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Critical role for Death-Receptor Mediated Apoptotic Signaling in Viral Myocarditis

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Abstract

Background—Apoptosis of cardiac myocytes plays a key role in the pathogenesis of many cardiac diseases including viral myocarditis. The apoptotic signaling pathways that are activated during viral myocarditis and the role that these pathways play in disease pathogenesis have not been clearly delineated.

Methods and Results—We investigated the role of apoptotic signaling pathways following virus infection of primary cardiac myocytes. The death receptor associated initiator caspase, caspase 8, and the effector caspase, caspase 3, were significantly activated following infection of primary cardiac myocytes with myocarditic, but not non-myocarditic, reovirus strains. Furthermore, reovirus-induced cardiac myocyte apoptosis was significantly inhibited by soluble death receptors. In contrast, the mitochondrial membrane potential remained unaltered and caspase 9, the initiator caspase associated with mitochondrial apoptotic signaling, was only weakly activated in cardiac myocytes following infection with myocarditic reovirus strains. Inhibition of mitochondrial apoptotic signaling had no effect on reovirus-induced cardiac myocyte apoptosis. In accordance with our *in vitro* data, caspase 8, but not caspase 9, was significantly activated in the hearts of reovirus-infected mice.

Conclusions—Death receptor, but not mitochondrial, apoptotic signaling plays a key role in apoptosis following infection of cardiac myocytes with myocarditic reovirus strains.

Keywords

Apoptosis; Myocarditis; Virus; Death Receptors

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Introduction

Viral myocarditis remains a disease without reliable or effective treatment, resulting in chronic dilated cardiomyopathy or death in up to 20% of affected children and 50% of affected adults.¹ Events following viral attachment and replication that produce cytopathic effect and lead to cardiac dysfunction are not clearly understood. Multiple mechanisms have been implicated (reviewed²), including direct viral injury and persistence, autoimmune phenomena, cytokine fluxes and T-cell mediated inflammatory responses. Direct damage to cardiac myocytes plays a key role in the pathogenesis of nearly all models of viral myocarditis

Apoptosis, a distinct mechanism of active cellular death, has been increasingly implicated in multiple forms of cardiac pathology (reviewed³⁻⁵), including *in vivo* models of hypertrophic and dilated failure,⁶⁻⁹ myocardial infarction,¹⁰ ischemia-reperfusion^{11,12} and beta adrenergic stimulation.⁸ Apoptosis of cardiac myocytes *in vitro* has also been demonstrated in response to serum and glucose deprivation (components of ischemia *in vivo*).¹⁰

Increasing evidence indicates that apoptosis plays a central role in microbial pathogenesis, particularly in viral infection.¹³ The role of apoptosis in the pathogenesis of viral myocarditis has been demonstrated more recently in experimental animal models of infection, as well as human endomyocardial biopsy specimens.¹⁴⁻¹⁸ Murine models of viral infection utilizing coxsackievirus, murine encephalomyelitis virus and reovirus, have been invaluable in defining key events in the pathogenesis of viral myocarditis. Reovirus strain 8B produces lethal myocarditis in infected neonatal mice¹⁹⁻²¹ and provides a useful model for delineating direct virus-mediated events leading to myocarditis. We have previously shown that apoptosis occurs in cardiac tissue following infection of animals with the myocarditic reovirus 8B and that *in vivo* inhibition of calpain and caspases, cysteine proteases implicated in apoptotic signaling, is protective against virus-induced myocardial injury.^{14, 22} Taken together, these results indicate that apoptosis plays a central role as a mechanism of reovirus-induced myocardial tissue injury, as has been suggested in several animal models of viral myocarditis.²³

