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Inhibition of Theiler's Virus-Induced Apoptosis in Infected Murine Macrophages Results in Necroptosis

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Abstract

In mice Theiler's murine encephalomyelitis virus (TMEV) persists in macrophages that eventually undergo apoptosis. TMEV infection of macrophages in culture induces apoptosis through the intrinsic pathway, restricting virus yields. We show that inhibition of TMEV-induced apoptosis leads to phosphorylation of receptor interacting protein 1 (RIP1), localization of RIP1 and RIP3 to mitochondria, ROS production independent of MAPK activation and programmed necrosis (necroptosis). Blocking both apoptosis and necroptosis restored virus yields.

Keywords

Theiler's virus; apoptosis; necroptosis; RIP1; virus titers

Low-neurovirulence Theiler's murine encephalomyelitis virus (TMEV) strains persist in the central nervous system (CNS) of mice after experimental infection. Persistent infection results in cytolytic death of oligodendrocytes (early) and immunemediated tissue damage (late), both resulting in demyelination (Blakemore et al., 1988; Gerety et al., 1994; Rodriguez et al., 1983). Macrophages appear to bear the predominant virus burden during the persistent phase of the infection (Christophi et al., 2009; Lipton et al., 1995; Pena-Rossi et al., 1997), harboring low levels of infectious virus (10^3 pfu/mouse spinal cord (Chamorro et al., 1986; Lipton and Melvold, 1984) but high levels of virus RNA copies ($>10^8$ copies/spinal cord (Trottier et al., 2001; Trottier et al., 2002).

Macrophages in spinal cords of TMEV-infected mice (Schlitt et al., 2003), as do infected macrophages in culture, undergo apoptosis which occurs late in infection *in vitro* after assembly of virions, reducing virus yields (Jelachich et al., 1999; Son et al., 2008). Treatment of infected macrophages with qVD-OPh, a broad-spectrum caspase inhibitor, increases macrophage survival and virus yields (Son et al., 2008). Possible alternate forms of cell death upon inhibition of apoptosis have suggested a role for necrosis, previously believed to be a passive form of cell death. Necroptosis is a form of programmed necrosis in

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which the kinase activity of receptor-interacting protein 1 (RIP1) plays a central role in regulating apoptosis and necroptosis (Degterev et al., 2008; Hitomi et al., 2008). When apoptotic signaling is blocked RIP1 forms an intracellular complex with receptor RIP3, termed the necrosome. Activated RIP3 phosphorylates the downstream mixed lineage kinase domain-like protein, causing programmed necrosis by a still unclear mechanism (Sun et al., 2012; Wu et al., 2013).

Programmed necrosis has been studied mostly based on initiation through the death receptors TNF receptor 1 (TNFR1), Fas receptor, TNF-related apoptosis-inducing ligand (TRAIL) receptor 1 (TRAILR1) and -2 (TRAILR2) in the context of apoptotic cell death inhibition. Until recently, there was no precedent for an RNA virus initiating programmed necrosis other than through death or toll-like receptors; however, TMEV-induced apoptosis is mediated through the intrinsic or mitochondrial pathway. Thapa et al. (Thapa et al., 2013) have now shown that type I and II interferons (IFN) activate RIP1 via a transcription-dependent mechanism requiring Jak/STAT signaling through dsRNA-dependent protein kinase (PKR) which interacts with RIP1 to form the necrosome complex. Caspase-8, the adaptor protein FADD, and c-FLIP, a regulator of caspase-8 activity, negatively regulate programmed necrosis (Kaiser et al., 2011; Oberst et al., 2011; Zhang et al., 2011), and in that study, FADD was genetically inactivated or disabled by phosphorylation (Thapa et al., 2013).

Persistence of highly cytolytic RNA viruses, such as TMEV, where infected cells do not survive requires continuous infection of new cells. Phagocytosis of infected apoptotic blebs is a potential mechanism whereby TMEV can evade virus-specific immune responses in the extracellular milieu. Thus, inhibiting apoptosis in mice to block TMEV persistence requires knowledge of the repercussions from alternative forms of cell death. Here we used necrostatin-1 (nec-1), a potent inhibitor of RIP1 kinase (Degterev et al., 2008) and qVD-OPh to determine whether infected macrophages *in vitro* in which apoptosis is inhibited die by programmed necrosis.

