Video Article

Activation and Measurement of NLRP3 Inflammasome Activity Using IL-1β in Human Monocyte-derived Dendritic Cells

Melissa V. Fernandez[1], Elizabeth A. Miller[2], Nina Bhardwaj[3]

[1]Department of Pathology, New York University School of Medicine

[2]Division of Infectious Diseases, Department of Medicine, Mount Sinai Medical Center

[3]Division of Hematology and Oncology, Hess Center for Science and Medicine, Mount Sinai Medical Center

Correspondence to: Nina Bhardwaj at nina.bhardwaj@mssm.edu

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Abstract

Inflammatory processes resulting from the secretion of Interleukin (IL)-1 family cytokines by immune cells lead to local or systemic inflammation, tissue remodeling and repair, and virologic control[1,2] . Interleukin-1β is an essential element of the innate immune response and contributes to eliminate invading pathogens while preventing the establishment of persistent infection[1-5].

Inflammasomes are the key signaling platform for the activation of interleukin 1 converting enzyme (ICE or Caspase-1). The NLRP3 inflammasome requires at least two signals in DCs to cause IL-1β secretion[6]. Pro-IL-1β protein expression is limited in resting cells; therefore a priming signal is required for IL-1β transcription and protein expression. A second signal sensed by NLRP3 results in the formation of the multi-protein NLRP3 inflammasome. The ability of dendritic cells to respond to the signals required for IL-1β secretion can be tested using a synthetic purine, R848, which is sensed by TLR8 in human monocyte derived dendritic cells (moDCs) to prime cells, followed by activation of the NLRP3 inflammasome with the bacterial toxin and potassium ionophore, nigericin.

Monocyte derived DCs are easily produced in culture and provide significantly more cells than purified human myeloid DCs. The method presented here differs from other inflammasome assays in that it uses *in vitro* human, instead of mouse derived, DCs thus allowing for the study of the inflammasome in human disease and infection.

Video Link

The video component of this article can be found at https://www.jove.com/video/51284/

Introduction

Activation of innate immune system is required to steer adaptive immune responses during infection, disease, and vaccination[7]. Dendritic cells are the most potent antigen presenting cells of the innate immune system; they are specialized for uptake of antigens, migration to lymph nodes, and activation of naïve $CD4^+$ and cytolytic $CD8^+$ T-cells[8-10]. To enable rapid pathogen detection the innate immune system utilizes numerous germline encoded pattern recognition receptors (PRR) that recognize conserved pathogen derived motifs or host derived markers of cell stress and damage. Toll like receptors (TLRs) are membrane bound pattern recognition receptors that recognize certain extracellular phagocytized pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). By contrast nod like receptors (NLRs) are cytosolic and respond to a diverse range of PAMPs and DAMPs. Nod like receptors represent a second line of defense against pathogens that evade cell surface and endocytic PRRs. The interaction of pathogen derived, or “danger” associated, factors with TLR and NLR ligands leads to a state of DC maturation resulting in increased DC interaction with other immune cells and promotion of T cell and natural killer cell activation[11].

Interleukin-1β is a crucial component of the host defense against infection. Upon recognition of a microorganism, the highly proinflammatory cytokine, IL-1β, is secreted and functions as a chemo attractant and activator of innate and adaptive immune cells. *In vivo* IL-1β is largely responsible for the acute phase response including fever and inflammatory cytokine synthesis[12].

Most NLRs contain a C terminal leucine rich repeat domain that is thought to function in ligand sensing, a central nucleotide binding domain (NACHT) that is important for NLRP3 oligomerization, and an N terminal effector domain (PYD in NLRP3) that mediates signal transduction to downstream targets through protein protein interactions. The NLRP3 protein defines the most intensely studied inflammasome complex. This protein is a member of the NLR family and has the ability to form a multi molecular protein complex composed of NLRP3, the adaptor protein PYCARD (also known as ASC), and ICE. Upon inflammasome activation PYCARD binds to NLRP3 N terminal domains and recruits ICE via caspase activation and recruitment domain (CARD) domains. Interleukin-1 converting enzyme is initially generated as a zymogen containing

a CARD motif at its N-terminus. Inflammasome formation results in bringing two ICE molecules sufficiently close to induce their autocatalytic activation. The inflammasome complex is necessary for activating ICE thus allowing it to convert cytoplasmic pro-IL-1β to mature cytokine.