Programmed cell death may occur by activation of death receptor (extrinsic) or mitochondrial (intrinsic)-mediated signaling pathways. Extrinsic apoptotic signaling involves the activation of cell surface death receptors belonging to the Tumor Necrosis Factor Receptor (TNFR) family of proteins, including Fas/APO-1, tumor necrosis factor receptor 1 (TNFR-1) and TNF-related apoptosis inducing ligand receptors 1 and 2 (TRAIL-R1 and TRAIL-R2) [24]. These receptors are activated following binding of their cognate ligands namely, Fas ligand (FasL), TNF and TRAIL. Death receptors contain a cytoplasmic death domain (DD) that serves as a docking site for homotypic DD interactions with DD-containing adaptor proteins.²⁵⁻²⁶ Fas-associating protein with a death domain (FADD) is the adaptor protein for Fas and is recruited to the activated receptor along with pro-caspase 8 to form a death induced signaling complex (DISC). Caspase 8 is activated at the DISC and can then activate downstream effector caspases (such as caspase 3), resulting in apoptosis. The crucial role of FADD and caspase 8 in Fas signaling is shown in FADD- or caspase 8-deficient mice that are resistant to Fas-induced apoptosis.²⁷⁻²⁹

The intrinsic apoptotic pathway involves the release of pro-apoptotic factors through pores in the mitochondrial membrane.³⁰ Pro-apoptotic factors released through mitochondrial pores include cytochrome *c*, which triggers the activation of caspase 9, and SMAC (second mitochondrion-derived activator of caspases) which down-regulates cellular inhibitor of

apoptosis proteins (IAPs). Mitochondrial pores consist of dimers of pro-apoptotic members of the Bcl-2 family of proteins (Bax and Bak) and are tightly regulated by interactions with other (anti-apoptotic and BH3-only) Bcl-2 family proteins.^{31, 32}

Reovirus induced apoptosis in epithelial cell lines and during reovirus encephalitis has been associated with the activation of both death receptor and mitochondrial signaling pathways, with greater or lesser roles for each pathway depending on cell-type.^{33–38} In the present study, we identify a critical role for death receptor-mediated apoptotic signaling following myocarditic reovirus infection of primary cardiac myocytes. In contrast, mitochondrial apoptotic signaling is dispensable for reovirus-induced apoptosis in primary cardiac myocytes. Caspase 8 (the initiator caspase associated with death receptor apoptotic signaling), but not caspase 9 (the initiator caspase associated with mitochondrial apoptotic signaling) is also activated in the hearts of reovirus infected mice. Our results provide a clearer understanding of pathogenic mechanisms and key signaling pathways operative during virus-induced cardiac myocyte death. This information is crucial for the development of novel therapeutic strategies, since currently employed treatment strategies have not led to significant improvements in clinical outcome.

Methods

Reovirus stocks

Reovirus stocks were passaged 3 times in L929 cells prior to use. Growth of reovirus by propagation in L929 cells has been previously described.³⁹ Viral titers (in plaque forming units, pfu/ml) were determined by plaque assays in monolayer cultures of L929 cells. Following titration, viral samples were stored in aliquots at -80°C . Aliquots were then thawed and placed at 4°C for use. Myocarditic viruses utilized were the prototypic reovirus serotype 3 strain Abney (T3A) and reassortant reovirus strain 8B. Nonmyocarditic viruses utilized were the prototypic reovirus serotype 3 strain Dearing (T3D) and the serotype 1 strain Lang (T1L). Reovirus strain 8B is a highly myocarditic strain that was originally derived as an in vivo reassortant following simultaneous inoculation of mice with the non-myocarditic strains T1L and T3D.^{19–21} All infections were performed at a multiplicity of infection (MOI) of 100. All assays were performed in conjunction with mock-infected cardiac myocytes and staurosporine-treated myocytes as negative and positive controls, respectively.

