Hematopoietic RIPK1 deficiency results in bone marrow failure caused by apoptosis and RIPK3-mediated necroptosis

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The proinflammatory cytokine TNF stimulates receptor-interacting serine/threonine-protein kinase 1 (RIPK1) ubiquitination, NFκB and MAPK activation, and induction of apoptosis or necroptosis (1, 2). TNF signaling via TNF receptor 1 (TNFRI) is highly regulated and results in the recruitment of several adapter proteins including TNFRI-associated death domain (TRADD) protein, the E3 ubiquitin ligase cellular death substrate 3 (cDSS3), TNFR-associated factor 2 (TRAF2) or 5, and the serine threonine death domain-containing kinase RIPK1 (complex I) (1). We have demonstrated that the kinase activity of RIPK1 is not required for NFκB activation (3); rather, RIPK1 is modified by the addition of Lys63-linked and linear polyubiquitin chains (3–6). Polynuqueicated RIPK1 then recruits NEMO/IκB kinase-γ (IKKγ) to mediate IKK activation and TAK1/TAB2/3 to mediate MAPK activation, resulting in antiapoptotic and proinflammatory gene expression (7, 8). Deubiquitination of RIPK1 by cylindromatosis (CYLD) results in the formation of a cytosolic complex containing TRADD, Fas-associated death domain protein (FADD), caspase-8, and RIPK1 (complex IIa) (2). Caspase-8 cleaves and inactivates RIPK1 and CYLD and stimulates apoptosis (9–11). In the absence of caspase-8 or the presence of caspase inhibitors, TNF family members and potentially other ligands stimulate the kinase activity of RIPK1 to induce necroptosis (9, 11–16). RIPK1 also is recruited to the Toll-like receptor adapter TRIF via the Rip homotypic interaction motif (RHIM) to mediate NFκB activation (17) and, under conditions of caspase-8 inhibition, initiates necroptosis (14, 16). Necrostatin-1 (Nec-1), an allosteric RIPK1 inhibitor, inhibits necroptosis induced by TNF or the TLR3 ligand poly I:C and abolishes the formation and activation of an RIPK1/3 complex (13–16, 18). Although the molecular details whereby RIPK1 initiates necroptosis are unclear, RIPK3 and the pseudo kinase MLKL appear to be required (2).

Genetic studies in mice have revealed cross-regulation between the apoptotic and necroptotic pathways. For example, the FADD/caspase-8/FLICE-like inhibitory protein long form (FLIL) complex regulates RIPK1 and RIPK3 activity during development, because the embryonic lethality associated with a caspase-8 deficiency is completely rescued by the absence of RIPK3 (19, 20). Similarly, RIPK1 deficiency rescues FADD-associated embryonic lethality (21). Thus, in the absence of FADD or caspase-8, embryos succumb to RIPK1- and RIPK3-dependent necroptosis. However, Fadd−/−/Ripk1−/− mice, die perinatally (21, 22), as do Ripk1−/− mice, revealing that RIPK1 has prosurvival roles beyond the regulation of the FADD/caspase-8/FLIL complex.

Significance

Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) is involved in TNF signaling and interacts with the related RIPK3 to regulate cell death and inflammation. RIPK1 has kinase-independent prosurvival and kinase-dependent prodeath functions. To identify the lineages that depend on RIPK1 for survival, we generated conditional Ripk1 mice. Acute Ripk1 deletion results in rapid death of the animal caused by extensive cell death in the intestinal and hematopoietic lineages. A hematopoietic RIPK1 deficiency stimulates proinflammatory cytokine/chemokine production and cell death, resulting in bone marrow failure. Hematopoietic failure is partially rescued by a RIPK3 deficiency, indicating that RIPK1-deficient hematopoietic cells undergo RIPK3-mediated necroptosis. These findings show that in the hematopoietic lineage RIPK1 suppresses RIPK3 activity and suggest that RIPK1-dependent necroptosis may contribute to human bone marrow failure syndromes.

