Apoptosis in animal models of virus-induced disease

Penny Clarke and Kenneth L. Tyler*‡§||¶*

Abstract | Apoptosis is associated with virus-induced human diseases of the central nervous system, heart and liver, and causes substantial morbidity and mortality. Although virus-induced apoptosis is well characterized in individual cells in cell culture, virus-induced apoptosis *in vivo* and the role of apoptosis in virus-induced disease is not well established. This Review focuses on animal models of virus-induced diseases of the central nervous system, heart and liver that provide insights into the role of apoptosis in pathogenesis, the pathways involved and the potential therapeutic implications.

Extrinsic apoptotic pathway
An apoptotic signalling pathway that is triggered by the binding of ligands to cell-surface death receptors and culminates in the activation of the initiator caspase, caspase 8.

Intrinsic apoptotic pathway
An apoptotic signalling pathway that involves the release of pro-apoptotic factors from the mitochondria and activation of the initiator caspase, caspase 9.

Cytotoxic T lymphocyte
A T lymphocyte that induces the death of pathogen-infected cells.

**Departments of Neurology, ‡Immunology, §Medicine and ||Microbiology, University of Colorado, Denver Health Sciences Programs, Anschutz Medical Campus, Aurora, Colorado 80045, USA. ¶Denver Vetaren's Administration, Denver, Colorado 80220, USA. Correspondence to P.C. e-mail: Penny.Clarke@uchsc.edu*
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Apoptosis plays an essential part in homeostasis, development and human disease by facilitating the removal of unwanted, damaged or infected cells. However, apoptosis may also have a pathogenic role by contributing to cell death and tissue injury. Apoptotic cell death occurs in a wide range of human viral infections, including infections of the central nervous system (CNS), heart and liver. For example, apoptosis can be detected in the brains of patients with virus-induced CNS disease, including patients with HIV-1-associated dementia[1], herpes simplex virus (HSV)[2,3] and cytomegalovirus (CMV)[3] encephalitis. Furthermore, apoptotic cells can be observed in the diseased human heart during both active and chronic viral myocarditis[4], and hepatocyte apoptosis is emerging as an important feature of liver injury in patients with hepatitis B virus (HBV) and hepatitis C virus (HCV) infections[5–10].

The morphological and nuclear changes associated with apoptosis are typically caused by the sequential activation of a family of aspartate-specific cysteine proteases called caspases[11–13] (BOX 1). Caspase 3, the main executioner caspase, can be activated by the initiator caspase, caspase 8, following activation of cell-surface death receptors in the extrinsic apoptotic pathway (BOX 1; FIG. 1). Alternatively, the intrinsic apoptotic pathway can activate caspase 3 following release of pro-apoptotic factors from the mitochondria and activation of the initiator caspase, caspase 9 (BOX 1; FIG. 1). In some cell types (type I cells), activation of caspase 8 is sufficient for apoptosis. However, in other cell types (type II cells), apoptosis requires intrinsic apoptotic signalling, which can be activated directly or through extrinsic apoptotic signalling through the caspase 8-dependent cleavage of BCL-2 interacting domain death agonist (BID), a member of the BH3-only B-cell lymphoma protein 2 family (BOX 2; FIG. 1). Caspase 3 can also be activated by granzyme B, a serine protease found in cytotoxic T lymphocytes (CTLs) (FIG. 1). Granzyme B can also cleave BID, forming the truncated protein gtBID and resulting in the activation of intrinsic apoptotic signalling[14]. Granzyme B and extrinsic apoptotic signalling constitute the two major components of CTL-induced cell death.

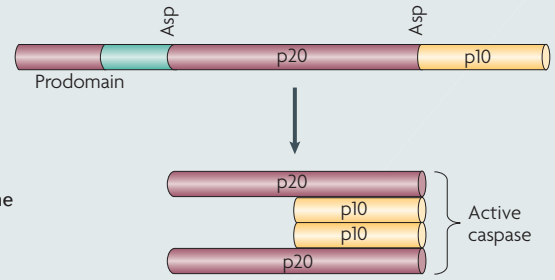
Our understanding of the potential role of apoptosis in the pathogenesis of human diseases has been facilitated by the availability of animal models. This Review focuses on representative animal models that have been used to determine the role of apoptosis in virus-induced pathogenesis in the CNS, heart and liver (TABLE 1), to define the specific pathways involved and to assess the potential of anti-apoptotic treatment strategies.

Viruses induce apoptosis *in vivo*

In many experimental animal models of apoptosis there is an association between the severity of apoptosis and the amount of virus or virus antigen, suggesting that apoptosis is a consequence of viral replication.

Apoptosis and viral replication. Peak virus titres in the CNS are associated with the appearance of activated caspase 3 following infection of mice with West Nile virus (WNV)[15]. Viral titre also correlates with caspase 3-related apoptotic events following virus infection of the CNS (BOX 1). For example, increased viral titre correlates with increased oligonucleosomal DNA laddering following infection with reovirus[16] and with TUNEL staining following infection with sindbis virus (SINV)[17]. Increased apoptosis also parallels viral replication in the hearts of mice infected with coxsackievirus B3 (CVB3)[18–20], reovirus[21,22], encephalomyocarditis virus[23] and murine CMV[24], and in the livers of mice infected with herpes simplex virus 1 (HSV-1)[25].

Box 1 | Caspase activation



Caspases are a class of cysteine-aspartyl proteases that are synthesized as inactive precursor enzymes or proenzymes (see the figure). These proteases typically lie dormant in the healthy cell and, in response to cell-death stimuli, are converted, either by proteolytic cleavage or by recruitment into large complexes, into active enzymes. Once activated, caspases cleave their substrates after conserved aspartate residues. The effects of caspases on cellular substrates bring about the biochemical and morphological features of apoptosis (FIG. 1).

Caspase proenzymes contain three domains: an amino-terminal prodomain; a large subunit that contains the active-site cysteine within a conserved QACXG motif (p20); and a carboxy-terminal small subunit (p10). Two cleavage events at aspartate (Asp) residues are required to activate caspases. The first divides the proenzyme into large and small caspase subunits, and the second removes the N-terminal prodomain. The resulting functional caspase is a tetramer of two large (p20) and two small (p10) subunits.

Caspases are divided into two groups. Initiator caspases (such as caspase 8 and caspase 9) activate the effector caspases and have long prodomains that allow them to interact with death effector domains (DEDs) or caspase recruitment domains (CARDs) in adaptor proteins such as FAS-associated death domain protein (FADD) and apoptotic protease-activating factor 1 (APAF1) (reviewed in REF. 106). Effector caspases (such as caspase 3) function primarily to cause the morphological features of apoptosis. Specifically, activation of caspase 3 leads to the breakdown of several cytoskeletal proteins, cleavage of poly (ADP-ribose) polymerase (PARP) and degradation of ICAD (inhibitor of caspase-activated DNase), thereby releasing CAD, which cleaves cellular DNA. Activation of caspase 3 and PARP cleavage, by western blot and immunohistochemistry, and DNA fragmentation, by gel electrophoresis and TUNEL, are frequently used as apoptosis assays *in vivo*.

Apoptosis and virus antigen. Animal models of virus-induced disease have also shown that there is a temporal and spatial correlation between the appearance of virus antigen and the onset of apoptosis. Examples of temporal correlations include time courses of reovirus and WNV infection, which show that apoptosis occurs concurrently with, or slightly after, the appearance of viral antigen[16,26]. In addition, in the livers of transgenic mice that express HBV and HCV proteins, the expression or recognition of viral antigen triggers apoptosis[27,28].

