Phosphorylation-Driven Assembly of the RIP1-RIP3 Complex Regulates Programmed Necrosis and Virus-Induced Inflammation

YoungSik Cho,1,2,4 Sreerupa Challa,1,2 David Moquin,1,2 Ryan Genga,1 Tathagat Dutta Ray,1,2 Melissa Guildford,1 and Francis Ka-Ming Chan1,2,3,*

1Department of Pathology
2Immunology and Virology Program
3Center for AIDS Research
University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655, USA
4Center for Metabolic Syndrome Therapeutics, Bio-Organic Science Division, Korea Research Institute of Chemical Technology, Yuseong-gu, Daejeon 305-600, Korea
*Correspondence: francis.chan@umassmed.edu
DOI 10.1016/j.cell.2009.05.037

SUMMARY

Programmed necrosis is a form of caspase-independent cell death whose molecular regulation is poorly understood. The kinase RIP1 is crucial for programmed necrosis, but also mediates activation of the prosurvival transcription factor NF-κB. We postulated that additional molecules are required to specifically activate programmed necrosis. Using a RNA interference screen, we identified the kinase RIP3 as a crucial activator for programmed necrosis induced by TNF and during virus infection. RIP3 regulates necrosis-specific RIP1 phosphorylation. The phosphorylation of RIP1 and RIP3 stabilizes their association within the pronecrotic complex, activates the pronecrotic kinase activity, and triggers downstream reactive oxygen species production. The pronecrotic RIP1-RIP3 complex is induced during vaccinia virus infection. Consequently, RIP3−/− mice exhibited severely impaired virus-induced tissue necrosis, inflammation, and control of viral replication. Our findings suggest that RIP3 controls programmed necrosis by initiating the pronecrotic kinase cascade, and that this is necessary for the inflammatory response against virus infections.

INTRODUCTION

Cell death by programmed necrosis (also known as caspase-independent cell death or necroptosis) is characterized by rapid loss of plasma membrane integrity prior to the exposure of phagocytic signal (Golstein and Kroemer, 2007). The release of endogenous "danger signals" from necrotic cells induces inflammation and can activate immune responses, trigger inflammatory diseases, and promote cancer growth (Kono and Rock, 2008). In addition, nonapoptotic or necrotic cell death has been shown to critically regulate disease pathologies in animal models of hypoxic/ischemic injury (Degterev et al., 2005), acute pancreatitis (Mareninova et al., 2006), and septic shock (Cauwels et al., 2003). Consistent with these observations, blockade of necrosis was effective in slowing or reducing cell injury in models of cardiac infarct (Smith et al., 2007) and ischemic brain injury (Degterev et al., 2005). Despite its biological importance, the molecular components regulating programmed necrosis are not well defined.

Tumor necrosis factor (TNF)-like cytokines are potent inducers of programmed necrosis. We and others have previously identified an obligate role for the protein serine/threonine kinase receptor interacting protein 1 (RIP/RIP1/RIPK1) in programmed necrosis (Chan et al., 2003; Holler et al., 2000; Lin et al., 2004). RIP1 is also a crucial adaptor that mediates activation of the prosurvival transcription factor NF-κB by TNF, TLR3, and TLR4 (Cusson-Hermance et al., 2005; Hsu et al., 1996; Meylan et al., 2004; Ting et al., 1996). The kinase function of RIP1 is essential for programmed necrosis but dispensable for NF-κB activation (Chan et al., 2003; Holler et al., 2000; Lin et al., 2004), suggesting that programmed necrosis might be regulated at the level of activation of RIP1 kinase activity. However, the molecular mechanism that activates the pronecrotic RIP1 kinase activity has remained elusive.

Necrosis distinguishes itself from apoptosis by its proinflammatory effects. The release of endogenous adjuvants from necrotic cells can activate the inflammasomes, induce cytokine production, facilitate inflammatory cell recruitment to the site of infection, stimulate dendritic cell maturation, and promote the subsequent virus-specific T cell responses (Kono and Rock, 2008). These proinflammatory effects of necrosis are important for successful antiviral immune responses. In addition, programmed necrosis may control the viral factory by eliminating the infected host cells. An antiviral role for programmed necrosis is further bolstered by our previous findings that certain viral FLIPs (FLICE-like inhibitor proteins) are potent inhibitors of TNF-induced programmed necrosis (Chan et al., 2003). These
results suggest that viral inhibition of programmed necrosis is an important immune evasion strategy for certain viruses. However, because genetic ablation of the only known programmed necrosis mediator RIP1 resulted in neonatal lethality (Kelliher et al., 1998), the physiological roles of programmed necrosis in inflammation and antiviral host defense have not been tested.

In this report, we identify RIP3 as a crucial upstream activating kinase that regulates RIP1-dependent programmed necrosis. We show that RIP3 acts upstream to phosphorylate RIP1. Interestingly, the RIP1 kinase activity is also required for necrosis-specific RIP3 phosphorylation. Both RIP3 and the kinase activity of RIP1 are essential for stable formation of the RIP1-RIP3 pro-necrotic complex, which critically controls downstream reactive oxygen species (ROS) production. Strikingly, the pro-necrotic RIP1-RIP3 complex was specifically induced in the liver upon vaccinia virus (VV) infection. RIP3−/− mice failed to initiate virus-induced tissue necrosis and inflammation, resulting in highly elevated viral replication and mortality. These results show that RIP3-dependent programmed necrosis is important for virus-induced inflammation and innate immune control of viral infections.

