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ABSTRACT

Caspase-8 (Casp8) suppresses receptor-interacting protein kinase-3 (RIPK3)/mixed lineage kinase domain-like protein (MLKL)-dependent necroptosis, demonstrated by the genetic evidence that deletion of Ripk3 or Mlkl prevented embryonic lethality of Casp8-deficient mice. However, the detailed mechanisms by which Casp8 deficiency triggers necroptosis during embryonic development remain unclear. In this article, we show that Casp8 deletion caused formation of the RIPK1-RIPK3 necrosome in the yolk sac, leading to vascularization defects, prevented by MLKL and RIPK3 deficiency, or RIPK3 RHIM mutant (RIPK3 V448P), but not by the RIPK1 kinase-dead mutant (RIPK1 K45A). In addition, Ripk1K45A/K45ACasp8/2 mice died on embryonic day 14.5, which was delayed to embryonic day 17.5 by ablation of one allele in Ripk1 and was completely rescued by ablation of Mlkl. Our results revealed an in vivo role of RIPK3 RHIM and RIPK1K45A scaffold-mediated necroptosis in Casp8 deficiency embryonic development and suggested that the Casp8-deficient yolk sac might be implicated in identifying novel regulators as an in vivo necroptotic model.

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Abbreviations used in this article: Casp, caspase; Cat#, catalog number; E, embryonic day; IP, immunoprecipitation; MEF, mouse embryonic fibroblast; MLKL, mixed lineage kinase domain-like protein; Nec-1, necrostatin-1; NP-40, Nonidet P-40; RHIM, RIP homotypic interaction motif; RIPK, receptor-interacting protein kinase; WT, wild-type; ZBP1, Z-DNA–binding protein 1.

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catalytic activity. Although the absence of Casp8 leads to embryonic lethality, mice may survive past weaning if either of the necroptosis-mediated genes, Ripk3 or Mlkl, is coablated (24–27). Mutation in the catalytic site of Casp8 (C362S or C362A), similar to disruption of Casp8, leads to embryonic lethality (28, 29), whereas mice harboring oligomerization-deficient (F122G and L123G) and noncleavable mutants (D387A or D212A/D218A/D225A/D387A) are viable (28, 30–32). In the specific absence of catalytic activity of Casp8 or the Casp8 scaffold in endothelial cells, Casp8$^{fl/fl}$, Casp8$^{R362S/ R362S}$, and Casp8$^{R225A/ R225A}$ embryos showed a similar gross pathology associated with a defect in yolk sac vascularization, which causes embryonic lethality at the same developmental stage as Casp8 deficiency (28, 33). However, the mechanisms of necroptosis in yolk sac triggered by Casp8 deficiency during embryogenesis remain to be established.

In this study, we generated Casp8 null mutation mice and showed embryonic lethality of Casp8-deficient mice by inducing RIPK1-RIPK3–mediated necroptosis in yolk sac vascularization, which is prevented by coablation of Ripk3, Mlkl, or RIPK3 RHIM mutant (RIPK3 V448P), but not by the RIPK1 kinase–dead mutant (RIPK1 K45A), indicating that Casp8 deficiency triggers RIPK1 kinase–independent necroptosis during embryonic development. Furthermore, the death of Ripk1$^{K45A/K45A}$ Casp8$^{−/−}$ on embryonic day 14.5 (E14.5) was delayed to E17.5 by ablation of one allele in Ripk1 or was fully rescued by coablation of Mlkl, suggesting that RIPK1 scaffold-dependent necroosome formation in yolk sac mediated by the RHIM interaction of RIPK1-RIPK3 is a mechanism of embryonic lethality in Casp8-deficient mice.