Spatial correlations are revealed by the appearance of apoptotic cell death in the same regions as viral antigen following infection of the CNS with reovirus[16,29] and WNV[26], and following infection of the heart with reovirus[21,30,31] and CVB3. Colocalization of apoptosis and virus antigen in individual cells can also be detected in the CNS following infection with Theiler's murine encephalomyelitis virus (TMEV)[32], SINV[33] and reovirus[34,35], in the hearts of CVB3-infected mice[19] and in the livers of transgenic mice that express HCV and HBV proteins[28,36].

Apoptosis in non-infected cells. Non-infected cells also undergo apoptosis in some animal models of virus-induced disease. In these cases, apoptosis typically occurs in proximity to infected cells (bystander apoptosis), as exemplified in the mouse CNS during acute TMEV and reovirus encephalitis and during WNV-induced acute flaccid polio-like paralysis[16,32,37]. By contrast, following infection of the mouse CNS with Venezuelan equine encephalitis virus (VEE) or the rat CNS with Borna disease virus (BDV), apoptosis occurs in areas that lack viral antigen but do show evidence of astrogliosis and inflammation (in the case of VEE)[38] or microglial proliferation (in the case of BDV)[39] . It has been proposed that virus-induced upregulation of proinflammatory genes, including those that encode inducible nitric oxide synthase (iNOS) and tumour necrosis factor (TNF), may be responsible for apoptosis in areas that do not contain viral antigen[38]. Similarly, although Murray Valley encephalitis virus infects neurons, most apoptotic cells are infiltrating inflammatory cells, including macrophages, lymphocytes and neutrophils, and the death of these cells may contribute to the eventual death of the host through the release of inflammatory mediators, such as iNOS[40,41].

Apoptosis and pathogenicity

In addition to showing that the severity of apoptosis is associated with increased viral titre and that apoptosis commonly occurs in anatomical areas targeted by viral infection, animal models have also revealed a positive correlation between apoptosis and disease severity, suggesting that apoptosis is a pathogenic mechanism in virus-induced disease.

For example, in a mouse model of SINV CNS infection, the rates of both apoptosis and mortality are high in young mice, but not in old mice[17,42]. The neurovirulence of viral strains also affects the level of apoptosis and mortality. For example, few apoptotic cells are observed in the CNSs of 2-week-old mice following infection with SINV strain 663, and these animals survive infection. By contrast, infection of 2-week-old mice with a more neurovirulent recombinant SINV strain (TE) induces apoptosis in the brains and spinal cords and results in death of the animals[33]. Similarly, infection of mice with the highly virulent TMEV GDVII strain produces an acute, fatal polioencephalomyelitis that is associated with high levels of neuronal apoptosis, whereas mice infected with the attenuated TMEV DA strain have lower levels of neuronal apoptosis and survive acute infection[32]. It is thought that differences in the capsid protein VP2 contribute to the differences in apoptosis that are induced by the GDVII and DA TMEV strains[43].

Increased apoptosis is also associated with increased severity of CNS disease following infection with WNV, as the brain and spinal cord from non-paralyzed mice showed virtually no apoptosis, whereas significant numbers of apoptotic cells were detected in the brain and spinal cord of paralyzed mice[44]. Increased apoptosis also correlates with increased severity of disease in CVB3-induced and murine CMV-induced myocarditis in infiltrating mononuclear cells and myocytes[18,24,45].

Although apoptosis plays a major part in disease severity in several virus-induced diseases, there are exceptions. For example, an inverse relationship between pathogenicity and apoptosis is usually observed following rabies virus infection of the CNS, indicating that apoptosis is not an essential component of rabies virus-induced CNS disease[46–52].

Oligonucleosomal DNA laddering
A generally accepted biochemical criterion of apoptosis that involves cleavage of DNA into 180 bp fragments by a caspase-activated DNase.

TUNEL
(Terminal deoxynucleotidyl transferase dUTP nick end labelling). A method used to label free ends of DNA, which increase during apoptosis owing to the action of a caspase-activated DNase.

Astrogliosis
An abnormal increase in the number of astrocytes.

Microglial proliferation
An abnormal increase in the number of microglia.

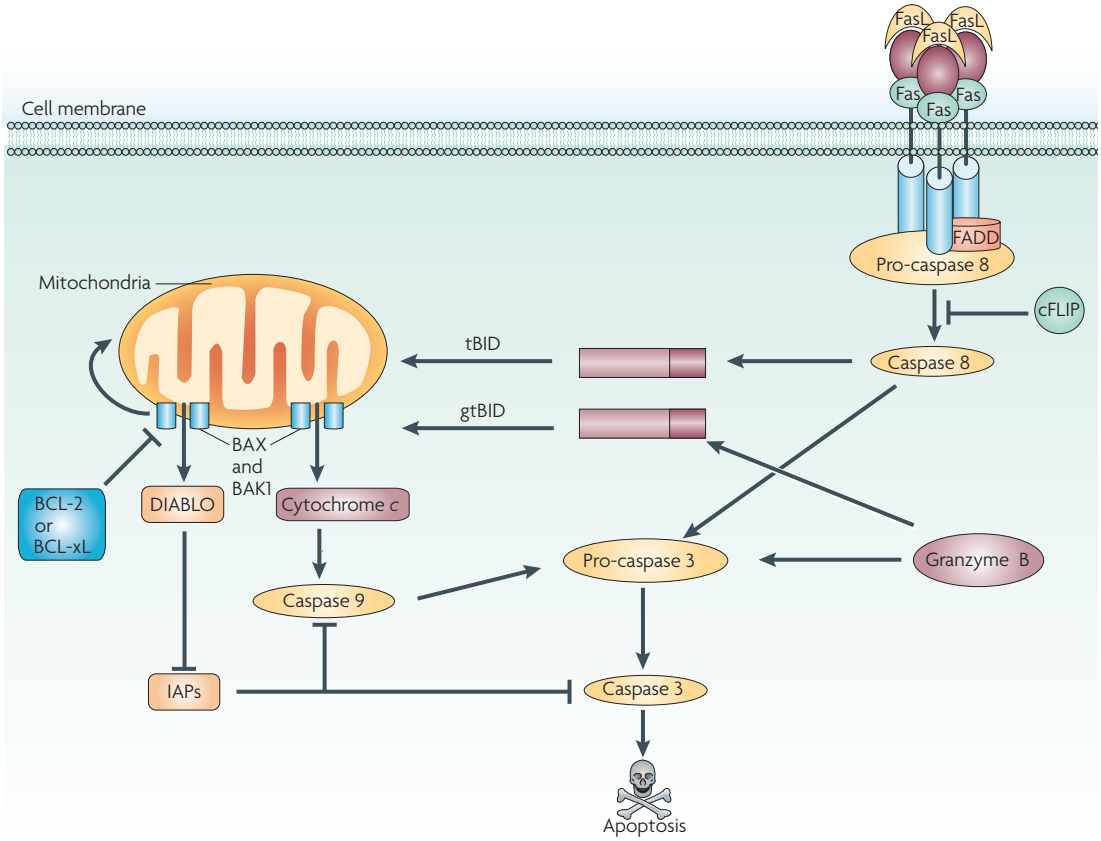


Figure 1 | **Caspase-dependent apoptosis.** Activation of caspase 3 and caspase-dependent apoptosis are caused by the initiator caspases, caspase 8 and caspase 9, as part of the extrinsic and intrinsic apoptotic signalling pathways, or by granzyme B. In the extrinsic pathway, death ligands, including Fas Ligand (FasL), bind and activate their cognate receptors (Fas). Activated receptors, an adaptor molecule, such as Fas-associated death domain protein (FADD), and pro-caspase 8 then form a death-inducing signalling complex (DISC) in which caspase 8 is activated. Caspase 8 can activate caspase 3 and can cleave the BH3-only protein BCL-2 interacting domain death agonist (BID), forming truncated BID (tBID), which can activate the intrinsic apoptotic pathway. In the intrinsic apoptotic pathway, pro-apoptotic B-cell lymphoma 2 (BCL-2) family members BCL-2 associated x protein (BAX) and BCL-2 antagonist killer 1 (BAK1) form pores in the mitochondrial membrane. Pro-apoptotic mitochondrial factors, including DIABLO (direct inhibitor of apoptosis protein-binding protein with low pI; also known as SMAC) and cytochrome *c*, are released and contribute to apoptosis by activating caspase 9 or inhibiting cellular inhibitor of apoptosis proteins (IAPs). Granzyme B can directly cleave and activate pro-caspase 3. Granzyme B can also cleave BID, resulting in granzyme tBID, which can activate the intrinsic apoptotic pathway. cFLIP (cellular FLIP/caspase 8 inhibitor protein) can block the activation of caspase 8. Bcl-xL, BCL-extra large.

