Necroptosis restricts influenza A virus as a stand-alone cell death mechanism

Maria Shubina1**, Bart Tummers2**, David F. Boyd2**, Ting Zhang1**, Chaoran Yin3, Avishekh Gautam1**, Xi-zhi J. Guo4**, Diego A. Rodriguez5, William J. Kaiser6, Peter Vogel7, Douglas R. Green3, Paul G. Thomas8**, and Siddharth Balachandran1**

Influenza A virus (IAV) activates ZBP1-initiated RIPK3-dependent parallel pathways of necroptosis and apoptosis in infected cells. Although mice deficient in both pathways fail to control IAV and succumb to lethal respiratory infection, RIPK3-mediated apoptosis by itself can limit IAV, without need for necroptosis. However, whether necroptosis, conventionally considered a fail-safe cell death mechanism to apoptosis, can restrict IAV—or indeed any virus—in the absence of apoptosis is not known. Here, we use mice selectively deficient in IAV-activated apoptosis to show that necroptosis drives robust antiviral immune responses and promotes effective virus clearance from infected lungs when apoptosis is absent. We also demonstrate that apoptosis and necroptosis are mutually exclusive fates in IAV-infected cells. Thus, necroptosis is an independent, “stand-alone” cell death mechanism that fully compensates for the absence of apoptosis in antiviral host defense.

**BRIEF DEFINITIVE REPORT**

Introduction

Influenza A virus (IAV) replication is typically accompanied by death of the infected cell (Herold et al., 2012; Upton et al., 2017). We and others have recently described a signaling pathway that appears to represent the dominant mechanism by which IAV triggers death in lung epithelial cells and other primary cell types. This pathway is initiated when the host sensor protein Z-DNA–binding protein 1 (ZBP1; also called “DAI” [DNA-dependent activator of IFN regulatory factors]) detects IAV Z-RNAs, activates receptor-interacting serine/threonine-protein kinase 3 (RIPK3), and drives parallel pathways of necroptosis and apoptosis in airway epithelial cells and mouse embryonic fibroblasts (MEFs; Fig. 1 A; Kuriakose et al., 2016; Nogusa et al., 2016; Thapa et al., 2016; Zhang et al., 2020).

Complete elimination of this branched cell death pathway in mice, such as via germline deletion of Zbp1 or Ripk3, renders the animal very susceptible to pulmonary viral replication and lethality (Nogusa et al., 2016; Thapa et al., 2016). Fascinatingly, eliminating only necroptosis signaling, by germline ablation of mixed lineage kinase domain–like (Mlkl), does not impinge on the apoptosis axis downstream of RIPK3 and does not significantly impact the anti-IAV immune response (Nogusa et al., 2016). That is, RIPK3-activated apoptosis signaling is largely capable of controlling pulmonary IAV infection on its own. It is unknown, however, if necroptosis can similarly limit IAV as a stand-alone mechanism when apoptosis is absent. This is a critical unanswered question because necroptosis is conventionally considered a “backup” form of fail-safe cell death that is activated when apoptosis pathways are compromised (e.g., by virus-encoded inhibitors of caspase activity; Mocarski et al., 2014; Upton et al., 2010; Vanden Berghe et al., 2016) and because RIPK3, in some cases, regulates the replication of viruses and other pathogens without triggering cell death (Daniels et al., 2017, 2019; Harris et al., 2015; Sai et al., 2019).

Unfortunately, germline ablation of genes encoding the apoptosis effectors in this pathway (i.e., RIPK1, Fas-associated death domain [FADD], and caspase 8 [Casp8]) results in perinatal (Ripk3−/−) or embryonic (Fadd−/−, Casp8−/−) lethality because each of these proteins also has nonapoptotic roles: they restrict RIPK3 activity and prevent necroptosis during early mammalian development (Dillon et al., 2016).

We have produced a knock-in mouse in which endogenous Casp8 has been replaced with a mutant allele (Casp8tm1) encoding a Casp8 variant selectively incapable of undergoing the autoprocessing requisite for apoptosis but that preserves Casp8 survival function during mammalian development (Dillon et al., 2012; Kang et al., 2008; Oberst et al., 2011; Philip et al., 2016). Mice homozygous for this allele of Casp8 are viable and fertile but selectively deficient in Casp8-driven apoptosis (Tummers et al., 2020), and thus they provide the first mammalian system in which to test the sole contribution of necroptosis to ZBP1/RIPK3-mediated IAV clearance.

*M. Shubina, B. Tummers, D.F. Boyd, and T. Zhang contributed equally to this paper; Correspondence to Siddharth Balachandran: siddharth.balachandran@fccc.edu.

© 2020 Shubina et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.nupress.org/terms/). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 4.0 International license, as described at https://creativecommons.org/licenses/by-nc-sa/4.0/).

J. Exp. Med. 2020 Vol. 217 No. 11 e20191259
Using Casp8\textsuperscript{DA} mice, we show here that necroptosis on its own limits and clears IAV infection in a manner that is largely indistinguishable from—and in some measures surpasses—IAV clearance in WT control animals. Thus, apoptosis is dispensable for IAV clearance, and necroptosis, often thought of as secondary to apoptosis in host defense, is a potent stand-alone antiviral cell death mechanism in vivo.

