To kill or be killed: viral evasion of apoptosis

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In the struggle between virus and host, control over the cell's death machinery is crucial for survival. Viruses are obligatory intracellular parasites and, as such, must modulate apoptotic pathways to control the lifespan of their host in order to complete their replication cycle. Many of the counter-assaults mounted by the immune system incorporate activation of the apoptotic pathway—particularly by members of the tumor necrosis factor cytokine family—as a mechanism to restrict viral replication. Thus, apoptosis serves as a powerful selective pressure for the virus to evade. However, for the host, success is harsh and potentially costly, as apoptosis often contributes to pathogenesis. Here we examine some of the molecular mechanisms by which viruses manipulate the apoptotic machinery to their advantage and how we (as vertebrates) have evolved and learned to cope with viral evasion.

The apoptotic machinery

Apoptosis results from a collapse of cellular infrastructure through regulated internal proteolytic digestion, which leads to cytoskeletal disintegration, metabolic derangement and genomic fragmentation. Members of the cytosolic caspase family of proteinases (cysteine-based, aspartate-directed) form "the engine" of the apoptotic pathway[1] (**Fig. 1**). The caspases (11 in total) represent one of more than 20 distinct components involved in initiation, execution and regulatory phases of the pathway, which indicates the extensive regulation this process has engendered over time. The apoptotic machinery shows sensitivity to a variety of agents by coordinating signals initiated by both internal sensors (intrinsic pathway, mitochondria-dependent) and external stimuli (extrinsic pathway, death receptor–mediated). When triggered, internal sensors (for example, p53) can initiate processes that result in the ultimate loss of mitochondrial integrity and apoptosis. Signals from the internal sensors are propagated to the mitochondria *via* pro-apoptotic Bcl-2 subfamily members (BH3 only), such as Bid, to oligomerize Bax and Bak, which are outer mitochondrial membrane proteins that promote the release of cytochrome c. Cytochrome c oligomerizes Apaf1 and recruits pro-caspase-9 (forming the apoptosome), which results in proteolytic conversion of pro-caspase-9 to an active enzyme. Caspase-9 then converts pro-caspase-3 to its active form, which, with other executioner caspases, such as caspase-7, then cleaves key substrates in the cell to orchestrate the cell's fatal collapse.

In contrast, the extrinsic pathway starts with members of the tumor necrosis factor (TNF) superfamily of death receptors transmitting external signals provided by immune effector cells to the virus-infected cell. Innate effector cells—for example, natural killer (NK) cells and dendritic cells (DCs)—can mount rapid antiviral responses through direct detection of viral products by pattern recognition receptors—such as binding of double-stranded RNA (dsRNA) by Toll-like receptor 3 (TLR3)—or by up-regulating death receptor ligands—for example, Fas ligand (FasL), TNF receptor–related apoptosis-inducing ligand (TRAIL) and TNF[2]. Later during adaptive immunity, these same death ligands are produced by antigen-specific cytotoxic T cells (CTLs).

The lethal program set in motion by these ligands mandates that their expression is transient and highly regulated at the transcriptional and post-translational steps. Upon binding TNF-family ligands, death receptors recruit adaptors and initiator caspases in a stepwise sequence based on specific interaction domains and form the death-inducing signaling complex (DISC) at the plasma membrane. In the case of Fas, the cytosolic adaptor FADD (Fas-associated death domain–containing protein) contains two interaction motifs: a death domain (DD) that associates with the homologous structure in Fas and a death effector domain (DED) that interacts with a homologous DED in pro-caspase-8 or pro-caspase-10. The proximal positioning of pro-caspase-8 and pro-caspase-10 in the DISC is thought to lead to autocatalysis and conversion to an active enzyme. Activated caspase-8 or caspase-10 can then directly convert pro-caspase-3 to its active form, completing the initiation phase of the pathway. There is cross-talk between the death receptor pathways and the mitochrondria-dependent arm through cleavage of the BH3-only protein Bid by caspase-8. In addition, the cytotoxic granule–associated proteinase granzyme B can bypass caspase-8 and, upon delivery by CTLs and NK effector cells, can directly cleave and activate caspase-3. Caspase-3 and other executioner caspases cleave numerous substrates, such as ICAD (inhibitor of caspase-activated DNase), which leads to genome fragmentation, collapse of the cell and preparation of cellular remnants for phagocytosis. Thus, vertebrates have evolved several distinct strategies to initiate apoptosis that lead to a common execution phase of the pathway.

