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Viral infection and the evolution of caspase 8-regulated apoptotic and necrotic death pathways

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Abstract

Pathogens specifically target both the caspase 8-dependent apoptotic cell death pathway and the necrotic cell death pathway that is dependent on receptor-interacting protein 1 (RIP1; also known as RIPK1) and RIP3 (also known as RIPK3). The fundamental co-regulation of these two cell death pathways emerged when the midgestational death of mice deficient in FAS-associated death domain protein (FADD) or caspase 8 was reversed by elimination of RIP1 or RIP3, indicating a far more entwined relationship than previously appreciated. Thus, mammals require caspase 8 activity during embryogenesis to suppress the kinases RIP1 and RIP3 as part of the dialogue between two distinct cell death processes that together fulfil reinforcing roles in the host defence against intracellular pathogens such as herpesviruses.

Apoptotic and necrotic cell death pathways determine the fate of mammalian cells. Apoptosis follows well-defined pathways that centre around a caspase-dependent proteolytic cascade that coordinates cell-membrane blebbing, nuclear condensation and DNA fragmentation, while maintaining membrane integrity[1,2]. By contrast, necrosis is caspase-independent and involves cell rounding and cytoplasmic swelling, terminating with the loss of membrane integrity and cytoplasmic leakage[3]. Necrosis has long been associated with incidental (passive) death in damaged or diseased tissues; however, programmed necrotic death in specific contexts is orchestrated in a cell-autonomous manner via receptor-interacting protein 1 (RIP1; also known as RIPK1)[4] and/or RIP3 (also known as RIPK3)[5–7]. The best-characterized form of programmed necrosis, known as necroptosis, requires the assembly of a RIP homotypic interaction motif (RHIM)-dependent[8] signalling complex of RIP1 and RIP3 (REFS 5–7).

Diverse cell-intrinsic and cell-extrinsic signals converge on the activation of executioner caspases that mediate apoptosis. Particularly in the case of intracellular pathogens (such as

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viruses), apoptosis contributes to host defence by eliminating infected cells. Intrinsic apoptotic machinery exists in metazoan organisms to eliminate excess cells during embryonic development and to sustain tissue homeostasis, as well as to purge stressed, damaged or infected cells. By contrast, extrinsic death pathways evolved more recently and facilitate host defence against pathogens. Intrinsic apoptosis depends on mitochondrial outer membrane permeabilization by the pro-apoptotic B cell lymphoma 2 (BCL-2) family members BAX and BAK[9,10]. Following mitochondrial permeabilization, pro-apoptotic factors — such as cytochrome *c* and second mitochondrial activator of caspases (SMAC; also known as DIABLO) — are released into the cytosol, triggering the activation of caspase 9 and of downstream effector caspases, such as caspase 3 and caspase 7. These effector caspases dismantle the cell through the proteolytic destruction of vital substrates[11,12]. In contrast to intrinsic apoptosis, extrinsic apoptosis is initiated by ligands of the tumour necrosis factor (TNF) family that engage death receptors to activate caspase 8. Caspase 8 activation ultimately drives the activation of caspase 3 and/or caspase 7, either directly, or indirectly by initiating a mitochondrial amplification pathway via the pro-apoptotic BCL-2 family member BID[13].

RIP1- and RIP3-dependent programmed necrosis (necroptosis) is unveiled when caspase 8 activity becomes compromised[14]. Investigators struggled for a decade to explain why mice with a germline disruption of the caspase 8 gene, the FAS-associated death domain protein (FADD) gene or the cellular FLICE-like inhibitory protein (cFLIP; also known as CFLAR) gene die during gestation at embryonic day 10 or 11 (see BOX 1). This pattern of death suggested a crucial non-apoptotic activity for caspase 8–FADD–cFLIP complexes[15–21]. Rescue of this embryonic lethality, as observed in either C*asp8*$^{-/-}$*Rip3*$^{-/-}$ or *Fadd*$^{-/-}$*Rip1*$^{-/-}$ mice, clarified the developmental role of caspase 8, strongly implicating this enzyme in the physiological suppression of necroptosis[22–24]. This interpretation was facilitated by evidence that death receptor-dependent signalling regulates the choice between caspase 8-directed apoptosis and the promotion of programmed necrosis by caspase inhibitors[14,25,26]. Moreover, accumulating evidence has shown that RIP1- and/or RIP3-dependent programmed necrosis can be initiated independently of death receptors of the TNF receptor (TNFR) superfamily during virus infection[27] or following the activation of Toll-like receptors (TLRs)[28,29], as well as in settings of genotoxic stress[30].

The choice between apoptotic and necrotic pathways following the ligation of death receptors has been extensively reviewed[14,25,26]. Likewise, a discussion of the inflammatory consequences of TNFR super family signalling and the roles of apoptosis and necrosis in inflammation are beyond the scope of this Review, although recent reports indicate an intimate association between inflammation and dysregulated programmed necrosis[31–35]. This Review focuses on the molecular pathways involved in the regulation of programmed necrosis, discusses the potential contribution of viral infections to the evolution of programmed necrosis as it is currently understood in mammals, and summarizes the latest evidence on the contribution of cell death pathways to immune homeostasis.

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The players in death receptor signalling

Death receptors, including TNFR1 and FAS (also known as CD95), control three cellular responses in mice and humans: first, a pro-inflammatory cytokine response that is dependent on nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs); second, apoptosis; and third, necroptosis[36–38]. Activation of NF-κB transcription factors contributes to inflammation and the suppression of cell death[39], and the interplay between the pro-inflammatory and the cell death processes that are under the control of death receptors probably contributes to disease pathology in many settings. Additional TNFR superfamily members drive the activation of NF-κB and inflammation without inducing cell death[40]. These, together with several classes of pattern recognition receptors (PRRs), sculpt inflammatory responses. These inflammatory responses contribute to the recognition and clearance of microbial infections through the activity of cytokines that initiate the elimination of infected cells via programmed cell death. Overlapping functions remain a major theme when considering pathogen recognition and death receptor signalling in host defence.