**Results and discussion**

**Casp8 activity is required for RIPK3-mediated apoptosis triggered by IAV**

Replicating IAV produces Z-RNA species, which are detected by the host sensor ZBP1 (Kuriakose et al., 2016; Thapa et al., 2016; Zhang et al., 2020). ZBP1 then activates RIPK3 to initiate parallel pathways of necroptosis and apoptosis (Nogusa et al., 2016). Apoptosis downstream of RIPK3 is mediated by RIPK1– and FADD-dependent autoproteolytic activation of the Casp8 zymogen into active homodimers consisting of p18 and p10 fragments (Fig. 1 A). We produced Casp8 knock-in mice (Casp8\textsuperscript{DA}) in which the initiation site for autoproteolytic processing of Casp8, essential for its capacity to stimulate apoptosis, was abolished by mutation of a critical aspartic acid to alanine (D387A; Fig. 1 B and Fig. S1, A and B; Kang et al., 2008; Oberst et al., 2011; Philip et al., 2016). Primary early-passage MEFs from Casp8\textsuperscript{DA} mice, but not those from either Zbp1\textsuperscript{−/−} or Mlkl\textsuperscript{−/−} mice, were resistant to TNF-related apoptosis-inducing ligand (TRAIL)–induced, Casp8-dependent apoptosis (Fig. 1 C). The resistance of Casp8\textsuperscript{DA} MEFs to TRAIL-induced apoptosis paralleled the degree of inhibition afforded by the pan-caspase blocker zVAD in WT MEFs (Fig. 1 C). The Casp8 D387A mutation thus effectively abrogates Casp8–dependent apoptosis signaling in cells.

Casp8\textsuperscript{DA} MEFs were as susceptible as WT MEFs to IAV-induced death, but a RIPK3 kinase inhibitor, which selectively blocks necroptosis without impeding apoptosis (Mandal et al., 2014), almost completely protected infected Casp8\textsuperscript{DA} MEFs from death (Fig. 1, D–F). In contrast, necroptosis-deficient Mlkl\textsuperscript{−/−} MEFs were not protected from IAV-induced death by a RIPK3 kinase inhibitor, but they were rescued by the pan-caspase inhibitor zVAD in WT MEFs (Fig. 1 C). The Casp8 D387A mutation thus effectively abrogates Casp8–driven apoptosis signaling in cells.

**Casp8\textsuperscript{DA} MEFs are selectively resistant to IAV-induced apoptosis.** (A) ZBP1–RIPK3–dependent cell death pathways activated by IAV. Apoptosis is mediated by RIPK1/FADD-dependent activation of Casp8. (B) Casp8 domain organization and knock-in mutation (D387A) in Casp8\textsuperscript{DA} mice. DED, death effector domain. (C) MEFs were treated with murine TRAIL (100 ng/ml) in the presence of cycloheximide (CHX, 250 ng/ml), and cell viability was determined at 24 h. n = 3 replicates per condition; data are from one of four experiments with similar results. (D) Photomicrographs of WT and Casp8\textsuperscript{DA} MEFs infected with PR8 (multiplicity of infection [MOI]), 2) in the presence or absence of RIPK3 inhibitor GS483 (5 µM) or treated with TRAIL (100 ng/ml) + CHX (250 ng/ml) for 24 h. Scale bar = 100 µm. (E) WT, Casp8\textsuperscript{DA}, or Mlkl\textsuperscript{−/−} MEFs were infected with PR8 (MOI, 2) and exposed to the indicated inhibitors, and cell viability was determined at 24 h postinfection (h.p.i.). n = 3 replicates per condition; data are from one of four experiments with similar results. (F) Cell death kinetics after PR8 infection (MOI, 2) of MEFs from the indicated genotypes. n = 3 replicates per condition; data are from one of six experiments with similar results. (G) WT and Casp8\textsuperscript{DA} MEFs were infected with PR8 (MOI, 2 or 5) and examined for the indicated proteins at 24 h. Molecular weights in kilodaltons are shown to the left. Results are representative of three independent experiments. Unpaired Student’s t test (C and E); two-way ANOVA and Tukey’s multiple comparisons test (F). Error bars represent mean ± SD; **, P < 0.005; ****, P < 0.0005.
protein 1 [NS1]) between WT and Casp8ΔA MEFs (Fig. 1 G). Collectively, these results demonstrate that cells from the Casp8ΔA mouse are selectively deficient in activating apoptosis upon IAV infection.

Apoptosis and necroptosis activation are mutually exclusive events in infected cells

In death receptor–mediated apoptosis (e.g., those stimulated by TRAIL and Fas ligand), Casp8 triggers activation of "executioner" caspases, predominantly Casp3, that mediate disassembly and eventual demise of the cell (Ashkenazi and Dixit, 1998). To test if the ZBP1/RIPK3-initiated apoptosis signal also proceeded by similar Casp3-driven mechanisms, we infected WT and Casp8ΔA MEFs with IAV and monitored Casp3 activity in infected nucleoprotein+ (NP+) cells by staining for cleaved caspase 3 (CC3). Although 30–40% of infected WT MEFs displayed a readily detectable CC3 signal at 18 h, only ~5% of similarly infected Casp8ΔA MEFs were CC3+ at this time point, indicating that Casp8 was required for activation of Casp3 upon IAV infection (Fig. 2, A and B).

Interestingly, the fraction of NP+ cells also staining positive for CC3 was rarely more than half, although all these cells eventually died. Indeed, dying WT cells displayed either apoptotic or necrotic morphologies; cells displaying features of both necrosis and apoptosis (e.g., both cell swelling and shrunken nuclei) were rarely seen. Because RIPK3 can induce both apoptosis and necroptosis downstream of ZBP1, these observations suggested that, once activated, RIPK3 in any given individual cell triggers either apoptosis or necroptosis and does not activate both pathways simultaneously. To explore this idea in more detail, we first defined IAV-infected cells undergoing apoptosis as those that were positive for both CC3 and the cell death marker SYTOX Green (CC3+SG+). These cells displayed classical features of apoptosis, including shrinkage and condensed chromatin (Fig. 2 C, middle panels). We next defined cells that were CC3− but SG+ as undergoing necroptosis; these cells typically had a swollen, balloon-like appearance (Fig. 2 C, bottom panels). Live cells were defined as CC3−SG− (Fig. 2 C, top panels). By these criteria, cells exposed to a canonical necroptosis stimulus (TNF + cycloheximide + zVAD-fmk; TCZ) expectedly underwent only necroptosis (green), whereas cells treated with a caspase-activating stimulus (TRAIL) succumbed primarily by apoptosis (red; Fig. 2 D).

