2017

Viral RNA at two stages of reovirus infection is required for the induction of necroptosis

Angela K. Berger
Emory University

Bradley E. Hiller
Washington University School of Medicine

Deepti Thete
Indiana University

Anthony J. Snyder
Indiana University

Encarnacion Perez Jr
University of Texas at Austin

See next page for additional authors

Viral RNA at two stages of reovirus infection is required for the induction of necroptosis

Angela K. Berger[1‡], Bradley E. Hiller[1#], Deepti Thete[1], Anthony J. Snyder[1], Encarnacion Perez Jr[2]., Jason W. Upton[2] and Pranav Danthi[1]*

[1]Department of Biology, Indiana University, Bloomington, Indiana 47405, USA
[2]Department of Molecular Biosciences, LaMontagne Center for Infectious Disease, University of Texas, Austin, TX 78712, USA

*, To whom correspondence should be addressed: Department of Biology, Indiana University, Bloomington, IN 47405. Tel: 812-856-2449, Fax: 812-856-5710, E-mail: pdanthi@indiana.edu

‡ Current address: Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322, USA.

Current address: Washington University in St Louis, 660 South Euclid Ave. St. Louis, MO 63110, USA.

ABSTRACT

Necroptosis, a regulated form of necrotic cell death requires the activation of the RIP3 kinase. Here, we identify that infection of host cells with reovirus can result in necroptosis. We find that necroptosis requires sensing of the genomic RNA within incoming virus particles via cytoplasmic RNA sensors to produce type I IFN. While these events that occur prior to de novo synthesis of viral RNA are required for induction of necroptosis, they are not sufficient. Induction of necroptosis also requires late stages of reovirus infection. Specifically, efficient synthesis of dsRNA within infected cells is required for necroptosis. These data indicate that viral RNA interfaces with host components at two different stages of infection to induce necroptosis. This work provides new molecular details about events in the viral replication cycle that contribute to the induction of necroptosis following infection with an RNA virus.

37 **IMPORTANCE**

38 An appreciation of how cell death pathways are regulated following viral infection
39 may reveal strategies to limit tissue destruction and prevent the onset of disease.
40 Cell death following virus infection can occur by apoptosis or a regulated form of
41 necrosis, known as necroptosis. Apoptotic cells are typically disposed of without
42 activating the immune system. In contrast, necroptotic cells alert the immune
43 system, resulting in inflammation and tissue damage. While apoptosis following
44 virus infection has been extensively investigated, how necroptosis is unleashed
45 following virus infection is only understood for a small group of viruses. Here,
46 using mammalian reovirus, we highlight the molecular mechanism by which
47 infection with a dsRNA virus results in necroptosis.

INTRODUCTION

Host cell death is a common outcome of virus infection (1). One form of cell death, necroptosis, has been described following infection with influenza A virus (IAV), herpes simplex virus 1 and 2 (HSV1 or 2), murine cytomegalovirus (MCMV), and vaccinia virus (VV). In each of these cases, necroptosis protects the infected animal (2-7). Examples also exist where increased necroptosis contributes to tissue injury and exacerbates viral disease (7, 8). The impact of necroptosis on these viral diseases may be due to premature death of the infected cell or as a consequence of inflammation induced by leakage of molecules from necrotic cells (9, 10).

Necroptosis requires the activation of receptor interacting protein 3 (RIP3) kinase (6, 11, 12). Once activated, RIP3 kinase signals via the pseudokinase, mixed lineage kinase-like (MLKL) protein to promote a necrotic form of cell death that is characterized by loss of membrane integrity and leakage of cellular contents (13-23). RIP3 contains a receptor-interacting protein homotypic interacting motif (RHIM) and is activated via interactions with other cellular RHIM-containing proteins - TRIF (TIR-domain-containing adapter-inducing interferon-β), RIP1, or DAI (DNA-dependent activator of IFN-regulatory factors) (24). TRIF activation by Toll-like receptor 3 (TLR3) and TLR4 ligands can evoke necroptosis but necroptosis by this mechanism has not yet been demonstrated following virus infection (25, 26). RIP1 activation by tumor necrosis factor α (TNFα) induces RIP3-dependent necroptosis following VV infection (6). The pathogen sensor,

DAI is required for necroptosis in cells infected with a MCMV variant (5). Ribonucleotide reductases, ICP6 and ICP10, respectively encoded by HSV1 and HSV2 contain a RHIM-like domain. These ribonucleotide reductases interact with murine RIP1 and RIP3, promote RIP1-RIP3 or RIP3-RIP3 oligomerization, and induce necroptosis (2, 3).

In contrast to these studies on DNA viruses, mechanisms by which RNA viruses induce necroptosis are less understood. IAV induces necroptosis in the lungs of cIAP2-deficient mice (8). Because uninfected cells also undergo cell death in this model, it is thought that cell death is a consequence of alteration in cellular homeostasis rather than induced by viral replication events. In wild-type cells, IAV activates a RIP3-containing signaling platform that can induce either apoptosis or necroptosis (7). Recent evidence suggests that DAI, which was previously thought to be a sensor for cytoplasmic DNA interacts with IAV components to engage RIP3 and induce necroptosis (27, 28). RNA viruses such as Coxsackievirus B (CVB), coronavirus, mammalian reovirus (Reovirus), Theiler's murine encephalomyelitis virus (TMEV), and West Nile virus (WNV) also have been demonstrated to evoke cell death with morphologic features resembling necrosis (29-32). However, the events in viral replication that initiate pronecrotic signaling pathways have not been defined for these RNA viruses.

In this study, we investigated the mechanism by which reovirus infection culminates in necroptosis. Our results indicate that IFNβ produced by detection

of genomic RNA of incoming virus particles is required, but not sufficient for eliciting necroptosis. In addition to IFNβ expression, de novo synthesis of viral dsRNA is also required for necroptosis induction. These results suggest that detection of viral components at two distinct stages is required for the induction of necroptosis following infection with an RNA virus.

Journal of Virology JVI Journal of Virology

MATERIAL AND METHODS

Cells and viruses. Spinner-adapted L929 cells (obtained from Dr. T. Dermody's laboratory) were maintained in Joklik's MEM (Lonza) supplemented to contain 5% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 µg/mL streptomycin, and 25 ng/mL of amphotericin B. Spinner-adapted L929 cells were used for cultivating and purifying viruses and for plaque assays. Prototype reovirus strain T3D was regenerated by plasmid based reverse genetics (33, 34). Viral particles were purified by Vertrel XF-extraction and CsCl gradient centrifugation (35). Viral titer was determined by plaque assay using spinner-adapted L929 cells (36). UV-inactivated virus was generated using a UV cross-linker (CL-1000 UV Crosslinker; UVP). Virus diluted in PBS was placed in a 60-mm tissue culture dish and irradiated with short-wave (254-nm) UV on ice at a distance of 10 cm for 1 min at 120,000 µJ/cm2. Murine L929 cells (ATCC CCL-1) were maintained in Eagle's MEM (Lonza) supplemented with 10% fetal bovine serum (FBS), and 2 mM L-glutamine. ATCC L929 cells were used for all experiments to assess cell death, viral RNA and protein synthesis, and cell signaling. Distinct from some L929 cell lines, the ATCC L929 cells used for this study do not undergo TNFα or zVAD-mediated cell death (37, 38). Wild-type and mutant bone marrow derived macrophages were obtained from Drs. Edward Mocarski and Mehul Suthar (Emory University) and were maintained in DMEM with 20% FBS, 10% filtered conditioned medium from L929 cells, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 µg/mL streptomycin.

122 **Reagents.** Z-VAD-FMK and Q-VD-OPh were purchased from Enzo Life
123 Sciences or R & D Systems, Necrostatin-1 was purchased from Calbiochem.
124 Ammonium chloride (AC), GuHCl, poly I:C, and human TNFα were purchased
125 from Sigma-Aldrich. siRNAs were purchased from Dharmacon as SMARTpools
126 of ON-TARGET plus siRNA. Non-targeting siRNA control pool or siRNA targeting
127 β-galactosidase were used as controls. Antisera raised against reovirus were
128 obtained from T. Dermody. Monoclonal antibody against IFNAR and rabbit
129 antisera against RIP3 were purchased from Santa Cruz Biotechnology, those
130 against TRIF, phospho-MLKL and total MLKL were purchased from Abcam, and
131 those against RIG-I and MDA5 were purchased from Cell Signaling. Mouse
132 antiserum specific for PSTAIR was purchased from Sigma, specific for KDEL
133 was purchased from Enzo Life Sciences. Alexa Fluor-conjugated anti-mouse
134 IgG, anti-rabbit IgG, and anti-goat IgG secondary antibodies were purchased
135 from Invitrogen. IRDye-conjugated anti-guinea pig IgG was purchased from LI-
136 COR.
137
138 **Fixing, embedding, and sectioning of infected cells.** L929 cells grown on 100
139 mm dishes were either mock infected or infected with 10 PFU/cell of T3D for 1 h
140 at room temperature. Following the viral attachment incubation, the cells were
141 washed twice with PBS and then overlayed with fresh medium. At 34 h post
142 infection, uninfected and infected cells were washed with PBS, trypsinized,
143 pelleted for 5 min at 800 × *g*, and washed again with PBS. The pelleted cells
144 were then fixed with 2.5% glutaraldehyde diluted in sodium cacodylate buffer

(100 mM sodium cacodylate [pH 7.5], 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5% NaCl) for 60 min at room temperature. Following fixation, the cells were washed twice with sodium cacodylate buffer. The washed cells were post fixed with 1% osmium tetroxide diluted in sodium cacodylate buffer for 60 min at room temperature. The fixed cells were washed twice with sodium cacodylate buffer followed by one wash with 100 mM sodium acetate [pH 4.2]. The cells were then stained with 0.5% uranyl acetate diluted in 100 mM sodium acetate [pH 4.2] for 60 min at room temperature. After staining, the cells were washed twice with 100 mM sodium acetate [pH 4.2]. Prior to embedding, the fixed and stained cells were dehydrated with sequential concentrations of ethanol (EtOH): 35% EtOH once for 5 min, 50% EtOH once for 5 min, 70% EtOH once for 5 min, 90% EtOH once for 5 min, 95% EtOH once for 5 min, and 100% EtOH four times for 5 min each. The dehydrated cells were incubated in a solution composed of 50% EMbed 812 resin and 50% EtOH for 2 h at room temperature. The cells were then incubated in 100% EMbed 812 resin overnight at room temperature. The next day, the resin was replaced with fresh EMbed 812 resin, which was allowed to harden for 18 h at 65°C. Thin-sections (85 nm thick) were collected using a diamond knife on a Leica Biosystems microtome.