Caspase 8 activation

Ligation of TNFR superfamily death receptors promotes the assembly of a caspase 8- and FADD-containing complex that initiates apoptosis. Signalling via FAS, TRAIL receptor 1 (TRAILR1) or TRAILR2 results in interactions between the receptor death domains and FADD, leading to the formation of a receptor-associated death-inducing signalling complex (DISC)[41–43]. The DISC recruits caspase 8 via death effector domain (DED)-dependent interactions with FADD. Apoptosis ensues following caspase 8 homodimerization and self-cleavage, which unleashes the full activity of the enzyme[44]. The two isoforms of cFLIP — cFLIP long ($cFLIP_L$) and cFLIP short ($cFLIP_S$) — are non-catalytic paralogues of caspase 8 that heterodimerize with caspase 8 to suppress the self-processing that is necessary for the induction of apoptosis[45] (FIG. 1).

TNFR1 signalling involves the recruitment of TNFR1-associated death domain protein (TRADD), together with RIP1 (REFS 46–48), into complex I. This drives the activation of NF-κB and MAPKs, which induce pro-survival genes[49], including those encoding cFLIP and cellular inhibitor of apoptosis proteins (cIAP1 and cIAP2; collectively referred to as cIAP here). Within the TNFR1 signalling complex, cIAP and the linear ubiquitin chain assembly complex (LUBAC) polyubiquitylate RIP1 to promote the engagement of IκB kinase-β (IKKβ) by NF-κB essential modulator (NEMO; also known as IKKγ), which results in NF-κB-mediated gene transcription. Polyubiquitylated RIP1 does not support apoptosis[50–54] or programmed necrosis[6,55,56]. When cIAP is inhibited — either naturally by SMAC or experimentally through the application of SMAC mimetics — or when the LUBAC component SHARPIN is absent, extrinsic death pathways that contribute to apoptosis and programmed necrosis become activated[35,57]. The deubiquitylation of RIP1 by enzymes such as cylindromatosis (CYLD) downregulates NF-κB activation and enables the kinase activity of RIP1 to direct the formation of a DISC-like cytosolic caspase 8-activating platform[53] known as complex II, which is composed of caspase 8, FADD and cFLIP[58] (FIG. 1).

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When caspase 8 activity is compromised, programmed necrosis is initiated by a RIP1–RIP3 complex[5–7] that has been called a necrosome[14,26]. Importantly, catalytically active but non-cleavable caspase 8 retains the ability to suppress programmed necrosis[23] and can prevent the embryonic lethality caused by caspase 8 deficiency[16]. Caspase 8 orchestrates apoptosis and prevents necroptosis in association with its activator, FADD (FIG. 1b). Both isoforms of cFLIP inhibit caspase 8-induced apoptosis (FIG. 1c,d); however, in the presence of sufficient levels of RIP3, $cFLIP_S$ promotes RIP1- and RIP3-dependent necroptosis[29] (FIG. 1f), whereas $cFLIP_L$ blocks necroptosis[23]. In contrast to a caspase 8–$cFLIP_S$ heterodimer, caspase 8–$cFLIP_L$ retains sufficient proteolytic activity[59–62] to inactivate the key adaptors, RIP1 (REFS 63–65) and RIP3 (REF. 66), as well as the deubiquitylating enzyme CYLD[67]. This prevents RHIM-dependent oligomerization[5–8] of RIP1 and RIP3 into a necrosome[14,26] (FIG. 1e).

The ripoptosome

Recent investigations have examined the roles of caspase 8 and FADD in regulating necroptosis following stimulation via PRRs (such as TLR3)[29] or the induction of genotoxic stress[30]. These studies have shown that TLR3-dependent or DNA damage-induced signals promote the formation of a high molecular weight complex called a ripoptosome[29,30], which contains RIP1, FADD, caspase 8 and cFLIP. This complex regulates cell fate by functioning as a caspase 8 activation platform reminiscent of TNFR1-induced complex II, although it is assembled independently of death receptor signalling. In TLR3 signalling, TIR domain-containing adaptor protein inducing interferon-β (TRIF) recruits the ripoptosome through a RHIM-dependent interaction with RIP1. Here, as in death receptor pathways, the proteolytic activity of caspase 8–$cFLIP_L$ suppresses necroptosis. TRIF-mediated necroptosis in response to the stimulation of TLR3 or TLR4 may underlie the pathogenesis of acute bacterial inflammation under conditions in which caspase 8 activity is compromised[33]. Similarly, genotoxic stress can induce cIAP degradation, driving ripoptosome formation[30]. Growing evidence suggests that TCR stimulation also results in the formation of a ripoptosome-like complex[65,68]. Thus, a variety of extracellular and intra cellular stimuli appear to regulate ripoptosome formation, expanding the number of settings in which cell death choices are controlled by caspase 8 activity. Settings in which the caspase 8–$cFLIP_S$ hetero dimer dominates during infection or inflammation may be predicted to favour necroptosis (FIG. 1f).

