

## Inflammasome Activation and Inhibition in Primary Murine Bone Marrow-Derived Cells, and Assays for IL-1 $\alpha$ , IL-1 $\beta$ , and Caspase-1

Katharina S. Schneider, Christina J. Thomas, and Olaf Groß

### Abstract

Through its ability to control the proteolytic maturation and secretion of interleukin-1 family cytokines, the inflammasome occupies a central role in the activation of inflammation and also influences the shaping of adaptive immunity. Since it affects a multitude of different immune responses from autoinflammatory diseases to host defense, vaccine efficacy, and even cancer, it has become of interest to many researchers. Here, we describe a straightforward method for inflammasome assays in primary murine bone marrow-derived myeloid cells. The protocol encompasses cell handling, inflammasome activation and inhibition, as well as the detection of IL-1 $\beta$ , caspase-1, and IL-1 $\alpha$  by ELISA and Western blot.

**Key words** Inflammasome, NLRP3, Caspase-1, Interleukin-1 $\beta$ , Interleukin-1 $\alpha$

---

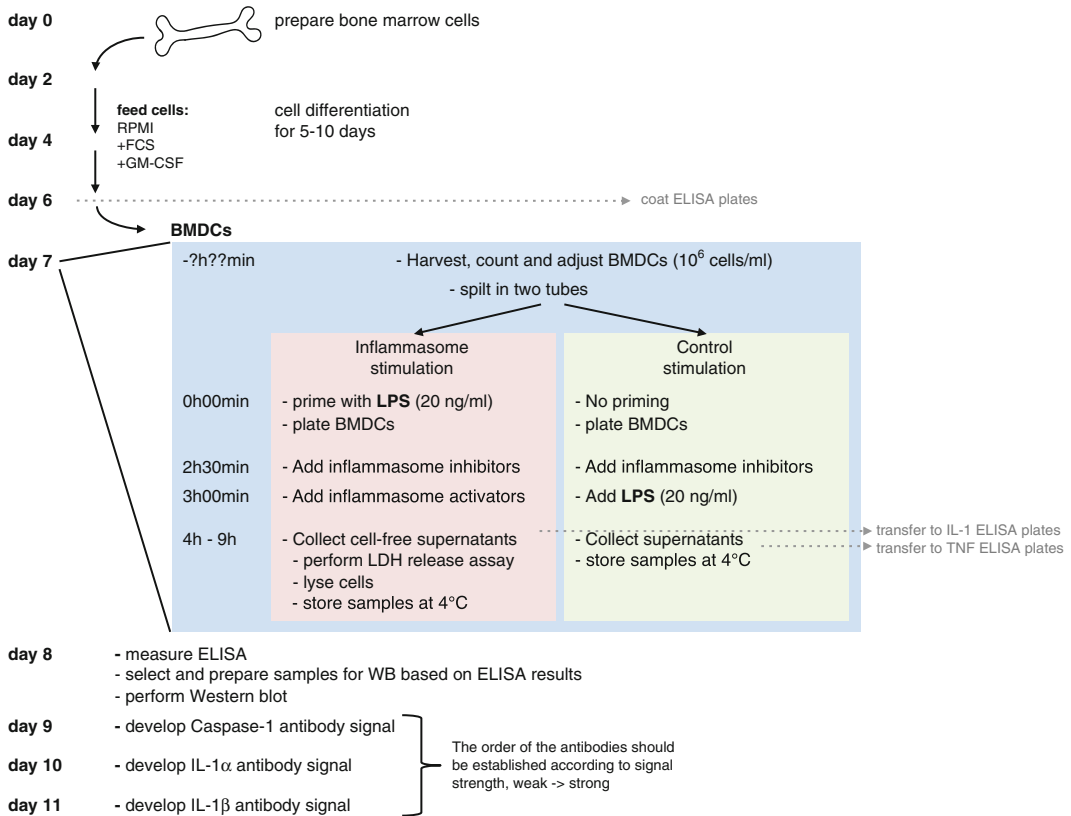
### 1 Introduction

Autocatalytic cleavage of caspase-1, subsequent cleavage of IL-1 $\beta$ , and secretion of both factors are hallmarks of inflammasome activation [1]. IL-1 $\alpha$ , while not a caspase-1 substrate, can also be secreted alongside IL-1 $\beta$  in an inflammasome-dependent manner [2–5]. Since both cytokines activate the same receptor [6], IL-1 $\alpha$  might contribute to the beneficial or adverse effects of inflammasome activity. Thus, the direct comparison of IL-1 $\alpha$  and IL-1 $\beta$  secretion in vitro and in vivo can be informative. Both IL-1 cytokines are encoded on the same genomic locus, and share a very similar protein structure [7]. A priming signal is required to induce their expression in innate immune cells of the myeloid lineage [3]. As both IL-1 $\alpha$  and IL-1 $\beta$  lack a signal peptide, they are not released by the default, ER-Golgi-dependent secretion pathway, but are instead retained in the cytoplasm until a second signal, i.e., inflammasome activation, prompts their secretion [8].

Caspase-1-mediated removal of the pro-domain of IL-1 $\beta$  is required for its binding to the IL-1 receptor. In contrast to IL-1 $\beta$ , IL-1 $\alpha$  is not a substrate of caspase-1, which could explain why IL-1 $\alpha$  has been largely overlooked in the inflammasome field. Instead, the pro-domain of IL-1 $\alpha$  is proteolytically processed by calpains [9, 10]. In the context of inflammasome activation, calpain activity can be induced in an inflammasome-dependent manner, indicating that the inflammasome can control IL-1 $\alpha$  cleavage, albeit indirectly [3]. However, IL-1 $\alpha$  cleavage is not required for receptor activation [7]. Therefore, any manner of IL-1 $\alpha$  release (i.e., active release after inflammasome activation, or passive release after cell injury) is sufficient for receptor activation on neighboring cells. This has encouraged the view that IL-1 $\alpha$  is a danger signal that passively leaks from the cytoplasm upon cell death [6], and IL-1 $\alpha$  release has even been used as a readout for cell death. However, since the inflammasome can control its release, IL-1 $\alpha$  appears to have a dual function as both a passively released danger signal and an actively secreted cytokine in the context of inflammasome activity.

Since its discovery over 10 years ago, the inflammasome has become one of the fastest growing areas of research in immunology [11, 12]. Initial studies were performed using cell lines such as THP1 monocytic cells and J774 macrophage-like cells. Aside from these two cell lines, most myeloid cell lines are hyporesponsive to inflammasome activators, presumably due to the low expression of certain inflammasome components. The generation of knockout mice deficient in the key inflammasome components caspase-1, Asc, and Nlrp3, and the use of these mice in *in vivo* disease models, has allowed identification of central roles of the different types of inflammasomes in host defense and in the pathogenesis of a multitude of diseases [1, 13–21]. In addition, primary myeloid cells derived from these mice are widely used in *in vitro* inflammasome research and have largely replaced the use of cell lines. Murine peritoneal macrophages, bone marrow neutrophils, bone marrow-derived macrophages (BMDMs), and bone marrow-derived dendritic cells (BMDCs) are all responsive to inflammasome activators.