RESULTS

Identification of RIP3 as a Mediator for Programmed Necrosis

RIP1 is a pleiotropic adaptor that mediates both NF-κB activation and programmed necrosis by TNF (Chan et al., 2003; Holler et al., 2000; Lin et al., 2004). The kinase activity of RIP1 is essential for programmed necrosis, but dispensable for NF-κB activation (Chan et al., 2003; Holler et al., 2000). We hypothesized that induction of programmed necrosis requires additional components that specifically turn on the RIP1 kinase activity. To identify such molecules, we screened a 21-mer small interfering RNA (siRNA) library consisting of 691 human kinase genes in FADD-deficient Jurkat cells (Table S1 available online, and the Supplemental Experimental Procedures), which rapidly undergo programmed necrosis in response to TNF (Chan et al., 2003). From the screen, we identified ten clones that potently inhibited TNF-induced programmed necrosis (% survival > average survival of all clones + 3 SD) (Table S2). Significantly, RIP1 was one of the protective clones, demonstrating the fidelity of our screen. Transfection of siRNA against RIP3 also conferred protection against TNF-induced programmed necrosis (% survival > average survival of all clones + 3 SD) (Table S2). Significantly, RIP1 was one of the protective clones, demonstrating the fidelity of our screen. Transfection of siRNA against RIP3 also conferred protection against TNF-induced programmed necrosis at a level comparable to that by RIP1 siRNA (Figure S1). The protection against TNF-induced programmed necrosis by RIP1 and RIP3 siRNAs were specific, since other RIP family kinases including rip2, rip4, and rip5 that were represented in the library did not protect against programmed necrosis (data not shown). We validated these results in TNFR-2−/− wild-type Jurkat cells (clone 4E3) and found that individual RIP3-specific siRNAs efficiently reduced RIP3 protein expression (Figure 1A) and inhibited TNF/zVAD-fmk-induced programmed necrosis (Figure 1B), but had little or no effects on apoptosis induced by TNF or FasL (Figure 1C-D). Consistent with a previous report (Newton et al., 2004), RIP3 deficiency did not alter TNF-induced NF-κB activation (Figure 1E). Thus, unlike RIP1, RIP3 is a specific activator for programmed necrotic cell death.

The Kinase and RHIM Domains of RIP3 Are Required to Induce Programmed Necrosis

RIP3 contains an N-terminal kinase domain with ~40% identity with the kinase domain of RIP1 (Figure 2A). A kinase-defective (KD) RIP3 mutant failed to restore TNF-induced programmed necrosis in Jurkat cells whose endogenous RIP3 expression was silenced by siRNA (Figure 2B, RIP3 null Jurkat cells), indicating that the RIP3 kinase activity is crucial for programmed necrosis. RIP1 and RIP3 have been reported to interact with each other via the RIP homotypic interaction motif (RHIM)
Figure 2. Requirement of Kinase and RHIM Domains of RIP3 for Programmed Necrosis

(A) Domain structures of RIP1 and RIP3.

(B and C) The RIP3 kinase and RHIM domains are required for programmed necrosis. Wild-type TNFR2+ Jurkat cells were transfected with siRNA against human RIP3. After 24 hr, cells were transfected with the indicated GFP-tagged plasmids: kinase-dead (KD, D161N) mouse RIP3 (B) and RIP3 RHIM mutant (C). TNF/zVAD-induced programmed necrosis was determined in the transfected GFP+ population.

(D) The kinase and RHIM domains of RIP1 are required for programmed necrosis. RIP1-deficient Jurkat cells were transfected with the indicated GFP-tagged RIP1 plasmids. TNF/zVAD-fmk induced programmed necrosis was determined as in (B) and (C). Data are presented as mean ± SEM of triplicates.

(E) RIP3 is recruited to complex II. Complex II was isolated with caspase-8 specific antibody from wild-type TNFR2+ Jurkat cells stimulated with TNF ± zVAD-fmk, and the recruitment of RIP1 and RIP3 was determined by western blot. WCE, whole-cell extract; C8, caspase-8.

(F) RIP3 is not recruited to the TNFR-1 signaling complex. TNFR-1 immune complexes from TNFR2+ Jurkat cells stimulated with TNF ± zVAD-fmk for the indicated times were examined for the recruitment of RIP1 and RIP3 by western blot. RIP1-Ub, polyubiquitinated RIP1.

(Figure 2A) (Sun et al., 2002). Tetra-alanine substitutions in the RHIM of RIP1 or RIP3 abolished their association (Figure S2) and the ability of RIP3 to restore TNF-induced programmed necrosis in RIP3 null Jurkat cells (Figure 2C). Similar mutations in the kinase and RHIM domains of RIP1 also abolished the RIP1-mediated rescue of programmed necrosis in RIP1-deficient Jurkat cells (Figure 2D). Thus, both the kinase and RHIM domains of RIP1 and RIP3 are important for programmed necrosis.

