

Functional Reconstruction of NLRs in HEK293 Cells

Vincent Compan and Gloria López-Castejón

Abstract

Inflammasomes are molecular complexes that initiate innate immune response. They are mainly expressed by immune cells; however, molecular manipulations in these cells remain very difficult. Here, we describe a simple protocol to overexpress and activate functional NLRP3 inflammasomes in HEK293 cells.

Key words NLR, HEK293, ASC, Inflammasome, Active caspase-1

1 Introduction

Members of the NOD-like receptor (NLR) family are key components in the initiation of innate immune response to tissue injury or pathogen infection. These proteins trigger cellular responses by forming supramolecular complexes called inflammasomes [1]. The best characterized inflammasome contains the NLR protein NLRP3, the adaptor protein ASC, and the cysteine protease caspase-1. Activation of this complex leads to the processing and the release of the pro-inflammatory cytokine IL-1 β in order to initiate and propagate the inflammatory response. NLRP3 inflammasome responds to a broad range of stimuli including different pathogens or pathogen associated toxin (as the bacterial ionophore nigericin [2]), endogenous danger signals (as extracellular ATP [2]), or variation in homeostatic parameter (for example, variation in extracellular osmolarity [3]). Immune cells are the main cells expressing the NLRP3 inflammasome, including macrophages and monocytes [4]. Characterization of inflammasome activation remains rather challenging as immune cells are hard to transfect cells and they express endogenous inflammasome components that could interact with the variations under study. Thus, we developed a simple protocol for expression of NLRP3 components in HEK293 cells that allows reconstituting a functional NLR complex. We found that in response to low osmolarity or nigericin, overexpressed NLRP3 inflammasome trigger caspase-1 activation and IL-1 β

processing with similar features to an endogenous inflammasome, hence validating the use of this approach in experimental research settings [5].

2 Materials

2.1 Transfection of NLRP3 Inflammasome in HEK293 Cells

1. Lipofectamine2000 reagent® (Life Technologies) or equivalent can be used to transfect HEK293 cells.
2. Complete cell culture media: Dulbecco's modified Eagle's medium (DMEM) high glucose with glutamine and complemented with 10 % decompeted fetal bovine serum. Antibiotics as penicillin/streptomycin can be used.
3. Opti-MEM® medium (Life Technologies).
4. Mammalian expressing plasmids coding for NLRP3, caspase-1, and ASC (*see Note 1*).

2.2 Detection of Inflammasome Functionality/Activation by Western Blot

1. Antibodies for detection of caspase-1 (inactive form and one of the active forms p10 or p20 of caspase-1), ASC, and NLRP3 (*see Note 2*).
2. Cell lysis buffer (CLB): 20 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM EDTA, 1 % Nonidet P-40, and supplemented on day of use with protease inhibitors.
3. HBS solution: 147 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 13 mM d-glucose (pH 7.4).
4. Hypotonic solution: HBS solution diluted in sterile ultrapure H₂O to get a 90 mOsmol solution.
5. Tris-glycine gels (resolving gel 15 % and stacking gel 5 %, *see Note 3*).
6. SDS-PAGE running buffer (TGS; 1×): 25 mM Tris-HCl, 192 mM Glycine, 0.1 % (w/v) SDS (pH 8.3).
7. Laemmli Sample Buffer 4× supplemented with 10 % β-mercaptoethanol.
8. Mini-PROTEAN Tetra Cell electrophoresis system (Bio-Rad) or equivalent.
9. Trans-Blot Turbo transfer system (Bio-Rad) or equivalent.
10. Nitrocellulose membrane pore size 0.22 μm.
11. Transfer buffer, for 1 L: 200 mL of 5× Transfer buffer (Bio-Rad), 600 mL of nanopure water, and 200 mL of ethanol 100 %.
12. Powdered skim milk.
13. Bovine serum albumin (BSA).
14. Tris-HCl buffered saline (TBS; 10×): 1.5 M NaCl, 0.1 M Tris-HCl (pH 7.4).

15. TTBS solution: TBS containing 0.1 % Tween-20.
16. Blocking solution: 5 % milk in TBST. Store at 4 °C.
17. Secondary immunoglobulins coupled to HRP.
18. Enhance chemoluminescent (ECL) substrate for Western blot.
19. ChemiDoc MP Imaging System (Bio-Rad) or equivalent.

3 Methods

Carry out all procedures with sterile and pyrogen-free material in biological safety cabinets Class II at room temperature unless otherwise specified.

3.1 Transfection of NLRP3 Inflammasome in HEK293

1. Plate HEK293 on 35 mm dishes to get 60 % confluence. Use one dish per condition to be tested.
2. Transfect cells 4–5 h later using Lipofectamine 2000[®] manufacturer's instructions with few exceptions. For one 35 mm dish, in a first tube prepare 0.1 µg of each DNA coding for NLRP3 and caspase-1, and 0.2 µg DNA coding for ASC in 100 µL Opti-MEM. In a second tube, gently mix 2.0 µL Lipofectamine 2000[®] transfection reagent in 100 µL Opti-MEM. Incubate for 5 min at room temperature then add tube 1 into tube 2. After 20 min at room temperature, add the previous mix (DNA-transfection reagent complexes) on dish containing 1 mL of complete cell culture media.
3. The following day, remove transfection reagent containing media and replace by 2 mL of fresh cell media.

