Chapter 14

Cell-Free Assay for Inflammasome Activation

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Abstract

Inflammasomes are multiprotein complexes, which assembly results in caspase-1 activation and subsequent IL-1β and IL-18 activation and secretion. In a cell-free system, based on cytosols of normally growing cells, the disruption of the cell membrane spontaneously activates the inflammasome. Studying the activation of the inflammasome in cytosolic extracts provides multiple advantages, as it is synchronized, rapid, strong, and mostly plasma membrane-free. This protocol covers the methods required to prepare cell lysates and study inflammasome activation using different read-outs. General considerations are provided that may help in the design of modified methods. This assay can be useful to study potential inflammasome interactors and the signaling pathways involved in its activation.

Key words Cell-free assay, Inflammasome, ASC, NLRP3, IL-1β

1 Introduction

Inflammasomes are multiprotein complexes, which are typically composed of a cytoplasmic sensor and an effector protease, caspase-1, which constitutes the catalytic active core of the complex. Most inflammasomes also require the adaptor ASC (apoptosis-associated speck-like protein containing a CARD) that recruits caspase-1 to the oligomeric sensor [1, 2]. The assembly of these complexes depends on the recognition of infectious stimuli or danger signals and results in caspase-1 activation and subsequent IL-1β activation and secretion. The first characterization of an inflammasome complex was made in 2002 in a cell-free system using THP-1 cell extracts [1]. This assay, which is based on the hypotonic lysis of cells in low-potassium buffers (<70 mmol/L), had been adapted from previous studies that characterized and studied the apoptosome [3, 4]. The apoptosome is formed upon binding of cytochrome c to APAF-1, leading to its oligomerization and further recruitment and activation of the apoptotic protease, caspase-9. While the assembly and activation of the apoptosome in cell-free extracts requires the addition of exogenous cytochrome c or its release

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from damaged mitochondria, the activation of the inflammasome occurs spontaneously, suggesting that its ligand or trigger is activated or released by mechanical disruption of the cell integrity.

Because the study of the inflammasome using the cell-free assay requires a relatively large amount of cells, the use of immortalized cells facilitates the setup of this assay. However, in most immortalized cell lines the ASC promoter is highly methylated and the transcription of ASC is repressed [5]. One exception is the human monocytic cell line, THP-1, that expresses ASC and is therefore, among human cell lines, the one that best responds to inflammasome activation, including in cell-free conditions [1]. Interestingly, caspase-1 was first detected and purified from THP-1 lysates prepared in buffers containing low-potassium, years before the first description of the inflammasome [6–9]. It is therefore likely that the preparation of the cell extracts in these landmark papers led to the spontaneous assembly and activation of the inflammasome that resulted in the observed caspase-1 activity. Indeed THP-1 cells do not activate caspase-1 or secrete processed IL-1β at basal conditions.

Over the years, the cell-free assay has been used in a few studies that investigated some aspects of the inflammasome. For example, it was used to demonstrate the key role of ASC in THP-1 cells as well as in primary mouse macrophages [1, 10]. It was then used to study the rates of caspase-1 activation in THP-1 cells and Bac1 mouse macrophages [11, 12]. This system has also been used to demonstrate the role of low-potassium concentration on inflammasome activation [13].

This system remains of interest for the study of inflammasome activation and regulation since it allows: (1) rapid, synchronized, and strong detection of inflammasome activation, caspase-1 cleavage, and IL-1β processing; (2) easy manipulation of the system with the addition of putative activators or inhibitors in the cell lysates; and (3) analysis of the biochemistry of the complex including its composition at definite time points following activation, without need for further membrane crossing.

Here, we describe the successive steps required to activate the inflammasome in cell-free extracts obtained from THP-1 monocytes and give examples of available read-outs.

2 Materials

Solutions are to be prepared using ultrapure and deionized water. All reagents, including solutions, tubes, and syringes, should be brought to a temperature of 4 °C unless indicated otherwise.

1. 22G needles.
2. 1 mL syringes.

