Supporting Information

SI Text

Mice. Mice genetically deficient for IRAK-1, IRAK-2, IRAK-1/2, MyD88, TRIF, NLRP3, MyD88/TRIF, TLR2/TLR4, TLR7, caspase-1, IL-1R1 and IL-18R, control C57BL/6 mice as well as OT-I transgenic mice (CD45.1background) were bred and maintained at the animal facility of UT Southwestern Medical Center.

Antibodies and reagents. Antibodies for ASC, NLRP3 (goat), IRAK-1 (mouse or rabbit), IRAK-2, IRAK-4, IκBα, HMGB-1, IL-18, IL-1β and GAPDH were obtained from Santa Cruz Biotechnology. Anti-NLRP3 (Cryo-2) was purchased from Adipogen. Anti-MyD88 antibody was purchased from R&D Systems. Phospho-IκBα and β-Tubulin antibodies were purchased from Cell Signalling. Secondary antibodies-conjugated with horseradish peroxidase (HRP), $F(ab')_2$ fragment of donkey secondary antibodies conjugated with fluorochromes (FITC (fluorescein isothiocyanate), TRITC (tetramethyl rhodamine isothiocyanate), cy5 (cyanine dye 5), and normal donkey serum (NDS) were from Jackson ImmunoResearch Laboratory. Mouse BD Fc Block was purchased from BD Pharmingen, and Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories. Anti-caspase-1 p20 rat monoclonal antibody was kindly provided by V. Dixit (Genentech). Chemiluminescence reagent was purchased from PerkinElmer. Reagents used to stimulate macrophages were LPS, poly I:C, CpG, poly dA:dT, R837, BLP (Invivogen), and ATP (Alexis Biochemicals). Cycloheximide and protein G sepharose beads were from Sigma-Aldrich. DPI was from EMD Millipore. Fetal bovine serum was purchased from HyClone, and other cell culture media and supplements were from Invitrogen. FITC anti-CD45, FITC anti-CD45.1, FITC anti-CD44, PE anti-IFN-γ, Alexa 647 anti-F4/80, PerCP anti-CD8, biotin anti-NK1.1 and APC streptavidin were purchased from Biolegend. Differentiated BMDMs were cultured in complete RPMI-1640 supplemented with 10% or 1% FCS, 2mM L-glutamine, 1mM sodium pyruvate, and 50μM β-mercaptoethanol. 100 U/ml penicillin and 100μg/ml streptomycin was added to the media with the exception of antibiotics-free media.

Preparation of bone marrow derived macrophages (BMDMs). Bone marrow was flushed from bones, erythrocytes were lysed, and the white cells were seeded in tissue culture dishes in media containing 10% fetal bovine serum and conditioned media containing mouse M-CSF. The remaining suspension cells were harvested the next day, and plated in fresh plates for selection by growth and adhesion. After 6 days, >99% of the surviving cells were macrophages and ready for stimulation.

Preparation of peritoneal resident macrophages (PCMs). Peritoneal cells were collected by flushing the peritoneal cavity with 10 ml of ice-cold HBSS and plated at 1 x 10^6 cells per well in 12-well plates. After 2 hours, cells in suspension were removed and the remaining adherent cells (PCMs) were washed and used for caspase-1 activation.

Caspase-1 activation assays. BMDMs were plated in 12-well tissue culture plates at 5 x 10^5 cells per well and allowed to attach. For acute caspase-1 activation studies, BMDMs were stimulated simultaneously with TLR ligands and ATP (5 mM) for 30 min. For delayed activation studies, BMDMs were incubated with LPS for 2 to 4 h and then pulsed with ATP (5 mM) during the last 30 min of incubation. In some cases, cells were pre-incubated with cycloheximide or Bay11-7082 for 1 h prior to stimulation with TLR ligands. After stimulation, cells were washed with cold PBS, SDS-sample buffer was then added directly to each well, and cell lysates were harvested, centrifuged, and incubated at 95°C for 20 min. The cell lysates were then subjected to western blot analysis using rat anti-mouse caspase-1 monoclonal antibody.

Pyroptosis Assays. BMDMs were harvested and suspended at 1x10^6 cells/ml in 1% FCS containing RPMI media. Cells were stimulated with LPS (100 ng/ml) and ATP (5 mM) or *Listeria monocytogenes* (1x10^7 CFU) for 1 h. Cells were spun down at 1,500 rpm for 5 min, resuspended in FACS buffer (0.5% (w/v) BSA in PBS) containing 0.5 μg/ml propidium iodide (PI, Sigma), and analyzed for PI incorporation using a FACS Calibur flow cytometer. LDH activity in the supernatants was measured using the LDH Cytotoxicity Detection Kit (Clontech) according to the user manual. For western blots, 500 μl of supernatant was concentrated to 20 μl using the Vivaspin 500 centrifugal filter units (10K) (Bioexpress), and subjected to western blot

analysis for HMGB-1 and Caspase-1. Ponceau S Staining Solution (0.1% (w/v) Ponceau S in 5%(v/v) acetic acid) was used to verify equal loading.

***In vivo Listeria* infection.** Mice were injected with $1x10^7$ CFU of *L. monocytogenes* intraperitoneally. After 20 minutes, peritoneal cells were stained with anti-CD45 and anti-F4/80 on ice for 10 min, washed once and resuspended in FACS buffer containing 0.5 μg/ml PI and analyzed by flow cytometry immediately. Alternatively, mice were injected with $1x10^6$ CFU of *L. monocytogenes* intravenously. After 12 hours, sera were collected via cardiac puncture and cytokine concentrations were measured by ELISA. Splenocytes were cultured at $2x10^6$ cells/ml for 6 hours in the presence of 1μg/ml brefeldin A and 20μg/ml gentamicin. IFN-γ production by memory CD8 T cells and NK cells was assayed by intracellular staining using the Foxp3 staining buffer set from Biolegend.

Immunoprecipitation. BMDMs were plated in 100-mm dishes the day before the experiments. Cells were stimulated with TLR and NLRP3 activators for the indicated periods of time, followed by two washes with ice cold PBS. Cells were lysed with 0.5% NP-40 in PBS supplemented with protease (Roche) and phosphatase (Sigma) inhibitors for 30 min at 4°C on an end-over-end rotator, followed by centrifugation at 14,000 x g for 30 min at 4°C. The lysates were stored at -80°C until use. After preclearing of lysates with protein G-sepharose (Sigma-Aldrich), primary antibodies were added to the supernatants and incubated overnight at 4°C on an end-over-end rotator. Protein G sepharose was added to the mixture and incubated at 4°C for 4 h. The protein G-sepharose beads were washed 4 times with PBS-0.5% NP-40, and then proteins were eluted from the beads by boiling in SDS-sample buffer, followed by western blot analysis.

