

Assessing Caspase-1 Activation

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Abstract

The caspase-1 enzymatic activity plays a major role in the innate immune response as it regulates the maturation of two major proinflammatory cytokines, the interleukin-1beta (IL-1 β) and IL-18. In this chapter, we describe the technique of Western blot to assess caspase-1 activation. This method provides multiple information within one experiment. It allows the detection of both unprocessed and processed caspase-1 and substrates.

Key words Western blot, Inflammasome, Interleukin-1beta (IL-1 β), Caspase-1

1 Introduction

Caspase-1 is an enzyme belonging to a family of cysteine proteases that cleave their substrates after an aspartic acid residue. Like apoptotic initiator caspases (e.g., caspase-8 or caspase-9), it is synthesized as a zymogen. Caspase-1 consists of three main domains, a Caspase Activation and Recruitment Domain (CARD) at the N-terminus of the protein followed by two catalytic subunits, the p20 and the p10 (Fig. 1). As an initiator caspase, caspase-1 gets activated within a multi-protein complex, named the “inflammasome.” The inflammasome acts as a molecular platform inducing the zymogen dimerization to initiate caspase-1 activation by auto-proteolysis. The freed subunits assemble into dimers of p20/p10 to form the active caspase-1 [1, 2]. The best-characterized substrates of caspase-1 are the proinflammatory cytokines, the pro-interleukin-1 β (pro-IL-1 β) and pro-IL-18. Their cleavage by caspase-1 results in the production and secretion of the biologically active cytokines IL-1 β and IL-18. Of note, upon inflammasome activation, the p20/p10 dimers of caspase-1 are secreted together with the mature substrates. This is the reason why cell supernatants are analyzed by Western blot.

Inflammasomes are major actors of the innate immune response. They are expressed by myeloid cells such as macrophages



Fig. 1 Schematic representation of the full length caspase-1, size 45 kDa, and of the active dimers

and dendritic cells, but also by epithelial cells such as keratinocytes or intestinal cells. Different cytosolic pattern recognition receptors such as nucleotide-binding domain and leucine rich repeat pyrin containing 1 (NLRP1), NLRP3, NLRC4, and absent in melanoma 2 (AIM2) are able to trigger the assembly of an inflammasome upon sensing of specific pathogen-associated molecular pattern (PAMP) or danger-associated molecular pattern (DAMP). The NLRP3 receptor is able to detect a wide range of DAMP, e.g., extracellular ATP and monosodium urate crystals, and PAMP, e.g., *Staphylococcus aureus*, influenza virus, adenovirus and bacterial toxins such as nigericin. The mouse NLRP1b is activated by the lethal toxin of *Bacillus anthracis*, while the NLRC4 detects bacteria like *Salmonella* or *Shigella* [3]. Inflammasome activation is often associated with a specific form of cell death dependent on caspase-1 activity called pyroptosis.

A convenient method to monitor caspase-1 activation is the detection of the autoproteolysis of the protein using the technique of Western blotting. Separation of the different forms of caspase-1 protein using denaturing polyacrylamide gel electrophoresis followed by Western blot allows the visualization of the full length caspase-1 at 45 kDa (zymogen) and of the cleaved fragments of the protein resulting from its autoprocessing, for instance p20 or p10 depending on the epitope recognized by the antibody. Similarly this technique allows the detection of both the immature and mature form of the substrate of caspase-1, for instance the pro-IL-1 β at 35 kDa and the mature IL-1 β at 17 kDa. However, recent studies identified specific situations where caspase-1 is active despite the absence of autoprocessing. Thus, for some situations, to assess caspase-1 activity, monitoring its autoprocessing is not sufficient and assessing substrate cleavage and the induction of cell death are also required [4, 5]. We therefore include a simple method to monitor cell death, the measurement of lactate dehydrogenase released in the medium.

In this chapter, we activate caspase-1 by triggering the formation of the NLRP3 and NLRP1b inflammasomes in mouse peritoneal macrophages. This method can be applied to bone marrow derived

macrophages and dendritic cells, and even to human macrophages, using adequate antibodies. It can also be applied to monitor the activation of other inflammasomes using cognate activators.