This protocol describes our standard technique for priming, stimulating and inhibiting the inflammasome in primary murine BMDCs, as well as the measurement of IL-1 $\alpha$ , IL-1 $\beta$ , and caspase-1 cleavage and secretion by ELISA and Western blot. It is optimized for the use of BMDMs and BMDCs, which are most commonly used in the inflammasome field because they have the distinct advantage that they can be generated in large quantities. We favor BMDCs due to the higher amounts of IL-1 produced [22]. Protocols for the differentiation of BMDCs and BMDMs have been reported [22–24]. An advantage of using primary cells is that inflammasome-deficient mouse lines are readily available commercially (e.g., caspase-1 $^{-/-}$  mice [5] from The Jackson



**Fig. 1** Flowchart summarizing the method. *WB* Western blot

Laboratory) or can be obtained from the originating laboratories. It is therefore often easier to acquire inflammasome knockout mice (or bone marrow thereof) and to generate primary knockout cells, as compared to performing RNA interference in cell lines. An alternative to or an extension of this method is the generation of inflammasome deficient cell lines (and wildtype controls) through immortalization of primary mouse macrophages, for example using the J2 virus [25] (e.g., produced by AMJ2 cells). This can be especially helpful for researchers who want to use knockout cells, but want to avoid the expense of housing mice that are only used for in vitro studies.

The NLRP3 inflammasome is the most widely studied member of the inflammasome family, and responds to a multitude of readily available activators, making it the focus of this protocol. However, this protocol can also be applied to studies of the NLRP1, NLRC4, AIM2, and RIG-I inflammasomes. We propose a two-step process in which all supernatant samples are first measured by ELISA for the secretion of IL-1 $\alpha$  and IL-1 $\beta$ . In a second step, both supernatants and cell lysates of selected samples are subjected to analysis by Western blot for IL-1 $\alpha$ , IL-1 $\beta$ , and caspase-1 (see Fig. 1). ELISA allows more precise quantification, while

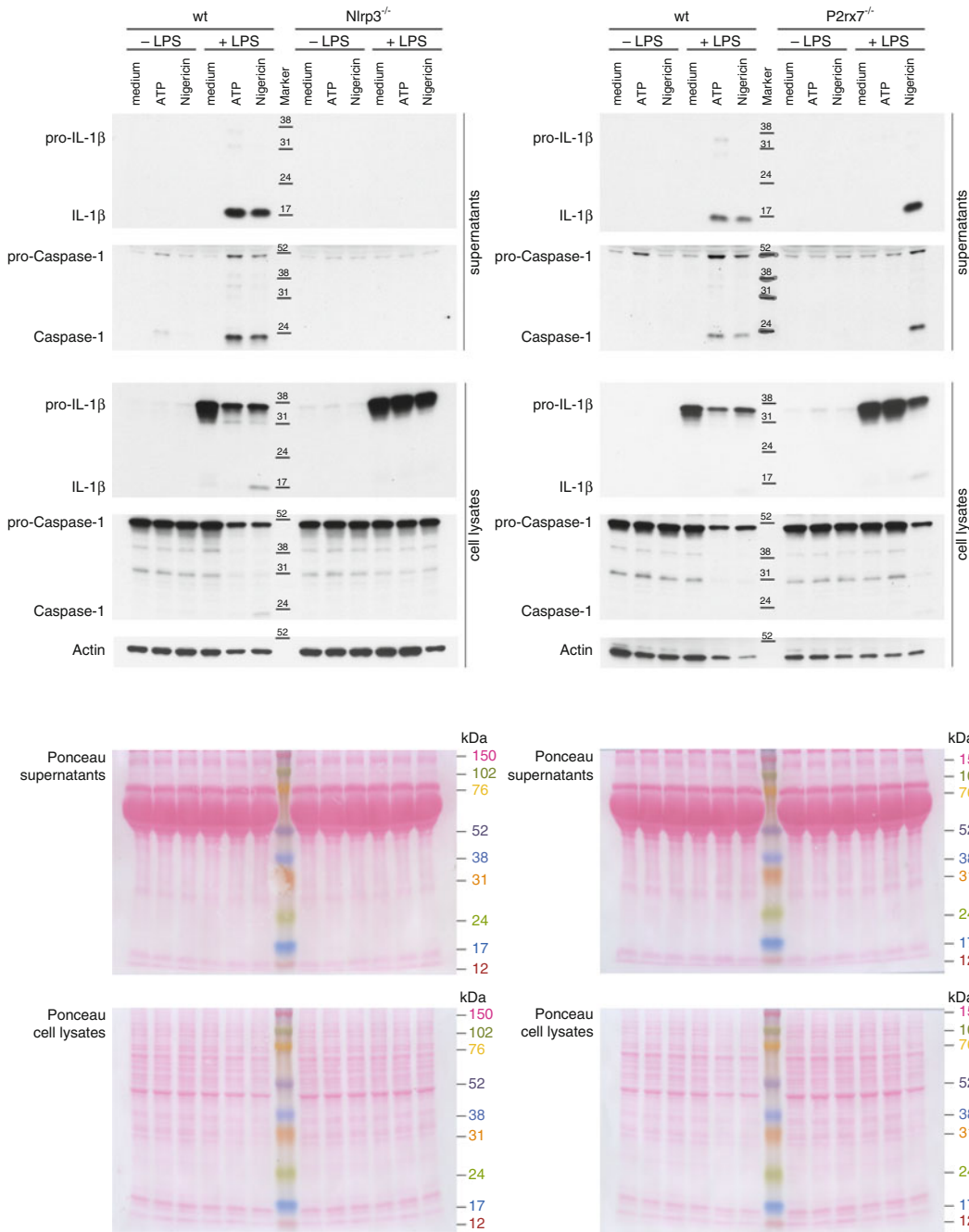
Western blot provides information about protein cleavage. Figures 2 and 4 show typical Western blots probed for these proteins. In addition, the protocol contains two different control measurements. We suggest combining the measurement of IL-1 $\alpha$  with a quantification of cell death through a lactose dehydrogenase (LDH) release assay, in order to allow differentiation of the two possible causes of IL-1 $\alpha$  release. Furthermore, stimulation with an activator that does not trigger the inflammasome but instead induces the secretion of inflammasome-independent cytokines can serve as an independent specificity control. As outlined above, there are several substantial differences between IL-1 $\alpha$  and IL-1 $\beta$  that have to be taken into consideration when extending inflammasome measurements to IL-1 $\alpha$ . The specific caveats in the measurement of IL-1 $\alpha$  are discussed in Subheading 4.

