# Supporting Information

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### SI Materials and Methods

**Mice.** *Rip3*$^{-/-}$ (1), *Rip1*$^{+/-}$ (2), *Casp8*$^{-/-}$ (3), *Tnf*$^{-/-}$ (4), and *Casp8*$^{-/-}$*Rip3*$^{-/-}$ (5) mice have been described previously. WT (C57BL/6J) mice were from The Jackson Laboratory. *Rip1*$^{+/-}$ mice and *Casp8*$^{+/-}$ were crossed to generate *Rip1*$^{+/-}$*Casp8*$^{+/-}$ mice. *Rip1*$^{+/-}$*Casp8*$^{+/-}$ mice were intercrossed to generate *Rip1*$^{-/-}$*Casp8*$^{-/-}$ mice. *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice were bred with *Rip1*$^{+/-}$ mice to generate *Rip1*$^{+/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice; *Casp8*$^{+/-}$*Rip3*$^{-/-}$ were bred with *Rip1*$^{+/-}$ mice to generate *Rip1*$^{+/-}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$. *Rip1*$^{+/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ and *Rip1*$^{+/-}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$ mice were intercrossed to generate *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ (triple knockout, TKO). *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice crossed with WT gave *Rip1*$^{+/-}$*Casp8*$^{+/-}$*Rip3*$^{+/-}$ and were used to generate *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{+/-}$ mice. RIP1 kinase-dead (KD) knockin (KI) (*Rip1*$^{KD/KD}$) mice were generated on a C57BL/6 background by homologous recombination using a targeting construct that mutated the catalytic lysine residue to alanine (K45A) to eliminate all kinase activity (performed at Genoway). *Rip1*$^{KD/KD}$ mice were viable, born at the expected Mendelian ratios, and showed no gross or histological abnormalities (6). PCR genotyping primers for *Casp8*$^{-/-}$ mice were 5′-TTGAGAACAAGACCTGGGGACTG and 5′-GGATGTCCAGGAAAAGATTTGTGTC. In mice containing wild-type Casp8, PCR amplification produces a 750-bp band. Casp8$^{\Delta 3-4}$ allele produces a 200-bp band. Genotyping of *Rip3*$^{-/-}$ mice was performed with the primers 5′-CGCTTTAGAAGCCTTCAGGTTGAC, 5′-GCAGGCTCTGGTGACAAGATTCATGG, and 5′-CCAGAGGCCACTTGTGTAGCG. PCR produces a 700-bp band (wild-type *Rip3* allele) or a 450-bp band (*Rip3* mutant). *Rip1*$^{-/-}$ mice were genotyped using the primers 5′-CTGCTAAAGCGCATGCTC, 5′-TGTGTCAAGTCTCCCTGCAG, and 5′-CACGGTCCTTTTGCCCTG. PCR amplification produces a 500-bp band (wild-type *Rip1*) and a 400-bp band (*Rip1 mutant*). *Rip1*$^{KD/KD}$ mice were genotyped using the primers 5′-CTCTGATTGCTTTATAGGACACAGC and 5′-GTCTTCAGTGATGTCTTCCTCGTA. PCR amplification produces a 575-bp band (knockin) and a 473-bp band (WT). Mice were bred and maintained by Emory University Division of Animal Resources where all procedures were approved by the Emory University Institutional Animal Care and Use Committee.