MATERIALS AND METHODS

Mice

Casp8$^{−/−}$ knockin mice were generated by mutating “TT” to “A” in exon 8 of mouse Casp8 locus via the CRISPR/Cas9 system (Bioray Laboratories, Shanghai, China). The Casp8$^{−/−}$ mice were backcrossed with C57BL/6 for eight generations. Genotyping of Casp8$^{−/−}$ mice was conducted with mouse-tail DNAs by PCR (95°C, 4 min; 95°C, 30 s; 58°C, 30 s; 72°C, 30 s; 72°C, 5 min; 35 cycles) and confirmed by sequencing analysis. The PCR primers used for genotyping were the following: forward primer 5’-CA GAGGCTCTGAGTAAAGCC-3’; reverse primer 5’-CTGAGGA CATCTTCCCTCAG-3’; and sequencing primer 5’-CAGAGG CTCTGAGTAAAGCC-3’. Mlkl$^{−/−}$, Ripk1$^{−/−}$, Ripk3$^{K45A/K45A}$, and Ripk3$^{V448P/V448P}$ mice have been previously described and maintained on C57BL/6 genetic background (9, 14, 34, 35). All mice were housed and cared for in a specific pathogen-free environment. Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute of Nutrition and Health, Shanghai Institutes for Biological Sciences, University of Chinese Academy of Sciences.

Abs and reagents

The primary Abs used were PARP1 (catalog number [Cat# 9542s; CST]), Casp3 (Cat#96628; CST), Casp8 (Cat#8592s; CST), RIP1 (Cat#610459; BD), P-RIP1 (Ser166) (Cat#311225; CST), RIP3 (Cat#ab72106; Abcam), P-RIP3 (Ser232) (Cat#ab195117; Abcam), MLKL (Cat#AP14272b; Abgent), P-MLKL (Ser345) (Cat#ab196436; Abcam), GAPDH (Cat#G9545; Sigma), Z-DNA-binding protein 1 (ZBP1; Cat#AG-20B-0010; AdipoGen), CD3-FITC (Cat#11-0031-82; eBioscience), and B220-allophycocyanin (Cat#17-0452-83; eBioscience). The compounds used were Z-VAD-FMK (Cat#HY-16658; MCE), Nec-1 (Cat#HY-15750; MCE), GSK’872 (Cat#HY-101872; MCE), and Mouse TNF-α (Cat#410-MT-050; R&D).

Cell culture

Mouse embryonic fibroblast (MEF) cells were maintained in high-glucose DMEM (Cat#SH30243; Hyclone) supplemented with 10% FBS (Cat#04-001A-1A; Bioind) and 100 U penicillin/streptomycin (Cat#15140122; Life Technologies). Cells were maintained at 37°C and 5% CO₂. Ripk3$^{−/−}$, Ripk3$^{V448P/V448P}$, Casp8$^{−/−}$, Mlkl$^{−/−}$Casp8$^{−/−}$, and Ripk1$^{K45A/K45A}$Casp8$^{−/−}$ MEFs were isolated from E12.5 embryos. Casp8$^{−/−}$ MEFs were isolated from E10.5 embryos, head and visceral tissues were dissected, and remaining bodies were incubated with 4 ml trypsin/EDTA solution (Life Technologies) per embryo at 37°C for 1 h. After trypsinization, an equal amount of medium was mixed and pipetted up and down a few times cultured in DMEM medium (10% FBS, 1% penicillin/streptomycin) and transformed with an SV40 large T Ag-expressing lentivirus.

Mice embryos survival assay

Male and female mice at 8 wk old were crossed in one box; mice were designated E0.5 on the morning a vaginal plug was detected. Embryos ranging in age from E9.5 to E17.5 were analyzed. Yolk sacs were harvested multiple times with PBS and then Nonidet P-40 (NP-40) buffer lysis for 30 min at 4°C. For Casp8 expression test, ~E10.5–E12.5 embryos were harvested in 6 M urea lysis buffer (6 M urea, 20 mM Tris–HCl [pH 7.5], 135 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100) supplemented with 1 mM PMSF and 1× protease inhibitor mixture (Cat#469-3132001; Roche). Lysates were centrifuged at 16,000 × g for 30 min at 4°C, and supernatants were diluted with 4× SDS-PAGE sample loading buffer (240 mM Tris–HCl [pH 6.8], 40% [v/v] glycerol, 8% [v/v] SDS, 0.04% bromophenol blue, and 5% [v/v] 2-ME).

Immunohistochemistry and TUNEL staining

E11.5 or E15.5 embryos were fixed in 4% paraformaldehyde; paraffin-embedded tissue sections were stained with 0.05 μg/ml anti-cleaved Casp3 Ab (Cell Signaling Technology). TUNEL staining was performed using the Roche Kit with Proteinase K digestion. Stained slides were digitized using a Nanozoomer digital slide scanner.

