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Non-lethal Outcomes of Engaging Regulated Cell Death Pathways in Cancer

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Abstract

Regulated cell death (RCD) is essential for successful systemic cancer therapy. Yet, the engagement of RCD pathways does not inevitably result in cell death. Instead, RCD pathways can take part in diverse biological processes if the cells survive. Consequently, these surviving cells, for which we propose the term 'flatliners', harbor important functions. These evolutionarily conserved responses can be exploited by cancer cells to promote their own survival and growth, with challenges and opportunities for cancer therapy.

Introduction

The term regulated cell death (RCD), occurring as a result of a molecular pathway, has replaced "programmed cell death," as the latter was specifically coined to refer to cell death that occurs at defined times during development[1]. RCD shapes the physiological development of tissues and organs, maintains homeostasis, and plays various roles in multiple disease processes[2,3]. The molecular machineries of RCD can be initiated by diverse mechanical, biological, physical, and chemical stresses, and are influenced by various cell intrinsic and extrinsic signals. This is opposed to "accidental" cell death, which is an immediate fatal response to severe physical, mechanical, or chemical damage, and is often referred to as "necrosis"[4]. The molecular events of RCD pathways and the resulting phenotypic changes have been related to various clinical outcomes, particularly in the context of cancer.

While evading cell death has been identified as a hallmark of cancer[5], this is often mis-interpreted as suggesting that cancer cells are resistant to RCD. This is incorrect, as

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highlighted by studies showing that cancer cells can be “primed for death,” and the response to conventional therapy correlates with such priming[6]. It is therefore understood that a hallmark of cancer is an ability of the cell to evade those RCD mechanisms that are engaged to suppress the oncogenic process, not necessarily RCD in general.

The engagement of RCD does not inevitably result in cell death. Cells that survive the activation of an RCD pathway can undergo changes that influence their behavior and/or that of surrounding cells. These may include genomic instability leading to high mutational burden[7,8] and pro- or anti-tumor immune responses[9]. Moreover, preclinical research provides evidence that sublethal engagement of RCD leads to phenotypic adaptations including epithelial mesenchymal transition (EMT) and altered interaction within the cell’s microenvironment[10–13]. Consequently, sublethal engagement of cell death contributes to metastasis, invasiveness, and therapy-unresponsiveness, but can also present vulnerabilities that might be harnessed for cancer treatment[13–16].

Herein, we introduce the term “flatliner” to represent a cell that has engaged a core RCD mechanism but manages to survive, in analogy to a patient who “flatlines” but is resuscitated. For cells, this is distinct from resistance to or evasion from signals that normally induce cell death. We propose that engaged cell death pathways do not always lead to cell death, and flatliners that survive may have altered properties. We elaborate how cancer cells resist therapy, with particular focus on the molecular mechanisms that underly the evasion of cell death following activation of an RCD pathway, and how survival of flatliners may account for phenomena associated with cancer persistence. Further, we assess how our knowledge and recent advances in the cell death field translate into our understanding of cancer progression and relapse, and how this may undercover novel therapeutic opportunities.

Finally, we discuss the potential relationship of flatliners to drug-tolerant persister cells, characterized as cancer cells without resistance-associated mutations that survive treatment[14,17]. These definitions are distinct: while both processes are transient (cells revert to the parental drug sensitivity over time), flatliners have demonstrably engaged a core cell death pathway and survived, while persister cells are defined only by their transient drug-tolerant state. While we argue that, at least in some cases, engagement of a core cell death machinery can induce the persister cell phenotype, the distinct definitions of each are important.

RCD pathways in cancer

In the following section we briefly survey four common RCD pathways: apoptosis, necroptosis, pyroptosis and ferroptosis, and discuss the molecular mechanisms that underlie their evasion.

Apoptosis

Apoptosis refers to cell death associated with the activation of Cysteine-Aspartate proteases (caspases) mediating cleavage of target proteins, leading to fragmentation of cellular DNA, nuclear condensation, membrane blebbing, and rapid clearance prior to loss of

plasma membrane integrity[18]. The biochemical and morphological changes associated with apoptosis are orchestrated by the activity of the executioner caspases, caspase-3 and −7, which cleave hundreds of substrates, including those responsible for the changes mentioned above[19,20]. For example, inter-nucleosomal double strand DNA breaks during apoptosis are caused by the caspase-activated nuclease (CAD). Another protein, inhibitor of CAD (iCAD) acts as a chaperone to bind and inhibit CAD while folding it into an active nuclease. The executioner caspases cleave iCAD, allowing the active CAD to cause DNA fragmentation[21].

The executioner caspases are activated by initiator caspases (e.g., caspase-8 and −9), which cleave the inactive, dimeric executioners to activate them. The initiator caspases are not activated by cleavage, but instead, by binding and oligomerization of the inactive monomers on activated adapter proteins. These adapters and initiator caspases define the apoptotic pathways (Figure 1).

In the mitochondrial, or intrinsic pathway of apoptosis, the adapter is apoptotic protease activating factor-1 (APAF1), which binds and thereby activates the initiator caspase, caspase-9, which in turn cleaves and thereby activates the executioner caspases[19]. The latter are inhibited, however, by X-linked inhibitor of apoptosis (XIAP). The activation of APAF1 occurs following mitochondrial outer membrane permeabilization (MOMP), releasing cytochrome c from the mitochondrial inter-membrane space, which induces the activation and oligomerization of APAF1. In addition, proteins that interfere with XIAP are also released upon MOMP, de-repressing the executioner caspases and allowing apoptosis to proceed[22]. Extensive MOMP in a cell can result in a mitochondrial energetic catastrophe that usually ends in cell death even if the executioner caspase activation is insufficient[23].

MOMP is caused by the action of the pro-apoptotic effectors of the BCL-2 family, e.g. BAX and BAK. These are antagonized by the anti-apoptotic BCL-2 proteins, e.g., BCL-2, BCL-XL, and MCL-1. A third type of BCL-2 protein, the BH3-only proteins function to inhibit the anti-apoptotic proteins and/or activate the pro-apoptotic effectors. The functions of the BCL-2 proteins have been reviewed elsewhere[24]. Engagement of apoptosis in cancer cells is triggered by many chemotherapeutic drugs and radiation therapies, which results in the activation of BAX and BAK through increased function of BH3-only proteins and decreased anti-apoptotic BCL-2 protein function[25]. Cells that are poised to undergo MOMP (“primed for death”) are associated with better prognosis in response to conventional therapy[6] and dynamic BH3 profiling, in which drugs are tested for their ability to prime cells for induction of MOMP by BH3 peptides, has shown promise in predicting therapeutic efficacy[26].

One way for cancer cells to survive in the face of chemotherapeutic insult is to prevent engagement of the cell death pathways via mutation or other mechanisms that permanently disrupt the action of the drug, referred to as resistance. This is distinct from survival *following* engagement of such a pathway and is not considered further herein. Previously, MOMP was widely considered a “point of no return” for cells, but we now know that cells can survive a degree of MOMP and sublethal caspase activation[16,27] (Figure 2). It has been observed that not all mitochondria necessarily release cytochrome c upon stimulation with chemotherapeutic agents (incomplete MOMP; iMOMP). iMOMP allows for repopulation of

cells with healthy mitochondria and increased clonogenic survival once the death-inducing stress is removed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) overexpression has also been shown to promote clonogenic survival in cells displaying iMOMP promoting increased glycolysis and a transient increase in mitochondrial mass[28]. In another study, the repair of double-strand DNA breaks induced upon caspase-mediated activation of CAD was noted as a requirement for cancer cell survival[8]. Widespread MOMP, followed by extensive apoptotic caspase activation normally is lethal, but cells that engage the mitochondrial apoptosis pathway can survive.

