Chapter 15

Measuring the Inflammasome

Olaf Groß

Abstract

Inflammasomes are multiprotein complexes whose activity has been implicated in physiological and pathological inflammation. The hallmarks of inflammasome activation are the secretion of the mature forms of Caspase-1 and IL-1β from cells of the innate immune system. This protocol covers the methods required to study inflammasome activation using mouse bone marrow-derived dendritic cells (BMDCs) as a model system. The protocol includes the generation and handling of BMDCs, the stimulation of BMDCs with established Nlrp3 inflammasome activators, and the measurement of activation by both ELISA and western blot. These methods can be useful for the study of potential inflammasome activators, and of the signaling pathways involved in inflammasome activation. General considerations are provided that may help in the design and optimization of modified methods for the study of other types of inflammasomes and in other cell types.

Key words:, Inflammasome, Nlrp3, Caspase-1, Interleukin-1, Bone marrow-derived dendritic cells

1. Introduction

Inflammasome activity has been causally linked to the induction of numerous inflammatory responses, which can be either beneficial or harmful for the organism (1). Among the harmful inflammatory responses are particle-induced sterile inflammation, caused by host-derived particles such as monosodium urate (MSU) crystals (2), which are involved in the pathogenesis of gout, as well as environmental and industrial particles such as asbestos, silica (3), and metallic nanoparticles (4), which induce lung inflammation upon inhalation. In addition, dominant gain-of-function mutations in inflammasome components are associated with certain hereditary periodic fever syndromes (5). Accumulating evidence also implicates inflammasome activity in numerous other disease conditions involving chronic inflammation, including cancer (6, 7) and the development of metabolic diseases such as type 2 diabetes (8, 9), atherosclerosis (10), and inflammatory bowel diseases (11).

Robert B. Ashman (ed.), *Leucocytes: Methods and Protocols*, Methods in Molecular Biology, vol. 844,
DOI 10.1007/978-1-61779-527-5_15, © Springer Science+Business Media, LLC 2012

Conversely, inflammasome activation after pathogen recognition can have beneficial effects for the host (12). Likewise, inflammasome activation may enhance vaccine efficacy (13). Though the inflammasome can mediate cleavage of other cellular targets, most of the effects mentioned above have been attributed to the ability of the inflammasome to cause the maturation and secretion of the proinflammatory cytokine IL-1β (14).

The inflammasome has largely been studied in innate immune cells of the myeloid lineage, such as macrophages and dendritic cells. Inflammasomes typically consists of three components: (1) a cytoplasmic sensor molecule, (2) Caspase-1, and (3) in most cases, the adaptor protein ASC (1). Several distinct inflammasomes, containing different sensor molecules and activated by distinct stimuli, have been described: the Aim2-inflammasome is activated by cytoplasmic DNA (15–18), while a Rig-I inflammasome has been suggested to be active in response to viral RNA (19). Nlrp1 responds to anthrax lethal toxin (20), whereas Nlrc4 is activated by *Salmonella* species and other Gram-negative bacteria with type III or IV secretion systems (21). These activators, either directly or indirectly, are thought to induce changes in their respective sensor molecules (1). The activated sensor can then recruit the other components of the inflammasome, leading to the formation of a multiprotein complex containing clustered Caspase-1. This leads to the autoproteolytic activation of Caspase-1, which can then cleave its various targets, including IL-1β (1). The best studied and also the most remarkable inflammasome complex contains the sensor molecule Nlrp3 (Cryopyrin/Nalp3/Cias1/Pypaf1) (22). It is unusual in that it is activated in response to an ever-expanding list of pathogen- and damage-associated molecular patterns (PAMPs and DAMPs). This includes not only live pathogens as well as individual pathogen-derived toxins, but also the aforementioned environmental and endogenous particles, and extracellular ATP (23). As the actual mechanisms of Nlrp3 inflammasome activation remain largely obscure, it is unclear how Nlrp3 is able to integrate signals derived from so many diverse activators.

As pro-IL-1β is absent in resting cells, inflammasome activation represents the second step in the generation of the cleaved, bioactive IL-1β (14). The first step is the upregulation of the expression of the IL-1β gene, a process that in this context is referred to as priming. Depending on the cell type, priming can also augment the expression of Nlrp3 and potentially other factors involved in inflammasome activation (24). This occurs, for example, in response to the activation of membrane-bound pattern recognition receptors (PRRs) and involves the activation of transcription factors, including NF-κB (14, 25). Figure 1 shows an example of the upregulation of intracellular IL-1 (but not the constitutively expressed Caspase-1) by bone marrow-derived dendritic cells (BMDCs) after a 3-h priming period with various amounts of LPS.

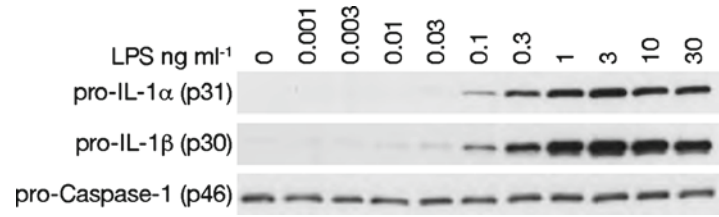

Fig. 1. **Priming effect of LPS treatment on pro-IL-1α and pro-IL-1β production**. Bone marrow-derived dendritic cells (BMDCs) were prepared as described in this protocol and treated for 3 h with increasing doses of LPS, as indicated. The cells were washed with PBS, lysed in SDS sample buffer, and subjected to western blot analysis for the presence of pro-IL-1α, proIL-1β, and pro-Caspase-1.

In the absence of Caspase-1 activity, the inactive pro-form of IL-1β is retained in the cytoplasm. Following cleavage, both mature IL-1β and mature Caspase-1 are secreted from the cells by a mechanism that is poorly understood (26). The release of these two proteins represents the hallmark of inflammasome activation. This protocol describes a standardized method to induce and measure this process.

As it is not feasible to foresee the objectives of all readers, this protocol is written as an outline. Modifications may have to be made in order to suit the goals of each experiment, and modified protocols may require further optimization. These may include not only the choice of stimuli and mouse genotypes and inhibitors, but might also involve the cell type used or even the selection of another method of measurement. Should the latter be required, a paragraph at the end of Subheading 4 summarizes some currently available alternative read-out methods.

The rationale for the key parameters of the method presented here are summarized below.

