Review

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Cell death pathways: intricate connections and disease implications

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Abstract

Various forms of cell death have been identified over the last decades with each relying on a different subset of proteins for the activation and execution of their respective pathway(s). In addition to the three best characterized pathways—apoptosis, necroptosis, and pyroptosis—other forms of regulated cell death including autophagy-dependent cell death (ADCD), mitochondrial permeability transition pore (MPTP)-mediated necrosis, parthanatos, NETosis and ferroptosis, and their relevance for organismal homeostasis are becoming better understood. Importantly, it is increasingly clear that none of these pathways operate alone. Instead, a more complex picture is emerging with many pathways sharing components and signaling principles. Finally, a number of cell death regulators are implicated in human diseases and represent attractive therapeutic targets. Therefore, better understanding of physiological and mechanistic aspects of cell death signaling should yield improved reagents for addressing unmet medical needs.

Keywords apoptosis; caspase; necroptosis; pyroptosis; RIPK1
Subject Categories Autophagy & Cell Death; Signal Transduction
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Introduction

Cell death plays a central role in all aspects of life. It is involved in the development of multicellular organisms and tissue homeostasis where cell death depletes dispensable cells. Moreover, it is critical for fighting off infections and is associated with multiple diseases that are caused by deregulated or dysfunctional cell death signaling. Consequentially, there is a growing interest in modulating cell death to treat diseases. Various forms of cell death have been described so far, with apoptosis, necroptosis, and pyroptosis being the best understood. In recent years, a more complex picture of cell death modalities has been established as crosstalk and backup mechanisms between different pathways were identified. This review will focus on different forms of cell death, their interconnectivity, and validated targets for treating diseases.

Apoptosis

Apoptosis is the first described form of programmed cell death (Kerr *et al*, 1972), and it plays a critical role in tissue homeostasis. It contributes to cell turnover, the proper functioning of the immune system, and embryonic development (Voss & Strasser, 2020). There are several key characteristics of apoptosis. Cells undergo morphological changes which lead to cellular, organelle, and DNA fragmentation as well as the formation of apoptotic bodies (Kerr *et al*, 1972; Zakeri *et al*, 1993). This is an active, energy consuming process executed by a subset of cellular proteins. Even though, in general, this process is immunological silent, apoptosis has been shown to be involved in inflammatory pathologies as well (Rickard *et al*, 2014; Yang *et al*, 2015; Singh *et al*, 2019).

There are two major pathways that mediate apoptosis: intrinsic and extrinsic pathways. Intrinsic apoptosis is controlled by the equilibrium of the different Bcl-2 (B-cell lymphoma 2) family members which can be disrupted by various stimuli leading to cell death. During extrinsic apoptosis, members of the TNF (tumor necrosis factor) superfamily (TNFSF) can induce cell death by binding to their cell surface receptors and activating a deathly signaling cascade causing extrinsic apoptosis. The third modality of apoptosis induction is cell-based. Cytotoxic T cells can engage cells that present non-self-antigens leading to cell death induction by proteases called granzymes. All apoptotic pathways converge on the central proteases of this pathway: caspases, which are either playing a role in transmitting cell death stimulus (initiator caspases) or in the execution (effector caspases).

Intrinsic apoptosis

Intrinsic apoptosis is engaged by cells that are obsolete, deprived from growth factors or damaged (e.g., UV) (Fig 1). These diverse stimuli can tip the balance between different groups of the Bcl-2 (B-cell lymphoma 2) proteins leading to the activation of cell death (Kale *et al*, 2018) The Bcl-2 superfamily can be divided into three subfamilies: the anti-apoptotic Bcl-2 proteins, the pro-apoptotic BH3-only (BH: Bcl-2 homology) proteins, and the death effectors Bax (Bcl-2-associated X protein), Bak (Bcl-2 homologous antagonist/killer), and Bok (Bcl-2-related ovarian killer). Normally, the cells keep Bax and Bak in check by the expression of anti-apoptotic

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Bcl-2 family members (Bcl-2, Bcl-XL, Mcl-1, A1, Bcl-w) which inhibit Bax and Bak pore forming ability (Kale *et al*, 2018). The BH3-only family members can inhibit the anti-apoptotic Bcl-2 proteins or in some cases also directly engage Bax and Bak (for example Bim) (Kim *et al*, 2006; Czabotar *et al*, 2014). Some BH3-only proteins are regulated by transcriptional regulation (PUMA regulated by p53, DNA damage) (Nakano & Vousden, 2001) or by post-translational modifications (BIM, BID) (Li *et al*, 1998; Lei & Davis, 2003). Tipping the equilibrium in favor of pro-apoptotic Bcl-2 proteins leads to activation of Bax and Bak and results in the MOMP (mitochondrial outer membrane permeabilization) (Wei *et al*, 2001). Bok, which can induce MOMP in a constitutively fashion, is regulated differently—by proteasomal degradation pathways (Llambi *et al*, 2016; Ke *et al*, 2018). MOMP causes the release of the key mediators of intrinsic apoptosis, cytochrome c (Stein & Hansen, 1999), and endogenous IAP (inhibitor of apoptosis) antagonist, SMAC/ Diablo (second mitochondria derived activator of caspases/ direct IAP binding protein with low pI) (Du *et al*, 2000; Verhagen *et al*, 2000). Cytochrome c-bound Apaf1 (apoptotic protease activating factor 1) (Zou *et al*, 1997) recruits initiator caspase caspase-9 to form the apoptosome, a platform for the activation of the executioner caspases caspase-3 and -7 (Li *et al*, 1997). Caspases-3, -7, and -9 can be blocked by the major endogenous caspase inhibitor, XIAP (X chromosome-linked IAP). SMAC can antagonize XIAP and other IAPs thus allowing full caspase activation and apoptosis execution (Du *et al*, 2000). Caspases cleave a wide variety of cellular proteins to induce characteristic changes of apoptotic death (cellular and nuclear fragmentation, DNA laddering, etc.). For example, ICAD (inhibitor of caspase-activated DNase) cleavage leads to the activation of CAD (caspase-activated DNase) that induces genome fragmentation (Enari *et al*, 1998; Sakahira *et al*, 1998), whereas cleavage of ROCK-I (Rho associated protein kinase) induces the contraction and blebbing of cells (Coleman *et al*, 2001; Sebbagh *et al*, 2001). A specific form of intrinsic apoptosis is anoikis, which is induced by the loss of pro-survival signals via integrin binding to the ECM (extracellular matrix) (Frisch & Francis, 1994). Integrins can signal via different pathways (PI3K or FAK), which modulate the Bcl-2 or BH3-only family members (Gilmore, 2005). In this fashion, anoikis ensures that cells can only survive in the appropriate compartments/organs within the body. Overcoming this checkpoint plays a major role in metastasis/ invasiveness of cancer (Paoli *et al*, 2013).

Extrinsic apoptosis

Extrinsic apoptosis is triggered by TNF family ligand-receptor interactions, most prominently by TNF family ligands: TNF, FasL, TRAIL, and TL1A. The receptor complexes either recruit FADD (Fas-associated protein with death domain) or TRADD (TNFRSF1A-associated via death domain) to the oligomerized complex (Wilson *et al*, 2009). FasL-mediated signaling will be used to describe extrinsic apoptotic signaling (Fig 1), and TNF signaling will be described for necroptotic signaling. FasL binds to its transmembrane receptor Fas, which recruits FADD (Fas-associated death domain protein) via death domain (DD) interactions (Chinnaiyan *et al*, 1995; Boldin *et al*, 1996). FADD contains a DD and also a death effector domain (DED), which allows the recruitment of caspase-8 via homotopic domain interaction, forming the death inducing signaling complex—DISC (Boldin *et al*, 1996; Muzio *et al*, 1996; Medema *et al*, 1997). The proximity of multiple caspase-8 molecules induces the transactivation by proteolytic cleavage (Muzio *et al*, 1998; Yang *et al*, 1998). Cleavage results in the p18 and p10 fragments which activate caspase-3 and caspase-7 (type I apoptosis) (Stennicke *et al*, 1998). Insufficient activation of caspase-3 leads to type II apoptosis in which caspase-8 cleaves the BH3-only protein BID (BH3 interacting domain death agonist) to generate its activated form: truncated BID (tBID) (Li *et al*, 1998). tBID stimulates intrinsic apoptotic pathway by binding directly binding to Bax/Bak inducing MOMP (type II apoptosis) (Desagher *et al*, 1999; Wei *et al*, 2000). The two pathways are cell line dependent, and their activation is differentially regulated by XIAP expression (Jost *et al*, 2009; Varfolomeev *et al*, 2009).

Granzyme-mediated apoptosis

Cytotoxic lymphoid cells (predominantly NK cells and cytotoxic T cells) can induce cell death via death receptor ligands (see above) or the granzyme/perforin system (Pardo *et al*, 2008; Martinez-Lostao *et al*, 2015). After recognition of transformed or infected cells, cytotoxic cells release secretory granules that contain perforin and granzyme B (Fig 1). These secreted factors are taken up by endocytosis and released to the cytosol by the perforin-dependent or -independent pathways (Voskoboinik *et al*, 2015). Once released to the cytosol, granzyme B cleaves caspases and Bid activating apoptotic pathways described above (Boivin *et al*, 2009). However, human granzyme B can also directly cleave ICAD, a known caspase-3 target, to induce DNA fragmentation, thereby circumventing the need of caspases (Thomas *et al*, 2000).

Necroptosis

Necroptosis is a regulated, pro-inflammatory, and caspase-independent form of necrotic cell death. Death receptors (DRs), toll-like receptors (TLRs), and nucleic acid sensing protein ZBP1 (Z-DNA binding protein 1) are prototypic inducer of necroptosis whose stimulation converges on the activation of RIP3 (receptor-interacting protein 3) (Newton & Manning, 2016). Following autophosphorylation, RIP3 engages the pseudokinase MLKL (mixed lineage kinase like) and phosphorylates it (Sun *et al*, 2012; Murphy *et al*, 2013). Activated MLKL oligomerizes and is transported to the cytoplasmic membrane, where it induces membrane permeabilization and cell death (Cai *et al*, 2014; Chen *et al*, 2014; Dondelinger *et al*, 2014; Samson *et al*, 2020). It is important to note that activation of MLKL is not the point of no return as several mechanisms have been found that can modulate necroptosis after MLKL activation, which are reviewed by Murphy (Murphy, 2020).

The key mediators for necroptosis contain a signature RHIM domain (RIP homotypic interaction motif): RIP1 is key for death receptor signaling (Sun *et al*, 2002), TRIF (TIR-domain-containing adapter inducing interferon-β) for toll-like receptors (Kaiser & Offermann, 2005), ZBP1 for virally encoded nucleic acid induced necroptosis (Kaiser *et al*, 2008), and RIP3 (Sun *et al*, 2002) as activator of MLKL.

