THE EVOLUTION OF REGULATED CELL DEATH PATHWAYS IN ANIMALS AND THEIR EVASION BY PATHOGENS

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CLINICAL HIGHLIGHTS
Regulated cell death is a fundamental biological process that underlies many physiological traits, including the response to infections. The regulated cell death pathways of pyroptosis, apoptosis, and necroptosis play an integral part in the immune defense against invading pathogens. We provide a comprehensive review of the molecular pathways of regulated cell death, the strategies contrived by pathogens to perturb these signaling cascades, and the evolutionary aspects of regulated cell death.
THE EVOLUTION OF REGULATED CELL DEATH PATHWAYS IN ANIMALS AND THEIR EVASION BY PATHOGENS

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Abstract
The coevolution of host-pathogen interactions underlies many human physiological traits associated with protection from or susceptibility to infections. Among the mechanisms that animals utilize to control infections are the regulated cell death pathways of pyroptosis, apoptosis, and necroptosis. Over the course of evolution these pathways have become intricate and complex, coevolving with microbes that infect animal hosts. Microbes, in turn, have evolved strategies to interfere with the pathways of regulated cell death to avoid eradication by the host. Here, we present an overview of the mechanisms of regulated cell death in Animalia and the strategies devised by pathogens to interfere with these processes. We review the molecular pathways of regulated cell death, their roles in infection, and how they are perturbed by viruses and bacteria, providing insights into the coevolution of host-pathogen interactions and cell death pathways.

1. INTRODUCTION
Regulated cell death (RCD), cellular demise through a molecular program, is a fundamental biological process that underlies many physiological traits. RCD is involved in embryonic development, where it controls the formation of bodily structures such as the digits of the fingers, the timing of neural tube and palate closure, the selection of neuronal connections, and lymphocyte specificity. RCD mediates metamorphosis, such as the loss of the tail by developing tadpoles, and regulates homeostasis of many tissues in adult animals, including the rapid turnover of the intestinal epithelium and the cells of the blood. Moreover, RCD plays an integral part in the immune defense against invading pathogens and the control of activated immune cells (1).
(DAMPs) and include a variety of proteins, nucleic acids, ATP, and metabolites (9), which attract innate immune cells to combat the infection.

Any organism can be infected by pathogens. Obligate, intracellular pathogens must gain access to the host cytoplasm. Viruses do this by binding to the extracellular region of transmembrane proteins present on the host’s cell membrane (10). Depending on the invading virus, receptor engagement induces subsequent events, such as membrane fusion or endocytosis, that eventually result in the delivery of the viral genetic material into the host cytoplasm. Intracellular bacteria, fungi, and protozoa use host cells to acquire nutrients and to shelter themselves from elimination by the host immune system (11, 12). Bacteria gain access by engaging cellular transmembrane proteins to induce their internalization or can be engulfed by phagocytes, after which they either escape to the cytosol or create a niche within the phagocytic vacuole.

For the host, the basis for the recognition and control of invading pathogens is the ability to distinguish between “self” and “nonself.” Over the course of evolution, eukaryotic cells have equipped themselves with many types of cell surface and intracellular receptors able to differentiate the wide variety of infectious organisms from “self.” Collectively, these pattern-recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) associated with many different types of microbes (13–15).

A primary function of many PRRs is to induce proinflammatory gene expression profiles that counteract an infection either directly (e.g., interferons, antibacterial peptides) or by attracting innate immune cells (e.g., cytokines, chemokines) to the site of infection to combat the invading pathogen. Moreover, this allows infected cells to “warn” neighboring cells to arm against incoming infectious agents. Another way that invading pathogens are detected is by the cellular stress associated with infection, owing to interference with cellular homeostasis. The response to such stress can be similar to that of PRR engagement, in that both can lead to inflammatory signals (9, 16, 17). Moreover, the engagement of specific PRRs and cellular stress can induce regulated cell death to destroy an infected cell (18).

PRRs are not the only receptors able to induce regulated cell death. Animal cells express death receptors (DRs), a subset of the tumor necrosis factor receptor (TNFR) superfamily, whose ligation can induce apoptosis or necroptosis, depending on the engaged receptor and the cellular context (19). Death receptors are not PRRs but recognize signals (death receptor ligands) from or on other cells. Death receptor ligation can induce cell death to control pathogenic spread and also functions in immune regulation and inflammation (20). Whether regulated cell death is activated through engagement of PRRs or DRs, by removing an infected nonessential cell the host eliminates the invading pathogens’ replicative niche, thereby halting pathogenic spread and limiting disease to improve host survival.

### 1.1. Scope of the Review

Over the course of evolution, many forms of regulated cell death have evolved (2). In this review, we consider only three: pyroptosis, apoptosis, and necroptosis. Although others certainly exist, our focus on these three is for several reasons. Perhaps most importantly, all three of these forms of regulated cell death have arguably evolved as forms of cellular “suicide”; that is, cells die as a consequence of engaged protein “executioners” that function to mediate the cell death. In contrast, many other forms of regulated cell death represent externally mediated subversions of cellular mechanisms to disrupt essential survival functions, such as scavenging of lipid peroxides [resulting in ferroptosis (21)] or control of vesicular transport [leading to death as a result of retrograde transport (22)]. The latter cell death modalities may be considered forms of cellular “sabotage” (23). Although it is possible that pathogens can act to cause such sabotage and thus drive evolution of survival mechanisms, we currently do not have evidence of coevolution in such interactions, and therefore they are not considered further here.

A relevant aspect of the interplay between cell death and infection is the engagement of adaptive immune responses. For example, dead and dying cells are engulfed by conventional dendritic cells, and, in a subset of the latter, proteins associated with the corpse (including microbial proteins) are degraded and their peptides presented on class I major histocompatibility molecules to CD8+ T cells (24, 25). The mode of cell death influences this “antigen cross presentation” process (25). There are excellent reviews on cell death and cross presentation (24–27), and we do not consider this further in this review.

An additional form of regulated cell death, called NETosis, appears to function in host defense, and it is quite possible that host-pathogen coevolution played important roles in its mechanisms. During NETosis, cells release DNA “nets” that trap bacteria and perhaps other microbes to control infections. We direct interested readers to other, excellent reviews of this process (28, 29).

This review describes the mechanisms of regulated cell death (here apoptosis, necroptosis, and pyroptosis) in the context of infection and host-pathogen coevolution. We begin with caspases, the regulators of the forms of RCD discussed here. The mechanisms of RCD are
explored, starting with the simplest way for a cell to sense a pathogen and induce cell death (pyroptosis) and proceeding to increasingly more intricate and complex mechanisms of RCD (apoptosis and necroptosis). Next, we explore the strategies developed by pathogens to disrupt the various RCD pathways. We then investigate the evolutionary aspects of the pathways of RCD. We close by exploring the relation between coronaviruses and RCD and future directions for the field. Excellent additional reviews that consider the origins and mechanistic control of the regulated cell death pathways are available for the interested reader (30–35).

2. CASPASES CONTROL REGULATED CELL DEATH

Before we can understand how regulated cell death functions in the context of infections, we first must appreciate the proteins and their interactions that control RCD. Pyroptosis, apoptosis, and necroptosis are regulated by a family of aspartate-specific cysteine proteases called caspases (reviewed in Refs. 36, 37). Caspases function as endopeptidases that cut proteins at aspartate residues that are surrounded by other residues (defining the broad specificity of the individual caspase) (38–40).

Caspases are usually divided into three subfamilies based on their physiological functions and genomic organization: initiator, executioner (or effector), and inflammatory caspases (36, 37) (FIGURE 1). These enzymes are expressed as inactive zymogens that contain a prodomain and a caspase catalytic region, consisting of a large and a small subunit connected by a linker sequence. The short prodomains of effector caspases, in general, do not have major functions, whereas the prodomains of initiator and inflammatory caspases are required for their activation. These contain protein-
protein interaction motifs that are structurally related [so-called “death folds” (41)], including a caspase-recruitment domain (CARD) (caspases-1, -2, -4, -5, -9, and -11), two tandem death effector domains (DEDs) (caspases-8 and -10), a death domain (DD) (Hydra HyDDCasp) (42), a pyrin domain (PYD) (Donia reio caspy and caspy2) (43), or another domain that allows their activation (Hydra HyCaspA) (42) (FIGURE 1). As discussed below, these interaction domains participate in the interaction with caspase-activation platforms that assemble in response to cellular signaling events.

Executioner caspases are expressed as inactive dimers that require cleavage to become active. Such cleavage is predominantly mediated by initiator caspses but can also be performed by granzyme B upon its intracellular delivery by cytotoxic lymphocytes (44, 45). Cleavage occurs at a site between the resulting large and small subunits of the procaspase. In contrast, initiator and inflammatory caspases are monomeric zymogens (procaspases) that are activated by enforced dimerization [known as induced proximity (46)]. Upstream signals induce the formation of “caspase-activation platforms,” leading to the recruitment and dimerization of procaspases via their prodomains. Dimerization endows the procaspase with enzymatic activity. The caspases then cleave their substrates, including, but not restricted to, executioner caspases (32). While initiator and inflammatory caspases undergo a series of autocleavage events that result in their release from the activation platform, the released enzyme may be active or inactive, depending on the caspase.

Caspases are solely found among the Animalia and are present throughout all animal phyla. It is therefore not surprising that caspase genes have undergone extensive diversification over the course of evolution. Caspase genes have duplicated, and some have been lost or have been rendered effectively inactive in different phyla (47–49). Caspases are members of the C14 class of cysteine proteases, which includes paracaspases and metacaspases, although the latter are not aspartic acid but arginine/lysine specific. Metacaspases and paracaspases have been found in fungi and plants (the paracaspases are also found in animals; the metacaspases are not), but their biochemistry is such that the types of pathways that activate the caspases are unlikely to function in the activation of these proteases. The functions of metacaspases and paracaspases have been reviewed elsewhere (50–54) and are not further considered here.

Humans express 12 known caspases. These include inflammatory caspases-1, -4, and -5, initiator caspases-2, -8, -9, and -10, executioner caspases-3, -6, and -7, caspase-12, and caspase-14. Caspase-10 is a gene duplication of caspase-8, although both proteins can be functionally distinguished (55), and is absent in rodents. Similarly, caspase-5 is a gene duplication of caspase-4 (56). Mice do not encode caspases-4 and -5, but instead express caspase-11, an ortholog of human caspase-4 (57). The csp12 gene has acquired multiple mutations over evolutionary time, including a single-nucleotide polymorphism that introduces a premature stop codon resulting in the expression of a truncated form of the protein, Csp12-s (58). This mutation was spread and nearly fixed in humans because of positive selection (59). However, the genomes of some individuals do not harbor the stop codon, allowing the expression of the full-length protein, apparently conferring a hypersusceptibility to sepsis (58). Nonhuman primates encode an intact csp12 gene, indicating that the nonfunctional variant evolved in humans. This is thought to coincide with prehistoric human behavioral changes that led to growth of population size and density, increasing the susceptibility for infection and sepsis (59, 60). Caspase-14 has functions in skin development (61), but no role in cell death has been identified for this caspase.

3. INFLAMMATORY CASPASES AND PYROPTOSIS

What would be the simplest way for a cell to sense a pathogen and induce cell death? This might be a protein that contains a receptor domain that upon ligation unlocks the molecule’s ability to kill the cell (a “receptor-executioner” protein). Although such a protein is not found in animals, fungi such as Podospora anserina express the HET-S/HET-s heterokaryon incompatibility system, which could be seen as one of the simplest ways to induce death upon the recognition of nonself. P. anserina expresses the HET-S protein, which contains a prion domain that upon activation folds into a β-solennoid conformation and causes cell death by forming pores in the plasma membrane (62). When P. anserina encounters another P. anserina strain that expresses the HET-S homolog HET-s, the proteins interact and form pores. The resulting death of the cell is believed to limit pathogen transmission between the strains (63).

A single-molecule receptor-executioner protein is not known to exist in animals. The shortest way to induce cell death upon pathogen recognition in animals is a two-step system in which a caspase contains a receptor domain that upon ligation activates the protease to cleave and thereby activate a substrate protein that causes death. Murine caspase-11 and human caspase-4 and caspase-5 have the ability to recognize intracellular Gram-negative bacteria through the binding of bacterial hexa-acetylated lipopolysaccharide (LPS) to a motif in their prodomains (64). LPS binding induces
the dimerization and subsequent activation of the protease (65–69). The caspases then cleave the pore-forming protein gasdermin D (GSDMD), which inserts into the plasma membrane to lyse the cell (70, 71). This two-step “from recognition to death” system is known as the noncanonical inflammasome and leads to the inflammatory RCD type pyroptosis (FIGURE 2A).