Cells

Cardiac myocytes were isolated from neonatal rats following mincing and trypsin dissociation of the hearts. Myocytes were cultured in DMEM with Hanks salts supplemented with 5% Fetal Bovine Serum, 50 units/ml Penicillin-G (Sigma PENK), $2\mu\text{g/ml}$ vitamin B-12 (Sigma V6629), $10\mu\text{g/ml}$ transferrin (Sigma T8158), $10\mu\text{g/ml}$ insulin (Sigma I1882), and 0.1mM bromodeoxyuridine (BrdU) (Sigma B5002) to inhibit fibroblast proliferation, and maintained at 37°C with 1% CO_2 . The following day, cultures were washed and changed to serum-free MEM containing $10\mu\text{g/ml}$ each of transferrin and insulin, 1mg/ml of bovine serum albumin (BSA) and 0.1mM bromodeoxyuridine (BrdU) to inhibit fibroblast proliferation. The addition of BrdU to the myocyte media resulted in nonmyocyte (fibroblast) contamination of $\leq 10\%$ of total cell population. Reovirus infection of cardiac myocytes was initiated on culture day 1 post-isolation.

Bcl-2 over-expression vector

The adenovirus Bcl-2 over-expression vector (a generous gift from Dr. Jerome Schaak, UCDenver) encodes the transgene and a green fluorescent protein (GFP) under the control of the human cytomegalovirus (CMV) major immediate-early promoter, and is deleted for

E1B, E1A, and E3. The adenovirus Bcl-2 over-expression vector was introduced to cells 24 hours prior to infection with reoviruses to allow for maximal expression prior to exposure to myocarditic and nonmyocarditic reovirus strains.

Immunocytochemistry

Primary cardiac myocytes were grown on 8-well chamber slides coated with rat-tail collagen (Becton Dickinson 354630), and infected with reovirus 48h prior to fixation with 3.7% formaldehyde/phosphate-buffered saline (PBS) (15min, rt). Cells were subsequently permeabilized/blocked with 5% normal goat serum (Vector S1000) in PBS with 0.1% tween20 (2–4h, rt). Cells were probed for reovirus antigen with a rabbit polyclonal T3D antibody (Terry Dermody, Vanderbilt), cleaved caspase 3 (Cell Signaling Technology 9661), cleaved caspase 9 (CST 9507), or cleaved caspase 8 (GlaxoSmithKline Beecham) at a 1:50 dilution in blocking solution (O/N, 4°C). After washing in PBS/0.1% tween 20, cells were incubated with secondary anti-rabbit IgG conjugated to FITC or Texas Red (Vector FI 1200) (1h, rt), then counterstained with Hoechst 33342 (Molecular Probes H 3570) (3min, rt) before final washes in PBS/.1% tween 20. Slides were mounted with vectashield (Vector H1000) and digitally imaged using a Zeiss Axioplan2 epifluorescence microscope.

Apoptosis assays and reagents

Apoptotic nuclear morphology and cell viability were determined by staining with acridine orange and ethidium bromide at a final concentration of 1 µg/ml each. Following staining, cells were examined by epifluorescence microscopy (Nikon Labophot-2: B-2A filter, excitation, 450–490 nm; barrier, 520 nm; dichroic mirror, 505 nm). The percentage of cells containing condensed nuclei and/or marginated chromatin in a population of 100 cells was recorded. Cells which contained condensed nuclei that stained green (acridine orange) were considered to be early apoptotic whereas cells which contained condensed nuclei that stained red (ethidium bromide) were considered to be late apoptotic. The specificity of this assay has been previously established in reovirus-infected cells using DNA laddering techniques and electron microscopy.^{40, 41} Cell populations were also analyzed by flow cytometry to determine intracellular levels of active caspases (8, 9 and 3) using a fluorochrome inhibitor of caspases (FLICA, Immunochemistry Technologies, Bloomington, MN). Mitochondrial Membrane Potential (MMP) was determined using a 63X water-dipping lens. Live cells were analyzed at 24h post-infection for changes in mitochondrial membrane potential. A cationic dye, tetramethylrhodamine ethylester, in the MitoShift Kit (Trevigen 6305-100-K), was used to visualize loss of membrane potential, which is characterized as diffuse staining versus the bright, punctate staining seen in healthy cells. Valinomycin was used as a positive control.

