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Detection of pyroptosis by measuring released lactate dehydrogenase activity

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Summary

Pyroptosis is a form of programmed, inflammatory cell death that is dependent on the activation of a cysteine protease Caspase-1. In this chapter, we describe an enzymatic assay for the detection of lactate dehydrogenase (LDH) released by dead or dying cells during pyroptosis using a commercially available kit. We also discuss another simple and cost-effective method to measure LDH adapted from Decker *et al.*(1).

Keywords

Pyroptosis; LDH; inflammasome; Caspase-1

1 Introduction

Cellular response to pathogens is initiated through the engagement and recognition of unique pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) such as toll like receptors (TLRs) and Nod-like receptors (NLRs). A subset of the NLRs, when activated lead to the formation of a multiprotein complex known as inflammasomes in the cytosol. Inflammasomes form the platform for the cleavage of pro-caspase-1 into its active caspases-1 form. Caspase-1 activation through a NLRP3, NLRC4, NLRP1b, and AIM2 have been shown to induce pyroptosis and concomitant release of two inflammatory cytokines – IL-1 β and IL-18. NLRC4 and NLRP1b are expressed in sufficient levels in bone marrow derived macrophages and dendritic cells. On the other hand, NLRP3 expression is not sufficient at basal levels, and requires priming via TLRs or TNF (2, 3). AIM2 expression is induced by type I interferon signaling (4). Further care should be taken with examination of human monocytes, as NLRP3 signaling is short circuited by secretion of ATP in response to TLR agonists (5, 6). Over the years a variety of microbial and danger associated ligands capable of inducing pyroptosis via these inflammasomes have been identified. The most well-studied among them is the activation of NLRC4 inflammasome by flagellin in the cytosol leading to caspases-1 activation and cell death via pyroptosis (7).

Pyroptotic cell death is distinct from some other known forms of cell death such as apoptosis, autophagy and necrosis in its dependence on caspase-1 (7). While caspase-1 dependency is the hallmark of pyroptosis, a recent report suggests that caspase-11 can also trigger pyroptosis(8). Pyroptosis has been best described in macrophages and dendritic cells. While the exact mechanisms of pyroptosis remain unclear, it is known that caspases-1 activation results in rapid formation of membrane pores, significant cell swelling followed

by membrane rupture, and release of intracellular content into the extracellular space, including cytosolic proteins such as lactate dehydrogenase (LDH). After strong stimulation of inflammasomes, the entire process can occur in less than one hour. Compounds such as glycine and polyethylene glycol can block membrane rupture release from pyroptotic cells(9) through poorly understood mechanisms.

Here we describe a simple method to measure the release of LDH into culture supernatants during pyroptosis. Amongst the myriad of released cytosolic proteins, LDH is used as a proxy for cell death only because of the ease of measuring its enzymatic activity. LDH is a key glycolytic enzyme that oxidizes lactate to pyruvate, transferring electrons to the cofactor nicotinamide adenine dinucleotide (NAD^+) to form NADH. Both of the methods discussed here utilize the ability of NADH to donate electrons to FAD^+ complexed with the enzyme diaphorase (purified diaphorase contains FAD^+). Diaphorase is a ubiquitous enzyme that detoxifies small molecules, including quinolones as well as catalyzing the reduction of tetrazolium salt (INT) into formazan. While INT is yellow, formazan is red, absorbing light at 492 nm. The increased absorption is readily detected with a spectrophotometer (Fig. 1). Thus, the amount of light absorbed is directly proportional to the number of lysed cells or cells with compromised membrane permeability. Therefore, early LDH measurement is indicative of pyroptotic cell death.

However, LDH measurement is not sufficient to qualify cell death as pyroptosis. Dependence of Caspase-1 is absolutely essential to distinguish pyroptotic cell death from necrotic cell death or apoptosis followed by secondary necrosis (lysis of apoptotic blebs). Although the caspase-1 inhibitor (z-YVAD-fmk) and shRNA/siRNA knockdown have been extensively used in the literature, we recommend the use of cells derived from knockout mice wherever possible. . Inhibitors or knock-down methods carry the potential caveats of off target effects and impenetrant inhibition at longer time-points, for example reduction of Caspase-1 activity by 90% could still result in the binary event of cell death after longer incubation periods. Experiments using inhibitors or knockdown should examine cell death at several time-points.

The method described below is routinely used in our laboratory to assess pyroptotic cell death in bone marrow derived macrophages (BMDMs) in response to *Salmonella typhimurium* infection. However, it can be adapted to assess pyroptosis in any macrophage or dendritic cells to the desired known or unknown stimuli. We describe a commercially available LDH assay kit as well as a noncommercial method adapted from Dekker *et al.*¹ that is approximately one third the cost of the commercial kit.