---

## 2 Materials

### 2.1 Inflammasome Activation

1. BMDCs from wildtype and, as required, inflammasome-deficient mouse lines, day 5–10 of differentiation (*see Note 1*).
2. BMDC medium: RPMI1640 containing GlutaMAX™ (Life Technologies) supplemented with 10 % FCS, 100 units/mL penicillin/streptomycin, 50  $\mu$ M  $\beta$ -mercaptoethanol, 10 mM HEPES, and 20 ng/mL recombinant murine GM-CSF (*see Note 2*).
3. 50 mL tubes.
4. Hank's Balanced Salt Solution containing 5 mM EDTA (HBSS/EDTA).
5. Trypan blue.
6. Hemocytometer.
7. 12-tip multichannel pipette with a volume range of at least 50–200  $\mu$ L per tip.
8. 10 mL serological pipettes.
9. 1.5 mL tubes.
10. 95 °C heat block.
11. Cell culture incubator at 37 °C and 5 % CO<sub>2</sub>.
12. 96-well flat-bottom cell culture plates.
13. Inflammasome activators, e.g.,
  - 1 M ATP stock (Sigma). Dissolve 1 g ATP in 1.8 mL H<sub>2</sub>O. Store at –20 °C. Dilution factor 1:33.3 for a 6 $\times$  working stock and 1:200 final = 5 mM.



**Fig. 2** Representative Western blots of cell-free supernatants and cell lysates of murine BMDCs. BMDCs from wildtype (wt) or Nlrp3-deficient (Nlrp3<sup>-/-</sup>, left half) or ATP receptor P2rx7-deficient (P2rx7<sup>-/-</sup>, right half) mice were LPS-primed and inflammasome-stimulated as indicated. Upregulation and processing of IL-1β and caspase-1 were analyzed by Western blot (upper part). Actin is shown as a loading control for lysates. Ponceau stainings of immunoblots (lower part) serve as a reference

- 10 mM nigericin stock (Sigma). Dissolve 5 mg in 670  $\mu$ L EtOH. Store at 4 °C. Dilution factor 1:333.3 for a 6 $\times$  working stock and 1:2,000 final = 5  $\mu$ M.
  - 40 mg/mL Imject alum stock (Pierce). Store at 4 or -20 °C. Dilution factor 1:33.3 for a 6 $\times$  working stock and 1:200 final = 200  $\mu$ g/mL.
14. LPS: 100  $\mu$ g/mL *E. coli* K14 ultrapure LPS in water (Invivogen). Store at -20 °C. Dilution factor 1:833.3 for a 6 $\times$  working stock and 1:5,000 final = 20 ng/mL.
  15. Inhibitors of choice (*see Note 3*), e.g.,
    - 0.1 M Ebselen stock (Enzo). Dissolve 10 mg in 365  $\mu$ L water. Store at -20 °C. Dilution factor 1:333.3 for a 6 $\times$  working stock and 1:2,000 final = 50  $\mu$ M.
    - 2.5 M KCl stock. Dissolve 1 g in 5.4 mL RPMI. Store at -20 °C. Dilution factor 1:8.3 for a 6 $\times$  working stock and 1:50 final = 50 mM.
    - 50 mM Z-VAD-fmk stock (Merck). Dissolve 10 mg in 441  $\mu$ L DMSO. Store at -20 °C. Dilution factor 1:416.7 for a 6 $\times$  working stock and 1:2,500 final = 20  $\mu$ M.

## **2.2 Inflammasome Measurement**

1. Cell culture centrifuge with swing-out buckets to hold 96-well plates.
2. Phosphate-buffered saline (PBS).
3. ELISA kits for IL-1 $\alpha$ , IL-1 $\beta$ , and TNF.
4. LDH release assay kit (Promega).
5. 96-well MaxiSorb ELISA plates (Nunc).
6. ELISA plate reader and analysis software.
7. 12 % SDS-PAGE gels (Mini-PROTEAN TGX precast gels, Bio-Rad).
8. Western blot equipment (Mini-PROTEAN Tetra cell, Bio-Rad).
9. Nitrocellulose membrane 0.45  $\mu$ m pores.
10. 5 % sodium azide in water.
11. Enhanced chemiluminescence solution.
12. Film, developer, dark room or a camera-based documentation system.
13. 3 $\times$  SDS PAGE sample buffer: 187.5 mM Tris-HCl, pH 6.8, 6 % w/v SDS, 0.03 % w/v Phenol Red, 30 % w/v Glycerol. Make a 1 $\times$  stock by diluting the 3 $\times$  stock with distilled water.
14. 5 $\times$  running buffer: 15 g/L Tris base, 72 g/L Glycine, 5 g/L SDS.
15. 1 $\times$  blotting buffer: 2.5 g/L Tris base, 12 g/L Glycine, 15 % denatured ethanol.

16. 1× Ponceau staining solution: 0.05 % Ponceau, 3 % Trichloroacetic acid.
17. Wash buffer: PBS containing 0.1% Tween-20.
18. Blocking buffer: wash buffer containing 2% skim milk powder.
19. Antibodies, e.g.,
  - Polyclonal anti-mouse IL-1 $\beta$  (goat, AF-401, R&D Systems).
  - Monoclonal anti-mouse caspase-1 p20 (mouse, Casper-1, AdipoGen).
  - Monoclonal anti-mouse IL-1 $\alpha$  (hamster, ALF-161, eBioscience).
  - HRP-conjugated anti-goat IgG (rabbit, #6026-05, Southern Biotech).
  - HRP-conjugated anti-mouse IgG (goat, #115-035-146, Jackson IR).
  - HRP-conjugated anti-hamster IgG (mouse, #554012, BD Biosciences).

---

### 3 Methods

#### 3.1 Stimulation of BMDCs

1. Plan the experiment and calculate the required number/volume of cells per mouse/genotype (*see Note 4*). Especially when inhibitors are used, it is crucial to perform a control experiment (*see Note 5*).
2. Harvest BMDCs between day 5 and day 10 of culture, by first transferring the suspension cells into 50 mL tubes (*see Note 6*).
3. Add 10 mL of HBSS/EDTA to the adherent cells remaining on the cell culture plate(s) and incubate for 10 min at 37 °C, to dislodge these cells.
4. Rinse the plate(s) thoroughly with the HBSS/EDTA by pipetting up and down with a serological pipette and add the collected cells to the respective 50 mL tube containing the suspension cells of the same plate/genotype.
5. Centrifuge the cells at 300–350  $\times g$  for 5–7 min and discard the supernatant.
6. Gently resuspended the cells in 10 mL of fresh BMDC medium using a serological pipette. Do not vortex (*see Note 7*).
7. Determine the total number of cells using trypan blue and a hemocytometer and adjust them to 10<sup>6</sup> per mL with fresh BMDC medium (*see Note 8*).
8. Split the cells into two tubes containing the volumes for inflammasome stimulation and control experiment (calculated in **step 1** of Subheading 3.1).