RIP3 Is Recruited to Complex II during Programmed Necrosis

TNFR signaling involves at least two spatially and temporally distinct signaling complexes: the transient membrane-associated TNFR-1 signaling complex (complex I) and the cytoplasmic signaling complex termed complex II (Micheau and Tschopp, 2003). Complex II is formed upon TNFR-1 internalization, when TNFR-1 dissociates from complex I to allow recruitment of additional factors. Because RIP1 is recruited to both complex I and complex II, we examined RIP3 recruitment to these signaling complexes. In TNFR-2+ Jurkat 4E3 cells, TNF triggers both apoptosis and programmed necrosis, although apoptosis predominates because of its faster kinetics unless caspases are inhibited (Zheng et al., 2006). In TNFR-2+ Jurkat 4E3 cells, RIP3 was recruited to caspase-8-associated complex II (Figure 2E), but not to TNFR-1 associated complex I (Figure 2F). On short exposure, zVAD-fmk moderately enhanced RIP1 and RIP3 binding to caspase-8-associated complex II (Figure 2E). However, the difference was minor and not distinguishable on longer exposure (Figure S3). On longer exposure, RIP1 cleavage in complex II was also visible (Figure S3). Although RIP1 cleavage by caspase-8 at D324 might inactivate the RIP1 kinase function and contribute to the bifurcation of apoptosis and programmed necrosis (Lin et al., 1999), zVAD-fmk probably sensitizes cells to programmed necrosis via additional regulatory events because full-length RIP1 was still readily detected without caspase inhibition (Figure S3).

Induction of Pronecrotic Complex II Kinase Activity

The recruitment of RIP1 and RIP3 to complex II suggests that their pronecrotic kinase activity might be activated within this complex. When caspase-8-associated complex II was examined, we did not observe strong TNF-inducible kinase activity (data not shown). Hence, although RIP1 and RIP3 were recruited to caspase-8, their activities were not regulated in this compartment. In contrast, complex II isolated by immunoprecipitation with FADD-specific antibody exhibited a transient induction of kinase activity as measured by phosphorylation of an ~60 kDa
protein and several other species (Figure 3A, top panel). We confirmed the identity of the 60 kDa phospho-protein to be RIP3 when we boiled the TNF-activated FADD complex in SDS and subjected it to a second immunoprecipitation with RIP3-specific antibody (Figure 3B, lane 5). In addition to RIP3, complex II kinase activity could also be measured with the artificial substrate MBP (Figure 3B, lane 3). Similar complex II kinase activity could also be detected in caspase-8-deficient Jurkat cells undergoing programmed necrosis (Figure S4). Interestingly, FADD complex kinase activity was detected in TNF-treated TNFR2+ Jurkat cells when apoptosis was the dominant cell death mode (Figure 3A, lane 2). Although zVAD-fmk slightly increased complex II kinase activity (Figure 3A, lane 5), these results suggest that zVAD-fmk might exert its necrosis-promoting effects subsequent to the induction of complex II kinase activity.

Consistent with the lack of RIP3 binding to complex I, TNF did not activate kinase activity in TNFR-1 complexes (Figure 3C). Moreover, TNFR-2+ Jurkat cells that did not undergo programmed necrosis in response to TNF (Chan and Lenardo, 2000) also exhibited no inducible FADD complex II kinase activity regardless of whether zVAD-fmk was present (Figure 3D). Thus, the induction of complex II kinase activity was specific to programmed necrosis. Complex II kinase activity was also not detected in TNFR2+ RIP1-deficient Jurkat cells, which undergo apoptosis in response to TNF (Chan et al., 2003) (Figure 3E). In addition, the RIP1 kinase inhibitor necrostatin-1 (Nec-1) potently inhibited complex II kinase activity (Figure S4). Interestingly, while RIP1 was recruited to FADD in a TNF-dependent manner, RIP3 was constitutively associated with FADD (Figure 3A, lower panels), although this interaction may be indirect (Figure S4).

Taken together, our results show that RIP1 recruitment to complex II is crucial for the induction of pronecrotic complex II kinase activity.

### RIP3 Controls RIP1 Binding to Complex II

Although the induction of complex II kinase activity appears to correlate with programmed necrosis, it could not be explained entirely by RIP1 recruitment to complex II because RIP1 was recruited to complex II in cells that did not undergo necrotic death (Figure 3D, lower panels). To further define the molecular regulation of programmed necrosis, we examined the recruitment of RIP1 and RIP3 to complex II in mouse embryonic fibroblasts (MEFs) because the role of RIP3 in the assembly of the pronecrotic complex could be evaluated with RIP3-deficient MEFs. Similar to Jurkat cells, constitutive association between RIP3 and FADD was also detected in MEFs (Figure 4A). When programmed necrosis was induced in wild-type MEFs with TNF, cycloheximide and zVAD-fmk (T/C/Z) (Figure S5), RIP1 was recruited to FADD in a TNF-dependent manner (Figure 4A, lane 3). Moreover, TNF treatment led to further recruitment of RIP3 to the FADD-associated complex II and RIP3 modification that resembles polyubiquitination (Figure 4A, lane 3). Preliminary evidence suggests that these modifications were indeed polyubiquitination (F.K.C., unpublished data). Strikingly, RIP1 recruitment to complex II was severely impaired in RIP3−/− MEFs (Figure 4A, lanes 5–8). The RIP1 kinase inhibitor Nec-1 (Degterev et al., 2008) also
abolished RIP1 recruitment to FADD and inhibited additional RIP3 binding to the complex (Figure 4A, lane 4, and Figure S6). These results indicate that both RIP1 and RIP3 are critical for the assembly of the pronecrotic complex II.

