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Viral manipulation of host cell necroptosis and pyroptosis

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Abstract

Cell death forms an essential component of the antiviral immune response. Viral infection elicits different forms of host cell death including the lytic and inflammatory cell death modes necroptosis or pyroptosis. The induction of necroptosis and pyroptosis not only eliminates virus-infected cells but also contributes to the development of innate and adaptive immunity through the release of inflammatory mediators. The importance of both necroptosis and pyroptosis in host defence is evident from the numerous viral evasion mechanisms that suppress these cell death pathways. Here, we review the emerging principles by which viruses antagonise host cell necroptosis and pyroptosis to promote their spread and block host immunity.

Highlights

- Necroptosis and pyroptosis of virus-infected cells are important host defence strategies.
- While viral antagonists that block host cell necroptosis are relatively well-documented, viral strategies to subvert pyroptosis remain poorly characterised.
- Large DNA viruses such as herpesviruses and poxviruses interfere with different stages of the necroptotic and pyroptotic signalling cascades. Elimination of one of the viral inhibitors of cell death severely attenuates virulence.
- Detailed insight into the molecular pathways that block host cell death are opening up new opportunities for the development of attenuated vaccine strains and the rational design of new antivirals.

Regulated cell death as an antiviral defence strategy

Within hours after infection, viruses reshape the host cell into a viral factory with the sole purpose of producing new infectious particles. An effective way to control viral spread is through the removal of infected cells *via* a process called regulated cell death (Box 1). Although cell death as a consequence of stress induced by the viral replication process has been reported to aid the release of viral progeny at the terminal stages of infection, the early induction of host cell death forms a formidable antiviral defence mechanism (Box 2, *Proviral vs. antiviral cell death*). Firstly, cell death destroys the viral niche of replication. Secondly, antiviral cell death promotes host innate immune responses through the release of intracellular immunostimulatory components collectively termed danger-associated molecular patterns (**DAMPs**, see Glossary). Thirdly, cell death facilitates the uptake and presentation of viral antigens by dendritic cells to T cells [1, 2]. The three major modes of antiviral cell death are apoptosis, necroptosis and pyroptosis that are each driven by unique genetically imprinted signalling cascades [3-10]. These cell death pathways initiate morphologically distinct cellular disintegration processes. Apoptosis proceeds through a proteolytic signalling cascade mediated by the family of cysteine aspartyl proteases (**caspases**) and occurs *via* two signalling pathways: the caspase-9-dependent intrinsic or the caspase-8-dependent extrinsic pathways [11, 12]. Apoptosis leads to the ordered disassembly of the cell involving nuclear fragmentation, cell shrinkage and breakdown of the cell into apoptotic bodies [13]. It is important to state that apoptosis of virus-infected cells should not be regarded as an immunologically inert process as is the case for the programmed cell death processes that support normal development and tissue homeostasis (see Box 1). Cell-intrinsic innate immune activation triggers an inflammatory response that often precedes or coincides with the death process. In contrast to apoptosis, both necroptosis and pyroptosis are not involved in maintaining normal physiology as demonstrated by the overtly normal development of mice that lack one or more key regulators of the necroptotic and/or pyroptotic

signalling cascades [14-16]. This suggests that these cell death pathways have evolved to deal with conditions of stress including virus infection. Activation of necroptosis and pyroptosis results in cellular swelling followed by plasma membrane perturbation through the action of the pore-forming proteins mixed lineage kinase domain like pseudokinase (**MLKL**) in the case of necroptosis and **gasdermin** (**GSDM**) D during pyroptosis. Cell membrane pore formation in necroptotic and pyroptotic cells causes the release of intracellular material including DAMPs into the extracellular space. As a result, these types of cell death are highly inflammatory even in non-infectious conditions [17, 18]. In addition, pyroptosis of myeloid cells goes hand-in-hand with secretion of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 [15].

Given its major role in antiviral immunity, viruses have evolved intricate mechanisms to block host cell death. In this review, we focus on the emerging molecular mechanisms by which viral pathogens manipulate the signalling pathways that induce necroptosis and pyroptosis. For an overview on the viral mechanisms that restrict host cell apoptosis we refer to excellent reviews on this topic [10, 19-21]. The relationship between viral cell death antagonists and the host cell death machinery is complex. A notable example is the capacity of large DNA viruses to block the activity of the initiator caspase of cell-extrinsic apoptosis caspase-8 (Box 3), which in turn renders cells highly sensitive to necroptosis [3, 21]. This important observation paved the way for the molecular characterisation of the necroptotic signalling pathway [14, 16]. In light of these findings, we also discuss how viruses manipulate caspase-8 activity and ask whether necroptosis acts as a primary antiviral defence mechanism acting in parallel to apoptosis or serves as a secondary molecular backup system.

Viral evasion of necroptosis

Necroptosis is a lytic and highly inflammatory form of cell death with important antiviral functions [4]. Proteins that contain receptor-interacting protein (RIP) homotypic interaction

motifs (**RHIMs**) play central roles in necroptotic signalling (Figure 1) [14, 16]. Humans and mice express four RHIM-containing proteins: RIP kinase 1 (RIPK1), RIPK3, TIR-domain-containing adapter-inducing IFN-β (TRIF) and Z-DNA binding protein 1 (**ZBP1**). Necroptosis crucially depends on the kinase activity of RIPK3, which phosphorylates the pore-forming protein MLKL resulting in its oligomerisation at the plasma membrane leading to loss of membrane integrity. RIPK3 activation requires upstream interaction with one of the three other RHIM-containing necroptosis-activating proteins RIPK1, TRIF or ZBP1. The induction of necroptosis has been extensively characterised upon stimulation of the cell surface tumour necrosis factor (TNF) receptor 1 (TNFR1), a member of the **death receptor** superfamily [16]. Concurrent TNFR1 stimulation and caspase-8 inhibition results in RIPK1 kinase-dependent assembly of a megadalton cytosolic protein complex called the necrosome, which contains RIPK1 and RIPK3 as its core constituents (see *Necroptosis: a backup mechanism or a stand-alone process?*) [16]. Similarly, activation of TRIF, an adaptor protein for Toll-like receptor (**TLR**) 3 and TLR4, triggers necroptosis when caspase-8 activity is blocked [22, 23]. Finally, the RHIM-containing innate immune sensor ZBP1 is emerging as an important inducer of antiviral necroptosis. ZBP1 binds double-stranded (ds)RNA molecules in the atypical left-handed Z-conformation referred to as Z-RNA, which accumulates in virus-infected cells [24, 25]. In the case of influenza A virus and members of the herpesviruses and of the poxviruses such as vaccinia virus, ZBP1 is the dominant necroptosis inducer [26-30]. In influenza A-infected cells Z-RNAs first appear in the nucleus resulting in activation of a nuclear ZBP1-RIPK3-MLKL signalling axis. This causes rupture of the nuclear membrane and release of nuclear DAMPs including HMGB1 [24]. Overactivation of this pathway may contribute to influenza A-induced lung immunopathology. In contrast, infection with the vaccinia poxvirus causes cytosolic accumulation of Z-RNA [25], indicating that ZBP1 surveys both nuclear and cytosolic compartments to induce necroptosis of virus-infected cells. Truncated recombinantly

