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Review

Vaccinia virus evasion of regulated cell death

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ABSTRACT

Regulated cell death is a powerful anti-viral mechanism capable of aborting the virus replicative cycle and alerting neighbouring cells to the threat of infection. The biological importance of regulated cell death is illustrated by the rich repertoire of host signalling cascades causing cell death and by the multiple strategies exhibited by viruses to block death signal transduction and preserve cell viability. Vaccinia virus (VACV), a poxvirus and the vaccine used to eradicate smallpox, encodes multiple proteins that interfere with apoptotic, necroptotic and pyroptotic signalling. Here the current knowledge on cell death pathways and how VACV proteins interact with them is reviewed. Studying the mechanisms evolved by VACV to counteract host programmed cell death has implications for its successful use as a vector for vaccination and as an oncolytic agent against cancer.

Contents

1. Introduction

Since the word apoptosis was coined [1] a great deal has been learnt about regulated cell death. Cell death is regulated by several well-defined molecular mechanisms that can be altered genetically or pharmacologically. Regulated cell death, as opposed to accidental cell death, occurs in response to modifications in the intracellular environment, such as DNA damage, energy stress or hypoxia, or changes in the extracellular milieu. Intrinsic challenges to cell survival initiate signalling pathways that converge on mitochondria, which are responsible for oxygen-dependent synthesis of ATP. Mitochondrial sensing of threats originating from within the

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cell leads to cytochrome *c* release and the irreversible activation of proteolytic enzymes known as caspases [2]. Mitochondrial-induced cell death thus constitutes an ancient regulatory mechanism to ensure organ development and tissue homeostasis. However, it is also a host defence mechanism against intracellular pathogens, such as viruses, because early, regulated cell suicide restricts completion of the viral life cycle and thereby viral production and spread. Indeed, the interplay between viruses and regulated cell death depicts a fascinating evolutionary arms race. Viral targeting of mitochondrial cell death is common and is likely to have exerted evolutionary pressure on the host to establish a mitochondrion-independent pathway for the activation of caspases. The 'extrinsic' pathway is triggered by extracellular ligands such as cytokines of the tumour necrosis factor (TNF) family, which are released rapidly in response to viral infection. The anti-viral potency of apoptosis is illustrated by the evolution of viral inhibitors targeting both extracellular and intracellular components of the signalling cascade including the initiator caspase-8 [3]. Experiments using vaccinia virus (VACV) have, however, demonstrated that viral countermeasures can be overturned by regulated necrosis, also known as necroptosis [4], a caspase-independent cell death mechanism that is likely to have evolved to counteract viral inhibition of caspases. Necroptosis, as opposed to apoptosis, has a clear pro-inflammatory nature. It is followed by the release of pathogen- and endogenous danger-associated molecular patterns (PAMPs and DAMPs) that are recognised by pattern recognition receptors (PRRs) that, for instance, activate inflammasomes and the production of pro-inflammatory cytokines, chemokines and interferons and thereby facilitate inflammatory cell recruitment to the site of infection [5,6]. Inflammasome activation promotes maturation and release of the pro-inflammatory cytokines interleukin (IL)-1β, IL-18 and IL-33 and the induction of pyroptosis, an inflammatory form of cell death mediated by caspase-1 [7].

VACV is the prototypic and best studied member of the *Poxviridae* and is a large DNA virus that replicates in the cell cytoplasm and dedicates between one third and one half of its coding capacity to counteract the host response to infection [8]. The means by which VACV interferes with the immune system is extensive, just as it is with host cell death, and studies on VACV-host interactions have contributed insights into the biology of infection. In this review, we describe the mechanisms evolved by VACV to evade host regulated cell death, namely apoptosis, necroptosis and pyroptosis, and discuss the importance of understanding such evasion strategies for the development of more effective vaccines and oncolytic agents. Although VACV encodes a number of extracellular proteins targeting IL-1β [9,10], IL-18 [11–14], or TNF [15], and hence with the capacity to modulate apoptosis, necroptosis and pyroptosis, these have been reviewed elsewhere [8,16] and are not considered here.

1.1. Activation of mitochondrial apoptotic cell death

Apoptosis, or programmed cell death, mediates the ordered disassembly of a cell from within, keeping the plasma membrane intact, thus avoiding the release of potentially damaging intracellular components and minimising damage to neighbouring cells [17]. The ensuing small vesicles called apoptotic bodies, which contain cellular debris, are subsequently taken up and destroyed by phagocytes. Cell disassembly is performed by caspases and orchestrated at the mitochondrion by B-cell lymphoma (Bcl)-2 and Bcl-2-like proteins. The Bcl-2 family is composed of anti-apoptotic proteins sharing similarity in Bcl-2-homology (BH) domains 1, 2, 3 and 4, and pro-apoptotic proteins, which only share homology to BH3 domains [18]. A complex protein–protein interaction network is established between these proteins to regulate cell suicide. Upon apoptotic challenge pro-apoptotic BH3-only proteins Bid, Bim and Puma activate effectors Bax and Bak [19], which oligomerise and assemble pores in the mitochondrial membrane. These pores allow the efflux of cytochrome *c*, amongst other proteins, from the mitochondrial intermembrane space to the cytosol. Cytosolic cytochrome *c* can assemble the apoptosome complex, composed of the apoptosis protease-activating factor-1 (APAF-1) and procaspase 9, which induces activation of executioner caspase-3 and −7, ultimately leading to cell death [20] (Fig. 1). Anti-apoptotic proteins (Bcl-2, Bcl-xl, Bcl-w, Mcl-1, and Bfl-1) interfere with activator BH3-only proteins to prevent Bax/Bak activation, although a direct interaction with the latter can also occur [21]. The final outcome is also modulated by other BH3-only proteins (e.g. Bad, Noxa, Bik) that sequester anti-apoptotic proteins liberating activator and effector pro-apoptotic factors. Recently, crystallographic data has provided a model to understand Bax/Bak oligomerisation. BH3-only proteins interact with Bak/Bax hydrophobic pocket triggering a conformational change in Bax/Bak that exposes their BH3 domains [22,23]. Exposed BH3 domains from these 'activated' Bax/Bak monomers can be recognised by hydrophobic pockets of other Bax/Bak monomers, effectively forming dimers and becoming themselves 'activated'. This process leads eventually to the formation of oligomers of Bax and Bak [24]. Despite this insight, how the intricate Bcl-2 protein network is organised remains the focus of ongoing research. Binding affinities between Bcl-2 members have been established, but mostly in solution using *in vitro* BH3 peptides that might not reflect the environment encountered at the mitochondrial membrane with whole proteins [25] and the temporal dynamics of the interactions [21,26]. In addition, many family members are tissue or cell type specific [27]. These factors contribute to the diversity of apoptotic thresholds observed in experimental and clinical settings.