BeAn virus-infected macrophages undergo necroptosis

TMEV strain BeAn infection at high moi (one-step growth kinetics) induces apoptosis in M1-D macrophages (Jelachich et al., 1999; Jelachich and Lipton, 2001; Son et al., 2008). TMEV-infected M1-D cells die at 24 h pi, but only 50–75% of cells die by apoptosis (Son et al., 2008), suggesting a contribution from another form of cell death. In the present study, virus-infected cells began dying after 8 h pi, with 67% of cells dead by 12 h pi (28% by apoptosis as determined DAPI staining for nuclear condensation and fragmentation) and 90% of cells dead by 16 h pi (60% by apoptosis) (Fig. 1A–C). At 24 h pi, no cells survived, such that the percent undergoing apoptosis could not be determined by DAPI staining. As anticipated, addition of nec-1 to virus-infected cell cultures had no effect on cell survival since nec-1 only blocks necroptosis only when apoptosis is also inhibited (Fig. 1A). Addition of qVD-OPh protected ~45% of cells from death as compared to virus-only control cultures at 24 h pi ($p = 0.01$), and addition of both qVD-OPh and nec-1 resulted in ~70% cell survival at 16 to 30 h pi ($p < 0.002$ vs. virus plus nec-1) (Fig. 1A); protection was also seen in the apoptosis assay at 12 and 16 h pi ($p < 0.05$). Flow cytometry analysis of

propidium iodide (PI)-and Annexin V-stained cells provided similar findings (not shown). These results indicate that while apoptosis is the dominant mode of infected cell death, infected cells die by necroptosis when apoptosis is inhibited.

RIP1 and RIP3 expression during necroptosis

RIP1 and RIP3 are constitutively expressed in untreated cells but during programmed necrosis certain stimuli, such as TNF α , may increase their level of expression (McComb et al., 2012; Ye et al., 2012). Immunoblotting analysis to assess expression of RIP1 and RIP3 and cleavage of PARP and caspase-3 to their active forms showed that RIP1 and RIP3 were constitutively expressed in infected macrophages and that addition of nec-1 did not alter expression levels; cleavage of caspase-3 and PARP began at 10 h pi (Fig. 2A,B). RIP1 and RIP3 expression was unchanged by the addition of qVD-OPh or qVD-OPh plus nec-1, but cleavage of caspase-3 and PARP was reduced (Fig. 2C,D). Levels of these proteins, and of β -actin, decreased at 16 and 20 h pi due to increasing cell death (Fig. 2A–D). Overall, no increases in RIP1 and RIP3 expression resulting necroptosis were observed.

Since RIP1 phospho-antibodies were not available, RIP1 phosphorylation was detected by immunoprecipitation of RIP1 with mouse anti-RIP1 (BD Pharmingen, San Diego, CA) in infected cells incubated with γ [32 P] orthophosphate in the presence of qVD-OPh at 12 and 16 h pi (Fig. 2E). RIP1 phosphorylation was observed at both times and inhibited in infected cells incubated with qVD-OPh plus nec-1 (Fig. 2G). Fig. 2F and H show the densitometric analysis of these data. Together, these results demonstrate the activation of RIP1 required for the formation of the necrosome complex, supporting the occurrence of necroptosis during infection when apoptosis is inhibited.