The roles of caspase 8 in TCR signalling

Caspase 8- or FADD-deficient T cells die when stimulated by antigens. The successful rescue of T cell function through the deletion of *Rip1* or *Rip3* — as observed in $Casp8^{-/-}Rip3^{-/-}$ mice[22,23], in mice with $Fadd^{-/-}Rip1^{-/-}$ haematopoietic cells[24], and in mice with a T cell-specific caspase 8 or FADD deficiency on a $Rip3^{-/-}$ background[65,68] — indicates that necroptosis underlies this T cell loss. Early evidence that linked NF-κB activation, as well as autophagy, with this type of T cell death is not supported by current data[69]. It has become very clear that TCR activation in caspase 8- or FADD-deficient T cells promotes RIP1- and RIP3-dependent necroptosis[65,68–70]. The CARMA1–BCL-10–MALT1 complex (FIG. 1a) activates NF-κB following antigen recognition via the TCR[71–73], while

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also directing the formation of a complex containing caspase 8, $cFLIP_L$ and RIP1 (REFS 74,75). Despite the fact that FADD and caspase 8 are essential for T cell proliferation in response to antigens, T cell activation does not result in apoptosis. Instead, an explanation posited with experimental support in 2008 (REF. 70), that necroptosis follows antigen engagement in caspase 8- or FADD-deficient T cells, continues to implicate an inactivation of RIP1 and/or RIP3 mediated by a caspase 8–$cFLIP_L$ complex following TCR stimulation. It remains to be fully elucidated whether TCR stimulation induces necroptotic pathways in non-transgenic T cells or more physiological settings.

Programmed necrosis mediated by RIP1 and RIP3

RIP1- and RIP3-dependent programmed necrosis (necroptosis)

Necroptosis is observed when caspase 8 activity is compromised. In *Drosophila melanogaster* and zebrafish, the homologues of caspase 8 and FADD do not have a developmental role[76–78]. By contrast, the embryonic lethality in mice that have a germline mutation in *Casp8* or *Fadd* indicates that a key developmental step in mammals requires caspase 8 activity[15–20]. Endothelial and haematopoietic cells are most affected in the absence of caspase 8, suggesting that normal development is predicated on the survival of these cells. A complex of FADD, caspase 8 (REF. 16) and $cFLIP_L$[23,29] restrains aberrant activation of RIP1–RIP3 during mammalian development, although the precise signals, cell-extrinsic or cell-intrinsic, that trigger caspase 8 and RIP1–RIP3 activation in these cell types remain to be defined (BOX 1). Although death receptor signalling may be involved, the recognition that ripoptosome formation follows cell-intrinsic as well as cell-extrinsic cues suggests new possible mechanisms for the activation of caspase 8 and the elimination of RIP1–RIP3 signalling (FIG. 1). The key players that promote embryonic lethality are clear; however, the downstream events in the execution of necroptosis and fetal loss remain vague. Several candidate targets of RIP1–RIP3 kinase activity have emerged from screens[7], but these still need to be fitted into the cell death pathways[14].

In addition to contributing to host defence during viral infections (see below)[5,27,63], necroptosis is involved in bacterium-driven chronic inflammation in the intestine (as shown in mice that lack FADD or caspase 8 in intestinal epithelial cells[33,34]) and in the spontaneous inflammation that occurs in the skin of mice that have a keratinocyte-specific deficiency in FADD[32]. These findings suggest that necroptosis may underlie inflammatory diseases that affect the gut and skin of humans.

RIP1-independent, RIP3-dependent programmed necrosis

RIP3 is necessary for necroptosis, as well as for murine cytomegalovirus (MCMV)-induced programmed necrosis[27], but does not contribute directly to TNFR- or TLR-induced NF-κB activation or apoptosis[79]. Despite their inability to support necroptosis[5–7] or MCMV-induced programmed necrosis[27], *Rip3*$^{-/-}$ mice lack obvious developmental or immunological abnormalities[79]. These animals show a full level of resistance to natural mouse pathogens, including MCMV[27], murine hepatitis virus[65] and lymphocytic choriomeningitis virus[68]. However, RIP3-deficient mice are remarkably susceptible to infection with vaccinia virus, a member of the poxvirus family[5]. Thus, RIP3-dependent

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pathways probably contribute to host defence against viral infection. However, these RIP3-dependent pathways may have evolved at a cost, as abnormal levels of necrosis may underlie sterile[80], as well as pathogen-induced[33,34], inflammatory diseases.

Although MCMV-induced programmed necrosis requires RIP3 kinase activity and RHIM-dependent interactions[27], it is independent of RIP1 and thus distinct from necroptosis[5–7]. MCMV-induced necrotic death rapidly eliminates infected cells, thereby removing the infection before viral replication can occur; these findings reinforce the idea that this pathway has an important role in host defence[27]. Given that RIP1 and TRIF are not involved in this pathway[27], RIP3 may either form a homotypic complex or interact with an additional cellular RHIM-containing protein. DNA-dependent activator of interferon regulatory factors (DAI; also known as ZBP1)[81] remains an attractive candidate partner for RIP3 (FIG. 1a). DAI and RIP3 form a RHIM-dependent complex, and the MCMV-encoded viral inhibitor of RIP activation (vIRA) blocks this inter action[82,83]. DAI is a cytosolic DNA sensor[81] that potentially contributes to host defence against human cytomegalo virus[84]. Although DAI-deficient mice lack any obvious developmental phenotype and retain the ability to recognize cytosolic DNA[85], these mice will help to clarify the role of this adaptor in MCMV-induced programmed necrosis.

Viral control of programmed necrosis

Viruses are heavily reliant on the fate of infected cells and have evolved to encode suppressors of cell death that increase viral spread by preventing cell clearance[86–88]. The existence of these suppressors provides tangible evidence that both apoptotic and necrotic death pathways are a benefit to host defence. It is possible to appreciate the compendium of crucial biological pathways encountered by each viral pathogen through the suppressors that the pathogen encodes. Programmed necrosis can occur when caspase 8 activity is compromised, suggesting that the abundance of viral caspase 8 inhibitors (TABLE 1) may have driven the evolution of programmed necrosis as a counteradaptation for host defence[27,63,89]. Few viral inhibitors of programmed necrosis have been identified (FIG. 2; TABLE 1), which at first glance may suggest a potential recent emergence. However, as programmed necrosis has only recently joined the ranks of bona fide host defence pathways, additional mechanisms and inhibitors of programmed necrosis may soon be recognized.