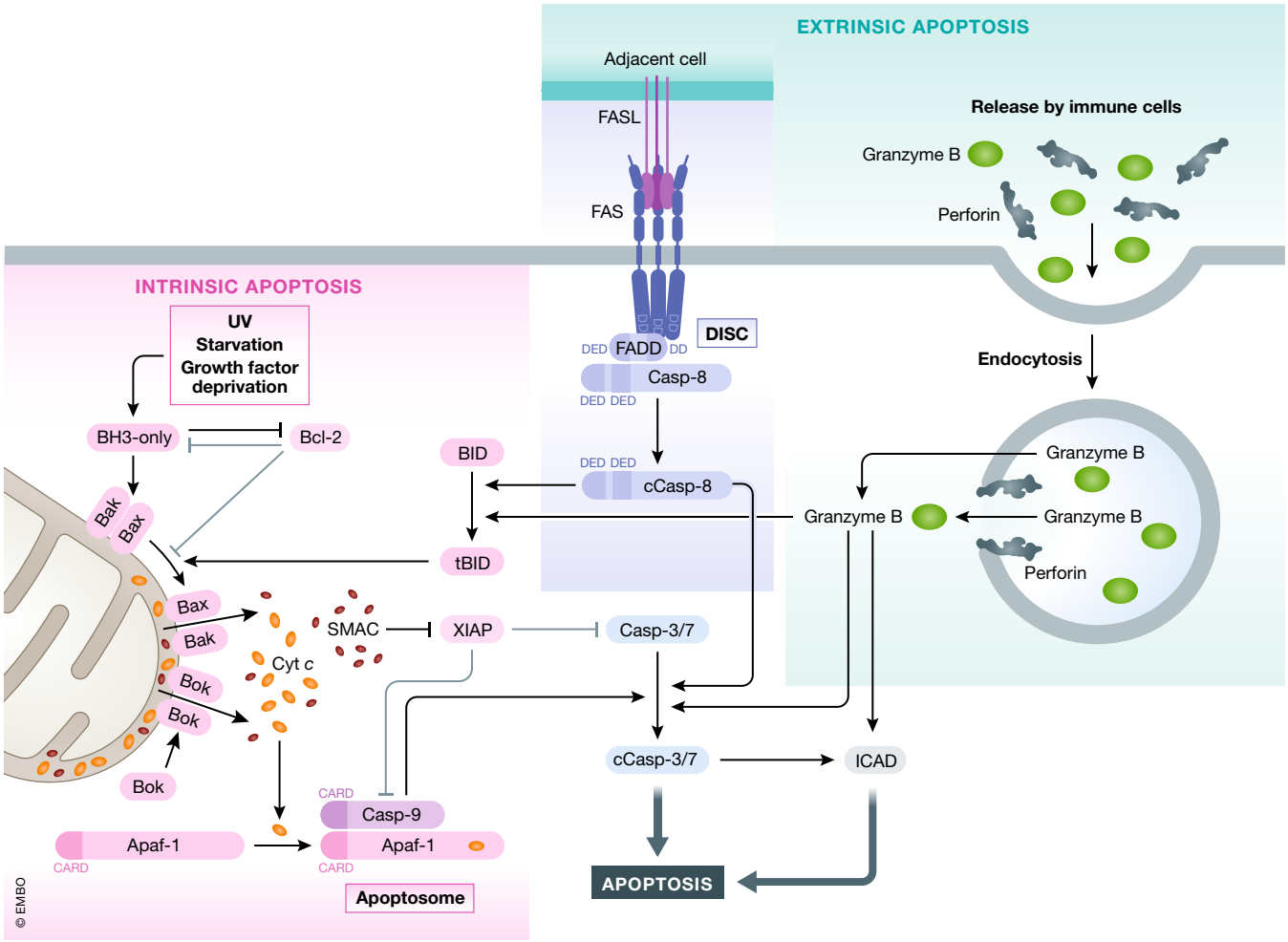

Figure 1. Intrinsic and extrinsic apoptosis.
Intrinsic apoptosis can be induced by various stimuli (e.g., GF (growth factor) withdrawal) by shifting the equilibrium of pro-survival Bcl-2 and BH3-only proteins. Sequestering of pro-survival Bcl-2 proteins or directed binding of BH3-only proteins to Bax and Bak induces oligomerization of Bax and Bak leading to MOMP and cytochrome c and Smac release. Bok can lead to MOMP independent of Bcl-2 family members. Released cytochrome c binds Apaf-1 and induces apoptosome formation which recruits caspase-9. Activated caspase-9 induces caspase-3/7 cleavage and activation (cCasp3/7) leading to apoptosis. Extrinsic apoptosis can be induced by binding of select group of TNF family ligands to, their receptors leading to DISC formation by recruitment of adapter FADD/TRADD and caspase-8. Caspase-8 autoprocesses itself (cCasp8—cleaved/activated caspase-8) and can directly activate caspase-3 or cleave Bid to generated tBid and triggers intrinsic apoptosis. Caspase-3/7/9 activity can be inhibited by XIAP, which itself can be antagonized by Smac. Lastly, apoptosis can also be induced by granzyme B and Perforin released form immune cells. Once taken up by the target cell, granzyme B can induces cell death by caspase-3 or by directly activating apoptosis effectors (e.g., CAD).

Necroptosis has been studied extensively based on the TNF receptor-mediated signaling (Fig 2). TNF binding to TNFR1 triggers the recruitment of RIP1, TRADD, TRAF2 (TNF receptor-associated factor 2), and c-IAP1/2 to the receptor-associated complex (complex I) (Newton, 2020). c-IAPs then ubiquitinate several proteins, including RIP1 and themselves, in complex I with K11- and K63-linked ubiquitin chains (Bertrand *et al*, 2008; Mahoney *et al*, 2008; Varfolomeev *et al*, 2008; Dynek *et al*, 2010; Moulin *et al*, 2012), resulting in LUBAC (linear ubiquitin chain assembly complex) recruitment, and addition of linear ubiquitin chains (Haas *et al*, 2009). The different ubiquitin chains serve as a platform for recruitment of the kinase complexes IKK and TAK1/TAB2/3 leading to the NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) and MAPK (mitogen-activated protein kinase) pathway activation (Hayden & Ghosh, 2014). These signaling events induce the expression of pro-survival and pro-inflammatory genes. Countering these effects are ubiquitin hydrolases, deubiquitinases, which restrict signaling by removing the different ubiquitin chains from the protein (Lork *et al*, 2017).

Once deubiquitinated, RIP1 is released into the cytosol, where it can be auto-activated by autophosphorylation. Interestingly, in mouse cells TNF-mediated activation of necroptosis can stimulate RIP1 autophosphorylation already in the TNFR1 complex (Newton *et al*, 2016b). Nevertheless, a protein complex composed of FADD, caspase-8, and c-FLIP (cellular FLICE-inhibitory protein) can bind RIP1 and regulate RIP1 necroptotic and apoptotic potential by

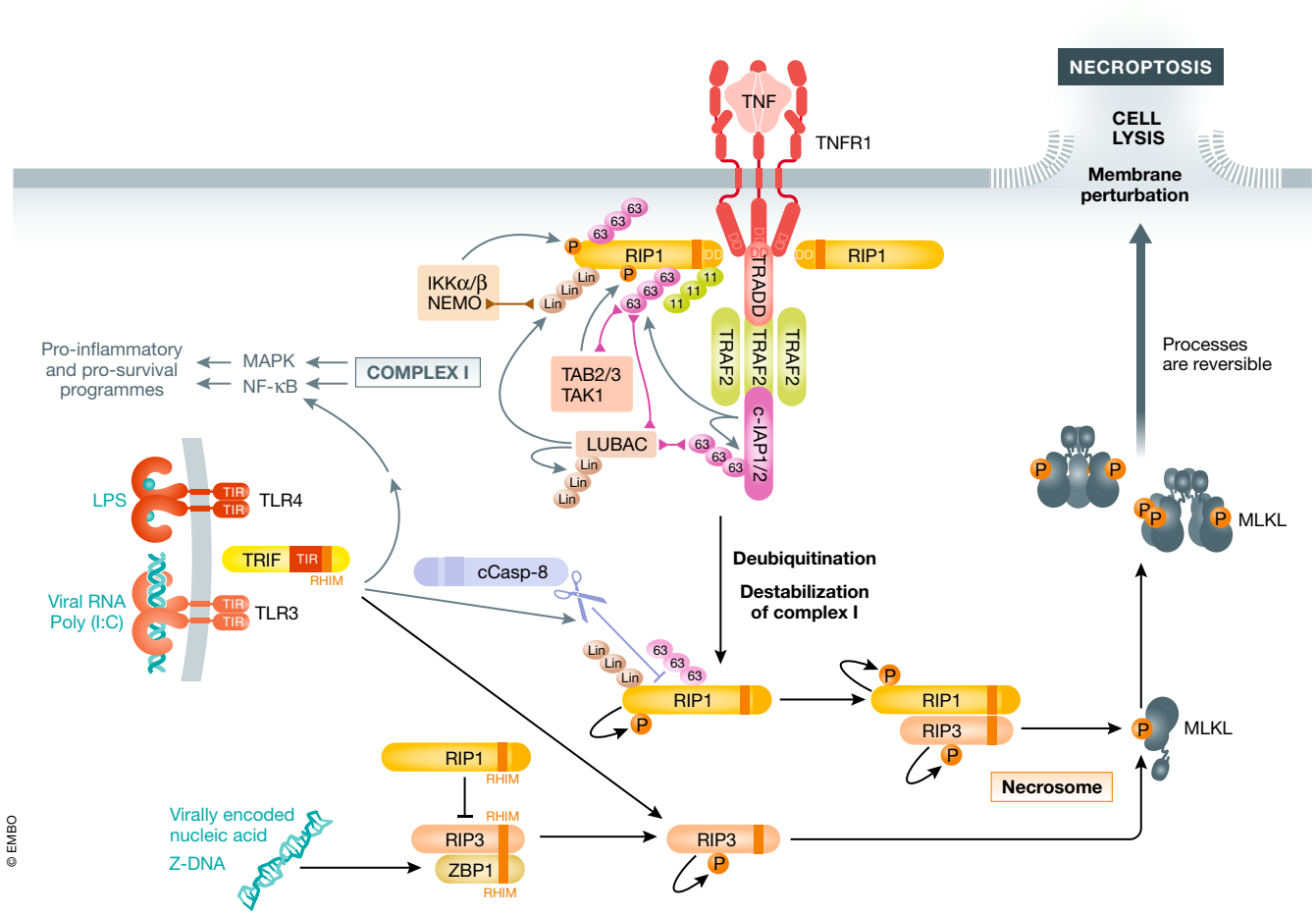

Figure 2. Necroptosis.
Necroptosis can be induced by different stimuli. Upon binding to its receptor, TNF induces complex formation leading to NF-κB and MAPK activation. Prolonged signaling and inhibition of caspases leads to RIP1 translocation to the cytosol forming complex II. RIP1 autophosphorylates recruiting RIP3. RIP3 phosphorylates itself as well as MLKL leading to MLKL oligomerization which induces membrane perturbation and cell lysis. LPS or Poly(I:C)-induced TLR3/4 signaling also can stimulate necroptosis through adaptor TRIF, which engages RIP1 or RIP3. Sensing of Z-DNA by ZBP1 leads to binding to RIP3 and cell death, which can be inhibited by RIP1.

caspase-8-mediated cleavage of RIP1 at D324 (Lin *et al*, 1999; Zhang *et al*, 2019; Newton *et al*, 2019a; Lalaoui *et al*, 2020). Stoichiometry of caspase-8 and c-FLIP isoforms determine if cells will survive or undergo apoptosis or necroptosis (discussed in more detail in another section). Only when caspase-8 is inhibited, depleted, or insufficiently activated, necroptotic signaling can proceed, leading to RIP1 binding to RIP3 and subsequent RIP3 autophosphorylation (Cho *et al*, 2009; He *et al*, 2009). RIP1 auto-activation and cell death induction can be restricted by inhibitory phosphorylation (e.g., on S321 of RIP1) (reviewed in (Delanghe *et al*, 2020).

The second key mediator for necroptosis induction is TRIF (Fig 2). Upon sensing of viral RNA (TLR3) or LPS (TLR4), TRIF can be recruited to activated TLR3/4 complexes via its TIR (Toll/interleukin-1 receptor) domain. TRIF can then induce NF-κB signaling via TRAF2/6-RIP1 axis, which leads to TNF expression (Kawasaki & Kawai, 2014). However, TRIF can also trigger apoptosis by engaging RIP1 and caspase-8 (McAllister *et al*, 2013) or necroptosis by directly activating RIP3 (Kaiser *et al*, 2013).

