Supporting Information

Weng et al. 10.1073/pnas.1403477111

SI Methods

Mice. Mice were maintained in specific-pathogen-free conditions at University of Massachusetts (UMass) Medical School, and all experiments involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the UMass Medical School. RIP3$^{-/-}$ caspase-8$^{-/-}$ double-deficient mice (*Rip3*$^{-/-}$*xCasp8*$^{-/-}$) [double knockout (dKO)] (1) mice were generated by W.J.K. and E.S.M. and were bred 6–10 generations into C57BL/6. RIP3 deficient (*Rip3*$^{-/-}$) mice were generated by V. Dixit (Genentech, South San Francisco, CA) (2). Mice lacking IFNαβR1 (*Ifnar1*$^{-/-}$) were from J. Sprent (The Scripps Research Institute). *Fasl*^{gld} and TNF-α$^{-/-}$ (*Tnf*$^{-/-}$ on a Lymphocytic Choriomeningitis Virus CTL P14 background, with corresponding WT controls) mice were from A. Marshak-Rothstein, R. Welsh (both UMass Medical School), and The Jackson Laboratories Bar Harbor, ME. Mice lacking caspase-1 (*Casp1*$^{-/-}$; also containing a caspase-11 mutation), NLRP3 (*Nlrp3*$^{-/-}$), NLRP3/NLRC4 (*Nlrp3*$^{-/-}$*xNlrc4*$^{-/-}$), NLRP12 (*Nlrp12*$^{-/-}$), TLR4 (*Tlr4*$^{-/-}$), TRIF (*Ticam1*$^{-/-}$), or MyD88 (*Myd88*$^{-/-}$) were from S. Akira, M. Starnback, or Milennium Inc. as previously reported (3, 4). *Caspase-8*$^{fl/fl}$ *LysM cre*$^{+/+}$ [conditional KO (cKO)] mice on a C57BL/6 background were generated by D.M.S. with a *Caspase-8*$^{fl/+}$ allele provided by S. Hedrick (University of California, San Diego, La Jolla, CA) (5). C57BL/6 mice were bred in-house or from The Jackson Laboratory. Bone marrow transplantation (BMT) was performed on lethally (900 rads) irradiated C57BL/6 mice that were reconstituted with 4×10^6 cells from the indicated genotypes of mice. Mice were used for infections 6–10 wk after transplantation or when 6 to 12 wk old (age/sex matched, similar numbers of each sex were used). Mice were infected s.c. in the nape of the neck or i.v. with 500 cfu of KIM1001-pEcLpxL as previously reported (3) and monitored for survival twice daily for up to 30 d; or killed at ∼42 h after infection for cytokine, cfu, histology, and cell death analysis. Sample collection was performed as previously described (4). Spleens from mice infected s.c. with 300 cfu of KIM1001 were harvested 68 h after infection. Cytokine amounts from the spleen were normalized by spleen weight to account for any increases in tissue size during active infection, because RIP3$^{-/-}$Caspase8$^{-/-}$ mice show signs of markedly enlarged spleens by 6–8 wk of age owing to an increase in T-cell numbers (1). All experiments were approved by the UMass Medical School IACUC and were performed according to ethical regulations regarding humane treatment of animals.

Reagents. Necrostatin-1 (Nec-1) was purchased from Enzo Life Sciences. Nigericin was from Sigma-Aldrich. z-VAD-fmk was from Promega. Antibodies were purchased from the following vendors: RIP1 and β-actin (BD Pharmingen); caspase-8 and cIAP1 (Enzo); caspase-3, IκBα, pIκBα, pIKKα/β, IKKα, and p65 (Cell Signaling); caspase-1 (eBioscience); IL-1β (R&D Systsems), RIP3 (ProSci), ASC (Santa Cruz), and histone H3 (Abcam). Pam3Cys was obtained from Invivogen.

Bacterial Strains and Growth Conditions. *Yersinia pestis* KIM5 [containing the complete type III secretion system (T3SS)] was grown in tryptose-beef extract (TB) broth with 2.5 mM $CaCl_2$ overnight at 26 °C, then diluted 1:8 into fresh media, cultured for 1 h at 26 °C, and shifted to 37 °C for 2 h. KIM5-EcLpxL was grown similarly, but culture medium was supplemented with 100 μg/mL ampicillin. *Yersinia pseudotuberculosis* IP2666 and *Yersinia enterocolitica* 8081 (4) were grown at 26 °C in TB overnight, diluted, and further cultured for 3 h at 37 °C. *Salmonella enterica* serovar *typhimurium* strain SL1344 was provided by M. O'Riordan and grown at 37 °C with shaking. For generation of *Y. pseudotuberculosis* IP2666Δ*YopJ*, an in-frame deletion removing codons 4–287 was created via allelic exchange. PCR products made with primer sets A (ATAGAGCTCCACTACTGATTCAACTTGGACG) and B (TCCGATCATTTATTTATCCTTATTCA), and C (TGAATAAGGATAAATAAATGATCGGATAATGTATTTTGGAAATCTTGCT) and D (GGGTCTAGACTGATGTCGTTTATTTCTGGGTAT), respectfully, were used to make a fused product by overlap PCR using primers A and D (6). For generation of *Y. pestis* KIM5 ΔCaF1 (F1), an in-frame deletion removing codons 11–164 was created via allelic exchange. PCR products made with primer sets A (ATCGATGAGCTCCGTCACAGTAAGAGCACAACTT), B (GCAGCAGCAGCAGCATGGCGATAACGGAACTGAT) and C (TGCTGCTGCTGCTGCGTAACCGTATCTAACCAATAA), D (ATCGATGGTACCCTTCCAGTATCAGTGGGTTC), respectfully, were used to make a fused product by overlap PCR using primers A and D (6). For both strains, the PCR product was cloned in the allelic exchange vector pRE107 (7) in *Esherichia coli* K12 strain B2155, transferred to parental strains by conjugation, and recombinants selected on TB medium containing 100 μg/mL ampicillin with no diaminopimelic acid. After counter selection with 5% (wt/vol) sucrose, deletion mutants were identified by PCR.

KIM5ΔYopJ was as previously described (4). Fully virulent KIM1001, and the attenuated strains KIM5-EcLpxL and KIM1001-EcLpxL, expressing pBR322 containing *E. coli lpxL* and constitutively generating a TLR4-stimulating hexa-acylated lipid A, were as indicated (3). *Pseudomonas aeruginosa* was a gift from S. Wong and B. Akerley (UMass Medical School) and was grown in LB medium with shaking at 37 °C.