Most pathogens, however, do not contain LPS or have evolved ways to restrict their presence in the cytosol, e.g., by “hiding” in vacuoles, and can therefore not be controlled by the noncanonical inflammasome. In response to intracellular infections or perturbations in cellular homeostasis, some cytosolic PRRs can induce the activation of a cytosolic macromolecular platform known as the canonical inflammasome (6) (FIGURE 2B). Canonical inflammasome activation is more elaborate than noncanonical inflammasome activation as it requires specialized sensors and the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC, also known as PYCARD) to recruit and activate the inflammatory caspase-1 (72). Caspases-1, -4, -5, and -11 can all cleave GSDMD. However, unlike caspases-4, -5, and -11, caspase-1 has the additional ability to process the inflammatory cytokines pro-interleukin (IL)-1β and pro-IL-18 into their biologically active forms to further aid the immune response to control an infection. The ability of an inflammatory caspase to cleave pro-IL-1β depends on a small set of charged amino acids near the catalytic site, which are present in caspase-1 but absent in human caspase-4 (73). GSDMD cleavage does not depend on these charged

**FIGURE 2.** Pathways of pyroptosis. A: the noncanonical inflammasome is activated upon recognition of LPS by the inflammatory caspases-4, -5, and -11, resulting in dimerization, activation, and processing of gasdermin D (GSDMD). Activated GSDMD induces plasma membrane pore formation by oligomerization to execute pyroptosis. B: the canonical inflammasome is activated upon engagement of inflammasome-inducing receptors, which recruit the inflammatory caspase-1, either directly via caspase-recruitment domain (CARD) interactions or indirectly via pyrin domain (PYD) and CARD interactions mediated by the adaptor protein ASC, forming the inflammasome. Activated caspase-1 cleaves pro-IL-1β and pro-IL-18 into their biologically active forms and activates GSDMD to execute pyroptosis.
residues but rather on hydrophobic interactions that are present between the COOH-terminal domain of GSDMD and all the inflammatory caspases (67, 69).

3.1. Induction of Inflammasomes

Inflammasomes can be activated by various microbial PAMPs, including flagellin, components of the bacterial type 3 secretion system (T3SS), RNA, DNA, toxins, and metabolic changes. These microbial elements are recognized by diverse inflammasome-inducing sensors, which include receptors of the Absent In Melanoma 2 (AIM2)-like receptor (ALR, aka PYHIN) gene family [the DNA sensors AIM2 and Interferon Gamma Inducible Protein 16 (IFI16), Pyrin, and members of the nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) family (74, 75)]. Inflammasome-inducing receptors contain a PYD or CARD through which they recruit procaspase-1 (76). PYD-containing receptors require ASC, which contains both PYD and CARD, to recruit caspase-1, whereas CARD-containing receptors can do so directly or through ASC.

Inflammasome-inducing sensors are activated through direct interaction with their respective ligands, by proteolytic processing, or by engaging and/or disrupting binding partner interactions (reviewed in Refs. 6, 76–79). AIM2, NLRP3, NLRP6, NLRP7, and NLRP9b are activated upon direct interaction with their respective ligands. NLRP1 activation can be activated by proteolytic processing, although human NLRP1 can also be activated by dsRNA (80). NLRC4 is activated upon binding activated NAIP proteins that function as the actual sensors, and sequestered Pyrin is released after disruption of 14-3-3 protein interactions (all detailed below).

The best studied inflammasome-inducing sensor is NLRP3, a member of the NLR receptor family. NLRP3 is activated by perturbations in cellular homeostasis. In most cases, activation of the NLRP3 inflammasome is a two-step process. First, a priming step is required in which PAMPs, DAMPs, and certain cytokines (TNF-α, IL-1β) induce the transcriptional upregulation of inflammasome components. Priming also readies NLRP3 via post-translational modifications to be activated (81). The second step is the activation of NLRP3. Changes in ion flux (e.g., K+ efflux or Ca2+ signaling), mitochondrial dysfunction or lysosomal rupture, and possibly other stresses activate the NLRP3 inflammasome. Cellular stress can be induced by a wide variety of stimulis, including pathogens, endogenous DAMPs during sterile inflammation, or environmental irritating factors (81–84). As such, the NLRP3 inflammasome does not appear to sense pathogens or other stressors directly but responds to their perturbing effects.

Although the exact mechanisms of NLRP3 activation by cellular stress remain to be elucidated, activated NLRP3 recruits ASC and caspase-1 to form the NLRP3 inflammasome, resulting in the processing of GSDMD and pro-IL-1β and pro-IL-18 and pyroptosis accompanied by IL-1β and IL-18 release. NLRP3 inflammasome formation occurs at the microtubule-organizing center (MTOC) (85) and further depends on the interaction with NIMA-related kinase 7 (NEK7) (86) and is promoted by DEAD-box helicase 3 X-linked (DDX3X) (87).

Cellular ion flux changes can occur as a result of disrupted plasma membrane integrity. This happens, for instance, after pore-forming proteins insert into the plasma membrane. Caspase-4 (or -11)-mediated pyroptosis following GSDMD activation causes K+ efflux, which then activates the NLRP3 inflammasome, thereby allowing the release of biologically active IL-1β and IL-18 (88). Similarly, insertion of MLKL into the plasma membrane during necroptosis (detailed below) causes K+ efflux and activates the NLRP3 inflammasome and IL-1β release independent of GSDMD (89, 90).

The NLR family member NLRP1 has been known to be a sensor for pathogen-encoded proteases, which activate NLRP1 through proteolytic cleavage. Cleavage releases the COOH-terminal CARD-containing fragment to recruit caspase-1 and induces the degradation of the formed NH2-terminal fragment (91–93). Details of the active NLRP1 structure have recently been elucidated (94, 95). The presence of ASC enhances, but is not required for, NLRP1-mediated caspase-1 activation (96).

Human NLRP1 can be activated by the 3C protease protein of enteroviruses, such as human rhinovirus (HRV) (97, 98). Rodent NLRP1 can be cleaved by lethal toxin of Bacillus anthracis (99, 100), but the cleavage site has not been conserved in human NLRP1 and lethal toxin does not activate the NLRP1 inflammasome in human macrophages (101). Murine NLRP1b is also cleaved by proteases of Shigella flexneri (102), Listeria monocytogenes (102), and Toxoplasma gondii (103) and can be activated by reduction of cytosolic ATP levels (104) or possibly by autoprocessing (101, 105), although the latter remains unclear (96). No viral proteases have been identified to activate rodent NLRP1, and no identified bacterial proteases cleave human NLRP1. In addition, dsRNA has recently been observed to activate NLRP1 in human cells (80). Human NLRP1, but not murine NLRP1b, binds cytosolic dsRNA to its leucine-rich region (LRR) domain and is activated upon delivery of long dsRNA or infection with Semliki Forest virus (SMV) but not after infection with modified vaccinia virus ankara (MVA), herpes simplex virus-1 (HSV-1), or measles virus (MV) (80). Rodent NLRP1 thus seems to mainly control bacterial...
infections, whereas human NLRP1 controls viral infections.

The ALR family members absent in melanoma 2 (AIM2) and gamma-interferon-inducible protein II-16 (IFI16) recognize pathogenic dsDNA, mainly from viruses. ALR family members contain an NH$_2$-terminal Pyrin domain (PYD) and a DNA-sensing COOH-terminal HIN200 domain (106, 107) that under homeostatic conditions maintain an autoinhibitory conformation. Ligand binding induces the release of the autoinhibitory conformation allowing for the recruitment of ASC via PYD-PYD interactions and the subsequent ASC-mediated recruitment of caspase-1 through CARD-CARD interactions (108).

NLRC4 is activated through binding with NAIP proteins that recognize components of the T3SS of Gram-negative bacteria. Primates, including humans, express one NAIP ortholog that recognizes T3SS needle proteins. Rodents encode multiple NAIP paralogs, which recognize flagellin (NAIP5/6) or the T3SS inner rod (NAIP2) or needle (NAIP1) proteins (109). Ligand specificity of rodent NAIPs depends on the central nucleotide-binding domain (NBD)-associated domains, which have evolved under positive selection in both rodents and primates (109). Upon ligation, NAIPs undergo a conformational change that initiates the recruitment, activation, and oligomerization of the inflammasome receptor NLRC4, which contains a CARD and can recruit caspase-1 directly or through ASC (110–112).

Activation of the Pyrin inflammasome is regulated by perturbations in the phosphorylation status of Pyrin. Phosphorylation by the kinases PKN1/2 induces Pyrin’s interaction with the chaperone proteins 14-3-3 and 14-3-3, resulting in restricted Pyrin activity (113, 114). Rho GTPases are required for the activity of PKN1/2. Rho GTPases can be modified or inactivated by bacterial toxins, resulting in reduced Pyrin phosphorylation and 14-3-3-mediated inhibition of PKN1/2 (115).

Other inflammasome-inducing sensors are known but have not been well characterized. NLRP6 is activated by lipoteichoic acid (LTA) derived from Gram-positive bacteria such as _L. monocytogenes_ (116), and direct binding of LTA to NLRP6 induces dimer formation, which can oligomerize in the presence of ATP to recruit ASC (117). NLRP7 can be activated by acylated lipopeptides, which are found on microbial cell walls (118), and NLRP9b recognizes short dsRNA strands via the RNA helicase Dhx9 (119).

### 3.2. Execution of Pyroptosis

The best-known executioner of pyroptosis is GSDMD, but this is not the only gasdermin that can execute pyroptotic cell death (reviewed in Refs. 120, 121). The human genome encodes six gasdermin family paralogs, _gsdmc, gsdmb, gsdmc, gsdmd, gsdme_, and _pjvk_. With the exception of pejvakin (PJVK), gasdermins are comprised of an NH$_2$-terminal cytotoxic domain and a COOH-terminal inhibitory domain that are connected by a linker region. In GSDMD, this linker can be cleaved by caspase-1, -4, -5, or -11. Cleavage of the linker results in the release of the cytotoxic NH$_2$-terminal domain, which undergoes a conformational change exposing a hydrophobic part of the protein that inserts itself into membranes and allows oligomerization with additional molecules, resulting in the formation of plasma membrane pores and pyroptotic cell death (120, 121). The NH$_2$-terminal domains of GSDMA, GSDMB, GSDMC, GSDMD, and GSDME are all able to form such pores (122), and the structures for the GSDMA and GSDMD pores have recently been solved (122–124).

Although expression of the NH$_2$-terminal fragments of gasdermin A, B, and C induces pyroptosis, the mechanisms underlying their activation are incompletely understood. GSDMB can be cleaved by the protease granzyme A (125), released into target cells by cytotoxic lymphocytes, and GSDMB may aid pyroptosis by enhancing the ability of caspase-4 to cleave GSDMD (126), but these observations remain to be confirmed by others. Pejvakin has a short COOH-terminal domain that does not share sequence homology with the other gasdermin family members, and the exact role for PJVK and its ability to form pores remains unclear, although mutations in _pjvk_ have been associated with hearing loss in mice and humans (127–129) due to altered functioning of peroxisomes (127).

Gasdermin D is activated by caspase-1 and caspases-4, -5, and -11 and can be cleaved by activated caspase-3, an executioner caspase in apoptosis (discussed below). The latter cleavage, however, renders GSDMD inactive, since it happens at a distinct site in the NH$_2$-terminal domain that disrupts the ability of GSDMD to insert into membrane and thus execute pyroptosis (130). By inhibiting GSDMD, caspase-3 ensures that apoptosis remains nonlytic. However, under certain conditions, apoptotic cells can undergo secondary necrosis, which may aid in the clearance of debris at the site of infection by enhancing the attraction of innate immune cells. In GSDME-expressing apoptotic cells, activated caspase-3 can cleave the linker region of GSDME, thereby activating gasdermin E to mediate pyroptosis (131, 132). Similarly, natural killer (NK) cell-derived granzyme B, but not granzyme A, can apparently activate GSDME (133). The formation of gasdermin D or E pores not only leads to the release of intracellular contents to induce inflammation and attract immune cells, it also traps intracellular pathogens in so-called pore-induced intracellular traps (PITs), which can lead to the clearance of the infection by...
efferocytosis—the engulfment of dead and dying cells by phagocytes—of the cell corpses and entrapped pathogens (134).