A second pathway of apoptosis involves the activation of the initiator caspase, caspase-8, by its adapter, FADD. This can occur upon ligation of death receptors of the TNF receptor family, e.g. TNFR1, FAS (CD95), and TRAIL receptors, and is often referred to as the death receptor, or extrinsic pathway of apoptosis (although other, intrinsic mechanisms, including non-death receptor processes exist to activate FADD-caspase-8 [29]). Active caspase-8 cleaves and thereby activates the executioner caspases.

Flice-like inhibitor of apoptosis, $cFLIP_L$, (herein, FLIP) resembles caspase-8 but lacks a catalytic cysteine. If FLIP is present, it binds to a monomer of FADD-bound caspase-8, preventing oligomerization of the latter and thus prevents apoptosis[30]. However, the caspase-8-FLIP heterodimer is proteolytically active and performs other functions, as discussed below. The extent to which caspase-8 and death receptor signaling contribute to cancer is not well understood.

The death receptor pathway of apoptosis appears to be an important mechanism for anti-tumor immunity, as cytotoxic lymphocytes deploy death receptor ligands (FAS/CD95, TRAIL) as one way they kill cancer cells. Caspase-8 is mutated or silenced in some cancers[31]. However, whether this represents an immune evasion mechanism or an escape from a tumor suppressor mechanism is not clear. Intriguingly, most cancers express FAS[32] and many express receptors for TRAIL[33], suggesting that such receptors may have roles beyond cell death that are important in cancer maintenance[34].

Cells that activate the death receptor pathway can survive, suggesting that low levels of executioner caspase activation are tolerated[35]. Survival following engagement of apoptosis and activation of executioner caspases has been termed anastasis (defined as cell survival despite activation of executioner caspases)[36]. Using a fluorescent marker of caspase-mediated cleavage, studies in flies indicated that many cells in the developing animals display evidence of caspase activation without apparent cell death[37]. Studies in primary and transformed mammalian cells revealed features of anastasis following induction of apoptosis, including DNA damage, oncogenic transformation, and induced gene signatures[36]. Although MOMP was not assessed in these studies, evidence (discussed above) strongly suggests that cells can survive MOMP, and thus might be considered to have undergone anastasis[15,16].

Necroptosis

While necrosis often refers to uncontrolled cell death, we now recognize that there are regulated forms of necrosis. Among these is necroptosis[38], in which receptor-interacting

kinase-3 (RIPK3) phosphorylates mixed-lineage kinase-like (MLKL) (Figure 1), which then oligomerizes and incorporates into the cell membrane forming a large pore, inducing necroptosis. Three proteins are known to bind and thereby activate RIPK3: RIPK1, TRIF, and ZBP1[39]. These, in turn, are activated by ligation of death receptors, some toll-like receptors, and interferon receptors, respectively. RIPK1 however, associates not only with RIPK3 but also with FADD, and in the absence of FLIP, can cause apoptosis via activation of caspase-8 [40]. If FLIP is present, apoptosis is blocked, while the catalytic activity of FADD-caspase-8-FLIP cleaves RIPK1 and associated RIPK3, preventing necroptosis. Inhibition of caspase-8 or genetic ablation of either FADD or caspase-8 allows necroptosis to proceed. Such inhibition can occur due to viral caspase inhibitors[41]. That necroptosis proceeds in the presence of intact caspase-8 signaling has been noted in pathological settings, but the precise mechanisms remain unclear.

Multiple key necroptotic proteins are downregulated in various cancers. MLKL expression is often decreased in AML[42], while RIPK3 protein levels are decreased in breast cancer and colorectal cancer and low levels correlate with worse overall survival in colorectal cancer[43,44]. Further, RIPK1, RIPK3, and phosphorylated MLKL levels correlate with better overall survival and $CD8^+$ T cell infiltration in hepatocellular carcinoma[45]. Conversely, cancer cells can apparently induce necroptosis in host endothelial cells via DR6 and thereby promote metastasis[46].

Negative regulation of necroptosis occurs via post-translational modifications on necroptotic proteins, reviewed elsewhere[47]. In addition, cells can delay or prevent necroptosis through membrane repair mechanisms. One membrane repair mechanism is mediated by endosomal sorting complexes required for transport (ESCRT) proteins. The ESCRT subcomplexes (ESCRT-0, -I, -II, -III) are the only machinery known in eukaryotic cells that can deform membrane on the distal side[48]. This machinery has multiple functions in the cell in addition to membrane repair[49]. Recruitment of the ESCRT-III machinery mediates abscission of the damaged plasma membrane to restore plasma membrane integrity. Cells undergoing necroptosis were able to survive in the presence of ESCRT-mediated membrane repair when the necroptotic stimulus was removed (Figure 3), whereas if ESCRT activity was blocked, cells which had activated MLKL were not able to survive (i.e., become necroptotic flatliners)[50]. This prolonged survival was necessary for production of cytokines and allowed for enhanced CD8 T-cell cross-priming by necroptotic tumor cells *in vivo*[10,12].

Pyroptosis

Pyroptosis refers to a lytic cell death driven by inflammatory caspase activation leading to gasdermin-dependent pore formation in the plasma membrane, mostly in the context of infection and inflammation[51] (Figure 1). Certain bacterial components such as cytosolic LPS can directly activate the inflammatory caspase 11 (caspase-4 and caspase-5 in humans), while other stimuli can cause the formation of various inflammasomes that can activate caspase-1[52]. Gasdermin D was identified as the crucial cleaved substrate downstream of caspase-11, and caspase-1, which mediates pore formation in the plasma membrane and initiates lytic cell death[51]. Other gasdermin family members have been reported to be activated by caspase-3 and −7[51], caspase-8[53], Granzyme A[54], or a streptococcal

exotoxin[55,56]. The cleaved gasdermin then inserts into the plasma membrane creating pores[57,58] which, in the case of Gasdermin D, selectively release mature IL-1 cytokines, which are cleaved by active caspase-1[51,58].

Gasdermin E, which is activated by caspase-3 and -7, was identified as a tumor suppressor downregulated in many tumors[59–61]. Chemotherapeutic drugs are able to activate Gasdermin E via caspase-3-dependent cleavage to induce cancer cell death[60]. Induction of pyroptosis in tumor cells promoted induction of a robust anti-tumor immune response and protective immunity against a re-challenge with the same tumor cells[61,62]. While gasdermin-mediated pore formation leads to water influx and collapse of the ionic gradient across the plasma membrane, rupture of the cells and the release of large cytosolic molecules is mediated by ninjurin-1 (NINJ1)[63]. Plasma membrane rupture (PMR) occurs downstream of gasdermin or MLKL activation as well as late after apoptosis induction upon cleavage of Gasdermin E by executioner caspases (also called secondary necrosis). Deletion of NINJ1 delays PMR after MLKL activation but does not block it. The mechanisms of how NINJ1 is activated and induces PMR are unknown[63]. It is possible that cells lacking NINJ1 can survive the activation of gasdermins (and thus, pyroptosis), although this has not been formally tested.