Cell Stimulations. Mouse bone marrow-derived macrophages (BMDMs) were prepared by maturing bone marrow cells for 6–7 d in the presence of M-CSF-containing supernatant from L929 cells. Some cell stimulations were also conducted with peritoneal macrophages and immortalized BMDM, and similar results to stimulations done with BMDM were obtained. RIP1$^{+/+}$ and RIP1$^{-/-}$ immortalized fetal liver macrophages were generated by M.A.K. and K.A.F. Mouse peritoneal macrophages were harvested from mice 4 d after injection of 3 mL thioglycollate (i.p. injected). Mouse macrophages were plated at a density of 5×10^4 cells per well in 96-well dishes and incubated overnight. Bacterial infections were performed at the multiplicities of infection (MOIs) indicated, and gentamicin (40 μg/mL) was added 1 h after infection. Supernatants were collected for cytokine analysis or lactate dehydrogenase (LDH) assays at the indicated time points. In some experiments, cells were pretreated with 1 μM of the receptor-interacting protein (RIP) 1 inhibitor GSK'963 or its inactive enantiomer GSK'962, 3 μM of the RIP3 inhibitor GSK'872 (RIP inhibitors provided by P.A.H., J.B., and P.J.G. at GlaxoSmithKline, Collegeville, PA), 20 μM Nec-1 (Enzo Life Sciences), 20 μM zIETD, zYVAD, or zVAD (Promega) 1 h before infection. Nigericin (10 μM; Sigma-Aldrich), 10 ng/mL of hexa-acylated LPS from *Y. pestis* grown at 26 °C or from *E. coli*, and 300 ng/mL Pam3CysSK4 (Pam3Cys; Invitrogen) were used as controls. Cytokines and caspase-1 cleavage were measured by ELISA and Western blot according to the manufacturer's instructions or as previously reported (4). ELISA antibodies or kits were from R&D systems (mouse TNF, IL-6, IL-18, and IL-1β).

Western Blot Analysis. Protein from cell supernatants was precipitated by methanol-chloroform extraction. Adherent cells were lysed with 1% Nonidet P-40 lysis buffer [10% (vol/vol) glycerol,

1% Nonidet P-40, 20 mM Tris·HCl (pH 7.4), 137 mM NaCl, and 2 mM EDTA] containing protease inhibitors: 1 mM sodium orthovanadate, 1 mM PMSF and cOmplete Protease Inhibitor Mixture (Roche). Immunoblot analysis was mainly as previously described (4, 8) with antibodies to mouse caspase-8 (clone 1G12, Enzo Life Sciences), caspase-3 (clone 5A1E, Cell Signaling), mouse caspase-1 p20 (clone 5B10, eBioscience), mouse IL-1β (catalog no. AF-401-NA, R&D Systems), RIP1 (clone 38/RIP, BD Transduction Laboratories), IκBα (catalog no. 9242, Cell Signaling), phospho-IκBα (catalog no. 2859, Cell Signaling), β-actin (BD Pharmingen), RIP3 (catalog no. 2283, ProSci), p65 (Cell Signaling), or histone 3 (Abcam).

LDH Assays. Cell death was measured by detecting the release of LDH by the CytoTox-96 nonradioactive cytotoxicity kit (Promega) according to the manufacturer's instructions. Background LDH release was determined by assaying supernatants from uninfected cells, and total LDH was determined from uninfected cells that had been lysed by lysis buffer. The percentage of cell death was calculated as (sample LDH − background LDH)/(total LDH − background LDH) × 100%.

FACS Analysis. Single-cell suspensions of splenocytes from infected animals or mouse macrophages were treated with Fc block (clone 93, rat anti-CD16/CD32, eBiosciences) for 15 min on ice, then stained with the indicated antibodies: CD40-APC (clone 1C10, eBiosciences) and CD11b –PE (clone M1/70, eBiosciences). Where applicable, dead cells were enumerated using the live/dead blue stain (Invitrogen) or propidium iodide (PI staining solution, BD) according to the manufacturer's instructions. Cells from infected mice were fixed with 2% (vol/vol) formaldehyde before analysis by flow cytometry. Data acquisition was performed with an LSRII instrument (BD) and BD FACSDiva software, and analysis was conducted with FlowJo.

Caspase-8 Activation Assays. To determine the status of caspase-8 enzymatic activity, 1×10^5 cells were plated into each well of a 96-well plate and stimulated with *Y. pestis* at an MOI of 40 for 2 h. Caspase-8 activation in cell lysates was detected using Caspase-Glo 8 Assay (Promega) according to the manufacturer's instructions.

qPCR Analysis of *Il1b* Expression in Primary Mouse Macrophages. RNA from resting or stimulated (with *Y. pestis* or LPS) BMDM was purified with the RNeasy Mini Kit (Qiagen), and cDNA was generated with the iScript cDNA Synthesis Kit (BioRad). PCR was performed as previously reported (4) on transcribed cDNA with primers for detection of mouse *Il1b* (5′-GCCCATCCTCTGTGACTCAT-3′, 5′-AGGCCACAGGTATTTTGTCG-3′) using SYBR green (BioRad) according to the manufacturer's instructions.

Staining and Microscopy. Tissue samples for histology were fixed in 10% (vol/vol) buffered formalin for a minimum of 72 h with three changes of fixative. H&E staining and microscopy were performed as previously described (3, 4).

Electron Microscopy. Wild-type, RIP3$^{-/-}$caspase8$^{-/-}$, or RIP3$^{-/-}$ immortalized mouse macrophages were infected with *Y. pestis* KIM5 for 2.5 h and subjected to electron microscopy analysis by the UMass Medical School Core Electron Microscopy Facility. Cells were fixed for 10 min by adding 1 mL of 2.5% glutaraldehyde (vol/vol) in 0.75 M sodium phosphate buffer (pH 7.2) to each of the medium-containing wells of the culture plate. Fixative was removed and added at full strength overnight at 4 °C. The next day the fixed samples were washed three times in 0.75 M sodium phosphate buffer (pH 7.2).The cells were then scraped off the bottom of the wells with a soft plastic spatula, collected in a microcentrifuge tube, pelleted, briefly rinsed in deionized water, postfixed for 1 h in 1% osmium tetroxide (wt/vol) in deionized water, washed, and dehydrated through a graded series of ethanol concentration increases in 20% increments before two changes in 100% ethanol. Samples were then infiltrated first with two changes of propylene oxide and then a mixture of 50% propylene oxide/50% SPIpon 812/Araldite epoxy resin overnight. Cell pellets were transferred through three changes of fresh SPIpon 812/Araldite epoxy resin, embedded into molds filled with the same resin, and polymerized for 48 h at 70 °C. The epoxy blocks were then trimmed and ultrathin sections were cut on a Reichart-Jung ultramicrotome using a diamond knife. The sections were collected and mounted on copper support grids and contrasted with lead citrate and uranyl acetate. The samples were examined on a FEI Tecnai 12 BT transmission electron microscope using 80 kV accelerating voltage. Images were captured using a Gatan TEM CCD camera.

