Virus Inhibition of RIP3-Dependent Necrosis

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SUMMARY

Viral infection activates cytokine expression and triggers cell death, the modulation of which is important for successful pathogenesis. Necroptosis is a form of programmed necrosis dependent on two related RIP homotypic interaction motif (RHIM)-containing signaling adaptors, receptor-interacting protein kinases (RIP) 1 and 3. We find that murine cytomegalovirus infection induces RIP3-dependent necrosis. Whereas RIP3 kinase activity and RHIM-dependent interactions control virus-associated necrosis, virus-induced death proceeds independently of RIP1 and is therefore distinct from TNFα-dependent necroptosis. Viral M45-encoded inhibitor of RIP activation (vIRA) targets RIP3 during infection and disrupts RIP3-RIP1 interactions characteristic of TNFα-induced necroptosis, thereby suppressing both death pathways. Importantly, attenuation of vIRA mutant virus in wild-type mice is normalized in RIP3-deficient mice. Thus, vIRA function validates necrosis as central to host defense against viral infections and highlights the benefit of multiple virus-encoded cell-death suppressors that inhibit not only apoptotic, but also necrotic mechanisms of virus clearance.

INTRODUCTION

Cellular sensing of viral pathogens by the host activates inflammatory gene expression and triggers cell death. These distinct cell-intrinsic response pathways directly control viral spread from the portal of entry and influence the quality of pathogen-specific adaptive immunity. Innate antiviral cytokines such as interferon (IFN) have long been considered central to control of viral spread (Roy and Mocarski, 2007), with caspase-dependent apoptotic cell death viewed as a complementary, evolutionarily conserved clearance mechanism triggered by distinct signals. The importance of death has been reinforced by the widespread existence of apoptotic cell-death suppressors, including those that viruses employ to subvert intrinsic clearance. These include (1) caspase inhibitors, such as baculovirus p35 (Clem et al., 1991), viral inhibitors of apoptosis (Crook et al., 1993), poxvirus crmA (Ray et al., 1992), and viral FLICE (caspase-8) inhibitory proteins (Thome et al., 1997), as well as (2) mitochondrial cell-death suppressors such as viral Bcl-2 homologs and other proteins encoded by large DNA viruses that block cytochrome c release from mitochondria (Galluzzi et al., 2008). Although apoptosis is a well-established cell-intrinsic response to pathogens, caspase-independent cell death, or programmed necrosis, has emerged as an alternative death pathway that dominates under specific conditions (Festjens et al., 2006). Necroptosis is a form of programmed necrosis induced by death receptors (DR) that is independent of caspases but dependent on receptor-interacting protein kinase (PK) 1 (RIP1) (Festjens et al., 2007; Meylan and Tschopp, 2005) and RIP3 (Cho et al., 2009; He et al., 2009; Zhang et al., 2009), two related RIP homotypic interaction motif (RHIM) -containing signaling adaptors (Sun et al., 2002). Necroptosis has been viewed as an alternative to apoptosis, and is triggered by the same signals under conditions where caspase-8 activation has been suppressed or is absent (Festjens et al., 2007). This alternative death pathway could come into play as a host countermeasure against invading pathogens (Cho et al., 2009); however, evidence that this pathway is suppressed by any microbe for pathogenesis in vivo has not yet been revealed.

RIP1 is the founding member of a serine-threonine PK family that transduces inflammatory and cell-death signals following DR ligation, activation of pattern recognition receptors (PRRs), and DNA damage (Festjens et al., 2007). An amino-terminal PK domain in RIP1 is critical for DR-induced necroptosis (Holler et al., 2000) and is the target of the inhibitor necrostatin-1 (Nec-1) (Degterev et al., 2008). RIP1 has a carboxy-terminal death domain (DD) that engages other DD-containing proteins as well as a central (intermediate) domain important for NF-κB activation (Festjens et al., 2007) and RHIM-dependent signaling (Sun et al., 2002). Three other cellular RHIM-containing adaptors interact with RIP1 to initiate gene activation and cell death: (1) RIP3, the only other RHIM-containing RIP family member (Sun et al., 2002), (2) the Toll-like receptor (TLR) 3 and TLR4 adaptor TIR domain-containing adaptor-inducing IFNβ (TRIF) (Kaiser and Oppermann, 2005; Meylan et al., 2004), and (3) DNA-dependent activator of IFN-regulatory factors (DAI, also termed DLM-1 or ZBP1) (Kaiser et al., 2008; Rebsamen et al., 2009), a candidate DNA sensor (Takaoka et al., 2007). Thus, RIP1 is a central adaptor, balancing inflammatory cytokine activation as well as the initiation of programmed cell-death pathways.

When first evaluated, RIP3 appeared to exert a negative modulatory role in TLR3- or DR-dependent NF-κB activation (Meylan et al., 2004; Sun et al., 2002). More recently, RIP3 has been shown to have a positive impact on DAI-induced, RIP1 RHIM-dependent NF-κB activation (Kaiser et al., 2008; Rebsamen et al., 2009), as well as in RHIM-dependent necroptosis induced by TNFα (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). The requirement for caspase inhibition to unveil
necroptosis has limited observations on RIP3- and RIP1-dependent necroptosis in natural biological settings (Declercq et al., 2009). Whereas mice lacking RIP1 die within 3 days postnata tally (Kelliker et al., 1998), RIP3-deficient mice are viable and display neither developmental defects nor altered NF-κB signaling triggered by lipopolysaccharide (LPS) or TNFx (Newton et al., 2004). One recent report described a decrease in inflammatory liver pathology in vaccinia virus-infected RIP3-deficient mice, processes that were ascribed to altered TNFα-induced necroptosis (Cho et al., 2009).