BeAn virus-induced necroptosis is associated with localization of RIP1 and RIP3 to mitochondria

Immunoblot analysis of RIP1 and RIP3 after fractionation of uninfected M1-D macrophages revealed their localization in the cytosol and organelle fractions (Fig. 3A). Digital confocal immunofluorescence microscopy analysis of uninfected cells revealed co-localization of RIP1 and RIP3 with Mitotracker (Fig. 3C a–d), indicating that both were present in mitochondria. Cell fractionation of infected M1-D macrophages at 12 h pi showed loss of RIP1 and RIP3 from the organelle fraction (Fig. 3B, lane 6), suggesting that the loss had occurred during virus-induced apoptosis. When apoptosis was inhibited by qVD-OPh, RIP1 and RIP3 were again detected in the organelle fraction (Fig. 3B, lane 7). Finally, when both apoptosis and programmed necrosis were inhibited by incubation with qVD-OPh and nec-1, RIP1 and RIP3 were no longer detected in the organelle fraction (Fig. 3B, lane 8). Immunofluorescence analysis was consistent with localization of RIP1 and RIP3 in mitochondria when apoptosis was inhibited (16 h pi) (Fig. 3C e), but not during infection or inhibition of necroptosis (not shown). These data suggest that RIP1 and RIP3 were present in mitochondria during necroptosis at a time when RIP1 was activated (Fig 2E–H), consistent with previous reports of necroptosis activation by TNF α through death receptors (Kasof et al., 2000; Temkin et al., 2006; Zaman et al., 2013).

Mitogen-activated protein kinases (MAPK) do not contribute to production of reactive oxygen species (ROS)

In some instances, programmed necrosis has been reported to involve signaling through c-Jun N-terminal kinases (JNK) which stimulates ROS production (Kamata et al., 2005; Ventura et al., 2004; Xu et al., 2006). In immunoblotting analysis of BeAn virus-infected M1-D macrophages treated with qVD-Oph alone or together with nec-1 for MAPK activation, phospho-p38, -JNK and -ERK were detected early (2h pi) with only modest increases over time pi (Fig. 3D), consistent with previous findings (Son et al., 2009). However, inhibition of necroptosis with nec-1 did not alter MAPK activation. ROS production as assessed by flow cytometry using a ROS-sensitive dye, increased minimally in infected or untreated infected monolayers at 8–12 h pi (not shown) but increased significantly between 16 and 24 h pi in the presence of qVD-Oph ($p < 0.01$) (Fig. 3E). Addition of nec-1 to the medium of infected monolayers significantly reduced ROS production ($p < 0.01$) (Fig. 3E), confirming a role for increased ROS production in necroptosis in the infection but without apparent signaling through MAPK. However, RIP3 has also been shown to mediate ROS production by activating several other metabolic enzymes (Vandenabeele et al., 2010).

Necroptosis and BeAn viral yields

One-step growth kinetics of BeAn virus in M1-D macrophages ($\text{moi} = 10$) peak at 10 to 12 h pi, when virion assembly is complete, yielding ~150 pfu/cell before the onset of apoptosis, followed by almost total loss of infectious virus (< 5 pfu/cell) by 20 to 24 h pi (Son et al., 2008; Yildiz Arslan et al., 2012). Treatment of infected cell monolayers with qVD-Oph restored $> 70\%$ of viral titers at 16 and 20 h pi ($p > 0.05$; $p < 0.001$ at 24 and $p < 0.01$ at 30 h pi), when RIP1 had been activated and necroptosis was underway (Fig. 4), suggesting that initiation of necroptosis *per se* may not have initially impacted viral titers. Loss of some infectious virus ensued at later times pi due to continued cell death (Fig. 4). Addition of qVD-Oph and nec-1 to the medium of infected monolayers led to longer cell survival (Fig. 1A) and significantly higher viral yields compared to incubation with only qVD-Oph at 24 and 30 h pi ($p < 0.05$) (Fig. 4). These results indicate that inhibition of apoptosis maintains virus yields while allowing necroptosis to proceed. The even higher virus yields upon inhibition of necroptosis likely reflect prolonged survival of healthy cells, allowing maximal viral production.