The activation of apoptosis induced by stress in the secretory pathway, namely the endoplasmic reticulum (ER) and the Golgi apparatus, is also relevant to this review. The ER is where nascent polypeptide strands are folded and first post-translationally modified. These proteins are then sorted and packaged into cargo vesicles destined for export or transport to different parts of the cell. Stress within the secretory pathway is an alternative 'from-within' cell suicide signal deriving from the accumulation of misfolded proteins, viral infection, depletion of luminal Ca^{2+} stores, or transmembrane protein interactions leading to apoptosis [28,29]. Although ER stress has been studied predominantly and no Golgi-specific stress sensors have been well-characterised, the continuous mixing of ER and Golgi contents is likely to contribute to the integration of stress signals detected in either of these organelles [28]. In the event that the ER and/or Golgi apparatus become stressed, an unfolded protein response (UPR) is initiated as a pro-survival signal to activate several repair systems. However, a prolonged UPR leads to apoptosis [30]. Amongst the different transmembrane kinases activated by an UPR, inositol-requiring enzyme 1 (IRE-1) is crucial in recruiting c-Jun N-terminal kinase (JNK) [31]. JNK regulates several Bcl-2 family members by phosphorylation and can ultimately lead to Bax/Bak activation and mediate cytochrome *c* release (reviewed in [32]). In addition to IRE-1, Ca^{2+} levels also contribute to the UPR because the protein folding monitoring function of Ca^{2+}-binding chaperones such as calnexin and calreticulin is inactivated by reduction in Ca^{2+} concentration [33].

1.2. Death receptor-mediated apoptosis

Extrinsic receptor-mediated apoptosis is triggered by extracellular death ligands such as Fas ligand (FasL) or TNFα (Fig. 1). Engagement of their cognate receptors causes the recruitment of several adapter proteins such as Fas-associated death domain protein (FADD), TNF-receptor [TNFR]-associated death domain protein (TRADD), TNFR-associated factor 2 (TRAF2) or receptor-interacting

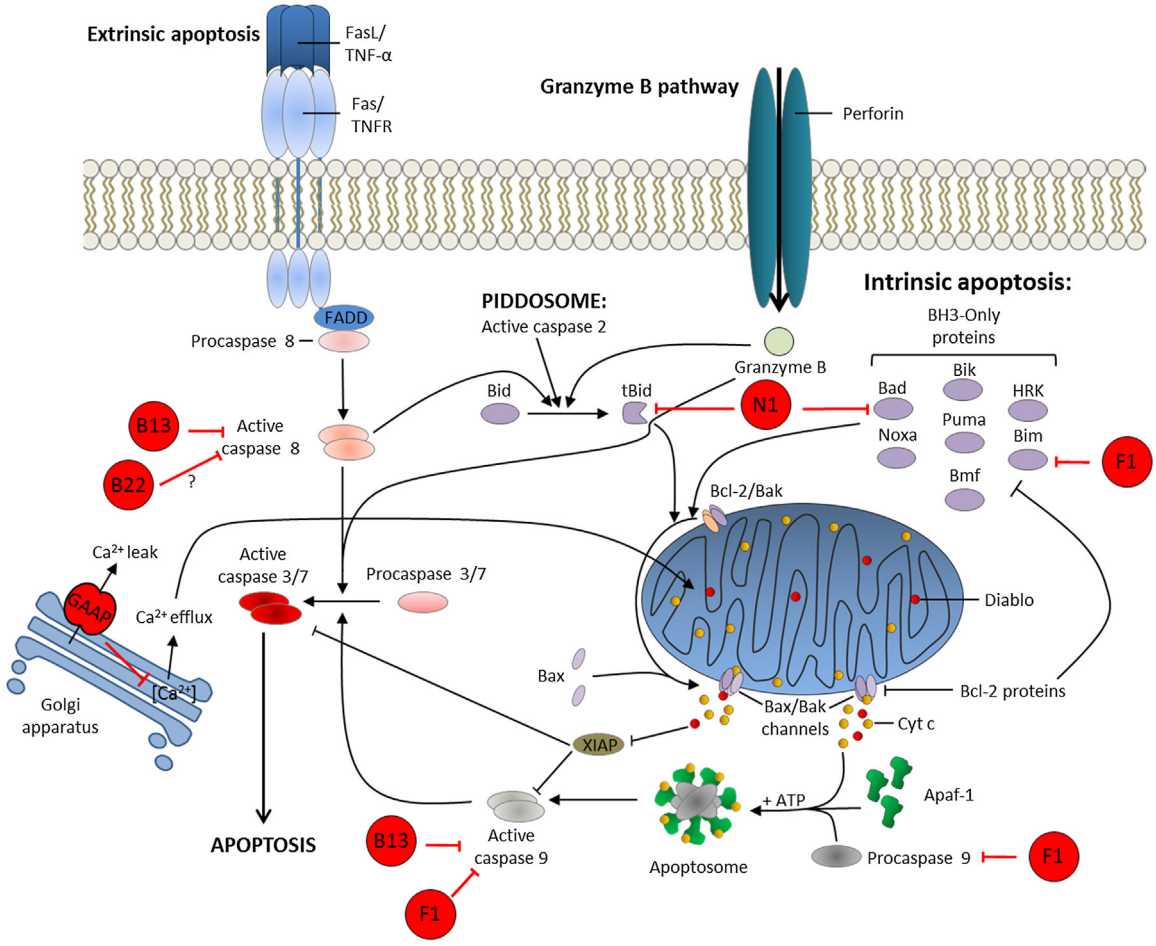

Fig. 1. Inhibition of apoptotic cell death by VACV proteins. The apoptosome complex consisting in APAF-1 and pro-caspase-9 is formed in response to the release of cytochrome *c* from the mitochondrion and leads to the activation of executioner caspases 3 and 7. In normal conditions, cytochrome c is stored inside the mitochondrial membrane, but upon cell intrinsic challenges and activation of BH3-only proteins, a conformational change occurs in effector proteins Bax and Bak, which oligomerise on the mitochondrial membrane forming channels. VACV protein F1 blocks this signalling cascade by targeting Bim, Bak and caspase-9, whereas protein N1 does so targeting Bad and Bid. The mitochondrion is also sensitive to other intrinsic challenges independent of the BH3-only family. Sudden increases in Ca^{2+} release from the ER and Golgi apparatus activate JNK and ultimately lead to Bax/Bak activation. Some VACV strains prevent this by expression of the channel-like protein vGAAP and depleting the levels of Ca^{2+} in the intracellular stores. Cell extrinsic challenges can also trigger activation of caspase-3 and −7 via the receptor mediating death signalling pathway and the activation of caspase-8. Proteins B13 and B22 target caspase-8, thus blocking extrinsic apoptosis. Inhibition of intrinsic apoptosis by B13 has also been demonstrated due to its ability to act as a pan-inhibitor of caspases.