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We have demonstrated that complete RIPK1 deficiency results in increased TNF-induced cell death that can be rescued, in part, by the absence of the TNFR1 (22, 23). However, Ripk1−/− Tnfr1−/− animals still succumb (23), indicating that other death ligands/pathways contribute to the RIPK1-associated lethality. Consistent with this hypothesis, RIPK3 deficiency recently has been shown to rescue the perinatal lethality observed in Ripk1−/− Tnfr1−/− mice (24, 25). Similarly, combined caspase-8 and RIPK3 deficiency also rescues the RIPK1-associated lethality (24–26). Collectively, these genetic studies in mice reveal that the perinatal death of Ripk1−/− mice reflects TNF-induced apoptosis and RIPK3-mediated necroptosis. The nature of the ligand(s) or the trigger(s) of RIPK3-mediated necroptosis in vivo remain unclear. However, Ripk1−/− MEFs are prone to necroptosis induced by poly IC or by treatment with type I or type II IFN (24, 25), suggesting that these pathways contribute. Although these studies reveal a regulatory role for RIPK1, the multorgan cell death and inflammation observed in the complete and compound RIPK1-knockout strains have made it difficult to discern the specific tissues that require RIPK1 for survival.

**Results**

To identify the cell types and lineages in the adult dependent on RIPK1 for survival, we mated conditional Ripk1 mice to the tamoxifen-inducible Rosa26CreER<sup>T2</sup> transgenic mice (27) (Fig. 1A and Fig. S1A). Administration of three daily injections of 1 mg of tamoxifen resulted in efficient RIPK1 deletion in RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> mice and in the rapid induction of pathology in the intestinal and hematopoietic lineages (Fig. 1 and Fig. S1). RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> mice treated with tamoxifen developed diarrhea and weight loss and were killed 24 h after the third tamoxifen injection (Fig. 1 B and C). Histological analysis revealed loss of bone marrow cellularity and erosion of villus structures with pronounced loss of intestinal epithelial cells in the ileum and colon (Fig. 1B). Increased numbers of dying cells that stained positive for cleaved caspase-3 were detected in the thymus, spleen, and small and large intestines of RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> mice, indicating that RIPK1 deficiency results in caspase-dependent apoptosis in these lineages (Fig. S1 E and F). Cell death in thymus and spleen resulted in significant decreases in overall thymic and splenic cellularity (Fig. S1H), features observed in mice with complete RIPK1 deficiency (22). Cell death and/or inflammation was not evident in the other organs examined (Fig. S1G), nor was there evidence of anemia, indicating that intestinal epithelial cell loss is likely the major contributing factor in the death of the tamoxifen-treated RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> mice. Genomic PCR confirmed Ripk1 deletion in the intestines and the hematopoietic organs of tamoxifen-treated RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> mice (Fig. S1 B–D). Tamoxifen-treated RosaCreER<sup>T2</sup> mice did not develop intestinal or hematopoietic pathology.

RIPK1 deficiency resulted in decreases in the absolute number of lymphoid and myeloid cells (Fig. S1 I–K). The Lin−Sca1<sup>+</sup>cKit<sup>+</sup> (L-SK) hematopoietic stem and progenitor cells (HSPCs) and the Lin−Sca1<sup>+</sup>cKit<sup>+</sup>high (L−SK) myeloid-enriched progenitors also were depleted significantly (ninefold) in tamoxifen-treated RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> mice as compared with tamoxifen-treated RosaCreER<sup>T2</sup> controls (Fig. 1D). The LSK population was analyzed further by staining with CD34 and fetal liver kinase 2 (Flk2) antibodies to quantify the long- and short-term hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs), all of which were reduced significantly in RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> mice (Fig. 1D). To examine stem and progenitor cell function, we flared bone marrow from tamoxifen-treated RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> control mice in an in vitro hematopoietic colony assay and observed >75% reduction in progenitor activity in the RIPK1-deficient cultures (Fig. 1E). Collectively, these data show that acute ablation of RIPK1 in adult mice results in intestinal epithelial and hematopoietic cell death, revealing crucial prosurvival roles for RIPK1 in these lineages.