Successful secretion of IL-1β in DCs requires sensing of two different and independent danger signals. First, TLR sensing of PAMPs, DAMPs, or cytokine signaling (TNFα or IL-1β) causes an upregulation of cytoplasmic pro-IL-1β protein expression. A second, often different, signal is required for inflammasome complex formation upstream of ICE maturation. A few inflammasome stimulating signals include bacterial membrane pore forming toxins (such as nigericin), lysosomal disrupting crystals (such as monosodium urate crystals, MSU), and extracellular ATP. The upstream mechanism leading to NLRP3 inflammasome activation by these diverse activators is unclear. Studies investigating signaling upstream of inflammasome formation proposes that intracellular events, such as induction of hypokalemia or reactive oxygen species (ROS) indirectly activate the inflammasome[13-28].

Amongst the different viral activators of the NLRP3 inflammasome is influenza, which provides both the primary and secondary signal required for IL-1β secretion[3,29-33] . Using mouse NLRP3 knockout models it was found that IL-1β secretion in DCs is NLRP3 dependent[32]. Additionally, NLRP3 knockout mice attracted fewer leukocytes to the site of infection and experienced higher mortality[2,5]. Two recent papers suggest a mechanism for NLRP3 inflammasome activation during Influenza virus infection; first, priming through recognition of viral RNA by TLR7 or TLR8 (depending on TLR expression of the responding cell) or through sensing of commensal bacteria by other TLRs to induce cytoplasmic pro-IL-1β expression, followed by a second signal, activation of NLRP3 inflammasome formation by viral ion channel protein M2 on the trans Golgi network[33,34]. In the latter step, triggering of the NLRP3 inflammasome is accomplished by disturbance of the intracellular ionic *milieu* leading to ROS production, which is, simply, sensed by NLPR3 as a signal to form the inflammasome. However, the precise mechanism of inflammasome activation upstream of ICE activity during Influenza infection still remains unclear.

This work describes a technique valuable for studying the NLRP3 inflammasome in human moDCs that can be used as a foundation for further investigation of the pathway underlying DC based IL-1β secretion in response to TLR8 ligation with R848 followed by activation of the inflammasome by a well known activator of NLRP3, nigericin. Variations of this method can be used with other cell types including, but not limited to: monocytes, macrophages, other DC subsets, and epithelial cells.

Protocol

Ethics Statement: Research samples are obtained and stored for research with donors' consent. All samples should be coded or anonymized prior to use. This protocol follows the guidelines of our Institutional Review Board.

1. Differentiation of Human Peripheral Blood Monocytes into Monocyte Derived Dendritic Cells.

Note: Human buffy coats serve as the source of human peripheral blood cells (PBMCs) and were obtained from the New York Blood Center (New York, NY). Blood donors are healthy volunteers. The 5 day procedure begins with the plating of human peripheral blood mononuclear cells (PBMCs) onto tissue culture flasks[35,36]. Notable differences from the published protocols are the following:

1. Use 225 cm^2 non pyrogenic polystyrene tissue culture flasks with a filter cap to adhere a total of $2x10^8$ PBMCs per flask instead of 10 cm polystyrene tissue culture plates (58 cm^2) (step 1).
2. Prepare media with 5% pooled human serum (PHS; 30 ml) in 500 ml RPMI-1640 + L-glutamine, 5 ml 1 M HEPES buffer, and 1.4 ml 50 mg/ml Gentamicin (5% PHS media) followed by filtration through a 20 µM Filter. Add a total of 50 ml 5% PHS media per 225 cm^2 tissue culture flask.
3. Wash adhered cells three times with 25 ml fresh RPMI-1640. Shake vigorously for 5 sec during each wash and aspirate non adherent cells (step 4).
4. Add 190 µl of 400 IU/µl IL-4 and 380 µl of 100 IU/µl GM-CSF per flask (step 3) on day 0, 2, and 4. Day 0 is defined as the day PBMCs are initially plated in Step 1 (Step 8).
5. Harvest day 5 moDCs at a concentration of $1x10^6$ cells/ml (step 15).
6. Use moDCs immediately in their resting state. Steps 17-22 are never performed. Note: Samples should be processed as soon as possible after collection for best results.
7. Aliquot moDCs at a concentration of $2x10^5$ cells/well (200 µl/well of the $1x10^6$ cells/ml) in a 96 well round bottomed plate (western blot, ELISA, FACs) or onto a poly-L-lysine treated chamberslide (microscopy) for experimentation. Note: There are at least 4 conditions: Completely unstimulated (negative control), priming only, activation only, and priming followed by activation. Conditions can be expanded to include diluent controls for R848 and nigericin if desired. If the downstream assay is ICS, duplicates are necessary for isotype controls.

