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## Programmed cell death as a defence against infection

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### Abstract

Eukaryotic cells can die from physical trauma, resulting in necrosis. Alternately, they can die via programmed cell death upon stimulation of specific signalling pathways. Here we discuss the utility of four cell death pathways in innate immune defence against bacterial and viral infection: apoptosis, necroptosis, pyroptosis and NETosis. We describe the interactions that interweave different programmed cell death pathways, which create complex signalling networks that cross-guard each other in the evolutionary arms race with pathogens. Finally, we describe how the resulting cell corpses — apoptotic bodies, pore-induced intracellular traps (PITs) and neutrophil extracellular traps (NETs) — promote clearance of infection.

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### Highlighted References

- 
- 21,22 Discovery of GSDMD as the effector of pyroptosis cleaved by caspase-1 and -11
- 26,28 GSDMD forms a pore in membranes.
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- 52 ZBP1 detects MCMV to trigger necroptosis, but is counteracted by MCMV vIRA.
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- 56 RIPK3 critical for necroptosis and *Ripk3*<sup>-/-</sup> mice susceptible to vaccinia infection.
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- 85,86 cGAS as guard for MOMP / caspase-9, triggering a type I IFN response when apoptosis fails to complete.
- 88,89 The embryonic lethality of *Casp8*<sup>-/-</sup> mice is complemented by crossing with *Ripk3*<sup>-/-</sup>, demonstrating that necroptosis guards caspase-8.
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- 55–57 RIPK3 activation drives necroptosis.
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### Competing interests statement

The authors declare no competing interests.

## Introduction

Cell death can be either programmed or accidental. Programmed cell death results in either lytic or non-lytic morphology, depending upon the signalling pathway. For example, **apoptosis**<sup>1,2</sup> is a non-lytic and typically immunologically silent form of cell death. On the other hand, programmed lytic cell death is highly inflammatory, and includes **necroptosis**<sup>3–5</sup>, **pyroptosis**<sup>6</sup>, and the rapid release of so-called **neutrophil extracellular traps** (NETs) in a process known as **NETosis**<sup>7</sup>. The elimination of host immune cells by programmed cell death can be thought to benefit an infecting pathogen<sup>8</sup>. However, programmed cell death is increasingly understood to benefit the host, for example, by eliminating the intracellular niche of certain pathogens<sup>9</sup>. Furthermore, the resulting cellular corpses coordinate an appropriate innate immune response to promote the resolution of infection. However, since our understanding of innate immune defences is mainly based on work with pathogens that evade these defences, it is often difficult to recognize how programmed cell death functions in response to infections. Indeed, many discoveries of cell death functions are based on experiments with pathogens that have been genetically modified to remove their normal host evasion strategies.

Significant crosstalk exists between different cell death pathways, and as a result, innate immune signalling pathways are well guarded against pathogen attack. The ‘guard hypothesis’ was first developed in plant immunity, where studies have shown that innate immune sensors monitor for perturbation of other innate immune signalling pathways<sup>10</sup>. For example, in *Arabidopsis thaliana*, pathogens often attempt to inhibit key proteins that are involved in innate immune signalling. When pathogens attack one such protein, RPM1-interacting protein 4 (RIN4), the perturbation is detected by two different nucleotide oligomerization domain (NOD)-like receptors (NLRs), which activate programmed cell death — a phenomenon known as the hypersensitive response in plants — to combat the pathogen<sup>11,12</sup>. This guard mechanism is echoed in mammalian programmed cell death pathways, which function to guard each other as well as other innate immune signalling pathways.

In this Review, we discuss how the host utilizes programmed cell death to fight infections with a focus on in vivo studies. We also discuss how difficult, but not impossible, it can be for pathogens to evade programmed cell death because of the guard functions built into the signalling pathways. We discuss the role of pyroptosis, necroptosis and apoptosis during infection, and how their signalling pathways are functionally interwoven to make them guard each other. Finally, we discuss the fate of dead cell corpses such as apoptotic bodies, NETs, and pore-induced intracellular traps (PITs), and how their physical properties are beneficial to fight infection.

## Inflammasomes trigger pyroptosis

Inflammasomes are cytosolic sensors that activate caspase 1<sup>13</sup>. Once activated, caspase 1 cleaves pro-interleukin-1 $\beta$  (IL-1 $\beta$ ) and pro-IL-18 into their mature forms, and cleaves gasdermin D (encoded by *Gsdmd*) to induce pore opening and pyroptosis. (Figure 1). Inflammasomes are cytoplasmic platforms composed of either NLR, AIM2-like receptor

(ALR), or tripartite motif-containing (TRIM) proteins that detect contamination of the cytosol by DNA, bacterial flagellin, the type 3 secretion system (T3SS) needle and rod subunits, toxins and cellular perturbations. Mouse caspase-11 — which is duplicated in humans as caspase-4 and caspase-5 — also induces pyroptosis<sup>14</sup>, but does not cleave pro-IL-1 $\beta$  or pro-IL-18 on its own. Caspase-11 directly detects lipopolysaccharide (LPS) when it enters the cytosol<sup>15–17</sup>, and thus protects against infection by cytosol-invasive Gram-negative bacteria such as *Burkholderia thailandensis*<sup>18,19</sup>, but can be evaded by pathogens such as *Francisella novicida*<sup>15</sup> and *Shigella flexneri*<sup>20</sup>. Caspases-11, -4 and -5 are often called the non-canonical inflammasome<sup>14</sup>. The role of inflammasomes in pyroptosis has been reviewed in detail<sup>6</sup> and is summarized in Fig. 1.

Recently, two research groups independently discovered that caspases-1 and -11 cleave gasdermin D and this cleavage is required to trigger pyroptosis<sup>21,22</sup>. This finding was later confirmed by a third research group<sup>23</sup>. Interestingly, cleavage of gasdermin D was observed a few years earlier, but its importance remained uninvestigated<sup>24</sup>. Gasdermin D is a member of the greater gasdermin-domain protein family, consisting of 6 members in humans and 10 in mice<sup>25</sup>. The function of other gasdermin family members remains to be further elucidated, and while many probably induce pyroptosis<sup>21</sup>, it remains unclear how they are activated.

Gasdermins are bipartite proteins whose N- and C-terminal domains are connected by a linker, and have several proposed roles in immune-related diseases<sup>25</sup>. Caspases-1 or -11 cleave this linker of gasdermin D, releasing the N-terminal domain<sup>21,22</sup>, which then associates with the inner leaflet of the plasma membrane and oligomerizes to form pores. These pores range in size from 10–33 nm<sup>26–29</sup>, just large enough to allow passage of mature IL-1 $\beta$  (which is 4.5 nm)<sup>26,28</sup>; this could explain how IL-1 $\beta$  can be released from cells that do not lyse, which might occur, hypothetically, if a very low number gasdermin D pores form. Small, but detectable, amounts of IL-1 $\beta$  may escape through these pores, while small amounts of sodium and water enter. The resulting mild cell swelling should be counteracted by regulatory volume decrease mechanisms, preventing membrane rupture<sup>30</sup>. Later, the cell could remove the pores by normal membrane repair processes<sup>31</sup>. Conversely, if more gasdermin D pores form, significant swelling should overwhelm the regulatory volume decrease, resulting in membrane rupture that we call pyroptosis, and the release of any remaining processed IL-1 $\beta$  from the cytosol. The N-terminal domain of gasdermin D has affinity not only for the inner leaflet of the plasma membrane, but also for other phospholipids and cardiolipin<sup>26–28,32</sup>, which suggests that it may also target organelles. In this regard, permeabilization of the endoplasmic reticulum may explain the calcium flux that occurs during pyroptosis<sup>33,34</sup>.

Although numerous pathogens trigger pyroptosis in vitro, the role of pyroptosis in vivo is much less studied. Mice deficient in caspase 1 have an increased susceptibility to a variety of pathogens (Table S1). The importance of pyroptosis in vivo is primarily inferred from comparing the resistance to infection of *Casp1*<sup>−/−</sup> mice with *Il1b*<sup>−/−</sup> *Il18*<sup>−/−</sup> mice. This comparison remains to be performed for many pathogens. The recently generated *Gsdmd*<sup>−/−</sup> mice are defective for both pyroptosis and release of IL-1 $\beta$  and IL-18 (at least in vitro)<sup>21–23</sup>, so a genetic approach to inhibit pyroptosis while maintaining cytokine secretion remains

elusive. Finally, it is important to remember that defence against infection may require IL-1 $\beta$ , IL-18 and pyroptosis in conjunction<sup>35</sup>.

### Pyroptosis defends against intracellular bacteria

The most direct evidence for pyroptosis in clearing infections comes from the investigation of bacterial strains engineered to activate the NLR family, CARD-containing 4 (NLRC4) inflammasome and induce pyroptosis *in vivo*. Normally, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Listeria monocytogenes* efficiently evade inflammasomes during the systemic phase of infection in mice<sup>9,36–39</sup>. By contrast, strains engineered to persistently express flagellin are detected by NLRC4 and are rapidly cleared. The observation that mice deficient in IL-1 $\beta$  and IL-18 could still clear these flagellin-engineered bacteria suggests that pyroptosis is the primary mechanism for bacterial clearance<sup>9,36–38</sup>. Similarly, caspase 11 provides *in vivo* defence against *S. Typhimurium* strains (*sifA* mutants) that have lost the ability to maintain the integrity of the *Salmonella*-containing vacuole and thus aberrantly enter the cytosol<sup>18</sup>. After pyroptosis *in vitro*, bacteria are damaged<sup>40</sup>, perhaps by gasdermin D pores<sup>28</sup> and are more susceptible to secondary insults such as hydrogen peroxide or antibiotics<sup>40</sup>. However, the physiologic relevance of this observation remains unclear, since pyroptosis and gasdermin D are insufficient to kill intracellular bacteria *in vivo*<sup>9</sup>. Instead, after pyroptosis in mice, bacteria are transferred to neutrophils, where they are killed by reactive oxygen species (ROS)<sup>9,40</sup>.