Immunofluorescence microscopy. BMDMs were plated in 60-mm dishes containing sterile glass cover slips the day before the experiments. Cells were treated with ATP (5 mM) and LPS (100 ng/ml) for the indicated periods of time, followed by two washes with PBS. Cells were fixed with ice-cold methanol for 5 min at -10 °C, and then methanol was removed and cells left to air-dry. Cells were then washed three times with PBS. Cells used for staining of actin and HMGB-1 were fixed with 4% PFA for 15 min at room temperature followed by three washes with PBS. Cover slips were first incubated with 20% normal donkey serum (NDS) in PBS containing 1 μg/ml of

mouse BD Fc Block (BD Pharmingen) for 30 min to suppress non-specific binding of IgG. Primary antibodies to IRAK-1 (rabbit or mouse), ASC (rabbit), NLRP3 (goat or mouse), MyD88 (goat), IRAK-2 (rabbit), IRAK-4 (goat) and HMGB-1 (mouse) diluted in PBS containing Fc Block and 10% NDS were added to the cover slips and incubated for 60 min at room temperature. For staining of actin, coverslips were incubated with diluted Phalloidin-TRITC for 60 min at room temperature. Following washes with PBS, cover slips were incubated with appropriate $F(ab')_2$ fragment of donkey secondary antibodies conjugated with fluorochromes for 60 min at room temperature. After three washes with PBS, cover slips were mounted on glass slides with Vectashield mounting medium containing DAPI. The signal was examined by fluorescent microscopy at the appropriate wavelength for the secondary antibody on an IX81 Olympus microscope or Zeiss LSM 510 meta confocal microscope, and images were obtained using a Hamamatsu Orca digital camera with a DSU confocal unit using Slidebook software (Intelligent Imaging Innovations (3i)). The images were then processed with ImageJ software (National Institutes of Health, http://imagej.nih.gov/ij/, 1997-2012) followed by Adobe Photoshop.

Listeria monocytogenes **culture.** LM 10403 serotype 1(2) was provided by Dr. James Forman at UT Southwestern Medical Center and had been repeatedly passaged through B6 mice to maintain virulent stocks. Bacteria were first grown on LB agar plates, and a single colony was inoculated to LB broth and cultured at 37˚C overnight without shaking. For *in vitro* infection, the bacterial culture was added directly to the cells without washing. For *in vivo* infection, the bacterial culture was diluted in PBS.

Cytokine measurements. Cell-free supernatants were measured for TNFα, IL-6, and IL-12(p40) using a custom-prepared Bioplex cytokine assay kit (Bio-Rad Laboratories) according to the manufacture's direction. IL-1β and IL-18 were measured using antibody pairs purchased from R&D Systems. IFN-γ was measured using an antibody pair from BD Pharmigen.

1. Kofoed EM & Vance RE (2011) Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* 477(7366):592-595.
2. Seaman MS, Perarnau B, Lindahl KF, Lemonnier FA, & Forman J (1999) Response to Listeria monocytogenes in mice lacking MHC class Ia molecules. *J Immunol* 162(9):5429-5436.

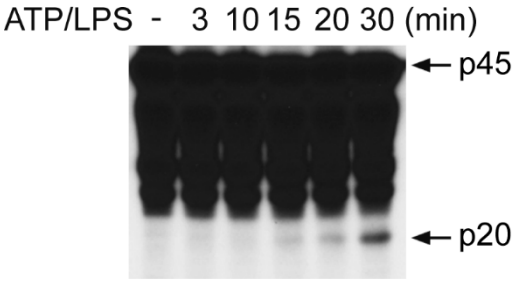

Fig. S1. Kinetics of rapid caspase-1 activation. BMDMs from wild type mice were stimulated with LPS (100 ng/ml) together with ATP (5 mM) for indicated periods of time and cell lysates were examined for caspase-1 cleavage by western blot analysis. Data are representative of three independent experiments.

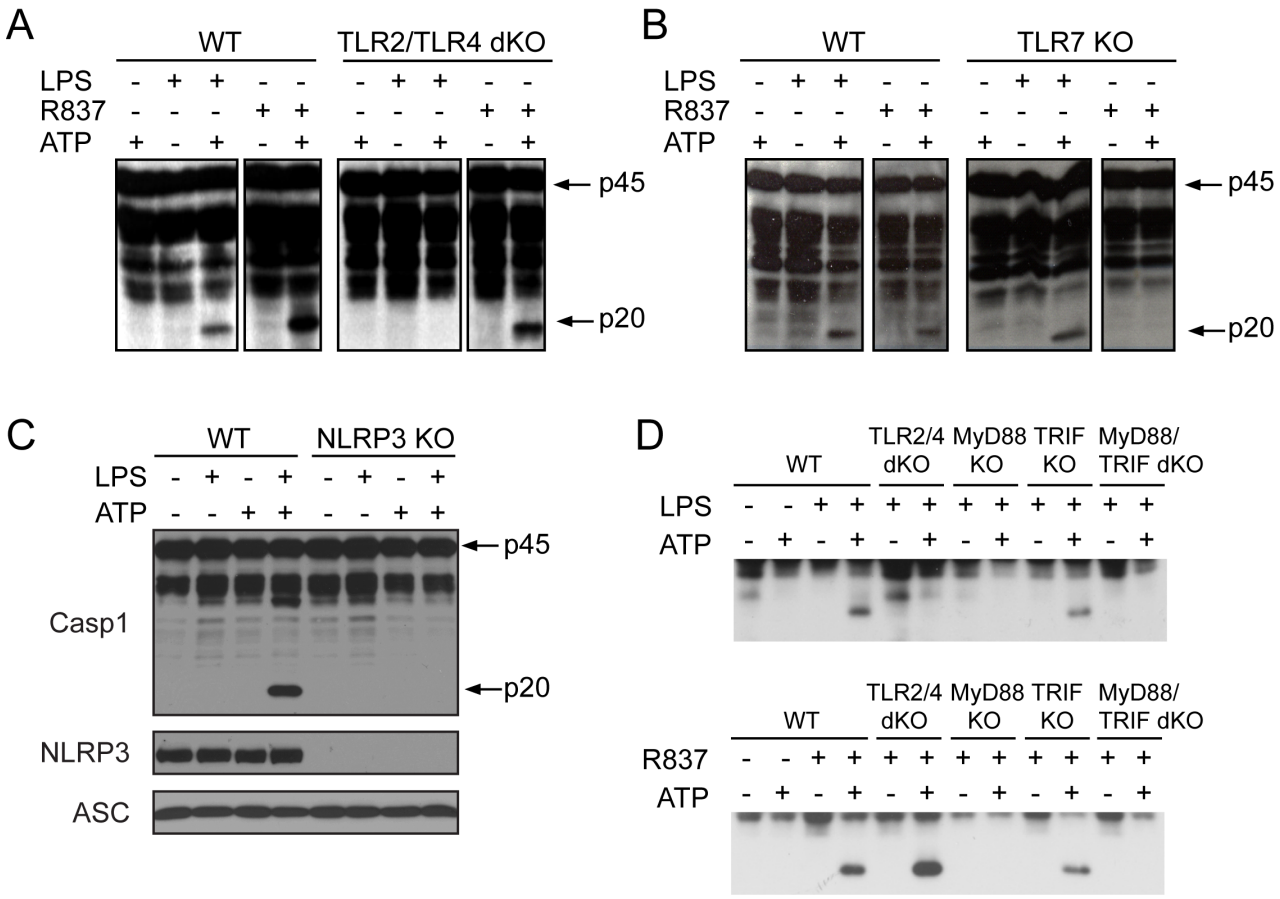

Fig. S2. Rapid Caspase-1 cleavage is abrogated in macrophages lacking TLR signaling and NLRP3. BMDMs from mice of indicated genotypes were stimulated with the TLR4 ligand LPS and ATP or the TLR7 ligand R837 and ATP for 30 minutes and cell lysates were analyzed for caspase-1 cleavage by western blot analysis. Bone marrow derived macrophages express constitutive NLRP3 protein that is sufficient for rapid caspase-1 cleavage but requires signals from both TLRs and NLRP3 (C). Data are representative of two to four independent experiments.

