Chapter 9

Immunoblotting for Active Caspase-1

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Abstract

Immunoblotting for caspase-1 is the gold-standard method of detecting inflammasome activation. In contrast to IL-1β-based readouts, it can be used in an experimental setup independent of de novo gene expression. Here, we present protocols for the preparation and precipitation of supernatant samples containing activated caspase-1 as well as protocols for polyacrylamide gel electrophoresis (PAGE) and protein immunoblotting.

Key words Caspase-1, Immunoblot, Western blot, Inflammasome

1 Introduction

A widely used readout for inflammasome and thus caspase-1 activation is the measurement of IL-1β release by ELISA, or the detection of IL-1β cleavage by immunoblotting. The major disadvantage of this approach is that assessment of mature cleaved IL-1β requires the induction of pro-IL-1β expression by a pro-inflammatory priming signal, such as a TLR ligand. However, priming can critically interfere with the subject under study, as various inflammasome complexes also require a pro-inflammatory priming signal [1]. As such, assessing caspase-1 activation directly remains the gold standard readout of inflammasome activation.

Since the proteolytic activation of caspase-1 via the inflammasome is the central step regulating its own proteolytic activity, detection of its subunits p20 or p10 is a good surrogate marker for caspase-1 activity. Currently, the standard technique of assessing caspase-1 cleavage is SDS-PAGE electrophoresis followed by immunoblotting. The activated caspase-1 is blotted from cell

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supernatants after protein precipitation whereas the uncleaved pro-form can be detected directly from cell lysates.

Detecting the caspase-1 subunits p20 or p10 by immunoblot presents certain technical difficulties, particularly with the choice of protein precipitation, protein separation and transfer protocols. Here, we describe several methods and provide specific recommendations for reliable analysis of caspase-1 cleavage by immunoblotting. To illustrate our approach, we use a murine immortalized macrophage cell line stimulated with defined NLRP3 activators (LPS priming followed by stimulation with ATP or Nigercin) [2]. However, this protocol is also amenable to other species, cell types, and of course other stimuli.

2 Materials

Prepare all reagents with ultrapure water and store them at room temperature (RT) unless otherwise specified. Please follow all waste disposal guidelines when disposing of reagents.

2.1 Cell Stimulation

1. 12-well tissue culture (TC) plates.
2. Immortalized murine macrophages (iMØ).
3. Cell culture medium: DMEM supplemented with 1 mM sodium pyruvate and 10 µg/mL ciprofloxacin (*see* **Note 1**). Store at 4 °C.
4. ATP: 500 mM Disodium-ATP dissolved in 500 mM NaOH. Store at −20 °C, and avoid freeze/thaw cycles.
5. Nigericin: 6.5 mM Nigericin stored in ethanol at −20 °C.
6. LPS: 5 mg/mL.
7. 2× SDS sample buffer: 150 mM Tris–HCl, pH 6.8, 200 mM DTT, 4 % SDS, 0.02 % bromophenol blue, 20 % glycerol. Store at −20 °C.

2.2 Protein Precipitation

2.2.1 Methanol–Chloroform Precipitation [3]

1. Methanol.
2. Chloroform.
3. SDS sample buffer (*see* **item 7** of Subheading 2.1).
4. 1.5 mL tubes.

2.2.2 Trichloroacetate Precipitation [4]

1. 20 % Trichloroacetate (TCA).
2. Ice-cold Acetone.
3. 1 M Tris buffer, pH 6.8.
4. SDS sample buffer (*see* **item 7** of Subheading 2.1).
5. Centrifuge at 4 °C.

2.3 Gel Electrophoresis

1. Ammonium persulfate (APS): 10 % solution in H_2O. Store at 4 °C (*see* **Note 2**).
2. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). Store at 4–8 °C.
3. Propanol.
4. 8 cm (W) × 7.3 cm (H) mini gel casting system with plates and combs for a 1.5 mm gel (e.g., Bio-Rad Mini-PROTEAN 3 electrophoresis system).
5. Vertical gel electrophoresis and transfer tank system (e.g., Bio-Rad Mini-PROTEAN 3 electrophoresis system).

