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

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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.
IMPORTANCE

An appreciation of how cell death pathways are regulated following viral infection may reveal strategies to limit tissue destruction and prevent the onset of disease.

Cell death following virus infection can occur by apoptosis or a regulated form of necrosis, known as necroptosis. Apoptotic cells are typically disposed of without activating the immune system. In contrast, necrotic cells alert the immune system, resulting in inflammation and tissue damage. While apoptosis following virus infection has been extensively investigated, how necroptosis is unleashed following virus infection is only understood for a small group of viruses. Here, using mammalian reovirus, we highlight the molecular mechanism by which 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.
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.
Reagents. Z-VAD-FMK and Q-VD-OPh were purchased from Enzo Life Sciences or R & D Systems, Necrostatin-1 was purchased from Calbiochem. Ammonium chloride (AC), GuHCl, poly I:C, and human TNFα were purchased from Sigma-Aldrich. siRNAs were purchased from Dharmacon as SMARTpools of ON-TARGET plus siRNA. Non-targeting siRNA control pool or siRNA targeting β-galactosidase were used as controls. Antisera raised against reovirus were obtained from T. Dermody. Monoclonal antibody against IFNAR and rabbit antisera against RIP3 were purchased from Santa Cruz Biotechnology, those against TRIF, phospho-MLKL and total MLKL were purchased from Abcam, and those against RIG-I and MDA5 were purchased from Cell Signaling. Mouse antiserum specific for PSTAIR was purchased from Sigma, specific for KDEL was purchased from Enzo Life Sciences. Alexa Fluor-conjugated anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG secondary antibodies were purchased from Invitrogen. IRDye-conjugated anti-guinea pig IgG was purchased from LI-COR.

Fixing, embedding, and sectioning of infected cells. L929 cells grown on 100 mm dishes were either mock infected or infected with 10 PFU/cell of T3D for 1 h at room temperature. Following the viral attachment incubation, the cells were washed twice with PBS and then overlayed with fresh medium. At 34 h post infection, uninfected and infected cells were washed with PBS, trypsinized, pelleted for 5 min at 800 × g, and washed again with PBS. The pelleted cells were then fixed with 2.5% glutaraldehyde diluted in sodium cacodylate buffer.
(100 mM sodium cacodylate [pH 7.5], 2 mM MgCl₂, 2 mM CaCl₂, 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
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 37°C 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 4°C 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 transflect 15 pmoles of siRNA. Cells (1 × 10⁴) were seeded on top of the siRNA-lipofectamine mixture. In 24-well plates, 0.75 μl Lipofectamine 2000 was used to transflect 45 pmoles of siRNA. Cells (5 × 10⁴) 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 × 10⁴) 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 mm² 48 h following infection are shown.

Assessment of caspase-3/7 activity. ATCC L929 cells (1 × 10⁴) 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). \( \Delta C_t \) values for each cDNA sample were
calculated by subtracting \( C_t \) values of T3DS1, ZBP1, or IFNβ and \( C_t \) values for
GAPDH. Fold increase in gene expression with respect to control sample
(indicated in each figure legend) was measured using the \( \Delta\Delta C_t \) method (42).

Statistical analysis. Statistical significance between experimental groups was
determined using the unpaired \( t \)-test function of the Graphpad Prism software.
Statistical analyses for differences in gene expression by RT-qPCR were done on the ΔCt values.
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
significantly decreased cell death (Figure 1E, 1F). The effect of RIP3 siRNA against reovirus-induced cell death matched the effect of RIP3 siRNA on necroptosis-inducing treatment of TNFα and Z-VAD-FMK (Figure 1G). These data indicate a role for RIP3 in the induction of cell death following reovirus infection. RIP3 can participate in the induction of both apoptosis and necroptosis (7, 44, 45). Because cell death following reovirus infection is unaffected by diminishment of caspase activity (Figure 1B, 1C), these data suggest that reovirus induces RIP3-dependent necroptosis in L929 cells.