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**Immunoprecipitation of RIPK1**

Embryo tissue, yolk sac, and MEF cells were lysed with NP-40 buffer (120 mM NaCl, 10 mM Tris–HCl [pH 7.4], 1 mM EDTA, 0.2% NP-40, 10% glycerol) supplemented with 1 mM PMSF and 1× protease inhibitor mixture (Cat#4693132001; Roche). For immunoprecipitation (IP) of RIPK1–RIPK3–associated complexes, the lysate was incubated with anti-RIPK1 overnight at 4°C. The immunocomplex was captured by protein A/G agarose (Cat#16-1001; Millipore). Beads were washed four times, and the immunocomplex was eluted from beads by loading buffer. Immune complexes were eluted by boiling in reducing Western blot loading buffer and resolved by Western blot using the Abs described.

**Flow cytometry**

Lymphocytes were isolated from the spleen, lymph nodes, and blood of mice. Abs against mouse CD3 (Cat#11-0031-82; eBioscience) and B220 (Cat#17-0452-83; eBioscience) were fluorescence conjugated and used for flow cytometry analysis. Single-cell suspensions of lymphocytes were stained on ice for half an hour with fluorescence-conjugated Abs in the staining buffer. After staining, cells were immediately analyzed by flow cytometry (FACSaria III; BD Biosciences).

**Statistical analysis**

Data in this study are representative results of at least three independent experiments. The in vitro results were presented as the mean ± SD of triplicate wells. The statistical significance of data was evaluated by Student t test in which p < 0.01 was considered significant and p < 0.001 was highly significant. The statistical calculations were performed with GraphPad Prism software.

**RESULTS**

**Apoptosis and necroptosis during embryogenesis in mice with Casp8 deletion**

Both tie-1-Cre– and tie-2-Cre–specific deletion of Casp8 in endothelial cells caused embryonic lethality at around E11.5, coinciding with formation of an abnormal yolk sac vasculature, which resembles the phenotype of Casp8−/− embryos (28, 33). When the necroptosis-mediating gene Ripk3 or Mlkl was co-deleted, Ripk3−/-/Casp8−/- or Mlkl−/-/Casp8−/- mice survived past weaning (24–26). To determine the mechanism of necroptosis in vivo, we generated a Casp8 null allele by replacing AA with T in exon 8 using the CRISPR-Cas9 system (Supplemental Fig. 1A, 1B). Consistent with previous reports, we crossed Casp8−/+ mice to generate Casp8−/- mutant animals and found that Casp8−/- mice died during embryogenesis, because interconnected of heterozygous mice generated only Casp8−/+ and Casp8−/+ offspring (Supplemental Fig. 1C). We confirmed the absence of Casp8 protein using E10.5, E11.5, and E12.5 embryo blots with anti-Casp8, which revealed loss of Casp8 expression in Casp8−/- deletion embryos (Supplemental Fig. 1D).

Casp8−/- mice died at around E11.5 because of hyperemia in the abdominal areas and an abnormal yolk sac vasculature (Fig. 1A). Immunoblotting of cleaved PARP1 and cleaved Casp3, markers of apoptosis, revealed significantly increased levels of apoptosis in Casp8−/-/Casp8−/- embryos (Fig. 1C, Supplemental Fig. 1D). Immunolabeling of cleaved Casp3 also suggested that apoptosis occurred in the abdominal areas of the Casp8−/-/Casp8−/- embryo (Fig. 1B). In addition, the Casp8−/-/Casp8−/- yolk sac did not show increased apoptosis with detectable levels of markers of apoptosis (data not shown). RIPK1 or RIPK3 phosphorylation and MLKL phosphorylation are hallmarks of necroptosis. Notably, the Casp8−/-/Casp8−/- yolk sac exhibited RIPK1 or RIPK3 phosphorylation as detected by an upshift in the anti-RIPK3 blotting band and MLKL phosphorylation (Supplemental Fig. 2G), coinciding with vascular defects (Fig. 1D). To confirm necroptosis occurred in the yolk sac, we lysed the yolk sac of individual embryos. Immunoblot analysis of RIPK1 immunoprecipitates revealed that RIPK3 strongly interacted with RIPK1 in the Casp8−/-/yolk sac (Fig. 1E), indicating that necroosome formation through RIPK1– RIPK3 interaction was involved in triggering necroptosis in the Casp8−/- yolk sac. Thus, these data demonstrated that RIPK1– RIPK3–MLKL signaling in the yolk sac drove the embryonic lethality of Casp8−/- mice.

