Whole-cell lysis buffer: 66 mM Tris pH 8.0, 2% sodium dodecyl sulfate.

3 Activation of the Non-canonical Inflammasome in Mouse Macrophages

3.1 Production of Primary Mouse Bone Marrow-Derived Macrophages

1. Euthanize the mouse in a CO_2 gas chamber (or according to the recommendation of your ethics committee; see Note 5).
2. Put the mouse on its back and pin down the legs on a board using a 21-G needle.
3. With dissecting scissors, cut the posterior legs at the hip level, carefully to not damage the bone or expose the bone marrow. Remove the skin and muscle around the bones with the scissors and put bones in ethanol 70%. Transfer the bones to a cell culture hood.
4. Cut the end joints of each bone with scissors to gain access to the bone marrow. With a 27-G needle, flush the bones using 5 mL RPMI media (unsupplemented) into a 50-mL tube through a 70-μM cell strainer to remove aggregates and tissue.
5. Rinse the cell strainer with 5 mL of RPMI media.
6. Centrifuge the 50-mL tube containing the flushed bone marrow for 5 min at 500 x g at room temperature (RT).
7. Discard the supernatant, resuspend the pellet in 2 mL of red blood cell lysis buffer, incubate 2–3 min at RT, and then fill up to 50 mL with cBMM media. Centrifuge again at 5 min at 500 x g at RT (see Note 6).
8. Plate the bone marrow cells in four Sterilin non-TC treated plates (15 mL per plate). Put the cells in a cell culture incubator at 37 °C, 5% CO_2. This is about seven million cells per plate when working from freshly isolated bone marrow (see Note 6). The plating day is counted as day zero of differentiation.
9. On day 4 of the differentiation, add 5 mL of fresh cBMM media to each plate, without removing any media, and put back the cells in the incubator.
10. On day 6 of the differentiation, remove the media and add 10 mL of cold sterile DPBS. Leave the cold DPBS on cells for 3–5 min.
11. Using an 18-G blunt syringe, flush cells to detach them from the plates and transfer to a 50-mL Falcon tube.
12. Rinse each plate with 5 mL of cold sterile DPBS.

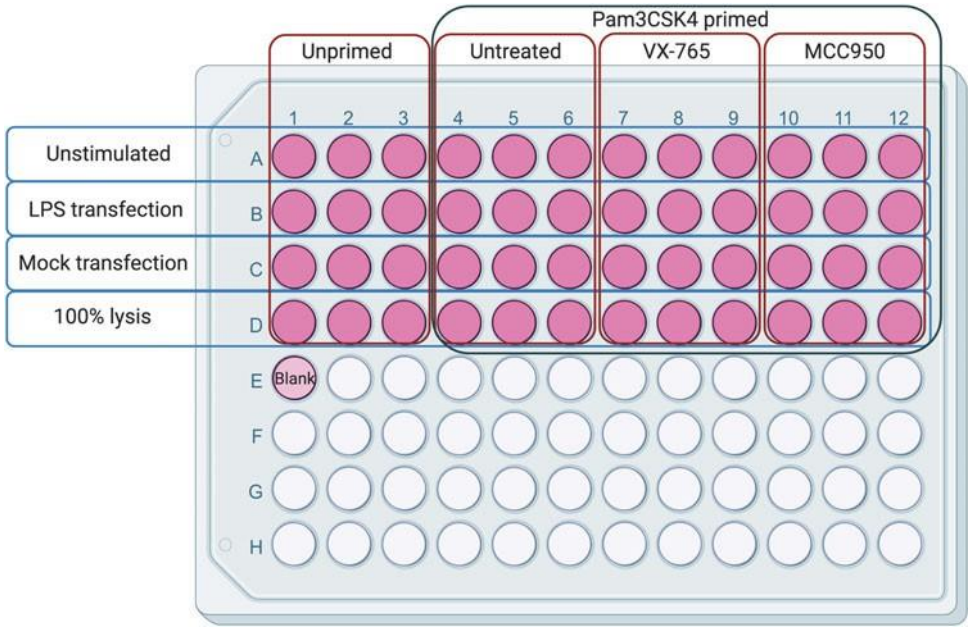

Fig. 2 Schematic 96-well plate layout for a non-canonical inflammasome experiment

13. Centrifuge for 5 min at 500 x g at RT.
14. Count the cells (using a cell counter or hemocytometer).
15. Add 100,000 cells per well of a flat bottom 96-well cell culture plate in 100 μL of cBMM media and place in the cell culture incubator overnight. For a properly controlled experiment, we suggest plating cells as illustrated in Fig. 2. The cells are now ready to be used for non-canonical inflammasome activation.