**Cell Culture, Plasmids, and Transductions.** Mouse embryonic fibroblasts (MEFs) and National Institutes of Health (NIH) 3T3 cells were maintained in DMEM containing 4.5 g/mL glucose, 10% (vol/vol) FBS (Atlanta Biologicals), 2 mM L-glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin (Invitrogen). For bone-marrow–derived macrophage (BMDM) culture, pooled bone marrow cells from flushed tibias and femurs of indicated animals were differentiated for 5–7 d in DMEM containing 20% serum and 20% filtered L929 medium as a source of macrophage colony-stimulating factor (M-CSF). Cells were harvested with PBS containing 0.5 mM EDTA, seeded at a density of $5 \times 10^3$ cells per well in a 96-well plate, and then cultured 18 h in DMEM containing 10% FBS before stimulation. Inhibition of RIP3 and MLKL expression in *Rip1*$^{-/-}$*Casp8*$^{-/-}$ and in simian virus 40 (SV40)-immortalized *Rip1*$^{-/-}$ fibroblasts was achieved by either Dharmacon SMARTpool RIP3 siRNA (L043044-00-0005) or SMARTpool MLKL siRNA (L061420-00-0005) in comparison with OnTarget Plus Non-Targeting Pool (D-001810-10) siRNA control. *Rip1*$^{-/-}$*Casp8*$^{-/-}$ or SV40 immortalized *Rip1*$^{-/-}$ cells were seeded on 60-mm tissue culture plates and plates were transfected with 166 pmol of siRNA using Lipofectamine RNAimax (Invitrogen) according to the manufacturer's protocol. For dsRNA transfections, *Ripk1*$^{-/-}$ MEFs ($8 \times 10^4$/well) seeded into 6-well plates were transfected with poly(I:C) (2 μg/mL) using Lipofectamine 2000 (Invitrogen) (6 μg/mL). Poly (I:C) and Lipofectamine 2000 were diluted separately in 250 μL of Opti-MEM I medium (Invitrogen) without serum and mixed gently. After 5 min of incubation, diluted solutions were mixed and incubated for an additional 25 min to allow complex formation. A total of 500 μL of complexes was added to each well and mixed gently by rocking the plate back and forth.

**Immunoblots.** Immunoblotting (IB) and preparation of protein extracts were performed as previously described (7). The following antibodies were used in IB analyses: mouse anti–β-actin (clone AC-74; Sigma), mouse anti-RIP1 (clone 38; BD Biosciences), rabbit anti-RIP3 (Imgenex), rabbit anti-mouse cleaved-caspase 8 (Cell Signaling Technology), rabbit anti-mouse cleaved caspase 3 (Cell Signaling Technology), rabbit anti-mouse MLKL (Abgent), anti-mouse IgG-HRP (Vector Laboratories), and anti-rabbit IgG-HRP (Vector Laboratories).

**Cell Viability Assays.** For viability MEFs (5,000 cells per well) or BMDMs (30,000 cells per well) were cultured in 96-well plates for 18 h and then stimulated as indicated in the text. IL-1β, IL-6, and TNF were purchased from Peprotech. IFNβ was from Millipore or PBL Assay Science. LPS and heat-killed Salmonella typhimurium (HKST) were from Sigma and InvivoGen, respectively. Necrostatin-1 (Nec-1) and the pan-caspase inhibitor zVAD-fmk were from Calbiochem and SM Biochemicals, respectively. GSK'872 and GSK'843 (8) were from GlaxoSmithKline (GSK), BV6 was from Genentech, and polyI:C was from GE Healthcare or InvivoGen. Cell viability was determined indirectly by measuring the intracellular levels of ATP using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's instructions and was graphed relative to control cultures. Luminescence was measured on a Synergy HT Multi-Detection Microplate Reader (BioTek). To determine the quantity of cell impermeable green nucleic acid staining dye Sytox Green (Invitrogen), cells were plated in 24-well ($10^5$ cells per well) or 48-well ($5 \times 10^4$ cells per well) plates. Plates were placed in an IncuCyte machine (Essen Biosystems) and the nuclear uptake of Sytox Green dye was detected over time. Graphs were constructed based on the number of Sytox Green positive nuclei per square millimeter in each well.

**Caspase3/7 Activation Assay.** Cells were seeded onto 96-well plates and stimulated as indicated in the text. Effector caspase activity was determined by measuring the DEVDase proteolytic activity in cell lysates using the Caspase 3/7 Activity Assay kit (Promega) according to the manufacturer's instructions and graphed relative to the basal activity in untreated wells. Luminescence was measured on a Synergy HT Multi-Detection Microplate Reader (BioTek).