The fourth RHIM domain containing protein is ZBP1 or DAI (Fig 2). ZBP1 is activated by binding to viral Z-DNA or Z-RNA, a left-handed fold of DNA/RNA, to mediate immune response against certain viruses (reviewed by (Kuriakose & Kanneganti, 2018)). ZBP1 can directly bind RIP3 to induce necroptosis, while RIP1 inhibits necroptotic signaling by RHIM-RHIM domain interactions (Lin *et al*, 2016; Newton *et al*, 2016b). Most recently, ZBP1 activation has been linked to sensing endogenous Z-double-strand RNA and induction of necroptosis in the context of RIP1 RHIM mutation, epithelial cell-specific knockout of RIP1 (*Ripk1*$^{E\text{-}KO}$), or intestinal epithelial cell knockout of FADD (*Fadd*$^{IEC\text{-}KO}$) deficiency (Jiao *et al*, 2020).

Pyroptosis

Pyroptosis is a Gasdermin-dependent form of pro-inflammatory necrotic cell death. Stimulation of caspase-1/4/5/11 (caspase-4/5 are the human homologs to murine caspase-11) by different

inflammasome pathways lead to their activation by autoprocessing. Active caspases can then cleave Gasdermin-D (GSDMD) into its N-terminal and C-terminal domains, GSDMD-N and GSDMD-C (He *et al*, 2015; Kayagaki *et al*, 2015; Shi *et al*, 2015). GSDMD-C is the auto-inhibitory domain that inhibits the cell lytic properties of GSDMD-N (Kayagaki *et al*, 2015; Shi *et al*, 2015; Kuang *et al*, 2017). Once the two domains are separated by cleavage, GSDMD-N translocates to the membrane by binding to phosphatidylinositol phosphates and phosphatidylserine and oligomerizes inducing a lytic cell death by forming multi subunit pores (Aglietti *et al*, 2016; Ding *et al*, 2016; Liu *et al*, 2016; Sborgi *et al*, 2016). Besides targeting GSDMD, caspase-1 also processes the pro-inflammatory cytokines pro-IL-1β (interleukin 1β) and pro-IL-18 (interleukin 18) to their mature forms (Thornberry *et al*, 1992; Ghayur *et al*, 1997; Gu *et al*, 1997). Neither IL-1β nor IL-18 have a secretion sequence, so they are leaking through GSDMD pores (Evavold *et al*, 2018; Heilig *et al*, 2018).

Signal for GSDMD cleavage by caspases 1/4/5/11 is propagated from different inflammasomes and mediated by the canonical or the non-canonical inflammasome pathways (Fig 3). Generally, canonical inflammasomes consist of a sensor for DAMP (damage-associated molecular pattern) or PAMP (pathogen-associated molecular pattern) detection (AIM2, NLRP1, NLRP3, NLRC4, and Pyrin), which interact via Pyrin domains (PYD) with the bridging molecule ASC (apoptosis-associated speck-like protein containing a CARD). ASC then interacts with caspase-1 via CARD (caspase activation and recruitment domains) and oligomerizes caspase-1 to induce its activation (Martinon *et al*, 2002; Srinivasula *et al*, 2002; Chauhan *et al*, 2020).

Inflammasome activation is a two-step process. In a priming step, TLRs recognize PAMPs and induce a NF-κB and IFN type I (interferon type I)-dependent gene expression (Rathinam *et al*, 2012). This leads to upregulation of NLRP3 and caspase-11. The second step in the activation of non-canonical inflammasome signaling is triggered by intracellular LPS which binds murine caspase-11 or human caspase-4/5 leading to their activation (Kayagaki *et al*, 2011; Shi *et al*, 2014). Activated caspase-11 cleaves GSDMD to trigger pyroptosis (Kayagaki *et al*, 2015). The process of inflammasome activation is reviewed in more detail elsewhere (Kelley *et al*, 2019).

Besides GSDMD, other Gasdermins also have reported roles in cell death. For example, Gasdermin E (GSDME) has been shown to be cleaved by caspase-3 as well as granzyme B (Rogers *et al*, 2017; Wang *et al*, 2017; Lee *et al*, 2018). The interconnectivity of various cell death modalities will be discussed in more detail in following sections.

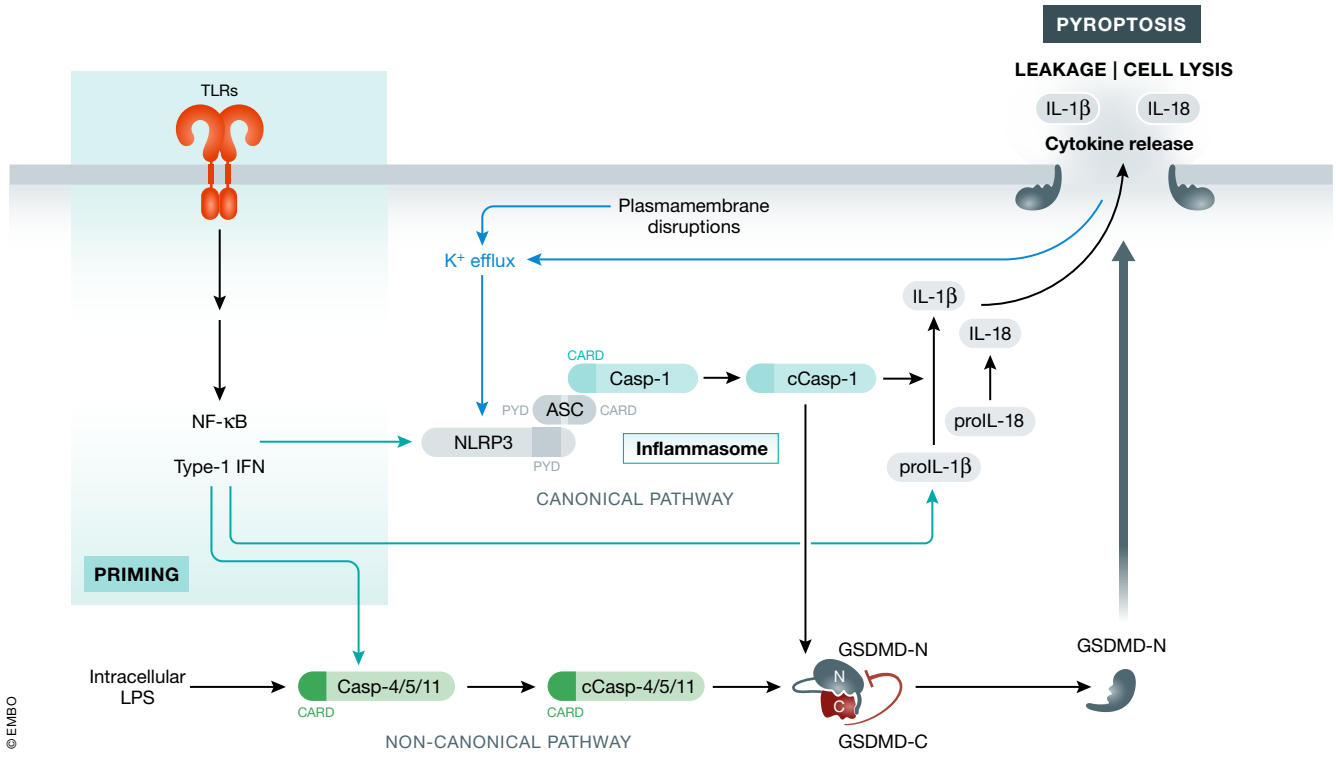

Figure 3. Pyroptosis.
Pyroptotic cell death can be induced by various stimuli that activate inflammasome. The activation of NLRP3 prompts its binding to ASC and caspase-1 forming the inflammasome. Caspase-1 processes pro-IL-1β and pro-IL-18 to their active forms. In parallel, caspase-1 cleaves GSDMD separating the inhibitory C- and active N-terminal domains. GSDMD-N then translocates to the membrane inducing cell lysis and cytokine release. Non-canonical inflammasome activation consists of priming which induces expression of several pathway genes (caspase-11). Intracellular LPS can be sensed by caspase-11 leading to its activation and processing of GSDMD and caspase-1. Cell lysis can then also activate canonical inflammasome signaling. Green arrows indicate upregulation by gene expression, while black arrows indicate interactions/cleavage events or inhibition.

Other forms of regulated cell death

Other forms of cell death have been identified in the recent years. While some signaling components and pathways are already well understood, others still need further research. The key characteristics of each cell death pathway described below are summarized in Fig 4.

Autophagy-dependent cell death (ADCD)

Autophagy defines the degradation of cellular components by the lysosomal pathway. This can include organelles, like mitochondria and ER, as well as other cytoplasmic content. Autophagy is a critical process for cellular homeostasis and knockout of autophagy mediators often results in perinatal lethality (Kuma *et al*, 2017). The process of autophagy can be divided into several steps which are regulated by individual protein complexes. The most common way for autophagy activation is starvation, which initiates autophagy by a kinase complex consisting of Ulk1-ATG13/101 and FIP200 (Zachari & Ganley, 2017). After initiation of autophagy, nucleation, elongation, and phagophore formation are regulated by different protein complexes. The phagophore is labeled with LC3 to eventually mature to the autophagosome. Upon fusion of those vesicles with lysosomes, the autolysosome is formed and the content is degraded. A detailed description of the process can be found in the following review (Dikic & Elazar, 2018). Interestingly, most of the pathway components seem to have additional non-autophagic functions (reviewed in (Galluzzi & Green, 2019)). Autophagy-dependent cell death (ADCD) has been defined as a form of cell death that relies exclusively on the autophagic pathway components, which is an important distinction given that autophagy can also coincide with other forms of cell death (Galluzzi *et al*, 2018). ADCD can proceed by two different pathways. The first pathway is induced by extensive degradation of organelles which is dependent on the

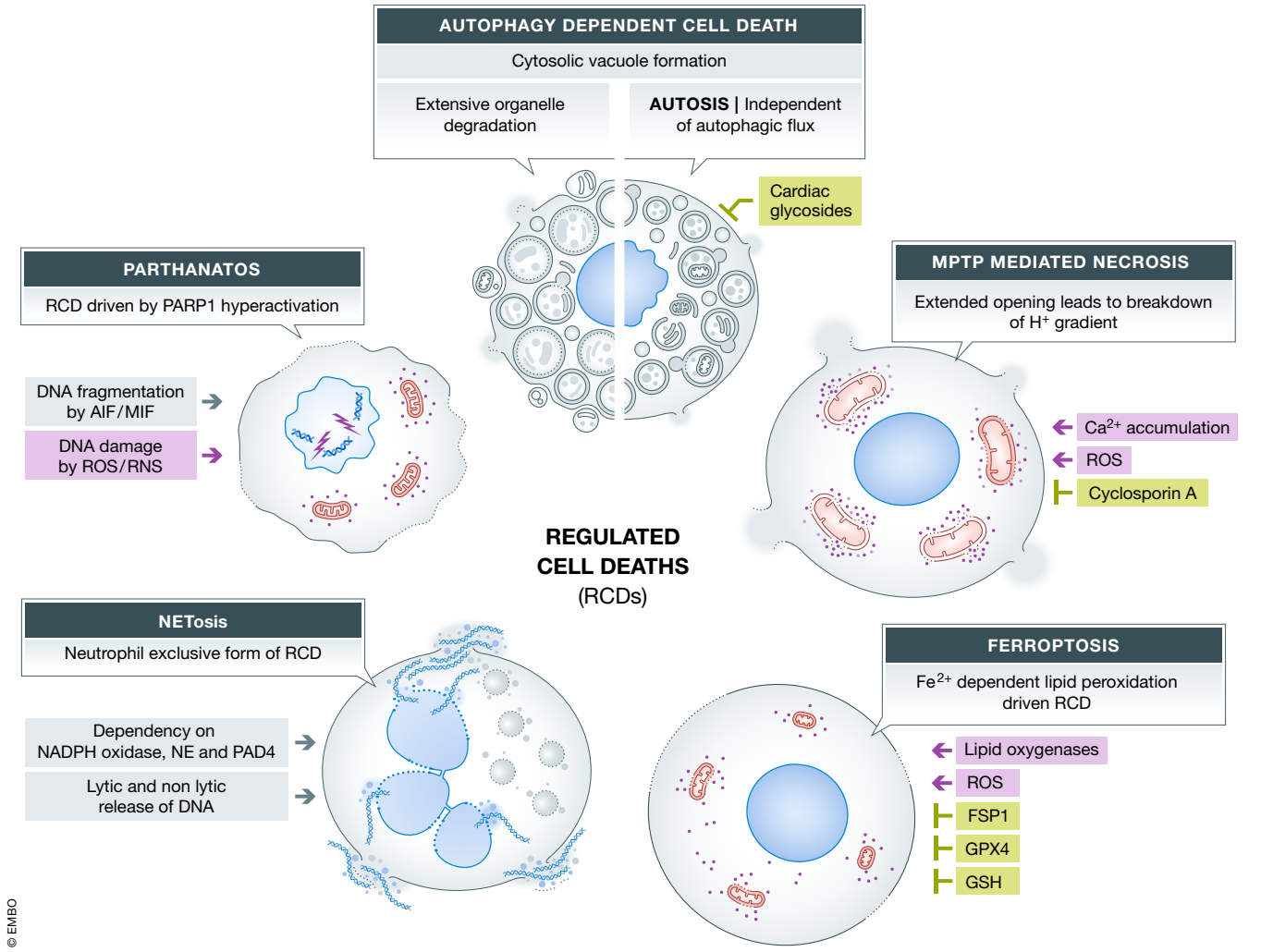

Figure 4. Key features of different forms of regulated cell death.
Overview of regulated cell death pathways highlighting the stimuli, key features as well as positive and negative regulators of the pathways. Gray boxes indicate cell death pathway, green boxes show negative regulators/inhibitors, while red boxes indicate activators.

