RIP1 suppresses innate immune necrotic as well as apoptotic cell death during mammalian parturition

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The pronecrotic kinase, receptor interacting protein (RIP1, also called RIPK1) mediates programmed necrosis and, together with its partner, RIP3 (RIPK3), drives midgestational death of caspase 8 (Casp8)-deficient embryos. RIP1 controls a second vital step in mammalian development immediately after birth, the mechanism of which remains unresolved. *Rip1*$^{-/-}$ mice display perinatal lethality, accompanied by gross immune system abnormalities. Here we show that RIP1 K45A (kinase dead) knockin mice develop normally into adulthood, indicating that development does not require RIP1 kinase activity. In the face of complete RIP1 deficiency, cells develop sensitivity to RIP3-mixed lineage kinase domain-like–mediated necroptosis as well as to Casp8-mediated apoptosis activated by diverse innate immune stimuli (e.g., TNF, IFN, double-stranded RNA). When either RIP3 or Casp8 is disrupted in combination with RIP1, the resulting double knockout mice exhibit slightly prolonged survival over RIP1-deficient animals. Surprisingly, triple knockout mice with combined RIP1, RIP3, and Casp8 deficiency develop into viable and fertile adults, with the capacity to produce normal levels of myeloid and lymphoid lineage cells. Despite the combined deficiency, these mice sustain a functional immune system that responds robustly to viral challenge. A single allele of *Rip3* is tolerated in *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{+/-}$ mice, contrasting the need to eliminate both alleles of either *Rip1* or *Rip3* to rescue midgestational death of *Casp8*-deficient mice. These observations reveal a vital kinase-independent role for RIP1 in preventing pronecrotic as well as proapoptotic signaling events associated with life-threatening innate immune activation at the time of mammalian parturition.

interferon | MLKL | herpesvirus

Receptor interacting protein (RIP) kinase RIP1 (RIPK1) functions as an essential adapter in a number of innate immune signal transduction pathways, including those initiated by Toll-like receptor (TLR)3, TLR4, and retinoic acid-inducible gene 1 (RIG-I)-like receptors, in addition to death receptors (1–4). Signaling via these pathways bifurcates at the level of RIP1 to produce opposing outcomes, a prosurvival inflammatory response counterbalanced by extrinsic cell death signaling that drives either apoptosis or necroptosis. Despite the normal development of many organs and neuromuscular architecture, RIP1-null mice die within a few days of birth with signs of edema as well as significant levels of cell death within lymphoid tissues, particularly immature thymocytes (5). Although TNF-signaling contributes to this perinatal death (6) and implicates the prosurvival role of RIP1 in activating nuclear factor κB (NF-κB) (5), the precise mechanism responsible for developmental failure of RIP1-deficient mice remains unresolved. It seems likely that dysregulation of additional signaling pathways contributes to this phenotype, given that deficiency in TNF receptor 1 (TNFR1) only modestly extends the lifespan of RIP1-null mice and deficiency in TNFR2 only rescues thymocytes from death (7).

RIP1 orchestrates assembly of distinct signaling platforms via two C-terminal protein–protein binding domains: a death domain and a RIP homotypic interaction motif (RHIM) (3, 4). This unique architecture facilitates convergent death domain-dependent and RHIM-dependent pathways. RIP1 partners with death domain-containing proteins, particularly fas-associated death domain protein (FADD), as well as RHIM-containing proteins, such as the pronecrotic kinase RIP3 and the TLR3/TLR4 adapter TIR-domain–containing adapter-inducing IFN (TRIF) (8, 9). RIP1 is essential for TNF-induced necroptosis but dispensable for other forms of RIP3 kinase-dependent death (10, 11). Oligomerization of RIP1 through either domain promotes activation of its N-terminal serine/threonine kinase and triggers either of two distinct cell death pathways: (*i*) apoptosis following assembly of a cytosolic FADD–Casp8–cellular FLICE-like inhibitory protein (cFLIP)-containing complex or (*ii*) necroptosis via RIP3-dependent, mixed lineage kinase domain-like (MLKL)-mediated membrane permeabilization (1–4).

In addition to death, RIP1 activation downstream of either TNFR1 or TNFR2 facilitates prosurvival NF-κB gene expression contingent on the balance of ubiquitination and deubiquitination (12). In this context, deubiquitination converts RIP1 into a death-inducing adapter within the TNFR-signaling complex (12). RIP1 remains a component of a death receptor-free cytosolic complex, termed complex II (also called the ripoptosome) (1–3), together with FADD, Casp8, and cFLIP where cFLIP levels control Casp8 activation (13) and death (14). When Casp8 or FADD are absent or Casp8 activity is inhibited (14–17), RIP1

Significance

The protein kinase receptor interacting protein 1 controls signaling via death receptors, Toll-like receptors, and retinoic acid-inducible gene 1-like receptors, dictating inflammatory outcomes as broad as cytokine activation and cell death. RIP1 makes a vital contribution during development, evident from the fact that RIP1-deficient mice die soon after birth. Here, we show that a kinase-independent function of RIP1 dampens the consequences of innate immune cell death. During parturition, RIP1 prevents the lethal consequences of RIP3-dependent necroptosis as well as caspase 8 (Casp8)-dependent apoptosis. In contrast to the RIP1-deficient phenotype, mice lacking a combination of RIP1, RIP3, and Casp8 are born and mature into viable, fertile, and immunocompetent adults. These results demonstrate the important protective role of RIP1 against physiologic and microbial death cues encountered at birth.

