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Reconstruction of the Mouse Inflammasome System in HEK293T Cells

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[Abstract] The NLRP3 (NLR family, Pyrin domain containing 3) inflammasome is a multiprotein complex comprised of NLRP3, pro-caspase-1, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and the protein kinase NIMA related kinase 7 (NEK7) (Shi *et al.*, 2016; He *et al.*, 2016; Schmid-Burgk *et al.*, 2016). When cells are exposed to microbes and/or danger signals, the inflammasome assembles and serves as a platform for the activation of caspase-1. Caspase-1 activation promotes the processing and secretion of the pro-inflammatory cytokines interleukin-1β (IL-1β), IL-18, and IL-33 as well as pyroptosis induction (Gross *et al.*, 2011; Arend *et al.*, 2008), which elicit inflammatory responses. Here, we describe how to co-transfect the NLRP3 inflammasome components into HEK293T cells, which enables inflammasome activation and the production of IL-1β upon stimulation with nigericin.

[Background] Inflammasomes are multiprotein complexes that control inflammatory responses and coordinate immune responses against invading microbes. Reconstitution of the NLRP3 inflammasome *in vitro* provides an easy and efficient way to study the regulation of inflammasome activation. In this protocol, NEK7 was introduced into the *in vitro* NLRP3 inflammasome system and the ratio among the NLRP3 inflammasome components was optimized, making the reconstituted NLRP3 inflammasome more similar to the physiological inflammasome *in vivo*. Nigericin was used to activate the inflammasome as we have observed that it induces a rapid rate of IL-1β secretion compared to other inflammasome activators. Using this protocol, the levels of IL-1β can be assayed to determine NLRP3 inflammasome function under physiological conditions as well as after gene knockdown or overexpression.

Materials and Reagents

1. Costar 24 well clear TC-treated multiple well plate (Corning, Costar®, catalog number: 3527)
2. 1.5 ml Eppendorf tubes
3. HEK293T cells (ATCC, catalog number: CRL-11268)
4. Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Gibco™, catalog number: 10569-044)
5. Fetal bovine serum (FBS) (Gemini Bio-Products, catalog number: 100-106)

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6. Penicillin-streptomycin (10,000 U/ml; 10,000 µg/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15140-163)
7. Pro-IL-1β-Flag, NLRP3-Flag, ASC1-Flag, pro-caspase-1-Flag and NEK7-HA plasmids
   Note: Pro-IL-1β, NLRP3, ASC1, pro-caspase-1, and NEK7 were amplified by standard PCR techniques and were subsequently inserted into mammalian expression vectors using the In-Fusion® HD cloning kit per manufacturer's instructions (click here for a detailed protocol and Shi et al., 2016). All plasmids were submitted to Addgene.
   pCMV-pro-IL1β-C-Flag (Addgene, catalog number: 75131)
   pcDNA3-N-Flag-NLRP3 (Addgene, catalog number: 75127)
   pcDNA3-N-Flag-ASC1 (Addgene, catalog number: 75134)
   pcDNA3-N-Flag-Caspase-1 (Addgene, catalog number: 75128)
   pcDNA3-N-HA-NEK7 (Addgene, catalog number: 75142)
8. IL-1β ELISA Kit (affymetrix ,eBioscience, catalog number: 88-7013-76)
9. Plasmid Plus Midi Kit (QIAGEN, catalog number: 12943)
10. LB medium (MP Bio, catalog number: 3002-31)
11. Opti-MEM® (Thermo Fisher Scientific, Gibco™, catalog number: 51985-034)
12. Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Invitrogen™, catalog number: 11668-019)
13. Nigericin (Sigma-Aldrich, catalog number: N7143-10MG)
14. Ethanol
15. SuperSignal™ West Pico chemiluminescent substrate (Thermo Fisher Scientific)
16. DMEM cell culture medium (see Recipes)
17. Nigericin stock solution (see Recipes)

Equipment

1. 37 °C, 5% CO_2 forced-air incubator (Thermo Fisher Scientific, Fisher Scientific, model: Sanyo Incubator Panasonic MCO-19AIC(UV) CO_2)
2. Shaker incubator (Eppendorf, model: New Brunswick I24)
3. Pipettes (Mettler-Toledo, ShopRainin)
4. Microcentrifuge (Eppendorf, model: Centrifuge 5424)
5. ELISA plate reader (Bio Tek Instruments, model: Synergy Neo2 Multi-Mode Reader)
6. Spectrophotometer (Thermo Fisher Scientific, Thermo Scientific™, model: NanoDrop 2000c Spectrophotometer)