autophagic flux (Dasari *et al*, 2017). The second form, referred to as Autosis, does not depend on the fusion of autophagosomes and lysosomes (Liu *et al*, 2013). In both cases, vacuole formation in the cytoplasm can be detected (Bialik *et al*, 2018). Treatment of cancer cells with resveratrol triggers the autophagic flux-dependent ADCD, without activating apoptosis or necroptosis (Dasari *et al*, 2017). The massive degradation by lysosome fusion leads to a breakdown of the cytoplasmic organization with loss of organelles such as endoplasmic reticulum or mitochondria. Autosis can be induced by treatment with TAT-Beclin-1 peptides, starvation or hypoxia, which leads to cell swelling and eventually rupture of the plasma membrane. These conditions result in cell death mediated by Na^+/K^+-ATPase and can be inhibited by cardiac glycosides (Liu *et al*, 2013). Autotic cells were also identified in samples of patients with severe anorexia nervosa (Kheloufi *et al*, 2015). In general, ADCD has been shown in association with physiological process as well as various pathologies including reperfusion injuries and various forms of cancer (Bialik *et al*, 2018; Denton & Kumar, 2019).

Mitochondrial permeability transition pore (MPTP)-mediated necrosis

The mitochondrial permeability transition pore can mediate necrosis based on changes in the intracellular microenvironment. Two factors that can induce opening of the pores are oxidative stress and cytosolic/ mitochondrial Ca^{2+} accumulation. The pores allow the flux of molecules up to 1.5 kDa in size leading to breakdown of the H^+ gradient and subsequently halting the ATP synthesis (Lemasters *et al*, 2009; Izzo *et al*, 2016). The pore opening has been shown to be reversible and meant to regulate mitochondrial Ca^{2+} levels while prolonged opening induces cell death (Baines *et al*, 2005; Korge *et al*, 2011). Cyclophilin D (CypD) so far is the only protein that has been shown to be critical for MPTP *in vivo* and *in vitro*. Accordingly, *Ppif*$^{-/-}$ mice (gene coding for CypD) showed reduced infarct size after ischemia/reperfusion injury of heart or brain (Baines *et al*, 2005; Nakagawa *et al*, 2005; Schinzel *et al*, 2005). In addition, mitochondria isolated from *Ppif*$^{-/-}$ showed a reduced swelling upon treatment with Ca^{2+}. Cyclosporin A, a MPTP inhibitor, did not show an additional effect in knockout mitochondria indicating CypD as the target (Basso *et al*, 2005; Nakagawa *et al*, 2005; Schinzel *et al*, 2005). Yet, another study detected pore opening in mitochondria lacking CypD with increasing Ca^{2+} concentration, which indicates that blocking CypD might not be sufficient (Basso *et al*, 2005). So far, the structure and components of the pore are still not completely known (Nesci, 2020), but the F_1F_0 ATP synthase has been shown to be part of it (Bonora *et al*, 2013; Giorgio *et al*, 2013). These findings also suggest the F_1F_0 ATP synthase may be a potential drug target for various pathologies, such as myocardial infarct, reperfusion injuries, and neurodegenerative diseases (Sileikyte & Forte, 2019).

Parthanatos

Parthanatos is a form of regulated cell death dependent on poly (ADP) ribose polymerase 1 (PARP1) (Andrabi *et al*, 2008). PARP1 is part of the DNA repair machinery which binds DNA single breaks and PARylates itself and other proteins to recruit other components of the machinery (reviewed in (Ray Chaudhuri & Nussenzweig, 2017)). Severe DNA damage by prolonged generation of reactive oxygen species or reactive nitrogen species (RNS) induces recruitment and activation of PARP1 to the DNA (Zhang *et al*, 1994) leading to the formation of PAR polymers and depletion of NAD^+ and ATP, which might be fatal for the cell (Robinson *et al*, 2019). However, extensive generation of PAR polymers can promote AIF (apoptosis inducing factor mitochondria associated 1) -MIF (macrophage migration inhibitory factor) interaction to facilitate MIF catalyzed DNA fragmentation (Yu *et al*, 2002; Wang *et al*, 2016). Nevertheless, a PARP-dependent cell death driving retinal degradation *in vivo* can be AIF-independent (Jang *et al*, 2017). RNS associated with parthanatos have been show to play a role in neural pathologies (Virag & Szabo, 2002; Fatokun *et al*, 2014).

NETosis

Neutrophils are part of the innate immune system, and their main task is to neutralize pathogens by phagocytosis or degranulation (Segal, 2005). Another form of host defense is the formation of NET (neutrophil extracellular traps). NETosis describes the process of neutrophil DNA release into the extracellular space (Brinkmann *et al*, 2004). The release of neutrophil DNA containing different proteins with anti-pathogenic activity can be associated with cell death, but can be independent of it as well (Yipp & Kubes, 2013; Yousefi *et al*, 2019). For both processes, NADPH oxidase and ROS, including mitochondrial ROS, have been reported to be critical for actin depolarization and NET release (Papayannopoulos *et al*, 2010; Douda *et al*, 2015; Stojkov *et al*, 2017). Elevated ROS leads to myeloperoxidase activation, which leads to activation of neutrophil elastase (NE) (Papayannopoulos *et al*, 2010). NE associates with actin and processes it thus leading to depolarization and loss of actin dynamics (Metzler *et al*, 2014). Subsequently, NE can translocate to the nucleus, cleaves histones, and nuclear envelope proteins (Papayannopoulos *et al*, 2010). In combination with histone processing, histones are also citrullinated by PAD4 (protein arginine deiminase 4), which leads to chromatin decondensation and eventually NET release (Wang *et al*, 2009; Thiam *et al*, 2020). A detailed discussion of the pathway can be found in this review (Papayannopoulos, 2018). Further research is needed to understand the molecular details of non-lytic and lytic forms of NET formation. The role of other forms of cell death and autophagy in the context of NETosis will be discussed below.

Ferroptosis

Ferroptosis is a form of regulated cell death that depends on iron (Fe^{2+})-mediated lipid peroxidation induced by ROS (Yang & Stockwell, 2008; Dixon *et al*, 2012). Reactive oxygen species are constantly generated by different physiological processes. In combination with cellular labile iron, this can lead to ferroptosis (Snezhkina *et al*, 2019). Fe^{2+} can act as a catalyst to convert H_2O_2 to $OH^{\bullet}$ radicals (Fenton reaction) which react with polyunsaturated fatty acids. Further, Fe^{2+} is a cofactor for lipoxygenases which catalyze the generation of lipid hydroperoxides (Yang *et al*, 2016;

Stoyanovsky *et al*, 2019). To protect cells from ROS, hydroperoxides are neutralized stepwise by different enzyme families, namely superoxide dismutases, glutathione peroxidases, catalases, and peroxiredoxins. Ferroptotic cell death can be induced by either increased ROS generation or dysregulation of ROS neutralization (Li *et al*, 2020). Ferroptosis can be negatively regulated by glutathione and GPX4 (glutathione peroxidase 4). Cystine uptake is critical for glutathione synthesis, and interference with its transporter (X_c^- Cys/Glu anti-porter) induces ferroptosis by indirectly reducing GPX4 activity (Dixon *et al*, 2012). Loss of GPX4 resulted in sensitization to ferroptosis *in vitro* and *in vivo* (Friedmann Angeli *et al*, 2014; Yang *et al*, 2014). Besides a glutathione dependent system, ferroptosis suppressor protein 1 (FSP1) was identified to protect cells from ferroptosis by reducing coenzyme Q10, which acts as a radical scavenger (Bersuker *et al*, 2019; Doll *et al*, 2019). Ferroptosis has been associated with several pathologies as reviewed elsewhere (Bebber *et al*, 2020; Belavgeni *et al*, 2020; Li *et al*, 2020).

The interplay of cell death pathways

Caspase-8: Between cell survival, apoptosis, and necroptosis

Caspase-8 was initially identified as a component of extrinsic apoptotic signaling platform DISC (death inducing signaling complex) (Boldin, 1996; Muzio *et al*, 1996) and later as part of the cytosolic TNF-induced complex II (Micheau & Tschopp, 2003). Soon, it became obvious that caspase-8 has a more complex role, especially in the regulation of other cell death pathways (Degterev *et al*, 2005) (Fig 5).