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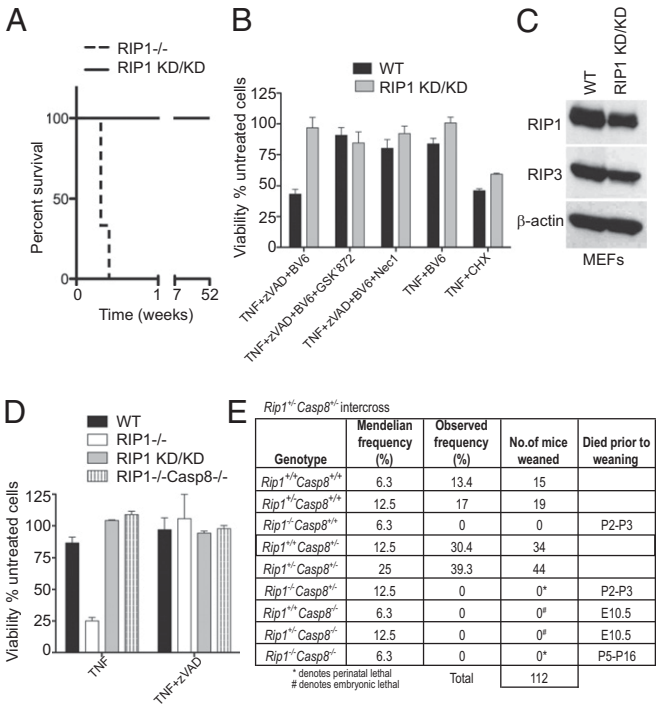
mediates RHIM-dependent recruitment of RIP3. Then, RIP1 kinase activity facilitates RIP3 kinase-dependent phosphorylation of MLKL to drive necroptosis (18, 19). Importantly, basal Casp8 activity conferred by cFLIP blocks this process (14), and in vivo, this translates into a unique requirement for Casp8 to prevent RIP3-dependent embryonic lethality and tissue inflammation triggered by Casp8 or FADD compromise (14–17). Recently, the importance of Casp8 suppression of necroptosis has been extended to diverse innate signaling pathways, including those activated by TLR3 as well as type I or II interferon (IFN) (11, 20, 21), broadening a concept that first emerged in death receptor signaling (3, 4). Once TLR3 becomes activated, the adapter protein TRIF recruits RIP1 or RIP3 via RHIM interactions (8). In this context, the RIP1 death domain ensures the suppression of necrotic death by recruiting FADD, Casp8, and cFLIP. Necroptosis is unleashed whenever Casp8 or FADD is compromised. Likewise, IFN activation of protein kinase R sets up a similar relationship with the FADD–Casp8–cFLIP–RIP1 complex (21). Thus, innate immunity elicits dueling signals that both potentiate and suppress programmed necrosis.

In this study, we implicate multiple innate immune signaling pathways in the death of RIP1-deficient mice. Once dysregulated by disruption of RIP1, RIP3-mediated necroptosis and Casp8-dependent apoptosis contribute to death at the time of birth. Our observations bring to light the consequences of diverse innate immune stimuli arising from TNF, IFN, and/or nucleic acids that play out during mammalian parturition. RIP1 plays a vital role suppressing cell death consequences of this innate signaling. RIP3 and Casp8 must be eliminated to rescue RIP1-null mice from perinatal death and produce fully viable, fertile, and immunocompetent triple-knockout (TKO) mice.

Results

Perinatal Lethality Is Independent of RIP1 Kinase Activity. Although RIP1-deficient mice fail to survive beyond birth (5), the relative contribution of kinase activity, RHIM function, or death domain interactions have not been investigated. The expectation that RIP1 kinase activity is necessary to form a FADD–Casp8–cFLIP signaling platform (1) lead us to evaluate the phenotype of *Rip1* knockin (KI), kinase-dead (*Rip1*$^{KD/KD}$) mice expressing an ATP binding site (K45A) mutant. Remarkably, *Rip1*$^{KD/KD}$ mice were viable and fertile (Fig. 1*A*) and showed the ability to reverse inflammatory disease (22). RIP1 kinase activity is dispensable for the steps that support extrinsic apoptosis (Fig. 1*B*), consistent with a recent report using a different *Rip1*$^{KD/KD}$ strategy (23). To develop the understanding of RIP1 kinase as a partner of RIP3, we showed that the sensitivity of WT mouse embryonic fibroblasts (MEFs) to TNF-induced necroptosis was reversed by addition of RIP1 kinase inhibitor necrostatin-1 (Nec-1) or RIP3 kinase inhibitor GSK'872 [from GlaxoSmithKline (GSK)] (Fig. 1*B*) (11, 24). In accord with a recent report (23), *Rip1*$^{KD/KD}$ mice resisted this death (Fig. 1*B*) despite the presence of mutant protein at levels similar to WT RIP1 (Fig. 1*C*). These studies revealed a pattern that was reminiscent of the full viability of *Rip3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice (25–27). Thus, RIP1 kinase activity, like pronecrotic RIP3 and MLKL, is not involved in mammalian development but provides a necrotic trap door in host defense (3, 4).