protein 1 (RIP1). The formation of this death-inducing signalling complex (DISC) is essential to create a scaffold complex that recruits and activates pro-caspase-8 (Fig. 2). This complex is commonly known as ripoptosome or complex II [34,35]. After it has been cleaved proteolytically, free caspase-8 activates caspase-3, −6 and −7 to initiate apoptosis. The extrinsic receptor-mediated pathway is regulated at different stages. First, the silencer of death domain protein (SODD), an intracellular TNFR inhibitor, prevents constitutive signalling [36]. Secondly, cellular inhibitor of apoptosis proteins (cIAPs) prevent the release of DISC and stimulate TNFα-mediated activation of nuclear factor κB (NF-κB) [37]. Lastly, cFLIP (FLICE/caspase-8 inhibitory protein) sequesters pro-caspase-8 and negatively regulates its protease activity [38]. Similar strategies for interfering with TNF-induced cell death are used by several viruses, particularly herpes viruses [3].

Double stranded (ds)RNA also has the potential to induce mitochondria-independent apoptosis, via toll-like receptor 3 (TLR3) and the recruitment of caspase-8 [39]. Viral inhibitors acting at this level can therefore block both TLR3- and TNF-induced death. Conversely, in certain cell types, commonly known as type II cells, death receptor signalling is amplified via the mitochondrion. Active caspase-8 cleaves Bid and generates a 15-kDa protein named truncated Bid (tBid), which translocates into the mitochondrion and induces Bax/Bak oligomerisation [40,41] (Fig. 1). Coexpression of Bcl-xl inhibits tBid-mediated pro-apoptotic effects, thus explaining why viral anti-apoptotic proteins acting at the mitochondrion have also the potential to diminish extrinsically-induced apoptosis.

1.3. The alternative, caspase-independent cell death: necroptosis

Besides extrinsic apoptosis, death receptor signalling unleashes a second, alternative regulated cell death programme that becomes activated when caspase-8 activity is compromised (reviewed in [34]) (Fig. 2). Caspase-independent programmed necrosis (necroptosis) follows the formation of a ripoptosome complex lacking caspase-8 (e.g. deficient cells) or containing catalytically inactive caspase-8 (e.g. pharmacological inhibition, viral infection). In this complex, caspase-8 susceptible, pro-necrotic kinases RIP1 and RIP3 become stabilised, interact through RIP homotypic interaction motif (RHIM) and establish a phosphorylation-dependent complex able to activate downstream effectors [4,42,43] (Fig. 2).

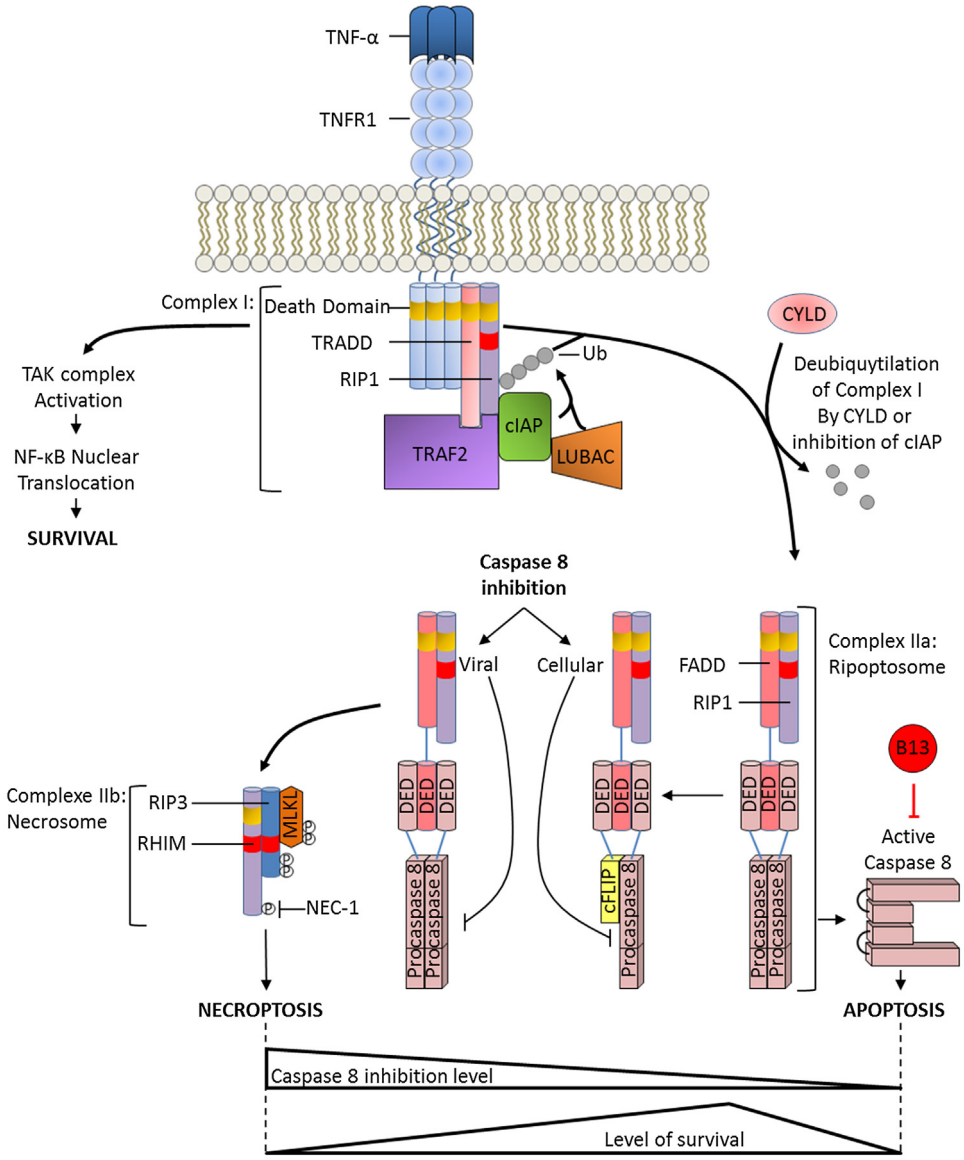

Fig. 2. Activation and regulation of the death receptor signalling cascade. Members of the TNF superfamily can trigger survival or death signals. Upon receptor engagement they induce the formation of a protein complex (I) that leads to activation of the transforming growth factor β-activated kinase 1 (TAK1) and a pro-survival signal that includes NF-κB activation. This complex is characterised by extensive ubiquitin chains. In the absence of these or in the presence of deubiquitylases, the receptor complex derives in the formation of an alternative complex known as ripoptosome or complex II that leads to activation of caspase-8 and apoptotic death. VACV protein B13 inhibits caspase-8 and consequently blocks the death signal deriving from complex II. This action, however, has the potential to unleash the necroptotic death pathway that is activated when extrinsic apoptotic agonists that cannot signal due to compromised caspase activity.