3.2 Inflammasome Activation

1. 48 h after transfection, cells expressing functional NLRP3 inflammasome can be used (*see Note 4*).
2. Wash cells twice with HBS.
3. Add 1 ml of HBS to each dish.
4. To activate the inflammasome add 5 µM nigericin for 30 min or apply the hypotonic solution (1 mL) for 40 min.
5. Remove supernatants, add 100 µL of CLB to the cells.
6. Scrape cells and transfer cell lysate to a tube. Store on ice.
7. After 30 min on ice, spin tubes at 13,000 ×g at 4 °C to remove insolubilized materials and keep supernatant.

3.3 Western Blot to Detect Inflammasome Functionality/Activation

1. Determine protein concentration using a BCA kit (alternative protein concentration methods can be used).
2. Load 30 µg of protein from each condition per lane.
3. Run the SDS-PAGE gels and transfer proteins on nitrocellulose membrane (*see Note 5*).

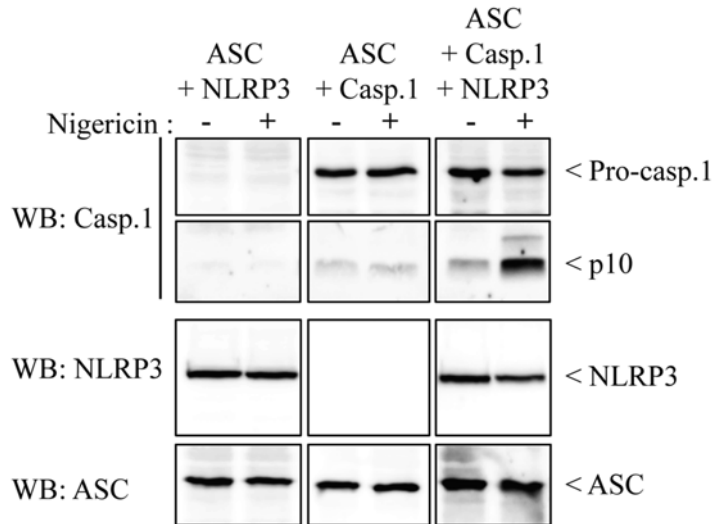


Fig. 1 Reconstruction of functional NLRP3 inflammasome. HEK293 cells overexpressing inflammasome components (NLRP3, Caspase-1, and ASC) were stimulated with the K^+ ionophore nigericin ($5 \mu\text{M}$ for 40 min). Activation of caspase-1 (detection of p10 subunit) and expression of NLRP3 and ASC were detected by WB after protein separation on 4–12 % gradient gels. Note that in this experiment, cells were also transfected with the IL-1 β BRET sensor described in Chap. 5 (not shown) (adapted from [5])

4. Block the membrane for 30 min in 5 % milk/TBST.
5. Probe membranes with caspase-1, NLRP3, and ASC antibodies accordingly, overnight at 4°C .
6. Wash the membrane $3\times$ with TBST during 30 min.
7. Incubate membrane with appropriate secondary antibodies in 5 % milk/TBST for 1 h at room temperature.
8. Wash the membrane $3\times$ with TBST during 30 min.
9. Develop using ECL substrate according to manufacturer instructions.
10. Check for the active form of caspase-1 p10 or p20 to determine inflammasome activation. Check for the presence of NLRP3 and ASC to confirm their expression (Fig. 1).

4 Notes

1. Similar protocol described here has been used to reconstitute functional NLRPC4 inflammasome (data not shown).
2. References and dilution of antibodies working by WB: caspase-1 (Santa Cruz, sc-515, dilution 1:300), ASC (Santa Cruz sc-22514-R, dilution 1:1000), and NLRP3 (Adipogen, AG-20B-0014-C100, dilution 1:1000).

3. Alternatively to the current protocol describe here, NLRP3 inflammasome activation can be detected in 96-well plate using the IL-1 β BRET biosensor described in this book (*see* Chap. 5).
4. 15 % acrylamide homemade gels can be employed to visualize active caspase-1. Alternatively, 4–12 % precast gradient gels allow large separation of proteins with different molecular weight as NLRP3 (MW = 118 kDa) and p10 fragment of active caspase-1 (MW = 10 kDa).
5. To improve caspase-1 p10 detection, transfer proteins on nitrocellulose membrane with pore size of 0.22 μ m.

Acknowledgments

This work was supported by funds from the Manchester Collaborative Centre for Inflammation Research and a Sir Henry Dale fellowship from the Wellcome Trust (UK) to G.L.-C. V.C. was supported by the *Institut National de la Santé et de la Recherche Médicale*.

References

1. 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
2. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440(7081):228–232. doi:10.1038/nature04515
3. Compan V, Baroja-Mazo A, Lopez-Castejon G, Gomez AI, Martinez CM, Angosto D, Montero MT, Herranz AS, Bazan E, Reimers D, Mulero V, Pelegrin P (2012) Cell volume regulation modulates NLRP3 inflammasome activation. *Immunity* 37(3):487–500. doi:10.1016/j.immuni.2012.06.013
4. Guarda G, Zenger M, Yazdi AS, Schroder K, Ferrero I, Menu P, Tardivel A, Mattmann C, Tschopp J (2011) Differential expression of NLRP3 among hematopoietic cells. *J Immunol* 186(4):2529–2534. doi:10.4049/jimmunol.1002720
5. Compan V, Baroja-Mazo A, Bragg L, Verkhratsky A, Perroy J, Pelegrin P (2012) A genetically encoded IL-1beta bioluminescence resonance energy transfer sensor to monitor inflammasome activity. *J Immunol* 189(5):2131–2137. doi:10.4049/jimmunol.1201349