Transmission electron microscopy (TEM). Thin-sections of uninfected and infected cells were applied to 300-mesh copper grids and stained with Reynold's lead citrate and 2% uranyl acetate (40). The stained grids were analyzed using a JEOL 1010 transmission electron microscope operating at 80 kV. Images were

recorded using a Gatan MegaScan 794 charge-coupled-device camera. Micrographs were processed and analyzed using ImageJ software.

Infections and preparation of extracts. Cells were either adsorbed with PBS or T3D at room temperature for 1 h, followed by incubation with media at 37ºC for the indicated time interval. Ribavirin, GuHCl, Z-VAD-FMK, Q-VD-OPh, Necrostatin-1, or anti-IFNAR Ab was added to the media immediately after the 1 h adsorption period. For preparation of whole cell lysates, cells were washed in phosphate-buffered saline (PBS) and lysed with 1X RIPA (50 mM Tris [pH 7.5], 50 mM NaCl, 1% TX-100, 1% DOC, 0.1% SDS, and 1 mM EDTA) containing a protease inhibitor cocktail (Roche), 500 μM DTT, and 500 μM PMSF, followed by centrifugation at 15000 × *g* for 10 min to remove debris. For detection of phosphorylated MLKL, cells were lysed in 1X RIPA supplemented with 10 mM NaF.

RNA transfection and cell death. L929 cells were mock infected or infected with 10 PFU/cell of T3D for 24 h. Total RNA was extracted using Tri-reagent (Molecular Research Center). When needed, the RNA was mock treated or CIP treated for 1 h at 37°C and repurified using Tri-reagent**.** 100 ng of RNA was introduced into the cells by Lipofectamine 2000 transfection. Cell death was measured 21-24 h following transfection.

Immunoblot assay. Cell lysates were resolved by electrophoresis in polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked for at least 1 h in blocking buffer (PBS containing 5% milk or 2.5% BSA) and incubated with antisera against MLKL (1:2000), phosho-MLKL (1:750), RIP3 (1:1000), MAVS (1:1000), RIG-I (1:1000), MDA5 (1:1000), TRIF (1:1000), or PSTAIR (1:10000) at 4ºC overnight. Membranes were washed three times for 5 min each with washing buffer (TBS containing 0.1% Tween-20) and incubated with 1:20000 dilution of Alexa Fluor conjugated goat anti-rabbit IgG (for RIP3, RIG-I, and MDA5), donkey anti-goat IgG (for RIP3), goat anti-mouse IgG (for KDEL and PSTAIR), or IRDye-conjugated anti-guinea pig IgG (for σNS) in blocking buffer. Following three washes, membranes were scanned using an Odyssey Infrared Imager (LI-COR).

Knockdown of host proteins by siRNA. In 96-well plates, 0.25 µl Lipofectamine 2000 was used to transfect 15 pmoles of siRNA. Cells (1×10^4) were seeded on top of the siRNA-lipofectamine mixture. In 24-well plates, 0.75 µl Lipofectamine 2000 was used to transfect 45 pmoles of siRNA. Cells (5×10^4) were seeded on top of the siRNA-lipofectamine mixture. Virus infection was performed 48 h following siRNA transfection.

Assessment of cell death by measuring cellular ATP levels. Cells (1×10^4) grown in black, clear-bottom 96-well plates were mock infected with PBS or adsorbed with 10 PFU/cell of T3D at room temperature for 1 h. Following

incubation of cells at 37°C for 42 h, ATP levels were assessed using the Cell titer-Glo assay system (Promega).

Assessment of cell death by acridine orange ethidium bromide staining. Cells grown in 24-well plates or 96-well plates were adsorbed with the indicated amount of virus. Inhibitors were added immediately following adsorption. The percentage of dead cells after 48 h incubation was determined using AOEB staining as described (41). For identifying host regulators of cell death, cells were transfected with siRNA as described above and incubated for 48 h prior to infection with T3D. For each experiment, >250 cells were counted by a blinded researchers, and the percentage of isolated cells exhibiting orange staining (EB positivity) was determined by epi-illumination fluorescence microscopy using a fluorescein filter set on an Olympus IX71 microscope. < 5% of uninfected cells were EB positive following treatment with each inhibitor or siRNA.

Assessment of cell death by IncuCyte automated cell imaging. Cells grown in 48-well plates were mock infected with PBS or adsorbed with the indicated amount of virus. Inhibitors were added immediately following adsorption in addition to 500 nM Sytox green. The cells were imaged over a time course of 48 h. Values of Sytox positive cells per mm^2 48 h following infection are shown.

Assessment of caspase-3/7 activity. ATCC L929 cells (1×10^4) were seeded into black clear-bottom 96-well plates, adsorbed with 10 PFU/cell of reovirus in

serum-free medium at room temperature for 1 h. Following incubation of cells at 37°C for 48 h, caspase-3/7 activity was quantified using the Caspase-Glo-3/7 assay system (Promega).

Assessment of viral yield. BMDMs in 24-well plates were adsorbed in triplicate with 50 PFU/cell of T3D for 1 h. Cells were washed once with PBS, and incubated for 0 h or 24 h. Cells were frozen and thawed twice prior to determination of viral titer by plaque assay. Viral yields were calculated according to the following formula: $\log_{10}\text{yield}_{24h} = \log_{10}(\text{PFU/ml})_{24h} - \log_{10}(\text{PFU/ml})_{0h}$.

RT-qPCR. RNA was extracted from infected cells at various time intervals after infection using Tri-reagent or an RNAeasy kit (Qiagen). For RT-qPCR, 0.5 to 2 μg of RNA was reverse transcribed using random hexamers or gene specific primers using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A 1:10 dilution of the cDNA was subjected to PCR using SYBR Select Master Mix (Applied Biosystems). ΔCt values for each cDNA sample were calculated by subtracting Ct values of T3DS1, ZBP1, or IFNβ and Ct values for GAPDH. Fold increase in gene expression with respect to control sample (indicated in each figure legend) was measured using the ΔΔCt method (42).

Statistical analysis. Statistical significance between experimental groups was determined using the unpaired *t*-test function of the Graphpad Prism software.

258 Statistical analyses for differences in gene expression by RT-qPCR were done
259 on the ΔCt values.
260

RESULTS

Reovirus-induces necroptosis. Upon ultrastructural evaluation of L929 cells infected with prototype reovirus strain Type 3 Dearing (T3D) 34 h following infection (a time point conducive for recovery and processing of dying cells for microscopy), we observed cells with normal nuclear morphology, the absence of apoptotic blebs, swelling of the cellular cytoplasm and early stages of disruption of the plasma membrane (Figure 1A). These features are not characteristic of apoptosis and suggested that reovirus may elicit an alternate form of cell death, such as necrosis. Cell death can be assessed by measurement of cellular ATP levels or by evaluating the permeability of cellular nuclei to DNA-staining vital dyes. These treatments do not distinguish between cell death by apoptosis or necrosis and therefore need to be coupled with pharmacologic blockade of molecules specifically involved in cell death pathways leading to apoptosis or necrosis (43). Consistent with the absence of apoptotic features, although pancaspase inhibitors Z-VAD-FMK or Q-VD-OPh abolish effector caspase activation in L929 cells infected with reovirus, they fail to block cell death (Figure 1B and 1C) (30). Instead, cell death following reovirus infection of L929 cells exhibits features of necrosis and is diminished by Nec1, a RIP1 kinase inhibitor (30). The kinase activity of RIP1 can potentiate the activation of RIP3 to promote necroptosis (6). To determine if reovirus-induced cell death occurs via this mechanism, we assessed the capacity of reovirus to elicit necrosis in cells expressing reduced levels of RIP3 (Figure 1D). We found that in comparison to cells treated with control siRNA, treatment of cells with siRNAs against RIP3