Amongst several suggested RIP1-RIP3 targets, the pseudo-kinase mixed lineage kinase domain-like protein (MLKL) has emerged as a critical effector of necroptosis [44,45]. When MLKL is doubly phosphorylated by RIP3, it oligomerises and associates with membrane compartments leading to cytosolic leakage. MLKL activation and necroptosis can also occur downstream of a non-canonical ripoptosome deriving from TLR3 and TLR4 signalling. In this case, TIR domain-containing adaptor protein inducing interferon-β (TRIF) mediates recruitment of RIP1 through its RHIM domains [34]. Necroptosis can be reversed with necrostatin-1, a chemical inhibitor of RIP1 [46], or necrosulphonamide, which prevents MLKL oligomerisation [44] (Fig. 2). No specific inhibitors of RIP3 have, however, been described, perhaps because they activate caspase-8 apoptosis, as reported recently with a kinase-dead RIP3 mutant [47]. These observations reflect the tight balance established between apoptotic and necroptotic pathways.

1.4. Pyroptotic cell death

Pyroptosis is a more recently identified form of cell death characterised by plasma membrane rupture and release of pro-inflammatory intracellular contents. Its dependence on caspases has complicated its discrimination from apoptosis, but pyroptosis is highly inflammatory and morphologically and mechanistically distinct from other forms of cell death [48]. Pyroptosis occurs upon activation of inflammatory caspases: caspase-1 (known formerly as IL-1-converting enzyme [ICE]) and the human homologues of murine caspase-11 (caspase-4 and −5) [49,50]. Caspase-1 activation derives from of a multiprotein complex named the inflammasome (Fig. 3). Members of this complex belong to the nucleotide-binding domain and leucine-rich repeat containing receptor (NLR) family, the absent in melanoma 2 (AIM2)-like family, or the tripartite motif (TRIM) family [7,51,52]. Members

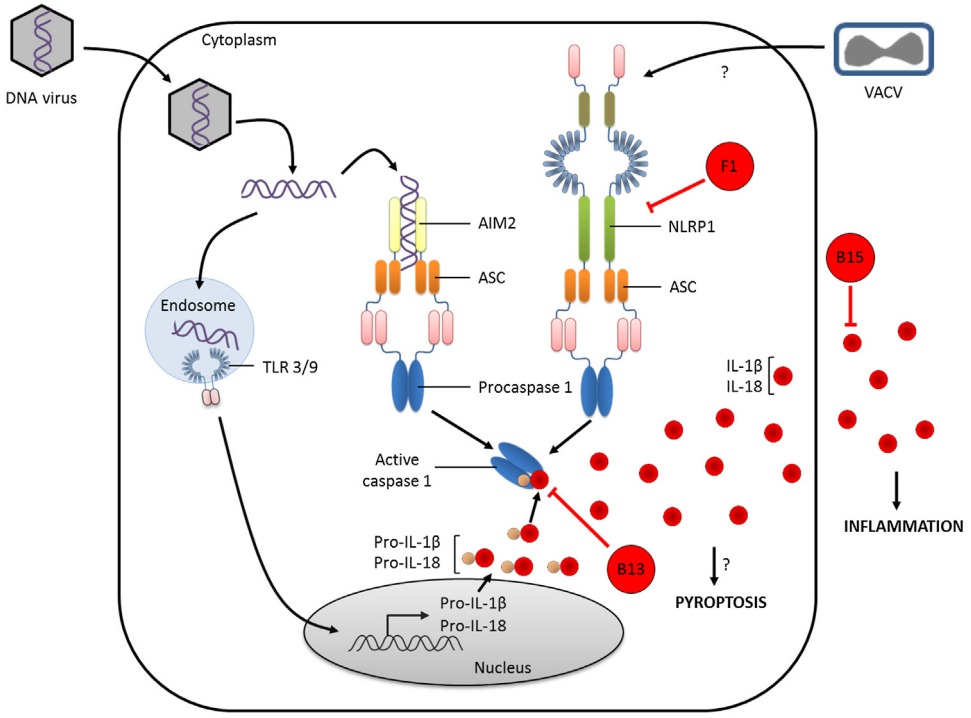

Fig. 3. Inhibition of pyroptotic cell death by VACV proteins. Activation of caspase-1 can induce cell death and the maturation and release of pro-inflammatory intracellular contents. A variety of intracellular complexes known as inflammasomes can become activated in response to viral infection and promote the maturation of pro-caspase-1 into active caspase-1. VACV can counteract the pyroptotic machinery using protein B13, which acts intracellularly inhibiting caspase-1 directly, and protein B15, which acts extracellularly as a soluble IL-1β receptor. In addition, protein F1 has the capacity to block the NLRP1 inflammasome and consequently prevent the upstream activation of caspase-1.

of these families act as PRRs in response to a variety of infectious agents and signal through either a caspase activation and recruitment domain (CARD) or a pyrin domain, which requires the adapter ASC (apoptosis-associated speck-like protein containing a CARD). Inflammasomes recognise multiple PAMPs including those produced during VACV infection [53,54]. Unlike canonical inflammasomes, recent work has demonstrated that caspase-11, −4 and −5 bind directly to PAMPs such as lipopolysaccharide (LPS), which illustrated a new mode of caspase activation not observed for apoptotic caspases [55]. Whether inflammatory caspases can recognise viral PAMPs directly is unknown. Irrespective of how they become activated, inflammatory caspases promote the proteolytic maturation of pro-IL-1β, pro-IL-18 and pro-IL-33 to the mature forms of these cytokines and their release from the cell. These events also trigger the induction of pyroptotic cell death, which causes the release of IL-1α and high-mobility group box chromosomal protein B1 (HMGB1) [49,56].