Gasdermin D-mediated pore formation during pyroptosis is also regulated by ESCRT-mediated membrane repair. Calcium flux through gasdermin pores recruits the ESCRT machinery to the site of pore formation where membrane pieces containing pores are shed to repair the plasma membrane, prolonging survival. ESCRT-deficient macrophages secreted more pro-inflammatory cytokines upon activation of caspase-1 compared to membrane repair proficient cells[64]. This mechanism can be exploited to increase anti-tumor immunity. Co-delivery of gasdermin and a calcium-chelating agent (to inhibit membrane repair) markedly increased efficacy of PD1-blocking antibodies in various murine tumor models[65].

The induction of pyroptosis may be an avenue towards therapeutic treatment of AML[66]. Further, evidence suggests that the activation of Gasdermin E promotes effective anti-cancer immunity[62]. One study suggests that one of the inflammasomes (NLRP3) is active in glucocorticoid-resistant B-ALL[67]. The active Caspase-1 cleaves and thereby inactivates the glucocorticoid receptor, rendering the cells resistant to glucocorticoid treatment. Inhibition of Caspase-1 or disruption of the NLRP3 inflammasome restored glucocorticoid sensitivity in these cells, yet how they remain alive despite demonstrable Caspase-1 activity is unknown.

Ferroptosis

Another form of RCD is ferroptosis, resulting from accumulation of toxic lipid peroxides as a consequence of the iron-dependent Fenton reaction[68,69]. Most of the regulation of ferroptosis centers around activity of the selenoenzyme, glutathione peroxidase 4 (GPX4)[70]. GPX4 detoxifies oxidized lipid species (L-OOH) into nontoxic lipid alcohols (L-OH), a mechanism to prevent lipid peroxidation in the plasma membrane and subsequent cell death. Thus, GPX4 is a potent inhibitor of ferroptosis, and GPX4 inhibition drives ferroptosis. One mechanism of GPX4 inhibition occurs via depletion of its regenerative substrate, glutathione (GSH)[70] which occurs through inhibition of the upstream cysteine/glutamate transporter (x_c-)[71]. In addition to GPX4, tetrahydrobiopterin (BH4), synthesized by GTP-

cyclohydrolase-1 (GCH1), serves as an antioxidant which synergizes with vitamin E to prevent lipid oxidation and subsequent cell death from ferroptosis[72]. Another negative regulator of ferroptosis, ferroptosis suppressor protein 1 (FSP1) can protect from GPX4 inhibition by regenerating CoQ_{10}, allowing suppression of peroxy radicals in lipid bilayers [73,74].

Many experimental and approved cancer therapeutics are thought to kill cancer cells via ferroptosis[75], although in the majority of such reports, the term is invoked when lipid peroxidation is observed, and reactive oxygen scavengers reduce killing *in vitro*.

While ferroptosis does not involve a dedicated membrane perforation mechanism, ESCRT-mediated membrane repair delays the kinetics of ferroptotic cell death[76]. Although little is known about sub-lethal activation of ferroptosis in cancer, various reports (elaborated below) suggest that cancer cells which survive activation of other cell death pathways have increased sensitivity to ferroptosis[13,77]. Thus, the interconnectivity between RCD pathways (reviewed in[78]) allows for acquired vulnerabilities.

Drug Tolerant Persister cells

The concepts described for antibiotic-tolerant bacterial ‘persisters’ [79] translate to drug-tolerant persister cancer cells (DTPs), defined as a population of genetically identical cells that survive a cell death-inducing, therapeutic treatment. The phenomenon of persistence is distinct from that of drug resistance, in that drug tolerance in persister cells is transient and reverts to the parental, drug-sensitive state over time. These DTPs have been described in cell lines representing a wide variety of cancers in response to a variety of treatments, primarily targeted therapies and chemotherapies[80]. For example, DTPs have been described in AML patient samples and mouse models in response to BET inhibitors[81,82], and in patient melanoma samples and cell lines following treatment with BRAF and/or MAP kinase inhibitors[83–85].

There are several characteristics displayed by DTPs, including decreased cell proliferation, a change in cell identity (usually defined by gene expression changes), adaptation of cellular metabolism, and modification of the tumor microenvironment. For most settings, it is unknown if the persisting population was preexistent (i.e. developed independent of the selective pressure) or if they are a response to drug treatment. Slow cycling persister cells which survive treatment with an EGFR inhibitor are pre-existing in a population of PC9 lung cancer cells. Using a single cell barcode tracking system coupled to a fluorescent cell cycle tracker, the proliferative capacity was largely predetermined in the same population of PC9 lung cancer cells and the size of clones surviving and expanding upon treatment was similar across experiments. This suggests the presence of a pre-existing state in a population of cells, existing independently of selection pressure, which allows for persistence in the face of therapy[14]. It is important to note, however, that this does not preclude an inductive effect of the treatment on the persister phenotype; a pre-existing population may be in a state that is responsive to such an effect. This idea is further supported by considering another feature of DTPs, sensitivity to ferroptosis.

Some cancer cells are constitutively sensitive to ferroptosis induction by inhibition of GPX4, while others are not. Studies of DTPs derived from a wide variety of human cancer cell lines showed that DTPs generated by a variety of therapeutics become dependent on GPX4 for survival[70,86]. A GPX4 inhibitor induced ferroptosis in these DTPs, however, pretreatment of the parental cells with a GPX4 inhibitor had no effect on their generation[87]. Therefore, the GPX4-dependent DTPs were not a pre-existing population in the parental cells, but instead, were induced by the therapeutic treatment. Validation of such an effect from patient samples is lacking, however.

Changes in metabolism are another feature of DTPs that survive anti-cancer therapies. DTPs change their metabolism towards mitochondrial oxidative respiration rather than glycolysis, which resembles respiration in untransformed cells[88]. $KRAS^{G12D}$-mutated mouse pancreatic ductal adenocarcinoma cancer persister cells that survive ablation of the oncogene increase mitochondrial biogenesis and oxidative phosphorylation[89]. Similar results were obtained in $BRAF^{V600E}$-mutated melanoma cells that persist following treatment with cisplatin or the BRAF inhibitor, vemurafenib[90] or AML cells that persist following cytarabine treatment[91]. Utilization of alternate metabolic pathways such as fatty acid oxidation has been described in triple negative breast cancer cells[92,93], HER2 positive breast cancer cells[94] and melanoma cells[95]. For different cells, upregulation of the fatty acid transporter CD36 seems to be a key mechanism to allow changes in metabolism[94,95]. These results suggest that a switch in metabolism towards a more oxidative state is associated with dormancy and chemotherapy resistance in DTPs.

In general, all treatments that result in DTPs induce apoptosis via the mitochondrial pathway. This raises the possibility that DTPs are a result of non-lethal effects of engaging this apoptosis pathway. However, activation of apoptosis in surviving cells was not measured in any of these studies. A recent review provided evidence in support of the hypothesis that anastasis may contribute to tumor relapse following therapy, suggesting that drug persistent cancer cells may represent cells that had undergone anastasis[96]. Cells that “recover” from apoptosis induced by ethyl alcohol can display stem-like properties[97], and cells that survive MOMP following treatment with BH3 mimetic drugs display transient, increased drug tolerance in vitro, and increased invasiveness and metastasis in vivo[13].

The consequences of sublethal cell death engagement: Hallmarks of flatliners

In the following section we discuss the features of flatliners and their possible relationship to DTPs.

Genomic instability

A frequently described consequence of flatliner survival is genetic alteration that can take place upon sublethal activation of caspases, mainly via the caspase-activated nuclease, CAD (Figure 4). Emerging evidence revealed that limited caspase activation, initiated by death receptor signaling[98,99] or by iMOMP[16,100] can result in the sublethal activation of CAD and

cause mutagenesis. As noted above, survival following activation of CAD has been shown to depend on the repair of double-stranded DNA breaks[8].