Inducible nitric oxide synthase
An enzyme involved in the generation of nitric oxide, which can result in cell death through apoptosis or necrosis.

Macrophage
A cell within tissues that originates from specific white blood cells called monocytes. Monocytes and macrophages are phagocytes that engulf and then digest cellular debris and pathogens. They also stimulate lymphocytes to respond to the pathogen.

Lymphocyte
A white blood cell from the vertebrate immune system. Lymphocytes include large granular lymphocytes, commonly known as natural killer (NK) cells, and small lymphocytes (T and B cells). NK cells are a part of the innate immune system and help defend the host from tumours and virally infected cells by releasing cytotoxic granules. T cells and B cells are the main cellular components of the adaptive immune response. B lymphocytes respond to pathogens by producing large quantities of antibodies that neutralize foreign objects, such as bacteria and viruses. T cells include helper T cells, which produce cytokines that direct the immune response, and cytotoxic T cells, which induce the death of pathogen-infected cells.

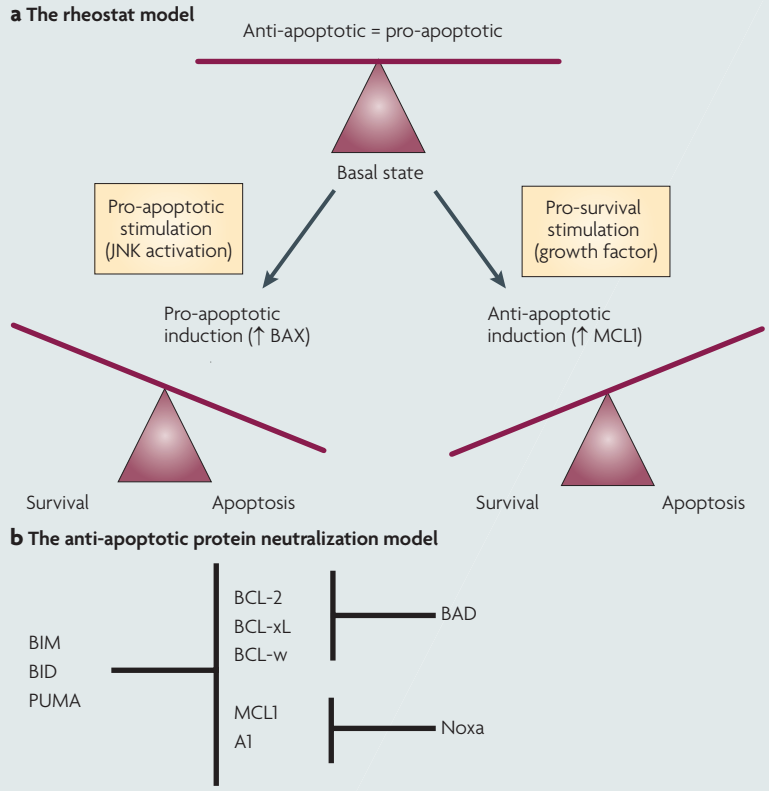
Neutrophil
The most abundant type of white blood cell in humans. Neutrophils are normally found in the bloodstream and form an essential part of the immune system. However, during the acute phase of inflammation, neutrophils migrate towards the site of inflammation, usually as a result of bacterial infection.

The immune system in virus-induced apoptosis

The evidence indicates that cellular immune responses are not always necessary for the induction of apoptosis in infected cells in the CNS, heart and liver. For example, *in vitro* studies show that many viruses induce apoptosis following infection of cultured neurons, myocytes and hepatocytes in the absence of immune cells. In addition, *in vivo* evidence supporting direct viral induction of apoptosis has come from models in which apoptotic injury occurs in the absence of, or prior to, an obvious immune response. For example, apoptosis occurs following infection of the CNS and heart with reovirus[16,21,30], and shortly after the induction of protein expression in transgenic mice that conditionally express HCV structural proteins in the liver[28] in the presence of little, or no, infiltrating immune cells. This shows that immune cells are not required for apoptosis in these models.

The ability of individual viral proteins to interact with cellular apoptotic signalling molecules also suggests that viruses can directly induce apoptosis without the involvement of immune cells. For example, virus proteins have been directly linked to apoptosis or pro-apoptotic proteins during virus-induced myocarditis. Specifically, the pro-apoptotic protein Siva, which binds to the CVB3 capsid protein VP2 (a determinant of CVB3-induced myocarditis), is strongly upregulated in the same region where apoptosis occurs during acute CVB3-induced myocarditis[53]. In addition, Reoviruses that are deficient in the expression of the non-structural reovirus protein σ1small (σ1s) induce less CNS and cardiac apoptosis than parental virus strains[31], and a single amino-acid change at position 55 in the SINV E2 glycoprotein results in different levels of CNS apoptosis[33]. Although it has not yet been shown that σ1s or E2 binds the apoptotic machinery, mutations in these

Box 2 | **BCL-2 family proteins**



Mitochondrial apoptotic signalling and mitochondrial outer-membrane permeabilization (MOMP) controlled by the B-cell lymphoma 2 (BCL-2) family of proteins, which is divided into three groups based on the presence of BCL-2 homology (BH) domains. Anti-apoptotic BCL-2 family proteins, such as BCL-2, BCL-w (BCL-2 related gene, long isoform), BCL-xL, BCL-extra large., A1 and myeloid cell leukaemia 1 (MCL1), contain BH domains 1–4 and are generally integrated into the outer mitochondrial membrane[107]. These proteins function to directly bind and inhibit the pro-apoptotic BCL-2 proteins. The pro-apoptotic BCL-2 proteins are functionally divided into two classes. The effector molecules, such as BCL-2 associated x protein (BAX) and BCL-2 antagonist killer 1 (BAK1), contain BH domains 1–3 and permeabilize the outer mitochondrial membrane by creating a proteolipid pore. The BH3-only proteins, such as BCL-2 antagonist of cell death (BAD), BCL-2 interacting domain death agonist (BID), BCL-2 interacting killer (BIK), BCL-2-like protein 11 (BCL2L11; also known as BIM), BCL-2 modifying factor (BMF), BCL-2/adenovirus E1B 19 KD protein-interacting protein 3 (bNIP3), Harakiri (HRK), Noxa NOXA1 and BCL-2-binding component 3 (BBC3; also known as PUMA), function in distinct cellular stress pathways and signal that a stress has occurred through interactions with other BCL-2 family members.

The combined signalling in the BCL-2 family dictates the immediate fate of the cell, and two models have been described. In the rheostat model (see the figure, part **a**), a basal state is described in which the number of anti- and pro-apoptotic BCL-2 family proteins is equal and tipping this balance dictates cell fate. In the anti-apoptotic protein neutralization model (see the figure, part **b**), the BH3-only proteins BID, BIM and PUMA engage MOMP because they bind and engage all anti-apoptotic BCL-2 family members. A combination of other BH3-only proteins is required to promote apoptosis because each neutralizes only a subset of anti-apoptotic BCL-2 family proteins (reviewed in REF. 108). JNK, Jun N-terminal kinase.

proteins do not seem to alter the host immune response following infection, suggesting that they have a direct effect on cellular apoptotic signalling.