All of the key pro-apoptotic components are preformed in the cell, enabling the pathway to respond rapidly to apoptosis-inducing signals. In contrast, the cellular regulatory elements of the death pathway often require new gene expression. The transcription factor NF-κB controls several genes, including those of the Bcl-2 and inhibitors of apoptosis (IAP) families, whose functions regulate apoptosis and promote cell survival[3]. This is especially pertinent when a virus has compromised the biosynthetic capacity of the host cell: death becomes the default outcome and, predictably, viral counter-strategies have evolved that mimic the key cellular regulatory elements. The key regulatory steps in the cell death pathway targeted by viral pathogens include control over the expression of internal sensors, cytochrome c release by Bcl-2

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family members, inhibition of caspases and regulation of death receptor signaling. Indeed, the discovery of several of the key regulatory points in the apoptotic pathway have emerged directly from studies of the death-escape mechanisms used by viruses.

Blinding the sensors

The tumor suppressor p53 limits cellular proliferation by inducing apoptosis or cell cycle arrest in response to cellular stresses and is intimately linked to cancer development. p53-deficient mice are more prone to certain viral infections, indicating a role beyond tumor suppression[4]. Some of the genes whose transcription is stimulated by p53 include those that encode the death receptors Fas and TRAIL receptor 2 (TRAILR2), although the importance of their up-regulation by p53 in triggering apoptosis is unclear. Additionally, p53 induces the transcription of genes such as *Bax*, *Bak1*, *Bbc3* and *Pmaip1* whose products are involved in death signal propagation through the mitochondria[5–9]. p53 represses transcription of the anti-apoptotic protein Bcl-2, an antagonist of Bax and Bak[10]. Viruses can also disrupt apoptosis by inactivating p53. The SV40 large T antigen binds to p53 and sequesters it in an inactive complex[11,12]. The human papillomavirus E6 protein and adenovirus E1B-55K protein, in concert with E4orf6, promote ubiquitination and degradation of p53, albeit *via* different mechanisms[13–16]. Additionally, the pX protein encoded by hepatitis B virus complexes with p53 and inhibits p53-mediated transcriptional activation as well as p53-dependent apoptosis[17].

Mimicking Bcl-2

Viral encoded orthologs of the anti-apoptotic regulator Bcl-2 are a widely used immune evasion strategy (**Fig. 2**). A pertinent example is adenovirus E1B-19K, which is similar both in sequence and function to Bcl-2[18,19]. Unlike cell death signaled through cell-surface receptors, p53-mediated apoptosis in response to adenovirus infection does not require the cleavage of Bid into t-Bid to achieve Bax-Bak oligomerization and the subsequent release of cytochrome c. Instead, p53 stimulates a conformational change in Bax that is required for Bax-Bak interaction. These events are blocked by adenovirus E1B-19K as a result of its binding to Bak and the abrogation of Bax-Bak oligomerization[20]. E1B-19K also affects the apoptotic process signaled *via* TNF at the level of Bax and Bak activation. Treatment of cells with TNF produces a death signal that results in the cleavage of Bid to t-Bid followed by the recruitment of monomeric, pro-apoptotic Bax into a 500-kD protein complex and the release of cytochrome c from the mitochondria. In the case of TNF-mediated apoptosis, E1B-19K appears to interact primarily with Bax, inhibiting oligomerization and the subsequent release of cytochrome c[21].

Oncogenic human herpesviruses use Bcl-2 orthologs to block mitochondrial release of cytochrome c. This is also true for Epstein-Barr virus (EBV), which encodes two Bcl-2 orthologs (BHRF1 and BALF-1[22,23]), and Kaposi's sarcoma–associated γ-herpesvirus (KSHV), which expresses KSbcl-2[24,25]. Mouse γ-herpesvirus MHV-68, a virus that serves as a model for human EBV infection, encodes a Bcl-2 ortholog (MHVBcl-2) that protects against TNF-mediated apoptosis in cell culture. In addition, MHVBcl-2 is important for chronic infection, as demonstrated by the impaired virulence of MHV-68 virus lacking the Bcl-2 ortholog in interferon-γ (IFN-γ)–deficient mice[26]. In contrast, the human cytomegalovirus (CMV) UL37 gene product vMIA shares no sequence homology to Bcl-2; however, it resides in the mitochondria and appears to be functionally similar to Bcl-2, as it associates with the adenine nucleotide translocator and inhibits Fas-mediated apoptosis[27].