Reactive oxygen species (ROS) are potential contributors to DNA damage-induced genomic alterations during sublethal mitochondrial cell death. Mechanistically, MOMP and the activation of executioner caspases can result in the cleavage of NADH–ubiquinone oxidoreductase 75 kDa subunit (NDUFS1), an essential part of complex I, resulting in a drop in $\Delta\Psi m$, a rapid reduction in ATP synthesis and an increase in ROS[101,102]. The tumorigenic effects of mitochondrial ROS have been demonstrated in mouse models with heterogeneous deletion of genes that encode crucial mitochondrial proteins, causing an increase in ROS[103–105].

It is likely that a combination of DNA damage by activation of CAD and/or ROS production, together with an impaired DNA-damage response (DDR) increases the risk for oncogenic genomic changes[106]. Proteins involved in nonhomologous end joining (NHEJ) and homologous recombination (HR) are potential substrates of caspases and might be degraded during limited caspase activity[107]. Tumor-specific expression of transcription factors that are involved in both DDR and downregulation of apoptosis-associated genes can also potentially promote flatliners with high mutational burden. For example, the transcription factor BRN2 reprograms the DDR, promoting the more error-prone Ku-dependent NHEJ at the expense of HR, and simultaneously suppresses apoptosis in malignant melanoma cells upon various treatments such as UVB, chemotherapy, and vemurafenib[108]. If BRN2 contributes to the survival of some flatliners, reprogramming could contribute to genomic instability in these cells, a possibility that has not yet been tested.

Proliferation versus dormancy

In vitro and ex vivo data point to distinct heterogeneous subpopulations in untreated and treated cancers, including fast cycling/proliferative, slow cycling/quiescent, or non-cycling/senescent cells[109]. Apoptosis-induced flatliners can display a pro-proliferative state which relies on the caspase 3-dependent upregulation of prostaglandin E2 (PGE2)[110,111]. Conversely, patient-derived xenografts of colorectal cancer which were treated with chemotherapy revealed cancer cells that entered the DTP state, characterized by slow cycling tumor cells[112]. Other evidence suggests that subpopulations of DTP cells with different proliferative states exist, which mainly differ in their antioxidant gene programs and fatty acid oxidation[14].

Cellular senescence can be evoked by diverse intra- or extracellular stresses, including mitochondrial damage, oxidative stress, irreparable DNA damage, and oncogene activation[113]. The permeabilization of the mitochondrial inner membrane in some mitochondria undergoing MOMP[114,115] can lead to release of mitochondrial DNA (mtDNA) to the cytoplasm. This causes activation of the cytosolic cGAS-STING pathway, which connects DNA damage and cytosolic DNA-sensing to senescence, and the senescence associated secretory phenotype (SASP)[116]. Apoptosis-inducing treatments, such as radiation and chemotherapy, often result in a cGAS -dependent release of cytokines and chemokines involved in SASP, such as IFN-β, IL1β, IL-6 and IL-8[117] and induce type I interferon (IFN) responses (Figure 4). The removal of mitochondria abrogates the senescent phenotype

induced by various drugs or irradiation[118]. In addition, cleavage and inactivation of mediators in the IFN pathway by caspases can prevent such activation, and cells induced to undergo the mitochondrial pathway of apoptosis activate STING and IFN responses if caspases are inhibited[119–122]. It is therefore possible that flatliners with limited caspase activation engage STING signaling. The sublethal engagement of MOMP via BH3-mimetics can engage cytokine production by human epithelial cells[123] and the inhibition of caspase activation enabled type I IFN responses and anti-tumoral immunity in *in vivo* experiments of cancer treatment[11,124].

Metastatic potential

An increased metastatic potential of flatliners following engagement of the death receptor pathway of apoptosis has been studied in the context of diverse cancers[125–127]. Commonly, a crucial role of canonical NF-κB signaling for invasion and migration has been found in this setting. Death receptor-induced and cellular IAP (cIAP)-mediated activation of RIPK1 leads to the stabilization and activation of the NFκB1/RelA complex (Figure 5).

Interestingly, highly metastatic apoptosis flatliners engage a pro-invasion program that is promoted by a sublethal caspase-activation[15,100,128] (Figure 4). An aggressive phenotype of apoptosis flatliners with increased metastatic capacity can also be promoted by iMOMP independently of caspase activation via the activation of the induced stress response (ISR) and ATF4[13]. The ISR pathway generates transcription signatures consistent with invasiveness and metastasis or that have a documented impact on cell motility.

Intriguingly, there are indications that sublethal apoptosis engagement has a role in tissue regeneration and wound healing for metazoans[129,130]. This feature of apoptosis flatliners can rely not only on the auto- and paracrine activity of factors released, such as FGF2 or PGE2, but also on the increased motility of cells that survived the engagement of apoptosis[15,129–131]. It is possible that at the limits of damage in a wounded tissue, cells that engage apoptosis but survive play important roles in the repair process.

Inflammatory outcomes

The three forms of regulated necrosis considered herein (necroptosis, pyroptosis and ferroptosis) have been recognized for their role in inflammatory signaling[132]. In the absence of pathogens, dying cells release pro-inflammatory damage-associated molecular patterns (DAMPs, which can act as an adjuvant for adaptive immune responses when combined with neo-epitope mediated antigenicity[133]. This might elicit superior anti-tumor immunity, as combinations of immune checkpoint blockade and chemotherapies or targeted therapies indicate[134,135]. However, little is known about the impact of sublethal engagement of these pathways on inflammatory signaling, without cell death.

The role of ferroptosis in cancer immunity remains hypothetical in the context of flatliners. If, indeed, apoptotic flatliners and/or persister cells (which may often be the same thing, see above) are more dependent on GPX4 for survival, perturbations in GPX4 function may induce ferroptosis in such cells *in vivo*. One consideration is that a decrease in GPX4 activity leads to an upregulation of prostaglandins, including prostaglandin E2 (PGE2)[136]. While PGE2 has not been studied in the context of immunogenicity of ferroptotic cancer

cells, there is a body of evidence for its immunosuppressive functions[137–139]. Ferroptotic cancer cells suppress dendritic cell function and adaptive immune responses despite the release of DAMPs[140], and while the molecular events that shape this response have yet to be defined, it is possible that these are mediated via PGE2 or other prostaglandins.

More evident is the contribution of sublethal necroptosis to anti-cancer immunity. RIPK1 activates NF-κB signaling within a dying cell, leading to transcriptional upregulation of inflammatory cytokines, including IL6 and CXCL1, and promoting anti-tumor immunity[12] (Figure 5). For efficient cross-priming cells relied on both RIPK1 and NF-κB activation. The ESCRTIII machinery allows cells that have engaged necroptosis to continue this intracellular signaling, leading to production and release of immune mediators and inducing adaptive immunity[50].

In stark contrast to regulated necrosis, apoptosis has been considered as an immunologically silent cell death program[141]. This dogmatic view has been challenged in recent years, owing to evolved experimental approaches and increasing refinement of the characteristics of immunogenic cell death[133]. Early upstream initiation of the pro-inflammatory and pro-survival signals via the TNFR death receptor family offers an intriguing opportunity to prevent lethal events, and instead to function as a messenger to its environment. Interestingly, FAS/CD95-induced cytokine production requires RIPK1 as well as the downstream XIAP[142]. Cell survival and tumorigenesis have been widely associated with cIAPs, but this function is mostly attributed to their potential to activate the canonical NF-κB pathway[143,144].