9. For inflammasome stimulation: Prime cells with 20 ng/mL LPS (1:5,000 of 100 µg/mL stock), and mix by inversion. Leave the cells intended for the LPS-induced TNF production assay (used to test for toxicity and off-target effects of the inhibitors) unprimed.
10. Plate 200 µL of both primed and unprimed cells per well in separate 96-well flat-bottom plates to perform triplicates of each condition.
11. Incubate the plates for 2.5 h at 37 °C to allow cellular priming to occur.
12. Prepare 6× stocks of inhibitors using dilution information in **item 15** of Subheading 2.1 (*see Note 9*).
13. Add 50 µL of the 6× inhibitor stocks (or medium) to the LPS-primed cells (to be treated with inflammasome activators) and unprimed cells (to be treated with LPS) and incubate for 30 min at 37 °C (*see Note 10*).
14. Prepare 6× concentrated inflammasome activators and 6× concentrated LPS (*see Note 11*).
15. Add 50 µL of 6× inflammasome activator to the primed cells and 50 µL of 6× LPS to the unprimed cells.
16. Incubate for 1–6 h (for inflammasome activators) or 4–6 h (for LPS) at 37 °C (*see Note 12*).
17. Centrifuge the inflammasome-stimulated plate(s) for 7 min at 300 × *g* at 4 °C.
18. Carefully transfer the uppermost 200 µL of the supernatants to a fresh 96-well plate using a multichannel pipette (*see Note 13*).
19. Discard the remaining supernatant in the inflammasome-stimulated plate(s) 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 to wick away excess supernatant without losing cells.
20. Add 200 µL of cold PBS to each well, centrifuge for 7 min at 300 × *g* at 4 °C, and repeat **step 19**.
21. Next, prepare the cell pellet for subsequent analysis by ELISA or Western blot as follows:  
To Prepare the Cell Pellet for Analysis by ELISA
  - (a) Add 200 µL of medium containing 10 % FCS.
  - (b) Subject the cells to three freeze–thaw cycles by transferring the plate between a freezer (–20 or –80 °C) and a 37 °C incubator. Make sure that the medium is completely frozen or thawed after each step.



- (c) After the last thaw, centrifuge the plates at  $300\text{--}350\times g$  to pellet debris, transfer the cell lysate supernatant to a new plate and perform a pro-IL-1 $\beta$  (and potentially IL-1 $\alpha$ ) ELISA in parallel to the measurement of IL-1 in the cell culture supernatants as described in Subheading 3.2 (*see Note 14*).

To Prepare the Cell Pellet for Analysis by Western Blot

- (a) Add 40  $\mu\text{L}$  of  $1\times$  SDS sample buffer to each well.
  - (b) Store the plate(s) at 4  $^{\circ}\text{C}$  overnight for next day Western blot analysis or at  $-20$  or  $-80$   $^{\circ}\text{C}$  for longer storage.
22. Transfer up to 50  $\mu\text{L}$  per well of the supernatants from the inflammasome stimulation from the fresh plate (from **step 18** of Subheading 3.1) to another fresh 96-well cell culture plate and perform a LDH release assay according to manufacturer's instructions (*see Note 15*).
  23. Store the fresh plate with the supernatants (from **step 18** of Subheading 3.1) at 4  $^{\circ}\text{C}$  overnight for selection of Western blot samples on the next day (or at  $-20$  or  $-80$   $^{\circ}\text{C}$  for longer storage).

### 3.2 ELISA

Carry out IL-1 $\alpha$ , IL-1 $\beta$ , and TNF ELISAs according to the manufacturer's instructions. This section suggests time management of your ELISA experiment(s).

1. Coat the required amounts of IL-1 $\alpha$ , IL-1 $\beta$ , and TNF ELISA plates with 50  $\mu\text{L}$  of capture antibody solution the day before BMDC stimulation in Subheading 3.1, according to manufacturer's specifications (*see Note 16*).
2. During the incubation in **step 16** of Subheading 3.1, block ELISA plates and prepare a standard dilution using a high standard of 10 ng/mL and an 11-point standard curve of  $1/2$  dilutions with a blank.
3. Apply supernatants (from **step 18** of Subheading 3.1) and/or freeze-thaw cell lysates (from **step 21** of Subheading 3.1) to the ELISA plate (*see Note 14*). Most samples must be diluted for the ELISA measurement (*see Note 17*).
4. Following overnight incubation of the plates, develop ELISA for IL-1 $\beta$ , IL-1 $\alpha$ , and TNF according to manufacturer's recommendations (*see Note 18*) and analyze the results.

### 3.3 Western Blot

1. Based on the ELISA results, choose samples from the inflammasome stimulation for further analysis by Western blot.
2. Bring the plate with the cell lysates from **step 21** of Subheading 3.1 to room temperature. Pool the triplicates into 1.5 mL tubes (*see Note 19*).

3. Remove undiluted supernatants from **step 18** of Subheading 3.1 from the refrigerator/freezer. Pool 30  $\mu\text{L}$  of each well of a triplicate into one well of a new 96-well plate using a multichannel pipette. Add 45  $\mu\text{L}$  of 3 $\times$  SDS sample buffer to each pooled well, and transfer the contents of each well to a 1.5 mL tube.
4. Incubate samples from **steps 2** and **3** of this section in a 95  $^{\circ}\text{C}$  heat block for 5 min.
5. Subject the samples to SDS-PAGE using 12 % precast gels (*see Note 20*), or 15 % gels, if IL-1 $\alpha$  will be analyzed (*see Note 21*).
6. Transfer the proteins to a nitrocellulose membrane at 100 V for 1.5 h (*see Note 22*).
7. Incubate the membrane in 10–15 mL Ponceau red solution for 2 min with mild shaking to confirm successful transfer.
8. Decant Ponceau red solution and wash the membrane for 2 min in distilled water with mild shaking until excess dye is removed. Ponceau red solution can be repeatedly reused.
9. Make a photocopy or scan of the blot for your documentation, and note any loading differences between samples. Figure 2 shows an example (*see Note 23*).
10. Block the membranes by incubating in blocking buffer for 0.5–1 h at room temperature with mild shaking (*see Note 24*).
11. Discard blocking buffer, add the primary antibody (0.5–1  $\mu\text{g}/\text{mL}$  in blocking buffer containing 0.05 % azide), and incubate overnight at 4  $^{\circ}\text{C}$  with mild shaking (*see Note 25*).
12. Remove primary antibody and store at  $-20^{\circ}\text{C}$  for reuse. Wash the blots four times in wash buffer for 5 min each, at room temperature with mild shaking.
13. Dilute the appropriate HRP-conjugated secondary antibody 1:3,000–1:10,000 in blocking buffer (no azide) and add to the blots (*see Note 24*).
14. Incubate for 2 h at room temperature with mild shaking.
15. Wash for 2 h in wash buffer at room temperature with mild shaking, changing the buffer at least five times during the wash.
16. 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 foil) for 1–2 min, and tap dry again (*see Note 26*).
17. Develop the blot using standard techniques and equipment. For troubleshooting on background problems, (*see Note 27*).
18. Wash blots briefly in wash buffer. Repeat **steps 11–17** with different primary and secondary antibodies. Example blots are provided in Figures 2 and 4 (*see Note 28*).