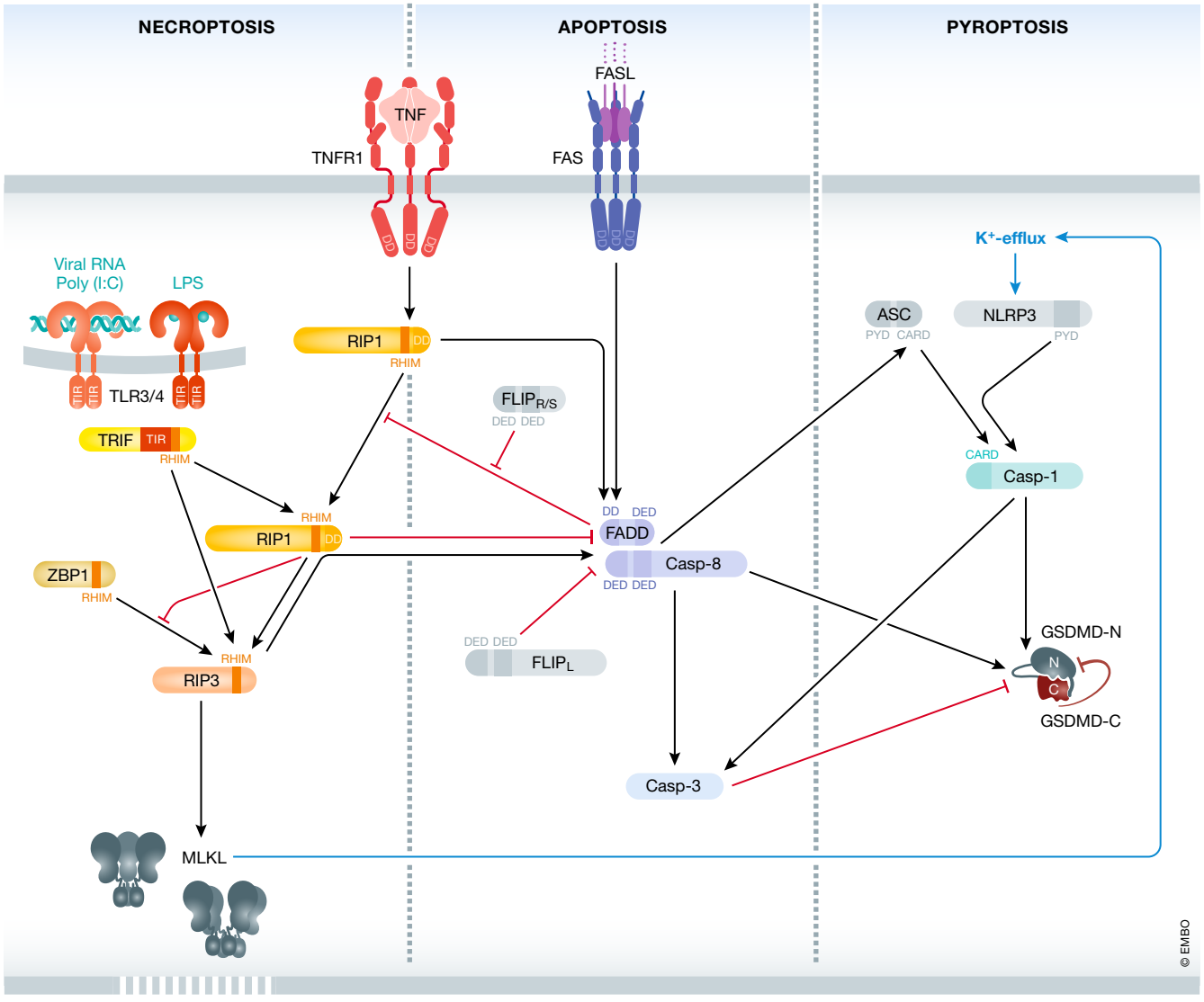

Figure 5. Crosstalk between different forms of cell death.
A more complex network between the different cell death pathways has been established over the years. Arrows indicate established interconnectivity between apoptosis, necroptosis, and pyroptosis. For details, please refer to the main text. Black arrows indicate crosstalk events, while red arrows indicate inhibition.

Caspase-8 lethality can be rescued by deletion of RIP3 or MLKL (Kaiser *et al*, 2011; Oberst *et al*, 2011), which indicated that caspase-8 restricts necroptotic signaling. The enzymatic activity of caspase-8 determines if cells survive or die via apoptosis or necroptosis. One way of regulating catalytic activity of caspase-8 in complex II is achieved by its catalytically inactive paralog c-FLIP (Goltsev *et al*, 1997; Han *et al*, 1997; Hu *et al*, 1997; Inohara *et al*, 1997; Irmler *et al*, 1997; Shu *et al*, 1997; Srinivasula *et al*, 1997; Rasper *et al*, 1998). Low levels of c-FLIP$_L$ lead to the formation of caspase-8 homodimers resulting in self-processing and apoptosis (Hughes *et al*, 2016). Pharmacological inhibition of caspase-8 (e.g., by zVAD-FMK or Emricasan) or inhibition by FLIP(S/R) leads to necroptosis and RIP1 activation (Fricker *et al*, 2010). Besides the cellular isoforms of FLIP, some viruses carry their own versions of FLIP (viral FLIP—vFLIP), which can inhibit caspase-8 (Thome *et al*, 1997). Overexpression of vFLIP MC159 in combination with IAP antagonist induces necroptosis (Feoktistova *et al*, 2012). Heterodimers of c-FLIP and caspase-8 partially activate caspase-8 and restrict necroptosis by cleaving RIP1 (at Asp324 human or at Asp325 mouse RIP1) (Oberst *et al*, 2011; Pop *et al*, 2011). Cleavage of RIP1 is critical for cell homeostasis, and several patients have been identified with heterozygous mutation at the caspase-8 cleavage site resulting in a disease called cleavage-resistant RIP1-induced auto-inflammatory (CRIA) syndrome (Lalaoui *et al*, 2020; Tao *et al*, 2020). CRIA patients suffer periodic fever with elevated cytokine and chemokine levels (Lalaoui *et al*, 2020; Tao *et al*, 2020). Analogous mutation in mice drew a similar picture, with *Ripk1*$^{D325A/D325A}$ animals being embryonic lethal (Zhang *et al*, 2019; Newton *et al*, 2019a; Lalaoui *et al*, 2020) and heterozygous animals being sensitive in TNF-induced systemic inflammatory response syndrome (SIRS) (Newton *et al*, 2019a).

Interestingly, RIP1 also restricts caspase-8-mediated cell death as loss of RIP1 sensitized to TNF-induced apoptosis (Dillon *et al*, 2014; Rickard *et al*, 2014). In addition, RIP1 restricts ZBP1-mediated necroptosis by interfering with RHIM-mediated interaction of ZBP1 and RIP3 (Lin *et al*, 2016; Newton *et al*, 2016b). The interplay of apoptosis and necroptosis is apparent in many inflammatory *in vivo* models where they are frequently concomitantly activated (Webster & Vucic, 2020). This is not surprising given that majority of signaling proteins are common to both pathways, and the balance of expression or activation of critical factors (RIP3, caspase-8) can tip the balance in favor of apoptosis or necroptosis.

The addition of pyroptosis to the crosstalk

Caspases are involved in apoptotic, necroptotic, and pyroptotic signaling, and in recent years, a more complex interconnectivity has been revealed. As mentioned before, caspase-3 can cleave GSDME, and there are conflicting reports of GSDME playing a role during secondary necrosis of apoptotic macrophages (Rogers *et al*, 2017; Lee *et al*, 2018). GSDME has also been implicated in mitochondrial pore formation and enhancement of apoptotic signaling (Rogers *et al*, 2019). GSDMD forms pores at the mitochondrial membranes preceding plasma membrane rupture (de Vasconcelos *et al*, 2019). Caspase-3 can inactivate GSDMD by cleavage at Asp84 though the physiological meaning remains unknown (Rogers *et al*, 2017; Taabazuing *et al*, 2017). On the other hand, caspase-1 has been reported to induce apoptosis by cleaving caspase-3 in *Gsdmd*$^{-/-}$ cells (Tsuchiya *et al*, 2019).

Apart from mediating the activation of apoptotic cell death and regulation of necroptosis, caspase-8 was found to be important for pyroptotic signaling. Several studies provided evidence that caspase-8 plays a central role inducing cell death, inflammasome activation, and IL-1β/ IL-18 processing in *Yersinia* infection models (Weng *et al*, 2014; Orning *et al*, 2018; Sarhan *et al*, 2018). Caspase-8 has been shown to activate GSDMD by cleavage, which resulted in K^+-efflux inducing NLRP3 inflammasome activation (Orning *et al*, 2018; Sarhan *et al*, 2018). In this setting, *Asc*$^{-/-}$ or *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ cells showed reduced IL-1β release without altering cell death. On the other hand, caspase-8 can be recruited to the inflammasome. Cytosolic DNA and nigericin were able to induce caspase-8 activation and apoptosis in an ASC-dependent fashion (Sagulenko *et al*, 2013). Further studies from Sagulenko *et al* showed that caspase-1/11 can process caspase-3 during DNA transfection when caspase-8 is deleted (Sagulenko *et al*, 2018). IAP antagonism or deletion of XIAP, cIAP1, and cIAP2 led to release of IL-1β in LPS primed cells in a process that was also dependent on caspase-1, caspase-8, and RIP3 (Vince *et al*, 2012). The interaction of caspase-8 and ASC is mediated by DED1 and DED2 of caspase-8 and PYD of ASC, as was shown with recombinant proteins as well as overexpression studies (Vajjhala *et al*, 2015). This heterotypic interaction also has been shown to be critical for NLRC4-induced apoptotic signaling when cells do not express caspase-1 (Lee *et al*, 2018).

Besides caspase-8, RIP1 inactivation (*Ripk1*$^{KD/KD}$—RIP1 kinase-dead bone marrow derived macrophages (BMDM)) can reduce cell death induced by *Yersinia* infection or LPS + TAK1 inhibitor (Peterson *et al*, 2017; Sarhan *et al*, 2018). Reduced GSDMD cleavage comparable to *Casp8*$^{-/-}$ *Ripk3*$^{-/-}$ BMDMs was also detected by Orning *et al* in *Ripk1*$^{KD/KD}$ BMDMs (Orning *et al*, 2018). This indicates that not only caspase-8 but probably RIP1 also plays an important role in mediating pyroptosis. This is underlined by another publication that implicated FADD in the regulation of inflammasome activation. *C. rodentium* infection of *Fadd*$^{-/-}$ *Ripk3*$^{-/-}$ BMDMs lead to reduced processing of caspase-1/8, IL-1β secretion, and cell death compared to WT or *Ripk3*$^{-/-}$ BMDMs (Gurung *et al*, 2014). Necroptotic cell death also leads to NLRP3 activation by K^+ efflux mediated by MLKL in cell intrinsic manner (Conos *et al*, 2017). *In vivo*, reduced IL-1β was detected in *Fadd*$^{-/-}$ *Ripk3*$^{-/-}$ mice compared to WT or *Ripk3*$^{-/-}$ animals when treated with LPS, but increased bacterial load when infected with *C. rodentium* (Gurung *et al*, 2014). Similar findings were made for *Fadd*$^{-/-}$ *Mlkl*$^{-/-}$ BMDMs and mice. Treatment with LPS + ATP resulted in reduced caspase-1 cleavage and IL-1β secretion. *In vivo* challenge with LPS resulted in decreased IL-1β serum levels in *Fadd*$^{-/-}$ *Mlkl*$^{-/-}$ mice (Zhang *et al*, 2016). However, bacterial infection of *Casp8*$^{-/-}$ *Ripk3*$^{-/-}$ mice resulted in higher morbidity compared to WT mice. These DKO mice showed reduced cytokine levels, but increased bacterial load indicating that a proper clearance was not achieved (Weng *et al*, 2014). The same finding was made in *Ripk1*$^{KD/KD}$ mice when they were infected with *Yersinia* by oral gavage (Peterson *et al*, 2017). During LPS-induced shock, *Casp11*$^{-/-}$ and *Casp8*$^{-/-}$ *Ripk3*$^{-/-}$ mice showed reduced morbidity compared to WT mice, due to the involvement of TNF-mediated signaling in LPS-mediated shock (Mandal *et al*, 2018). The described findings could be explained by the fact that bacterial infection is a long-term challenge, which becomes worse with a defective clearance. For the

LPS challenge, reduced cytokines result in healthier animals as they do not have to fight an infection.

Further understanding of caspase-8 in the context of pyroptosis was gained by analysis of various transgenic mouse models (Fritsch *et al*, 2019; Newton *et al*, 2019b; Schwarzer *et al*, 2020; Tummers *et al*, 2020) (Fig 5, Table 1). Mice expressing catalytic-dead caspase-8, *Casp8*$^{C362A/C362A}$ (Newton *et al*, 2019a) and *Casp8*$^{C362S/C362S}$ (Fritsch *et al*, 2019), are embryonic lethal at E11.5, just like *Casp8*$^{-/-}$ mice (Varfolomeev *et al*, 1998). While *Casp8*$^{-/-}$ mice can be rescued by loss of RIP3 or MLKL, *Mlkl*$^{-/-}$ only delayed lethality of *Casp8*$^{C362A/C362A}$ to birth and *Ripk3*$^{-/-}$ in some of the animals to after weaning. Interestingly, processing of caspase-3, caspase-7, and cleavage of RIP1 and RIP3 was detectable in caspase-1-dependent manner in intestines of *Casp8*$^{C362A/C362A}$ *Mlkl*$^{-/-}$ E18.5 embryos (Newton *et al*, 2019b). This might draw a link to papers reporting caspase-1-mediated apoptosis in *Gsdmd*$^{-/-}$ (Tsuchiya *et al*, 2019). Fritsch *et al* and Newton *et al* showed ASC specks in the intestine of E18.5 *Casp8*$^{C362A/C362A}$ *Mlkl*$^{-/-}$ *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ embryos or 5-week-old *Casp8*$^{C362S/C362S}$ *Mlkl*$^{-/-}$ *Casp1*$^{-/-}$ mice suggesting that caspase-8 can induce ASC-dependent inflammasome activation (Fritsch *et al*, 2019; Newton *et al*, 2019b). Deletion of ASC in *Casp8*$^{C362A/C362A}$ *Mlkl*$^{-/-}$/*Casp8*$^{C362S/C362S}$ *Mlkl*$^{-/-}$ mice *lead* to survival beyond weaning comparable to *Casp8*$^{C362A/C362A}$ *Mlkl*$^{-/-}$ *Casp1*$^{-/-}$/*Casp8*$^{C362S/C362S}$ *Mlkl*$^{-/-}$ *Casp1*$^{-/-}$ (Fritsch *et al*, 2019; Newton *et al*, 2019b). Combined loss of caspase-1/11 in *Casp8*$^{C362A/C362A}$ *Mlkl*$^{-/-}$ mice had an additional protective effect suggesting an interplay of the different cell death signaling pathways. Most mice survived when RIP3 and caspase-1/11 were deleted in *Casp8*$^{C362A/C362A}$ (Newton *et al*, 2019b).