284 significantly decreased cell death (Figure 1E, 1F). The effect of RIP3 siRNA
285 against reovirus-induced cell death matched the effect of RIP3 siRNA on
286 necroptosis-inducing treatment of TNFα and Z-VAD-FMK (Figure 1G). These
287 data indicate a role for RIP3 in the induction of cell death following reovirus
288 infection. RIP3 can participate in the induction of both apoptosis and necroptosis
289 (7, 44, 45). Because cell death following reovirus infection is unaffected by
290 diminishment of caspase activity (Figure 1B,1C), these data suggest that reovirus
291 induces RIP3-dependent necroptosis in L929 cells.
292 RIP3-dependent necroptosis requires the activation of the effector protein
293 MLKL (13-23). MLKL is directly phosphorylated by RIP3 and MLKL
294 phosphorylation is considered to be a hallmark of the activation of necroptosis
295 signaling cascade (13, 46). To determine if reovirus infection leads to the
296 activation of MLKL, we immunoblotted extracts from reovirus-infected cells using
297 a phospho-MLKL antibody (Figure 1H). Our results indicate that MLKL is
298 activated within 24 h following reovirus infection and remains activated until 48 h
299 post infection, when a significant proportion of cells are undergoing cell death.
300 The detection of this biochemical marker along with the genetic and
301 pharmacologic experiments described above indicating that cell death is blocked
302 by loss of RIP3 function but not of caspase function, meet the criteria to
303 demonstrate that reovirus infection of L929 cells results in necroptosis (15).
304
305 Reovirus infects cells in a variety of tissues in newborn mice. Previous
306 work on reovirus-induced apoptosis has utilized primary neurons or mouse

307 embryo fibroblasts (MEFs) to evaluate cell death pathways in primary cells. Since
308 both neurons and MEFs succumb to reovirus via apoptosis (47-54), we used
309 bone marrow-derived macrophages (BMDMs) to determine whether reovirus can
310 induce necroptosis in primary cells. While it is not known if cells within the bone
311 marrow are infected in reovirus-infected animals, identification of primary cells
312 that undergo necroptosis following reovirus infection would allow us to
313 complement our siRNA studies with work in cells from mice genetically deficient
314 in important regulators of necroptosis. We found that cell death following reovirus
315 infection of BMDMs occurred in the absence of caspase activity (Z-VAD-FMK-
316 treated cells) or RIP1 kinase activity (Nec1-treated cells) but was diminished
317 when the activity of caspases and RIP1 kinase were simultaneously blocked
318 (Figure 2A). Consistent with this, cell death was not blocked by the genetic
319 absence of RIP3 but was reduced by blockade of caspases in the absence of
320 RIP3 (Figure 2B, 2C). Cells lacking both caspase-8 and RIP3 were also resistant
321 to death following reovirus infection (Figure 2D). These data indicate that
322 reovirus can induce necroptosis in BMDMs when apoptosis is blocked. These
323 findings match previous work in other systems where necroptosis is evident
324 when caspases have been rendered non-functional (55-57).

325 In the context of infection by other viruses, necroptosis is antiviral (2, 4-7).
326 To determine if necroptosis affects replication of reovirus, we measured viral
327 yield over 24 h of infection in wild-type and RIP3-deficient BMDMs in the
328 presence and absence of Z-VAD-FMK. Viral yields in wild-type cells treated with
329 DMSO or Z-VAD-FMK were ~ 1 $\log_{10}$ (Figure 2E). The genetic absence of RIP3

330 enhanced viral yield to ~ 1.7 $\log_{10}$. Importantly, viral yield did not change in RIP3-
331 deficient BMDMs in conditions where apoptosis was blocked using Z-VAD-FMK.
332 While the basis for the slight increase in viral yield in absence of RIP3 is unclear
333 and was not further investigated, our data suggest that the capacity of cells to
334 undergo necroptosis does not influence viral yield in cell culture. These data are
335 reminiscent of previous evidence indicating that blockade of apoptosis does not
336 influence reovirus replication in cell culture (47, 48). The absence of effect of cell
337 death on reovirus replication in cell culture may be due to the differences in
338 timing of the reovirus replication cycle and the induction of cell death. Whereas
339 reovirus completes its replication cycle in 18 h, cell death following infection is
340 not detected until 36-48 h following infection.
341
342 **Transfection of reovirus RNA can elicit necroptosis.** Reovirus strains that
343 exhibit a greater level of gene expression are more potent inducers of necrosis
344 (58). Blockade of reovirus + strand RNA synthesis using ribavirin blocks necrosis,
345 suggesting a possible role for viral RNA in the induction of necrosis (58).
346 Transfection of dsRNA mimic poly I:C in L929 cells treated with either type I or
347 type II IFNs results in cell death by necrosis (25, 26, 59). Because our data
348 suggested a role for reovirus RNA in the induction of necroptosis in infected cells,
349 we sought to determine if viral RNA was sufficient for the induction of
350 necroptosis. For these experiments, we purified total RNA from mock- or
351 reovirus-infected cells 24 h following infection. We found that in comparison to
352 RNA from mock-infected cells, RNA extracted from T3D-infected cells induced a

353 significantly greater amount of cell death following transfection into cells (Figure
354 3A, 3B). Cell death by transfected RNA was diminished by treatment with Nec1
355 but not Q-VD-OPh (Figure 3C), analogous to what we have reported in L929
356 cells infected with reovirus (30). These data are also consistent with previous
357 work indicating that poly I:C-induced cell death is blocked by Nec1 (26). Our
358 results presented above suggest that RNA isolated from reovirus-infected cells
359 elicits necroptosis following introduction into L929 cells. Interestingly, unlike
360 previous work with transfection of dsRNA into cells (25, 59), cell death following
361 transfection of RNA extracted from reovirus-infected cells did not require priming
362 of the cells with exogenous IFN.

363 We reasoned that necroptosis was induced without addition of exogenous
364 IFN because transfection of reovirus RNA obtained from infected cells can
365 induce the expression of IFNβ (60-62)(Figure 3D). Indeed, treatment with an
366 IFNAR-blocking antibody MAR1-5A3 (63), resulted in a reduction in cell death
367 (Figure 3D). IFNβ production following transfection of reovirus RNA occurs via
368 RIG-I-mediated detection of the RNA (60). Consistent with this, removal of the 5'
369 phosphates using CIP resulted in a reduction in the expression of IFNβ (Figure
370 3E) and the induction of cell death (Figure 3F, 3G). Interestingly, if cells were
371 primed with exogenous IFNβ before transfection, the capacity of CIP-treated
372 RNA to elicit necroptosis was restored (Figure 3F, 3G). These data suggest that
373 though RIG-I mediated detection of viral RNA is required for IFNβ production, it is
374 not sufficient for the induction of cell death. Thus, cell death induction occurs by
375 sensing of viral RNA via an alternate pathway.

376 Based on the evidence that poly I:C elicits necroptosis by TLR3 detection
377 and signaling to RIP3 via TRIF (25, 26), we examined whether reovirus RNA-
378 induced necroptosis could be blocked by treatment of cells with ammonium
379 chloride (AC), an agent that blocks TLR3-mediated detection of dsRNA (64). We
380 found that though AC did not negatively impact IFNβ expression following RNA
381 transfection (Figure 3H), it blocked cell death induction by transfected RNA
382 (Figure 3I). Consistent with previous work, these data indicate that detection of
383 reovirus RNA via RIG-I produces IFNβ. In addition, these data suggest that IFNβ
384 primes reovirus RNA transfected cell to undergo TLR3-dependent necroptosis.
385 Thus, the IFN- and TLR3-dependent pathway for induction of necroptosis
386 following transfection of reovirus RNA into L929 cells is similar to that previously
387 described for transfection of synthetic dsRNA (25, 59).
388
389 **Sensing of reovirus RNA during infection is required for necroptosis.** We
390 next sought to determine if detection of viral RNA in infected cells contributes to
391 cell death induction in reovirus-infected cells. During infection, reovirus RNA can
392 be detected by both RIG-I-like receptors (RLRs), RIG-I and MDA5 (65).
393 Simultaneous reduction of both RLRs, or their common downstream signaling
394 adaptor, MAVS, led to a significant reduction in cell death following reovirus
395 infection (Figure 4A, 4B, 4C). The susceptibility of cells to TNFα and Z-VAD-FMK
396 induced necroptosis was not changed following MAVS knockdown indicating that
397 MAVS is not required for the function of the core necroptosis machinery (Figure
398 4D). BMDMs deficient in either both RLRs or MAVS were also protected from

399 reovirus-induced necroptosis (Figure 4E, 4F). These data suggest that
400 necroptosis following reovirus infection requires detection and signaling by RLRs.
401
402 **IFN signaling is required for necroptosis.** To determine whether type I IFNs
403 produced by RLR-MAVS signaling are required for reovirus-induced necroptosis,
404 we quantified the capacity of reovirus to induce necroptosis in the presence of an
405 IFNAR-blocking antibody (63). We found that this antibody diminished the
406 expression of a representative interferon-stimulated gene (ISG), ZBP1, which is
407 potently induced following reovirus infection (66), and diminished the capacity of
408 reovirus to induce necroptosis (Figure 5A, 5B, 5C). This reduction in necroptosis
409 was not due to a deleterious effect of the antibody on the capacity of reovirus to
410 establish infection (Figure 5D). Blocking IFNAR signaling did not alter the
411 capacity of TNFα and Z-VAD-FMK cotreatment to induce necroptosis, suggesting
412 that this treatment did not affect the function of the core necroptosis machinery
413 (Figure 5E). IFNAR-deficient BMDMs treated with Z-VAD-FMK also were
414 resistant to reovirus-induced necroptosis (Figure 5E, 5F), indicating a role for IFN
415 signaling in the induction of necroptosis following reovirus infection.
416 Based on the role for TLR3 in necroptosis following transfection of RNA
417 obtained from reovirus-infected cells (Figure 3), we next sought to evaluate
418 whether TLR3 is also required for cell death in reovirus-infected cells. Toward
419 this goal, we tested the effect of AC on cell death induction in reovirus-infected
420 cells. Because treatment of cells with AC prevents reovirus infection by blocking
421 capsid disassembly, we initiated infection of AC-treated cells with infectious