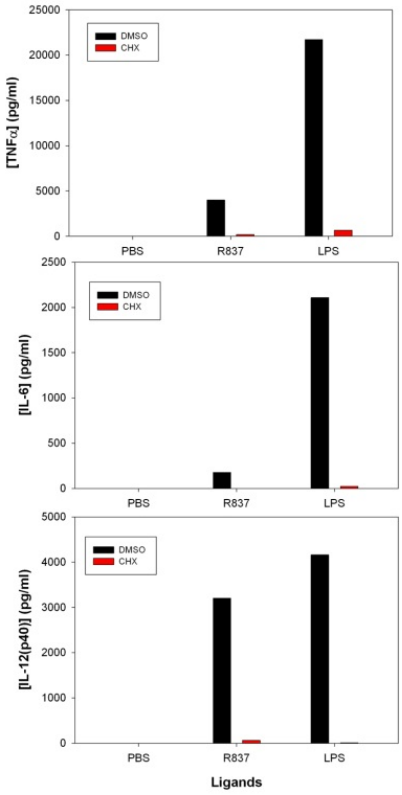

Fig. S3. Cycloheximide treatment of macrophages inhibits pro-inflammatory cytokine production in response to TLR ligands. BMDMs were treated with cycloheximide (CHX, 50 ng/ml) for 60 min prior to stimulation with LPS (100 ng/ml) or R837 (5 μg/ml) for 4 hours, and then supernatants were analyzed for the presence of TNF-α, IL-6 or IL-12. Bar graphs represent the average from duplicated measurements for each sample. Cell lysates from duplicate cultures, with or without addition of ATP, were analyzed for caspase-1 cleavage (Figure 1e). Data are representative of three independent experiments.

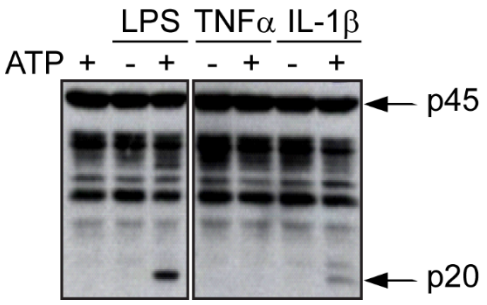

Fig. S4. IL-1β but not TNF-α can induce rapid NLRP3 inflammasome activation. BMDMs from WT mice were stimulated with LPS, TNF-α and IL-1β with and without ATP for 30 minutes. Only LPS or IL-1β were able to induce caspase-1 cleavage. Macrophages express very low levels of IL-1R and this could explain the lower level of caspase-1 cleavage induced by IL-1 when compared to LPS. Data are representative of three independent experiments.

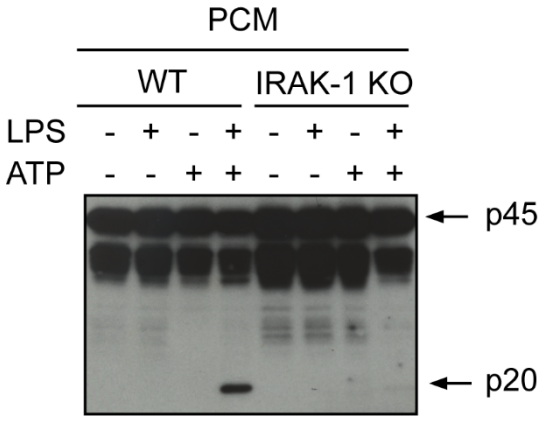

Fig. S5. Rapid NLRP3 inflammasome mediated caspase-1 activation pathway is functional in peritoneal cavity resident macrophages (PCM) and depends on IRAK-1. PCMs from WT and IRAK-1 KO mice were stimulated with the TLR4 ligand LPS and ATP for 30 minutes. Cell lysates were subjected to western blot analysis to detect caspase-1 cleavage. Data are representative of two independent experiments.

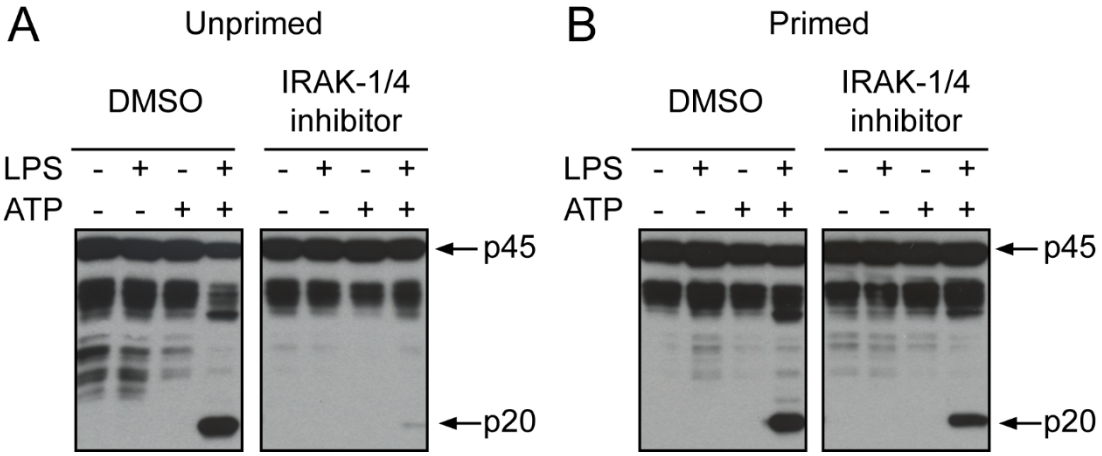

Fig. S6. Rapid NLRP3 inflammmasome activation depends on kinase activity of IRAKs, but priming dependent NLRP3 inflammasome activation does not require kinase activity of IRAKs. BMDMs from WT mice were activated using LPS and ATP for 30 minutes (A) or were first stimulated with LPS for 4 hours before addition of ATP for an additional 30 minutes (B). Stimulations were done either in the presence of DMSO or the IRAK 1 and 4 kinase inhibitor (5 μM CAS 509093-47-4, Millipore) dissolved in DMSO. Cell lystes were subjected to western blot analysis to detect caspase-1 cleavage. Data are representative of four independent experiments.