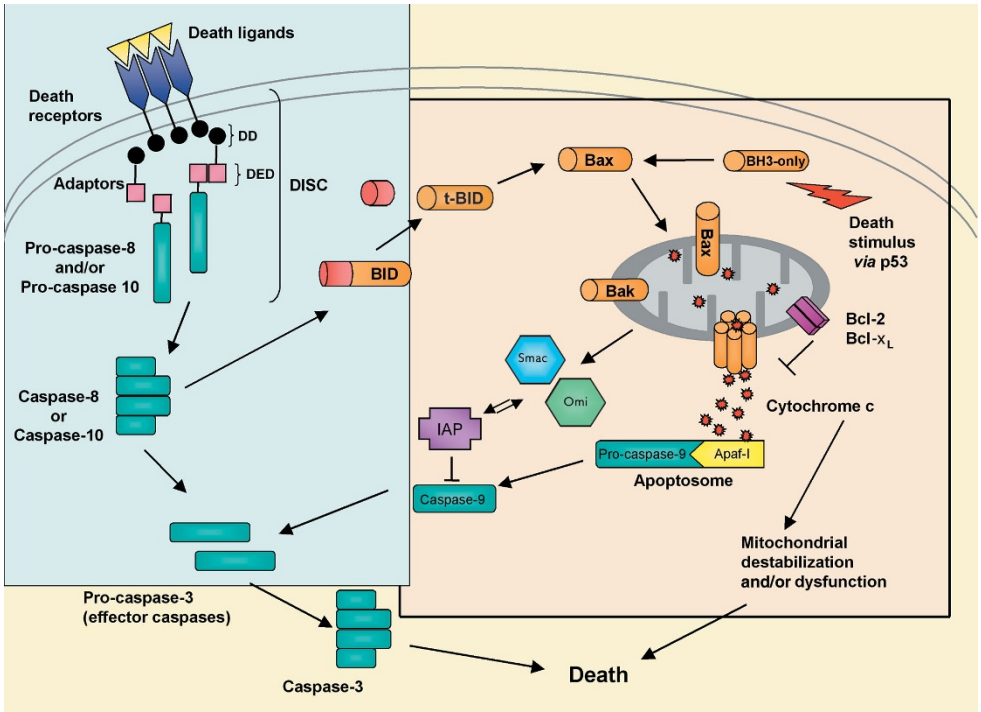

Figure 1. Molecular pathways of apoptosis. The intrinsic apoptotic pathway is initiated by internal sensors that monitor cellular stresses such as viral infection *via* activation of BH3 domain–only members of the Bcl-2 family. Activated BH3-only proteins are thought to mediate the assembly of pro-apoptotic members of the Bcl-2 family (Bax, Bak, Bcl-rambo, Bok) into hetero-oligomeric "pores" in the outer membrane of the mitochondria; this results in the release of factors such as cytochrome c, Smac (also known as Diablo) and Omi (also known as HtrA2) into the cytoplasm. The loss of mitochondrial membrane integrity can be blocked by the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and Bcl-B. Release of cytochrome c promotes formation of the apoptosome, which contains Apaf-1 and pro-caspase-9. Autocatalytic activation of caspase-9 initiates the effector caspase cascade, which includes caspase-2, -3, -6 and/or -7. Caspase activation is negatively regulated by the IAPs, which are counter-balanced by the release of pro-apoptotic Smac and Omi from the mitochondria. The extrinsic pathway of apoptosis is triggered by TNF family death ligands binding to their cognate death receptors. *Via* their DDs, multimerized receptors interact with the DDs of adaptor proteins such as FADD. These adaptor proteins also contain DEDs that facilitate their binding to pro-caspase-8 and/or pro-caspase-10 to form the DISC. As part of the DISC, the pro-caspases are cleaved into their active forms and initiate the intrinsic pathway of apoptosis by cleaving Bid into t-Bid and activate the effector caspase cascade (caspase-3 is shown).

