SUPPLEMENTAL FIGURE 1

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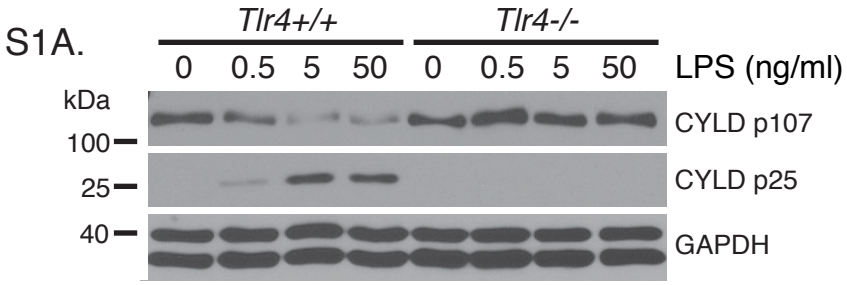

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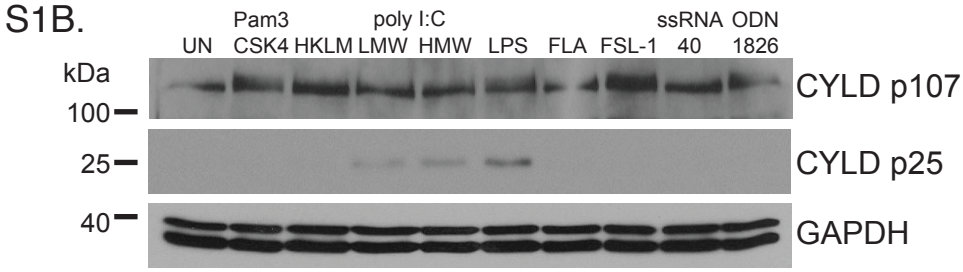

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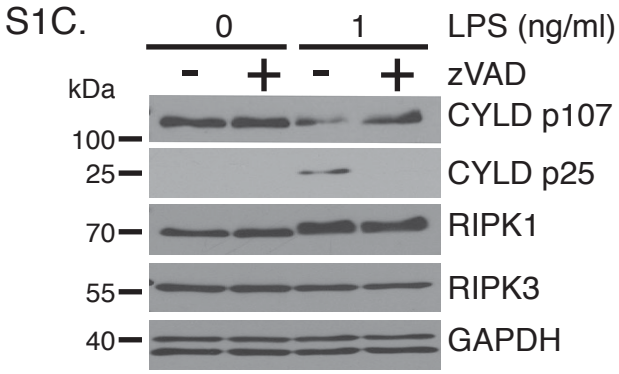

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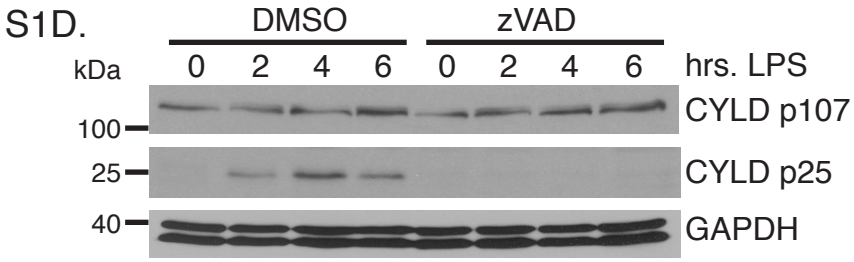

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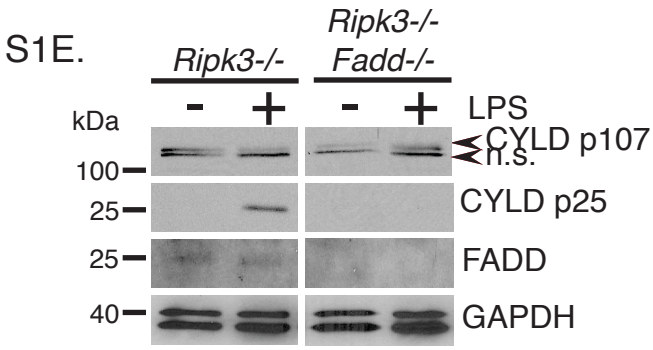
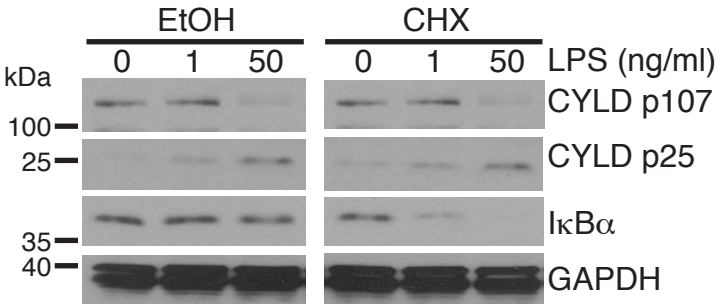


SUPPLEMENTAL FIGURE 1 (Related to Figure 1)

Analysis of CYLD cleavage. (S1A) *Tlr4*$^{+/+}$ and *Tlr4*$^{-/-}$ BMDMs were treated with the indicated concentrations of LPS for 4 hours. (S1B) Wild type BMDMs were stimulated with TLR agonists Pam3CSK4, heat-killed *Lysteria monocytogenes* (HKLM), poly I:C low molecular weight (LMW), poly I:C high molecular weight (HMW), LPS, flagellin (FLA), synthetic diacylated lipoprotein (FSL-1), ssRNA 40, and ODN1826 for 4 hours. (S1C) Wild type BMDMs were pre-treated with 25 uM zVAD-fmk prior to stimulation with LPS for 4 hours. (S1D) Human monocytic THP-1 cells were pre-treated with 25 uM zVAD-fmk prior to stimulation with 100 ng/ml of LPS for the indicated times. (S1E) BMDMs from *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$ *Fadd*$^{-/-}$ mice were stimulated with LPS (100 ng/ml) for 6 hours. (S1A-S1E) Cell lysates were subject to SDS-PAGE and western blot analysis using antibodies for CYLD, RIPK1, RIPK3, FADD, and GAPDH. All experiments are representative of at least three experiments with similar results that were conducted.