Human cytomegalovirus (HCMV), the prototypic, medically significant β-herpesvirus, and murine cytomegalovirus (MCMV), a surrogate used to model viral pathogenesis, encode several cell-death suppressors (McCormick, 2008). The MCMV M45-encoded viral inhibitor of RIP activation (vIRA) is a viral structural protein (Lembo et al., 2004) originally identified as a tropism determinant required for endothelial cell-specific viral replication (Brune et al., 2001). MCMV mutants lacking vIRA induce premature death in some cell lines, although the mechanism of death remains unresolved. Caspase-dependent and -independent cell-death pathways have been implicated (Brune et al., 2001; Mack et al., 2008). Recent reports showing the critical role of RIP1 and RIP3 in DR-induced necroptosis (Cho et al., 2009; He et al., 2009; Zhang et al., 2009), together with previous evidence that vIRA physically interacts efficiently with RIP3 as well as RIP1 (Upton et al., 2008), suggest that vIRA may interact with RIP3 and/or RIP1 to suppress cell death in sensitive cell types; however, the specific contributions of these adaptors to the mutant virus phenotype have not been established. Here we demonstrate that vIRA disrupts formation of a RHIM-dependent RIP3-RIP1 kinase complex in TNFα-induced necroptosis as well as a RIP3 RHIM-dependent, RIP1-independent step in virus-induced necrosis. Importantly, the severe attenuation of vIRA-deficient virus is normalized in mice that lack RIP3 function. Thus, RIP3 mediates necrotic death independently of RIP1, and viral suppression of RIP3 provides a benefit during viral pathogenesis.

**RESULTS**

**vIRA RHIM-Dependent Interactions Are Necessary for Suppression of Virus-Induced Programmed Necrosis during Pathogenesis**

To explore the biological consequences of vIRA RHIM-specific interactions during infection, a recombinant mutant MCMV was evaluated in cells and animals. M45mutRHIM virus carried a substitution mutation (see Figures S1A and S1B available online) previously shown to disrupt RHIM-dependent interactions (Upton et al., 2008). Viral stocks were recovered from transfected NIH 3T3 fibroblasts with mutant and parental viruses exhibiting equivalent replication properties, as elaborated previously (Brune et al., 2001). Viral genome integrity was affirmed by restriction enzyme digestion (Figure S1C; data not shown). Immunoblot analysis indicated M45mutRHIM and parental viruses produced M45 proteins of comparable size and levels of expression (Figure 1A). Infected cells also expressed similar levels of viral immediate early (IE) 1 protein. To investigate whether the in vivo behavior of vIRA mutant virus recapitulated the severe attenuation previously demonstrated for vIRA null mutants (Lembo et al., 2004), we evaluated mutant and parental virus replication in immunodeficient and immunocompetent mice. To evaluate viral replication, dissemination, and virulence independently of innate natural killer (NK) as well as adaptive T and B lymphocyte function, the behavior of these viruses was compared in nonobese diabetic (NOD), severe combined immunodeficient (scid), IL2 common γ chain−/− (NSG) mice (Shultz et al., 2003). Mutant virus exhibited 102-fold reduced levels in spleen, 103-fold reduced levels in liver, and 106-fold reduced levels in salivary glands (SGs) compared with parental virus (Figure 1B). To investigate viral pathogenesis, cohorts of NSG mice inoculated with either virus were followed for 6 weeks. Whereas mice infected with parental virus succumbed between 20 and 22 days postinfection, mutant virus-infected animals showed no signs of CMV disease up to 42 days (Figure 1C) and beyond (data not shown). We extended in vivo studies to include immunocompetent mice, demonstrating that parental virus was detected in the spleens of BALB/c mice by day 3 post-inoculation when virus titers normally peak (Figure 1D) whereas mutant virus remained undetectable at this time (Figure 1D) as well as at day 5 (data not shown). The phenotype of M45mutRHIM mutant virus was similar to previously characterized vIRA-deficient viruses (Lembo et al., 2004). Importantly, the attenuated phenotype of M45mutRHIM in vivo establishes the critical role of RHIM-dependent interactions in vIRA function, extending prior investigations that relied upon truncation or deletion mutants that eliminated vIRA expression, and demonstrates the significance of RHIM-dependent signaling in antiviral host defense.

Consistent with prior reports using vIRA null mutants (Brune et al., 2001; Mack et al., 2008; Upton et al., 2008), M45mutRHIM virus was severely attenuated for growth in SVEC4-10 cells (Figure 1E), which died within 18 hr after exposure to mutant (Figure 1F). Parental virus replicated at levels similar to NIH 3T3 cells, where there was no impact on cell viability, and uninfected NIH 3T3 fibroblasts were sensitive to TNFα-induced apoptosis. As expected from previous investigations (Mack et al., 2008), mutant virus-infected cell death was unaffected by addition of the caspase inhibitor zVAD-fmk (Figure 1F). Additionally, the phenotype of two independently derived M45mutRHIM isolates was comparable (Figure S2A). Thus, the behavior of RHIM mutant virus recapitulates that of previously characterized M45-deficient viruses, and clearly demonstrates that RHIM-dependent interactions are central to vIRA-mediated cell-death suppression, consistent with a model (Upton et al., 2008) implicating a RHIM-dependent interaction(s) in control of an antiviral cell-death pathway triggered by infection. Thus, MCMV induces programmed caspase-independent death in susceptible cells, and virus-encoded vIRA naturally suppresses this pathway by interfering with RHIM-dependent pathways.