422 subvirion particles (ISVPs), a viral entry intermediate that bypasses the inhibitory
423 effect of AC (67). We found that necroptosis following infection by ISVPs was
424 unaffected by treatment with AC (Figure 5G). Parallel transfection of cells with
425 reovirus RNA in control and AC treated cells yielded results that matched those
426 described in Figure 3H (data not shown), indicating that AC treatment was
427 effective. siRNA-mediated reduction in the expression of TRIF, the TLR3 adaptor
428 also did not block cell death following reovirus infection (Figure 5H, 5I). These
429 data indicate that although RLR-mediated IFNβ expression and signaling is
430 required for necroptosis following both, RNA transfection and viral infection,
431 TLR3-mediated signaling is only required for cell death after viral RNA
432 transfection.
433
434 **Two-stage detection of reovirus infection is required for necroptosis.**
435 We next sought to determine the stage of infection required for the induction of
436 necroptosis. Blockade of viral + strand RNA synthesis using ribavirin diminishes
437 reovirus-induced necrosis (58). The reovirus + sense RNA can direct protein
438 synthesis or can be packaged into progeny core particles and serve as the
439 template for minus strand RNA synthesis to generate viral genomic dsRNA (68).
440 Progeny cores containing genomic dsRNA undergo secondary transcription to
441 produce additional viral mRNAs. Thus, the diminishment of necroptosis by
442 ribavirin treatment may be due to blockade of any of these steps in reovirus
443 replication. To define the stage of infection required for necroptosis further, we
444 used Guanidine hydrochloride (GuHCl). GuHCl does not affect reovirus + strand

RNA synthesis but prevents the generation of genomic dsRNA within infected cells (Figure 6A, 6C)(69). Under the conditions used, perhaps because sufficient translation occurs from primary transcripts, we did not observe a diminishment in viral protein synthesis in the presence of GuHCl (Figure 6B). Treatment of reovirus-infected cells with GuHCl led to diminishment in necroptosis (Figure 6D, 6E). Because GuHCl does not affect necroptosis induced by TNFα and Z-VAD-FMK treatment (Figure 6F), our results point to the importance of the synthesis of viral genomic dsRNA for the induction of necroptosis following reovirus infection.

It is not known when during infection reovirus RNA is detected to produce IFNβ. The type of reovirus RNA that activates the expression of IFNβ in the context of infection also remains undefined. Ribavirin and GuHCl may thus indirectly prevent cell death because they affect the synthesis of RNA required for IFNβ synthesis. To better understand the effect of ribavirin and GuHCl on reovirus-induced cell death, we measured the expression of the IFNβ mRNA at different times following infection of L929 cells with reovirus. We observed a ~ 10 fold increase in IFNβ mRNA levels 12 h following infection (Figure 7A). No further increase in IFNβ mRNA was observed at 18 or 24 h following infection. We found that though IFNβ mRNA expression was diminished by blockade of virus disassembly using AC, it was not decreased by either ribavirin or GuHCl treatment (Figure 7B). Although UV-inactivated virus failed to produce detectable levels of reovirus S1 + strand RNA (> 3 $\log_{10}$-fold reduction), it remained capable of eliciting the same level of IFNβ mRNA expression as control, infectious virus (Figure 7C, 7D). These data suggest that genomic RNA present within incoming

viral particles is sufficient for the induction of IFNβ expression. These results are consistent with data describing IFN induction by a UV inactivated reovirus mutant, IRF3 activation following reovirus infection in absence of RNA synthesis and recent studies on IFN expression following avian reovirus infection (49, 70, 71). We observed that an infection-induced increase in IFNβ expression was diminished in cells transfected with MAVS siRNA (Figure 7E). Importantly, MAVS was also required for efficient induction of IFNβ expression in reovirus-infected cells when viral + strand RNA synthesis was blocked using ribavirin (Figure 7E). These data suggest that genomic RNA within incoming virus particles is detected by cytoplasmically localized RLRs and signals via MAVS to produce IFNβ. Because necroptosis is blocked by GuHCl under conditions where IFNβ is produced but viral dsRNA synthesis is diminished (Figure 6, 7), these studies indicate that IFNβ signaling is required but not sufficient for the induction of cell death. Together, our data indicate a role for reovirus RNA at two different stages of infection to induce necroptosis. First, viral genomic dsRNA is detected during entry to activate type I IFN signaling. Second, generation of newly synthesized viral dsRNA is required for the induction of necroptosis.

DISCUSSION

In this manuscript, we demonstrate that reovirus infection of both cultured cells and primary murine macrophages evokes necroptosis. Our results point to a role for viral components at two stages of infection to evoke necroptosis (Figure 8). First, detection of the incoming viral genomic RNA by host cell cytoplasmic sensors to produce IFNβ is required for necroptosis (Figure 4). In addition, synthesis of new viral genomic dsRNA also is required for the induction of necroptosis (Figure 6). This work indicates that the type I IFN signaling pathway functions in the induction of necroptosis following infection by an RNA virus. These data provide evidence for a previously unknown signaling cascade by which infection with an RNA virus culminates in necroptosis.

IFN signaling has been previously implicated in the induction of necroptosis. In *Salmonella typhimurium* infected mice, murine macrophages undergo necroptosis (72). In this context, the IFNAR is internalized and complexes with RIP1 and RIP3 to elicit necroptosis (72). ISGF3, a protein complex that drives the expression of ISGs following IFN signaling is required for sustained activation of RIP3 following ligation of TNFR or TLRs (73). However, whether a particular ISG modulates the basal activity of RIP3 has not been defined. Multiple ISGs are implicated in the induction of necroptosis. These include ZBP1/DAI, which may sense either viral DNA, viral RNA, or viral proteins, and those that recognize viral dsRNA (TLR3 and PKR)(5, 25, 27, 28, 74). Based on the role of DAI in the induction of necroptosis following IAV infection (27, 28), we tested the contribution of DAI to reovirus induced cell death. We found that

508 reovirus remained capable of inducing cell death in ZBP1-deficient BMDMs (data
509 not shown). Our results suggest that TLR3 does not participate in necroptosis
510 induction following reovirus infection (Figure 5). PKR can promote necroptosis in
511 cells lacking functional FADD (74). Reovirus induces necroptosis in wild-type
512 cells expressing FADD (Figure 1, 2). Moreover, because reovirus encodes a
513 well-described PKR inhibitor, we think it unlikely that PKR is involved in this
514 process (75). Thus, the identity of the ISGs that control necroptosis following
515 reovirus infection remains to be determined. Because IAV induced necroptosis is
516 unaffected by the genetic absence of MAVS or IFNAR (7) and requires ZBP1
517 (27, 28), whereas reovirus-induced necroptosis requires MAVS and IFNAR
518 (Figure 4, 5) but is not affected by the absence of ZBP1, the mechanism
519 underlying necroptosis following reovirus infection appears distinct from that
520 reported for IAV.

521 Investigations into reovirus-induced cell death indicate that reovirus
522 infection can initiate cell death signaling from distinct stages of replication and
523 elicit cell death via a variety of pathways. The precise pathway that executes cell
524 death likely varies with cell type. One model suggests that events initiated during
525 cell entry that occur after virus disassembly but prior to de novo synthesis of viral
526 RNA and proteins can elicit cell death by apoptosis (76). Apoptosis by this
527 mechanism is thought to occur independently of the presence of viral genomic
528 RNA but relies on the function of the μ1 capsid protein and the host transcription
529 factor NFκB (47, 77). Another set of studies implicates a role for viral genomic
530 RNA, viral RNA sensors, and IRF3 in the induction of apoptosis. However, cell

death by this pathway does not appear to require viral replication or type I IFN signaling (49, 78). Two BH3-only members of the Bcl-2 family, Bid and Noxa appear to be involved in the induction of apoptosis and their function is downstream of transcription factors NFκB and IRF3 (48, 78). Our studies presented highlight an additional way in which reovirus infection leads to cell death. First, distinct from previous work on reovirus-induced apoptosis, we show that IFN signaling is required for necroptosis. Though we have not directly tested its requirement, IRF3, which is required for IFNβ expression (79), likely also plays a role in necroptosis. Thus the requirement for IRF3 in reovirus induced apoptosis and necroptosis is likely shared. Unlike for apoptosis, we demonstrate that the generation of viral genomic dsRNA late in infection is required for necroptosis (Figure 5). The requirement for genomic dsRNA synthesis may be direct, similar to the detection of viral RNA during transfection (Figure 2). Alternatively, synthesis of genomic dsRNA may be required to produce secondary transcripts, which in turn are detected by the host cell to induce necroptosis. Secondary transcripts generated following reovirus infection are qualitatively different than primary transcripts, and therefore, it is possible that secondary transcripts are detected in a manner distinct from primary transcripts (80). Though our studies indicate that protein synthesis in absence of ongoing dsRNA synthesis is not sufficient for necroptosis induction (Figure 6), it remains possible that viral proteins modulate necroptosis following reovirus infection.