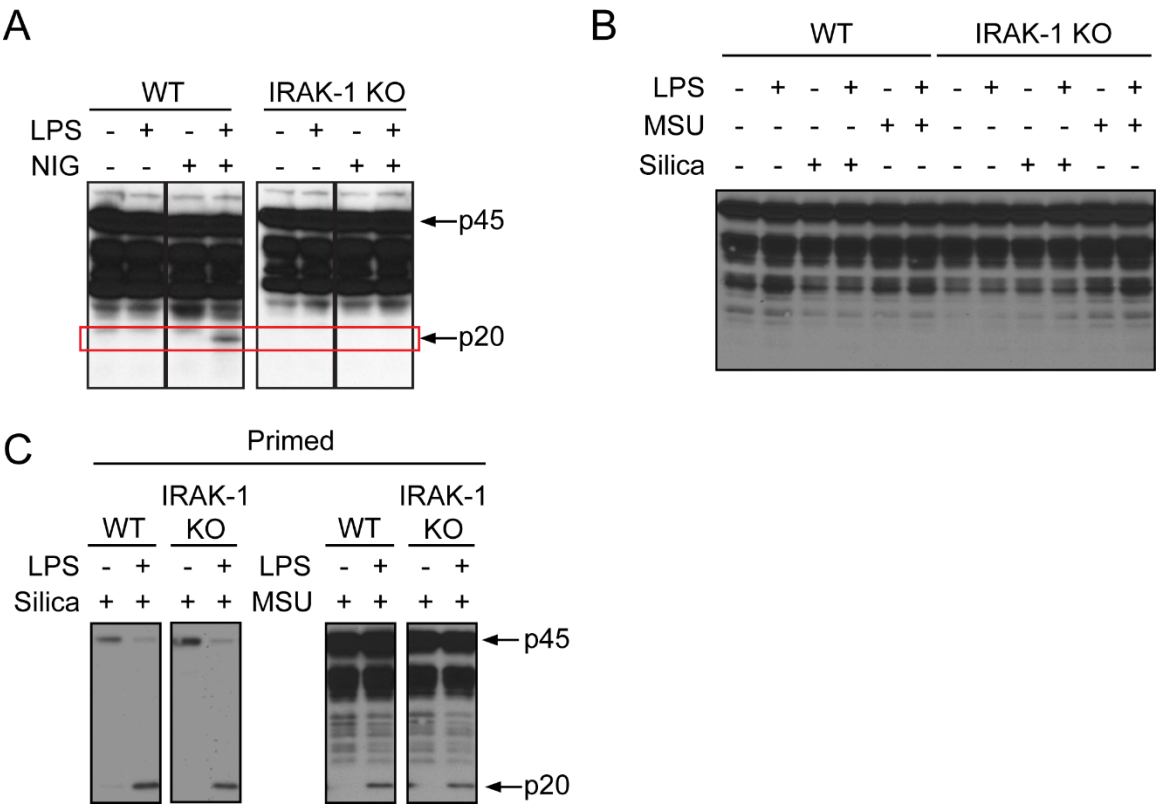

Fig. S7. Differential ability of NLRP3 ligands to induce rapid caspase-1 activation. WT or IRAK-1 KO BMDMs were stimulated with LPS plus nigericin for 30 minutes (A) or with LPS plus monosodium urate (MSU) and LPS plus silica crystals (B) for 30 minutes. WT or IRAK-1 KO BMDMs were primed using LPS for 4 hours before stimulation with MSU and silica crystals for an additional 6 hours (C). Cell lystates were examined for caspase-1 cleavage by western blot analysis. Data are representative of two to three independent experiments.

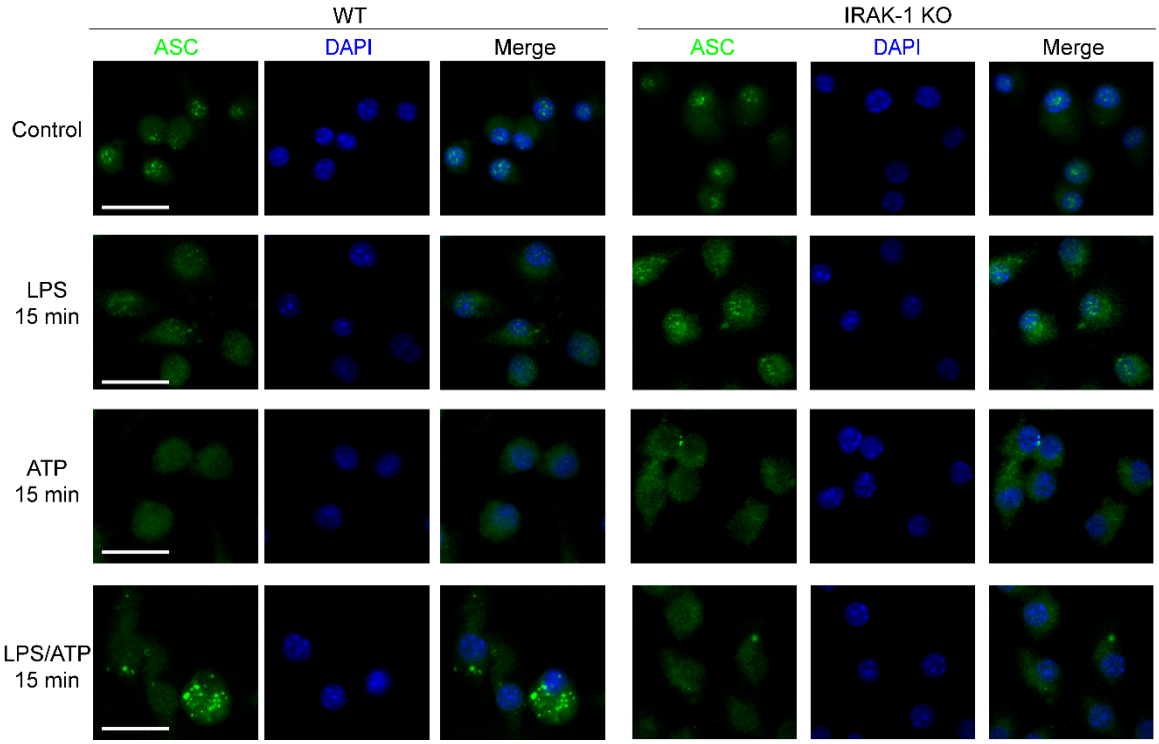

Fig. S8. Relocalization and speck formation by ASC following TLR and NLRP3 stimulation. WT (left panels) or IRAK-1 KO (right panels) BMDMs underwent nuclear staining with DAPI and immunofluorescence of endogenous ASC after stimulation with LPS alone, ATP alone, or LPS and ATP simultaneously. Scale bar, 20 μm.

