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Review

Cell death

Kim Newton,[1,*] Andreas Strasser,[2,3,*] Nobuhiko Kayagaki,[1,*] and Vishva M. Dixit[1,*]
[1]Physiological Chemistry Department, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA
[2]WEHI: Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia
[3]Department of Medical Biology, The University of Melbourne, Melbourne, VIC 3010, Australia
*Correspondence: knewton@gene.com (K.N.), strasser@wehi.edu.au (A.S.), kayagaki@gene.com (N.K.), dixit@gene.com (V.M.D.)
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SUMMARY

Cell death supports morphogenesis during development and homeostasis after birth by removing damaged or obsolete cells. It also curtails the spread of pathogens by eliminating infected cells. Cell death can be induced by the genetically programmed suicide mechanisms of apoptosis, necroptosis, and pyroptosis, or it can be a consequence of dysregulated metabolism, as in ferroptosis. Here, we review the signaling mechanisms underlying each cell-death pathway, discuss how impaired or excessive activation of the distinct cell-death processes can promote disease, and highlight existing and potential therapies for redressing imbalances in cell death in cancer and other diseases.

INTRODUCTION

Cell death can be non-lytic and largely immunologically silent (apoptosis) or lytic and pro-inflammatory (necrosis). Genetically programmed cell death is recognized as an important pillar of homeostasis in multicellular organisms, but even unicellular organisms can use cell death to fend off pathogens or to limit colony size for adaptation to nutrient deprivation.[1] The non-lytic cell-death program of apoptosis was first defined on the basis of its morphological features,[2] which include cytoplasmic vacuolation, nuclear condensation, and plasma membrane blebbing. The underlying molecular mechanisms emerged later after advances in three disparate fields. First came the realization that certain cells in the genetically tractable nematode *Caenorhabditis elegans* reproducibly undergo apoptosis.[3] Subsequent genetic screens identified both mediators of cell death (*egl-1*, *ced-3*, and *ced-4*) and an inhibitor of cell death (*ced-9*), although at that time they could not be ascribed recognizable biochemical activities.[4,5] Insight on *ced-3* function came from the identification of the mammalian protease responsible for maturation of pro-inflammatory interleukin-1β (IL-1β).[6,7] Homology between CED-3 and this IL-1β converting enzyme, now called caspase-1, prompted the realization that CED-3 is a protease[8] and that programmed cell death in *C. elegans* is driven by proteolysis.

The *ced-9* gene had a functional counterpart in mammalian B cell lymphoma gene 2 (*BCL-2*), a proto-oncogene activated by chromosomal translocation in human follicular lymphoma.[9] Rather than inducing proliferation like many other oncogenes, *BCL-2* inhibited cell death[10] and could functionally substitute for *ced-9* in the worm.[5,11] Following these pivotal discoveries, additional mammalian caspases (cysteine-dependent aspartate-directed proteases) were identified. Moreover, *BCL-2* turned out to be part of a larger family of adjudicators controlling activation of caspases in what is referred to as the intrinsic apoptosis pathway. Further complexity emerged in the form of an extrinsic apoptosis pathway and the commandeering of death pathway components by pathogens. Studies of infected cells showed that certain forms of necrosis, where cells burst and release pro-inflammatory contents, were also genetically programmed. These cell-death pathways are referred to as pyroptosis[12] and necroptosis.[13] Having many ways to die is an effective host strategy against pathogens seeking to preserve their replicative niche.

It is estimated that a staggering 10^{11} cells undergo programmed cell death each day in an adult human, which is equivalent to our entire body weight in the course of a year.[14] Cell proliferation maintains the *status quo* as the body rids itself of old, dysfunctional, infected, or mutated cells. Therefore, it makes intuitive sense that disrupting the homeostatic balance between cell proliferation and cell death will result in organismal dysfunction. Indeed, too little cell death contributes to diseases of excess proliferation, such as cancer, and too much cell death to degenerative disorders, like neurodegenerative diseases.[15] Here, we review our present understanding of the various pathways to cell death. The literature is extensive and, at times, confusing and even contradictory. As such, at the risk of missing certain advances, we have chosen to emphasize concepts that have been pressure-tested through a combination of biochemistry and genetics and are therefore likely to stand the test of time.

INTRINSIC APOPTOSIS—FROM WORMS TO MAMMALS

Many aspects of intrinsic apoptosis signaling in *C. elegans* are conserved in mammals, but there is greater complexity in the mammalian cell death pathway (Figure 1). Similarities include the use of a scaffolding protein to co-ordinate caspase activation. CED-4 serves this function for *C. elegans* caspase CED-3,[16] whereas apoptotic peptidase activating factor 1

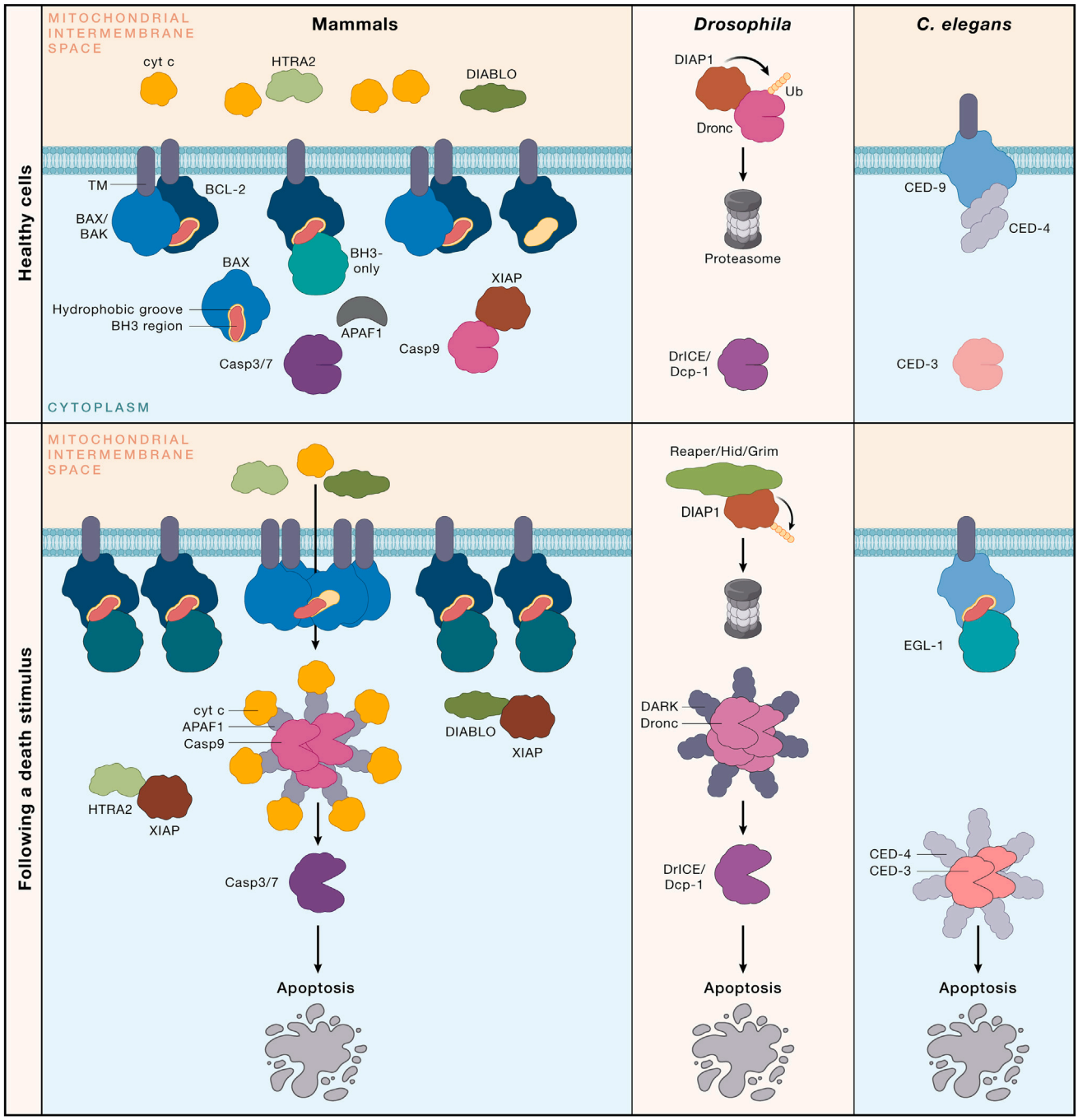


Figure 1. The intrinsic apoptosis signaling pathway in mammals compared with apoptosis signaling in *C. elegans* and *Drosophila*

Pro-survival BCL-2 family proteins restrain BAX and BAK in healthy mammalian cells. Following an apoptotic stimulus, upregulated BH3-only proteins bind to the pro-survival BCL-2 proteins, liberating BAX and BAK from the restraint of the pro-survival BCL-2 proteins. BAX and BAK then form oligomers that cause mitochondrial outer membrane permeabilization (MOMP). Cytochrome *c* released into the cytoplasm binds to APAF-1, triggering assembly of the apoptosome scaffold that activates the initiator caspase, caspase-9. Caspase-3 and -7, which are proteolytically activated by caspase-9, execute the cleavage events that dismantle the apoptotic cell. XIAP, an inhibitor of caspase-3, -7, and -9, is neutralized by DIABLO or HTRA2, which like cytochrome *c*, are released from permeabilized mitochondria. In *Drosophila*, the ubiquitin ligase death-associated inhibitor of apoptosis 1 (DIAP1) suppresses apoptosis by targeting the initiator caspase Dronc for proteasomal degradation. Apoptotic stimuli upregulate Reaper, Hid, and Grim, which bind to DIAP1 and liberate Dronc for activation by the APAF-1 homolog death-associated APAF-1-related killer (DARK). Active Dronc then cleaves and activates the executioner caspases death-related ICE-like caspase (DrICE) and death caspase-1 (Dcp-1). In *C. elegans*, live cells use the BCL-2 homolog CED-9 to restrain the APAF-1 homolog CED-4. Apoptotic stimuli upregulate the BH3-only protein EGL-1, which binds to CED-9 and thereby frees CED-4 to activate CED-3.