2 Materials

1. CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, catalog number G1780)
2. Lactate (Sigma, catalog number L7022)
3. Iodonitrotetrazolium chloride (INT) (Sigma, catalog number I8377; 20mg/ml stock solutions in DMSO can be stored at -20°C)
4. Diaphorase (purified from *Clostridium kluyveri*; Worthington Biochemical, catalog number LS004327; sold as 1 ku, or 1 kilo unit = 1000 Units; light sensitive; note that diaphorase from other vendors is considerably more expensive)
5. NAD^+ (Sigma, catalog number N7004)
6. Sucrose (Sigma)

7. Bovine serum albumin (BSA) (Fisher)
8. Triton-X100
9. Acetic acid
10. Cells (macrophages or dendritic cells)
11. 96-well flat bottom plates, half volume wells (Costar, catalog number 3690)
12. Plate reader

3 Methods

3.1 Assay substrate

1. Assay substrate mix—

12 mg/ml lactate
 0.66 mg/ml INT
 4.5 U/ml Diaphorase
 0.01% BSA
 0.4% sucrose
 in PBS
 Freeze in 12 ml aliquots.

2. NAD⁺ (100X stock)—100mg/ml in ddH₂O, freeze in single use aliquots (working concentration is 1mg/ml). NAD⁺ is light sensitive and should be stored in –80°C. Freeze thaw should be strictly avoided. Note that NAD⁺ degrades at neutral pH, especially in the presence of phosphate (Sigma). We have found that NAD⁺ stock solutions in PBS stored at –20°C degrade in one week.

3. Lysis buffer—9% Triton-X100 in dH₂O. Keep Refrigerated until use.

4. Stop solution—1M acetic acid. Store at 4°C or room temperature.

3.2 Infection conditions

1. Plate BMDMs in 96-well plate at a density of 5×10^4 cells per well in 100μl of complete media in triplicates (DMEM+10%FBS+10%L-cell conditioned media). [Note: reserve BMDM seeded wells for untreated and lysis controls, plus empty wells for media only controls and media + lysis buffer]
2. Infect cells with *Salmonella typhimurium* for the desired amount of time. The plates can be spun at $500 \times g$ for 5 min at room temperature to ensure uniform infectivity and to correct for amotile strains. [Note that serum free media or media containing 5% serum can be used to reduce background signal arising from LDH enzyme that is present within FBS.]
3. Add 10μl of 9% Triton-X100 to lyse the cells in the lysis wells. Pipette up and down to make sure all cells have lysed.
4. Collect supernatants. They can also be frozen at –80°C without loss in LDH. However, make sure that all reagents and sample supernatants are at room temperature before doing the assay.

LDH Assay, commercial kit—

1. If using commercially available kit, follow the manufacturer's instructions. We routinely scaled down to half the volumes suggested (25µl of cell supernatant, 25µl of reagent and 25µl of stop solution in half volume 96 well plates). Development takes about 8 minutes.

LDH Assay, noncommercial—

1. Thaw assay substrate mix to room temperature in a room temperature water bath. Once the assay substrate is fully thawed, add 25µl of 100× NAD⁺ stock to 2.5ml of assay reagent mix. This is enough substrate mix for one 96 well plate.
5. Transfer 25µl of your sample to the flat bottom assay plates.
6. Add 25µl of assay substrate (containing the NAD⁺) to the wells using a multichannel pipette.
7. Incubate at room temperature for about 15 minutes. (Note: The incubation time may vary depending on the number of cells plated. When the lysis wells change color to deep maroon, the reaction can be stopped.)
8. To stop the reaction, add 25µl of the stop solution.
9. Read the absorbance at 490 or 492nm using appropriate plate reader. [Note: Air bubbles in the wells can interfere with the absorbance readings. Bubbles can be popped by spraying the plate lightly with 70% ethanol or by lancing each bubble with a needle]
10. Calculate cytotoxicity (% LDH release) using the following formula:

$$\%LDH \text{ release} = \frac{(\text{Sample} - \text{Media}) - (\text{Untreated Sample} - \text{Media})}{(\text{Lysis} - \text{Lysis Media}) - (\text{Untreated Sample} - \text{Media})} \times 100$$

Sample – experimental wells

Media – cell culture media (background absorbance)

Untreated sample – supernatants from uninfected or untreated cells

Lysis – supernatants from cells lysed using Triton-X

Lysis Media – cell culture media + lysis solution

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Figure 1. Schematic of assay for LDH activity. LDH oxidizes lactate to pyruvate, reducing NAD⁺ to NADH. NADH is then oxidized back to NAD⁺, while reducing the diaphorase-FAD⁺ complex to diaphorase FADH₂. Diaphorase FADH₂ then reduces tetrazolium to formazan,