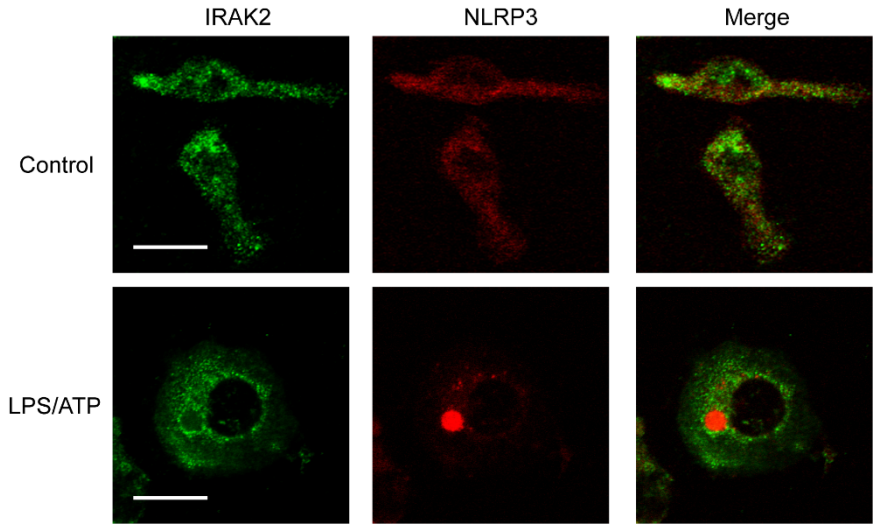

Fig. S9. IRAK-2 does not associate with NLRP3 during rapid inflammasome activation. Immunostaining of endogenous IRAK-2 and NLRP3 in WT BMDMs stimulated with LPS and ATP for 15 minutes. Scale bar, 10 μm. Data are representative of two independent experiments.

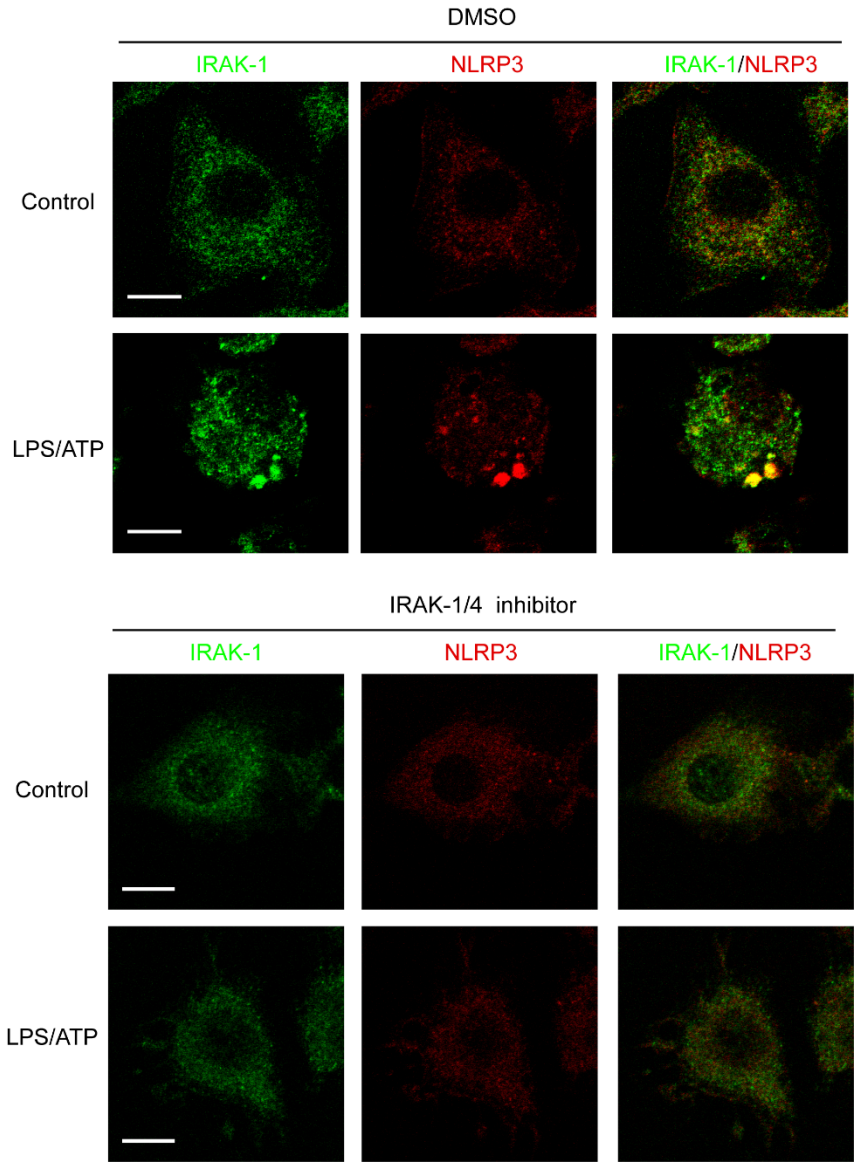

Fig. S10. Inhibition of the kinase activity of IRAKs prevent NLRP3 speck formation. Immunostaining of endogenous IRAK-1 and NLRP3 in WT BMDMs stimulated with LPS and ATP for 15 minutes in the presence of an IRAK-1/4 kinase inhibitor (5 μM CAS 509093-47-4, Millipore) dissolved in DMSO. Scale bar, 10 μm. Data are representative of two independent experiments.

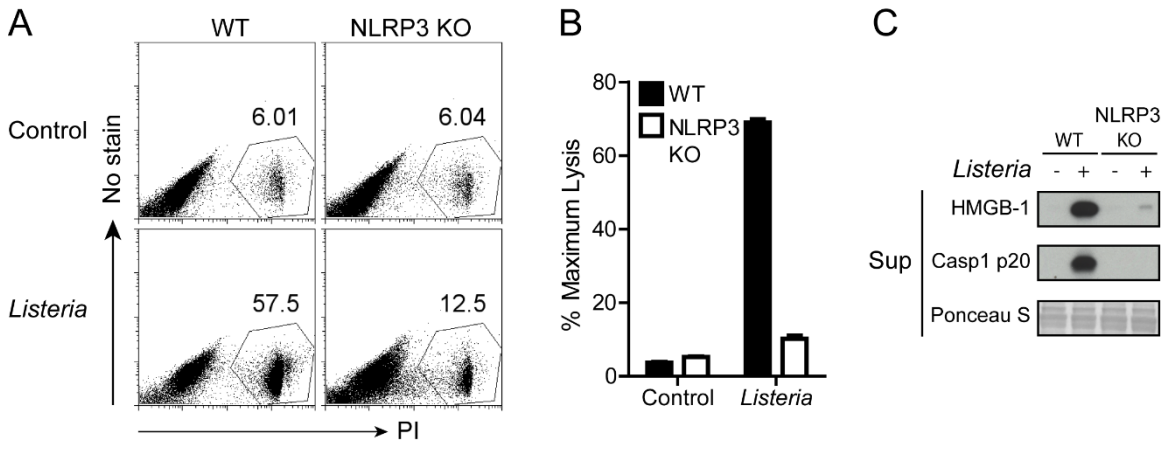

Fig. S11. *Listeria monocytogenes* infection induces NLRP3-dependent inflammasome activation and pyroptosis (MOI=40). WT or NLRP3-deficient BMDMs were infected with *L. monocytogenes* for 1 h and analyzed for pyroptosis by (A) PI incorporation, (B) LDH release and (C) cleaved caspase-1 release and HMGB-1 release. Data are representative of two independent experiments.