The activation of caspase 8 is prevented by various pathogen-encoded molecules, including the baculovirus p35 protein[90], the adenovirus E3 14.7 kDa protein[91], several poxvirus serpins and vFLIPs (viral FLIPs)[92], the gammaherpesvirus vFLIPs[93] and the cytomegalovirus protein vICA (viral inhibitor of caspase 8 activation)[94] (FIG. 2). Vaccinia virus-infected cells become susceptible to death receptor-induced necroptosis owing to inhibition of caspase 8 by a viral caspase inhibitor[95] in host cells that have sufficient levels of RIP3 (REF. 5). These features help to explain the susceptibility of RIP3-deficient mice to lethal vaccinia virus infection[5], as well as why necroptosis can be viewed as a trap door to eliminate cells when caspase 8 activity is compromised.

Cytomegaloviruses are evolutionarily ancient herpesviruses that give rise to a complex pathogenesis and can persist in targeted host epithelial, myeloid and endothelial cells[96]. Like

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other mammalian viruses, herpesviruses differ in pathogenicity and virulence in large part owing to their expression of immune modulators that facilitate infection by undermining host innate and adaptive immunity. The presence in all mammalian cytomegaloviruses of well-conserved inhibitors of caspase 8 activation and of mitochondrial inhibitors of apoptosis that target the activation of BAX and BAK demonstrate that apoptotic cell death pathways contribute to viral clearance[94,97–100].

By targeting caspase 8, cytomegalovirus-derived vICA reduces the impact of extrinsic, death receptor-associated host control over the virus. Moreover, MCMV counteracts necrotic death by encoding vIRA, which is a product of the *M45* gene that blocks RHIM-dependent signalling pathways, including death receptor-induced apoptosis and necroptosis[27,83,101], TRIF-dependent apoptosis[27] and TRIF- or DAI-dependent NF-κB activation[82,83,102]. The region of *M45* encoding the RHIM domain, which is crucial for vIRA function, is not present in primate cytomegaloviruses, even though the gene is otherwise conserved. Rat cytomegalovirus encodes a close homologue of vIRA and, interestingly, an evolutionarily distant orthologue may be carried by herpes simplex virus 2 (REF. 103).

An understanding of the role of vIRA in viral pathogenesis emerged from studies of a precise mutation that affects only the RHIM domain[27,101]. Wild-type MCMV replicates in an equivalent manner in both wild-type and *Rip3*$^{-/-}$ mice, demonstrating that RIP3 pathways make little difference during infection by a fully armed herpesvirus that has a full complement of immune modulators. By contrast, MCMV with the specific *M45* mutation is completely attenuated by RIP3-dependent pathways in C57BL/6 mice. Importantly, the replication and pathogenesis of the mutant virus are completely normalized in RIP3-deficient mice, a fact that establishes the direct relationship between RIP3 as the target and vIRA as a RHIM-disrupting suppressor of cell death.

The evolutionary adaptation of necrotic death into a host defence pathway may have driven the acquisition of vIRA by a progenitor of MCMV (and rat cytomegalovirus), even though this acquisition has not apparently affected primate cytomegaloviruses. Once a primordial vertebrate evolved to execute programmed necrosis in response to pathogen-encoded caspase inhibitors, the arms race was heightened while the pathogen evolved to counteract the additional death pathway. Each pathogen–host pairing reaches détente, but solutions vary. The fact that MCMV vICA targets caspase 8 (REFS 98,104) and that this sensitizes cells to MCMV-induced necrosis, which can be suppressed by vIRA[27], fits with such an evolutionary scenario. Cycles of pathogen–host adaptation and counteradaptation play out through evolution[105] and, in the case of herpesviruses, the survival of both the pathogen and the host involves a continually changing battle over the lifetime of an individual, as well as a war that wages over evolutionary time in populations. In addition to vIRA, several vFLIPs — namely, MC159 from molluscum contagiosum virus, E8 from equine herpesvirus 1 and K13 from Kaposi's sarcoma-associated herpesvirus (also known as human herpesvirus 8) — also have the capacity to block both apoptosis and necroptosis[26,63]. These vFLIPs are structurally most similar to the $cFLIP_S$ isoform and likewise inhibit the enzymatic activity of caspase 8; thus, the biochemical mechanisms by which these viral inhibitors prevent necroptosis remain to be established. Given that only two viral antinecrotic strategies have been described, the absence of a RHIM-dependent inhibitor similar to vIRA in primate

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cytomegaloviruses may be due to an independent adaptation, such as a mechanism that suppresses RIP3 kinase activity, and this is worth exploring.

The role of cell death pathways in immunity

In addition to its role in natural host defence through the elimination of infected cells, programmed cell death plays an important part in shaping host immune responses[106]. Although the general consensus is that apoptosis is immunologically tolerogenic and necrosis is immunogenic, questions remain as to how the different modes of death, particularly programmed necrosis, influence adaptive immune responses. Despite the absence of caspase 8-dependent and RIP3-dependent death pathways, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice mount immune responses during viral infections[22], so these mice provide a tool for understanding the contributions of extrinsic apoptosis and of danger signals that result from necrosome-dependent cell death[107] to innate and adaptive immunity. In addition to the control of MCMV infection by *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice[22], RNA virus infections can be controlled by mice reconstituted with *Fadd*$^{-/-}$ *Rip1*$^{-/-}$ haematopoietic progenitors[24] and by *Rip3*$^{-/-}$ mice in which T cells express a dominant-negative mutant of FADD[65] or lack caspase 8 (REF. 68). These observations indicate that basic innate and adaptive immune responses to natural pathogens are sustained in these mice despite the absence of extrinsic apoptosis and programmed necrosis pathways. In *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice, intrinsic apoptosis remains intact. A combination of intrinsic death and inflammatory responses appears to be adequate for the elimination of infected cells, antiviral interferon action and the cross-presentation of antigens. These events are sufficient for the initiation of an adaptive immune response and the elimination of pathogen-infected cells by activated T cells, despite the absence of extrinsic apoptosis and programmed necrosis. Moreover, intrinsic apoptosis appears to be sufficient for the contraction phase of the immune response, as well as for immune memory. It will be important to determine the precise apoptotic and inflammatory responses that contribute to immune control of infections and immune regulation in the absence of both caspase 8-dependent apoptosis and RIP3-dependent necrosis, given the potential for either pathway to influence adaptive immunity[86,108].