SUPPLEMENTAL FIGURE 2

S2A.



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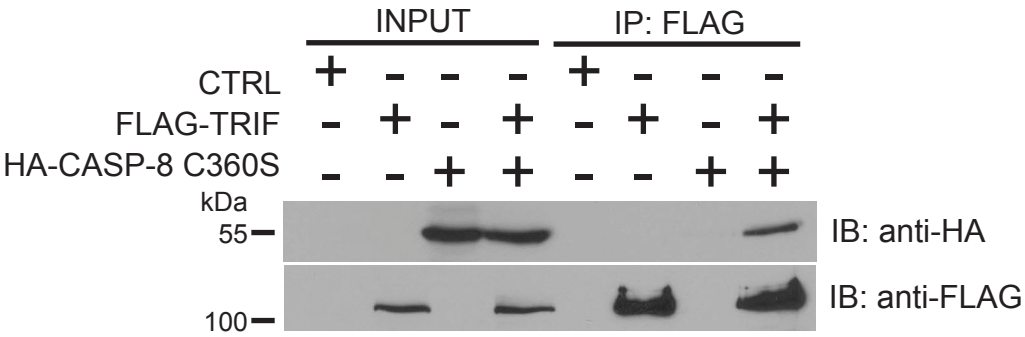

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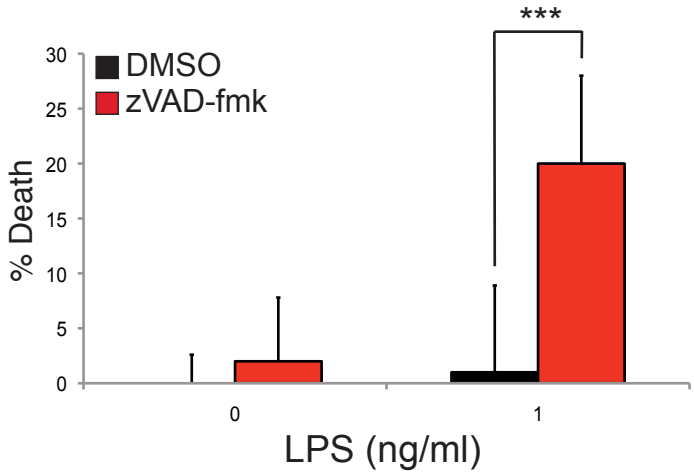
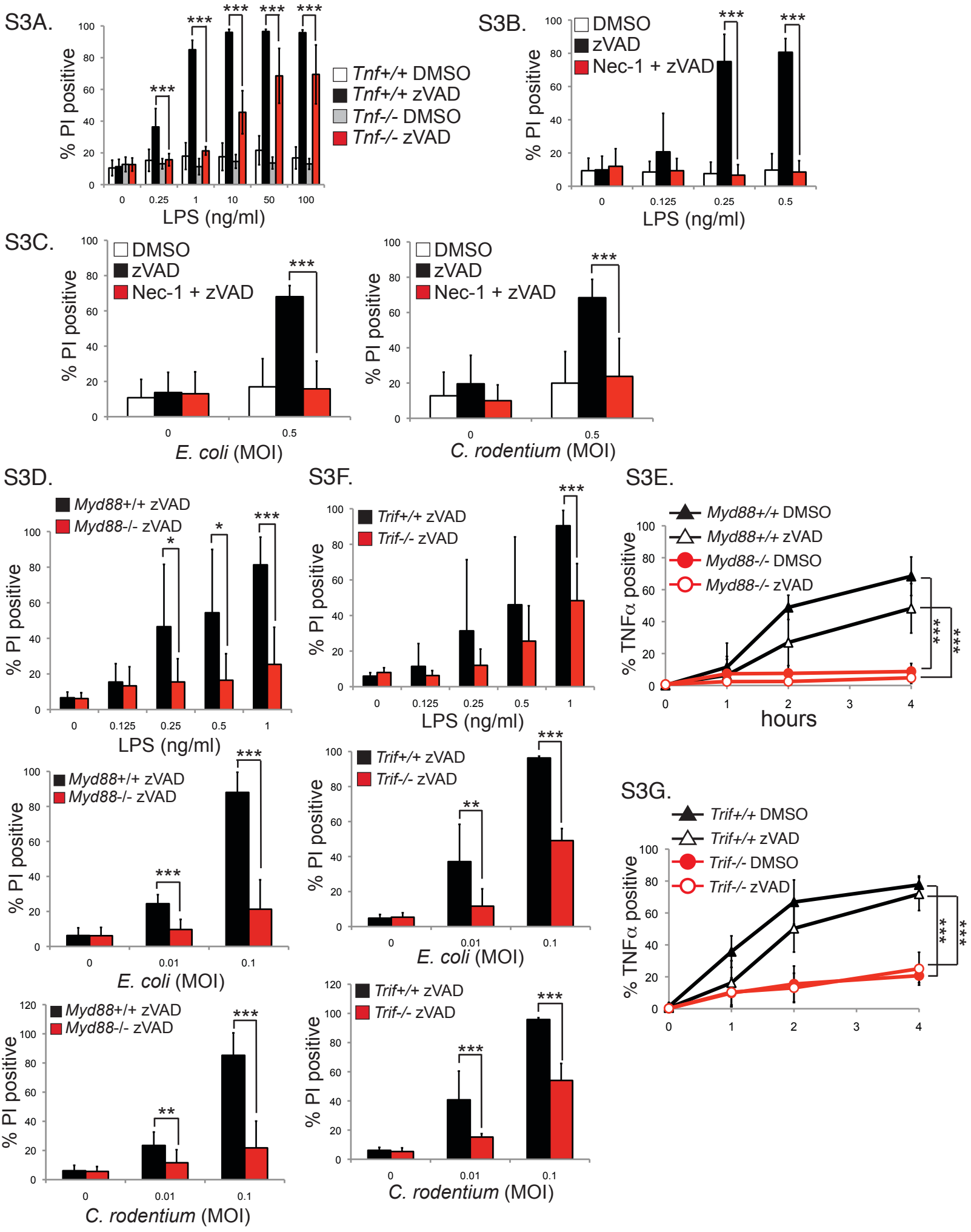