2. Priming the Inflammasome - Signal 1

1. Reconstitute lyophilized R848 in DMSO according to the manufacturer's instructions. Dilute working stock at RT with RPMI-1640.
2. Add R848 at a 10 µM final concentration to appropriate wells for 18 hr. Place cells in an incubator at 37 °C and 5% CO_2.

3. Activating the NLRP3 Inflammasome - Signal 2

1. Reconstitute lyophilized nigericin in ethanol according to the manufacturer's instructions. Dilute working stock at RT with RPMI-1640 before adding to the appropriate conditions.
2. Add nigericin at a 20 µM final concentration and return to the incubator at 37 °C and 5% CO_2 for 6 hr. No washing steps occur between priming and during activation. Note: Secretion of IL-1β is increased by the presence of calcium ionophores, brefeldin A, monensin,

dinitrophenol, or carbonyl cyanide chlorophenylhydrazone[37,38]. Therefore, addition of these secretory inhibitors are not recommended when analyzing inflammasome priming or activity since it will alter secretion of IL-1β.

4. IL-1ß Sample Collection

1. Centrifuge the plate with cells and supernatants at 974 x g for 3 min.
2. Without disturbing the cell pellet, aspirate the supernatant and transfer to a separate round bottomed plate in order to measure cytokine secretion from the supernatants. Note: Supernatants can be temporarily stored at -20 °C or -80 °C for long term storage.
3. Wash the cellular pellets three times with 200 µl of 1x PBS at 974 x g for 3 min to remove any extracellular IL-1β from the cellular samples. Note: When washing cell pellets from a 96 well plate, remove the wash by swift plate inversion into a collection bin so as to not disturb the sample.

5. Measuring IL-1ß From Cellular and Supernatant Samples

1. Intracellular Cytokine Staining (ICS): The ICS protocol is described below[39,40]. Note: do not suspend the cells if the downstream assay is microscopy. All washes and aspirations should be done without disturbing the cell layer/pellet (depending on the downstream assay).
    1. Add 100 µl of 5% PHS media into appropriate wells. Add appropriate volume (approximately 1 µl/2x10^5 cells, or 1 µl/well) of fluorescently labeled α-CD11c and α-CD14 (phenotype markers; clone B-ly6 and MφP9, respectively) or isotype control to the cells for 10 min at RT in the dark.
    2. Wash three times with 1x PBS at 974 x g for 3 min.
    3. Fix the cells by adding 100 µl of 4% PFA for 20 min at RT in the dark.
    4. Pause Point: Wash with 100 µl 1x PBS at 974 x g for 3 min and store at 4 °C O/N in 1x PBS.
    5. Add 100 µl of permeabilization buffer to cells for 30 min. Note: permeabilization buffer is composed of 1x PBS with 0.3% Triton X-100, and 1% bovine serum albumin (BSA) for permeabilization. Do not disturb adherent cells when downstream assay is microscopy.
    6. Add appropriate volume (approximately 62 ng antibody/2x10^5 cells, approximately or 2.5 µl/well) of α-IL-1β-FITC (clone 8516) or isotype control for 2 hr in an incubator at 37 °C.
    7. Wash cells three times with 200 µl of permeabilization buffer at 974 x g for 3 min in the dark.
    8. Optional: Stain with DAPI, add mountant, and place coverslip gently on top of a glass slide. Allow mountant to cure O/N before capturing microscopy images.
    9. Acquire the data by FACs or microscopy. Note: Compare the isotype for each condition to the appropriate staining when analyzing by FACs or microscopy.
        1. FACs: Acquire one sample at a time. Set the FSC/SSC gate on the live cells, followed by gating on $CD11c^+CD14^-$ cells before analyzing the moDC population pro-IL-1β staining.
        2. Microscopy: Set the exposure time using the positive staining sample - R848 treated. Use $DAPI^+$pro-IL-$1\beta^+$ and $DAPI^+$pro-IL-$1\beta^-$ to determine percentage of pro-IL-1β+ expressing moDCs.