RIP1 Protects from TNF-Induced Apoptosis Independent of Its Kinase Activity. Consistent with previous observations (5), *Rip1*$^{-/-}$ MEFs were hypersensitive to TNF-induced apoptosis (Fig. 1*D* and Fig. S1*A*). Death was suppressed by pretreatment with the pan-caspase inhibitor zVAD-fmk (Fig. S1*B*) and was accompanied by increased Casp8 and Casp3 processing and activity (Fig. S1*C*). As expected, *Rip1*$^{-/-}$*Casp8*$^{-/-}$ MEFs were insensitive to TNF-induced apoptosis (Fig. 1*D*), reinforcing the direct contribution of Casp8 to this striking phenotype (5). *Rip1*$^{KD/KD}$ MEFs were also insensitive to TNF-induced apoptosis (Fig. 1*D*), indicating



E

Rip1$^{+/-}$*Casp8*$^{+/-}$ intercross

Genotype	Mendelian frequency (%)	Observed frequency (%)	No.of mice weaned	Died prior to weaning
Rip1$^{+/+}$*Casp8*$^{+/+}$	6.3	13.4	15	
Rip1$^{+/-}$*Casp8*$^{+/+}$	12.5	17	19	
Rip1$^{-/-}$*Casp8*$^{+/+}$	6.3	0	0	P2-P3
Rip1$^{+/+}$*Casp8*$^{+/-}$	12.5	30.4	34	
Rip1$^{+/-}$*Casp8*$^{+/-}$	25	39.3	44	
Rip1$^{-/-}$*Casp8*$^{+/-}$	12.5	0	0*	P2-P3
Rip1$^{+/+}$*Casp8*$^{-/-}$	6.3	0	0#	E10.5
Rip1$^{+/-}$*Casp8*$^{-/-}$	12.5	0	0#	E10.5
Rip1$^{-/-}$*Casp8*$^{-/-}$	6.3	0	0*	P5-P16
		Total	112	

* denotes perinatal lethal
\# denotes embryonic lethal

Fig. 1. Survival of *Rip1*$^{KD/KD}$ but not *Rip1*$^{-/-}$*Casp8*$^{-/-}$ mice implicates programmed necrosis in perinatal death of *Rip1*$^{-/-}$ mice. (*A*) Kaplan–Meier survival plots of *Rip1*$^{KD/KD}$ and *Rip1*$^{-/-}$ mice. (*B*) Viability of WT and *Rip1*$^{KD/KD}$ MEFs by Cell Titer-Glo (Promega) assay (10), determined 12 h after stimulation with necrotic or apoptotic stimuli. Necroptosis was induced by treatment with TNF (25 ng/mL) in the presence of zVAD-fmk (zVAD, 25 μM) and BV6 (1 μM) with or without inhibitors GSK'872 (3 μM) or Nec-1 (30 μM). Apoptosis was induced by treatment with TNF in the presence of cyclohexamide (5 μg/mL). (*C*) Immunoblot of RIP1, RIP3, and β-actin levels in WT and RIP1$^{KD/KD}$ MEFs. (*D*) Viability of indicated genotypes of primary MEFs at 18 h after treatment with TNF in the presence or absence of zVAD-fmk. (*E*) Epistatic analysis of mice born after intercross of *Rip1*$^{+/-}$*Casp8*$^{+/-}$ mice, with the day of embryonic (E) or perinatal (P) death before weaning indicated in the last column.

RIP1 function was independent of its kinase activity. To determine the contribution of Casp8 to perinatal death of RIP1-deficient mice, we performed a *Rip1*$^{+/-}$*Casp8*$^{+/-}$ intercross and found that RIP1 rescued the embryonic lethality of *Casp8*$^{-/-}$ mice, although none of the resulting RIP1-deficient progeny (*Rip1*$^{-/-}$*Casp8*$^{-/-}$, *Rip1*$^{-/-}$*Casp8*$^{+/-}$, or *Rip1*$^{-/-}$*Casp8*$^{+/+}$) survived to weaning at 21 d of age (Fig. 1*E*). *Rip1*$^{-/-}$*Casp8*$^{+/+}$ and *Rip1*$^{-/-}$*Casp8*$^{+/-}$ pups died at perinatal day 2 (P2) and *Rip1*$^{-/-}$*Casp8*$^{-/-}$ pups died somewhat later (P5–P16). This pattern revealed a very limited contribution of Casp8 to perinatal lethality underlying RIP1 deficiency, results that phenocopied *Fadd*$^{-/-}$*Rip1*$^{-/-}$ mice (15). Any Casp8-deficient embryos that expressed RIP1 showed the expected midgestational death phenotype (16, 28, 29) due to unleashed RIP1–RIP3 death (14–17). Whereas these data affirm a contribution of Casp8-dependent apoptosis to perinatal lethality of RIP1-deficient mice (5), the failure to rescue fully viable *Rip1*$^{-/-}$*Casp8*$^{-/-}$ mice strongly implicates an additional pathway in this striking phenotype.

