SUPPORTING INFORMATION (SI) APPENDIX

Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation

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This PDF file includes:

SI Materials and Methods

Mice and cell culture

C57BL/6 wild-type mice were from Vital River Laboratory Animal Technology Co. Ltd. *Nlrc*4$^{-/-}$ mice were kindly provided by Dr. Vishva Dixit (Genentech Inc., USA). Primary bone marrow derived macrophages (BMDMs) were prepared as described (1). Mouse BMDC cells were derived from bone marrow cells according to the published protocol (2). The supernatant of X63-GM-CSF-producing cell line (provided by Dr. Fu Guo at The Scripps Research Institute, USA) was used as the source of GM-CSF, and the medium used for culturing BMDC cells was RPMI-1640 with 10% fetal bovine serum, 10% supernatant of X63-GM-CSF-producing cells and 2 mM L-glutamine. Briefly, primary bone marrow cells were seeded at 2 X 10^6 per 10 cm dish at Day 0. At Day 3, another 10 mL of medium was added to the plates and three days later the old medium was replaced with another 10 mL of fresh BMDC culture medium. At Day 8, the cells were used for the inflammasome activation assay as indicated. 293T cells were cultured following a standard protocol provided by ATCC. Human U937 and THP-1 monocytes obtained from ATCC and mouse dendritic cell DC2.4 (kindly provided by Dr. Wenyue Xu at the Third Military Medical University of China) were cultured in RPMI-1640 medium containing 10% FBS and 2 mM L-glutamine at 37°C in a 5% CO2 incubator. THP1 and U937 were differentiated into macrophages as described previously (3-4).

RNAi knockdown and lentiviral infection

The target sequence for RNAi knockdown of hNAIP, mouse NAIP1/2 and NLRC4 are listed in Table S1 (SI Appendix). For siRNA knockdown, primary BMDMs or immortalized DC2.4 cells were cultured in 24-well plates (2.5×10^5/well for BMDM, 4×10^4/well for DC2.4) and siRNA transfection was performed using the INTERFERinTM reagent (Polyplus Transfection Inc.) by following the manufacturer's instructions. 60 h after transfection, knockdown efficiency was monitored by quantitative Real-Time PCR (qRT-PCR) and casapse-1 activation was analyzed by western blotting as previously described (5). pLKO.1 lentiviral shRNA plasmids for stable knockdown of hNAIP in U937 cells were purchased from Sigma (Sigma MISSION shRNA). The puromycin resistance gene in pLKO.1 was replaced by the GFP-coding sequence. Lentivirus-infected GFP-positive U937 cells were sorted out by flow cytometry as previously described (5).

Generation of bacterial deletion strains and bacterial infections

prgI-deficient *S. typhimurium* SL1344 strain and *mxiH*-deficient *S. flexneri* strain were generated by the standard homologous recombination method using the suicide plasmids (pDM4 for *S. typhimurium* and pCVD442 for *S. flexneri*). A PCR fragment containing flanking sequences of the target gene was cloned into pDM4 or pCVD442. The resulting targeting vector was transferred into *S. typhimurium* or *S. flexneri* through *E. coli* SM10 (λpir)-mediated conjugation. The transconjugants were selected on LB agar medium containing 50 μg/mL chloramphenicol and 100 μg/mL streptomycin (for *S. flexneri*, use 100 μg/mL ampicillin instead of chloramphenicol). The integrants were further screened for markerless in-frame deletion by growth on

LB agar plates containing 16% sucrose without NaCl and deletion of the target gene was confirmed by PCR analyses.

To infect U937 cells, *S. typhimurium*, *S. flexneri* or *L. monocytogenes* EGD strain were cultured overnight at 37°C in LB broth under conditions of vigorous shaking. Bacterial cultures were then diluted by 1:100 in fresh LB broth, and grown until OD_{600} reached 0.8. Prior to infection, the bacteria were diluted in serum-free RPMI-1640 medium to achieve the desired MOI (multiplicity of infection) (50 for *S. typhimurium*, 10 for *S. flexneri*, 10 for *L. monocytogenes*). Infection was started by centrifugation at 500 g for 10 min for infection followed by incubation at 37°C for another 30 min. Infected U937 cells were washed three times with PBS and replaced with fresh media containing 100 ng/ml gentamycin to kill the extracellular bacteria. 2 h later, the supernatants were harvested and subjected to assays for inflammasome activation. *L. pneumophila* strains were cultured as described (6). pJB908 vectors expressing indicated needle proteins were introduced into *L. pneumophila* by electroporation (2.5 Kv, 200 Ω, 25 μF, and 5 ms). For infection, fresh single bacterial colonies were streaked onto culture plates 2 days before infection. Bacteria were scraped off, diluted in sterile water, and added to cells at MOI of 50. Infection was facilitated by a centrifugation of 300 x g for 10 min. 3 h later, the supernatants were collected and analyzed for caspase-1 activation and or IL-1β maturation by immunoblotting.