Studies thus far have indicated a pathogenic role for apoptosis in reovirus-induced encephalitis and myocarditis (81). Cell death pathways in reovirus-

infected animals are thought to be tissue specific but precisely how these cell death pathways differ in a tissue-specific manner has not been defined (82, 83). It is possible that in some cases, cell death via IFN-dependent pathways we have described in this study contribute to tissue injury. Due to its natural preference for infecting and killing transformed cells and its innocuousness to human adults, reovirus is currently in phase III clinical trials as a cancer therapeutic (84). The capacity of reovirus to elicit cell death via multiple mechanisms may therefore underlie its efficacy as an effective therapeutic.

Journal of Virology

JVI

Journal of Virology

562 **ACKNOWLEDGMENTS**

563 We are grateful to Bernardo Mainou, Indiana University Virology colleagues, and

564 members of our laboratory for helpful discussions and review of the manuscript.

565 Transmission electron microscopy was performed in the Indiana University

566 Bloomington Electron Microscopy Center with assistance of Dr. Barry Stein.

FIGURE LEGENDS

Figure 1. Reovirus-induces necroptosis in L929 cells. (A) L929 cells infected with 10 PFU/cell of T3D for 34 h were fixed, stained, and imaged using transmission electron microscopy. (B) Cell death in L929 cells 48 h following mock infection or infection with 10 PFU/cell of T3D and treatment with DMSO or Q-VD-OPh (20 μM) was assessed by Cell Titer Glo. Luminescence measurement in similarly treated, uninfected cells was considered to represent 100% viability. (C) Caspase-3/7 activity 48 h following infection of L929 cells with 10 PFU/cell of T3D and treatment with DMSO or Q-VD-OPh was assessed by a chemiluminescent enzymatic assay. Caspase activity in mock-infected cells was set to 1. Data are represented as relative caspase-3/7 activity in comparison to similarly treated, uninfected cells. *, $P < 0.05$ compared to cells treated with DMSO. (D, E, F, G) L929 cells were transfected with non-targeting siRNAs or siRNAs specific for RIP3 (D) Efficiency of knockdown was assessed by immunoblotting for RIP3 and PSTAIR loading control. (E) Cell death 48 h following mock infection or infection with 10 PFU/cell of T3D was assessed by Cell Titer Glo. Luminescence measurement in similarly treated, uninfected cells was considered to represent 100% viability. *, $P < 0.05$ compared to cells transfected with non-targeting siRNAs. (F) Cell death 48 h following infection with 10 PFU/cell of T3D was assessed by AOEB staining. *, $P < 0.05$ compared to cells transfected with non-targeting siRNAs. (G) Cell death 3 h following treatment with TNFα and Z-VAD-FMK treatment was assessed by Cell Titer Glo. Luminescence measurement in similarly siRNA treated, DMSO treated cells was

considered to represent 100% viability. (H) Whole cell extracts from L929 cells infected with 10 PFU/cell of T3D at the indicated time points were immunoblotted for phosphorylated MLKL, total MLKL, and PSTAIR loading control.

Figure 2. Reovirus can induce necroptosis in primary BMDMs. (A) BMDMs from wild-type mice were mock infected or infected with 50 PFU/cell of T3D in the presence of DMSO, Z-VAD-FMK (25 μM) or Nec1 (50 μM) or both inhibitors. Cell death 48 h following infection was assessed by Cell Titer Glo. Luminescence measurement in similarly treated, uninfected cells was considered to represent 100% viability. *, $P < 0.05$ compared to DMSO treated cells. (B) BMDMs from wild-type (left panel) or RIP3 -/- (right panel) mice were mock infected or infected with 50 PFU/cell of T3D in the presence DMSO or Z-VAD-FMK (25 μM). Cell death 48 h following infection was assessed by Cell Titer Glo. Luminescence measurement in uninfected cells of the same genotype that were similarly treated was considered to represent 100% viability. *, $P < 0.05$ compared to DMSO treated cells of the same genotype. (C) BMDMs were infected with 50 PFU/cell of T3D in the presence DMSO or Z-VAD-FMK (25 μM). Cell viability was assessed by Sytox green staining. *, $P < 0.05$ compared to DMSO treated cells of the same genotype. (D) BMDMs from wild-type, RIP3 -/-, or Casp8 -/- x RIP3 -/- mice were infected with 50 PFU/cell of T3D. Cell death 48 h following infection was assessed by Cell Titer Glo. Luminescence measurement in mock-infected cells of the same genotype was considered to represent 100% viability. *, $P < 0.05$ compared to wild-type cells. (E) BMDMs from wild-type or RIP3 -/- mice were

613 infected with 50 PFU/cell of T3D in the presence or absence of Z-VAD-FMK (25
614 μM). Virus yield 24 h following infection was measured using plaque assay.
615
616 **Figure 3. Reovirus RNA is sufficient for the induction of necroptosis.** (A,B)
617 L929 cells were transfected with 100 ng of RNA extracted from mock-infected or
618 reovirus-infected cells. (A) Cell death 24 h following transfection was assessed
619 by Cell Titer Glo. Luminescence measurement in untransfected cells was
620 considered to represent 100% viability. *, $P < 0.05$ compared to cells transfected
621 with RNA extracted from mock-infected cells. (B) Cell death 24 h following
622 transfection of RNA was assessed by AOEB staining. *, $P < 0.05$ compared to
623 cells transfected with RNA extracted from mock-infected cells (C) L929 cells
624 were transfected with 100 ng of RNA extracted from mock infected or reovirus-
625 infected cells in the presence of DMSO, Q-VD-OPh (25 μM) or Nec1 (50 μM).
626 Cell death 24 h following transfection was assessed by Cell Titer Glo.
627 Luminescence measurement in similarly treated cells transfected with RNA from
628 mock-infected cells was considered to represent 100% viability. *, $P < 0.05$
629 compared to cells transfected with DMSO treated cells transfected with the same
630 type of RNA (D) L929 cells were transfected with 100 ng of RNA extracted from
631 mock-infected or reovirus-infected cells. Levels of IFNβ mRNA were assessed by
632 RT-qPCR at 18 h following transfection. IFNβ:GAPDH ratio for cells transfected
633 with RNA from mock-infected cells was considered 1. *, $P < 0.05$ compared to
634 cells transfected with RNA from mock-infected cells. (E) L929 cells were
635 transfected with 100 ng of RNA extracted from mock-infected or reovirus-infected

636 cells in the presence and absence of 0.1 μg/ml anti-IFNAR Ab. Cell death 24 h
637 following transfection was assessed by Cell Titer Glo. Luminescence
638 measurement in similarly treated cells transfected with RNA from mock-infected
639 cells was considered to represent 100% viability. *, $P < 0.05$ compared to cells
640 transfected with same type of RNA without anti-IFNAR Ab. (F) L929 cells were
641 transfected 100 ng of untreated or CIP-treated RNA extracted from reovirus-
642 infected cells. Levels of IFNβ mRNA were assessed by RT-qPCR at 18 h
643 following transfection. IFNβ:GAPDH ratio for cells transfected with untreated
644 RNA from reovirus-infected cells was considered 1. *, $P < 0.05$ compared to cells
645 transfected with untreated RNA from reovirus-infected cells. (G) Cells treated
646 with 0 or 100 units/ml IFNβ were transfected with 100 ng of untreated RNA from
647 mock-infected cells or untreated or CIP-treated RNA from T3D-infected cells. Cell
648 death 24 h following transfection was assessed by Cell Titer Glo. Luminescence
649 measurement in similarly treated cells transfected with RNA from mock-infected
650 cells was considered to represent 100% viability. *, $P < 0.05$ compared to
651 similarly treated cells transfected with untreated RNA from T3D-infected cells. **,
652 $P < 0.05$ compared to cells transfected with similarly treated RNA in the presence
653 of 0 units/ml of IFNβ. (H) Cells treated with 0 or 100 units/ml IFNβ were
654 transfected with 100 ng of untreated or CIP-treated RNA from T3D-infected cells.
655 Cell death 24 h following transfection of RNA was assessed by AOEB staining. *,
656 $P < 0.05$ compared to cells transfected with similarly treated RNA in the presence
657 of 0 mU/ml of IFNβ. (I) Cells pretreated with 0 or 20 mM AC were transfected
658 with RNA from T3D-infected cells. Levels of IFNβ mRNA were assessed by RT-

qPCR at 18 h following transfection. IFNβ:GAPDH ratio for cells 0 mM AC treated cells transfected with RNA from T3D-infected cells was considered 1. (J) Cells pretreated with 0 or 20 mM AC were transfected with 100 ng RNA from mock-infected or T3D-infected cells. Cell death 24 h following transfection was assessed by Cell Titer Glo. Luminescence measurement in similarly treated cells transfected with RNA from mock-infected cells was considered to represent 100% viability. *, $P < 0.05$ compared to transfection of cells treated with 0 mM AC.