RIP3 Acts Upstream to Regulate RIP1 Phosphorylation

We then ascertained the possibility that RIP3 might regulate RIP1 recruitment to the pronecrotic complex II via phosphorylation by labeling of MEFs with [32P]-orthophosphate. Indeed, RIP1 phosphorylation was detected when programmed necrosis was induced (Figure 4B, lane 2). Although low levels of basal RIP1 phosphorylation could sometimes be detected, TNF treatment alone, which did not result in apoptosis or programmed necrosis (Figure S6), did not induce RIP1 phosphorylation (Figure 4B, lanes 3 and 4). Strikingly, ligand-dependent RIP1 phosphorylation was completely abrogated in RIP3−/− MEFs (Figure 4B, lanes 5 and 6). Similar TNF-dependent RIP1 phosphorylation was observed in FADD-deficient Jurkat cells (Figure 4C, lane 2). Importantly, Nec-1 did not inhibit RIP1 phosphorylation (Figure 4C, compare lanes 2 and 3), indicating that TNF-dependent RIP1 phosphorylation was not due to autophosphorylation. To determine whether RIP1 might directly phosphorylate RIP3, we subjected wild-type or kinase-defective RIP1 or RIP3 to in vitro kinase assays. Indeed, WT-RIP3 phosphorylated KD-RIP1 to a level comparable to that achieved via RIP1 autophosphorylation (Figure 4D, compare lanes 6 and 7). In contrast, although WT-RIP1 could undergo autophosphorylation, it did not phosphorylate KD-RIP3 (Figure 4D, lane 6). However, the level of RIP3-mediated RIP1 phosphorylation was weak, suggesting that other kinases might be involved in phosphorylating RIP1 and stabilizing its association with the pronecrotic complex II.
The Kinase Activity of RIP1 Is Required for Stable Assembly of the Pronecrotic Complex II

Recent evidence indicates that signal transduction by TNFR-like receptors may involve distinct complex IIs (Wang et al., 2008; Wilson et al., 2009). We reasoned that RIP1 and RIP3 might interact specifically within a pronecrotic complex II. In MEFs, TNF stimulation in the presence of cycloheximide (T/C) or T/C and zVAD-fmk (T/C/Z) for 6 hr. ROS production was quantified using the fluorescent dye H2DCFDA on flow cytometry. Data are presented as mean ± SEM of triplicates.

Flow cytometric analysis of RIP3-dependent ROS production during programmed necrosis. The numbers represent the percentages of cells in each quadrant. (D) RIP3 is not required for oxidative stress-induced cell injury. (E) RIP3 is required for zVAD-fmk-induced, autocrine TNF-dependent necrosis. The inset shows the reduction of RIP1 or RIP3 protein expression by the respective siRNAs. Data are presented as mean ± SEM of triplicates. *p = 0.01, **p = 0.002, ***p < 0.001. (G) Dose-dependent response to zVAD-fmk induced necrosis of L929 cells transfected with the RIP3 siRNA. Cell death was measured by MTS assay 24 hr later. Data are presented as mean ± SEM of triplicates. *p = 0.027, **p = 0.018, ***p = 0.001.

The Kinase Activity of RIP1 Is Required for Stable Assembly of the Pronecrotic Complex II

Recent evidence indicates that signal transduction by TNFR-like receptors may involve distinct complex IIs (Wang et al., 2008; Wilson et al., 2009). We reasoned that RIP1 and RIP3 might interact specifically within a pronecrotic complex II. In MEFs, TNF stimulation in the presence of cycloheximide induces apoptosis (T/C), but not programmed necrosis (Lin et al., 2004) (Figure S6). Under these conditions, no interaction between RIP1 and RIP3 was detected (Figure 4E, lanes 1–4). By contrast, programmed necrosis induced by T/C/Z specifically induced formation of the RIP1-RIP3 complex (Figure 4E, lanes 5–8). Similar to the recruitment of RIP1 and RIP3 to FADD, the interaction between RIP1 and RIP3 was abolished by Nec-1 (Figure 4F, compare lanes 7 and 8, and Figure 4G, lanes 2 and 3), indicating that the RIP1 kinase activity was also required for stable interaction between RIP1 and RIP3. The effect of Nec-1 on RIP1-RIP3 association might be due to inhibition of RIP3 phosphorylation, since necrosis-specific RIP3 phosphorylation was abolished by Nec-1 (Figure 4H, compare lanes 2 and 3). Because RIP1 did not directly phosphorylate RIP3 (Figure 4D), another downstream kinase might be responsible for RIP3 phosphorylation. Taken together, our data strongly implicate that phosphorylation of RIP1 and RIP3 plays a crucial role in the stable assembly of the pronecrotic complex II.