2. Inhibition of programmed cell death by vaccinia virus (VACV)

VACV is a large DNA virus with a broad cell tropism and a rapid replication cycle. VACV modulates many biological properties of the infected cell to aid virus replication and spread and suppress the host innate immune response. In this context an early and efficient inhibition of host cell death is paramount for the virus and accordingly, VACV expresses several proteins to either suppress upstream signals leading to death or inhibit the activity of downstream effectors, such as caspases. All these viral factors are expressed early after infection, before viral genome replication is initiated, so that cell viability during infection is ensured. A list of VACV proteins affecting programmed cell death is presented alongside their location in the viral genome (Fig. 4) and their site of action in each death signalling pathway (Figs. 1–3 and Table 1). For earlier reviews on this topic see [57,58].

2.1. Protein B13

VACV strain Western Reserve (WR) protein B13 (also known as serine protease inhibitor [serpin] 2 or SPI-2) belongs to the serpin family, a group of protease inhibitors with multiple roles in inflammation, blood clotting and complement activation [59]. The presence of genes encoding serpins in poxvirus genomes was established in the late 1980s [60–63]. One of these is a 38-kDa protein called cytokine response modifier (Crm)A from cowpox virus (CPXV), and has been studied intensively. Initially, CrmA was demonstrated to induce the formation of haemorrhagic pocks on the chorioallantoic membranes of CPXV-infected chicken embryos [62]. Thereafter, CrmA was shown to be an inhibitor of ICE [64], now called caspase-1, and of extrinsic apoptosis induced by Fas or TNF, leading to the conclusion that ICE/caspase-1 was the key enzyme to trigger extrinsic apoptosis [65–69]. These studies established that despite being a serpin inhibitor, CrmA could also interact with another family of proteins, the so-called caspases, allowing inhibition of apoptosis.

The first characterisation of the VACV orthologue of CPXV CrmA, B13 (encoded by gene *B13R* in VACV strain WR), showed that B13 shared 92% amino acid identity with CrmA [63,70] and was expressed in some strains of VACV such as WR, International Health Department (IHD)-J, Wyeth and rabbitpox virus (RPXV, a VACV strain), but not in others such as Copenhagen, Tashkent, Lister or Tian Tian [71]. This was consistent with the subsequent observation that VACV strain WR, but not Copenhagen, could inhibit Fas-induced apoptosis [72–74]. Deletion of *B13R* from the VACV strain WR genome did not affect virus growth *in vitro* or alter virulence in a murine intranasal model of infection [71], but produced

Table 1
VACV factors modulating regulated cell death.

VACV protein	Mass (kDa)	Structure/Oligomerisation	Conserved in VACV strains	Cellular localisation	Role on regulated cell death	Mechanism	Other roles	Role in Virulence
B13 (SPI-2)	38.5	Serine protease inhibitor/No	No	Cytosolic	• Inhibition of extrinsic and intrinsic apoptosis • Inhibition of pyroptosis • Activation of necroptosis?	• Inhibition of caspase-8, -9, -10 • Inhibition of caspase-1	• Unknown	in: no id: yes, ↘
B22 (SPI-1)	40	Serine protease inhibitor/No	Yes	Cytosolic	• Inhibition of extrinsic apoptosis	• Inhibition of caspase-8	• Host restriction	in: no
F1	26	Bcl-2-like/Dimer	Yes	Mitochondrion	• Inhibition of intrinsic apoptosis • Inhibition of pyroptosis	• Targetting Bak, Bim • Targetting NLRP1	• Unknown	in: yes, ↗
N1	14	Bcl-2-like/Dimer	Yes	Cytosolic	• Inhibition of intrinsic apoptosis	• Targetting Bid, Bad	• NF-κB pathway • IRF3 pathway	in: yes, ↗ id: yes, ↗
GAAP	26.5	Transmembrane/Oligomer	No	Golgi apparatus	• Inhibition of extrinsic and intrinsic apoptosis	• Unknown	• Cation channel • Intracellular Ca^{2+} flux regulation • SOCE regulation • Cell adhesion and migration	in: yes, ↘
E3	20–25	Unknown/dimer	Yes	Cytosolicnuclear	• Inhibition of intrinsic apoptosis	• Binding to dsRNA, Z-DNA, PKR	• PKR inhibition • Host range	in: yes, ↗

Abbreviations: VACV, vaccinia virus; in, intranasal model of infection; id, intradermal model of infection; ↗, presence of the protein increases virulence; ↘, presence of the protein decreases virulence; SOCE, store-operated calcium entry.

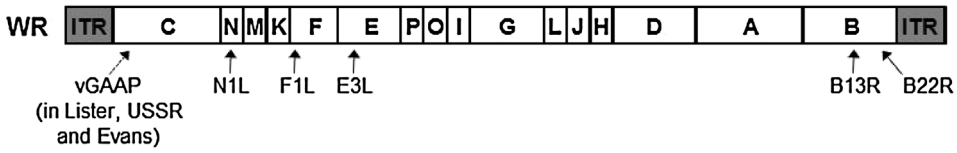

Fig. 4. Genomic location of VACV genes inhibiting regulated cell death.

larger lesion sizes after intradermal inoculation [75]. Loss of B13 also enhanced the antibody response to the HPV L1 protein following immunisation with a recombinant VACV expressing this protein [76]. B13 inhibited the cleavage of pro-IL-1β to IL-1β like CrmA, but did not affect the febrile response to infection or influence animal weight loss after intranasal inoculation even in the context of a virus lacking protein B15 [74], the VACV soluble IL-1β receptor [9,77]. Analyses of the response to intranasal infection with VACV strains WR and Copenhagen that do (WR) or do not (Copenhagen) express B15 showed that the presence of B15 was sufficient to inhibit fever following infection. Taken together, these observations reinforced the notion that IL-1β is the main endogenous pyrogen leading to febrile episodes [9,10,77].

After the harmonisation of caspase classification and nomenclature [78], the spectrum of action of CrmA was established and despite small discrepancies due to different experimental conditions, it was accepted that CrmA and, by extension, B13 were broad spectrum inhibitors of caspases with clear inhibitory activity for caspase-1, −4, −5, −8, −9 and −10 [79–82] (Figs. 1–3). Inhibition of caspase 8 explains the ability of B13 to block extrinsic apoptosis (Figs. 1 and 2), and inhibition of caspase 1 would predict an ability to block pyroptosis (Fig. 3), although this has never been formally demonstrated. Pan-inhibition of caspases by B13 is consistent with the activation of the necroptotic response following VACV infection [4,83,84]. Given that B13 is the most potent inhibitor of apoptosis in VACV [85], it is presumed that B13 is the main inhibitor of caspase activity and, consequently, the main contributor to viral-induced necroptosis in response to extrinsic apoptotic challenges, although this has not been tested with viruses engineered to lack B13.