Caspase-1 activation, IL-1β maturation and pyroptotic cell death assays

To assay caspase-1 activation, the supernatants of stimulated macrophages or dendritic cells were subjected to trichloroacetic acid (TCA) precipitation by adding TCA to the final concentration of 15% (w/v). The precipitates were re-suspended in SDS loading buffer and analyzed by Western blotting for caspase-1 or IL-1β using the corresponding antibodies. Cell lysates were blotted with actin to control equal loading. Pyroptotic cell death was measured by the lactate dehydrogenase (LDH) assay using CytoTox96 Non-Radioactive Cytotoxicity Assay kit (Promega) following the manufacturer's instructions.

Quantitative Real-Time PCR (qRT-PCR) analysis

For qRT-PCR analysis, total RNA was extracted by TRIzol (Invitrogen) and digested with DNaseI (Invitrogen).1 μg of total RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega). qRT-PCR analysis was performed using the SYBR Premix Ex Taq (TaKaRa) on Applied Biosystems 7500 Fast Real-Time PCR System. Primers used for qRT-PCR analysis were listed in Table S2 (SI Appendix). The mRNA level of targeted genes was normalized to that of GAPDH for mouse cells or to that of actin for U937 cells.

Assay for the needle protein-stimulated oligomeric NAIP-NLRC4 inflammasome complex formation

293T cells were seeded into a 6-well plate 12 h before transfection with plasmids encoding Flag-NLRC4, HA-NAIPs and myc-FliC/PrgJ/MxiH/PrgI using Vigofect Reagent (Vigorous Inc.). 24 h later, transfected cells were washed with PBS for 3 times and lysed in the native sample buffer. The lysates were then subjected to blue native (BN) polyacrylamide gel electrophoresis (PAGE) analysis (Invitrogen), as

previously described (7), to analyze the oligomerization of NLRC4 and also the presence of NAIPs in the NLRC4 inflammasome complex.

Inflammasome reconstitution in 293T cells

293T cells were transfected with indicated combinations of plasmids expressing NLRC4, different NAIPs, pro-caspase-1 and pro-IL-1β with the details described previously (5). 24 h after transfection, 2 μg of LFn-FlaA, BsaK, or indicated needle proteins, together with 1 μg of PA, were added to the culture media followed by incubation for 12 h. Alternatively, transfected cells were also subjected to infection with *S. typhimurium* or *S. flexneri*. Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl and 1% TritonX-100. Lysates were resolved onto SDS-PAGE gels followed by anti-IL-1β immunoblotting analyses for inflammasome activation. All the reconstitution experiments were performed for more than three times and representative results are shown in the figures.

References

1. Boyden ED & Dietrich WF (2006) Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet* 38(2):240-244.
2. Lutz MB, *et al.* (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 223(1):77-92.
3. Ge J, Gong YN, Xu Y, & Shao F (2012) Preventing bacterial DNA release and absent in melanoma 2 inflammasome activation by a Legionella effector functioning in membrane trafficking. *Proc Natl Acad Sci U S A* 109(16):6193-6198.
4. Gong YN, *et al.* (2010) Chemical probing reveals insights into the signaling mechanism of inflammasome activation. *Cell Res* 20(12):1289-1305.
5. Zhao Y, *et al.* (2011) The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* 477(7366):596-600.

6. Ge J, *et al.* (2009) A Legionella type IV effector activates the NF-kappaB pathway by phosphorylating the IkappaB family of inhibitors. *Proc Natl Acad Sci U S A* 106(33):13725-13730.