RIP3-dependent necroptosis requires the activation of the effector protein MLKL (13-23). MLKL is directly phosphorylated by RIP3 and MLKL phosphorylation is considered to be a hallmark of the activation of necroptosis signaling cascade (13, 46). To determine if reovirus infection leads to the activation of MLKL, we immunoblotted extracts from reovirus-infected cells using a phospho-MLKL antibody (Figure 1H). Our results indicate that MLKL is activated within 24 h following reovirus infection and remains activated until 48 h post infection, when a significant proportion of cells are undergoing cell death. The detection of this biochemical marker along with the genetic and pharmacologic experiments described above indicating that cell death is blocked by loss of RIP3 function but not of caspase function, meet the criteria to demonstrate that reovirus infection of L929 cells results in necroptosis (15).

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

In the context of infection by other viruses, necroptosis is antiviral (2, 4-7). To determine if necroptosis affects replication of reovirus, we measured viral yield over 24 h of infection in wild-type and RIP3-deficient BMDMs in the presence and absence of Z-VAD-FMK. Viral yields in wild-type cells treated with DMSO or Z-VAD-FMK were ~ 1 log10 (Figure 2E). The genetic absence of RIP3
enhanced viral yield to ~ 1.7 log₁₀. Importantly, viral yield did not change in RIP3-
deficient BMDMs in conditions where apoptosis was blocked using Z-VAD-FMK. 
While the basis for the slight increase in viral yield in absence of RIP3 is unclear 
and was not further investigated, our data suggest that the capacity of cells to 
undergo necroptosis does not influence viral yield in cell culture. These data are 
reminiscent of previous evidence indicating that blockade of apoptosis does not 
influence reovirus replication in cell culture (47, 48). The absence of effect of cell 
death on reovirus replication in cell culture may be due to the differences in 
timing of the reovirus replication cycle and the induction of cell death. Whereas 
reovirus completes its replication cycle in 18 h, cell death following infection is 
not detected until 36-48 h following infection.

**Transfection of reovirus RNA can elicit necroptosis.** Reovirus strains that 
exhibit a greater level of gene expression are more potent inducers of necrosis 
(58). Blockade of reovirus + strand RNA synthesis using ribavirin blocks necrosis, 
suggesting a possible role for viral RNA in the induction of necrosis (58). 
Transfection of dsRNA mimic poly I:C in L929 cells treated with either type I or 

type II IFNs results in cell death by necrosis (25, 26, 59). Because our data 
suggested a role for reovirus RNA in the induction of necroptosis in infected cells, 
we sought to determine if viral RNA was sufficient for the induction of 
necroptosis. For these experiments, we purified total RNA from mock- or 
reovirus-infected cells 24 h following infection. We found that in comparison to 
RNA from mock-infected cells, RNA extracted from T3D-infected cells induced a
significantly greater amount of cell death following transfection into cells (Figure 3A, 3B). Cell death by transfected RNA was diminished by treatment with Nec1 but not Q-VD-OPh (Figure 3C), analogous to what we have reported in L929 cells infected with reovirus (30). These data are also consistent with previous work indicating that poly I:C-induced cell death is blocked by Nec1 (26). Our results presented above suggest that RNA isolated from reovirus-infected cells elicits necroptosis following introduction into L929 cells. Interestingly, unlike previous work with transfection of dsRNA into cells (25, 59), cell death following transfection of RNA extracted from reovirus-infected cells did not require priming of the cells with exogenous IFN.