**Necroptosis in Casp8−/- embryos was dependent on necroosome formation**

Next, we examined necrototic signaling in primary MEFs by comparing Casp8−/-, Casp8−/+ , and Casp8−/+ MEFs treated with TNF-α plus Smac mimic and the pan-caspase inhibitor Z-VAD-FMK. Casp8−/- MEFs exhibited RIPK1 and RIPK3 phosphorylation in medium alone, whereas the level of RIPK3 and MLKL proteins decreased compared with Casp8−/+ or Casp8−/+ MEFs (Fig. 2A), suggesting that some cells spontaneously died because of the loss of Casp8. Casp8−/- MEFs were more sensitive than Casp8−/+ MEFs to TNF-α plus Smac mimic and the pan-caspase inhibitor Z-VAD-FMK treatment, whereas phosphorylation of RIPK3 and MLKL was increased (Fig. 2A). Embryonic lethality of Casp8−/- mice was notably prevented by either Ripk3 (Fig. 2B, Supplemental Fig. 2A, 2B) or Mlkl (Fig. 2B, Supplemental Fig. 2C, 2D) deletion, suggesting that increased apoptosis in Casp8−/- embryos was a secondary consequence and drove the pathology. Notably, Casp8−/- mice expressing RIPK3 with an RHIM mutation (Ripk3V448P/V448P Casp8−/-) also survived beyond weaning (Fig. 2B, Supplemental Fig. 2E, 2F). Ripk3−/-/Casp8−/-, Ripk3−/-/Casp8−/-, Mlkl−/-/Casp8−/- (Supplemental Fig. 3A, 3D), and Mlkl−/-/Casp8−/- (Supplemental Fig. 4A–E) mice were viable but developed lymphadenopathy and splenomegaly similar to Ripk3−/-/Casp8−/-, Ripk3−/-/Casp8−/-, and Mlkl−/-/Casp8−/- mice (24, 29, 35). The data show that necrosomes formation is the primary driver of necroptosis in Casp8−/- mice because RHIM of RIPK3 is critical for RIPK1– RIPK3–mediated necroptotic formation.

Next, we investigated the effects of RIPK kinase activity on necrototic signaling in primary Ripk3−/-/Casp8−/-, Ripk3−/-/Casp8−/-, and Mlkl−/-/Casp8−/- MEFs in the presence or absence of the RIPK1 kinase inhibitor Nec-1.
FIGURE 1. Casp8 prevents yolk sacs necroptosis during mouse embryogenesis. (A) E11.5 yolk sacs and embryos representative of WT (n = 7) and Casp8/−/− (n = 5). Arrows denote sites of hemorrhage. (B) Hematoxylin and eosin (H&E)-stained section of E11.5 embryos, representative of WT and Casp8/−/− embryos. (C) Western blots of E11.5 embryos (Casp8+/+, Casp8+/−/−, Casp8−/−). GAPDH was used as a loading control. Lanes, individual mice. Results are representative of three independent experiments. (D) Western blots of E11.5 yolk sacs (Casp8+/+, Casp8+/−/−, Casp8−/−). GAPDH was used as a loading control. Lanes, individual mice. Results are representative of three independent experiments. (E) Western blots of E11.5 yolk sac lysates from mice of the indicated genotypes (Casp8+/+, Casp8+/−/−, Casp8−/−) before (Input) and after IP with anti-RIPK1 Ab. GAPDH was used as a loading control. Lanes, individual mice. Results are representative of three independent experiments.