3. Pasteur pipettes.
4. Thermoshaker block for microtubes, as the ThermoMixer® (Eppendorf) or equivalent such as the Thermo-Shaker® (Biosan) or the ThermoCell® Mixing Block (Bioer).
5. THP-1 human monocytic cell line. (THP-1 cells are cultured to a density of about 1.5×10^6 cells/mL.)
6. 150-cm^2 plastic flasks for cell culture or, alternatively, 2-L roller bottles system for cell culture.
7. Sterile 50 mL conical tubes.
8. 1.5 mL microfuge tube.
9. Refrigerated centrifuge and microfuge.
10. Complete cell culture media: Roswell Park Memorial Institute (RPMI) 1640 media complemented with 10 % heat-inactivated fetal calf serum (FCS) and 100 μg/mL each of penicillin/streptomycin (*see* **Note 1**).
11. Sterile phosphate buffered saline solution (PBS): 137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4.
12. Buffer W: 20 mM HEPES–KOH at pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, and 0.1 mM PMSF (*see* **Note 2**). Since PMSF is unstable in water, it should be added freshly for each experiment (PMSF can be stored for longer time in isopropanol or ethanol). Store Buffer W at 4 °C (*see* **Note 3**).
13. Buffer A: 20 mM HEPES–KOH at pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, and 320 mM sucrose.
14. CHAPS buffer: 20 mM HEPES–KOH at pH 7.5, 5 mM $MgCl_2$, 0.5 mM sodium EGTA, 0.1 mM PMSF, and 0.1 % CHAPS. PMSF should be added freshly for each experiment.
15. Disuccinimidyl suberate (DSS), prepare fresh each time in dimethyl sulfoxide (DMSO) according to the manufacturer's instructions.
16. Antibody dilution buffer: 5 % skimmed milk.
17. 1:1000 dilution of each primary antibody: mouse monoclonal antihuman caspase-1 (#AG-20B-0048-C100, AdipoGen), rabbit polyclonal anti-ASC (AL177, #AG-25B-0006-C100, AdipoGen), and mouse monoclonal antihuman IL-1β (MAB201, R&D Systems).
18. 1:10,000 dilution of appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies.
19. Nitrocellulose membranes.
20. 1 M DTT stock solution: dissolve 1.5 g of DTT in 8 mL of water, adjust the final volume to 10 mL with water, and

dispense into 1 mL aliquots. Store the stock solution in the dark, at −20 °C.

21. 4× Laemmli Sample Buffer: for 100 mL mix 3 g of Tris–HCl adjust to pH 6.8, 40 mL of a 40 % solution of glycerol, 5 g of 5 % SDS, and 5 mg of 0.005 % bromophenol blue. Complete with water to 100 mL. Store at room temperature and before use complement with 400 mM DTT from stock solution.
22. 15 % SDS-polyacrylamide resolving gel: for 8 mL mix 2.8 mL of water, 3 mL of 40 % acrylamide/bis-acrylamide solution, 2 mL of 1.5 M Tris–HCl pH 8.8, 80 µL of 10 % SDS, 80 µL of 10 % ammonium persulfate (APS), and 8 µL of TEMED immediately before pouring the gel.
23. Stacking SDS-polyacrylamide gel: for 5 mL mix 2.9 mL of water, 750 µL of 40 % acrylamide/bis-acrylamide solution, 1.25 mL of 0.5 M Tris–HCl pH 6.8, 50 µL of 10 % SDS, 50 µL of 10 % APS, and 5 µL of TEMED immediately before pouring the stacking gel.
24. Acrylamide electrophoresis system.
25. Semidry or wet transfer system.

3 Methods

3.1 Cell-Free Lysates Preparation and Inflammasome Activation

Carry out all procedures on ice unless otherwise specified. A schematic diagram of the cell-free assay is provided in Fig. 1.

1. Harvest 100 million of THP-1 cells (*see* **Note 4**) in 50 mL tubes and centrifuge the cells at 4 °C for 5 min at 400 × *g*. Wash the pellets by aspirating the supernatant, adding 2 mL of ice-cold PBS, and centrifuging the tubes again 5 min at 400 × *g*. Discard the supernatant, centrifuge the pellets one more time

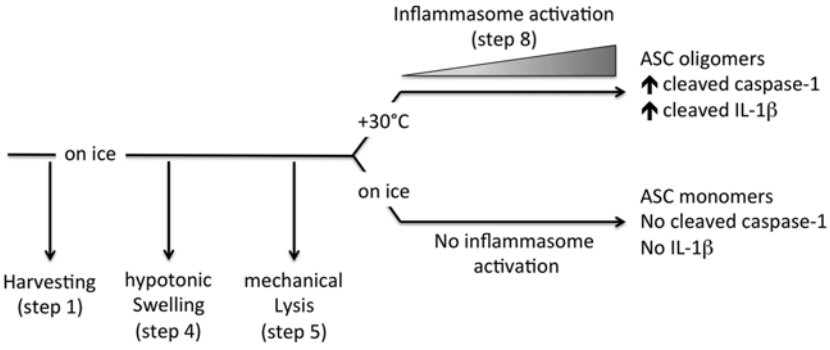

Fig. 1 Schematic diagram of the cell-free assay. After hypotonic swelling and mechanical disruption of the plasmatic membrane, the inflammasome complex is spontaneously activated in the lysates of THP-1 cells, upon incubation at 30 °C

for 2 min at 400 × *g* and remove the remaining liquid with a Pasteur pipette (*see* **Note 5**).