Given initial observations on vIRA as an endothelial growth determinant (Brune et al., 2001), we were surprised when M45mutRHIM virus replicated poorly (Figure 2A) and induced premature death in 3T3-Swiss albino (3T3-SA) fibroblasts (Figure S2A), another immortal cell line fully permissive for parental MCMV (Figure S2B; data not shown). Exposure to mutant virus resulted in premature death comparable to SVEC4-10 cells, based on three independent lines of evidence: morphological evaluation (Figure 2B; Figure S2), intracellular ATP levels (Figure 2C), and release of intracellular proteases (Figure 2D).
Death of mutant virus-infected cells first occurred between 5 and 9 hr postinfection (hpi) and approached maximal levels by 18 hpi. Death was accompanied by cytoplasmic swelling, cell detachment, propidium iodide inclusion, and membrane rupture (Figure S2C; data not shown), morphological evidence most consistent with necrosis (Festjens et al., 2006). To demonstrate that the collapse in ATP levels (Figure 2C) was due to necrotic death and not to viral modulation of cellular respiration, we showed intracellular proteases were released into the culture medium (Figure 2D), consistent with loss of plasma membrane integrity, rather than changes in cellular physiology during infection. In contrast, when TNFα plus cycloheximide (CHX) was employed to induce apoptosis, ATP levels decreased without a dramatic rise in extracellular protease activity. To assure that the virus-induced cell death occurred independently of apoptosis or autophagy, infected cells failed to exhibit evidence of caspase-3 activation at any time from 6 through 18 hpi with mutant virus, and levels of LC3 II in virus-infected cells remained similar to uninfected cells and lower than Bafilomycin A1-treated controls (Figure 2E). Ultrastructural analysis by electron microscopy (EM) revealed no evidence of membrane blebbing, nuclear condensation, or autophagosome formation in mutant-infected cells compared with wild-type (WT) virus infection (Figure 2F; data not shown). Taken together, these observations were most consistent with induction of programmed necrosis during mutant virus infection.
As recent studies have implicated high levels of RIP3 as a determinant in necroptosis (Cho et al., 2009; He et al., 2009; Zhang et al., 2009), we evaluated levels of known vIRA-interacting proteins, RIP1 and RIP3, in susceptible cells (3T3-SA and SVEC4-10). Immunoblot (IB) analysis revealed RIP3 levels were elevated compared with NIH 3T3 controls (Figure 2G), whereas RIP1 levels were similar in all cell lines. SVEC4-10 and 3T3-SA cells were sensitive to DR-induced necroptosis (Figure 2H), paralleling susceptibility to virus-induced programmed necrosis. Thus, high levels of RIP3 are a defining characteristic of cells sensitive to either form of necrotic death, implicating this adaptor in necrosis induced by different stimuli.

vIRA Suppresses Necroptosis in a RHIM-Dependent Fashion

To establish the contribution of RIP1 and RIP3 to necroptosis in SVEC4-10 and 3T3-SA cells, we employed specific shRNAs (RIP3-A and RIP3-B) to reduce expression levels of RIP3. This RNAi approach showed RIP3 played a critical role in TNFα-induced necroptosis (Figures 3A and 3B). Addition of the RIP1 kinase inhibitor Nec-1 restored cell viability, showing necroptosis to be RIP1 kinase dependent (Figure 3B; Figures S3E and S3F) (Degterev et al., 2008). The contribution of RIP3 and RIP1 to necroptosis was further evaluated in RIP1−/− and control RIP1+/− mouse embryonic fibroblasts (MEFs). RIP1−/− MEFs were insensitive to necroptosis (Figure 3C), but, as expected
Figure 3. MCMV RHIM-Dependent Suppression of RIP3-Mediated Necrosis

(A) IB analysis (top) to detect RIP3, RIP1, and β-actin, and viability (bottom) of 3T3-SA fibroblasts expressing a scramble control (Sc) or one of two RIP3-specific shRNAs (RIP3-A and RIP3-B) and treated to induce necroptosis as described in Figure 2H or to induce apoptosis as described in Figure 1F.

(B) IB analysis (top) to detect RIP1, RIP3, and β-actin, and viability (bottom) of SVEC4-10 cells expressing Sc or RIP3-A shRNA. Cells were treated to induce necroptosis as described in Figure 2H in the absence or presence of Nec-1 (30 μM).

(C) Viability (top) and IB analysis to detect RIP1, RIP3, β-actin, and Flag-epitope-tagged proteins (bottom) in RIP1+/+ and RIP1−/− MEFs transduced with empty vector (EV), Flag-tagged RIP3 (RIP3), or Flag-tagged kinase-deficient RIP3 (RIP3-KD) retroviral constructs. Transduced cells were treated as described in (B). The vertical line shows where lanes from the original gel were brought adjacent.