3.2 LPS Transfection in Mouse Cells

1. To each well, add 10 μL of Pam3CSK4 (10 μg/mL, diluted in cBMM media). The final concentration of Pam3CSK4 in the well is 1 μg/mL. Put the plate back in the cell culture incubator for 4 h.
2. Preheat Opti-MEM media (37 ºC).
3. Remove cBMM media from all the wells, and add 90 μL of preheated Opti-MEM to each well.
4. Prepare the LPS transfection mix as follows. In a 1.5-mL microcentrifuge tube, combine 95.5 μL of Opti-MEM and 2 μL of ultrapure LPS stock (stock concentration is 1 mg/mL). Vortex vigorously for 30 s.
5. Add 2.5 μL of FuGENE HD. Mix by flicking the tube. Incubate at RT for 15 min.
6. Prepare a no FuGENE HD and a no LPS transfection control by omitting each respective component.

7. At the end of the incubation, add 10 μL of the transfection mix (or control) to each well. The final concentration in each well is 0.25% FuGENE and 2 μg/mL LPS.
8. Centrifuge the 96-well plate at 500 ×g for 5 min at RT (see Note 7).
9. After 4 h, collect supernatants, and lyse cells in 50 μL of whole-cell lysis buffer per well.
10. Measure cell death using LDH release assay (see Note 8), as described in the protocol in Subheading 5.
11. Measure IL-1β and IL-18 secretion using ELISA, according to the manufacturer's recommendation.
12. Measure cleavage of Caspase-11 and Caspase-1 by immuno-blotting, in cell lysates and supernatants, as described in the protocol in Subheading 5.

4 Activation of NCI in Human Cell Lines

This section of the protocol will describe how to activate the NCI in human cell lines.

4.1 Preparation of THP-1 Cells

1. Culture THP-1 cells in THP-1 media (see details in Subheading 2) in T75 flasks. When the cells reach a density of 1,000,000 cells/mL, dilute them in fresh media to a density of 0.2×10^6 cells/mL (see Note 9).
2. Using a sterile pipette, centrifuge 15 mL of cells at 500×g for 5 min at RT.
3. Discard the supernatant and resuspend the cells in 1 mL of THP-1 media.
4. Count the cells and adjust the total volume to a concentration of 700,000 cells/mL.
5. Add PMA to a final concentration of 10 ng/mL and mix gently using a sterile pipette.
6. Plate 100 μL of the cell suspension (70,000 cells) per well, into a flat bottom 96-well cell culture plate.
7. Put the cells back in a cell culture incubator and allow to differentiate for 48 h.
8. After differentiation, remove the media and replace with fresh THP-1 media without PMA. Put the cells back in the incubator overnight.

The cells are now ready to be used for inflammasome activation (see Subheading 4.3).

4.2 Preparation of HeLa Cells

1. Culture HeLa cells in complete HeLa medium in a T75 flask. Passage the cells when they are 70–75% confluent.
2. The day prior to the experiment, remove the cell media and gently rinse the cells with 5 mL of prewarmed (37 °C) DPBS. Do not detach the cells.
3. Remove the DPBS and add 2 mL of Trypsin/EDTA (37 °C). Incubate for 3 min at RT.
4. Add 5 mL of complete HeLa medium. With a serological pipette, detach the remaining cells and transfer into a 15-mL conical tube.
5. Centrifuge the cells at 500 × g for 5 min at RT.
6. Discard the media and resuspend the cell pellet in 1 mL of complete HeLa media.
7. Using a hemocytometer, count the cells and resuspend to 200,000 cells/mL.
8. Plate 100 μL of cells (20,000 cells) per well, into a flat bottom 96-well cell culture plate.
9. Where indicated, add 5 μL of Interferon-γ (stock 200 ng/mL; final concentration 10 ng/mL).
10. Put the cells back in the cell culture incubator for 16 h.