(APAF-1) mediates oligomerization of mammalian caspase-9.[17] This process is unleashed when BCL-2 homology region 3 (BH3)-only proteins (EGL-1 in *C. elegans*[18]; BCL-2 associated agonist of cell death [BAD], BH3-interacting domain death agonist [BID], BCL-2 interacting killer [BIK], BCL-2 interacting mediator of cell death [BIM], BCL-2 modifying factor [BMF], harakiri [HRK], NOXA [also called phorbol-12-myristate-13-acetate induced protein 1], and p53 upregulated modulator of apoptosis [PUMA] in mammals[19]) bind to and thereby inhibit pro-survival members of the BCL-2 protein family (CED-9 in *C. elegans*[5]; BCL-2, BCL-XL, myeloid cell leukemia gene 1 [MCL-1], BCL-W, and BCL-2 related gene expressed in fetal liver [BFL-1; called A1 in mice] in mammals[19]). Expression of these BH3-only proteins is increased in response to developmental cues or stress stimuli (for example, nutrient deprivation, DNA damage, and endoplasmic reticulum [ER] stress) through diverse transcriptional and post-translational mechanisms.[19]

In *C. elegans*, CED-9 keeps cells alive by preventing the adaptor CED-4 from activating the caspase CED-3.[16] By contrast, mammalian pro-survival BCL-2 family proteins prevent caspase activation by restraining their pro-apoptotic relatives, BCL-2 associated X (BAX) and BCL-2 antagonist/killer 1 (BAK). Unchecked BAX and/or BAK oligomerize and cause mitochondrial outer membrane permeabilization (MOMP), thereby releasing mitochondrial factors into the cytoplasm that promote apoptosis.[20] Cytochrome *c* released into the cytoplasm interacts with the adaptor APAF-1, promoting assembly of the apoptosome complex that facilitates dimerization and activation of the initiator caspase, caspase-9.[17] The mitochondrial release of DIABLO (also called second mitochondria-derived activator of caspase [SMAC]) or HtrA serine peptidase 2 (HTRA2) prevents caspase inhibition by x-linked inhibitor of apoptosis protein (XIAP).[21] Although *C. elegans* does not have a BAK- or BAX-like protein, other worms do.[22] Therefore, *C. elegans* may be an oddity of evolution that dispensed with mitochondrial regulation of apoptosis because CED-9 acquired the ability to curtail caspase activation in a more direct manner through binding to CED-4. Alternatively, CED-9 bound by the BH3-only protein EGL-1 may function like BAX or BAK. There is evidence for CED-9 associating with the mitochondrial outer membrane,[23] but the subcellular localization of EGL-1 is less clear.

Mammalian caspase-9 proteolytically activates the so-called executioner caspases (caspase-3, -7, and possibly -6), resulting in the cleavage of hundreds of cellular proteins.[24] Some of these cleavage events help dismantle the cell, while others activate processes that promote phagocytic engulfment of apoptotic cells and their membrane-enveloped fragments (called apoptotic bodies). An example of the former is caspase-3 or -7 cleavage of inhibitor of caspase-activated deoxyribonuclease (ICAD), which releases caspase-activated deoxyribonuclease (CAD) to mediate inter-nucleosomal cleavage of chromosomal DNA.[25] An example of the latter is proteolytic activation of the scramblase XK related 8 (XKR8), which causes phosphatidylserine to be displayed on the cell surface as an "eat me" signal to phagocytes.[26]

Phagocytosis is important for the non-inflammatory nature of apoptosis. If apoptotic cells fail to be "eaten," they can eventually develop membrane damage.[2] The release of intracellular damage-associated molecular patterns (DAMPs) sounds the "alarm" to neighboring cells and triggers a pro-inflammatory response. DAMPs include DNA, ATP, and proteins such as high mobility group box 1 (HMGB1) and IL-1α. The processes regulating the phagocytosis of apoptotic cells are conserved through evolution,[26] underscoring their importance for normal development and adult tissue homeostasis.

The fruit fly *Drosophila melanogaster* is another model organism that has been used to study programmed cell death. Debcl, Buffy, and Sayonara in *Drosophila* belong to the BCL-2 protein family, but they have a more limited role in programmed cell death than their mammalian and *C. elegans* counterparts.[27,28] *Drosophila* caspases are largely held in check by inhibitor of apoptosis proteins (IAPs) (Figure 1). Programmed cell death in *Drosophila* is triggered by the induced expression of the IAP inhibitors, Reaper, Hid, and Grim.[27] Mammalian IAPs also play important roles in restraining cell death, but XIAP is the only mammalian IAP to target caspases directly, inhibiting caspase-3, -7, and -9.[21]

THE BCL-2 PROTEIN FAMILY

Members of the BCL-2 family can be divided into three subgroups: the pro-apoptotic BH3-only proteins, the pro-survival proteins, and the pro-apoptotic effector proteins. The mammalian pro-survival proteins have four BH regions, a C-terminal transmembrane (TM) domain, and a hydrophobic surface groove that mediates interactions with the BH3 domain of the two pro-apoptotic sub-groups of the BCL-2 family.[19] The effectors, BAX, BAK, and BCL-2-related ovarian killer (BOK), have a very similar structure to the pro-survival proteins.[20] They too have four BH regions (the BH4 domain defined by a conserved sequence motif[29]), a TM domain, and a BH3-binding surface groove. By contrast, many of the BH3-only proteins are unstructured unless bound to a pro-survival family member.[19]

PRO-APOPTOTIC EFFECTORS BAX, BAK, AND BOK

Most healthy cells express detectable amounts of BAX and BAK, although some cells express one much more than the other. Mice lacking BAX exhibit male sterility and minor splenomegaly,[30] whereas BAK-deficient mice are largely normal.[31] However, the combined loss of BAX and BAK typically produces severe craniofacial abnormalities that are lethal at birth.[31,32] This phenotype is exacerbated in mice that also lack BOK.[32] BOK differs from BAX and BAK in that it does not appear to be inhibited by pro-survival BCL-2 proteins.[33] The abundance and activity of BOK are instead suppressed by gp78, a ubiquitin ligase that resides on the ER and targets BOK for proteasomal degradation.[33] Cells from $Bax^{-/-}$ $Bak^{-/-}$ or $Bax^{-/-}$ $Bak^{-/-}$ $Bok^{-/-}$ mice are profoundly resistant to all intrinsic apoptosis stimuli tested,[31,32] demonstrating that BAX and BAK have largely overlapping functions as essential effectors of apoptosis, whereas BOK has an ancillary role.

Despite their extensive functional overlap, there are notable differences between BAX and BAK. Although both interact with pro-survival BCL-2 proteins on the outer mitochondrial membrane, most BAX in healthy cells is cytoplasmic with its TM

domain embedded within its hydrophobic groove.[20] How the TM domain is displaced to allow BAX activation is not well understood. Another difference between BAX and BAK is that BCL-2 is thought to mainly restrain BAX, with MCL-1 inhibiting BAK, whereas BCL-XL can effectively inhibit both proteins.[34] Curiously, voltage-dependent anion channel 2 (VDAC2) allows BAX to localize to the outer mitochondrial membrane to kill cells but, conversely, can inhibit activation of BAK.[20] Despite many years of intense study, the structures of the BAX or BAK pores that mediate MOMP remain elusive.

BH3-ONLY PROTEINS

BIM, PUMA, and proteolytically cleaved BID (termed truncated BID or tBID) bind with high affinity to all pro-survival BCL-2 proteins and therefore are potent initiators of apoptosis.[19] The other BH3-only proteins bind in a more selective manner. NOXA only binds to MCL-1 and A1/BFL-1, whereas BAD, BIK, and HRK mainly bind to BCL-XL and, to a lesser extent, BCL-2 and BCL-W. Therefore, these BH3-only proteins tend to be less potent killers because they only inhibit some of the pro-survival BCL-2 proteins safeguarding a cell. Certain BH3-only proteins, particularly BIM, PUMA, and tBID, may also trigger apoptosis by interacting directly with BAX and BAK.[19,35] However, analyses of cell lines lacking all BH3-only proteins indicate that these interactions are dispensable for initiating apoptosis. The cells still undergo BAX- or BAK-mediated apoptosis when the pro-survival BCL-2 proteins safeguarding cells are removed genetically or inhibited with small-molecule BH3 mimetics.[36] Collectively, these findings are consistent with a model in which BAX and BAK need to be restrained by pro-survival BCL-2 proteins to maintain cell viability. Apoptosis is initiated when the pro-survival BCL-2 proteins are neutralized by BH3-only proteins or proteasomal degradation (described below).

The BH3-only proteins that initiate apoptosis depend on the stress stimulus and cell type. BIM plays a major role in immunological tolerance by deleting self-reactive B and T cells.[19] It also aids immune cell homeostasis by removing B and T cells that are no longer needed after an immune response.[37,38] BIM and PUMA contribute to apoptosis triggered by growth factor deprivation, deregulated calcium flux, glucocorticoids, or ER stress. The transcription factor and tumor suppressor p53 (also called transformation related protein 53 [TRP53] in mice and tumor protein p53 [TP53] in humans) promotes apoptosis by inducing expression of *Puma* and *Noxa*.[19] BID is activated upon proteolytic cleavage by either caspase-8 in the extrinsic apoptosis pathway (described below)[39] or granzyme B, the latter entering the cell through perforin pores after its release from cytotoxic T cells or natural killer cells.[40] Analyses of gene-targeted mice suggest that BMF, BAD, BIK, and HRK have less prominent roles in the initiation of apoptosis.[19]

PRO-SURVIVAL BCL-2 PROTEINS

All pro-survival BCL-2 proteins localize to the outer aspect of the mitochondrial outer membrane, but BCL-2 is also found on the ER and the nuclear envelope. A substantial portion of BCL-XL is cytoplasmic. BCL-2, BCL-XL, and BCL-W are relatively stable, taking many hours to turn over, whereas MCL-1 and A1/BFL-1 are labile proteins, their half-lives measured in minutes rather than hours because they are ubiquitinated and targeted for proteasomal degradation.[41] Consequently, when RNA or protein synthesis cease, levels of MCL-1 and A1/BFL-1 drop precipitously, and cells that are dependent on them will die. Many viruses also make pro-survival BCL-2 homologs or structural mimics,[42] highlighting the important role that intrinsic apoptosis plays in eliminating virus-infected cells.