Other viral proteins inhibit apoptosis by modulating Bcl-2 family members at the transcriptional level or *via* post-translational modification. The human T cell leukemia virus type 1 (HTLV-1) Tax protein transcriptionally activates the Bcl-x_L promoter while repressing transcription of Bax[28]. HIV-1 Nef mediates phosphorylation of the pro-apoptotic Bad protein, abrogating its activity and suppressing apoptosis in T cells[29]. Similarly, the U(S)3 protein kinase encoded by herpes simplex virus 1 (HSV-1) mediates a post-translational modification of Bad and blocks its cleavage and subsequent activation of apoptosis[30].

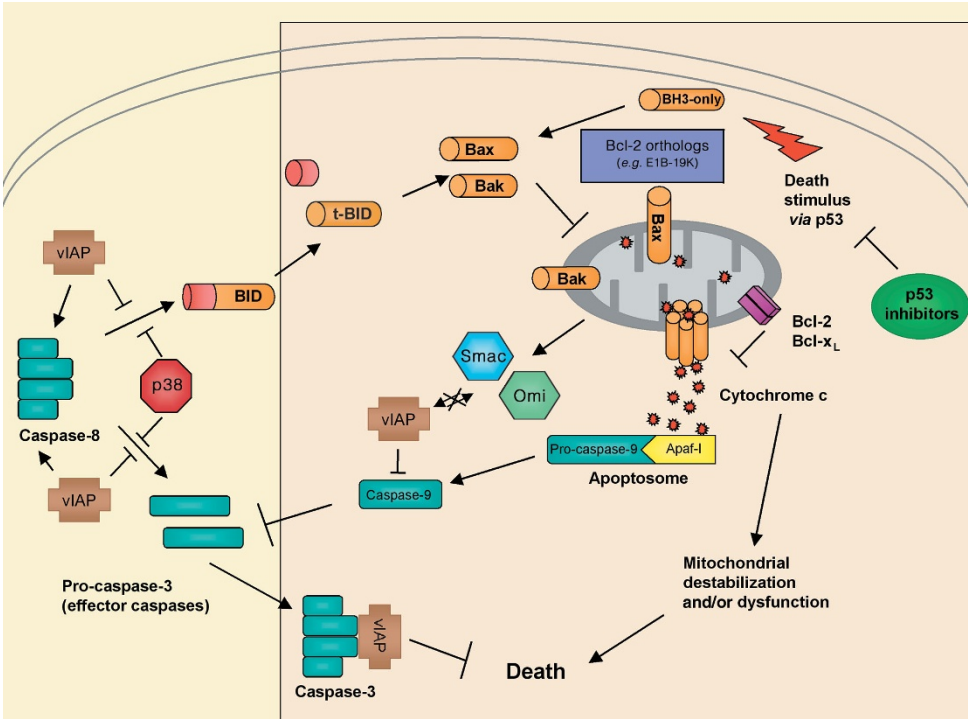

Figure 2. Viral regulation of the intrinsic apoptotic pathway. Cellular sensors, such as p53, that detect stress and initiate the apoptotic process are inactivated by the proteins adenovirus E1B-55K and human papillomavirus E6. Several viruses encode orthologs of Bcl-2 family proteins that antagonize their pro-apoptotic activity. Several viral strategies target the caspases. The vIAPs contain conserved BIR domains and their expression may shift the equilibrium of Smac to favor cell survival. Non-BIR–containing proteins, such as p35 and the serpins (including CrmA), also inhibit caspase activation.

Caspase regulation

Caspases play a central role in apoptosis and are regulated in several ways[31–33]. The enzymatic activity of caspases is inhibited by a conserved family of inhibitor of apoptosis proteins (IAPs)[34,35] that were originally defined in baculovirus based on the suppression of apoptosis and presence of a zinc-binding motif called a BIR (baculoviral IAP repeat)[36]. To date, eight cellular IAPs have been identified that regulate both the effector and initiator caspases[37]. For example, XIAP, c-IAP1, c-IAP2 and ML-IAP (livin) target the initiator caspase, caspase-9, and the effector caspases, caspase-3 and caspase-7. XIAP, c-IAP1 and c-IAP2 contain three BIR domains, each with a different function. The third BIR domain (BIR3) inhibits the activity of processed caspase-9, whereas the linker region between BIR1 and BIR2 abrogates the activity of caspase-3 and caspase-7. An IAP-ortholog strategy is used infrequently in mammalian viruses, with the exception of African swine fever virus, which encodes a viral IAP (vIAP) that does not contribute to virulence[38]. In mammals, the serpin CrmA—which is derived from cowpox and is present in most poxviruses—also inhibits several caspases, likely through covalent modification of caspase-8, and blocks or delays apoptosis in response to TNF and Fas signaling or CTLs[39–41]. Also, CrmA inhibits caspase-1, a critical processing enzyme for the inflammatory cytokine interleukin 1β (IL-1β).