expressed proteins containing β-sheet sequences surrounding the core (I/V)Q(I/V)G RHIM residues of RIPK1 and RIPK3 stack together into an alternating pattern to form **amyloid** structures [31, 32]. Further *in vitro* experimentation shows that RHIM-dependent interactions between RIPK1 and RIPK3 seed the formation of RIPK3-only amyloidal fibres that serve as MLKL activation platforms [33, 34] and may form important targets of viral antagonism (see below). It is well established that RIPK3-containing necrosomes separate into large insoluble complexes in cells [16]. However, whether RIPK1/RIPK3 amyloid complexes are formed under natural conditions and/or whether amyloid formation is functionally required for necroptosis induction and thus represent a target of viral antagonism *in vivo* is less clear.

RHIM-dependent inhibition of necroptosis

Multiple members of the herpesvirus family encode RHIM-containing proteins that interfere with host cell RIPK3 amyloid formation and downstream MLKL activation. The prototypical viral RHIM protein is the enzymatically inactive viral ribonuclease reductase subunit 1 (R1) homologue M45 from mouse cytomegalovirus (MCMV) [29, 35]. M45 directly interacts and blocks signalling of RIPK1, TRIF and ZBP1 *via* its N-terminal RHIM [29, 36-38]. An N-terminal fragment of M45 encompassing the minimal RHIM region, which is still capable of inhibiting necroptosis, is amyloidogenic, similar to the RHIMs of RIPK1 and RIPK3[39]. This supports a model whereby M45 prevents necroptosis induction by forming dysfunctional ZBP1-M45 and/or RIPK3-M45 host-viral heteroamyloids that are unable to activate MLKL. The viral UL39-encoded R1 analogues from herpes simplex virus (HSV)-1 and HSV-2, termed ICP6 and ICP10 respectively, similarly contain a RHIM at their N-termini to inhibit TNF- and ZBP1-driven necroptosis [30, 40, 41]. While MCMV M45 lacks ribonucleotide reductase activity, both HSV-1 and HSV-2 R1 proteins are enzymatically active and promote the synthesis of deoxynucleotide building blocks required for DNA genome replication. The ribonucleotide

reductase domain of ICP6 additionally interacts with and inhibits caspase-8 (see Box 3) and contains a short C-terminal amino acid sequence, termed induced protein aggregation motif (IPAM), which induces RIPK1 aggregation and autophagosome-mediated degradation [42]. IPAM sequences are conserved in MCMV M45 and at least 70 other viral R1 proteins from herpesviruses, baculoviruses and giant viruses and their targets extend beyond necroptosis-inducing proteins including the NF-κB activating protein NEMO [42]. ICP6 thus acts as a functional tetrad simultaneously supporting ribonucleotide reductase activity, caspase-8 blockade, RIPK1 degradation and RHIM-mediated necroptosis inhibition. Similar to that of M45, the RHIM of ICP6 also forms heteroamyloids with the RHIMs of host proteins at least *in vitro*, which may block necroptotic signalling [43]. In contrast to MCMV M45, which efficiently blocks necroptosis in both mouse and human cells [36], ICP6 promotes rather than inhibits necroptosis independently from RIPK1, TRIF or ZBP1 in mouse cells by directly interacting with and activating mouse RIPK3 [40, 41, 44]. This emphasises the necessity to study the function of viral proteins within the context of their natural host. The adaptation of viral R1 proteins such as M45, ICP6 and ICP10 through the incorporation of viral RHIMs appears to be an important evasion strategy to limit necroptosis. However, this scenario does not apply to all herpesvirus family members. Varicella zoster virus (VZV), which causes chickenpox and shingles, encodes a RHIM in its ORF20 capsid triplex protein, which forms heteroamyloids with the RHIMs of host the proteins RIPK3 and ZBP1 [45]. Of note, ZBP1-restricted growth of an ORF20 RHIM-mutant VZV strain was restored by chemical inhibition of caspase activity, indicating that the RHIM of ORF20 acts as an antagonist of ZBP1-mediated apoptosis rather than necroptosis [45].

RHIM-independent evasion of necroptosis

Some herpesviruses including human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) and members of the poxviruses do not express RHIM-containing proteins altogether. Instead these viruses developed RHIM-independent strategies to subvert necroptosis. The EBV latent membrane protein 1 (LMP1) blocks necroptosis at two levels: it interferes with normal ubiquitin attachment to RIPK1 and RIPK3 thereby blocking formation of the necrosome and reduces RIPK3 expression by inducing *RIPK3* promoter hypermethylation [46, 47]. HCMV-infected RIPK3-expressing human fibroblasts are resistant to necroptosis induced by TNF or by secondary infection with M45 RHIM-mutant MCMV *via* a mechanism that was reported to interfere downstream of phosphorylation of MLKL [48]. Although this work did not assign a role to UL36 as a viral inhibitor of necroptosis, another study reported that the UL36-encoded viral tegument protein and inhibitor of caspase-8 activation (see Box 3) binds to human MLKL and promotes its proteasome-mediated degradation, thereby inhibiting necroptosis [49]. A single mutation of cysteine 131 to arginine in UL36 abrogates both caspase-8 and MLKL binding, making it difficult to separate anti-apoptotic from anti-necroptotic functions of UL36 as has been done before by mutating the RHIM in the HSV1 ICP6 [40, 41, 49]. To work around this problem, Muscolino *et al.* engineered a chimeric M45 RHIM-mutant MCMV in which the caspase-8 inhibitor M36 has been replaced with UL36 from HCMV, showing that UL36 could substitute the necroptosis-inhibitory activity of M45 [50].