(D) IB of myc-tagged proteins following IP with anti-myc-conjugated agarose (top) of SVEC4-10 cells transduced with EV, M45-myc, or M45mutRHIM-myc retroviral constructs, and viability (bottom) of these cells after treatment as described in (B).
(Kelliher et al., 1998; Wong et al., 2010), exhibited an increased sensitivity to TNFα-induced apoptosis (Figure 3C). IB analysis of empty vector (EV) transduced RIP1+/− and control MEFs showed similar expression of endogenous RIP3 (Figure 3C). To evaluate the impact of increasing RIP3 in the absence of RIP1, RIP1−/− and control MEFs were transduced with epitope-tagged WT or kinase-deficient (KD) RIP3 (Figure 3C, bottom). Elevated levels of WT, but not KD, RIP3 conferred increased sensitivity of WT MEFs to TNFα plus zVAD-fmk-induced necroptosis (Figure 3C). This was shown also to be dependent on RIP1 kinase activity using Nec-1. In contrast, overexpression of RIP3 in RIP1−/− MEFs failed to enhance susceptibility to either necroptosis or apoptosis. Interestingly, elevated expression of RIP3, but not RIP3-KD, in RIP1−/− MEFs resulted in a dramatic RIP3 mobility shift, suggesting RIP1 may suppress a RIP3 kinase-dependent posttranslational modification of RIP3 (Figure 3C). Together, these results indicate that RIP1 plays an essential role in induction of DR-associated necrotic death, elevated levels of RIP3 are necessary for this pathway, and overexpression of RIP3 cannot overcome the requirement for RIP1 in DR-induced cell death. Furthermore, these data establish that the PK activity of both RIP1 and RIP3 is required in necrotic death induced following DR activation in sensitive cells. These independently derived results reaffirm the essential role of an active RIP3–RIP1 kinase complex to drive DR-induced necroptosis (Cho et al., 2009; He et al., 2009; Zhang et al., 2009).

To investigate the role of the vIRA RHIM in suppression of necroptosis, SVEC4-10 cells stably expressing epitope-tagged WT (M45-myc) or mutant (M45mutRHIM-myc) vIRA were generated (Figure 3D) and compared to cells carrying an EV as control. As expected, expression of WT vIRA conferred protection to treatment with TNFα plus zVAD-fmk (Figure 3D) (Mack et al., 2008). Cells expressing M45mutRHIM-myc were as sensitive to death as control cells, indicating viRA is a potent RHIM-dependent suppressor of necroptosis. Because both RIP1 and RIP3 contributed to the necrotic pathway (Cho et al., 2009; He et al., 2009; Zhang et al., 2009), we sought to evaluate the potential role of viRA as a competitor of RIP1–RIP3 complex formation. Flag-RIP3 and myc-RIP1 were coexpressed in 293T cells in the presence of increasing amounts of either WT M45-Flag or mutant M45mutRHIIM-Flag. Immunoprecipitation (IP) followed by IB analysis revealed a RHIM-dependent viRA-mediated inhibition of RIP1–RIP3 complex formation (Figure 3E). This demonstration provides mechanistic insight into how viRA suppresses the execution of necroptosis initiated by DR signaling but leaves open whether RIP1, RIP3, or both are directly bound by the viral cell-death suppressor.

**MCMV-Induced Programmed Necrosis Is RIP3 Dependent but RIP1 Independent**

Having shown viRA suppresses RIP1/3-dependent necroptosis, we characterized the cellular requirements for MCMV-associated programmed necrosis. RIP3 knockdown rendered either 3T3-SA or SVEC4-10 cells resistant to virus-induced programmed necrosis (Figures 4A and 4B). To clarify the role of RIP3, control (RIP3+/+), heterozygous (RIP3+/−), and homozygous (RIP3−/−) deficient MEF cells were isolated and assessed for virus replication and sensitivity to programmed necrosis. RIP3 levels in cells reflected gene copy (Figure 4D). Parental MCMV replicated to comparable high titers in all MEF lines tested, whereas M45mutRHIIM virus was attenuated in RIP3−/− cells (Figure 4E), where RIP3 levels were highest (Figure 4D), and normalized in RIP3−/− MEFs (Figure 4E). Consistent with a relationship between RIP3 levels and sensitivity to necrotic death (Figure 4D), the mutant virus phenotype was intermediate in RIP3+/− cells (Figures 4E and 4G). Furthermore, RIP3+/− MEFs died following mutant virus infection, whereas RIP3−/− cells survived, sustaining the requirement for RIP3 in virus-induced death (Figure 4G). RIP3 levels correlated with cellular sensitivity to virus-induced necrosis as well as to the concomitant attenuation of viral growth. When RIP3−/− MEFs were reconstituted with WT, KD, or mutant RHIM (mRHIM) RIP3 (Figure 4F) and infected with mutant virus, only WT RIP3-reconstituted cells exhibited increased sensitivity to virus-induced necrosis (Figure 4F). These data are consistent with a direct role of RIP3 as well as a requirement for an intact RIP3 kinase domain and RHIM for virus-induced necrotic cell death.