The choice of cell type: This protocol utilizes primary, murine BMDCs for several reasons. First, BMDCs are highly sensitive to inflammasome activators, respond quickly, and secrete large amounts of IL-1 and Caspase-1. In direct comparison, bone marrow-derived macrophages (BMDMs) secrete less than 10% of the amount of IL-1β produced by BMDCs (Fig. 2a). Another benefit of using primary murine cells is that gene-targeted mouse lines are available for inflammasome components, as well as many candidate inflammasome regulators. Finally, primary cells are preferred as they are more physiologically relevant. Immortalized cell lines can behave quite different from primary cells as they harbor alterations in many signal transduction pathways. Notably, inflammasome- and apoptosis-activating pathways both lead to the activation of caspases, and there is a form of inflammatory cell death termed pyroptosis, that is driven by Caspase-1 (27). The caspase-activating cell death pathways of many cell lines are reprogrammed, and this may influence

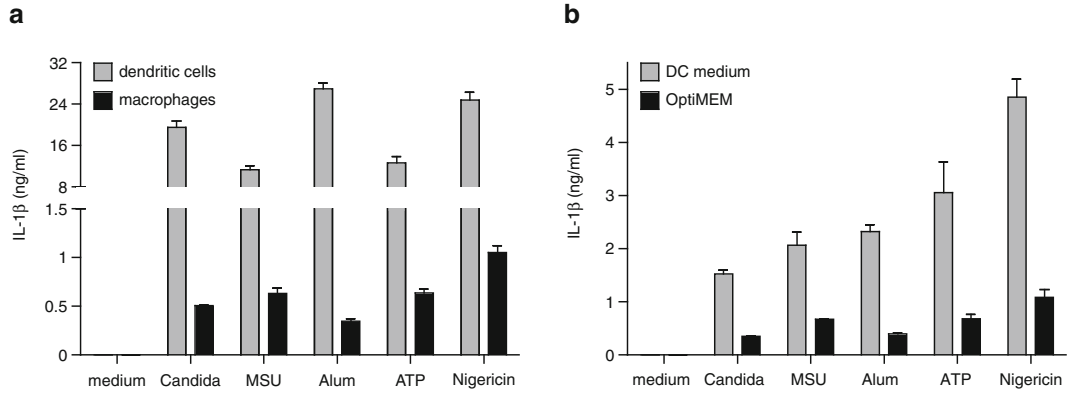

Fig. 2. **Comparison of cell types and conditions for inflammasome activation.** (**a**) BMDCs were prepared as described here and bone marrow-derived macrophages (BMDMs) were generated in the same manner from the bone marrow of the same mouse by using 10 ng/ml of recombinant murine M-CSF instead of GM-CSF. Cells were differentiated for 8 days and, for BMDMs only adherent cells were used while for BMDCs, both floating and adherent cells were included in the assay. Cells were stimulated with 10^6 cells/ml of live *Candida albicans*, 300 μg/ml of MSU or alum for 5 h or with 5 mM ATP or 5 μM nigericin for 1 h as described in this protocol. IL-1β was measured from cell-free undiluted and 1:5 diluted supernatants by ELISA. (**b**) BMDCs were collected at day 5 of differentiation, then primed and stimulated either in DC medium or in OptiMEM (without additional supplements) as in (**a**).

inflammasome activation, and could give misleading results. For example, the macrophage cell line RAW264.7 does not express ASC, while the neutrophil-like HL-60 cells do not express Nlrp3 or Caspase-1. Not surprisingly, these cell lines are irresponsive to many inflammasome activators. In contrast, human monocyte-like THP-1 cells do express inflammasome components and are very sensitive to some inflammasome activators, but rather unresponsive to others such as ATP.

The importance of detecting cleavage and secretion: Both cleavage AND secretion of Caspase-1 and IL-1β are important events in inflammasome activation, especially since cleavage is currently thought to be a prerequisite for secretion (28). In principle, the commercially available ELISA kits should detect both pro- and cleaved IL-1β. Therefore, they may not distinguish between specific, inflammasome-dependent release of the cleaved form and the accidental release of the pro-form (e.g., due to cell death). In order to specifically demonstrate inflammasome activation, one should perform a western blot to show that both cleaved IL-1β and cleaved Caspase-1 are present in the cellular supernatants. However, if multiple experiments with similar conditions and activators are performed, it may be sufficient to show both western blot and ELISA data for just one key experiment. This would demonstrate that under your experimental conditions, the ELISA data for IL-1β correlate with the secretion of cleaved IL-1β and Caspase-1. Herein, however, I suggest a two-step analysis, in which all supernatant samples are first measured by ELISA, then both supernatants and lysates of selected samples (based on ELISA data) are

analyzed by western blot. This approach combines the advantages of both methods. While ELISA allows precise quantification of IL-1β, western blots provide information about the inflammasome-dependent cleavage of IL-1β.

Measurability: A major challenge in establishing inflammasome assays can be to reach a point of reliable, reproducible measurability. Two approaches can be applied: increasing the amount of IL-1β and Caspase-1 secreted, and/or increasing the sensitivity of the assay. An important consideration is the cell type used, as the amount of IL-1β and Caspase-1 secreted is highly variable between different cell types. Furthermore, optimizing the stimulus concentration and duration of stimulation may be necessary when characterizing new inflammasome activators. Up to a certain point, increasing the cell density will also increase the concentrations of Caspase-1 and IL-1β in the supernatant. Another approach used to increase the amount of IL-1β and Caspase-1 per sample is to precipitate the supernatants before western blotting. However, this requires the use of serum-reduced medium, which can decrease the amount IL-1β and Caspase-1 secreted and thereby counteract the gain achieved by this approach (Fig. 2b). A summary of this method is provided at the end of Subheading 4.

The sensitivity of an ELISA can be increased by shifting the IL-1β standard curve samples to lower concentrations than recommended by the manufacturer. This method requires incubation of the samples on the ELISA plate overnight at 4°C, a more thorough final wash step before adding the substrate, and a longer development time. Background problems also affect the sensitivity of the western blot. Here, longer washing, or the use of more stringent wash buffers or different secondary antibodies can help. The type and protein loading capacity of the polyacrylamide gel used, as well as the blotting conditions and the substrate and development systems used can also influence the signal strength. Some of the options for increasing sensitivity at this point in the procedure are mentioned in Subheading 4. However, the most important factor for western blot sensitivity is probably the choice of a primary antibody. Unfortunately, some of the published antibodies for mouse IL-1β and Caspase-1 are polyclonal, and not all are commercially available. Figure 3 shows a direct comparison of some antibodies commonly used.

Useful controls: Any experiment is only as good as its controls. The choice of appropriate controls is also crucial when performing inflammasome experiments. A simple way to ensure equal cell numbers and equal priming is to perform control western blots from cell lysates and probe for pro-caspase-1 (constitutively expressed) and for pro-IL-1 or Nlrp3 (induced upon priming). A caveat here is that some inflammasome activators can induce cell death, or are otherwise stressful to the cells. Also, secretion of IL-1