Hematopoietic cell death observed upon acute ablation of RIPK1 may be a secondary effect resulting from the inflammation induced by cell death in the intestine and potentially other lineages. Consistent with this possibility, serum proinflammatory chemokine and cytokine levels were increased significantly in tamoxifen-treated RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> mice but not in controls (Fig. S2). To exclude the potential confounding effects of a proinflammatory microenvironment on HSPC survival, we used the Vav-iCre transgene to delete Ripk1 in hematopoietic cells. Half of the Vav-iCre Ripk1<sup>fl/fl</sup> mice exhibited histopathological features associated with complete RIPK1 deficiency and died during the postnatal period, likely as the result of reported Vav-iCre activity in the germ line (28). The remaining Vav-iCre Ripk1<sup>fl/fl</sup> mice survived beyond the postnatal period (average survival 35 d) but appeared smaller and weighed significantly less than Vav-iCre Ripk1<sup>fl/fl+</sup> littermate controls (Fig. 2 A and B). Ripk1<sup>fl</sup> deletion was detected in bone marrow, spleen, and thymus of these mice but not in nonhematopoietic tissues (Fig. S3A). The Vav-iCre Ripk1<sup>fl/fl</sup> mice developed marked pancytopenia and anemia with an average 38-fold decrease in bone marrow cellularity (Fig. 2 C–E and Fig. S3). Histological examination revealed hypocellular bone marrow and extensive cell death in the thymus and spleen of Vav-iCre Ripk1<sup>fl/fl</sup> mice compared with Vav-iCre Ripk1<sup>fl/fl+</sup> controls (Fig. 2 D and E and Fig. S3E). Lineage
CXC chemokine (LIX) (the mouse ortholog of CXCL5) was upregulated, whereas the IFN-γ, IL-6, and G-CSF (Fig. S5). The anti-inflammatory cytokine IL-10 also was up-regulated in these mice compared with littermate controls (Fig. S3 A). The hematopoietic cell loss observed in 2-wk-old Vav-iCre Ripk1fl/+ mice could reflect RIPK3-deficient pancytopenia, anemia, and bone marrow hypocellularity associated with morbidities also are observed in human patients with bone marrow failure (29–31). These data suggest that RIPK1 deficiency sensitizes HSPCs to cytokine-mediated cell death. To test this hypothesis, we isolated bone marrow from 14-d-old Vav-iCre Ripk1fl/+ and added TNF, TNF-related apoptosis-inducing ligand (TRAIL), or type I or type II IFN to the hematopoietic colony assays. Colony number and size were reduced significantly when the RIPK1-deficient progenitors were cultured with TNF or with type I or type II IFN but not when cultured with TRAIL; these findings indicate that, in addition to TNF, RIPK1-deficient hematopoietic progenitors also are sensitive to cell death induced by type I or type II IFN (Fig. 3D).

RIPK1 deficiency had clear effects on all the hematopoietic lineages examined (Figs. 2 and 3 and Fig. S3). Mice deficient in RIPK1 also were significantly smaller than littermate controls (Fig. 2A), and most of these mice exhibited focal areas of inflammation (Fig. S3). To determine whether RIPK1 has a hematopoietic cell-intrinsic survival function, we transplanted embryonic day 14.5 fetal liver cells from Vav-iCre Ripk1fl/+ mice and littermate controls into lethally irradiated syngeneic recipients and monitored the relative contributions of donor and host cells to hematopoiesis (Fig. 4A). Mice transplanted with RIPK1-deficient fetal liver cells exhibited significant reductions in the HSPC population as early as 2 wk posttransplantation (Fig. 4B). Eight weeks after transplantation, the RIPK1-deficient HSPC populations were nearly undetectable in the transplanted mice (Fig. 4C), confirming that RIPK1 has a cell-intrinsic survival function in HSPC.