In contrast to their activating role in the canonical NF-κB pathway, cIAPs negatively regulate non-canonical NF-κB signaling[145], which was demonstrated in the context of pharmaceutical IAP antagonism using SMAC-mimetic drugs [146] and as a consequence of MOMP in the absence of caspases[11]. In the latter setting, the release of IAP antagonists (such as SMAC/DIABLO) upon MOMP act to inhibit not only XIAP, but also cIAPs, and can thereby engage non-canonical NF-κB signaling (Figure 5). In this pathway, inhibition of cIAPs (e.g. via CD40 or LTbR-receptor) leads to NF-κB-inducing kinase (NIK) stabilization, followed by its binding and activation of the kinase IKKα and thereby initiation of the NFκB2/RelB complex.

NF-κB stabilization can also be a direct effect of translation reprogramming in apoptosis flatliners, where upon eIF2α phosphorylation the short-lived NFκB inhibitor IκB is no longer synthesized[147–149] (Figure 5). In addition, cells also trigger inflammatory signaling mediated directly by ATF4, which drives the transcription of several cytokines including IL-8 and CCL2[13,150,151]. However, *in vivo* experiments so far indicate that a pro-inflammatory role for flatliners of the intrinsic pathway of apoptosis can only be achieved in the absence of caspase activity[152] or if apoptosis is followed by secondary necrosis via Gasdermin E[60,153] (Figure 1). Yet, Gasdermin E is frequently downregulated in cancers[154–156].

Acquired sensitivities of flatliners enable therapies

Does the survival of flatliners contribute to minimal residual disease in treated cancer patients? If so, then acquired vulnerabilities of such cells may invigorate cancer therapies. In a study of BH3 mimetic-induced DTPs, a BAX, BAK, and ATF4 gene signature in these surviving flatliners was identified in a publicly available data set from lung cancers with minimal residual disease, but not in those that were treatment naïve or progressed post-treatment[13]. Therefore, targeting flatliner survival, may impact minimal residual disease.

In this regard, the expression of ATF4 as a consequence of the ISR may represent a therapeutic target. Studies have indicated that ATF4 is required for the DTP phenotypes[13,157,158] and an inhibitor of the ISR, by stabilizing eIF2B, was effective in preventing DTP generation *in vitro*[13]. Activators of eIF2B are in preclinical development and may have benefit to limit persistence and residual disease[159–161].

The observation that repair of double-strand DNA breaks is necessary for survival of cells that have engaged caspase activation and CAD function[8] suggests an approach to limiting the emergence of DTPs. Studies of EGFR mutant non-small cell lung cancer cell lines and patient xenografts showed that the generation of DTPs following targeted therapies was blocked by an inhibitor of ataxia-telangiectasia mutated (ATM). Rare patients who harbor ATM mutations in such cancers showed better prognosis than those whose cancers had functional ATM, highlighting the possible application of ATM inhibitors to prevent relapse[8].

Another approach is to harness the increased sensitivity towards ferroptosis in DTPs, which has been established in experimental settings for kinase inhibitor-treated cancer cells[86,87,162]. While the regulation of ferroptosis appears to be an attractive target to treat susceptible cancer types[70] and metastatic outburst[163], several hurdles, including the bioavailability and drug stability of specific ferroptosis-inducing small molecules[164], must be addressed before clinical use is possible.

Preclinical research on organoids and xenografts revealed that diapause-like adaptation of DTP cancer cells is associated with suppressed MYC activity and can be targeted by CDK9 inhibition, a cyclin-dependent kinase that is involved in transcriptional control[165]. Diverse CDK9 inhibitors are currently in clinical trials[166] and combinational therapy with the BH3-mimetic Venetoclax showed promising results in certain murine tumor models[167].

Inhibition of the PGE2 signaling pathway, which may be increased in surviving flatliners (see above) appears to be promising due to its involvement in many processes, including tumor cell proliferation and repopulation as well as chemoresistance and pro-tumoral immunity. In murine experimental settings, neutralizing antibodies against PGE2 or the administration of the cyclooxygenase-2 (COX2) inhibitor celecoxib could successfully abrogate chemoresistance-associated, early tumor repopulation[111]. Celecoxib has been beneficial in the context of sporadic colorectal adenomas[168] and its value in combinational therapies for cancer treatment is under clinical investigation[169].

CONCLUSIONS

Flatliner cells that survive the engagement of RCD pathways in response to therapy have the properties ascribed to cancer DTPs, and thus, the goal of most cancer therapies (to induce RCD in cancer cells) directly brings about the cellular changes that we seek to avoid. By recognizing this relationship and how it occurs, we can identify ways to prevent flatliners and thus limit DTP generation and, at least in some cases, minimal residual disease and relapse in cancer.

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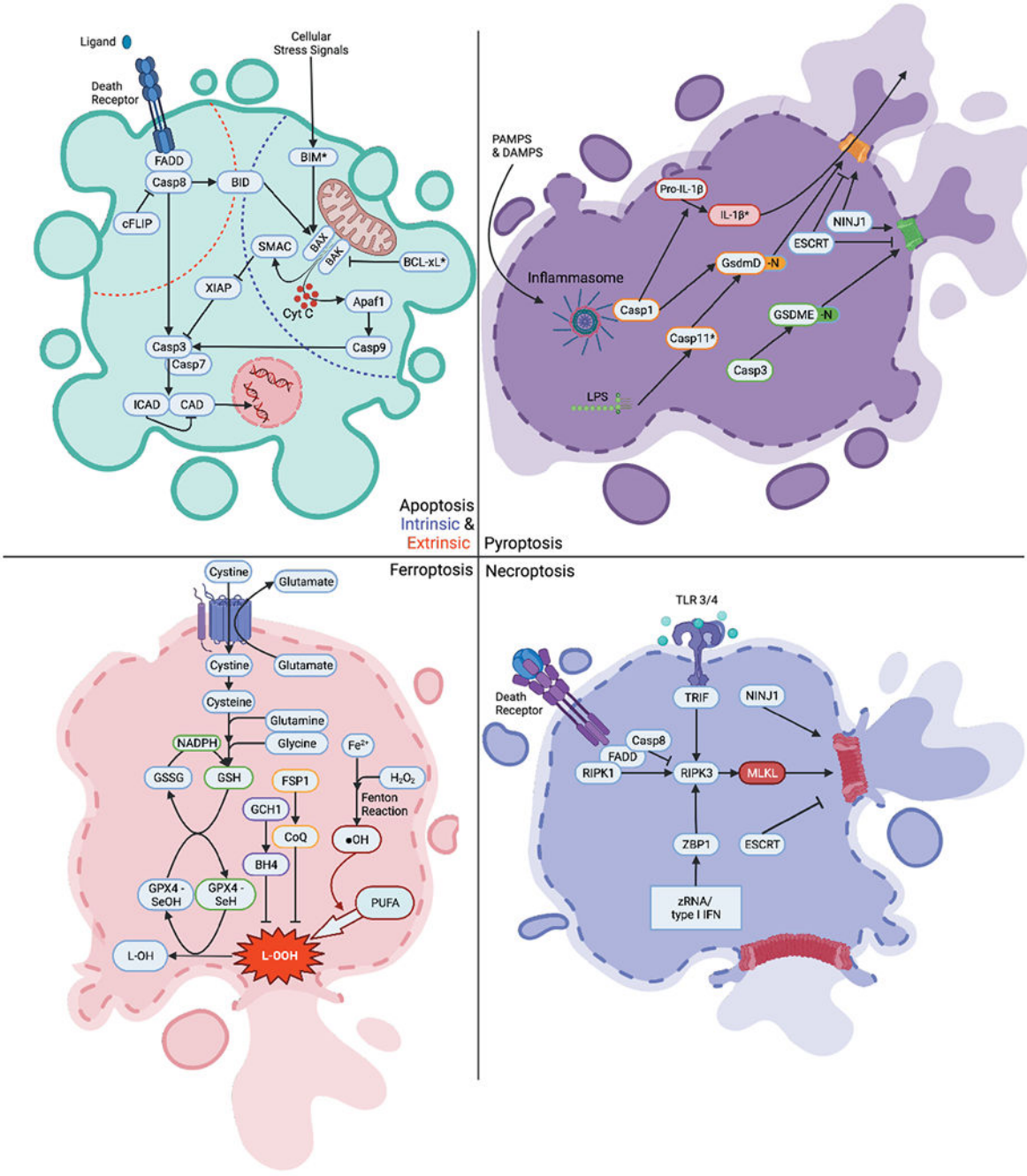