4. APOPTOSIS AND THE ACTIVATION OF INITIATOR AND EXECUTIONER CASPASES

A common response of cells to invading pathogens is the induction of apoptotic cell death. Apoptosis as a cell death modality is unique to animals, and its features rely on the activity of caspases (2). The initiator caspases that regulate apoptosis, caspase-8 and caspase-9, cleave and thereby activate the executioner caspases-3 and -7 (FIGURES 3A and 4). The executioner caspases induce apoptosis by cleaving approximately a thousand substrates, such as the chaperone and inhibitor of Caspase-Activated DNase (iCAD, which is in a complex with the DNase CAD), gelsolin, Pac21, Xkr8, and ATP11c (32), cumulatively resulting in the characteristic phenotype of apoptotic cells: DNA fragmentation, loss of

| A Most animals (including chordates) | B Arthropods | C Nematodes |
|-------------------------------------|-------------|-------------|
| ![Diagram A](image1)                 | ![Diagram B](image2) | ![Diagram C](image3) |

**FIGURE 3.** The caspase-9 pathway of apoptosis. Comparison between the caspase-9 pathway of apoptosis in most animals, arthropods, and nematodes. **A:** in most animals, the caspase-9 pathway of apoptosis is mediated by the BCL-2 effector proteins BAX and BAK to induce mitochondrial outer membrane permeabilization (MOMP). Activation of BCL-2 effector proteins is regulated by the balance between proapoptotic and antiapoptotic Bcl-2 proteins. MOMP releases cytochrome c and inhibitor of apoptosis (IAP) inhibitors (IAPI) into the cytosol, where cytochrome c binds and activates Apoptotic Protease Activating Factor 1 (APAF-1) to recruit the initiator caspase-9, forming the apoptosome. Activated caspase-9 cleaves and activates the executioner caspases-3 and -7 to mediate apoptosis. IAPI released by MOMP block the suppressive effects of the caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP), allowing the caspases to function. **B:** in arthropods, the APAF-1 homolog ARK is constitutively active. The IAP inhibitors Hid, Grim, and Reaper inhibit DIAP1 to release the initiator caspase DRONC to form the apoptosome with ARK. Activated DRONC cleaves and activates the executioner caspases DRICE and DCP1 to induce apoptosis. **C:** in nematodes, the APAF-1 homolog CED-4 is suppressed by the anti-apoptotic protein CED-9. Binding of the BH-3-only protein EGL-1 to CED-9 releases CED-4 to form the apoptosome with the executioner caspase CED-3, resulting in CED-3 activation and apoptosis.
We generally think of two major pathways of apoptosis, the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. Although useful, we know that these pathways can be intertwined, as components of the extrinsic pathway can engage the intrinsic pathway. The terms “intrinsic” and “extrinsic” are problematic, since the “intrinsic pathway” can be engaged by extrinsic signals and the “extrinsic pathway” by intrinsic signals. For this reason, we instead present the pathways in terms of how the initiator caspases specific to each are engaged. This focus on mechanism rather than characteristic description has emerged as a useful approach to terminology (135).

For our purposes, we consider the activation and functions of two of the initiator caspases, caspase-9 and caspase-8 (which correspond to the “traditional” mitochondrial and death receptor pathways, respectively).

### 4.1. The Caspase-9 Pathway of Apoptosis

The caspase-9 pathway of apoptosis relies on the formation of an activation platform termed the apoptosome (FIGURE 3A). The adaptor protein in this complex, Apoptotic Protease Activating Factor 1 (APAF-1), is a cytosolic monomeric protein that belongs to the NLR protein family (see above) and consists of an NH2-terminal CARD, an NB-ARC ATPase domain, and a COOH-terminal domain containing WD40 repeats (WD40 domain). APAF-1 is activated by binding of holocytochrome c to...
the WD40 domain (136). This induces a conformational change that allows the exchange of deoxyADP for deoxyATP in the NB-ARC domain, initiating exposure of the CARD, oligomerization into septameric structures, and the recruitment of caspase-9 via CARD-CARD interactions (137, 138). Caspase-9 then cleaves and activates the executioner caspases-3 and -7 that mediate apoptosis (136).

Caspase-9 activation thus relies on the interaction between APAF-1 and cytochrome c. Under homeostatic conditions, cytochrome c resides between the inner and outer mitochondrial membranes, where it functions as an electron shuttle of the respiratory chain. During apoptosis, the mitochondrial outer membrane is permeabilized in a process called mitochondrial outer membrane permeabilization (MOMP). This results in the release of the mitochondrial intermembrane space proteins, including cytochrome c, into the cytosol. Cytochrome c then binds to APAF-1, which induces the formation of the apoptosome (136, 139, 140).

Besides cytochrome c, MOMP releases the mitochondrial intermembrane space proteins Smac/Diablo and Omi/Htra2, which are antagonists of inhibitor of apoptosis (IAP) proteins (141–144). Activated caspases-3 and -7, as well as caspase-9, are inhibited by X-linked inhibitor of apoptosis protein (XIAP). MOMP thus not only results in apoptosis activation but also induces the release of XIAP-mediated inhibition of caspases-3, -7, and -9.

The process of MOMP is regulated by the Bcl-2 family of proteins, which contain proapoptotic (MOMP promoting) and antiapoptotic (MOMP inhibiting) members (145, 146). Upon activation, the effectors of MOMP, BCL2-associated X protein (BAX) and BCL2-antagonist/killer 1 (BAK1, known as BAK), insert themselves into the outer mitochondrial membrane and oligomerize to form pores, leading to mitochondrial ultrastructure perturbation and the leakage of the intermembrane content into the cytosol. The activation of the MOMP effector proteins is tightly regulated by an interplay between the antiapoptotic Bcl-2 proteins (e.g., BCL-2, BCL-XL, and MCL-1) and the proapoptotic BH-3-only subfamily that can either neutralize the antiapoptotic proteins (e.g., BAD, BMF, and NOXA) or activate BAX and BAK (e.g., BID, BIM, and PUMA). Apoptotic stimuli, such as cellular stress, DNA damage, or pathogen recognition, can change this delicate balance in favor of the proapoptotic proteins, resulting in the initiation of MOMP (145, 146). Another Bcl-2 family protein is BCL2-like Ovarian Killer (BOK), which does not appear to be regulated by other Bcl-2 family proteins but instead is controlled by the endoplasmic reticulum (ER)-associated degradation machinery (147). Like BAX and BAK, BOK can effect MOMP to promote caspase-9 activation.

Thus, the caspase-9 pathway of apoptosis depends on the induction of the apoptosome and the balance between proapoptotic and antiapoptotic Bcl-2 proteins to harness MOMP.

4.2. The Caspase-8 Pathway of Apoptosis

The caspase-8 pathway of apoptosis depends on the interaction between caspase-8 and the adaptor protein Fas-associated Death Domain (FADD) (FIGURE 4). FADD consists of a COOH-terminal death domain (DD) and an NH2-terminal death effector domain (DED) and mediates the activation of caspase-8 by recruiting procaspase-8 via DED-DED interactions upon upstream signals that recruit FADD to DD-containing proteins (detailed below) (35, 148). Binding of procaspase-8 to FADD induces procaspase-8 oligomerization, filament formation, and proximity-induced activation. Autoproteolytic processing then yields the fully active, mature caspase-8 (35).

Caspase-8 directly cleaves and activates the executioner caspases (caspases-3 and -7). In cells that express XIAP (see above), however, the function of the activated executioner caspases is inhibited. Therefore, when caspase-8 cleaves another substrate, the proapoptotic Bcl2 family member BH3 Interacting Domain Death Agonist (BID), the cleaved BID (cBID) induces BAX-BAK-mediated MOMP (detailed above) (149, 150). The liberation of the IAP antagonists SMAC and Omi then promotes apoptosis by inhibiting the antiapoptotic function of XIAP. In such cells, antiapoptotic Bcl-2 proteins can inhibit the caspase-8 pathway of apoptosis.

FADD is engaged by DD-DD interactions. Death receptors (DRs) are a subset of the tumor necrosis factor superfamily (TNFRSF) that includes tumor necrosis factor (TNF) receptor-1 (TNFR1; TNFRSF1a), CD95 (TNFRSF6, Apo-1, Fas), TNF-related apoptosis-inducing ligand (TRAIL) receptor-1 and -2 (TRAIL-R1/2; DR4/5, TNFRSF10a/b), DR3 (TNFRSF25), and DR6 (TNFRSF21) and contains a COOH-terminal cytosolic DD (19). With the exception of TNFR1, ligation of these DRs by their cognate ligands or by agonistic antibodies results in the DD-mediated engagement of the protein FADD and the subsequent recruitment of procaspase-8 through DD interactions. DR ligation thus induces the formation of a FADD-caspase-8 activation platform at the cytosolic tail of the receptor, known as the death-inducing signaling complex (DISC) (151). TNFR1-induced apoptosis is also mediated by FADD-caspase-8, but unlike other DRs that recruit FADD directly, TNFR1 first engages the protein TNFR1-associated Death Domain (TRADD), which in turn recruits FADD (152).

FADD can also be recruited to a cytosolic protein complex called the ripoptosome that further consists of the receptor interacting serine/threonine kinase (RIPK)-1.
(RIPK1) (153). RIPK1 contains an NH₂-terminal kinase domain (KD), an intermediate domain (ID), a RIP homotypic interaction motif (RHIM) required for RHIM-containing protein interactions, and a COOH-terminal DD through which it interacts with FADD to promote caspase-8 activation (154).

TNFR1 ligation recruits RIPK1 to the cytoplasmic tail of the receptor, where RIPK1 is modified by phosphorylation and ubiquitylation to mediate downstream signaling, resulting in gene expression. However, when the functioning of E3 ligases that ubiquitylate RIPK1, such as cIAP1 and cIAP2, is impaired or RIPK1 debiquitylation is enhanced, RIPK1 is released from the TNFR1 complex and then recruits the FADD-procaspase-8 complex to form the ripoptosome (155). RIPK1 may also induce the formation of the ripoptosome when engaged by retinoic acid receptor gamma (RARγ) when this protein translocates from the nucleus to the cytosol in response to DNA damage (156). Ripoptosome formation can also be induced by another protein kinase, RIPK3. RIPK3 is a homolog of RIPK1 and contains an NH₂-terminal KD, an ID, and a RHIM but lacks the COOH-terminal DD found in RIPK1 (157). RIPK3 recruits RIPK1 through RHIM-RHIM interactions of the RHIMs present in both proteins, which is induced by ligation of Toll-like receptor (TLR)3, TLR4, and the cytosolic zDNA/zRNA receptor zDNA binding protein 1 [ZBP1, also known as DNA-dependent activator of IFN-regulatory factors (DAI)] (153, 158, 159). Ligation of TLR3 or TLR4 induces the recruitment of the adaptor molecule TIR-domain-containing adapter-inducing interferon-β (TRIF) to the engaged receptors. TRIF contains a RHIM through which it interacts with RIPK3 (158). Similarly, ZBP1 contains two RHIMs and can induce the ripoptosome by directly recruiting RIPK3 (159). The outcome of ripoptosome formation can be FADD-caspase-8-mediated apoptosis, although other outcomes are possible, as detailed below.

Thus, the caspase-8 pathway of apoptosis depends on FADD engagement through DD interactions with DRs, TRADD, or RIPK1 and is activated by death-inducing signals coming from within the cell or from the extracellular environment.

4.3. Regulation of the Caspase-8 Pathway of Apoptosis by cFLIP

Many death receptors, TLRs, and cytosolic receptors induce transcriptional programs upon their ligation. TNFR1 ligation induces the MAPK and NF-κB signaling pathways that induce gene expression of proinflammatory and prosurvival factors. Among the expressed genes is cflar, which encodes FLICE-like inhibitory protein (cFLIP). Depending on posttranslational mRNA splicing, two major isoforms of cFLIP are generated (160, 161). cFLIP short (cFLIPₜ) is a truncated form that blocks DISC-dependent procaspase-8 activation by halting DED-mediated procaspase-8 oligomer assembly and thereby blocks apoptosis. The second isoform, cFLIP long (cFLIPₐ), is a procaspase-8-like protein that lacks a catalytic site and thus does not possess proteolytic activity. Procaspase-8 favors heterodimerization with cFLIPₜ over homodimerization with other procaspase-8 zymogens (162, 163). While heterodimerization ablates the autoproteolytic processing of procaspase-8, the procaspase-8-cFLIPₐ complex retains proteolytic capacity, although substrate specificity is slightly changed compared to procaspase-8-procaspase-8 homodimers (164). Low cFLIPₜ levels can enhance apoptotic signaling by enhancing the specific activity of the heterodimer and procaspase-8 homodimer activation without disrupting the filament. High cFLIPₐ levels, however, disrupt procaspase-8 homodimer formation, thereby reducing the activity of procaspase-8 and blocking apoptosis (165). Thus, cFLIP isoforms and expression levels determine whether caspase-8-mediated apoptosis ensues or is inhibited.

4.4. The Caspase-2 Pathway of Apoptosis

The caspase-2 pathway of apoptosis is more elusive. Caspase-2 is an initiator caspase that has been implicated in apoptosis in response to a diverse array of triggers including genotoxic stress, heat shock, β-amyloid accumulation, and irregularities in cytoskeleton arrangement and the mitotic spindle (166). The best-known platform for the activation of caspase-2 is termed the PIDDosome, although caspase-2 can be activated outside of this complex as well (167). The PIDDosome consists of the receptor protein PIDD, which contains a COOH-terminal DD through which it interacts with the adaptor protein RAIDD. RAIDD recruits procaspase-2 via CARD-CARD interactions leading to caspase-2 activation. Activated caspase-2 likely does not directly cleave executioner caspases; rather, it induces apoptosis by cleaving substrates that mediate the activation of caspases-3 and -7. For instance, activated caspase-2 can cleave Bid, resulting in activation of the caspase-9 pathway of apoptosis (168). It is therefore not clear whether the primary function of caspase-2 is to mediate apoptosis or it has other functions (169–172). Probably the best-characterized function of the caspase-2 pathway is in mitotic quality control. The presence of supernumerary centrioles in a dividing cell activates the caspase-2 pathway (173) via the binding of PIDD to the distal appendage protein ANKRD26 (174). In this setting, activated caspase-2 functions to cleave the E3-ligase MDM2, resulting in p53 stabilization (which in turn can lead to...
cellular senescence or apoptosis) (173, 174). The caspase-2 pathway is also engaged in the nucleus upon DNA damage, in this case by the binding of PIDD to nucleolar phosphoprotein nucleophosmin-1 (NPM1) (175).