Intriguingly, roughly equal proportions of IAV-infected WT cells showed evidence of either apoptosis or necroptosis but rarely manifested signs of mixed apoptotic-necrotic death (black; Fig. 2 D). When RIPK3 kinase activity was blocked or when MLKL was absent, almost all dying cells showed apoptotic morphology (Fig. 2 D). In contrast, when caspase activity was prevented, or in Casp8ΔA cells, cells died primarily by necroptosis (Fig. 2 D). Only when both RIPK3 kinase activity and caspase activity were inhibited, or in Zbpt−/− MEFs, was cell death prevented. In agreement with these results, concluding WT MEFs for CC3 (apoptosis) and phosphorylated MLKL (pMLKL; necroptosis) showed that IAV-infected cells were either CC3+ (red arrows) or pMLKL+ (green arrows); again, cells double positive for CC3 and pMLKL were almost never seen (Fig. 2, E and F). As controls, cells were almost only pMLKL+ when treated with TCZ and primarily CC3+ when exposed to TRAIL (Fig. 2, E and F). Over a time course of 12 h, the proportion of cells that were either CC3+ or pMLKL+ gradually increased to 40–50% of all infected (NP+) cells, but the ratio of CC3+ to pMLKL+ cells remained largely equivalent (Fig. 2 F). Notably, there was no significant difference between CC3+ and pMLKL+ cells in the intensity of the NP signal (Fig. S2 A), suggesting that the extent of virus replication may not be a major determinant of whether the cell undergoes apoptosis or necroptosis. In human HT-29 cells made competent for IAV-induced ZBP1/RIPK3-dependent cell death by stable expression of FLAG-ZBP1 (Fig. S2 B), IAV infection resulted in either necroptosis or apoptosis but not both outcomes (Fig. 2, G and H). IAV-infected cells in lung sections from WT mice were also either pMLKL+ or CC3+ but rarely both (Fig. 2, I and J).

Interestingly, necroptosis appears to manifest notably earlier than apoptosis in HT-29 FLAG-ZBP1 cells (Fig. 2 H) and in infected lungs (Fig. 2 J). Taken together, these findings demonstrate that, although both apoptosis and necroptosis fates are theoretically available to RIPK3 in IAV-infected cells, only one of these two forms of death ultimately manifests.

Necroptosis can clear IAV from infected lungs in the absence of apoptosis

Following infection with IAV (2,500 50% egg infectious dose [EID50]; ~LD50 for this virus in C57BL/6 mice), ~20% of WT MEFs succumbed by 12 d postinfection (d.p.i.); surviving mice made full recoveries (Fig. 3 A). Mlkl−/− mice displayed survival and mortality rates that were mostly indistinguishable from those of WT mice in both kinetics and magnitude, demonstrating, as we have previously shown (Nogusa et al., 2016), that necroptosis is dispensable for preventing IAV-induced lethality in mice (Fig. 3 A). Importantly, >80% of Casp8ΔA mice also survived this dose of IAV (Fig. 3 A); these mice made complete recoveries by 3 wk after infection. Only ~10% of Casp8ΔA Mlkl−/− mice, however, survived past 12 d.p.i. (Fig. 3 A). Comparing weights of infected Casp8ΔA mice with those of Casp8ΔA Mlkl−/− mice showed that both genotypes initially lost weight with roughly equivalent kinetics, but whereas most Casp8ΔA mice eventually rebounded to within 10% of their starting weights by 18 d.p.i., the majority of Casp8ΔA Mlkl−/− mice continued to lose weight and had to be killed by 12 d.p.i. (Fig. 3 B). Casp8ΔA (and Mlkl−/−) animals were able to control virus replication at least as effectively as WT mice at both early (3 and 5 d.p.i.; Fig. S2 C) and late (9 d.p.i.; Fig. 3 C) time points; indeed, lungs from Casp8ΔA mice contained even lower levels of virus than lungs of either WT mice or Mlkl−/− animals at 9 d.p.i. (Fig. 3 C), and IAV replication rates at early time points were modestly but significantly reduced in Casp8ΔA MEFs compared with either WT or Mlkl−/− cells (Fig. 2 D). In contrast, Casp8ΔA Mlkl−/− mice were unable to effectively curb IAV output and continued to support high levels of progeny virus production in their lungs late into infection (Fig. 3 C). These findings were corroborated by staining lung sections from each genotype for virus protein. Whereas only residual virus debris (black arrows) was observed in WT, Casp8ΔA, and Mlkl−/− mice at 9 d.p.i., similar sections from Casp8ΔA Mlkl−/− mice demonstrated persistence of virus in bronchiolar and alveolar epithelial cells (Fig. 3 D, red arrows). In agreement with these
Figure 2. IAV-induced apoptosis and necroptosis do not occur in the same infected cell. (A) WT and Casp8ΔMEFs infected with PR8 (MOI, 2) were examined for apoptosis using antibodies to CC3 (red) at 18 h.p.i. Virus replication was determined by staining for NP (green). Scale bar = 20 µm. (B) Quantification of CC3 in virus-positive infected cells at the indicated times after infection. Data are pooled from four fields with 30–60 cells/field; data are from one of four experiments with similar results. (C) MEFs were infected with PR8 (MOI, 2), harvested at 18 h.p.i., and stained for CC3 (red) and SYTOX Green. Scale bar = 10 µm. (D) Quantification of live, apoptotic, and necroptotic cells following the indicated treatments at 18 h (PR8; MOI, 2) or 12 h (TCZ, TRAIL + CHX). Data are pooled from four fields with 30–60 cells/field; data are from one of four experiments with similar results. (E) Primary MEFs infected with PR8 (MOI, 2; 18 h) or treated with either TCZ (12 h) or TRAIL + CHX (12 h) were examined for apoptosis and necroptosis by immunofluorescence using antibodies to CC3 (red) and pMLKL (green). Scale bar = 20 µm. (F) Quantification of CC3 and/or pMLKL positivity at the indicated times after infection (PR8) or at 12 h (TCZ, TRAIL + CHX). Data are pooled from four fields with 30–60 cells/field; data are from one of four experiments with similar results. (G) Human HT-29 cells made competent for IAV-activated cell death by stable retroviral expression of FLAG-tagged hZBP1 were infected with PR8 (MOI, 2; 9 h.p.i.) or treated with either TCZ (6 h) or TRAIL + CHX (9 h) and examined for apoptosis and necroptosis by immunofluorescence using antibodies to CC3 (red) and pMLKL (green). Scale bar = 10 µm. (H) Quantification of CC3 and/or pMLKL positivity at the indicated times after infection (PR8; MOI, 2). TCZ- and TRAIL + CHX–treated cells were evaluated at 6 h and 9 h after treatment, respectively. Data are pooled from four fields with 30–60 cells/field; data are from one of four experiments with similar results. (I) Mock- or IAV-infected lungs were stained for CC3 (red) or pMLKL (green) at 5 d.p.i. Nuclei are stained with DAPI (blue), and IAV HA is shown in yellow. Scale bar = 20 µm. (J) Quantification of pMLKL- and CC3-positive cells in IAV-infected lungs at the indicated days after infection. Arrows in E, G, and I point to pMLKL+ (green) or CC3+ (red) cells. Data are pooled from seven fields with 60–130 cells/field; data are from one of two experiments with similar results. Unpaired Student’s t test (B). Error bars represent mean ± SD. ***, P < 0.005.
findings, Casp8\textsuperscript{DAMlkl}−/− mice at 9 d.p.i. displayed clear zones of ongoing virus replication (red) in cells along the entire periphery of the lesioned lung (yellow), whereas both Casp8\textsuperscript{DA} and Casp8\textsuperscript{DAMlkl}−/− mice, like their WT counterparts, showed no evidence of active virus replication by this time point (Fig. 3 E, quantified in Fig. 3, F and G). Collectively, these data also demonstrate that necroptosis on its own can protect against IAV and, in fact, may represent a more efficient means of viral clearance than apoptosis alone.