We reasoned that necroptosis was induced without addition of exogenous IFN because transfection of reovirus RNA obtained from infected cells can induce the expression of IFNβ (60-62) (Figure 3D). Indeed, treatment with an IFNAR-blocking antibody MAR1-5A3 (63), resulted in a reduction in cell death (Figure 3D). IFNβ production following transfection of reovirus RNA occurs via RIG-I-mediated detection of the RNA (60). Consistent with this, removal of the 5’ phosphates using CIP resulted in a reduction in the expression of IFNβ (Figure 3E) and the induction of cell death (Figure 3F, 3G). Interestingly, if cells were primed with exogenous IFNβ before transfection, the capacity of CIP-treated RNA to elicit necroptosis was restored (Figure 3F, 3G). These data suggest that though RIG-I mediated detection of viral RNA is required for IFNβ production, it is not sufficient for the induction of cell death. Thus, cell death induction occurs by sensing of viral RNA via an alternate pathway.
Based on the evidence that poly I:C elicits necroptosis by TLR3 detection and signaling to RIP3 via TRIF (25, 26), we examined whether reovirus RNA-induced necroptosis could be blocked by treatment of cells with ammonium chloride (AC), an agent that blocks TLR3-mediated detection of dsRNA (64). We found that though AC did not negatively impact IFNβ expression following RNA transfection (Figure 3H), it blocked cell death induction by transfected RNA (Figure 3I). Consistent with previous work, these data indicate that detection of reovirus RNA via RIG-I produces IFNβ. In addition, these data suggest that IFNβ primes reovirus RNA transfected cell to undergo TLR3-dependent necroptosis. Thus, the IFN- and TLR3-dependent pathway for induction of necroptosis following transfection of reovirus RNA into L929 cells is similar to that previously described for transfection of synthetic dsRNA (25, 59).

Sensing of reovirus RNA during infection is required for necroptosis. We next sought to determine if detection of viral RNA in infected cells contributes to cell death induction in reovirus-infected cells. During infection, reovirus RNA can be detected by both RIG-I-like receptors (RLRs), RIG-I and MDA5 (65). Simultaneous reduction of both RLRs, or their common downstream signaling adaptor, MAVS, led to a significant reduction in cell death following reovirus infection (Figure 4A, 4B, 4C). The susceptibility of cells to TNFα and Z-VAD-FMK induced necroptosis was not changed following MAVS knockdown indicating that MAVS is not required for the function of the core necroptosis machinery (Figure 4D). BMDMs deficient in either both RLRs or MAVS were also protected from...
reovirus-induced necroptosis (Figure 4E, 4F). These data suggest that necroptosis following reovirus infection requires detection and signaling by RLRs.

**IFN signaling is required for necroptosis.** To determine whether type I IFNs produced by RLR-MAVS signaling are required for reovirus-induced necroptosis, we quantified the capacity of reovirus to induce necroptosis in the presence of an IFNAR-blocking antibody (63). We found that this antibody diminished the expression of a representative interferon-stimulated gene (ISG), ZBP1, which is potently induced following reovirus infection (66), and diminished the capacity of reovirus to induce necroptosis (Figure 5A, 5B, 5C). This reduction in necroptosis was not due to a deleterious effect of the antibody on the capacity of reovirus to establish infection (Figure 5D). Blocking IFNAR signaling did not alter the capacity of TNFα and Z-VAD-FMK cotreatment to induce necroptosis, suggesting that this treatment did not affect the function of the core necroptosis machinery (Figure 5E). IFNAR-deficient BMDMs treated with Z-VAD-FMK also were resistant to reovirus-induced necroptosis (Figure 5E, 5F), indicating a role for IFN signaling in the induction of necroptosis following reovirus infection.

Based on the role for TLR3 in necroptosis following transfection of RNA obtained from reovirus-infected cells (Figure 3), we next sought to evaluate whether TLR3 is also required for cell death in reovirus-infected cells. Toward this goal, we tested the effect of AC on cell death induction in reovirus-infected cells. Because treatment of cells with AC prevents reovirus infection by blocking capsid disassembly, we initiated infection of AC-treated cells with infectious
subvirion particles (ISVPs), a viral entry intermediate that bypasses the inhibitory effect of AC (67). We found that necroptosis following infection by ISVPs was unaffected by treatment with AC (Figure 5G). Parallel transfection of cells with reovirus RNA in control and AC treated cells yielded results that matched those described in Figure 3H (data not shown), indicating that AC treatment was effective. siRNA-mediated reduction in the expression of TRIF, the TLR3 adaptor also did not block cell death following reovirus infection (Figure 5H, 5I). These data indicate that although RLR-mediated IFNβ expression and signaling is required for necroptosis following both, RNA transfection and viral infection, TLR3-mediated signaling is only required for cell death after viral RNA transfection.