T cells naturally exhibit the greatest proliferative capacity of any mammalian cell type. Cell death pathways have been implicated in the homeostasis, activation and contraction of T cell populations. FAS-dependent apoptosis mediates the elimination of excess T cells that accumulate over the course of life[109]. The importance of FAS signalling in homeostatic T cell control has been evident since lymphadenopathy, splenomegaly and abnormal $CD3^+CD4^-CD8^-B220^+$ lymphocytes were first observed in ageing *lpr/lpr* mice (which are deficient in FAS)[110] and *gld/gld* mice (which are deficient in FAS ligand)[111]. A deficit in caspase 8 or FADD alone in T cells does not recapitulate this phenotype[69], although modest lymphoproliferation is sometimes noted[112]. Progressive abnormal T cell accumulation similar to that observed in mice deficient in FAS signalling is observed in *Casp8*$^{-/-}$ *Rip3*$^{-/-}$ mice[22,23], presumably because caspase 8 activation lies directly downstream of FAS in the apoptotic pathway[109]. Similar defects are observed in mice that have a T cell-specific disruption of caspase 8 or FADD on a *Rip3*$^{-/-}$ background[65,68]. These defects do not compromise T cell responses towards a variety of viral infections[22,65,68], but they clearly indicate that caspase 8 has a crucial role in T cell homeostasis downstream of FAS.

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RIP1- and RIP3-dependent necroptosis underlies mouse T cell loss in caspase 8- or FADD-deficient settings[69,70]. The T cell response is rescued when necrosome components are eliminated[22–24,65,68], suggesting that necroptosis may regulate the antigen-specific T cell response. It is tempting to extrapolate these findings to human biology, where the presence of caspase 10 — a caspase 8 paralogue not present in mice — contributes to outcomes along with caspase 8. Interestingly, the T cell defects in individuals with mutant caspase 8 are different from those found in individuals with mutant caspase 10. *Casp8* mutations result in immunodeficiency[113], suggesting a role in inhibiting necroptosis in lymphocytes, whereas caspase 10 deficiency results in lymphadenopathy, splenomegaly and auto immunity[114], which is most aligned with a role downstream of FAS in apoptosis. Moreover, human caspase 8 and caspase 10 may have only partially overlapping functions during human embryonic development, as individuals with mutations in either gene have different outcomes; caspase 8 is predicted to control apoptosis and necroptosis, whereas caspase 10 is possibly restricted to the control of apoptosis[68]. The contributions that the two human orthologues make to apoptotic and necrotic death during development, host defence, cancer, immunity and disease require closer comparative evaluation.

Conclusions

It is now clear that programmed necrosis and apoptosis have complementary roles in host defence against pathogens. One form of programmed necrosis, necroptosis, is dependent on RIP1 and RIP3 and is triggered via death receptors, PRRs, the TCR or genotoxic stress, as well as during midgestational development, in settings when caspase 8 activity is compromised. Necroptosis has a role in host defence during infection with intra-cellular pathogens that encode caspase 8 inhibitors, but it also contributes to disease pathogenesis in acute or chronic bacterium-induced inflammation. By contrast, MCMV-induced programmed necrosis is independent of RIP1 but dependent on RIP3. MCMV encodes not only a caspase 8 inhibitor that sensitizes infected cells to programmed necrosis, but also a RHIM-dependent inhibitor of necroptosis and MCMV-induced programmed necrosis. The identification of MCMV-induced programmed necrosis implicates viral infection — and viral suppressors of cell death — in the evolution of programmed necrosis as an alternative mechanism of host defence.

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Glossary

Apoptosis The most common form of developmental cell death that regulates cell numbers, drives morphogenesis, deletes structures and eliminates unneeded and harmful cells. Apoptosis is a cell-autonomous death pathway mediated by caspases that dismantles the cell but maintains

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	membrane integrity and is characterized by cell shrinkage, membrane blebbing and DNA fragmentation.
RIP1	(Receptor-interacting protein 1; also known as RIPK1). A signalling adaptor and protein kinase that regulates the activation of gene expression via the NF-κB and MAPK pathways and controls the initiation of necroptosis and apoptosis via death domain- and RHIM-dependent complexes.
RIP3	(Receptor-interacting protein 3; also known as RIPK3). A RHIM-containing signalling adaptor and protein kinase that mediates necroptosis and MCMV-induced necrosis.
Programmed necrosis	A cell-autonomous, regulated cell death pathway that is characterized by cell swelling, membrane rupture and cytoplasmic leakage, but not by membrane blebbing.
Necroptosis	A form of programmed necrosis that is executed by the kinase activities of RIP1 and RIP3. This pathway is inhibited by RIP1 kinase inhibitors, such as necrostatin 1 (5-(1H-indol-3-ylmethyl)-3-methyl-2-thioxo-4-imidazolidinone).
RIP homotypic interaction motif	(RHIM). A protein–protein interaction motif containing the core sequence (I/V/L)-(Q/M)-(I/V/L)-G that mediates homophilic interactions between four cellular proteins (namely, RIP1, RIP3, TRIF and DAI) and one viral protein (MCMV vIRA).
Death receptors	A subset of receptors belonging to the TNF receptor superfamily that transmit cell death signals initiated by their cognate ligands. These receptors include TNFR1, FAS, DR3, TRAILR1 and TRAILR2.
Toll-like receptors	(TLRs). A family of pattern recognition receptors that recognize unique structures derived from microorganisms. TLR signalling promotes inflammatory and cytokine responses, as well as cell proliferation or cell death pathways.
FAS	Also known as CD95). A death receptor of the TNF receptor superfamily. FAS ligand binding induces cell death. FAS signalling controls the homeostatic elimination of T cells.
Death-inducing signalling complex	(DISC). A death receptor-bound complex that contains FADD and caspase 8 (or caspase 10). The DISC assembles following ligand binding and drives autocatalytic caspase 8 (or caspase 10) activation.
Complex I	A TNFR1-bound complex that contains TRADD, TRAF2 or TRAF5, cIAP and RIP1. This complex drives the activation of gene expression via the NF-κB and MAPK pathways.
Cellular inhibitor of	(cIAP1 and cIAP2; collectively referred to as cIAP here). Members of a family of functionally and structurally related E3 ubiquitin ligases that regulate canonical and non-canonical activation of NF-