RIP1 Prevents IFN- and Double-Stranded RNA-Induced Necroptosis. In addition to the known contribution of TNF to necroptosis, type I IFN, type II IFN, and the double-stranded RNA (dsRNA) mimic poly(I:C) show the capacity to trigger this pathway in susceptible simian virus 40 (SV40)-immortalized cells (21, 30–32). Greater than 50% of *Rip1*$^{-/-}$ cells treated with either IFNβ, IFNγ, TNF, or dsRNA died within 48 h (Fig. 2 *A* and *B* and Fig. S2*A*). In contrast, WT fibroblasts resisted these innate immune/proinflammatory cell

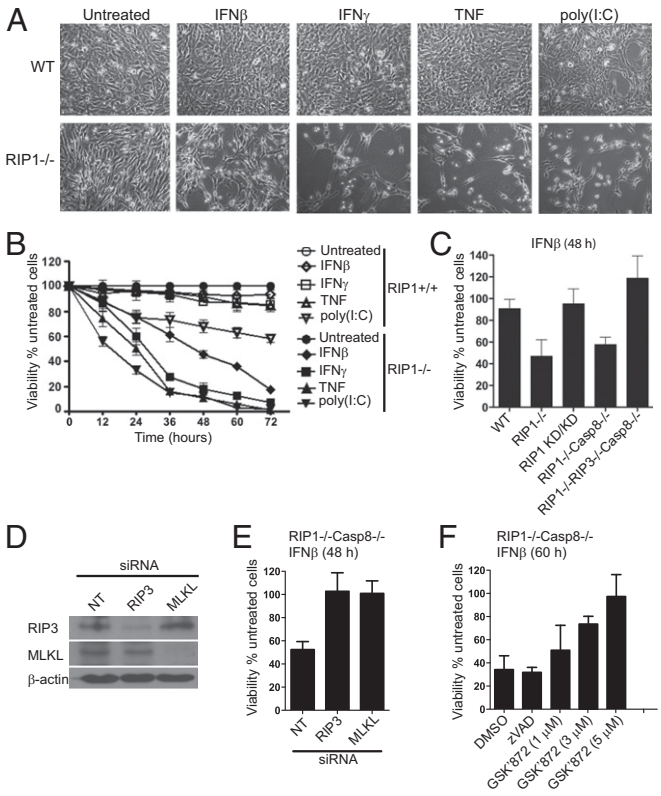


Fig. 2. *Rip1*$^{-/-}$ and *Rip1*$^{-/-}$*Casp8*$^{-/-}$ fibroblasts exhibit sensitivity to innate immune signaling death. (*A*) Photomicrographs of SV40 immortalized WT and *Rip1*$^{-/-}$ fibroblasts treated with IFNβ (5 ng/mL), IFNγ (5 ng/mL), TNF (50 ng/mL), or cytosolic poly(I:C) (2 μg/mL transfected in 6 μL Lipofectamine 2000) for 48 h. (*B*) Time course of viability of immortalized WT and *Rip1*$^{-/-}$ fibroblasts treated with IFNγ, IFNβ, TNF, or poly(I:C). (*Inset*) Immunoblot of RIP1 and β-actin levels in immortalized WT and *Rip1*$^{-/-}$ fibroblasts. (*C*) Viability of MEFs with the indicated genotypes at 48 h posttreatment with IFNβ (5 ng/mL). (*D*) Immunoblot of MLKL and RIP3 levels in *Rip1*$^{-/-}$*Casp8*$^{-/-}$ MEFs transfected with nontargeting (NT), RIP3, or MLKL siRNA. (*E*) Viability assay of *Rip1*$^{-/-}$*Casp8*$^{-/-}$ MEFs 48 h posttransfection with NT, RIP3, or MLKL siRNA treated with IFNβ (5 ng/mL) for 48 h. (*F*) Viability assay of *Rip1*$^{-/-}$*Casp8*$^{-/-}$ MEFs in the presence or absence of zVAD-fmk (25 μM), GSK'872 (1, 3, or 5 μM) at 60 h posttreatment. Viability was determined by Cell Titer-Glo assay.

death inducers. Consistent with a contribution of RIP3-dependent necroptosis in these settings, IFNβ-induced death of SV40-immortalized *Rip1*$^{-/-}$ fibroblasts was blocked by RIP3-specific RNAi (Fig. S2*B*). Thus, sensitivity to diverse innate immune pathways known to signal via FADD–Casp8 increased dramatically in the absence of RIP1. Interestingly, RIP1-deficient cells were insensitive to IL-1β, IL-6, *Escherichia coli* LPS, or heat-killed *Salmonella typhimurium* (Fig. S2*C*), indicating that the RIP1-regulated prosurvival response is selective to a subset of innate immune stimuli.

Rip1$^{-/-}$*Casp8*$^{-/-}$ MEFs exhibited striking hypersensitivity to treatment with IFNβ (Fig. 2*C*), a pattern that contrasted their resistance to TNF (Fig. 1*D*). Time-lapse imaging indicated that dying cells lost membrane integrity without signs of blebbing or nuclear fragmentation, showing a clear necrotic death pattern. Consistent with this process, the death induced by IFNβ was eliminated by genetic ablation of RIP3 in *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ MEFs (Fig. 2*C*), by knockdown of RIP3 or MLKL (Fig. 2 *D* and *E*), or by treatment with RIP3 kinase inhibitor GSK'872 (Fig. 2*F*). In contrast to the important role of RIP3 kinase, caspases and RIP1 kinase activity were dispensable (Fig. 2*F* and Fig. S2*D*). The contribution of RIP3 kinase, as well as its downstream target, MLKL (18, 19), demonstrates that IFNβ induces a conventional RIP3-dependent necroptosis in *Rip1*$^{-/-}$*Casp8*$^{-/-}$ MEFs (Fig. 2 *D* and *E*), albeit independent of RIP1 (Fig. 1). These results unveil an unexpected, cytoprotective role for RIP1 in suppressing RIP3–MLKL-mediated necroptosis following stimulation with IFN or dsRNA, contrasting the established contribution of RIP1 kinase activity to TNF-induced necroptosis (1–4).