7. Kofoed EM & Vance RE (2011) Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* 477(7366):592-595.

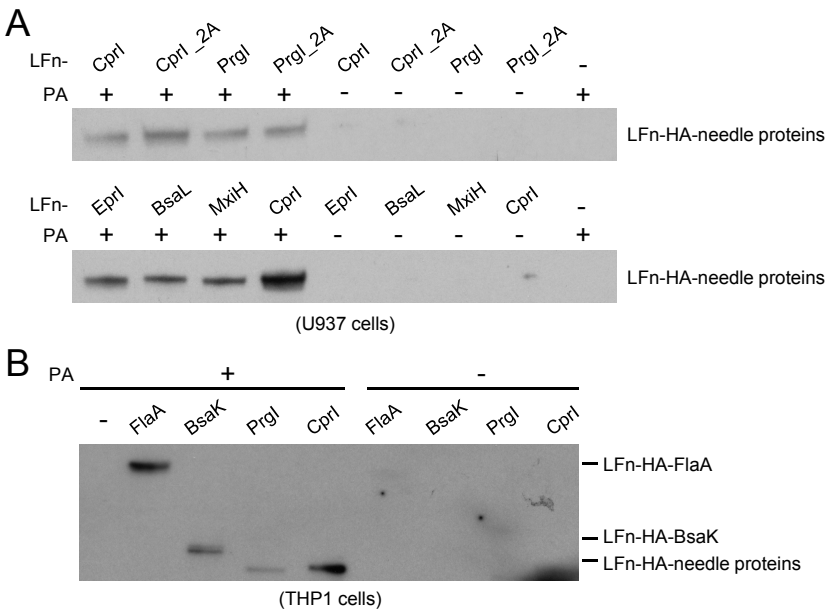


Fig. S1. Cytoplasmic delivery of T3SS needle proteins into human monocytes using the LFn-PA protein delivery system. All LFn-fusion proteins also have an HA (in the linker region) epitope and an N-terminal His tag. The indicated LFn-proteins were added to PMA-differentiated U937 (A) or THP1 cells (B) in the presence or absence of PA. 1.5 h later, cells were harvested and lysated by sonication. The LFn-proteins delivered into the cytoplasm were pulled down by Ni-NTA beads and followed by anti-HA immunoblotting.

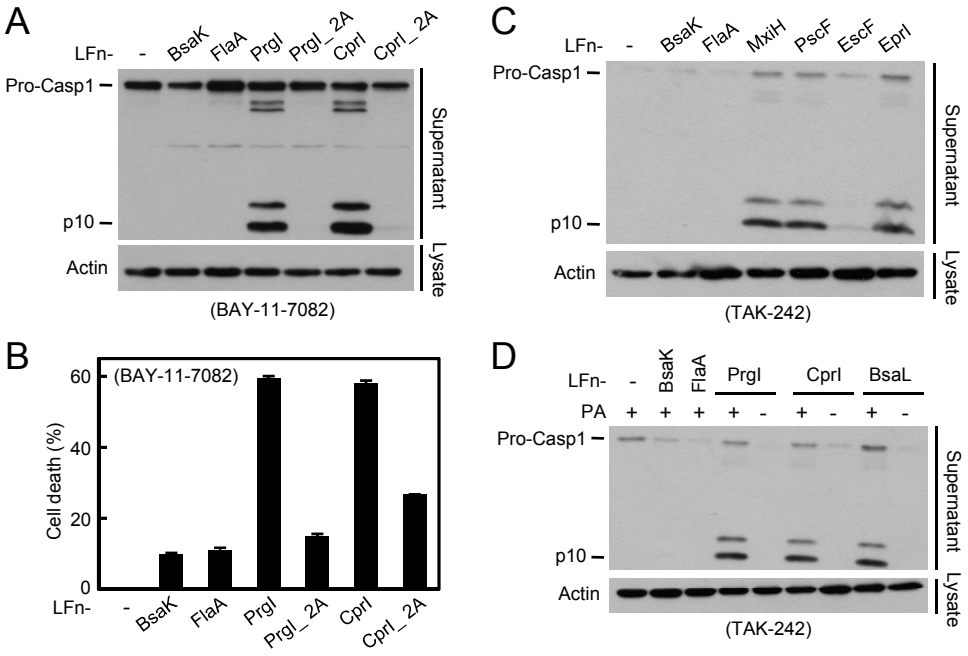


Fig. S2. Inflammasome activation by cytoplasmic needle proteins in THP-1-derived macrophages. Human monocyte THP-1 cells were differentiated into macrophages in the presence of 33 ng/ml PMA for 36 h. Indicated LFn-needle proteins (100 ng/ml) and PA (1 μg/ml) were added into cell culture media in the presence of 30 μM BAY-11-7082 (*A* and *B*) or 20 mM TAK-242 (*C* and *D*). Anti-caspase-1 and anti-actin immunoblots of culture supernatants (top) and total cell lysates (bottom) are shown in (*A*, *C* and *D*). Percentages of pyroptosis in (*B*) were determined by LDH release as mean values ± SD (error bar) from three independent determinations.