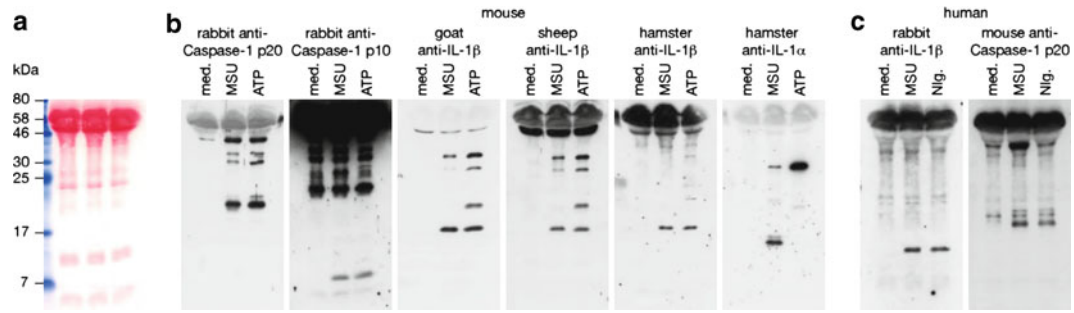

Fig. 3. **Example western blots from inflammasome assays, comparing some currently available antibodies.** (**a**) An example of a Ponceau stain of a western blot performed with samples containing 10% FCS. (**b**) Western blot analysis of murine BMDC supernatants in DC medium from cells either left unstimulated (med.) or stimulated with 300 μg/ml of MSU for 5 h or with 5 mM ATP for 1 h. Antibodies used were (from *left* to *right*): rabbit polyclonal anti-mouse Caspase-1 p20 (generated by Peter Vandanabeele, Ghent); rabbit polyclonal anti-mouse Caspase-1 p10 (Santa Cruz, SC514); goat polyclonal anti-mouse IL-1β (R&D Systems, AF-401-NA); sheep polyclonal anti-mouse IL-1β (generated by GSK); hamster monoclonal anti-mouse IL-1β (eBioscience, clone B122); hamster monoclonal anti-mouse IL-1α (eBioscience, clone ALF-161). (**c**) Human primary blood monocytes were rested after purification in the presence of 100 ng/ml recombinant human M-CSF (Immunotools) in complete RPMI. The cells were stimulated in the same medium with 300 μg/ml of MSU for 5 h or with 5 μM nigericin for 1 h or left unstimulated (med.). Cell-free supernatants were subjected to western blot analysis using the following antibodies (from *left* to *right*): rabbit polyclonal anti-human IL-1β (Cell Signaling; #2022); mouse monoclonal anti-human Caspase-1 p20 (Adipogen). Note: In addition to the antibodies used here, monoclonal antibodies directed against the p20 and p10 fragments of murine caspase-1 (clones Casper-1 and Casper-2, respectively, from Adipogen) recently became available, and also work well in these assays.

and Caspase-1 after inflammasome activation can significantly reduce the amount of the pro-forms of these proteins left intracellularly. These factors can lead a reduction of the signal from stimulated as compared to primed but unstimulated cells.

Another informative control can be to perform, *in a parallel experiment*, stimulation and measurement of an inflammasome-independent pathway such as LPS-induced TNF production. This is especially useful when working with cells derived from uncharacterized mouse model systems, or with inhibitors that display toxicity or other side effects.

Finally, it might be useful to monitor the secretion of IL-1α. IL-1α and IL-1β activate a common receptor and, under certain conditions, IL-1α is cosecreted along with IL-1β and can contribute to an inflammatory response (4). Figures 1 and 3, therefore, also show blots probed with an antibody against IL-1α. These measurements can help to connect in vivo and in vitro findings, and may give insight toward the possible contribution of IL-1α to the observed phenomena. In addition, monitoring inflammasome-independent IL-1α secretion in response to inflammasome activating stimuli can be a suitable control to evaluate side effects or toxicity of potential inflammasome inhibitors. As the antibodies for Caspase-1, IL-1β, and IL-1α were generated in different host species, the panel used here offers the attractive option to detect all of these secreted factors on the same membrane.

As it is the nature of a protocol, the following instructions are specific. However, it is most likely not critical if, for example, the

wash buffer contains 0.5% or 0.2% Tween-20. It is therefore advisable that laboratories, in which some of the methods presented here are already in use, stay with their established methods, reagents, and equipment. The recipes for the standard buffers used in this protocol are nonetheless provided to serve as a reference for troubleshooting. Any aspect that has appeared to be critical for the performance of the method is discussed in Subheading 4.

2. Materials

2.1. Differentiation of Murine BMDCs

1. Mice.
2. Ice.
3. 12-Well cell culture plates.
4. Flushing medium: RPMI1640, containing 100 units/ml penicillin/streptomycin (Invitrogen).
5. Scissors and forceps.
6. Paper towels.
7. 70% Ethanol.
8. $n+2$ sterile 50-ml tubes (n being the number of mice).
9. n 100-μm cells strainers.
10. n 20-ml syringes.
11. n 2-ml syringes.
12. n 22-G needles.
13. n ml of red blood cell (RBC) lysis buffer (eBioscience).
14. Trypan blue solution (Invitrogen).
15. Hemocytometer (Neubauer improved).
16. $n\times50$ ml of DC medium (see Note 9) RPMI1640 with Glutamax (Invitrogen), containing 10% FCS, 100 units/ml penicillin/streptomycin, 50 μM β-mercaptoethanol, 10 mM HEPES, and 20 ng/ml of recombinant murine GM-CSF (Immunotools).
17. $n\times5$ sterile, non-tissue culture treated 10-cm Petri dishes.
18. Cell culture facility and equipment including a 37°C, 5% CO_2, water-jacketed cell culture incubator and a laminar flow hood.

2.2. Stimulation

1. 12-Tip multichannel pipette with a volume of at least 50–200 μl per tip.
2. 96-Well flat-bottom cell culture plates.
3. 100 μg/ml *E. coli* K14 ultrapure LPS (Invivogen, in water, store at −20°C).

4. Stimuli of choice, e.g., 1 M ATP (Sigma; 1 g ATP in 1.8 ml water; store at −20°C), 10 mM nigericin (Sigma; 5 mg in 670 µl Et-OH; store at 4°C), 40 mg/ml alum (Pierce; store at 4°C or −20°C), 40 mg/ml silica (Alfa Aesar; sterilize with EtOH and resuspend in water; store at −20°C).
5. Inhibitors of choice, e.g., 1 M APDC (Enzo or Sigma; 10 mg in 60 µl water; make fresh), 2.5 M KCl (Sigma; 1 g in 5.3 ml RPMI; store at −20°C), 200 mM Glibenclamide (Enzo or Sigma; 10 mg in 100 µl DMSO; make fresh), 50 mM Z-VAD-fmk (Merck; 10 mg in 100 µl DMSO).
6. DC medium (see Subheading 2.1).
7. Cell culture centrifuge with swing-out buckets to hold 96-well plates.
8. 3× and 1× SDS sample buffer (see below, dilute in phosphate-buffered saline (PBS)).
9. PBS (Invitrogen).

2.3. Measurement

1. ELISA Kits for IL-1 and TNF as required (R&D, BD, or eBioscience).
2. NUNC MaxiSorb ELISA plates.
3. ELISA plate reader and analysis software.
4. Western blot equipment (Protean mini 1 mm, BioRad).
5. Nitrocellulose membrane (GE Healthcare, Hybond 0.45 µm).
6. 5% sodium azide in water (Sigma).
7. Skim milk powder (Sigma, or your local grocer).
8. PBS.
9. ECL solution (Pierce or GE).
10. Film, developer, dark room or equivalent development equipment.
11.

3× Western blot sample buffer	(For 500 ml)
187.5 mM Tris–HCl (pH 6.8)	178 ml of a 0.5-M Tris–HCl pH 6.8 stock solution
6% w/v SDS	150 ml of a 20% stock solution
0.03% w/v Phenol Red	150 mg
30% w/v Glycerol	172 ml of 87% stock solution

Adjust to pH 6.8 with 0.1N HCl, if necessary.