Although cleaved caspase-3+ hematopoietic cells were detected in the thymus and spleen, dying cells that were negative for caspase-3 were observed in the RIPK1-deficient bone marrow and spleen, suggesting that some hematopoietic cells die from necrosis rather than apoptosis. We hypothesized that the HSPC death in Vav-iCre Ripk1fl/+ mice could reflect RIPK3-dependent necroptosis. To address the potential role of RIPK3, we generated and analyzed Vav-iCre Ripk1fl/+ Ripk3−/− mice. In contrast to Ripk1fl/+ Ripk3−/− double-knockout mice, which die during the postnatal period (24–26), Vav-iCre Ripk1fl/+ Ripk3−/− mice develop normally and reach adulthood without showing any macroscopic or histologic abnormalities (Fig. 5A, B, and E). Remarkably, RIPK3 deficiency rescued the pancytopenia, anemia, and bone marrow hypocellularity associated with significantly down-regulated in Vav-iCre Ripk1fl/+ mice; these features also are observed in human patients with bone marrow failure (29–31). This analysis by flow cytometry confirmed decreases in the absolute numbers of lymphoid cells and myeloid cells in Vav-iCre Ripk1fl/+ mice compared with littermate controls (Fig. S3 B–D). Despite the hematopoietic cell loss, some of the Vav-iCre Ripk1fl/+ mice exhibited mild to moderate inflammation characterized by focal areas of granulocyte infiltration in skin or liver (Fig. S3F). These histologic findings indicate that hematopoietic RIPK1 deficiency can result in tissue inflammation.

These mouse genetic studies using hematopoietic or inducible Cre recombinase reveal crucial roles for RIPK1 in hematopoietic cell survival. To reveal the kinetics of hematopoietic cell death, we examined 2-wk-old Vav-iCre Ripk1fl/+ mice for evidence of hematopoietic deficiency. We detected no significant decreases in peripheral blood counts or in the cellularity of the bone marrow or spleen, although modest decreases in the absolute numbers of T and B cells were evident in thymus, spleen, and bone marrow (Fig. S4). However, statistically significant decreases in absolute number of HSPCs and in colony-forming activity were observed (Fig. 3 A and C). HSPC number and colony formation in Vav-iCre Ripk1fl/+ mice were reduced further at 5 wk (Fig. 3 B and C). The hematopoietic cell loss observed in these mice was associated with increases in serum chemokines including interferon gamma-induced protein-10 (IP-10), keratinocyte chemotactic factor (KC), monocyte chemotactic protein-1 (MCP-1), and monocline induced by γIFN (MIG), and proinflammatory cytokines (TNF-α, IFN-γ, IL-6, and G-CSF) (Fig. S5). The anti-inflammatory cytokine IL-10 also was up-regulated, whereas the IFN-γ-regulated chemokine LPS-induced CXC chemokine (LIX) (the mouse ortholog of CXCL5) was

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hematopoietic RIPK1 deficiency (Fig. 5 C–E). These genetic data reveal that, in the absence of RIPK1, hematopoietic cells undergo RIPK3-mediated necroptosis. Thus, in contrast to published studies showing that RIPK1 is essential for TNF- and Fas-mediated (9, 12, 13, 24, 25, 32) as well as for TLR3/4-mediated necroptosis (14, 33), we found that RIPK1 is not required to execute necroptotic hematopoietic cell death in vivo. Rather, our data suggest that RIPK1 functions in the hematopoietic lineage to prevent RIPK3-initiated necroptosis. Consistent with the notion that necroptosis constitutes an inflammatory form of cell death, we found serum cytokines and chemokines (TNF-α, IFN-γ, IL-6, IP-10, KC, MCP-1, MIG, IL-10, and G-CSF) up-regulated in Vav-iCre Ripk1fl/fl mice (Fig. S5); however, cytokine/chemokine levels were significantly reduced in Vav-iCre Ripk1fl/fl Ripk3–/– mice, in which necroptotic death was prevented (Fig. 5 F and G). Furthermore, Vav-iCre Ripk1fl/fl Ripk3–/– HSPCs no longer appeared as sensitive to γ- or IFN-induced cell death but retained sensitivity to TNF-induced apoptosis (Fig. 5H). These in vitro colony data suggest that RIPK1-deficient HSPCs undergo TNF-induced apoptosis and RIPK3-mediated necroptosis potentially triggered by TNF and/or type I or type II interferons.