2 Materials

2.1 Cells and Inflammasome Stimulation

All buffers and solutions must be prepared using ultrapure water.

1. Mouse peritoneal macrophages from WT, and ideally, from *caspase-1* deficient (KO) mice that will serve as negative control (for method *see* ref. 5). If no caspase-1 KO mice are available, the caspase-1 inhibitor Z-YVAD-fmk can be used.
2. 12-well plates treated for tissue culture.
3. Dulbecco's modified Eagle's medium (DMEM) cell culture medium complemented with 10 % (vol/vol) decomplemented fetal bovine serum, 1 mM sodium pyruvate, 100 IU/ml penicillin/streptomycin, and 2 mM glutamine.
4. Opti-MEM® medium (this medium is exclusive of Life Technologies); other serum free media could be also used.
5. Phosphate buffered saline solution (PBS): 137 mM NaCl, 2.7 mM KCl, 1 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4.
6. Ultrapure *Escherichia coli* 0111:B4 lipopolysaccharide (LPS).
7. Stock solution of 10 mM nigericin in ethanol.
8. 1 mM adenosine triphosphate (ATP) stock solution in water (*see* **Note 1**).
9. Lethal toxin (LT) from anthrax: the stock is composed of 1 µg/µl of lethal factor mixed with 1 µg/µl of protective antigen [5].
10. 6 mM EDTA in PBS buffer: dilute 0.5 M EDTA stock solution in PBS.

2.2 Protein Extractions

1. RIPA lysis buffer stock 2×: Mix 5 ml of 1 M Tris-HCl pH 7.5, 3 ml 5 M of NaCl, 10 ml of 10 % sodium deoxycholate, 0.15 ml of 20 % SDS, 1 ml of 0.5 % Triton X-100, 1 ml of 0.5 M EDTA, and 0.2 ml of 1 M sodium orthovanadate. Add two tablets of Roche protein inhibitor cocktail; other protein inhibitors cocktails are also suitable. Make the volume up to 50 ml with H₂O. Mix well. Aliquot 0.5 ml in 1.5 ml microtubes and store at -80 °C.

2.3 SDS-PAGE

1. Bio-Rad mini-PROTEAN Tetra cell electrophoretic system (or equivalent).
2. Glass plates with 1.5 mm spacer, 15-well combs.
3. Solution of premixed 30 % ratio 37.5:1 acrylamide-bis-acrylamide.

4. 10 % (wt/vol) ammonium persulfate (APS) solution in water.
5. *N,N,N,N'*-tetramethyl-ethylenediamine (TEMED).
6. Stacking gel buffer: 0.5 M Tris-HCl pH 6.8: Weigh 60 g of tris base, add 0.5 L of H₂O, mix using magnetic stirrer, adjust pH to 6.8 using HCl (*see Note 2*) and add H₂O up to 1 L. Store at +4 °C.
7. Separating gel buffer 1.5 M Tris-HCl pH 8.8: Weigh 181.5 g tris base, add 0.7 L of H₂O, mix, adjust pH to 8.8 using HCl (*see Note 2*), make up to 1 L with H₂O. Store at +4 °C.
8. Separating gels: for two 15 % acrylamide mini-gels mix 4.8 ml of H₂O, 10 ml of 30 % acrylamide-bis-acrylamide, 5 ml of separating gel buffer, 0.1 ml of 20 % SDS, 0.2 ml of 10 % APS, 0.01 ml TEMED.
9. Stacking gels: for two 5 % acrylamide mini-gels mix 5 ml H₂O, 1.7 ml of 30 % acrylamide-bis-acrylamide, 2.5 ml of staking gel buffer, 0.05 ml of 20 % SDS, 0.1 ml of 10 % APS, 0.01 ml TEMED.
10. Running (migration) buffer 10× stock solution: Weigh 60.6 g of tris base, 288 g of glycine, add 0.1 L of 20 % SDS, make up to 2 L with H₂O. Mix.
11. 3× Laemmli buffer (LB): Weigh 60 mg phenol red (*see Note 3*), add 69 ml of glycerol, 37.5 ml of 1 M Tris-HCl pH 6.8 and 60 ml of 20 % SDS. Make up to 200 ml using H₂O. Mix well. Verify final pH is 6.8 and adjust if necessary. Just before adding the LB to the protein extracts, add 100 mM DTT to reach final concentration.
12. 1 M dithiothreitol (DTT): Dissolve 15.45 g of DTT in H₂O, aliquot into microtubes and store at -20 °C.
13. Protein ladder.