Although RIPK3 deficiency or RHIM mutation compromised RIPK1 phosphorylation, it occurred normally in Mlkl−/−Casp8−/− MEFs (Fig. 2C), suggesting that RIPK1 and RIPK3 phosphorylation in Casp8−/− MEFs does not require MLKL. Mlkl−/−Casp8−/− MEFs treated with Nec-1 also maintained normal levels of RIPK3 phosphorylation, whereas RIPK1 phosphorylation was blocked (Fig. 2C). We examined RIPK1 phosphorylation in Mlkl−/−Casp8−/− MEFs treated with the RIPK3 kinase inhibitor GSK’872 (36), which revealed that RIPK1 phosphorylation occurred after GSK’872 treatment (Fig. 2D). Immunoblot analysis of RIPK1 immunoprecipitates revealed that RIPK3 strongly interacted with RIPK1 in Mlkl−/−Casp8−/− MEFs, and GSK’872, but not Nec-1, treatment reduced the RIPK1-RIPK3 interaction (Fig. 2E). Thus, Casp8 deficiency can trigger the RIPK1 kinase–independent interaction of RIPK1 with RIPK3 to form necrosomes in vitro. Notably, considering the mechanisms here that Casp8 deficiency can trigger the kinase activity–independent interaction between RIPK1 and RIPK3 to form necrosomes was revealed in MEFs, we agree that these mechanisms might not be applied to the cases in embryos. Therefore, we need to further investigate the contribution of RIPK1 kinase activity–independent function on Casp8−/− mice by utility of more in vivo mouse models.

**RIPK1 kinase–independent signaling led to necroptosis in Casp8−/− mice**

Because RIPK1 kinase activity has a critical role in regulating necroptosis in vitro and in vivo, we examined whether RIPK1 kinase activity contributes to necroptosis in Casp8−/− mice. To examine RIPK1 catalytic activity, autophosphorylation site Ab (anti–p-S166-RIPK1) was blotted in wild-type (WT), Ripk3−/−, Mlkl−/−Casp8−/−, and Ripk1K45A/K45A Casp8−/− immortalized MEFs. We found that RIPK1 autophosphorylation (S166) was induced in Mlkl−/−Casp8−/− MEFs, which was caused by the deficiency of Casp8, while p–S166-RIPK1 was completely blocked by the RIPK1 K45A mutation in Ripk1K45A/K45A Casp8−/− MEFs (Supplemental Fig. 4F). These results confirmed that the
FIGURE 2. Caspase-8 prevents the formation of the RIPK1–RIPK3 complex that was dependent on RIPK3 RHIM dependent but not RIPK1 kinase activity.

(A) Western blots of Casp8+/+, Casp8+/−, and Casp8−/− immortalized MEFs treated with 1 μM Smac mimic, 20 ng/ml TNF-α plus 20 μM zVAD for 6 h (+TSZ) or 0 h (−TSZ) as indicated. Results are representative of three independent experiments. (B) E12.5 yolk sacs and embryos representative of WT (n = 2), Casp8−/− (n = 3), Ripk3−/−Casp8−/− (n = 6), Mltk−/−Casp8−/− (n = 2), and Ripk3v448p/v448pCasp8−/− (n = 2). Diagram depicting the extent of viability of different strains of Casp8−/− mice was shown below. (C) Western blots of Casp8+/+, Casp8−/−, Ripk3−/−Casp8−/−, Mltk−/−Casp8−/−, and Ripk3v448p/v448pCasp8−/− immortalized MEFs after 24-h Nec-1 treatment. Results are representative of three independent experiments. Nec-1, 30 μM (−, untreated; +, treated). (D) Western blots of Casp8+/+, Ripk3−/−, and Mltk−/−Casp8−/− immortalized MEFs after 24-h treatment. Nec-1 ([IRIP1], 30 μM, GSK872 ([IRIP3]), 10 nM (−, untreated). Results are representative of three independent experiments. (E) Western blots of Mltk−/−Casp8−/− immortalized MEFs lysates before (input) and after IP with anti-RIPK1 Ab. GAPDH was used as a loading control. Results are representative of three independent experiments.
K45A mutation indeed impaired RIPK1 kinase activity, which is further consistent with previous reports from us and others (6, 9, 37). We generated Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) embryos by Ripk1\(^{K45A/K45A}\)/Casp8\(^{+/+}\) intercrossing. Notably, Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) mice died during embryogenesis as intercrossing of heterozygous mice produced only Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) and Ripk1\(^{K45A/K45A}\)/Casp8\(^{+/+}\) offspring (Fig. 3A). RIPK1 kinase inactive (K45A) resulted in delayed lethality in Casp8\(^{-/-}\) embryos; however, this trend was not consistent in control embryos at E15.5 (Fig. 3C) and suggests that RIPK1 kinase activity is independent of embryonic lethality of Casp8\(^{-/-}\) mice.