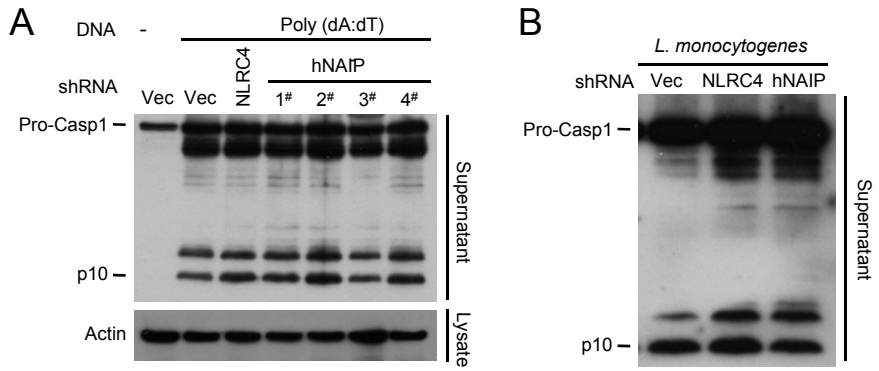


Fig. S3. Knockdown of hNAIP in U937 cells does not affect DNA transfection and *L. monocytogenes*-induced caspase-1 activation. (*A*) Poly (dA:dT) was transfected into PMA-differentiated NLRC4 and hNAIP knockdown U937 cells (generated in Fig. 2*A*) to induce AIM2 inflammasome activation. (*B*) NLRC4 and hNAIP ($1^{\#}$) stable knockdown U937 macrophages were infected with *L. monocytogenes.* Shown are anti-caspase-1/IL-1β and anti-actin immunoblots of culture supernatants (top) and total cell lysates (bottom).

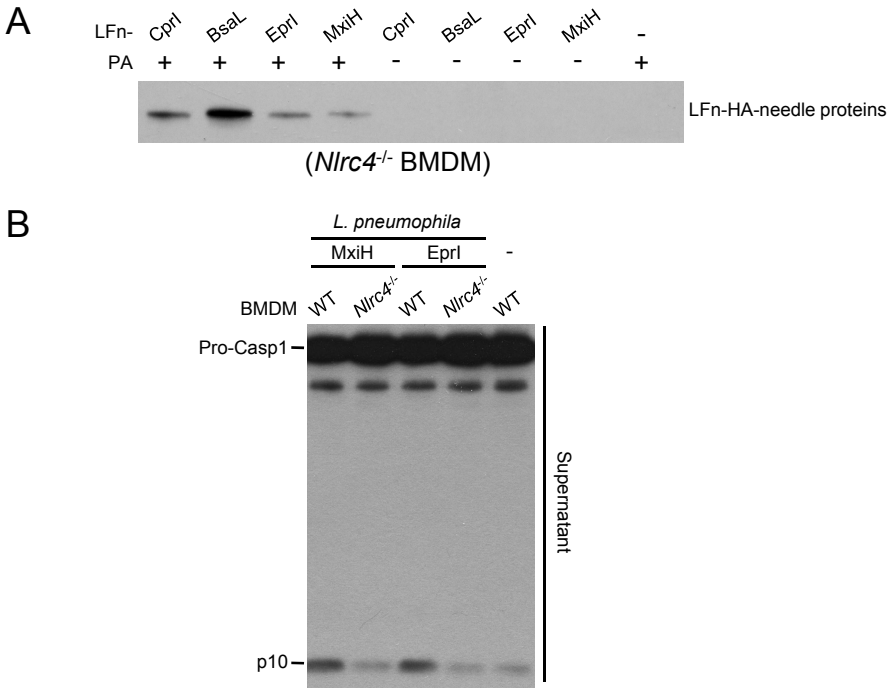


Fig. S4. Activation of the NLRC4 inflammasome by T3SS needle proteins in mouse BMDM cells. (*A*) Delivery of T3SS needle proteins into primary mouse BMDM cells using the LFn-PA protein delivery system. All LFn-fusion proteins have an HA (in the linker region) epitope and an N-terminal His tag. The indicated LFn-proteins were added to primary mouse BMDM cells (*Nlrc4*$^{-/-}$) in the presence or absence of PA. Proteins delivered into the cytoplasm were pulled down by Ni-NTA beads and followed by anti-HA immunoblotting. (*B*) Wild-type or *Nlrc4*$^{-/-}$ BMDM cells were infected with *L. pneumophila* ΔflaA strain ectopically expressing MxiH or EprI. Shown is the anti-caspase-1 immunoblot of culture supernatants.