To formally demonstrate that viRA directly targets RIP3 to modulate this death process during infection, we infected necrosis-sensitive 3T3-SA cells. M45 protein interacted with RIP3 but not mutRHIM M45 protein when infected cell lysates were subjected to co-IP at 12 and 18 hpi with antibody to RIP3, consistent with a RHIM-dependent interaction of viRA with endogenous RIP3 (Figure 4C). We were unable to detect an interaction between endogenous RIP3 and RIP1 during viral infection with either WT or mutant viruses (data not shown), suggesting that virus-associated programmed necrosis makes distinct use of adaptors compared with DR-induced necroptosis. To investigate the role of TNFα as well as RIP1 in virus-associated programmed necrosis, infected cells were treated with TNFα-neutralizing antibody over a range of effective concentrations (Figure S3D) without observing any impact on virus-induced necrosis (Figures S3A–S3C). Infected cells were also treated with the RIP1-specific kinase inhibitor Nec-1 to determine whether RIP1 kinase activity contributes to virus-induced programmed necrosis. Treatment with Nec-1 over a range of doses failed to restore viability of infected primary MEF, 3T3-SA, or SVEC4-10 cells (Figure 4G, gray bars; Figures S3E–S3G). In contrast, Nec-1 treatment was sufficient to suppress TNFα-induced necroptosis in these same cell lines (Figure 4G; Figures S3E and S3F), confirming both the sensitivity of these cells to necroptosis and the efficacy of Nec-1 as a RIP1 inhibitor when this adaptor is involved in death. Thus, RIP1 kinase activity is dispensable for virus-associated programmed necrosis, in contrast to its critical role in necroptosis. Because SV40-transformed MEFs, including both the RIP1+/+ and RIP1−/− MEFs used in Figure 3C, were resistant to virus-induced cell death (data not shown), we employed a number of different hairpin RNAi constructs to suppress RIP1 in primary
Figure 4. RIP3 Is Required, and RIP1 Is Dispensable, for MCMV-Associated Programmed Necrosis

(A) Viability of 3T3-SA cells expressing Sc, RIP3-A, or RIP3-B shRNAs determined 18 hpi with parental WT or M45mutRHIM virus (MOI of 10).

(B) Viability of SVEC4-10 cells using a subset of conditions described in (A).

(C) IB of 3T3-SA cells infected with parental WT or M45mutRHIM virus (MOI of 5), harvested at indicated times for IP of RIP3 followed by detection of vIRA (M45) and RIP3. IB using 5% of cell lysate to detect vIRA (M45) and β-actin.

(D) IB of RIP3+/+, RIP3+/−, and RIP3−/− MEFs to detect RIP3, RIP1, and β-actin.

(E) Replication of WT and M45mutRHIM viruses (MOI of 5) on RIP3+/+, RIP3−/− (left), RIP3+/− (middle), and RIP3−/− (right) MEFs over a 72 hr time course. Viral titers were determined by plaque assay with the first (0 h) time point representing the amount of virus in the inoculum.

(F) IB analysis for Flag-tagged proteins as well as β-actin (left) in RIP3−/− MEFs expressing Flag-tagged RIP3, RIP3-KD, or RIP3-mRHIM, and viability of reconstituted cells (right) infected with M45mutRHIM and WT virus.

(G) Viability of RIP3+/+, RIP3+/−, and RIP3−/− MEFs infected with WT or M45mutRHIM virus in the presence or absence of Nec-1 (30 μM).
MEFs as well as SVEC4-10 cells (Figures 4I and 4J), but these failed to suppress virus-induced necrosis induced by independently derived M45mutRHIM mutant viruses (Figures 4I and 4J). Thus, unlike TNFα-induced necroptosis, virus-induced necrosis relies on RIP3 but not RIP1.

**Normalization of M45 Mutant Virus Phenotype in RIP3-Deficient Mice**

Given that M45mutRHIM virus induced a form of RIP3-dependent programmed necrosis that attenuates viral pathogenesis, we inoculated RIP3−/− and control mice to follow initial inflammatory events induced by mutant or parental virus. Footpad inoculation allows assessment of virus-induced inflammation at the site of inoculation, characterized by swelling of the footpad compared with uninfected controls, which is influenced by the MCMV-encoded CC chemokine homolog MCK-2 (Sæderup et al., 2001). M45mutRHIM virus infection induced swelling in RIP3−/− mice comparable to parental virus infection of either RIP3+/− or RIP3−/− mice, reaching a greater than 50% increase in size over uninfected paws (Figure 5A). In contrast, mutant virus did not induce significant swelling in RIP3−/− mice, consistent with an attenuated phenotype. Thus, in the absence of RIP3 function, mutant virus infection recapitulated early inflammatory events characteristic of WT infection.

TRIF is known to influence WT MCMV infection (Tabeta et al., 2004). Given the importance of RHIM-mediated activity in suppressing virus-induced death as well as the observation that RIP3 associates with TRIF, we evaluated any role of this cellular RHIM adaptor (Kaiser and Offermann, 2005; Meylan et al., 2004). Despite previous demonstration that vIRA suppresses RHIM-dependent cell-death signaling mediated via TRIF (Upton et al., 2008), footpad-inoculated Trif−/−/Lps2−/− mice (Hoebe et al., 2003) failed to exhibit any difference in behavior from WT mice (Figure 5A). When explanted RIP3+/−, RIP3−/−, and Trif−/−/Lps2−/− peritoneal exudate cells (PECs) were infected ex vivo with either mutant or parental virus, only RIP3−/− PECs were resistant to mutant virus-induced programmed necrosis (Figure 5B). Because PECs express TLR3 and TLR4, this experiment excluded any contribution of TRIF-dependent TLR signaling to virus-induced necrosis. Thus, vIRA plays a critical RIP3-dependent, TRIF-independent step in viral pathogenesis.