RIP3 Regulates Downstream ROS Production during Programmed Necrosis

A recent report shows that the NADPH oxidase Nox1 acts within the membrane-associated receptor complex to generate superoxide anions during programmed necrosis (Kim et al., 2007). Since RIP3 acts within the cytoplasmic signaling complex, we asked whether RIP3 might function downstream of ROS to promote programmed necrosis. Surprisingly, ROS production as detected by staining with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) (Figure 5A), and necrotic cell death as
detected by propidium iodide (PI) uptake were completely inhibited in RIP3−/− MEFs (Figures 5B and 5C). Similar results were obtained with the murine fibrosarcoma L929 transfected with RIP3-specific siRNA (Figure S7). Unlike programmed necrosis, silencing of RIP1 or RIP3 expression in L929 cells had no effect on hydrogen peroxide-induced oxidative cell injury (Figures 5D and 5E). By contrast, siRNA-mediated silencing of RIP1 or RIP3 inhibited zVAD-fmk-induced necrosis in L929 cells (Figures 5F and 5G), which was recently shown to be driven by autocrine TNF production (Hitomi et al., 2008). Thus, our results indicate that RIP3 acts upstream to regulate ROS production during programmed necrosis.

Figure 6. RIP3 Regulates Cell Death during Virus Infections
(A and B) RIP3 is essential for AICD- and TNF-induced death in VV-infected T cells. Wild-type (white bars) or RIP3−/− (black bars) T cells were infected with GFP-VV (moi = 10) and stimulated with plate-bound anti-CD3 antibody (A) or mTNF (B). Cell death in the GFP+ infected population and the GFP− uninfected population was determined by PI staining and flow cytometry. Data are presented as mean ± SEM of triplicates.
(C) RIP3 is required for T cell AICD when caspases are inhibited. Wild-type or RIP3−/− T cells were stimulated with different doses of plate-bound anti-CD3 antibody in the presence or absence of zVAD-fmk. Cell death was assessed by PI uptake on flow cytometry. Data are presented as mean ± SEM of triplicates.
(D) VV infection sensitizes MEFs to TNF-induced programmed necrosis in a RIP3-dependent manner. RIP3+/+ and RIP3−/− MEFs were infected with GFP-VV (moi = 0.5). Infected cells were treated with TNF, and cell death was determined by PI uptake in the infected GFP+ populations by flow cytometry. Data are presented as mean ± SEM of triplicates.
(E) Electron microscopy shows that VV-infected MEFs underwent necrosis in response to TNF. Panel a: uninfected cell. Panel b: VV-infected cell (no TNF). The electron-dense structures indicated by the arrows are viral particles. Panel c: VV-infected cell treated with TNF. Scale bars represent 2 μm.
(F) Formation of the pronecrotic RIP1-RIP3 complex in VV-infected MEFs. Uninfected MEFs (lanes 1–4) or VV-infected MEFs (lanes 5–8) were treated with TNF ± zVAD-fmk as indicated. RIP1 was immunoprecipitated, and the presence of RIP3 in the immune complex was determined by western blot.

RIP3-Dependent Programmed Necrosis Is an Important Cell Death Mechanism during Vaccinia Virus Infection
One physiological situation in which caspases are inhibited and programmed necrosis might be important is during viral infections (Benedict et al., 2002). To test this hypothesis, we examined cell death responses in activated wild-type and RIP3−/− T cells infected with vaccinia virus (VV), which encodes the viral caspase inhibitor B13R/Spi2. VV-infected cells were resistant to death receptor-induced apoptosis but become sensitized to TNF-induced necrosis (Li and Beg, 2000). Wild-type T cells infected with recombinant GFP-VV exhibited normal T cell activation-induced cell death (AICD) induced by anti-CD3 antibody (Figure 6A, compare the white bars). In contrast, AICD in GFP-VV-infected RIP3−/− T cells was significantly reduced compared with uninfected GFP− cells in the same culture (Figure 6A, compare the black bars). VV-infected RIP3−/− cells were also protected from TNF-induced cell death (Figure 6B, black bars). In fact, TNF stimulated the proliferation of GFP+ VV-infected RIP3−/− cells, which accounted for the negative cell loss. VV-infected wild-type T cells also exhibited a modest decrease in TNF-induced cell death because of the inhibition of caspase-dependent apoptosis (Figure 6B, white bars).

The effect of VV infection on T cell death was recapitulated with zVAD-fmk. While AICD was only moderately impaired by zVAD-fmk in wild-type T cells, it was significantly reduced in RIP3−/− cells (Figure 6C). Geldanamycin (GA), which was thought to inhibit programmed necrosis by destabilizing RIP1 (Lewis
et al., 2000), also suppressed RIP3 protein expression (Figure S8). Hence, GA and Nec-1 also synergized with zVAD-fmk to suppress T cell death (Figure S8). The sensitization to programmed necrosis by VV infection was also observed in MEFs. While TNF alone did not cause cell death in MEFs (Figure S6), VV-infected MEFs were highly sensitive to TNF-induced necrosis in a RIP3-dependent manner (Figure 6D). Electron microscopy confirmed that VV-infected MEFs underwent necrotic-like cell death in response to TNF (Figure 6E, panel c). Importantly, while RIP1-RIP3 complex was only detected when cells were stimulated with T/C/Z (Figures 4E–4G) or TNF/zVAD (Figure 6F, lanes 3 and 4), TNF alone was sufficient to trigger RIP1-RIP3 association in VV-infected MEFs (Figure 6F, lanes 5–8). This is in stark contrast to uninfected cells, in which TNF did not trigger RIP1 and RIP3 interaction (Figure 6F, lanes 1 and 2). Taken together, these results show that RIP3-dependent programmed necrosis is an important cell death mechanism when the cellular apoptosis machinery was inhibited by viral inhibitors.