2.2. Protein B22

The ability to induce haemorrhagic pocks on chorioallantoic membranes could not be ascribed to CrmA/B13 in all cases. Contrary to the case with CPXV, the equivalent of B13 in RPXV was dispensable for red pock formation and host cell range, and this was rather determined by the product of the gene *B22R* [86,87]. These results showed that RPXV B13 and CPXV CrmA are not functionally equivalent and that in certain settings, not all B13 counterparts were able to inhibit apoptosis [88].

VACV protein B22 (also known as serpin inhibitor 1 or SPI-1) is encoded by gene *B22R* in strain WR and *C12L* in strain Copenhagen [63,89–91], and also belongs to the serpin family. Its presence in poxvirus genomes was demonstrated at the same time as B13 [60–62]. B22 is a 40-kDa protein expressed early during infection that shares 46% amino acid identity with CrmA [63]. B22 was detected in every orthopoxvirus tested including CPXV and VACV strains WR, IHD-J, IHD-W, Copenhagen, Tashkent, Tian Tian, Lister, rabbitpox virus and Wyeth [71]. The *B22R* gene is also present in multiple strains of variola virus [92–94] and in ectromelia virus [95], suggesting an important role in infection. However, as for B13, no difference in virus replication *in vitro* or virulence in a murine intranasal model of infection was observed for viruses lacking B22 [71]. The activity of B22 in preventing cell death was demonstrated in two studies showing that B22 had a role in host restriction and that its deletion could trigger apoptosis in the pig kidney cell line PK-15 and in the human lung carcinoma A549 cell line [86,87], presumably via targeting caspase-8 (Fig. 1). Moreover, another group demonstrated that the complete inhibition of extrinsic apoptosis in certain cell types required both B13 and B22 [88].

2.3. Protein F1

VACV protein F1 is a 26-kDa protein expressed early after infection that is widely conserved amongst VACV strains [96]. There are also more distantly related counterparts in avipoxviruses such as fowlpox virus (FPXV) where it is known as FPXV039 [97]. F1 is a potent inhibitor of intrinsic mitochondrial apoptosis (Fig. 1) and its existence was suggested initially by the ability of the VACV strain Copenhagen to block apoptosis despite lacking B13 [98]. The inhibitory effect of F1 on apoptosis was demonstrated clearly when ectopically expressed F1 was capable of protecting human monocyte THP-1 cells from apoptosis after infection with VACV strains 811 (vv811) and 759, which contain large genomic deletions including F1 and B13 [96]. Similar results were obtained in human osteosarcoma U2-OS cells and human cervical carcinoma HeLa cells infected with recombinant vv811 engineered to express F1 [85]. F1 localised at the mitochondrion by virtue of an anchor domain at its C terminus, and this localisation was necessary for its anti-apoptotic activity [99]. Initially, F1 was found to interact with Bak, but not Bax, preventing its oligomerisation and the release of cytochrome *c* following viral infection [100–102]. Soon thereafter, an inhibitory effect on Bax activation was also noticed and this was attributed to the ability of F1 to bind the pro-apoptotic BH3-only protein Bim [103]. The crystal structure of F1 revealed that despite the absence of amino acid sequence similarity to other Bcl-2 proteins, F1 had a Bcl-2 fold and formed a dimer with a canonical BH3-binding groove and an unusual N-terminal extension [104]. This surface groove interacted with the BH3 peptide of Bim and, to a lower extent, Bak *in vitro*. More recently, the structures of the F1-Bak and F1-Bim complexes were solved and demonstrated that F1 can engage two BH3 ligands simultaneously via its surface groove to prevent Bax and Bak homo-oligomerisation and apoptosis [105]. In 2010 another group reported the role of F1 as a direct inhibitor of caspase-9 and hence, of the formation of the apoptosome [106] (Fig. 1). This function was independent of the Bcl-2-like core and was ascribed to the N-terminal extension [107] despite previous reports indicating that this region was dispensable for apoptosis inhibition [101].

F1 has also been reported to inhibit the formation of inflammasomes via targetting of the NLR family member NLRP1 [53] (Fig. 3). The absence of F1 in *in vitro* infected macrophages led to increased activation of caspase 1 and elevated IL-1β secretion. This activity was mapped to the N-terminal sequence upstream of the Bcl-2-like core, and a recombinant hexapeptide mimicking the F1 binding site supressed NLRP1 activity *in vitro*. More importantly, mice intranasally infected with recombinant viruses expressing an F1 allele unable to target NLRP1, but retaining its anti-apoptotic activity, showed a smaller drop in body temperature, which correlated with increased levels of proteolytically cleaved caspase-1 in the bronchial alveolar lavage (BAL) fluids of the animals [53]. This confirmed a net increase of IL-1β production in the absence of F1. Interestingly, this mutant virus displayed almost identical virulence to a virus lacking the entire *F1L* gene, suggesting that the

anti-apoptotic activity of F1 did not drive virulence in this model of infection. At present, it is unknown whether F1 contributes to virulence in the intradermal model of infection like B13, or whether F1 delays the appearance of illness symptoms and mortality in the context of a virus lacking B15. B13 and F1 are so far the only VACV intracellular proteins blocking inflammasome activation, IL-1β production and hence pyroptosis (Fig. 3). Studies using the highly attenuated strain MVA have suggested a role for the NLPR3 inflammasome in detecting VACV infection [108], but a viral NLPR3 specific inhibitor is yet to be identified. Irrespective of the number of intracellular strategies, it is likely that these determine minor changes to the overall systemic levels of IL-1β and that the latter are mostly regulated by protein B15, an extracellular protein that binds IL-1β produced by both infected and uninfected cells recruited to the site of infection [9,10] (Fig. 3). Intracellular strategies must, conversely, be crucial in preventing pyroptotic cell death.

2.4. Protein N1

VACV gene N1L is a widely conserved gene encoding a small intracellular protein that contributes to virulence [109–112]. Similar to F1, N1 did not share any sequence similarity to Bcl-2 proteins, but its crystal structure revealed a homodimeric protein exposing a conserved surface groove capable of binding BH3 peptides [113,114]. In fact, the number of VACV proteins adopting a Bcl-2-like fold extends beyond F1 and N1. Besides these, the structure of 5 other VACV proteins has shown to be Bcl-2-like [115–118], and 4 more are predicted to share structural similarity with the Bcl-2 family [115,119]. Despite their shape, most of these proteins have evolved to inhibit innate immune signalling cascades [8,115], and only F1 and N1 seem to have retained some anti-apoptotic activity.