**RIPK1 scaffold function promoted lethality of Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) embryos**

To further characterize the mechanisms underlying the RIPK1 kinase–independent embryonic lethality observed in Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) mice, we isolated primary MEFs from Ripk1\(^{K45A/K45A}\)/Casp8\(^{+/+}\), Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\), and Ripk1\(^{K45A/K45A}\)/Casp8\(^{+/+}\)/Casp8\(^{-/-}\) embryos. Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) MEFs also exhibited RIPK3 phosphorylation in medium alone, whereas MLKL expression decreased compared with expression levels in Ripk1\(^{K45A/K45A}\)/Casp8\(^{+/+}\) or Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) MEFs (Fig. 4A). Immunoblot analysis of RIPK1 immunoprecipitates revealed that RIPK3 strongly interacted with RIPK1\(^{K45A}\) in the Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) MEFs (Fig. 4A). Because Ripk1\(^{-/-}\)/Casp8\(^{-/-}\) mice survived until birth, we hypothesized that RIPK1 scaffold-dependent and kinase activity–independent function promoted necroptosis in Casp8\(^{-/-}\) mice.

To determine the impact of RIPK1 scaffold on necroptosis formation of yolk sac of Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) mice, we deleted an allele of RIPK1 (Ripk1\(^{+/+}\)) in the background of Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) mice through intercrossing. Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) mice were viable at E14.5 with normal-appearing embryos and yolk sac vasculature as Ripk1\(^{+/+}\)/Casp8\(^{-/-}\) embryos (Fig. 4B). Furthermore, immunoblot analysis of RIPK1 immunoprecipitates revealed that the RIPK3-RIPK1\(^{K45A}\) interaction was slightly decreased by the absence of one allele of RIPK1 in Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) mice (Fig. 3C).

### FIGURE 3. Kinase-dead mutant RIPK1\(^{K45A}\) cannot rescue Casp8\(^{-/-}\) lethality.

**A** Observed numbers of offspring from Ripk1\(^{K45A/K45A}\)/Casp8\(^{+/+}\) intercrosses and numbers expected from Mendelian ratios at the indicated stage of development. (B) E11.5 yolk sacs and embryos representative of Ripk1\(^{K45A/K45A}\)/Casp8\(^{+/+}\) \((n = 1)\), Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) \((n = 5)\), and Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) \((n = 3)\); E12.5 yolk sacs and embryos representative of Ripk1\(^{K45A/K45A}\)/Casp8\(^{+/+}\) \((n = 2)\), Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) \((n = 9)\), and Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) \((n = 4)\); E13.5 yolk sacs and embryos representative of Ripk1\(^{K45A/K45A}\)/Casp8\(^{+/+}\) \((n = 1)\), Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) \((n = 4)\), and Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) \((n = 3)\); E14.5 yolk sacs and embryos representative of Ripk1\(^{K45A/K45A}\)/Casp8\(^{+/+}\) \((n = 4)\), Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) \((n = 13)\), and Ripk1\(^{K45A/K45A}\)/Casp8\(^{-/-}\) \((n = 3)\). (Continued)
FIGURE 4. RIPK1 interacts with RIPK3 to trigger necroptosis in Ripk1<sup>K45A/K45A</sup>Casp8<sup>+/−</sup> mice.
(A) The cell lysates of immortalized MEFs derived from mice of the indicated genotypes (Ripk1<sup>K45A/K45A</sup>Casp8<sup>+/−</sup>, Ripk1<sup>K45A/K45A</sup>Casp8<sup>+/−</sup>, Ripk1<sup>K45A/K45A</sup>Casp8<sup>+/−</sup>) were immunoprecipitated using anti-RIPK1 and were analyzed by Western blotting using the indicated Abs. GAPDH was used as a loading control. Results are representative of three independent experiments. (B) E14.5 yolk sacs and embryos representative of Ripk1<sup>K45A/K45A</sup>Casp8<sup>+/−</sup> (<i>n</i> = 2), Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup> (<i>n</i> = 3), Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup> (<i>n</i> = 5), and Ripk1<sup>−/−</sup>Casp8<sup>−/−</sup> (<i>n</i> = 2). (C) The E14.5 yolk sac lysates from embryos of the indicated genotypes (Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup>, Ripk1<sup>K45A/K45A</sup>Casp8<sup>+/−</sup>, Ripk1<sup>−/−</sup>Casp8<sup>−/−</sup>, Ripk1<sup>−/−</sup>Casp8<sup>−/−</sup>, Ripk1<sup>−/−</sup>Casp8<sup>−/−</sup>, Ripk1<sup>−/−</sup>Casp8<sup>−/−</sup>) were immunoprecipitated using anti-RIPK1 and were analyzed by Western blotting using the indicated Abs. GAPDH was used as a loading control. Lanes, individual mice.