2.3.1 Tris-Glycine PAGE

1. 30 % Acrylamide/Bisacrylamide solution (37.5:1) (e.g., Carl Roth: Rotiphorese Gel 30, 3029.X). Store at 4–8 °C.
2. Sodium dodecyl sulfate (SDS): 10 % solution in H_2O (*see* **Note 3**).
3. 1 M Tris–HCl, pH 6.8.
4. 1.5 M Tris–HCl, pH 8.8.
5. 10× SDS Running Buffer: 250 mM Tris, 1.92 M Glycine, 1 % SDS in H_2O. For 1 L: 30.2 g Tris, 144 g Glycine, 10 g SDS and fill up to 1 L with H_2O.

2.3.2 Bis–Tris PAGE

1. 30 % Acrylamide Solution. Store at 4–8 °C.
2. 2 % Bisacrylamide Solution. Store at 4–8 °C.
3. 3.5× Bis–Tris Gel Buffer: 1.25 M Bis–Tris HCl, pH 6.6. To make: 26.16 g Bis–Tris made up to 100 mL with H_2O. Adjust pH to 6.6 with HCl.
4. 10× MES-SDS Running Buffer: 500 mM MES, 500 mM Tris–HCl, 1 % SDS and 10 mM EDTA. To make: 97.6 g MES, 60.58 g Tris–HCl, 10 g SDS made up to 1 L with H_2O (*see* **Note 4**).
5. 200× Running Buffer Reducing Agent: 1 M Na-Bisulfite in H_2O. For 50 mL: Dissolve 9.506 g Na-Bisulfite in 50 mL H_2O. Store at 4 °C.

2.4 Immunoblotting Reagents

1. 0.2 μm Nitrocellulose Membrane (*see* **Note 5**).
2. General-purpose blotting paper or Whatman paper.
3. 1 large measuring cylinder.
4. 1 small plastic container (e.g., the cover of a pipette tip box) for each blot.
5. Wet gel transfer system (e.g., Bio-Rad Protean) and an ice block which fits into the chamber.
6. 10× Tris–Glycine running buffer: 250 mM Tris, 1.92 M Glycine in H_2O. For 1 L: 30.2 g Tris, 144 g Glycine, fill to 1 L with H_2O.
7. 96 % Ethanol, denatured is acceptable for most systems (*see* **Note 6**).

8. Nonfat milk powder to make blocking solution.
9. Tween 20 %.
10. 10× PBS: For 1 L Add 80 g NaCl, 2 g KCl, 26.8 g $NA_2HPO_4{\cdot}7H_2O$, 2.4 g KH_2PO_4 and fill to a final volume of 1 L with H_2O. Adjust pH to 7.4 with HCl.
11. PBS–Tween Solution: 100 mL 10× PBS, 900 mL H_2O, 500 µL 20 % Tween (*see* **Note 7**).

2.5 Antibodies and Developing Tools

1. Primary antibodies detecting murine caspase-1 (*see* **Note 8**).
   - sc-514 (Santa Cruz): Rabbit polyclonal IgG anti p10 subunit, provided at 200 µg/ mL.
   - Casper-1 (Adipogen): Mouse mAb IgG1 anti p20 subunit, provided at 1,000 µg/mL.
2. Secondary antibodies (*see* **Note 9**).
   - sc-2004 (Santa Cruz): goat anti-rabbit IgG-HRP provided at 400 µg/mL.
   - sc-2005 (Santa Cruz): goat anti-mouse IgG-HRP provided at 400 µg/mL.
3. 50 mL conical tubes.
4. ECL Substrate kit.
5. Photographic films.
6. Film developing cassette.
7. Developing machine (*see* **Note 10**).

3 Methods

3.1 Cell Stimulation

1. Seed 5×10^5 immortalized murine macrophages [2] in 12-well plates in culture medium (500 µL/well).
2. Incubate cells overnight.
3. Stimulate the cells as follows.
   - Control: 6 h exposure to 200 ng/mL LPS.
   - ATP: 4 h priming with LPS, then stimulation with 5 mM ATP for 2 h.
   - Nigericin: 4 h priming with LPS, then stimulation with 650 µM Nigericin for 2 h.