Analysis of the LSK population in Vav-iCre Ripk1fl/fl Ripk3–/– mice revealed that the absence of RIPK3 did not completely rescue the absolute numbers of HSPC or in vitro colony formation (Fig. S1 and Fig. S6F), indicating that hematopoietic stem and progenitor repopulating capability may be compromised. To test this notion directly, we transplanted bone marrow from Vav-iCre Ripk1fl/fl Ripk3–/– mice and littermate controls into lethally irradiated syngeneic recipients and monitored survival. In contrast to controls, five of six of the mice transplanted with

Fig. 5. RIPK3 deficiency partially rescues the lethal bone marrow failure of Vav-iCre Ripk1fl/fl mice. (A) Bone marrow cellularity for Vav-iCre Ripk1fl/fl mice (n = 10) and Vav-iCre Ripk1fl/fl Ripk3–/– (n = 5) mice. (B) Blood cell counts and hematocrits of diseased Vav-iCre Ripk1fl/fl mice (n = 14) compared with age-matched Vav-iCre Ripk1fl/fl Ripk3–/– mice (n = 5) and littermate controls Vav-iCre Ripk1fl/fl (n = 11) and Vav-iCre Ripk1fl/fl Ripk3–/– (n = 3). (B) Bone marrow cellularity for Vav-iCre Ripk1fl/fl mice (n = 11), Vav-iCre Ripk1fl/fl Ripk3–/– mice (n = 5) and littermate controls Vav-iCre Ripk1fl/fl (n = 11) and Vav-iCre Ripk1fl/fl Ripk3–/– (n = 3). (B) Bone marrow cellularity for Vav-iCre Ripk1fl/fl mice (n = 11), Vav-iCre Ripk1fl/fl Ripk3–/– mice (n = 5) and littermate controls Vav-iCre Ripk1fl/fl (n = 11) and Vav-iCre Ripk1fl/fl Ripk3–/– (n = 3). (B) Bone marrow cellularity for Vav-iCre Ripk1fl/fl mice (n = 11), Vav-iCre Ripk1fl/fl Ripk3–/– mice (n = 5) and littermate controls Vav-iCre Ripk1fl/fl (n = 11) and Vav-iCre Ripk1fl/fl Ripk3–/– (n = 3). (B) Bone marrow cellularity for Vav-iCre Ripk1fl/fl mice (n = 11), Vav-iCre Ripk1fl/fl Ripk3–/– mice (n = 5) and littermate controls Vav-iCre Ripk1fl/fl (n = 11) and Vav-iCre Ripk1fl/fl Ripk3–/– (n = 3).
Vav-iCre Ripk1 D138N/D138N bone marrow cells died (Fig. 5J). Although RIPK3 deficiency reduced inflammation and prevented bone marrow failure in Vav-iCre Ripk1 D138N/D138N mice, hematopoietic precursors lacking both RIPK1 and RIPK3 lacked long-term multilineage repopulating activity. Based on our in vitro data, we speculate that Vav-iCre Ripk1 D138N/D138N RIPK3−/− HSPCs remain sensitive to TNF-induced apoptosis and this sensitivity compromises HSPC function in transplanted mice.

Discussion

We demonstrate that hematopoietic RIPK1 deficiency results in HSPC loss and subsequent pancytopenia, anemia, and bone marrow failure. An absence of RIPK3 reduces the inflammation and hematopoietic cell death, and consequently normal numbers of erythroid, lymphoid, and myeloid cells are observed in Vav-iCre Ripk1 D138N/D138N mice (37). Collectively, these data indicate that RIPK1-deficient hematopoietic cells undergo RIPK3-mediated necroptosis, showing that RIPK1 is not absolutely required for necroptotic death in vivo. These findings are supported by transplants with Ripk1 D138N/D138N Ripk3−/− progenitors (26) and in vitro studies demonstrating that necroptosis can be RIPK1 independent (34–36). Importantly, we provide evidence that pancytopenia and bone marrow failure reflect cell autonomous effect(s), because mice reconstituted with Vav-iCre Ripk1 D138N/D138N fetal liver cells recapitulate the HSPC and hematopoietic lineage loss observed in Vav-iCre Ripk1 D138N/D138N mice. These data demonstrate that RIPK1 loss in hematopoietic cells results in RIPK3 activation and induction of necroptosis, revealing clear anti-inflammatory roles for RIPK1 in the hematopoietic lineage.

How RIPK1 regulates RIPK3 activity is unclear; however, steady-state hematopoiesis appears normal in kinase-inactive Ripk1 D138N/D138N mice (37), suggesting that the kinase activity of RIPK1 is not required to regulate RIPK3 activity. Moreover, genetic rescue appears specific to the hematopoietic and skin lineages (26, 38), because RIPK3 deficiency has marginal effects on the overall survival of RIPK1-deficient mice (24–26).