Viral clearance. For most viral infections, the immune system of the host probably induces apoptosis to promote virus clearance. During acute viral infections, the number of virus-specific T cells, including CTLs, increases, resulting in apoptosis of virus-infected cells and viral clearance. For example, the appearance of $CD3^+$ CTLs coincides with a drop in TMEV titre during acute disease, which suggests that these cells are involved in the apoptotic death of TMEV-infected cells and the partial clearance of TMEV from the CNS[54]. CTLs secrete cytotoxic cytokines, including TNF, and express high levels of surface Fas ligand (FasL) and TRAIL, which can induce apoptosis by interacting with death receptors on target cells and activating the extrinsic apoptotic pathway (FIG. 1). Alternatively, cytotoxic immune cells can induce apoptosis by the action of granzyme B (FIG. 1), which can directly activate caspase 8 and caspase 3, thereby activating apoptosis. Immune-cell-mediated Fas-induced apoptosis thus plays a part in $CD8^+$ CTL-induced death of WNV-infected cells, leading to viral clearance in mice. In these mice, Fas is strongly upregulated in neurons in the same areas where virus antigen is present. In addition, in the generalized lymphoproliferative disease (*gld*) mouse model, in which mice are defective in the expression of functional FasL, there was an increase in susceptibility to WNV with increased virus burden and delayed clearance. Finally, WNV-primed $CD8^+$ T cells from wild-type, but not of *gld*, mice limited WNV infection in the CNS and enhanced survival in recipient mice[55]. Fas-induced apoptosis is not only involved in the action of perforin and granzymes, but also promotes virus clearance by infiltrating T cells that express the γδ T-cell receptor[56,57].

Interestingly, transgenic mice that expressed HCV core, E1, E2 and NS2 proteins were resistant to Fas antibody-stimulated apoptosis and lethality, as they inhibited Fas antibody-induced release of cytochrome *c* and activated caspase 9, and caspase 3 or caspase 7, but not caspase 8 (REF. 58). It has been proposed that the suppression of Fas-mediated death of hepatocytes that express HCV proteins protects these cells from immune-cell-mediated apoptosis and may contribute to the development of persistent infection.

Apoptosis and chronic disease. Activated T cells generated during viral infections produce potentially toxic molecules (discussed above) and must be silenced following virus clearance. This feat is accomplished by a process known as activation-induced cell death, which results in the survival of only a small number of virus-specific T cells that become memory cells. Activation-induced cell death following virus infection is mediated, at least in part, by Fas[59], BCL-2-like protein 11 (BCL2L11; also known as BIM)[59] and BCL-2-binding component 3 (BBC3; also known as PUMA)[60]. Furthermore, the expression of both Fas and FasL are induced following T-cell activation[61]. Defects in activation-induced cell death have been proposed to contribute to the autoimmune-mediated, apoptotic spinal cord injury that occurs during chronic TMEV infection. As a result, inflammation in the brain and grey matter of the spinal cord subsides following acute TMEV CNS infection, and TUNEL- (BOX 1) and virus-positive cells are seldom seen. Instead, TUNEL-positive oligodendrocytes, microglia and macrophages can be observed in

Fas ligand
A member of the tumour necrosis factor family of cytokines that triggers apoptosis following the binding of cell-surface receptors.

Table 1 | **Apoptosis in representative animal models of human disease**

Virus family	Virus	Human disease	Animal model	Apoptosis	
				Acute disease	Chronic disease
Central nervous system (CNS)					
Picornaviridae	TMEV	No	Infects neurons, leading to acute CNS disease in mice, which can progress to chronic inflammatory demyelinating disease with a low level of viral persistence in macrophages, astrocytes and oligodendrocytes; serves as a model for multiple sclerosis[125–127]	• Infected neurons; uninfected neurons in regions that contain viral antigen[32] • T cells (activation induced cell death)[54]	Oligodendrocytes with no evidence of colocalization with virus antigen[32]
Togaviridae	SINV	SINV is the prototype alphavirus; alphaviruses cause fever, rash, arthritis, meningitis and encephalomyelitis in humans.	Infects neurons, leading to acute CNS disease in mice[128]; in surviving mice, persistent SINV RNA, but not infectious virus, is associated with neurological damage in the hippocampus[64]	Infected neurons[33]	Immune-mediated apoptosis of uninfected hippocampal cells[64]
Flaviviridae	WNV	Infection can be associated with a febrile illness that can progress to severe neuroinvasive disease, including encephalitis, meningitis or myelitis	Infects neurons, leading to encephalitis and myelitis[15,26,129,130]	Apoptosis in neurons in same areas as viral antigen[15,26]	Not applicable
Reoviridae	Reovirus (type 3)	No	Infects neurons, leading to acute meningoencephalitis or, in newborn mice, acute flaccid paralysis[16,35]	Apoptosis of infected neurons[16,34,35,131]	Not applicable
Heart					
Picornaviridae	CVB3	Myocarditis; early acute disease followed by chronic autoimmune-mediated disease	Acute myocarditis associated with a low level of infected myocytes (1–3%); can be followed by chronic disease in some strains of mice[65]	Myocytes and infiltrating mononuclear cells[19,45,132]	Massive necrosis and fibrosis owing to cardiac-specific autoimmune responses in the absence of infectious virus[65]
Reoviridae	Reovirus strain 8B	No	Infects myocytes, leading to acute myocarditis in newborn mice[133]	Apoptosis of infected myocytes[21,134]	Not applicable
Liver					
Hepadnaviridae	HBV	Chronic hepatitis and hepatocellular carcinoma	Transgenic mice that conditionally express viral proteins[28,36]	Not applicable	Apoptosis of hepatocytes that express viral antigen[28,36]
Flaviviridae	HCV	None	None	None	None

CVB3, coxsackievirus B3; HBV, hepatitis B virus; HCV, hepatitis C virus; SINV, Sindbis virus; TMEV, Theiler's murine encephalomyelitis virus; WNV, West Nile virus.

the demyelinated white matter. These TUNEL-positive cells localize near antigen-positive cells (macrophages, astrocytes and oligodendrocytes) but do not appear to colocalize in specific cells[32,54,62,63].

Autoimmune-mediated apoptosis also has a role in the chronic CNS disease that can follow acute CNS infection with SINV and TMEV. For example, although SINV is cleared from the CNS following acute, non-fatal encephalomyelitis, virus RNA persists. The brains of recovered mice show marked destruction of regions of the hippocampus and extensive loss of brain tissue in the surrounding area that is associated with mononuclear cell infiltration. In these animals, the number of $CD4^+$ T cells and macrophages or microglia that infiltrate the hippocampal gyrus correlates with the number of apoptotic pyramidal neurons. This indicates that in chronic SINV-induced CNS disease, $CD4^+$ cells promote progressive apoptotic neuronal damage in the brain despite clearance of the virus[64].

Apoptosis is also an important pathogenic mechanism in the chronic immune-mediated phase of virus-induced myocarditis apoptosis following infection with CVB3. The persistence of apoptosis and inflammatory infiltrates in the severe disease form of CVB3-induced myocarditis is associated with CVB3-stimulated autoimmune T-cell responses to cardiac antigens, resulting in large areas of necrosis and fibrosis[19]. Induction of autoimmunity depends on $CD4^+$ T helper 1 (T_H1) cells, whereas T_H2 responses promote disease resistance. It has also been shown that T lymphocytes which express the γδ T-cell receptor selectively induce Fas-mediated apoptosis in T_H2 cells and are therefore crucial for the maintenance of a dominant T_H1 response[45,65].