RIPK1 scaffolds contribute to lethality of Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup> embryos at midgestation rather than RIPK1 kinase function.

Death of Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup> embryos depends on MLKL

Further, we observed that Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup> mice did not survive until birth and had an abnormal yolk sac at E17.5 (Fig. 5A). Immunoblot analysis of RIPK1 immunoprecipitates revealed that RIPK3 still interacted with RIPK1<sup>K45A</sup> in the Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup> yolk sac lysate (Fig. 5B), indicating that one allele of RIPK1<sup>K45A</sup> is sufficient to promote necroptosome formation at later stages of embryonic development. ZBP1, also identified as DAI/DLM-1, is known to function upstream of RIPK3 and interacts with RIPK3 through their RIP homotypic interaction motif (RHIM) domains. These domains assist in the formation of the RIPK1-RIPK3-ZBP1 complex (38). Interestingly, immunoblot analysis of yolk sac extracts from E11.5 Casp8<sup>−/−</sup> (Fig. 1D), E14.5 Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup> (Fig. 4C), and E17.5 Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup> (Fig. 5B) mice showed that ZBP1 expression was significantly increased. We determined whether Mlkl deficiency rescues the lethality of Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup> mice to confirm necroptotic-dependent embryonic lethality in Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup> mice. Mlkl<sup>−/−</sup>Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup> mice survived normally past weaning (Fig. 5C, 5D), which was similar to trends in Mlkl<sup>−/−</sup>Casp8<sup>−/−</sup> mice (data not shown). Casp8<sup>−/−</sup> caused RIPK1-RIPK3 complex formation in yolk sac, while ablation of MLKL had no impact on the formation of the RIPK1-RIPK3 complex (Fig. 2E). We also observed the RIPK1<sup>K45A</sup>-RIPK3 complex in Ripk1<sup>K45A/K45A</sup>Casp8<sup>−/−</sup>Mlkl<sup>−/−</sup> mice (data not shown) where the formation of the RIPK1-RIPK3 complex is dependent on the RIPK1/RIPK1<sup>K45A</sup>
FIGURE 5. MLKL deficiency rescues lethality in Ripk1K45A/K45ACasp8−/− mice.
(A) E15.5 yolk sacs and embryos representative of Ripk1K45A/K45ACasp8+/+ (n = 2), Ripk1K45A/K45ACasp8−/− (n = 6), Ripk1K45A/Casp8−/− (n = 3), Ripk1K45A/K45ACasp8−/− (n = 2), Ripk1K45A/Casp8−/− (n = 2); E17.5 yolk sacs and embryos representative of Ripk1K45A/K45ACasp8+/+ (n = 2), Ripk1K45A/Casp8−/− (n = 2), Ripk1K45A/Casp8−/− (n = 3).
(B) Western blots of E17.5 yolk sac lysates from mice of the indicated genotypes (Ripk1+/+, Ripk1K45A/K45A, Ripk1K45A/Casp8−/−, Ripk1K45A/Casp8−/−, Ripk1K45A/Casp8−/−) before (Input) and after IP with anti-RIPK1 Ab. GAPDH was used as a loading control. Lanes, individual mice. (C) Observed numbers of offspring from Mlkl−/−/Ripk1K45A/K45ACasp8−/− intercrosses and numbers expected from Mendelian ratios at weaning. (D) Diagram depicting the extent of viability of different strains of Casp8−/− mice. (E) Proposed model for Casp8 deficiency–triggered necroptosis in vivo. (Left) Lack of Casp8 could result in necroptosis in yolk sac during embryogenesis. RIPK1 can recruit RIPK3 through the RHIM motif to form necrosomes, resulting in autophosphorylation of RIPK1 and autophosphorylation of RIPK3 leading to MLKL-dependent necroptosis. (Right) When RIPK1 kinase activity is disrupted in Casp8-deficient mice, RIPK1K45A is not autophosphorylatable, which could trigger RIPK1 kinase–independent recruitment and autophosphorylation of RIPK3. This suggests that the recruitment of RIPK3 to RIPK1 is independent of RIPK1 kinase activity, but scaffold function is dependent on Casp8 deficiency–triggered necroptosis in yolk sac during embryonic development.