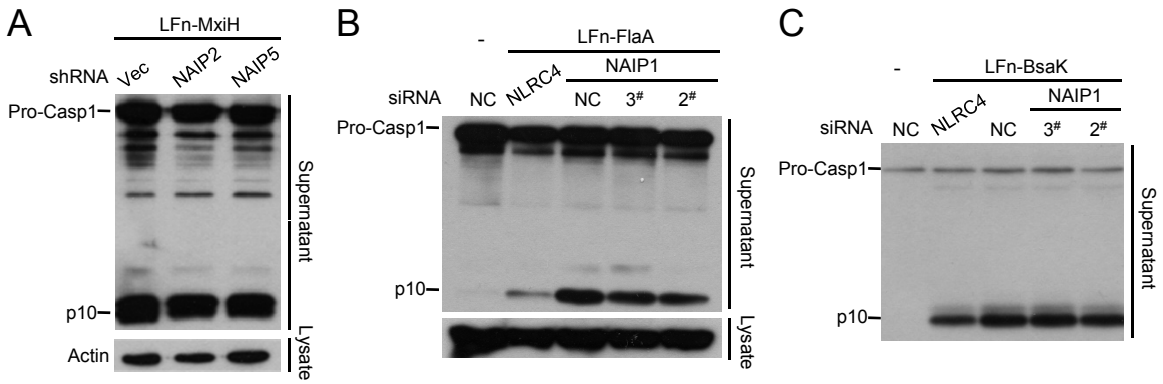


Fig. S5. Effects of RNAi knockdown of different NAIP family members on T3SS needle/rod proteins and flagellin-induced caspase-1 activation in mouse BMDM cells. Purified recombinant MxiH protein was transduced into NAIP2 and NAIP5 shRNA stable knockdown BMDM cells in (*A*); *L. pneumophila* flagellin (*B*) and *B. thailandensis* rod protein BsaK (*C*) were delivered into NAIP1 or NLRC4 siRNA knockdown BMDM cells. Stable knockdown was performed with immortalized BMDMs (*A*) while primary BMDMs were used for transient siRNA knockdown (*B* and *C*). Shown are anti-caspase-1 and anti-actin immunoblots of culture supernatants (top) and total cell lysates (bottom).

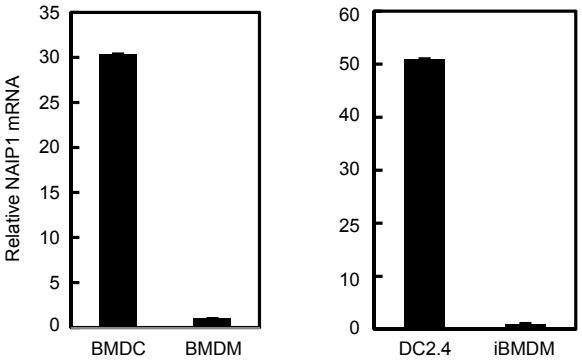


Fig. S6. qRT-PCR measurements of NAIP1 expression in primary mouse BMDM, BMDC, immortalized BMDM (iBMDM) and DC2.4 cells. The transcript level of NAIP1 was normalized to that of actin. The NAIP1 expression levels in primary BMDM and iBMDM were arbitrarily set as 1 for comparison. Shown are mean values ± SD (error bar) from three independent determinations.