SUPPLEMENTAL FIGURE 2 (Related to Figure 2)

CYLD cleavage occurs independent of cytokine production. (S2A) Wild type BMDMs were pre-treated with either ethanol (EtOH) or 1 ug/ml of cycloheximide (CHX) prior to stimulation with the indicated concentrations of LPS for 6 hours. Cell lysates were subject to SDS-PAGE and western blot analysis using antibodies for CYLD (D1A10), IκBα, and GAPDH. (S2B) HEK 293 EBNA cells were co-transfected with FLAG-TRIF, HA-CASPASE-8 C360S, or a control expression plasmid, as indicated. Fourty-eight hours after transfection, FLAG-TRIF was immunoprecipitated from total cell lysate using anti-FLAG M2 affinity beads. The 2% of the total cell lysate and the immunoprecipitate were subject to SDS-PAGE and western blot analysis using anti-HA and anti-FLAG antibodies. The experiments shown are representative of at least three experiments that were conducted independently, with similar results. (S2C) THP-1 cells were pre-treated with 25uM of zVAD-fmk followed by stimulation with the indicated concentrations of LPS for 16 hours. Cell death was determined by measuring ATP levels. Data is presented as the mean of three independent experiments, each performed in triplicate, ± standard deviation. Statistics were performed using T-test. *** $p<0.001$.

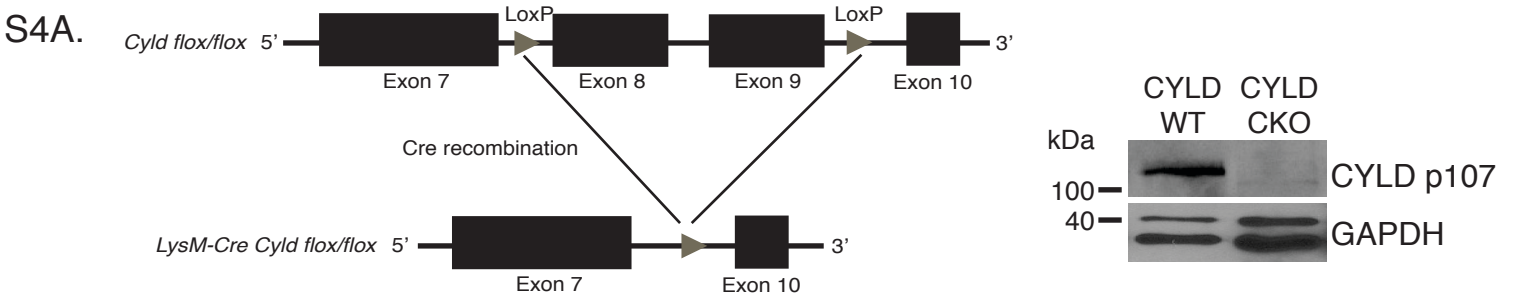
SUPPLEMENTAL FIGURE 3

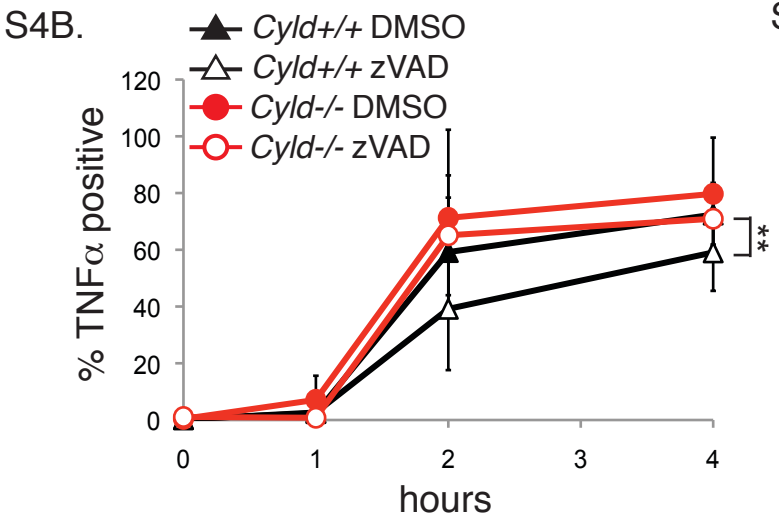


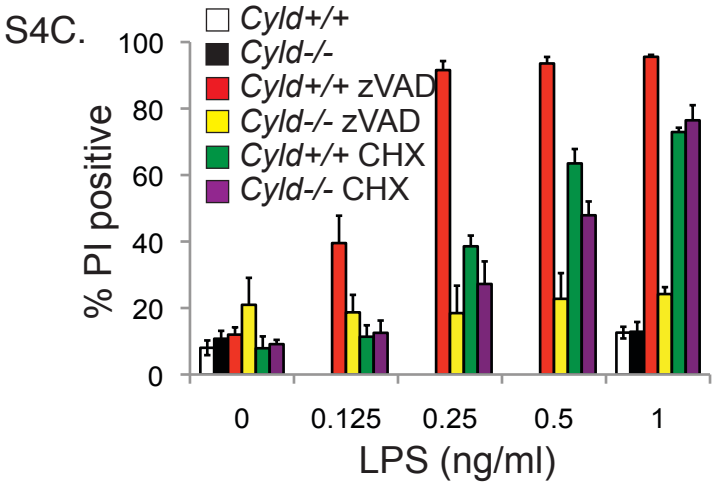
SUPPLEMENTAL FIGURE 3 (Related to Figure 3)