Mice and inoculations

Two-day-old Swiss Webster mice were infected intramuscularly (in the left hind limb) with 10⁴ PFU of virus administered in 20 µl of gel saline (137 mM NaCl, 0.2 mM CaCl₂, 0.8 mM MgCl₂, 19 mM H₃BO₃, 0.1 mM Na₂B₄O₇, 0.3% gelatin).

Results

Infection of cardiomyocytes with myocarditic strains of reovirus results in activation of caspase 3

We have previously demonstrated that reovirus myocarditis is associated with the activation of the effector caspase, caspase 3 and the apoptotic death of infected cells in areas of myocardial tissue injury.²² Furthermore, reovirus-induced myocardial tissue injury is markedly reduced and survival improved following *in vivo* pharmacologic inhibition of

caspase activity and in caspase 3 $-/-$ mice.²² These results indicate that the activation of caspase 3 and resultant apoptotic cell death are important mechanisms of reovirus-induced cardiac injury. We have also recently shown that apoptosis is activated in primary rat myocytes infected with myocarditic reovirus strains.⁴² We therefore wished to investigate activation of caspase 3 in this *in vitro* model of viral myocarditis. Primary cardiac myocytes were infected with myocarditic (8B and T3A) and non-myocarditic (T1L and T3D) strains of reovirus (MOI 100). Forty eight hours following infection, cells were analyzed by fluorescent microscopy using an antibody directed against the active fragment of caspase 3. Sixty percent of cardiac myocytes infected with the myocarditic reovirus strain 8B contained active caspase 3, which was significantly increased (6-fold increase; $P < 0.05$) compared to mock-infected controls (Figure 1A). Similar results were also obtained following infection with a second myocarditic reovirus strain, T3A (Figure 1A). In contrast, infection of cardiac myocytes with non-myocarditic reovirus strains (T3D, T1L) did not result in significant activation of caspase 3 (Figure 1A). We next assessed the time course of caspase 3 activation using caspase 3 activation (FLICA) assays. Significant caspase 3 activity was first detected at 45 hours post infection (pi) with myocarditic virus T3A, but not at earlier times (Figure 1B). In congruence with our prior *in vivo* data, these results demonstrate that caspase 3 is activated in primary cardiac myocytes infected with myocarditic reovirus strains.

Infection of cardiomyocytes with myocarditic strains of reovirus results in activation of caspase 8

Having shown that caspase 3 is activated following reovirus infection of primary cardiac myocytes, we next wished to determine the initiator caspase or caspases involved in reovirus-induced myocyte apoptosis. We first evaluated activation of caspase 8, the initiator caspase associated with death receptor signaling, in primary cardiac myocytes infected with myocarditic and non-myocarditic reovirus strains. Primary cardiac myocytes were infected with myocarditic (8B and T3A) and non-myocarditic (T1L and T3D) strains of reovirus (MOI 100). Forty eight hours following infection, cells were analyzed by fluorescent microscopy using an antibody directed against cleaved caspase 8. Seventy-five percent of cardiac myocytes infected with myocarditic reovirus 8B contained cleaved caspase 8, which was significantly higher (15 fold increase) than that observed in mock-infected controls (Figure 2A). Similar results were observed following infection of myocytes with myocarditic virus T3A. However, in accordance with our caspase 3 activation results, no significant cleavage of caspase 8 was observed following infection of myocytes with non-myocarditic reovirus strains (Figure 2A). The time course of caspase 8 activation was also assessed using caspase 8 activation (FLICA) assays. Following infection of cardiac myocytes with T3A, significant caspase 8 activity was detected starting at 24 h pi and continuing throughout the duration of infection (Figure 2B). The proportion of cells expressing activated caspase 8 (FLICA assay) was less than those expressing cleaved caspase 8 (immunocytochemistry). This likely represents the fact that cleaved caspase 8 may not be fully active.⁴³ These results demonstrate that caspase 8 activity is triggered in cardiac myocytes infected with myocarditic reovirus strains. The early activation of caspase 8, preceding activation of caspase 3, suggests that caspase 8 activity contributes to activation of caspase 3 in reovirus infected myocytes.