Pyroptosis also protects against non-engineered, opportunistic microorganisms *in vivo*, including *Chromobacterium violaceum*, *B. thailandensis*, *Burkholderia pseudomallei* and *F. novicida*<sup>18,19,39,41,42</sup>. However, additional supporting data is needed to confirm this protection<sup>43</sup>. Amongst these bacteria, *B. thailandensis* and *C. violaceum* have the strongest phenotypes of all tested pathogens in inflammasome-deficient mice<sup>19,39</sup> (Table S1)<sup>44</sup>. As few as 100 colony forming units (CFUs) of *C. violaceum* or *B. thailandensis* are lethal to inflammasome-deficient mice, whereas wild-type mice survive challenges by 1,000,000 to 20,000,000 CFU. The strength of these phenotypes leads us to propose the Red Pawn Hypothesis, a corollary to the Red Queen Hypothesis. We propose that despite their evasion by host-adapted pathogens, inflammasomes may be maintained over evolutionary time due to their critical role in defence against pathogens that are co-evolving with a non-mammalian host that lacks inflammasome defences. Thus, some ubiquitous environmental bacteria have specific virulence traits capable of manipulating a generic eukaryotic cell, making them potentially lethal pathogens. However, they are unprepared for the mammalian innate immune system, including inflammasome-driven responses (Box 1 and further discussion in reference <sup>44</sup>).

#### Box 1

##### Hypothesis to explain inflammasome importance despite evasion by pathogens

In Lewis Carol's *Through the Looking Glass*, Alice finds herself in a race with the Red Queen, in which "it takes all the running you can do, to keep in the same place." This inspired the evolutionary hypothesis that constant change is required to survive within a

constantly changing environmental niche alongside constantly changing competitors. But in the end, each organism tends to maintain its place within the niche. This concept applies to the host-pathogen interaction: as hosts (Alice) evolve new immune responses, pathogens (the Red Queen) evolve virulence factors to overcome them. For example, numerous pathogens evade inflammasome detection<sup>156</sup>. Thus, many successful pathogens appear to be perpetually one step ahead in the evolutionary equilibrium between inflammasome detection and evasion. On the other hand, aberrant inflammasome activation can be lethal<sup>15,16,34</sup>, which might select for loss of these sensors over time. One must ask, then, why would Alice run with inflammasomes that fail to fight the Red Queen?

Interestingly, there are two pathogens which inflammasomes decisively control in vivo, *Burkholderia thailandensis*<sup>19</sup> and *Chromobacterium violaceum*<sup>39</sup> (Table S1)<sup>44</sup>. Both employ significant virulence traits that make them lethal to inflammasome-deficient mice and immunocompromised people. But in normal animals, inflammasomes render them harmless. *B. thailandensis* and *C. violaceum* are ubiquitous soil/mud bacteria whose natural host remains unknown; perhaps it is a lower eukaryote that lacks inflammasomes, such as insects or nematodes.

This leads us to propose a corollary to the Red Queen Hypothesis. We propose that *C. violaceum* and *B. thailandensis* are not running an evolutionary race alongside humans because they are not natural human pathogens. Instead, they run a completely separate race altogether, alongside a non-human host. They may occasionally lose their way and stumble clumsily onto the human racecourse, where they find themselves brushed aside by inflammasomes. Hence, the mammalian innate immune response is perpetually winning the evolutionary race against environmental pathogens that may be dangerous, but that lack sophisticated immune evasion strategies.

This could explain why inflammasomes are maintained over evolutionary time despite the prevalence of evasive pathogens. Inflammasomes may act as a barrier to prevent opportunistic infection. Such a barrier may not be perfect since *B. thailandensis* has two close relatives that have advanced on the pathway to mammalian pathogenesis – environmental bacteria that have evolved into Red Queens. *Burkholderia pseudomallei* is broadly pathogenic to mammals and causes melioidosis<sup>157</sup>. Further along the adaptation course is *B. mallei*, which is the highly virulent equine-adapted cause of glanders<sup>158</sup>. Further caveats to the this hypothesis are described in ref <sup>44</sup>.

Our hypothesis thus predicts that certain innate immune sensors provide a near-permanent victory for the host in defence against a large reservoir of potentially deadly environmental pathogens, while host-adapted pathogens must evade these sensors.

Additionally, inflammasomes provide defence against microorganisms that have adapted to mammalian hosts. For example, although *S. Typhimurium* efficiently evades inflammasomes during systemic infection, they are detected in the gut<sup>45</sup>. Intestinal epithelial cell NLRC4 triggers epithelial cell shedding during infection. Whether this process is a precursor stage during pyroptosis, or is fully independent of pyroptosis requires further study<sup>45</sup>. Caspase-11

appears to play a similar, parallel role in epithelial shedding to eliminate the low frequency but rapidly replicating sub-population of *S. Typhimurium* that aberrantly enter the cytosol<sup>46</sup>.

### Pyroptosis during viral infection

Inflammasomes also induce pyroptosis in vitro following the detection of viruses, such as murine cytomegalovirus (MCMV) via the double-stranded DNA-detecting cytosolic sensor AIM2. Parallel in vivo studies show that *Aim2*<sup>-/-</sup> mice are susceptible to MCMV infection<sup>47</sup>. However, whether this susceptibility arises from pyroptosis, IL-1 $\beta$  or IL-18 remains to be explored. Conversely, a detrimental effect of pyroptosis has been described during HIV infection. Interferon- $\gamma$  (IFN $\gamma$ )-inducible protein 16 (IFI16) — which is an AIM2-like receptor that detects viral nucleic acids — induces pyroptosis in CD4<sup>+</sup> T cells abortively infected by HIV<sup>48</sup>. This is proposed as the major pathway by which HIV depletes CD4<sup>+</sup> T cells in vivo<sup>49,50</sup>. Conversely, IFI16 has also been suggested to play a beneficial role in the defence against HIV infection, although pyroptosis was not examined as a possible mechanism<sup>51</sup>.

### RIPK3 triggers necroptosis

Necroptosis, like pyroptosis, is a form of programmed lytic cell death. Necroptosis can be triggered by a variety of intricate pathways (discussed below) and perhaps the simplest pathway is through Z-DNA binding protein 1 (ZBP1; also known as DAI and DLM-1).

### Induction of necroptosis by ZBP1

ZBP1 binds either Z-DNA or Z-RNA via two Z-DNA binding motifs, and can respond to MCMV (a DNA virus) or Influenza (a RNA virus)<sup>52–54</sup>. Unlike the normal B-form of DNA, Z-form DNA results from torsional strain that may occur during rapid DNA synthesis that could occur during viral infection. Once activated, its receptor-interacting protein (RIP) homotypic interaction motif (RHIM) binds the RHIM of RIP kinase 3 (RIPK3)<sup>52</sup>. This interaction stimulates RIPK3 kinase activity, auto-phosphorylation and oligomerization<sup>55–57</sup> and RIPK3 phosphorylates the necroptosis effector mixed lineage kinase domain-like (MLKL)<sup>58</sup> (Figure 2). Following phosphorylation by RIPK3, the MLKL pseudokinase undergoes a conformational switch<sup>59</sup>, licensing interaction with the inner leaflet of the plasma membrane where MLKL oligomerizes to form the necroptotic pore<sup>60–62</sup>, which is analogous to gasdermin D for pyroptosis. Additional steps may also regulate MLKL<sup>63</sup>. After pore formation, the terminal events in necroptosis are similar to those in pyroptosis; osmotic pressure from ion and water influx results in rupture of the plasma membrane. In our hands pyroptosis and necroptosis (both at 2h post treatment) are morphologically identical in primary macrophages<sup>40</sup>, with the exception of slightly different nuclear morphology (I.J. and E.A.M, unpublished observation). Other research laboratories visualize differences in RAW264.7 macrophages in settings where pyroptosis is fast (2h) while necroptosis is slow (4h)<sup>32</sup>. The morphologies in comparison deserve further study. Necroptosis can be investigated in vivo by using *Ripk3*<sup>-/-</sup> or *Mlkl*<sup>-/-</sup> mice; however, RIPK3 has been suggested to have non-necroptotic functions during influenza infection<sup>64</sup> and during inflammatory disease models<sup>65</sup>. Therefore, before attributing a phenotype to necroptosis, it is important to verify that phenotypes observed in *Ripk3*<sup>-/-</sup> mice are also seen in *Mlkl*<sup>-/-</sup> mice.



## Necroptosis defends against viral infections

The clearest phenotypes for necroptosis during in vivo infection highlight an antiviral role. MCMV encodes a viral inhibitor of RIP activation (vIRA), whose RHIM mimic inhibits RIPK3 signalling, and thereby prevents necroptosis<sup>52</sup>. A RHIM domain mutant virus is attenuated, but this attenuation is reversed in *Ripk3*<sup>-/-</sup> and *Zbp1*<sup>-/-</sup> mice<sup>52</sup>. Similarly, human-adapted herpes simplex virus 1 (HSV-1) and HSV-2 express two protein homologs, ICP6 and ICP10, that inhibit human RIPK3<sup>66,67</sup>. However, there appears to be some degree of species tropism; human HSV-1 ICP6 interacts with mouse RIPK3, presumably attempting to inhibit it, but due to the species mismatch, RIPK3 is activated, which triggers necroptosis to prevent viral replication<sup>66–68</sup>. Accordingly, *Ripk3*<sup>-/-</sup> mice are susceptible to HSV-1 infection<sup>69</sup>. This idea does not hold true for MCMV, which inhibits necroptosis in both human and mouse cells<sup>66</sup>.