Software

1. GraphPad Prism 6 software (http://www.graphpad.com/scientific-software/prism/)

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Procedure

1. Plate HEK293T cells in 24-well microplates at a density of 2 x 10^5 cells per well in 0.5 ml complete DMEM cell culture medium and incubate at 37 °C in a forced-air incubator overnight.
2. Extract the pro-IL-1β-Flag, NLRP3, ASC, pro-caspase-1 and NEK7 plasmids from their respective bacterial cultures (35 ml) by using a QIAGEN Plasmid Plus Midi Kit according to the manufacturer's instructions.
   Note: Click here for a detailed protocol on how to prepare a liquid culture of the Addgene plasmid. Briefly, add 35 ml of LB medium (with the appropriate antibiotic) to a tube or flask for each Addgene plasmid. Inoculate the LB medium using a sterile pipette tip and incubate 12-18 h at 37 °C in a shaking incubator.
3. In 1.5 ml Eppendorf tubes, dilute 1.5 µl of Lipofectamine® 2000 in 100 µl Opti-MEM per well.
4. In 1.5 ml Eppendorf tubes, dilute the pro-IL-1β-Flag (200 ng/well), ASC (20 ng/well), pro-caspase-1 (100 ng/well), NLRP3 (200 ng/well) and NEK7 (200 ng/well) plasmids in 100 µl Opti-MEM.
   Note: Avoid transfecting more than the amount indicated for the ASC plasmid. Excess ASC promotes self-aggregation and induces NLRP3-independent caspase-1 activation.
5. Add the diluted DNA to the diluted Lipofectamine® 2000 (1:1 ratio), incubate for 5 min at room temperature, and then add the DNA-lipid complex to the HEK293T cells in 0.5 ml DMEM cell culture medium.
6. 24-36 h after transfection, replace the medium with 250 µl DMEM cell culture medium.
7. Supernatants are collected 6-12 h after media change.
8. Optional: To stimulate the NLRP3 inflammasome, 10 µg/ml nigericin can be directly added to the 250 µl DMEM media one hour before supernatant collection (step 7).
9. ELISA (or Western blot) to assay the amount of mature IL-1β in the supernatants. Perform ELISA according to the manufacturer's instructions.
   Note: When using Western blot to detect the mature IL-1β, the supernatants do not need to be concentrated. Mature IL-1β in 15 µl of supernatant could be detected by SuperSignal™ West Pico chemiluminescent substrate (Thermo Fisher Scientific).

Data analysis

ELISA data should be collected from a minimum of two independent experiments with at least 3 replicates per treatment. Figure 1 shows representative ELISA data after transfection of the indicated plasmids into HEK293T cells. The statistical significance of the differences between samples can be determined with GraphPad Prism 6 software and Student's *t*-test (unpaired, two-tailed). A *P* value of < 0.05 is considered statistically significant.

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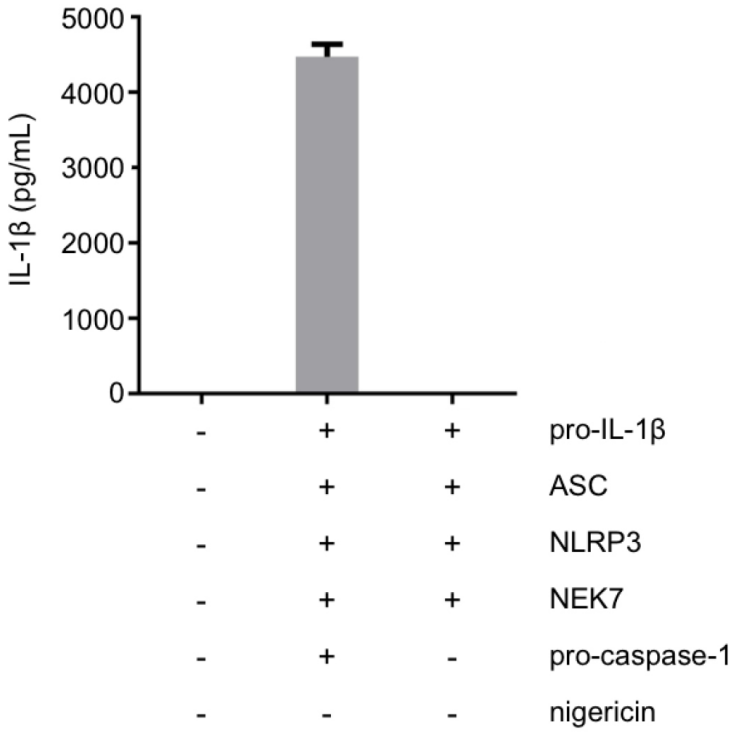

Figure 1. Levels of secreted IL-1β from reconstituted HEK293T cells. Transfected components in each sample are indicated below the X-axis. 50 µl supernatants of each HEK293T cell culture sample were used for the IL-1β ELISA. Representative data were graphed using GraphPad Prism 6 software.

Recipes

1. DMEM cell culture medium
   Mix 500 ml DMEM with 50 ml FBS and 5 ml penicillin-streptomycin (10,000 U/ml; 10,000 µg/ml)
   Store at 4 °C until use
2. Nigericin stock solution
   Dissolve 10 mg nigericin in 1 ml pure ethanol and mix thoroughly
   Store at -80 °C until use

Acknowledgments

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