**Flow Cytometry.** Single-cell suspensions were prepared from spleens and lymph nodes by mechanical disruption. Red blood cells were lysed using ammonium chloride solution (0.15 M $NH_4Cl$, 10 mM $NaHCO_3$, and 1.0 mM $Na_2$ EDTA in $H_2O$, pH 7.4). Cells were resuspended in FACS staining buffer (PBS containing 0.2% BSA and 0.09% sodium azide), filtered through 40-μm screens, and viable cells were enumerated using trypan blue exclusion. Cells ($10^6$) were incubated with 10% normal rat serum (Pel-Freez) and anti-mouse CD16/CD32 (2.4G2; BD Biosciences) to reduce nonspecific antibody interactions before incubating with lineage-specific antibodies. For evaluation of anti-murine cytomegalovirus

(MCMV) T cell cytokine production, cells were incubated at 37 °C for 5 h with $10^{-9}$ M M45 (HGIRNASFI) peptide (JPT Technologies) in the presence of GolgiStop (BD Biosciences) before staining.

Antibodies used were as follows: CD8-FITC, B220-PECy7, IFNγ-FITC, TNF-PECy7, B220-FITC, Ly6C-FITC, CD8 PerCP-Cy5.5, NK1.1-PE-Cy7, CD11b-APC-Cy7, CD3-Pacifc Blue, CD19-FITC, FAS-PE, B220-PerCp, and CD138-PE were purchased from BD Biosciences; CD3-PECy7, B220-APC, CD3-PE-Cy7, and GL7-eFluor 660 from eBioscience; CD8-APC and CD4-PE purchased from BioLegend; and CD45-PE-TexasRed from Invitrogen. Data were acquired using an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software.

**MCMV Infections.** Mice were inoculated by i.p. injection with $10^6$ pfu tissue culture-derived K181-BAC strain of MCMV (9). Organs harvested from mice at 7 d postinfection were homogenized and virus titer was determined by standard plaque assay on NIH 3T3 cells. Statistical differences between groups were calculated by one-way ANOVA with Bonferroni's multiple comparison posttest using GraphPad Prism 5 software.

**ELISA for Serum Anti-dsDNA Ab.** Immulon 1B plates (Thermo Scientific) were UV irradiated overnight and subsequently coated with 0.2 μg/well of calf thymus DNA (Sigma) for 1 h at room temperature. Plates were washed with PBS and blocked with PBS-1% BSA and washed again. Threefold serum dilutions starting at 1/100 were prepared in PBS-0.5% BSA-0.05% Tween and added to plates. After a 4-h incubation at room temperature, the plates were washed extensively with PBS and bound Abs were detected by overnight incubation with alkaline-phosphatase conjugated goat anti-mouse IgG antibodies (Southern Biotech) diluted in PBS-1% BSA. Plates were washed extensively with PBS and developed with 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) dissolved in diethanolamine buffer. The absorbance was read at 405 nm using BioTek Synergy HT microplate reader and the associated Gen5 data analysis software.


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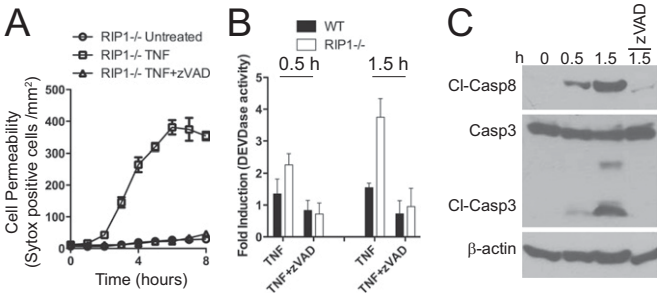



**Fig. S1.** (*A*) Time course (IncuCyte) cell viability analysis of immortalized *Rip1*$^{-/-}$ cells treated with in the presence or absence of zVAD-fmk and measured by appearance of cells that become permeable to the nucleic-acid detecting stain Sytox Green (50 nM). (*B*) DEVDase assay to detect Casp3/Casp7 activation in SV40 immortalized WT and *Rip1*$^{-/-}$ cells treated with TNF in presence or absence of zVAD-fmk for the indicated times. (*C*) Immunoblot detection of Casp8 and Casp3 processing in immortalized *Rip1*$^{-/-}$ cells treated with TNF in the presence or absence of zVAD-fmk for indicated times.