---

## 4 Notes

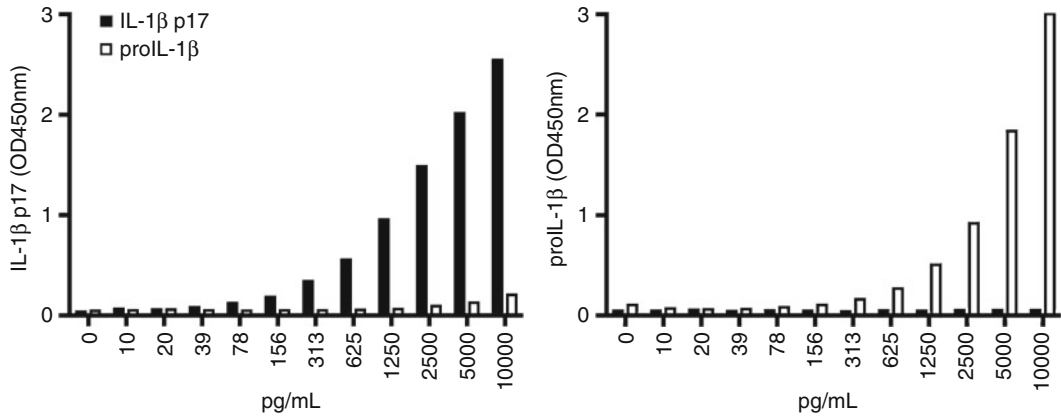
1. Several protocols are available for the generation of BMDCs [22, 23]. Most of them aim at a maximum yield of immature (but differentiated) BMDCs for use in antigen presentation and T cell activation assays. As we prime the cells for inflammasome activation anyway, we are not as concerned about their preactivation status. Approximately  $0.5\text{--}1 \times 10^8$  cells per mouse can be expected. Seed cells on day 0 at a density of  $5 \times 10^5\text{--}10^6$  cells per mL (typically 5–10 non-tissue culture treated 10 cm dishes per mouse). On day 2, add 10 mL of medium, on day 4, 6, and 8, remove 10 mL of cell suspension from each plate, centrifuge at  $300\text{--}350 \times g$  for 5–7 min and discard the medium. Resuspend the cells in the same volume of fresh BMDC medium and add back to the plates. The cell density should not exceed  $2 \times 10^6$  per mL. Laboratories working with bone marrow-derived myeloid cells may choose to adhere to their established protocols for generating these cells.
2. The quality of the FCS used is critical. It may be necessary to test several batches of low endotoxin FCS to find one that gives a good yield of differentiated (CD11c<sup>+</sup>) immature DCs. Regular RPMI medium without GlutaMAX™ but supplemented with 2 mM glutamine can also be used for BMDC medium.
3. Typically, inhibitors will only be used on wildtype cells. Here, Ebselen, KCl, and Z-VAD-fmk are used. Ebselen is a ROS inhibitor, sometimes referred to as a glutathione mimetic. KCl is used to prevent potassium efflux from cells by increasing the extracellular levels of potassium. Z-VAD-fmk is a pan-caspase inhibitor that irreversibly binds to the active site of caspases including caspase-1. Since the activation mechanism of NLRP3 is still unclear, some debate has arisen as to the specificity of certain inhibitors of inflammasome activation. For example, increasing extracellular potassium levels to cytoplasmic concentration has significant off-target effects, presumably due to effects on membrane polarization. Furthermore, many ROS inhibitors can also affect the activity of the transcription factor NF- $\kappa$ B and might therefore interfere with priming [26]. If inhibitors are to be used, it is important to include controls for off-target effects or toxicity (*see Note 5*) and to ensure sufficient priming before adding the inhibitor. It can also be useful to determine NLRP3 levels in cell lysates, since priming can boost NLRP3 expression [27].
4. It is useful to label the 96-well plates before the experiment in order to visualize the set-up and ensure that the calculation is correct. Leave wells empty for ELISA standards if a complete

plate of the stimulation is to be transferred to one ELISA plate. Here is an example calculation for an experiment using the stimuli listed in Subheading 2:

- *For inflammasome activation—wildtype*: (3 stimuli (ATP, nigericin, alum) + 1 unstimulated = 4) × (3 inhibitors (Ebselen, KCl, Z-VAD-fmk) + 1 uninhibited = 4) × (replicates = 3) × (volume per well = 200  $\mu$ L) × (pipetting loss = 1.1) = 10.56 mL of cells with a density of  $10^6$  per mL.
  - *For inflammasome activation—knockout*: (3 stimuli (ATP, nigericin, alum) + 1 unstimulated = 4) × (replicates = 3) × (volume per well = 200  $\mu$ L) × (pipetting loss = 1.1) = 2.64 mL of cells with a density of  $10^6$  per mL.
  - *For control stimulation—wildtype*: (1 stimulus (LPS) + 1 unstimulated = 2) × (3 inhibitors (Ebselen, KCl, Z-VAD-fmk) + 1 uninhibited = 4) × (replicates = 3) × (volume per well = 200  $\mu$ L) × (pipetting loss = 1.1) = 5.28 mL of cells with a density of  $10^6$  per mL.
  - *For control stimulation—knockout*: (1 stimulus (LPS) + 1 unstimulated = 2) × (replicates = 3) × (volume per well = 200  $\mu$ L) × (pipetting loss = 1.1) = 1.32 mL of cells with a density of  $10^6$  per mL.
5. To control for unexpected effects of inhibitors or gene deficiency, and also for potential differences in plating density, a good option is to do a parallel experiment in which a different signaling pathway is activated. Here, LPS-induced TNF production is used for this purpose. We suggest taking an aliquot of the same cells used for inflammasome stimulation and leave them unprimed (*see* Fig. 1). These should be plated and treated in parallel to the primed cells with the inhibitor(s). Instead of treatment with inflammasome activators, stimulate the control cells with LPS for a similar duration as the primed cells are subjected to inflammasome activators (4–6 h). Measuring TNF production from this stimulation gives an indication as to whether a compound or genetic deficiency interferes with TLR4 signaling in addition to inflammasome activation. Furthermore, determining the levels of pro-IL-1 $\alpha$ , pro-IL-1 $\beta$ , and pro-caspase-1 (as well as NLRP3 or ASC) in cell lysates by Western blot will give an indication as to a possible influence on priming in comparison to actual inflammasome activation.
  6. The longer the DCs are in culture, the more IL-1 and caspase-1 they secrete upon inflammasome activation. This may in part be due to the higher proportion of differentiated (CD11c<sup>+</sup>) cells. However, the longer the DCs are in culture, the more cells will become adherent. Classically, these cells would be deemed either mis-differentiated cells that drifted into a macrophage lineage or preactivated, mature dendritic cells.