Orthopoxviruses including vaccinia virus encode the E3L protein, which contains two distinct dsRNA binding domains that are both essential for causing virulence [51]: the N-terminus of E3L includes a Zα domain, which specifically binds to Z-RNA and the C-terminus contains a classical dsRNA binding motif, which interacts with dsRNA in the typical A-conformation. While the C-terminal dsRNA binding motif is required to limit activation of the A-form dsRNA-activated innate immune receptors PKR and the OAS, the Zα domain sequesters viral

Z-RNA and specifically limits ZBP1-mediated necroptosis [25, 28]. Interestingly, E3L binding to A-form dsRNA stimulates formation of the Z-RNA agonist of ZBP1 [25]. This reveals a unique scenario whereby a viral protein designed to antagonise host innate immune responses generates another pathogen-associated pattern under the form of an alternate dsRNA structure. DsRNA binding proteins are expressed by many viruses to suppress innate immune responses [52]. Whether these viral proteins also stimulate Z-RNA formation and thus predispose to ZBP1-dependent necroptosis remains to be addressed.

Cowpox virus, mousepox ectromelia virus and the variola orthopoxvirus, the causative agent of smallpox, express yet another necroptosis inhibitor dubbed viral inducer of RIPK3 degradation (vIRD) [53]. Mechanistically, the ankyrin repeats of vIRD bind to RIPK3 and its F-box recruits the SKP1-Cullin1-F-box complex, which cooperate to promote the ubiquitin-mediated proteasomal degradation of RIPK3. Notably, vaccinia virus, which was used as a vaccine to eradicate smallpox, does not encode a functional vIRD and this may at least in part explain its attenuation and its success as a vaccine strain [53]. It should be noted that laboratory strains of vaccinia virus including the commonly used “wild type” Western Reserve have lost many genes compared to variola virus. Perhaps most notably are the viral decoy receptors of TNF [54], which could further sensitise vaccine strains to cell death. Finally, viral MLKL (vMLKL) homologues lacking cell membrane binding domains are present in many bird poxviruses and some mammalian poxviruses [55]. The vMLKL proteins from the related Cotia and Bean 58058 poxviruses inhibit necroptosis in human and mouse cells by binding to RIPK3, thereby preventing the interaction and activation of host MLKL. The function of vMLKL proteins from these viruses in the natural host species such as birds, which do not express RIPK3, remains unclear [56].

Necroptosis: a backup mechanism or a stand-alone process?

Blockade of the proteolytic activity of caspase-8, the initiator caspase of extrinsic apoptosis, greatly sensitises cells to the induction of necroptosis [14]. This phenomenon was first reported in pig cells that were infected with cowpox virus. Infection of these cells with wild type cowpox expressing the pan-caspase inhibitor CrmA resulted in necrosis-like death while CrmA-deficient cowpox induced apoptosis [14, 57]. At the time, this necrosis-inducing phenotype could not be ascribed to the capacity of CrmA to block caspase-8 activity. Detailed genetic studies of caspase-8 deficient mice now unequivocally show that the proteolytic activity of caspase-8 is required to block lethal activation of necroptosis during embryo development [58-62]. The precise molecular mechanism(s) by which caspase-8 limits necroptosis remain(s) incompletely understood and deactivating cleavage of RIPK1 and/or RIPK3 is thought to contribute to the necroptotic suppressive effect of caspase-8 [60, 63-66]. Importantly, caspase-8 inhibition is a strategy employed by large DNA viruses to prevent host cell extrinsic apoptosis (Box 3) and thus naturally sensitises virus-infected cells to necroptosis [67]. The emergence of the RIPK3-MLKL-dependent necroptotic host cell death pathway in vertebrates may have resulted from an evolutionary tug-of-war driven by the success of many large DNA viruses including herpesviruses and poxviruses to subvert caspase-8-mediated extrinsic apoptosis [3, 56, 67]. Indeed, the induction of necroptosis is an incredibly efficient way to halt viral replication as MCMV expressing M45 with a mutated RHIM fails to replicate in wild type hosts, while its replication is restored in ZBP1- or RIPK3-deficient mice [29]. Similarly, infection with E3L Zα domain-deficient vaccinia virus is efficiently cut short by the ZBP1-RIPK3 necroptotic signalling axis [28]. These infection models, which assigned crucial roles for necroptosis in antiviral immunity, however, all rely on the use of mutant viruses that fail to inhibit the necroptotic signalling axis. Together with the observation that caspase-8 deficient mice succumb to necroptosis-driven lethal inflammation this led to the conclusion that

necroptosis acts as a molecular backup system only when caspase-8 activity is perturbed. In other words, inhibition of caspase-8 activation is a prerequisite for the induction of necroptosis. The fact that many DNA viruses that deliberately block caspase-8 have incorporated parallel mechanisms to inhibit necroptosis, indicates that viruses seem to have prevailed and that necroptosis acting as a backup mechanism is an evolutionary dead end. Why then is this cell death pathway preserved in so many animal species? A clue may come from the study of viruses that do not express any (known) caspase-8 and necroptosis inhibitors. Influenza A virus infection engages both caspase-8-dependent apoptosis and MLKL-mediated necroptosis in parallel to each other and both host cell death pathways are required to mount protective immunity [26, 27]. Interestingly, cells either commit to an apoptotic or a necroptotic cell death programme and rarely do cells show features of activation of both forms of cell death [68]. Mouse macrophages that are infected with an M36/M45 double mutant MCMV strain, which is unable to block caspase-8 activation and necroptosis induction, exhibit the biochemical and morphological characteristics of both apoptosis and necroptosis [69]. In this case, both signalling pathways are activated within the same cell in a process called secondary necroptosis (not to be confused with **secondary necrosis**) and infection with this virus is only restored in caspase-8/RIPK3-doubly deficient mice.

Together, this shows that necroptosis not only proceeds under conditions when caspase-8 activity is blocked, but may act as a stand-alone mechanism to inhibit viral infection.