2.4 Western Blot and Antibody Incubation

1. Bio-Rad Mini Transblot electrophoretic system (or equivalent).
2. Transfer buffer×1 (store at +4 °C): weigh 25 g of tris base, 120 g of glycine, add 8 L of H₂O, mix. Adjust pH to 8.3 using HCl, add 1.6 L of pure ethanol, make up the volume to 10 L with H₂O.
3. 10× stock solution of tris buffer saline Tween (TBST) (store at +4 °C): 500 ml of 1 M tris base pH 7.5, 10 ml of Tween 20, 300 ml of 5 M NaCl, and 190 ml of H₂O.
4. Nitrocellulose membranes with 0.22 μm pore size.
5. Whatman paper, cut into 7.5×7.5 cm pieces.
6. Ponceau S.
7. Nonfat dry milk.
8. Blocking buffer: 5 % (wt/vol) nonfat dry milk in TBST 1×.

9. Primary antibodies: 1:500 dilution of anti-caspase-1 Casper-1 mouse (Adipogen), 1:1000 dilution of anti-IL-1 β IL-1F2 (R&D Systems).
10. Secondary antibodies coupled to the horseradish peroxidase (HRP): 1:5000 dilution of donkey anti-goat or donkey anti-mouse.
11. Chemoluminescence substrate for HRP.
12. Frozen ice pack for the transfer.

2.5 Cell Death Assay

1. Lactate dehydrogenase (LDH) activity assay kit, such as the CytoTox 96[®] Non-Radioactive Cytotoxicity Assay (Promega). However, other brands are also suitable. Prepare LDH substrate mix following manufacturer's instructions.
2. Flat-bottom 96-well plate.

3 Methods

3.1 Activation of the Inflammasome

1. Plate the WT and *caspase-1* KO macrophages at a density ranging from 8×10^5 to 10^6 cells in a 12 well-plate in 1 ml of complete DMEM. Make sure to seed the adequate number of wells; do not forget to seed an extra control well that will be used as a positive control for the LDH assay. After 2 h, change the medium to remove the non-adherent cells.
2. Prime the cells overnight by stimulating them in complete DMEM containing 0.5 $\mu\text{g}/\text{ml}$ of LPS. This step allows the induction of the production of the pro-IL-1 β and of the NLRP3 proteins.
3. The following morning, remove the medium and replace it with 1 ml of Opti-MEM (*see Note 4*). Then stimulate the cells to activate the inflammasome by adding either 10 μM nigericin per well for 2–4 h or 5 mM ATP for 30 min or 1 $\mu\text{g}/\text{ml}$ LT for 6 h, or the solvent to the control wells. If using Z-YVAD-fmk, add the inhibitor at 50 μM 30 min prior to the stimulation.

3.2 Sample Preparation

1. Collect each 1 ml of cell supernatant into labeled microtubes and centrifuge gently at $1500 \times g$ to get rid of any dead cells. Remove the supernatant and store it in 2 aliquots of 0.5 ml at -20°C (1 for protein precipitation and 1 for other applications such as LDH assay or ELISA)
2. Wash the cells with PBS and then incubate the cells in 0.5 ml of 6 mM EDTA in PBS for 5 min on ice. Scrape the cells and collect the cells into a clean microtube. Pellet the cells at $1500 \times g$ in a refrigerated centrifuge. Discard the supernatant, store the dry cell pellet at -80°C or proceed directly to the cell lysis.