Reovirus-induced myocyte apoptosis is inhibited by soluble death receptors

Having shown that caspase 8 is activated during reovirus-induced myocyte apoptosis we next investigated the mechanism of caspase 8 activation. Caspase 8 is activated following the binding of death receptors (usually TNF, FasL or TRAIL) to their cognate ligands (TNFR, Fas or TRAIL-R2). We investigated the role of death receptors in reovirus-induced apoptosis in primary mouse myocytes using soluble death receptors. Myocarditic reovirus-induced myocyte apoptosis was significantly inhibited ($P < 0.05$) by soluble DR5 (Fc:DR5)

and soluble Fas (Fc:Fas), but not by soluble TNFR (Fc:TNFR) (Figure 3). These results indicate that the binding of TRAIL and FasL to their cell surface receptors is a critical factor resulting in apoptosis of reovirus-infected cardiac myocytes.

In order to determine whether the increased apoptosis in reovirus infected primary myocytes was due to an increase in the expression of death ligands or death receptors we performed quantitative PCR. However, we were unable to show a significant increase in the expression of death ligands (FasL, TRAIL, TNF) or their receptors (Fas, TNFR, DR5) in primary myocytes infected with myocarditic or non-myocarditic reovirus strains. These results were consistent with microarray analysis performed on RNA extracted from 8B-infected myocytes (unpublished).

Mitochondrial apoptotic signaling is not required for reovirus-induced myocyte apoptosis

Having shown that caspase 8 is activated following reovirus infection of primary cardiac myocytes and that death receptor signaling is required for virus-induced myocyte apoptosis, we next wished to investigate the potential role of mitochondrial apoptotic signaling pathways. Reovirus infection did not alter the mitochondrial membrane potential of cardiac myocytes (Figure 4), which can indicate the release of pro-apoptotic molecules associated with the intrinsic pathway. Furthermore we detected no translocation of cytochrome *c* or SMAC from mitochondria to cytoplasm of reovirus-infected cardiac myocytes (data not shown). Despite the apparent lack of requirement for mitochondrial apoptotic signaling in reovirus-induced myocyte apoptosis, we detected a limited, but significant, activation of caspase 9 in cardiac myocytes infected with myocarditic virus (8B), compared to mock-infected cells (Figure 5A). However, evaluation of the time course of caspase 9 activation indicated that caspase 9 was only activated at a late time point pi, concomitant with caspase 3, rather than preceding it (Figure 5B). This suggests that caspase 9 activation may be a consequence, rather than initiator, of, caspase 3 activation following reovirus infection of primary myocytes. Finally, reovirus-induced myocyte apoptosis was not blocked following inhibition of mitochondrial apoptotic signaling by over-expression of the anti-apoptotic protein Bcl-2. For these experiments myocytes were infected with adenovirus expressing Bcl-2 (MOI 5). Twenty four hours following adenovirus vector infection, cells were infected with myocarditic reovirus strain 8B (MOI 100). After a further 48 hours, cells were harvested and the percentage of cells with apoptotic nuclear morphology was determined by microscopy. Over-expression of Bcl-2 had no effect on 8B-induced myocyte apoptosis (Figure 6). However, Bcl-2 over-expression significantly blocked cardiac myocyte apoptosis in control cells treated with staurosporine and etoposide, agents that are known to induce mitochondrial apoptotic signaling. Taken together, these results indicate that mitochondrial (intrinsic) apoptotic signaling does not play a critical role in reovirus-induced apoptosis of infected cardiac myocytes.