In both the inducible and hematopoietic lineage knockout models, the HSPC population appears particularly dependent on RIPK1 for survival. Twenty-four hours after the final tamoxifen injection, we observe an 8- to 10-fold decrease in HSPCs with more modest reductions in lineage-restricted cells (Fig. 1D and Fig. S1 A–C). Consistently, the absolute numbers of HSPCs are reduced in 2-wk-old Vav-iCre Ripk1 D138N/D138N mice and become nearly undetectable by age 5 wk.

Our data suggest that hematopoietic RIPK1 deficiency is sufficient to stimulate necroptosis and cytokine/chemokine production and/or release; however, these mice develop only mild tissue inflammation before succumbing to hematopoietic failure. Therefore, it remains unclear from these studies whether hematopoietic RIPK1 deficiency is sufficient to trigger systemic inflammation. What triggers hematopoietic necroptosis in Vav-iCre Ripk1 D138N/D138N mice also is unknown. Hematopoietic cell death may reflect unregulated RIPK3 activity and be independent of receptor signaling (as suggested in ref. 39). Alternatively, necroptosis may be triggered by infection, stress, and/or injury or by the transition from the fetal liver to the bone marrow microenvironment. It also is possible that hematopoietic RIPK1 deficiency stimulates the degradation of prosurvival proteins cIAP1/2 and TRAF2, thereby making cell death more likely. In addition to mediating survival, the IAPs have prosurvival roles for RIPK1 in the hematopoietic lineage.

Materials and Methods

Mice. Ripk1 conditional mice (Ripk1 D138N/D138N mice; a generous gift from Vishva Dixit, Genentech, San Francisco, Vav-iCre) were crossed with Rosa26-CreERT2 (Rosa26-CreERT2) mice (obtained from Jackson Laboratory). When mice became moribund, they were weighed and killed humane. Complete blood counts and hematocrits were performed on a Hemavet 950FS analyzer (Drew Scientific). For bone marrow transplantation studies, recipient C57BL/6 CD4.1 mice received 11 Gy of total body irradiation in a split dose (550 rads) with a 4-h rest between doses using a CES173 irradiator. Irradiated recipients were reconstituted by i.v. injection of 2 × 106 E14.5 fetal liver cells or bone marrow cells. Recipients were maintained on medicated water and monitored daily for signs of failed engraftment. For Vav-iCre Ripk1 transplants mice were killed at 2 and 8 wk after transplantation. All animal procedures used in this study were approved by The University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Histology. Tissues were fixed in 10% formalin (Fisher Scientific), and bone marrow was decalcified in Cal-Rite (Richard Allen Scientific) for 48 h. Samples were stained with H&E or with a cleaved caspase-3 antibody (Cell Signaling) at a 1:200 dilution. Images were taken at 10–20× magnification on an Olympus BX41 microscope using an Evolution MP 5.0 Mega-Pixel Camera (MediaCybernetic) and QCapture Pro software (Qimaging).

Colony-Forming Assay. Bone marrow cells were seeded in MethoCult medium M3434 (STEMCELL Technologies), and total colony number was determined following the manufacturer’s protocol. Ligands were added at the time of plating at the following concentrations: mTRAIL, 200 ng/mL (Pepro Tech); mTNF-α, 10 ng/mL (R&D); mIFN-α 100 units/mL (PBL Interferon Source); mIFN-γ, 10 ng/mL (Pepro Tech).
Flow Cytometry. Single-cell suspensions were stained with cell-surface antibodies for myeloid (Gr-1 and CD11b) and lymphoid (CD3, CD4, CD8, B220) markers. For LSX analysis, bone marrow cells were stained with a biotin lineage mixture, Sca-1, c-Kit, CD34, and Fik2. To distinguish between donor and host hematopoietic cells in the transplant studies, an anti-CD45.2 antibody was added to the LSX staining mixture. All samples were run on a BD LSRII flow cytometer (BD Bioscience) and analyzed using FlowJo software (Tree Star). A complete list of antibodies including clone numbers is given in Table 51.