Statistical Analysis. Statistical analysis was performed with GraphPad Prism 5.0. To evaluate the differences between two groups (cell death and BMDM cytokine release), the two-tailed *t* test was used. When more than two groups were compared, two-way ANOVA with Tukey's posttest was used. Differences between cytokine levels in infected mice were analyzed by the Mann-Whitney *U* test. Group size for animal experiments was calculated by power analysis. Survival analysis was performed using the log–rank test.

1. Kaiser WJ, et al. (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* 471(7338):368–372.
2. Newton K, Sun X, Dixit VM (2004) Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *Mol Cell Biol* 24(4):1464–1469.
3. Montminy SW, et al. (2006) Virulence factors of *Yersinia pestis* are overcome by a strong lipopolysaccharide response. *Nat Immunol* 7(10):1066–1073.
4. Vladimer GI, et al. (2012) The NLRP12 inflammasome recognizes *Yersinia pestis*. *Immunity* 37(1):96–107.
5. Beisner DR, Ch'en IL, Kolla RV, Hoffmann A, Hedrick SM (2005) Cutting edge: Innate immunity conferred by B cells is regulated by caspase-8. *J Immunol* 175(6):3469–3473.
6. Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: Gene splicing by overlap extension. *Gene* 77(1):61–68.
7. Edwards RA, Keller LH, Schifferli DM (1998) Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. *Gene* 207(2):149–157.
8. Hornung V, et al. (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9(8):847–856.

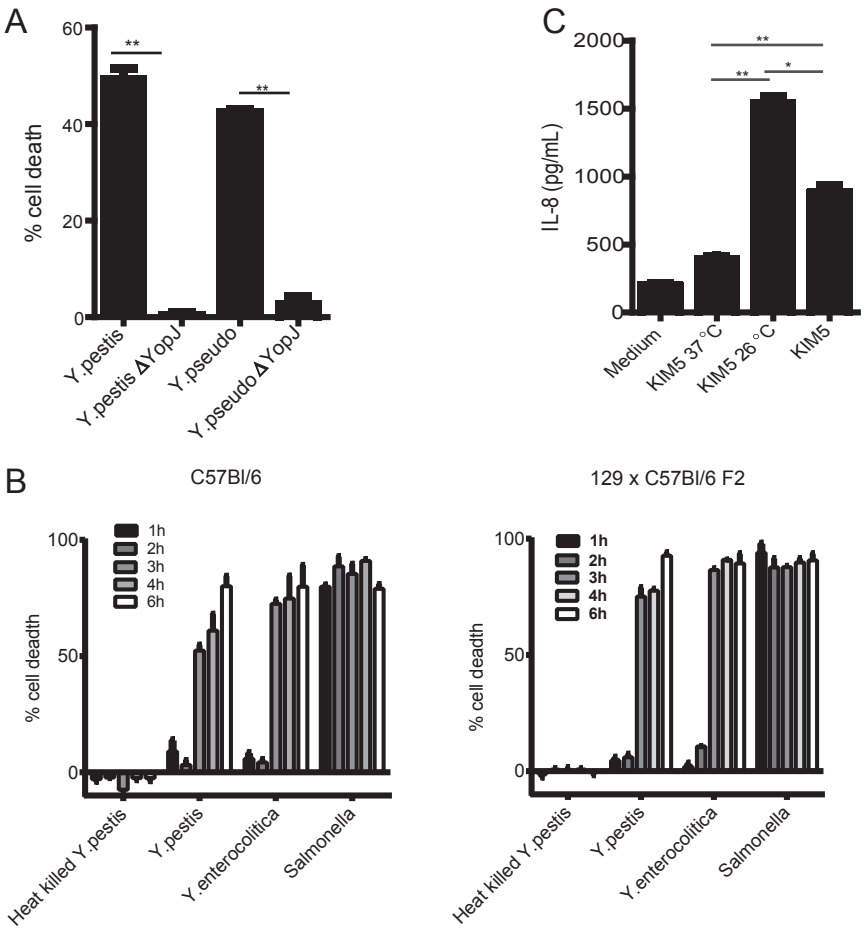

Fig. S1. Live *Yersinia* induces rapid cell death that is dependent on YopJ and *Y. pestis* shows TLR4-stimulating activity when grown at different temperatures. (*A*) BMDMs were stimulated with the indicated *Yersinia* strains (MOI 40) for 4 h, and cell death was determined by LDH assay. BMDMs were killed by *Y. pestis* and *Y. pseudotuberculosis*, but not by bacteria lacking the type III secretion effector YopJ. (*B*) C57BL/6 or 129xC57Bl/6 F2 immortalized macrophages were infected with live *Y. pestis*, *Y. enterocolitica* (MOI 40), *Salmonella* (MOI 1.5), or heat killed (56 °C, 2 h) *Y. pestis* (equivalent to MOI of 40) for the indicated time points. Cell death is comparable between C57BL/6 cells and 129 x C57BL/6 F2 macrophages. (*C*) HEK293 cells that stably express human TLR4/MD2 were stimulated with heat-killed *Y. pestis* KIM5 (MOI 10) for 18 h. KIM5 37 °C bacteria were grown continuously at 37 °C, and KIM5 26 °C were grown continuously at 26 °C before being heat-killed. IL-8 in supernatant was detected by ELISA.