### Necroptosis by itself can mediate effective anti-IAV adaptive immune responses

Lung sections from Casp8\textsuperscript{DA} mice were overtly similar to those of WT control animals (and to Mlkl−/− mice) when evaluated for classical indicators of IAV-mediated pathology (e.g., presence of hyaline membranes, septal thickening, and inflamed alveoli), whereas similar sections from Casp8\textsuperscript{DAMlkl}−/− mice showed markedly increased evidence of inflammatory injury, including significantly elevated levels of alveolar inflammation (Fig. 4 A), compared with any of the other genotypes. These observations are consistent with increased virus replication in Casp8\textsuperscript{DAMlkl}−/− lungs and reminiscent of our previous results in Ripk3−/− mice (Nogusa et al., 2016). Notably, our earlier work also showed that Ripk3−/− mice failed to mount effective CD8+ T cell–driven adaptive immune responses to IAV (Nogusa et al., 2016). To examine if Casp8\textsuperscript{DA} mice were similarly compromised in their CD8+ T cell responses, we first evaluated the location and abundance of lymphoid cells in infected lungs. Casp8\textsuperscript{DA} mice displayed numbers and distribution of lymphoid cells that were mostly indistinguishable from those of WT or Mlkl−/− mice on the basis of H&E staining (Fig. 4 B). Compared with the other genotypes, however, Casp8\textsuperscript{DAMlkl}−/− mice had markedly
diminished evidence of lymphoid inflammation (both T and B cells; Fig. 4 B). When we examined bronchoalveolar lavage (BAL) fluid from IAV-infected mice of each genotype, we found that Casp8<sup>DA</sup> mice displayed frequencies of CD8<sup>+</sup> T cells that were equivalent to those of WT animals (Fig. 4 C), whereas Casp8<sup>DA</sup>Mlkl<sup>−/−</sup> mice had a significantly lower proportion (among immune cells) of CD8<sup>+</sup> T cells than any of the other genotypes (Fig. 4 C). Following stimulation with IAV-specific peptides ex vivo, Casp8<sup>DA</sup> mice mounted anti-IAV CD8<sup>+</sup> T cell responses that were indistinguishable from those of WT mice, while Casp8<sup>DA</sup>Mlkl<sup>−/−</sup> mice had significantly lower numbers of IFNγ-producing, IAV-specific CD8<sup>+</sup> T cells as compared with Casp8<sup>DA</sup> mice (Fig. 4 D), paralleling our results from Ripk3<sup>−/−</sup> mice (Nogusa et al., 2016). Of note, activation of CD8<sup>+</sup> T cells by a nonspecific stimulus (PMA/ionomycin) ex vivo was not dampened in Casp8<sup>DA</sup>Mlkl<sup>−/−</sup> mice, demonstrating that Casp8<sup>DA</sup>Mlkl<sup>−/−</sup> CD8<sup>+</sup> T cells are not intrinsically encumbered in their capacity to survive and proliferate once activated (Fig. S3 A). Moreover, whereas Casp8<sup>DA</sup>Mlkl<sup>−/−</sup> mice showed significantly lower frequencies (Fig. 4 E) and numbers (Fig. S3 B) of IAV-specific CD8<sup>+</sup> T cells in BAL fluid, the frequencies (Fig. 4 F) and numbers (Fig. S3 C) of these cells were not diminished in the spleen compared with Casp8<sup>DA</sup> mice. Altogether, these results show that apoptosis is not required for effective anti-IAV CD8<sup>+</sup> T cell responses in pulmonary tissue, as long as necroptosis signaling is intact. They also demonstrate that necroptosis is a bona fide stand-alone antiviral mechanism in vivo.