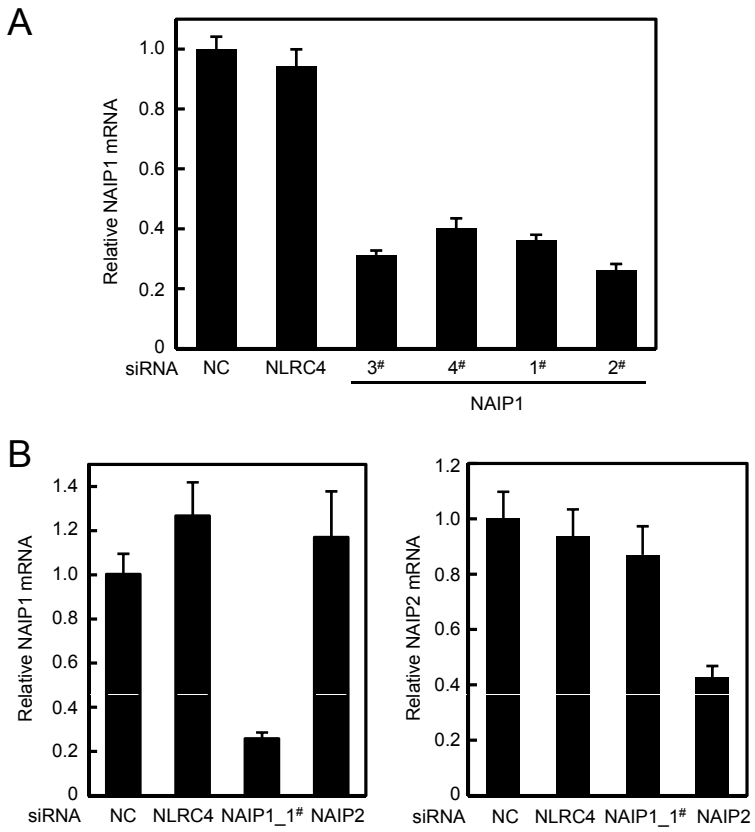
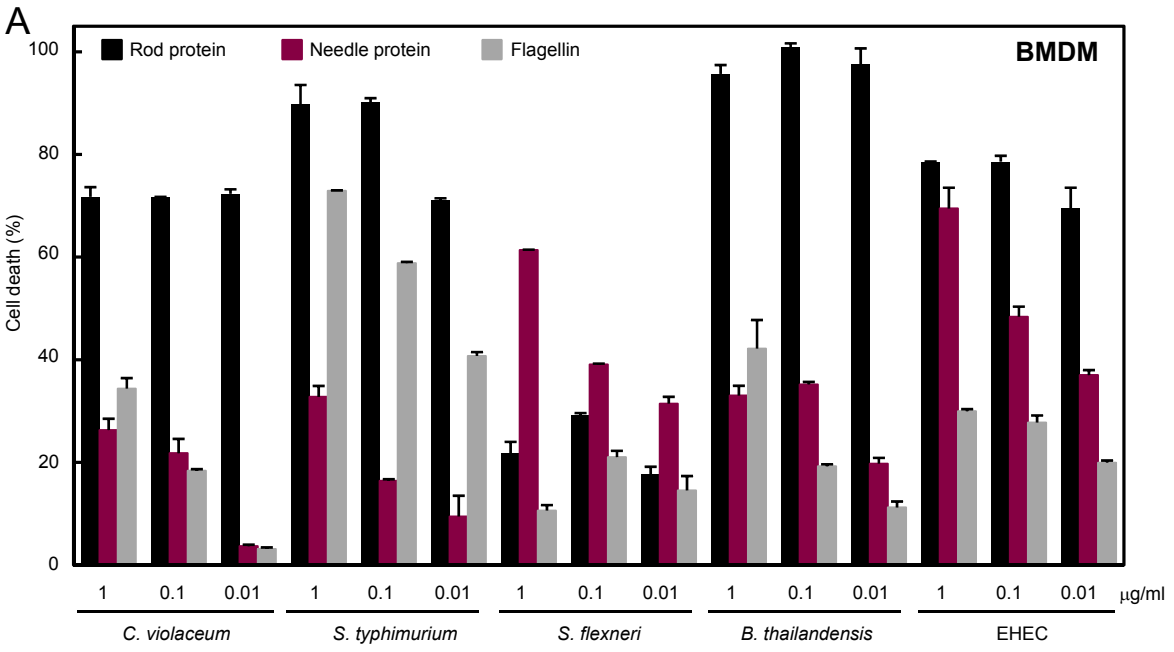
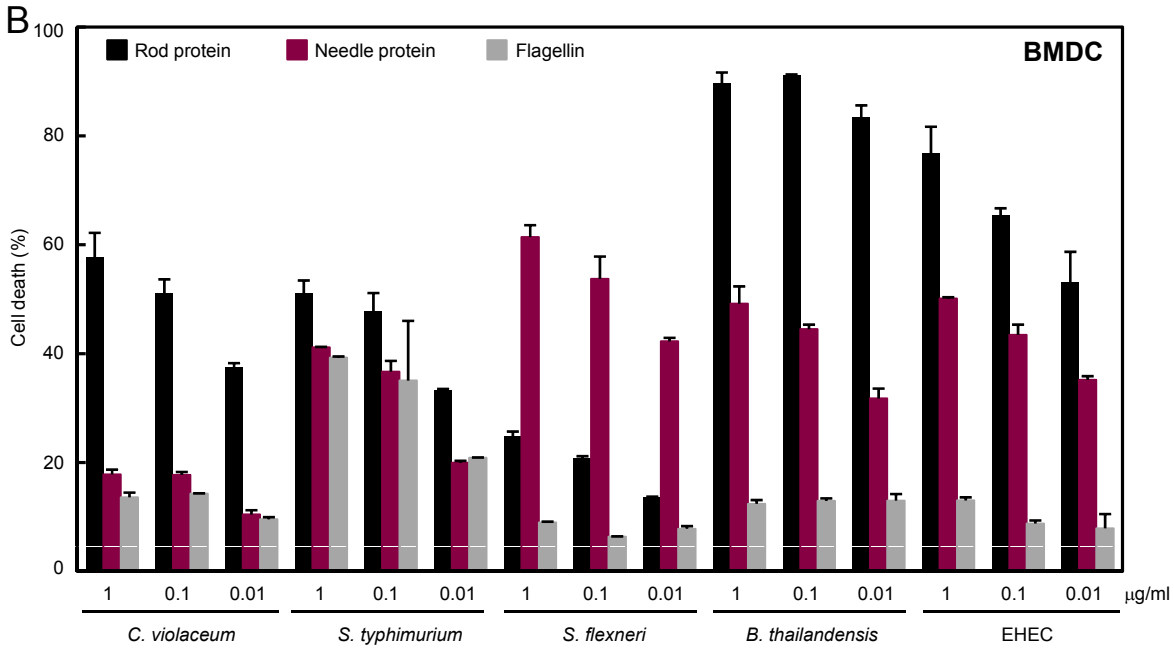


Fig. S7. qRT-PCR analysis of siRNA knockdown efficiency of NAIP1/2 in DC2.4 dendritic cells. A non-targeting (NC) or an NLRC4-targeting siRNA or one of the four independent NAIP1-targeting siRNAs ($1^{\#}$-$4^{\#}$) was transfected into DC2.4 cells in (*A*). In (*B*), an NAIP2-targeting siRNA was included as a control and also for comparison. The transcript level of NAIP1/2 was normalized to that of actin. Shown are mean values ± SD (error bar) from three independent determinations.