12.

15% separation gel	**(For 12 gels = 120 ml):**
Tris–HCl pH 8.8	30 ml of a 1.5-M Tris–HCl pH 8.8 stock solution
SDS	0.6 ml of a 20% stock solution
Acrylamide (37.5:1)	60 ml of a 30% stock solution
dH_2O	30 ml
TEMED	75 µl, mix well
APS	600 µl of a 10% stock solution, mix well, and cast immediately

13.

6% stacking gel	**(For 12 gels = 50 ml)**
Tris–HCl pH 6.8	12.5 ml of a 0.5-M Tris–HCl pH 6.8 stock solution
SDS	0.25 ml of a 20% stock solution
Acrylamide (37.5:1)	10.5 ml of a 30% stock solution
dH_2O	27 ml
TEMED	62.5 µl
Pyronine Y (red)	150 µl of a 1% w/v stock solution, mix well
APS	500 µl of a 10% stock solution, mix well, and cast immediately

14.

Running buffer	**(For 5 l)**
Tris base	75 g
Glycine	360 g
SDS (20%)	125 ml
dH_2O	To 5 l; mix well

15.

Blotting buffer	**(For 20 l)**
Tris base	50 g
Glycine	238 g
Ethanol	3.3 l
dH_2O	To 20 l; mix well

16.

Ponceau staining solution	(For 500 ml)
0.05% Ponceau S	250 mg
3% Trichloroacetic acid	15 g
dH_2O	To 500 ml

17. Wash buffer: PBS containing 0.5% Tween 20.
18. Blocking buffer: wash buffer containing 2% skim milk powder.

3. Methods

3.1. Differentiation of Murine BMDCs (see Note 1)

1. Prepare a 12-well plate with 2 ml of flushing medium per well in as many wells as you have mice, and keep it on ice.
2. Kill a mouse by cervical dislocation or CO_2 inhalation.
3. Lay the mouse down on its back (on some paper towels), pull up the fur at the center of the belly with two fingers, and make a small cut into the skin caudal of your fingers, without cutting into the peritoneum.
4. Grab the fur above (cranial) and below (caudal) the cut and pull it open, approximately half way around the body of the mouse.
5. Pull the fur over the hind legs and over the ankle (see Note 2).
6. Dislodge the ankle joint by repeatedly moving and twisting the foot into unnatural directions.
7. Put the knee on the table with the foot standing up and pull the foot with the attached muscles down along the tibia (see Note 3).
8. Remove the tibia by hyperextending it over the knee. The tibia should come off the knee cleanly and without muscles or the meniscus (cartilage) attached. Put the bone in a well of the 12-well plate.
9. With your thumbnail flip the cartilage off of the femur, and push the muscle surrounding the bone down toward the hip joint (see Note 4).
10. Grab the end of the femur in two fingers and hold the mouse up with it. Using sharp, pointy scissors, place several cuts in the muscles of the hip area until the hip-end of the femur comes loose from the rest of the mouse (see Note 5).
11. Remove the remaining muscle tissue at the end of the bone first with scissors, and then by rubbing the bone with paper towel between your fingers. Put it in the 12-well plate in the

same well as the tibia. Repeat the process with the other leg, and the other mice.

12. Move to a sterile work place, such as a laminar flow hood.
13. Put a pair of scissors and forceps into a 50-ml tube filled with 70% ethanol.
14. Using a new, sterile 12-well plate containing 1 ml of 70% ethanol per well, place the bones of each mouse in a separate well of for 1 min. Afterward, put them into cold, sterile flushing medium in a third, sterile 12-well plate.
15. Place a 100-μm cell strainer into a 50-ml tube.
16. Pour flushing medium into a 50-ml tube and use it to fill a 20-ml syringe. Attach a 22-G needle to the syringe.
17. Take one bone with the forceps and cut open the hip- or foot-side of the bone (see Note 6).
18. Insert the needle from knee-side of the bone and carefully flush the marrow into the cell strainer (see Note 7). Repeat the process with the other three bones of the same mouse.
19. Using the plunger of a 2-ml syringe, press the marrow through the mesh of the cell strainer. Use the remaining medium in the syringe to flush remaining cells (red clumps) out of the mesh. Put the tube on ice and repeat the process with the remaining bones.
20. Spin the cells down at 300 × *g*, resuspend them in 1 ml of RBC lysis buffer, and incubate them for 5 min at room temperature.
21. Add 9 ml of flushing medium to quench the RBC lysis buffer, and mix by inversion. Remove a 10-μl sample, dilute it in 40 μl trypan blue solution, and count the cells using the hemocytometer (see Note 8).
22. Spin the cells down and resuspend them in DC medium to a density of 10^6 per ml (see Note 9).
23. Plate the cells in 10 ml medium per plate, using 10-cm non-tissue culture treated Petri dishes (see Note 10).
24. Move plates to a 37°C, 5% CO_2 incubator and allow them to differentiate for 5–9 days (see Note 11).
25. On days 3 and 6, add 5 ml of fresh DC medium to the culture.

3.2. Stimulation

1. Harvest BMDCs between days 5 and 9 of culture by transferring them into 50-ml Falcon tubes (see Note 12).
2. Count the cells and adjust them to 10^6 per ml (see Note 13). For the experiment outlined in Fig. 4, put 5.5 ml in a 15-ml tube and 14 ml in another 15-ml tube.
3. Add 2.8 μl of LPS (100 μg/ml) to 14 ml of cells (final LPS concentration of 20 ng/ml) in order to prime them, and mix

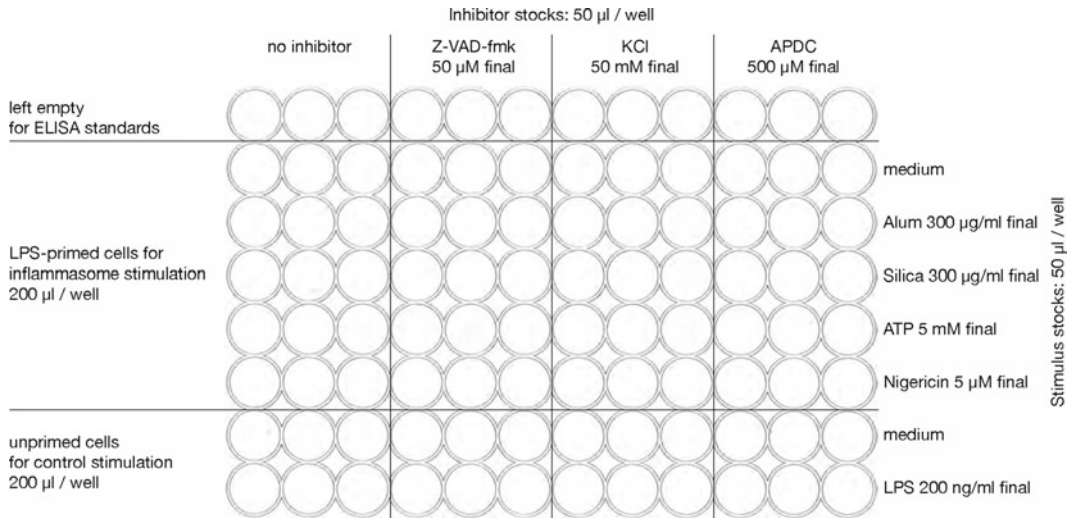

Fig. 4. **Overview of the example experiment outlined in the text.** First, cells are added to the plate (*left label*) and primed for 3 h. Second, inhibitors (*top label*) are added for 30 min. Third, inflammasome activators (*right label*) are added for a final incubation of 5 h. For details *see* Subheading 2 in Chapter 3.

by inverting. Leave 5.5 ml of cells unprimed for testing toxicity and side effects of the inhibitors (see Note 14).