Two-stage detection of reovirus infection is required for necroptosis.

We next sought to determine the stage of infection required for the induction of necroptosis. Blockade of viral + strand RNA synthesis using ribavirin diminishes reovirus-induced necrosis (58). The reovirus + sense RNA can direct protein synthesis or can be packaged into progeny core particles and serve as the template for minus strand RNA synthesis to generate viral genomic dsRNA (68). Progeny cores containing genomic dsRNA undergo secondary transcription to produce additional viral mRNAs. Thus, the diminishment of necroptosis by ribavirin treatment may be due to blockade of any of these steps in reovirus replication. To define the stage of infection required for necroptosis further, we 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 log10-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.
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...
reovirus remained capable of inducing cell death in ZBP1-deficient BMDMs (data not shown). Our results suggest that TLR3 does not participate in necroptosis induction following reovirus infection (Figure 5). PKR can promote necroptosis in cells lacking functional FADD (74). Reovirus induces necroptosis in wild-type cells expressing FADD (Figure 1, 2). Moreover, because reovirus encodes a well-described PKR inhibitor, we think it unlikely that PKR is involved in this process (75). Thus, the identity of the ISGs that control necroptosis following reovirus infection remains to be determined. Because IAV induced necroptosis is unaffected by the genetic absence of MAVS or IFNAR (7) and requires ZBP1 (27, 28), whereas reovirus-induced necroptosis requires MAVS and IFNAR (Figure 4, 5) but is not affected by the absence of ZBP1, the mechanism underlying necroptosis following reovirus infection appears distinct from that reported for IAV.

Investigations into reovirus-induced cell death indicate that reovirus infection can initiate cell death signaling from distinct stages of replication and elicit cell death via a variety of pathways. The precise pathway that executes cell death likely varies with cell type. One model suggests that events initiated during cell entry that occur after virus disassembly but prior to de novo synthesis of viral RNA and proteins can elicit cell death by apoptosis (76). Apoptosis by this mechanism is thought to occur independently of the presence of viral genomic RNA but relies on the function of the μ1 capsid protein and the host transcription factor NFκB (47, 77). Another set of studies implicates a role for viral genomic 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
definition 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.
We are grateful to Bernardo Mainou, Indiana University Virology colleagues, and members of our laboratory for helpful discussions and review of the manuscript. Transmission electron microscopy was performed in the Indiana University 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
infected with 50 PFU/cell of T3D in the presence or absence of Z-VAD-FMK (25 μM). Virus yield 24 h following infection was measured using plaque assay.