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.

7. It is particularly important that cells are resuspended in fresh medium if cell death will be determined by LDH release assay.
8. Counting and adjusting BMDCs is subject to inaccuracy. To avoid cells aggregating or sticking to the wall of the tube, it is best to count for each mouse/genotype immediately either after pooling adherent and non-adherent cells or after resuspending the cells in fresh BMDC medium. The cells should be carefully resuspended by pipetting up and down several times with a serological pipette (not by vortexing) right before taking a sample. Aliquots should not be left in a 96-well plate for later counting, since the cells might become adherent. The cells should also be counted immediately after trypan blue is added. Count the cells again after readjusting, allowing a tolerance of  $\pm 10\%$ . The control stimulation will help to estimate the error derived from any differences in cell density.
9. Use 1.5 mL tubes containing the required volume of BMDC medium. Leave one tube without inhibitor and add the required amount of each inhibitor stock to the other tubes and mix well. Calculation: [(number of inflammasome stimuli + 1 control stimulus + 2 unstimulated)  $\times$  (3 replicates)  $\times$  (50  $\mu$ L)  $\times$  (1.1 for pipetting loss)]. Typical dilution factors and stock concentrations are listed in Subheading 2.1, **item 15**. Note that cell culture medium contains 5 mM KCl, while cytoplasmic concentrations are around 130 mM. Therefore, concentrations tested should stay within these boundaries and 50 mM is sufficient for inflammasome inhibition. ROS inhibitors might become spontaneously oxidized over time, so stock solutions should not be stored for extended periods of more than 6 weeks.
10. 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 performing a time course experiment (after the optimal time point for the stimulus has been previously determined, *see Note 12*) to find the optimal preincubation period for optimal inhibition and minimal off-target effects or toxicity.
11. Use 1.5 mL tubes containing the required volume of BMDC medium. Leave one tube without stimulus and add the required amount of each stimulus stock to the other tubes, mix well by vortexing. Note that particulate stimuli tend to aggregate, but a short sonication (with either a bath or probe sonicator) will help to dissociate them. Calculation: [(number of mice + number of inhibitors)  $\times$  (3 replicates)  $\times$  (50  $\mu$ L)  $\times$  (1.1 for pipetting loss)]. Typical stock concentrations and dilution factors are listed in **items 13** and **14** of Subheading 2.1.



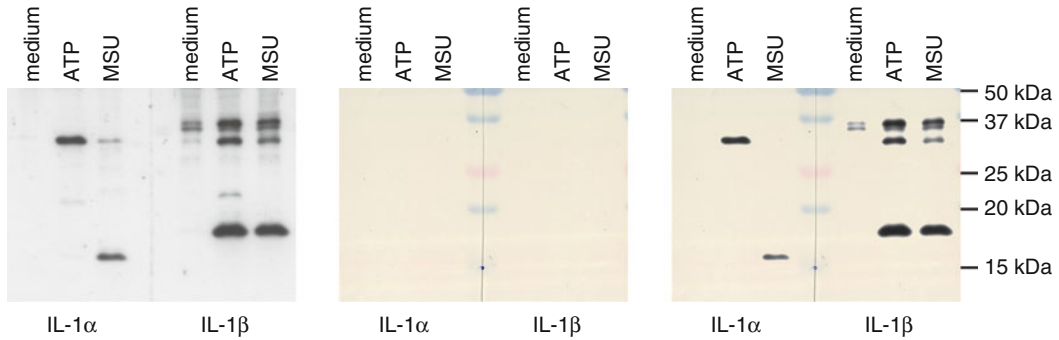
**Fig. 3** Specificity of ELISAs for pro-IL-1 $\beta$  and IL-1 $\beta$  p17. Serial  $\frac{1}{2}$  dilutions of recombinant pro-IL-1 $\beta$  and IL-1 $\beta$  p17 were subjected to ELISA for both pro-IL-1 $\beta$  and IL-1 $\beta$  p17 according to the manufacturer's instructions. Data is presented as absorbance at 450 nm

12. The duration of the stimulation is stimulus-dependent. 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. It might be worth performing 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 the effects of feedback loops, cell death, or overgrowth of a pathogen. It might be useful to harvest fast-acting stimuli earlier than slow ones, especially when measuring cell death by LDH release, since that signal will increase over time [3]. In contrast, the IL-1 signal does not change if fast-acting stimulants are left on the cells for longer time periods [3]. Therefore, in a larger experiment, samples treated with fast-acting stimuli and samples treated with slow-acting stimuli should be cultured on separate plates, while cells of different genotype or various inhibitors can be cultured on the same plate.
13. For supernatant samples that will be analyzed for IL-1 secretion, be careful not to disturb the pelleted cells. 200  $\mu$ L per replicate should be enough for subsequent analysis. If the control stimulation (i.e., LPS stimulation-induced TNF production after treatment with inhibitors) was done on a separate plate, the supernatants can be diluted and the plate can be stored (at 4  $^{\circ}$ C overnight or at  $-20$  or  $-80$   $^{\circ}$ C for longer storage) without separating the supernatants from the cells.
14. In contrast to IL-1 $\alpha$ , the commercially available IL-1 $\beta$  ELISA kits do not detect pro-IL-1 $\beta$ , and should therefore not be used to monitor intracellular pro-IL-1 $\beta$  levels [3, 28]. This is illustrated in Fig. 3. However, intracellular amounts of pro-IL-1 $\beta$

can be detected by an ELISA kit designed to detect only the pro-form (eBioscience). By subjecting the cells to repeated freeze-thaw cycles, the intracellular pro-IL-1 is released and is thereby made available for ELISA testing. This allows direct comparison of the amount of pro-IL-1 inside the cells to the amount of secreted IL-1 in the supernatants.