2. SDS-Page: Immunoblotting is performed to detect pro-IL-1β. Note: The technique described below combines standard immunoblotting technique, gradient 4-20% polyacrylamide gel, and fluorescent or chemiluminescent detection[41,42] .
    1. Lyse cells directly in 10 µl denaturing lysis buffer (5 µl β-mercaptoethanol/950 µl Laemmli sample buffer followed by diluting 1:1 with cell lysis buffer). Transfer lysate to 1.5 ml Eppendorf tubes.
    2. Heat lysate on a dry plate for 10 min at 100 °C.
    3. Spin lysate at 20,800 x g on a minicentrifuge for 1 min to concentrate the liquid volume. Pause point: samples can be frozen at -20 °C for short term storage.
    4. Load the total volume onto the polyacrylamide gel. Run 140 V through the gel box for approximately 1 hr, or until the dye front runs off the bottom of the gel.
    5. Transfer the protein from the polyacrylamide gel onto a PVDF Immobilon-FL membrane for 90 min at 100 V. Block the membrane with 5% BSA in Tris Buffered Saline/ 0.1% Tween-20 (TBS-T) for 1 hr. Note: PVDF Immobilon-FL Is best for use with fluorescent detection. A membrane with a different composition is recommended for other detection techniques to increase the signal to background ratio.
    6. Dilute primary and secondary antibodies in 10 ml of 5% BSA/TBS-T. Perform primary antibody incubations at 4 °C O/N while shaking and secondary antibody at RT for 1 hr while shaking.
    7. Wash the membrane 3 times with TBS-T for 5 min while shaking between primary and secondary antibody incubations and again between secondary incubations and imaging. Note: Bands can be detected using fluorescent or chemiluminescent detection. Follow the manufacturer's instructions for detection.

3. ELISA: Samples that are frozen for storage need to equilibrate to RT before analysis. Spin down samples in a centrifuge to consolidate supernatant condensation. Follow the manufacturer's instructions for measurement of IL-1β. Note: In this protocol IL-1β is specifically measured; however, simultaneous measurement of TNFα, IL-6, and the immunomodulatory cytokine IL-10 ensure the appropriate conditions were primed.

Representative Results

These techniques measure TLR8 priming with R848. Intracellular cytokine staining for pro-IL-1β allows for microscopy and FACs readouts from $CD14^-CD11c^+$ moDCs. Both techniques can be quantified relative to a non primed, or resting, cell control as well as an isotype control (**Figures 1 and 2**). Percent of pro-IL-$1\beta^+$ staining cells is multiplied by the geometric median of this population to provide the median fluorescent intensity (MFI). The MFI is comparable to the amount of pro-IL-1β present in the positive staining cells.

Immunoblotting measures pro-IL-1β from cell lysate, which is then quantified relative to an internal cellular control, such as β-tubulin or β-actin (**Figure 3**). Immunoblotting for pro-IL-1β in nigericin treated cells should reveal a decrease in pro-IL-1β. This is complemented by a concurrent increase in IL-1β in supernatants, measured by ELISA, only in R848 followed by nigericin conditions (**Figures 3 and 4**). All other conditions should result in no extracellular IL-1β present. Simultaneous measure of other inflammatory cytokines, such as TNFα, IL-10, and IL-6, ensure that nigericin is specific in causing the secretion of IL-1β. The level of priming is time (**Figure 3**) and dose (**Figure 4**) dependent, a response reflected in the degree of intracellular pro-IL-1β in R848 primed and extracellular IL-1β (as well as TNFα, IL-10, and IL-6) secretion in all R848 treated conditions (**Figure 4**).

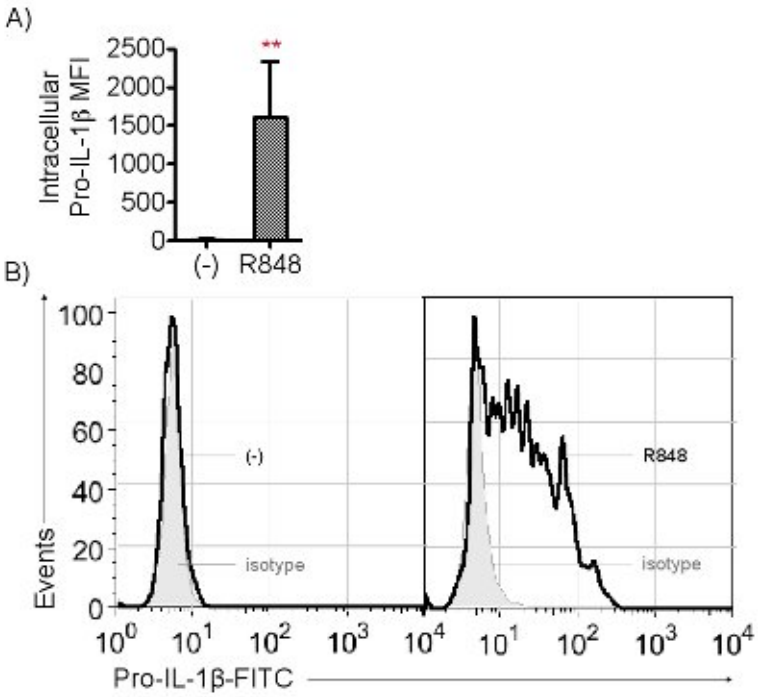

Figure 1. Cytoplasmic pro-IL-1β is detected by flow cytometry. Please click here to view a larger version of this figure.