Figure 3. Reovirus RNA is sufficient for the induction of necroptosis. (A,B) L929 cells were transfected with 100 ng of RNA extracted from mock-infected or reovirus-infected cells. (A) Cell death 24 h following transfection was assessed by Cell Titer Glo. Luminescence measurement in untransfected cells was considered to represent 100% viability. *, P < 0.05 compared to cells transfected with RNA extracted from mock-infected cells. (B) Cell death 24 h following transfection of RNA was assessed by AOEB staining. *, P < 0.05 compared to cells transfected with RNA extracted from mock-infected cells (C) L929 cells were transfected with 100 ng of RNA extracted from mock infected or reovirus-infected cells in the presence of DMSO, Q-VD-OPh (25 μM) or Nec1 (50 μM). 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 cells transfected with DMSO treated cells transfected with the same type of RNA (D) L929 cells were transfected with 100 ng of RNA extracted from mock-infected or reovirus-infected cells. Levels of IFNβ mRNA were assessed by RT-qPCR at 18 h following transfection. IFNβ:GAPDH ratio for cells transfected with RNA from mock-infected cells was considered 1. *, P < 0.05 compared to cells transfected with RNA from mock-infected cells. (E) L929 cells were transfected with 100 ng of RNA extracted from mock-infected or reovirus-infected
cells in the presence and absence of 0.1 μg/ml anti-IFNAR Ab. 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 cells transfected with same type of RNA without anti-IFNAR Ab. (F) L929 cells were transfected 100 ng of untreated or CIP-treated RNA extracted from reovirus-infected cells. Levels of IFNβ mRNA were assessed by RT-qPCR at 18 h following transfection. IFNβ:GAPDH ratio for cells transfected with untreated RNA from reovirus-infected cells was considered 1. *, $P < 0.05$ compared to cells transfected with untreated RNA from reovirus-infected cells. (G) Cells treated with 0 or 100 units/ml IFNβ were transfected with 100 ng of untreated RNA from mock-infected cells or untreated or CIP-treated RNA from 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 similarly treated cells transfected with untreated RNA from T3D-infected cells. **, $P < 0.05$ compared to cells transfected with similarly treated RNA in the presence of 0 units/ml of IFNβ. (H) Cells treated with 0 or 100 units/ml IFNβ were transfected with 100 ng of untreated or CIP-treated RNA from T3D-infected cells. Cell death 24 h following transfection of RNA was assessed by AOEB staining. *, $P < 0.05$ compared to cells transfected with similarly treated RNA in the presence of 0 mU/ml of IFNβ. (I) Cells pretreated with 0 or 20 mM AC were transfected 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 transfected with untreated RNA from T3D-infected cells was considered 1. *, $P < 0.05$ compared to cells transfected with untreated RNA from T3D-infected cells.
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
treated cells was considered to represent 100% viability. (E) Cell death in wild-type, RIG-I -/- x MDA5 -/- or MAVS -/- BMDMs treated with Z-VAD-FMK following mock infection or infection with 50 PFU/cell of T3D 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. (F) Cell death in wild-type, RIG-I -/- x MDA5 -/- or MAVS -/- BMDMs treated with Z-VAD-FMK (25 μM) following infection with 50 PFU/cell of T3D was assessed by Sytox green staining. *, $P < 0.05$ compared to wild-type cells.

Figure 5. Signaling via IFNAR is required for necroptosis (A,B,C,D) L929 cells were infected with 10 PFU/cell of T3D in the presence of 0.1 μg/ml of anti-IFNAR Ab. (A) Levels of ZBP1 mRNA were assessed using RT-qPCR at 24 h post infection. ZBP1:GAPDH ratio at 0 h post infection was set to 1. *, $P < 0.05$ compared to cells infected without IFNAR antibody. (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 similarly treated, mock-infected cells was considered to represent 100% viability. *, $P < 0.05$ compared to cells infected without IFNAR antibody. (C) Cell death 48 h following infection with 10 PFU/cell of T3D was assessed by AOEB staining. *, $P < 0.05$ compared to cells infected without IFNAR antibody. (D) Viral infectivity 18 h following infection with 2 PFU/cell T3D was assessed by indirect immunofluorescence. (E) Cell death 3 h following treatment with TNFα and Z-VAD-FMK treatment was assessed by Cell Titer Glo. Luminescence measurement cells treated without IFNAR antibody was.
considered to represent 100% viability. (F) Cell death in wild-type and IFNAR-
deficient BMDMs treated with Z-VAD-FMK following mock infection or infection
with 50 PFU/cell of T3D. Cell viability was assessed by Cell titer Glo. Luminiscence measurement in mock-infected cells of the same genotype was
considered to represent 100% viability. *, $P < 0.05$ compared to wild-type cells. (G) Cell death in wild-type and IFNAR-deficient BMDMs treated with Z-VAD-FMK
following infection with 50 PFU/cell of T3D was assessed by Sytox green staining. *, $P < 0.05$ compared to wild-type cells. (H) L929 cells were mock
infected or infected with 100 PFU/cell of T3D ISVPs in the presence or absence
of 20 mM AC. Cell death 48 h following infection was assessed by Cell Titer Glo. Luminiscence measurement in similarly treated, mock-infected cells was
considered to represent 100% viability. *, $P < 0.05$ compared to cells infected
without AC. (H, I) L929 cells were transfected with non-targeting siRNAs or
siRNAs specific for TRIF. (H) Efficiency of knockdown was assessed by
immunoblotting for TRIF and PSTAIR loading control. (I) 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.