The idea that necroptosis is a “backup” fail-safe cell death mechanism secondary to apoptosis emerged from studies with the cytokine TNF-α, where inactivation of apoptosis signals is a prerequisite for licensing necroptosis, and from work with murine CMV, which encodes an inhibitor of Casp8-dependent apoptosis (Mocarski et al., 2014; Moriwaki and Chan, 2013; Upton et al., 2010, 2017). The substantial number of viruses encoding Casp8 inhibitors suggests that necroptosis might have evolved as a counteradaptation to eliminate cells with compromised apoptosis signaling (Mocarski et al., 2011). Using the Casp8<sup>DA</sup> mouse, we now show that necroptosis can fully compensate for the absence of apoptosis to efficiently limit virus spread, mobilize adaptive immunity, and ultimately clear the virus (in this case, IAV) from the host.

It is noteworthy that necroptosis appears to peak earlier than apoptosis in vivo (Fig. 2 J), and viral titers were statistically lower in Casp8<sup>DA</sup> mice at 9 d.p.i. than in Mlkl<sup>−/−</sup> mice or WT controls (Fig. 3 C). These findings suggest that necroptosis may in fact be a more efficient means of controlling IAV than apoptosis, perhaps by virtue of its earlier deployment during the host anti-IAV response. Interestingly, the rates of IAV RNA synthesis were moderately reduced in Casp8<sup>DA</sup> MEFs compared with either Mlkl<sup>−/−</sup> or WT MEFs (Fig. S2 D), although the kinetics of IAV-triggered cell death were largely similar between all three genotypes (Fig. 1 F), indicating that triggering the necroptosis machinery in the absence of apoptosis signaling may limit virus replication even before the cell dies.

IAV is the first physiological stimulus, to our knowledge, that activates both apoptosis and necroptosis in parallel downstream of RIPK3 (Nogusa et al., 2016). These observations raise the question of how RIPK3, at the single-cell level, “decides” between apoptosis and necroptosis. Is the decision to trigger either cell fate a stochastic one, in which both outcomes are equally probable and determined simply by levels of downstream effectors (i.e., RIPK1 for apoptosis and MLKL for necroptosis) in
the vicinity of activated RIPK3 (Cook et al., 2014)? Or is cell death downstream of RIPK3 governed by active mechanisms (such as post-translational modifications of RIPK3 and/or its effectors) that determine whether the individual cell dies by apoptosis or by necroptosis? Our finding that IAV-infected cells positive for phosphorylated MLKL are almost always negative for activated effector caspases, and vice versa, strongly suggests that once a cell commits to a form of death, the other fate becomes unavailable. That is, although the “decision” at the level of activated RIPK3 to trigger apoptosis or necroptosis might be stochastic, once this decision is made, active mechanisms are likely invoked to prevent parallel triggering of the other death modality. An initial stochastic “choice” of a particular fate, followed by active suppression of the alternative death pathway, supplies a compelling explanation for why cells positive for phosphorylated MLKL or active Casp3 are found in roughly equal proportions, but cells positive for both signals, or cells undergoing death with morphological features of both apoptosis and necroptosis, are rarely observed. Regulated ubiquitylation of ZBP1, RIPK3, and/or downstream proteins may be an attractive mechanistic explanation for how this cell fate switch might be controlled (Dondelinger et al., 2016; Kesavardhana et al., 2017; Rodriguez-Gervais et al., 2014; Seo et al., 2016; Witt and Vucic, 2017).

Materials and methods

Mice and cells

Mice were housed in specific pathogen-free facilities at the Fox Chase Cancer Center and at St. Jude Children’s Research Hospital, and all in vivo experiments were conducted under protocols approved by the Committees on Use and Care of Animals at these institutions. MEFs were generated from embryonic day 14.5 embryos and used within five passages in experiments. All these institutions. MEFs were generated from embryonic day cols approved by the Committees on Use and Care of Animals at Chase Cancer Center and at St. Jude Children Cancer Research Center. IAV strain A/Puerto Rico/8/1934 (PR8, H1NI) was propagated by allantoic inoculation of embryonated hen eggs with diluted (1:10³) seed virus. Stock virus titers were determined as EID₅₀.

Virus infection and titration

Age-matched (8-12-wk-old) and sex-matched mice were anesthetized with Avertin (2,2,2-tribromoethanol; Winthrop Laboratories) or isoflurane and infected intranasally with virus inoculum diluted in endotoxin-free saline. Mice were either monitored for survival and weight loss over a period of 18 d or sacrificed at defined time points for analysis of histology and virus replication. Mice losing >35% body weight were considered moribund and euthanized by CO₂ asphyxiation. For cell culture experiments, near-confluent monolayers of cells were infected with virus in serum-free DMEM for 1 h, with occasional gentle rocking, in a humidified tissue culture incubator maintained at 37°C and 5% CO₂. Following infection, the inoculum was removed and replaced with growth medium. In conditions involving small-molecule inhibitors, cells were preincubated for 1 h with inhibitors before infection; after removal of inoculum and washing, inhibitors were added back to the medium. Cell viability was determined by Trypan Blue exclusion or SYTOX Green uptake. Titration of virus was conducted by standard plaque assay of diluted lung homogenates on monolayers of Madin-Darby canine kidney cells. Plaques were scored after 3 d of incubation.