After harvesting the supernatants, the remaining cells were washed with PBS and then directly lysed in 150 µL 1× SDS sample buffer per well. For each condition, the supernatants and lysates were pooled separately to minimize loading differences while comparing protocols. Pooled supernatants were centrifuged at 1,000 × *g* for 5 min to remove cellular debris. Both supernatants and lysates were stored at −20 °C until use.

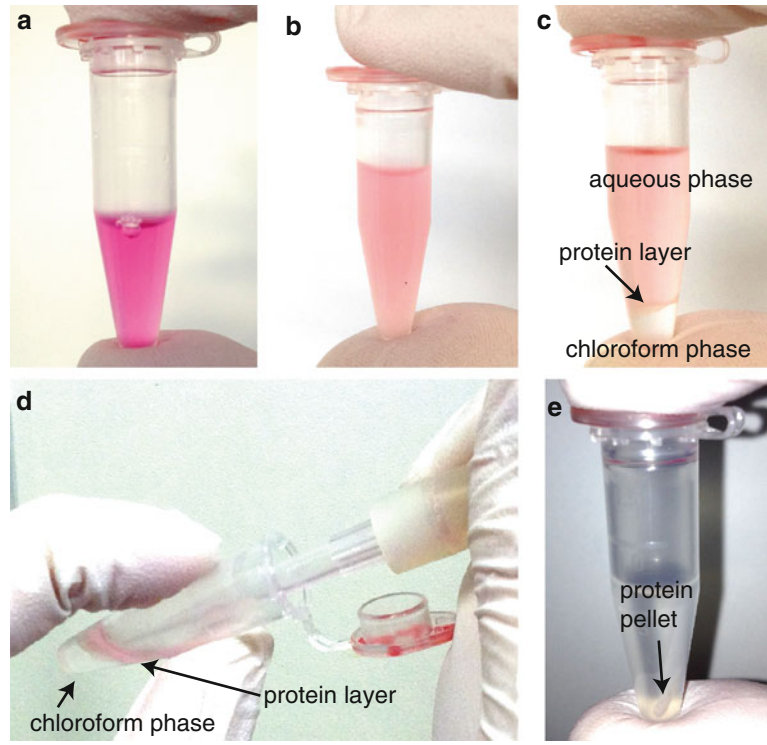

Fig. 1 Chloroform–methanol precipitation. (**a**) 1.5 mL tube containing 500 μL of supernatant from cultured macrophages. (**b**) 500 μL supernatant is mixed with 500 μL of methanol and 125 μL of chloroform and vortexed. (**c**) After centrifugation a protein layer between the aqueous and organic phase is visible. (**d**) The aqueous phase is removed without disturbing the protein layer using a suction device. (**e**) After centrifugation with an additional 500 μL of methanol a protein pellet forms at the site of maximal centrifugation force

3.2 Sample Precipitation

In order to detect cleaved caspase-1 in the supernatant, it is advisable to concentrate the sample via precipitation. From one 12-well TC plate, use 500 μL of supernatant for precipitation (*see* **Note 11**). The samples can be used fresh or thawed after storage at −20 °C.

Although most standard protein precipitation protocols work sufficiently well (*data not shown*), we found that methanol–chloroform precipitation and TCA-precipitation provide the most reliable results (*see* **Note 12**). Thus, we have only included adapted versions of these two protocols in detail. All centrifugation steps are carried out at room temperature unless otherwise indicated.

3.2.1 Methanol/Choloroform Precipitation

1. Add 500 μL methanol and 125 μL chloroform to 500 μL supernatant.
2. Vortex the sample vigorously for at least 30 s. The sample should be a homogenous, milky white suspension (*see* Fig. 1b).
3. Centrifuge the sample at 13,000 × *g* for 5 min.