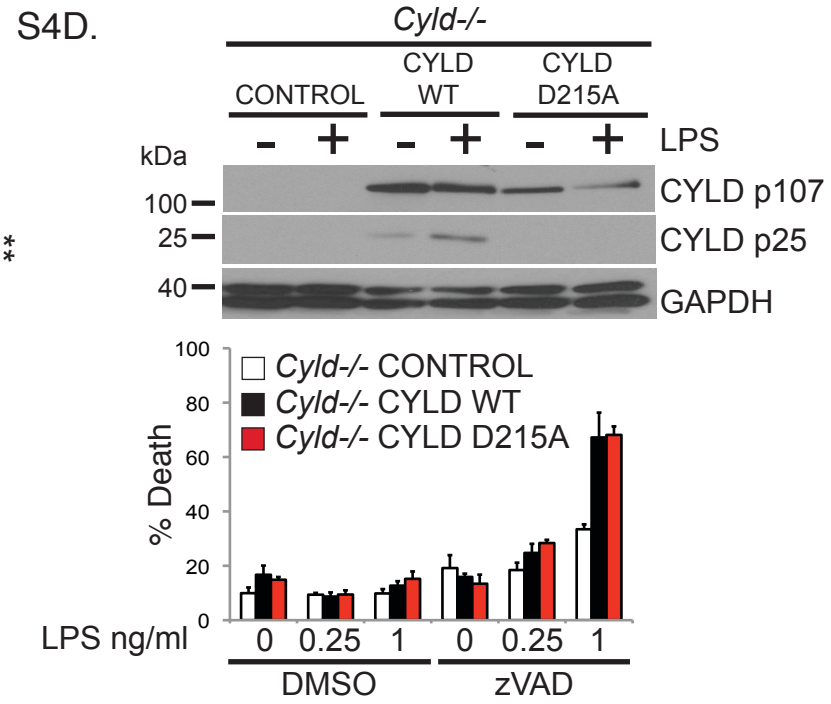
Examining the role of TNF and the TLR signaling adaptor proteins MyD88 and TRIF in TLR4-induced necroptosis. (S3A) BMDMs from *Tnf*$^{+/+}$ or *Tnf*$^{-/-}$ mice were pre-treated with 25 μM zVAD-fmk or DMSO (vehicle control) prior to stimulation with the indicated concentrations of LPS for 16 hours. (S3B-C) Wild type BMDMs were pre-treated DMSO, zVAD-fmk, or Necrostatin-1 (Nec-1), as indicated, prior to stimulation with the indicated concentrations of LPS (S3B) or with viable *E. coli* or *C. rodentium* at the indicated MOI (S3C) for 16 hours. Cell death was measured by staining with propidium iodide and flow cytometry analysis. (S3D, F) BMDMs from *MyD88*$^{+/+}$, *MyD88*$^{-/-}$, *Trif*$^{+/+}$ *or Trif*$^{-/-}$ mice were pre-treated with zVAD-fmk prior to stimulation with LPS or with viable *E. coli* or *C. rodentium* at the indicated MOI for 16 hours. Cell death was measured by staining with propidium iodide and flow cytometry analysis. (S3E, G) BMDMs from *MyD88*$^{+/+}$, *MyD88*$^{-/-}$, *Trif*$^{+/+}$ *or Trif*$^{-/-}$ mice were pre-treated with either DMSO or zVAD-fmk prior to stimulation with 10 ng/ml of LPS for the indicated times. Cells were then permeabilized, stained for intracellular TNF, and analyzed by flow cytometry. All experiments shown are representative of at least three experiments performed independently, with similar results. Data is presented as the mean of three independent experiments performed in triplicate, ± standard deviation. Statistics were performed using T-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

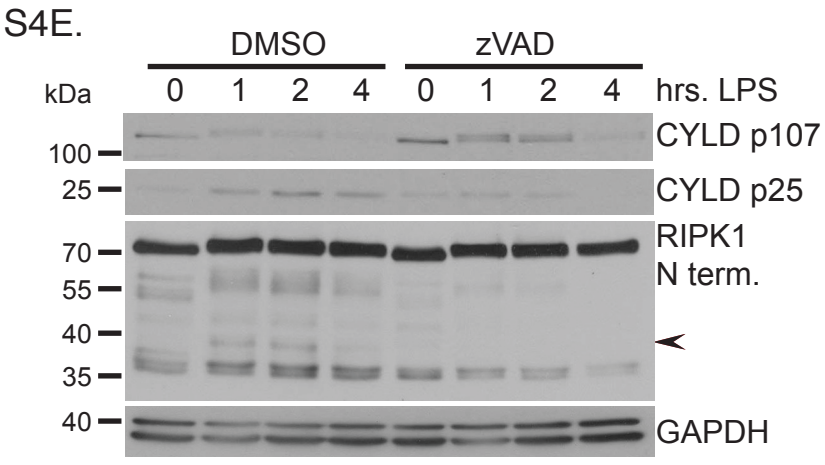
SUPPLEMENTAL FIGURE 4









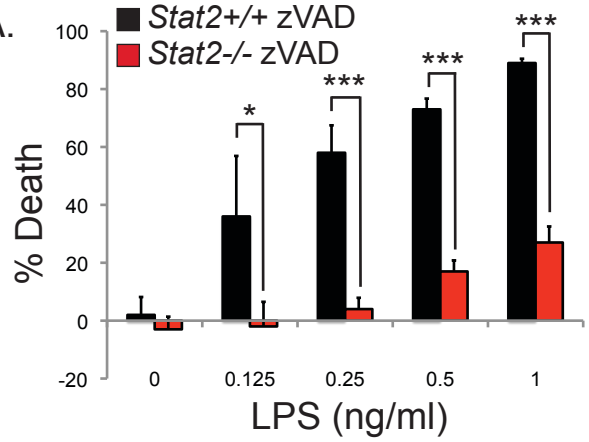


SUPPLEMENTAL FIGURE 4 (Related to Figure 4)