A
Rod protein
Needle protein
Flagellin
BMDM
Cell death (%)
100
80
60
40
20
0
1
0.1
0.01
μg/ml
C. violaceum
S. typhimurium
S. flexneri
B. thailandensis
EHEC


B
Rod protein
Needle protein
Flagellin
BMDC
Cell death (%)
100
80
60
40
20
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1
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C. violaceum
S. typhimurium
S. flexneri
B. thailandensis
EHEC

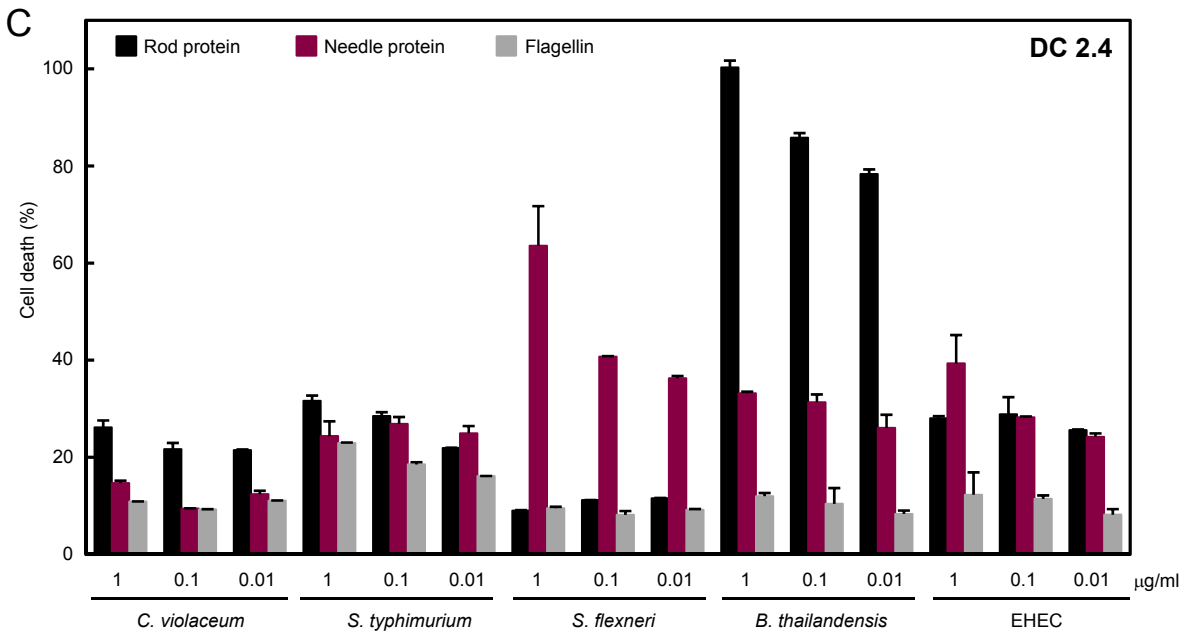

Fig. S8. Profiling and comparison of the inflammasome stimulation activity of flagellin, T3SS rod and needle proteins from five different bacterial pathogens in primary mouse BMDM (*A*), BMDC (*B*) and DC2.4 cells (*C*). 1.0, 0.1, or 0.01 μg/ml purified recombinant flagellin, T3SS rod and needle proteins from *C. violaceum*, *S. typhimurium*, *S. flexneri*, *B. thailandensis*, and EHEC were transduced into indicated mouse macrophage or dendritic cells using the LFn-PA delivery system. Percentages of pyroptotic cell death measured by the lactate dehydrogenase (LDH) release are shown as mean values ± SD (error bar) from three independent determinations.

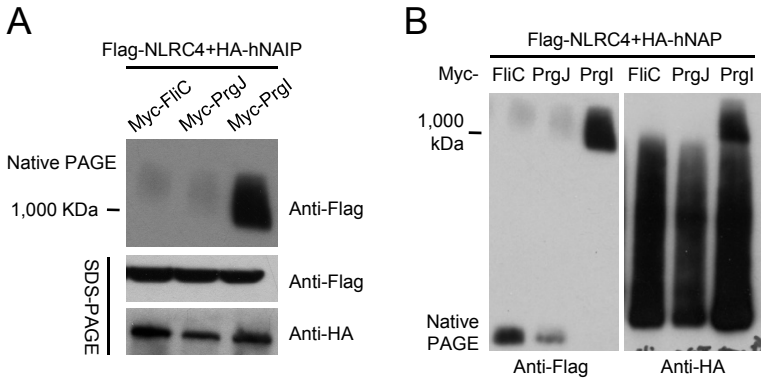

Fig. S9. Recognition of T3SS needle protein by hNAIP stimulates formation of the oligomeric needle protein-hNAIP-NLRC4 inflammasome complex. 293T cells were transfected with indicated combinations of Flag-NLRC4, HA-hNAIP and Myc-FliC/PrgJ/PrgI. Anti-Flag and anti-HA immunoblotting of blue native (BN) polyacrylamide gel electrophoresis (PAGE) gels was performed to assay formation of the large hNAIP-NLRC4 inflammasome complex (>1,000 kDa). Total cell lysates were also separated on the SDS-PAGE gels followed by anti-Flag and HA immunoblotting in (A).