Figure 4. Detection of viral RNA by cytoplasmic sensors is required for necroptosis. (A) L929 cells were transfected with non-targeting siRNAs or siRNAs specific for RIG-I, MDA5, or MAVS. Efficiency of knockdown was assessed by immunoblotting for RIG-I, MDA5, MAVS and KDEL or PSTAIR loading controls. (B, C, D) L929 cells were transfected with non-targeting siRNAs or siRNAs specific for both RIG-I and MDA5, or MAVS. (B) Cell death 48 h following mock infection or infection with 10 PFU/cell of T3D was assessed by Cell Titer Glo. Luminescence measurement in uninfected cells transfected with the same siRNA was considered to represent 100% viability. *, $P < 0.05$ compared to cells transfected with non-targeting siRNAs. (C) Cell death 48 h following infection with 10 PFU/cell of T3D was assessed by AOEB staining. *, $P < 0.05$ compared to cells transfected with non-targeting siRNAs. (D) Cell death 3 h following treatment with TNFα and Z-VAD-FMK treatment was assessed by Cell Titer Glo. Luminescence measurement in similarly siRNA treated, DMSO

682 treated cells was considered to represent 100% viability. (E) Cell death in wild-
683 type, RIG-I -/- x MDA5 -/- or MAVS -/- BMDMs treated with Z-VAD-FMK following
684 mock infection or infection with 50 PFU/cell of T3D was assessed by Cell Titer
685 Glo. Luminescence measurement in mock-infected cells of the same genotype
686 was considered to represent 100% viability. *, $P < 0.05$ compared to wild-type
687 cells. (F) Cell death in wild-type, RIG-I -/- x MDA5 -/- or MAVS -/- BMDMs treated
688 with Z-VAD-FMK (25 μM) following infection with 50 PFU/cell of T3D was
689 assessed by Sytox green staining. *, $P < 0.05$ compared to wild-type cells.
690
691 **Figure 5. Signaling via IFNAR is required for necroptosis** (A,B,C,D) L929
692 cells were infected with 10 PFU/cell of T3D in the presence of 0.1 μg/ml of anti-
693 IFNAR Ab. (A) Levels of ZBP1 mRNA were assessed using RT-qPCR at 24 h
694 post infection. ZBP1:GAPDH ratio at 0 h post infection was set to 1. *, $P < 0.05$
695 compared to cells infected without IFNAR antibody. (B) Cell death 48 h following
696 mock infection or infection with 10 PFU/cell of T3D was assessed by Cell Titer
697 Glo. Luminescence measurement in similarly treated, mock-infected cells was
698 considered to represent 100% viability. *, $P < 0.05$ compared to cells infected
699 without IFNAR antibody. (C) Cell death 48 h following infection with 10 PFU/cell
700 of T3D was assessed by AOEB staining. *, $P < 0.05$ compared to cells infected
701 without IFNAR antibody. (D) Viral infectivity 18 h following infection with 2
702 PFU/cell T3D was assessed by indirect immunofluorescence. (E) Cell death 3 h
703 following treatment with TNFα and Z-VAD-FMK treatment was assessed by Cell
704 Titer Glo. Luminescence measurement cells treated without IFNAR antibody was

705 considered to represent 100% viability. (F) Cell death in wild-type and IFNAR-
706 deficient BMDMs treated with Z-VAD-FMK following mock infection or infection
707 with 50 PFU/cell of T3D. Cell viability was assessed by Cell titer Glo.
708 Luminiscence measurement in mock-infected cells of the same genotype was
709 considered to represent 100% viability. *, $P < 0.05$ compared to wild-type cells.
710 (G) Cell death in wild-type and IFNAR-deficient BMDMs treated with Z-VAD-FMK
711 following infection with 50 PFU/cell of T3D was assessed by Sytox green
712 staining. *, $P < 0.05$ compared to wild-type cells. (H) L929 cells were mock
713 infected or infected with 100 PFU/cell of T3D ISVPs in the presence or absence
714 of 20 mM AC. Cell death 48 h following infection was assessed by Cell Titer Glo.
715 Luminiscence measurement in similarly treated, mock-infected cells was
716 considered to represent 100% viability. *, $P < 0.05$ compared to cells infected
717 without AC. (H, I) L929 cells were transfected with non-targeting siRNAs or
718 siRNAs specific for TRIF. (H) Efficiency of knockdown was assessed by
719 immunoblotting for TRIF and PSTAIR loading control. (I) Cell death 48 h
720 following mock infection or infection with 10 PFU/cell of T3D was assessed by
721 Cell Titer Glo. Luminescence measurement in uninfected cells transfected with
722 the same siRNA was considered to represent 100% viability.
723
724 **Figure 6. Synthesis for genomic dsRNA is required for necroptosis.** L929
725 cells were infected with 10 PFU/cell of T3D in the presence of ribavirin (200 μM)
726 or GuHCl (15 mM). (A) Levels of reovirus + strand RNA corresponding to the viral
727 S1 gene segment were measured by RT-qPCR 24 h post infection. Reovirus

728 T3D S1 +:GAPDH ratio in untreated cells infected for 0 h was considered 1.*, *P* <
729 0.05 compared to cells infected with T3D in absence of inhibitor. (B) Levels of
730 reovirus μ1C protein and PSTAIR loading control 24 h following infection with 10
731 PFU/cell of T3D were assessed by immunoblotting. (C) Generation of reovirus
732 genomic dsRNA at 24 h following infection was evaluated by electropherotyping.
733 (D) Cell death 48 h following mock infection or infection with 10 PFU/cell of T3D
734 was assessed by Cell Titer Glo. Luminescence measurement in similarly treated,
735 uninfected cells was considered to represent 100% viability. *, *P* < 0.05
736 compared to control treated cells. (E) Cell death 48 h following infection with 10
737 PFU/cell of T3D was assessed by AOEB staining. *, *P* < 0.05 compared to
738 control treated cells. (F) Cell death 4 h following treatment with TNFα and Z-
739 VAD-FMK treatment was assessed by Cell Titer Glo. Cell viability in similarly
740 treated cells in absence of TNFα and Z-VAD-FMK was considered 100%.
741
742 **Figure 7. De novo synthesis of viral RNA is not required for IFN expression.**
743 (A) L929 cells were infected with 10 PFU/cell of T3D. Levels of IFNβ mRNA were
744 assessed at the indicated time intervals using RT-qPCR. IFNβ:GAPDH ratio at 0
745 h post infection was set to 1. *, *P* < 0.05 compared to cells infected for 0 h. (B)
746 L929 cells treated with AC (20 mM), ribavirin (200 μM), or GuHCl (15 mM) were
747 infected with 10 PFU/cell of T3D. Levels of IFNβ mRNA were assessed by RT-
748 qPCR at 24 h post infection. IFNβ:GAPDH ratio for untreated, T3D-infected cells
749 was set to 1. *, *P* < 0.05 compared to control treated, infected cells. (C,D) L929
750 cells were infected with 10 PFU/cell of T3D and equivalent particles of UV-

751 treated T3D. (C) Levels of reovirus + strand RNA corresponding to the viral S1
752 gene segment were measured by RT-qPCR 24 h post infection. Reovirus T3D
753 S1 +:GAPDH ratio in cells infected for 0 h was considered 1. UD, undetectable,
754 value below that detected at 0 h (D) Levels of reovirus IFNβ RNA corresponding
755 were measured by RT-qPCR 24 h post infection. IFNβ:GAPDH ratio in cells
756 infected with infectious T3D was considered 1. (E) L929 cells were transfected
757 with non-targeting siRNAs or siRNAs specific for MAVS. Levels of IFNβ mRNA in
758 cells infected with 10 PFU/cell of T3D in the presence of absence of ribavirin
759 (200 μM) were assessed by RT-qPCR. IFNβ:GAPDH ratio for untreated, T3D
760 infected non-targeting siRNA treated was set to 1. *, $P < 0.05$ compared to
761 untreated, T3D infected non-targeting siRNA treated cells.
762
763 **Figure 8. Model for reovirus-induced necroptosis.** Genomic RNA from
764 incoming viral particles is sensed by RLRs to produce type I IFN in a MAVS-
765 dependent manner. De novo synthesized viral genomic dsRNA or viral
766 secondary transcripts produced from newly synthesized genomic dsRNA (GuHCl
767 sensitive replication events) are detected by an as yet unidentified ISG to elicit
768 RIP3-dependent necrotic cell death.

REFERENCES

1. **Danthi P.** 2016. Viruses and the Diversity of Cell Death. Annu Rev Virol **3:**533-553.

2. **Wang X, Li Y, Liu S, Yu X, Li L, Shi C, He W, Li J, Xu L, Hu Z, Yu L, Yang Z, Chen Q, Ge L, Zhang Z, Zhou B, Jiang X, Chen S, He S.** 2014. Direct activation of RIP3/MLKL-dependent necrosis by herpes simplex virus 1 (HSV-1) protein ICP6 triggers host antiviral defense. Proc Natl Acad Sci U S A **111:**15438-15443.

3. **Huang Z, Wu SQ, Liang Y, Zhou X, Chen W, Li L, Wu J, Zhuang Q, Chen C, Li J, Zhong CQ, Xia W, Zhou R, Zheng C, Han J.** 2015. RIP1/RIP3 binding to HSV-1 ICP6 initiates necroptosis to restrict virus propagation in mice. Cell Host Microbe **17:**229-242.

4. **Upton JW, Kaiser WJ, Mocarski ES.** 2010. Virus inhibition of RIP3-dependent necrosis. Cell host & microbe **7:**302-313.

5. **Upton JW, Kaiser WJ, Mocarski ES.** 2012. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell Host Microbe **11:**290-297.

6. **Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK.** 2009. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell **137:**1112-1123.

7. **Nogusa S, Thapa RJ, Dillon CP, Liedmann S, Oguin TH, 3rd, Ingram JP, Rodriguez DA, Kosoff R, Sharma S, Sturm O, Verbist K, Gough PJ, Bertin J, Hartmann BM, Sealfon SC, Kaiser WJ, Mocarski ES, Lopez CB, Thomas PG, Oberst A, Green DR, Balachandran S.** 2016. RIPK3 Activates Parallel Pathways of MLKL-Driven Necroptosis and FADD-Mediated Apoptosis to Protect against Influenza A Virus. Cell Host Microbe **20:**13-24.