Quantitative PCR

Total RNA from IAV-infected cells was isolated and purified using the NucleoSpin RNA isolation kit (MACHEREY-NAGEL). RNA was reverse transcribed into cDNA using SuperScript IV VILO Master Mix (Thermo Fisher Scientific). Quantitative PCR was performed to detect polymerase acidic protein (PA) defective viral genomes using SYBR Green (Thermo Fisher Scientific) and the following primers: PA-(forward) 5'-GGGATCATTAATCAGGCA-3' and the following primers: PA-(reverse) 5'-GGGATCATTAATCAGGCA-3'.

Fluorescence microscopy

Cells were plated on 8-well glass slides (EMD Millipore) and allowed to adhere for at least 24 h before use in experiments. Following treatment or virus infection, cells were fixed, stained, and imaged as previously described (Zhang et al., 2020). For immunofluorescence studies on lung sections, lungs were fixed by intratracheal perfusion and subsequent immersion in freshly prepared 4% paraformaldehyde for 24 h. Fixed lungs were then immersed in 30% sucrose, embedded in optimal cutting temperature compound (Thermo Fisher Scientific), frozen, and sectioned. Frozen sections were stained and imaged as described previously (Zhang et al., 2020). In some experiments, cells were...
incubated with SYTOX Green for 15 min before fixation. Primary antibodies were diluted as follows: anti-CC3, 1:200; anti-NP, 1:1,000; anti-HA, 1:500; anti-pMLKL (murine), 1:10,000; and anti-pMLKL (human), 1:200.

**Histology and morphometry**

Lungs from IAV-infected mice were fixed via intratracheal infusion and subsequent immersion in freshly prepared 4% paraformaldehyde. Tissues were paraffin embedded, sectioned, and stained for virus with anti-IAV H1N1 antibody (1:1,000) and a secondary biotinylated donkey anti-goat antibody (Santa Cruz Biotechnology; 1:200 on tissue sections subjected to antigen retrieval for 30 min at 98°C). The extent of virus spread was quantified by first capturing digital images of whole-lung sections stained for viral antigen by using an Aperio ScanScope XT Slide Scanner (Aperio Technologies), then manually outlining fields with the alveolar areas containing virus antigen-positive pneumocytes highlighted in red (defined as “active” infection), whereas lesioned areas containing no antigen-positive cells and no/minimal antigen-positive debris were highlighted in yellow (defined as “inactive” infection). The percentage of each lung field with infection/lesions was calculated using the Aperio ImageScope software. The extent and severity of pulmonary inflammation were determined from blinded sections examined by a pathologist and scored on a severity scale as follows: 0 = no lesions; 1 = minimal, focal to multifocal, barely detectable; 15 = mild, multifocal, small but conspicuous; 40 = moderate, multifocal, prominent; 80 = marked, multifocal coalescing, lobar; 100 = severe, diffuse, with extensive disruption of normal architecture and function. These scores were converted to a semiquantitative scale as described previously (Matute-Bello et al., 2011).

**CD8+ T cell responses**

BAL fluid was collected by inserting a catheter into the trachea and washing with 3 × 1 ml of 1× PBS. Cells from BAL fluid or spleens were pelleted by centrifuging at 1,500 rpm for 5 min. RBC lysis was performed, cells were washed with HBSS, and counted using a Vi-CELL XR cell counter (Beckman Coulter). For tetramer staining, cells were incubated with Fc block (1:100; BioLegend) at room temperature, washed, and incubated with IAV peptide:MHC tetramers conjugated to PE. Cells were washed with tetramers for 1 h on ice and washed twice with FACS buffer. Following tetramer staining, cells were stained with the following antibodies and dyes on ice for 30 min: Ghost Dye Violet 510 (1:100), CD45-V450 (1:100), CD3-BV785 (1:100), CD8-BV650 (1:200), and CD4-APC Fire 750 (1:200). Cells were washed twice in FACS buffer, resuspended in 100 µl of FACS buffer, and analyzed on a BD LSR Fortessa Cell Analyzer.

**Online supplemental material**

Fig. S1 describes the generation and characterization of the Casp8DA mouse. Fig. S2 provides additional information on the influence of IAV replication rates on cell death outcomes (and vice versa) and demonstrates that ZBP1-expressing HT-29 cells support IAV-activated death signaling. Fig. S3 provides additional characterization of CD8+ T cell responses in mice lacking apoptosis and/or necroptosis signaling and outlines the gating strategy used to evaluate these and other immunological parameters by flow cytometry.

**Acknowledgments**

This work was supported by National Institutes of Health grants CA168621, CA190542, and AI135025 to S. Balachandran; National Institutes of Health grants AI44828 and CA231620 to D.R. Green; and National Institutes of Health grant R01 AI21832 and the St. Jude Center of Excellence for Influenza Research and Surveillance/National Institute of Allergy and Infectious Diseases contract HHSN272201400006C to P.G. Thomas. Additional funds were provided by National Institutes of Health Cancer Center support grant P30CA06927 to S. Balachandran.