Elimination of Both Casp8 and RIP3 Rescues RIP1 Perinatal Lethality. The sensitivity of *Rip1*$^{-/-}$ cells to both Casp8-dependent apoptosis and RIP3-mediated necroptosis implicates the combined pathways in perinatal death of RIP1-deficient mice. To directly evaluate the contribution of RIP3 and Casp8 to this phenotype, we bred *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ TKO progeny from a *Rip1*$^{+/-}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$ intercross. Remarkably, TKO mice survived to weaning and matured into fertile adults (Fig. 3*A*) that were indistinguishable in physical appearance from double-knockout (DKO) or WT C57BL/6 mice (Fig. 3*B*). In contrast, *Rip1*$^{-/-}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$ and *Rip1*$^{-/-}$*Casp8*$^{+/+}$*Rip3*$^{-/-}$ newborns died within a short time of birth, demonstrating unequivocally that the phenotype imposed by RIP1 deficiency was due to RIP3 as well as Casp8 death pathways.

Rip1$^{+/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice were subsequently crossed with *Rip1*$^{+/-}$*Casp8*$^{+/-}$*Rip3*$^{+/-}$ mice to generate *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{+/-}$ (KKH) offspring. Combined RIP1- and Casp8-deficient mice were born at the expected Mendelian frequency and grew into viable and fertile adults (Fig. 3*B* and Fig. S3*A*). This observation indicates that one allele of *Rip3* is tolerated by mice lacking RIP1 and Casp8, although two *Rip3* alleles are lethal (Fig. 1*E*). There was no such copy number tolerance of the *Casp8* allele, as *Rip1*$^{-/-}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$ mice died shortly after birth (Fig. 3*A* and Fig. S1*B*). These results demonstrate that concurrent ablation of Casp8 along with one allele of RIP3 confers full viability on RIP1-deficient mice, highlighting the benefits of reducing RIP3 below a lethal pronecrotic threshold.

Elimination of TNFR1 extends the lifespan of *Rip1*$^{-/-}$ mice for up to 2 wk, implicating TNF signaling in the perinatal death phenotype (7). To directly investigate the survival benefit of eliminating TNF signaling, we generated mice lacking TNF and RIP1 in combination with either Casp8 or RIP3. Elimination of Casp8 in *Casp8*$^{-/-}$*Rip1*$^{-/-}$*Tnf*$^{-/-}$ mice failed to extend the lifespan of *Rip1*$^{-/-}$*Tnf*$^{-/-}$ mice, although elimination of RIP3 provided a more pronounced benefit, such that *Rip1*$^{-/-}$*Rip3*$^{-/-}$*Tnf*$^{-/-}$ mice and *Rip1*$^{-/-}$*Rip3*$^{+/-}$*Tnf*$^{-/-}$ mice typically survived between 2 and 4 wk (Fig. S3*C*). These data are consistent with previous studies (7) as well as with our evidence implicating additional innate immune cell death signals in RIP3 activation.

Development of the Immune System Independent of RIP1. Thymic cell death and perturbation of immune homeostasis in secondary lymphoid organs are hallmarks in E18 *Rip1*$^{-/-}$ mice (5), consistent with a role of RIP1 in immune development at the final stages of gestation before parturition. We therefore examined the impact of combined elimination of RIP1, RIP3, and Casp8

A

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

Genotype	Mendelian frequency (%)	Observed frequency (%)	No.of mice weaned
Rip1$^{+/+}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$	12.5	12.5	7
Rip1$^{+/-}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$	25	44.64	25
Rip1$^{-/-}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$	12.5	0	0*
Rip1$^{+/+}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$	12.5	3.57	2
Rip1$^{+/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$	25	25	14
Rip1$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$	12.5	14.29	8
		Total	56

* denotes perinatal lethal

B

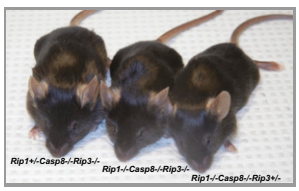


Fig. 3. *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ and the *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{+/-}$ mice are viable. (*A*) Epistatic analysis of mice born following *Rip1*$^{+/-}$*Casp8*$^{+/-}$*Rip3*$^{-/-}$ intercross. (*B*) Image of 5-wk-old TKO, KKH, and *Rip1*$^{+/-}$*Casp8*$^{-/-}$*RIP3*$^{-/-}$ mice.

(Fig. S3*B*) on the immune system. We found that adult TKO mice displayed normal numbers of myeloid and lymphoid cells in spleens and lymph nodes (LNs) at 6 wk of age (Fig. S4*A*). When $CD45^+$ leukocyte cell populations were evaluated, inflammatory monocyte ($Ly6C^{hi}CD11b^+$) and neutrophil ($Ly6C^{int}CD11b^+$) numbers in TKO mice were comparable to WT mice. Likewise, TKO mice possessed robust levels of natural killer (NK) ($CD3^-NK1.1^+$), T ($CD3^+$), and B ($CD19^+$) cells, with an increased number of germinal center ($CD95^+GL7^+$) B cells (Fig. S4*A*). T-cell development in younger TKO mice was comparable to WT mice (Fig. S4 *B* and *C*) such that naive TKO mice maintained normal numbers of CD4 T cells as well as antigen-inexperienced ($CD44^-$), antigen-experienced ($CD44^+$), effector (T_{eff}; $CD44^+KLRG^{hi}$ $CD62L^-$), and central memory (T_{CM}; $CD44^+KLRG^-CD62L^+$) CD8 T-cell subsets (Fig. S4*C*). Notably, these leukocyte lineages were all detected at comparable levels in KKH mice where Casp8 and RIP1 are absent but low levels of RIP3 are present (Fig. S3*B*). These data support the proposed prosurvival role of TNFR2 signaling in the immune system defects of *Rip1*$^{-/-}$ mice (7). Altogether, these observations reveal a remarkable fact that RIP1 fails to contribute to development or homeostatic maintenance of key myeloid and lymphoid populations, so long as Casp8 is eliminated and RIP3 levels are reduced.