Further analysis of CYLD elimination. (S4A) A loxP/FRT flanked Neo cassette was inserted on the 3' side of exon 9 of CYLD, and a single loxP site was inserted at the 5' side of exon 8, so that the target region includes exon 8 and 9. Cre-mediated recombination would result in the deletion of CYLD exons 8 and 9. To confirm deletion of CYLD expression in macrophages, BMDMs from age and gender-matched *Cyld*$^{flox/flox}$ and LysM-Cre x *Cyld*$^{flox/flox}$ conditional knockout (CKO) mice were isolated, and triton soluble lysates were subject to SDS-PAGE and western blot analysis using CYLD (D1A10) and GAPDH antibodies. (S4B) BMDMs from *Cyld*$^{+/+}$ and *Cyld*$^{-/-}$ mice were pre-treated with either DMSO or zVAD-fmk prior to stimulation with 10 ng/ml of LPS for the indicated times. Cells were then permeabilized, stained for intracellular TNF, and analyzed by flow cytometry. Data is presented as the mean of three independent experiments performed in triplicate, ± standard deviation. Statistics were performed using T-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (S4C) *Cyld*$^{+/+}$ and *Cyld*$^{-/-}$ BMDMs were either left untreated or treated with zVAD-fmk or cycloheximide prior to stimulation with the indicated concentrations of LPS for 16 hours. Cell death was measured by staining with propidium iodide and flow cytometry analysis. The data shown is presented as the mean of an experiment performed in triplicate, ± standard deviation. This is representative of three independent experiments. (S4D) CYLD-deficient BMDMs were reconstituted with either an irrelevant control protein, wild type CYLD (CYLD WT), or the cleavage-resistant CYLD (CYLD D215A). These cells were stimulated with 100 ng/ml of LPS for 6 hours, and triton-soluble lysates were analyzed by SDS-PAGE and western blot analysis to confirm CYLD expression. Concurrently, cells were treated with either DMSO or zVAD-fmk prior to stimulation with the indicated concentrations of LPS for 16 hours. Cell death was determined by measuring ATP levels. The data shown is presented as the mean of an experiment performed in triplicate, ± standard deviation. This is representative of three independent experiments. (S4E) Wild type BMDMs were treated with either DMSO or zVAD-fmk prior to stimulation with 100 ng/ml of LPS for the indicated times. Triton soluble lysates were subject to SDS-PAGE and western blot analysis using antibodies for CYLD, RIPK1 N-terminus, and GAPDH. The experiment shown is representative of at least three experiments with similar results.

SUPPLEMENTAL FIGURE 5

S5A.



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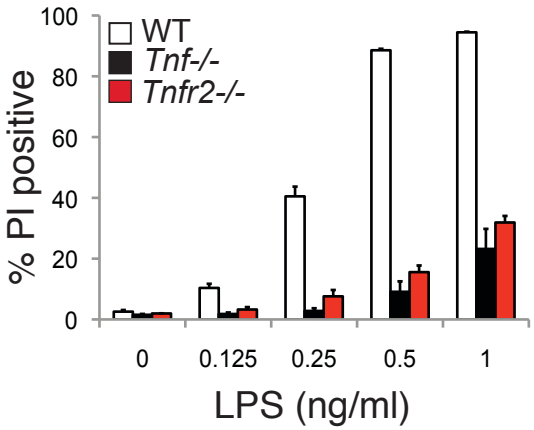

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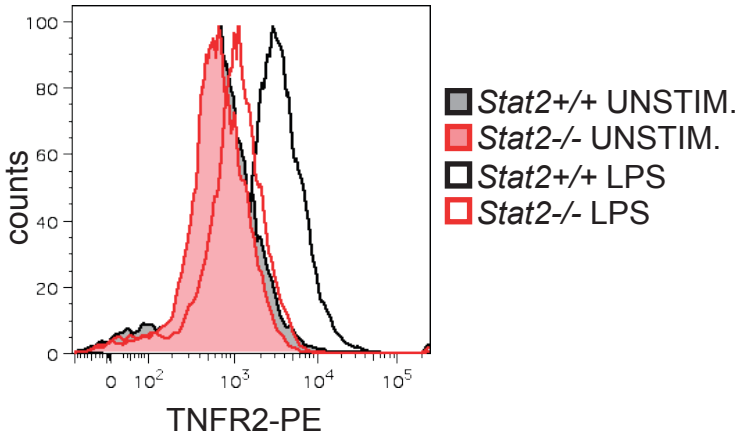