3. Cell extracts: Make the RIPA buffer 1× by adding 0.5 ml of water, and lyse the cells in 40 μ l of buffer. Incubate on ice for 30 min and centrifuge at +4 °C at 15,800×*g*. Collect the cell protein extract into a clean tube (optional: measure the protein concentration using the Bradford assay and use bovine serum albumin as a standard). Add Laemmli buffer containing DTT to the extracts, heat for 5 min at 95 °C. Load directly onto SDS-PAGE or store at -80 °C.
4. Supernatants: to analyze the protein contents of cell supernatants, we use the methanol/chloroform protein precipitation method. This part should be conducted under a fume hood. To 0.5 ml of cell supernatant, add 0.1 ml of chloroform and 0.5 ml of methanol. Vortex. Centrifuge for 3 min at 15,800×*g*. The white protein disk is visible at the interphase between the aqueous and organic phase. Discard most of the upper phase without disturbing the protein pellet. Add 0.5 ml of methanol, vortex, and centrifuge for 3 min at 15,800×*g*. The protein pellet should be stacked at the bottom of the tubes. Discard the supernatant. Leave the pellets to air-dry under a fume hood to remove any trace of solvent. Resuspend the proteins in 50 μ l of LB 3× containing DTT (*see Note 5*). Heat at 95 °C for 5 min. Load directly onto gels or store at -80 °C.

3.3 SDS-Polyacrylamide Gel Casting and Electrophoresis

1. Assemble the glass plates (1.5 mm spacer) using the casting system of miniPROTEAN electrophoretic system.
2. Prepare Separating gels.
3. Mix well.
4. Pour 7.5–8 ml of gel preparation into the plates and gently overlay the gel with 0.5 ml water. Leave to polymerize (20 min).
5. Prepare the stacking gels.
6. Mix well.
7. Remove the water from the surface of the gels and pour the stacking gels on top of the separating gels. Quickly add the combs to the stacking gel. Wait until gels are polymerized (*see Note 6*).
8. Install the glass plates containing the gels into the migration tank, fill with running buffer 1× and gently remove the combs.
9. Load the gels: add the protein ladder to one well and load 20 μ g of samples (or 15–20 μ l) per well. Top up the tank with running buffer 1× if necessary (*see Note 7*).
10. Start the electrophoresis at 70 V for 20 min then increase the voltage to 100 V for 90 min. Regularly check the migration. Another method is to electrophorese by applying 35 mA/gel.

3.4 Western Blot, Wet Method

1. Prepare a tank containing transfer buffer and soak the foam pads and membranes in this buffer.
2. Uncast the gels and remove the gels from the glass plates carefully.
3. Prepare the transfer sandwich by stacking 1 foam pad, 2 Whatman papers soaked into the transfer buffer, the gel (we remove the stacking gel before the transfer), the nitrocellulose membrane, and 2 Whatman papers soaked in the transfer buffer, on the black lid of the transfer cassette (Fig. 2). Get rid of any bubbles by gently rolling the top of the pile with half of a 5 ml pipette. Add another foam pad and close the cassette, place it into the transfer cell, taking care to put the cassette in the proper orientation with regards to the electrodes (with the Bio-Rad system the black side of the cassette toward the black side of the transfer cell). Add one ice block to the transfer tank and fill with transfer buffer (*see Note 8*).
4. Transfer at 100 V for 1 h.