4. Carefully remove the tubes from the centrifuge. Three phases should be visible: the upper aqueous/methanol phase, a protein layer, and the lower chloroform phase (*see* Fig. 1c).
5. Remove the aqueous phase using a suction device with the tube tilted forward and the tip of the suction device directed to the front portion of the tube (*see* **Note 13**). Be careful not to remove the protein phase or protein clinging to the back portion of the tube (*see* Fig. 1d).
6. Add 500 µL of methanol to each sample.
7. Vortex vigorously. The protein layer should break up into small white flakes.
8. Centrifuge at 13,000 × *g* for 5 min.
9. Carefully remove tubes from the centrifuge. A small, white protein pellet should be visible in all samples (*see* Fig. 1e).
10. As above, using a suction device, remove as much methanol as possible without disturbing the pellet. Try to leave the same amount of methanol in all probes (*see* **Note 13**).
11. Dry the probes for 5–10 min at 55 °C. The pellet will become transparent.
12. Reconstitute the pellet in 40 µL of 1× SDS sample buffer. Do not forget to scrape the back wall of the tube when resuspending the pellet.
13. Vortex the samples vigorously for 1 min.
14. Centrifuge for a few seconds at 13,000 × *g* to settle the contents of the tube.
15. Incubate the samples at 95 °C for 5 min.
16. Vortex the samples vigorously for 1 min.
17. Cool the samples by placing them on ice.
18. Quickly spin tubes (e.g., 30 s centrifugation at 10,000 × *g*) to collect the samples at the bottom of the tubes.

3.2.2 Trichloroacetate Precipitation

1. Add 500 µL of 20 % TCA to 500 µL of supernatant (1:1).
2. Incubate on ice for 30 min.
3. Centrifuge at 12,000 × *g*, 4 °C for 15 min.
4. Wash the samples 3×.
   - Carefully remove the supernatant
   - Add 500 µL ice cold acetone
   - Centrifuge at 12, 000 × *g*, 4 °C for 5 min
5. Remove supernatant and air-dry pellet.
6. Reconstitute the pellet in 40 µL SDS sample buffer (*see* **Note 14**).
7. Vortex and boil samples as in **steps 14–18** of Subheading 3.2.1.

3.3 Polyacrylamide Gel (PAGE) Electrophoresis

Bis–Tris PAGE offers a superior resolution for the separation of low-molecular weight proteins. This is achieved by the operating pH of 7.0 (as opposed to a pH of 9.5 of Tris–Glycine gels), which protects protein samples from deamination and alkylation and therefore increases protein stability during electrophoresis [5] (*see* **Note 15**). Moreover, Bis–Tris gels have the advantage of shorter running times and a longer shelf life. Nevertheless, we have also included a standard Tris–Glycine PAGE protocol because it is a good alternative for labs where Bis–Tris gels are not available. To blot the 10 kDa and 20 kDa activated caspase-1 from supernatants, we recommend using a 10 % Bis–Tris PAGE or a 15 % Tris–Glycine PAGE. For 40 kDa pro-caspase-1 electrophoresis, we recommend using an 8 % Bis–Tris gel or a 12 % Tris–Glycine PAGE.

3.3.1 Bis–Tris-PAGE Electrophoresis

1. Prepare 8 mL of 10 % resolving gel by mixing 656 µL H_2O, 2.5 mL 30 % acrylamide, 2.5 mL 2 % Bisacrylamide, 2.286 mL 3.5× Bis–Tris gel buffer, 50 µL APS and 8 µL TEMED (*see* **Note 16**). Vortex the mixture.
2. Cast gel in the assembled mini-gel chamber with 1.5 mm spacers. Layer the gel with 500 µL isopropanol. The gel will need around 10 min to harden.
3. Prepare 4 mL of 4 % stacking gel by mixing 1.83 mL H_2O, 500 µL 30 % Acrylamide, 500 µL 2 % Bisacrylamide, 1.14 mL 3.5× Bis–Tris gel buffer, 25 µL APS and 4 µL TEMED (*see* **Note 16**).
4. Remove isopropanol and layer the stacking gel over the resolving gel. Immediately add the 1.5 mm, 15-well comb to the gel without introducing air bubbles.
5. Prepare 1× MES-SDS running buffer from 10× MES-SDS stock solution (*see* **item 4** of Subheading 2.3.2) and add 0.005 % of 200× reducing agent immediately before use.
6. When the gel has hardened, remove the comb and assemble the electrophoresis chamber. Pour the running buffer in both chambers and load the samples.
7. Run the gel for ~1 h at 100 V.