Figure 6. Synthesis for genomic dsRNA is required for necroptosis. L929
cells were infected with 10 PFU/cell of T3D in the presence of ribavirin (200 μM)
or GuHCl (15 mM). (A) Levels of reovirus + strand RNA corresponding to the viral
S1 gene segment were measured by RT-qPCR 24 h post infection. Reovirus
T3D S1 +:GAPDH ratio in untreated cells infected for 0 h was considered 1.*, \( P < 0.05 \) compared to cells infected with T3D in absence of inhibitor. (B) Levels of reovirus \( \mu \)1C protein and PSTAIR loading control 24 h following infection with 10 PFU/cell of T3D were assessed by immunoblotting. (C) Generation of reovirus genomic dsRNA at 24 h following infection was evaluated by electropherotyping. (D) 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 control treated cells. (E) Cell death 48 h following infection with 10 PFU/cell of T3D was assessed by AOEB staining. *, \( P < 0.05 \) compared to control treated cells. (F) Cell death 4 h following treatment with TNF\( \alpha \) and Z-VAD-FMK treatment was assessed by Cell Titer Glo. Cell viability in similarly treated cells in absence of TNF\( \alpha \) and Z-VAD-FMK was considered 100%.

Figure 7. De novo synthesis of viral RNA is not required for IFN expression.

(A) L929 cells were infected with 10 PFU/cell of T3D. Levels of IFN\( \beta \) mRNA were assessed at the indicated time intervals using RT-qPCR. IFN\( \beta \):GAPDH ratio at 0 h post infection was set to 1. *, \( P < 0.05 \) compared to cells infected for 0 h. (B) L929 cells treated with AC (20 mM), ribavirin (200 \( \mu \)M), or GuHCl (15 mM) were infected with 10 PFU/cell of T3D. Levels of IFN\( \beta \) mRNA were assessed by RT-qPCR at 24 h post infection. IFN\( \beta \):GAPDH ratio for untreated, T3D-infected cells was set to 1. *, \( P < 0.05 \) compared to control treated, infected cells. (C,D) L929 cells were infected with 10 PFU/cell of T3D and equivalent particles of UV-
treated T3D. (C) Levels of reovirus + strand RNA corresponding to the viral S1
gene segment were measured by RT-qPCR 24 h post infection. Reovirus T3D
S1 +:GAPDH ratio in cells infected for 0 h was considered 1. UD, undetectable,
value below that detected at 0 h (D) Levels of reovirus IFNβ RNA corresponding
were measured by RT-qPCR 24 h post infection. IFNβ:GAPDH ratio in cells
infected with infectious T3D was considered 1. (E) L929 cells were transfected
with non-targeting siRNAs or siRNAs specific for MAVS. Levels of IFNβ mRNA in
cells infected with 10 PFU/cell of T3D in the presence of absence of ribavirin
(200 μM) were assessed by RT-qPCR. IFNβ:GAPDH ratio for untreated, T3D
infected non-targeting siRNA treated was set to 1. *, P < 0.05 compared to
untreated, T3D infected non-targeting siRNA treated cells.

Figure 8. Model for reovirus-induced necroptosis. Genomic RNA from
incoming viral particles is sensed by RLRs to produce type I IFN in a MAVS-
dependent manner. De novo synthesized viral genomic dsRNA or viral
secondary transcripts produced from newly synthesized genomic dsRNA (GuHCl
sensitive replication events) are detected by an as yet unidentified ISG to elicit
RIP3-dependent necrotic cell death.
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Controlling UV-treated virus 

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Treatment of virus

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Relative expression of IFN

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Downloaded from http://jvi.asm.org on March 15, 2017 by Washington University in St. Louis
Genomic RNA from incoming virus → RIG-I/MDA5 → MAVS → NFκB and IRF3 → IFN

NFκB and IRF3 → MLKL → RIP3 → ISG

Viral dsRNA or secondary viral transcripts (GuHCl sensitive events) → ISG

Cell death

Downloaded from http://jvi.asm.org on March 15, 2017 by Washington University in St. Louis