The immune response is also thought to be involved in the development of chronic hepatitis and hepatocellular carcinoma. In transgenic mice that contain the entire HBV-envelope coding region, HBV-antigen recognition

TRAIL
(Tumour necrosis factor (TNF)-related apoptosis inducing ligand). A member of the TNF family of cytokines that triggers apoptosis following the binding of cell-surface receptors.

Microglia
A type of glial cell that acts as the first and main form of active immune defence in the central nervous system.

by $CD8^+$ cells[27] induces hepatocellular apoptosis by activating FasL-dependent death pathways that are inhibited by the administration of soluble Fas[66] and by the neutralization of FasL activity[27].

Immune evasion. Unsurprisingly, given the involvement of the immune system in the onset of apoptosis in infected cells, many viruses have developed strategies to avoid host immunity to prevent the apoptotic death of infected cells and thereby increase viral growth. For example, in addition to infecting hepatocytes, HCV can infect and replicate in immune cells, which adversely affects immune-cell function. In transgenic mice with directed expression of the HCV core to T cells, T-cell responses are inhibited, possibly owing to the increased sensitivity of these T cells to Fas-mediated apoptosis. This suggests that, in contrast to the inhibition of Fas-mediated apoptosis in hepatocytes (discussed above), HCV protein expression in mononuclear cells contributes to liver injury by increasing Fas-mediated mononuclear cell apoptosis and may also contribute to persistent infection[67]. Increased Fas-mediated apoptosis of concanavalin-activated T cells is also observed in transgenic mice with hepatic expression of HCV core, E1 and E2 proteins following adoptive transfer[68]. Furthermore, FasL expression is increased in these livers, supporting the role of Fas signalling in HCV-mediated T-cell apoptosis. Similarly, evasion of the virus-specific T-cell response by increased lymphocyte apoptosis is also observed following exposure of woodchucks to woodchuck hepatitis virus (WHV) in the woodchuck model of HBV infection. In this case, lymphocytes derived from the early acute period of infection, when non-specific T-cell responses profoundly decline but the WHV-specific response has not yet increased, are significantly more prone to activation-induced death and delay the virus-specific T-cell response[69].

The apoptotic death of immune effector cells can also result from the expression of FasL by virus-infected cells. FasL may 'arm' the infected cell and allow it to kill infiltrating, uninfected bystander cells, including immune effector cells (which themselves express death receptors). This has been shown following rabies virus infection of the mouse CNS[70].

The immune response can also be evaded by inhibiting extrinsic apoptotic signalling in infected cells, as occurs following infection of mice with murine CMV. Four murine CMV genes are involved in regulating murine CMV-induced apoptosis. These include the gene that encodes M36, which interacts and prevents the activation of pro-caspase 8, the initiator caspase involved in extrinsic apoptotic signalling. *In vivo*, the growth of murine CMV recombinants that lack M36 results in more apoptosis in the liver and reduced replication, a phenotype that can be rescued by expression of dominant-negative Fas-associated death domain protein (FADD), which also blocks caspase 8-dependent apoptosis[71].

Interferon signalling. Interferons (IFNs) are secreted cytokines that are part of the innate immune response to virus infection. They elicit distinct antiviral effects and can control most virus infections in the absence of adaptive immunity (BOX 3). IFNs also establish a pro-apoptotic state in cells, which helps to remove infected cells[72]. Although the molecular basis for the role of IFNs in apoptosis remains unclear, several mechanisms are probably involved (BOX 3). For example, IFN induces the expression of several genes, including TRAIL, and NK cells can use TRAIL to kill virus-infected cells in an IFN-dependent manner[73]. The IFN-inducible proteins PKR (protein kinase R; also known as EIF2AK2) and 2′-5′-oligoadenylate synthetase may also affect virus-induced apoptosis[74].

In addition to triggering pro-apoptotic events, IFN signalling may inhibit virus-induced apoptosis by inhibiting viral replication. IFN signalling involves the Janus kinase (JAK) family of cytoplasmic tyrosine kinases and the signal transducers and activators of transcription (STAT), which are phosphorylated by JAKs and bind to DNA. A role for interferon signalling in the prevention of virus-induced apoptosis has been demonstrated following infection of mice with murine norovirus[75]. In these experiments, infection and virus-induced apoptosis were inhibited in STAT1-deficient mice[75]. Mice that lack the *Stat1* gene are also more susceptible to reovirus CNS infection, suggesting that IFN signalling can prevent reovirus-induced apoptosis[76]. Differential IFN and JAK or STAT signalling also plays a part in cell type-specific immune responses in reovirus-infected murine cardiac cells[77]. IFN signalling is activated following the detection of viruses by several viral sensors, including Toll-like receptor 3 (TLR3), which increases the expression of genes that express IFN through the actions of IFN regulatory factor 3 (IRF3) and IRF7 (REF. 74). The role of IFN signalling in the inhibition of viral replication in the CNS and the limitation of virus-induced apoptotic disease has also been shown following WNV infection of mice deficient in TLR3, IRF3 and IRF7 (REFS 78–80).

Taken together, the studies discussed in this section suggest that virus infection or specific viral proteins can induce apoptosis without the involvement of the immune system, but that in the vast majority of cases the immune system plays a part in the onset of apoptosis in infected cells in animal models of virus-induced disease.

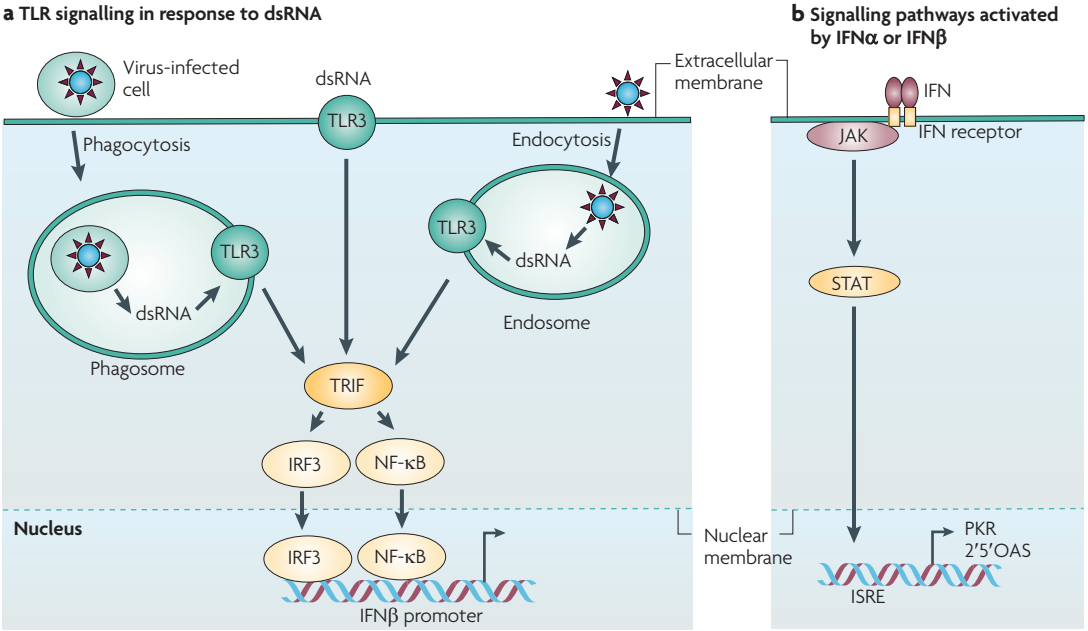
Triggering apoptosis

Both the extrinsic and the intrinsic apoptosis pathways, as well as Jun N-terminal kinase (JNK) signalling (BOX 4), have been shown to be involved in the triggering of apoptosis in animal models of virus-induced disease. Studies on this topic have typically been performed *in vitro*, but in some cases questions regarding specific apoptotic signalling pathways have also been addressed *in vivo*.