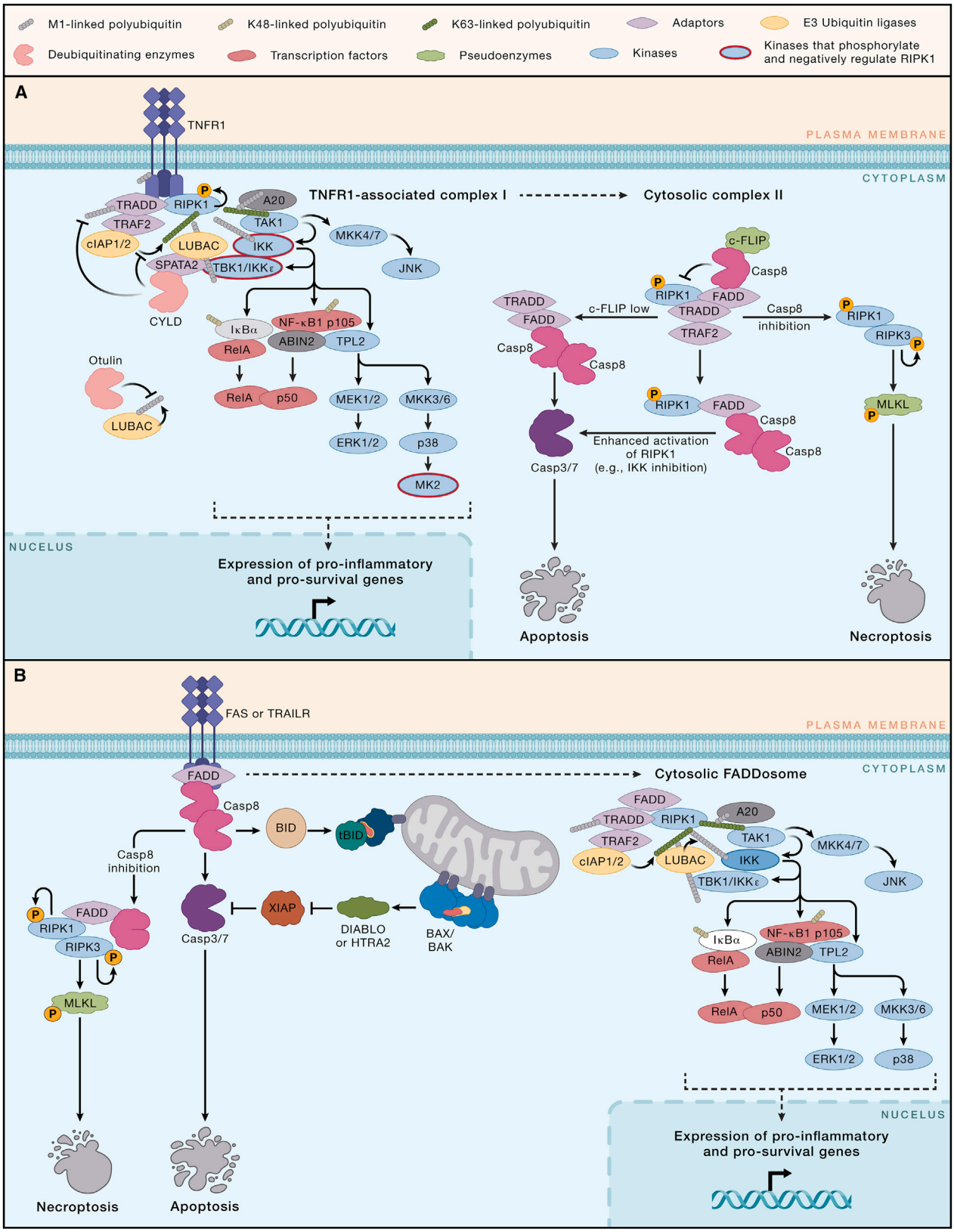
Genetic studies in mice have identified critical functions of the different pro-survival BCL-2 proteins.[41] Loss of A1 causes only minor defects in select hematopoietic cell subsets. BCL-W-deficient mice are also mostly normal, with the exception of male sterility. In mice lacking BCL-2, excessive BIM-driven apoptosis reduces mature B and T lymphocyte numbers and causes premature graying as well as fatal polycystic kidney disease. BCL-XL deficiency is lethal around embryonic day 13 of mouse development because this compromises the survival of platelets as well as certain neuronal and erythroid cell populations. MCL-1 loss has the most notable impact, being essential for the survival of pre-implantation embryos and a broad range of cell types, including hematopoietic populations, cardiomyocytes, and intestinal epithelial cells.[41]

In certain cell types, two pro-survival BCL-2 proteins must be eliminated to observe severe defects. For example, BCL-XL or MCL-1 can sustain hepatocytes[43] and certain neuronal populations.[44] Levels of the pro-survival BCL-2 proteins and BH3-only proteins are exquisitely balanced because halving the gene dosage can have dramatic consequences. For example, *Mcl-1*$^{+/-}$ *Bcl-x*$^{+/-}$ mice die soon after birth with severe craniofacial abnormalities, but *Mcl-1*$^{+/-}$ *Bcl-x*$^{+/-}$ *Bim*$^{+/-}$ mice are healthy.[45]

DISEASES CAUSED BY DEFECTIVE INTRINSIC APOPTOSIS

As mentioned above, enforced expression of *BCL-2* suppresses intrinsic apoptosis and can cause human follicular lymphoma.[9] In mice, transgenic over-expression of *BCL-2* in lymphocytes causes a fatal systemic lupus erythematosus (SLE)-like autoimmune disease and a low incidence of lymphoma.[46] These findings highlight the importance of intrinsic apoptosis in safeguarding immunological tolerance as well as tumorigenesis. Of note, *BCL-2* is a more potent oncogene when hematopoietic cells also have deregulated expression of *MYC*, a transcription factor that drives aberrant cell division.[47]

Abundant BCL-2 renders malignant and non-transformed cells resistant to a broad range of anti-cancer drugs, regardless of whether the drugs kill in a p53-dependent or p53-independent manner.[48] BH3-only proteins, in particular BIM and PUMA, fail to neutralize the excess BCL-2, and this prevents intrinsic apoptosis. Hence, small-molecule BH3 mimetics that emulate the function of BH3-only proteins were developed for cancer therapy. The BCL-2-specific BH3 mimetic Venetoclax (also called ABT-199) is currently approved by many regulatory authorities for the treatment of chronic lymphocytic leukemia (CLL)[49] and acute myeloid leukemia (AML),[50] malignancies that rely largely on BCL-2 rather than other pro-survival BCL-2 proteins for their survival. BH3 mimetics inhibiting MCL-1 and



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BCL-XL have also been developed, but their on-target toxicity in normal, healthy cells presents a challenge.[51] Targeting BCL-XL can cause thrombocytopenia, whereas cardiomyocytes, intestinal epithelial cells, and hematopoietic subsets are among the cell types that may not tolerate MCL-1 inhibitors. Strategies to limit toxicity in normal, healthy cells may include the use of antibody conjugates that selectively deliver BH3 mimetics to cancer cells.

There are scenarios when inhibition of intrinsic apoptosis prevents rather than promotes tumor development. For example, low-dose γ-radiation-induced thymic lymphoma in mice is prevented by *Puma* deficiency.[52,53] The explanation is that stress-induced apoptosis of mature leukocytes triggers massive mobilization and proliferation of hematopoietic progenitors, which facilitates the acquisition of oncogenic lesions. Enhanced apoptosis owing to MCL-1 deficiency promotes tumorigenesis in the mouse intestine.[54] Therefore, it will be important to understand whether BH3 mimetic drug-induced apoptosis of normal cells might lay the seed for secondary therapy-related cancers.

Excessive intrinsic apoptosis has been linked to acute and chronic degenerative diseases, including ischemia-reperfusion injury and neurodegenerative diseases.[15] Targeting caspases has not proven a fruitful therapeutic strategy to date, with inhibitors exhibiting limited efficacy as well as toxicity in clinical trials.[55] Importantly, inhibiting caspases in the intrinsic apoptosis pathway does not prevent mitochondrial dysfunction and cell death after MOMP. There are efforts to identify inhibitors of BAX or BAK,[15] but whether there is a therapeutic window for disease intervention is unclear given the potential for on-target toxicity.

EXTRINSIC APOPTOSIS AND NECROPTOSIS

The extrinsic apoptosis and necroptosis pathways are largely triggered by extracellular ligands that engage death receptors on the cell surface. An apoptotic signal is transduced through the protease caspase-8, whereas necroptosis, a lytic form of cell death,[13] may occur when caspase-8 is inhibited. Necroptosis probably evolved as an anti-viral defense mechanism because certain viruses, including cytomegalovirus, herpes simplex viruses, vaccinia virus, and adenovirus, encode inhibitors of caspase-8 to prevent apoptosis.[56] Consistent with this notion, necroptosis-deficient mice are more susceptible than wild-type (WT) mice to infection with vaccinia virus or mutant cowpox virus lacking the necroptosis inhibitor viral inducer of RIPK3 degradation (vIRD).[57,58] Like caspase-9 in the intrinsic apoptosis pathway, caspase-8 cleaves and thereby activates caspase-3 and -7 to execute the apoptotic program.[59]

Ligand and death receptor pairings include FAS ligand (FASL) with FAS, tumor necrosis factor (TNF) or lymphotoxin-α with TNF receptor 1 (TNFR1), TNF-like cytokine 1A (TL1A) with death receptor 3 (DR3), and TNF-related apoptosis-inducing ligand (TRAIL) with TRAILR1 or TRAILR2 (mice have only one TRAILR).[60] Despite their name, death receptors do not exclusively signal cell death. Many cells respond to TNFR1 engagement not by dying but by activating the nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways that promote the expression of pro-inflammatory and pro-survival genes.[61] Perturbations to the TNFR1 signaling machinery can favor the induction of cell death and are discussed below.

Beyond death receptors, the extrinsic apoptosis or necroptosis machineries can also be engaged by Toll-like receptor 3 (TLR3),[62] TLR4,[63] or Z-DNA binding protein 1 (ZBP1).[64,65] TLR3 is activated by double-stranded RNA in the endosomal compartment, whereas TLR4 senses extracellular bacterial lipopolysaccharide (LPS) and then traffics to the endosomal compartment. ZBP1 is an intracellular sensor of Z-form nucleic acids, which have a left-handed double-stranded helical structure and are produced by some viruses and endogenous retroviral elements.[66] The typical output of TLR3 or TLR4 signaling is pro-inflammatory and pro-survival gene expression, but as seen for TNFR1 signaling, certain cellular deficiencies can skew signaling toward cell death. Thus, a theme emerges where cells exposed to some death ligands or pathogen-associated molecular patterns (PAMPs) can escalate their response from pro-inflammatory and pro-survival gene expression to either extrinsic apoptosis or necroptosis if there is an immediate threat, reflected in the compromise of key signaling components.