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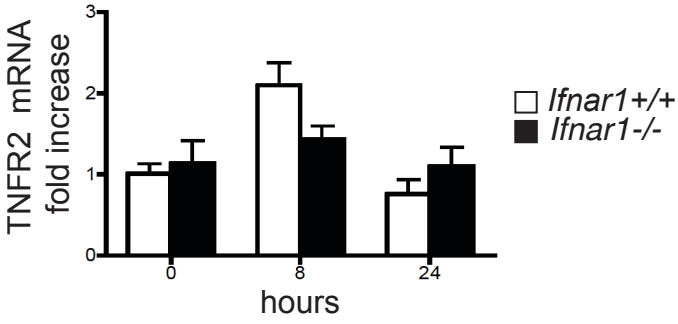
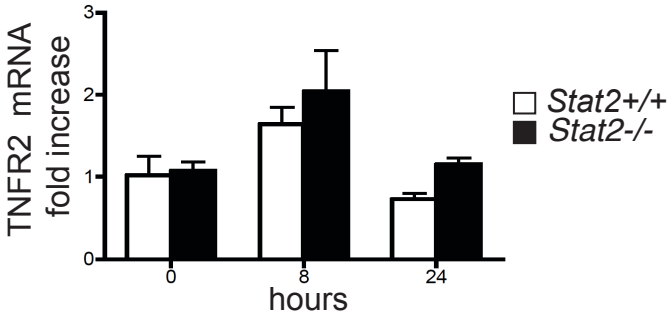


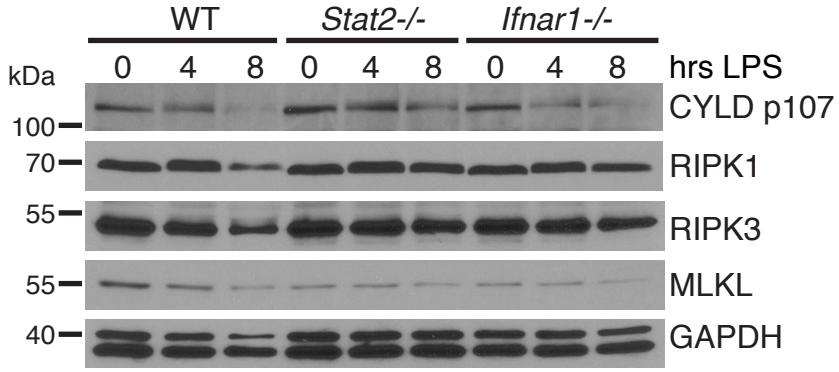


SUPPLEMENTAL FIGURE 5 (Related to Figure 5 and Figure 6)

STAT2 regulates TNFR2 expression and TLR4- induced necroptosis. (S5A) BMDMs from *Stat2*$^{+/+}$ and *Stat2*$^{-/-}$ mice were pre-treated with zVAD-fmk prior to stimulation with the indicated concentrations of LPS for 16 hours. Cell death was determined by measuring ATP levels. Data is presented as the mean of three independent experiments performed in triplicate, ± standard deviation. Statistics were performed using T-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (S5B) *Stat2*$^{+/+}$ and *Stat2*$^{-/-}$ BMDMs were stimulated with 1 ng/ml of LPS for 24 hours. Cells were then stained with a PE conjugated antibody against TNFR2 for flow cytometry analysis. Data presented is representative of at least three independent experiments with similar results. (S5C) BMDMs from *Ifnar1*$^{-/-}$, *Stat2*$^{-/-}$ and wild type mice (n=3) were treated with 10 ng/ml of LPS for the indicated times. *Tnfr2* expression from total mRNA was examined by quantitative real-time PCR and expression was normalized to its respective ubiquitin levels. The data is presented as the mean, ± standard deviation. (S5D) WT, *Tnf*$^{-/-}$, and *Tnfr2*$^{-/-}$ BMDMs were pre-treated with zVAD-fmk prior to stimulation with the indicated concentrations of LPS for 16 hours. Cell death was determined by propidium iodide uptake and flow cytometry analysis. The data shown is presented as the mean of an experiment performed in triplicate, ± standard deviation. This is representative of three independent experiments with similar results.

SUPPLEMENTAL FIGURE 6

S6A.



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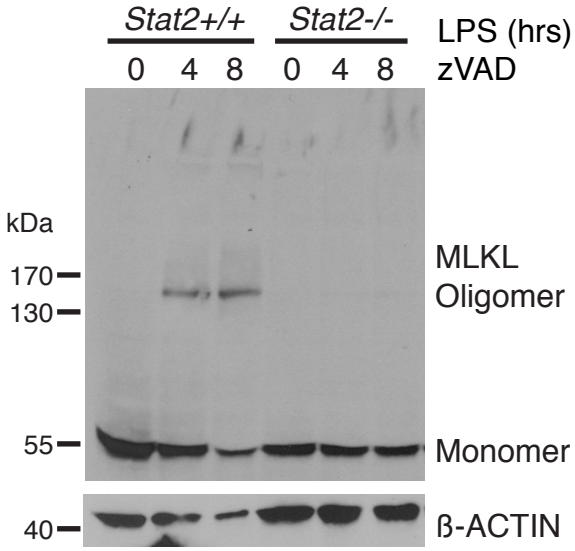

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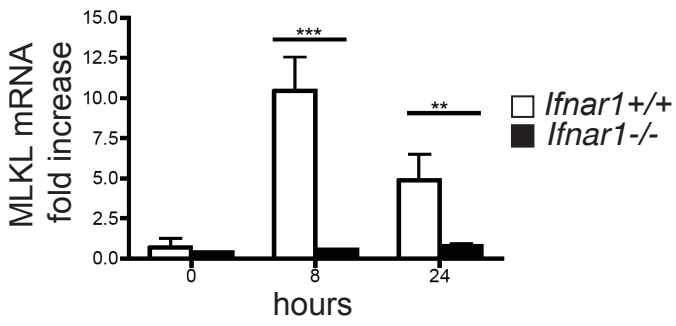

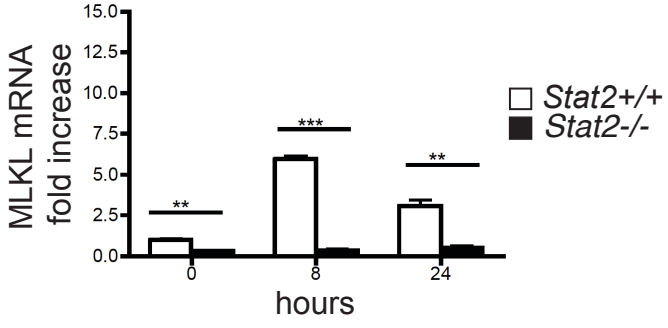

SUPPLEMENTAL FIGURE 6 (Related to Figure 6)