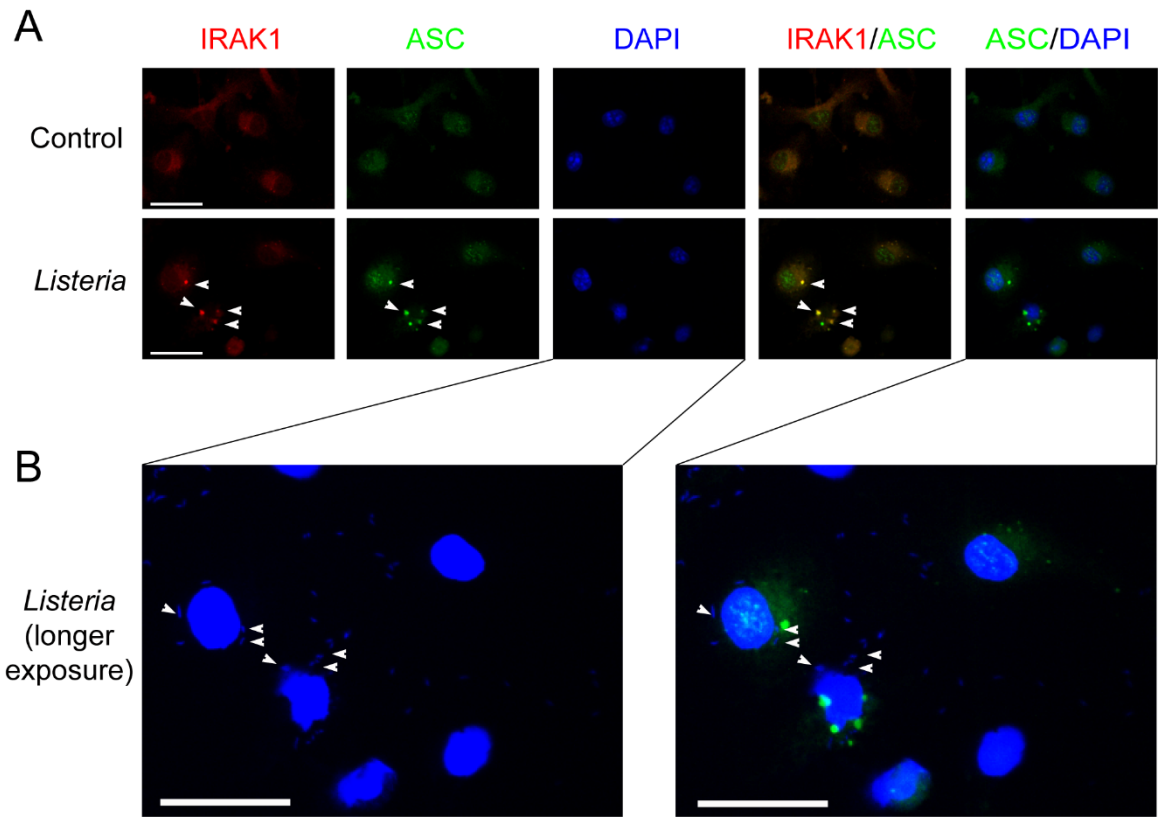

Fig. S12. *Listeria monocytogenes* infection causes formation of ASC and IRAK1 specks. (A) Immunostaining of endogenous ASC and IRAK-1 in control (upper panels) WT BMDMs or BMDM infected with *L. monocytogenes for* 1h (lower panels). Arrowheads show specks formed by indicated proteins. (B) Enlarged images with longer exposure of DAPI and merged ASC/DAPI staining of BMDM infected with *L. monocytogenes*. Arrowheads show the DAPI staining of *L. monocytogenes.* Scale bar, 20 μm. Data are representative of two independent experiments.

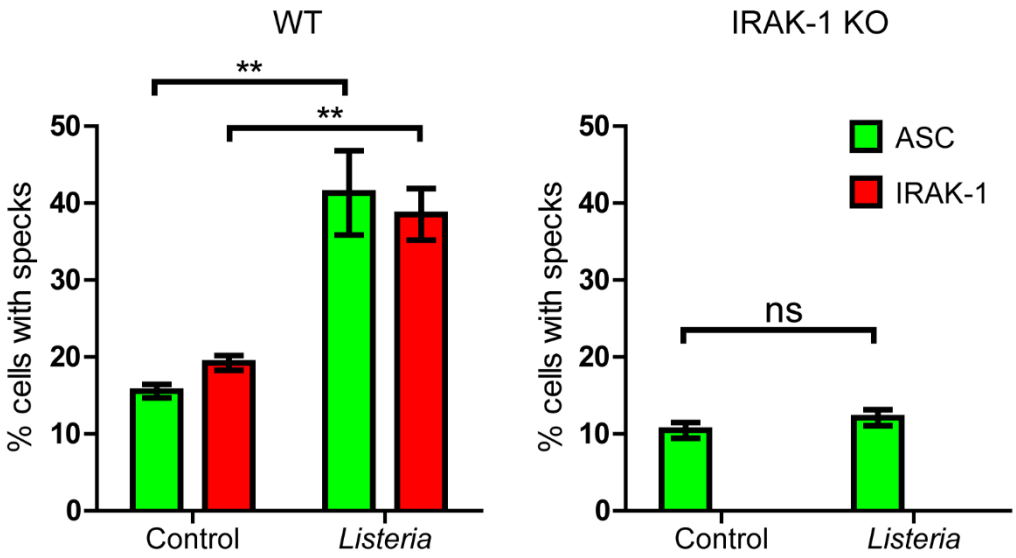

Fig. S13. Quantification of percentages of cells containing ASC (green bars) or IRAK-1 (red bars) specks following Listeria infection. Bar graphs represent mean ± SEM from three experiments and at least 100 cells were counted in every condition. *$p < 0.05$, **$p < 0.01$, ns, non-significant, one-way ANOVA test.

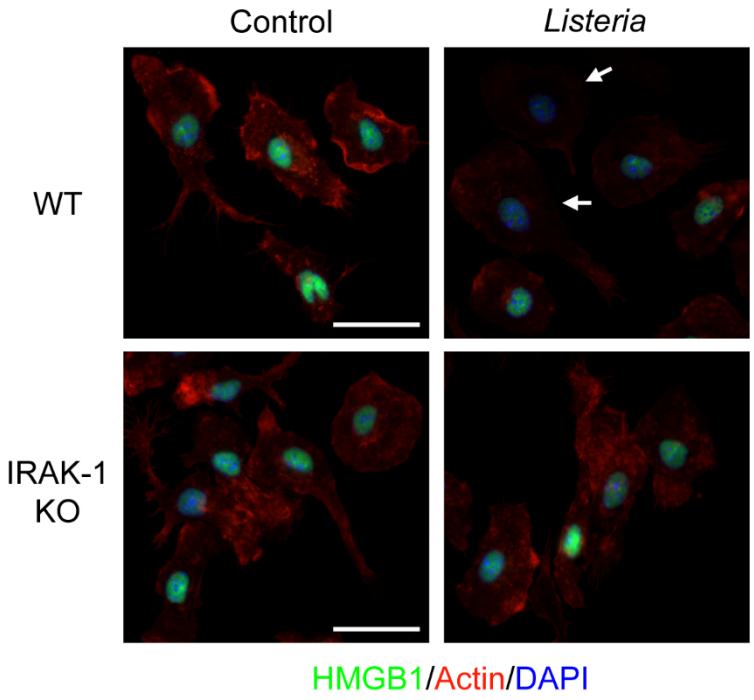

Fig. S14. *L. monocytogenes* infection leads to IRAK-1 dependent HMGB-1 release. Immunostaining of endogenous HMGB-1 and actin in WT or IRAK-1-deficient BMDMs infected with *L. monocytogenes* for 1 h. Arrows show cells that have lost HMGB-1 in the nucleus. Scale bar, 20 μm. Data are representative of two independent experiments.