The antiviral role for necroptosis is further supported by the observation that *Ripk3*<sup>-/-</sup> mice are susceptible to vaccinia virus infection<sup>56</sup>. The RIPK3 pathway also defends against a mouse adapted influenza infection. *Zbp1*<sup>-/-</sup> and *Ripk3*<sup>-/-</sup> mice have increased mortality during infection influenza A PR8<sup>54,64</sup>. However, the susceptibility of *Ripk3*<sup>-/-</sup> mice to influenza virus was not replicated in another study<sup>70</sup> and a third study shows the reverse, where *Zbp1*<sup>-/-</sup> mice have higher viral titers but reduced tissue damage and mortality<sup>53</sup>, suggesting a simultaneously beneficial role in defense paired with a detrimental role in immunopathology.

Finally, *Ripk3*<sup>-/-</sup> mice have normal resistance to mouse hepatitis virus<sup>71</sup>. Many other mouse viral infection models remain to be examined in *Ripk3*<sup>-/-</sup> and *Mlkl*<sup>-/-</sup> mice. All reported lethality phenotypes of *Ripk3*<sup>-/-</sup> and related necroptosis pathway mice are summarized in Table S1.

## Necroptosis during bacterial infections

To date, in vivo data does not support an important role for necroptosis against bacterial infections. The most prominent report proposed that reduced necroptosis leads to increased susceptibility to *S. Typhimurium* infection in mice lacking *Ifnar1* (which encodes interferon  $\alpha/\beta$ -receptor subunit 1)<sup>72</sup>. However, this study did not compare other competing hypotheses, most importantly the hyper-responsiveness of *Ifnar1*<sup>-/-</sup> mice to IFN $\gamma$  (which combats intracellular bacteria such as *S. Typhimurium*) due to increased expression of the IFN $\gamma$  receptor<sup>73</sup>. Wild-type and *Ripk3*<sup>-/-</sup> mice showed similar susceptibility to *S. Typhimurium* infection<sup>72</sup>, demonstrating that necroptosis plays little role in IFNAR1-sufficient mice. Similarly, *Ripk3*<sup>-/-</sup> mice and wild-type mice are comparably sensitive to *Yersinia pseudotuberculosis*<sup>74</sup> and *Citrobacter rodentium* infections<sup>75</sup>. *Ripk3*<sup>-/-</sup> mice may have a subtle (but statistically insignificant) susceptibility to an LPS engineered *Yersinia pestis* strain that cannot evade TLR4<sup>76</sup> (Table S1). Interestingly, *Mlkl*<sup>-/-</sup> mice are susceptible to *Staphylococcus aureus* skin infection<sup>77</sup>, but paradoxically *Ripk3*<sup>-/-</sup> mice are resistant to both skin and lung infection<sup>77,78</sup>. Inhibitor treatment suggests that necroptosis may be pathologic during *Serratia marcescens* infection<sup>79</sup>, but this needs further study using knockout mice. Specific inhibition of RIPK3 by bacteria has not yet been described. Many more bacterial infection models remain to be examined in *Ripk3*<sup>-/-</sup> and/or *Mlkl*<sup>-/-</sup> mice, and

it seems likely that some will establish a role for necroptosis in defence against specific bacteria.

## Apoptosis during infections

Apoptosis is programmed cell death characterized by cytoplasmic shrinking, cell rounding, chromatin condensation, DNA fragmentation and membrane blebbing<sup>1,2</sup>. Apoptosis can be initiated by cell extrinsic pathways (which are mediated by death receptors) or cell intrinsic (mitochondrial) pathways, both of which culminate in the activation of the effector caspases 3, 6 and 7 (Figure 2). Apoptosis can also be initiated by cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells that deliver granzymes, which activate apoptotic caspases. Upon completion of apoptosis, cellular contents are encapsulated within membrane bound apoptotic bodies. If these bodies are not cleared, they will undergo secondary necrosis (rupture), releasing cytosolic damage-associated molecular patterns (DAMPs) to the extracellular space<sup>80</sup>. The role of apoptosis against infection is perhaps best appreciated by inference, since numerous viruses and bacteria encode apoptosis inhibitors that are essential for virulence (reviewed in <sup>81,82</sup>). This again raises the conundrums posed in the Red Pawn Hypothesis, if most viruses evade apoptosis, are there pathogens that apoptosis effectively counters, providing fully penetrant innate immunity (Box 1). Here, we focus on some of the recent advances in apoptosis that are relevant to infection.

### Guarding cell intrinsic apoptosis with interferons

Intrinsic apoptosis is often driven by cell intrinsic stresses such as DNA damage and growth factor withdrawal, which leads to mitochondrial outer-membrane permeabilization (MOMP)<sup>83,84</sup>. Although its structure remains to be fully elucidated, the MOMP pore appears to be formed by BAX and BAK<sup>83,84</sup>. MOMP leads to release of mitochondrial contents, including cytochrome c, into the cytosol where it binds to the NLR protein apoptotic protease activating factor 1 (APAF1), which oligomerizes into a hub to form the apoptosome — an activating platform for the initiator caspase 9. Activated caspase 9 in turn cleaves and activates the effector caspases 3 and 7<sup>2</sup>. Since apoptosis destroys the replicative niche of intracellular pathogens, particularly viruses, those that prevent apoptosis will replicate better as the infected cell lives longer.

Historically, apoptosis is considered immunologically silent owing to the collective efforts of caspases. For example, apoptotic DAMPs that can induce a robust type I interferon-mediated proinflammatory response are dampened by caspases<sup>85,86</sup>. During viral infection of *Casp9*<sup>-/-</sup> or *Casp3*<sup>-/-</sup>*Casp7*<sup>-/-</sup> cells, MOMP is initiated, but apoptosis fails to proceed. MOMP eventually releases mitochondrial DNA (mtDNA) into the cytosol, where it is detected by the DNA sensor cGAS, resulting in a type I IFN response<sup>85,86</sup>. Thus, pathogens that only block caspases will encounter a cGAS-driven type I IFN response that effectively guards events downstream of MOMP. This interferon response promotes resistance to viral infections including encephalomyocarditis virus (EMCV) and vesicular stomatitis virus (VSV). By contrast, blockade upstream of MOMP — which can be studied in *Bax*<sup>-/-</sup>*Bak*<sup>-/-</sup> mice — does not trigger cGAS because MOMP is not initiated and mitochondria retain their DNA<sup>85,86</sup>.



## Apoptosis/necroptosis guard innate immune signalling

Cell extrinsic apoptosis is typically initiated by extracellular stress signals that engage a subset of TNF-superfamily of receptors called death receptors<sup>2</sup>. Prototypical extrinsic stimuli include FasL, TNF and TNF-related apoptosis-inducing ligand (TRAIL). Following death receptor engagement, caspase 8 (human caspase 10) are recruited and activated via the death inducing signalling complex (DISC). In certain cell types, caspase 8 can also cleave BID into tBID, initiating MOMP in a positive feedback loop that leads to caspase 9, another initiator caspase, thus ensuring a strong initiation of. Thus, caspase 8 activation connects extrinsic and intrinsic apoptosis (Figure 2).

Additionally, caspase 8 is ensconced within the TNF receptor 1 (TNFR1), TLR3, and TLR4 signalling pathways<sup>3–5,87</sup>. Caspase 8 interacts with RIPK1, which is a key scaffolding protein within the signalling cascade<sup>3–5,87</sup>. Thus, caspase 8 is often interpreted as a node that regulates the divergence of signalling towards either transcriptional responses or apoptosis. For example, it is frequently stated that caspase 8 regulates TNF-dependent transcriptional responses and TNF-driven apoptosis<sup>3–5,87</sup>. In this section, we will present this pathway from the perspective of a pathogen.

TNFR1, TLR3, and TLR4 are important innate immune sensors — which respond to TNF, double-stranded RNA and LPS, respectively — that drive potent transcriptional responses (but do not trigger cell death under normal circumstances)<sup>3</sup>. Hence, these sensors are attractive targets for pathogens to inhibit, particularly since all three use RIPK1 as a scaffolding node to assemble signalling complexes (Figure 2). However, when signalling at or below RIPK1 is pharmacologically inhibited, this causes an alteration in the flow of signals through the pathway, which triggers a counter response that activates caspase 8<sup>3,5</sup>. In other words, if pathogens target these signalling pathways they would be met by a caspase 8 tripwire that triggers apoptosis. If pathogens possessed virulence strategies to simultaneously target RIPK1 and caspase 8, they would encounter a second tripwire in RIPK3, which also interacts with RIPK1. As the first caspase 8 tripwire fails to function, signalling build-up at RIPK1 results in the activation of RIPK3, triggering this second guard pathway that drives the response to infection to necroptosis rather than apoptosis (Figure 2). Thus, caspase 8, RIPK1 and RIPK3 essentially function as a triangular guard system.

This cross-guarded system becomes activated during embryogenesis in *Casp8*<sup>-/-</sup> mice, causing embryonic lethality<sup>4</sup>. In line with the cross-guard model, *Casp8*<sup>-/-</sup> lethality is rescued by simultaneous deletion of *Ripk3*<sup>88,89</sup>. Thus, caspase-8 is guarded by RIPK1-RIPK3 driven necroptosis.