5. NECROPTOSIS AND ITS REGULATION BY THE FADD-CASPASE-8 COMPLEX

Upon recognition of pathogens by certain PRRs or as a result of exacerbated interferon or TNF signaling, cells can die by necroptosis (FIGURE 5). The necrototic pathway does not depend on the functioning of caspases but instead relies on the activity of RIPK3. Upon activation, RIPK3 autophosphorylates, oligomerizes into RHIM domain-mediated amyloid structures (176), and recruits the necroptosis executioner protein Mixed lineage kinase domain-like (MLKL), which consists of an NH$_2$-terminal helix bundle domain (HBD), a brace region, and a COOH-terminal pseudokinase domain (psKD) (177). RIPK3 phosphorylates the psKD of MLKL, which induces a conformational change in the brace region of MLKL, allowing for oligomerization and translocation to the plasma membrane, where the HBD inserts into the lipid bilayer to induce pore formation and rupture of the cell (178–181).

Necroptosis is induced by TNFR1 ligation (182–184), engagement of TLR3 or TLR4 (both via TRIF) (185–188), interferons (185), or after RAR$\gamma$ translocation to the cytosol in response to DNA damage (156). Interferon signaling does not directly induce necroptosis but promotes the expression of the interferon response gene ZBP1 (189). It remains unclear how ZBP1 is engaged in uninfected cells, and endogenous ligands are yet to be defined. Activation of these receptors leads to the

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**FIGURE 5.** Pathways of necroptosis. Ligation of TNFR1 induces the recruitment of TRADD, TRAF2, cIAP1/2, and RIPK1. Within this complex, RIPK1 is ubiquitylated by cIAP1/2 to mediate the expression of proinflammatory and prosurvival genes, including cFLIPL. When RIPK1 is deubiquitylated by CYLD, it leaves the complex and associates with RIPK3 through homotypic RIP homotypic interaction motif (RHIM) domain interactions, resulting in RIPK3 activation. IFN receptor signaling induces the expression of ZBP1. ZBP1 directly recruits RIPK3 by RHIM domain interactions. Ligated TLR3 and 4 engage the RHIM domain-containing protein TRIF to recruit RIPK3. RIPK3 autoactivates and forms amyloid structures to recruit and activate MLKL, which induces plasma membrane pore formation by oligomerization to execute necroptosis. Engagement of ZBP1 and TLR3 and 4 also results in the expression of proinflammatory and prosurvival genes. The inhibitory FADD-caspase-8-cFLIPL complex is recruited to RIPK3 via RIPK1 and inhibits the activation of RIPK3 and thereby necroptosis. DD, death domain; DED, death effector domain.
formation of the ripoptosome (described above) and induces FADD-caspase-8-mediated apoptosis or RIPK3-mediated necroptosis.

The engaged cell death pathways are controlled by the presence of cFLIPL and the enzymatic activity of caspase-8. Low cellular levels of cFLIPL allow for the homo-dimerization of caspase-8 and lead to apoptosis, whereas high cFLIPL levels induce procaspase-8-cFLIPL heterodimerization. RIPK1 and RIPK3 both contain caspase-8 cleavage sites that are processed by the catalytically active procaspase-8-cFLIPL heterodimer (164). This cleavage disrupts the ripoptosome and thereby blocks necroptosis. The procaspase-8-cFLIPL heterodimer thus inhibits both apoptosis and necroptosis upon ripoptosome formation. Inhibition of caspase-8, e.g., by pathogenic caspase inhibitors (described below) or by pharmacological inhibitors, abrogates the ability of the procaspase-8-cFLIPL heterodimer to cleave RIPK1 and RIPK3, allowing the autophosphorylation and oligomerization of RIPK3 and the induction of necroptosis.

RIPK1 has a dual role in necroptosis (154). On one hand RIPK1 mediates necroptosis induced by TNFR1 and RARγ, where FADD-mediated recruitment of the procaspase-8-cFLIPL complex blocks necroptosis, whereas on the other hand RIPK1 blocks necroptosis by recruiting the FADD-procaspase-8-cFLIPL complex to RIPK3 upon activation of TLR3/4 and ZBP1. The kinase activity of RIPK1 is required for TNFR1-induced RIPK1-mediated necroptosis but is dispensable for the inhibition of TLR- and ZBP1-induced necroptosis (154).

Thus, the ligation of TNFR1, TLR3, TLR4, or ZBP1 induces three distinct signaling pathways: gene expression, apoptosis, and necroptosis. Where apoptosis depends on the catalytic activity of initiator and executioner caspases, necroptosis relies on RHIM domain interactions and the activation of the pore-forming executioner protein MLKL. In the presence of cFLIPL, both apoptosis and necroptosis are inhibited, signifying that the apoptotic and necroptotic pathways are interconnected.

6. CROSS TALK BETWEEN RCD PATHWAYS

The mechanisms that shape the individual apoptotic, necroptotic, and pyroptotic signaling cascades are well understood, and it has been recognized that interplay between the pathways occurs (190), the best example being that necroptosis is inhibited by the apoptosis-initiating caspase-8 (FIGURE 5). However, it is now becoming apparent that the three cell death pathways are far more intertwined and intricately regulated than has previously been appreciated (FIGURE 6).

The apoptotic and pyroptotic pathways can interact with each other. The apoptosis executioner caspase-3 cleaves and activates GSDMD to mediate pyroptosis or cleaves and inactivates GSDMD to block pyroptosis (130–132), and caspase-8 has been suggested to cleave and activate GSDMD (191, 192). FADD and caspase-8 are required for the optimal activation of the NLRP3 inflammasome in macrophages (193), whereas in ileal epithelial cells FADD blocks caspase-8 from its involvement in inflammasome activation and pyroptosis (194, 195). In ileal epithelial cells in which FADD is present but caspase-8 is catalytically inactive, inflammasome activation is also induced (196, 197). Moreover, apoptotic caspases can activate the NLRP3 inflammasome by activating the channel-forming protein pannexin-1 (198), and the anti-inflammatory proteins BCL-2 and BCL-XL bind and inhibit NLRP1 activation, thereby reducing activation of caspase-1 and IL-1β (199). Conversely, inflammasome-activated caspase-1 can cleave caspase-3 to induce apoptosis (130, 200, 201) and Bid to release mitochondrial SMAC and drive secondary necrosis (202), and activated gasdermin pores permeabilize mitochondria to enhance caspase-3 activation (203).

The necroptotic and pyroptotic pathways also influence each other. RIPK3 can mediate NLRP3 inflammasome activation in the absence of MLKL (204) or in the presence of MLKL by inducing MLKL pores in the plasma membrane (90). In human monocytes, NLRP3 can be engaged by a TRIF-RIPK1-FADD-caspase-8 pathway, which leads to IL-1β secretion independently of inflammasome formation, K⁺ efflux, and pyroptosis in a process known as the alternative NLRP3 inflammasome pathway (205). ZBP1 may induce inflammasome activation, which involves a noncatalytic function of caspase-6 (206). ZBP1 activation may thus result in apoptosis, necroptosis, or pyroptosis, but how a cell mechanistically chooses its fate is not understood (207).

Recent studies indicate that proteins that mediate apoptosis, necroptosis, and/or pyroptosis may also be involved in the regulation of gene expression. Ligation of the death receptor TRAIL-R leads to the engagement of gene expression programs, which depends on the formation of a complex consisting of FADD, caspase-8, and RIPK1, which was dubbed the FADDosome (208–210). Similarly, engagement of the death receptor CD95 (also Fas-R) induces the FADDosome and gene expression (195). Thus, death receptor engagement may lead to DISC formation and apoptosis or FADDosome formation and gene expression that may lead to inflammation (194, 195).

Thus, regulated cell death pathways are not linear but interconnected, complex, and plastic signaling cascades (FIGURE 6). The plasticity between the pathways allows cells to overcome blockage posed by pathogens and as such has important implications for the control of infections and survival of the host organism.
7. PATHOGEN STRATEGIES TO INHIBIT CELL DEATH

Preventing host cells from dying secures the ability of pathogens to proliferate, spread, and survive, and pathogens have evolved a plethora of ways to dysregulate pyroptotic (FIGURE 7; TABLES 1 and 2) and apoptotic and necroptotic (FIGURE 8; TABLES 3 and 4) cell death programs in their hosts, varying from the modulation of gene expression profiles to direct interaction with cellular death-mediating proteins.

Many viral species, especially large DNA viruses, dedicate sizable parts of their genomes to the inhibition of cell death pathways. Six bacterial species, *Burkholderia* spp., enteroinvasive *Escherichia coli*, *Francisella* spp., *Listeria* spp., *Rickettsia* spp., and *Shigella* spp., have been identified to proliferate within the cytosol (211), and other bacteria invade host cells but reside and multiply within vacuoles or other structures (212) and deliver their effector proteins that regulate host cellular mechanisms delivered via a T3SS or other secretion systems into the host's cytosol. Regardless of their cellular localization, the intricate cross talk between RCD pathways means that pathogens must simultaneously block multiple RCD pathways to effectively inhibit cell death.

7.1. Pathogen Blockade of Pyroptosis

One of the first defenses against intracellular bacteria is the activation of the noncanonical inflammasome upon...
the detection of LPS. To avoid detection by caspase-4, -5, or -11, *Francisella* and *Shigella* modulate their LPS composition by decreasing the degree of acetylation of the lipid A moiety (66, 213). Interestingly, hypoacetylation of *Francisella* LPS abrogates detection by caspase-11, whereas caspase-4 remains able to detect hypoacetylated LPS, albeit less efficiently (214). This indicates that human cells are better equipped to control cytosolic bacteria than murine cells and suggests that intracellular bacteria that modulate their LPS composition either must do so in such a way that can no longer lead to recognition by caspase-4 or must have evolved additional mechanisms to block the noncanonical inflammasome in human cells. *Shigella* does so by preventing the LPS-induced dimerization of caspase-4, but not caspase-1 or caspase-11, by its T3SS-delivered OspC effector protein (215).

Microbes modulate various aspects of inflammasome signaling (FIGURE 7, TABLE 1). The virulence factors of pathogenic *Yersinia* species, named *Yersinia* outer protein (Yop) proteins, are delivered via the T3SS, which can be recognized by NAIps to activate the NLRC4 inflammasome. YopK of *Yersinia pseudotuberculosis* prevents the recognition of the T3SS by NAIps and also blocks the activation of the NLRP3 inflammasome (216). *Yersinia enterocolitica* YopE and YopT proteins inactivate Rho GTPases and thereby induce the Pyrin inflammasome (217, 218). YopM, however, recruits and activates PRK1/2 (219, 220), effectively taking over one of the functions of Rho GTPases to keep Pyrin inactive.
Furthermore, YopM interacts with caspase-1 to restrict IL-1β maturation and pyroptosis (221).

Proteases secreted by *Pseudomonas aeruginosa* into the extracellular space degrade host defense proteins, including complement components and proinflammatory cytokines, and degrade extracellular inflammasome components and flagellin, which would trigger the NLRC4 inflammasome upon cellular uptake. The *P. aeruginosa* virulence factors pyocyanin and C120HSL prevent inflammasome activation by directly inhibiting NLRC4 and NLRP3 (222). The effector proteins ExoU and ExoS inhibit caspase-1 activation and thereby prevent the processing of pro-IL-1β and pro-IL-18 (223), although precise mechanisms are unclear.

Viruses also disrupt the functioning of multiple inflammasome components (FIGURE 7; TABLE 2). Poxviral serpins block the catalytic site of caspase-1, thereby blocking pyroptosis and the release of IL-1β and IL-18 (224). Poxviruses also neutralize bioactive IL-1β and IL-18 by expressing scavenger receptors and soluble proteins that bind these cytokines, preventing them from activating their cognate receptors (225). Poxviruses Myxoma virus (M013L) and Shope fibroma virus (S013L) express pyrin-only proteins (POPs) that resemble human POPs and inhibit inflammasome activation by binding the PYDs of ASC and inflammasome-inducing receptors, thereby disrupting the ability of these proteins to effectively form an inflammasome and activate caspase-1 (226, 227). Human papillomaviruses dampen pyroptotic cell death responses by enhancing TRIM21-mediated proteasomal degradation of the IFI16 inflammasome (228). Kaposi’s sarcoma-associated herpesvirus (KSHV) inhibits the formation of NLRP1 and NLRP3 inflammasomes by expressing a viral NLRP1 homolog, Orf63, that lacks the NH2-terminal PYD and COOH-terminal CARD of human NLRP1 but can bind to NLRP1 and NLRP3 (229). The Measles virus V protein (230) and NS1 protein of Influenza virus (231, 232) also interact with NLRP3, whereas the viral proteases 2A and 3C of Enterovirus 71 inactivate NLRP3 and GSDMD by cleavage (233–235).