Viral evasion of pyroptosis

The execution of pyroptosis depends on the formation of large cytosolic protein complexes termed **inflammasomes**, which function as activation platforms for caspase-1 (Figure 2). Activated caspase-1 cleaves the inflammatory cytokines IL-1β and IL-18 into their biologically active forms and cleaves off a C-terminal inhibitory fragment from the pore-forming protein

GSDMD. The N-terminal part of GSDMD traffics to the plasma membrane and forms oligomeric pores, ultimately resulting in cell lysis [15]. Of note, detection of the bacterial cell wall component lipopolysaccharide by the human caspases-4 and -5 (or the mouse orthologue caspase-11), leads to the assembly of a complex called the non-canonical inflammasome. Activation of the non-canonical inflammasome plays a major role in antibacterial immunity and is likely not involved in antiviral defence [15]. In contrast to the well-defined viral mechanisms that suppress host cell apoptosis and necroptosis and aside from the early observations that poxvirus CrmA potently inhibits caspase-1 activity (see Box 3) [70], the viral evasion mechanisms of inflammasome activation are only slowly beginning to emerge and hint towards an important antiviral role for pyroptosis.

An ever-expanding group of innate immune sensors initiate inflammasome activation and trigger pyroptosis upon viral infection. These include the NOD-like receptor and pyrin domain containing (NLRP) family members NLRP1 and NLRP3 and the HIN200-containing proteins Absent in melanoma 2 (AIM2) and IFN-γ inducible protein 16 (IFI16; p204 in mice) [5, 71-74]. While AIM2 and IFI16 trigger pyroptosis upon recognition of viral dsDNA, both NLRP1 and NLRP3 seem to detect viral activities rather than directly sensing viral molecules in a process called effector-triggered immunity. For example, enteroviral proteases, which are essential for maturation of the viral polyprotein, inadvertently cleave and activate NLRP1 (see below) [72, 74]. Similarly, viroporins such as the influenza A virus M2 proton channel alter host cell membranes to promote viral replication and this change in membrane permeability leads to NLRP3-dependent pyroptosis [75]. In many cases, the exact mechanisms by which viruses trigger pyroptosis remains incompletely understood and often differ between humans and mice. For instance, viral dsRNA is capable of activating human NLRP1 but not its mouse orthologue and viral dsDNA triggers pyroptosis via the **cGAS-STING** signalling axis in human myeloid cells, while in mouse cells this function is primarily reserved for AIM2 [73, 76]. The

contribution of pyroptosis to antiviral resistance is complex and does not always benefit the host. Abortive human immunodeficiency virus (HIV)-1 infection induces IFI16-mediated pyroptosis and loss of CD4 T cells leading to progression to AIDS [77, 78]. Furthermore, excessive SARS-CoV-2-induced inflammasome activation and pyroptosis may underlie the development of damaging hyperinflammatory immune responses seen in severe COVID-19 cases [79, 80].

Viral antagonism of pyroptosis

While research on pyroptosis has mainly centred around NLRP3, recent findings assert important antiviral functions to NLRP1. Kaposi's sarcoma-associated herpesvirus (KSHV) encodes the viral NLRP1 homologue ORF63, which blocks NLRP1 inflammasome activation and subsequent caspase-1-mediated pyroptosis [81]. NLRP1 is highly expressed in keratinocytes and ORF63 may therefore specifically promote KSHV propagation in the skin. The vaccinia virus **vBCL2** protein F1L has a dual function and blocks NLRP1-mediated caspase-1 activation independently from its capacity to inhibit cell intrinsic apoptosis [82]. Viral proteases encoded by multiple picornaviruses cleave NLRP3 and GSDMD [83-85]. While these cleavage events inactivate both NLRP3 and GSDMD, cleavage of NLRP1 by the same viral proteases results in N-glycine-specific degron-mediated proteolysis of the autoinhibitory N-terminal fragment resulting in NLRP1 activation [72, 74]. The viral protease cleavage sites of both human NLRP1 and the mouse orthologue NLRP1B evolve rapidly and mimic the viral polyprotein cleavage sites, suggesting that NLRP1 proteins coevolved with enteroviral proteases to limit viral replication [72].

HSV-1 antagonises both AIM2- and IFI16-mediated inflammasome activation. The HSV-1 protein VP22 interacts with the AIM2 inflammasome preventing oligomerisation and activation, while the ubiquitin ligase activity of ICP0 targets IFI16 for proteasomal degradation

[86, 87]. The related rabbit poxviruses myxomavirus and Shope fibroma virus express viral pyrin-only proteins (vPOPs). Like their cellular orthologues, these vPOPs directly interact with apoptosis-associated speck like protein containing a CARD domain (ASC), a crucial inflammasome-adaptor protein, via Pyrin-Pyrin interactions. While host ASC promotes inflammasome activation, vPOPs inhibit the assembly of the NLRP3 inflammasome [87-89].

RNA viruses have also developed ways to block pyroptosis. NS1 from influenza A virus and the V protein from the paramyxovirus family members measles, Sendai and Nipah virus have been reported to block NLRP3 inflammasome activation [90-92]. The paramyxovirus human parainfluenza virus 3 (HPIV3) does not express a V protein. Instead, its C protein is able to prevent inflammasome signalling by inducing NLRP3 proteasomal degradation [93]. The coronavirus SARS-CoV-2 nucleocapsid protein was shown to block pyroptosis and IL-1β secretion from human monocytes by preventing caspase-1-mediated cleavage and activation of the pyroptosis executioner GSDMD [94]. To complicate matters, others have reported that the SARS-CoV-2 nucleocapsid promotes rather than inhibits pyroptosis by inducing the formation of an NLRP3-ASC complex [95]. Both studies, however, relied on SARS-CoV-2 nucleoprotein overexpression systems and it will be important to verify these findings using *in vivo* infection models to reconcile these conflicting results.

Finally, some viruses neutralise the biological activity of the inflammatory cytokines IL-1β and IL-18 that are released by pyroptotic host cells rather than preventing pyroptotic cell death itself. Examples are the B15 protein, which is secreted from vaccinia virus and cowpox virus infected cells and which acts as a scavenger for IL-1β [96], and IL-18 scavengers encoded by ectromelia virus p13 and Yaba monkey tumour virus 14L [97, 98]. Together, this multitude of viral evasion mechanisms shows that pyroptosis plays important roles in host immunity.