2. Add 2 mL of ice-cold buffer W, without resuspending cells. Centrifuge the tubes for 5 min at 400 × *g*. Discard the supernatant.
3. Estimate the volume of the dry pellet (*see* **Note 6**) and add 3× that volume of ice-cold buffer W (*see* **Note 7**). Resuspend the cells by gentle pipetting up and down. Transfer the resuspended cells to a prechilled 1.5 mL microfuge tube. Keep cells on ice.
4. Incubate 10 min on ice to allow the swelling of the cells.
5. Mechanically disrupt cell membranes by 15 passages (*see* **Note 8**) through a 22G needle, with the 1 mL syringe. Work rapidly and stay on ice to avoid the activation of the inflammasome and avoid the generation of air bubbles.
6. Centrifuge the tubes 5 min at 13,000 × *g*, at 4 °C.
7. Harvest the supernatant in a prechilled 1.5 mL microfuge tube. The negative control tube is to be left on ice or at −20 °C. At this time, protein concentration can be checked and adjusted using commercial kits (*see* **Note 9**).
8. Aliquot the samples in experimental tubes using at least 25 µL of lysate per condition (*see* **Note 10**).
9. Incubate the sample tubes during 30–120 min at 30–37 °C (*see* **Note 11**), with agitation set at 400 rpm, in a thermoshaker block (*see* **Note 12**).
10. The process can be stopped at any time, either by freezing the lysates at −20 °C or by adding denaturing buffers (Laemmli sample buffer or buffer A) (*see* **Note 13**).

3.2 Read-Outs

3.2.1 Caspase-1 Cleavage and Pro-IL1β Processing

1. Dilute 4× Laemmli sample buffer (containing 400 mM DTT) to 1× with the samples and the negative control from **step 10** of Subheading 3.1.
2. Boil samples for 5 min.
3. Fractionate samples by electrophoresis on a 15 % SDS-polyacrylamide gel.
4. Transfer proteins into a nitrocellulose membrane.
5. Probe membranes with antibodies for caspase-1 and IL-1β. Processing of these two proteins can then be analyzed, pro-caspase-1 cleavage results in 20 kDa (p20) fragment of active caspase-1 (Fig. 2), and pro-IL-1β processing results in 17 kDa (p17) active fragment of IL-1β.

3.2.2 ASC Assembly Assay

1. Save a 10 % aliquot of each incubated sample from **step 10** of Subheading 3.1 for future Western blot analysis of total ASC.

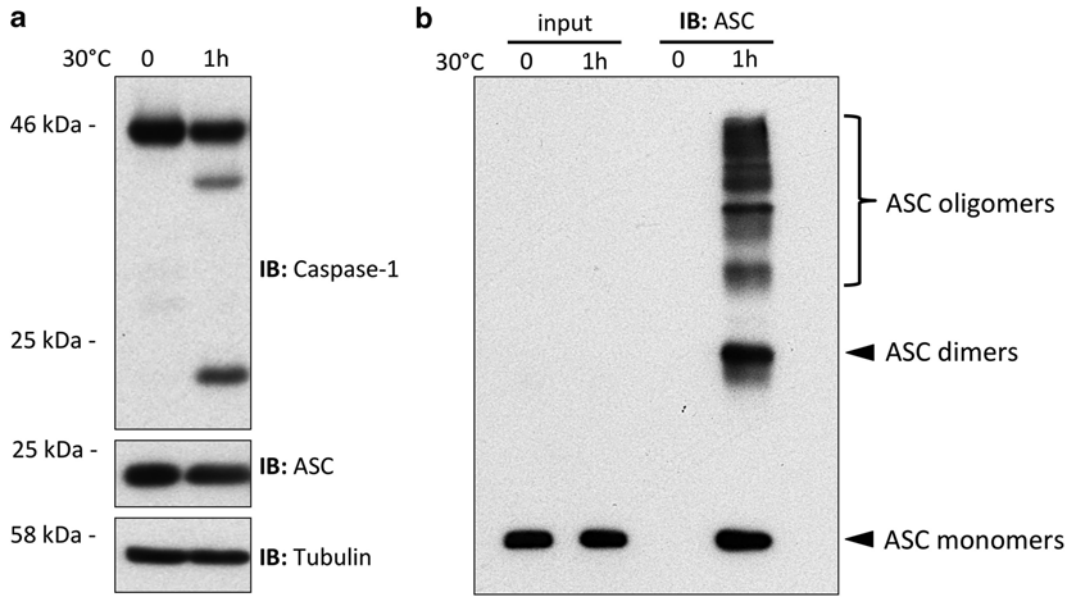

Fig. 2 Possible read-outs for inflammasome activation, using the cell-free assay. (**a**) In THP-1 cell lysates, obtained after hypotonic swelling and mechanical disruption, the inflammasome was activated upon incubation at 30 °C during 1 h, resulting in caspase-1 cleavage (20 kDa band). (**b**) Inflammasome activation can also be demonstrated by the formation of ASC aggregates (oligomers) upon incubation at 30 °C during 1 h

