Supporting Information

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SI Materials and Methods

Bacterial Strains. *L. pneumophila* was cultured for 48 h on charcoal–yeast extract agar plates [1% yeast extract, 1% N-(2-acetamido)-2-aminoethanesulfonic acid (pH 6.9), 3.3 mM L-cysteine, 0.33 mM Fe(NO3)3, 1.5% (wt/vol) bacto agar, and 0.2% activated charcoal] supplemented with thymidine (100 μg/mL). *E. coli* DH5α was cultured on LB agar plates for 24 h.

Mice. C57BL/6 mice were purchased from Jackson Laboratories. $Nlrc4^{-/-}$, $Asc^{-/-}$, $Nlrp3^{-/-}$, and $Casp1/11^{-/-}$ mice have been described previously and were kindly provided by Richard Flavell (Yale University, New Haven, CT) (1–4). $Myd88^{-/-}$ mice have been described previously and were kindly provide by Ruslan Medzhitov (Yale University, New Haven, CT) (5). $Ifnar1^{-/-}$ mice have been described previously and were kindly provide by Akiko Iwasaki (Yale University, New Haven, CT) (6). $Casp11^{-/-}$ mice have been described previously and were kindly provided by Junying Yuan (Harvard Medical School, Boston) (7). $Casp11^{-/-}$ mice have been back-crossed at least eight times to the C57BL/6 background. $Trif^{Lps2/Lps2}$ mice have been described previously (8) and were purchased from Jackson Laboratories. C57BL/6 and Myd88/TRIF double-knockout immortalized macrophages were a kind gift from Jonathan Kagan (Harvard Medical School, Boston).

To generate caspase-1 knockout mice, caspase-1 gene fragments were cloned from genomic DNA of C57BL/6J mice. Three fragments, the 5′ long arm (4 kb), a 1.8-kb internal region spanning exons 6 and 7, and the 3′ short arm (3 kb) were cloned into PGKneoF2L2 vector (from P. Soriano, Mount Sinai Hospital, New York). This targeting vector has both Flp recombinase recognition sequences (Frt) flanking the neomycin cassette and Cre recombinase sites (loxP). We chose exons 6 and 7 of caspase-1 to flank with these loxP sites, because this encodes the catalytic domain of caspase-1 and was the region deleted in the viable caspase-1 complete knockout line generated (1). This construct has been electroporated into JM8 C57BL/6 embryonic stem cells. Appropriate targeting was confirmed by long-range PCR in several clones. After germline transmission, mice were crossed to mice expressing Cre recombinase under control of the E2A promoter leading to deletion of the floxed region. Mice were crossed to C57BL/6 mice to segregate Cre recombinase from the deleted allele, and we subsequently intercrossed $Casp1^{+/-}$ to generate $Casp1^{-/-}$ mice.

Cell Culturing. Bone marrow was collected from the femurs and tibiae of mice and cultured for 7 d in RPMI 1640 containing 20% (vol/vol) FBS, 25% (vol/vol) M-CSF, and penicillin/streptomycin (100 U/mL). M-CSF used in macrophage culture media was from supernatants of L-929 fibroblast cells (ATCC). After differentiation, macrophages were replated in RPMI 1640 containing 10% (vol/vol) FBS and 5% (vol/vol) M-CSF, unless indicated otherwise. When indicated, cells were pretreated with 100 ng/mL of *E. coli* LPS (Sigma) or 10 ng/mL of TNF-α (eBioscience) for 4 h, washed, and incubated with fresh media just before infection. Infections were performed by centrifuging bacteria onto macrophages for 5 min at 400 × *g*.

Cytokine Assays. Supernatants were harvested 8 h after infection and cleared by centrifugation before cytokine measurements. IL-1α and IL-1β were measured using a mouse IL-1α and mouse IL-1β ELISA kit, respectively (BD Biosciences). IL-18 was captured using an antibody specific to mature mouse IL-18 (R&D Systems) and detected with a biotinylated antibody to mouse IL-18 (R&D Systems).

Western Blot Analysis. Macrophages were seeded in 48-well plates (2×10^5 per well) and infected with bacterial strains at an MOI of 10 for indicated times. Infections were carried out in RPMI medium 1640 containing 5% (vol/vol) FCS. For 18-h infections, gentamicin was added to cultures (50 μg/mL) after 4 h to prevent further bacterial uptake. Cell lysates and supernatants were subjected to SDS/PAGE analysis and Western blot. Primary antibodies used include a rabbit polyclonal against the p10 subunit of caspase-1 (Santa Cruz Biotechnology), a rat monoclonal antibody against caspase-11 (Novus Biologicals), a goat polyclonal antibody against IL-1α (Sigma), a mouse monoclonal antibody against actin (Sigma), and a rat polyclonal antibody against α-tubulin (Accurate Chemical & Scientific Corporation). Secondary antibodies conjugated to horseradish peroxidase were used for detection (Invitrogen). Supernatants were harvested and diluted with 2× sample buffer, and remaining cells were lysed with 1× sample buffer. Supernatant and cell lysate samples were then subjected to SDS/PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) and blocked overnight at 4 °C in PBS with 0.1% Tween-20 and 5% (wt/vol) nonfat dry milk, followed by incubation in PBS with 0.1% Tween-20 and 5% (wt/vol) BSA at room temperature for 1 h.