Caspase 8 is activated in the hearts of reovirus infected mice

To determine whether extrinsic, rather than intrinsic, apoptotic signaling is important for reovirus myocarditis *in vivo*, we inoculated 2 day old mice with 10^4 PFU of the myocarditic reovirus (8B). Eight days pi, we performed caspase activity assays on lysates from the harvested hearts of infected animals (Figure 7). Compared to mock-infected controls, we detected a 5-fold increase in caspase 8 activity in hearts from virus-infected animals ($P < 0.05$) and a 20-fold increase in caspase 3 activity ($P < 0.001$). In contrast, there was no significant difference in caspase 9 activity in the hearts from virus-infected compared to mock-infected animals. These results are consistent with our *in vitro* data and suggest that extrinsic apoptotic signaling mediates apoptosis of cardiac cells during myocarditic viral infection *in vivo*.

Discussion

Viral myocarditis remains a significant clinical challenge, since the pathogenesis of the disease is poorly understood, morbidity and mortality are high, and currently employed treatment strategies have little impact on improving outcome. Direct virus-induced injury early in infection, indirect immune-mediated injury at later times following infection, and aberrant remodeling responses have all been implicated in the pathogenesis of viral myocarditis. Regardless of the models employed for these studies, a central role for direct cardiac myocyte death has been recognized.

Apoptosis has been identified as an important mechanism of cardiac myocyte death in experimental models of viral myocarditis, as well as in endomyocardial biopsies from patients with viral myocarditis.² We have previously demonstrated that apoptosis occurs in the setting of reovirus myocarditis and co-localizes with viral antigen and tissue injury. In addition, reovirus-induced injury can be effectively ameliorated by inhibition of apoptosis-associated cysteine protease activation (calpain and caspase) *in vitro* and *in vivo*.^{4, 14} However, the specific apoptotic signaling pathways that are activated during viral myocarditis have not been clearly defined. In this report we demonstrate that reovirus infection of primary cardiac myocytes with myocarditic, but not non-myocarditic, reovirus strains results in activation of caspase 8, the initiator caspase associated with death receptor apoptotic signaling.

Caspase 8 is also activated in the hearts of mice following infection with myocarditic reovirus strains. In reovirus infected primary cardiac myocytes, caspase 8 activation precedes the activation of the effector, caspase 3, suggesting a functional role of caspase 8 as an initiator caspase in reovirus-induced myocyte apoptosis. Reovirus-induced myocyte apoptosis is inhibited by soluble death receptors, including soluble Fas (FC:Fas) and soluble TRAIL-R2 (FC:TRAILR2). Taken together these results indicate that extrinsic apoptotic signaling, mediated by death receptors and caspase 8, plays a critical role in reovirus-induced cardiomyocyte death.

We were unable to demonstrate a difference in the expression of death ligands or receptors following reovirus infection of primary myocytes. This suggests that reovirus infection modulates extrinsic apoptotic signaling by an alternate mechanism. We have previously shown that myocarditic, but not non-myocarditic, reovirus strains inhibit stimulus-induced degradation of I κ B resulting in decreased levels of NF- κ B and decreased expression of the NF- κ B dependent gene encoding cFLIP (FLICE inhibitory protein), the cellular inhibitor of caspase 8.⁴⁴ We have also shown that inhibition of cFLIP contributes to apoptosis in reovirus- infected cell lines.⁴⁴⁻⁴⁵ Decreased levels of cFLIP may thus promote extrinsic apoptotic signaling in reovirus infected myocytes.