Further, recent evidence suggests that RIPK3 is guarded by caspase 8, essentially reverse guarding the guard. Although *Ripk3*<sup>-/-</sup> mice are viable and healthy, mice engineered to carry some (but a not all) point mutations within the *Ripk3* kinase enzymatic site suffer from neonatal lethality as a caspase 8 guard program becomes activated, triggering lethal apoptosis during development<sup>90,91</sup>. In this guard system, the RIPK3 protein must be present and abnormal in order to trigger caspase 8, whereas simple absence of RIPK3 does not trip this guard pathway (as it does when caspase 8 is absent. This could explain instances where stimuli cause necroptosis, but *Mik1*<sup>-/-</sup> cells still die whereas *Ripk3*<sup>-/-</sup> cells live; in *Mik1*<sup>-/-</sup>

cells signalling events may accumulate at RIPK3, triggering the guard function of caspase 8, but this does not happen if *Ripk3* is deleted<sup>53</sup>.

Finally, RIPK1 is guarded by both caspase 8 and RIPK3. Deletion of *Ripk1* can only be rescued by simultaneous deletion of both *Casp8* and *Ripk3*<sup>92–94</sup>, illustrating redundant guarding. On the necroptosis side, there are two pathways that guard RIPK1. The first is direct, where RIPK3 activates autonomously in response to deleted or abnormal RIPK1. The second is upstream, when RIPK1 is absent or its RHIM domain is mutated, this triggers activation of ZBP1, which normally docks on RIPK1 to hold it in check<sup>95,96</sup>. Thus, *Ripk1*<sup>RHIM</sup> mice can be rescued by crossing with either *Mlt*<sup>−/−</sup>, *Ripk3*<sup>−/−</sup> or *Zbp1*<sup>−/−</sup> mice<sup>95,96</sup>. ZBP1 then activates RIPK3. These essentially double guard RIPK1.

To add to the complexity, the caspase 8 guard function is built into the RIPK1 scaffold function; TNFR1–TLR3–TLR4 signalling fails to drive transcription in *Casp8*<sup>−/−</sup> cells<sup>97,98</sup>. It seems that RIPK1 fails to function when it is unable to interact with its guard caspase 8. The same may be true for *Ripk3*<sup>−/−</sup> cells but only in specific cell types<sup>99</sup>. In contrast, TLR2 signalling, which does not use TRIF–RIPK1, remains intact<sup>76,100</sup> (although one study did see defects in TLR2 signaling<sup>75</sup>, perhaps due to autocrine TNF signalling; also see Figure 2 legend and Box 2).

## Box 2

### Compound defects in *Casp8*<sup>−/−</sup> mice

Investigators generated mice lacking caspase 8 to study cell extrinsic apoptosis in vivo, but these mice turned out not to be viable<sup>4</sup>. A caspase 8 deletion induces necroptosis and destruction of the yolk sac vasculature, and this circulatory failure leads to embryonic death. However, introduction of *Ripk3*<sup>−/−</sup> complements the lethality, and *Casp8*<sup>−/−</sup> *Ripk3*<sup>−/−</sup> mice are viable since cells in these mice cannot undergo necroptosis<sup>88,89</sup>. Comparing *Casp8*<sup>−/−</sup> *Ripk3*<sup>−/−</sup> mice to *Ripk3*<sup>−/−</sup> controls allows study of caspase 8 genetic deficiency in vivo. However, these mice must be interpreted with great caution since the *Casp8*<sup>−/−</sup> mutation eliminates multiple signalling pathways: i) it eliminates extrinsic apoptosis driven by TNF, Fas, and TNF-related apoptosis-inducing ligand (TRAIL), which could be beneficial during infection<sup>88,89,159</sup>; ii) it alters homeostasis in adaptive immune cells, resulting in a lymphoproliferative disorder that is apparent at 2 months of age<sup>88,89</sup>; iii) it significantly impedes or fully abrogates transcriptional responses via tumour necrosis factor receptor (TNFR), Toll-like receptor 3 (TLR3) and TLR4 due to loss of the caspase 8–RIPK1 scaffolding platform<sup>97,98</sup>; iv) its effect on TNF, TLR3 and TLR4 also prevent these receptors from post-translational priming of the NLRP3 inflammasome<sup>75,76,100,107–109</sup>. *Fadd*<sup>−/−</sup> *Ripk3*<sup>−/−</sup> mice are similarly affected<sup>71,74,75,160</sup>. This makes it difficult to assign a precise caspase 8 function to phenotypes observed by comparing *Casp8*<sup>−/−</sup> *Ripk3*<sup>−/−</sup> to *Ripk3*<sup>−/−</sup> mice. In order to do this, one could start by comparing various *Ripk3*<sup>−/−</sup> background mice. For example, the resistance of *Casp8*<sup>−/−</sup> *Ripk3*<sup>−/−</sup> mice to endotoxic shock<sup>88</sup> may be similar to *Tlr4*<sup>−/−</sup> *Ripk3*<sup>−/−</sup> mice but not *Nlrp3*<sup>−/−</sup> *Ripk3*<sup>−/−</sup> mice. This would begin to ascribe the

mechanism to the effect of *Casp8* deletion upon TLR4 signalling and not to its influence upon NLRP3.

One way to try to study cell extrinsic apoptosis in vivo is to compare *Casp8*<sup>-/-</sup>*Ripk3*<sup>-/-</sup> mice to *Ripk3*<sup>-/-</sup> control mice. However, given the compound effects of deleting *Casp8* (elaborated in Box 2), it is not surprising that *Casp8*<sup>-/-</sup>*Ripk3*<sup>-/-</sup> mice are highly susceptible to multiple infectious challenges. *Casp8*<sup>-/-</sup>*Ripk3*<sup>-/-</sup> mice have more rapid dissemination and/or lethality of fully virulent *Y. pestis* or *Y. pseudotuberculosis*<sup>74,76</sup>. These mice also have higher stool burdens during *C. rodentium* infection<sup>75</sup>. Furthermore, they succumb to infections by *Y. pestis* strains engineered to enhance TLR4 detection<sup>76</sup>, perhaps due to defective TLR4 signalling. Defective TLR4 signalling would also explain the resistance of *Casp8*<sup>-/-</sup> background mice to endotoxic shock<sup>88</sup>. *Fadd*<sup>-/-</sup>*Mlkt*<sup>-/-</sup> mice (which are similar to *Casp8*<sup>-/-</sup>*Ripk3*<sup>-/-</sup> mice, see Box 2) have increased mortality to influenza A virus infection<sup>64</sup>, perhaps due to defects in TLR3 signalling.

In summary, multiple innate immune defence pathways intersect at the caspase 8–RIPK1–RIPK3 triangle. It seems likely that the intricate cross wiring of these pathways plays an important role in preventing pathogens from inhibiting the function of TLR3, TLR4, TNFR1, extrinsic apoptosis and necroptosis.

## Neutrophil NETosis makes NETs

Whereas necroptosis and apoptosis are seen in many cell types, another form of cell death is uniquely observed in neutrophils — NETosis, which primarily functions to extrude NETs<sup>101</sup> (Figure 3). NETosis is triggered by various microbial and sterile activators — a process that was recently shown to differentiate between microbes based on their size<sup>102</sup> — or by ligation of specific receptors including complement, antibody, cytokine, and TLRs<sup>103</sup>. During NETosis, ROS-dependent release of elastase and myeloperoxidase from neutrophil granules to the nucleus (though not under all conditions<sup>104</sup>) promotes histone degradation and chromatin decondensation<sup>105</sup>. The effects of ROS and proteases on the nuclear material leads to extrusion of a meshwork of chromatin fibers dotted with granules containing anti-microbial molecules, which traps and kills extracellular bacteria and fungi.

NETosis is accompanied by ruptures in the plasma membrane and neutrophil lysis<sup>101</sup>. However, a mounting body of evidence demonstrates that neutrophils can release NETs while maintaining an intact plasma membrane<sup>103</sup>; this is paradoxically referred to as ‘vital NETosis’, in contrast to the typical ‘suicidal NETosis’. After vital NETosis, neutrophils continue to function in chemotaxis, phagocytosis and bacterial killing<sup>106</sup>. Whether a neutrophil undergoes suicidal or vital NETosis appears to be dependent on the NET-inducing stimulus. It is unknown how NETs are extruded from neutrophils without lysing the cell.

Whether NETosis has antimicrobial functions that are independent of NET extrusion remains unknown, but one could imagine that pathogens within neutrophils during NETosis could be killed by the release of granule contents into the nucleus or cytosol (see REF<sup>7</sup> for a comprehensive review on NET signalling mechanisms). How NETs combat infection is described further below in our discussion of consequences of cell death.

## Cross-talk between cell death pathways

There are many cross-pathway signalling events between apoptosis, necroptosis and pyroptosis (Figure 4). Most prominent amongst these are NLR family, pyrin domain-containing 3 (NLRP3) interactions with the different cell death pathways. Although the precise signal detected by NLRP3 is uncertain, NLRP3 can be considered as the “sensor for cellular catastrophe” (Box 3), activating when normal cellular physiology breaks down.

### Box 3

#### As the sensor of cellular catastrophe, NLRP3 detects the pyroptotic and necroptotic pores

Although NLRP3 is the most extensively investigated inflammasome, it remains the most enigmatic. NLRP3 is activated in response to numerous insults, including pore-forming toxins, ionophores and phagocytosis of indigestible particulates. As such, NLRP3 detects catastrophic events, including the inability to maintain a cytosolic potassium concentration<sup>161</sup> via an unknown biochemical mechanism. Besides loss of cytosolic potassium, other mechanisms have also been proposed to explain NLRP3 activation, including ER stress and mitochondrial dysfunction<sup>162</sup>.