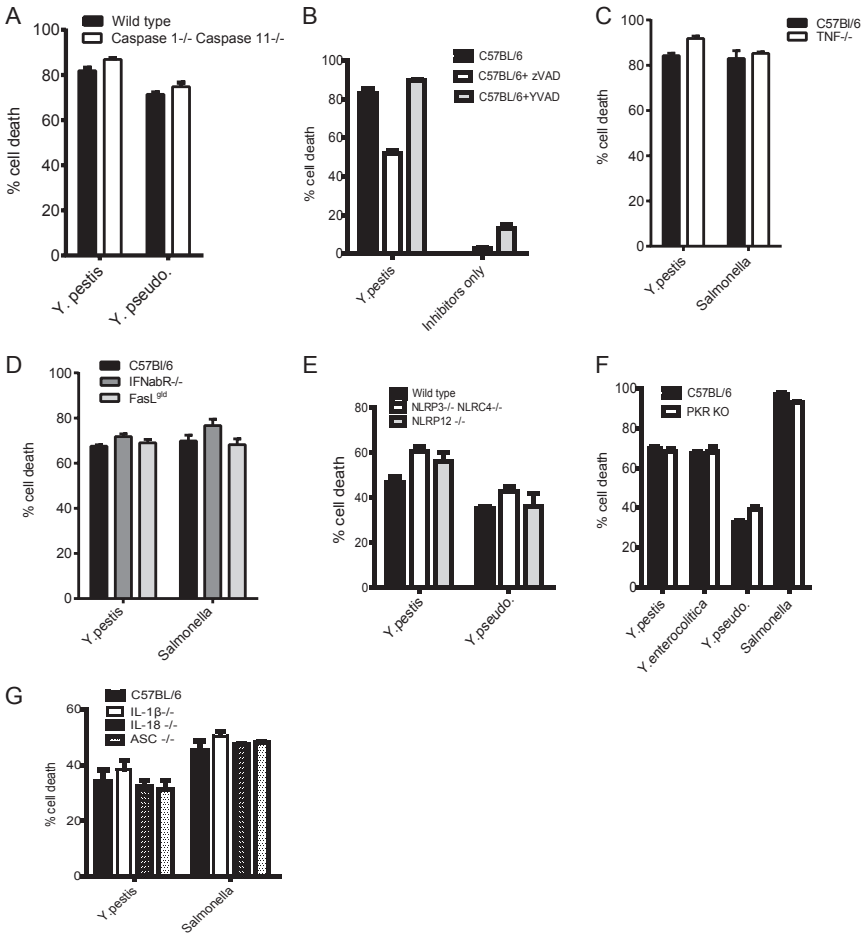

Fig. S2. *Yersinia*-induced macrophage death does not require caspase-1, caspase-11, TNF, IFN-αβR1, FasL, NLRP3, NLRC4, NLRP12, PKR, IL-1β, IL-18, or ASC. (*A*–*G*) BMDMs were infected with *Y. pestis*, *Y. enterocolitica*, *Y. pseudotuberculosis* (MOI 40), or *Salmonella* (MOI 1.5) for 4 h as indicated. In *B*, cells were pretreated for 1 h before infection with 20 μM of the pan-caspase inhibitor zVAD or the caspase-1 inhibitor YVAD. Cell death was determined by measuring LDH release into the supernatant.

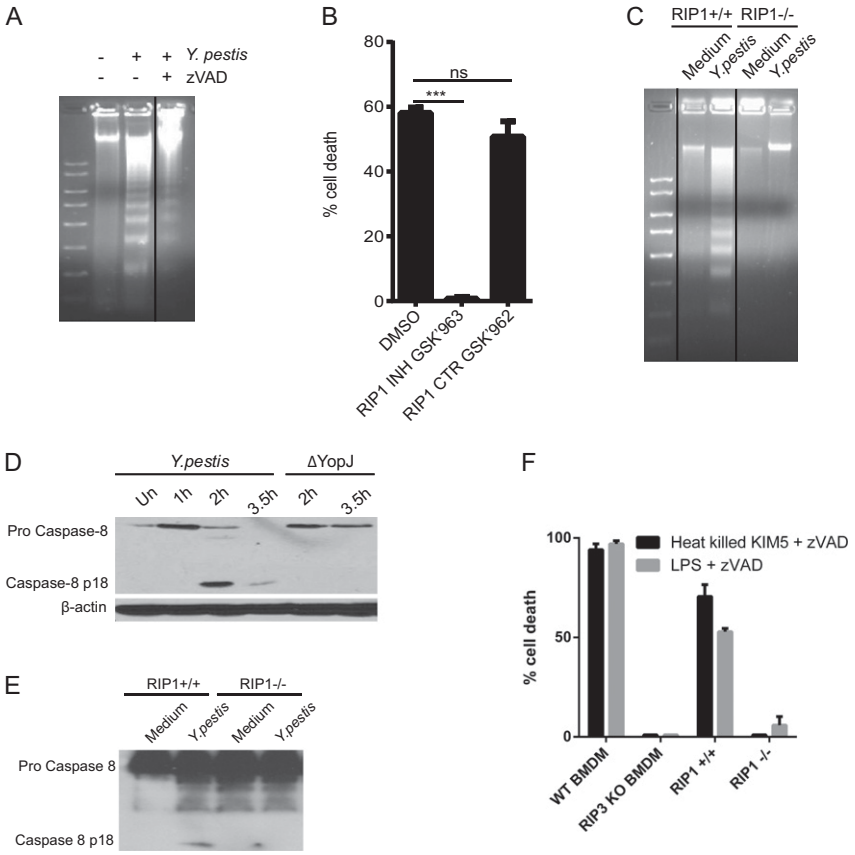

Fig. S3. *Y. pestis* induces macrophage apoptotic death, which is inhibited in RIP1$^{-/-}$ fetal liver macrophage or by RIP1 specific kinase inhibitor GSK'963 other than the inactive enantiomer GSK'962. (*A*) WT immortalized macrophages were treated with zVAD (20 μM) for 1 h before *Y. pestis* infection (MOI 40, 3 h). A Calbiochem Suicide-track DNA ladder isolation kit was used to detect DNA fragmentation according to the manufacturer's instructions. (*B*) WT BMDMs were treated with the RIP1 inhibitor GSK'963 or inactive control compound GSK'962 or vehicle control DMSO for 1 h before *Y. pestis* infection (MOI 40, 4 h), then cell death was determined by LDH assay. (*C*) DNA fragmentation was detected in both RIP1$^{+/+}$ and RIP1$^{-/-}$ fetal liver macrophages after 3 h of *Y. pestis* infection. (*D* and *E*) Cleavage of caspase-8 induced by *Y. pestis* is dependent on YopJ and absent in RIP1-deficient macrophages. (*F*) Necroptosis induced by zVAD (20 μM) plus LPS (50 ng/mL) or zVAD plus heat-killed *Y. pestis* KIM5 (equal to MOI 10) (18 h stimulation) is greatly decreased in RIP3$^{-/-}$ BMDMs or RIP1$^{-/-}$ fetal liver macrophages.