RIP3 Is Required for Protection against Vaccinia Virus Infection In Vivo

TNF-TNFR interactions are known to be important for the innate immune defense against VV infection (Chan et al., 2003; Ruby et al., 1997). The requirement for RIP3 to induce cell death in VV-infected cells suggests that TNF might confer protection against VV via RIP3-dependent programmed necrosis. To test this hypothesis, we infected wild-type and RIP3+/− mice with VV. Consistent with its innate immune protective role, TNF expression was detected in peritoneal exudate cells (PECs) 24 hr after infection (Figure 7A). Moreover, TNF expression was induced in the liver and spleen of infected wild-type and RIP3+/− mice (Figure 7B). Thus, virus-induced TNF expression was not impaired in the RIP3+/− mice.

In wild-type mice, VV infection causes inflammation marked by neutrophil/macrophage infiltration in the visceral fat pads (Figure 7C, panel a, red arrows), which was conspicuously absent in the RIP3−/− mice (Figure 7C, panels c and d). Interestingly, the inflammatory foci were concentrated in areas of extensive fat cell necrosis in the wild-type mice (Figure 7C, panels a and b), suggesting that necrosis might have promoted the inflammatory reaction. The PECs might provide a source of TNF to the visceral fat tissues (Figure 7A). In contrast, necrotic tissue injury was significantly reduced in VV-infected RIP3−/− mice (Figure 7C, panels c and d). The impairment in virus-induced inflammation was observed on day 3 prior to the peak of CD8+ T cell responses on days 7 and 8, consistent with defective innate immune responses in the RIP3−/− mice. Similarly, extensive inflammation and necrosis were detected in the liver of infected RIP3+/− mice (Figure 7D, panels a and b), but were absent in RIP3−/− and TNFR2−/− mice (Figure 7D, panels c and d). Remarkably, the pro necrotic RIP1-RIP3 complex was detected in infected liver cell extracts 12–24 hr after infection (Figure 7E), indicating that liver cells underwent programmed necrosis in response to VV infection. The reduced inflammation correlated with dramatic increases in viral titers in the visceral fat pad (~120x), liver (~50x), and spleen (~2000x) of RIP3−/− mice (Figure 7F). Consequently, the RIP3−/− mice succumbed to the infection (Figure 7G). Collectively, our results strongly support a role for RIP3 in promoting programmed necrosis and virus-induced inflammation (Figure S9).

DISCUSSION

RIP3 was originally identified as a NF-κB and apoptosis regulator (Kasof et al., 2000; Pazdernik et al., 1999; Sun et al., 1999; Yu et al., 1999). However, RIP3−/− mice exhibit no remarkable phenotypes and responded normally to apoptosis and NF-κB activation signals (Newton et al., 2004). Thus, the biological role of RIP3 was unknown until now. Although RIP3 was not identified as a necrosis mediator in a recent genome-wide RNAi screen (Hitomi et al., 2008), we show that formation of a unique pro necrotic complex II composed of RIP1 and RIP3 is a crucial first step in the induction of programmed necrosis. RIP3 acts upstream to regulate necrosis-specific RIP1 phosphorylation. However, since the level of RIP1 phosphorylation by ectopically expressed RIP3 was low, it remains possible that RIP3 may activate another kinase that directly phosphorylates RIP1.

Interestingly, the RIP1 kinase activity is also required for RIP3 phosphorylation during programmed necrosis. Because ectopically expressed RIP1 did not phosphorylate RIP3, RIP1 may facilitate RIP3 phosphorylation by activating another downstream kinase. In this regard, it is tempting to speculate that the other kinases we identified in the siRNA screen might fulfill this function. Alternatively, RIP1 within the pro necrotic complex II might directly phosphorylate RIP3, since in vitro phosphorylation of RIP3 by complex II was inhibited by the RIP1-specific inhibitor Nec-1 (Figure S4). In addition, ectopically expressed RIP3, but not RIP3 present within the endogenous FADD complex, efficiently phosphorylated the artificial substrate MBP. Thus, the kinase activities of RIP1 and RIP3 are tightly controlled within the context of the pro necrotic complex II.

Our results implicate a crucial role for RIP1 and RIP3 phosphorylation in the stable assembly of the pro necrotic RIP1-RIP3 complex. Intriguingly, transfection of KD-RIP1 or KD-RIP3 did not dominantly inhibit programmed necrosis in cells expressing endogenous RIP1 and RIP3, but rather enhanced TNF-induced programmed necrosis (F.K.C., unpublished data). The lack of dominant inhibition by KD-RIP1 or KD-RIP3 might suggest oligomerization as a crucial first step in activating the pro necrotic kinase complex. In such scenario, the RHIM might facilitate oligomerization of RIP1 and RIP3. Phosphorylation of RIP1 and RIP3 may stabilize the structural scaffold of the pro necrotic complex. However, once the oligomer is formed, a single copy of kinase active RIP1 and RIP3 within the oligomer might be sufficient to activate downstream function. Consistent with this model, the RIP3 RHIM mutant was kinase inactive and failed to sensitize programmed necrosis in wild-type Jurkat cells (F.K.C., unpublished data).