4.3 LPS Transfection in Human Cells

1. To each well, add 10 μL of Pam3CSK4 (diluted in complete THP-1 or HeLa media at a concentration of 10 μg/mL). The final concentration of Pam3CSK4 in the well is 1 μg/mL. Put the plate back into the cell culture incubator for 4 h (see Note 10).
2. Preheat Opti-MEM media (37 °C).
3. Remove the media in each well and add 90 μL of preheated Opti-MEM.
4. Prepare the transfection mix as follows. To 87.5 μL of Opti-MEM, add 10 μL of ultrapure LPS (stock concentration is 1 mg/mL). Vortex vigorously for 30 s.
5. Add 2.5 μL of Lipofectamine LTX. Mix by flicking the tube. Incubate at RT for 15 min.
6. Prepare a no lipofectamine and a no LPS transfection control by omitting each respective component.
7. At the end of the incubation, add 10 μL of the transfection mix (or control) to each well. The final concentration in each well is 0.25% Lipofectamine LTX and 10 μg/mL LPS.
8. Centrifuge the 96-well plate at 500 × g for 5 min at RT (see Note 7).
9. Put the plate back in the cell incubator for 5 h.

10. After 5 h, collect supernatants, and lyse cells in 25 μL whole cell lysis buffer per well.
11. Measure cell death using LDH release assay, as described in the protocol in Subheading 5.
12. Measure IL-1β and IL-18 secretion using ELISA, according to the manufacturer's recommendation. For HeLa cells, we recommend measuring only IL-18 as those cells produce only little IL-1β.
13. Measure cleavage of Caspase-4 and Caspase-1 in lysates and supernatants by immunoblotting, as described in the protocol in Subheading 5. The cleavage of Gasdermin D can also be monitored, as described by Kamalaya and Boucher in this volume (Chapter 4).

5 Analysis of NCI Activation

NCI activation can be assessed using several assays. In this section, we will describe how to monitor NCI activation by detecting the autoprocessing of Caspase-11 (in mice) and Caspase-4 (in humans) and by measuring pyroptosis. IL-1β and IL-18 secretion can be measured by ELISA following manufacturer's protocol.

5.1 Immunoblotting for Inflammatory Caspase Cleavage

1. Combine cell lysis extracts from triplicate wells. Total volume is 150 μL from mouse cells and 75 μL from human cells. Add 50 μL or 25 μL of 4× LDS loading dye, to mouse and human lysates, respectively. Add DTT to a final concentration 10 mM.
2. Heat samples for 3 min at 100 °C.
3. Combine cell supernatants and precipitate using methanol–chloroform extraction, as previously described [24] and as follows:
   (a) To 300 μL of supernatants, add 300 μL of MeOH and 100 μL of Chloroform. Vortex vigorously for 15 s.
   (b) Centrifuge at 17,000 x g for 15 min at RT.
   (c) Discard the upper phase carefully (do not disturb the protein interphase).
   (d) Add 500 μL of MeOH and vortex 1 s.
   (e) Centrifuge at 17,000 x g for 15 min at RT.
   (f) Discard the soluble phase and let the protein pellet dry 15 min.
   (g) Resuspend the protein pellet in 30 μL of lysis buffer. Add 10 μL of 4× LDS loading buffer. Add DTT to a final concentration 10 mM.

Table 1
Recommended antibodies for NCI activation analysis

Target	Antibody	Dilution	Expected molecular weight (kDa)
hCasp1	D7F10	1/1000	45 (FL); 33 (active); 20 (active)
mCasp1	casper-1	1/1000	45 (FL); 33 (active); 20 (active)
hCasp4	4B9	1/750	43 (FL); 32 (active); 24 (active)
mCasp11	EPR18628	1/1000	43 (FL); 38 (ALT), 32 (active)
Tubulin	B-5-1-2	1/5000	52

Human (h) and mouse (m) antibodies recommended to detect full length (FL), alternative caspase splicing (ALT), and their active cleaved species

4. On a 4–20% SDS PAGE gel, load 15 μL of cell extract or supernatant fraction.
5. Run the gels in the Tris–glycine buffer at 150 V until the dye front exits the gel.
6. Transfer proteins from the gel to a nitrocellulose membrane using your preferred transfer method and probe by immunoblot using standard methods to detect full-length caspases and their cleaved forms. Note that cleaved forms can be detected in both the cell lysate and the supernatant as a result of pyroptosis. The expected molecular weights are presented in Table 1.