### 7.2. Pathogen Blockade of the Caspase-9 Pathway of Apoptosis

The presence of pathogens in a cell disturbs cellular homeostasis, and the associated stress can trigger the caspase-9 pathway of apoptosis. Pathogens therefore developed several strategies to block the caspase-9 pathway of apoptosis (FIGURE 8; TABLES 3 and 4). *Shigella* infection, for instance, induces necrotic death and provokes cellular genotoxic stress, which induces p53-mediated apoptosis. *Shigella* counteracts this by activating a prosurvival program that induces expression of BCL-2 (236). Moreover, *Shigella* mediates the degradation of p53 by its T3SS-delivered virulence factor VirA, which induces calpain proteases to cleave p53, and its virulence factor LpgD that induces MDM2 to ubiquitylate p53 for proteasomal degradation (237). LpgD

| Bacterial Species | Protein | Mode of Action | Reference |
|-------------------|---------|----------------|-----------|
| Enteropathogenic *Escherichia coli* | NleF | Inhibits caspase-4 activation | (293) |
| *Francisella* spp. | LPS | Inhibits caspase-4 to recognize LPS | (66) |
| *Pseudomonas aeruginosa* | C120HSL | Inhibits NLRC4 and NLRP3 | (222) |
| | ExoS | Inhibits caspase-1 activation | (223) |
| | ExoU | Inhibits caspase-1 activation | (223) |
| | Pyocyanin | Inhibits NLRC4 and NLRP3 | (222) |
| *Shigella* spp. | LPS | Inhibits caspase-4 to recognize LPS | (213) |
| | Osp3C | Prevents caspase-4 dimerization | (215) |
| *Yersinia* spp. | YopK | Blocks NLRP3 inflammasome activation | (216) |
| | YopK | Blocks NLRC4 inflammasome activation | (216) |
| | YopM | Activates PRK1/2 to keep Pyrin inactive | (219) |
| | YopM | Blocks caspase-1 | (221) |
also generates phosphatidylidylinositol 5-phosphate (PI5P) to activate additional prosurvival signaling (238). VirA-activated calpain proteases cleave and activate BID, leading to mitochondrial permeabilization and the release of proapoptotic factors including SMAC to block the inflammatory effects of XIAP (236, 239). However, mitochondrial permeabilization does not lead to apoptosis, since the O-antigen moiety of LPS from *Shigella* inhibits the activity of caspases-3 and -7 by directly binding to these executioner caspases (240, 241).

Many bacterial species regulate the expression and degradation of cellular pro- and antiapoptotic Bcl-2 proteins to inhibit the activation of MOMP. *Chlamydia*, *Mycobacterium tuberculosis*, and *Coxiella burnetii* induce the transcription and block the degradation of the antiapoptotic Bcl-2 protein MCL-1 (242–245), whereas *Helicobacter pylori* upregulates MCL-1 (246) and BCL-2 and decreases BAX (247). *Chlamydia* degrades proapoptotic BH-3-only proteins, including Bad, Bim, and PUMA (248), and recruits Bad to the inclusion vacuole, thereby sequestering Bad from mitochondria (249). The *Anaplasma phagocytophilum* T4SS effector Ats-1 enters mitochondria, where it inhibits redistribution of BAX, thereby inhibiting MOMP and cytochrome c release (250). *Coxiella burnetii* employs three T4SS effector proteins, AnG, CaeA, and CaeB, to inhibit apoptosis. AnG and CaeB act at the mitochondrial level (251, 252), whereas CaeA prevents caspase-7 activation (253).

A similar approach to block MOMP is employed by viruses belonging to the families of *Adenoviridae*, *Bimaviridae*, *Herpesviridae*, and *Poxviridae*. These

| TABLES 3 and 4 | respective. Cyt. C, cytochrome c; DD, death domain; DED, death effector domain. |
|----------------|----------------------------------------------------------------------------------|

![Figure 8](image-url)
viruses express viral BCL-2 (vBcl2) proteins that show structural similarities with antiapoptotic BCL-2 proteins and block MOMP by inhibiting the activation of BAK and BAX (254). vBcl2 proteins from *Herpesviridae* and *Poxviridae* contain transmembrane domains through which they integrate into the mitochondrial membrane, where they interact with cellular pro- and antiapoptotic Bcl2 proteins to modulate apoptosis (255). Among the retroviruses, human T cell leukemia virus type 1 (HTLV-1) promotes BCL-XL expression and represses BAX expression via its TAX protein (256), whereas human immunodeficiency virus (HIV) Nef and HSV-1 U(S)3 proteins inhibit the proapoptotic BH-3-only protein Bad by mediating its phosphorylation (257, 258). Human cytomegalovirus (HCMV) vMIA and murine cytomegalovirus (MCMV) m38.5 inhibit the proapoptotic activity of BAX, but not BAK, by sequestering BAX at mitochondria (259–261). MCMV also inhibits BAK-mediated MOMP by its vIBO protein (262). Epstein–Barr virus (EBV) blocks apoptosis by sequestering BH-3-only proteins by its viral BCL-2 homolog protein BHRF-1 (263–265) and expresses viral miRNAs that repress the transcriptional levels of caspase-3 (266).

Apoptosis in insect cell does not rely on MOMP but rather on expression of IAP antagonist proteins (detailed below). Therefore, viruses that infect insect cells would not benefit from expressing vBcl2 proteins. The insect poxvirus *Amsacta moorei* Entomopoxvirus (AmEPV)
does not contain the apoptosis suppressor genes expressed by vertebrate poxviruses but encodes a viral IAP (vIAP), AMV-IAP, that binds and impairs two IAP antagonist proteins in *D. melanogaster* cells (267). AmEPV AMV-IAP is not present in vertebrate poxviruses, even though AMV-IAPs can inhibit the enzymatic activities of human caspases-3 and -9 in vitro (267). In fact, with the exception of African swine fever virus, vIAPs have not been identified in mammalian viruses, indicating that viral mimicry of IAPs is not an evolutionarily selected strategy to block apoptosis in mammalian cells.

### 7.3. Pathogen Blockade of Receptor-Induced Apoptosis and Necroptosis

Pathogens employ many strategies to inhibit apoptosis and necroptosis (*FIGURE 8; TABLES 3 and 4*). One

| Bacterial Species                     | Protein | Mode of Action                           | Reference |
|---------------------------------------|---------|------------------------------------------|-----------|
| **Anaplasma phagocytophilum**         | Ats-1   | Inhibits BAX activation                  | (250)     |
| **Chlamydia spp.**                    | Cdu1    | Blocks degradation of MCL-1              | (244)     |
|                                       | Unknown | Induces expression of MCL-1              | (245)     |
|                                       | Unknown | Degrades proapoptotic BH-3-only proteins | (248)     |
|                                       | Unknown | Sequesters BAD                           | (249)     |
|                                       | Unknown | Blocks the internalization of TNF-TNFR complexes | (268) |
| **Coxiella burnetii**                 | Unknown | Induces expression of MCL-1              | (243)     |
| **Coxiella burnetii**                 | AnkG    | Blocks MOMP                              | (252)     |
| **Coxiella burnetii**                 | CaeA    | Blocks caspase-7 activation              | (253)     |
| **Coxiella burnetii**                 | CaeB    | Blocks MOMP                              | (251)     |
| **Coxiella burnetii**                 | EspL    | Cleaves RHIM domain-containing proteins  | (296)     |
| **Coxiella burnetii**                 | NieB1   | Blocks FADD recruitment                   | (278)     |
| **Coxiella burnetii**                 | NieF    | Inhibits caspase-9 activation            | (292)     |
| **Coxiella burnetii**                 | NieF    | Inhibits caspase-8 activation            | (292)     |
| **Coxiella burnetii**                 | Unknown | Induces expression of MCL-1              | (243)     |
| **Coxiella burnetii**                 | Unknown | Induces expression of BCL-2              | (247)     |
| **Coxiella burnetii**                 | Unknown | Decreases BAX                            | (247)     |
| **Helicobacter pylori**               | Unknown | Induces expression of MCL-1              | (242)     |
| **Helicobacter pylori**               | Unknown | Promotes secretion of soluble TNFR2      | (269)     |
| **Mycobacterium tuberculosis**        | Unknown | Induces expression of MCL-1              | (242)     |
| **Mycobacterium tuberculosis**        | Unknown | Promotes secretion of soluble TNFR2      | (269)     |
| **Shigella spp.**                     | IpgD    | Activates prosurvival signaling          | (238)     |
| **Shigella spp.**                     | IpgD    | Induces expression of BCL-2              | (237)     |
| **Shigella spp.**                     | VirA    | Induces expression of BCL-2              | (237)     |
| **Shigella spp.**                     | OspC1   | Inhibits caspase-8 activation            | (291)     |
| **Shigella spp.**                     | OspD3   | Degrades RIPK1 and RIPK3                 | (291)     |

MOMP, mitochondrial outer membrane permeabilization; RHIM, RIP homotypic interaction motif.
### Table 4. Viral inhibition of apoptosis and necroptosis

| Virus Family   | Virus       | Protein | Mode of Action                                                                 | Reference |
|---------------|-------------|---------|--------------------------------------------------------------------------------|-----------|
| Adenoviridae  | Adenovirus  | E1B 19K | Viral BCL-2 homolog                                                             | (381)     |
| Birnaviridae  | IPNV        | VP5     | Viral BCL-2 homolog                                                             | (382)     |
| Flaviviridae  | HCV         | NS5A    | Viral BCL-2 homolog                                                             | (383)     |
| Herpesviridae | EBV         | BALF1   | Viral BCL-2 homolog                                                             | (384)     |
|               |             | BHRF1   | Viral BCL-2 homolog                                                             | (385)     |
|               |             | EBNA3A and EBNA3C | Block expression of the proapoptotic BCL-2 protein BIM | (386)     |
|               |             | BART miRNAs | Block caspase-3 expression                                                     | (266)     |
|               |             | LMP1    | Blocks RIPK1 and RIPK3                                                          | (303)     |
| γHV-68        | M11         |         | Viral BCL-2 homolog                                                             | (387)     |
|               | vMAP        |         | Blocks BAX activation                                                          | (388)     |
| HCMV          | vMIA        |         | Blocks BAX activation                                                          | (259)     |
|               | UL36 (aka vlCA) |     | Blocks caspase-8 activation                                                     | (286)     |
|               | UL45        |         | Blocks necroptosis downstream of MLKL activation                                 | (304)     |
| HHV8 (KSHV)   | KSBcl-2     |         | Viral BCL-2 homolog                                                             | (389)     |
|               | K7          |         | Inhibits caspase-3 activation                                                   | (390)     |
|               | vFLIP       |         | Blocks caspase-8 oligomerization                                                | (284)     |
| HSV-1         | U(S)3       |         | Blocks BAD                                                                      | (257)     |
|               | LAT         |         | Stabilizes AKT to inactivate proapoptotic BCL-2 proteins                        | (391)     |
|               | ICP6        |         | Blocks necroptosis                                                             | (302)     |
| HSV-2         | ICP10       |         | Blocks caspase-8 activity                                                      | (392)     |
|               | ICP10       |         | Blocks necroptosis                                                             | (302)     |
| HVS           | Orf6        |         | Viral BCL-2 homolog                                                             | (393)     |
| MCMV          | M38.5       |         | Blocks BAX activation                                                          | (260)     |
|               | M41.1 (aka vBIO) |     | Blocks BAK activation                                                          | (262)     |
|               | M36 (aka vlCA) |     | Blocks caspase-8 activation                                                    | (286)     |
|               | M45 (aka vlRA) |     | Inhibits interaction of RHIM domain-containing proteins                         | (300)     |

