**Supporting Information**

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**SI Materials and Methods**

**Plasmids and Reagents.** NOD2FL, NOD2ΔCARD1, and NOD2ΔCARDs were cloned into p3XFLAG-CMV-14 (Sigma). NOD2ΔLRR, NOD2CARDs, caspase-1CARD, caspase-1ΔCARD, and NOD2FL fused to different epitope tags (FLAG, c-Myc, or HA), were cloned into pcDNA3 (Invitrogen). Full-length NOD2 was cloned into the retroviral vector pMSCVpuro (Clontech). Recombinant LF and PA were obtained as described and combined to generate LT (1). LPD (Eschericia coli, 055:B5) and MDP were purchased from Sigma-Aldrich and Bachem, respectively.

**Cytokine Secretion and Cell Signaling.** Whole-cell extracts were separated by SDS/PAGE, transferred to Immobilon membranes (Millipore), and analyzed by immunoblotting. Cytokines were quantitated by ELISA by using duoSet kits from R&D Systems. IL-1β (Cell Signaling), and caspase-1 were measured (2).

**Transfections and Retroviral Infection.** HEK293T cells were transfected by using Lipofectamine 2000 (Invitrogen) in the absence or presence of MDP. Cell lysates were analyzed by immunoprecipitation and immunoblotting as described (3). Bone marrow-derived macrophages (BMDMs) were retrovirally transduced as described (4). Culture supernatants were analyzed for IL-1β release and caspase-1 content.

**shRNA-Mediated Gene Silencing.** Oligonucleotides (sequence available upon request) corresponding to NALP1-specific shRNA were cloned into pLSLPw (lentivirus vector) and lentiviruses were prepared. TDM were infected with lentivirus as described (1) and cultured for 72 hr before incubation with 10 μg/ml MDP for 12 hr.

**Size-Exclusion Chromatography.** TDM were treated with 10 μg/ml MDP for 2 hr or left untreated. Cell pellets were resuspended in lysis buffer W (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF) supplemented with protease inhibitors and sonicated three times at 10-sec pulses with 30-sec intervals. Approximately 5 mg of protein lysate was loaded onto a precalibrated Superdex 200 column (Amersham-Pharmacia Biotech) and eluted in buffer W.

**Analysis of Gene Expression and NF-κB Activity Assay.** Total cellular RNA was prepared using TRIzol (Invitrogen), quantitated by UV absorption, and analyzed by real-time Q-PCR (3). Primer sequences are available upon request. All values were normalized to cyclophilin mRNA levels. NF-κB DNA binding was determined as described (5).