Serum Cytokines. Serum cytokines were measured using a 12-plex protein/peptide multiplex analysis (Luminex Technology) conducted by the National Mouse Metabolic Phenotyping Center at the University of Massachusetts Medical School. Chemokines and cytokines that were below the level of detection were assigned the value of zero.


Statistical Measures. Statistical analyses were performed using GraphPad Prism software, version 6.0. Kaplan–Meier survival curves were analyzed with a logrank test with the use of the log-rank confidence interval. A two-sided P < 0.05 was considered statistically significant for Student’s t tests and non-parametric Mann–Whitney tests.
Supporting Information

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SI Materials and Methods

DNA was isolated using a DNAeasy kit (Qiagen) following the manufacturer’s protocol. Two hundred nanograms of DNA was used per reaction using the following program: 95 °C for 5 m, 35 cycles of 95 °C for 1 m, 58 °C for 1 m, 72 °C for 1 m for 35 cycles, followed by 72 °C for 5 m. DNA was amplified using the following primers: primer 1: 5′-GGA GGC AAT GAG AAG AAA ACA GC-3′; primer 2: 5′-ATA GCA GAG GGC TGG ATC TG-3′; primer 3: 5′-CCC CTC TTC CAA GAG AAC CT-3′. PCR products were run on a 2% agarose gel, and images were taken on a 2UV Transilluminator (UVP) using LabWorks image acquisition and analysis software.
**Fig. S1.** Acute ablation of receptor-interacting serine/threonine-protein kinase 1 (RIPK1) results in the loss of the intestinal and hematopoietic lineages. (A) Experimental design. (B) PCR of tail DNA from the indicated genotypes taken before and after tamoxifen treatment. (C) PCR of DNA isolated from ileum and colon of the indicated genotypes after tamoxifen treatment. (D) PCR of DNA isolated from the thymus, spleen, and bone marrow of the indicated genotypes after tamoxifen treatment. (E, Left) Representative images of colon sections stained with cleaved caspase-3 from RosaCreER<sup>T2</sup> and RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> tamoxifen-treated mice. (Right) Quantification of cleaved caspase-3<sup>+</sup> cells per 40x field of tamoxifen-treated RosaCreER<sup>T2</sup> (n = 2) and RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> mice (n = 4). (F, Left) Representative images of thymus and spleen stained with cleaved caspase-3 from RosaCreER<sup>T2</sup> and RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> tamoxifen-treated mice. (Right) Quantification of cleaved caspase-3<sup>+</sup> cells per 40x field of tamoxifen-treated RosaCreER<sup>T2</sup> (n = 2) and RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> (n = 4) mice. (G) Representative images of H&E-stained lung, kidney, liver, and skin sections from RosaCreER<sup>T2</sup> and RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> tamoxifen-treated mice. (H) Total cellularity of thymus, spleen, and bone marrow from RosaCreER<sup>T2</sup> (n = 6-8) and RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> (n = 10) mice. (I-K) Lineage analysis of thymus (I), spleen (J), and bone marrow (K) of RosaCreER<sup>T2</sup> (n = 4) and RosaCreER<sup>T2</sup> Ripk1<sup>fl/fl</sup> (n = 6) tamoxifen-treated mice. Error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.0001 (Student’s t tests). DN, CD4 and CD8-negative; DP, CD4 and CD8-positive; Gr/Mac, Gr-1-positive and Mac-1-positive; Mac, Mac-1-positive.
Fig. S2. Conditional deletion of RIPK1 results in the production of proinflammatory chemokines and cytokines. (A and B) Levels of chemokines (A) and cytokines (B) in the serum of RosaCreER^{T2} (n = 4) and RosaCreER^{T2} Ripk1^{fl/fl} (n = 10) tamoxifen-treated mice. Error bars represent SEM. *P < 0.05, **P < 0.01 (Mann–Whitney test). IP-10, interferon gamma-induced protein-10; KC, keratinocyte chemoattractant; LIX, LPS-induced CXC chemokine; MCP-1: monocyte chemoattractant protein-1; MIG: monokine induced by γ-IFN.