3.3.2 Tris–Glycine-PAGE Electrophoresis

1. Prepare 8 mL of 15 % resolving gel by mixing 1.84 mL H_2O, 4 mL 30 % Acrylamide/Bisacrylamide solution, 2 mL 1.5 M Tris–HCl pH 8.8, 80 µL SDS solution, 80 µL APS solution and 8 µL TEMED (*see* **Note 16**).
2. Cast gel in the assembled mini-gel chamber with 1.5 mm spacers. Layer the gel with 500 µL isopropanol. The gel will need around 10 min to harden.
3. Prepare 4 mL of 5 % stacking gel by mixing 2.72 mL H_2O, 0.68 mL 30 % Acrylamide/Bisacrylamide solution, 0.52 mL 1 M Tris–HCl pH 6.8, 40 µL SDS solution, 40 µL APS solution and 4 µL TEMED (*see* **Note 16**).

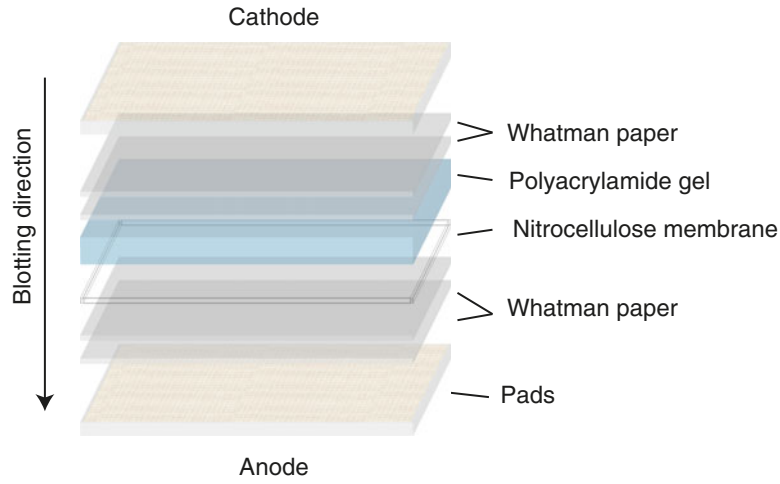

Fig. 2 Assembly for blotting. A schematic view of the assembly setup for the blotting procedure is shown

4. Remove isopropanol and layer the stacking gel over the resolving gel. Immediately add the 1.5 mm, 15-well comb to the gel without introducing air bubbles.
5. Prepare 1 L of 1× SDS running buffer from the 10× stock solution (*see* **item 5** of Subheading 2.3.1).
6. When the gel has hardened, remove the comb and assemble the electrophoresis chamber. Pour the running buffer in both chambers and load the samples.
7. Run the gel for ~2 h at 100 V.

3.4 Gel Transfer

We recommend using a short transfer (30 min) for the 15 % Tris–Glycine gel, especially if the small p10 subunit of caspase-1 is to be detected. For the 10 % Bis–Tris gel, we recommend a transfer time of 45–60 min (*see* Fig. 3).

1. Following electrophoresis, pry the glass plates open so that the gel will remain on one of the glass plates. Remove the stacking gel.
2. For both gel types, prepare the transfer buffer by mixing 200 mL 10× Tris–Glycine, 400 mL Ethanol and 1,400 mL H_2O (*see* **Note 6**).
3. Incubate the gel for 10 min in the transfer buffer.
4. Prepare two pieces of Whatman paper per gel.
5. Prepare a piece of nitrocellulose membrane large enough to cover the lanes of interest and small enough to fit into the transfer cassette.
6. Assemble the transfer as displayed in Fig. 2.

7. Set up the transfer chamber and pour at least 500 mL of transfer buffer.
8. Place the cassette and two sponges in the buffer.
9. Carefully open the gel mold and assemble the gel cassette as in Fig. 2 (*see* **Note 17**).
10. Place the cassette in the transfer chamber and fill the chamber with transfer buffer. For cooling during transfer add a chilled cool pack into the chamber or place the chamber in a cooling box surrounded by ice.
11. Transfer for 30 min (15 % Tris–Glycine gel) or 45 min (10 % Bis–Tris gel) at 100 V.

3.5 Incubation with Antibodies and Blot Exposure

We have found that the commercially available antibodies sc-514 (Santa Cruz) and Casper-1 (Adipogen) work well for immunoblotting of the p10 and p20 subunit respectively (*see* Fig. 3 and **Note 8**). Wash and block the membranes separately using one small plastic container for each blot.