Concluding remarks

It is clear that viral antagonism of apoptosis, necroptosis and pyroptosis greatly contributes to virulence. The success of viruses to evade these cell death pathways may have driven the emergence of alternative routes leading to host cell death. Indeed, recent reports show that virus-induced caspase-3 activation results in the activation of GSDME, another member of the gasdermin family of pore-forming proteins [99, 100]. This lytic process, which is called apoptosis-driven secondary necrosis, results in membrane perturbation and the release of cellular components from apoptotic cells [100, 101]. Recent studies demonstrate that human granzyme A and granzyme B cleave and activate GSDMB and GSDME, respectively, thereby causing cancer cell lysis [80, 102]. Whether the granzyme A/GSDMB and granzyme B/GSDME cell death axes also eliminate virus-infected cells has not been tested. It will be interesting to determine whether inhibition of GSDME- and GSDMB-mediated host cell death is broadly employed by viruses to promote their propagation (see Outstanding Questions).

Given the vastness of the viral gene pool, it is reasonable to assume that many viral strategies to evade cell death remain unexplored. Viral antagonists of host cell death such as viral RHIM proteins, vPOPs and vFLIPs (see Box 3), were identified through protein sequence homology with their cellular counterparts. Many proteins adopt similar domain structures despite having unrelated amino acid sequences. Improvements of *in silico* structure prediction will greatly aid in the discovery of novel viral mimics of cellular protein folds that inhibit host cell death. Repeated passaging of viruses in cultured cells often results in loss of virulence factors and viral attenuation *in vivo*. Deliberate passaging of viruses into cell lines that lack cell death pathways may select for attenuated and clinically relevant viral strains that fail to limit host cell death. For example, the attenuated vaccinia poxvirus has lost the necroptosis inhibitory protein vIRD thereby restricting its growth. At the same time, vaccinia virus still greatly boosts an immune response through the release of DAMPs and antigens from infected necroptotic cells [53]. A

similar concept is emerging with the use of oncolytic viruses that specifically kill cancer cells and stimulate an anti-cancer immune response [103]. A clear understanding of the molecular mechanism by which viral proteins manipulate host cell death will inspire the rational design of new classes of antivirals that selectively interfere with viral antagonists of host cell death.

Outstanding Questions

- What is the contribution of the lytic cell death modes secondary necrosis, necroptosis and pyroptosis to activation of the innate and adaptive immune response?
- What is the function of GSDMB- and GSDME-mediated cell death in antiviral immunity and do viruses antagonise pore formation by GSDMs?
- Is there crosstalk between the different modes of antiviral cell death and what determines the switch between caspase-8-driven extrinsic apoptosis and necroptosis?
- Which inflammasomes restrict viral infection in humans and mice, how are they activated and which viral mechanisms block pyroptosis induced by activation of these inflammasomes?
- Is viral attenuation *in vivo* the result of loss of function of viral inhibitors of cell death and can we exploit this to generate novel vaccine strains with enhanced immunogenicity or improve the efficacy of oncolytic viruses?

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645

Glossary

Amyloid: An aggregation of proteins forming fibrillary structures generated by stacking of β-sheet structures.

Caspases: Family of cysteine aspartic proteases with important roles in regulated cell death. Caspases have a cysteine residue in their active site and predominantly cleave their substrates on the C-terminal site of an aspartate residue.

cGAS-STING: The main innate immune pathway by which viral dsDNA is detected. Upon binding to dsDNA, cyclic GMP-AMP synthase (cGAS) catalyses the formation of the secondary messenger cyclic GMP-AMP (cGAMP), which binds to and activates stimulator of interferon genes (STING). STING activation then induces the production of type I interferons, cytokines with potent antiviral activity.

DAMPs: Intracellular molecules that are released during lytic cell death and which are recognised by innate immune receptors to trigger an inflammatory response.

Death receptors: Protein members of the TNF superfamily characterised by the presence of a so-called death domain that exerts important functions in immunity.

Gasdermin (GSDM): GSDMs are characterized by the presence of a conserved N-terminal GSDM domain (N-GSDM). Proteolytic cleavage results in release of the N-GSMD from the autoinhibitory C-terminal end, followed by membrane translocation, oligomerisation and pore formation.

Inflammasome: Large multiprotein complexes consisting of pathogen recognition receptors (e.g. NLRP1, NLRP3, AIM2 or IFI16), caspase-1 and in most cases the adaptor protein ASC. They function as activation platforms for caspase-1, which processes IL-1β and IL-18 into their mature forms and cleaves and activates GSDMD resulting in membrane perforation and cytokine secretion.

MLKL: The executioner protein of necroptosis. Upon activation through phosphorylation by RIPK3, MLKL oligomerises and forms pores in the cell membrane.

RHIM: Receptor-interacting protein homotypic interaction motif is a conserved motif consisting of a core amino acid tetrad I/V-Q-V/I/L/C-G surrounded by β-sheet-formin hydrophobic residues. RHIM-RHIM interactions stimulate amyloid structure formation of host and viral RHIM-containing proteins.

Secondary necrosis: A mode of autolytic regulated necrotic cell death that occurs in cells that underwent caspase-dependent apoptosis, but have not been cleared by phagocytes. Recent evidence shows that this process is - at least in some cell types - driven by GSDME.

TLR: A class of pattern recognition receptors present at the cell surface or within endosomes, which specifically recognise microbial nucleic acids or proteins.

vBCL2: Viral homologs of the cellular antiapoptotic BCL2 proteins, encoded by a multitude of DNA viruses. vBCL2s block the intrinsic apoptosis pathway to favour viral replication.

ZBP1: Z-DNA binding protein 1, also known as DAI is an innate immune receptor for Z-form dsRNA, which accumulates upon infection with RNA and DNA viruses. ZBP1 signals via RIPK3 to induce apoptosis and/or necroptosis.