Deletion of RIPK1 in the hematopoietic lineage results in lymphoid and myeloid lineage loss with modest effects in nonhematopoietic tissues. (A) PCR of DNA isolated from the thymus, spleen, bone marrow (B.M.), and tail of 6-d-old Vav-iCre Ripk1^{fl/+} and Vav-iCre Ripk1^{fl/fl} mice. (B–D) Lineage analysis of thymus (B), spleen (C), and bone marrow (D) of Vav-iCre Ripk1^{fl/+} mice (n = 7) and littermate Vav-iCre Ripk1^{fl/+} controls (n = 5). (E. Upper) Representative images of thymus and spleen sections stained with cleaved caspase-3. (Lower) Quantification of cleaved caspase-3^{+} cells per 40x field of thymus and spleen of Vav-iCre Ripk1^{fl/+} (n = 4) and Vav-iCre Ripk1^{fl/fl} (n = 5) mice. (F) Representative images of H&E-stained heart, small intestine, colon, kidney, liver, lung, and skin from Vav-iCre Ripk1^{fl/+} and Vav-iCre Ripk1^{fl/fl} mice. Error bars represent SEM. *P < 0.05, **P < 0.01 ***P < 0.0001 (Student's t test).
Fig. S4. Bone marrow failure of Vav-iCre Ripk1fl/fl mice is progressive. (A) Total body weight of Vav-iCre Ripk1fl/+ (n = 4) and Vav-iCre Ripk1fl/fl (n = 3) mice at age 2 wk. (B) Complete blood counts of Vav-iCre Ripk1fl/+ (n = 5) and Vav-iCre Ripk1fl/fl (n = 5) mice at age 2 wk. (C) Representative images of H&E-stained thymus, spleen, and bone marrow sections from the indicated genotypes. (D) Single-cell suspensions of the thymus, spleen, and bone marrow were counted by trypan blue exclusion. Total viable cell number is shown for Vav-iCre Ripk1fl/+ (n = 5) and Vav-iCre Ripk1fl/fl (n = 5) mice. (E–G) Lineage analysis of thymus (E), spleen (F), and bone marrow (G) of Vav-iCre Ripk1fl/+ (n = 5) and Vav-iCre Ripk1fl/fl (n = 5) mice. Error bars represent SE. *P < 0.05, **P < 0.01 ***P < 0.0001 (Student’s t tests).
Fig. S5. Loss of RIPK1 in the hematopoietic system results in the production of proinflammatory chemokines and cytokines. Levels of chemokines (A) and cytokines (B) in the serum of 14-d-old Vav-iCre Ripk1\textsuperscript{fl/+} (n = 4) and in Vav-iCre Ripk1\textsuperscript{fl/fl} (n = 6) mice and 35-d-old Vav-iCre Ripk1\textsuperscript{fl/+} (n = 5) and Vav-iCre Ripk1\textsuperscript{fl/fl} (n = 9) mice. Error bars represent SEM. *P < 0.05, **P < 0.01 ***P < 0.001 (Mann–Whitney test). Black bars represent Vav-iCre Ripk1\textsuperscript{fl/+} mice and white bars represent Vav-iCre Ripk1\textsuperscript{fl/fl} mice.
Fig. S6. RIPK3 deficiency partially rescues the lymphoid and myeloid lineage loss in Vav-iCre Ripk1fl/fl mice. (A) Representative images of thymus and spleen sections from the indicated genotypes at age 5 wk stained with cleaved caspase-3. (B) Total cellularity from thymus and spleen was calculated by trypan blue exclusion assay in 5-wk-old Vav-iCre Ripk1fl/+ Ripk3−/− (n = 3) and Vav-iCre Ripk1fl/fl Ripk3−/− (n = 5) mice. (C–E) Lineage analysis of thymus (C), spleen (D), and bone marrow (E) of Vav-iCre Ripk1fl/+ Ripk3−/− (n = 3) and Vav-iCre Ripk1fl/fl Ripk3−/− (n = 5) mice. (F) Colony-forming assay of bone marrow cells isolated from 35-d-old Vav-iCre Ripk1fl/+ Ripk3−/− (n = 3) and Vav-iCre Ripk1fl/fl Ripk3−/− (n = 5) mice. Error bars represent SEM. *P < 0.05, **P < 0.01 ***P < 0.0001 (Student’s t tests).
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APC, allophycocyanin; PE, phycoerythrin.