4. Plate 200 µl of cells per well in a 96-well flat-bottom plate as indicated in Fig. 4 to perform triplicates of each condition (see Note 15).
5. Incubate the plates for 3 h at 37°C.
6. Prepare sixfold concentrated inhibitors: prepare four 1.5-ml tubes containing 1.2 ml of DC medium and add: (1) nothing; (2) 7.2 µl Z-VAD-fmk stock solution; (3) 164 µl KCl stock solution; (4) 3.6 µl APDC stock solution; vortex (see Note 16).
7. Add 50 µl of sixfold concentrated inhibitors (or medium) to the cells as indicated in Fig. 4 and incubate for 30 min to 1 h at 37°C (see Note 17).
8. Prepare sixfold concentrated stimuli: prepare seven 1.5-ml tubes containing 0.8 ml of DC medium and add: (1) nothing; (2) 36 µl of alum stock suspension; (3) 36 µl of silica stock suspension; (4) 24 µl of ATP stock solution; (5) 2.4 µl of nigericin stock solution; (6) nothing; (7) 9.6 µl of LPS stock solution; vortex and sonicate particles (see Note 18).
9. Add 50 µl of sixfold concentrated stimulus to the cells and incubate for 5 h at 37°C (see Note 19 for details about optimal incubation times for different activators).
10. In the meanwhile, coat an appropriate number of IL-1β and/or IL-1α and TNF ELISA plates according to manufacturer's instructions (see Note 20).

11. At the end of the incubation period, spin the inflammasome-stimulated plate(s) down at 300×*g* and carefully transfer the uppermost 200 µl of the supernatants to a fresh 96-well plate using a multichannel pipette (see Note 21). Store the fresh plate with the supernatants at 4°C if you plan to run ELISAs or western blots on the samples the next day, or at −20°C or −80°C until use.
12. Discard the remaining supernatant in the plate by decanting the contents into the sink and then, without flipping the plate back, tap the plate once firmly on a stack of paper towels.
13. Add 200 µl of cold PBS to each well, spin down, and repeat step 12.
14. Add 40 µl of 1× SDS sample buffer to each well and store the plate(s) at −20°C until use (see Note 22). See alternative method of analyzing cell lysates by ELISA in Subheading 4.4.3.

3.3. Measurement

1. Subject 50–100 µl of each well of the supernatants to ELISA for IL-1β and/or IL-1α according to manufacturer's recommendations (see Note 23).
2. Choose supernatant samples for western blot.
3. Using a multichannel pipette, pool 30 µl of each triplet into one well on a fresh 96-well plate.
4. Add 45 µl of 3× SDS sample buffer to each well, transfer the content of each well to a 1.5-ml tube, and incubate in a 95°C heat block for 5 min. Pool and boil the cell lysates accordingly (see Note 24).
5. Subject the samples to polyacrylamide gel electrophoresis using a 15% gel (see Note 25).
6. Blot the proteins on a nitrocellulose membrane (see Note 26).
7. Put the membrane for 2 min in Ponceau red solution with mild shaking. Wash for 2 min with distilled water. Make a photocopy or scan of the blot for your documentation, and note any loading differences between samples. Figure 3a shows an example (see Note 27).
8. Block the membranes by incubating in blocking buffer for 1 h at room temperature with mild shaking.
9. Add your antibody (1:1,000 to 1:2,000 (0.5 µg/ml final concentration) in blocking buffer containing 0.05% azide) and incubate over night at 4°C with mild shaking (see Note 28).
10. Wash 4×5 min with wash buffer at room temperature with mild shaking.
11. Dilute your HRP-conjugated secondary antibody 1:3,000 to 1:10,000 in blocking buffer (no azide!), add to the blots, and incubate for 2 h at room temperature with mild shaking.

12. Wash for 2 h in wash buffer (changing the buffer at least five times) at room temperature with mild shaking.
13. Tap the membrane dry using a tissue, immediately lay it over a 1-ml drop of regular or high fidelity ECL solution (on a piece of Parafilm or plastic) for 1 min, and tap dry again (see Note 29).
14. Develop your blot using standard techniques and equipments (see Note 30).
15. Repeat steps 9–14 with different primary and secondary antibodies. Example blots are provided in Fig. 3 (see Note 31).

4. Notes

4.1. Differentiation of Murine BMDCs

1. There are several published protocols on how to make BMDCs (29). They usually aim at a maximum yield of immature (but differentiated) BMDCs to use them, for example, for antigen presentation and T cell activation. As we prime the cells for inflammasome activation anyway, we are not as concerned about their preactivation status. If you want to use BMDCs for other purposes than inflammasome activation, you might want to consider using a different protocol.

   The method described here employs scissors to remove muscle from the bones only where absolutely necessary. It relies on the fact that the muscles are not attached directly to the bones, but rather to the knee and ankle joint, except at the proximal end of the femur. The method has the advantage that it is fast and yields bones with very little muscle attached. The procedure is, however, a bit hard to describe without actually demonstrating what you have to do. If you know someone who routinely takes bones from mice, you might want to ask for a demonstration of their method.
2. As soon as the knee is exposed, pinch the area under the knee and pull the fur further off. If the fur rips off before it is pulled clear over the ankle, make a cut into the fur over the ankle and pull it further.
3. The foot and lower leg muscles should come off with ease. If they do not, turn and twist the foot a bit more to completely dislodge the ankle joint. If you do not do this properly, the tibia bone might brake. Should this happen, continue with the protocol nonetheless. The bone usually breaks in the position where the fibula meets the tibia, and you will cut the bone at this point later in the protocol. I have never experienced a significant loss in yield or contamination due to breaking of a bone.