8. **Rodrigue-Gervais IG, Labbe K, Dagenais M, Dupaul-Chicoine J, Champagne C, Morizot A, Skeldon A, Brincks EL, Vidal SM, Griffith TS, Saleh M.** 2014. Cellular inhibitor of apoptosis protein cIAP2 protects against pulmonary tissue necrosis during influenza virus infection to promote host survival. Cell Host Microbe **15:**23-35.

9. **Chan FK.** 2012. Fueling the flames: Mammalian programmed necrosis in inflammatory diseases. Cold Spring Harb Perspect Biol **4**.

10. **Mocarski ES, Kaiser WJ, Livingston-Rosanoff D, Upton JW, Daley-Bauer LP.** 2014. True grit: programmed necrosis in antiviral host defense, inflammation, and immunogenicity. J Immunol **192:**2019-2026.

11. **Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J.** 2009. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science **325:**332-336.

12. **He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X.** 2009. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell **137:**1100-1111.

13. **Wang H, Sun L, Su L, Rizo J, Liu L, Wang LF, Wang FS, Wang X.** 2014. Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. Mol Cell **54:**133-146.

14. **Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, Liu ZG.** 2012. Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proceedings of the National Academy of Sciences of the United States of America **109:**5322-5327.

15. **Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nunez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon HU, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G.** 2012. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ **19:**107-120.

16. **Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X.** 2012. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell **148:**213-227.

17. **Quarato G, Guy CS, Grace CR, Llambi F, Nourse A, Rodriguez DA, Wakefield R, Frase S, Moldoveanu T, Green DR.** 2016. Sequential Engagement of Distinct MLKL Phosphatidylinositol-Binding Sites Executes Necroptosis. Mol Cell **61:**589-601.

18. **Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang JG, Alvarez-Diaz S, Lewis R, Lalaoui N, Metcalf D, Webb AI, Young SN, Varghese LN, Tannahill GM, Hatchell EC, Majewski IJ, Okamoto T, Dobson RC, Hilton DJ, Babon JJ, Nicola NA, Strasser A, Silke J, Alexander WS.** 2013. The Pseudokinase MLKL Mediates Necroptosis via a Molecular Switch Mechanism. Immunity **39:**443-453.

19. **Moujalled DM, Cook WD, Murphy JM, Vaux DL.** 2014. Necroptosis induced by RIPK3 requires MLKL but not Drp1. Cell Death Dis **5:**e1086.

20. **Hildebrand JM, Tanzer MC, Lucet IS, Young SN, Spall SK, Sharma P, Pierotti C, Garnier JM, Dobson RC, Webb AI, Tripaydonis A, Babon JJ, Mulcair MD, Scanlon MJ, Alexander WS, Wilks AF, Czabotar PE, Lessene G, Murphy JM, Silke J.** 2014. Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death. Proc Natl Acad Sci U S A **111:**15072-15077.

21. **Galluzzi L, Kepp O, Kroemer G.** 2014. MLKL regulates necrotic plasma membrane permeabilization. Cell Res **24:**139-140.

22. **Dondelinger Y, Declercq W, Montessuit S, Roelandt R, Goncalves A, Bruggeman I, Hulpiau P, Weber K, Sehon CA, Marquis RW, Bertin J, Gough PJ, Savvides S, Martinou JC, Bertrand MJ, Vandenabeele P.** 2014. MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. Cell Rep **7:**971-981.

23. **Cai Z, Jitkaew S, Zhao J, Chiang HC, Choksi S, Liu J, Ward Y, Wu LG, Liu ZG.** 2014. Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. Nat Cell Biol **16:**55-65.

24. **Newton K.** 2015. RIPK1 and RIPK3: critical regulators of inflammation and cell death. Trends Cell Biol **25:**347-353.

25. **Kaiser WJ, Sridharan H, Huang C, Mandal P, Upton JW, Gough PJ, Sehon CA, Marquis RW, Bertin J, Mocarski ES.** 2013. Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. J Biol Chem **288:**31268-31279.

26. **He S, Liang Y, Shao F, Wang X.** 2011. Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. Proc Natl Acad Sci U S A **108:**20054-20059.

27. **Kuriakose T, Man, S.M., Malireddi, R.S., Karki, R., Kesavardhana, S., Place, D.E., Neale, G., Vogel, P. and Kanneganti, T.D.** 2016. ZBP1/DAI is an innate sensor of influenza virus triggering the NLRP3 inflammasome and programmed cell death pathways. Science Immunology **1:**aag2045.

28. **Thapa RJ, Ingram JP, Ragan KB, Nogusa S, Boyd DF, Benitez AA, Sridharan H, Kosoff R, Shubina M, Landsteiner VJ, Andrake M, Vogel P, Sigal LJ, tenOever BR, Thomas PG, Upton JW, Balachandran S.** 2016. DAI Senses Influenza A Virus Genomic RNA and Activates RIPK3-Dependent Cell Death. Cell Host Microbe doi:10.1016/j.chom.2016.09.014.

29. **Bozym RA, Patel K, White C, Cheung KH, Bergelson JM, Morosky SA, Coyne CB.** 2011. Calcium signals and calpain-dependent necrosis are

essential for release of coxsackievirus B from polarized intestinal epithelial cells. Mol Biol Cell **22:**3010-3021.

30. **Berger AK, Danthi P.** 2013. Reovirus activates a caspase-independent cell death pathway. mBio **4**.

31. **Chu JJ, Ng ML.** 2003. The mechanism of cell death during West Nile virus infection is dependent on initial infectious dose. J Gen Virol **84:**3305-3314.

32. **Meessen-Pinard M, Le Coupanec A, Desforges M, Talbot PJ.** 2016. Pivotal role of RIP1 and MLKL in neuronal cell death induced by the human neuroinvasive coronavirus OC43. J Virol doi:10.1128/JVI.01513-16.

33. **Kobayashi T, Antar AAR, Boehme KW, Danthi P, Eby EA, Guglielmi KM, Holm GH, Johnson EM, Maginnis MS, Naik S, Skelton WB, Wetzel JD, Wilson GJ, Chappell JD, Dermody TS.** 2007. A plasmid-based reverse genetics system for animal double-stranded RNA viruses. Cell Host Microbe **1:**147-157.

34. **Danthi P, Kobayashi T, Holm GH, Hansberger MW, Abel TW, Dermody TS.** 2008. Reovirus apoptosis and virulence are regulated by host cell membrane penetration efficiency. J Virol **82:**161-172.

35. **Berard A, Coombs KM.** 2009. Mammalian reoviruses: propagation, quantification, and storage. Curr Protoc Microbiol **Chapter 15:**Unit15C 11.

36. **Virgin HW, IV, Bassel-Duby R, Fields BN, Tyler KL.** 1988. Antibody protects against lethal infection with the neurally spreading reovirus type 3 (Dearing). Journal of Virology **62:**4594-4604.

37. **Wu YT, Tan HL, Huang Q, Sun XJ, Zhu X, Shen HM.** 2011. zVAD-induced necroptosis in L929 cells depends on autocrine production of TNFalpha mediated by the PKC-MAPKs-AP-1 pathway. Cell death and differentiation **18:**26-37.

38. **Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J.** 2000. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol **1:**489-495.

39. **Becker MM, Peters TR, Dermody TS.** 2003. Reovirus sigma NS and mu NS proteins form cytoplasmic inclusion structures in the absence of viral infection. J Virol **77:**5948-5963.

40. **Reynolds ES.** 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol **17:**208-212.

41. **Tyler KL, Squier MK, Rodgers SE, Schneider BE, Oberhaus SM, Grdina TA, Cohen JJ, Dermody TS.** 1995. Differences in the capacity of reovirus strains to induce apoptosis are determined by the viral attachment protein sigma 1. J Virol **69:**6972-6979.

42. **Schmittgen TD, Livak KJ.** 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc **3:**1101-1108.

43. **Vanden Berghe T, Grootjans S, Goossens V, Dondelinger Y, Krysko DV, Takahashi N, Vandenabeele P.** 2013. Determination of apoptotic and necrotic cell death in vitro and in vivo. Methods **61:**117-129.

44. **Newton K, Dugger DL, Wickliffe KE, Kapoor N, de Almagro MC, Vucic D, Komuves L, Ferrando RE, French DM, Webster J, Roose-Girma M, Warming S, Dixit VM.** 2014. Activity of protein kinase RIPK3 determines whether cells die by necroptosis or apoptosis. Science **343:**1357-1360.

45. **Mandal P, Berger SB, Pillay S, Moriwaki K, Huang C, Guo H, Lich JD, Finger J, Kasparcova V, Votta B, Ouellette M, King BW, Wisnoski D, Lakdawala AS, DeMartino MP, Casillas LN, Haile PA, Sehon CA, Marquis RW, Upton J, Daley-Bauer LP, Roback L, Ramia N, Dovey CM, Carette JE, Chan FK, Bertin J, Gough PJ, Mocarski ES, Kaiser WJ.** 2014. RIP3 induces apoptosis independent of pronecrotic kinase activity. Mol Cell **56:**481-495.

46. **Rodriguez DA, Weinlich R, Brown S, Guy C, Fitzgerald P, Dillon CP, Oberst A, Quarato G, Low J, Cripps JG, Chen T, Green DR.** 2016. Characterization of RIPK3-mediated phosphorylation of the activation loop of MLKL during necroptosis. Cell Death Differ **23:**76-88.