TNFR1

TNFR1 is expressed on many cell types and has important roles in pathogen defense,[67,68] but sustained activation of the receptor can fuel chronic inflammation.[69,70] Indeed, TNF inhibitors, which block both TNF-TNFR1 and TNF-TNFR2 signaling, are widely used to treat auto-inflammatory disorders, such as inflammatory bowel disease, rheumatoid arthritis, and ankylosing spondylitis.[71] Like all death receptors, TNFR1 has a cytoplasmic homotypic protein interaction motif called a death domain (DD). Upon ligand-induced TNFR1 oligomerization, the DD initiates the assembly of what is known as complex I by recruiting the adaptor TNFRSF1A associated via death domain (TRADD) and receptor interacting protein kinase 1 (RIPK1) (Figure 2A). TRADD binds to TNF receptor associated factor 2 (TRAF2), an

Figure 2. The extrinsic apoptosis signaling pathway

(A) Binding of TNF to TNFR1 on the cell surface promotes assembly of TNFR1-associated complex I, resulting in NF-κB and MAPK signaling that induces the expression of pro-inflammatory and pro-survival genes. A secondary, cytosolic complex II is nucleated by either TRADD or RIPK1. Complex II only triggers apoptosis when levels of cFLIP are low, or the mechanisms that suppress the activation of RIPK1 are disabled (see main text). If caspase-8 is eliminated or inhibited, then complex II becomes more stable because RIPK1 is not cleaved. Active RIPK1 can then trigger necroptosis by promoting oligomerization and activation of RIPK3, which in turn phosphorylates MLKL. The oligomerization and translocation of phosphorylated MLKL to the plasma membrane promotes cell lysis.

(B) Binding of FASL or TRAIL to their cognate death receptors, FAS or TRAILR, promotes the assembly of a primary death-inducing signaling complex. In "type 1" cells (for example, lymphocytes), direct activation of caspase-3 and -7 by caspase-8 is sufficient to cause apoptosis. By contrast, type 2 cells (for example, hepatocytes) fail to die unless caspase-8 also cleaves BID. The resulting tBID causes BAX/BAK-dependent release of HTRA2 and DIABLO from mitochondria and thereby relieves caspase inhibition by XIAP. Like TNF, FASL and TRAIL can trigger necroptosis when caspase-8 is inhibited, and this death is dependent on the kinase activities of RIPK1 and RIPK3. If the cells lack RIPK3, however, then formation of a secondary, cytoplasmic FADDosome complex can drive gene expression programs that increase, for example, the levels of cytokines and chemokines.

adaptor for the ubiquitin ligases cIAP1 and cIAP2. By adding K63-linked polyubiquitin chains to RIPK1 and other components of complex I, cIAP1 and cIAP2 help dock the linear ubiquitin chain assembly complex (LUBAC) and TGF-beta activated kinase 1 (TAK1). LUBAC then adds M1-linked polyubiquitin chains to TNFR1, TRADD, and RIPK1, which enhances recruitment of the canonical IκB kinase (IKK) complex. Activation of TAK1 and IKK within complex I engages the NF-κB and MAPK-signaling pathways that drive the expression of pro-inflammatory genes (for example, *Ccl2*, *Ccl3*, *Ccl5*, *Csf2*, *Cxcl1*, *Cxcl2*, *Il1b*, *Il6*, *Nos2*, and *Tnf*) and pro-survival genes (for example, *Bclx* and *Cflar*, the gene encoding cellular FLICE-inhibitory protein [cFLIP]).[61]

LUBAC is also required for TANK binding kinase 1 (TBK1) and IKKε to enter complex I. These kinases phosphorylate RIPK1 and thereby suppress its death-inducing kinase activity.[72,73] Other kinases activated by TNFR1 signaling, including IKK and MK2 (also called MAPK-activated protein kinase 2), promote cell survival by phosphorylating distinct sites on RIPK1.[74] K63-linked ubiquitination of RIPK1 also appears to suppress its kinase activity.[75–77] Disabling any of these post-translational modifications on RIPK1 skews TNFR1 signaling toward cell death.

Precisely how activation of RIPK1 downstream of TNFR1 promotes cell death is still being elucidated. The kinase activity of RIPK1 is dispensable for TNF-induced NF-κB or MAPK signaling,[78] and the main substrate of RIPK1 appears to be itself.[79] RIPK1 autophosphorylation following DD-mediated dimerization of the kinase[80] licenses RIPK1 to enter a secondary, cytoplasmic death-inducing complex II.[81] Transition of RIPK1 from complex I to complex II is enhanced by cylindromatosis (CYLD), a deubiquitinating enzyme that removes M1- or K63-linked polyubiquitin.[82] CYLD is recruited to complex I via its adaptor protein spermatogenesis associated 2 (SPATA2), which in turn binds to LUBAC.[74] Whether CYLD can access its substrates appears to be further dictated by the ubiquitin-binding proteins A20 (also called TNF alpha-induced protein 3) and A20 binding and inhibitor of NF-κB (ABIN-1). Both proteins are recruited into complex I and may shield polyubiquitin chains from cleavage by CYLD.[83–85] Consistent with this model, mouse intestinal epithelial cells lacking ABIN-1 and A20 are more susceptible than their WT counterparts to TNF-induced cell death that is driven by the activation of RIPK1.[86,87]

Inhibiting protein translation with cycloheximide can also sensitize to TNF-induced cell death, but in this instance, the kinase activity of RIPK1 is dispensable.[74] TRADD can nucleate complex II independent of RIPK1.[82,88] Regardless of whether TRADD or RIPK1 forms the complex II scaffold, both proteins can engage FAS associated via death domain (FADD), the adaptor for caspase-8. The death effector domain (DED) in FADD then interacts with one of two DEDs in the caspase-8 zymogen. Tandem DEDs in caspase-8 support the assembly of helical DED filaments composed of caspase-8 and its catalytically inactive paralog cFLIP.[89,90] The less studied human caspase-10, which has no murine counterpart, may also enter these structures.[91]

Proximity-induced caspase-8 homodimers, or heterodimers of caspase-8 and the long isoform of cFLIP ($cFLIP_L$), auto-proteolytically process to yield fully active caspase-8. Cleavage between the catalytic subunits of caspase-8 allows conformational changes that stabilize the active site, but this is less crucial in caspase-8/$cFLIP_L$ heterodimers.[92–94] Heterodimer-mediated cleavage of caspase-8 homodimers may promote caspase-8 activation, at least in the initial stages. The ratio of caspase-8 to $cFLIP_L$ is important because abundant $cFLIP_L$ can suppress activation of caspase-8, presumably by limiting the overall amount of caspase-8 incorporated into DED oligomers. Shorter isoforms of cFLIP (for example, human $cFLIP_S$ and viral FLIP proteins) inhibit caspase-8 activation by perturbing the helical DED filaments that orient caspase-8 dimers.[90] Cycloheximide appears to promote TNF-induced apoptosis, in large part by reducing the amount of highly labile $cFLIP_L$.[88] Tight regulation of $cFLIP_L$ abundance may have evolved to facilitate the rapid killing of virus-infected cells that have compromised protein translation. Some viruses have acquired their own version of $cFLIP_S$ as a means of blocking extrinsic apoptosis.[56] Other pathogens, including enteropathogenic *Escherichia coli* (EPEC), have acquired other means of thwarting death receptor signaling. For example, the EPEC virulence factor NleB1 is an acetylglucosamine transferase that modifies and disables DDs, including those in TNFR1, TRADD, RIPK1, and FADD.[95,96]

Many uninfected, primary cells respond to TNF with low-level caspase-8 activation that does not trigger apoptosis. Available evidence suggests that in this scenario caspase-8 cleaves the RIPK1 scaffold within complex II, thereby disrupting complex II before there is enough active caspase-8 to effectively cleave and activate caspase-3 and -7. Consistent with this model, heterozygous mutations that eliminate the caspase-8 cleavage site in RIPK1 enhance TNF-induced caspase-8 activation and apoptosis.[97–99] XIAP, an inhibitor of caspase-3, -7, and -9, probably adds an extra layer of protection against the induction of apoptosis.[100] Other mechanisms reported to limit complex II involve lysosomal targeting of the complex by autophagy related 9A (ATG9A),[101] and proteasomal degradation of the complex following its poly-ADP-ribosylation by tankyrase-1 and subsequent ubiquitination.[102]

The "tickling" of caspase-8 by TNFR1 in healthy cells is not a wasted effort because it sets the stage for stabilization of a death-inducing complex II when pathogens inhibit caspase-8. If active RIPK1 is not cleaved by caspase-8 in complex II, then it can engage the kinase RIPK3 to unleash caspase-independent mixed lineage kinase domain like (MLKL)-mediated necroptosis (described below). Accordingly, caspase-8 inhibition by vaccinia virus B13R, cowpox virus CrmA, or small-molecule pan-caspase inhibitors sensitizes some cells to TNF-induced cell death.[103,104] RIPK1 and RIPK3 interact by virtue of their RIP homotypic interaction motifs (RHIMs). RHIM-driven oligomerization of RIPK3 activates its kinase activity, resulting in phosphorylation of the pseudo-kinase MLKL. MLKL then forms oligomers that translocate to membranes to elicit cell lysis.[105] The importance of necroptosis as an anti-viral defense mechanism is highlighted by the discovery of viral MLKL-like decoys that prevent RIPK3 from activating MLKL.[106] Viral and bacterial proteins that block necroptosis by interfering with RHIM-dependent activation of RIPK3 or by targeting RIPK3 for proteasomal degradation have also been identified.[58,107,108]

Although loss-of-function mutations in human *RIPK3* have been linked to herpes simplex encephalitis,[109] it is uncertain if

the disease stems from impaired necroptosis of virus-infected cells because RIPK3 can have necroptosis-independent functions.[110–112] Consistent with a necroptosis-independent role of RIPK3, rare cases of *MLKL* deficiency did not display increased susceptibility to infectious pathogens.[113]

In mice, excessive TNFR1-driven cell death in intestinal epithelial cells or keratinocytes is a potent driver of inflammation. For example, chronic proliferative dermatitis in LUBAC-impaired *Sharpin*^{cpdm} mutant mice is prevented by eliminating TNFR1, CYLD, FADD, caspase-8, or the kinase activity of RIPK1.[114–116] Colitis caused by loss of the IKK subunit NEMO from intestinal epithelial cells is also prevented by loss of TNFR1, FADD, or the kinase activity of RIPK1.[117] Therefore, TNFR1-induced cell death can drive inflammation even in the absence of pro-inflammatory NF-κB signaling. Excessive apoptosis in the skin or intestine may disrupt these barriers and allow an influx of microbes that then drive inflammation.