Author contributions: M. Shubina, D.F. Boyd, and T. Zhang conducted most of the experiments. B. Tummers and D.R. Green produced Casp8DA mice and performed mouse crosses. C. Yin, A. Gautam, and X.J. Guo assisted with experiments. W.J. Kaiser produced HT-29 cells expressing FLAG-ZBP1. D.A. Rodriguez generated pMLKL antibodies for immunofluorescence. P. Vogel carried out histological assessments. M. Shubina, B. Tummers, D.F. Boyd, D.R. Green, P.G. Thomas, and S. Balachandran designed experiments. D.R. Green, P.G. Thomas, and S. Balachandran supervised experiments and analyzed data. S. Balachandran wrote the manuscript with assistance from M. Shubina, B. Tummers, D.F. Boyd, D.R. Green, and P.G. Thomas. All authors participated in editing the manuscript.

Disclosures: P.G. Thomas reported other from Cytoagents outside the submitted work; in addition, P.G. Thomas had a patent to US20146206856P pending. No other disclosures were reported.

Submitted: 9 July 2019
Revised: 8 May 2020
Accepted: 2 July 2020

Shubina et al.
Necroptosis is a stand-alone antiviral mechanism

https://doi.org/10.1084/jem.20191259
Necroptosis is a stand-alone antiviral mechanism

References

Ashkenazi, A., and V.M. Dixit. 1998. Death receptors: signaling and modula-
tion. Science. 281:1305–1308. https://doi.org/10.1126/science.281.5381.
1305

Cook, W.D., D.M. Moujalled, T.J. Ralph, P. Lock, S.N. Young, J.M. Murphy, and
D.L. Vaux. 2014. RIPK1- and RIPK3-induced cell death mode is
determined by target availability. Cell Death Differ. 21:1600–1612.
https://doi.org/10.1038/cdd.2014.70

Daniels, B.P., A.G. Snyder, T.M. Olsen, S. Orozco, T.H. Oguin, III, S.W.G. Tait,
and A.G. Snyder. 2019. The nucleotide
sensor ZBP1 and kinase RIPK3 induce the enzyme IRG1 to promote an
antiviral metabolic state in neurons. Immunity. 50:64–76.e4. https://doi.
.org/10.1016/j.immuni.2018.11.017

Dillon, C.P., A. Oberst, R. Weinlich, L.J. Janke, T.B. Kang, T. Ben-Moshe, T.W.
Daniels, B.P., S.B. Kofman, J.R. Smith, G.T. Norris, A.G. Snyder, J.P. Kolb, X.
Daniels, B.P., A.G. Snyder, T.M. Olsen, S. Orozco, T.H. Oguin, III, S.W.G. Tait,
Cook, W.D., D.M. Moujalled, T.J. Ralph, P. Lock, S.N. Young, J.M. Murphy, et al. 2013.
The pseudokinase MLKL mediates necroptosis via a molecular switch
mechanism. Immunity. 39:443–453. https://doi.org/10.1016/j.immuni.
2013.06.018

Nogusa, S., R.J. Thapa, C.P. Dillon, S. Liedmann, T.H. Oguin, III, J.P. Ingram,
D.A. Rodriguez, R. Kosoff, S. Sharma, O. Sturm, et al. 2016. RIPK3 ac-
tivates parallel pathways of MLKL-driven necroptosis and FADD-
mediated apoptosis to protect against influenza A virus. Cell Host
Microbe. 20:33–24. https://doi.org/10.1016/j.chom.2016.05.011

Oberst, A., C.P. Dillon, R. Weinlich, L.L. McCormick, P. Fitzgerald, C. Pop, R.
Hakem, G.S. Salvesen, and D.R. Green. 2011. Catalytic activity of the
raspase-8-FLIP(−) complex inhibits RIPK3-dependent necrosis. Nature.
471:363–367. https://doi.org/10.1038/nature09852

Philip, N.H., A. DeLaney, L.W. Peterson, M. Santos-Marrero, J.T. Grier, Y.
Sun, M.A. Wynosky-Dolfi, E.E. Zwack, B. Hu, T.M. Olsen, et al. 2016.
Activity of uncleaved caspase-8 controls anti-bacterial immune defense
and TLR-induced cytokine production independent of cell death. PLoS
Pathog. 12. e1005910. https://doi.org/10.1371/journal.ppat.1005910

Rodrigue-Cervais, I.G., K. Labbé, M. Dagenais, J. Dupaul-Chiloin, C.
Champagne, A. Morizot, A. Skeldon, E.L. Brincks, S.M. Vidal, T.S.
Griffith, et al. 2014. Cellular inhibitor of apoptosis protein cIAP2 pro-
tects against pulmonary tissue necrosis during influenza virus infection
to promote host survival. Cell Host Microbe. 15:23–35. https://doi.
org/10.1016/j.chom.2013.12.002

Rodrigues, D.A., R. Weinlich, S. Brown, C. Guy, P. Fitzgerald, C.P. Dillon, A. Oberst,
G. Quarato, J. Low, J.G. Cripps, et al. 2016. Characterization of RIPK3-
mediated phosphorylation of the activation loop of MLKL during
necroptosis. Cell Death Differ. 23:76–88. https://doi.org/10.1038/cdd.2015.70

Sai, K., C. Parsons, J.S. House, S. Kathariou, and J. Ninomiya-Tsuji. 2019.
Necroptosis mediators RIPK3 and MLKL suppress intracellular Listeria
replication independently of host cell killing. J. Cell Biol. 218:1994–2005.
https://doi.org/10.1083/jcb.201811014

Seo, J., E.W. Lee, H. Sung, D. Seong, Y. Dondelinger, J. Shin, M. Jeong, H.K.
Lee, J.H. Kim, S.Y. Han, et al. 2016. CHIP controls necroptosis through
ubiquitylation- and lysosome-dependent degradation of RIPK3. Nat.
Cell Biol. 18:291–302. https://doi.org/10.1038/ncb3314