Figure 1: Cell death pathways.

Intrinsic Apoptosis: Upon cellular stress, Bax & Bak permeabilize the outer membrane of the mitochondria releasing many proteins including cytochrome c and SMAC into the cytoplasm. Pro-survival BCL-2 family members (represented by BCL-XL*) can prevent MOMP. Upon the release of cytochrome c, Apaf-1 binds to initiator caspase (caspase-9) forming the “apoptosome”. This apoptosome then cleaves the executioner caspases (caspase-3 and −7) leading the proteolytic cleavage of many substrates including the ICAD which activates CAD. CAD then cleaves nuclear DNA and induces apoptotic cell

death. Additionally, MOMP-induced release of SMAC inhibits XIAP and thereby releases the break on caspase-3. **Extrinsic Apoptosis:** After activation of a death receptor (e.g. FAS), the FADD adaptor recruits and activates caspase-8. Activated caspase-8, if not inhibited by cFLIP binding, then directly cleaves caspase-3 and induces cell death as described above. Additionally, caspase-8 can also cleave BID which induces MOMP directly or by activation of BAX. **Pyroptosis:** Direct binding of LPS to inflammatory caspase-11 (caspase 4,5 in humans) leads to cleavage of GsdmD releasing the N-terminus (GsdmD-N). The GsdmD-N then forms an oligomer on phospholipid-membranes, permeabilizing it and inducing pyroptotic cell death. Alternatively, PAMPs and DAMPs can activate the inflammasome which can mediate inflammatory caspase-1 cleavage of GsdmD termed the "canonical inflammasome" pathway. Caspase-1 can also process Pro-IL-1β into IL-1β which is released via GsdmD pores. In addition to GsdmD-dependent pyroptosis, GsdmE, after cleavage from executioner caspase-3, can also lead to pore formation via it's released N-terminal fragment (GsdmE-N). Both GsdmD and GsdmE pores can be repaired via the ESCRT complex. NINJ1 mediates cell rupture. **Necroptosis:** Upon stimulation of a death receptor, RIPK1 is activated and forms an oligomer with RIPK3, activating it (if Caspase 8 is inhibited). RIPK3 then phosphorylates MLKL which oligomerizes and forms a pore in the plasma membrane. Alternatively, activation of ZBP1 or TRIF can also activate RIPK3 leading to necroptosis. ESCRT proteins can repair MLKL pores. NINJ1 mediates cell rupture. **Ferroptosis:** Fe^{2+} reacts with hydrogen peroxide during fenton reaction, thereby generating hydroxyl radicals (•OH), which oxidize PUFAs. The resulting toxic oxidized lipids (L-OOH) can integrate into phospholipid membranes and thereby lead to necrotic cell death. The selenoenzyme GPX4 can directly detoxify L-OOH. Glutathione reduces GPX4 to replenish the antioxidant pool, thereby getting oxidized itself into glutathione disulfate (GSSG). NADPH from the pentose phosphate pathway reduces GSH. The xc^- transporter exports glutamate and imports cystine into the cytosol. Cystine is then converted into cysteine, and then synthesized together with glutamine and glycine to glutathione. BH4, as produced by GCH1, is also able to decrease levels of oxidized lipids. FSP1 is able to generate CoQ which acts as an antioxidant capable of inhibiting ferroptosis.

* Bcl-xL is shown as a representative of "pro-survival" BCL-2 family members

* Caspase-11 in mice, Caspase-4,5 in humans.

