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Short communication

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.

1. Introduction

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 immune-mediated 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, 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 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. (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

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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.

2. 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 16–30 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.

3. RIP1 and RIP3 expression during necroptosis

RIP1 and RIP3 are constitutively expressed in untreated cells but during programmed necrosis certain stimuli, such as TNFα, 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 and 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 and 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 shows 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.

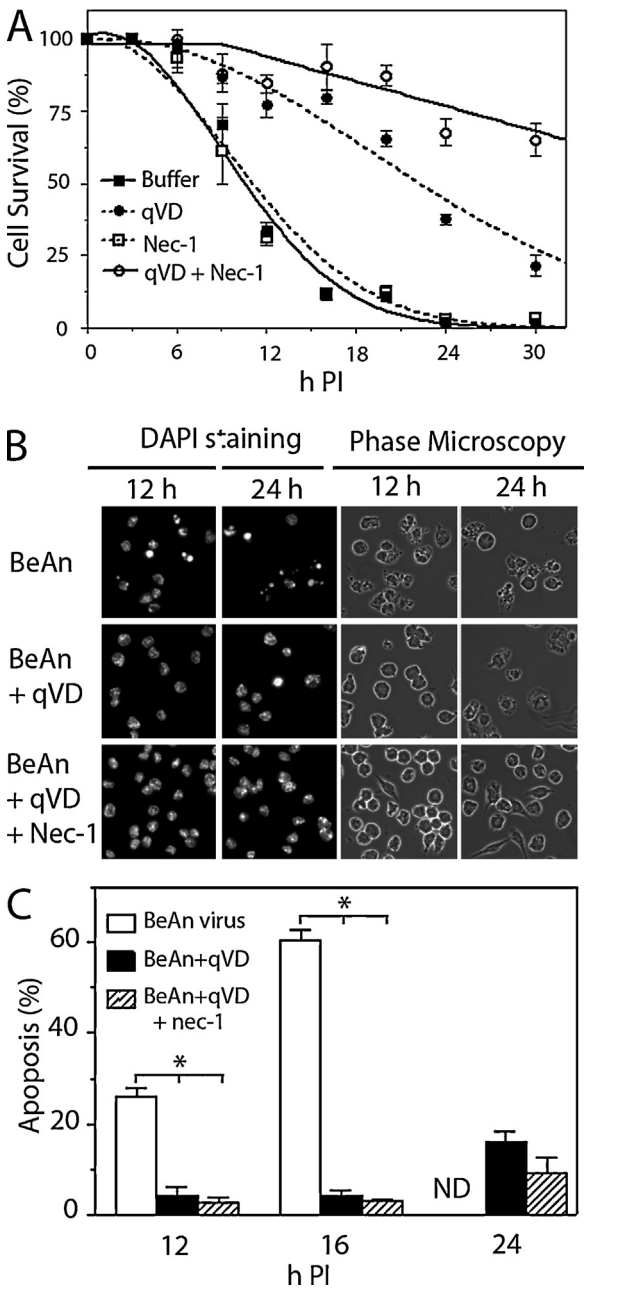

Fig. 1. Survival of BeAn virus-infected M1-D macrophages undergoing apoptosis and necroptosis. (A) Survival of infected macrophages by the WST-1® cell proliferation assay (Roche Diagnostics Corps, Indianapolis, IN) in the presence of the RIP1 kinase inhibitor, nec-1, alone remained unchanged compared 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, $n = 3$.

4. 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