Although little has been reported regarding the role of TRAIL in myocarditis, the importance of Fas in myocarditis has been implicated both in animal models and humans. For example, an increase in the expression of Fas has been reported in the myocardium of patients with myocarditis or dilated cardiomyopathy.⁴⁶⁻⁴⁷ It has also been proposed that elevation of sFas and sFasL can serve as predictors of prognosis in the setting of acute myocarditis of humans.⁴⁸ Similar observations have been reported in animal models of infection such as coxsackievirus (CV), in which increased Fas expression in myocytes was demonstrated *in vitro* and *in vivo*.^{49, 50} Decreases in Fas expression have also been associated with decreased CV-induced cardiomyocyte apoptosis *in vivo*.⁵¹ Furthermore, myocardial inflammation is reduced in mice treated with anti FasL mAb, or in Fas(-/-) or FasL(-/-) mice.^{49, 52} These findings suggest that extrinsic apoptotic signaling pathways may also be important in other instances of heart disease, including that induced by CV.

In contrast to caspase 8 activation, our results demonstrate that caspase 9 is minimally activated in reovirus infected myocytes and that significant activation of caspase 9 is only seen at late time points pi, concurrent with the significant activation of caspase 3. These results suggest that caspase 8, rather than caspase 9, is the initiator caspase involved in the activation of caspase 3 following infection of myocytes with reovirus. Furthermore, reovirus-induced myocyte apoptosis is not inhibited by over-expression of the anti-apoptotic protein Bcl-2. These results suggest that mitochondrial apoptotic signaling is not required for reovirus-induced myocyte apoptosis. Increased levels of the pro-apoptotic Bcl-2 family protein, Bax, and decreased levels of the anti-apoptotic Bcl-2 family proteins, Bcl-2 and Bcl-XL have been reported in association with increased pathology during CV-induced myocarditis.^{51, 53, 54} However, none of these studies demonstrated the requirement of Bcl-2 family proteins for virus-induced cardiac disease.

During CVB3-induced myocarditis Fas may contribute both to myocyte apoptosis⁵⁰ and to the development of massive myocardial necrosis through activation of infiltrating immune cells.⁴⁹ In contrast, reovirus-induced myocarditis occurs without infiltrating cells and Fas-mediated cardiac damage appears to be a direct consequence of viral-induced myocyte apoptosis. Inhibition of Fas signaling in both these settings appears to reduce virus-induced pathology. Our results thus substantiate the need for further studies to evaluate the role of death receptor signaling, including Fas, as a potential therapeutic target for viral myocarditis. Targeting virus-activated cell death signaling pathways rather than the myriad of inciting viruses, may provide a novel and promising strategy for treatment of this devastating disease.

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Fig. 1.

Caspase 3 is activated following infection of myocytes with myocarditic reovirus strains. Primary cardiac myocytes were infected with myocarditic (8B and T3A) and non-myocarditic (T1L and T3D) reovirus strains. (A) 48 h following infection cells were fixed for immunocytochemical evaluation. Red/Cy3 staining in representative images indicates cells containing cleaved caspase 3. Blue/DAPI staining identifies nuclei. The mean percentage of cells expressing the cleaved/active fragment of caspase 3 is shown in the graph. Error bars represent standard errors of the mean. Statistically significant differences ($P < 0.005$) in caspase 3 activation between reovirus-infected and mock-infected cells are indicated (*). (B) The graph depicts the degree of caspase 3 activation in primary cardiac

myocytes, as determined by FLICA assay, over a time course following reovirus infection. Data are expressed as mean percentage of cardiac myocytes expressing active caspase 3 (reo – mock). Error bars represent standard errors of the mean. Statistically significant differences ($P < 0.05$) in caspase 3 activation, between late and early (21 hpi) time points are indicated (*).

Fig. 2.