Modulation of TNFR signaling

Several members of the TNF receptor (TNFR) superfamily, including Fas, TNFR1 and TRAILR2, are potent inducers of apoptosis[42]. Not surprisingly many viruses specifically target these cytokine receptors (**Fig. 3**). Neutralization of TNF by soluble decoy receptors was one of the first-described evasion tactics, as shown by the secreted TNFR2 ortholog expressed by Shope fibroma virus (rabbit poxvirus)[43]. Several TNFR orthologs have been identified in the genomes of lepri- and orthopoxviruses, including smallpox, which indicates its impact on the success of poxviruses[44]. The T2 protein of myxoma virus is a dimeric, high-affinity binding protein for TNF and virulence is attenuated when it is deleted from the viral genome[45]. Interestingly, the TNFR decoys in vaccinia, the attenuated form of smallpox, are mutated. Additionally, the poxviral TNFR ortholog T2 exists in an intracellular form that is required to inhibit apoptosis of lymphocytes[46]. Another TNFR ortholog is found in avian leukocytosis virus, which encodes an ortholog of TRAILR2 that serves as a virus entry factor[47]. Additionally, human CMV (but not mouse CMV) contains a TNFR ortholog encoded by the UL144 orf, although its functional significance remains obscure[48].

Herpes simplex virus 1, through its envelope glycoprotein D, uses a TNFR family member, herpesvirus entry mediator (HVEM), to gain access to the lymphoid compartment, where it can induce apoptosis of T cells[49] and block maturation of antigen-presenting DCs[50]. The major B cell–transforming protein in EBV, LMP1, behaves like a constitutively activated CD40 by engaging TRAFs and TNFR1-associated DD protein (TRADD), which are adaptors used by TNFR to activate the transcription of anti-apoptotic genes through NF-κB and c-Jun NH_2-terminal kinase (Jnk)-dependent signaling pathways. In a transgenic model, LMP1 expression prevents B cells from localizing to the follicle, thus protecting cells harboring latent virus from interactions with T cells[51].

Whereas poxviruses inhibit TNF ligand-receptor interactions by deploying a soluble receptor smoke screen, a distinct strategy is used by adenoviruses. The E3 region encodes several proteins that sweep the cell surface clear of the death-inducing receptors Fas, TRAILR1 and TRAILR2. The E3 proteins responsible for modulating cell surface amounts of Fas are E3-10.4K and E3-14.5K[52,53], which localize to various cellular membrane compartments, including the plasma membrane, as a heteromeric complex[54]. However, the ability of adenovirus