Extrinsic apoptotic signalling. In the extrinsic apoptotic pathway, death ligands, including FasL, TNF and TRAIL, bind and activate their cognate receptors (Fas, TNF receptors and TRAIL receptors, respectively)[81–83]. Activated receptors, an adaptor molecule, such as FADD or TNF receptor type 1-associated DEATH domain protein (TRADD), and pro-caspase 8 then form a death-inducing signalling complex, in which pro-caspase 8

Astrocyte
A type of non-neuronal glial cell that performs many functions, including biochemical support of endothelial cells, that form the blood–brain barrier, provision of nutrients to the nervous tissue and aiding the repair and scarring process in the brain.

Box 3 | **Interferon signalling**



Type I interferons (IFNs), including IFNα and IFNβ, are induced directly in response to viral infection (reviewed in REF. 74). They act through a common heterodimeric receptor on infected, and neighbouring uninfected, cells to activate a signal-transduction pathway that triggers the transcription of a diverse set of IFN-inducible genes that establish an antiviral response. IFNα and IFNβ also promote the development of the acquired immune response. IFN can be induced in cells following detection of viral proteins and nucleic acid. One of the most efficient inducers of IFNβ is double-stranded RNA (dsRNA), which can be presented to the cell in several ways, including presentation to the outside of the cell; presentation to endosomes by endocytosis of extracellular dsRNA; uncoating of viral particles; and degradation of engulfed apoptotic cells. One of the cellular detectors of dsRNA is TLR3 (see the figure, part,a). dsRNA binds Toll-like receptor 3 (TLR3), which recruits the adaptor protein TIR domain-containing adapter molecule 1 (TICAM1; also known as TRIF). TRIF acts as a scaffold to signalling components that feed into the IFN-regulatory factor 3 (IRF3) or nuclear factor-κB (NF-κB) pathways, resulting in the transcriptional activation of IFNβ. IFNα and IFNβ signalling involves the Janus kinase (JAK) family of cytoplasmic tyrosine kinases and the signal transducers and activators of transcription (STAT) that are phosphorylated by JAKs and bind to DNA (see the figure, part B).

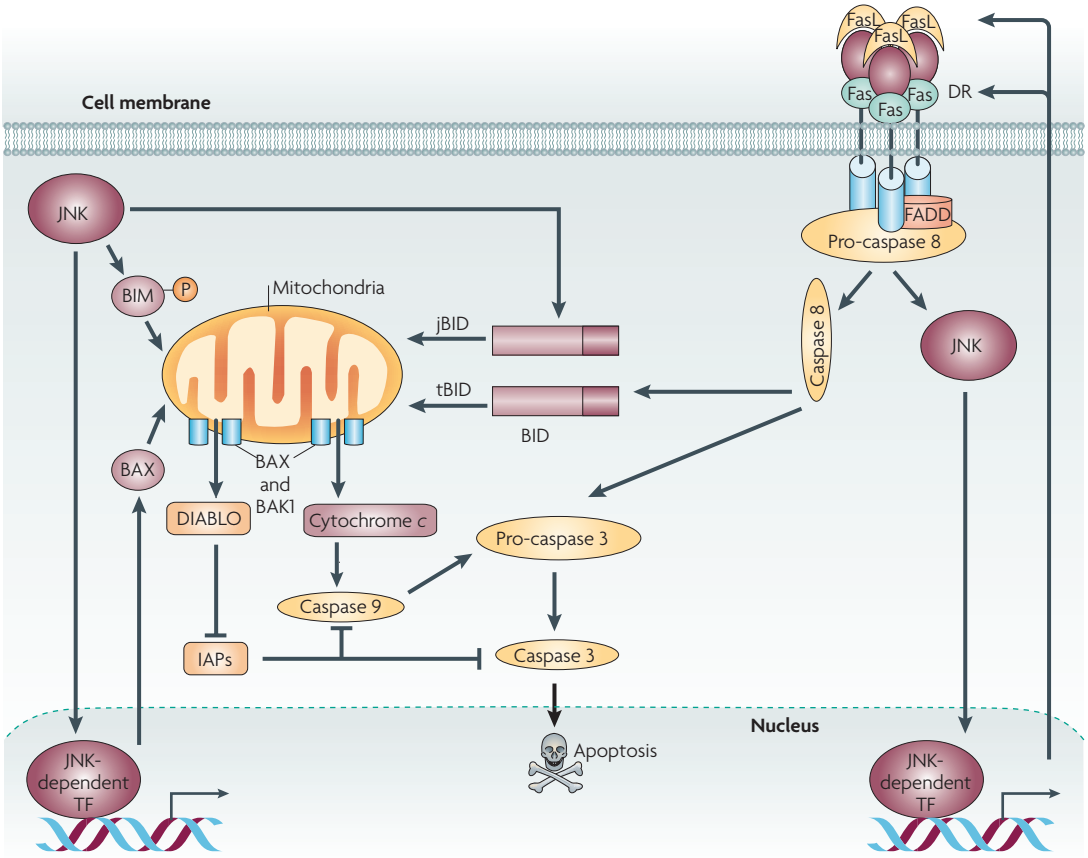
Treatment of cells with IFNα or IFNβ upregulates the expression of hundreds of genes that contain an IFN-stimulated response element. Some of the best-characterized genes include protein kinase R (PKR; also known as EIF2AK2) and 2′-5′-oligoadenylate synthetase (2′5′OAS), which are synthesized in an inactive form and undergo dimerization and activation in response to dsRNA. Activated PKR results in phosphorylation of the α-subunit of eukaryotic translational initiation factor 2 (eIF2α) and halts eukaryotic transcription. Activation of 2′5′OAS activates RNase L, which degrades cellular and viral RNAs. A simplified schematic of IFN induction and IFN signalling pathways is provided in the figure. ISRE, interferon-stimulated response element.

undergoes autoactivation. This results in activation of the initiator caspase, caspase 8, which can activate caspase 3 to bring about the apoptotic phenotype (FIG. 1). The extrinsic pathway of apoptosis is central to the process of immune-mediated viral clearance, which has already been discussed above. However, in some cases, viral-induced increases in death receptors and their ligands might also trigger apoptosis in infected cells without the involvement of infiltrating immune cells. For example, following reovirus infection, it seems that Fas is upregulated in infected cells, resulting in the activation of caspase 8 without any involvement of the immune system (P.C. and K.L.T., unpublished observations). In addition, in HBV-mediated acute liver failure, the expression of TRAIL in the liver is not dependent on immune cells and is thought to be a direct response of the infected cell[84].

Intrinsic apoptotic signalling. In the intrinsic apoptotic pathway, pro-apoptotic members of the B-cell lymphoma 2 (BCL-2) family of proteins (BCL-2 associated x protein (BAX) and BCL-2 antagonist killer 1 (BAK1)) form pores in the outer mitochondrial membrane (BOX 2; FIG. 1). Pro-apoptotic mitochondrial factors, including cytochrome *c* and DIABLO (direct inhibitor of apoptosis protein-binding protein with low pI; also known as SMAC), are released through these pores and contribute to apoptosis. Binding of cytochrome *c* and dATP (deoxyadenosine triphosphate) causes the adaptor molecule, apoptotic protease-activating factor 1 (APAF1), to form a large complex called the apoptosome. The apoptosome recruits pro-caspase 9, which is then processed by autocatalysis. DIABLO inhibits cellular inhibitor of apoptosis proteins (IAPs), which normally function to block the activity of caspase 3 and caspase 9 (REFS 85,86).