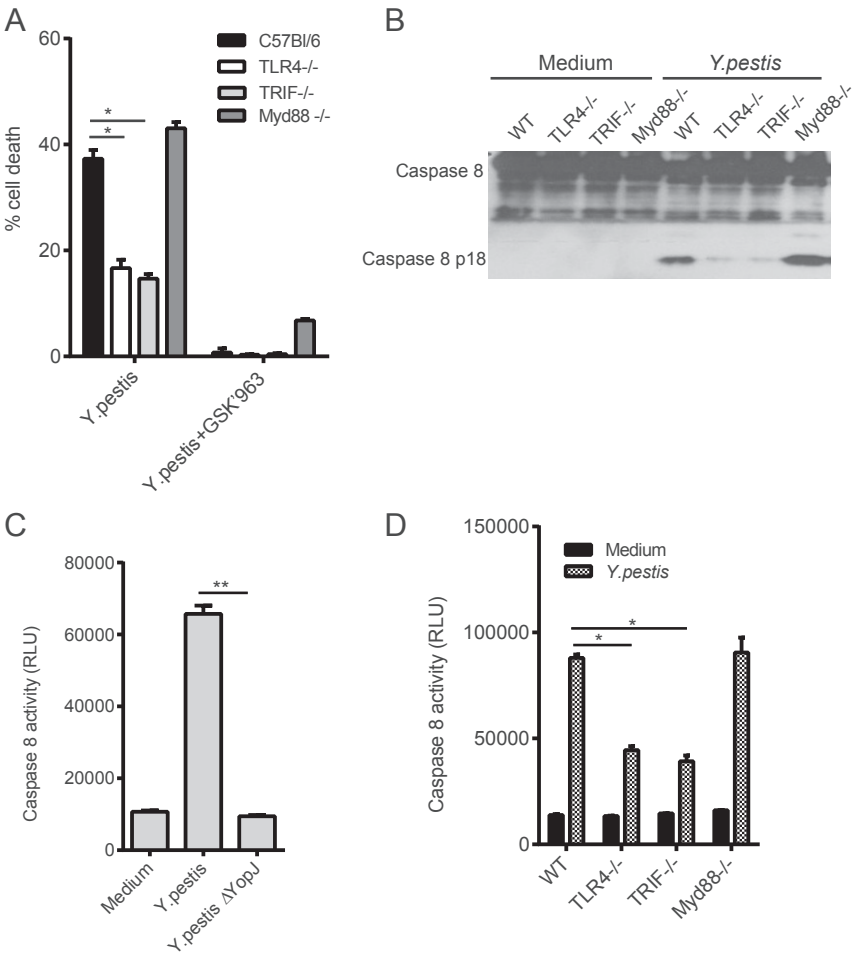

Fig. S4. *Y. pestis*-induced macrophage death, caspase-8 cleavage, and activation partially require TLR4 and TRIF but not Myd88. This has been proposed for other Yersiniae. (*A–D*) BMDMs from WT, TLR4$^{-/-}$, TRIF$^{-/-}$, or Myd88$^{-/-}$mice were infected with *Y. pestis* (MOI 40) or *Y. pestis* ΔYopJ (MOI 40) for 2 (*B–D*) or 4 h (*A*). In *A*, cells were pretreated for 1 h before infection with the RIP1 inhibitor GSK'963. Supernatants were assayed for LDH release to determine cell death. Caspase-8 cleavage was assessed by Western blot (*B*) and caspase-8 activity by Caspase-Glo 8 assay (*C* and *D*). (*A*) *Y. pestis*-induced BMDM death is partially dependent on TLR4 and TRIF, but not Myd88, and is further reduced when RIP1 is inhibited. (*B*) Cleavage of caspase-8 is impaired in TLR4- and TRIF-deficient BMDMs but not in Myd88$^{-/-}$ BMDMs. (*C*) Caspase-8 activity is dependent upon YopJ. (*D*) Caspase-8 activity induced by *Y. pestis* is impaired in TLR4- and TRIF-deficient BMDMs but not in Myd88$^{-/-}$ BMDMs.