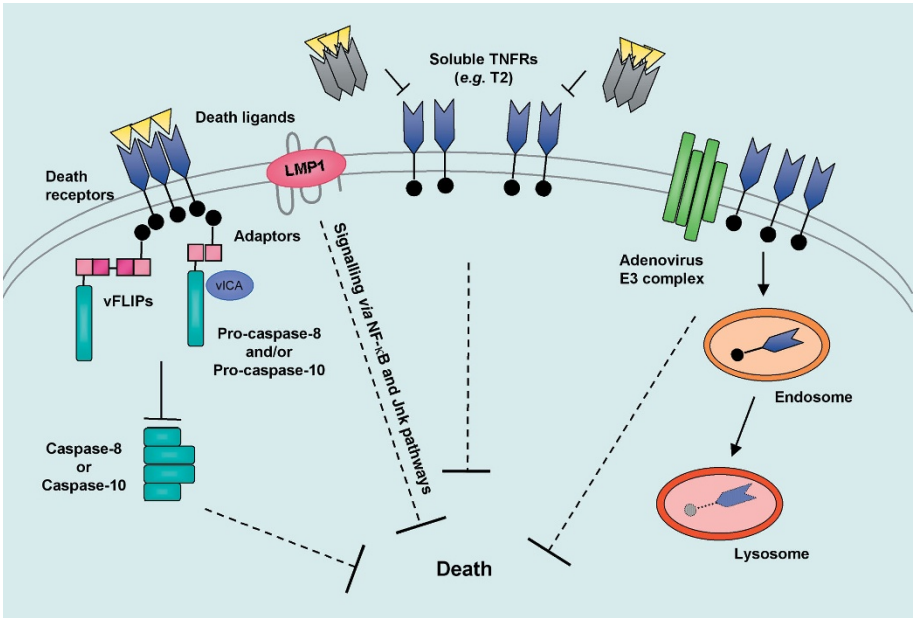

Figure 3. Viral regulation of the extrinsic apoptotic pathway. Poxviruses block signaling *via* TNF and lymphotoxin by producing soluble decoy receptors (for example, myxoma virus T2 protein). vFLIPs contain two DEDs that interact with the homologous DED of FADD and pro-caspase-8 or pro-caspase-10, blocking caspase activation and inhibiting death receptor–induced apoptosis. The human CMV protein vICA binds and inhibits pro-caspase-8 activation, but lacks a DED. The EBV protein LMP1 self-aggregates and engages TRAF and TRADD molecules, activating "anti-apoptotic" NF-κB– and Jnk-dependent pathways. A heterocomplex of proteins encoded by the E3 region of adenovirus (E3-10.4K, E3-14.5K and E3-6.7K) facilitates the removal of the death receptors Fas, TRAILR1 and TRAILR2 from the surface of infected cells. Consequently, receptors accumulate in late endocytic compartments, and cells are desensitized to killing.

to modulate TRAILR2 absolutely requires a third E3 protein, E3-6.7K, in addition to E3-10.4K and E3-14.5K[55]; this highlights the complexity of the mechanisms used by adenoviruses to inhibit signaling by these death receptors. Upon E3-induced down-regulation, death-inducing receptors accumulate in late endocytic compartments, resulting in the desensitization of infected cells to killing by FasL and TRAIL[53,55].

Prevailing wisdom is that the E3-10.4K–E3-14.5K–E3-6.7K complex pirates the cellular endocytic compartments that direct membrane protein trafficking. A similar strategy is used by the HIV Nef protein, which down-modulates CD4 and major histocompatibility complex (MHC) class I from the cell surface by cross-linking these proteins to the cellular endocytic machinery[56]. As a consequence of Nef's actions, the membrane forms of TNF and the related cytokine LIGHT show sustained expression on the surface of T cells, potentially contributing to the cytopathic effects of HIV on the T cell compartment[57]. The substantial death of bystander (uninfected) lymphocytes during HIV infection may stimulate lymphopoiesis, possibly by homeostatic control mechanisms, which could provide a pool of dividing (but not HIV-specific) T cells for HIV genome replication[58].

Viruses can down-modulate or enhance expression of death receptors and ligands to their own advantage. The loss of death receptors may function to protect infected cells from cytolysis by CTLs or NK cells, which express FasL or TRAIL upon activation[59]. In addition, this down-regulation may help inhibit apoptosis mediated by neighboring cells that are induced to express death receptor ligands upon infection. Induction of TRAIL has been observed in HCMV-[50,60] and reovirus-infected[61] cells and of FasL by HSV in T cells and HCMV in DCs[49,50]. It has even been proposed that this induction of FasL and TRAIL is another viral immune evasion tactic, through the killing of infiltrating host CTLs and DCs[49,50]. IFNs induce TRAIL in various cell types[60,62,63]; this suggests that a possible mechanism for up-regulation of TRAIL could be *via* production of IFNs by the virus-infected cell.

Several viruses have evolved a different strategy for blocking death receptor signaling at the level of DISC assembly; they do this through blockade of caspase-8 and caspase-10 processing. The viral FLICE (caspase-8) inhibitory proteins (FLIPs) contain DEDs, but lack caspase activity, and are present in the genomes of various γ-herpesviruses, including equine herpesvirus 2 (EHV-2), herpesvirus saimiri (HVS), KSHV, bovine herpesvirus 4 (BHV-4) and moluscum contagiosum virus (MCV)[64,65]. The cellular ortholog of vFLIP was subsequently cloned after identification of the vFLIPs[65] and exists in both a ~26-kD short ($cFLIP_S$) and ~55-kD long ($cFLIP_L$) form generated by alternative splicing. $cFLIP_S$ is essentially the cellular ortholog of vFLIP and encodes two DEDs, whereas $cFLIP_L$ encodes an additional COOH-terminal domain with high homology to caspase-8 and caspase-10. Both isoforms of cFLIP are recruited to the DISC and inhibit death receptor–induced apoptosis; however, their mechanisms of action are slightly different: $cFLIP_S$ completely inhibits proteolytic processing of caspase-8 (similar to vFLIP) and $cFLIP_L$ allows partial caspase-8 processing[66]. The HCMV UL36 gene product vICA also associates with caspase-8 and blocks its activation, but shows no sequence identity to its proposed cellular orthologs, the FLIPs[67].