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apoptosis proteins	κB, as well as MAPK activation by receptors of the TNF receptor superfamily. cIAP polyubiquitylates RIP1 to prevent the formation of the ripoptosome or TNFR1-dependent complex II.
Complex II	A TNFR1- and RIP1-dependent cytosolic complex that contains caspase 8, FADD, cFLIP and RIP1. Within this complex, caspase 8 and cFLIP regulate programmed cell death pathways.
Necrosome	An inducible cytosolic complex that contains oligomerized RIP1 and RIP3. This complex drives RIP1- and RIP3-dependent necroptosis.
Ripoptosome	A RIP1-dependent cytosolic complex that is similar in composition to TNFR1-dependent complex II and that controls programmed cell death pathways.
TIR domain-containing adaptor protein inducing interferon-β	(TRIF). A adaptor protein for TLR3 and TLR4 that organizes downstream signalling cascades leading to IRF3 and NF-κB activation, or cell death. TRIF mediates signalling through a TIR domain, TRAF-binding sites and RHIM-mediated interactions with RIP1 and RIP3.
CARMA1–BCL-10–MALT1 complex	A PKC-dependent specialized signalling complex that is formed during TCR-dependent antigen recognition and that triggers NF-κB activation.
DNA-dependent activator of interferon regulatory factors	(DAI; also known as ZBP1). A cytosolic, RHIM-containing sensor of double-stranded DNA that activates IRF3 and NF-κB.

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Box 1

Lessons from mice deficient in FADD or caspase 8

The striking phenotypes that emerge when caspase 8 or FAS-associated death domain protein (FADD) are eliminated in specific mouse tissues must now be viewed with the understanding that a caspase 8–FADD complex controls receptor-interacting protein 1 (RIP1)- and RIP3-mediated necroptosis[22–24]. Caspase 8-mediated control of apoptosis is important for T cell homeostasis, as revealed in adult *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice[22,23], as well as in mice with a T cell-specific disruption of *Casp8* or *Fadd* on a *Rip3*$^{-/-}$ background[65,68]. Moreover, the tissue-specific disruption of *Casp8* or *Fadd* has revealed many examples of conditions under which necroptosis may be unveiled during life. Caspase 8 deficiency in TIE1^{+} endothelial cells results in a phenotype that parallels germline disruption[15], reinforcing the conclusion that dysregulated RIP1–RIP3 underlies the vascular cell defects and embryonic death in *Casp8*-null mice. Interferon-inducible disruption of *Casp8* or *Fadd* leads to the elimination of cells in various lineages, and this suppresses early and mid-stage haematopoietic development[15,18,115]. The disruption of *Casp8* or *Fadd* in CD19^{+} B cells does not alter their response to antigens, although mutant cells fail to proliferate and die in response to Toll-like receptor 3 (TLR3) and TLR4 agonists that induce signalling through TIR domain-containing adaptor protein inducing IFNβ (TRIF)[116–118]. This provides a potential biological link to the recently identified ripoptosome[29]. The disruption of *Casp8* in the epidermis results in atopic dermatitis during the cornification process and has been used to model chronic skin disease[119–121]. Furthermore, mice that express a catalytically inactive form of caspase 8 along with a single wild-type allele develop inflammation in internal organs and skin[16]. Hepatocyte-specific caspase 8 deficiency results in a strong inflammatory response following partial hepatectomy and impaired liver regeneration[122], probably through the induction of necroptosis. Although a complete analysis is yet to be reported, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice continue through life without suffering any obvious deficits apart from abnormal T cell levels[22,23]. Remarkably, despite a pattern of midgestational death in C*asp8*$^{-/-}$ mice, the combined mutation of *Casp8* and *Rip3* results in embryos with functioning hearts, a correctly organized architecture of the yolk sac endothelia and normal levels of haematopoiesis. These mice appear normal and complete gestation to become fertile adults with no abnormal inflammation[22,23], in a similar manner to *Rip3*$^{-/-}$ mice[79]. *Fadd*$^{-/-}$*Rip1*$^{-/-}$ embryos appear normal throughout gestation but die soon after birth owing to the absence of RIP1 (REF. 24). The viability of *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice establishes that caspase 8 is largely dispensable for mammalian development and tissue homeostasis. Therefore, the many settings in which this caspase seemed to be crucial will require re-examination. The recent demonstration that RIP3 deficiency rescues epithelial necroptosis[32–34] reinforces this fact. Thus, severe inflammatory abnormalities that arise when caspase 8 (or FADD) is compromised[15,32–34,119,120,122] are likely to be the consequence of unleashed necroptosis.