Box 4 | **C-Jun N-terminal kinases**

Mitogen-activated protein kinases (MAPKs) are important mediators of signal-transduction pathways that serve to coordinate the cellular response to various extracellular stimuli. The MAPK superfamily includes the c-Jun N-terminal kinases (JNKs) (see the figure), which are activated in response to various environmental stresses and inflammatory signals and promote apoptosis and growth inhibition. JNK influences apoptosis by various mechanisms, including cytochrome *c* release[109] and requires the pro-apoptotic B-cell lymphoma 2 (BCL-2) family proteins BCL-2 associated x protein (BAX) and BCL-2 antagonist killer 1 (BAK1) (REF. 110). It has been proposed that JNK influences cytochrome *c* release in various ways, including post-translational modification of the BH3-only BCL-2 family proteins Bcl-2-like protein 11 (BCL2L11; also known as BIM) (phosphorylation) and BCL-2 interacting domain death agonist (BID) (cleavage to form jBid), by up-regulating BAX and by promoting accumulation of active BAX at the mitochondria[109,111–114]. In addition, multiple transcription factors (TFs), including Jun, p53 and activating transcription factor 2 (ATF2), are activated by JNK (JNK-dependent TFs), and these TFs may affect apoptosis by upregulating apoptosis-related genes. For example, JNK can trigger extrinsic apoptotic signalling by inducing the upregulation of death receptors and/or their ligands[115–121]. JNK itself can also be activated by death receptor (DR)-induced apoptotic signalling (see the figure), suggesting that a positive-feedback loop may occur to amplify apoptosis[122–124]. DIABLO, direct inhibitor of apoptosis protein-binding protein with low pI; FADD, Fas-associated death domain protein; IAP, inhibitor of apoptosis protein; tBID, truncated tBID..



It has been proposed that cytochrome *c* release from the mitochondria occurs by a two-step process[87]. In the first step, cytochrome *c* is released from the inner mitochondrial membrane, where it is bound by interactions with cardiolipin. Once released, cytochrome *c* is then free to leave the mitochondria following permeabilization of the outer mitochondrial membrane by pro-apoptotic BCL-2 family proteins (BOX 2). Release of cytochrome *c* is brought about by oxidation of cardiolipin by reactive oxygen species (ROS), which are generated through the activity of the mitochondrial respiratory chain.

Evidence that the intrinsic pathway of apoptosis can be involved in virus-induced apoptosis in animal models of virus-induced disease comes from studies which show that the upregulation or activation of pro-apoptotic BCL-2 family members is associated with apoptosis. For example, an increase in the amount of pro-apoptotic BAX mRNA correlates strongly with cardiomyocyte apoptosis in some[18,88], but not all[89], studies of CVB3-induced myocarditis. Furthermore, glutathione levels decrease during CVB3-induced myocarditis, suggesting that ROS are released from the mitochondria (as a by-product of BAX dimerization and activation). In this study, ROS were associated with high mortality and had a role in the pathogenesis of cardiac damage following CVB3 infection[88].

Increases in the ratio of BAX (pro-apoptotic) to BCL-2 (anti-apoptotic) and levels of cytosolic cytochrome *c* are also associated with apoptosis in the livers

Reactive oxygen species (ROS). Highly reactive ions or very small molecules that include oxygen ions, free radicals and peroxides. During oxidative stress, increased ROS levels can result in substantial damage to cell structures.

of animals infected with rabbit haemorrhagic disease virus, again suggesting that the intrinsic apoptotic signalling pathway is involved in virus-induced disease[90]. In addition, in HCV transgenic mice, HCV core protein localizes to the mitochondria and promotes ROS generation[91,92].

JNK signalling. The mitogen-activated protein kinase (MAPK) JNK is also thought to play a part in apoptosis either by post-translational modifications of BCL-2 family proteins or by its ability to activate transcription factors and promote transcription of apoptosis-related genes (BOX 4). Reovirus infection of the mouse CNS leads to the activation of JNK and JUN (also known as mitogen-activated protein kinase 8; MAPK8) in reovirus-infected neurons in regions that colocalize with areas of virus-induced injury and apoptosis, suggesting that JNK contributes to neuronal apoptosis in these animals[34]. JNK may contribute to extrinsic apoptotic signalling following reovirus infection of the mouse CNS (P.C. and K.L.T., unpublished observations). Alternatively, or additionally, JNK may mediate reovirus-induced apoptosis in the mouse CNS by activating intrinsic apoptotic signalling, as found following reovirus infection of epithelial cell lines[93]. JNK is activated following infection with various viruses and may therefore have a more widespread role in virus-induced apoptosis. For example, JNK has a role as a pro-apoptotic factor in HSV-1- and HSV-2-induced CNS disease, although precisely how JNK contributes to apoptosis is unclear[2,94].

Taken together, the studies discussed in this section show that multiple apoptotic signalling pathways can be induced in animal models of virus-induced disease. Much still remains unknown, however, including the virus-specific and cell-specific nature of the response and the interactions that exist between the various signalling pathways.

Therapeutic implications

In animal models of virus-induced disease, virus-infected cells thus appear to die by apoptosis. This process can facilitate viral clearance, but is also a mechanism of virus-induced tissue injury and resulting disease. The central role of apoptosis in virus-induced tissue injury has prompted studies designed to test the effect of inhibition of apoptosis on both viral growth and the severity of virus-induced disease.

Effect on viral replication. The use of apoptosis as a host antiviral strategy to eliminate virus-infected cells is counteracted by the capacity of many viruses to encode anti-apoptotic proteins that allow them to complete their replication cycles before destruction of the cell[95]. In these cases, therapeutic strategies designed to inhibit apoptosis might actually enhance viral replication and increase pathogenicity. The host can also use apoptosis to block viral spread. For example, virus-induced neuronal apoptosis of peripheral neuronal nervous-system cells could be a protective host response that blocks transmission of HSV-2 to the CNS[96]. Again, therapeutic inhibition of apoptosis under these circumstances might be expected to enhance viral spread and pathogenicity. However, viruses have evolved and adapted to use many cellular processes to their advantage and the same might have happened for apoptosis. The apoptotic death of a cell may facilitate virus egress, and chromatin margination may 'make room' for viral replication compartments[95]. In such cases, inhibition of apoptosis would be expected to inhibit viral replication and pathogenesis.

In light of these potentially contradicting effects, it is perhaps not surprising that inhibition of apoptosis has been reported to have mixed effects on viral replication. For example, in a mouse model of WNV, caspase $3^{-/-}$ mice have less-severe disease and apoptosis than the wild type, but the CNS titres of WNV do not change[15]. Similarly, the CNS titre of reovirus-infected mice does not change when virus-induced apoptosis and tissue injury are reduced. Consequently, the CNS titre of reovirus-infected mice does not change compared with wild-type controls following pharmacological inhibition of JNK[34], following infection with an apoptosis-deficient reovirus strain[31] or in mice that do not express the P50 subunit of the transcription factor nuclear factor-κB[22]. By contrast, apoptosis and disease severity are reduced and viral titre decreases following infection of mice with a recombinant SINV chimera that expresses BCL-2 compared with the virus from which it was derived[97]. Similarly, virus titre decreases in reovirus-infected hearts of caspase $3^{-/-}$ mice compared with wild-type mice[21]. In addition, inhibition of CVB3 replication *in vivo* with the drug WIN 54954 reduces both viral replication and apoptosis[98]. These differences are probably due to differences in viral replication strategies that have evolved to enable viruses to survive in the host or by organ-specific differences in antiviral responses.

Effect on severity of disease. Studies in animals have shown that conditions designed to inhibit apoptosis reduce disease severity, even though there were conflicting effects on viral titre[15,21,22,31,34,97,98] (discussed above). These studies provide strong evidence that apoptosis is an important mechanism of virus-induced disease and that anti-apoptotic treatments may be beneficial in human virus-induced diseases.