Finally, we sought to evaluate the role of RIP3 in viral pathogenesis. SGs are the major site of secondary MCMV replication and source of transmission between hosts. Virus levels in this organ remain a sensitive indicator of successful infection and dissemination to secondary organs in the host. Parental MCMV disseminated at similar levels to SGs of controls, RIP3+/−, and TRIF-defective C57BL/6 mice. Consistent with experiments in NSG and BALB/c mice, C57BL/6 mice failed to support infection and dissemination of mutant virus, and this characteristic was shared with TRIF-defective mice (Figure 5C). Mutant and parental virus titers in SGs of RIP3−/− mice were comparable (Figure 5C). In the absence of RIP3, vIRA function appears dispensable for WT levels of viral replication or dissemination. Normalization of mutant virus behavior by elimination of a host determinant provides compelling evidence that RIP3 is the target of vIRA-mediated modulation during MCMV infection, and suggests RHIM-dependent vIRA suppression of RIP3 function in virus-associated cell death is essential for MCMV pathogenesis.

**DISCUSSION**

Manipulation of host cytokine and cell-death pathways affords pathogens the opportunity to maintain an environment necessary for efficient replication to establish a foothold within the host organism. In this work, we demonstrate a critical role for RIP3 as a positive regulator of a cell-intrinsic antiviral programmed necrosis triggered during MCMV infection. Our results show a dependence on RIP3 function in both virus-associated programmed necrosis and DR-associated necroptosis. We also provide a clear mechanistic distinction between these two pathways. Whereas necroptosis is dependent on a RIP1-RIP3 signaling complex (Cho et al., 2009; He et al., 2009; Zhang et al., 2009), virus-induced death is dependent on RIP3 but independent of RIP1. MCMV-encoded vIRA suppresses either necrotic death pathway in a RHIM-dependent fashion, suggesting the common target of this viral cell-death suppressor is RIP3. The dramatic differences in behavior of viruses expressing WT or RHIM-deficient form of vIRA, assayed in vitro and in vivo, demonstrate this cell-death suppressor is critical for MCMV replication and pathogenesis. Normalization of mutant virus behavior when RIP3 function is eliminated in the host formally establishes the biological target of suppression as well as the significance of vIRA-RIP3 interactions. By revealing the commitment of a specific pathogen-encoded suppressor to necrotic death pathways, our data validate RIP3-dependent programmed necrosis as a central player in host defense and strongly argues that RIP3 may be the dominant adaptor controlling cellular necrotic pathways in viral pathogenesis.

Restoration of an attenuated vIRA viral mutant phenotype in vivo by the elimination of the host intracellular adaptor such as RIP3 has rarely been achieved in studies on viral pathogenesis. The most relevant to our studies is the demonstration that herpes simplex virus ICP34.5 uniquely targets PKR (Leib et al., 2000), an RNA binding adaptor protein that restricts translation in virus-infected cells. The attenuation of ICP34.5 mutant virus was completely normalized in PKR-deficient mice. Here we provide in vivo evidence that the MCMV M45 gene product vIRA mediates its biological function by antagonizing the host prof necrotic kinase RIP3 via RHIM-mediated interactions. Thus, our demonstration that virus carrying RHIM-deficient vIRA exhibited normalized replication in RIP3-deficient mice demonstrates the exclusive interplay of RIP3 and vIRA in MCMV pathogenesis.

High levels of RIP3 remain the common feature predicting sensitivity to either virus- or DR-induced necrotic death. Recent
findings that high RIP3 levels confer sensitivity of human and murine cell lines to TNFα-induced necroptosis (He et al., 2009; Zhang et al., 2009) are entirely consistent with the results presented here. One characteristic of necroptosis, the requirement of RIP1 kinase activity (Declercq et al., 2009; Festjens et al., 2007), distinguishes this necrotic pathway from virus-induced necrosis. Nec-1 treatment inhibits the formation of a RIP1-RIP3 complex, RIP3 activation, and cell death in response to TNFα (Cho et al., 2009; He et al., 2009), but treatment with this RIP1-specific inhibitor has no impact on virus-induced necrosis. Overexpression of RIP3 has previously been reported to induce cell death, including situations where RIP1 expression has been eliminated (Zhang et al., 2009). Although this work indicated RIP3 alone may be sufficient to drive necrotic death, the data reported here alone may show physiological relevance of a RIP3-dependent, RIP1-independent pathway.

Although we have identified RIP3 as the executioner of MCMV-induced programmed necrosis, the cellular processes leading to activation of this pathway by virus remain unidentified. UV-inactivated mutant virus does not induce cell death (Brune et al., 2001), and preliminary evaluation suggests that inhibition of viral DNA replication fails to protect from programmed necrosis. Thus, the RIP3-activating step likely originates from early events in virus-infected cells, perhaps through cellular processes altered or usurped by viral infection. Alternatively, PRRs initiate signals to contend with pathogens, and several of these sensor proteins are known to recruit RIP3. TLR3-TRIF contributes to the innate immune response to MCMV infection (Hoebe et al., 2003; Tabeta et al., 2004), and RIP3 binds to TRIF in a RHIM-dependent interaction (Kaiser and Offermann, 2005; Meylan et al., 2004), although the significance of this interaction remains unclear. The capacity of vIRA to suppress RHIM-dependent death signals relayed via TRIF (Upton et al., 2008) was not sustained here, as TRIF-deficient cells remained susceptible to killing by mutant virus and mice lacking TRIF were no more susceptible to mutant virus than were WT mice. Thus, suppression of programmed necrosis by vIRA during infection is unlikely to involve TRIF signaling. Our results raise the possibility that another sensor