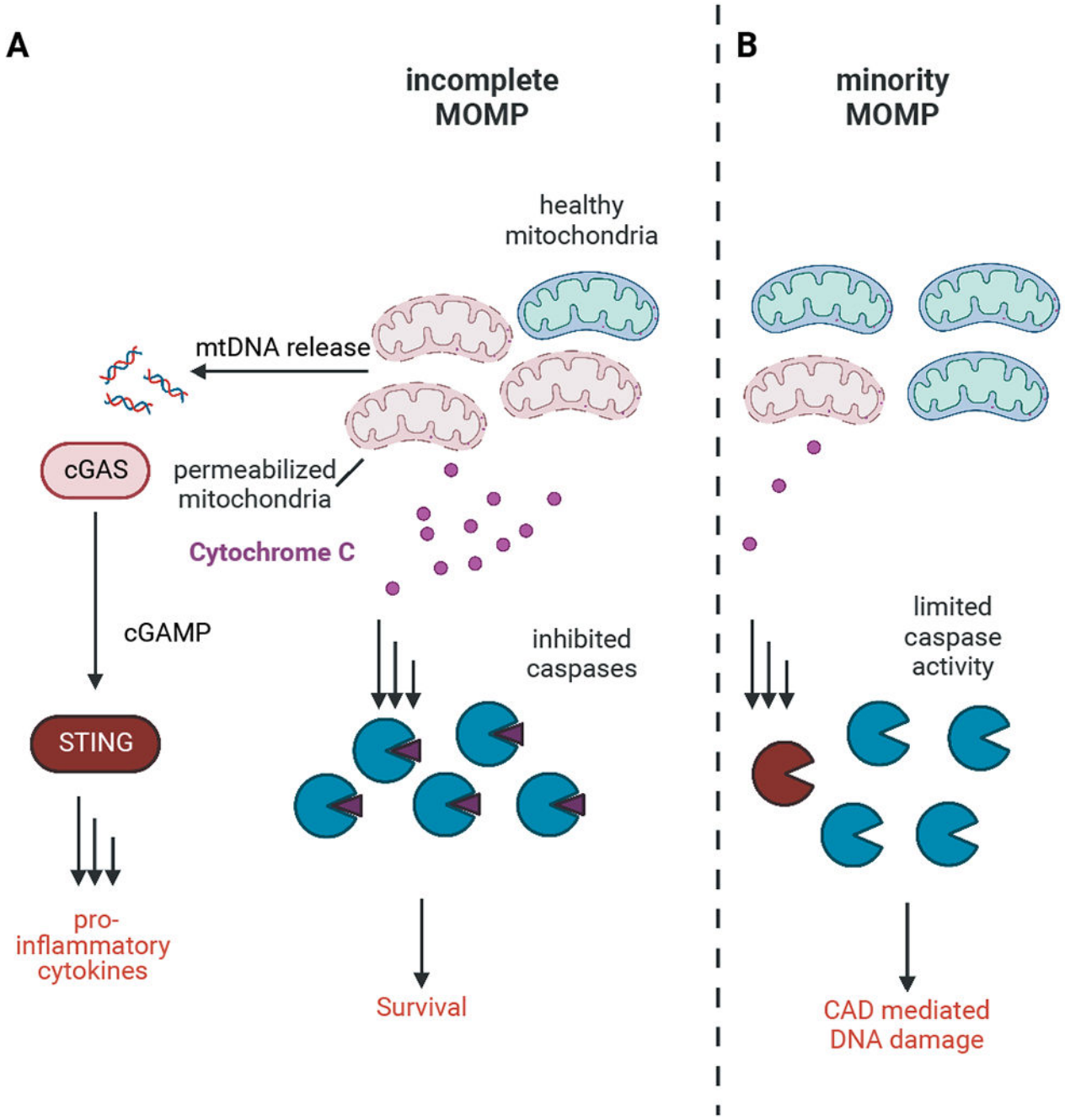

Figure 2: Apoptosis induction and survival

A If apoptotic caspases are inhibited induction of incomplete MOMP leads to release of mitochondrial DNA from permeabilized mitochondria. This stimulated activation of the cGAS-STING pathway and production of pro-inflammatory cytokines. Cells harboring enough healthy mitochondria can expand these mitochondria and survive even after engagement of apoptosis.

B Low levels of MOMP (miMOMP) engage sublethal levels of caspase activation, which can mediate DNA damage through activation of CAD.

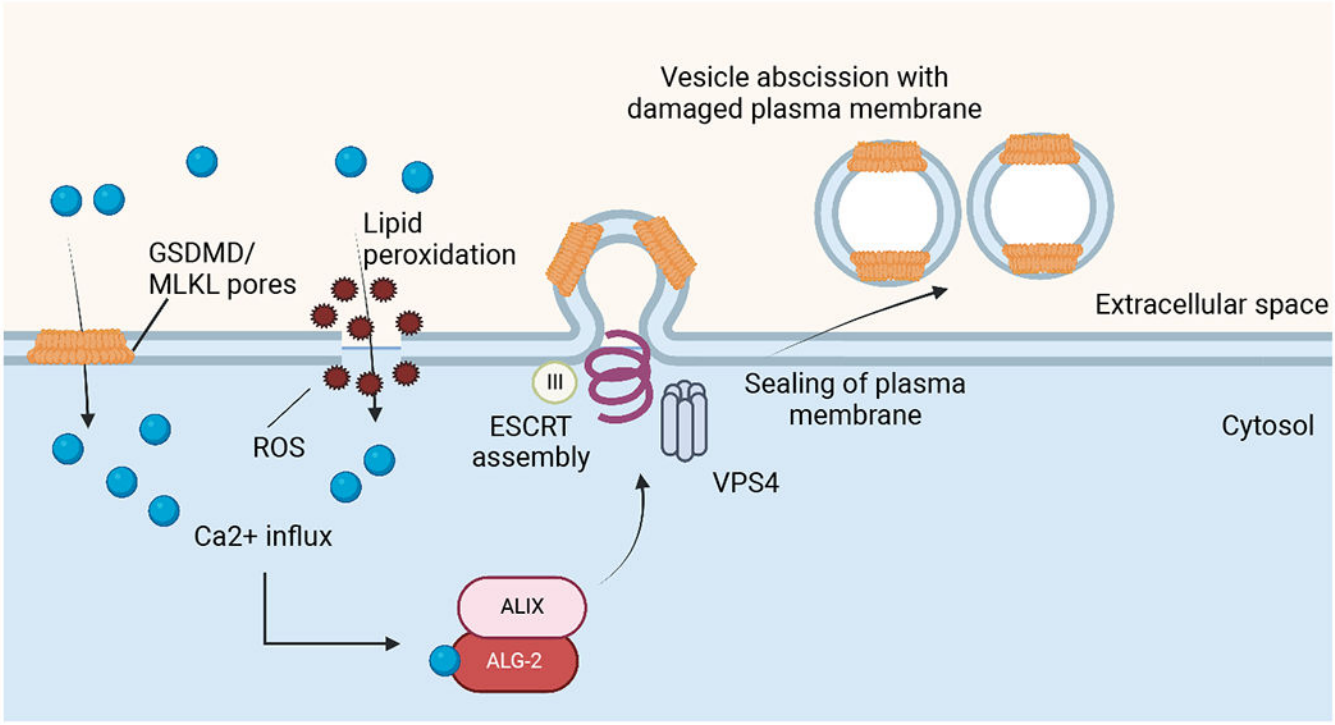

Figure 3: ESCRT mediated membrane repair of plasma membrane damage.

Calcium influx through gasdermin D or MLKL pores as well as plasma membrane (PM) damaged by lipid peroxidation leads to recruitment of ALIX through ALG-2. This promotes assembly of the ESCRT-III machinery at the site of calcium influx. Subsequently, VPS4 mediates abscission of the damaged pieces of PM, restoring its integrity.