NLRP3 is also activated downstream of lipopolysaccharide (LPS) detection by caspase 11<sup>14</sup>. When caspase 11 cleaves gasdermin D, it polymerizes into the pyroptotic pore. The resulting loss of cytosolic potassium triggers NLRP3 and consequent interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18 processing<sup>163,164</sup> (Fig. 3). Thus, NLRP3 has a very short activation window before cellular swelling causes rupture of the plasma membrane. IL-1 $\beta$  secretion downstream of cytosolic LPS is thus much lower than other direct inflammasome agonists. To date there is no in vivo evidence supporting physiologic relevance of NLRP3 activation downstream of caspase 11. In fact, caspase 11-dependent defence against *Burkholderia thailandensis* does not require NLRP3<sup>19</sup>, which argues against an in vivo role, at least in this model system.

Similarly, NLRP3 activation downstream of necroptosis<sup>53,100,110</sup> may be caused by the same mechanism. It is likely that potassium loss after opening of the MLKL pore is detected by NLRP3. This pathway may promote IL-1 $\beta$  secretion in vivo during influenza virus and vesicular stomatitis virus infection<sup>165</sup>, but more work is needed to determine whether MLKL is the primary pathway whereby NLRP3 is activated in vivo, or whether NLRP3 is actually responding to some other catastrophic event during infection in vivo.

## Role of caspase 8 upstream of inflammasome signalling

Caspase 8 is a component of the TNFR1–TLR3–TLR4 signalling scaffold. Therefore, loss of caspase 8 prevents these receptors from providing both post-translational and transcriptional priming of inflammasome components. For example, pro-IL-1 $\beta$  and NLRP3 transcriptional induction is compromised in *Casp8*-deficient cells<sup>75,76,100,107–109</sup>, although this may not hold true for all cell types<sup>110</sup>. Furthermore, NLRP3 requires TNFR1 or TLR signalling to provide post-translational priming<sup>111–113</sup>. Thus, *Casp8*<sup>-/-</sup> cells fail to prime NLRP3 after TNFR1, TLR3, or TLR4 stimulation, but can prime NLRP3 within 10 minutes of TLR2

stimulation<sup>76,100,108,114</sup>. However, when longer time points (4 hours) are examined, TLR2-driven TNF may require caspase 8 to cause paracrine stimulation<sup>75</sup>. In contrast, *Casp8*-deficiency does not prevent activation of inflammasomes that do not need priming, such as NLRC4<sup>75,107</sup>. The in vivo relevance of these interactions remains to be assessed.

### Caspase-8 downstream of inflammasomes ensures death

Caspase 8 can also function downstream of inflammasomes, which makes caspase 8 interactions more complicated. Caspase 8 may guard pyroptosis, and thereby ensure activation of cell death in a hypothetical situation where a pathogen attempt to inhibit pyroptosis. ASC is a small adaptor between most inflammasomes and caspase 1; the ASC pyrin domain (PYD) interacts with the PYD of inflammasomes, while its caspase activation and recruitment domain (CARD) interacts with the CARD of caspase 1<sup>13</sup>. Upon activation by inflammasomes, ASC polymerizes to provide a large surface area of activating CARD domains<sup>115,116</sup>. In the absence of caspase 1, cell death still occurs after ASC polymerization, but under slower kinetics and with apoptotic morphology. Here, caspase 8 is proposed to interact with ASC via an unusual heterotypic death domain fold interaction between the ASC CARD and the caspase 8 death effector domain<sup>117–119</sup> (Figure 4). This pathway may be most relevant when a pathogen directly inhibits caspase 1 without preventing ASC polymerization<sup>120</sup>. This situation is mimicked by comparing *Asc*<sup>-/-</sup> to *Casp1*<sup>-/-</sup> mice, which reveal an in vivo effect of cytokine responses via this pathway during a *F. novicida* infection<sup>117,121</sup>, but this has no effect on the *F. novicida* bacterial load<sup>117,121</sup>.

In addition, caspase 8 may be directly activated by caspase 1 in settings where gasdermin D is absent. Although cell death was fully ablated in *Gsdmd*<sup>-/-</sup> cells in one study<sup>21</sup>, a slower form of cell death was observed in another study<sup>22</sup> that was later suggested to be apoptosis associated with caspase 8 activation<sup>23</sup>. By contrast, this pathway to apoptosis was not observed after activation of caspase 1<sup>22</sup> (Figure 4).

Some researchers describe this pathway as “suppression of apoptosis by pyroptosis”<sup>23</sup>, or as a “balance between pyroptosis and apoptosis” that depends on the quantity of agonist<sup>118</sup>. However, we prefer the interpretation that this is a versatile pathway that can “switch from pyroptosis to apoptosis” in the hypothetical case where a pathogen inhibits pyroptosis, thus ensuring cell death<sup>117</sup>. Apoptosis could thus be considered as a backup guard pathway for pyroptosis, however the in vivo relevance in controlling pathogen burdens remains to be elucidated.

Finally, caspase 8 has been proposed to substitute for caspase 1 as an pro-IL-1 $\beta$  and pro-IL-18 cleaving enzyme<sup>121–125</sup>, although this function warrants further study.

In summary, caspase 8 has been proposed to function upstream of inflammasomes, but also downstream of inflammasomes, which creates a quite confusing picture when one attempts to integrate the multiple functions into a single model. In vivo validation of these interactions will hopefully reveal their physiological importance.

### Caspase 1 driven IL-18 primes caspase 11

Mouse caspase 11 activation is strictly dependent upon priming; *in vitro* this is commonly accomplished by studying cells previously primed by type I IFNs (via TLR3 or TLR4 driven type I IFN secretion)<sup>6</sup>, or by TLR2 stimulation<sup>16</sup>. The TLR3 pathway has been used *in vivo* to enable caspase 11 priming during LPS shock models<sup>15,16</sup>. Surprisingly, these pathways were insufficient to prime caspase 11 during infection with the cytosol-invasive bacterium *B. thailandensis* *in vivo*<sup>19</sup>. Instead, NLRC4-driven caspase 1 activation was required to drive IL-18 production, which in turn stimulated IFN $\gamma$  within the first day of infection. IFN $\gamma$  was absolutely essential for caspase 11 priming in this model, while endogenous TLR agonists and type I IFN were insufficient. The IL-18 requirement could be bypassed only under delayed kinetics after 3 days as alternate mediators eventually promoted IFN $\gamma$  production<sup>19</sup>. Thus, in the context of intracellular bacterial infection *in vivo*, caspase 1 acts upstream of caspase 11 priming, which was surprising as NLRP3 and caspase 1 are commonly thought to function downstream of caspase 11 (Box 3).

### IL-18 drives NK cytotoxicity vs intracellular bacteria

NK-derived IFN $\gamma$  is well established to combat intracellular bacterial infection. However, whereas NK cytotoxicity is critical in combating viruses such as MCMV, HSV-1, influenza virus, and cancer, initial *in vitro* studies suggesting that bacterially infected cells are killed by NK cells have been uniformly disproven by subsequent *in vivo* studies<sup>126,127</sup>. Collectively, this data led to the conclusion that cytokines rather than cytotoxicity is the primary mode of NK cell-mediated protection against intracellular pathogens. However, our group recently showed that NK lysis of infected hepatocytes is crucial for controlling infection by an intracellular bacterial pathogen<sup>39</sup>. In this model, infected phagocytes detected *C. violaceum* via NLRC4, which lead to production of IL-18 and activation of liver NK cells. Activated NK cells then directly lysed infected hepatocytes in a perforin-dependent manner. NK cytotoxicity also controlled the inflammasome-evasive *L. monocytogenes* infection, but only when mice were treated with exogenous IL-18<sup>39</sup>. This example highlights the importance of direct cytotoxicity against bacteria and crosstalk between two different cell death modalities — inflammasomes and apoptosis — to efficiently fight infection. Similarly, NK cytotoxicity initiated by inflammasome detection of *S. Typhimurium* in a colitis model was recently shown to promote inflammation<sup>128</sup> and *Prf1*<sup>-/-</sup> mice were also susceptible to *C. rodentium* during the innate phase of infection<sup>129</sup>. Interestingly, human, but not mouse, NK cells have additional means to directly kill bacteria, since granulysin delivered upon NK degranulation is directly toxic for both bacteria and parasites<sup>130,131</sup>.

### How programmed cell death clears infection

Recent studies have examined the fate of pathogens after apoptosis, necroptosis and pyroptosis, and how these cell death mechanisms promote the resolution of infection. Apoptosis converts cells into apoptotic bodies, which release a series of find-me and eat-me signals that promote efferocytosis (phagocytosis of dead cells). How the necroptotic and pyroptotic cells are cleared, is currently under thorough investigation. These mechanisms remove the niche for intracellular viruses and bacteria, but pathogens frequently survive host



cell death. Finally, NETosis generates structures that are specifically microbicidal. In this final section we discuss what happens to pathogens after programmed cell death (Figure 3).

### Efferocytosis of apoptotic bodies

The resolution phase of apoptosis is another example of how apoptotic caspases maintain immunological silence. Failure to clear apoptotic debris can contribute to inflammatory or autoimmune disease {Poon:2014ik}. During normal development and homeostasis, apoptotic bodies are efferocytosed by a secondary phagocyte, thereby preventing secondary necrosis and maintaining immunological quiescence<sup>80</sup>. Efferocytosis is currently an active area of research in the context of infection.