1. Dilute 1:10 of a sufficient quantity of 10× PBS in water. You will need 50–100 mL per blot and wash cycle.
2. Wash each membrane in 1× PBS (without Tween) 3× for 5 min.
3. Prepare 3 % milk blocking solution. You will need ~25 mL for each blot. For 50 mL: mix 5 mL 10× PBS, 30 mL H_2O, and 1.5 g milk powder until the milk dissolves and then fill to 50 mL with H_2O (*see* **Note 18**).
4. Block membranes in 3 % milk blocking solution for 1 h at RT.
5. Wash the membranes 4× in PBS with 0.05 % Tween for 5 min.
6. Prepare a 1 % milk blocking solution for Antibody Incubation (as in **step 3** of Subheading 3.5), but use only 0.5 g milk powder for 50 mL blocking solution. You will need ~3 mL solution for each blot (*see* **Note 18**).
7. Add the primary antibody to the 1 % milk blocking solution with the appropriate dilution.
   - Sc-514: 1:200 (final concentration 1 μg/mL).
   - Casper-1: 1:1,000 (final concentration 1 μg/mL).
8. Incubate the membrane in a 50 mL conical tube with at least 3 mL of antibody solution on a rotator overnight at 4 °C.
9. Wash the membranes as indicated in **step 5** of Subheading 3.5.
10. Prepare 1 % milk blocking solution for Antibody Incubation: as in **step 3** of Subheading 3.5 but use only 0.5 g milk powder for 50 mL blocking solution. You will need approx. 3 mL solution for each blot (*see* **Note 18**).
11. Dilute the appropriate conjugated secondary antibody 1:2,000 in the 1 % milk blocking solution (*see* **Note 9**).