Mechanistically, N1 was initially shown to interact with BH3 peptides of Bid, Bim and Bax in *in vitro* binding assays [113], and with Bad, Bid and Bax in co-immunoprecipitation experiments [114]. Later on, a group was unable to reproduce the N1-Bax interaction [120] and another one reported that, in addition, N1 had no anti-apoptotic activity [121]. The lack of interaction with Bax was also confirmed by a third report that demonstrated instead an interaction with Bad and Bid (Fig. 1). Moreover, this interaction was abrogated by introduction of specific point mutations in the BH3-binding groove, whilst the other known biological activity of N1, the inhibition of NF-κB, was retained [122]. This report also demonstrated that abolishing the anti-apoptotic activity of N1 did not have an impact on virulence in mice, a conclusion consonant with similar data for F1 [53]. Virulence relied instead on the ability of N1 to inhibit inflammatory signalling, such as blockage of the activation of the inflammatory transcription factors NF-κB and interferon regulatory factor 3 (IRF-3) [123]. However, although the capacity to block NF-κB activation has been confirmed by others [114,122,124], its role as IRF-3 inhibitor remains unclear [114,124] and a mechanistic understanding of such inhibitory activity is lacking. The effects of N1 on the host immune response are profound. Infection with WR lacking N1 or carrying an allele (I6E) unable to block NF-κB activation induced stronger effector and memory $CD8^+$ T cells [125]. More recently, a comprehensive study comparing the potency of all known VACV anti-apoptotic proteins concluded that although N1 prevented apoptosis in certain conditions, its potency after ectopic expression was lower than that observed for B13 or F1, and almost negligible in the context of infection [85]. These results indicated that NF-κB inhibition is the main function of N1 and is consistent with the demonstration that loss of N1 anti-apoptotic activity did not affect virulence [122]. Finally, it was shown recently that N1 is ubiquitylated during viral infection [126], but it remains unclear whether this constitutes another potential function or simply modulates reported ones.

2.5. Protein vGAAP

The *6L* gene from camelpox virus encodes a 26.5-kDa protein termed viral Golgi anti-apoptotic protein (vGAAP) that is also present in 3 VACV strains, Lister, USSR and Evans [127,128], and CPXV [129]. The discovery of vGAAP led to the characterisation of its human counterpart hGAAP due to the unusually high amino acid identity (73%) between them [127]. hGAAP is a member of the transmembrane Bax inhibitor-1 motif containing (TMBIM) family, whose most studied member is Bax inhibitor-1 (BI-1). BI-1 is located in the ER and inhibits Bax- and staurosporine-, but not Fas-, induced apoptosis, suggesting a role in preventing intrinsic apoptosis [130,131]. GAAP is also highly hydrophobic and contains 6 transmembrane domains and a C-terminal hydrophobic loop [132], but is located in the Golgi apparatus where it forms a cation-selective channel involved in several cellular pathways [129]. In addition, over-expression of GAAP can also cause its accumulation at the ER. GAAP reduces the Ca^{2+} loading of intracellular stores (Golgi and ER) and reduces the induction and frequency of oscillatory changes in cytosolic Ca^{2+} [133]. hGAAP also enhanced store-operated Ca^{2+} entry (SOCE) into the cell and consequently the activity of calpain 2, a Ca^{2+} sensitive protease involved in the recycling of cell adhesion components [134]. In this way, hGAAP promotes cell adhesion and migration. Although the mechanism through which alterations in cellular Ca^{2+} handling sensitises cells against apoptosis are not completely understood, the ability of GAAP to reduce Ca^{2+} available for release from the stores into the cytosol and regulate intracellular Ca^{2+} fluxes forms the basis of its current mechanistic link with its ability to suppress apoptosis [133] (Fig. 1). Indeed, mutation of a residue important for the channel pore activity abrogated the anti-apoptotic activity of GAAP against intrinsic and extrinsic pro-apoptotic stimuli [129].

vGAAP is the largest viral ion channel described so far and the only one identified in poxviruses. Deletion of vGAAP from the viral genome did not impact on viral replication, but did increase signs of illness and weight loss in an intranasal model of infection [127], demonstrating a negative impact on virulence. Unlike BI-1, vGAAP inhibited both intrinsic and extrinsic apoptosis in tissue culture [127,135], but was only capable of blocking intrinsic apoptosis in the context of a recombinant vv811 strain engineered to express vGAAP [85]. In addition, vGAAP can homo-oligomerise, although a vGAAP mutant that could only form monomers retained its ability to inhibit apoptosis and reduce the Ca^{2+} content of intracellular stores [135]. This indicates that vGAAP is functional as a monomer [135], although there is no direct evidence that monomeric vGAAP forms a functional ion channel and it does not exclude the possibility that oligomeric vGAAP can also form a functional channel.

2.6. Protein E3

VACV protein E3 is an important interferon antagonist that also affects VACV host range and contributes to virulence. E3 was characterised first as a 25-kDa dsRNA binding protein that antagonises the anti-viral activity of the interferon-induced dsRNA binding protein PKR [136,137] and possesses a C-terminal dsRNA binding domain [138,139]. E3 is present in both the cytoplasm and nucleus and is translated into 19- and 25-kDa forms by use of alternative AUG codons [140]. The N-terminal region of E3 forms a distinct domain [139] that has similarity with Z-DNA binding proteins [141] and both N- and C- terminal domains contribute to virus virulence [142–144]. Both domains can also contribute to resistance to interferon [145]. In addition to blocking activation of PKR, E3 was also described as an apoptosis inhibitor when HeLa cells infected with a mutant VACV lacking the *E3L* gene resulted in rapid cell death [146]. Uniting both observations, dsRNA was found subsequently to be a potent trigger for apoptosis in VACV-infected

cells, and this correlated with the ability of E3 to sequester dsRNA and inhibit PKR activity [136,147–149]. PKR is a serine-threonine kinase, expressed in mammalian cells constitutively at low levels and upregulated upon IFN treatment. Once activated, PKR phosphorylates the eukaryotic translation initiation factor eIF2α leading to inhibition of protein synthesis. Activated PKR can also phosphorylate the NF-κB inhibitor IκB leading to NF-κB-dependent gene expression. Both signalling cascades contribute to PKR-induced apoptosis [150–152], and although E3 was also described as a direct inhibitor of PKR via protein–protein interactions [153,154] and mutagenesis has suggested dsRNA binding is not essential for E3 function [155], the majority of evidence suggests that it is the ability of E3 to bind dsRNA that prevents PKR apoptotic effects. The C-terminal dsRNA binding domain is also necessary for inhibition of activation of NF-κB and transcription of the IFN-β gene induced by dsRNA transcribed by RNA polymerase III from A:T rich DNA [156,157].