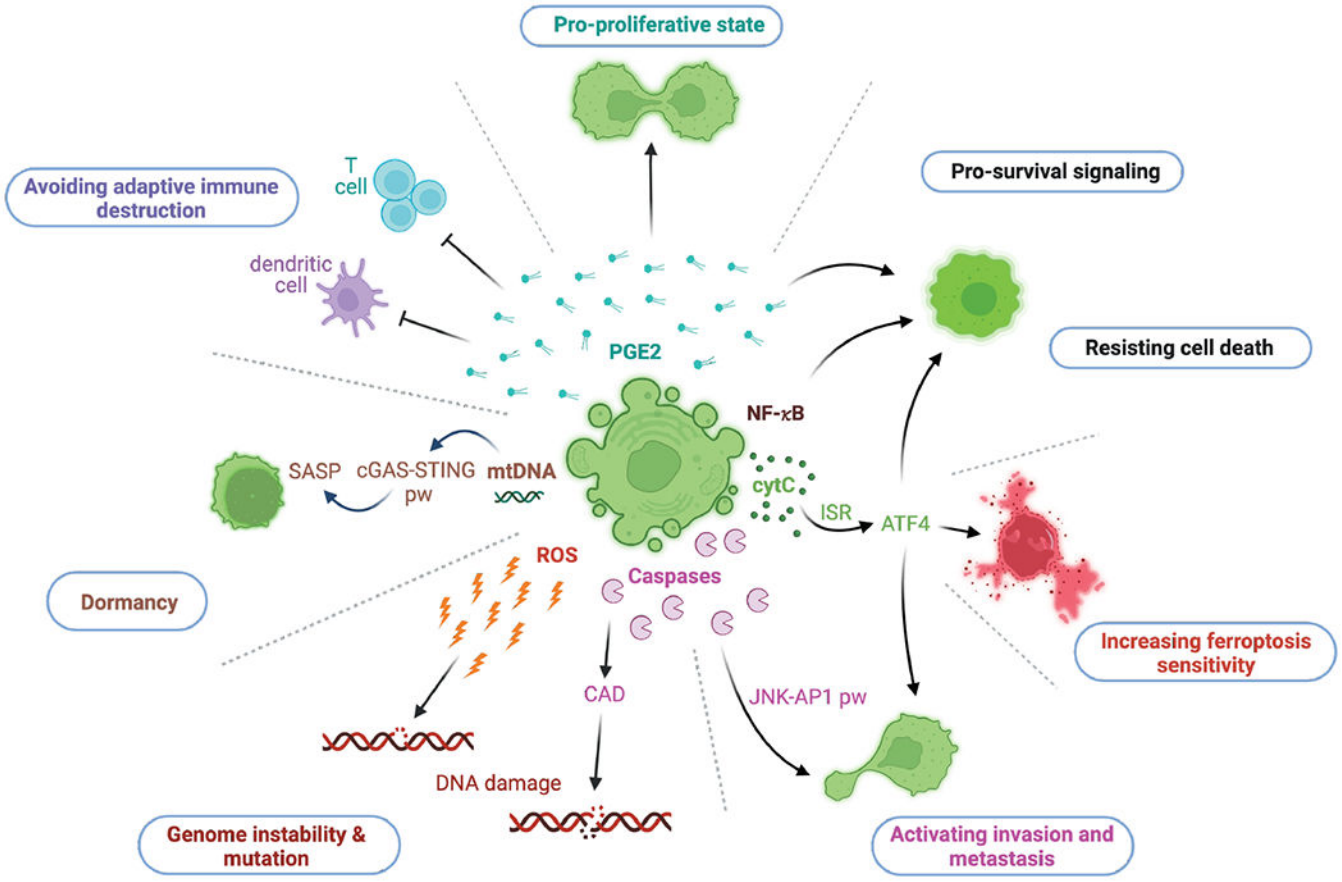

Figure 4: Non-lethal outcomes for apoptosis flatliners.

Illustration of evident consequences of the sublethal engagement of the intrinsic pathway of apoptosis and their main molecular players. The release of PGE2 relays inhibitory effects on T cells and dendritic cells. PGE2 can promote a pro-proliferative state and cell survival. NF-κB engages pro-survival signaling. Sublethal cytochrome c release can activate the ISR and thereby ATF4 translation. ATF4 impacts on various signaling pathways that lead to an escape from cell death, an increased sensitivity towards ferroptosis and a metastatic phenotype. Latter can also be promoted by sublethal caspase activation followed by subsequent initiation of the JNK-AP1 pathway. ROS and CAD can cause DNA damage, leading to genome instability and mutation. The cytosolic release of mtDNA can activate the cGAS-STING pathway, which promotes senescence and a senescence-associated secretory phenotype (SASP).

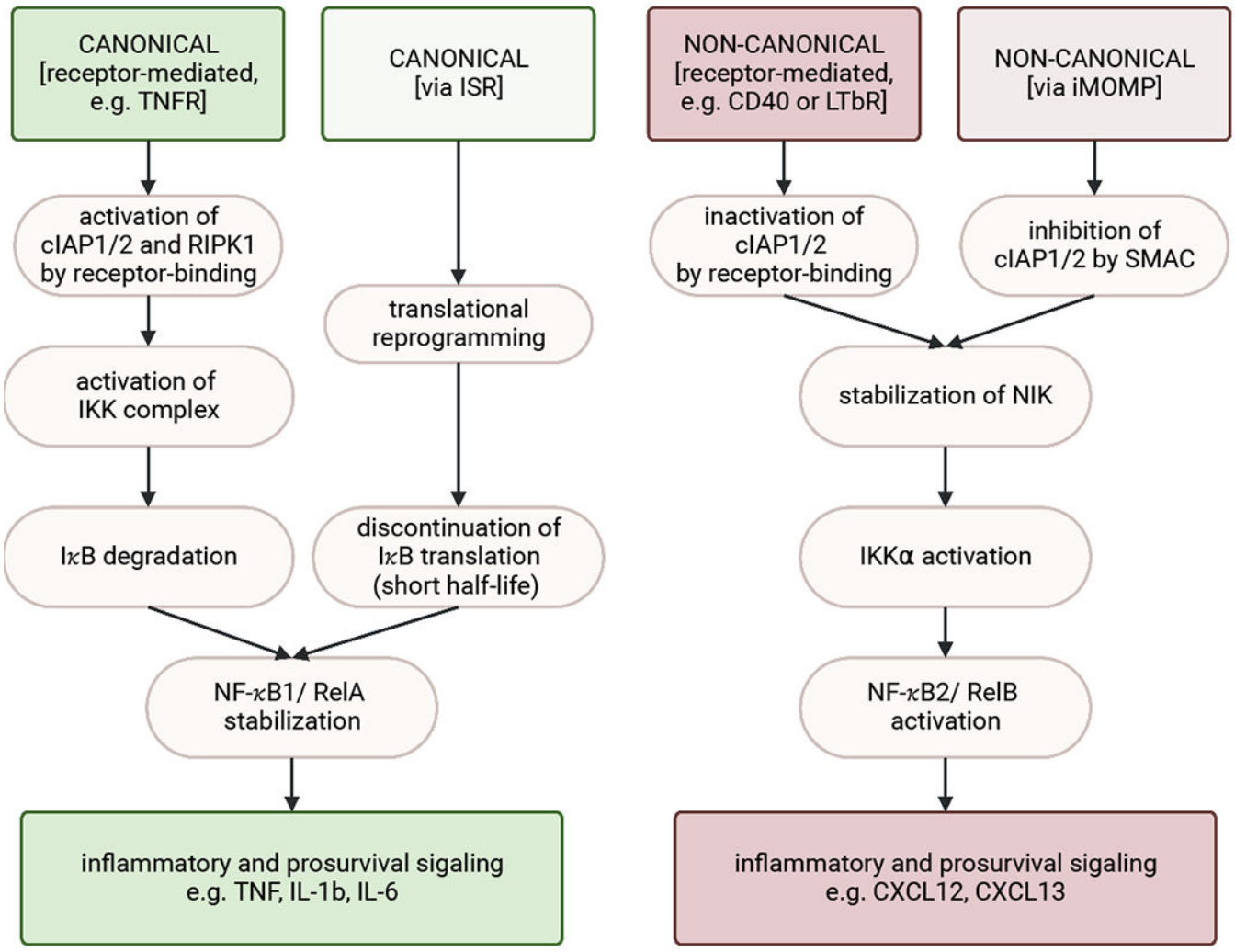

Figure 5: Activation of NF-κB signaling.

The canonical NF-κB signaling pathway can be promoted via receptor-mediated activation (e.g. via TNF-receptor) or engagement of the ISR. Receptor activation leads to the recruitment of adaptor proteins to form and activate a complex including cIAP and RIPK1. In turn, RIPK1 engages the IKK complex, which induces the proteasomal degradation of IkB. This then allows for the stabilization and nuclear localization of the NF-κB1/ RelA heterodimer. Nuclear localization leads to the transcription of proinflammatory cytokines such as TNF, IL-1b, and IL-6. Activation of the ISR followed by translational reprogramming can lead to NF-κB stabilization due to discontinuation of IκB generation, which is a short-lived NFκB-inhibitor.

The non-canonical NF-κB signaling pathway can be engaged by the inhibition of cIAPs, which occurs either upon receptor engagement (e.g. via CD40 or LTbR) or by SMAC, which is released during MOMP. This results in the stabilization of NIK, which is then free to activate IKKα, resulting in NF-κB2/RelB activation and transcription of noncanonical NF-κB associated genes, such as CXCL12 and CXCL13.

Conclusively, upon specific receptor activation cIAPs engage the canonical NF-κB signaling pathway while inhibiting the non-canonical. *Vice versa*, inactivation of cIAPs promotes non-canonical NF-κB signaling through NIK stabilization while inhibiting the canonical pathway.