3.5 Probing and Detection

1. Open the transfer cassettes to collect the membranes.
2. Optional: you may want to incubate your membrane in a Ponceau S solution before the blocking step. The Ponceau S stains the proteins and is useful to visualize the quality of the protein transfer and the protein load of each well. Incubate the membranes in a solution of Ponceau S for 5 min under agitation, then rinse briefly several times with water.
3. Block the membranes by incubating them in the blocking buffer for 1 h at room temperature and under gentle agitation.
4. Incubate the membranes overnight in the primary antibodies diluted in the blocking buffer at +4 °C under gentle agitation.
5. Proceed to the washing step: add at least 10 ml of TBST 1× for 15 min at room temperature under agitation. Discard the buffer and repeat this step three times.
6. Incubate the membranes in the secondary antibody solution. Dilute the appropriate horseradish-coupled antibody in 1 % milk TBST.
7. Wash the membranes with 10 ml of TBST 1× for 15 min at room temperature under agitation. Discard the buffer and repeat this step three times.
8. Incubate the membranes in HRP substrate. For detection of IL-1 β and caspase-1, we incubate the membranes for 5 min in the luminescence HRP substrate solution diluted 1:2 with water (*see Note 9*).
9. Expose the membranes to autoradiography films or to a gel imager (*see example results in Fig. 3*) (*see Note 10*).



Fig. 2 Schematic representation of the sandwich assembly for the Western blot

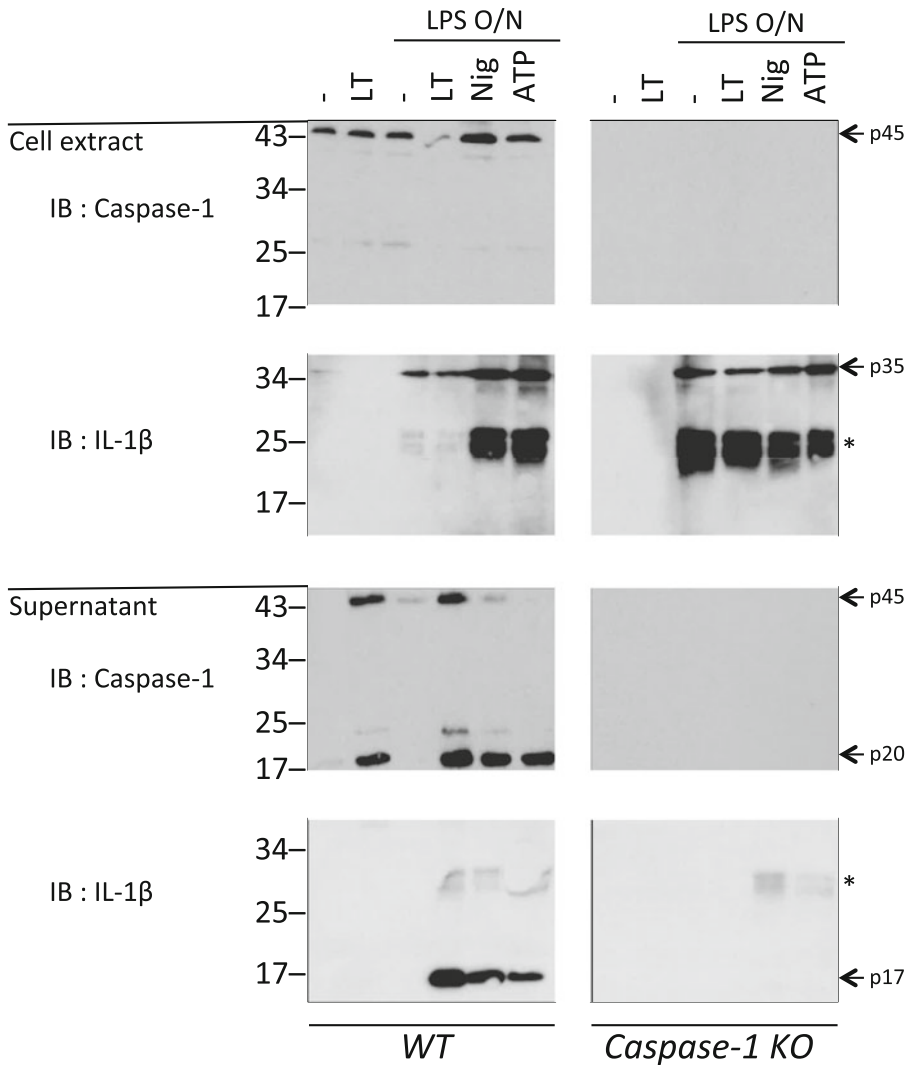


Fig. 3 Immunoblots showing caspase-1 autoproteolysis and IL-1 β cleavage in response to the NLRP1b and NLRP3 inflammasome activation in WT and *caspase-1* KO peritoneal macrophages. Caspase-1 and IL-1 β are shown in cell extracts and cell supernatants. O/N overnight, LT lethal toxin, Nig nigericin, – untreated, * unspecific signal

3.6 LDH Assay

This assay is carried out following the manufacturer's instructions. It is used to detect the cell death induced by caspase-1 activation.