MLKL expression and oligomerization is impaired in *Stat2*$^{-/-}$ BMDMs. (S6A) Wild type, *Stat2*$^{-/-}$, and *Ifnar1*$^{-/-}$ BMDMs were stimulated with 10 ng/ml of LPS for the indicated times. Triton-soluble lysates were then subject to SDS-PAGE and western blot analysis using antibodies for CYLD, RIPK1, RIPK3, MLKL, and GAPDH. (S6B) *Stat2*$^{+/+}$ and *Stat2*$^{-/-}$ BMDMs were pre-treated with 25 μM zVAD-fmk prior to stimulation with 10 ng/ml of LPS for the indicated periods of time. Cells were lysed in 2% SDS, subjected to SDS-PAGE under non-reducing conditions, and examined by western blot analysis using anti-MLKL and β-actin antibodies. The experiments shown are representative of three independent experiments with similar results. (S6C) BMDMs from *Ifnar1*$^{-/-}$, *Stat2*$^{-/-}$ and control mice (n=3) were treated with 10 ng/ml of LPS for the indicated times. *Mlkl* expression from total mRNA was examined by quantitative real-time PCR and expression was normalized to its respective ubiquitin levels. The data is presented as the mean, ± standard deviation. Statistics were performed using Two-Way ANOVA. Bonferroni posts-tests were performed to compare the triplicate means at different time points. ** $p<0.01$, *** $p<0.001$.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reagents and cells: LPS *E.coli* 0111:B4 was purchased from Sigma. Mouse TLR agonists were purchased from Invivogen. TNFR1-Fc was purchased from R&D. IFNAR1 neutralizing antibody was obtained from EMD Millipore. The Adherent and invasive *E. coli* (AIEC) reference strain LF82 that was isolated originlly from a chronic ileal lesion of a patient with Crohn's disease by Drs. Arlette Darfeuille-Michaud and Nicolas Barnich was provided by Dr. Emiko Mizoguchi (Boudeau J et al., 1999). Mouse and human recombinant TNF were purchased from Peprotech. zVAD-fmk was purchased from Calbiochem and Bachem Americas. Necrostatin-1 was purchased from Tocris Bioscience. Cycloheximide and 4-hydroxytamoxifen were purchased from Sigma. THP-1 cells were obtained from ATCC. HEK 293 EBNA cells were obtained from Invitrogen. Antibodies used in this study were purchased from Santa Cruz Biotechnology (GAPDH, CYLD clone E4, goat IgG HRP), Cell Signaling Technology (MYD88, CYLD clone D1A10, RIPK1, mouse IgG HRP, β-ACTIN), Jackson ImmunoResearch (rabbit IgG HRP, rabbit IgG (light-chain specific) HRP), Invitrogen (CYLD clone 733), BD Transduction Laboratories (RIPK1), ProSci (RIPK3), R&D Systems (CASPASE-8), Imgenex (IκBα), Biolegend (TNFR2-PE and isotype control), EMD Millipore (MLKL), Roche (HA), Sigma (FLAG), and eBioscience (mouse TNFα-PE and isotype control).

Mice: C57BL/6 (WT), *Tnf*$^{-/-}$, *Tnfr2*$^{-/-}$, and *Myd88*$^{-/-}$ were obtained from The Jackson Laboratory (Bar Harbor, ME). *Trif* $^{-/-}$ mice were a generous gift from Dr. Shizuo Akira (Osaka University, Japan). *Cyld*$^{-/-}$ mice were generously provided by Dr. Shao-Cong Sun (The University of Texas MD Anderson Cancer Center, Houston, TX). *Stat2-/-* mice were provided by Adolfo Garcia-Sastre (Icahn School of Medicine at Mount Sinai, NY, NY). Femurs and tibia from *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$ *Casp8*$^{-/-}$ mice were generously provided by Dr. Douglas Green (St. Jude Children's Research Hospital, Memphis, TN). All experiments involving the use of mice were performed in agreement with approved protocols by the Institutional Animal Care and Use Committee (IACUC) at the Icahn School of Medicine at Mount Sinai.

Generation of Bone Marrow-Derived Macrophages (BMDMs): BMDMs were obtained by flushing bone marrow cells from mouse femurs and tibias with sterile PBS and culturing in DMEM or RPMI containing 10% FBS and 30% L929 conditioned medium for 7-10 days in non-treated cell culture plates. Following differentiation, the expression of F4/80 and CD11b was determined by flow cytometry to confirm purity (>90%).

Generation of immortalized *RosaCreER*^{T2}*Ripk1*$^{fl/fl}$ BMDMs: BMDMs from *RosaCreER*^{T2}*Ripk1*$^{fl/fl}$ mice (Roderick et al., 2014) were isolated as described above. After 3-4 days of culturing in DMEM containing 10% FBS and 20% L929 conditioned medium, the cells were immortalized by infecting with the J2 recombinant retrovirus (Roberson and Walker, 1988). The amount of L929 supernatant in culture medium was gradually reduced until the macrophages

were growing in the absence of conditioned medium. To stably delete RIPK1, the immortalized line was treated twice with 4-OHT (200nM) for 24hrs, one week apart. Procedures involving the use of *RosaCreER*^{T2}*Ripk1*$^{fl/fl}$ mice were approved by The University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC).