Recently, programmed necrosis has been demonstrated as a cause of virus-induced cytopathology in a number of virus infections, including vaccinia virus (Cho et al., 2009), murine cytomegalovirus (CMV) (Upton et al., 2010), reovirus (Berger and Danthi, 2013), and human immunodeficiency 1 virus (Ting et al., 2014) (reviewed in (Kaiser et al., 2013)). Yet the effect of necrosis on virus titers has been reported for only vaccinia virus and murine CMV infections in cell cultures and mice. Interestingly, murine CMV-induced programmed necrosis occurs through a novel RIP3-dependent signaling pathway, initiated in the cytoplasm by the DNA-dependent activator of IFN regulatory factors (DAI, also known as ZBP1 or DLM-1) and mediated by a DAI-RIP3 complex formation with no requirement for RIP1 activation or necrosome formation (Upton et al., 2012). The larger genomes of many

dsDNA viruses transcribe genes that actively suppress cell death pathways, including caspase-8 inhibition by vaccinia virus (Cho et al., 2009) and RIP3 inhibition by murine CMV (Upton et al., 2010), while no RNA virus products have yet been shown to inhibit cell death. Importantly, the effect of virus growth in programmed necrosis of virus-infected cells *in vitro* as in the present study clearly differs from that in animal hosts which mount strong adaptive immune and inflammatory responses that contribute to the suppression of virus yields and virus clearance (Cho et al., 2009). BeAn virus-induced demyelination in mice deficient in RIP3 is on a resistant genetic background and awaits backcrossing to susceptible mice. Although the acute phase of CNS infection might well be potentiated from reduced inflammation, an effect on viral persistence and demyelinating disease is less predictable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Berger AK, Danthi P. Reovirus activates a caspase-independent cell death pathway. *MBio*. 2013; 4:e00178. [PubMed: 23674612]
- Blakemore WF, Welsh CJ, Tonks P, Nash AA. Observations on demyelinating lesions induced by Theiler's virus in CBA mice. *Acta Neuropathol*. 1988; 76:581–589. [PubMed: 3201921]
- Chamorro M, Aubert C, Brahic M. Demyelinating lesions due to Theiler's virus are associated with ongoing central nervous system infection. *J Virol*. 1986; 57:992–997. [PubMed: 3951022]
- Cho YS, Chalia S, Moquin D, George R, Ray TD, Guildford M, Chan FKM. Phosphorylation-driven assembly of the RIP1–RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell*. 2009; 137:1112–1123. [PubMed: 19524513]
- Christophi GP, Hudson CA, Panos M, Gruber R, Massa PT. Modulation of macrophage infiltration and inflammatory activity by the phosphatase SHP-1 in virus-induced demyelinating disease. *J Virol*. 2009; 83:522–539. [PubMed: 18987138]
- Degterev A, Hitomi J, Gemscheid M, Ch'en IL, Korkina O, Teng X, Abbot D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J. Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol*. 2008; 4:313–321. [PubMed: 18408713]
- Gerety SJ, Rundell KM, Dal Canto MC, Miller SD. Class II-restricted T cell responses in Theiler's murine encephalomyelitis virus-induced demyelinating disease. VI. Potentiation of demyelination with and characterization of an immunopathologic CD4 + T cell line specific for an immunodominant VP2 epitope. *J Immunol*. 1994; 152:919–929. [PubMed: 7506740]
- Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell*. 2008; 135:1311–1323. [PubMed: 19109899]
- Jelachich ML, Brumlage C, Lipton HL. Differentiation of M1 myeloid precursor cells into macrophages results in binding and infection by Theiler's murine encephalomyelitis virus (TMEV) and apoptosis. *J Virol*. 1999; 73:3227–3235. [PubMed: 10074176]
- Jelachich ML, Lipton HL. Theiler's murine encephalomyelitis virus induces apoptosis in gamma interferon activated M1 differentiated myelomonocytic cells through a mechanism involving tumor necrosis factor alpha (TNF- α) and TNF- α -related apoptosis-inducing ligand. *J Virol*. 2001; 75:5930–5938. [PubMed: 11390594]

- Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem T, Caspary T, Mocarski ES. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature*. 2011; 471:368–372. [PubMed: 21368762]
- Kaiser WJ, Upton JW, Mocarski ES. Viral modulation of programmed necrosis. *Curr Opin Virol*. 2013; 3:296–306.
- Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNF α -induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell*. 2005; 120:649–661. [PubMed: 15766528]
- Kasof GM, Prosser JC, Liu D, Lorenzi MV, Gomes BC. The RIP-like kinase, RIP3, induces apoptosis and NF- κ B nuclear translocation and localizes to mitochondria. *FEBS Lett*. 2000; 473:285–291. [PubMed: 10818227]
- Lipton HL, Melvold R. Genetic analysis of susceptibility to Theiler's virus-induced demyelinating disease in mice. *J Immunol*. 1984; 132:1821–1825. [PubMed: 6699403]
- Lipton HL, Twaddle G, Jelachich ML. The predominant virus antigen burden is present in macrophages in Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease. *J Virol*. 1995; 69:2525–2533. [PubMed: 7884902]
- McComb S, Cheung HH, Korneluk RG, Wang S, Krishnan L, Sad S. cIAP1 and cIAP2 limit macrophage necroptosis by inhibiting Rip1 and Rip3 activation. *Cell Death Differ*. 2012; 19:1791–1801. [PubMed: 22576661]
- Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, Green DR. Catalytic activity of the caspase-8-FLIP_L complex inhibits RIPK3-dependent necrosis. *Nature*. 2011; 471:363–367. [PubMed: 21368763]
- Pena-Rossi C, Delcroix M, Huitinga I, McAllister A, van Rooijen N, Claassen E, Brahic M. Role of macrophages during Theiler's virus infection. *J Virol*. 1997; 71:3336–3340. [PubMed: 9060706]
- Rodriguez M, Leibowitz JL, Lampert PW. Persistent infection of oligodendrocytes in Theiler's virus-induced demyelination. *Ann Neurol*. 1983; 13:426–433. [PubMed: 6340596]
- Schlitt BP, Felrice M, Jelachich ML, Lipton HL. Apoptotic cells, including macrophages, are prominent in Theiler's virus-induced inflammatory, demyelinating lesions. *J Virol*. 2003; 77:4383–4388. [PubMed: 12634394]
- Son KN, Becker RP, Kallio P, Lipton HL. Theiler's virus-induced apoptosis in M1-D macrophages is Bax-mediated through the mitochondrial pathway, resulting in loss of infectious virus: A model for persistence in the mouse central nervous system. *J Virol*. 2008; 82:4502–4510. [PubMed: 18287228]
- Son KN, Pugazhenth S, Lipton HL. Activation of tumor suppressor protein p53 is required for Theiler's murine encephalomyelitis virus-induced apoptosis in M1-D macrophages. *J Virol*. 2009; 83:10770–10777. [PubMed: 19656889]
- Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell*. 2012; 148:213–227. [PubMed: 22265413]
- Temkin V, Huang Q, Liu H, Osada H, Pope R. Inhibition of ADP/ATP exchange in receptor-interacting protein-mediate necrosis. *Mol Cell Biol*. 2006; 26:2215–2225. [PubMed: 16507998]
- Thapa RJ, Nogusa S, Chen P, Maki JL, Lerro A, Andrade M, Rall GF, Degterev A, Balachandran S. Interferon-induced RIP1/RIP3-mediated necrosis requires PKR and is licensed by FADD and caspases. *Proc Natl Acad Sci USA*. 2013; 2013:E3109–E3118. [PubMed: 23898178]
- Ting P, Wu SQ, He X, Luo H, Zhang Y, Fan M, Geng G, Ruiz VC, Zjang J, Mills L, Zhand H. Necroptosis takes place in human immunodeficiency virus type-1 (HIV-1)-infected CD4⁺ T lymphocytes. *PLoS One*. 2014; 9:e93944. [PubMed: 24714696]
- Trottier M, Kallio P, Wang W, Lipton HL. High numbers of viral RNA copies in the central nervous system of mice during persistent infection with Theiler's virus. *J Virol*. 2001; 75(7420):7428.
- Trottier M, Schlitt BP, Lipton HL. Enhanced detection of Theiler's virus RNA copy equivalents in the mouse central nervous system by real-time RT-PCR. *J Virol Methods*. 2002; 103:89–99. [PubMed: 11906736]
- Upton JW, Kaiser WJ, Mocarski ES. Virus inhibition of RIP3-dependent necrosis. *Cell Host Microbe*. 2010; 7:302–313. [PubMed: 20413098]