Pore-Formation Assays. Pore formation in macrophages was determined by quantification of propidium iodide uptake. Macrophages (1×10^5) were seeded in black 96-well plates. Macrophages were infected with *Legionella* at an MOI of 20 for 2 h. Infections were carried out in RPMI 1640 media lacking phenol red with 20 mM Hepes and propidium iodide at a concentration of 6 μg/mL. Throughout infections, plates were incubated at 37 °C in a Tecan Infinite M1000 fluorescent plate reader, and propidium iodide fluorescence was measured every 5 min.

Cell Death Assays. At the indicated times, supernatants were harvested for analysis of LDH released by dying cells. For 18-h infections, gentamicin was added to cultures (50 μg/mL) after 4 h. LDH levels in supernatants were quantified using the Cytotox 96 kit (Promega) and calculated by subtracting the LDH levels in noninfected samples from all other samples and dividing the sample value by the maximum value obtained when cells were lysed with Triton-X 100.

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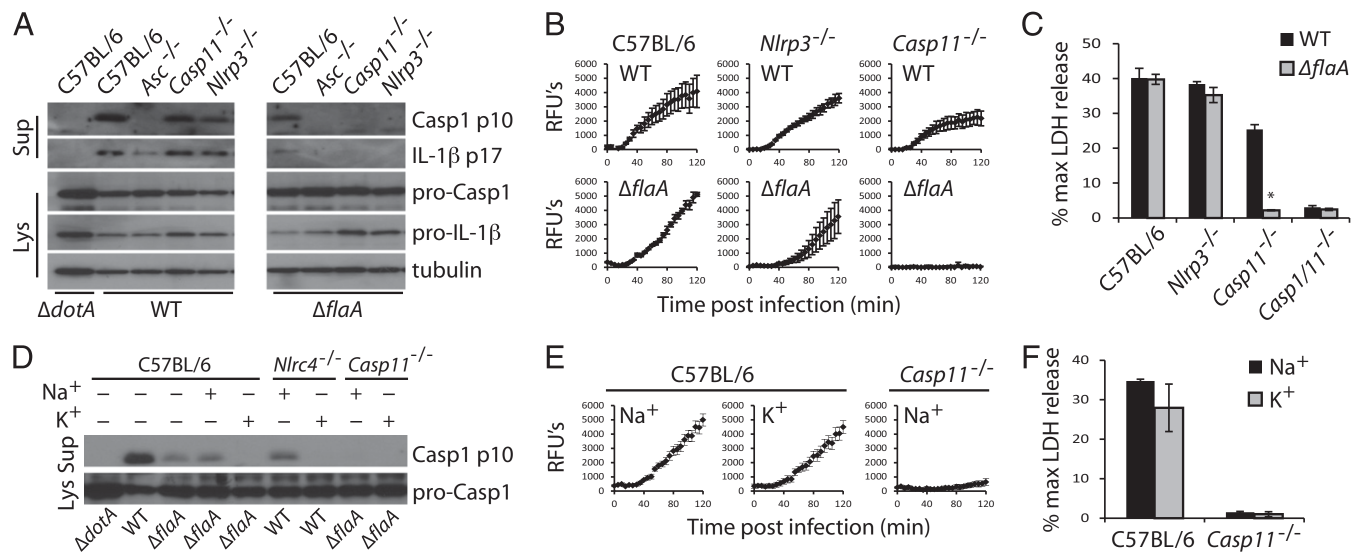

Fig. S1. NLRP3 is required for NAIP/NLRC4-independent activation of caspase-1 in primed macrophages. (*A*) BMMs derived from the indicated mouse strains were pretreated with LPS and then infected with the *Legionella* strains. Immunoblot analysis of the lysates and supernatants from the infected BMMs was conducted using antibodies specific for the proteins indicated on the right side of each panel set. (*B*) Fluorometric plots show propidium iodide uptake (RFUs) over time to reveal the kinetics of pore formation induced upon infection of LPS-treated BMMs with *Legionella*. Data are shown as averages ± SD of three independent wells after subtraction of values for noninfected samples. (*C*) Cell death of LPS-treated BMMs infected for 2 h. Values represent the percentage of LDH released compared with cells lysed with Triton X-100. Data are represented as averages ± SD. (*D*) Caspase-1 cleavage was measured 2 h after infection by the indicated strains of *Legionella* by immunoblot analysis in LPS-treated BMMs from the indicated mouse strains. (*E*) Fluorometric plots show propidium iodide uptake (RFUs) over time to reveal the kinetics of pore formation induced upon infection of LPS-treated BMMs with Δ*flaA Legionella*. (*F*) Cell death of LPS-treated BMMs infected with Δ*flaA Legionella* for 2 h was determined by LDH release. Where indicated, 50 mM NaCl (Na^+) or 50 mM KCl (K^+) were added to culture media before infection (*D*–*F*). $^*P < 0.05$ compared with WT *Legionella* infections.