**Continued**
| Virus Family | Virus | Protein | Mode of Action | Reference |
|-------------|-------|---------|---------------|-----------|
| Papillomaviridae | MCV | MC159 (aka vFLIP<sub>2</sub>) | Blocks caspase-8 oligomerization | (394) |
| Papillomaviridae | HPV16 and 18 | E6 | Inhibits BAK | (395) |
| Papillomaviridae | HPV16 and 18 | E6 | Mediates degradation of caspase-8 | (288) |
| Papillomaviridae | Unknown | Downregulates RIPK3 expression | (297) |
| Picornaviridae | Rhinovirus | 3C | Cleaves RIPK1 | (396) |
| Poxviridae | ASFV | A179L | Viral BCL-2 homolog | (397) |
| Poxviridae | ASFV | A224L | Viral IAP | (398) |
| Poxviridae | Cowpox | CrmA | Blocks caspase-8 activity, not cFLIP activity | (282) |
| Poxviridae | Cowpox | CrmB | Viral TNFR homolog | (270) |
| Poxviridae | FPV | FPV039 | Viral BCL-2 homolog | (399) |
| Poxviridae | Myxoma virus | M11L | Inhibits BAK | (400) |
| Poxviridae | Myxoma virus | M11L | Inhibits BAX | (401) |
| Poxviridae | Myxoma virus | M-T2 | Viral anti-TNF protein | (272) |
| Poxviridae | Myxoma virus | Serp2 | Blocks caspase-8 activity | (402) |
| Poxviridae | PPVO | OrfV125 | Viral BCL-2 homolog | (403) |
| Poxviridae | Shope fibroma virus | T2 orf | Viral TNFR homolog | (273) |
| Poxviridae | Smallpox | CrmE | Viral TNFR homolog | (271) |
| Poxviridae | VACV | NIL | Viral BCL-2 homolog | (404) |
| Retroviridae | HIV | Nef | Blocks the proapoptotic BCL-2 protein BAD | (258) |
| Retroviridae | HTLV-1 | TAX | Enhances expression of cFLIP and cIAP2 expression | (408) |
| Retroviridae | HTLV-1 | TAX | Enhances BCL-XL expression | (256) |
such strategy is to hamper the activation of death-inducing receptors. *Chlamydia* inhibits DR-mediated cell death by blocking the internalization of TNF-TNFR complexes (268), and *M. tuberculosis* promotes secretion of soluble TNFR2 to prevent TNF from binding to cellular receptors (269). The cowpox (270), smallpox (271), myxoma (272), Shope fibroma (273), and VACV (271) poxviruses and the herpesvirus HCMV (274) express viral TNFR orthologs that neutralize TNF-α, whereas adenoviruses promote the internalization and lysosomal degradation of Fas, TNFR, and TRAIL-R1 (275) and the herpesviruses HCMV and MCMV suppress the expression of Fas, TNFR, and TRAIL-R1 (275) and RIPK3 to block necroptosis (291). Human papillomavirus type 16 (HPV16) E6 binds to DEDs (280).

A frequently used approach to block the caspase-8 pathway of apoptosis is through inhibition of the proteolytic activity or the activation of caspase-8 at the DISC or in the ripoptosome. Serpins expressed by poxviruses, e.g., VACV B13R and Cowpox virus crmA, bind and block the catalytic sites of caspase-8 and caspase-1 (224, 281, 282), whereas the poxvirus Molluscum contagiosum virus (MCV) (283, 284) and several gamma-herpesviruses (284, 285) encode a viral ortholog of cFLIP (vFLIP) that blocks caspase-8 oligomerization and apoptosis. HCMV and MCMV express the viral inhibitor of caspase-8–induced apoptosis (vICA) (MCMV M36, HCMV UL36) (286), and HSV-1 and HSV-2 contain ICP-6 and ICP-10 (287), respectively, to bind and inhibit caspase-8. Human papillomavirus type 16 (HPV16) E6 binds to DEDs of caspase-8 and inhibits or activates caspase-8, depending on the E6 splice variant (288, 289). The TAX protein of HTLV-1 induces the expression of cIAP2 by enhancing NF-κB signaling (297), whereas Rhinovirus 3C cleaves RIPK1 (298) and HSV-1 TAX induces the expression of cIAP2 by enhancing NF-κB signaling (299) to dampen necroptotic cell death responses. Herpesviruses express functional RHOM domain-containing proteins to inhibit necroptosis. Alpha- and gamma-herpesviruses encode an enzymatically active large subunit (R1) of the ribonucleotide reductase (RNR) complex that functions in viral DNA synthesis, whereas beta-herpesviruses encode an enzymatically inactive R1 homolog. These R1 proteins contain RHIM domain-containing proteins that block signal transduction to RIPK3. Indeed, viral inhibitor of RIP activation (vIRA) proteins of MCMV (M45) can interact with TRIF, ZBP1, RIPK1, and RIPK3 (300, 301) to impair cell death signaling. Where MCMV expresses two proteins to inhibit caspase-8 and necroptosis, HSV-1 ICP-6 and HSV-2 ICP-10 can simultaneously inhibit both caspase-8 and the RIP kinases through direct interaction (302).

Although blocking the activity of caspase-8 is an efficient strategy to block the caspase-8 pathway of apoptosis, this also disrupts the ability of caspase-8 to inhibit RIPK1 and RIPK3, and consequently may result in the induction of necroptosis. The necroptotic pathway was therefore originally believed to have evolved as a “back-up” cell death mechanism for when the extrinsic apoptosis pathway is blocked by pathogenic caspase-8 inhibitors or vFLIPs. It is therefore interesting that the poxvirus crmA is more effective in inhibiting caspase-8 homodimer activity than caspase-8-cFLIP, heterodimer activity (294), allowing it to block caspase-8-mediated apoptosis without sensitizing for necroptosis (295).

Several pathogens express proteins that specifically target necroptosis by inhibiting RIPK1, RIPK3, MLKL, or effects downstream of MLKL (FIGURE 8; TABLES 3 and 4). The EPEC cysteine protease EspL degrades all cellular and viral RHIM domain-containing proteins (296), and *Shigella* employs its OspD3 protease to degrade RIPK1 and RIPK3 to block necroptosis (291). Human papillomaviruses downregulate the expression of ripk3 (297), whereas Rhinovirus 3C cleaves RIPK1 (298) and HTLV-1 TAX reduces BAX expression (256) without sensitizing for necroptosis (295).
Unlike its MCMV M45 counterpart, HCMV UL45 does not contain a RHIM domain and is not able to block RIPK3-mediated activation of MLKL. However, HCMV UL45 blocks necroptosis by acting downstream of MLKL activation, although it is currently unknown how this is mechanistically achieved (304).

Poxviruses have several ways to inhibit necroptosis, depending on the viral genus. Many orthopoxviruses, including Cowpox virus, express viral inducer of RIPK3 degradation (vIRD) proteins that bind to and promote K48-linked ubiquitylation of RIPK3, resulting in proteasomal degradation of RIPK3 and repression of necroptosis (305). Other poxviruses express viral MLKL-like proteins (vMLKL), which lack the NH2-terminal 4HB domain and the brace region but maintain the pseudokinase domain (306). vMLKL of BeAN 58058 poxvirus (BAV), originally isolated from an Oryzomys rodent (307), and mouse-infecting Cota poxvirus (COTV) block necroptosis, but not apoptosis, in human and mouse cells by sequestering RIPK3. Swine poxvirus (SPV) vMLKL, however, does not block necroptosis in human and mouse cells, indicating that vMLKLs have evolved to target necroptosis in specific hosts (306). Interestingly, avipoxviruses, which infect birds that do not harbor ripk3, express vMLKLs, whereas poxviruses that infect necroptosis-deficient mammals lacking RIPK3 or MLKL have lost the virulence factors that inhibit necroptosis (305, 306, 308), exemplifying how well poxviruses are adapted to their specific host. Such findings underscore the dynamic interactions between viral infection and the necroptosis machinery.

7.4. Is Necroptosis a Back-Up or Stand-Alone Cell Death Modality?

Studies using influenza A virus (IAV) suggest that necroptosis can be viewed as a stand-alone cell death mechanism to limit infections (207). IAV activates ZBP1-dependent RIPK3-mediated cell death, which can be necroptosis, apoptosis (207, 309–311), or pyroptosis (79, 312) and is dependent on the presence but not the kinase activity of RIPK3 (207, 309, 311). Interestingly, infected individual cells each seem to undergo only one type of cell death—that is, cells ultimately die by apoptosis or necroptosis. It is unknown how a cell mechanistically decides which type of cell death it will engage (207). Deletion of zbp1 or ripk3 renders cells resistant to IAV-induced death, and, as a result, zbp1- and ripk3-deficient mice cannot control viral replication and succumb to the infection (309–311), although this may depend on the viral dose (313). Abrogation of necroptosis by pharmacological inhibition of RIPK3 or mlkl deficiency does not block IAV-induced cell death, and mlkl-deficient mice control and survive IAV infection, since ZBP1 now engages apoptosis. Pharmacological or genetic inhibition of both caspase-8-mediated apoptosis and necroptosis renders cells unable to die upon IAV infection, resulting in apoptosis-necroptosis double-deficient mice that succumb to the infection (207, 309, 311). These data suggest that the apoptotic and necroptotic pathways can independently control IAV infection, indicating that necroptosis is a stand-alone cell death modality and should not be seen as “just” a back-up mechanism, engaged only when apoptosis fails.

8. EVOLUTIONARY ASPECTS OF REGULATED CELL DEATH

Host-pathogen interactions are among the major driving forces for organismal evolution. Hosts must adapt to survive infection with an invading pathogen, and, in turn, the pathogen must adapt to avoid destruction by the host’s immune defenses. The resulting arms race selects for increasingly complex defense mechanisms in the host, while the pathogen is selected to evolve equally complex evasion strategies. In evolutionary biology, this is known as the Red Queen effect (314), which states that an organism must continuously adapt and evolve to maintain its relative fitness among the systems with which it is coevolving.

In accordance with the Red Queen effect, pathogens that coevolved with vertebrates have gained a multitude of strategies to avoid detection or interfere with signaling leading to a cell’s demise, effectively rendering pathways of RCD incapable to control the infection. It therefore may seem curious that the components of regulated cell death remain present in the genomes of evolving vertebrates. It has been speculated that inflammasomes are not maintained for defense against vertebrate-adapted pathogens but to defend against ubiquitous opportunistic pathogens that reside in the environment (315, 316). Such microbes are not dependent on vertebrate hosts and therefore have not evolved strategies to inhibit pyroptotic cell death. Infection with these environmental pathogens could be harmful to a vertebrate host, but by retaining inflammasomes hosts can cope with ubiquitous opportunistic pathogens (315, 316). This hypothesis can be seen as a corollary to the Red Queen effect and was dubbed the Red Pawn effect (315). It should be noted that not all host-microbe interactions result in a Red Queen effect; some scenarios invoke a cooperative defense strategy that can result in commensalism (317, 318). Although much of our thinking, herein, relies on antagonistic coevolution as a driving force, it is likely that cooperative defense strategies and Red Pawn effects also played a major role in the evolution and maintenance of cell death mechanisms.
In light of the Red Queen effect, it is not surprising that the activation and execution mechanisms of RCD have evolved to be elaborate and tightly controlled. At first sight RCD may only seem advantageous to multicellular organisms. However, unicellular organisms also benefit from regulated cell death to control infection within a colony. Invading intracellular pathogens use the host’s resources to replicate and spread, often killing the host cell in the process. The individual cell is thus fated to die. By undergoing a more rapid death, the altruistic individual cell can limit the spread of the pathogen within its identical clone mates, effectively “saving” its genome. In a process called kin selection (319), colonies in which kin evolved to undergo rapid, regulated cell death upon pathogen recognition would thus be less susceptible to eradication by that pathogen. As such, RCD forms a first line of immune defense that protects the host organism and its kin from extinction. From this perspective, regulated cell death may have evolved as early immune defense mechanisms to allow organismal survival at the single cell level (33, 320).

Many studies have investigated the evolutionary origins of the components involved in regulated cell death signaling as present in humans today. The components of the regulated cell death signaling machinery have highly diversified over evolutionary time and continue to evolve, arguably as a consequence of the Red Queen effect as pathogens similarly evolve strategies to subvert the cell death pathways. Gene ontology studies have identified homologs of mammalian genes involved in regulated cell death in many animal phyla. However, it has to be noted that sequence similarity does not predict conserved functionality and cellular and biochemical approaches are required to understand whether conserved genes also have conserved functions. From this perspective, apoptosis and pyroptosis are functional in basal metazoans and therefore are ancient forms of regulated cell death, whereas necroptosis appears to be evolutionarily “younger,” arising with the vertebrates. Together, these studies have led to surprising insights into the origins, regulation, and conservation of the apoptotic, necrotic, and pyroptotic cell death pathways throughout the animal kingdom.

8.1. Pyroptosis in Animal Evolution

Pyroptosis in humans, as mediated by the inflammatory caspases-1, -4, and -5 and mainly executed by GSDMD, is a relatively recent evolutionary innovation, since gsdmd, like gsdmb and gsdmc, is only found in mammals (321). GSDMA homologs are present in many vertebrates, but no studies have been performed to confirm the functional conservation of gsdma in nonmammalian model systems. Pjvk and gsdme are evolutionarily ancient, and homologs have been identified in cnidaria, arthropods, and placozoans in addition to vertebrates (322). Phylogenetically, pjvk and gsdme from invertebrate species form distinct clusters. Moreover, both clusters are distinctly separated from clusters of vertebrate gsdme, gsdmb, and gsdmd. It is thus unclear whether all gasdermins are derived from gsdme or if the vertebrate GSDM family and invertebrate gsdme arose from a common (unidentified) ancestor. The possibility that the other gasdermins evolved from pejvakin seems unlikely because of a lack of sequence homology. Although the structure of GSDMA3 resembles structures formed by aerolysins (123), cytolytic pore-forming toxins secreted by the Gram-negative bacterium Aeromonas hydrophila, gasdermins and aerolysins are likely not related, since the proteins do not share any sequence homology (123).