RIP1 Deficiency Increases Autoimmune Markers in Casp8- and RIP3-Deficient Mice. Older (>8 wk) TKO mice developed splenomegaly and lymphadenopathy (Fig. 4*A* and Fig. S5 *A* and *B*). In addition to these phenotypic abnormalities, and, similar to DKO mice (16), all TKO and KKH showed levels of abnormal $B220^+CD3^+CD4^-CD8^-$ T cells by 20 wk of age (Figs. S4*B* and S5*C*), a population that increased as mice aged. This accumulation of abnormal $B220^+$ T cells occurs in settings where the midgestational death phenotype of Casp8 deficiency has been rescued by elimination of RIP3 (16) and is reminiscent of Fas/FasL deficiency where Casp8 controls steps downstream of Fas signal transduction in lymphocyte homeostasis (33). Although CD4:CD8 T-cell ratios in younger TKO mice were similar to WT mice, there was a 3.5-fold increase in this ratio in aging TKO mice (Fig. S4*D*), a higher ratio than observed in aging DKO mice. The most striking difference we observed in TKO mice, compared with DKO or WT mice, was increased levels of anti-dsDNA antibodies (Fig. 4*B*), a pattern that aligned with increased levels of germinal center B cells (Fig. S4*A*). It appears that the combined disruption of RIP1, Casp8, and RIP3 exacerbates an autoimmune lymphoproliferative syndrome-like condition (33) in mice that have aged in the absence of Casp8 function (16). High levels of autoimmune antibodies were also detected in KKH mice, indicating that RIP3 expression does not suppress this phenotype. KKH mice showed distinct phenotypic abnormalities. Although they sustained splenomegaly (Fig. S5*B*) with abnormal $B220^+$ T cells (Fig. S4*B*), KKH exhibited milder lymphadenopathy (Fig. 4*A* and Fig. S5*A*) with fewer $B220^+CD3^+CD4^-CD8^-$ T cells accumulating in LNs with age (Fig. S5*C*). These characteristics suggest that RIP3 contributes to the elimination of this abnormal population in LNs but not spleens. In addition, KKH mice accumulated very little body fat and weighed one-third less than age-matched WT or TKO mice (Fig. 4*C*). Whereas most TKO mice survived beyond 6 mo of age, only one of seven KKH mice survived to 6 mo (Fig. 4*D*). The shorter lifespan of KKH mice was associated with a highly pronounced perivascular inflammatory infiltrate in multiple organs including liver, lungs, pancreas, and intestine that appeared more severe than TKO or other genotypes (Fig. S5*D*). In aggregate, these data indicate that although below a lethal threshold, sustained RIP3 levels in KKH mice result in negative inflammatory consequences during life.

TKO Mice Control Viral Infection with a Robust CD8 T-Cell Response. To further probe immune competence, adult TKO mice were infected with the natural mouse herpesvirus, murine cytomegalovirus (MCMV), a pathogen that is normally controlled by innate NK and adaptive CD8 T cells (34). At 7 d post-infection, viral titers in the spleen, lungs, and salivary glands were all higher in TKO mice compared with WT or *Rip3*$^{-/-}$ mice but similar to DKO mice (Fig. 5 *A*–*C*). This pattern is consistent with a model in which Casp8-mediated apoptosis contributes to the pace with which virus levels are brought under control and is reminiscent of studies in mice with combined Fas and TNFR1 death receptor deficiency (35). Total numbers of splenic T cells, CD8 T cells, and MCMV M45 epitope-specific CD8 T cells appeared comparable across genotypes (Fig. 5*D* and Fig. S6 *A* and *B*). Based on analysis of this dominant viral epitope, CD8 T-cell expansion in response to virus infection appeared largely normal despite the combined absence of Casp8, RIP3, and RIP1. M45 peptide stimulation resulted in slightly fewer virus-specific $IFN\gamma^+$ and $INF\gamma^+TNF^+$ cells when CD8 T cells from infected TKO mice were compared with WT or *Rip3*$^{-/-}$ mice (Fig. 5 *E* and *F*). The capacity of TKO and DKO mice to generate a similar, bifunctional $INF\gamma^+TNF^+$ T-cell response against MCMV reflects the known ability of DKO mice to bring viral infection under immune control (16). Additional characterization is required to fully understand the quality of the immune response in settings where viable mutant mice have been derived; however, it is clear from these studies that Casp8 function contributes to the restriction of MCMV replication, but neither RIP1 nor RIP3 have a noticeable impact on this virus, likely due to the elaboration of virus-encoded cell death suppressors during infection (3, 36). It is remarkable that the complete absence of all RIP1, RIP3, and Casp8 signaling pathways, which compromises NF-κB signaling and completely eliminates the capacity for either extrinsic apoptosis or necroptosis, nevertheless leaves intact the necessary innate-to-adaptive immune signaling processes for a robust antigen-specific T-cell response to viral infection.