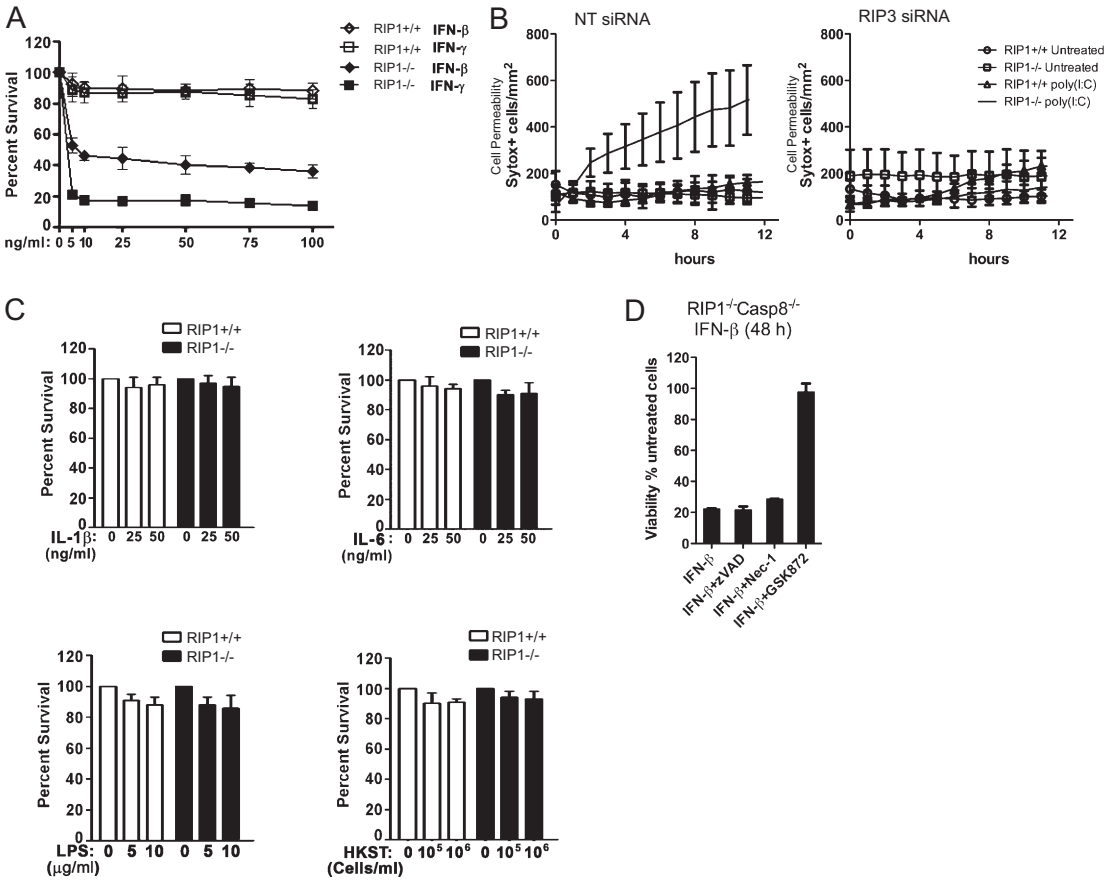



**Fig. S2.** (*A*) Viability of SV40-immortalized WT and *Rip1*$^{-/-}$ fibroblasts treated with increasing concentrations of IFNβ and IFNγ. (*B*) Time course IncuCyte analysis of cell viability of NT (*Left*) or RIP3 (*Right*) siRNA transfected, immortalized *Rip1*$^{+/+}$, or *Rip1*$^{-/-}$ fibroblasts treated with poly(I:C) (50 μg/mL) measured by appearance of nucleic-acid binding stain Sytox (50 nM). (*C*) Viability of SV40-immortalized WT and *Rip1*$^{-/-}$ fibroblasts treated with increasing concentrations of IL-1β, IL-6, LPS, and heat-killed *Salmonella typhimurium* (HKST) was determined by Cell Titer-Glo assay. (*D*) Viability assay of *Rip1*$^{-/-}$*Casp8*$^{-/-}$ MEFs in the presence or absence of zVAD-fmk (25 μM), GSK'872 (5 μM), or Nec-1 (30 μM) at 48 h posttreatment. Viability was determined by Cell Titer-Glo assay.

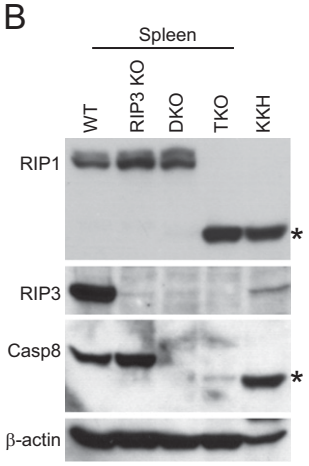
A

*Rip1*$^{-/-}$*Casp8*$^{-/-}$ *Rip3*$^{-/-}$ :: *Rip1*$^{+/-}$*Casp8*$^{+/-}$*Rip3*$^{+/-}$

| Genotype | Mendelian frequency (%) | Observed frequency (%) | No.of mice weaned |
|---|---|---|---|
| *Rip1*$^{+/-}$*Casp8*$^{+/-}$*Rip3*$^{+/-}$ | 12.5 | 17.39 | 8 |
| *Rip1*$^{+/-}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$ | 12.5 | 23.91 | 11 |
| *Rip1*$^{-/-}$*Casp8*$^{+/-}$*Rip3*$^{+/-}$ | 12.5 | 0 | 0* |
| *Rip1*$^{-/-}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$ | 12.5 | 0 | 0* |