Although the evolutionary relation between gsdme and the other gasdermins is unclear, GSDME-mediated cell death, and thus pyroptosis, is functional in Cnidaria. Cnidarian GSDME is able to mediate pyroptosis in coral species, including Orbicella faveolata (322). Similar to human caspase-3, coral caspase-3 is involved in apoptosis, and apoptotic and necrotic death have been observed to occur in parallel in corals (323). Coral GSDME can be cleaved and activated by both human and coral caspase-3 (322), indicating that the mechanisms of GSDME activation are evolutionarily conserved. In the coral P. damicornis, GSDME-mediated pyroptosis appears to control infection with the coral pathogen V. coralliilyticus. Infection with this pathogen induces caspase-dependent GSDME cleavage and tissue necrosis in coral polyps (322). Although further studies are required to elucidate the exact mechanisms of coral caspase-3-mediated coral GSDME activation, cnidarian and mammalian GSDME-mediated pyroptotic death are remarkably similar, suggesting that GSDME-mediated pyroptosis is an evolutionarily ancient form of regulated cell death.

Zebrafish (Danio rerio) contain all components of the pyroptotic machinery, including many NLRs, four inflammatory caspases, ASC, and three gasdermin genes (pjvk, gsdmea, and gsdmeb), and both the noncanonical and canonical inflammasomes are functional in these vertebrates (324). The inflammatory caspases caspy and caspy2 consist of an NH₂-terminal PYD followed by the catalytic domain, which is unlike mammalian inflammatory caspases that contain NH₂-terminal CARDs (FIGURE 1). The PYD of caspy2 is a receptor for cytotoxic LPS and activates the noncanonical inflammasome upon ligation, similar to human caspases-4 and -5 (325). The PYD of caspy, but not of caspy2, directly interacts with the zebrafish ortholog of ASC (43, 326). Similar to mammalian NLRP1 and NLRP3, zebrafish DrNLRP1 and DrNLRP3 induce the formation of a canonical, ASC-
dependent inflammasome leading to caspy and caspy2 recruitment and activation, zebrafish IL-1β processing, and pyroptosis (327, 328). DrNLRP3 can also recruit caspy2 without ASC and caspy, leading to caspy2 activity, but not autoproteolytic processing, which suffices to cleave GSDMEa/b and execute pyroptosis (328). Thus, zebrafish caspy and caspy2 are functional orthologs of human caspase-1 and caspase-5, respectively. Moreover, these studies in teleost fish signify that NLRP3 inflammasomes are conserved in vertebrates.

Although it remains unclear why the gasdermin family diversified only later in evolution, the functionality of GSDME-mediated pyroptosis in zebrafish and cnidaria indicates that pyroptosis can be seen as an ancient form of regulated cell death in response to infection. Furthermore, murine caspase-11 can be involved in canonical inflammasome-mediated pyroptosis, since, similar to the ability of caspy2 to be recruited to zebrafish NLRP3 inflammasomes, caspase-11 can be recruited to NLRP6-ASC inflammasomes (116). Thus, inflammatory caspasases that are involved in noncanonical inflammasome activation may have more elaborate functions that are yet to be fully understood.

Inflammasome-inducing sensors seem to continuously diversify. For instance, the ALR gene family, present in mammals, shows wide species diversity, and functional redundancy between family members can act as a compensatory mechanism when specific receptors are absent in a species (329). Similarly, NLRP1 molecules differ between mammalian species. The linker regions of primate NLRP1 proteins show high levels of sequence diversification, indicating that this region continues to evolve under positive selection (101, 330), likely increasing the chances for species-specific NLRP1 to detect pro-enzymes expressed by host-specific pathogens. Moreover, NLRP1 of primates and various other mammals contain an NH2-terminal PYD with currently unknown function, which has been lost in rodent NLRP1 (101). Pyrin is evolving under episodic positive selection in humans and primates, and the function of Pyrin seems to be modulating over evolutionary time (331). Interestingly, humans suffering from familial Mediterranean fever (FMF) contain an amino acid sequence similar to a domain sequence in Pyrins of nonhuman primates. These mutations lower the threshold for human Pyrin activation, although how this mechanistically works is not understood (332, 333). Since FMF has high carrier frequencies in people with origins associated with Mediterranean countries, the mutations are speculated to protect against endemic pathogens and as such became prevalent by selection in this population. It thus seems that the receptors that induce inflammasomes continue to evolve with harmful pathogens.

Not all mammalian species seem to benefit from all aspects of the pyroptotic pathway. Members of the order Carnivora have several alterations in inflammasome-related proteins. Felidae (cats and relatives) species do not encode NLRP1, whereas Canidae (dogs and relatives) and Mustelidae (weasels and relatives) lost NLR4 and NAIPs, and mustelids are additionally deficient for the dsRNA sensor NLRP9 (334). Cats, tigers, and dogs are also deficient for AIM2 and lack the GSDMB activation site, with cats and tigers additionally lacking the activation site of GSDME (335). All carnivores lost the individual genes for caspas1-4, -4, and -5. Instead, they encode a casp1/casp4 fusion gene that is made up of the CARD of the ancestral casp1 and the CARD and enzymatic domain of the ancestral casp4 gene. Alternative splicing provides two isoforms: casp1/4/a contains the casp1 CARD and the enzymatic domain, and casp1/4/b harbors both CARDs and the enzymatic domain (56). Although the enzymatic domains are homologs of human caspase-4, which is able to cleave GSDMD but not pro-IL-1β, both dog (Canis lupus familiaris) casp1/4a and -b isoforms can process GSDMD and pro-IL-1β upon multiple inflammasome stimuli (73). However, the casp1/4/b isoform is not able to sense cytosolic LPS and induce pyroptosis, indicating that canine casp1/4a and -b function like human caspase-1, although being homologs to human caspase-4 (73). Feline casp1/4/b is able to sense cytosolic LPS and induce subsequent pyroptosis and, unlike human caspase-4, can additionally process pro-IL-1β. Thus, pyroptosis functions in carnivores, although several inflammasome receptors are lacking. Carnivore species are known to carry many zoonotic pathogens (334, 336), which suggests that the lack of certain inflammasome-inducing receptors may contribute to the presence of these pathogens.

The best-known mammalian species to carry zoonotic pathogens are bats (336). Bats can tolerate high viral loads and live full life spans, asymptptomatically hosting viruses, including SARS-CoV-2, SARS-CoV-1, and Ebolavirus, that can be lethal to humans or other mammals. Bats have naturally dampened stress-related and virus-induced inflammatory responses, and it is therefore not surprising that bats evolved multiple ways to diminish inflammasome activation or the outcome thereof.

Bats lack the entire locus that harbors the AIM2-like receptor gene family, which includes the DNA sensors AIM2 and IFI16 (337), rendering bat cells incapable of cytosolic dsDNA-induced ASC-speck formation (338). Bats express four NLRP3 splice variants, including a splice variant that skips exon 7. This exon 7-deficient isoform is the most commonly expressed variant in Pteropus alecto tissues and contains an alteration in the LRR domain that results in a reduced ability of NLRP3 to induce ASC speck formation and IL-1β processing in response to ATP or the RNA viruses PRV3M, IAV, and MERS-CoV, known inducers of NLRP3 inflammasome activation. The
LRR domain alterations probably are conserved features in bats (339). Bats control the level of IL1β-associated inflammation. Caspase-1 of the bat species P. alecto and P. vampyrus contains two inactivating mutations that prevent substrate cleavage, presumably by weakening homodimerization. Two other bat species, E. spelaea and M. davidii, do not contain these mutations and, accordingly, retain caspase-1 activity. However, M. davidii harbors three mutations in IL-1β, resulting in defective IL-1β cleavage. Interestingly, IL-1β cleavage potential inversely correlates with caspase-1 activity in bats; bat species with high caspase-1 activity have low IL-1β cleavage potential and vice versa (338). Thus, bats harbor significant alterations in inflammasome-related proteins, thereby dampening the ability of inflammasomes to induce inflammation. Bats harbor a functional caspase-4 homolog, indicating that pyropoietosis in response to bacteria may be beneficial. It remains unclear, however, whether the loss of canonical inflammasomes evolved because of the selective pressure posed by the various residing viruses or other mechanisms that are unique to bats, such as their metabolically costly ability to fly or their relative longevity (338).

8.2. Apoptosis in Animal Evolution

The presence of the apoptotic machinery has been observed throughout the animal kingdom, and an increasing number of mechanistic studies, mainly by means of expression of apoptotic protein orthologs into mammalian cell culture systems, suggest that the apoptotic pathways are functionally present in the earliest metazoans. Bcl-2 proteins from the cnidarian species Acropora (coral) are able to inhibit cell death (340), and caspases and Bcl-2 family members from Hydra function similarly to their mammalian orthologs (42, 341). Platyhelminth (Planaria) Bak localizes to mitochondria and induces MOMP and cell death, and addition of cytochrome c to cytosolic extracts from Planaria and echinoderms (sea urchin and sand dollar) induces caspase activation (342). Functional TNF-induced apoptosis has been observed in the cnidarian Acropora (coral) (343) and seems to signal through FADD and caspase-8, since Acropora FADD and caspase-8 functionally interact (344). Moreover, all components of the caspase-8 and caspase-9 pathways of apoptosis are present and functional in zebrafish (345). Collectively, these studies suggest that the complex apoptotic machinery present in mammals is functionally conserved across many animal phyla.

However, not all animals execute apoptosis similarly. The apoptotic mechanisms in D. melanogaster (arthropod) and C. elegans (nematode) function differently than in other animals (346). The D. melanogaster APAF-1 homolog ARK is constitutively active (FIGURE 3B). Recruitment and activation of the initiator caspase DRONC (FIGURE 1) are controlled by DIAP1, a homolog of the mammalian IAP proteins (e.g., XIAP). Apoptotic signals regulate the expression of the DIAP1 inhibitors HID, Grim, and Reaper, which antagonize DIAP1, resulting in the release, recruitment to ARK, and activation of DRONC. DRONC then cleaves the executioner caspases DICE (FIGURE 1) and DCP-1, resulting in cellular demise (347).

Apoptosis in C. elegans is mediated by cell death defective (ced) genes ced-3, ced-4, and egg laying defective-1 (egl-1), whereas ced-9 inhibits death (348) (FIGURE 3C). CED-3 functions as the caspase (FIGURE 1) and is activated by the APAF-1 homolog CED-4. CED-9 is homologous to antiapoptotic Bcl-2 proteins and is anchored in the mitochondrial outer membrane, where it sequesters CED-4. Developmental signals induce the expression of the BH-3-only protein EGL-1, which disrupts the interaction between CED-4 and CED-9, permitting CED-4 to recruit CED-3 via CARD-CARD interactions, resulting in the direct demise of the cell. Thus, despite the similarities to the caspase-9 pathway in other phyla, including those basal to the arthropods and nematodes, the molecular mechanisms of caspase activation have diverged. MOMP and cytochrome c-induced activation of the APAF-1 homolog do not occur in these animals (342), and the ability of the BCL-2 homolog CED-9 to directly bind and suppress the APAF-1 homolog CED-4 is not observed in Bcl-2 family proteins from other phyla (including arthropods).

8.3. Necroptosis in Animal Evolution

Although components of the TNF signaling pathway are present in the most basal animals, the TNF-induced necroptotic pathway seems to have arisen only later in animal evolution. With the exception of RIPK1-like molecules, RHIM domain-containing proteins (RIPK3, TRIF, and ZBP1) are not found in nonvertebrates (349), and although RIPK1-like proteins are present throughout the Deuterostomia they are likely involved in mediating gene expression, not cell death. The necroptotic pathway, as defined by RIPK3-mediated activation of MLKL, therefore likely arose with the vertebrates (349). MLKL homologs are also found throughout the Deuterostomia, but since RIPK3 is the only known activator of MLKL, it seems unlikely that necroptosis is a cell death modality in invertebrate species (349). This view has been debated, however, based on homology between an MLKL domain and a domain of the fungal protein HELLP that acts similarly to HET-s to mediate cell death (350, 351). This speculative suggests that MLKL activation may occur in the absence of RIPK3 and implies that necroptosis should be defined.
as death induced upon domain-dependent activation of MLKL.