Until recently, apoptosis was often considered intrinsically bactericidal. For example, apoptosis was thought to kill *Mycobacteria spp.* in vitro<sup>132</sup> and in vivo<sup>133</sup>. However, recent studies suggest that *Mycobacteria tuberculosis* and *Mycobacterium marinum* survive apoptosis, but are subsequently killed after efferocytosis by a secondary macrophage<sup>134,135</sup>. This efferocytic phagosome matures, acidifies and kills the bacteria via ROS. Notably, pathogens that resist apoptosis, such as *Mycobacterium*, also interfere with efferocytosis, which increases their survival<sup>136</sup>. *L. monocytogenes*, on the other hand, exploits efferocytosis receptors in order to spread from cell to cell<sup>137</sup>. The Listeriolysin O toxin damages the plasma membrane, exposing phosphatidyl serine, which is recognized by TIM4. As *L. monocytogenes* propels itself with actin comets, TIM4 on the neighbouring cell promotes efferocytosis of the protruding membrane, enabling *L. monocytogenes* to enter the next cell<sup>137</sup>.

Virus-containing apoptotic bodies can also be efferocytosed; for example, influenza A virus induces apoptosis of infected epithelial cells, and neighbouring macrophages efferocytose apoptotic bodies containing viral progeny, which reduces viral titers<sup>138</sup>. On the other hand, some viruses use mimicry to induce apoptosis in order to spread to neighbouring cells<sup>139</sup>.

### NETs trap extracellular microbes

In addition to killing pathogens after phagocytosis, neutrophils can target extracellular pathogens using NETs<sup>101</sup>. NETs play two distinct anti-microbial roles by trapping and killing pathogens. Extensive intravital and confocal microscopy of neutrophils in infected tissue show in real time how bacterial and fungal pathogens can be trapped in NETs<sup>103,105,140</sup>. Moreover, additional pathogens are trapped in the extracellular chromatin in vitro. In fact, systemic DNase treatment in a mouse model of a *S. aureus* skin infection, which induces NET formation, resulted in the escape of the bacteria from the site of inoculation and increased vascular bacterial loads<sup>106</sup>.

The extent to which NETs are directly capable of killing bacterial and fungal pathogens remains controversial, since not all bacterial pathogens trapped in NETs are killed and there are no known specific inhibitors that unlink NET release from NET antimicrobial activity. For example, in vitro DNase treatment of *Streptococcus pneumonia* and *Candida albicans* trapped in NETs results in the release of viable bacteria<sup>141</sup>. This lack of microbial killing may stem from the loss of optimal protease activity of key bactericidal enzymes, such as elastase, under in vitro conditions<sup>142</sup>. Moreover, many research groups have clearly

demonstrated anti-microbial activity of NETs against a variety of microbes in vitro<sup>140</sup>, and trapping is obviously beneficial to the host<sup>103,143</sup>. For example, intravital microscopy of *Escherichia coli* trapped in NETs within liver sinusoids demonstrate that disruption of NETs via platelet depletion or DNase treatment prevents trapping and promotes dissemination<sup>143</sup>. The use of vital DNA-intercalating dyes demonstrate that bacteria trapped within NETs are killed<sup>101,144</sup>. A thorough discussion of the controversies regarding the composition and antimicrobial properties of NETs was recently published<sup>145</sup>.

Many pathogens directly disrupt NETs — for example group B *Streptococcus*<sup>146</sup>, *Vibrio cholera*<sup>147</sup>, *Yersinia enterocolitica*<sup>148</sup> and *Neisseria gonorrhoeae*<sup>149</sup> — by expressing nucleases that degrade the NET-associated chromatin, which enables them to escape from NETs and may result in systemic dissemination and sepsis. Other pathogens, such as *Haemophilus influenza*<sup>150</sup>, *Pseudomonas aeruginosa*<sup>151</sup> and *S. pneumoniae*<sup>152</sup>, evade NETs by incorporating NET-associated DNA into their biofilms, which promotes their replication in vivo. Hence, trapping of some microorganisms by NETs in the absence of bacterial killing could be advantageous to the pathogen. Finally, some pathogens evade killing by NETs by sidestepping the specific antimicrobial activity of NET components by expressing polysaccharide capsules<sup>153,154</sup>. A full overview of exotoxins that disrupt NETs and other evasion mechanisms are reviewed elsewhere<sup>155</sup>.

### Pore-induced intracellular traps

Recent evidence from our group establishes the fate of the pyroptotic cell corpse and its role in orchestrating a downstream innate immune response. Similar to apoptosis, pyroptosis does not kill bacteria<sup>9,40</sup>. We initially assumed that pyroptosis released intracellular bacteria to the extracellular space<sup>9</sup>. However, in a follow-up study we show that viable bacteria remain trapped within the cellular debris of pyroptotic macrophages<sup>40</sup>. This trapping appears to be mediated by the pyroptotic cell membrane, which remains largely intact despite containing discreet ruptures, encompassing collapsed organelles and cellular structures. We call this structure the pore-induced intracellular trap (PIT)<sup>35,40</sup> (Figure 3), which is conceptually parallel to NETs. Both PITs and NETs trap the pathogen and prevent microbial dissemination. In contrast to NETs, which act against extracellular microbes, PITs are trap intracellular microorganisms. In addition to pyroptosis, necroptosis and necrosis also result in formation of a PIT that is efferocytosed by neutrophils and macrophages<sup>40</sup>, although the in vivo relevance of necroptotic and necrotic PITs in the context of infection remains to be elucidated.

PITs coordinate innate immune responses to combat intracellular bacteria; PIT formation leads to complement activation, release of IL-1 $\beta$  and IL-18, and eicosanoid production. These mediators recruit neutrophils, which utilize phagocytic scavenger and complement receptors to efferocytose the PIT and entrapped bacteria<sup>35,40</sup>, which is conceptually parallel to efferocytosis of bacteria trapped within apoptotic bodies<sup>134</sup>. Ultimately, this secondary phagocyte kills the bacteria via ROS<sup>40</sup>.

We therefore propose that PITs are highly effective immunologically important structures with two key functions; PITs immobilize the pathogen to prevent dissemination and promote efferocytosis by a secondary phagocyte that kills the pathogen.

## Concluding remarks

Much work remains to be done to fully understand how pyroptosis, necroptosis, apoptosis and NETosis defend against infection in vivo. Still, genetic tools to study programmed cell death in vivo remain confounded by effects upon other aspects of host defence. For example, *Gsdmd* deletion prevents pyroptosis, but also prevents IL-1 $\beta$  and IL-18 secretion; similarly, *Casp8* deletion prevents cell extrinsic apoptosis, but also may trigger necroptosis, cause a lymphoproliferative disorder, and inhibit innate immune receptor signalling (Box 2). Despite such difficulties, our understanding of programmed cell death has significantly advanced in recent years, revealing the utility of programmed cell death during infection both to destroy intracellular niches, and to coordinate an appropriate innate immune response thereafter. Finally, if a programmed cell death modality is exquisitely effective against a specific virulence strategy, this exerts strong selective pressure for those pathogens to evade the response. Therefore, when interpreting the results of experimental infections it is important to keep in mind the competing possibilities that the host may be benefiting, the pathogen may be evading, or that host defence and pathogenic evasion may both be partially effective. In this regard, identification of new infectious agents that fail to evade specific cell death pathways can aid our understanding of the true function of programmed cell death during infection.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Glossary

### Apoptosis

A common form of cell death, which is also known as intrinsic or programmed cell death. Many physiological and developmental stimuli cause apoptosis, and this mechanism is frequently used to delete unwanted, superfluous or potentially harmful cells, such as those undergoing transformation

### Pyroptosis

Programmed lytic cell death via caspase-1/4/5/11 cleavage of GSDMD, and recently expanded to include lytic cell death caused by other gasdermin family proteins

### Necroptosis

Programmed lytic cell death via RIPK3 activation of MLKL

### Apoptosis

Programmed non-lytic cell death in which initiator caspases-2/8/9/10 activate executioner caspases-3/6/7

**NETosis**

Programmed neutrophil death that results in formation of NETs

**Apoptotic bodies**

Apoptotic bodies form after apoptosis

**NETs**

Neutrophil extracellular traps that form after NETosis

**pore-induced intracellular traps (PITs)**

Structures that form after pyroptosis and necroptosis to retain organelles and bacteria

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## Biographies

Dr. Jorgensen received her PhD from Duke University where she studied *Chlamydia* pathogenesis, and did post-doctoral research with Dr. Miao at the University of North Carolina at Chapel Hill studying how pyroptosis clears intracellular bacteria. She is now a researcher at Oslo University Hospital at Rikshospitalet in Oslo, Norway.