Table 1. Genotypes and phenotypes of cell death mediator mutations

Genotype	Phenotype
Casp8$^{C362A/C362A}$	Embryonic lethal (E12.5)
Casp8$^{C362A/C362A}$ *Mlkl*$^{-/-}$	Perinatal lethal
Casp8$^{C362A/C362A}$ *Ripk3*$^{-/-}$	75% of animals survive past weaning
Casp8$^{C362A/C362A}$ *Mlkl*$^{-/-}$ *Ripk1*$^{-/-}$	Lethal before weaning
Casp8$^{C362A/C362A}$ *Mlkl*$^{-/-}$ *Asc*$^{-/-}$	65% of animals survive past weaning
Casp8$^{C362A/C362A}$ *Mlkl*$^{-/-}$ *Casp1*$^{-/-}$	40% of animals survive past weaning
Casp8$^{C362A/C362A}$ *Mlkl*$^{-/-}$ *Casp1*$^{-/-}$ *Casp11*$^{-/-}$	75% of animals survive past weaning
Casp8$^{C362A/C362A}$ *Ripk3*$^{-/-}$ *Casp1*$^{-/-}$ *Casp11*$^{-/-}$	Survival past weaning, best survival
Casp8$^{C362S/C362S}$	Embryonic lethal (E12.5)
Casp8$^{C362S/C362S}$ *Mlkl*$^{-/-}$	Perinatal lethal
Casp8$^{C362S/C362S}$ *Mlkl*$^{-/-}$ *Casp1*$^{-/-}$	Survival beyond parturition
Casp8$^{C362S/C362S}$ *Mlkl*$^{-/-}$ *Asc*$^{-/-}$	Survival beyond parturition
Casp8$^{D387A/D387A}$	No overt phenotype
Casp8$^{D387A/D387A}$ *Mlkl*$^{-/-}$	Inflammatory phenotype
Casp8$^{D387A/D387A}$ *Mlkl*$^{-/-}$ *Fasl*$^{+/-}$	Rescues phenotype
Casp8$^{D387A/D387A}$ *Mlkl*$^{-/-}$ *Ripk1*$^{+/-}$	Rescues phenotype
Casp8$^{D387A/D387A}$ *Mlkl*$^{-/-}$ *Fadd*$^{+/-}$	Rescues phenotype
Casp8$^{D387A/D387A}$ *Mlkl*$^{-/-}$ *Fadd*$^{-/-}$	Lethal before weaning
Casp8$^{D387A/D387A}$ *Mlkl*$^{-/-}$ *Fadd*$^{-/-}$ *Ripk1*$^{-/-}$	Survival past weaning
Casp8$^{D387A/D387A}$ *Mlkl*$^{-/-}$ *Fadd*$^{-/-}$ *Casp1*$^{-/-}$	Survival past weaning
Casp8$^{IEC\text{-}KO}$	Ileitis
Casp8$^{IEC\text{-}KO}$ *Mlkl*$^{-/-}$	Rescues phenotype
Fadd$^{IEC\text{-}KO}$	Colitis and ileitis
Fadd$^{IEC\text{-}KO}$ *Ripk3*$^{-/-}$	Rescues phenotype
Fadd$^{IEC\text{-}KO}$ *Ripk3*$^{IEC\text{-}KO}$	Rescues phenotype
Fadd$^{IEC\text{-}KO}$ *Mlkl*$^{-/-}$	Casp8-driven pathology
Fadd$^{IEC\text{-}KO}$ *Mlkl*$^{-/-}$ *ASC*$^{-/-}$	Casp8-driven pathology
Fadd$^{IEC\text{-}KO}$ *Mlkl*$^{-/-}$ *Gsdmd*$^{-/-}$	Rescues phenotype

Summary of genotypes and main associated phenotypes discussed in the context of crosstalk between different cell death pathways. Please refer to the original publications for a complete list of crosses and their respective phenotypes. References for listed genotypes are indicated throughout the text.

Caspase-8 autoprocessing is key for it is full activation but mutagenesis of the cleavage site D387 to alanine, which separates the small and large catalytic subunit, did not cause a developmental phenotype (Philip *et al*, 2016; Newton *et al*, 2019a; Tummers *et al*, 2020). However, a reduced morbidity was observed in *in vivo* challenge with CD95. Crossing of *Casp8*$^{D378A/D378A}$ (from now on Casp8$^{DA/DA}$) to *Mlkl*$^{-/-}$ mice resulted in inflammation and splenomegaly as well as hypersensitivity to LPS injection (Tummers *et al*, 2020). The inflammatory phenotype of *Casp8*$^{DA/DA}$ *Mlkl*$^{-/-}$ mice was rescued by deletion of one allele of FADD, RIP1, or FASL (Tummers *et al*, 2020). Interestingly, *Casp8*$^{DA/DA}$ *Mlkl*$^{-/-}$ *Fadd*$^{-/-}$ died within 14 days after birth indicating that FADD has a bivalent role in this model. Lethality of *Casp8*$^{DA/DA}$ *Mlkl*$^{-/-}$ *Fadd*$^{-/-}$ mice was rescued by crossing to *Casp1*$^{-/-}$ or to *Ripk1*$^{-/-}$ mice but ASC specks were again revealed in ileal tissue (Tummers *et al*, 2020). Another study focused on ileitis and colitis driven by deletion of either FADD or caspase-8 in the intestinal epithelial cells (IEC) (Schwarzer *et al*, 2020). Caspase-8-induced pathologies were rescued by deletion of *Mlkl*$^{-/-}$; however, MLKL ablation was not significantly protective in *Fadd*$^{IEC\text{-}KO}$ mice (Schwarzer *et al*, 2020). Interestingly, *Fadd*$^{IEC\text{-}KO}$ ileitis was milder in *Ripk3*$^{IEC\text{-}KO}$ or in RIP1 kinase-dead knock-in mice (Schwarzer *et al*, 2020) and completely inhibited by whole body RIP3 ablation (Welz *et al*, 2011). But only the deletion of GSDMD, not of ASC, resulted in loss of the inflammatory phenotype in *Fadd*$^{IEC\text{-}KO}$ *Mlkl*$^{-/-}$ (Schwarzer *et al*, 2020).

In all caspase-8 knock-in models or *Fadd*$^{IEC\text{-}KO}$, RIP1 deletion or kinase inhibition resulted in attenuated phenotypes arguing that RIP1 plays important role in caspase-8-associated pathologies. Nevertheless, a large body of published work shows a complex role of caspase-8 in regulation of apoptosis, necroptosis, and pyroptosis. Thus, incompletely activated (either enzymatic dead or cleavage-resistant) caspase-8 in the context of *Mlkl*$^{-/-}$ can provide a scaffold for ASC binding and induce ASC-dependent inflammasome formation. In *Fadd*$^{IEC\text{-}KO}$ *Mlkl*$^{-/-}$ mice, it seems more likely that caspase-8 cleaves GSDMD to induce pyroptosis. These findings exemplify the complexity in the signaling crosstalk between different cell death pathways and raise additional questions concerning other pathway components and their role. For example, what is the role of apoptosis induced by caspase-3 in those pathologies? What are the proteins or pathways regulated by RIP3? Clearly, future studies are needed to answer these questions.

Autophagy during other forms of cell death

ADCD is defined as a form of cell death that does not share features with other forms of cell death. However, autophagy has been shown to coincide various forms of cell death. In the following, the role of autophagy-related genes in the context of other forms of cell death will be discussed.

Apoptotic and autophagic pathways can intercross at various levels. Most $Bax^{-/-}$ $Bak^{-/-}$mice die perinatally and show interdigital skin and increased white blood cells besides other phenotypes (Lindsten *et al*, 2000). Similarly, genetic deletion of autophagy regulator ATG5 also leads to perinatal lethality (Kuma *et al*, 2004). However, *Atg5* ablation in a $Bax^{-/-}$ $Bak^{-/-}$ mice causes a more severe phenotype with enhanced brain exencephaly and even more delayed reduction of interdigital webbing (Arakawa *et al*, 2017), compared to $Atg5^{-/-}$ (Kuma *et al*, 2004) or $Bax^{-/-}$ $Bak^{-/-}$. This suggests that autophagy serves as a partial backup mechanism for apoptosis during development.

In addition to *Atg5*, deletion of other critical mediators of autophagy *Atg16l1* or *Atg12* also results in perinatal lethality (Kuma *et al*, 2004; Saitoh *et al*, 2008; Malhotra *et al*, 2015). Several autophagy regulators are implicated in human pathologies, such as *Atg16l1* polymorphism, which is associated with Crohn's disease (Hampe *et al*, 2007). The most common ATG16L1 variant, T300A, is linked with a loss of Paneth cells in humans as well as mice (Cadwell *et al*, 2008). ATG16L1 T316A mutation (in mouse) introduces a new caspase-3 cleavage site, and mice harboring this mutation show reduced autophagy and pathogen clearance (Lassen *et al*, 2014; Murthy *et al*, 2014). Bacterial infections of these mice lead to increased cytokines levels, especially IL-1β, indicating a defective clearance of bacteria that results in more severe inflammation (Lassen *et al*, 2014; Murthy *et al*, 2014). Similar results have been reported for ATG16L1 conditional knockout mice. Mice with myeloid-specific deletion of ATG16L1 ($Atgl16l1^{\Delta Lyz2}$) succumbed faster than WT mice after LPS injection (Samie *et al*, 2018; Lim *et al*, 2019). Similarly, chimeric mice with transplanted $Atg16l1^{-/-}$ fetal liver cells treated with DSS (dextran sodium sulfate) and infected with MNV (murine norovirus) develop a lethal colitis while WT mice survived, indicating that ATG16L1 is necessary to restrict inflammatory signaling (Saitoh *et al*, 2008). Interestingly, mice with an intestinal-specific ATG16L1 knockout ($Atgl16l1^{IEC\text{-}KO}$) were also more sensitive in a DSS-induced colitis model (Matsuzawa-Ishimoto *et al*, 2017). Comparison of conditional knockout of ATG16L1 in intestinal epithelial cells or mononuclear cells showed that both have increased cytokine secretion; however, $Atgl16l1^{IEC\text{-}KO}$ show a more drastic increase compared to myeloid-specific KO (Conway *et al*, 2013). In DSS/MNV-induced colitis, TNF-blocking antibodies or RIP1 inhibitors had protective effects, which is in line with their finding that TNF-treated $Atg16l1^{-/-}$ organoids are more sensitive to TNF-induced and RIP1-mediated death compared to WT organoids (Matsuzawa-Ishimoto *et al*, 2017; Matsuzawa-Ishimoto *et al*, 2020). Similar findings were made in a different infection model. $Atg16l1^{IEC\text{-}KO}$ mice were more susceptible to *Helicobacter hepaticus* + αIL-10R-induced colitis compared to WT or myeloid-specific $Atg16l1^{-/-}$ mice. Epithelial cell-specific KO mice disease was preventable by administration of TNF (Pott *et al*, 2018), confirming a TNF-mediated pathology. Furthermore, mice with intestinal deletion of ATG16L1 were exquisitely sensitive to TNF-induced hypothermia and lethality in RIP1-dependent fashion (Patel *et al*, 2020). Similar results were obtained when *Atg5, Atg16l1, Fip200,* or *Becn1* were deleted in the myeloid cell compartment by LysM-Cre (Orvedahl *et al*, 2019). All 4 genotypes were more sensitive to TNF injections compared to their WT littermates. In the case of $Atg5^{\Delta LysM}$, the RIP1 kinase inhibitor Nec-1 protected the mice from death (Orvedahl *et al*, 2019). In $Atg16l1^{-/-}$ BMDMs, TRIF degradation by autophagy was shown to be critical for regulation of inflammatory signaling (Samie *et al*, 2018). In addition, ATG16L1 can restrict necroptotic signaling by regulating the turnover of RHIM-containing proteins, especially ZBP1 (Lim *et al*, 2019). ATG16L1 has been shown to play a critical role in the modulation of TNF-mediated cell death signal in various *in vitro* and *in vivo* systems. Several models proved that loss of ATG16L1 either in intestinal epithelial cells or myeloid cells leads contributes to a more severe phenotype. For this reason, further investigation of the pathways is critical to understand the differences and similarities of interplay between autophagy and TNF-mediated cell death signaling in different cell types.