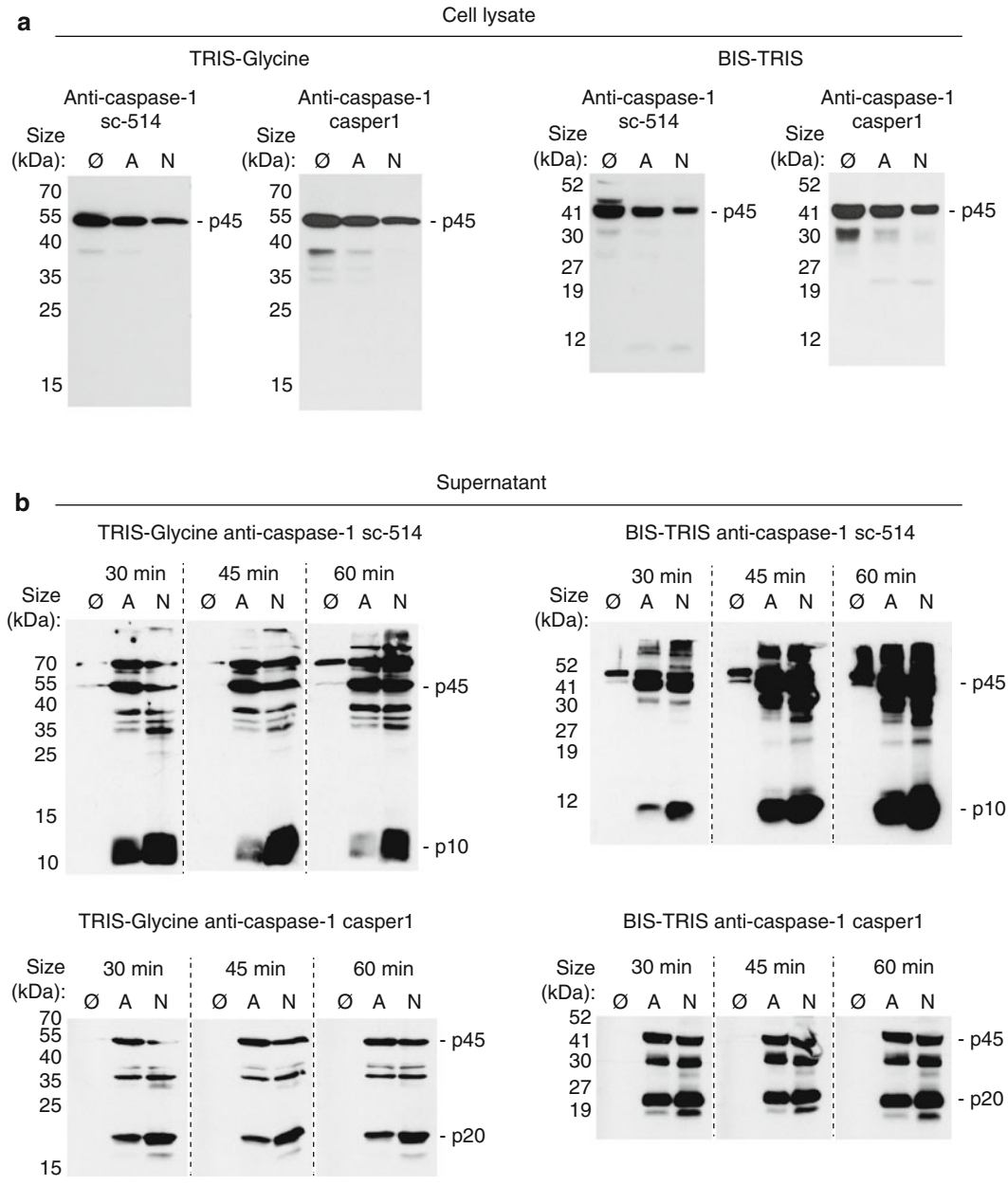

Fig. 3 Caspase-1 western blots. 5×10^5 LPS-primed macrophages were stimulated with either PBS (*Ø*) or ATP (*A*) or Nigericin (*N*) in 500 μL serum-free DMEM for 2 h. (**a**) After stimulation cells were lysed in Laemmli buffer and lysates were separated using Tris–Glycine or Bis–Tris gels. Following transfer, membranes were stained for caspase-1 by with anti-caspase-1 antibody sc-514 and subsequently stripped and re-probed with Casper-1. (**b**) Supernatants of stimulated cells were precipitated by chloroform–methanol and subsequently separated using Tris–Glycine or Bis–Tris gels. For each lane 40 % (16 μL) of a 500 μL supernatant precipitation was loaded. The gels were each divided into three parts and then blotted for 30′, 45′ or 60′ onto nitrocellulose. Following transfer, membranes were stained for caspase-1 by using anti-caspase-1 antibody sc-514 and subsequently stripped (*see* **Note 21**) and re-probed using Casper-1

- For sc-514 use anti-rabbit-HRP.
- For Casper-1 use anti-mouse-HRP.

12. Incubate the membrane in a 50 mL conical tube with at least 3 mL conjugated antibody solution on a rotator for 90 min at RT. Keep the tube protected from light.
13. Wash the membranes as indicated in **step 5** of Subheading 3.5.
14. Briefly dry the membrane by dipping the edges on cellulose and apply sufficient ECL substrate (e.g., 2 mL per mini blot) onto the membrane and wait 30 s (*see* **Note 19**).
15. Drain superfluous ECL substrate from the membrane and place it between two layers of clear foil. Carefully scrub the foil to remove remnants of substrate and to remove air bubbles and put it into a film exposure cassette box.
16. Apply a film onto the membrane in a dark room.
17. The amount of time needed for the film exposure varies considerably. For the figures shown (*see* Fig. 3b), the film was exposed for approximately 10 s (*see* **Note 20**).

4 Notes

1. It is critical not to use FCS-supplemented medium if supernatants are to be precipitated. The excess protein will overload the gel during electrophoresis, resulting in streaking bands. We recommend using serum-free medium or serum-reduced medium.
2. A new stock should be made every month. Alternatively, aliquots can be kept at –20 °C.
3. It is usually necessary to mix overnight with a magnetic stirrer.
4. We recommend using MES-SDS buffer instead of the commonly used MOPS-SDS buffer, as it is better suited for small proteins such as the p10 and p20 subunits of caspase-1 [6].
5. It is also possible to use membranes with 0.45 μm pores, yet different transfer times might be required.
6. Denatured 96 % ethanol can be used if the membrane is to be developed by conventional indirect film exposure. For infrared imaging systems that directly detect the protein on the membrane (e.g., Li-Cor Odyssey), pure ethanol should be used to avoid interference.
7. Tween 20 % has a rather high viscosity. Thus, it is best to cut off the narrow portion of the pipette tip and not to flush the tip after releasing the Tween.