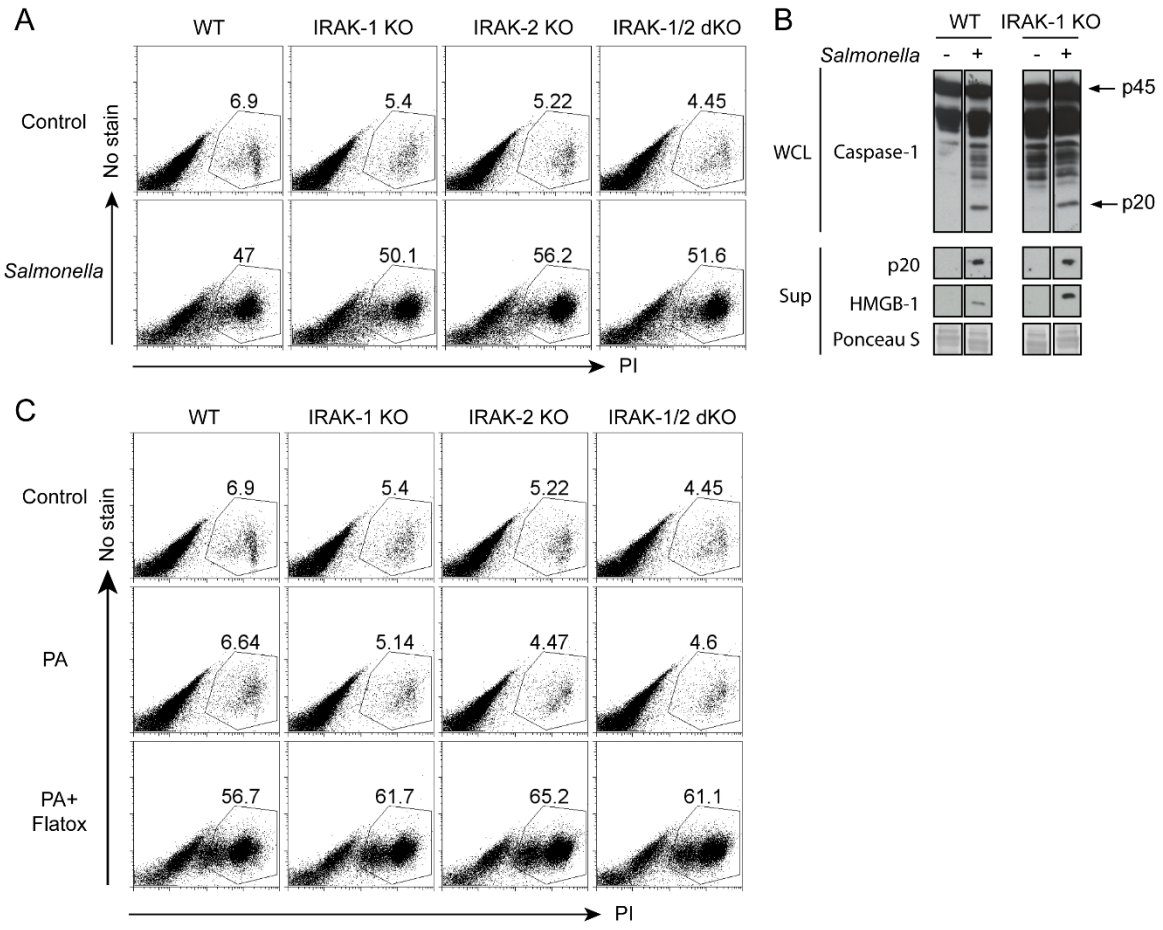

Fig. S15. IRAK-1 does not regulate NLRC4 inflammasome activation and pyroptosis. (A, B) BMDMs of the indicated genotypes were infected with *Salmonella typhimurium* SL1344 (MOI=2) for 1h and analyzed for pyroptosis by (A) PI incorporation and (B) cleaved caspase-1 release and HMGB-1 release. Ponceau S stain of total protein serves as the loading control for the supernatant (sup). (C) BMDMs were treated with 4 μg/ml of *B. anthracis* protective antigen (PA) alone, or together with 2 μg/ml of LFn-FlaA (Flatox)(1), a fusion protein encoding the first (non-enzymatically active) 263 amino acids of the lethal factor from *Bacillus anthracis* fused to full-length flagellin (FlaA) from *L. pneumophila*, which allows cytosolic delivery of flagellin. After 1h, cells were stained with PI to measure pyroptosis. Data are representative of five independent experiments.

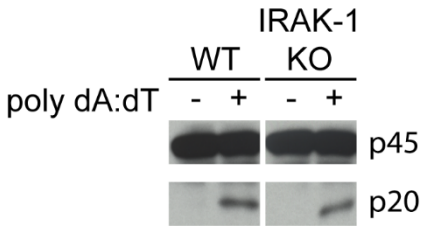

Fig. S16. AIM-2 inflammasome activation is independent of IRAK-1. WT and IRAK-1 deficient BMDMs were mock transfected or transfected with 5 μg/ml of poly dA:dT using lipofectamine 2000 for 1h and analyzed for caspase-1 cleavage by western blot. Data are representative of two independent experiments.

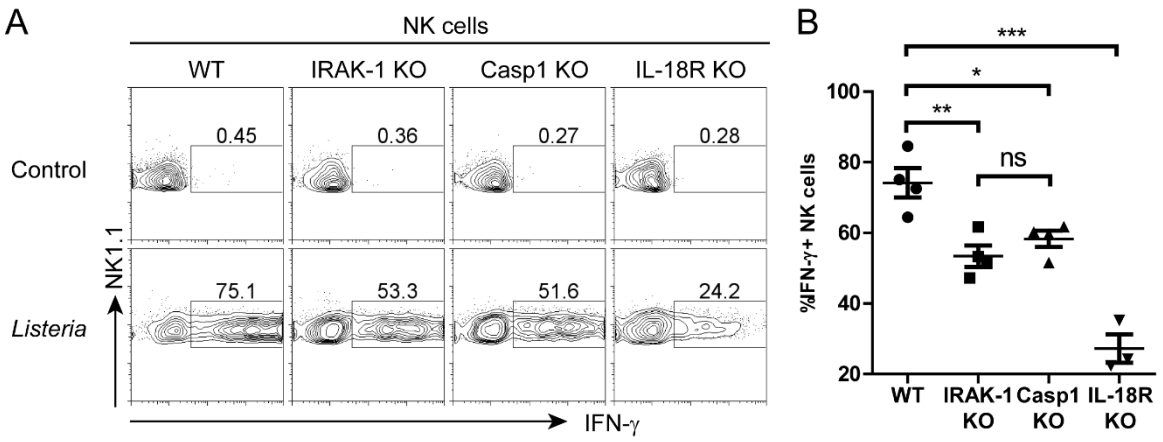

Fig. S17. IRAK-1 dependent IFN-γ production by NK cells during *L. monocytogenes* infection *in vivo*. (A) Representative plots and (B) combined data of IFN-γ production by NK cells from mice infected intravenously with *L. monocytogenes* for 12 hours.*p<0.05, **p<0.01, ***p<0.005, ns, non-significant, one-way ANOVA test.