Besides ATG16L1, other autophagy pathway components were also implicated in TNF and TRAIL-mediated apoptosis and necroptosis. Several studies suggested a scaffolding function of autophagy genes and autophagic structures, which will be discussed in more detail below. In Tak1-deficient cells, TRAIL-induced necroptotic cell death is dependent on p62, indicating that the autophagosome formation plays a critical role in complex formation (Goodall *et al*, 2016). The interplay of autophagy and cell death also has been implicated in HIV-infected T cells (HIV-TCM) treated with IAP antagonists (also referred to as SMAC mimetics). IAP antagonists induce cell death of HIV-TCMs in a caspase, RIP1, and autophagosome formation-dependent manner, while p62 knockdown protected from induced cell death (Campbell *et al*, 2018). Similarly, necroptosis in L929 cells could be inhibited by autophagy inhibitors wortmannin and pepstatin A or by the knockdown of RIP1 (Yu *et al*, 2006). In all studies described above, inhibition of autophagosome formation by wortmannin reduced cell death suggesting that autophagosome can directly affect cellular viability. The importance of autophagy as inducer of other forms of cell death was also shown for the clinically tested Bcl-2 antagonist GX15-070/obatoclax, which was shown to induce necroptotic cell death in different cancer cell lines depending on ATG5, ATG7, RIP1, and RIP3 (Bonapace *et al*, 2010; Basit *et al*, 2013). These studies provide a link between regulated cell death and autophagy and implicate dysfunctional autophagy in cell death activation in physiological settings linked to diseases.

NETosis in the context of inflammatory cell death

NET formation can also be associated with the activation different cell death pathways. Necroptosis has been shown in several studies to by critical for NETosis. Loss of the necroptotic effectors RIP3 or MLKL or the pharmacological inhibition of RIP1, RIP3, or MLKL let to reduced cell death and NET formation when cells were treated with LPS, PMA (Desai *et al*, 2016), RSV (respiratory syncytial virus) (Muraro *et al*, 2018), or various crystalline substances (Desai *et al*, 2017) for an extended period. NET formation was independent of RIP3 or MLKL when neutrophils were treated for shorter periods (Amini *et al*, 2016). These findings might be linked to the differences of lytic versus non-lytic NET formation. Injection of monosodium urate (MSU) crystals in air pouches has been shown to

promote NET formation and sterile inflammation *in vivo* (Schauer *et al*, 2014). In this model, *Ripk3*$^{-/-}$, *Mlkl*$^{-/-}$ mice, and Necrostatin-1-treated mice showed reduced tophus like NET aggregations (Desai *et al*, 2016; Desai *et al*, 2017). Similar findings were made using a murine AAV (anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis) disease model. *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice were protected against necrotizing crescentic glomerulonephritis detected in WT mice (Schreiber *et al*, 2017). Besides necroptosis, several studies showed that GSDMD plays a critical role during neutrophil cell death and NET formation (Chen *et al*, 2018; Kambara *et al*, 2018; Sollberger *et al*, 2018). Chen *et al* showed that non-canonical inflammasome activation can lead to GSDMD processing by caspase-11 leading to cell death resembling NETosis, which was independent of MPO, NE, and PAD4 (Chen *et al*, 2018). The other two publication that identified GSDMD as a target of the neutrophil elastase during PMA induced NET formation (Sollberger *et al*, 2018) or *E. coli* infection (Kambara *et al*, 2018). Infection with a cytosolic *Salmonella* strain (ΔsifA) lead to a more severe infection in *Gsdmd*$^{-/-}$ or *Casp11*$^{-/-}$ mice compared to WT mice. Interestingly, DNAse I treatment resulted in aggravation of the bacterial load only in WT mice (Chen *et al*, 2018), indicating potential defects of NET formation in *Gsdmd*$^{-/-}$ and *Casp11*$^{-/-}$ mice. A second study, however, reported that *Gsdmd*$^{-/-}$ lead to better bacterial clearance due to increased neutrophil numbers because of an increased lifespan (Kambara *et al*, 2018).

For a better understanding of necrotic and pyroptotic cell death in the context of NET formation, it would be interesting to perform infection or disease models in tissue specific knockouts. Additionally, it would be interesting to understand and clarify in which physiological contexts distinct cell death forms play a dominant role.

Ferroptosis and necroptosis

Ferroptosis and necroptosis have been implicated in kidney pathologies (Belavgeni *et al*, 2020), and inhibitors of ferroptosis and necroptosis showed protection in various disease models in mice. However, it is still not completely understood how both different mechanisms work together. Deletion of *Ripk3* or *Mlkl* has been shown to be protective in kidney reperfusion injury models (Linkermann *et al*, 2013; Newton *et al*, 2016a; Muller *et al*, 2017; von Massenhausen *et al*, 2018). *Ripk1*$^{KD/KD}$ mice (Newton *et al*, 2016a) or pharmacological inhibition of RIP1 showed protective effects in IRI (ischemia–reperfusion injury) as well (Linkermann *et al*, 2012). However, in AKI (acute kidney injury) induced by folic acid ferroptosis has been shown to be the driving form of inflammatory cell death (Martin-Sanchez *et al*, 2017). Further analysis of this model leads to better understanding of the interplay of ferroptosis and necroptosis. During later stages of disease, deletion of TWEAK receptor (Fn14) as well as treatment of Necrostatin-1 reduced the severity of the disease (Martin-Sanchez *et al*, 2018). As discussed by Martin-Sanchez *et al*, this leads to a model in which ferroptosis in critical for during the initial phase of AKI and necroptosis is taking over at the later stages leading to amplification of tubular cell death. Besides ferroptosis and necroptosis, MPTP and necroptosis have been shown to be critical mediators during IRI, as either genetic ablation of RIP3 and CypD showed complete rescue of lethality (Linkermann *et al*, 2013).

Clearly, ferroptosis and necroptosis can be triggered by shared stimuli (e.g., ROS) and are both involved in ischemia–reperfusion-driven pathologies. Consequently, further experimental validation of inhibitors or biomarkers selective for these pathways is needed for better understanding of ferroptosis/necroptosis biology and physiological importance.

Cell death pathways components as therapeutic targets

Cell death pathways play a pivotal role in homeostasis of the body, and their dysregulation can lead to many diseases ranging from autoimmune disease to neurodegeneration and cancer. As such, these pathways are attractive targets for therapeutic intervention. The initial validation of the relevance of cell death for human diseases came from identification of Bcl-2 from the genomic region with frequent chromosomal translocations t(14;18) in follicular lymphomas (Tsujimoto *et al*, 1984). Furthermore, expression of Bcl-2 protected cells from death and enabled lymphocyte accumulation often leading to cancer (Vaux *et al*, 1988; McDonnell *et al*, 1989; Strasser *et al*, 1991). Soon afterward, the importance of Bcl-xL, Mcl-1, and other Bcl-2 family members was recognized and development of antagonists of anti-apoptotic Bcl-2 proteins was started (Czabotar *et al*, 2014). These compounds are referred to as BH3 mimetics given that they emulate cell death promoting function of BH3 domains.

The first successful BH3 mimetic was ABT-737, a compound with partial selectivity for Bcl-2, Bcl-xL, and Bcl-w over Mcl-1 and A1 that efficiently inhibited tumor growth in numerous animal cancer models and validated this targeting approach (Oltersdorf *et al*, 2005). ABT-737 was followed by a related orally available BH3 mimetic ABT-263 or navitoclax (Tse *et al*, 2008). Navitoclax showed great promise in patients with chronic lymphocytic leukemia. In addition to its single-agent activity, navitoclax had a great potential for combination therapies, especially those that downregulated Mcl-1, Bcl-2 protein not affected by ABT-263 (Cragg *et al*, 2009). However, navitoclax clinical path was hindered because it stimulated precipitous loss of platelets (Roberts *et al*, 2012). Drop in platelet number was caused by Bcl-xL inhibition, which steered ongoing drug discovery efforts away from this important pro-survival protein (Czabotar *et al*, 2014). Thus, emerged Bcl-2 selective inhibitor ABT-199 or venetoclax, the first BH3 mimetic and the first cell death regulating small molecule to be approved by the FDA for the treatment of chronic lymphocytic leukemia or small lymphocytic lymphoma (Souers *et al*, 2013). Venetoclax is undergoing investigation in a number of clinical trials that aim to expand the number of malignancies where it can be beneficial (Strasser & Vaux, 2020). However, there is also increasing awareness that Mcl-1 is a major resistance factor for Bcl-2 targeting (Gong *et al*, 2016). To address this therapeutic need, several Mcl-1 selective inhibitors have been generated and some have been enrolled in clinical trials (Kotschy *et al*, 2016; Caenepeel *et al*, 2018; Tron *et al*, 2018), (ClinicalTrials.gov). Having selective antagonist to various pro-survival Bcl-2 protein would give oncologists a great set of tools to treat patients' tumors with potent and tolerable anti-cancer agents.

The favorite strategy for targeting IAP proteins involves SMAC-mimicking small-molecule IAP antagonists (Fulda & Vucic, 2012). IAP antagonists were meant to block caspase inhibition by XIAP (Sun *et al*, 2007). However, the key to single-agent pro-apoptotic activity of IAP antagonists results from c-IAP1/2 antagonism and TNF-dependent cell death (Gaither *et al*, 2007; Petersen *et al*, 2007; Varfolomeev *et al*, 2007; Vince *et al*, 2007). The monovalent IAP antagonists emulate one SMAC AVPI motif, while the bivalent antagonists comprise two AVPI-like motif mimetics connected by a

chemical linker (Sun *et al*, 2007; Varfolomeev *et al*, 2007). Binding of IAP antagonists triggers a conformational change that opens the c-IAP1 structure and enables c-IAP RING domain dimerization, a prerequisite feature of their E3 activation (Dueber *et al*, 2011; Feltham *et al*, 2011). This prompt activation of c-IAP1/2 E3 activity causes their K48-linked auto-ubiquitination, subsequent proteasomal degradation (Dueber *et al*, 2011; Feltham *et al*, 2011) and activation of canonical NF-κB signaling (Varfolomeev *et al*, 2007; Vince *et al*, 2007). Proteasomal degradation of c-IAPs leads to NIK stabilization and activation of the non-canonical NF-κB pathway (Varfolomeev *et al*, 2007; Vince *et al*, 2007). The stimulation of NF-κB as well as MAPK pathways induces TNF production and activation of TNFR1 signaling (Varfolomeev *et al*, 2007; Vince *et al*, 2007). However, with c-IAP1/2 degraded, RIP1 cannot be ubiquitinated during TNF-induced signaling and the canonical NF-κB pathway is poorly activated. Instead, RIP1 will complex with FADD/caspase-8 and provoke apoptosis, and if caspase-8 is inhibited or insufficiently activated, RIP3 and MLKL-dependent necroptotic cell death (Bertrand *et al*, 2008; He *et al*, 2009). TNF-blocking reagents efficiently inhibit IAP antagonist stimulated cell death further demonstrating its' TNF dependence (Petersen *et al*, 2007; Varfolomeev *et al*, 2007; Vince *et al*, 2007).