| *Rip1*$^{+/-}$*Casp8*$^{-/-}$*Rip3*$^{+/-}$ | 12.5 | 0 | 0# |
| *Rip1*$^{+/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ | 12.5 | 17.39 | 8 |
| *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{+/-}$ | 12.5 | 26.09 | 12 |
| *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ | 12.5 | 15.22 | 7 |
| | | Total | 46 |

* denotes perinatal lethal
\# denotes embryonic lethal

B




C

| Genotype | No.of mice | Died prior to weaning |
|---|---|---|
| *Rip1*$^{-/-}$*Rip3*$^{+/+}$ *TNF*$^{+/-}$ | 2 | p0 |
| *Rip1*$^{-/-}$*Rip3*$^{+/+}$ *TNF*$^{-/-}$ | 9 | p0, p0, p1, p1, p2, p4, p4, p14, p14 |
| *Rip1*$^{-/-}$*Rip3*$^{+/-}$*TNF*$^{+/-}$ | 1 | p5 |
| *Rip1*$^{-/-}$*Rip3*$^{+/-}$*TNF*$^{-/-}$ | 7 | p5, p14, p17, p19, p19, p20, p20 |
| *Rip1*$^{-/-}$*Rip3*$^{-/-}$ *TNF*$^{-/-}$ | 4 | p3, p20, p23, p28 |
| Total | 23 | |

**Fig. S3.** (*A*) Epistatic analysis of mice born after *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ and *Rip1*$^{+/-}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$ cross. (*B*) Immunoblot analysis of RIP1, RIP3, Casp8, and β-actin from the spleen of adult mice. The asterisk denotes elevated IgG heavy chain detected with secondary antibody in both TKO and Rip1$^{-/-}$Casp8$^{-/-}$Rip3$^{+/-}$ (KKH) samples. (*C*) Table indicating the survival of RIP1-null mice with combinations of mutations of TNF and RIP3.