15. A triplet of fresh BMDC medium should be included to determine the background of the medium without cells. Freeze-thaw or detergent-treated cells can be used to determine 100 % lysis (maximal LDH release).
16. One well in the stimulations corresponds to one well on the ELISA plate. Therefore, if wells are left empty on the stimulation plates for ELISA standards, the same number of plates is needed for the TNF and IL-1 ELISAs as are needed for the control stimulation and the inflammasome stimulation, respectively. The final triplets measured are replicates of the whole experimental procedure, not only of the ELISA method.
17. Many stimuli may induce the production of more than 10 ng/mL of IL-1 or TNF. This is above the measurable range of the ELISA, so the supernatants should be diluted at least 1/3 for measurement. Greater dilutions (1/10) may be necessary for some experiments. For cytokine measurement from the cell pellet, the samples should be diluted at 1/10.
18. The last washing steps after incubation with streptavidin-HRP and before adding the substrate are critical in reducing the background and minimizing variation between replicate samples.
19. The cell lysates serve two purposes: They represent a control to demonstrate that equal amounts of cells (e.g., from different genotypes) were used. Some stimuli like nigericin or ATP are quite toxic to the cells, which might reduce the total protein content in some samples. 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.
20. We use the Mini-PROTEAN gel system from Bio-Rad with 1 mm thick 12 % precast gels and 15 lanes per gel and the related wet blot system and run the gels at 100–160 V for 1–2 h until just before the dye front leaves the gel. We have also obtained good results with precast gradient gels from Invitrogen.
21. The predicted protein size of both mature IL-1 $\beta$  and cleaved IL-1 $\alpha$  are 17.4 kDa and 18.0 kDa, respectively. However, the molecular weight of cleaved IL-1 $\alpha$  has variably been reported to be between 15 and 17 kDa, while mature IL-1 $\beta$  is usually labelled as 17 kDa. Figure 4 shows a direct comparison between





**Fig. 4** Comparison between the migration of murine IL-1 $\alpha$  and IL-1 $\beta$  in an SDS-PAGE. Murine BMDCs were left unstimulated or stimulated with ATP or monosodium urate crystals (MSU). The supernatant samples were loaded twice on the same 15 % gel and blotted on a nitrocellulose membrane. This was cut in two after transfer; one half was probed for IL-1 $\alpha$ , the other for IL-1 $\beta$ . For the exposure to film, the two halves of the blot were arranged side by side so that the molecular weight markers lined up. The blots themselves were scanned directly after exposure to the film and in the exact same arrangement. A scan of the developed films (*left*) and a scan of the membranes (*middle*) were overlaid (*right*). The IL-1 $\alpha$  antibody has been raised against the full C-terminus of murine IL-1 $\alpha$  (aa 99–268) [30]

the migration of murine IL-1 $\alpha$  and IL-1 $\beta$  in an SDS-PAGE and Western blot from cell culture supernatants. Murine IL-1 $\alpha$  migrates below its predicted molecular weight (at ~16 kDa), while IL-1 $\beta$  migrates at approximately the same level as predicted. This could be because IL-1 $\alpha$  has a low theoretical isoelectric point (5.57) and is thus more negatively charged than IL-1 $\beta$  (pI 8.08) in the pH range that is commonly used in the separating gel of an SDS-PAGE. For such a small protein, this additional negative charge (on top of that provided by the SDS) could be sufficient to shift the charge to mass ratio of the unfolded peptide and allow IL-1 $\alpha$  to migrate faster in the gel. Similarly, human IL-1 $\alpha$  and IL-1 $\beta$  have different isoelectric points, and it was this difference that allowed the initial identification of the two IL-1 cytokines as distinct proteins [29]. The different behavior in an SDS-PAGE has consequences when measuring IL-1 $\alpha$  along with IL-1 $\beta$  and caspase-1 in a Western blot. We suggest here to use precast 12 % SDS PAGE gels for the analysis of IL-1 $\beta$  and caspase-1. These gels are easy to use and allow maximal resolution of the pro- and mature forms of these two proteins. However, IL-1 $\alpha$  will migrate in the running front of a 12 % gel, so 15 % gels should instead be used. These can be purchased (e.g., from Life Technologies) or made as previously described [22].

22. A wet blot system may lead to better transfer than a semidry system, and thus a stronger signal. The current should be no higher than 300 mA per tank. The blotting buffer might get



warm, but should not get hot. The cold packs that come with the system should be used to keep the temperature moderate. Alternatively, 2–3 water-filled 50 mL tubes frozen at  $-20^{\circ}\text{C}$  can be used as cold packs. Make sure that the blot buffer is thoroughly mixed before filling the tanks, since poor mixing might increase the resistance. If Western blots are performed infrequently, the blot buffer should be made without ethanol and it should be freshly added before each blot to avoid evaporation of the ethanol.

23. The most prominent band in the supernatants is around 60 kDa, and corresponds to the albumin in the FCS.
24. The volumes of blocking, washing and antibody solutions will vary greatly depending on the number of blots, the size of the blots, and the size of the vessel in which the incubations are performed. Washing and blocking steps should be carried out using generous volumes. Antibody incubation steps should use no more volume than is necessary to cover the blots and allow them to move freely against one another.
25. 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). Antibody solutions should be stored at  $-20^{\circ}\text{C}$  in the meantime.
26. Do not let the blots air-dry completely after tapping them but quickly cover them in transparent foil and move them to a developing cassette or put them back into ECL or wash buffer. 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. If normal ECL is too weak, but high sensitivity (“femto”) is too strong, femto, 1:3 diluted in normal ECL solution or water can be used. If a CCD camera system is used for documentation, the last tapping-dry step can be omitted and instead, the ECL-soaked membrane can be transferred to the CCD system without any covering foil to avoid potential scattering of the light signal by the foil.
27. Several methods for minimizing background are described here. First, longer or more intense washing steps could be performed. We have found washing overnight at  $4^{\circ}\text{C}$  to be helpful in some cases. Using a different wash buffer, containing 0.1 % Triton 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). The use of a lower concentration of primary and/or secondary antibodies, or a different secondary antibody, directed against the specific isotype of the primary antibody, may also help.

28. As the common antibodies used (Figures 2 and 4) were generated in different species (caspase-1: mouse, IL-1 $\beta$ : goat, IL-1 $\alpha$ : hamster), the same membrane can be probed for all three proteins. The order in which these three antibodies are used should be decided based on signal strength and the project-specific interest. Either the weakest signal or the most important readout for a given experimenter should be measured first.

---

## Acknowledgments

OG is supported by the Bavarian Ministry of Sciences, Research and the Arts in the Framework of the Bavarian Molecular Biosystems Research Network. C.J.T. is supported by an NSERC postgraduate scholarship. K.S.S. is supported by a Ph.D. scholarship by the faculty of medicine.