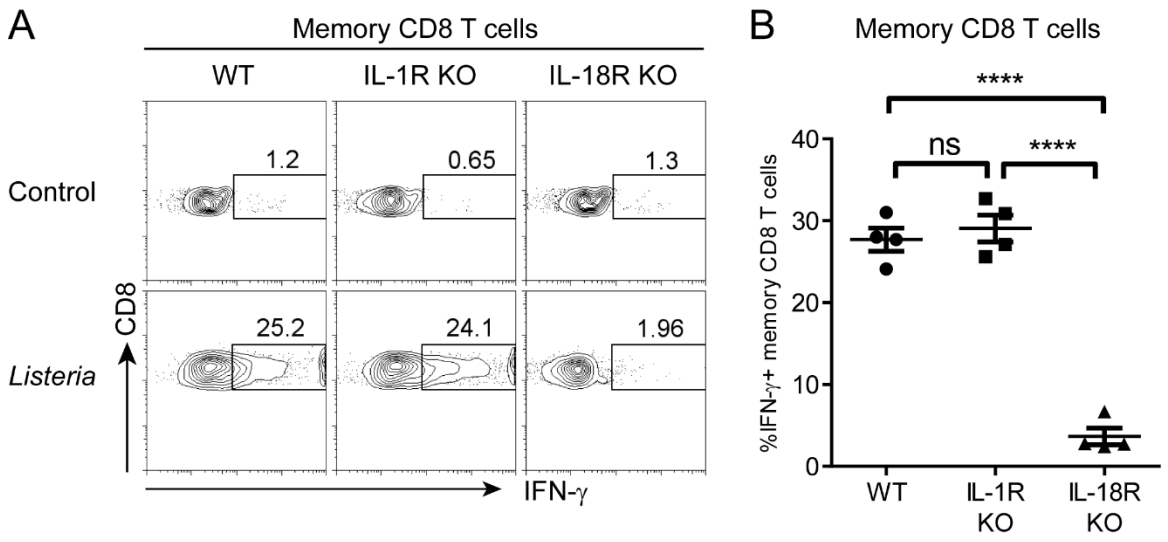

Fig. S18. IL-1 is dispensable for innate IFN-γ production by memory CD8 T cells during *L. monocytogenes* infection *in vivo*. (A) Representative plots and (B) combined data of IFN-γ production by memory CD8 T cells from mice infected intravenously with *L. monocytogenes* for 12 hours.****$p<0.001$, ns, non-significant, one-way ANOVA test.

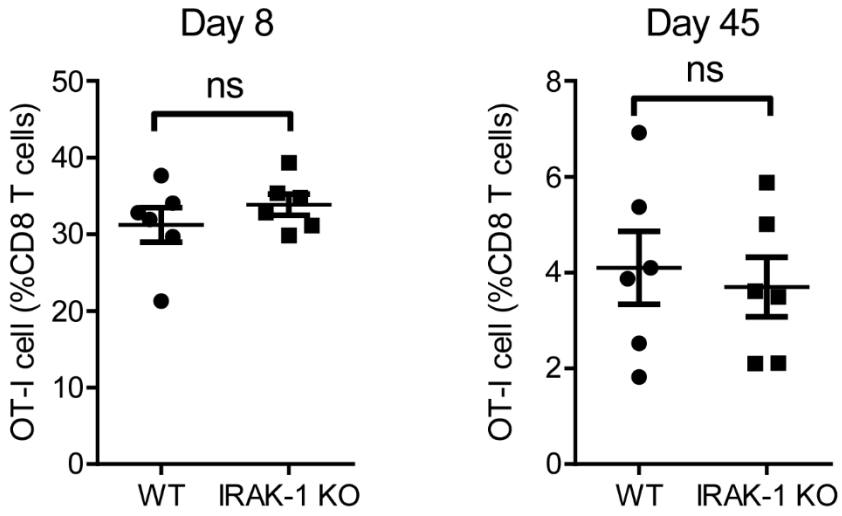

Fig. S19. Equivalent expansion and survival of OT-I T cells in WT and IRAK-1 KO hosts following infection. WT and IRAK-1 deficient mice, both on the CD45.2 background received 1000 OT-I T cells from transgenic mice on the CD45.1 background and were infected using vesicular stomatitis virus expressing ovalbumin ($2x10^6$ pfu per mouse). OT-I T cell expansion was measured in the peripheral blood at day 8 by staining for surface CD45.1 and CD8. Similar staining was done for cells from the spleen on day 45 after challenge to measure OT-I T cell survival. Scatter plots show mean$\pm$SEM. ns, non-significant, unpaired t test.

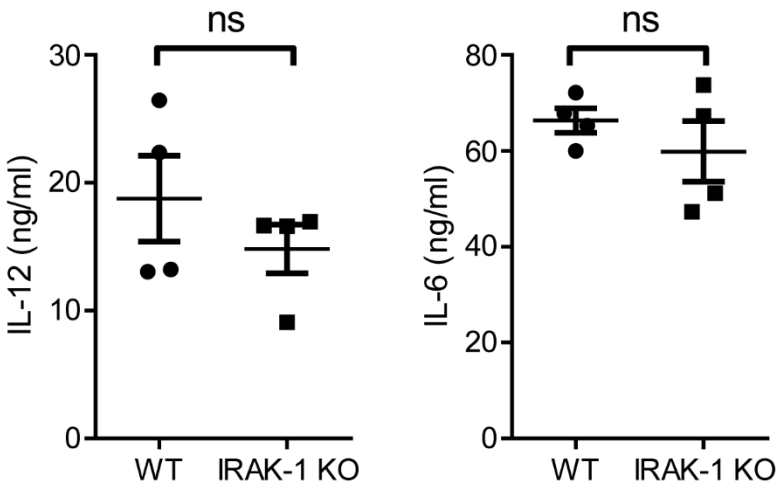

Fig. S20. Comparable levels of IL-12 and IL-6 induction by *Listeria monocytogenes* in WT and IRAK-1 deficient mice. WT and IRAK-1 KO mice were infected with $1x10^6$ *Listeria monocytogenes*. IL-12 and IL-6 concentrations in the serum were determined by ELISA at 12 hours following infection. Scatter plots show mean±SEM. ns, non-significant, unpaired t test.

	Early pathway	*Late pathway*
Activation of TLR and NLRP3	Simultaneous	Sequential
Participating TLRs	All except for TLR3	All
NF-κB activity	Not required	Required
New protein synthesis	Not required	Required
IRAK-1	Required	Not required
IRAK-1&IRAK-2	Required	Required

Table S1. Major differences in the early (rapid) and late (priming-dependent) pathways of NLRP3 inflammasome activation.