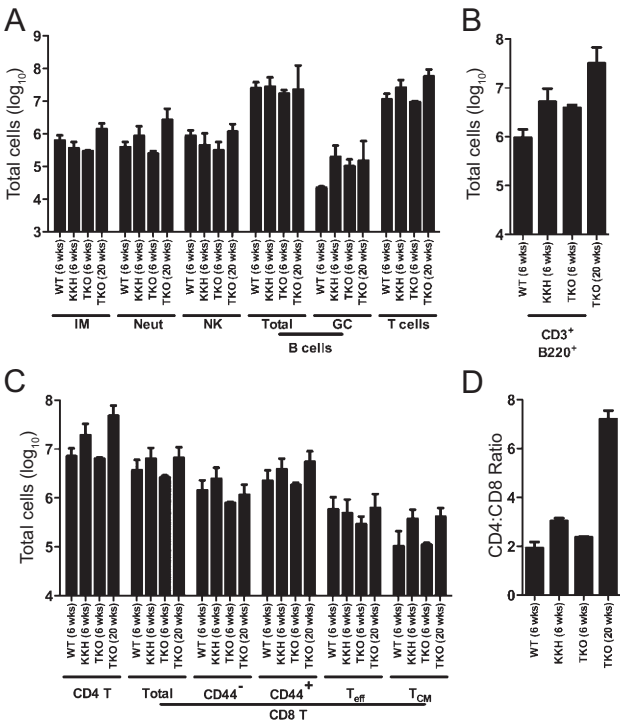



**Fig. S4.** (*A*) Numbers of splenic inflammatory monocytes (IMs) (Ly6C$^{hi}$CD11b$^{+}$), neutrophils (Neut) (Ly6C$^{int}$CD11b$^{+}$), natural killer (NK) cells (CD3$^{-}$NK1.1$^{+}$), total (CD19$^{+}$B220$^{+}$) as well as germinal center (GC) B cells (CD95$^{+}$GL7$^{+}$), and total T cells (CD3$^{+}$) in 6-wk-old WT, KKH, and TKO as well as 20-wk-old TKO mice. (*B*) Total number of abnormal splenic B220$^{+}$CD3$^{+}$ T cells at indicated ages. (*C*) CD3$^{+}$CD4$^{+}$ T cells and CD3$^{+}$CD8$^{+}$ T cells that were of an Ag-inexperienced (CD44$^{-}$), Ag-experienced (CD44$^{+}$), effector ($T_{eff}$; CD44$^{+}$KLRG$^{hi}$ CD62L$^{-}$), or central memory ($T_{CM}$; CD44$^{+}$KLRG$^{-}$CD62L$^{+}$) phenotype in WT, KKH, and TKO mice at the indicated ages. (*D*) Ratio of CD4 to CD8 T cells in spleens of WT, KKH, and TKO mice at indicated ages.