**Fig. S1.** RIP2 but not caspase-1 is required for elevated MDP-stimulated TNF-α secretion in macrophages. (A and B) Peritoneal macrophages from the indicated mouse strains were incubated with TiO₂ microparticles without or with MDP and TNF-α secretion was assayed by ELISA 16 h later. Results are averages of three separate experiments. Significant differences, **P < 0.01, *P < 0.05**
Fig. S2. RIP2 is required for TNF-α but not IL-1β secretion, whereas caspase-1 is essential for IL-1β but not TNF-α release in LPS-primed macrophages stimulated with MDP. LPS-primed peritoneal macrophages from the indicated mouse strains were incubated with TiO₂ microparticles without or with MDP. Secreted IL-1β and TNF-α were measured after 16 h by ELISA. Significant differences, **P < 0.01, *P < 0.05.
NF-κB activation by MDP in macrophages requires RIP2 but not caspase-1. (A) RIP2 is required for MDP-dependent IκB degradation in macrophages. Peritoneal macrophages from the indicated mice were incubated for 30 or 60 min with TiO2 particles without or with MDP (1 μg/ml) or peptidoglycan (PGN from Staphylococcus aureus, 10 μg/ml) for 30 min. Cell lysates were prepared and NF-κB DNA binding activity was analyzed by EMSA and IκBα degradation was examined by immunoblot analysis. Actin and NF-Y were used as loading controls. (B) RIP2 is required for induction of NF-κB target genes after MDP stimulation. Peritoneal macrophages were incubated with MDP with TiO2 for 4 h. Total RNA was prepared and expression of the indicated genes was examined by real time Q-PCR. Data are presented as fold-increase in mRNA expression relative to non-stimulated WT macrophages, which were assigned an arbitrary level of 1.0 for each gene. Significant differences, **P < 0.01, *P < 0.05. (C) Caspase-1 is not required for MDP-induced NF-κB activation. Peritoneal macrophages from the indicated mouse strains were treated as above. NF-κB DNA binding activity was measured by EMSA. NF-Y activity was used as a loading control.
Fig. S4. Roles of NOD2, RIP2 and caspase-1 in induction of pro-IL-1β synthesis. (A) Peritoneal macrophages from the indicated strains were incubated without or with LPS (0.5 ng/ml) for 6 h, and then stimulated with TiO₂ microparticles without or with MDP (10 μg/ml) for 4 h. Cell lysates were prepared and pro-IL-1β was examined by immunoblot analysis. (B) NOD2, caspase-1 and RIP2 dependence of IL-1β mRNA induction. Peritoneal macrophages from the indicated strains were preincubated with or without LPS (0.5 ng/ml) for 6 h followed by 4 h incubation with TiO₂ microparticles without or with MDP. Total cellular RNA was isolated and pro-IL-1β mRNA levels were determined by real time Q-PCR. Data are fold-increase in mRNA expression in stimulated macrophages relative to non-stimulated macrophages, which were given an arbitrary level of 1.0. Results are means ± s.d. of three independent experiments normalized to the level of cyclophilin mRNA. Significant differences, **P < 0.01, *P < 0.05.
ASC and NALP3 are not required for MDP-stimulated IL-1β secretion. Peritoneal macrophages from WT, Nalp3−/− and Asc−/− mice were incubated with TiO₂ microparticles without or with MDP or with LPS + ATP as indicated. After 16 h, secreted IL-1β and TNF-α were quantitated by ELISA.
Fig. S6. NOD2, but not RIP2, interacts with caspase-1. (A) Mouse and human NOD2 proteins bind caspase-1. FLAG-tagged caspase-1 was co-expressed in HEK293T cells with HA-tagged mouse (left panel) or FLAG-tagged human (right panel) NOD2 constructs. Caspase-1 was immunoprecipitated and presence of associated NOD2 proteins was examined by immunoblotting. (B) RIP2 does not interact with caspase-1. c-Myc-tagged caspase-1 was transfected with either RIP2 or NOD2 constructs into HEK293T cells. Caspase-1 was immunoprecipitated with anti-Myc and presence of associated NOD2 or RIP2 was examined by immunoblotting of cell lysates. RIP2 was analyzed by using an anti-HA antibody from Santa Cruz.
Fig. S7. NOD2 specifically interacts with caspase-1 and caspase-4, but not with other caspases. NOD2 was co-expressed in HEK293T cells with the indicated FLAG-tagged human caspase constructs. After 36 h, anti-FLAG-caspase immunoprecipitates were prepared and analyzed for presence of NOD2 and FLAG-caspases by immunoblotting. Expression of NOD2 was analyzed by immunoblotting.
Fig. S8. MDP disrupts binding of the NOD2 LRR to the N-terminal half of the protein. HEK293T cells were transfected with the indicated human NOD2 fragments in the absence or presence of different MDP concentrations. After 36 h, the Myc-tagged LRR fragment was immunoprecipitated and coprecipitation of the FLAG-tagged NOD2ΔLRR N-terminal fragment was examined by immunoblotting.
IL-1β, but not TNF-α, secretion in *B. anthracis*-infected macrophages is NOD2-dependent. (A and B) IL-1β secretion by *B. anthracis*-infected macrophages. Peritoneal macrophages from WT, Nod2−/− and caspase1−/− mice were infected or not with the indicated *B. anthracis* Sterne strains (BaWT, or BaΔpX01) at moi of 2. Macrophages were also pretreated with LPS and then pulsed with ATP as a positive control. Supernatants were collected 6 h postinfection and secreted IL-1β (A) and TNF-α (B) were measured by ELISA. Significant differences, **P < 0.01, *P < 0.05. (C) NOD2 or caspase-1 is not required for induction of pro-IL-1β. Macrophages from the indicated strains were infected as (A) and pro-IL-1β expression was examined by immunoblotting. (D) TNF-α secretion in *B. anthracis* infected mice does not require caspase-1 and NOD2. Mice (*n* = 5) were injected intraperitoneally with 10⁷ cfu of early log phase *B. anthracis* BaWT. TNF-α in plasm was measured 17 h after infection.