In humans, TNFR1-induced cell death is thought to be an important driver of the auto-inflammatory syndromes caused by *TBK1* or *OTULIN* deficiency. OTU deubiquitinase with linear linkage specificity (OTULIN) preserves LUBAC activity by removing M1-linked polyubiquitin from the ubiquitin ligase.[83,118] Patient cells lacking TBK1 or OTULIN show heightened sensitivity to TNF-induced death in culture, and notably, TNF inhibition in these patients can ameliorate disease.[119–121] In contrast to humans, mice with inactivating mutations in *Tbk1* or *Otulin* die as embryos. *Tbk1* deficiency unleashes cell death in the fetal liver that is driven by TNFR1 and the kinase activity of RIPK1,[73,122] whereas embryonic lethality caused by *Otulin* inactivation (or by loss of the core LUBAC genes *Hoil1* or *Hoip*) is only partly dependent on TNFR1 and active RIPK1.[118,123] Some of the inflammatory lesions in humans lacking NEMO respond to TNF blockade,[124] and seeing as IKK deficiency enhances TNFR1-stimulated cell death,[125] they too may reflect aberrant TNF-induced cell death.

Haploinsufficiency in *TNFAIP3* encoding A20 can cause autoinflammation and autoimmunity,[126] with some patients responding to TNF blockade.[127,128] Although A20 deficiency confers sensitivity to TNF-induced cell death,[85,129] mice completely lacking A20 develop severe early-onset inflammation independent of TNF or TNFR1 signaling.[130] The latter observation suggests that TNF-induced cell death is but one of several pro-inflammatory processes negatively regulated by A20. Mice expressing hypomorphic A20 with a disabled zinc finger 7 (ZnF7) ubiquitin-binding domain develop a TNF-driven arthritis,[131] whereas mutating both ZnF4 and ZnF7 in A20 elicits the more severe phenotype that is associated with A20 deficiency.[132] The impact of eliminating both caspase-8-dependent cell death and MLKL-dependent necroptosis in mice completely lacking A20 or in A20 ZnF4/ZnF7 mutant mice has not been reported. Therefore, it is unclear if aberrant cell death instigated by death receptors or the TRIF-dependent TLRs is a major driver of their lethality.

The extent to which TNFR1-stimulated cell death drives human inflammatory disease in the absence of mutational inactivation of a negative regulator of the pathway is unclear. Clinical trials with inhibitors of RIPK1 may provide insights, although as discussed in the sections below, inhibiting the kinase activity of RIPK1 can also block some forms of cell death triggered by FAS, TRAILR1, TRAILR2, TLR3, and TLR4. Rheumatoid arthritis and colitis patients treated with the RIPK1 inhibitor GSK2982772 did not show a benefit (ClinicalTrials.gov studies NCT02858492 and NCT02903966), but other RIPK1 inhibitors are being trialed in cutaneous lupus erythematosus (ClinicalTrials.gov study NCT04781816) and amyotrophic lateral sclerosis (ClinicalTrials.gov study NCT05237284).

DR3

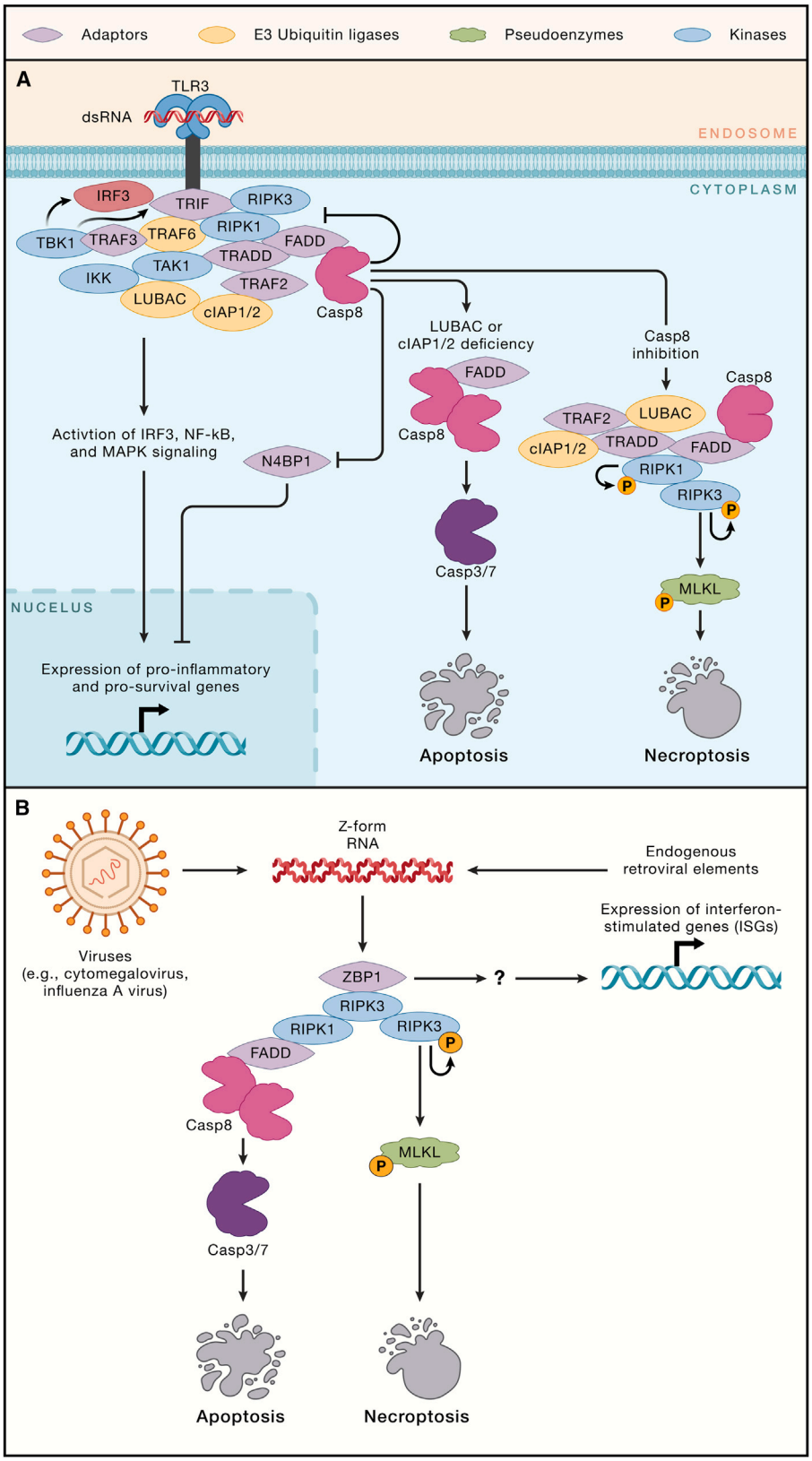
DR3 is not expressed as widely as TNFR1, being found predominantly on lymphoid cells, but it appears to signal in a similar fashion to TNFR1.[133] TL1A engagement of DR3 largely stimulates pro-inflammatory cytokine and chemokine production, but perturbations such as cIAP deficiency favor the assembly of a cytoplasmic death-inducing signaling complex. TL1A inhibitors have been shown to benefit patients with inflammatory bowel disease,[134,135] indicating that TL1A, like TNF, is an important driver of chronic inflammation.

FAS AND THE TRAIL RECEPTORS

The death receptors FAS, TRAILR1, and TRAILR2 also signal using two sequential complexes, but in the reverse order to TNFR1 and DR3. The receptor-associated complex signals cell death, whereas a secondary cytoplasmic complex termed the FADDosome can stimulate NF-κB and MAPK signaling in cells that fail to die.[136–138] The DD in FAS or the TRAIL receptors binds directly to FADD to activate caspase-8 (Figure 2B). Although caspase-8 can cleave and activate caspase-3 and -7 directly, FAS-induced apoptosis in "type 2" cells (for example, mouse hepatocytes) requires caspase-8-mediated proteolytic activation of the pro-apoptotic BH3-only protein BID, yielding tBID. Activation of the intrinsic pathway is needed to overcome XIAP-mediated inhibition of caspase-3 and -7.[100] FAS and the TRAIL receptors, like TNFR1, can signal necroptosis when caspase-8 is inhibited, and this cell death is dependent on the kinase activity of RIPK1.[139] RIPK1 is recruited to the receptor complex by FADD and subsequently engages RIPK3 to promote necroptosis.

Assembly of the cytoplasmic FADDosome requires FADD and caspase-8, but not caspase-8 catalytic activity.[136–138] The FADDosome appears to incorporate many of the components of TNFR1 complex I, including TRADD, cIAP1/2, RIPK1, TRAF2, IKK, TAK1, and A20. FADDosome-regulated gene expression in tumor cells that are resistant to TRAIL-induced cell death has been suggested to modulate anti-tumor immune cell responses.[138]

FASL and TNF are part of the arsenal deployed by cytotoxic T cells to kill their target cells, in addition to perforins and granzymes.[140,141] Just as excessive TNFR1-driven cell death can be deleterious, excessive cell death triggered by FAS or TRAILR can cause tissue damage and inflammation.[142,143] FASL also contributes to lymphoid homeostasis by killing lymphocytes that are no longer needed.[37,38] Mice and humans with compromised FASL or FAS develop autoimmune lymphoproliferative syndrome (ALPS), a disease in which normally rare, unconventional B and T cells accumulate aberrantly.[144–146] Loss of FADD or caspase-8 enzymatic activity, which prevents



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all extrinsic apoptosis, impacts mice and humans differently. Mice unable to activate caspase-8 die as embryos owing to unchecked TNFR1-, RIPK1-, RIPK3-, and MLKL-dependent necroptosis.[97,110,147–151] By contrast, humans lacking FADD or caspase-8 can exhibit ALPS, recurrent infections, and early-onset inflammatory bowel disease.[152–154] Whether necroptosis drives the human disease is unclear. Impaired caspase-8 cleavage of RIPK1 must promote inflammation because heterozygous *RIPK1* mutations that destroy the caspase-8 cleavage site cause a periodic fever syndrome.[98,99] Failure to cleave and inactivate another caspase-8 substrate, NEDD4 binding protein 1 (N4BP1), may contribute to impaired innate immunity in patients with FADD or caspase-8 deficiency. In human and mouse myeloid cells, N4BP1 suppresses cytokine and chemokine production by TLRs that signal solely through the adaptor myeloid differentiation gene 88 (MyD88).[155] N4BP1 is a ubiquitin-binding endoribonuclease, but precisely how it limits cytokine and chemokine responses requires further study.