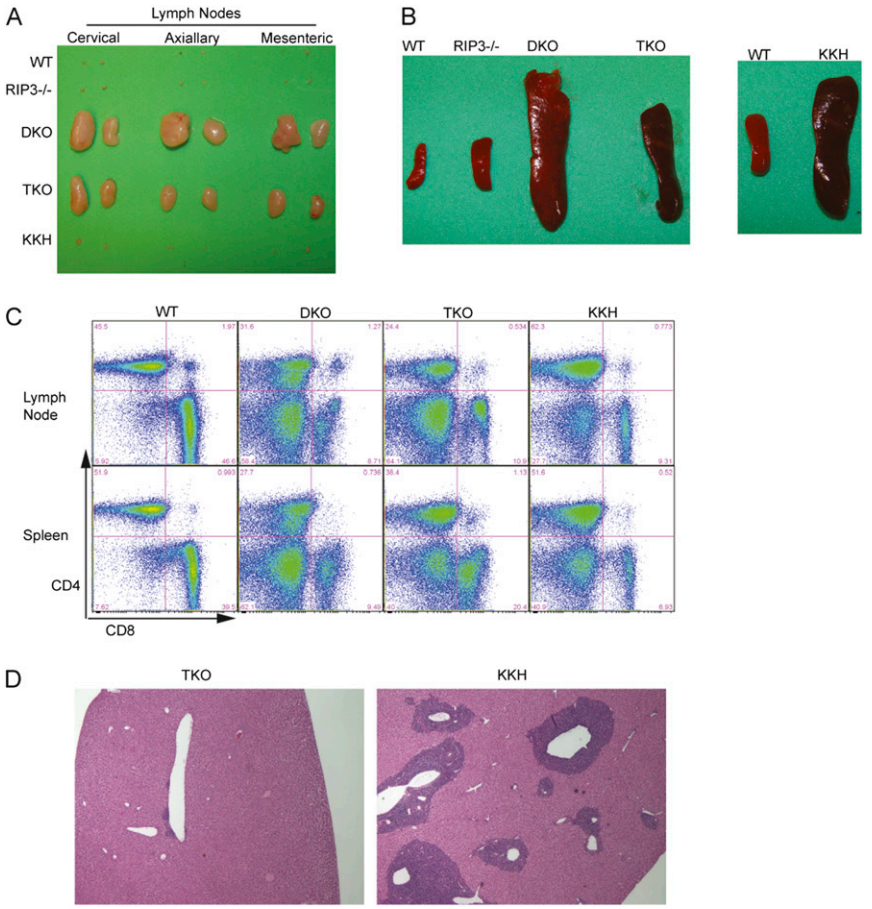



**Fig. S5.** (*A*) Images of cervical, axillary, and mesenteric lymph nodes and (*B*) spleens of 20-wk-old WT, *Rip3*$^{-/-}$, double-knockout (DKO), TKO, and KKH mice. (*C*) Flow cytometry plots showing patterns of CD4 and CD8 expression on CD3$^{+}$ cells collected from cervical lymph nodes of 20-wk-old WT, DKO, TKO, and KKH mice. (*D*) Histology images of liver sections from 20-wk-old TKO and KKH mice.

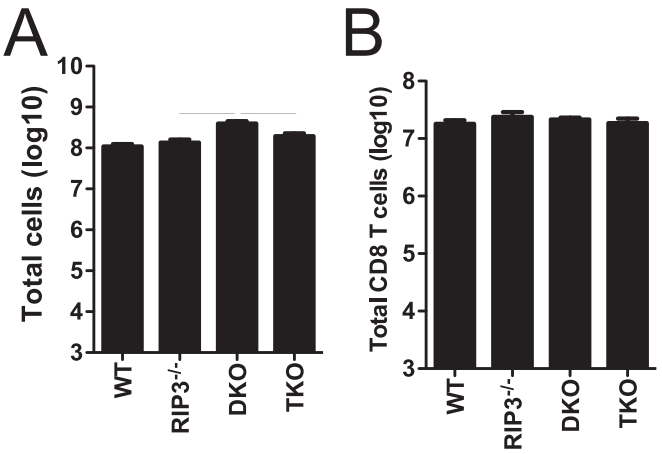



**Fig. S6.** Cell numbers in spleens from MCMV-infected mice. Total leukocytes (*A*) and CD8$^{+}$ T cells (*B*) in spleens of 12- to 16-wk-old WT, *Rip3*$^{-/-}$, DKO, or TKO mice at 7 d postinfection (same groups as in Fig. 5).