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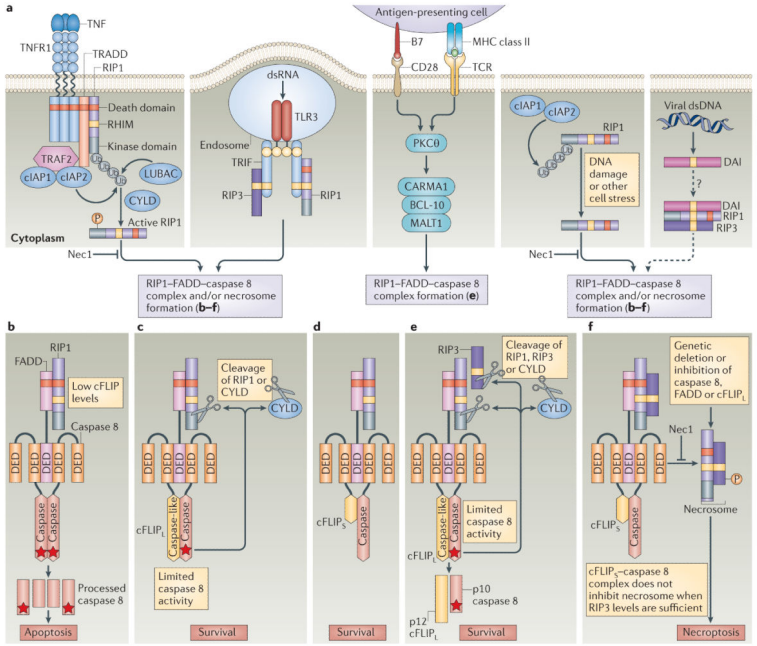

Figure 1. Caspase 8-mediated regulation of RIP1–RIP3 signalling pathways

a | Following tumour necrosis factor (TNF) binding, TNF receptor 1 (TNFR1) recruits receptor-interacting protein 1 (RIP1) via its death domain. When polyubiquitylated by the E3 ligases cellular inhibitor of apoptosis 1 (cIAP1), cIAP2 and linear ubiquitin chain assembly complex (LUBAC), RIP1 promotes the activation of nuclear factor-κB (NF- κB), which enhances cell survival by inducing the expression of cIAP1, cIAP2 and cellular FLICE-like inhibitory protein (cFLIP) (not shown). In the absence of RIP1 polyubiquitylation (for example, owing to insufficient cIAP levels or deubiquitylation by proteins such as cylindromatosis (CYLD)), RIP1 kinase activity downstream of TNFR1 facilitates the assembly of alternative signalling platforms comprising RIP1, FAS-associated death domain protein (FADD) and caspase 8. In an alternative scenario, the Toll-like receptor 3 (TLR3) and TLR4 adaptor protein TIR domain-containing adaptor protein inducing IFNβ (TRIF) binds to both RIP1 and RIP3 via RIP homotypic interaction motif (RHIM)-mediated interactions, bridging TLR3 signalling to the RIP1–FADD–caspase 8 complex (ripoptosome). Genotoxic damage or cell stress leads to the degradation of cIAP1 and cIAP2 and the formation of a ripoptosome. The cytosolic DNA sensor DAI (DNA-dependent activator of interferon regulatory factors) directly engages RIP1 and RIP3 in RHIM-dependent complexes to potentially drive the assembly and/or recruitment of a ripoptosome, in a similar manner to TRIF. The levels of cFLIP, the balance of cFLIP isoforms and the levels of caspase 8 activity determine whether apoptosis, necroptosis or cell survival ensues following the formation of the ripoptosome. **b** | When the levels of cFLIP long ($cFLIP_L$) or cFLIP short ($cFLIP_S$) are limiting, caspase 8 homodimerization promotes enzymatic activity, and this leads to caspase 8 autoprocessing and the execution of apoptosis through BID and/or caspase 3. **c**,**d** | In the presence of sufficient $cFLIP_L$ or $cFLIP_S$, a caspase 8–$cFLIP_L$ or caspase 8–$cFLIP_S$ heterodimer forms, and this supports cell survival. Importantly, the caspase 8–$cFLIP_L$ heterodimer retains sufficient proteolytic

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activity to cleave substrates such as RIP1 and CYLD to prevent necroptosis without allowing caspase 8 to induce apoptosis. **e** | T cell survival and proliferation following stimulation of the T cell receptor (TCR) and CD28 requires the inactivation of RIP1- and RIP3-mediated necroptosis by FADD–caspase 8–$cFLIP_L$, and this probably occurs downstream of the CARMA1–BCL-10–MALT1 signalling complex. **f** | When caspase 8 activity is blocked — under conditions of elevated $cFLIP_S$ levels or in the presence of a caspase 8 inhibitor — RIP1 binds to RIP3 to form a kinase-active necrosome to initiate necroptosis. RIP1 kinase activity drives the assembly of the cytosolic RIP1–FADD–caspase 8 signalling platform, as well as the necrosome. The RIP1 kinase inhibitor necrostatin 1 (Nec1) blocks both RIP1-dependent apoptosis and necroptosis. Stars indicate catalytically active caspase 8. DED, death effector domain; dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; PKCθ, protein kinase Cθ; TRADD, TNFR1-associated death domain protein; TRAF2, TNFR-associated factor 2; Ub, ubiquitin.