Text Boxes

Box 1: Programmed cell death, regulated cell death and inflammation. The terms programmed cell death and regulated cell death are often used interchangeably to refer to the genetically encoded and tightly regulated processes to remove a surplus of cells from an organism. The removal of cells, however, has different functions. On the one hand, cell death is an integral part of development (e.g. the removal of interdigital cells or dysfunctional neurons during brain development) and of tissue maintenance (e.g. during mammary gland involution or immune cell development). These processes are largely mediated by apoptosis and dead cells are immediately removed by tissue macrophages. The engulfment of apoptotic cells triggers an immunosuppressive programme to maintain an immunologically silent environment. These immunologically inert cell death processes that support normal physiology are referred to as programmed cell death [13]. On the other hand, cell death also occurs in response to stress imposed by factors that disrupt homeostasis including virus infection. In this case, the induction of cell death pathways including necroptosis and pyroptosis, but also apoptosis, often coincides with an inflammatory immune response. For example, the detection of virally-derived nucleic acids by the innate immune system results in the production of inflammatory cytokines including type I and type III interferons [104]. This renders virus-infected cells inherently inflammatory, regardless of the mode of cell death. Similarly, ionising radiation or chemotherapy induces cell death of malignant cells, which stimulates an anti-cancer immune response, a process referred to as immunogenic cell death [1, 105]. Finally, genetic mutation of regulatory components of cell death pathways results in chronic and excessive cell death including apoptosis and underlies the development of human autoinflammatory syndromes [17, 18]. To indicate that these (inflammatory) cell death processes, which are triggered by genetic or exogenous insults, are not disordered but still rely on tightly controlled molecular signalling cascades, these modes of cell death are collectively referred to as regulated cell death.

Box 2: Proviral vs. antiviral cell death. Historically, virus-induced cell death was thought to promote rather than restrict viral replication. Many viruses sustain a lytic life cycle causing the host cell to lose its integrity, thereby promoting egress of viral progeny. Prolonged perturbance of normal cell function by the viral replication process may eventually trigger host cell death [10]. A prime example is the adenovirus E1A protein which forces S-phase entry by inactivating pRb, thereby instructing the cell to produce deoxynucleotide building blocks to generate new viral DNA genomes [106]. This process causes p53 activation and induces intrinsic apoptosis [107]. At the same time, another adenoviral protein E1B-55K binds to p53 to block its pro-apoptotic activity [107]. It is likely that the activities of viral cell death-inducing proteins and of those that antagonise host cell death signalling strike a balance between survival to allow at least one replicative cycle and death allowing the release of new virions. Accordingly, viruses with fast replication kinetics including many RNA viruses and small DNA viruses may proportionally devote less of their protein coding capacity to counteract host cells death. Indeed, many studies that characterised the viral evasion strategies of apoptosis, necroptosis and pyroptosis have been performed in large DNA viruses including herpesviruses and poxviruses. These viruses support longer replication cycles and would benefit the most from prolonging host cell survival.

Box 3: Viral escape of caspase-8-mediated apoptosis activation. Caspase-8 is the critical initiator caspase of the extrinsic apoptotic pathway, also called the death receptor pathway. Caspase-8 activation is initiated by binding of death ligands of the tumour necrosis factor (TNF) superfamily including Fas ligand (FasL), TRAIL and TNF to their cognate membrane-bound receptors [12]. The signals for death receptor-mediated killing of virus-infected cells originate among others from cytotoxic T cells and NK cells, which express FasL, TRAIL or TNF on their

cell surface [9]. Viruses evolved many ways to block caspase-8 activity (Figure I) [67]. The first identified viral caspase(-8) inhibitor is the cowpox virus cytokine modifier A (CrmA), a serpin-like protein with broadly acting serine and cysteine protease inhibitory activity. CrmA was originally described as a caspase-1 inhibitor, but also potently inhibits caspases-8, -10 and granzyme B [70, 108]. Homologues of CrmA are found in most poxviruses and include the vaccinia virus B13R (SPI-2) and B22R proteins [20]. Viral inhibitor of apoptosis proteins (vIAPs) represent another class of broad-spectrum caspase inhibitors. Viral IAPs were identified before their cellular homologues in baculoviruses infecting butterflies, moths and flies [109, 110]. Most viral and cellular IAPs contains at least one baculovirus IAP Repeat (BIR) motif and a C-terminal really interesting new gene (RING) domain conferring ubiquitin ligase activity [111]. Nearly all of the ~200 vIAPs identified to date are encoded by large DNA viruses that infect insects, suggesting that mammalian viruses co-opted different strategies to evade apoptosis [109]. Given the fact that vIAPs coevolved with insect hosts, it came as a surprise that the prototypical vIAP p35 from *Autographa californica* nuclear polyhedrosis virus acts as a potent antagonist of human caspases-1, -3, -6, -7, -8, and -10 [112]. Structural analysis showed that p35 covalently binds to the catalytic site of caspase-8 thereby acting as an irreversible pseudo-substrate [113]. In humans, the name IAP is somewhat of a misnomer as only the X-linked IAP (XIAP) among the eight human IAP family members directly inhibits caspase activation [114]. Similarly, not all vIAPs have anti-apoptotic activity and may exert different functions to modulate host immunity [109]. Other viral proteins exhibit narrower specificity towards caspase-8. For instance, viral inhibitor of caspase-8 activation (vICA) encoded by UL36 in human and M36 in mouse CMV and the UL39-encoded HSV-1 ICP6 and HSV-2 ICP10 proteins, specifically interact with caspase-8 and inhibit its proteolytic activity thereby protecting CMV- and HSV-infected cells against TNF- and cytotoxic T cell-mediated killing [69, 115-118]. Finally, members of the γ-herpesviruses and the molluscipoxvirus express viral

761 homologues of c-FLIP (v-FLIPs) that resemble the short c-FLIP isoform lacking the
762 catalytically dead protease domain of caspase-8. Like their cellular counterpart, v-FLIPs are
763 potent inhibitors of caspase-8 activation downstream of Fas, TNF and TRAIL-induced
764 apoptosis [119].