3. Clinical and therapeutic implications

The study of VACV and its interplay with the host has important implications for its use as an oncolytic agent and as a vaccine vector. Oncolytic viral therapy started with the concept of engineering viruses that could replicate selectively in tumours leading to their destruction whilst not replicating in normal tissue. Evasion of cell death is a hallmark of cancer, and many tumours acquire genetic mutations in apoptotic pathways to become resistant to both intrinsic and extrinsic pro-apoptotic stimuli [158]. In such an environment viral anti-apoptotic strategies become redundant and can be eliminated from the oncolytic virus to generate an agent that is unable to replicate in normal cells, because these undergo apoptosis in response to infection, and replicates selectively in transformed cells. Although this was the basis for the development of the first oncolytic viral agents, several clinical trials have demonstrated the need to harness the immune response associated to the oncolytic agent to guarantee optimal therapeutic activity [159]. An attractive approach to enhance immunotherapeutic effects consists in promoting immunogenic cell death to induce a more robust anti-tumour immune response [160–162]. VACV represents an ideal candidate for oncolytic therapy because of its immune activation capacity, its rapid and cytocidal replicative cycle, broad tissue tropism, inability to integrate its genome in host chromosomes and the extensive clinical data collected from its use as the smallpox vaccine [159]. For instance, systemic delivery of the JX-594 virus, a thymidine kinase-negative VACV engineered to express granulocyte-macrophage colony stimulating factor, proved to be safe and efficacious against tumour tissue and has undergone phase II clinical trials for the treatment of solid tumours [163]. Despite these successes little is known about the mechanisms employed by VACV to induce tumour cell death. A study reported extensive apoptosis following MVA infection of melanoma cells [164], whilst another study reported necroptotic cell death in ovarian cancer cells infected with strain Lister [84]. Although cell death mode can be cell type specific, it can also be virus strain specific. For instance, B13, the main necroptotic inducer, is not expressed by strain Lister (used in [84]), although B22 is [71]. Interestingly, an engineered WR derivative lacking both B13R and B22R induced both apoptosis and necrosis in normal and cancer cells as revealed by HMGB1 release [165]. How these viruses triggered regulated necrosis whilst lacking both pan-caspase inhibitors remains intriguing, but it may indicate the existence of tumour-specific inhibitory mechanisms lowering the necroptotic threshold.

VACV also holds promise as a vaccine vector. VACV was the agent used for the eradication of smallpox more than 30 years ago [166,167]. Although the safety record of VACV does not meet the standards applied to current vaccines, attenuated VACV strains such as MVA and NYVAC do possess excellent safety records and are currently being tested as vaccines for several diseases [168]. MVA was generated after passaging the chorioallantois VACV Ankara strain ~570 times in chicken embryo fibroblasts and despite not replicating in most mammalian cell types, it retains the ability to induce strong immune responses [169]. NYVAC was generated by deletion of 18 open reading frames (ORF) from strain Copenhagen, and like MVA, it can drive a potent immune response [170]. How VACV elicits such potent immune responses remains unclear, particularly considering the large number of immunomodulatory proteins present in its genome. Similarly, the impact that VACV inhibition of programmed cell death may have on those responses has not been fully addressed.

The type of cell death can influence the outcome of an acquired immune response during infection. Apoptosis involves the organised disassembly of a cell and it is not associated with the release of PAMPs and DAMPs [5]. Antigens associated with dying cells can be engulfed by dendritic cells (DCs) and used to stimulate $CD8^+$ T-cell cross-priming [171], and the long-term fate of these cells is determined by the additional signals or 'help' from activated $CD4^+$ T-cells. DCs encountering apoptotic cells present antigen to $CD8^+$ T-cells, but not $CD4^+$ T-cells [172]. These 'helpless' $CD8^+$ T-cells can act as effector cells, but subsequently produce TNF-related apoptosis-inducing ligand (TRAIL) and die prematurely [173]. On the contrary, necroptosis and pyroptosis involve the release of PAMPs and DAMPs, and DCs encountering necroptotic cells present antigen to both $CD4^+$ and $CD8^+$ T-cells [172]. The subsequent 'helped' $CD8^+$ T-cells do not produce cytotoxic TRAIL and are long-lived. Interestingly, recent work has unveiled a crucial role for NF-κB signalling in dying cells for optimal $CD8^+$ T cell cross-priming [174], a pathway that is targeted by most viruses including VACV [8]. Necroptosis and pyroptosis are therefore more immunogenic cell death modes than apoptosis, and their activation has the potential to increase the immunogenicity of vaccine vectors. While necroptosis is critical to control VACV infection [4], no VACV inhibitors have so far been described. It is possible that necroptotic responses contribute to the immunogenicity of some VACV strains. However, this is unlikely to be the case for NYVAC as B13, the main caspase inhibitor and hence necroptotic trigger, is not expressed in its parental strain Copenhagen [71]. Likewise, although a B13 ORF is present in MVA, ORF 182, [175], it is truncated as in VACV Copenhagen and therefore non-functional. Similarly, MVA gene *183R*, the equivalent of VACV WR gene *B14R,* is mutated and non-functional in MVA [176].

Unlike necroptosis, VACV encodes a number of inhibitors that modulate pyroptosis, namely B13, F1 and B15, and deletion of these genes has the potential to unleash pyroptotic responses. Indeed, deletion of the *B15R* gene from MVA improved T-cell memory responses and protection against a respiratory challenge infection with WR [177]. Likewise, vaccination with MVA strains engineered to express human immunodeficiency virus (HIV)-1 antigens and to lack genes *A41L* and *B15R* [178] or *F1L* [179] showed enhanced immune correlates of protection against HIV-1 antigens. These reports demonstrate that the rational deletion of inhibitors of cell death has the potential to improve VACV vaccine vectors.

4. Conclusions

Programmed cell death encompasses a number of highly regulated signalling cascades invariably leading to cell arrest and cell disruption. Apoptosis is essential for tissue homeostasis and development and abortion of the replicative cycle of viruses. Necroptosis and pyroptosis are more inflammatory in nature and can shape immune responses against infection and cancer. VACV holds exten-

sive interest both as an oncolytic agent against cancer and as a vaccine vector against infectious diseases. However, as a pathogen, VACV has evolved a number of strategies to counteract host programmed cell death. Data collected over the past recent years has started to reveal the impact that the different forms of cell death have on virulence and immunogenicity, and the importance of triggering immunogenic versus non-immunogenic cell death. Identification and understanding of the mechanisms employed by VACV to prevent cell death not only has the potential to reveal the molecular basis underpinning anti-viral responses, but also offers the possibility of exploiting them to induce more immunogenic cell death and elicit stronger and more robust immune responses. This goal will aid in the development of more efficacious vaccines and anti-cancer agents.

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