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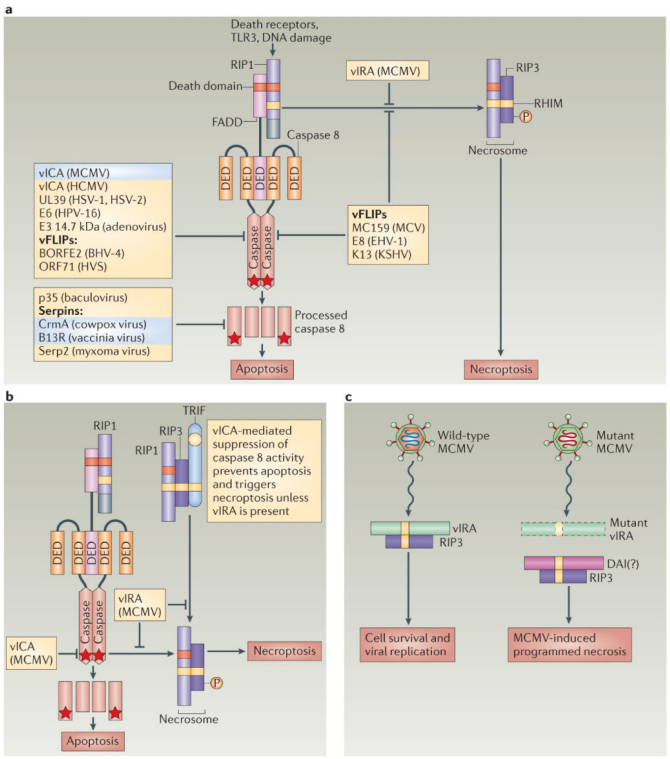

Figure 2. Viral modulation of cell death signals mediated by caspase 8 activation and RIP1–RIP3 pathways

a | Viral proteins that directly target receptor-interacting protein 1 (RIP1), FAS-associated death domain protein (FADD) and/or caspase 8 suppress signalling pathways that are activated by death receptors, pattern recognition receptors (PRRs; such as Toll-like receptor 3 (TLR3)) or cell stress (such as DNA damage). Many virus-encoded proteins block caspase 8-dependent apoptosis by interfering with caspase 8, FADD and/or RIP1 (see also TABLE 1). Several poxvirus proteins of the serpin family bind directly to fully processed caspase 8 to prevent apoptosis, whereas other viral inhibitors of caspase 8 — such as vICA (viral inhibitor of caspase 8 activation), CrmA and B13R (all highlighted in blue) — sensitize cells to death receptor-induced necroptosis by disrupting caspase 8-mediated suppression of RIP1–RIP3 activity. A subset of viral FLICE-like inhibitory proteins (vFLIPs) — including MC159, E8 and the murine cytomegalovirus (MCMV) protein vIRA (viral inhibitor of RIP activation) — block both apoptosis and RIP1- and RIP3-mediated necrosis. **b** | The MCMV protein vICA prevents caspase 8 activation, and this sensitizes cells to death receptor-induced necroptosis. In addition, the MCMV protein vIRA blocks necroptosis and MCMV-induced programmed necrosis by inhibiting RHIM (RIP homotypic interaction motif)-dependent interactions. vIRA also inhibits the activation of caspase 8 by RIP1 or TIR domain-containing adaptor protein inducing IFNβ (TRIF). **c** | A mutant MCMV that encodes vIRA with a mutant RHIM domain triggers programmed necrosis in cells with sufficient levels of RIP3. MCMV-induced programmed necrosis is independent of RIP1. The cellular RHIM-containing cytosolic sensor of double-stranded DNA DAI (DNA-dependent activator of interferon regulatory factors) may promote RIP3-dependent programmed necrosis during infection with mutant MCMV. Stars indicate catalytically active caspase 8. BHV-4, bovine

herpesvirus 4; DED, death effector domain; EHV-1, equine herpesvirus 1; HCMV, human cytomegalovirus; HPV-16, human papillomavirus 16; HSV, herpes simplex virus; HVS, herpesvirus saimiri; KSHV, Kaposi's sarcoma-associated herpesvirus; MCV, molluscum contagiosum virus.

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Table 1

Selected viral inhibitors of apoptosis and necrosis

Type of inhibitor	Inhibitor	Virus	Known targets	Mechanism	Gene ID or accession number
Inhibitors of apoptosis and programmed necrosis					
cFLIP homologue	MC159	MCV	Caspase 8, FADD	Inhibits oligomerization	1487017
cFLIP homologue	K13	KSHV	Caspase 8	Prevents activation	4961494
cFLIP homologue	E8	EHV-1	Caspase 8	–	1461076
RHIM inhibitor	vIRA	MCMV	RIP1, RIP3, TRIF, DAI	Inhibits RHIM-mediated interactions	CAP08092.1
Inhibitors of apoptosis					
Caspase 8 inhibitor	vICA	CMV	Caspase 8	Prevents activation	3077442
Caspase 8 inhibitor	BORFE2	BHV-4	Caspase 8	–	1684940
Caspase 8 inhibitor	E3 14.7 kDa	Adenovirus	Caspase 8	Prevents activation	1460862
Caspase 8 inhibitor	UL39	HSV-1, HSV-2	Caspase 8	Prevents activation	2703361, 1487325
Serpin	CrmA	Cowpox virus	Caspases 1, 4, 5, 8 and 10, granzyme B	Inhibits activity	1486086
Serpin	B13R	Vaccinia virus	Caspases	–	3707572
Serpin	Serp2	Myxoma virus	Caspases	–	932102
Other	E6	HPV-16	Caspase 8, FADD	Inhibits oligomerization, degrades	1489078
Other	p35	Baculovirus	Caspases	Inhibits activity	1403968

BHV-4, bovine herpesvirus 4; CMV, cytomegalovirus; DAI, DNA-dependent activator of interferon regulatory factors; EHV-1, equine herpesvirus 1; FADD, FAS-associated death domain protein; HPV-16, human papillomavirus 16; HSV, herpes simplex virus; KSHV, Kaposi's sarcoma-associated herpesvirus; MCMV, murine cytomegalovirus; MCV, molluscum contagiosum virus; RHIM, RIP homotypic interaction motif; RIP, receptor-interacting protein; TRIF, TIR domain-containing adaptor protein inducing IFNβ; vICA, viral inhibitor of caspase 8 activation; vIRA, viral inhibitor of RIP activation.