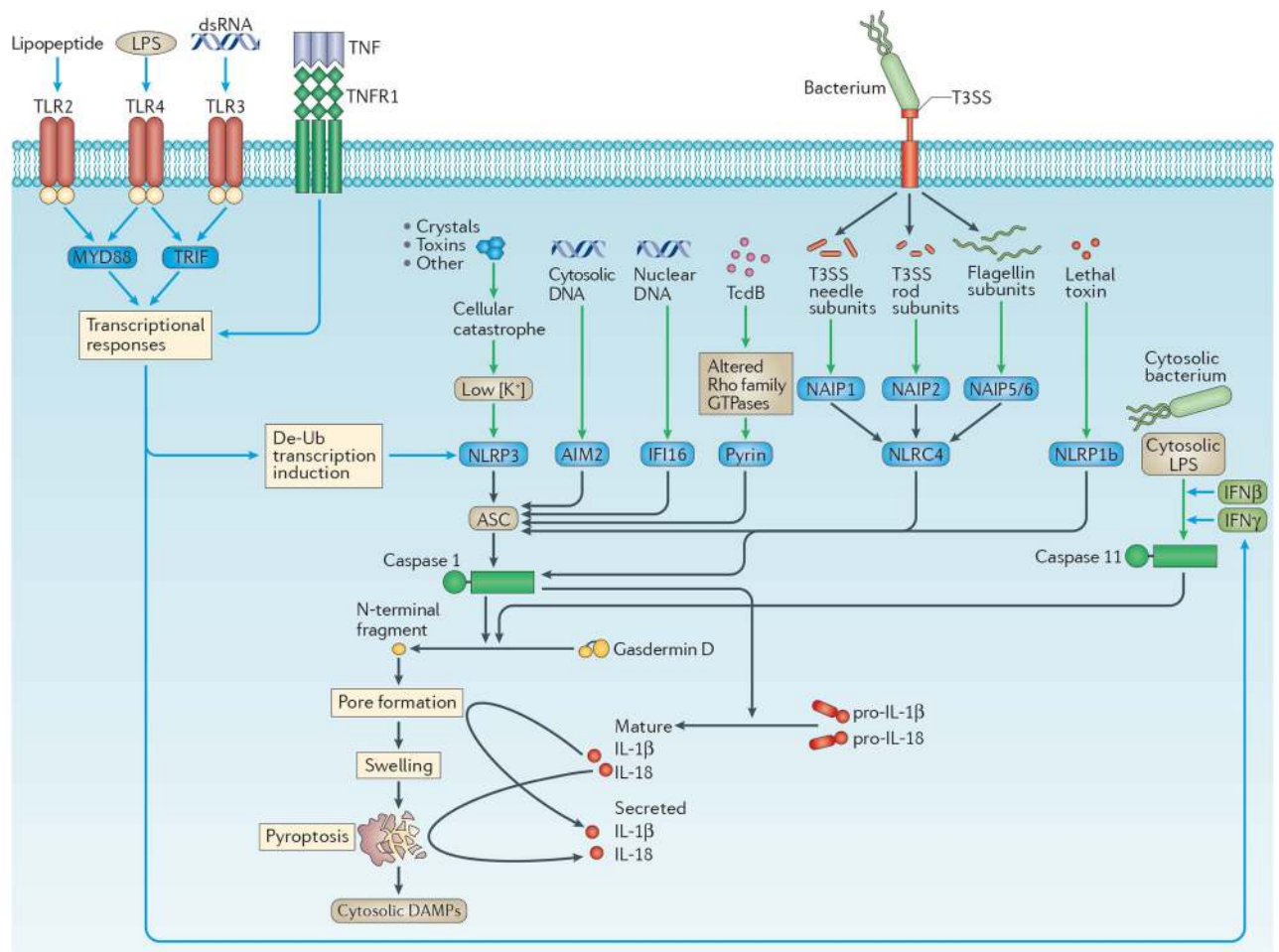
Dr. Rayamajhi received her PhD from the University of Colorado and National Jewish Health, then did post doctoral research with Dr. Miao at the University of North Carolina at Chapel Hill studying apoptotic and inflammatory caspase responses to bacterial infection. She is now a research scientist at Camargo Pharmaceuticals.

Dr. Miao received MD and PhD degrees from the University of Washington, and is now an Associate Professor at the University of North Carolina at Chapel Hill. His laboratory studies the mechanisms whereby the innate immune system differentiates pathogenic from non-pathogenic microbes with a focus on intracellular bacteria. His work considers the concepts within microbial pathogenesis to understand innate immune surveillance.



### Online summary

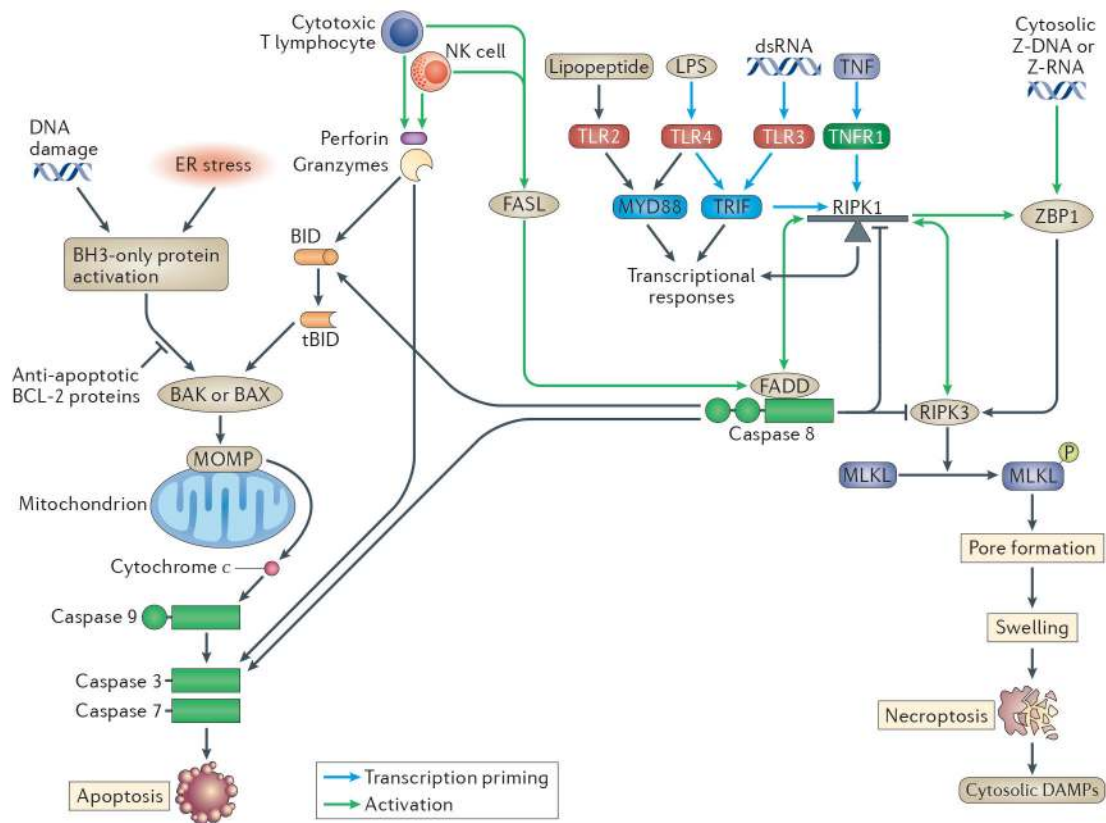
- Pyroptosis is programmed lytic cell death caused by caspase-1/4/5/11 cleavage of GSDMD. Recent work suggests that pyroptosis defends against infection by vacuolar or cytosol-invasive bacteria.
- Necroptosis is programmed lytic cell death caused by RIPK3 activation of MLKL. Recent work suggests that necroptosis defends against viral infection.
- Apoptosis can be activated by extrinsic and intrinsic signals. It is a critical host defence mechanism, and pathogens have evolved to both evade and co-opt apoptosis.
- Apoptosis and necroptosis guard each other to make it difficult for pathogens to inhibit these programmed cell death mechanisms. Despite this, pathogens such as MCMV seem to inhibit both pathways simultaneously.
- The signaling cascades leading to apoptosis, necroptosis, and pyroptosis have numerous interactions, creating a complex web of programmed cell death mechanisms to defend against infection. In this regard, physiologic relevance for apoptosis and necroptotic cross guarding is established, but most other interactions remain to be fully explored.
- Neutrophil extracellular traps (NETs) and pore-induced intracellular traps (PITs) are mechanism to physically restrain bacteria in the extracellular or intracellular space, respectively. These cellular corpses can be considered as structures that exist in parallel to apoptotic bodies (the corpse of an apoptotic cell).



**Figure 1. Pyroptosis**

Pyroptosis is initiated by either caspases 1 or 11. Caspase 1 is activated by one of several inflammasomes, NOD-, LRR- and pyrin domain-containing 3 (NLRP3), AIM2, interferon- $\gamma$  (IFN $\gamma$ )-inducible protein 16 (IFI16), pyrin, NOD-, LRR- and CARD-containing 4 (NLRC4) and NLRP1b<sup>6</sup>. NLRP3 responds to numerous agonists, which may converge upon low cellular potassium concentration, although this remains controversial. NLRP3 will not respond to these agonists unless it also receives a priming stimulus via various Toll-like receptors (TLRs) or from tumour necrosis factor (TNF), which trigger post-translational priming (probably de-ubiquitination), as well as boosting NLRP3 sensitivity by transcriptional induction<sup>162</sup>. AIM2 detects cytosolic DNA<sup>83,84</sup>, and IFI16, which lacks a clear mouse homolog<sup>166</sup>, also detects viral nucleic acids<sup>50</sup>. Pyrin detects modulation of Rho-family GTPases by bacterial toxins<sup>167</sup>. NLRC4 is activated by one of three bacterial flagellin or type III secretion rod or needle proteins that signal via an upstream NLR in the NAIP family<sup>9,36,168,169</sup>. NLRP1b detects the protease activity of the anthrax lethal toxin<sup>170,171</sup>. By contrast, caspase 11 itself is the sensor for cytosolic lipopolysaccharide (LPS)<sup>15–17</sup>. Similar to NLRP3, caspase 11 requires priming, but priming is by either type I interferon or interferon- $\gamma$  (IFN $\gamma$ ). Either caspases 1 or 11 independently cleaves gasdermin D, from which the released N-terminal fragment associates with the cell membrane and oligomerizes

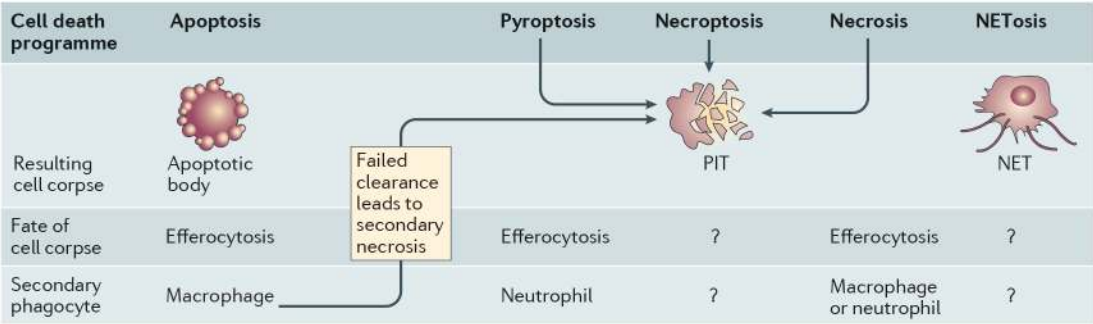
to form the pyroptotic pore<sup>26–29</sup>. The cell then swells, resulting in membrane rupture that is called pyroptosis. In addition, caspase 1 will cleave pro-interleukin-1 $\beta$  (pro-IL-1 $\beta$ ) and pro-IL-18 to their mature forms (caspase 1 cannot do this directly)<sup>14</sup>. Mature IL-1 $\beta$  and IL-18 can escape through the gasdermin D pore, or be released later by membrane rupture<sup>26,28</sup>. Red and blue lines represent initiating and priming events, respectively.