4. This requires a bit of fumbling around until you have gained some experience. A lip of cartilage projects off of the dorsal side of the femur. After you flipped if off, the femur ends bluntly, showing an X-like shape at its end. The older the mouse, the more tightly the cartilage is attached to the femur and the more difficult it is to remove. If you break open the bone, just continue anyway. Alternatively, you can use scissors to flip (but not cut!) the cartilage off.
5. Holding the mouse up by the exposed end of the femur allows you, by moving your hand in different angles with respect to the hanging mouse, to estimate where the bone is attached to the hip. It is in this area where you want to cut. The mouse will fall down once the femur is sufficiently dislodged from the hip.
6. For the tibia, cut at the site where tibia and fibula meet. For the femur, cut off just a little bit more than just the spherical femur-head.
7. The needle should easily enter the bone from this side. If it does not, the cartilage has not been properly removed. You can try to twist the needle to bore through the remaining cartilage, or carefully remove the remainder of the cartilage using scissors.
8. You can expect about 100 cells in one of the nine squares of the hemocytometer ($=10^6$ cells/ml in the trypan blue diluted cells and 5×10^7 cells per mouse).
9. There are various recipes for DC medium (29). Some laboratories add sodium pyruvate or nonessential amino acids, others use regular RPMI and add glutamine or leave out the HEPES buffer. These variables are relatively unimportant; as long as the medium contains GM-CSF and FCS, you should be able to generate DCs. However, the quality of the FCS used is critical. It might require testing several lots of FCS to find one that gives good yields of differentiated DCs. Those that contain measurable amounts of endotoxin according to the data sheet should not be considered. BMDCs in general do not proliferate much throughout the differentiation process. However, depending on the quality of the FCS, the cells will proliferate somewhat. Accordingly, you might have to alter the initial cell density to accommodate for that. The cell density should not exceed two million per ml.
10. The usual amount of bone marrow cells obtained per mouse is about 50 million, so you can expect to end up with five dishes of DCs per mouse.
11. During the differentiation process, the cells start to form floating clusters (after 2–3 days), and more cells become adherent and develop dendritic projections.

4.2. Stimulation

12. The longer you leave the DC culture, the higher is the amount of IL-1 and Caspase-1 secreted upon inflammasome stimulation, presumably, at least in part, due to the higher proportion of differentiated ($CD11c^+$) cells. However, the longer the DCs are in culture, the more cells that will become adherent. Classically, these cells would be deemed either mis-differentiated cells that drifted into a macrophage lineage or preactivated, mature dendritic cells. Both cell types are not useful for studying DC biology and would therefore be discarded. However, in terms of inflammasome activation, we have found that the adherent cells in a GM-CSF culture respond in the same way as the floating cells. Therefore, if you are short on cells or want to use a late DC culture in which a lot of cells have become adherent, these cells can also be used for the experiment. To dislodge the cells, add 10 ml of HBSS containing 5 mM EDTA to the plate after removing the floating cells in the medium. Incubate them for 10 min at 37°C, rinse the plate carefully, spin the cells down, discard the supernatant, and resuspend them in the medium containing the floating cells.
13. Counting and adjusting BMDCs is subject to inaccuracy. To avoid cells aggregating or sticking to the walls of the tube, it is best to count for each mouse/genotype immediately after pooling and resuspending the cells in one 50-ml tube. Do not put an aliquot in a 96-well plate for later counting, they might become adherent. Always carefully resuspend them by pipetting up and down several times with a 25-ml serological pipette before you take a sample. Count the cells again after readjusting, allowing a tolerance of ±10%. As outlined in the following note, a control stimulation will help to estimate the error derived from any differences in cell density.
14. If inhibitors are to be tested, it is important to include controls for off-target effects or toxicity. A good option is to do a parallel experiment in which a different signaling pathway is activated. I recommend using an aliquot of the cells you want to use for inflammasome stimulation but leave them unprimed. Plate and treat them with the inhibitor(s) in parallel with the primed cells. Instead of inflammasome activators, stimulate the control cells with LPS for the same duration as the inflammasome stimuli (4–6 h). Measuring TNF production from this stimulation gives an indication as to whether the compound is toxic to the cells, or whether it inhibits pathways unrelated to inflammasome activation.
15. A general formula to calculate the amount of cells *per genotype* you need is $(\#\text{ of stimuli} + 1) \times (\#\text{ of inhibitors} + 1) \times 3\text{ wells} \times 200\ \mu\text{l}$; the "+1" representing the negative controls. In the example used here, the numbers are $(4\text{ stimuli} + 1\text{ control}) \times (3\text{ inhibitors} + 1\text{ control}) \times 3\text{ wells} \times 200\ \mu\text{l} = 12\text{ ml}$; and for

the control stimulation (1 stimulus + 1 control) × (3 inhibitors +1 control) × 3 wells × 200 µl = 4.8 ml.

16. Note that cell culture medium contains 5 mM KCl, while cytoplasmic concentrations are around 130 mM. Therefore, concentrations tested should stay within these boundaries. APDC and other ROS scavengers might become spontaneously oxidized over time, so stock solutions should not be stored for extended periods.
17. The time required for an inhibitor to be effective should be evaluated for each inhibitor individually, but should generally be kept as short as possible. It might be worth doing a time course experiment (after you have found the optimal time point for the stimulus first, see Note 19) to find the optimal preincubation period for optimal inhibition AND minimal off-target effects/toxicity.
18. Particulate stimuli tend to aggregate, but a short sonication (with either a bath or probe sonicator) will help to dissociate them.
19. The duration of the stimulation depends on the stimulus. ATP or nigericin induce robust inflammasome activation after only 30 min to 1 h, whereas most particulate stimuli, but also many pathogens, require at least 3–4 h. If you are interested in a specific stimulus, it is worth doing a time course experiment in order to identify the minimum stimulation period for full activation. Stimulation for more than 6 h should be avoided, in order to minimize contribution of feedback loops, cell death, or overgrowth of a pathogen. It might be useful to harvest fast-acting stimuli earlier than slow ones. (However, it does not change the signal if you leave fast activators on for longer, as we suggest in this protocol in order to reduce the experiment to one 96-well plate.) In a larger experiment, plate stimuli that will be harvested at the same time point on the same plate. If you do not use inhibitors, use 100 µl of a 3× stimulus instead.
20. Several companies produce good but rather expensive ELISA kits for these cytokines. In order to make a kit go further, use 50 µl instead of the recommended 100 µl per well for all steps except the substrate. This is mentioned here, as IL-1β is usually measured from undiluted supernatant. If 100 µl of sample per well is used, the total amount of sample might not be enough for all measurements intended or for repeating a measurement should the ELISA not work. In contrast to the recommendations, I use a highest standard of 10 ng/ml and an 11-point standard curve of 1/2 dilutions with a blank.

    In order to save time, coat your ELISA plates the day before the stimulation, so that you can transfer the supernatants to the plate immediately after the experiment.

21. Be extra careful at this step not to disturb the pelleted cells. 200 µl per replicate should be enough to do the measurements. If the control experiment (e.g., LPS stimulation) was done on a separate plate, it can be moved to storage immediately, without separating the supernatants from the cells.
22. Some stimuli like nigericin or ATP are quite toxic to the cells, which might reduce the total protein content of some of the samples.

4.3. Measurement

23. Most stimuli will not induce the production of more than 10 ng/ml of IL-1, so you can measure your supernatants undiluted. For TNF, dilute the supernatants 1:10. One well of the plate holding the supernatants corresponds to one well on the ELISA plates. Therefore, the final triplets measured consist of replicates of the whole experimental procedure, not only of the ELISA method.