Expression Constructs: cDNA for mouse CYLD was obtained by PCR and its identity was confirmed by DNA sequencing. D215A point mutation was introduced by mutagenesis using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), and verified by DNA sequencing. CYLD wild-type and D215A mutant were FLAG-tagged at their N-termini and subcloned into the Moloney-murine-leukemia-virus-derived retroviral vector, pEAK12mmp. Mouse TNFR2 cDNA and mouse MLKL cDNA were obtained from Origene (catalog number MC216483 for TNFR2, MC206757 for MLKL) and subcloned into pEAK12mmp retroviral vector. pCDNA-HA-CASPASE-8 C360S was generated as described previously (O'Donnell et al., 2011). pCAGGS-FLAG-TRIF expression construct was a generous gift from Dr. Christopher F. Basler (Icahn School of Medicine at Mount Sinai, New York, NY).

Retrovirus pseudotyping and transduction of BMDMs: HEK 293 EBNA cells were transfected by calcium phosphate precipitation with plasmids encoding VSV-G and GAG-POL, together with retroviral expression construct encoding either irrelevant control or the indicated protein. Forty-eight hours after transfection, the viral supernatant was collected and used to infect bone marrow progenitors by spinoculation. Infected progenitors were then differentiated to BMDMs as described above.

Bacteria and treatment of macrophages with viable bacteria: AIEC strain LF82 was grown in LB liquid, standing at 37°C (Darfeuille-Michaud et al., 1998). *Citrobacter rodentium* strain DBS100 was grown in LB liquid at 37°C with aeration. Both bacteria were grown from a single colony to mid-log phase and CFUs were determined by plating *E. coli* onto LB agar supplemented with 80 ug ml^{-1} of ampicillin and *C. rodentium* onto LB agar, and incubating at 37°C overnight. BMDMs were re-plated in antibiotic-free media containing 10% FBS and 30% L929 conditioned media 16 hours before the experiment. Mid-log cultures of bacteria were washed with PBS and added to BMDMs cultures at the indicated multiplicity of infection (MOI). One hour after the addition of the bacteria, the culture media was replaced with 'complete media' containing penicillin, streptomycin, and gentamicin for the indicated time. When required, DMSO, zVAD-fmk, or Nec-1 was added at this time.

Protein lysis and western blot analysis: Cells were lysed in buffer containing 20 mM Tris pH 7.5, 40 mM sodium chloride, 5 mM EDTA, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 1% Triton X-100, and Protease Inhibitor Cocktail Set V (EMD Biosciences Inc.) for 10 min on ice. Lysates were clarified by centrifugation at 10 000 × *g* at 4°C. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with the indicated antibodies.

To visualize MLKL oligomerization, cells were lysed in either 2% SDS or in M2 buffer containing 50 mM Tris pH 7.4, 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 0.5 mM EDTA, 0.5 mM EGTA, and 50 mM sodium fluoride. Lysates were resolved by SDS-PAGE under non-reducing conditions (without beta-mercaptoethanol),

Cell death and cell viability assay: BMDM cell death was determined by propidium iodide (PI) staining or by cell viability assays. Briefly, 300,000 to 500,000 cells were seeded in 24-well suspension plates. Cells were then pretreated with zVAD-fmk for 30 minutes before stimulation. Cells were harvested, washed with PBS, and stained with propidium iodide. PI staining was determined using an Accuri C6 Flow Cytometer or BD LSRFortessa cell analyzer. Cell viability of BMDMs were determined using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega). THP-1 cells were pre-treated with 100 ng/ml human IFNγ prior to stimulation with LPS in the presence of zVAD-fmk. Assays were performed in triplicate and according to manufacturer's instructions. A POLARstar Omega plate reader was used to record luminescence.

Cell staining: For intracellular staining for cytokine expression, cells were treated as indicated in the presence of brefeldin A (eBioscience). After harvesting and washing, cells were fixed and permeabilized (eBioscience) at 4°C. Cells were then pre-treated with Fc block (eBioscience) at 4°C prior to staining for the indicated cytokine. For cell surface staining, cells were treated as indicated, pre-treated with Fc-block for 30 minutes at 4°C, and subsequently stained with the indicated surface marker for 30 minutes at 4°C. Staining was determined using an Accuri C6 Flow Cytometer or BD LSRFortessa cell analyzer.

Co-expression and Co-immunoprecipitation: HEK 293 EBNA cells were transfected by calcium phosphate precipitation with expression plasmids encoding FLAG-TRIF and HA-CASPASE-8 C360S by calcium phosphate precipitation. Forty-eight hours after transfection, cells were lysed in buffer containing 20 mM Tris pH 7.5, 40 mM sodium chloride, 5 mM EDTA, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 1% Triton X-100, and Protease Inhibitor Cocktail Set V (EMD Biosciences Inc.) for 10 min on ice. Lysates were clarified by centrifugation at 10,000 × *g* at 4°C. FLAG immunoprecipitation was performed by incubating lysate with anti-FLAG M2 agarose affinity beads (Sigma). Protein was eluted from the beads by competition with 300 mg/ml of FLAG peptide. Lysates were resolved by SDS-PAGE and analyzed by western blot.

Real-time quantitative PCR: Total mRNA was isolated using RNease Mini kit (Qiagen). cDNA was generated using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time PCR was performed on a LightCycler Real-Time PCR System (Roche), using 25 ng of cDNA and iQ SYBR Green Supermix (Bio-Rad). The primers used are as follows: TNFR2: forward, 5'-TGGCTATTAATTATTCGGTCTGCA-3' and reverse, 5'-

GCAAGTGGCTAGAGTGCAGAGTAA-3′; MLKL: forward, 5′-AATTGTACTCTGGGAAATTGCCA-3′ and reverse, 5′-TCTCCAAGATTCCGTCCACAG-3′; Ubiquitin: forward, 5′-TGGCTATTAATTATTCGGTCTGCA-3′ and reverse, 5′-GCAAGTGGCTAGAGTGCAGAGTAA-3′.