## References

- Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440(7081):237–241. doi:nature04516 [pii], 10.1038/nature04516
- Fettelschoss A, Kistowska M, LeibundGut-Landmann S, Beer HD, Johansen P, Senti G, Contassot E, Bachmann MF, French LE, Oxenius A, Kundig TM (2011) Inflammasome activation and IL-1 $\beta$  target IL-1 $\alpha$  for secretion as opposed to surface expression. *Proc Natl Acad Sci USA* 108(44):18055–18060. doi:10.1073/pnas.1109176108
- Gross O, Yazdi AS, Thomas CJ, Masin M, Heinz LX, Guarda G, Quadroni M, Drexler SK, Tschopp J (2012) Inflammasome activators induce interleukin-1 $\alpha$  secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity* 36(3):388–400. doi:10.1016/j.immuni.2012.01.018
- Keller M, Ruegg A, Werner S, Beer HD (2008) Active caspase-1 is a regulator of unconventional protein secretion. *Cell* 132(5):818–831. doi:10.1016/j.cell.2007.12.040
- Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS, Flavell RA (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1 $\beta$  converting enzyme. *Science* 267(5206):2000–2003
- Dinarello CA (2009) Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 27:519–550. doi:10.1146/annurev.immunol.021908.132612
- Sims JE, Smith DE (2010) The IL-1 family: regulators of immunity. *Nat Rev Immunol* 10(2):89–102. doi:10.1038/nri2691
- Nickel W, Seedorf M (2008) Unconventional mechanisms of protein transport to the cell surface of eukaryotic cells. *Annu Rev Cell Biol* 24:287–308. doi:10.1146/annurev.cellbio.24.110707.175320
- Carruth LM, Demczuk S, Mizel SB (1991) Involvement of a calpain-like protease in the processing of the murine interleukin 1 $\alpha$  precursor. *J Biol Chem* 266(19):12162–12167
- Kobayashi Y, Yamamoto K, Saïdo T, Kawasaki H, Oppenheim JJ, Matsushima K (1990) Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1 $\alpha$ . *Proc Natl Acad Sci USA* 87(14):5548–5552
- Schroder K, Tschopp J (2010) The inflammasomes. *Cell* 140(6):821–832. doi:S0092-8674(10)00075-9 [pii], 10.1016/j.cell.2010.01.040
- Martinon F, Burns K, Tschopp J (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL- $\beta$ . *Mol Cell* 10(2):417–426. doi:S1097276502005993 [pii]
- Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J (2008) Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320(5876):674–677. doi:1156995 [pii], 10.1126/science.1156995
- Henderson C, Goldbach-Mansky R (2010) Monogenic autoinflammatory diseases: new

- insights into clinical aspects and pathogenesis. *Curr Opin Rheumatol* 22(5):567–578. doi:[10.1097/BOR.0b013e32833ceff4](https://doi.org/10.1097/BOR.0b013e32833ceff4), 00002281-201009000-00017 [pii]
15. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G, Ullrich E, Perfettini JL, Schlemmer F, Tasmehir E, Uhl M, Genin P, Civas A, Ryffel B, Kanellopoulos J, Tschopp J, Andre F, Lidereau R, McLaughlin NM, Haynes NM, Smyth MJ, Kroemer G, Zitvogel L (2009) Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat Med* 15(10):1170–1178. doi:[nm.2028](https://doi.org/10.1038/nm.2028) [pii], [10.1038/nm.2028](https://doi.org/10.1038/nm.2028)
  16. Masters SL, Dunne A, Subramanian SL, Hull RL, Tannahill GM, Sharp FA, Becker C, Franchi L, Yoshihara E, Chen Z, Mullooly N, Mielke LA, Harris J, Coll RC, Mills KH, Mok KH, Newsholme P, Nunez G, Yodoi J, Kahn SE, Lavelle EC, O'Neill LA (2010) Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. *Nat Immunol* 11(10):897–904. doi:[ni.1935](https://doi.org/10.1038/ni.1935) [pii], [10.1038/ni.1935](https://doi.org/10.1038/ni.1935)
  17. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nunez G, Schnurr M, Espevik T, Lien E, Fitzgerald KA, Rock KL, Moore KJ, Wright SD, Hornung V, Latz E (2010) NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464(7293):1357–1361. doi:[nature08938](https://doi.org/10.1038/nature08938) [pii], [10.1038/nature08938](https://doi.org/10.1038/nature08938)
  18. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA (2009) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 458(7237):514–518. doi:[nature07725](https://doi.org/10.1038/nature07725) [pii], [10.1038/nature07725](https://doi.org/10.1038/nature07725)
  19. Poeck H, Bscheider M, Gross O, Finger K, Roth S, Rebsamen M, Hanneschlagger N, Schlee M, Rothenfusser S, Barchet W, Kato H, Akira S, Inoue S, Endres S, Peschel C, Hartmann G, Hornung V, Ruland J (2010) Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1 beta production. *Nat Immunol* 11(1):63–69. doi:[ni.1824](https://doi.org/10.1038/ni.1824) [pii], [10.1038/ni.1824](https://doi.org/10.1038/ni.1824)
  20. Boyden ED, Dietrich WF (2006) Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet* 38(2):240–244. doi:[ng1724](https://doi.org/10.1038/ng1724) [pii], [10.1038/ng1724](https://doi.org/10.1038/ng1724)
  21. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, Roose-Girma M, Erickson S, Dixit VM (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430(6996):213–218. doi:[10.1038/nature02664](https://doi.org/10.1038/nature02664), [nature02664](https://doi.org/10.1038/nature02664) [pii]
  22. Gross O (2012) Measuring the inflammasome. *Methods Mol Biol* 844:199–222. doi:[0.1007/978-1-61779-527-5\\_15](https://doi.org/10.1007/978-1-61779-527-5_15)
  23. Lutz MB (2004) IL-3 in dendritic cell development and function: a comparison with GM-CSF and IL-4. *Immunobiology* 209(1–2):79–87
  24. Manzanero S (2012) Generation of mouse bone marrow-derived macrophages. *Methods Mol Biol* 844:177–181. doi:[10.1007/978-1-61779-527-5\\_12](https://doi.org/10.1007/978-1-61779-527-5_12)
  25. Palleroni AV, Varesio L, Wright RB, Brunda MJ (1991) Tumoricidal alveolar macrophage and tumor infiltrating macrophage cell lines. *Int J Cancer* 49(2):296–302
  26. Murphy MP, Holmgren A, Larsson NG, Halliwell B, Chang CJ, Kalyanaraman B, Rhee SG, Thornalley PJ, Partridge L, Gems D, Nystrom T, Belousov V, Schumacker PT, Winterbourn CC (2011) Unraveling the biological roles of reactive oxygen species. *Cell Metab* 13(4):361–366. doi:[10.1016/j.cmet.2011.03.010](https://doi.org/10.1016/j.cmet.2011.03.010)
  27. 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:[jimmunol.0901363](https://doi.org/10.1093/jimmunol.0901363) [pii], [10.4049/jimmunol.0901363](https://doi.org/10.1093/jimmunol.0901363)
  28. Wewers MD, Pope HA, Miller DK (1993) Processing proIL-1 beta decreases detection by a proIL-1 beta specific ELISA but increases detection by a conventional ELISA. *J Immunol Methods* 165(2):269–278
  29. Dinarello CA, Goldin NP, Wolff SM (1974) Demonstration and characterization of two distinct human leukocytic pyrogens. *J Exp Med* 139(6):1369–1381
  30. Fuhlbrigge RC, Sheehan KC, Schreiber RD, Chaplin DD, Unanue ER (1988) Monoclonal antibodies to murine IL-1 alpha. Production, characterization, and inhibition of membrane-associated IL-1 activity. *J Immunol* 141(8):2643–2650