8. For immunoblotting of human caspase-1, we obtain good results using sc-515 (Santa Cruz) a rabbit polyclonal IgG antibody provided at 200 μg/mL.
9. These are HRP-conjugated antibodies for ECL substrate and a conventional developer. There are suitable conjugated antibodies for infrared imaging available on the market, as well. Please also consult the manual of the imaging system that will be used.
10. We employ conventional photographic film exposure in a cassette and a developing machine, yet other luminescence-based or fluorescence-based detection systems are also well suited.
11. With more experience, it is possible to use less supernatant, e.g., 100–200 μL. However, such changes should only be made after the immunoblotting protocol is well established.
12. Even if acetone and ethanol precipitation are both effective, they are less suitable for the precipitation of samples containing large quantities of ATP. The ATP is precipitated along with the protein and causes running difficulties.
13. The centrifuge will distribute some protein on the back wall of the tube, so be careful to always face all samples in the same direction, i.e., with the hinge of the tube to the back. When removing the methanol or TCA, be careful not to touch the back wall of the tube, so that the protein there will not be lost. Do not risk losing the pellet by taking out too much methanol, but try to only leave a small, uniform quantity, because the samples should all need the same amount of time to dry.
14. Bromophenol blue turns yellow in an acidic milieu (pH 3). If the sample turns yellowish, you can adjust the pH with a small volume of 1 M Tris, pH 6.8.
15. The neutral pH of the running front alters the migration behavior of some prestained markers, which are usually calibrated to run in Tris–Glycine gels (pH = 9.5). It is recommended to check the compatibility of the marker prior to electrophoresis or to use an unstained marker for correct determination of protein size. In our case, we used the PageRuler™ prestained marker (Fermentas) and calibrated it using the PageRuler™ Unstained Broad Range Protein Ladder (Fermentas).
16. Adding APS and TEMED will start gel polymerization and should only be done immediately before the gel is cast.
17. This is the description of a wet transfer. It is also possible to put together the cassette in a semidry fashion.
18. It is best to make a fresh milk blocking solution on the day of use.
19. We use the Pierce ECL Western Blotting Substrate or our self-made substrate as described [7] as an HRP-substrate. However, the substrate needed depends on the conjugated antibody and

general developing technique. Please also consult the manual of the imaging system that will be used.

20. The amount of exposure time depends on many variables and thus it is difficult to give a general recommendation. Although we only needed 10 s exposure for the blots shown, it may even be necessary to expose the film overnight. In the beginning we recommend trying out different exposure times in the range of 10 s to 30 min.
21. Stripping was performed by membrane incubation for 30 min at 55 °C in a 50 mL conical tube completely filled with stripping buffer (2 % SDS, 62.5 mM Tris–HCl pH 6.8, 0.8 vol.% of β-Mercaptoethanol in H_2O). During the incubation the tube was carefully inverted ten times. Subsequently, the membrane was thoroughly washed (*see* **step 2** of Subheading 3.5) and the membrane was furthermore processed starting from **step 3** of Subheading 3.5.

References

1. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol 183(2):787–791. doi:10.4049/jimmunol.0901363
2. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol 9(8):847–856. doi:10.1038/ni.1631
3. Wessel D, Flugge UI (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 138(1):141–143
4. Rajalingam D, Loftis C, Xu JJ, Kumar TK (2009) Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured intermediate. Protein Sci 18(5):980–993. doi:10.1002/pro.108
5. Moos M Jr, Nguyen NY, Liu TY (1988) Reproducible high yield sequencing of proteins electrophoretically separated and transferred to an inert support. J Biol Chem 263(13): 6005–6008
6. Good NE, Winget GD, Winter W, Connolly TN, Izawa S, Singh RM (1966) Hydrogen ion buffers for biological research. Biochemistry 5(2):467–477
7. Haan C, Behrmann I (2007) A cost effective non-commercial ECL-solution for Western blot detections yielding strong signals and low background. J Immunol Methods 318(1–2): 11–19. doi:10.1016/j.jim.2006.07.027