Reports show that caspase $3^{-/-}$ mice, or mice treated with pharmacological caspase inhibitors, are more resistant to virus-induced disease following infection of the CNS with WNV[15], following infection of the heart with reovirus[21] (FIG. 2) and following infection with rhesus rotavirus in a mouse model of biliary atresia[99]. Inhibition of specific apoptotic pathways can also inhibit virus-induced disease. For example, the anti-apoptotic mitochondrial proteins BCL-2 (REF. 97) and peripheral benzodiazepine receptor[100] are protective following CNS infection with SINV, and *N*-acetyl-cysteine[90] and astragaloside[101], both of which prevent oxidative stress, prevent virus-induced liver and heart injury, respectively. Furthermore, intracerebral injection of recombinant simian virus 40 (SV40) vectors that carry

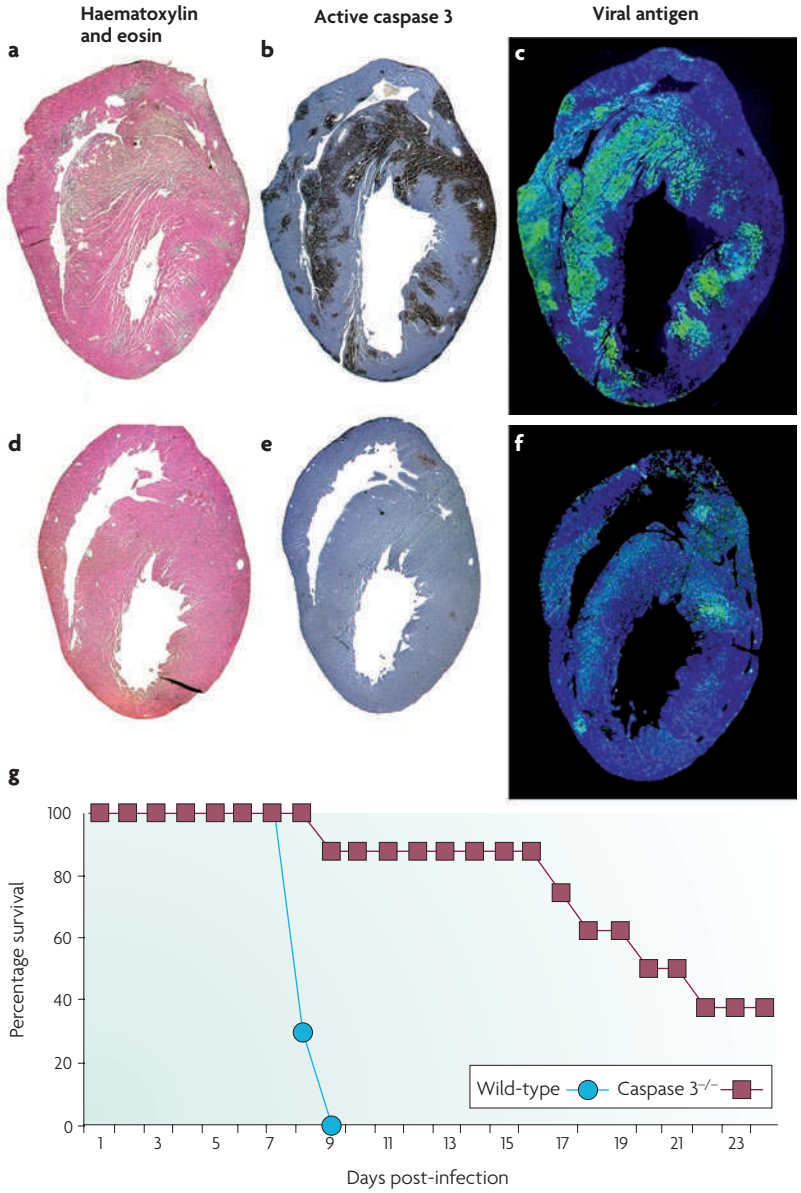


Figure 2 | **Inhibition of caspase 3, apoptosis (caspase 3 activation) and survival in reovirus-infected mice.** Consecutive sections from reovirus-infected hearts were analysed for histological injury (haematoxylin and eosin staining), active caspase 3 (brown diaminobenzadine staining) and virus antigen (fluorescent green staining) in mice treated with the pharmacological caspase inhibitor Q-VD-OPH (**a**–**c**) and in non-treated control mice (**d**–**f**). Survival curves for reovirus-infected caspase $3^{-/-}$ mice and wild-type controls are also shown (**g**). In the caspase $3^{-/-}$ mice, 37.5% of animals survived, and cardiac tissues from long-term survivors appeared to be normal (54 days post-infection). Figure modified, with permission, from REF. 21 © (2004) American Society for Microbiology.

Cu–Zn superoxide dismutase 1 (SOD1) or glutathione peroxidase 1 (GPX1) significantly protected neurons from HIV-1 envelope gp120 (REF. 102), and pharmacological inhibition of JNK signalling, which is upregulated following reovirus infection *in vivo* (discussed above), and has been shown to be required for reovirus-induced apoptosis *in vitro*, decreased disease severity following reovirus infection of the mouse CNS[34]. Anti-FasL antibody prevents hepatocyte apoptosis and proliferation, liver inflammation and the development of hepatocellular carcinoma in transgenic mice that express HBV proteins[27]. Finally, soluble TRAIL inhibits liver damage by blocking apoptosis in an acute hepatitis model in which BALB/c mice were transfected with pcDNA3-HBV1.1, which contains the entire HBV genome[103], and silencing TRADD expression with small-interfering RNA reduces neuronal apoptosis and subsequent microglial and astroglial activation[104,105].

Concluding remarks

Apoptotic cells occur in the CNS, heart and liver following infection with various viruses or expression of viral proteins. In many cases, these apoptotic cells colocalize to the same areas or specific cells as viral antigen. Apoptosis correlates with disease severity in a number of different animal models of virus-induced disease. Furthermore, several studies have shown that there is a decrease in disease severity following treatment with anti-apoptotic agents. These studies indicate that apoptosis is an important mechanism in viral-induced disease and suggest that apoptosis and apoptotic signalling pathways may provide novel targets for treatment of virus-induced human disease. However, it must be remembered that viruses have different strategies to benefit from or evade apoptosis. Accordingly, it will be important to define the specific role of apoptosis in the pathogenesis of individual viral infections. For example, in some cases, virus-induced chronic disease can be immunologically mediated and result from the inability to terminate the immune response, and therefore inhibition of apoptosis might actually exacerbate disease.

Further work is required to determine whether inhibition of apoptosis ameliorates or worsens virus disease in relevant animal models. Then, for cases in which the inhibition of apoptosis promotes survival or reduces tissue injury, it is crucial to define the apoptotic signalling pathways involved. With this knowledge, we could identify specific targets for potential antiviral therapy.

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This study shows that humoral immunity and cellular immunity are protective against, and not required for, reovirus-induced myocarditis and reveals that the potential to induce cardiac damage is independent of lymphocyte-based immunity.

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DATABASES

Entrez Genome: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genome
BDV | HBV | HCV | HIV-1 | HSV-1 | rabies virus | SINV | VEE | WNV

UniProtKB: http://www.uniprot.org
APAF1 | ATF2 | BAD | BAK1 | BAX | BBC3 | BCL2L11 | BID | BIK | BMF | BDV | bNIP3 | caspase 3 | caspase 8 | caspase 9 | DIABLO | eIF2α | EIF2AK2 | FADD | GPX1 | HRK | IRF3 | IRF7 | iNOS | MAPK8 | SOD1 | STAT1 | TICAM1 | TLR3 | TNF | TRADD

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