Caspase 8 is activated following infection of myocytes with myocarditic reovirus strains. Primary cardiac myocytes were infected with myocarditic (8B and T3A) and non-myocarditic (T1L and T3D) reovirus strains. (A) 48 h following infection cells were fixed for immunocytochemical evaluation. Green /FITC staining in representative images indicates cells containing cleaved caspase 8. Blue/DAPI staining identifies nuclei. The mean percentage of cells expressing cleaved caspase 8 is shown in the graph. Error bars represent standard errors of the mean. Statistically significant differences ($P < 0.05$) in caspase 8 activation between reovirus-infected and mock-infected cells are indicated (*). (B) The graph depicts the degree of caspase 8 activation in primary cardiac myocytes, as determined by FLICA assay, over a time course following reovirus infection. Data are expressed as mean percentage of cardiac myocytes expressing active caspase 8 (reo – mock). Error bars represent standard errors of the mean. Statistically significant differences ($P < 0.05$) in caspase 8 activation between late and early (21 hpi) time points are indicated (*).

Fig. 3.

Soluble death receptors inhibit reovirus-induced myocyte apoptosis. Primary cardiac myocytes were pre-treated for 1 h with soluble death receptors before being infected with a myocarditic (8B) reovirus strain. 72 h following infection, cells were examined by microscopy for apoptotic nuclear morphology. The graph shows mean percent reduction in apoptosis seen in infected cells treated with soluble receptors compared to infected, but untreated, controls. Error bars represent standard errors of the mean. Statistically significant differences ($P < 0.05$) in apoptosis between soluble death receptor-treated and untreated infected cells are indicated (*).

Fig. 4.

Reovirus infection of myocytes does not disrupt the mitochondrial membrane potential. Live cells were analyzed at 24h post-infection with myocarditic (8B) and non-myocarditic (T1L) reovirus strains for altered mitochondrial membrane potential. Loss of membrane potential, which is characterized as diffuse staining versus the bright, punctate staining seen in healthy cells, was demonstrated following treatment with valinomycin (positive control) but not following myocarditic (8B) or nonmyocarditic (T1L) reovirus infection.

Fig. 5.

Caspase 9 is activated at late time points following infection of cardiac myocytes with myocarditic reovirus strains. Primary cardiac myocytes were infected with myocarditic (8B) and non-myocarditic (T1L) reovirus strains. (A) 48 h following infection cells were fixed for immunocytochemical analysis. Red/Cy3 staining in representative images indicates cells containing cleaved caspase 9. Blue/DAPI staining represents nuclei. The mean percentage of cells expressing cleaved caspase 9 is shown in the graph. Error bars represent standard errors of the mean. Statistically significant differences ($P < 0.05$) in caspase 9 activation between reovirus-infected and mock-infected cells are indicated (*). (B) The graph depicts the degree of caspase 9 activation in primary cardiac myocytes, as determined by FLICA assay, over a time course following reovirus infection. Data are expressed as mean percentage of cardiac myocytes expressing active caspase 9 (reo – mock). Error bars represent standard errors of the mean. Statistically significant differences ($P < 0.05$) in caspase 9 activation between early (21 hpi) and later time points and are indicated (*).

Fig. 6.

Reovirus-induced apoptosis is not inhibited by Bcl-2. Primary rat myocytes were infected with adenovirus (MOI 5) designed to over-express Bcl-2. Twenty four h following adenovirus vector infection, cells were then infected with myocarditic reovirus (8B, MOI 100). After an additional 48 h, cells were harvested and the percentage of cells with apoptotic nuclear morphology was determined by microscopy. The graph shows the mean percentage of apoptotic cells. Error bars represent standard errors of the mean. Statistically significant ($P < 0.05$) differences in apoptosis between Bcl-2 over-expression and control conditions are indicated (*).

Fig. 7.

Caspase 8 is activated in myocardium of mice infected with the myocarditic reovirus 8B. 2 day old mice were inoculated with 10^4 PFU 8B virus. Eight days following infection hearts were harvested and fluorogenic caspase activity assays were performed. The graph shows the mean fold increase in activity (Fluorescence) of caspases 8 (extrinsic pathway initiator), 9 (intrinsic pathway initiator) and 3 (effector) in virus-infected, compared to mock-infected hearts. Error bars represent standard errors of the mean. N= 9 virus-infected mice and 4 mock-infected mice.