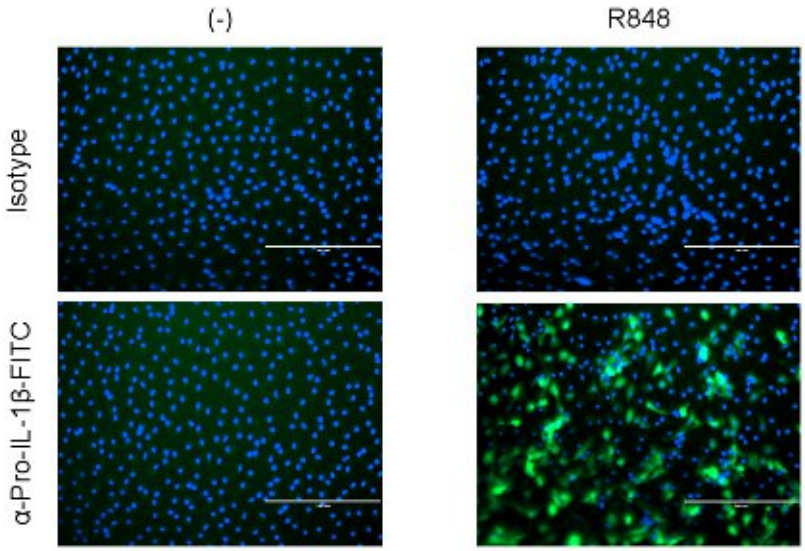

Figure 2. Cytoplasmic pro-IL-1β is detected by microscopy. Please click here to view a larger version of this figure.

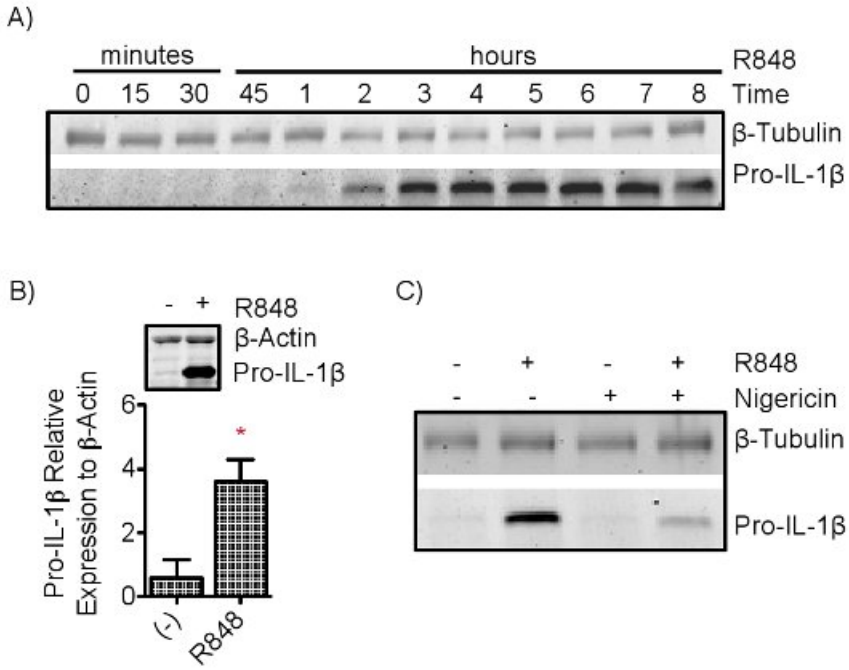

Figure 3. Cytoplasmic pro-IL-1β is detected by SDS-Page. Please click here to view a larger version of this figure.