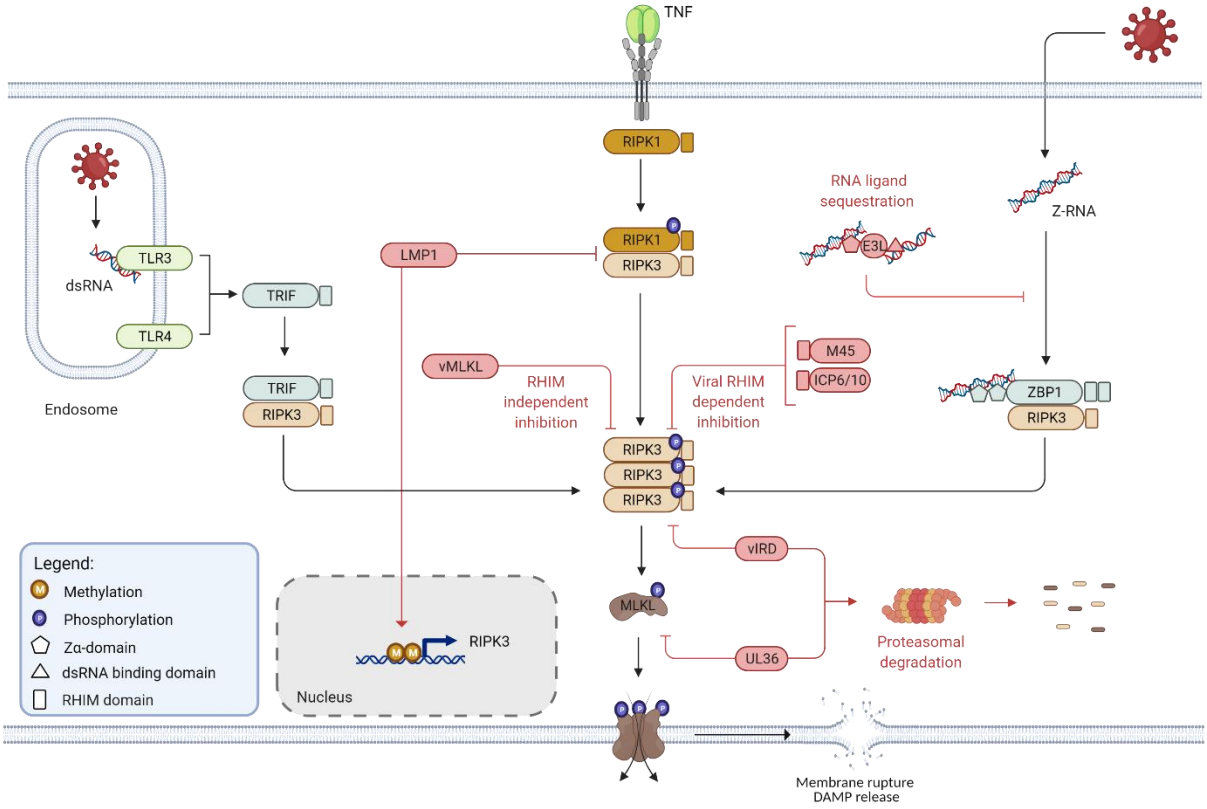
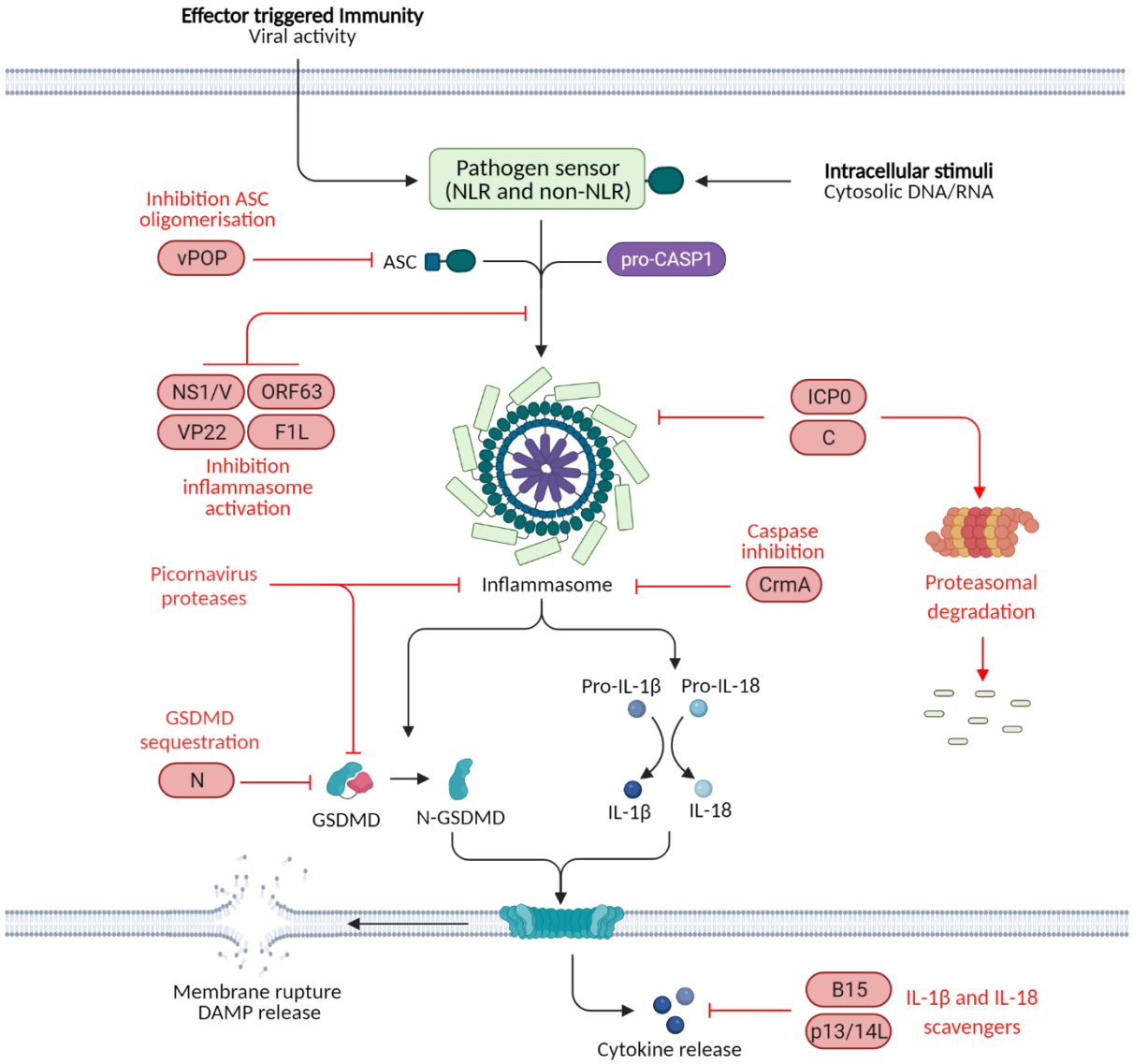