Figure 5. M45mutRHIM Attenuation In Vivo Is Specifically Normalized in RIP3-Deficient Mice
(A) Swelling induced by parental WT or M45mutRHIM virus infection of C57BL/6 (RIP3+/+), RIP3−/−, and TRIF-deficient (Lps2/Lps2) mice. Groups of five (C57BL/6 and RIP3−/−) or three (Lps2/Lps2) mice were inoculated (10^6 pfu) in footpads, thickness was measured with a digital caliper (Saederup et al., 2001), and mean values were plotted at the indicated times over a 14 day time course. Error bars indicate standard error of the mean.
(B) Viability of explanted, cultured RIP3−/−, Lps2/Lps2, or C57BL/6 (WT) PECs at 18 hpi with either WT or M45mutRHIM. Error bars indicate SD of the mean.
(C) SGs were harvested from euthanized mice (described in A) and titers were determined by plaque assay. Each symbol represents one mouse, and solid horizontal lines represent the mean for each group. The dotted line is the limit of detection in this assay.
pathway drives RIP3 activation, and the IFN-inducible, cytosolic DNA sensor DAI/ZBP1/DLM-1 (Takaoka et al., 2007) remains an attractive candidate to fulfill such a role, given that it is both a RHIM-containing adaptor and binding partner of RIP3 as well as vIR (Kaiser et al., 2008; Rebsamen et al., 2009). Defining the cellular processes required for induction of RIP3-dependent death during MCMV infections remains a significant future line of investigation.

Cytomegaloviruses encode an array of cell-death suppressors to counter apoptotic cell-death pathways that may dominate in different cell and tissue settings (McCormick, 2008). MCMV encodes three genes that target core components of the apoptotic machinery: viral mitochondrial inhibitor of apoptosis, vMIA (Goldmacher et al., 1999; McCormick et al., 2005), viral inhibitor of Bak oligomerization, vBO (Cam et al., 2010), and viral inhibitor of caspase-8 activation, vICA (Skaletskaya et al., 2001). Together, these functions antagonize caspase-dependent death pathways that would otherwise compromise viral replication. A recombinant viral mutant lacking the Bax inhibitor vMIA replicates comparably to parental virus in visceral organs of infected animals, but is attenuated for leukocyte-dependent dissemination of virus to the SGs (Manzur et al., 2009). Mutants lacking the caspase-8 inhibitor vICA are attenuated in vivo following systemic inoculation, and are severely attenuated for dissemination to SGs (Cinc-Sain et al., 2008). These results support the importance of modulating host apoptotic pathways in MCMV pathogenesis. It is worth noting that the attenuation of vIRA mutants in vivo is as critical to viral pathogenesis as the suppression of Bax activation or caspase-8 activation by vMIA or vICA, respectively.

Conclusions
The identification of vIRA as an inhibitor of RIP3-dependent programmed necrosis in a natural biological setting extends the role of RIP3 as a vital player in host antiviral defense, and underscores the critical role of vIRA RHIM-dependent activities in suppression of programmed necrosis in MCMV pathogenesis. This study also begins to illustrate the balance of necrotic and apoptotic pathways in host defense against viruses, supporting the growing evidence that viruses exploit multiple innate immune processes to influence a wide range of alternative immune effector activities.

EXPERIMENTAL PROCEDURES

Reagents
CHX, Bafilomycin A1, and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich. Nec-1 and z-VAD-fmk were from Calbiochem and Enzo Life Sciences, respectively. Recombinant mouse TNFα was from R&D Systems. The following antibodies were used in IB analyses: mouse anti-c-myc (clone 9E10; Sigma-Aldrich), rabbit anti-c-myc HRP conjugate (Sigma-Aldrich), anti-mouse IgG-HRP (Vector Laboratories), anti-rabbit IgG-HRP (Vector Laboratories), anti-MCMV M45 (gift from David Lembo, University of Turin), and anti-MCMV IE1 (gift from Stijn Jonnic, University of Rijeka). IP analyses were performed with rabbit anti-c-myc agarose conjugate (Sigma-Aldrich) or goat anti-RIP3 (clone C-16; Santa Cruz Biotechnology) and protein A/G agarose (Santa Cruz Biotechnology).

BAC Mutagenesis and Recombinant Viruses
Plasmid pSIH6 encoding a red recombination functions (Datta et al., 2006) was introduced into bacteria carrying a bacterial artificial chromosome (BAC) harboring the MCMV K181(Perth) strain genome (Redwood et al., 2005). Recombineering for K181-BAC mutagenesis and diagnostics were performed essentially as previously described (Tandon and Mocarski, 2008), and is outlined in Supplemental Information. BAC-derived mutant and parental K181 virus was generated in NIH 3T3 fibroblasts (CRL-1658; American Type Culture Collection) and purified as previously described (Redwood et al., 2005). Parental and mutant viruses were propagated, clarified, concentrated, and titered by plaque assay on NIH 3T3s as previously described (Saederup et al., 1999). Growth curves and viral yields were performed by infecting cells in six-well plates at the indicated multiplicity of infection (MOI) in 0.5 ml for 2 hr at 37°C with periodic rocking. Following adsorption, cells were washed three times with PBS and refed. Samples were analyzed at indicated times, and titered by plaque assay.