47. **Connolly JL, Rodgers SE, Clarke P, Ballard DW, Kerr LD, Tyler KL, Dermody TS.** 2000. Reovirus-induced apoptosis requires activation of transcription factor NF-kappaB. J Virol **74:**2981-2989.

48. **Danthi P, Pruijssers AJ, Berger AK, Holm GH, Zinkel SS, Dermody TS.** 2010. Bid regulates the pathogenesis of neurotropic reovirus. PLoS Pathog **6:**e1000980.

49. **Holm GH, Zurney J, Tumilasci V, Leveille S, Danthi P, Hiscott J, Sherry B, Dermody TS.** 2007. Retinoic acid-inducible gene-I and interferon-beta promoter stimulator-1 augment proapoptotic responses following mammalian reovirus infection via interferon regulatory factor-3. J Biol Chem **282:**21953-21961.

50. **Pruijssers AJ, Hengel H, Abel TW, Dermody TS.** 2013. Apoptosis induction influences reovirus replication and virulence in newborn mice. J Virol **87:**12980-12989.

51. **Clarke P, Beckham JD, Leser JS, Hoyt CC, Tyler KL.** 2009. Fas-mediated apoptotic signaling in the mouse brain following reovirus infection. J Virol **83:**6161-6170.

52. **Berens HM, Tyler KL.** 2011. The proapoptotic Bcl-2 protein Bax plays an important role in the pathogenesis of reovirus encephalitis. Journal of virology **85:**3858-3871.

53. **Dionne KR, Zhuang Y, Leser JS, Tyler KL, Clarke P.** 2013. Daxx upregulation within the cytoplasm of reovirus-infected cells is mediated by interferon and contributes to apoptosis. J Virol **87:**3447-3460.

54. **Zhuang Y, Berens-Norman HM, Leser JS, Clarke P, Tyler KL.** 2016. Mitochondrial p53 Contributes to Reovirus-Induced Neuronal Apoptosis and Central Nervous System Injury in a Mouse Model of Viral Encephalitis. J Virol **90:**7684-7691.

55. **Gunther C, Martini E, Wittkopf N, Amann K, Weigmann B, Neumann H, Waldner MJ, Hedrick SM, Tenzer S, Neurath MF, Becker C.** 2011. Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. Nature **477:**335-339.

56. **Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Caspary T, Mocarski ES.** 2011. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature **471:**368-372.

57. **Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, Green DR.** 2011. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature **471:**363-367.

58. **Hiller BE, Berger AK, Danthi P.** 2015. Viral gene expression potentiates reovirus-induced necrosis. Virology doi:10.1016/j.virol.2015.06.018.

59. **Kalai M, Van Loo G, Vanden Berghe T, Meeus A, Burm W, Saelens X, Vandenabeele P.** 2002. Tipping the balance between necrosis and apoptosis in human and murine cells treated with interferon and dsRNA. Cell Death Differ **9:**981-994.

60. **Goubau D, Schlee M, Deddouche S, Pruijssers AJ, Zillinger T, Goldeck M, Schuberth C, Van der Veen AG, Fujimura T, Rehwinkel J, Iskarpatyoti JA, Barchet W, Ludwig J, Dermody TS, Hartmann G, Reis ESC.** 2014. Antiviral immunity via RIG-I-mediated recognition of RNA bearing 5'-diphosphates. Nature doi:10.1038/nature13590.

61. **McAllister CS, Taghavi N, Samuel CE.** 2012. Protein kinase PKR amplification of interferon beta induction occurs through initiation factor eIF-2alpha-mediated translational control. J Biol Chem **287:**36384-36392.

62. **Kato H, Takeuchi O, Mikamo-Satoh E, Hirai R, Kawai T, Matsushita K, Hiiragi A, Dermody TS, Fujita T, Akira S.** 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J Exp Med **205:**1601-1610.

63. **Sheehan KC, Lai KS, Dunn GP, Bruce AT, Diamond MS, Heutel JD, Dungo-Arthur C, Carrero JA, White JM, Hertzog PJ, Schreiber RD.** 2006. Blocking monoclonal antibodies specific for mouse IFN-alpha/beta receptor subunit 1 (IFNAR-1) from mice immunized by in vivo hydrodynamic transfection. J Interferon Cytokine Res **26:**804-819.

64. **de Bouteiller O, Merck E, Hasan UA, Hubac S, Benguigui B, Trinchieri G, Bates EE, Caux C.** 2005. Recognition of double-stranded RNA by human toll-like receptor 3 and downstream receptor signaling requires multimerization and an acidic pH. J Biol Chem **280:**38133-38145.

65. **Loo YM, Fornek J, Crochet N, Bajwa G, Perwitasari O, Martinez-Sobrido L, Akira S, Gill MA, Garcia-Sastre A, Katze MG, Gale M, Jr.** 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J Virol **82:**335-345.

66. **Clarke P, Leser JS, Bowen RA, Tyler KL.** 2014. Virus-induced transcriptional changes in the brain include the differential expression of genes associated with interferon, apoptosis, interleukin 17 receptor A, and glutamate signaling as well as flavivirus-specific upregulation of tRNA synthetases. MBio **5:**e00902-00914.

67. **Sturzenbecker LJ, Nibert ML, Furlong DB, Fields BN.** 1987. Intracellular digestion of reovirus particles requires a low pH and is an essential step in the viral infectious cycle. Journal of Virology **61:**2351-2361.

68. **Dermody TS, Parker JC, Sherry B.** 2013. Orthoreoviruses. *In* Knipe DM, Howley PM (ed), Fields Virology, Sixth ed, vol 2. Lippincott Williams & Wilkins, Philadelphia.

69. **Murray KE, Nibert ML.** 2007. Guanidine hydrochloride inhibits mammalian orthoreovirus growth by reversibly blocking the synthesis of double-stranded RNA. J Virol **81:**4572-4584.

70. **Henderson DR, Joklik WK.** 1978. The mechanism of interferon induction by UV-irradiated reovirus. Virology **91:**389-406.

71. **Lostale-Seijo I, Martinez-Costas J, Benavente J.** 2016. Interferon induction by avian reovirus. Virology **487:**104-111.

72. **Robinson N, McComb S, Mulligan R, Dudani R, Krishnan L, Sad S.** 2012. Type I interferon induces necroptosis in macrophages during infection with Salmonella enterica serovar Typhimurium. Nat Immunol **13:**954-962.

73. **McComb S, Cessford E, Alturki NA, Joseph J, Shutinoski B, Startek JB, Gamero AM, Mossman KL, Sad S.** 2014. Type-I interferon signaling through ISGF3 complex is required for sustained Rip3 activation and necroptosis in macrophages. Proc Natl Acad Sci U S A doi:10.1073/pnas.1407068111.

74. **Thapa RJ, Nogusa S, Chen P, Maki JL, Lerro A, Andrake M, Rall GF, Degterev A, Balachandran S.** 2013. Interferon-induced RIP1/RIP3-mediated necrosis requires PKR and is licensed by FADD and caspases. Proc Natl Acad Sci U S A **110:**E3109-3118.

75. **Imani F, Jacobs BL.** 1988. Inhibitory activity for the interferon-induced protein kinase is associated with the reovirus serotype 1 sigma 3 protein. Proceedings of the National Academy of Sciences of the United States of America **85:**7887-7891.

76. **Connolly JL, Dermody TS.** 2002. Virion disassembly is required for apoptosis induced by reovirus. J Virol **76:**1632-1641.

77. **Danthi P, Coffey CM, Parker JS, Abel TW, Dermody TS.** 2008. Independent regulation of reovirus membrane penetration and apoptosis by the mu1 phi domain. PLoS Pathog **4:**e1000248.

78. **Knowlton JJ, Dermody TS, Holm GH.** 2012. Apoptosis induced by mammalian reovirus is beta interferon (IFN) independent and enhanced by IFN regulatory factor 3- and NF-kappaB-dependent expression of Noxa. J Virol **86:**1650-1660.

79. **Fensterl V, Chattopadhyay S, Sen GC.** 2015. No Love Lost Between Viruses and Interferons. Annu Rev Virol **2:**549-572.

80. **Zarbl H, Skup S, Millward S.** 1980. Reovirus progeny subviral particles synthesize uncapped mRNA. Journal of Virology **34:**497-505.

81. **Clarke P, Tyler KL.** 2009. Apoptosis in animal models of virus-induced disease. Nat Rev Microbiol **7:**144-155.

82. **O'Donnell SM, Hansberger MW, Connolly JL, Chappell JD, Watson MJ, Pierce JM, Wetzel JD, Han W, Barton ES, Forrest JC, Valyi-Nagy T, Yull FE, Blackwell TS, Rottman JN, Sherry B, Dermody TS.** 2005. Organ-specific roles for transcription factor NF-kappaB in reovirus-induced apoptosis and disease. J Clin Invest **115:**2341-2350.

1071 83. **Holm GH, Pruijssers AJ, Li L, Danthi P, Sherry B, Dermody TS.** 2010.
1072 Interferon regulatory factor 3 attenuates reovirus myocarditis and
1073 contributes to viral clearance. J Virol **84:**6900-6908.

1074 84. **Maitra R, Ghalib MH, Goel S.** 2012. Reovirus: A Targeted Therapeutic -
1075 Progress and Potential. Mol Cancer Res doi:10.1158/1541-7786.MCR-12-
1076 0157.
1077