    The sensitivity of the ELISA can be increased by using lower standard values than recommended (e.g., a 16-point standard curve ranging from 10 ng/ml to 0.6 pg/ml with a blank) and by incubating the supernatants overnight at 4°C rather than 2 h at room temperature. The last washing step before adding the substrate is critical in reducing the background and minimizing variation between replicate samples.
24. The cell lysates serve two purposes: They represent a control by which it can be demonstrated that equal amounts of cells (e.g., from different genotypes) were used. In addition, the pool of intracellular pro-IL-1 and pro-Caspase-1 and potential intracellular cleavage can be monitored. Pool the lysates from each triplet in one 1.5 ml tube. The samples are viscous until they have been boiled. Note that, as these are whole cell lysates, you might get more nonspecific bands than in samples prepared by methods that do not lyse organelles. In the case that the focus of a project lies in intracellular events that shall be monitored by western blot, you may wish to modify this protocol, do your stimulation in a larger plate format, and use a milder extraction buffer that solubilizes the cytoplasm but not the organelles.
25. We use the Mini-PROTEAN gel system from Bio-Rad with 1-mm thick gels and 15 lanes per gel and the related wet blot system. I let my gels run at 160 V for 1 h or until the dye front, but not the lowest marker in the protein molecular weight standard has left the gel. We have also obtained good results with precast gradient gels from Invitrogen, using the associated equipment and wet blot system.
26. A wet blot system may lead to better transfer than a semidry system, and thus a stronger signal. I blot at 100 V for 1 h–1.5 h. The current should be no higher than 300 mA per tank. The blotting buffer might get warm, but one should avoid letting

it get hot. Make sure that the blot buffer is thoroughly mixed, as undissolved salts and high ethanol concentrations will increase the resistance. If you do not frequently perform western blots, make the blot buffer without ethanol and add it freshly each time to avoid evaporation of the ethanol.

27. The most prominent bands are around 60 kDa, and correspond to albumin and the immunoglobulin heavy chain in the FCS.
28. Primary antibody solutions in blocking buffer with azide can be used until they are exhausted (i.e., when the signal becomes weaker and requires the use of stronger ECL solutions for detection).
29. Do not let your blots air-dry completely after tapping them but quickly move them again into ECL or your developing cassette. Use tissues or paper towels that have no pattern pressed into them, as this might lead to uneven removal of wash buffer or ECL solution. In case normal ECL is too weak, but high sensitivity ("femto") is too strong, you can dilute femto 1:3 in normal ECL solution.
30. Several methods for minimizing background are described here. First, you can try to wash longer or more intensely. Using a different wash buffer, containing 0.1% Trition X-100 and 1% skim milk powder in TBS or PBS seems to help for some antibodies, especially if used for the whole process from the first blocking of the membrane up to the second-last wash step before adding ECL solution (the last wash before ECL should be done without milk in the buffer). You can also try to use a lower concentration of primary and/or secondary antibodies, or a different secondary antibody.
31. As the common antibodies used (Fig. 3) were generated in different species (Caspase-1: rabbit, IL-1β: goat or sheep, IL-1α: hamster), the same membrane can be probed for all three proteins.

4.4. Supplementary and Alternative Methods

Below are methods for storage and shipment of bone marrow, as well as alternative methods for measuring inflammasome activation.

4.4.1. Storage of Bone Marrow in Liquid Nitrogen

The method described here uses a 96-well format and therefore, the amount of bone marrow obtained from one mouse might be more than sufficient for doing the intended experiment. Freezing the leftover bone marrow for long-term storage in liquid nitrogen is a good method to reduce mouse consumption and increase experimental flexibility. The whole freezing process should be done quickly, as DMSO is toxic to cells at room temperature.

1. Before performing erythrocyte lysis (after step 19 in Subheading 3.1 of the main protocol), set a portion of the bone marrow aside for freezing. Keep on ice.

2. Spin down the bone marrow set aside.
3. Discard the supernatant and gently but quickly resuspend the bone marrow in freezing medium (ice-cold FCS containing 10% DMSO). Use 1–3 ml freezing medium per mouse.
4. Immediately transfer 1 ml of the suspension per tube to prechilled cryo-tubes (NUNC) and transfer them into a prechilled (on ice) "Mr Frosty" (Nalgene) freezing container. It is important to label the tubes with a pencil or an alcohol-resistant pen.
5. Immediately transfer to −80°C.
6. The next day, transfer for long-term storage to liquid nitrogen. Do not store bone marrow at −80°C for longer periods as the cells will eventually die under these conditions.
7. In order to thaw the cells, put them in a 37°C water bath until the contents of the tube is half liquid but there is still some ice left.
8. Sterilize the outside of the tube with ethanol and quickly but gently transfer the content to 10 ml of medium.
9. Spin the cells down and resuspend them in DC medium.
10. Count the living cells using trypan blue and a hemocytometer and adjust them to 10^6 cells/ml. Continue at step 23 in Subheading 3.1 of the main protocol.

4.4.2. Shipping Bone Marrow

The easiest way to ship bone marrow to collaborators is to send frozen bone marrow on dry ice. An alternative is to ship bones or legs in medium in a 15-ml tube on ice. The cells inside the bones easily survive 24 h at 0°C. If the shipping takes a longer time it might be safer to flush the bone marrow and send it in complete medium in a 15-ml tube on ice. In any case, make sure that the sample does not freeze during shipping, as freezing in regular medium will kill the bone marrow cells. For this reason, do not put −20°C cold packs into a parcel containing bones or cells in medium. Instead, use blue ice or 0°C cold packs.

For the shipping itself, make sure you include the necessary paperwork, especially the dry ice declaration, declaration of hazards, and commercial value. Include enough dry ice or cold packs so the package will remain cool even if shipping is delayed.

4.4.3. Quantification of Intracellular Pro-IL-1β by ELISA

Commercially available ELISA kits also measure pro-IL-1, and therefore can be used to monitor intracellular pro-IL-1 levels. In addition, a specific mouse pro-IL-1β ELISA kit is available from eBioscience. By subjecting the cells to repeated freeze–thaw cycles, the intracellular pro-IL-1 is released and can be quantified by an additional ELISA test. This allows direct comparison of the amount of pro-IL-1 inside the cells to the amount of secreted IL-1 in the supernatants.

1. After step 13 in Subheading 3.2, instead of adding SDS sample buffer to the cells in the plate, add 200 µl of medium containing 10% FCS.
2. Subject the cells to three freeze–thaw cycles by transferring the plate between a freezer (–20°C or –80°C) and a 37°C incubator. Make sure that the medium is completely frozen or thawed after each step.
3. After the last thaw, spin the plates down at 300×*g* to pellet debris, transfer the cell lysate supernatant to a new plate, and perform an ELISA using the same method as was used to measure IL-1 in the cell culture supernatants (step 1 in Subheading 3.3).

4.4.4. Protein Precipitation from Supernatants

This protocol replaces complete medium with unsupplemented OptiMEM, which allows precipitation of the secreted proteins, and can be used to increase signal strength. Using the method presented here, the signal strength can be increased 20–30-fold as compared to unprecipitated samples. However, as cells primed in OptiMEM secrete 2–10-fold less IL-1β (Fig. 2b), the actual gain is reduced. Medium containing FCS should not be precipitated, as this will overload the gel. This protocol can be scaled up as much as necessary, by using more cells and precipitating from larger volumes of medium.