Mice, Infections, and Organ Harvests
C57BL/6, BALB/c, TRIF mutant (strain C57BL/6J-Ticam1<tm1(2ae)> (Hoebe et al., 2003), and NSG mutant (NOD.scID-PkR<tm1m1Myh2>Rag2<tm1Mm2>S2) mice were from Jackson Laboratory, RIP3<−/−> mice (Newton et al., 2004) were provided by Francis Chan (University of Massachusetts) with permission from Genentech. Eight- to twelve-week-old mice were inoculated with 10^6 pfu into a rear footpad or by intraperitoneal injection as previously described (Saederup et al., 1999). Upon sacrifice, organs were placed in 1 ml complete DMEM and stored at −80°C until they were thawed, disrupted by sonication, and titered by plaque assay. Resident peritoneal macrophages were collected as previously described (Saederup et al., 1999) and seeded at a density of 5 x 10^4 cells per well in 96-well plates 18 hr prior to infection. For virulence studies, animals losing 20% body weight or displaying severe signs of CMV disease (ruffled fur, hunched posture, dehydration, diminished responsiveness) were scored and euthanized. Mice were bred and maintained by the Emory University Division of Animal Resources, where all procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Cell Culture and Embryonic Fibroblast Isolation
NIH 3T3 fibroblasts, ST3-SA (ATCC CCL-92), SVEC4-10 (ATCC CRL-1218), HEK293T, L929, and MEFs were maintained in DMEM containing 4.5 g/ml glucose, 10% fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (Invitrogen). SV40T-transfected Rip1<−/−> and control WT MEFs (Wong et al., 2010) were provided by John Silke (LaTrobe University) with permission from Michelle Kelliher (University of Massachusetts). MEFs were isolated from timed pregnancies at days E14.5–16.5, as previously described (Pollock and Virgin, 1995).

Immunoblot and Immunoprecipitations
IPs were performed essentially as described previously (Kaiser and Offermann, 2005), with minor modifications. Clarified cell lysates were incubated overnight with anti-c-myc agarose or goat-anti-RIP3 antibody and protein A/G agarose and were washed four to six times prior to analysis. Cell lysates and IP samples were separated on Criterion gels (Bio-Rad), transferred to Immobilon PVDF membranes (Millipore), and subjected to IB analysis. Detection of c-myc and RIP3 immunoprecipitates was performed with mouse anti-c-myc (clone 9E10) or rabbit anti-RIP3 antibodies, respectively.

Plasmids, Transfections, and Transductions
Transfections were performed with Lipofectamine 2000 (Invitrogen) and DNA at a 1:1 ratio in Opti-MEM (Invitrogen). Carboxy-terminal Flag-epitope-tagged WT and murRHM M45 were generated by subcloning the respective open reading frame into pCMV-TAG4A (Staglujan). pCMV10-3xFlag-RIP3 and pCDNA3-6myc-RIP1 (Kaiser and Offermann, 2005) have been previously described. Flag-RIP3, Flag-RIP3-KD (Kaiser et al., 2008), and Flag-RIP3-mRHM1 (Kaiser and Offermann, 2005), as well as M45-myc and M45murfRHM-myc (Upton et al., 2008) were subcloned into the pCOXIIH (Clontech) retroviral construct. The pLKO.1-based RIPA-3 (TRCN0000022535), RIPA-3 (TRCN0000022538), RIPA-1 (TRCN0000022467), and RIPA-1B (TRCN0000022464) shRNA constructs were obtained from Open Biosystems. The pLKO.1-scramble control
shRNA vector (Sarbassov et al., 2005) as well as lentiviral and retroviral production, infection, and selection have all been described (Kaiser et al., 2008).

**Cell Viability Assays**

Cells (5000 cells/well) were seeded into 96-well plates. Sixteen to eighteen hours postseeding, medium was replaced with 50 μl of viral inoculum containing 10 pfu/cell (MOI = 10). Nec-1 at the indicated concentration was added 1 hr prior to infection, and maintained for the duration of the assay. Alternatively, cells were treated with TNF-α, CHX, zVAD-fmk, and/or Nec-1. The Nec-1 vehicle, DMSO, was held constant for all cells. Viability was determined 18 hr postinfection/treatment. Unless otherwise indicated, the number of viable cells per well was determined indirectly by measuring the intracellular levels of ATP using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer’s instructions. Released protease activity was measured using the CytoTox-Fluor Assay kit (Promega) according to the manufacturer’s instructions. Luminescence or fluorescence was measured on a Synergy HT Multi-Detection microplate reader (Bio-Tek).

**Microscopy**

3T3-SA cells were infected with WT or M45rhRHM viruses (MOI of 10) for the indicated times in 6- or 12-well dishes. Bright-field images were acquired with an AxioCam MRC5 camera with Zeiss Axio Imager A1 and processed with AxioVision Release 4.5 software. 3T3-SA cells infected with WT or M45rhRHM viruses (MOI of 10) for 18 hr were prepared for EM as described (Tandon and Mocarski, 2008).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at doi:10.1016/j.chom.2010.03.006.

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