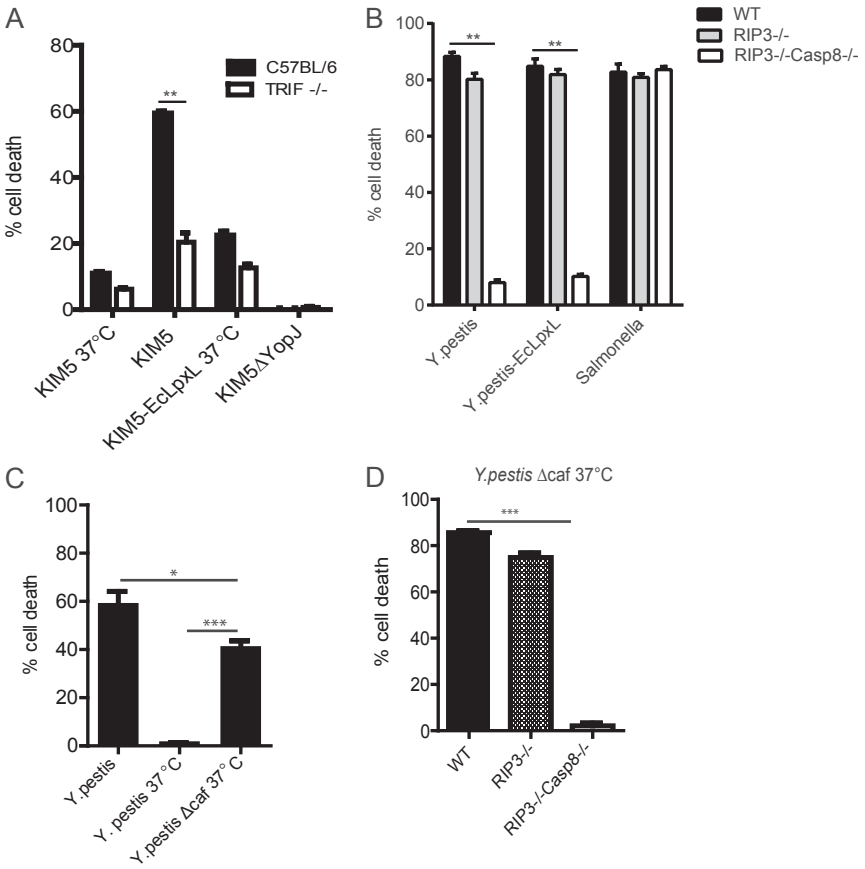

Fig. S5. *Y. pestis*-expressing *E. coli* LpxL (*Y.pestis*-EcLpxL strain), which produces a potent LPS, also induces RIP3/caspase-8–dependent cell death as WT *Y. pestis*. A capsule-deficient strain of *Y. pestis* triggers RIP3/caspase-8–dependent death when bacteria are grown at 37 °C. (*A* and *B*) BMDMs were infected with *Y. pestis* KIM5 or KIM5-EcLpxL grown at 37 °C (*A*) or grown as our normal temperature shift rationale (*B*) for 4 h. Then LDH release was detected. (*C* and *D*) Immortalized mouse macrophages were infected with WT *Y. pestis* or *Y. pestis* Δcaf1 (F1 antigen, protein capsule) grown at 37 °C (MOI 40). (*C*) Caf1 deficiency enables *Y. pestis* grown at 37 °C to induce cytotoxicity, in contrast to WT bacteria. (*D*) RIP3$^{-/-}$caspase-8$^{-/-}$ immortalized macrophages are resistant to *Y. pestis* Δcaf1-induced cytotoxicity. Cell death was measured by LDH release into the supernatant.

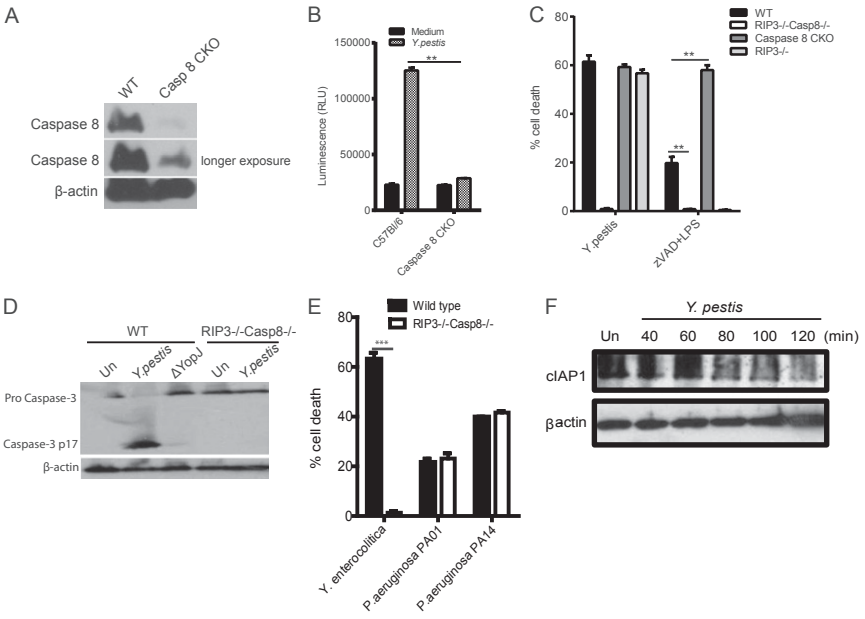

Fig. S6. Caspase-8 expression is strongly reduced in caspase-8 cKO BMDMs, and caspase-8 activation by *Y. pestis* is at background levels in caspase-8 cKO macrophages. (*A*) Western blot of BMDMs from the caspase-8 cKO mouse show a significant reduction in caspase-8. (*B*) BMDMs from the conditional caspase-8 knockout mouse infected with *Y. pestis* (MOI 40) for 2 h show reduced caspase-8 activity compared with WT cells. (*C*) BMDMs were treated with *Y. pestis* (MOI 8, 9 h) or zVAD (20 μM) plus LPS (100 ng/mL) for 10 h. zVAD plus LPS can induce necroptosis, which is inhibited when RIP3 is deficient. However, in caspase-8 cKO macrophages, the necroptosis induced by zVAD and LPS is significantly increased compared with WT cells. (*D*) WT or RIP3$^{-/-}$caspase-8$^{-/-}$ immortalized mouse macrophages were infected with WT bacteria or*Y. pestis* ΔYopJ for 2.5 h, and caspase-3 was determined in cell lysates by Western blot. Cleavage and activation of caspase-3 induced by *Y. pestis* is dependent upon YopJ and absent in caspase-8 RIP3 dKO macrophages. (*E*) WT or RIP3$^{-/-}$caspase-8$^{-/-}$ immortalized mouse macrophages were infected with (*E*) *Y. enterocolitica* (MOI 20) or *Pseudomonas aeruginosa* (MOI 40) for 4 h. Cell death was determined by measuring LDH in the supernatant. Strain PA01 expresses ExoS, ExoT, and ExoY but not ExoU. PA14 expresses ExoT, ExoY, and ExoU but not ExoS. (*F*) WT immortalized macrophages were infected with *Y. pestis* for the indicated time points, and gentamycin was added at time points beyond 1 h. Western blot was used to detect the *Y. pestis*-dependent degradation of cIAP1 during infection.