1. In contrast to the standard protocol, following step 2 in Subheading 3.2, spin down the cells after counting, and resuspend them in OptiMEM medium (Invitrogen) without any supplements at 10^6 cells/ml. Add LPS to 20 ng/ml final concentration for priming.
2. Seed 1 ml of cells 12-well plates without replicates (instead of 96-well plates as triplicates) and perform inflammasome stimulations analogous to the standard protocol using 10× inhibitors and stimuli in OptiMEM.
3. Harvest the supernatants (1.2 ml) into 1.5-ml tubes and spin for 5 min at 400×*g*.
4. Transfer two aliquots of 500 µl into fresh 1.5-ml tubes, leave some medium with the cells in the original tube to make sure you do not carry over any cells or debris.
5. Store one of the two parallel tubes at –20°C as a backup.
6. To the other tubes, add 500 µl methanol and 150 µl chloroform, vortex, and spin down in a microcentrifuge at maximum speed for 10 min at room temperature. Spinning will separate the sample into three phases: an organic phase at the bottom containing chloroform, an aqueous phase containing water and methanol, and a protein-containing interphase.

7. Discard the aqueous phase (at the top) without touching the interphase. Leave some of the aqueous phase to ensure that the interphase is not disturbed or removed. If you do not see a lower (organic) phase, add 50 μl more of chloroform, mix, and spin again.
8. Add 800 μl of methanol, mix, and spin again. (Now, the chloroform is dissolved and the protein is pelleted).
9. Remove the supernatant carefully, without disturbing the brittle pellet.
10. Dry the pellets for 10 min at 37°C. If the methanol is not completely removed, the sample will float out of the well when loading the gel.
11. Add 25–50 μl of SDS sample buffer, mix, and incubate for 5 min at 95°C.
12. Spin down and perform western blot following the standard protocol (step 5 in Subheading 3.3).

4.4.5. Caspase-1 ELISA

Various companies offer ELISA kits, primarily for the detection of human Caspase-1. In principle, these kits suffer from the same limitation as those for IL-1β as they do not allow distinguishing pro-Caspase-1 from cleaved and active Caspase-1 p10 and p20 subunits. However, they can be useful for the detection of Caspase-1 in serum and other body fluids.

4.4.6. Fluorescent Caspase-1 Substrates

FLICA™ (ImmunoChemistry Technologies) is a fluorescent probe that binds to active Caspase-1, thereby labeling cells in which the inflammasome is active. It consists of carboxyfluorescein (FAM) bound to the irreversible Caspase-1 inhibitor Y-VAD-FMK. This reagent can be useful for the determination of inflammasome activation by fluorescence microscopy and flow cytometry-based assays.

Acknowledgments

I would like to thank Michael Bscheider, Manuel Ritter, and James Vince for helpful suggestions, and Christina Thomas for proofreading and correcting the manuscript. My work is funded by an EMBO long-term fellowship. I declare no competing financial interests.

References

1. Schroder, K., Tschopp, J. (2010) The inflammasomes, *Cell* **140**, 821–832.
2. Martinon, F., Petrilli, V., Mayor, A. et al. (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome, *Nature* **440**, 237–241.
3. Dostert, C., Petrilli, V., Van Bruggen, R. et al. (2008) Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica, *Science* **320**, 674–677.
4. Yazdi, A. S., Guarda, G., Riteau, N. et al. (2010) Nanoparticles activate the NLR pyrin domain containing 3 (Nlrp3) inflammasome and cause pulmonary inflammation through release of IL-1alpha and IL-1beta, *Proc Natl Acad Sci USA* **107**, 19449–19454.
5. Henderson, C., Goldbach-Mansky, R. (2010) Monogenic autoinflammatory diseases: new insights into clinical aspects and pathogenesis, *Curr Opin Rheumatol* **22**, 567–578.
6. Okamoto, M., Liu, W., Luo, Y. et al. (2010) Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1beta, *J Biol Chem* **285**, 6477–6488.
7. Ghiringhelli, F., Apetoh, L., Tesniere, A. et al. (2009) Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors, *Nat Med* **15**, 1170–1178.
8. Zhou, R., Yazdi, A. S., Menu, P. et al. (2010) A role for mitochondria in NLRP3 inflammasome activation, *Nature* doi:10.1038/nature09663
9. Masters, S. L., Dunne, A., Subramanian, S. L. et al. (2010) Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes, *Nat Immunol* **11**, 897–904.
10. Duewell, P., Kono, H., Rayner, K. J. et al. (2010) NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals, *Nature* **464**, 1357–1361.
11. Bauer, C., Duewell, P., Mayer, C. et al. (2010) Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome, *Gut* **59**, 1192–1199.
12. Gross, O., Poeck, H., Bscheider, M. et al. (2009) Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence, *Nature* **459**, 433–436.
13. Hornung, V., Bauernfeind, F., Halle, A. et al. (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization, *Nat Immunol* **9**, 847–856.
14. Dinarello, C. A. (2009) Immunological and inflammatory functions of the interleukin-1 family, *Annu Rev Immunol* **27**, 519–550.
15. Burckstummer, T., Baumann, C., Bluml, S. et al. (2009) An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome, *Nat Immunol* 10, 266–272.
16. Fernandes-Alnemri, T., Yu, J. W., Datta, P. et al. (2009) AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA, *Nature* **458**, 509–513.
17. Hornung, V., Ablasser, A., Charrel-Dennis, M. et al. (2009) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC, *Nature* **458**, 514–518.
18. Roberts, T. L., Idris, A., Dunn, J. A. et al. (2009) HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA, *Science* **323**, 1057–1060.
19. Poeck, H., Bscheider, M., Gross, O. et al. (2010) Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1 beta production, *Nat Immunol* **11**, 63–69.
20. Boyden, E. D., and Dietrich, W. F. (2006) Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin, *Nat Genet* **38**, 240–244.
21. Mariathasan, S., Newton, K., Monack, D. M. et al. (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf, *Nature* **430**, 213–218.
22. Martinon, F., Burns, K., and Tschopp, J. (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta, *Mol Cell* **10**, 417–426.
23. Mariathasan, S., Weiss, D. S., Newton, K. et al. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP, *Nature* **440**, 228–232.
24. Bauernfeind, F. G., Horvath, G., Stutz, A. et al. (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression, *J Immunol* **183**, 787–791.
25. Greten, F. R., Arkan, M. C., Bollrath, J. et al. (2007) NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta, *Cell* **130**, 918–931.

26. Bauernfeind, F., Ablasser, A., Bartok, E. et al. (2011) Inflammasomes: current understanding and open questions, *Cell Mol Life Sci* **68**, 765–83.
27. Bergsbaken, T., Fink, S. L., Cookson, B. T. (2009) Pyroptosis: host cell death and inflammation, *Nat Rev Microbiol* **7**, 99–109.
28. Keller, M., Ruegg, A., Werner, S. et al. (2008) Active caspase-1 is a regulator of unconventional protein secretion, *Cell* **132**, 818–831.
29. Lutz, M. B. (2004) IL-3 in dendritic cell development and function: a comparison with GM-CSF and IL-4, *Immunobiology* **209**, 79–87.