vFLIPs interact with adaptor proteins that regulate the expression of NF-κB, including TNFR-associated factor 2 (TRAF2), receptor-interacting protein (RIP), NF-κB–inducing kinase (NIK) and the inhibitor of κB-kinase 2 (IKK2)[68], which indicates that the vFLIPs may also regulate activation of transcription factors important in inhibiting apoptosis.

Death taxes

Cell death comes at a potentially large cost for the host. However, it is becoming appreciated that IFN-dependent nonapoptotic mechanisms can result in the successful attenuation of viral spread[69]. IFNs play a critical role in mounting innate responses to viral infection. IFNs are typically considered nonapoptotic but, in some circumstances, can promote apoptosis of virus-infected cells and are especially potent when they act in concert with TNF-related ligands. IFNs signal *via* the Janus protein kinase–signal transducers and activators of transcription pathway (Jak-STAT)[70]. Well studied IFN-inducible signaling pathways include the RNA-dependent protein kinase (PKR)[71] and the 2′,5-oligoadenylate and RNase-L systems[72]. Upon interaction with virus-derived dsRNA, PKR is activated and can inhibit host-cell translation *via* phosphorylation of elongation initiation-factor 2α (eIF2α)[73]. More recently, it has been shown that PKR has additional activities, including the modulation of

NF-κB[74] and promotion of apoptosis by functional enhancement of the tumor suppressor genes p53 and IFN response factor 1 (IRF-1)[75]. The regulation of NF-κB by PKR places this protein at a critical crossroad in the coordination of apoptotic signals, potentially through the NF-κB–dependent induction of FasL and IFN-induced TRAIL.

Several noncytolytic antiviral programs activated by IFNs can arrest the viral life cycle at different steps, thereby attenuating virus spread and limiting the infection. However, IFNs and their response genes are themselves targeted by many viruses[76]. Replication-competent HCMV specifically inhibits induction of IFN-β transcription by an unknown mechanism[77] that may involve inhibition of IRF-3 activation[78]. This blockade of IFN-β transcription is overridden by signaling *via* TNFR1 or the lymphotoxin β receptor (LTβR), but not by Fas or TRAIL receptors[79]. Induction of IFN-β by these two TRAF-adapting receptors is NF-κB–dependent, but occurs only in HCMV-infected cells. Therefore, host and virus factors cooperate to induce IFN-β, establishing a state of détente in which the host cell survives and the viral genome persists, but cannot produce new virions. This differs from the case of hepatitis B, where IFN signaling results in protease-dependent clearance of the viral genome[69]. These two examples highlight the virus- and cell type–specific issues often seen with IFN's antiviral action.

The diversity of strategies used by viruses to modulate the apoptotic pathway is as varied as there are viruses. In addition, for each virus, these strategies are integrated into a wider scheme that manipulates other aspects of host defenses. For example, herpesviruses have large DNA genomes that have accumulated an extensive repertoire of immune-evasion tactics that target both the afferent (for example, antigen recognition) and effector phases (apoptosis) of host defenses[80]. The cumulative effect of these mechanisms is thought to contribute to the ability of herpesviruses to sustain life-long infection. As a cautionary note, nearly all the data gathered to date are on clinically important viruses, which unfortunately often lack comparable animal models with which we can assess the role of immune-modulatory genes in viral pathogenesis. Indeed, there are significant differences in the molecular mechanisms used to modulate immune function by mouse CMV compared with those of human CMV. It is also important to recognize that a particular viral evasion strategy may act differently in a non-native host compared to in tissue culture, which also contributes to the difficulty in defining mechanisms of action. Nonetheless, the elucidation of evasion strategies directed at apoptotic mechanisms can provide deeper insight into the host-virus relationship that hopefully will yield better vaccine strategies.

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