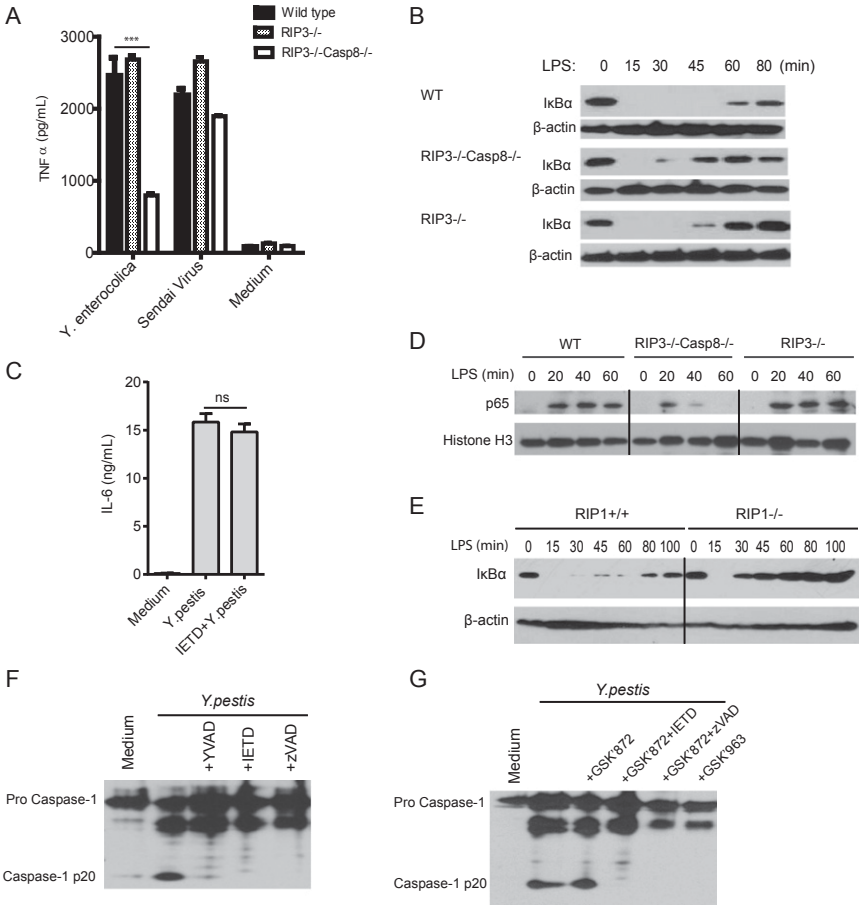

Fig. S7. Sendai virus-induced TNF-α production is not significantly decreased in RIP3$^{-/-}$caspase-8$^{-/-}$ macrophages, whereas LPS-induced late NF-κB activation is attenuated in RIP3$^{-/-}$caspase-8$^{-/-}$ macrophages. *Y. pestis*-induced caspase-1 processing is reduced by caspase-1 inhibitor YVAD, caspase-8 inhibitor IETD, pan-caspase inhibitor zVAD, and RIP1 inhibitor GSK'963. (*A*) Immortalized macrophages were infected with *Y. enterocolitica* (MOI 10) or Sendai virus (200 pfu) for 20 h. TNF-α production was detected by ELISA. (*B*) Immortalized macrophages were stimulated with LPS (100 ng/mL) for the indicated time points. The IκBα degradation in cell lysates was detected. In RIP3$^{-/-}$caspase-8$^{-/-}$ macrophages, the IκBα degratdation was affected at 30 min and 45 min, but not early 15-min points, compared with WT macrophages. (*C*) Caspase-8 enzymatic inhibitor IETD (40 μM) does not affect *Y. pestis*-induced IL-6 production. (*D*) Immortalized macrophages were stimulated with LPS (100 ng/mL) for the indicated time points. Nuclear extracts were probed by immunoblot for p65 and histone H3. (*E*) Fetal liver macrophages were treated with LPS (100 ng/mL) for the indicated time points. The IκBα degradation in cell lysates was detected. (*F* and *G*) WT BMDMs were pretreated with inhibitors (YVAD, 20 μM; IETD, 40 μM; zVAD, 20 μM; GSK'963, 1 μM; GSK'872, 10 μM) for 1 h before *Y. pestis* infection. The supernatants were harvested and assayed for caspase-1 processing.

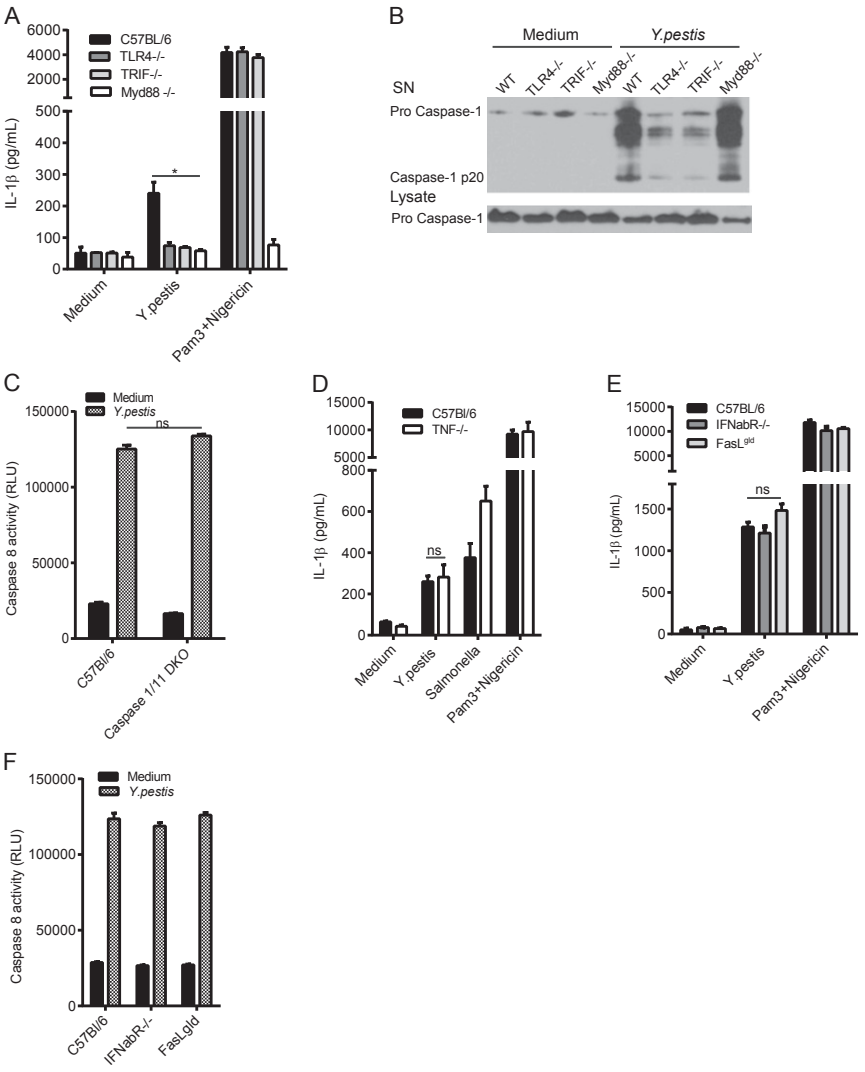

Fig. S8. *Y. pestis*-induced caspase-1 cleavage and IL-1β release are reduced in TLR4- and TRIF-deficient BMDMs, but not in Myd88$^{-/-}$, TNF$^{-/-}$, IFNαβR$^{-/-}$, and FasL^{gld} BMDMs. (*A*, *D*, and *E*) BMDMs from TLR4$^{-/-}$, TRIF$^{-/-}$, Myd88$^{-/-}$, TNF$^{-/-}$, IFNαβR$^{-/-}$, and FasL^{gld} mice were infected with *Y. pestis* (MOI 10) for 6 h, then the IL-1β in supernatant was detected via ELISA. (*B*) BMDMs were infected with *Y. pestis* (MOI 10) for 6 h. Then the supernatants (SN) or lysates were analyzed for caspase-1 processing by immunoblots. (*C* and *F*) Caspase-8 activity induced by *Y. pestis* is not dependent on caspase-1/11 (*C*), IFNαβR1 and FasL (*F*). Caspase-8 activation was detected in C57BL/6, caspase-1/11 dKO, IFNαβR1$^{-/-}$, and FasL^{gld} BMDMs, which were infected with 40 MOI *Y. pestis* for 2 h, using Caspase-Glo 8 assay.