Several IAP antagonists such as GDC-0152, TL3271, and SM-164 have demonstrated tumor-inhibiting activity in *in vivo* cancer models without showing any significant toxicity or weight loss in mice (Lu *et al*, 2008; Flygare & Fairbrother, 2010; Flygare *et al*, 2012; Fulda & Vucic, 2012; Morrish *et al*, 2020a). Based on the positive results from preclinical studies, a number of IAP antagonists have entered phase I/II clinical trials for people with a variety of malignancies (Fulda & Vucic, 2012; Jensen *et al*, 2020b). Clinical trials with GDC-0152, LCL161, HGS1029, and TL32711 reported target antagonism, dose proportional pharmacokinetics, and no dose-limiting toxicity (Morrish *et al*, 2020a). However, none of these trials reported significant anti-tumor activity of IAP antagonists and were not pursued further (Morrish *et al*, 2020a). Nevertheless, the ability of IAP antagonists to activate non-canonical NF-κB signaling is prompting an interest in combining them with checkpoint inhibitors (e.g., anti-PD1 antibody) and antiretroviral therapy (Chesi *et al*, 2016; Nixon *et al*, 2020; Morrish *et al*, 2020a). IAP antagonists have also shown a great potential in treating liver pathologies caused by HBV and Plasmodium infections (Ebert *et al*, 2020; Morrish *et al*, 2020b). These and other ongoing and future clinical trials will examine the safety and the efficacy of IAP antagonists for the treatment of human malignancies and infections in hopes of bringing new therapies to patients who need them.

Bcl-2 and IAP antagonists were developed to promote cell death in hematological and solid tumors. However, excessive cell death can be detrimental for healthy organism and cause tissue damage and neurodegeneration. For this reason, RIP1 has been proposed as a safe modality to treat inflammatory and neurodegenerative diseases with no known risk of immunosuppression (Yuan *et al*, 2019; Mifflin *et al*, 2020). While RIP3 kinase could also be considered an attractive target, genetic studies and RIP3 targeting efforts have demonstrated toxicity which precludes safe inhibition of RIP3 (Mandal *et al*, 2014; Newton *et al*, 2014). Contrarily, genetic inactivation or chemical inhibition of RIP1 kinase activity is well tolerated and pose no known risks (Berger *et al*, 2014; Newton *et al*, 2014; Polykratis *et al*, 2014; Patel *et al*, 2020; Webster *et al*, 2020).

Inhibiting RIP1 kinase activity is beneficial in joint and skin inflammation, ileocolitis as well as in the TNF-induced systemic inflammatory response syndrome (SIRS) model (Berger *et al*, 2014; Vlantis *et al*, 2016; Newton *et al*, 2016a; Patel *et al*, 2020; Webster *et al*, 2020). Similarly, the role of RIP1 kinase activity is also evident in the number of neurodegenerative and neuroinflammatory diseases (Yuan *et al*, 2019; Mifflin *et al*, 2020). In pancreatic cancers, lung metastases, pancreatitis, and certain viral infections however, the therapeutic effects of RIP1 inhibition have been disputed recently (Newton *et al*, 2016a; Patel *et al*, 2020; Webster *et al*, 2020). Clearly, more studies are needed to delineate the suitable diseases for RIP1 inhibition, with confirmation coming from testing RIP1 inhibitors in clinical trials.

So far, RIP1 inhibitors developed by GlaxoSmithKline (GSK) and Denali have been tested in clinical settings with initial reports indicating that GSK2982772 and DNL104 were generally well tolerated in people (Harris *et al*, 2017; Weisel *et al*, 2017b; Grievink *et al*, 2020; Jensen *et al*, 2020; Mifflin *et al*, 2020). Phase I trials with GSK2982772 showed no serious adverse events (AEs) and no suggestion of a safety concern (Weisel *et al*, 2017), which enabled GSK to initiate several small phase 2 clinical trials for psoriasis, rheumatoid arthritis, and ulcerative colitis. To date, GSK2982772 has not shown significant therapeutic benefit in psoriasis or rheumatoid arthritis, while the data from the ulcerative colitis trial are not yet available (ClinicaTrials.gov). In addition to inflammatory diseases, GSK tested RIP1 inhibitor, GSK3145095, in clinical trial intended to test RIP1 inhibition in pancreatic and other solid tumors (Harris *et al*, 2019), but was terminated during patient recruitment. DNL104, Denali's brain-penetrant RIP1 inhibitor, did not trigger any adverse effects in central nervous system, although they observed abnormal liver function in some healthy subjects (Grievink *et al*, 2020). Denali has abandoned clinical trials of DNL104 and entered into collaboration with Sanofi to examine another RIP1 inhibitor, DNL747, in clinical trials for Alzheimer's disease, amylotrophic lateral sclerosis, and multiple sclerosis (Martens *et al*, 2020). Overall, targeting RIP1 represents an attractive opportunity for therapeutic intervention in inflammatory diseases, and future preclinical and especially clinical studies should further define the optimal indication and patient populations,

An alternative way of targeting inflammatory diseases is to block inflammasome assembly and inflammatory cell death mediated by NLRP3 and GSDMD. GSDMD is a key component of pyroptotic cell death and targeting GSDMD cleavage, membrane association, and/or the ability to form membrane pores is attractive strategy for several devastating diseases such as sepsis or ARDS (acute respiratory distress syndrome) (Chauhan *et al*, 2020). As GSDMD is a relatively novel target with no known enzymatic activity, GSDMD targeting efforts are still nascent (Shi *et al*, 2017). Consequently, none of the GSDMD blocking reagents have advanced to clinical trials yet. However, several more established inflammasome regulators have been a focus of drug discovery for a long time. The best example of NLRP3 inflammasome has been implicated in a number of inflammatory, neurodegenerative, and metabolic diseases (Voet *et al*, 2019). Early verification of the feasibility of NLRP3 therapeutic targeting came from demonstration that chemical compound called glyburide effectively blocked IL-1b secretion and pyroptotic cell

death (Lamkanfi *et al*, 2009). Another agent, MCC950/CRID3, targets the NACHT domain of NLRP3 and has a higher potency and selectivity for NLRP3 (Coll *et al*, 2019; Tapia-Abellan *et al*, 2019). MCC950/CRID3 has shown efficacy in a number of animal disease models including myocardial infarction, atherosclerosis, dermal and pulmonary inflammation, and multiple sclerosis (Primiano *et al*, 2016; van der Heijden *et al*, 2017; van Hout *et al*, 2017; Perera *et al*, 2018; Voet *et al*, 2019). Currently, two MCC950/CRID3-related compounds (IZD334 and Inzomelid) are undergoing clinical trials to evaluate their safety and tolerability (Clinicaltrials.gov). These and future trials could pave the way for efficacious and safe NLRP3 targeting in patients with CAPS (cryopyrin-associated periodic syndromes) and other inflammatory diseases.

Caspases play a central role in various cell death pathways and therefore were/are an interesting target for drug development. Several pan-caspase inhibitors mimicking peptide substrates have been developed with few reaching clinical trials. Emricasan is an irreversible caspase inhibitor which accumulates in the liver, likely because of the first pass effect (Hoglen *et al*, 2004). In several mouse models (α-Fas models, D-Gln/LPS), Emricasan showed protective capacity and was also used in intervention animal studies (Hoglen *et al*, 2004). Combination therapy of Emricasan and IAP antagonist Birinapant promoted necroptosis in AML cells in vivo and prolonged survival in mouse models (Brumatti *et al*, 2016). Clinical trials of Emricasan did not raise any safety concerns and showed reduction of serum level transaminases in patients with prior diagnosis of mild hepatic impairment (Valentino *et al*, 2003). In several other liver pathologies, Emricasan showed promising results. During liver transplantation, Emricasan was tested in two different patients with non-alcoholic fatty liver acid disease and it showed reduced levels of ALT (alanine aminotransferase) compared to the placebo group (Shiffman *et al*, 2019). Similar findings were made for patients with hepatitis C virus infection where Emricasan reduced transaminase blood levels without affecting virus titers (Shiffman *et al*, 2010). However, Emricasan did not have a beneficial effect on portal hypertension in liver cirrhosis patients (Garcia-Tsao *et al*, 2020). Lastly, the inhibitor was tested during liver transplantation as cell death driven by reperfusion is a major concern. Treatment showed some therapeutic effect by reducing apoptosis (Baskin-Bey *et al*, 2007). As mentioned by the authors, more studies would need to be conducted to confirm these observations. Another caspase inhibitor, GS-9450, reduced ALT levels in patients suffering from non-alcoholic steatohepatitis (Ratziu *et al*, 2012). Therefore, although caspase inhibitors showed some potential in the treatment of various hepatic disease, more studies are needed to evaluate their therapeutic potential.

As described earlier, PARP1 plays a critical role in DNA damage repair pathways. PARP1 inhibitors induce cytotoxicity, especially in BRCA1/2 mutated (homologous recombination-deficient (HDR)) cancers cells (Bryant *et al*, 2005; Farmer *et al*, 2005). This is explained by synthetic lethal interaction between BRCA1/2 and PARP1 during DNA repair (Lord & Ashworth, 2017). PARP inhibitors "trap" PARP1 at the DNA inducing blockage of replication forks leading to cell death in HDR tumors (Murai *et al*, 2012). Several PARP inhibitors are approved in BRCA1/2 mutated ovarian cancer as well as BRCA mutated HER negative breast cancer with ongoing trials for other forms of cancer (Hoy, 2018; Jiang *et al*, 2019; Murthy & Muggia, 2019).

Ferroptosis has been implicated in several experimental models of reperfusion injury. In addition, chelation of iron by M30 or α-lipoic acid showed improvement in neurodegenerative disease models (Kupershmidt *et al*, 2012; Zhang *et al*, 2018; Han *et al*, 2020) suggesting a possible benefit in the clinical setting. In a clinical trials, iron chelator DFO (desferrioxamine mesylate) slowed the progression of dementia in Alzheimer's patients over the placebo group (McLachlan & Dalton, 1991). While inhibition of ferroptosis may be a promising strategy for neurodegenerative diseases, ferroptosis inducers showed a potential benefit for cancer. In mouse models, induction of ferroptosis reduced the tumor growth/size significantly compared to the controls (Kim *et al*, 2016). This process was reversible by cotreatment of DFO (Kim *et al*, 2016). There are several potential strategies targeting ferroptosis in cancer reviewed by (Dixon & Stockwell, 2019). Several agents which can potentially induce ferroptosis by interference with the GSH synthesis or GPX4 have been investigated in clinical trials. The GSH synthesis inhibitor (Buthionine sulfoximine-BSO) reduced GSH levels in tumor cells and was tolerated in patients in phase I clinical trials (Bailey *et al*, 1997). The X_c^- inhibitor Sulfasalazine (SAS) reduced targeted cancer stem cell populations in a phase I trial (Shitara *et al*, 2017). Interestingly, the approved cancer drug altretamine, which was originally classified as a alkylating agent, was identified as a potential GPX4 inhibitor (Woo *et al*, 2015). These examples show the perspective of targeting ferroptosis for various diseases but, clearly, further research is needed to understand its full potential.

In summary, various cell death pathways are implicated in numerous seminal physiological processes. Therefore, they represent attractive targets for therapeutic intervention with the hope of addressing unmet medical needs and helping patients suffering from cancers, neurodegenerative, inflammatory, and other diseases.

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Conflict of interest

The authors declare that they have no conflict of interest.

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