Figure 1: Necroptosis signalling and viral evasion strategies. Necroptosis is induced by phosphorylation and subsequent oligomerisation of the pore-forming protein MLKL at the plasma membrane. MLKL activation depends on its phosphorylation by RIPK3, which assembles into amyloid signalling complexes. Three RHIM-containing necroptosis-activating molecules induce RIPK3 activation: RIPK1, ZBP1 and TRIF. At least six different viral escape mechanisms counteract necroptosis. (1) The sequestration of Z-RNA ligands by poxvirus E3L, which prevents ZBP1 activation. (2) Prevention of the formation of the necrosome by EBV encoded LMP1 (3) Inhibition of functional RIPK3 amyloid formation through the herpesviral RHIM proteins M45, ICP6 and ICP10. (4) Inhibition of host MLKL interaction with RIPK3 through viral MLKL homologues (vMLKL). (5) Proteasome-mediated degradation of RIPK3 and MLKL by poxvirus vIRD and HCMV UL36 (6) Inhibition of RIPK3 transcription *via* hypermethylation of its promotor by EBV LMP1.

Abbreviations: EBV – Epstein-Barr virus; HCMV – Human Cytomegalovirus; RHIM – RIP homotypic interaction motif



780

781 **Figure 2. Pyroptosis signalling and modulation by viral proteins.** NLR (NLRP1 and
782 NLRP3) and non-NLR (AIM2 and IFI16) family members interact with caspase-1, either
783 directly (not illustrated) or indirectly *via* the ASC adaptor protein. The resulting complex is
784 termed the inflammasome and leads to caspase-1 activation. Active caspase-1 cleaves the pro-
785 inflammatory cytokines IL-1β and IL-18 and GSDMD. Cleavage of GSDMD releases the N-
786 terminal fragment (N-GSDMD) from the auto-inhibitory C-terminal fragment, followed by
787 plasma membrane translocation, oligomerisation and pore formation. Ultimately, this results in
788 export of IL-1β and IL-18, plasma membrane rupture and release of DAMPs. Several

mechanisms of viral antagonism of pyroptosis have been described: viral POPs (vPOPs) interact with ASC thereby preventing inflammasome assembly. IAV NS1 and the V protein of several paramyxoviruses block NLRP3 activation. KSHV ORF63 and VACV F1L prevent NLRP1 inflammasome assembly, while HSV-1 VP22 antagonises AIM2 activation. HSV-1 ICP0 and HPIV3 C inhibit the NLRP3 inflammasome by inducing its proteasomal degradation. SARS-CoV-2 N has been reported to interact with GSDMD and prevent proteolytic activation. Picornaviral proteases prevent inflammasome signalling by cleaving NLRP3 and GSDMD. Some viral proteins (e.g. cowpox CrmA) inhibit caspase-1 activity, preventing downstream inflammasome signalling. Lastly, vaccinia virus B15 functions as an extracellular IL-1β scavenger, while ectromelia virus p13 and Yaba monkey tumour virus 14L neutralize IL-18.

Abbreviations: ASC – apoptosis-associated speck-like; DAMP – Damage-associated molecular pattern; GSDMD – Gasdermin D; HSV-1 – Herpes Simplex virus 1; HPIV3 – Human parainfluenza virus type 3; KSHV – Kaposi's sarcoma-associated herpesvirus; NLR – Nod-like Receptor.

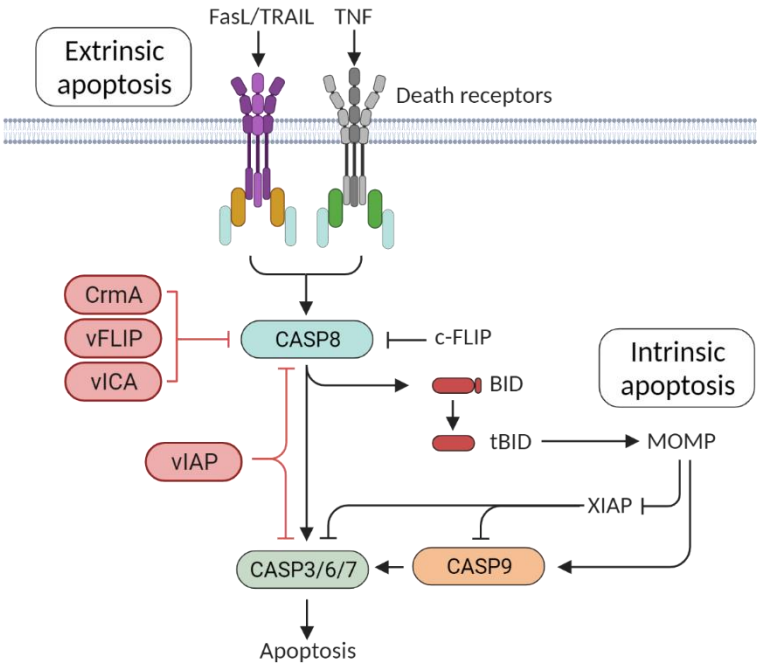


Figure I, related to Box 3: Viral escape of caspase-8 activation. Binding of death ligands (FasL, TRAIL, TNF) to their cognate death receptors results in assembly of caspase-8-containing signalling complexes, inducing extrinsic apoptosis signalling. This leads to activation of caspase-8, which cleaves executioner caspases-3, -6 and -7. In some cell types, caspase-8 cleaves BID to simultaneously engage the intrinsic apoptosis pathway. During intrinsic apoptosis signalling, tBID or intracellular stimuli lead to MOMP, ultimately resulting in inactivation of XIAP and activation of caspase-9. Activated caspase-9 eventually cleaves executioner caspases-3, -6 and -7. Several viral proteins interfere with different apoptotic caspases: e.g. vFLIPs, Cytomegalovirus vICA, vIAPs and cowpox CrmA.

Abbreviations: CASP – Caspase; MOMP – Mitochondrial outer membrane permeabilization; tBID – Truncated BID