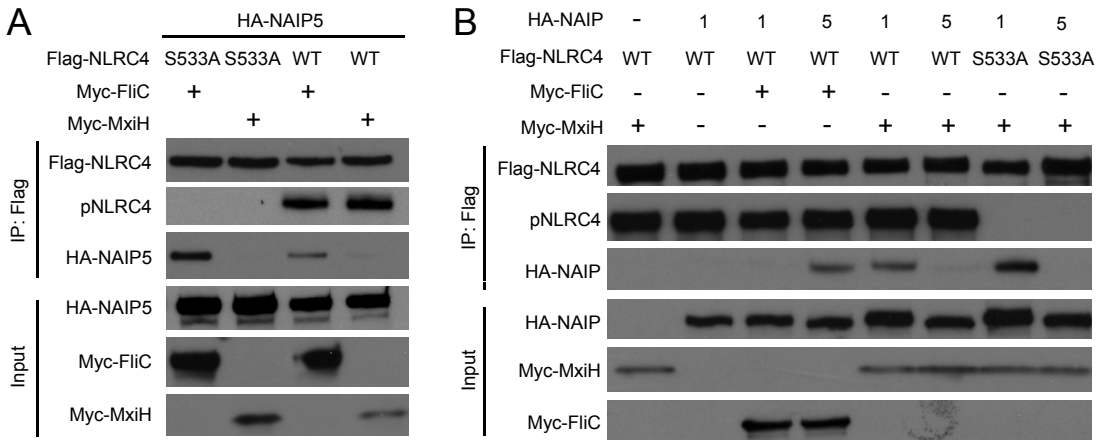

Fig. S10. The role of NLRC4 Ser-533 phosphorylation in formation of the NAIP-NLRC4 inflammasome complex in 293T cell reconstitution. 293T cells were co-transfected with indicated expression plasmid combinations of Flag-NLRC4 (WT or the S533A mutant), HA-NAIP (1 or 5) and Myc-tagged NAIP ligands (FliC or MxiH). The cells were lysed and subjected to anti-Flag M2 immunoprecipitation. The immunoprecipitates as well as the total cell lysates were subjected to immunoblotting analyses as shown.

Table S1

RNAi	**Target sequence (5'–3')**
siRNA	
mNlrc4-1#	TCGAAACACTGTACGATCA
mNlrc4-2#	GAACATCCCTGACTATTTA
Naip1-1#	GCCAAACTTGCAGAATCTGAA
Naip1-2#	CCTTGGAAAGAACTTAGAAAT
Naip1-3#	CGTCTACTAGAGTTTATGGTT
Naip1-4#	CACTGTGTTACTCATAATCTA
Naip2	GCCATTGCCTTTCAACCTATA
shRNA (in pLKO.1-GFP)	
hNAIP-1#	CGTGGTGAACTTTGTGAAT
hNAIP-2#	TGAGTATGATCCTTCCAAA
hNAIP-3#	CAGAGACTAAGACCATTCTA
hNAIP-4#	TTTCAATCAATCACAAGAT

Target sequences for siRNAs and shRNAs used in this study. The four shRNAs targeting hNAIP were purchased from Sigma MISSION shRNA library (clone number: TRCN0000063733, TRCN0000063734, TRCN0000063735 and TRCN0000063737). Puromycin encoding sequence in the shRNA plasmid was replaced with GFP coding sequence. The siRNA used to knockdown murine NLRC4 is a mixture of mNLRC4-1# and NLRC4-2# in the list, which are from the Dharmacon library. The sequences of the four siRNAs target NAIP1 were also taken from Sigma MISSION shRNA library.

Table S2

Primers	Sense (5'–3')	Antisense (5'–3')
hNAIP	**GAACTACGGCTGGACTCTTTT**	**AGCAAAGACATGTGGCGGAA**
NAIP1	**TGCCCAGTATATCCAAGGCTAT**	**AGACGCTGTCGTTGCAGTAAG**
NAIP2	**AGGCTATGAGCATCTACCACA**	**AAGACACTCAATCCACAGCAAA**
mNLRC4	**GCGGAGGTGGGAGATATG**	**CGTAGAAGGTTTGGAACAGC**
hActin	**CATGTACGTTGCTATCCAGGC**	**CTCCTTAATGTCACGCACGAT**
mActin	**CATGTACGTTGCTATCCAGGC**	**CTCCTTAATGTCACGCACGAT**

Primers used in quantitative Real-Time PCR (qRT-PCR) experiments.