- Upton JW, Kaiser WJ, Mocarski ES. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. *Cell Host Microbe*. 2012; 11:290–297. [PubMed: 22423968]
- Vandenabeele P, Galluzzi L, Vandem Berghe T, Kroemer G. Molecular mechanisms of necroptosis. *Nat Rev Mol Cell Biol*. 2010; 11
- Ventura JJ, Cogswell P, Flavell RA, Baldwin AS Jr, Davis RJ. JNK potentiates TNF-stimulated necrosis by increasing the production of cytotoxic reactive oxygen species. *Genes Dev*. 2004; 18:2905–2915. [PubMed: 15545623]
- Wu J, Huang Z, Ren J, Zhang Z, He P, Li Y, Ma J, Chen W, Zhang Y, Zhou X, Yang Z, Wu SQ, Chen L, Han J. Mkl1 knockout mice demonstrate the indispensable role of Mkl1 in necroptosis. *Cell Res*. 2013; 23:994–1006. [PubMed: 23835476]
- Xu Y, Zheng-Gand L, Han J. Poly (ADP-ribose) polymerase-1 signaling to mitochondria in necrotic cell death requires RIP1/TRAF2-mediated JNK1 activation. *J Biol Chem*. 2006; 281:8788–8795. [PubMed: 16446354]
- Ye YC, Wang HJ, Yu L, Tashiro SI, Onodera S, Ikejima T. RIP1-mediated mitochondrial dysfunction and ROS production contributed to tumor necrosis factor alpha-induced L929 cell necroptosis and autophagy. *Int Immunopharmacol*. 2012; 14:674–682. [PubMed: 23000518]
- Yildiz Arslan S, Son KN, Lipton HL. The antiapoptotic protein Mcl-1 controls the type of cell death in Theiler's virus-infected BHK-21 cells. *J Virol*. 2012; 86:1922–1929. [PubMed: 22130544]
- Zaman MMU, Nomura T, Takagi T, Okamura T, Jin W, Shinagawa T, Tanaka Y, Ishii S. Ubiquitination-deubiquitination by the TRIM27-USP7 complex regulates tumor necrosis factor alpha-induced apoptosis. *Mol Cell Biol*. 2013; 33:4971–4984. [PubMed: 24144979]
- Zhang H, Zhou X, McQuade T, Li J, Chan FKM, Zhang J. Functional complementation between FADD and RIP1 in embryos and lymphocytes. *Nature*. 2011; 471:373–376. [PubMed: 21368761]

Highlights

- Theiler's virus induces apoptosis in murine macrophages through the intrinsic pathway reducing virus yields late in infection.
- Inhibition of apoptosis led to the induction of necroptosis, a programmed form of necrosis.
- Signaling events preceding necroptosis included phosphorylation of receptor interacting protein 1 (RIP1) and localization of RIP1 and RIP3 to mitochondria.
- Blocking both apoptosis and necroptosis restored virus yields.

Figure 1.

Survival of BeAn virus-infected M1-D macrophages undergoing apoptosis and necroptosis.

(A) Survival of infected macrophages by the WST-1® cell proliferation assay (Roche Diagnostics Corps, Indianapolis, IN) in the presence of the RIP1 kinase inhibitor, nec-1, alone remained unchanged compared to buffer but was increased in the presence of the broad-spectrum caspase inhibitor qVD-Oph ($p=0.01$) and of both qVD-Oph and nec-1 ($p=0.002$). (B) DAPI-stained infected macrophages revealed nuclear chromatin condensation and fragmentation at 12 and 24 h pi, indicative of apoptosis; phase-contrast

images are shown for comparison. (C) The percentage of apoptotic cells was significantly reduced after incubation with qVD-OPh or qVD-OPh plus nec-1 at 12 and 16 h pi ($p<0.05$). The number of virus-infected cells remaining attached to monolayers in the presence of buffer only in the medium was insufficient for DAPI staining at 24 h pi. ND, not detected, *, $p<0.05$, error bars in A and C are SEM, $n=3$.