scaffold, but not MLKL. Therefore, embryonic lethality of Ripk1K45A/K45ACasp8−/− mice continued to be attributable to MLKL-mediated necroptosis in the yolk sac. This suggests that kinase activity of RIPK1 was not required for necroptosis in vivo. (A) Necroptosis in yolk sacs and embryos representative of Ripk1K45A/K45ACasp8+/+ (n = 2), Ripk1K45A/K45ACasp8−/− (n = 6), Ripk1K45A/Casp8−/− (n = 3), Ripk1K45A/K45ACasp8−/− (n = 2), Ripk1K45A/Casp8−/− (n = 2); E17.5 yolk sacs and embryos representative of Ripk1K45A/K45ACasp8+/+ (n = 2), Ripk1K45A/Casp8−/− (n = 2), Ripk1K45A/Casp8−/− (n = 3).
(B) Western blots of E17.5 yolk sac lysates from mice of the indicated genotypes (Ripk1+/+, Ripk1K45A/K45A, Ripk1K45A/Casp8−/−, Ripk1K45A/Casp8−/−, Ripk1K45A/Casp8−/−) before (Input) and after IP with anti-RIPK1 Ab. GAPDH was used as a loading control. Lanes, individual mice. (C) Observed numbers of offspring from Mlkl−/−/Ripk1K45A/K45ACasp8−/− intercrosses and numbers expected from Mendelian ratios at weaning. (D) Diagram depicting the extent of viability of different strains of Casp8−/− mice. (E) Proposed model for Casp8 deficiency–triggered necroptosis in vivo. (Left) Lack of Casp8 could result in necroptosis in yolk sac during embryogenesis. RIPK1 can recruit RIPK3 through the RHIM motif to form necrosomes, resulting in autophosphorylation of RIPK1 and autophosphorylation of RIPK3 leading to MLKL-dependent necroptosis. (Right) When RIPK1 kinase activity is disrupted in Casp8-deficient mice, RIPK1K45A is not autophosphorylatable, which could trigger RIPK1 kinase–independent recruitment and autophosphorylation of RIPK3. This suggests that the recruitment of RIPK3 to RIPK1 is independent of RIPK1 kinase activity, but scaffold function is dependent on Casp8 deficiency–triggered necroptosis in yolk sac during embryonic development.
**DISCUSSION**

Necroptosis is a form of regulated cell death defined morphologically by cell lysis with release of intracellular contents into the extracellular space. It has been implicated in embryonic development (39) and many human diseases such as Alzheimer’s disease (40), multiple sclerosis (41), acute kidney injury (42), and others (3). Mechanism studies showed that when cells fail to activate the apical apoptotic mediator Casp8, RIPK1 kinase activation promotes formation of the cytosolic amyloid-like necroosome complex, also known as complex IIb, which causes RIPK3 phosphorylation and eventually leads to phosphorylation of MLKL. This series of events results in lysis of the plasma membrane to execute cell death (14, 17, 23). Genetic evidence shows that the embryonic mortality of Fadd−/− or Casp8−/− mice can be rescued by codelletion of Ripk1, Ripk3, or Mlkl, supporting the notion that the FADD-Casp8 complex inhibits necroptosis and its underlying mechanisms. In this study, our results revealed that FADD-Casp8 complex inhibition promotes formation of the cytosolic amyloid-like necroosome complex, also known as complex IIb, which causes RIPK3 phosphorylation and eventually leads to phosphorylation of MLKL. This series of events results in lysis of the plasma membrane to execute cell death (14, 17, 23).

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**DISCLOSURES**

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