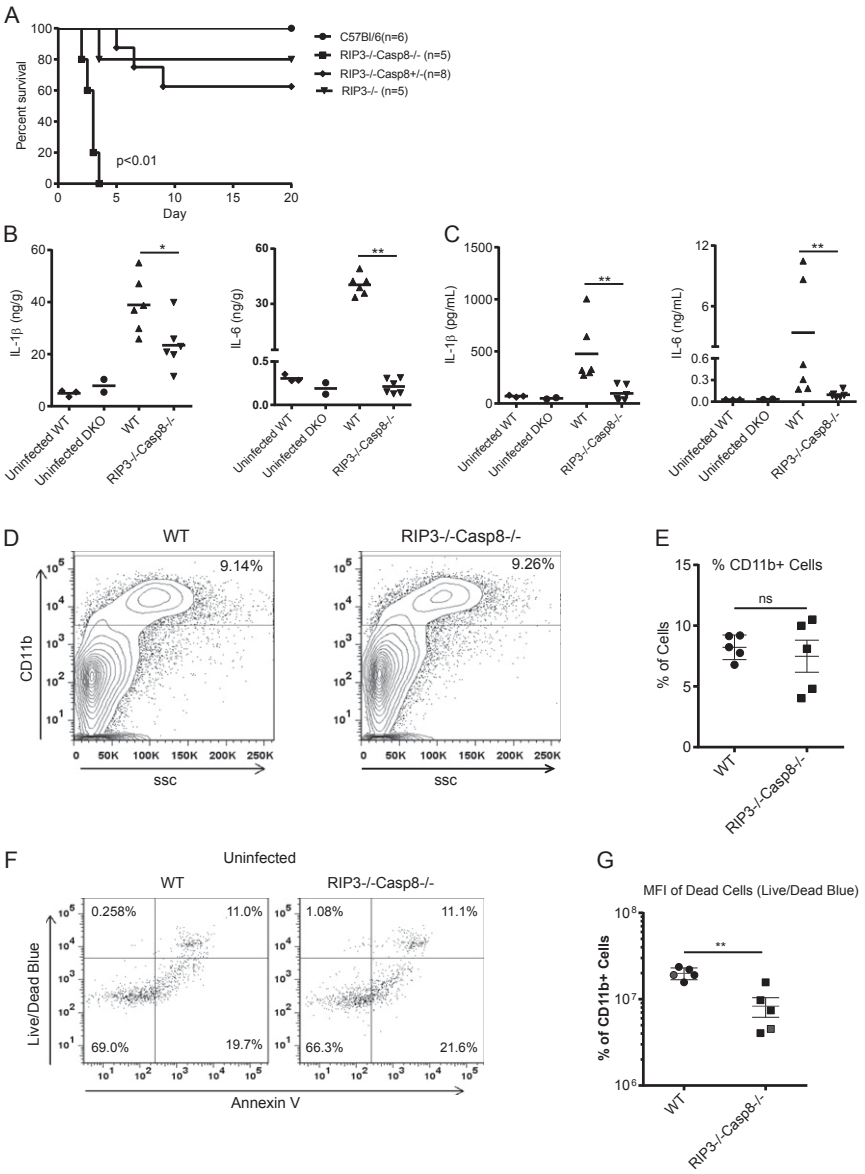

Fig. S9. RIP3 and caspase-8 are critical for survival and cytokine responses in mice infected with an attenuated strain of *Y. pestis*, KIM1001-EcLpxL. EcLpxL-expressing *Y. pestis* is an attenuated strain of KIM1001 that has TLR4-activating properties and uses the same RIP3/caspase-8–dependent cell death pathway (Fig. S5*B*), making this a good strain for survival studies. (*A*) C57BL/6, RIP3$^{-/-}$, RIP3$^{-/-}$caspase-8$^{+/-}$ or RIP3$^{-/-}$caspase-8$^{-/-}$ mice were infected s.c. with 500 cfu of KIM1001-EcLpxL and monitored for survival. RIP3$^{-/-}$caspase-8$^{-/-}$ mice were significantly more sensitive to infection, and all died before day 5. (*B* and *C*) WT or RIP3$^{-/-}$caspase-8$^{-/-}$ mice were uninfected or infected i.v. with 500 cfu of KIM1001-EcLpxL, and spleen homogenates (*B*) and serum (*C*) were collected for IL-1β and IL-6 measurement by ELISA. In the spleen (*B*) cytokine levels are displayed as ng cytokine per g of tissue. IL-1β and IL-6 were significantly reduced in RIP3$^{-/-}$caspase-8$^{-/-}$ mice in both the spleen and serum compared with WT animals. (*D*–*G*) Lethally irradiated mice, transplanted with bone marrow cells (BMT) from WT or RIP3$^{-/-}$caspase-8$^{-/-}$ mice were infected i.v. with 750 cfu of *Y. pestis* KIM1001-EcLpxL for 42 h. Single-cell suspensions were made from spleens and stained with CD11b-PE to identify myeloid cells and with live/dead blue and annexin V-FITC to determine cell viability. (*D*) Single cells were gated for CD11b expression, and there were no significant differences in the percentage of CD11b+ cells in WT or RIP3$^{-/-}$caspase-8$^{-/-}$ mice (*E*). (*F*) Of CD11b^{+} cells, there was no difference in the frequency of dead cells (live/dead blue^{+}, annexin V^{+}) in the uninfected WT and RIP3$^{-/-}$caspase-8$^{-/-}$ mice. (*G*) The mean fluorescent intensity (MFI) live/dead blue stain in CD11b^{+} cells was significantly higher in WT compared with RIP3$^{-/-}$caspase-8$^{-/-}$ mice. Asterisks indicate significant differences ($P < 0.01$).