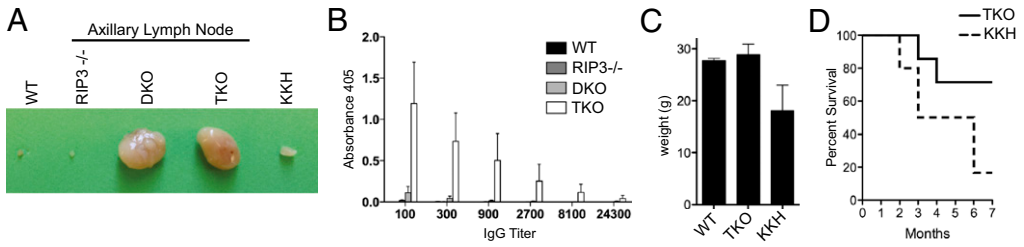


Fig. 4. Immune phenotype of *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ and *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{+/-}$ mice. (*A*) Axillary lymph nodes from WT, *Rip3*$^{-/-}$, DKO, TKO, and KKH mice. (*B*) Relative serum levels of double-stranded (ds) DNA-specific antibodies measured by ELISA in WT, *Rip3*$^{-/-}$, DKO, and TKO mice. (*C*) Weights of adult WT, TKO, and KKH mice. (*D*) Kaplan–Meier survival plots comparing survival of TKO and KKH mice through 7 mo of age.

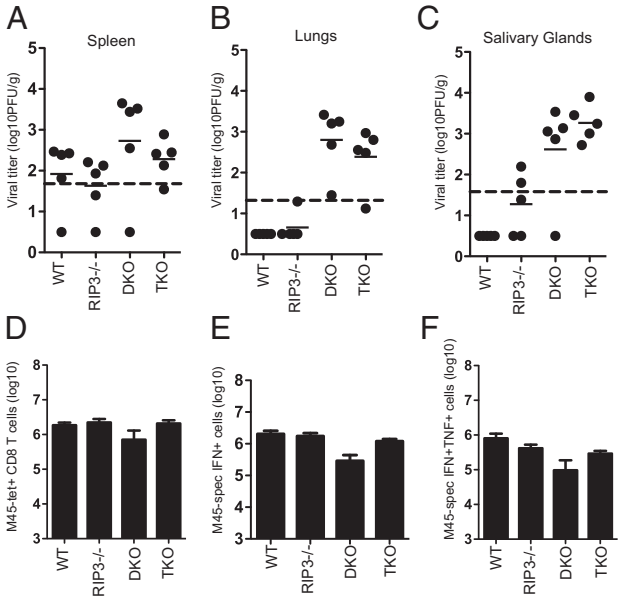


Fig. 5. *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice retain the ability to mount an adaptive immune response to virus infection. (*A*–*C*) MCMV titers in spleen (*A*), lung (*B*), and salivary glands (*C*) from 12- to 16-wk-old WT, *Rip3*$^{-/-}$, DKO, or TKO mice 7 d postinoculation with 10^6 pfu virus. Dashed line indicates limit of detection for each organ type. Shown is log titer of virus per gram of tissue from indvidual mice (five mice per group). (*D*) Total number of CD8 T cells in spleen recognized by M45-specific MHC class I tetramer in WT, *Rip3*$^{-/-}$, DKO, or TKO mice 7 d postinfection. (*E*) Frequency of splenic CD8 T cells producing IFNγ when stimulated with M45 peptide. (*F*) Frequency of splenic CD8 T cells producing both IFNγ and TNF when stimulated with M45 peptide.

Discussion

This investigation unveils the vital kinase-independent prosurvival role for RIP1 in preventing programmed necrosis in addition to suppressing extrinsic apoptosis (5). This comes as a surprise, given the well-established contribution of RIP1 in promoting TNF-induced necroptosis (1–4). The protection from apoptosis aligns with a long-recognized prosurvival role of RIP1 as an adapter that meters NF-κB activation dependent on polyubiquitylation state (12, 37). The diverse innate signaling pathways activated by TNF, IFN, or dsRNA that are implicated here in the perinatal death of RIP1-null newborns, all drive NF-κB activation. Although the precise spectrum and temporal relationship between RIP1 control of NF-κB activation and cell death remain to be dissected in detail, we observe a level of selectivity where RIP1 provides a vital role in the direct suppression of FADD–Casp8–cFLIP–RIP1 (complex II/ripoptosome) activity. IFN or dsRNA treatment induces necroptosis in cells with combined disruption of Casp8 and RIP1, settings where TNF, IL-1β, IL-6, or inactivated bacteria do not significantly affect cell viability even though these stimuli trigger NF-κB activation (36). Thus, our investigation reveals a kinase-independent cytoprotective activity of RIP1 above and beyond the expected contribution to NF-κB activation.