1. Make sure to set up the experimental controls: 1 well left untreated for cell spontaneous LDH release and 1 well treated with a lysis solution for cell maximum LDH release.
2. Transfer 50 μ l of cell supernatant to a flat-bottom 96-well plate.
3. Add 50 μ l of the reconstituted LDH Substrate Mix to each well of the plate, cover and incubate at room temperature, protected from light, up to 30 min.
4. Add 50 μ l of the Stop Solution to each well of the plate.
5. Read the absorbance at 490 nm.

LDH release percentage is calculated by the following formula:

$$\left(\frac{[\text{LDH treated}] - [\text{LDH untreated}]}{[\text{LDH total lysis}] - [\text{LDH untreated}]} \right) \times 100$$

4 Notes

1. Make small aliquots of ATP stock and always use a new aliquot for inflammasome stimulation.
2. Use 12 N HCl to get close to the desired pH then use 6 N HCl to finish the adjustment.
3. Phenol red can be replaced by bromophenol blue. We find convenient, for instance, to use a blue LB for cell extracts and a red LB for cell supernatants.
4. Opti-MEM[®] medium allows a cleaner protein electrophoresis as it contains very few proteins.
5. It is also possible to resuspend the protein pellet in 30 μ l of RIPA buffer 1 \times and then add LB.
6. The casted gels can be stored for 10 days immersed in a box containing running 1 \times buffer at +4 $^{\circ}$ C.
7. If many wells are empty, we recommend adding 15 μ l of LB 3 \times to these empty wells to ensure an even migration of the samples.
8. As the best transfer is obtained with cool buffer, do not remove buffer from the fridge until immediately prior to filling tanks. Some people of the team like to transfer in the cold room.
9. We use the LuminataTM Crescendo Western HRP substrate (Millipore). This HRP substrate is convenient as it is premixed. Furthermore, depending on the abundance of the protein detected and on the specificity of the primary antibody, it is possible to adapt the substrate concentration by diluting it

with water and to also adapt the time of membrane incubation to modulate the intensity of the signal.

10. The membranes can be re-hybridized with another antibody to detect another protein without being stripped if, the size of the other protein of interest to be detected is different and if the primary antibody species is different. To that purpose, we block the membranes as described in Subheading 3.5 and add 0.05 % of sodium azide to the buffer to inactivate the HRP. For instance, the anti-caspase-1 antibody can be re-hybridized following the hybridization with the anti-IL-1 β antibody.

Acknowledgement

We thank Sarah Karabani for proofreading the manuscript. V. Petrilli is supported by the ITMO cancer and the Ligue contre le cancer comité de l'Ain.

References

1. Walker NP et al (1994) Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a (p20/p10)₂ homodimer. *Cell* 78:343–352
2. Elliott JM, Rouge L, Wiesmann C, Scheer JM (2009) Crystal structure of procaspase-1 zymogen domain reveals insight into inflammatory caspase autoactivation. *J Biol Chem* 284:6546–6553
3. Lamkanfi M, Dixit VM (2012) Inflammasomes and their roles in health and disease. *Annu Rev Cell Dev Biol* 28:137–161
4. Broz P, von Moltke J, Jones JW, Vance RE, Monack DM (2010) Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. *Cell Host Microbe* 8:471–483
5. Guey B, Bodnar M, Manié SN, Tardivel A, Petrilli V (2014) Caspase-1 autoproteolysis is differentially required for NLRP1b and NLRP3 inflammasome function. *Proc Natl Acad Sci U S A* 111:17254–17259