**Figure 2. Apoptosis and Necroptosis**

(Left) Apoptosis can be triggered via intrinsic or extrinsic pathways, including by natural killer (NK) cells and cytotoxic T lymphocyte (CTL) granzymes. These stimulate the cleavage of BID to tBID, which causes BAX and/or BAK to trigger mitochondrial outer-membrane permeabilization (MOMP), resulting in release of cytochrome C (CytC), which binds to apoptotic protease activating factor 1 (APAF1). APAF1 then oligomerizes into the apoptosome, the platform for caspase 9 activation. Either caspases 8 or 9 will cleave the effector caspases 3 and 7 into their active forms, which leads to apoptosis<sup>2</sup>. (Right) Necroptosis can be triggered by DNA-sensing via ZBP1<sup>52</sup>, which activates receptor-interacting protein kinase 3 (RIPK3) followed by phosphorylation of MLKL. Phosphorylated MLKL binds to the inner leaflet of the plasma membrane and forms the necroptotic pore<sup>60–62</sup>. (Center) In the more complex necroptotic pathway, tumour necrosis factor receptor 1 (TNFR1), Toll-like receptor 3 (TLR3)–TRIF, or TLR4–TRIF signal via RIPK1 to activate NF- $\kappa$ B (but RIPK1 is not required for the TRIF-type I IFN response<sup>97,98</sup>). RIPK1 can be thought of as a finely balanced teeter-totter with two guards: caspase 8 on one side balanced by a duo of RIPK3 and ZBP1 on the other. When TNFR1–TLR3–TLR4 signalling proceed normally, RIPK1 is “balanced” and functions as a scaffolding protein that becomes poly-ubiquitinated, and serving as a platform for signalling complex assembly<sup>5</sup> driving transcriptional responses. When signalling is inhibited, for example by inhibition of cIAP1/2 pharmacologic inhibitors (with SMAC mimetics), by inhibiting TAK1, by inhibition of protein synthesis, or by blocking RIPK1 ubiquitination<sup>3</sup>, or potentially by virulence factors, RIPK1 becomes “unbalanced”. RIPK1 then recruits FADD (a DD-DED

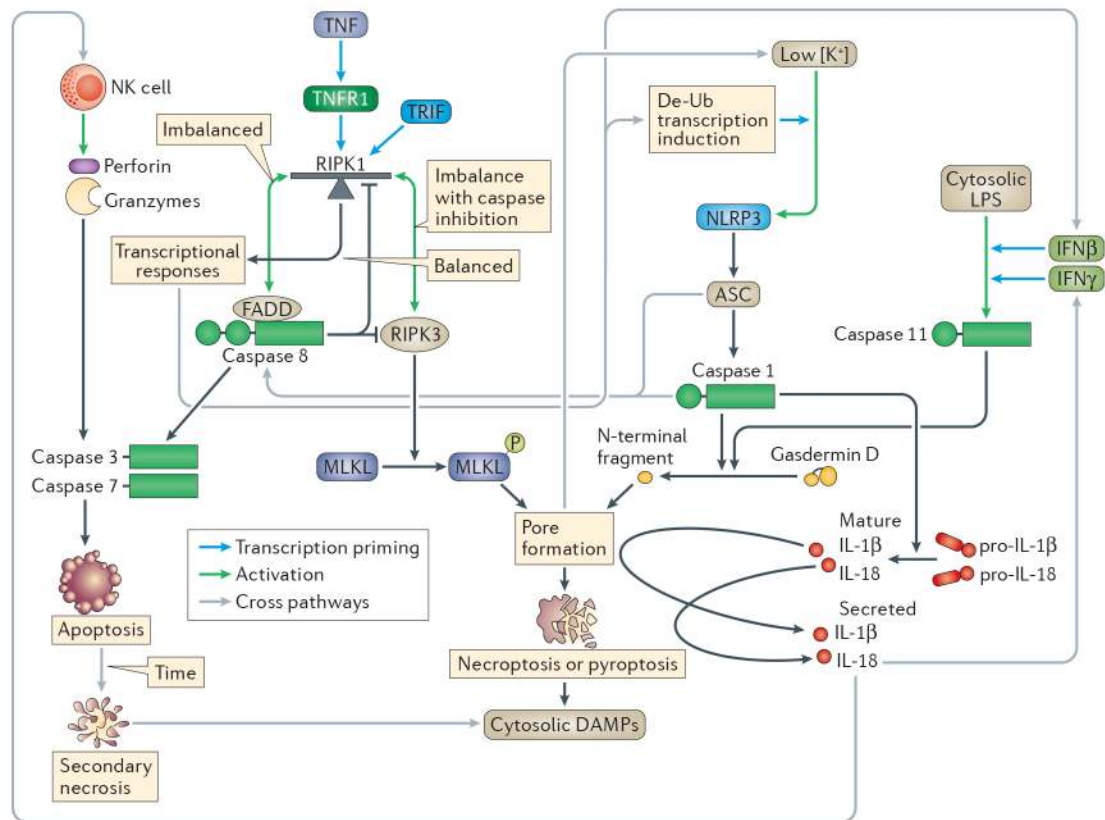
adaptor) via DD-DD interactions, and FADD recruits caspase 8 via DED-DED interaction thereby triggering extrinsic apoptosis<sup>172–174</sup>. Successful caspase 8 activation provides negative feedback to prevent necroptosis by cleaving RIPK1 and RIPK3 in the kinase domains<sup>175,176</sup>. However, inhibition of caspase-8 pharmacologically or by viral virulence factors permits continued RIPK1 kinase activity, and the teeter-totter swings the other direction. RIPK1 recruits RIPK3 via RHIM-RHIM interaction, and phosphorylates it, triggering RIPK3 kinase activity and necroptosis<sup>177–179</sup>. Thus, RIPK1, RIPK3, and caspase 8 act as a cross guard system for TNFR1, TLR3, and TLR4. TLRs such as TLR2 that do not signal through TRIF<sup>76,100,108,114</sup>, unless longer time points are examined<sup>75</sup> where TLR2-driven TNF could require caspase 8 to cause paracrine TNFR1 stimulation.



**Figure 3. Apoptotic bodies, neutrophil extracellular traps and pore-induced intracellular traps**

The fate of the cell corpse is dependent on the cell death pathway. Extrinsic or intrinsic apoptosis leads to the formation of apoptotic bodies that are engulfed by a secondary phagocyte, a process that is referred to as efferocytosis. In the absence of efferocytosis, apoptotic bodies can undergo secondary necrosis. Lytic death of cells infected with an intracellular pathogen, including pyroptosis, leads the formation of a pore-induced intracellular trap (PIT), which traps the pathogen within the cell corpse. The PIT also presents ligands that are recognized by neutrophils (and potentially macrophages), which efferocytose the PITs and associated bacteria, ultimately killing the pathogen. Efferocytosis of pathogen-containing apoptotic bodies also eliminates the pathogen. Necroptosis and necrosis also result in PITs in vitro, although the physiologic importance in vivo needs further study. Following detection of extracellular pathogens, neutrophils extrude a meshwork of chromatin dotted with granules loaded with antimicrobial molecules. These neutrophil extracellular traps (NETs) trap and kill extracellular pathogens.





**Figure 4. Interactions between apoptotic, necroptotic and pyroptotic pathways**

Cell death pathways can interact with each other, with the caveat that once a cell death pathway runs to completion, the interaction ends. The exception to this is the lysis of apoptotic bodies by secondary necrosis, which releases cytosolic contents, effectively converting apoptosis to lytic cell death. Signalling pathways running through RIPK1 will fail if *Casp8* is deleted, affecting priming of NOD-, LRR- and pyrin domain-containing 3 (NLRP3) and caspase 11<sup>75,76,100,107–109</sup>. Caspase 1 driven IL-18 can have two interactions with other pathways, first by inducing interferon- $\gamma$  (IFN $\gamma$ ) production to prime caspase 11<sup>19</sup>, and second by stimulating NK cytotoxic activity<sup>39</sup>. The terminal events occurring after the MLKL or gasdermin D pores open are catastrophic, resulting in loss of cellular potassium, which is the trigger for NLRP3 activation<sup>163,164</sup>. In the window between potassium loss and membrane rupture, NLRP3 activity will trigger caspase 1-dependent processing of interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18 processing. Finally, ASC and caspase 1 can activate caspase 8, triggering apoptosis<sup>22,23,117,118</sup>. Purple lines are interacting pathways.