Thapa, R.J., J.P. Ingram, K.B. Ragan, S. Nogusa, D.F. Boyd, A.A. Benitez, H.
Sridharan, R. Kosoff, M. Shubina, V.J. Landsteiner, et al. 2016. DAI senses influenza A virus genomic RNA and activates RIPK3-dependent
cell death. Cell Host Microbe. 20:674–681. https://doi.org/10.1016/j.chom.
2016.09.014

Tummers, B., L. Mari, C.S. Guy, B.L. Heckmann, D.A. Rodríguez, S. Rühl, J.
Moretti, J.C. Crawford, P. Fitzgerald, T.D. Kanneganti, et al. 2020.
Caspase-8-dependent inflammatory responses are controlled by its
adaptor, FADD, and necroptosis. Immunity. 52:994–1006.e8. https://doi.
org/10.1016/j.immuni.2020.04.010

Upton, J.W., W.J. Kaiser, and E.S. Mocarski. 2010. Virus inhibition of RIP3-
mediated inflammatory and cell death signaling.
Cell Host Microbe. 7:302–313. https://doi.org/10.1016/j.chom.2010.03.006

Upton, J.W., M. Shubina, and S. Balachandran. 2017. RIPK3-driven cell death
during virus infections. Immunol. Rev. 277:90–101. https://doi.org/10.
1111/imr.12539

Vandenberghe, T., B. Hassannia, and P. Vandenamee. 2016. An outline of
necrostatecrosis. Cell. Mol. Life Sci. 73:2137–2152. https://doi.org/10.
1007/s00018-016-1219-y

Witt, A., and D. Vucic. 2017. Diverse ubiquitin linkages regulate RIP kinase-
mediated inflammatory and cell death signaling. Cell Death Differ. 24:
1610–1617. https://doi.org/10.1038/cdd.2017.33

Zhang, T., C. Yin, D.F. Boyd, G. Quarato, J.P. Ingram, M. Shubina, K.B. Ragan,
T. Ishizuka, J.C. Crawford, B. Tummers, et al. 2020. Influenza virus
Z-RNAs induce ZBP1-mediated necroptosis. Cell. 180:1115–1129.e13.
https://doi.org/10.1016/j.cell.2020.02.050

Shubina et al.
Necroptosis is a stand-alone antiviral mechanism

Journal of Experimental Medicine
https://doi.org/10.1083/jem.20191259
Figure S1. **Generation of Casp8<sup>DA</sup> mice.** (A) Schematic of the Casp8<sup>DA</sup> allele showing primer sequences, location of the SacI cleavage site, and sizes of expected PCR amplicons after cleavage with SacI. (B) PCR results showing undigested (top) or SacI-digested (bottom) amplicon products produced from genomic tail DNA of mice from the indicated genotypes.
Characterization of virus replication in cells and in vivo. (A) Quantification of NP signal intensity in IAV-infected WT MEFs (PR8; MOI, 2; 18 h.p.i.) that are either pMLKL+ or CC3+. Of note, a few apoptotic (i.e., CC3+) cells showed a markedly intense NP signal, likely because they had shrunk in size (characteristic of apoptosis) and therefore harbor a more condensed pool of NP. Data are pooled from three fields with 6–10 cells/field; data are from one of four experiments with similar results. (B) HT-29 FLAG-ZBP1 cells were produced by retroviral transduction of an expression vector encoding FLAG-tagged human ZBP1 into the HT-29 cell line. These cells, but not control cells expressing an empty vector (EV), underwent rapid cell death upon infection with PR8 (MOI, 2). IAV-induced cell death was dependent on ZBP1-RIPK3 signaling, shown by rescue with the combination of RIPK3i (GSK843; 5 µM) + zVAD (50 µM). n = 3 replicates per condition; data are from one of six experiments with similar results. (C) Lung virus titers of mice of the indicated genotypes at 3 d.p.i. (left) or at 5 d.p.i (right) with PR8 (1,500 EID₅₀). WT, n = 4; Casp8ΔK, n = 3; Mlkl−/−, n = 5; Casp8ΔKMlkl−/−, n = 3 for D3. WT, n = 4; Casp8ΔK, n = 4; Mlkl−/−, n = 5; Casp8ΔKMlkl−/−, n = 4 for D5. (D) Kinetics of IAV (PR8; MOI, 2) replication in MEFs of the indicated genotypes as determined by quantitative RT-PCR analysis of PA segment levels. n = 3 replicates per condition; data are from one of six experiments with similar results. Unpaired Student’s t test (A and D); two-way ANOVA with Tukey’s multiple comparisons test (B); Mann-Whitney test (C). Error bars represent mean ± SD. *, P < 0.05; **, P < 0.005; ***, P < 0.0005.
Figure S3. **CD8+ T cell activation defects in Casp8DAMlkl−/− mice are lung specific.** (A) Proliferation of CD8+ T cells from Casp8DAMlkl−/− mice is not impeded in response to the nonspecific stimulus PMA/ionomycin ex vivo. WT, n = 8; Casp8D, n = 12; Mlkl−/−, n = 10; Casp8DAMlkl−/−, n = 6. (B) Total IAV PB1703-711 tetramer+ CD8+ T cells from the BAL of mice of the indicated genotypes collected at 8 d.p.i. with PR8 (1,500 EID50). Casp8D, n = 6; Casp8DAMlkl−/−, n = 11. (C) Total IAV PB1703-711 tetramer+ CD8+ T cells from the BAL of mice of the indicated genotypes collected at 8 d.p.i. with PR8 (1,500 EID50). Casp8D, n = 6; Casp8DAMlkl−/−, n = 11. (D) Gating strategy used in this figure and in Fig. 4. Data are representative of two independent experiments (A–C). One-way ANOVA comparing WT samples with every other genotype (A–C). * P < 0.05; ** P < 0.005. FSC, forward scatter; SSC, side scatter.