The possibility that MLKL may be activated independently of RIPK3 is supported by the finding that all sequenced avipoxviruses contain a viral homolog of MLKL (vMLKL), suggesting that inhibition of MLKL is a necessary step for these viruses to establish an infection. The fact that avipoxviruses infect birds, which lack the gene for ripk3 but encode mkl1, indicates that avian MLKL may be activated by one or more unidentified kinases (306). This suggests that MLKL of other vertebrate species, including human MLKL, may also be activated by such kinases. Although human and mouse MLKL can be directly phosphorylated by TAM kinases, this requires prior MLKL phosphorylation by RIPK3 (352), and as such TAM kinases are not RIPK3-independent MLKL activators. Thus, MLKL may be activated by RIPK3-independent mechanisms in some species, but whether human MLKL can be activated independently of RIPK3 remains to be determined. Identifying such a mechanism may have implications for the treatment of cancers, since many cancer types downregulate RIPK3 expression and protein levels and as such may overcome control by necroptosis (353, 354).

Although necroptosis likely arose with the vertebrates, several species have lost components of the necroptotic machinery. All species of the class Aves (birds) lack ripk3 but encode mkl1, whereas Marsupialia lack both genes. Although the evolutionary reasons for this are not understood, this suggested that MLKL functions independently of RIPK3, perhaps to promote necroptosis (349). ZBP1 is also lacking in birds and marsupials, suggesting that ZBP1- and RIPK3-induced necroptosis are evolutionarily linked (349). TRIF is present in these species, indicating that TLR-induced, TRIF-mediated activation of gene expression programs is required for organismal survival, whereas ZBP1-induced gene expression may be controlled by other nucleic acid sensors and can therefore be seen as redundant (349). Several other mammalian species lack mkl1 or harbor disrupting mutations in ripk3 and/or mkl1. Members of the order Carnivora, which also carry alterations in inflammasome-related genes (see above), lack the gene for mkl1. It has been speculated that their raw meat diet, comprising an abundant presence of microbes and viruses, would cause exuberant inflammation and excessive damage in the digestive tract of the foraging carnivore, which could have driven evolution to counterselect for the highly inflammatory necroptotic (and pyroptotic) pathways (349). A similar reasoning may explain why cetaceans (whales, orcas, dolphins, and narwhals), who are also strictly carnivorous, harbor mutations in mkl1 and ripk3 that introduce frameshifts or premature stop codons in these genes (308). However, several herbivorous species also lost necroptosis. Lagomorpha (such as hares, rabbits, and pikas) lack mkl1 and harbor a mutation in ripk3 that introduces a premature stop codon (308). An exception is Ochotona curzoniae (the plateau pika), which does harbor mkl1 and functional ripk3 genes but lacks ripk1 (308). A small number of species from afrotheria (Trichechus manatus) and rodentia (Heteroccephalus glaber, Fukomys damarensis, and Octodon degus) contain premature stop codons in ripk3 and mkl1, and Loxodonta africana (the African bush elephant) is deficient in mkl1 (308). Why these herbivorous species lack a necroptotic pathway is currently unclear, but the convergent loss of necroptosis in different species demonstrates that necroptosis is not necessary for organismal development and survival. This raises the question of why necroptosis evolved and is retained in other species. It may be that Red Pawn effects are responsible for the evolution and retention of necroptosis, although to date no opportunistic pathogens have been identified in animals in which necroptosis has been experimentally ablated. The reasons for the evolution and retention of necroptosis thus remain enigmatic.

9. REGULATED CELL DEATH AND CORONAVIRUSES

The causative agent for the current coronavirus disease-2019 (COVID-19) pandemic is the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Like SARS-CoV-1 (here SARS-1) and Middle East respiratory syndrome coronavirus (MERS-CoV), SARS-CoV-2 is a member of the Betacoronavirus genus within the Coronaviridae family. Infection with betacoronaviruses may remain asymptomatic in early stages but may progress to severe disease that encompasses inflammation, organ damage, and sometimes death of the infected individuals. Disease severity of COVID-19 has been associated with inflammasome activation (355), which occurs in various tissues including lung epithelium and circulating monocytes (355–357), and the ability of the lung epithelium to undergo apoptosis (358).

Studies investigating SARS-CoV-2-mediated inflammasome activation are currently ongoing, and our current knowledge of how betacoronaviruses activate inflammasomes mainly comes from studies on SARS-1. SARS-1 seems to actively engage inflammasomes and has several strategies to do so. The envelope (E) protein of SARS-1 forms Ca$^{2+}$-permeable ion channels in the endoplasmic reticulum (ER) Golgi apparatus intermediate compartment (ERGIC) and Golgi membranes, which activates the NLRP3 inflammasome (359). SARS-1 Orf8b activates the NLRP3 inflammasome by directly interacting with the LRR domain of NLRP3 (360). Moreover, Orf8b proteins can form insoluble intracellular aggregates that
induce ER stress and lysosomal damage that causes cellular demise (360). The viral Orf3a protein of SARS-1 is a Viroporin that forms potassium-sensitive ion channels upon oligomerization (361). Orf3a oligomerization and subsequent cell death depend on its interaction with RIPK3, which it binds through the NH2-terminal kinase domain of RIPK3, although the detailed mechanism of RIPK3-Orf3a pore formation remains unclear (362). The kinase activity of RIPK3 is not required for Orf3a oligomerization and subsequent cell death, and the latter is independent of MLKL or the activity of caspase-8, indicating that cell death is not apoptotic or necroptotic (362).

Instead, the Orf3a ion channel seems to activate the NLRP3-inflammasome by facilitating K⁺ efflux (362, 363). Orf3a-mediated NLRP3 inflammasome activation also depends on the production of mitochondrial reactive oxygen species (ROS) (363), and, independent of its ion channel activity, Orf3a directly binds to TRAFs and ASC, which mediates the ubiquitylation of ASC by TRAF3 (364).

Most of these studies focus on loss of plasma membrane integrity and IL-1β release and do not analyze cleavage of caspase-1 and/or gasdermin D. Although most studies conclude that inflammasomes mediate IL-1β release through pyroptosis, SARS-CoV-2 was suggested to activate caspase-8 to mediate IL-1β cleavage and necroptosis to facilitate IL-1β release (365). However, inflammasome activation does not necessarily lead to pyroptosis (defined by gasdermin-mediated membrane disruption), and other, currently unknown, mechanisms may lead to the release of IL-1β. For instance, ssRNA sequences of SARS-CoV-2 and SARS-1 can activate the NLRP3 inflammasome in macrophages, leading to IL-1β maturation and release while GSDMD remains uncleaved (366). IL-1β is apparently released through a noncanonical pathway that involves caspase-8, caspase-1, the NLRP3 inflammasome, potassium efflux, and autophagy (366). Although the complete sequence of events remains to be determined, the process is suggested to be activated by binding of coronavirus GU-rich ssRNA to TLR8 (366). However, since TLR8-MyD88-mediated activation of a FADD-caspase-8-RIPK1-RIPK3 complex has not been demonstrated to date, we speculate that the GU-rich ssRNA from SARS-CoV-2 and SARS-1 (and SARS-CoV) may activate ZBP1 to induce the NLRP3 inflammasome upon gaining access to the cytosol.

Betacoronaviruses not only induce inflammasome activation but also actively induce apoptosis. MERS-CoV M protein induces PERK-mediated apoptosis to augment viral titers, which contributes to disease severity and lethality (367). SARS-1 proteins Orf3b, Orf6, and Orf7a induce caspase-3-mediated apoptosis (368), whereas SARS-CoV-2 induces apoptosis by an ER stress response (358). Orf3a of SARS-CoV-2 and SARS-1 mediate activation of caspase-8, leading to apoptosis via tB499, caspase-9, and caspase-3 (369), although the exact mechanisms underlying apoptosis induction remain elusive, since, given the effects of Orf3a on inflammasome activation, it is unclear whether both the apoptotic and inflammasome pathways are activated by Orf3a or if apoptosis is an effect of activated inflammasomes.

Our current knowledge of coronaviral strategies to block the pathways of regulated cell death is limited. It seems that SARS-CoV-2 NSP1 and NSP13 are able to block the activation of caspase-1 and the subsequent release of cleaved IL-1β (370), but how this mechanistically occurs is not understood. SARS-1 Orf3a reduces phosphorylation of RIPK3 and MLKL, indicating that it may suppress necroptosis (362), but whether SARS-CoV-2 suppresses necroptosis remains to be determined. The extent of coronaviral blockage of regulated cell death currently is unclear, but we postulate that these potential mechanisms may have implications for disease severity.

The active engagement of inflammasomes and apoptosis by highly pathogenic betacoronaviruses suggests that betacoronavirus infections may be therapeutically controlled by targeting apoptosis and/or inflammasome activation (371). The disease severity and lethality of MERS-CoV infection can be controlled by inhibiting intrinsic apoptosis by PERK and caspase-3 and -7 inhibitors in murine infection models (367). The infection outcome of SARS-CoV-2 and SARS-1 was also improved by an inhibitor of caspases-3 and -7 in these murine models (367), but more studies are needed to confirm that apoptosis inhibition is a potent strategy for the treatment of COVID-19. Interestingly, lung cells in young individuals are more susceptible to apoptosis than cells of older individuals. Older individuals are at higher risk for developing severe COVID-19, possibly suggesting that the propensity for cells to undergo apoptosis helps to control the infection (358). These observations also indicate that the age of a patient should be considered when potential therapeutic strategies to combat COVID-19 and other diseases are developed.

The pronounced activation of cell death pathways during betacoronavirus infections seems paradoxical since these viruses risk losing their replicative niche by inducing rapid cellular demise. However, humans are not the natural reservoir for coronaviruses, and the current COVID-19 as well as the SARS-1 and MERS pandemics stem from zoonotic infections that likely originated from bats (372, 373). Bats are natural reservoirs for SARS-like coronaviruses (373) and are speculated to be the ancestral hosts for coronaviruses (372). The reductive inflammasome functioning in bats (see above and
Ref. 374) leads to an overall reduction in inflammation activation and an increase in tolerance of resident microbes, allowing bats to live healthy lives while hosting pathogens that are lethal to other species. How bats do this is unclear but of great interest (374). Bats have an intact necroptosis pathway, and that may be sufficient to control pathogenic infections, although these for now are speculations. Fully suppressing inflammatory responses in humans may lead to significantly increased pathogenic titers that may be detrimental for the patient. Learning from bats (374) as to how to fine-tune immune tolerance to keep infections in check will help us find therapeutic strategies to overcome the current COVID-19 pandemic and may be instrumental in defining approaches for the treatment of inflammatory diseases, autoimmune diseases, neuropathies, cancers, and other zoonotic diseases that are likely to arise in the future.

10. FUTURE PERSPECTIVES

Over evolutionary time, the human pathways of regulated cell death have become intricate and complex. But how effective are cell death strategies in combatting infections, and how well do pathogens evade these mechanisms? The coevolution of pathogens and their hosts provides clues, as does the existence of “Red Pawn” microbes that only manifest pathogenicity in the absence of cell death pathways (see above and Ref. 315). However, understanding the clinical significance of cell death and its evasion by pathogens will require new probes and markers for the activation of cell death pathways and the interrogation of clinical samples in the setting of infection. At present, such analyses are in their infancy. Unlike many biological events, cell death pathways cannot be elucidated from gene expression data, as our discussion of these pathways, their engagement, and their mechanisms of action makes clear. Caution should be exercised in evaluating any gene expression studies that purport to elucidate cell death mechanisms on this basis.

There is much to be learned about the extent to which the pathways of RCD interact and how these interactions affect organismal health in contexts of homeostasis and infection. This interplay is not only important for the control of infectious diseases but also affects immune homeostasis (190, 194, 195) and organismal development (196, 197). These studies show that deregulation of the genes involved in RCD may lead to severe inflammation that may prove lethal to an organism. Determining whether inflammation happens under sterile conditions or is induced by a resident microbiome (195) is of interest, and molecular and genetic studies aiming to understand how the RCD pathways interact are ongoing. Whether the effects of pathogenic virulence factors that interfere with the death-mediating functions of RCD proteins may also directly contribute to inflammation that is associated with a given pathogenic infection is an important area of ongoing study.

The recent realization that the pathways of regulated cell death extensively interact indicates that we are only at the beginning of fully understanding how infections are controlled by regulated cell death. We now know that the interplay between the RCD pathways may have important implications for the outcome of infections, exemplified by the recent observation that plasticity between the caspase-8 pathway of apoptosis and necroptosis is required for the control of influenza A virus infections (207). This is particularly relevant in settings of coinfections, where an established microbe may block certain pathways of RCD, potentially adding to the pathogenicity of a coinfecting pathogen. This illustrates the need for a deeper understanding of the interplay between pathogens and cell death, the specific signaling pathways that trigger one type of cell death over another, and whether they manifest as a benefit to the host or to the pathogen. This understanding will be vital for the development of therapeutics aimed to control infections and/or immunological diseases that are affected by RCD.

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**AUTHOR CONTRIBUTIONS**

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