The mitochondria generate ROS as a result of oxidative respiration. Disruption of mitochondrial function can further exacerbate ROS release. Interestingly, several components of the mitochondria permeability transition pore (mPTP) are kinases substrates (Le Mellay et al., 2002; Pastorino et al., 2005). It is therefore tempting to speculate that RIP1, RIP3, or other downstream kinases may phosphorylate components of the mPTP to disrupt their functions and to trigger an increase in ROS during...
programmed necrosis. Alternatively, the NADPH oxidase Nox-1 has been shown to mediate ROS production (Kim et al., 2007). Although Nox-1 was implicated to signal via the TNFR-1 associated complex, it is possible that RIP3 might also interact with Nox-1 within the pronecrotic complex II to mediate ROS generation.

Figure 7. RIP3-Dependent Programmed Necrosis Is Required for Control of Vaccinia Virus Replication In Vivo

(A) Induction of TNF expression in PECs upon VV infection. PECs from uninfected or VV-infected (24 hr after infection) wild-type mice were evaluated for TNF expression by intracellular cytokine staining and flow cytometry.

(B) Quantitative PCR analyses of TNF induction upon VV infection. Total RNA was isolated from the indicated tissues 24 hr after infection. Fold induction of TNF mRNA was determined by comparison with uninfected controls and normalization of the TNF message to that of the 18S RNA. Data are presented as mean ± SEM of triplicates.

(C) Visceral fat pads from VV-infected RIP3+/+ mice (panels a and b), but not RIP3−/− mice (panels c and d), exhibited massive fatty tissue necrosis and inflammation marked by macrophage and neutrophil infiltration. The red arrows denote the necrotic tissue mass and the inflammatory foci. Panels a and c: 4X objective. Panels b and d: 20X objective of the boxed area in panels a and c. Images shown are representative from eight RIP3+/+ and ten RIP3−/− mice.

(D) H&E staining of liver sections from RIP3+/+ (panels a and b), TNFR2−/− (panel c), and RIP3−/− (panel d) mice. In panels a and b, leukocyte infiltration and necrotic cell masses (arrows) were clearly seen.

(E) Time post-infection (h) Liver extracts

(F) Viral titers in the visceral fat pads, livers, and spleens of RIP3+/+ and RIP3−/− mice were determined on day 3 after infection.

(G) Kaplan-Meier survival plot of VV-infected RIP3+/+ and RIP3−/− mice.
TNF has long been known to be an important innate immune effector cytokine against bacterial and viral infections. TNF exerts its antimicrobial effects through induction of apoptosis and NF-κB. Using vaccinia virus infection as a model, we have now established RIP3-dependent programmed necrosis as a third mechanism by which TNF contributes to innate immune control of pathogens. TNF expression was rapidly induced upon VV infection in multiple cell types. The expression of TNF coincided with the formation of the pronecrotic RIP1-RIP3 complex in the liver of infected mice. In RIP3−/− mice, virus-induced tissue necrosis and inflammation was severely compromised. Strikingly, the resultant increase in viral replication was comparable to that observed in the TNFR1−/− and TNFR2−/− mice (Chan et al., 2003; Ruby et al., 1997) and much higher than that observed in MyD88−/− or TLR2−/− mice (Zhu et al., 2007). These results are consistent with the normal TLR responses of the RIP3−/− mice (Newton et al., 2004) and indicate that the viral disease observed in RIP3−/− mice was caused by defective TNF signaling rather than abnormal TLR signaling. Our results also support a role for RIP3-dependent programmed necrosis in promoting the subsequent virus-induced inflammation. An important role for RIP1/RIP3-mediated programmed necrosis in antiviral responses is further bolstered by our previous discovery of certain viral FLIPs that potently inhibit programmed necrosis (Chan et al., 2003). More recently, the M45 viral cell death inhibitor from murine cytomegalovirus was shown to interact with RIP1 and RIP3 via the RHIM (Upton et al., 2008). These results suggest that, like apoptosis, inhibition of programmed necrosis might be an emerging viral immune evasion strategy.

In addition to virus-induced inflammation, necrotic cell death may have broader roles in regulating other inflammatory processes through the release of “endogenous adjuvants” into the tissue milieu (Kono and Rock, 2008). For example, RIP3 expression was upregulated during wound healing (Adams et al., 2007), a biological process that shares some hallmarks of inflammation. In addition, programmed necrosis can directly induce cancer cell death or promote cancer growth and metastasis through its proinflammatory effects (Cousens and Web, 2002). In this light, it is noteworthy that Non–Hodgkin’s lymphomas-associated RIP3 SNPs have been identified (Cerhan et al., 2007). Thus, RIP3-dependent programmed necrosis may be a significant component in determining the outcome of viral diseases, trauma/injury-induced inflammation, and cancers.

**EXPERIMENTAL PROCEDURES**

**siRNA Library Screen**

The human kinase siRNA set V2.0 was purchased from QIAGEN. Each gene in the library was represented by four different siRNA duplexes. We pooled the library and transfected them into the FADD-deficient Jurkat cells using HiPerfect (QIAGEN) as per the manufacturer’s protocol. Transfected cells were tested for response to TNF 48 hr after transfection. In all cell death assays, the mean ± SEM of triplicates was presented. Statistical significance was determined with the Student’s t-test.