TLR3, TLR4, AND ZBP1

TLR3 and TLR4 both utilize the RHIM-containing adaptor Toll/IL-1 receptor domain-containing adaptor inducing interferon-beta (TRIF) to engage RIPK1 and RIPK3 (Figure 3A). RIPK1, in turn, probably recruits TRADD-TRAF2-cIAP1/2 and FADD-caspase-8, ultimately leading to NF-κB and MAPK activation[156] plus caspase-8-dependent cleavage of N4BP1.[155] Disabling LUBAC, or both cIAP1 and cIAP2, has been shown to push TLR3 signaling toward robust caspase-8 activation and cell death.[62,157] If caspase-8 is inhibited, then both TLR3 and TLR4 can stimulate necroptosis.[63] Inhibiting RIPK1 blocks TLR3- or TLR4-induced necroptosis in macrophages,[63,158] suggesting that the kinase activity of RIPK1 is normally required for TRIF-dependent necroptosis. However, genetic studies in mice have indicated that TRIF or ZBP1 can activate necroptosis even when RIPK1 is absent.[150,159] Indeed, mouse RIPK1 appears to use its RHIM to suppress TRIF- or ZBP1-induced necroptosis because inflammation and perinatal lethality in RIPK1 RHIM mutant mice are prevented by the loss of MLKL or the combined loss of TRIF and ZBP1.[159,160] Consistent with RIPK1 preventing TRIF from engaging the cell death machinery, primary human fibroblasts lacking *RIPK1* are more sensitive than control fibroblasts to cell death induced by LPS or the TLR3 agonist poly(I:C).[161] Collectively, these data suggest that activation of RIPK1 might relieve its RHIM-dependent, but otherwise ill-understood suppression of TRIF or ZBP1 signaling.

The RIPK1 scaffold also suppresses caspase-8-driven cell death because *Ripk1* deficient mice die in the perinatal period unless both the caspase-8 and MLKL-dependent death programs are eliminated.[150,162,163] By contrast, the kinase activity of RIPK1 is dispensable for mouse viability.[78] RIPK1 appears to limit apoptosis, at least in part, by blocking the assembly of a TRADD-FADD-caspase-8 signaling complex.[164] Given that RIPK1 and TRADD seem to act in parallel and bind to the same DD proteins, loss of RIPK1 probably allows more TRADD to enter signaling complexes, coincident with impaired NF-κB and MAPK signaling and reduced expression of $cFLIP_L$.[159,165] *RIPK1* deficiency in primary human fibroblasts also enhances TNF-induced cell death.[161] Although RIPK1 loss is lethal in mice, humans lacking RIPK1 largely exhibit immune cell dysfunction leading to lymphopenia, infections, arthritis, and early-onset inflammatory bowel disease.[161,166] The broader requirement for RIPK1 in mice versus humans may reflect species-specific differences in the expression patterns of RIPK1 or differences in other pathway components.

ZBP1 has two Zα domains for detecting Z-form nucleic acids and a RHIM to engage RIPK3.[66] The ZBP1-RIPK3 interaction can trigger necroptosis through MLKL or apoptosis via RIPK1, FADD, and caspase-8 (Figure 3B). The kinase activity of RIPK1 is dispensable for this cell death. Viruses that make Z-RNA include cytomegalovirus, influenza A virus, herpes simplex virus 1, and vaccinia virus.[66] Cytomegalovirus evades cell death by producing inhibitors of caspase-8 and RHIM-RHIM interactions, whereas vaccinia virus produces a caspase inhibitor and a Zα domain protein that shields viral Z-RNA from ZBP1.[56,167] Z-form nucleic acids produced by endogenous retroviral elements have been implicated in mouse models where skin or intestinal inflammation is caused by excessive ZBP1-dependent cell death.[168,169]

Intriguingly, the outputs of ZBP1 signaling are not limited to cell death. ZBP1 can also promote the expression of interferon-stimulated genes (ISGs) independently of RIPK3, the RIPK1 RHIM, or cell death.[170] Unraveling this signaling pathway will be important because ZBP1 contributes to ISG-driven pathology that is caused by inactivating *Adar1* mutations.[170–172] Human *ADAR1* mutations cause severe diseases characterized by increased ISG expression, including Aicardi-Goutieres syndrome. Adenosine deaminase RNA specific (ADAR1) has a Zα domain and appears to bind to and edit the Z-RNAs that would otherwise activate ZBP1. Given the pro-inflammatory nature of ZBP1-induced necroptosis and ISG expression, others have suggested utilizing ZBP1 agonists to enhance cancer immunotherapy.[173]

PYROPTOSIS

Pyroptosis refers to cell death that is induced by gasdermin pores in the plasma membrane.[174] It emerged as a distinct cell

Figure 3. Cell-death signaling by TLR3 and ZBP1

(A) Double-stranded RNA binding to endosomal TLR3 stimulates TRIF-dependent signaling. LPS binding to TLR4 (not shown) also stimulates TRIF signaling, the typical output of which is gene transcription induced by interferon regulatory factor 3 (IRF3), NF-κB, and MAPK signaling. Proteins that have been implicated in different aspects of TRIF signaling through genetic loss-of-function studies are indicated, but the post-translational modifications that regulate the activity of this complex and the enzymes responsible are still being elucidated. For example, the relative contributions of the ubiquitin ligases TRAF6, cIAP1, and cIAP2 to MAPK and IKK activation are unclear. TRIF has a RHIM to engage either RIPK1 or RIPK3, the former contributing to NF-κB activation. RIPK1 probably also recruits caspase-8 and cFLIP in a transient fashion via FADD, resulting in N4BP1 cleavage and enhanced pro-inflammatory gene expression. Loss of LUBAC or cIAP1/2 has been shown to favor caspase-8 driven apoptosis, whereas loss of RIPK1 or caspase-8 promotes RIPK3-dependent necroptosis.

(B) ZBP1 triggers RIPK3-dependent cell death after binding to Z-form nucleic acids, including Z-RNAs made by certain viruses or endogenous retroviral elements. ZBP1 can also promote RIPK3-independent ISG expression, but the signaling mechanism has yet to be defined.

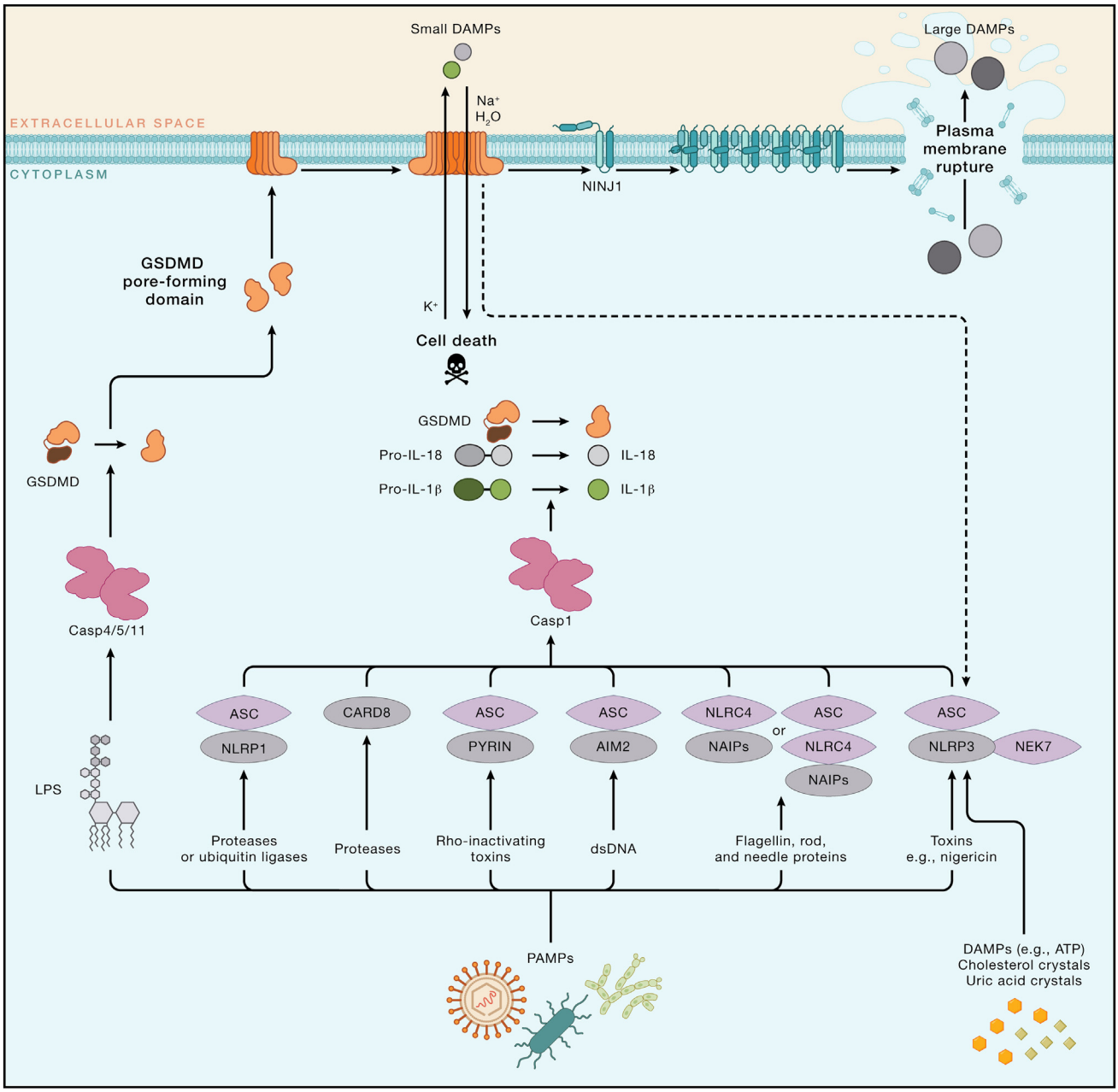

Figure 4. Inflammasome-induced GSDMD-dependent pyroptosis

Many DAMPs or PAMPs are sensed directly or indirectly by one of the inflammasome components indicated in gray. These sensors oligomerize and typically recruit an adaptor protein (ASC or NLRC4) to activate caspase-1. One exception is CARD8, which can bind to caspase-1 directly. CARD8 is found in humans but not mice. By contrast, human *NLRP1* and *NAIP* genes have several orthologs in mice. Some inflammasome sensors appear to have evolved to protect a species against specific pathogens. For example, the proteases that activate NLRP1 (or CARD8) in one species may not activate NLRP1 (or CARD8) in another. LPS forms a non-canonical inflammasome complex, binding directly to human caspase-4 and -5 (caspase-11 in mice). Active caspase-1, -4, -5, and -11 cleave GSDMD, releasing an N-terminal fragment that oligomerizes and forms pores in membranes. Caspase-1 proteolytically matures the leaderless cytokines IL-1β and IL-18 into their biologically active forms. GSDMD pores have several important consequences. They disrupt plasma membrane potential to kill the cell, allow mature IL-1β and IL-18 to exit the cell, and in a manner not well understood, promote NINJ1-dependent cell rupture. Mature IL-1β and IL-18 are also released following non-canonical inflammasome activation, owing to secondary activation of the NLRP3 inflammasome. Caspase-4 and -5 can also cleave IL-18 directly.[191,192]