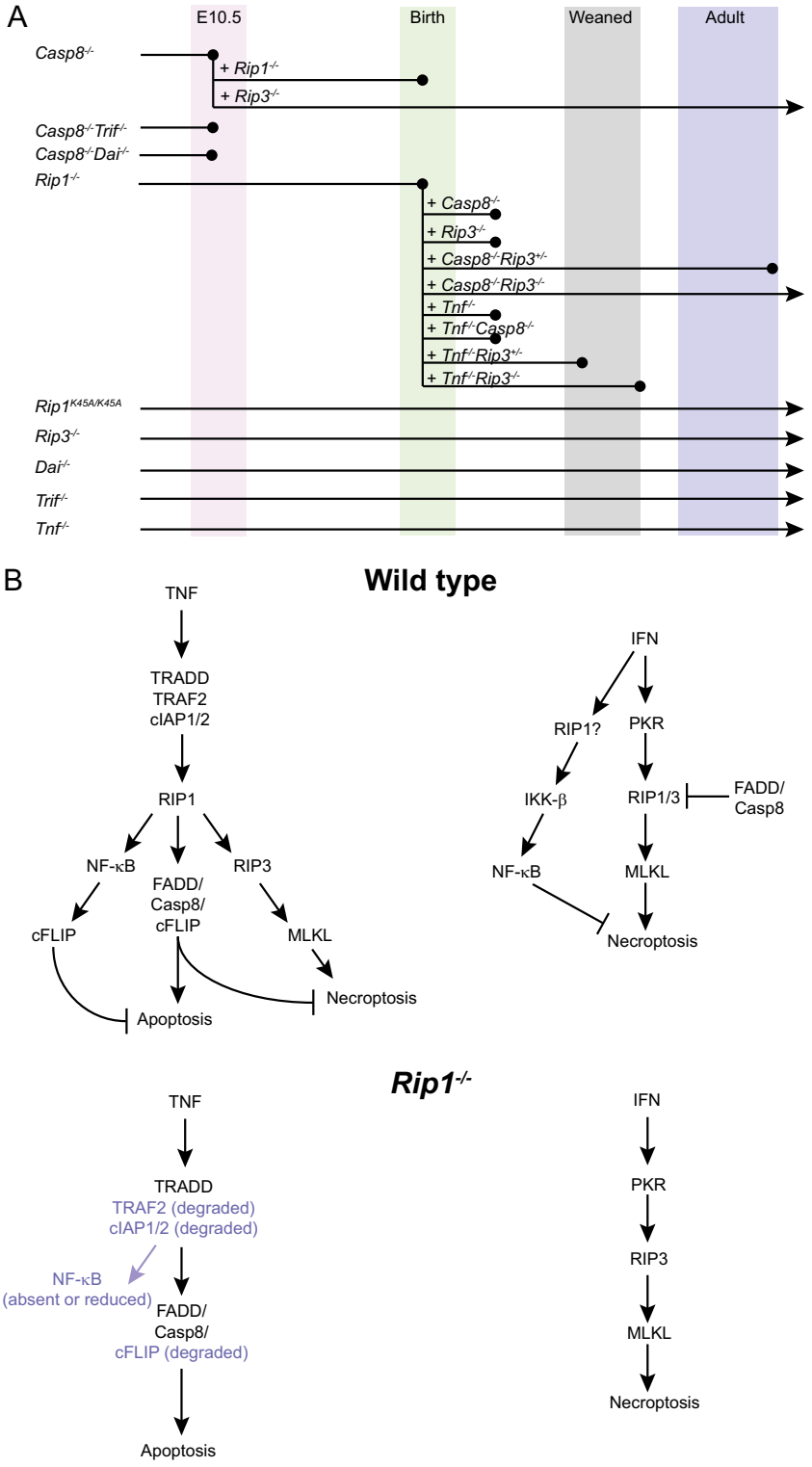



**Fig. S7.** (*A*) The role of key apoptotic and necroptotic genes in mouse development. (*B*) Schematic of TNF and IFN signaling pathways in WT and *Rip1*$^{-/-}$ cells.