5.2 Detecting Pyroptosis Using LDH Release Assay (See Note 8)

1. At the end of the cell stimulation, add 1 μL of 10% Triton X-100 solution to each well that will serve as 100% lysis control (see plate layout in Fig. 2). Mix and incubate for 5 min at 37 °C.
2. Spin the 96-well plate for 5 min at 500×g at RT to reduce floating cells.
3. Transfer 20 μL of cell-free supernatants and the 100% lysis controls into a new clear 96-well plate (non-sterile).
4. Add 20 μL of media to the blank control wells.
5. Using a multi-channel pipette, add 20 μL of LDH substrate (reconstituted as described in the manufacturer's instructions) to each well.
6. Incubate 15–30 min at RT in an opaque container or wrapped in foil to protect from light (see Note 11).
7. Add 20 μL of Stop solution (provided with the LDH kit) to stop the reaction.
8. Make sure there are no bubbles in the wells. Use a 27-G needle to gently remove them.
9. Measure absorbance at 490 nM in a spectrophotometer plate reader.

10. Determine the level of cell death as follows:

$$\text{Cell death (\% to 100\% control)} = \frac{(\text{Sample OD}^{490\text{nM}} - \text{Blank OD}^{490\text{nM}})}{(\text{100\% OD}^{490\text{nM}} - \text{Blank OD}^{490\text{nM}})} \times 100$$

11. Plot your data using GraphPad Prism or other data analysis software.

6 Notes

1. We recommend optimizing the concentration of hM-CSF-1 for bone marrow macrophage differentiation. While the concentration recommended here works well with hM-CSF-1 from Immunotools (Cat 11343118), hmCSF-1 from other suppliers might differentiate cells with a different efficiency.
2. The quality of the fetal calf serum is important for this type of experiment. The use of low-endotoxin fetal calf serum is essential. We recommend testing the endotoxin level of each fetal calf serum batch before purchasing a large amount. We used Thermo Fisher, Cat 16000044.
3. LPS quality is another critical determinant in the success of the experiments. LPS with lower purity can contain impurities that can also activate the canonical inflammasome. We highly recommend ultrapure LPS-EK from Invivogen (Cat tlrl-3pelps or Cat tlrl-peklps) to efficiently and specifically activate the NCI in mouse and human cells.
4. Human and murine interferon-γ are not cross-reactive. Human M-CSF is cross reactive with mouse and human cells. We used interferon-γ from Biolegend, Cat 570204.
5. Before beginning this protocol, it is essential that you have obtained ethical approval for this procedure and that your euthanasia protocol conforms to your institutional guidelines.
6. If needed, the bone marrow can be frozen after flushing the bones. Red blood cells do not need to be lysed for this procedure. We routinely resuspend bone marrow from one mouse in 2 mL of freezing media (10% DMSO, 90% FCS). When replating frozen bone marrow, replate one vial of cells in two Sterilin plates.
7. The temperature of centrifugation is a key element for the success of this procedure. Centrifugation at temperature colder than RT will lead to an unsuccessful transfection and will fail to activate the NCI.

8. Cell supernatants must be used fresh for LDH assays. Frozen supernatants lose LDH activity and cannot be used for this assay.
9. The density of cells plated in this experiment is critical for transfection success. We recommend plating cells to a density of 70–80% at the time of transfection.
10. HeLa cells do not require further priming as they are already primed with IFN-γ.
11. Certain cell types contain less LDH and will develop slower. It is recommended to incubate the LDH reaction for a longer period of time.

Acknowledgments

Figures were created with BioRender (www.biorender.com). J.S.B. is supported by Kennedy Trust, KTRR start-up fellowship (KENN 15 16 06), and M.R.C. New Investigator Grant (MR/S000623/1). R.C. is supported by an Academy of Medical Sciences Springboard Award (SBF005\1104), a Royal Society Research Grant (RGS\R1\201127), and institutional funding from the Wellcome-Wolfson Institute for Experimental Medicine and Queen's University Belfast. D.B. is supported by a Springboard award (SBF006\1025) from the Academy for Medical Science and institutional funding from the University of York.

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