RIP1 is the major target of a polyubiquitin-sensitive mechanism to activate NF-κB and regulate cell death (12) downstream of signals as diverse as TNF, DNA, RNA, and IFN (37). Whereas disruption of RIP1 compromises NF-κB activation downstream of TNFR1, TNFR2, and TLR3 in certain settings (5, 7, 38), RIP1 deficiency does not compromise NF-κB activation levels in all cell types (39). We and others have proposed that the FADD–Casp8–cFLIP–RIP1 complex functions as a pathogen supersensor (3) that evolved to trigger alternate innate cell death pathways and overcome pathogen-encoded cell death suppressors. The data presented here align with a potential role of RIP1 in modulating apoptotic cell death through (*i*) NF-κB–mediated activation of prosurvival functions such as cFLIP as well as (*ii*) preventing destabilization of the FADD–Casp8–cFLIP–RIP1 complex (40). Our study expands the contribution of RIP1 as an activator and as a crucial brake on this core death-promoting complex. The hypersensitivity of RIP1-deficient cells to necroptosis is reminiscent of Casp8- or FADD deficiency (14–17), where the vital role of preventing dysregulated cell death during development was first elaborated (Fig. S7*A*). RIP1 evolved as a vital adapter to protect cells and balance the alternate pathways of apoptosis and necroptosis. In the context of death receptors, signaling in the absence of RIP1 manifests as apoptosis likely through the combination of blunted NF-κB activation and cFLIP destabilization (40). In contrast, the RIP1 RHIM-dependent association with RIP3 likely prevents aberrant necroptosis in response to IFN and dsRNA, acting upstream of RIP3 as a link to harness the antinecrotic potential of Casp8 activity and short circuit programmed necrosis through a mammalian mechanism that remains to be defined. This process, in addition to its long-recognized role as an activator of NF-κB prosurvival responses downstream of pathogen sensors and IFN-receptors, makes RIP1 crucial for life (Fig. S7*B*) (37).

It is clear from the data assembled here that RIP1 tempers the lethal consequences of aberrant cell death. In the absence of RIP1, dysregulation of extrinsic apoptosis and programmed necrosis pathways combine to become uniformly fatal around the time of birth. Although all organs form, RIP1-deficient mice exhibit disrupted lymphoid organ architecture, lymphopenia, and increased thymocyte apoptosis (5). In contrast, once RIP3 and Casp8 pathways are eliminated, these defects are reversed. Resulting TKO mice are viable and fertile and mount a robust response to viral infection, indicating the remarkable fact that all three enzymes are collectively dispensable for development. Our previous characterization of *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice (16) demonstrated an inability to support either extrinsic apoptosis or necroptosis that extends to *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice described here. Additional, subtle roles for RIP1 in adult mice will likely emerge from further comparisons of *Rip1*$^{-/-}$*Casp8*$^{-/-}$*Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice. The essential prosurvival role of RIP1 is independent of protein kinase activity, given that K45A (this study) or D138N (23) kinase-dead knockin mice retain full viability despite the inability to support RIP1-dependent necroptosis. RIP1 kinase activity collaborates with RIP3 in the embryonic death of Casp8-deficient mice (14–17); whereas, closer to birth, RIP1 paradoxically represses RIP3. Thus, dysregulation of lethal RIP3 activity is a surprising common property of RIP1-, Casp8-, and FADD-deficient mice and extends to specific mutants of RIP3 as well (23). The perinatal death of mice lacking RIP1 and Casp8 is reversed by a single RIP3 allele, although RIP3-dependent pathways are clearly deleterious as KKH mice die prematurely with elevated levels of inflammation distributed widely in organs. Interestingly, KKH mice do not accumulate high levels of $B220^+$ T cells in the periphery, suggesting these animals eliminate abnormal T cells via necroptosis independent of RIP1.

It is clear from our data that diverse innate cell death pathways collaborate with TNFR1 to drive perinatal death (7). The modest extension in life following the combined elimination of RIP1 and Casp8 substantiates this benefit. *Rip1*$^{-/-}$*Casp8*$^{-/-}$ mice survive for a similar period (P5–P16) as mice with a combined elimination of RIP1 and TNF (7), and the additional absence of Casp8 (*Rip1*$^{-/-}$*Casp8*$^{-/-}$*Tnf*$^{-/-}$) does not extend life further. In contrast, *Rip1*$^{-/-}$*Rip3*$^{-/-}$*Tnf*$^{-/-}$ mice survive between 3 and 4 wk. We observed considerable scatter in the patterns of death observed, consistent with a range of environmental cues driving dysregulated Casp8 unleashed by TNF or necroptosis unleashed by IFN. Based on these parallels, we predict that tissue-specific disruption of RIP1 will trigger uncontrolled cell death and consequent inflammatory disease similar to that seen with Casp8 or FADD mutants (1). It

appears from our study that RIP1 protects against inflammatory cues that start in utero as a component of mammalian parturition, possibly in combination with physiological cues or microbial colonization accompanying birth (6). In the absence of RIP1, inflammatory events triggered by exposure to such environmental cues, viruses, bacteria, or microbiota become lethal encounters. Collectively, these results identify a critical gatekeeper role for RIP1 in repressing cell death and subsequent inflammatory processes that accompany late gestation and early life.

Materials and Methods

Rip3$^{-/-}$ (25), *Rip1*$^{+/-}$ (5), *Casp8*$^{+/-}$ (16), and *Tnf*$^{-/-}$ (41) mice have been described previously. All strains were subsequently intercrossed. Genotypes were determined by PCR from tail snips or fetal tissue as described in *SI Materials and Methods*. RIP1 kinase-dead knockin (RIP1 KD/KI) mice were generated by homologous recombination using a targeting construct that mutated the catalytic lysine residue to alanine (K45A) to eliminate all kinase activity. Tissue processing and staining was performed by Emory University Division of Animal Resources (EU-DAR). Mice were bred and maintained by EU-DAR where all procedures were approved by the Emory University Institutional Animal Care and Use Committee. Immunoblotting and preparation of protein extracts were as previously described (9). MEFs and bone-marrow–derived macrophages were generated and viability was determined as previously described (10). Cells for flow cytometry were harvested, processed, and stained with indicated antibodies by standard methods. Data were acquired using an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software.

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