2. In each sample, add twice the sample volume of buffer A and centrifuge the lysates at 2000 × *g* for 5 min at 4 °C.
3. Collect the supernatant in a clean 1.5 mL microfuge tube. Dilute the supernatant with 1 volume of CHAPS buffer and centrifuge at 5000 × *g* for 8 min to pellet the ASC oligomers. Discard the supernatant.
4. Wash the ASC aggregates by resuspending the crude pellets in 1 mL of CHAPS buffer, pipet up and down, and centrifuge the tubes for 8 min at 5000 × *g*. This step should be repeated twice.
5. After three washes, discard the supernatant and resuspend the pellets in 30 μL of CHAPS buffer.
6. Crosslink by adding 4 mM DSS and incubate 30 min at room temperature.
7. Quench the reaction with 30 μL of 2× Laemmli buffer without DTT. Add 2× Laemmli buffer without DTT to the 10 % aliquot from **step 1**.
8. The ASC assembly can then be analyzed by Western blot after boiling and fractionating on a 15 % SDS-polyacrylamide gel and using anti-ASC antibody. The assembly of aggregated ASC is detected as monomers (22 kDa), together with dimers, and high molecular weight oligomers (Fig. 2).

4 Notes

1. Other cells that express the inflammasome components (including ASC) can be used. For example, this assay has been successfully reported in thioglycolate-elicited mouse peritoneal macrophages [10], in mouse Bac1 macrophages [11], and in J2-immortalized mouse bone marrow-derived macrophages [[13]; *unpublished personal data*].
2. Buffer W refers to buffer A reported in Xiaodong Wang's landmark studies characterizing the apoptosome complex in a cell-free system [4, 14, 15].
3. A buffer W with 150 mM KCl can be used as negative control, since inflammasome is only activated with potassium concentration below 70 mmol/L. However because the addition of potassium may affect the hypotonic lysis, it is recommended to add the extra KCl after mechanical lysis of the cells (**step 5** of Subheading 3.1).
4. This amount of cells is optimized for this protocol to obtain a good lysis and a reproducible caspase-1 activity, however the assay can be scaled-up or down.
5. Remaining PBS and media may affect the efficiency of the hypotonic lysis.
6. To estimate the volume of the dry pellet, place an empty tube next to the tube containing the pellet, add the same volume of a solution (water) to reach the level of the pellet. Measure the volume of the solution with a pipette.
7. The volume of buffer W can be increased up to 5× the volume of the dry pellet. This may decrease the activity of the lysate but may facilitate downstream applications.
8. The number of passages can vary from 10 to 20, depending on the volume to lyse and the experimenter.
9. For example, the Quick Start™ Bradford Protein Assay (Bio-Rad) can be used. Use the "standard protocol" with 10–100-times dilutions of each sample in buffer W. Bovine serum albumin is used for the standard range. Pipet 5 μL of each sample into separate microplate wells. Dispense 250 μL of 1× dye reagent in each well with multichannel pipet. Depress the plunger repeatedly to mix the sample and reagent in the wells. Incubate 5 min at room temperature. Read the microplate using a spectrophotometer with wavelength set at 595 nm. Refer to manufacturer's protocol for data analysis.
10. We typically performed experiments using 25–500 μL of lysate per condition without observing obvious differences in the activity per μL. The protein concentration of the lysates typically ranges from 5 to 10 μg/μL.

11. In THP-1 lysates the activation occurs within minutes. A 30 °C incubation temperature should be preferred to limit protein degradation. Note that the assay can be performed at 37 °C, this may result in stronger activity depending on the cell line.
12. Strong activation can also be observed using a water bath incubator rather than with the thermoshaker block.
13. Laemmli sample buffer can be used if the samples are directly loaded on a polyacrylamide gel electrophoresis for Western blotting. Buffer A is used if the samples are analyzed for ASC oligomerization. Additionally, RIPA buffer can be used in other biochemical procedures such as immunoprecipitations and molecular pull-down assays.

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