death program from the study of macrophages infected with *Salmonella*.[12] The death of the infected macrophages was dependent on caspase-1,[12] the protease that proteolytically matures IL-18 and the endogenous pyrogen IL-1β.[6,7] Later studies revealed that caspase-1, -4, and -5 in humans, and caspase-1 and -11 in mice, each cause pyroptosis by cleaving gasdermin D (GSDMD).[175,176] The N-terminal GSDMD cleavage fragment promotes cell lysis by oligomerizing and forming pores in

membranes.[177,178] GSDMD pores in the plasma membrane collapse ion gradients and cause an influx of water, culminating in ninjurin 1 (NINJ1)-dependent plasma membrane rupture (PMR) and the release of large pro-inflammatory DAMPs.[179] However, small DAMPs, including IL-1β, can exit the cell through GSDMD pores.[180] In some settings, membrane repair mechanisms may limit the extent of GSDMD pore formation.[181] Whether transient GSDMD pores can release IL-1β from the cell without triggering pyroptosis is unresolved.[182]

INFLAMMASOMES

Caspase-1 forms active dimers within cytoplasmic complexes called inflammasomes[183] (Figure 4). Inflammasome assembly is triggered by proteins that sense specific DAMPs or PAMPs, and then form an oligomeric scaffold. Some "sensor" proteins are engaged directly by DAMPs or PAMPs, whereas others detect the perturbations that DAMPs or PAMPs elicit within the cell. The former category includes absent in melanoma 2 (AIM2), which binds to viral or bacterial double-stranded DNA,[184,185] and the NLR family apoptosis inhibitory proteins (NAIPs), which bind to the flagellin, needle, and inner rod proteins of bacteria.[186,187] NLR family pryin domain containing 1 (NLRP1) and caspase activation and recruitment domain 8 (CARD8) sense certain infections because they each have an inhibitory domain that can be modified by bacterial or viral enzymes and targeted for proteasomal degradation.[188–190] Removal of this inhibitory domain promotes inflammasome assembly.

The inflammasome sensors that detect PAMPs or DAMPs indirectly include PYRIN and NLRP3. PYRIN senses bacterial toxins that modify and inactivate Rho GTPases,[193] with dephosphorylation of PYRIN and microtubule polymerization implicated in inflammasome assembly.[194] NLRP3 responds to diverse stimuli, including the bacterial toxin nigericin, extracellular ATP, and gout-associated uric acid crystals.[195,196] Potassium efflux from the cell is proposed to be the unifying event that drives NEK7-dependent oligomerization of NLRP3,[197–200] but the mechanistic details of this process remain elusive. NLRP3 activation in settings of sterile inflammation has prompted the development of small-molecule inhibitors of NLRP3. Clinical trials of NLRP3 inhibitors have focused on auto-inflammatory diseases, including osteoarthritis and neuroinflammation, as well as COVID-19.[201] The PYRIN, NLRP1, and NLRP3-NIMA related kinase 7 (NEK7) inflammasomes incorporate the caspase-1 adaptor apoptosis-associated speck-like protein containing a CARD (ASC), which has a CARD that binds to the CARD in caspase-1. The NAIP and CARD8 inflammasomes do not require ASC because the CARD in the NAIP-interacting protein NLR family CARD-containing 4 (NLRC4) or in CARD8 itself can engage caspase-1.[186,187,202–204]

The CARD in caspase-4 or -5 (the human counterparts of rodent caspase-11) binds directly to cytoplasmic oligomers of bacterial LPS.[205] This binding does not require the surface-expressed LPS receptor TLR4.[206,207] These non-canonical inflammasome complexes yield active caspase dimers that trigger GSDMD-dependent pyroptosis.[175,176,208] The pyroptotic cells also release mature IL-1β because GSDMD cleavage elicits perturbations that activate the NLRP3 inflammasome and caspase-1[176] (Figure 4).

GSDMD-DEPENDENT PYROPTOSIS IN PATHOGEN DEFENSE AND INFLAMMATORY DISEASES

Genetic studies in mice have shown that GSDMD-dependent pyroptosis is an important mechanism for limiting the growth of intracellular pathogens such as *Salmonella*.[209] Other intracellular bacteria preserve their replicative niche by using virulence factors that suppress pyroptosis. *Shigella* has two effectors in its arsenal to foil pyroptosis, the caspase-4 and -11 inhibitor OspC3[210] and the ubiquitin ligase IpaH7.8.[211,212] The latter inhibits pyroptosis by targeting human GSDMD and GSDMB (described below) for proteasomal degradation.

The pro-inflammatory nature of pyroptosis is a boon in combating pathogens because immune cells, including neutrophils, are recruited to the site of infection. The downside is that excessive pyroptosis from dysregulation of the pathway can cause disease. Germline activating mutations in human *NLRP1*, *NLRP3*, *NLRC4*, and *MEFV* (encoding PYRIN) disable normal inflammasome regulation and cause a range of auto-inflammatory disorders.[213–216] Patients with these disorders often respond to IL-1β blockade, and aberrant IL-1β production may reflect enhanced pyroptosis. Consistent with this notion, *Gsdmd* loss prevents inflammatory lesions and elevated levels of active IL-1β in knockin mice expressing disease-associated PYRIN or NLRP3 mutants.[217,218] Of note, *Gsdmd* deficiency delays rather than prevents cell death and IL-1β release after canonical inflammasome activation in cultured cells.[176] Cell death still occurs because caspase-1 can also trigger the slower process of apoptosis by proteolytically activating caspase-3 and -7.[219] Therefore, the type of cell death is important *in vivo*. Presumably, *Gsdmd* deficiency is beneficial in the *Mefv* or *Nlrp3* knockin mutant mice because *Gsdmd*-deficient cells dying by apoptosis are cleared by phagocytes before secondary membrane damage can release the pro-inflammatory, mature form of IL-1β that is generated by caspase-1.

Gsdmd deficiency also ameliorates disease in mouse models of sepsis.[176,220] Caspase-1, IL-1β, and IL-18 are dispensable for LPS-induced septic shock in mice,[221] whereas caspase-11-dependent pyroptosis in endothelial cells appears to drive vascular leak and hypotension.[222] An influx of Ca^{2+} through endothelial GSDMD pores may promote lethal coagulation and clot formation in micro-vessels.[220] Collectively, these studies suggest that inhibitors of GSDMD might provide a therapeutic benefit in settings of fulminant inflammation. Intriguingly, exogenously added antagonist GSDMD nanobodies were recently reported to suppress GSDMD-dependent pyroptosis in cultured cells.[223] It was suggested that the GSDMD nanobodies entered cells through initial GSDMD pores and then inhibited further pore formation. The initial GSDMD pores were thought to be transient and removed from the plasma membrane by the membrane repair machinery. These findings suggest that GSDMD is druggable, but whether these nanobodies can inhibit GSDMD *in vivo* is unclear. Since the nanobodies could only bind to human GSDMD, they could not be assessed for efficacy in pre-clinical mouse models.

Proteases other than caspase-1, -4, -5, and -11 can also cleave and activate GSDMD. Pyroptosis induced by caspase-8 cleavage of GSDMD has been implicated in the host response to *Yersinia* infection.[224] Neutrophil elastase has also been shown

to cleave and activate GSDMD.[225] By contrast, caspase-3 reportedly disables GSDMD by cleaving within the N-terminal pore-forming domain.[226]

THE BROADER GASDERMIN FAMILY

Humans have six gasdermin family members (GSDMA, GSDMB, GSDMC, GSDMD, GSDME, and GSDMF), whereas mice lack *Gsdmb*, but have three *Gsdma* genes (*Gsdma1*, *Gsdma2*, and *Gsdma3*), four *Gsdmc* genes (*Gsdmc1*, *Gsdmc2*, *Gsdmc3*, and *Gsdmc4*), *Gsdmd*, *Gsdme*, and *Gsdmf*.[227] With the exception of GSDMF, each gasdermin has a conserved pore-forming domain at the N terminus that is held in check by the C terminus. Different proteases cleave the different gasdermins to liberate the pore-forming domain, which can then associate with the plasma membrane, organelle membranes, or the outer membrane of bacteria.[178,228,229] Mice lacking *Gsdma1* (or *Gsdma1-3*),[230,231] *Gsdmc1-4*,[232] or *Gsdme*[233] are more susceptible than WT controls to certain bacterial or helminth infections, suggesting that many gasdermins contribute to host defense against pathogens.