Figure 2.

Immunoblot analysis of RIP1 and RIP3 expression and activation of RIP1 during necroptosis in BeAn virus-infected M1-D macrophages. RIP1 and RIP3 were constitutively expressed, with no detectable differences when incubated with (A) buffer, (B) nec-1, (C) qVD-OPh or (D) qVD-OPh plus nec-1, except for decreased intensity of protein bands at 12 h pi commensurate with cell death (A,B). PARP and caspase-3 were cleaved by 10 h pi; incubation with nec-1 showed no effect, while incubation with qVD-OPh or qVD-OPh plus nec-1 inhibited these cleavages. (E) Anti-RIP1 antibody immunoprecipitation of RIP1

radiolabeled with [$\gamma^{32}\text{P}$] orthophosphate at 0 h pi revealed phosphorylated RIP1 at 12 and 16 h pi in the presence of qVD-Oph but not in the presence of buffer (M = mock). (G) RIP1 was not phosphorylated in the presence of buffer, but phosphorylation of RIP1 was decreased observed in the presence of qVD-Oph plus nec-1. (F,H) Densitometric analysis of autoradiograms in E,G, respectively.

Figure 3.

Intracellular localization of RIP1/RIP3, and analysis of MAPK activation and ROS production in infected M1-D cells. (A) RIP1 and RIP3 were localized to the heavy membrane/organelle fraction in the cytoplasm of uninfected M1-D macrophages using a commercial subcellular proteome extraction kit (Calbiochem). (B) Both RIP1 and RIP3 in the heavy membrane/organelle fraction decreased during infection resulting in apoptosis (lane 6) and when apoptosis and necroptosis were inhibited with qVD-OPh plus nec-1 in the medium (lane 8). The higher band above RIP3 was nonspecific. (C) (a–d)

Immunofluorescence microscopy analysis of uninfected cells showed co-localization of RIP1 and RIP3 with Mitotracker Red CMXRos® (Molecular Probes, Carlsbad, CA), indicating that both proteins were present in mitochondria. (e) Presence of RIP1 and RIP3 in mitochondria in infected cells with qVD-Oph plus nec-1 in the medium at 12 h pi during the time of programmed necrosis. (D) Temporal immunoblot profile of infected M1-D macrophage lysates showed no difference in P-JNK 1/2, P-ERK 1/2 or P-p38 in the presence or absence of nec-1, indicating that virus-induced necroptosis did not activate MAPKs. (E) At indicated late times pi, M1-D cell monolayers detached with Versene and stained with 5 µM CM-H₂DCF₂ (Molecular Probes, Eugene, OR) for 30 min in the dark were assessed for ROS expression by flow cytometry. ROS production was unchanged in infected cells plus buffer, reached maximal levels in the presence of qVD-Oph at 16 to 24 h pi, and fell in the presence of qVD-Oph plus nec-1 at 16–20 h pi. I = infected in B and C.

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Figure 4.

Effect of qVD-Oph and qVD-Oph plus nec-1 (added after virus adsorption) on virus titers of BeAn virus-infected M1-D macrophages (moi = 10). Maximal levels of ~150 to 200 pfu/cell were reached at 10 to 12 h pi during one-step growth kinetics (not shown but results similar to those in reference (13). Virus titers were higher after adding qVD-Oph to the medium compared to buffer ($p < 0.001$ at 20 and 24 h pi and < 0.01 at 30 h pi) and after qVD-Oph plus nec-1 vs. qVD-Oph alone ($p < 0.001$ at 16 h pi and $p < 0.05$ at 24 and 30 h pi). Virus titers after addition of nec-1 were not significantly different (ns) from buffer ($p > 0.05$). *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$. Error bars are SEM, $n = 3$.