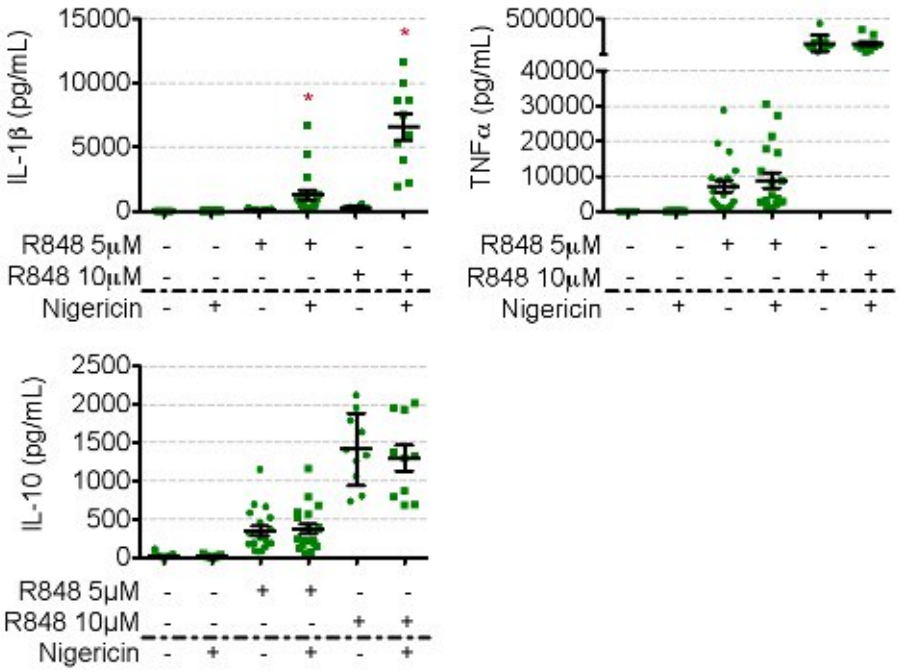

Figure 4. Secreted IL-1β is detected by ELISA. Please click here to view a larger version of this figure.

Discussion

Inflammatory cytokines are integral in steering the innate and adaptive immune response to fight viral infection. Secreted IL-1β has been shown to increase during Influenza infection[3,43,44] . The precise mechanisms by which these cytokines are processed in response to viral recognition in human dendritic cells are not fully understood. Myeloid DC isolation kits are expensive and time consuming. Isolation kits and FAC sorting may unintentionally stress or activate the cells. Additionally, there is frequently insufficient amount of isolated cells for experimentation. Fortunately, the biology of human moDCs closely models that of primary human myeloid DCs *in vitro*[45,46] . Both cell types require two signals, TLR priming

and NLRP3 activating, to achieve mature IL-1β secretion. Therefore, moDCs provide and affordable and simple DC model cell type to study and better understand the relevance and role of the NLRP3 inflammasome in human health and disease.

The detection of IL-1β utilizing the methods described here provides various simple and effective immunoassays to study diseases with inflammatory associated pathologies, including viral RNA infection; specifically, how to measure IL-1β in a variety of ways in response to R848 and nigericin stimulation. Other TLR agonists (such as Poly(I:C) and LPS) and NLRP3 inflammasome activators (such as ATP and MSU) can be used to measure this activity upon stimulation in the context of other disease pathologies; however, stimulation times and conditions may need to be adjusted. Reagent exposure times and concentrations would also need to be adjusted when modifying this protocol for use in other cell types and species.

All conditions should be run in triplicate and the response weighed carefully for quality control purposes; it is common for great donor variability to exist. Monocyte derived DCs are a cell type, not a cell line, and heterogeneity exists within a moDC culture.

Priming can be confirmed with ICS for pro-IL-1β and comparing resting moDCs to the R848-primed condition. Resting moDCs should not result in positive staining. Priming may also be validated by immunoblotting for the expression of pro-IL-1β. Successful R848 priming results in the secretion of proinflammatory cytokines TNFα, IL-6, and the immunomodulatory cytokine IL-10 with minimal secretion of IL-1β. Inflammasome activated cells should not show a further increase in TNFα, IL-6, and IL-10 secretion but will have an increase in secreted IL-1β concentrations compared to unactivated cells.

Pro-IL-1β may be passively released during necrotic cell death therefore bioavailability assays might be of interest. Alternatively, immunoblotting can be performed on supernatants to determine the molecular weight of secreted IL-1β to ensure active IL-1β is the form of the cytokine secreted; mature IL-1β is 17 kDa while the precursor is 31 kDa. To measure IL-1β from supernatants, cell concentration will have to be adjusted to achieve a positive signal above the detection limit. Caspase-1 activation can also be measured via a variety of methods to determine inflammasome activation.

Disclosures

The authors have no conflicts of interest to disclose.

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