Proteolytic activation of GSDME by caspase-3 in cultured cells is reported to induce either pyroptosis[234] or secondary membrane damage after apoptosis.[235] The physiological significance of the latter is less certain because of the normally rapid apoptotic cell clearance by phagocytes *in vivo*.[26] Regardless, GSDME has been shown to mediate tissue damage in mice dosed with chemotherapy drugs[234] or infected with enterovirus 71.[236]

Granzyme B released from cytotoxic lymphocytes can cleave and activate caspase-3 to cleave GSDME, but it can also cleave GSDME directly in targeted tumor cells.[237] Epigenetic silencing or mutational inactivation of *GSDME* in various tumor cells has implicated GSDME in tumor suppression.[234,237] Therefore, experiments to see if *Gsdme* deficiency enhances tumorigenesis in sporadic mouse tumor models would be informative. GSDME-driven tumor cell lysis instigated by chimeric antigen receptor T cells has been reported to trigger cytokine release syndrome in mice.[238]

GSDMA is largely expressed in epithelial cells. It appears to function as a direct pathogen sensor, inducing pyroptosis after proteolytic cleavage by streptococcal pyrogenic exotoxin B (SpeB).[230,231] GSDMB can be cleaved and activated by granzyme A, another effector protease that is released by cytotoxic lymphocytes.[239] Notably, multiple splice variants of *GSDMB* have been described across different cell types, some encoding non-functional GSDMB proteins that fail to induce pyroptosis.[240,241] It is unclear which isoforms are the most physiologically relevant, which complicates the interpretation of genome-wide association studies linking single nucleotide polymorphisms in *GSDMB* to asthma, Crohn's disease, and ulcerative colitis.[242,243] The less studied GSDMC may mediate anti-helminth defense in mice by releasing IL-33 from goblet and Paneth cells.[232]

NINJ1-DEPENDENT PMR

PMR responsible for the bursting of pyroptotic cells is mediated by NINJ1, a TM protein expressed on the cell surface.[179] Eliminating NINJ1 does not prevent cell death, but it does impair the release of large intracellular molecules like lactate dehydrogenase (LDH), a standard marker of PMR, and HMGB1. *Ninj1* deficient cells induced to undergo pyroptosis in culture stand out because of their persistent ballooned morphology. The expulsion of large DAMPs from dying cells is thought to "alert" immune cells because mice lacking *Ninj1* are more susceptible than WT controls to infection with *Citrobacter rodentium*[179] or *Yersinia pseudotuberculosis*.[244]

NINJ1 appears to be monomeric or dimeric in live cells but oligomeric in pyroptotic cells.[179] Purified NINJ1 forms filaments that are hydrophobic on one surface but hydrophilic on the other,[245–248] suggesting that NINJ1 disrupts cell membrane integrity by either capping the edges of membranes[245] or by forming nanodisc-like rings that rip out sections of the membrane.[247] It is unclear what triggers oligomerization of NINJ1 in dying cells. One possibility is that NINJ1 senses alterations in membrane tension or lipid packing as the dying cell becomes swollen.

In culture, NINJ1 also mediates secondary PMR in apoptotic cells.[179] The apoptotic cells eventually swell because of declining ATP levels and malfunction of the Na^+/K^+ ATPase pump. Caspase-3 cleavage of GSDME and the formation of GSDME pores may accelerate the swelling via oncotic pressure. Interestingly, NINJ1 is dispensable for PMR in cells undergoing necroptosis or ferroptosis.[179,249] Whether NINJ1-dependent DAMP release exacerbates inflammation and disease severity is an active area of investigation.[246]

FLEXIBLE ROUTES TO CELL DEATH

There is burgeoning evidence for flexible usage of the programmed cell-death pathways in mammalian cells. For example, inflammasomes in macrophages can engage either caspase-1 or -8, with pyroptosis typically prevailing over apoptosis because it is induced with faster kinetics.[219,250] Genetic experiments in mice have unveiled unexpected routes to cell death when the usually dominant death pathway is stymied. For example, expression of proteolytically inactive caspase-8 in mice unleashes lethal necroptosis, but if MLKL is eliminated to prevent necroptosis, then ASC and caspase-1 signaling contribute to lethality.[112,151] In another example, ileitis in mice with FADD-deficient intestinal epithelial cells is driven by either necroptosis or GSDMD-dependent pyroptosis.[251] Finally, necroptosis can trigger NLRP3 inflammasome activation within the dying cell, resulting in activation of caspase-1 and the release of active IL-1β.[252,253] Presumably, NLRP3 detects some form of cellular perturbation within the dying cell. Neither NLRP3 nor caspase-1 are required for the death of these cells, but they nonetheless contribute to the pro-inflammatory nature of the death by allowing the dying cells to release mature IL-1β.

FERROPTOSIS

Ferroptosis refers to the non-apoptotic death of cells that accumulate lethal levels of iron-dependent, phospholipid peroxides in cell membranes.[254] Cells undergoing ferroptosis rupture, but precisely how lipid peroxides compromise the integrity of the plasma membrane is unclear. Susceptibility to ferroptosis is conferred by metabolic enzymes that synthesize oxidizable membrane lipids (for example, acyl-CoA synthetase long-chain family member 4 [ASCL4] and lysophosphatidylcholine

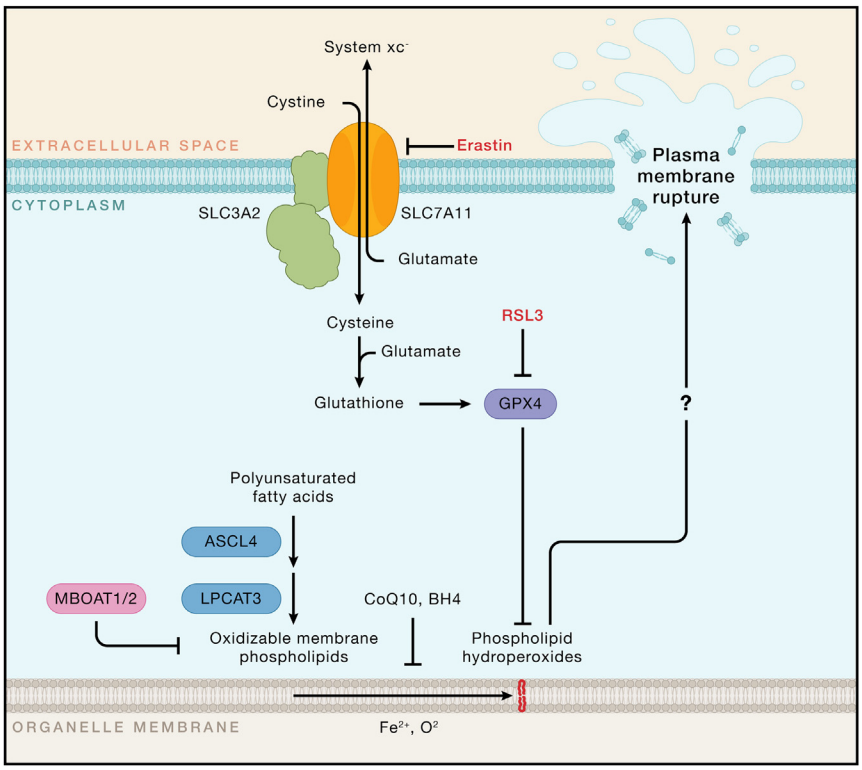

Figure 5. Cellular components determining sensitivity to ferroptosis

Cells die by ferroptosis when they accumulate lethal levels of phospholipid hydroperoxides in membranes, albeit the precise events causing cell rupture are unclear. Ferroptosis sensitivity is determined by (1) the availability of oxidizable membrane phospholipids, which is governed by enzymes like ASCL4, LPCAT3, membrane bound O-acyltransferase domain containing 1 (MBOAT1), and MBOAT2, (2) the availability of Fe^{2+} for membrane phospholipid oxidation, and (3) the endogenous mechanisms for limiting reactive oxygen species (for example, CoQ_{10}, BH4, and GPX4). Two commonly used inducers of ferroptosis, erastin and RSL3, interfere with the reduction of phospholipid hydroperoxides by GPX4 and glutathione.

acyltransferase 3 [LPCAT3]) and iron-dependent enzymes that oxidize membrane lipids (for example, arachidonate lipoxygenases) (Figure 5). Commonly used inducers of ferroptosis include the small molecules erastin and RSL3, which act by disrupting the mechanisms that limit membrane lipid peroxides. RSL3 inhibits glutathione peroxidase 4 (GPX4), a lipid hydroperoxidase that uses glutathione to reduce lipid peroxides to non-toxic lipid alcohols. Erastin depletes intracellular glutathione by inhibiting the xc^- system that imports cystine and makes cysteine available for glutathione synthesis. Treatments that suppress the production of other radical scavenging metabolites, including coenzyme Q_{10} (CoQ_{10}) and tetrahydrobiopterin (BH4), can also sensitize cells to ferroptosis. Increasing the abundance of labile iron is another way of priming cells for ferroptosis.[254]

Ferroptosis has been implicated in many diseases,[254] but it is difficult to test causality in pre-clinical disease models genetically because mediators of ferroptosis also have critical roles in normal cell metabolism. Detection of ferroptosis is another challenge because there is no one discriminating marker unique to this form of cell death. Lipid peroxidation must be observed, and a combination of markers evaluated to exclude other forms of cellular stress. Nonetheless, one area of active investigation is whether cancer cells can be eliminated selectively by inducers of ferroptosis. For example, some tumor cells may be more dependent than normal cells on the import of cystine.[255]

CONCLUSIONS AND FUTURE DIRECTIONS

Cell death is required for the survival and fitness of multicellular organisms but must be tightly regulated to prevent disease. A comprehensive understanding of cell-death signaling mechanisms has revealed opportunities to counter aberrant cell death in disease. In the case of the intrinsic apoptosis pathway, the success story has been the development of the BCL-2 inhibitor Venetoclax for the treatment of certain hematologic malignancies. Identification of inhibitors of the pro-inflammatory cell-death programs is a more recent endeavor based on the benefits of genetically eliminating these pathways in pre-clinical models of inflammatory disease. Effectors of pyroptosis, such as NLRP3 and GSDMD, are targets of interest, along with RIPK1 as a mediator of extrinsic apoptosis and necroptosis. Key to these efforts is knowing when and where the different cell-death pathways are activated in human disease. Molecular markers of the various forms of cell death have been defined, for example, phosphorylated RIPK3 or phosphorylated MLKL for necroptosis and cleaved GSDMD for inflammasome-induced pyroptosis, but their presence in tissues is likely ephemeral owing to the rapid clearance of dead cells by phagocytes. Therefore, detection of pathway activation markers can be challenging even in pre-clinical models where the genetics tell us the pathway is active.

What really matters is whether interruption of a death pathway will result in clinical benefit. If pyroptosis is the engine that drives runaway inflammation in sepsis, then specific inhibitors of pyroptosis, like those targeting GSDMD, should be efficacious. The promise of such therapies warrants further investigation. In a similar vein, the resounding clinical success of BCL-2 inhibition motivates the development of inhibitors of MCL-1 and BCL-XL, which are commonly overexpressed in solid malignancies. Targeting these inhibitors to cancer cells will be crucial to avoid on-target toxicity in normal, healthy cells, but progress is being made. For example, a BCL-XL inhibitor conjugated to an antibody specific for the tumor antigen CD276 seems to elicit minimal thrombocytopenia while retaining anti-tumor activity.[256] The hope is that the advances made in cell-death research over the past 50 years will continue to be translated into meaningful life-saving therapies.

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