**Transfections of DNA Plasmids and siRNAs**

For plasmid transfections, Jurkat cells were transfected with 20 μg of expression vectors using the Biorad 630 Electro Cell Manipulator (262V, 725 ohms, 1050 ±f0). After 16–20 hr, cells were transfected with the indicated stimuli and cell death was measured by flow cytometry using PI as an indicator of cell death. Cell death was determined in the GFP-positive transfected populations. Cell death was measured by MTS assay (Promega) and in some cases by PI uptake and 1 μM zVAD-fmk and 1 μM cycloheximide for 1 hr before TNF stimulation. For some experiments, 30 μM of necrostatin-1 was added to the cells 1 hr prior to TNF stimulation. For MEFs, 3,000,000 cells per IP sample were plated on day 0 before the labeling experiment. Cells were treated with TNF for 6 hr prior to cell lysis. Immunoprecipitations were performed with RIP1- or RIP3-specific antibody and resolved on SDS-PAGE.

**Induction of Programmed Necrosis**

To induce programmed necrosis in TNFR2− Jurkat cells and L929 cells, we pretreated cells with 50 μM zVAD-fmk and 1 μM cycloheximide for 1 hr prior to stimulation with 100 ng/ml TNFα (mTNFα for L929 cells). Programmed necrosis was induced in caspase-8− and FADD-deficient Jurkat cells with 100 ng/ml TNFα. Cell death was determined by propidium iodide (PI) staining and flow cytometry. In some experiments, we stained cells with Annexin V and FAM-FLICA (for active caspases) to confirm that the cells were undergoing programmed necrosis, but not apoptosis. For MEFs, cells were pretreated with 20 μM zVAD-fmk and 1 μM cycloheximide for 1 hr before TNF stimulation. Cell death was determined using the CellTiter 96 AQueous Non-Radioactive Assay (MTS assay, Promega) and in some cases by PI uptake and 1 μM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) on flow cytometry. For primary activated T cells, splenocytes from RIP3−/− or wild-type littermates were activated with 5 μg/ml Concanavalin A for 2 days, followed by another 2 days of culture in 100 U/ml IL-2. Where applicable, cells were treated with 50 μM zVAD-fmk or 1 μM geldanamycin for 1 hr prior to the induction of cell death. For the induction of zVAD-induced necrosis and oxidative stress cell death, L929 cells were transfected with siRNA and treated with different doses of zVAD-fmk (Sigma) for 24–48 hr to induce autophagic cell death, or 0.2 mM H2O2 for 6 hr to induce oxidative stress. Cell death was measured by MTS assay (Promega). Procedures for cell death assays with VV-infected cultures can be found in the Supplemental Experimental Procedures.
Vaccinia Virus Infections

All animal experiments were approved by the institutional animal care and use committee. The RIP3<sup>−/−</sup> mice were backcrossed to C57BL/6J background for at least five generations. Ten-week-old mice were infected with 2 x 10<sup>3</sup> plaque forming units (pfu) of Western Reserve strain of vaccinia virus via the intraperitoneal route. For intracellular TNF staining, PECs were harvested 24 hr after infection, incubated with Golgi plug for 4 hr, fixed and permeabilized with the BD IntraSureTM Kit (BD PharMingen). For quantitative PCR analyses, total RNA from tissues were isolated 24 hr after infection and real-time PCR was performed with IQ<sup>®</sup> SYBR Green Supermix on a Biorad iCycler. For in vitro virus infections, activated T cells and MEFs were infected with recombinant GFP-VV at a multiplicity of infection (moi) of 10 and 0.5, respectively. Cells were stimulated with TNF or anti-CD3 antibody 6-8 hr after infection. Cell death was determined using PI uptake by flow cytometry. For liver extract immunoprecipitations, liver extracts were obtained by homogenization in 0.5 M sucrose, 10 mM HEPES (pH 7.6), 25 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, and 1 x Complete Protease Inhibitor Cocktail (Roche) in a tissue grinder. The homogenates were further lysed by 1:10 dilution of crude homogenates in lysis buffer (150 mM NaCl, 20 mM Tris–Cl [pH 7.5], 0.2% NP-40, 1 mM EDTA, 3 mM NaF, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 10% glycerol) prior to immunoprecipitations with RIP1-specific antibody (BD PharMingen clone 38).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, nine figures, and two tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00642-4.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants A065877 (F.K.C.) and AI17672 (R. Welsh) and departmental startup fund (to F.K.C.). This work was supported by National Institutes of Health grants AI065877 (F.K.C.) and AI17672 (R. Welsh) and departmental startup fund (to F.K.C.). This work was supported by National Institutes of Health grants AI065877 (F.K.C.) and AI17672 (R. Welsh) and departmental startup fund (to F.K.C.). This work was supported by National Institutes of Health grants AI065877 (F.K.C.) and AI17672 (R. Welsh) and departmental startup fund (to F.K.C.). This work was supported by National Institutes of Health grants AI065877 (F.K.C.) and AI17672 (R. Welsh) and departmental startup fund (to F.K.C.).

REFERENCES


