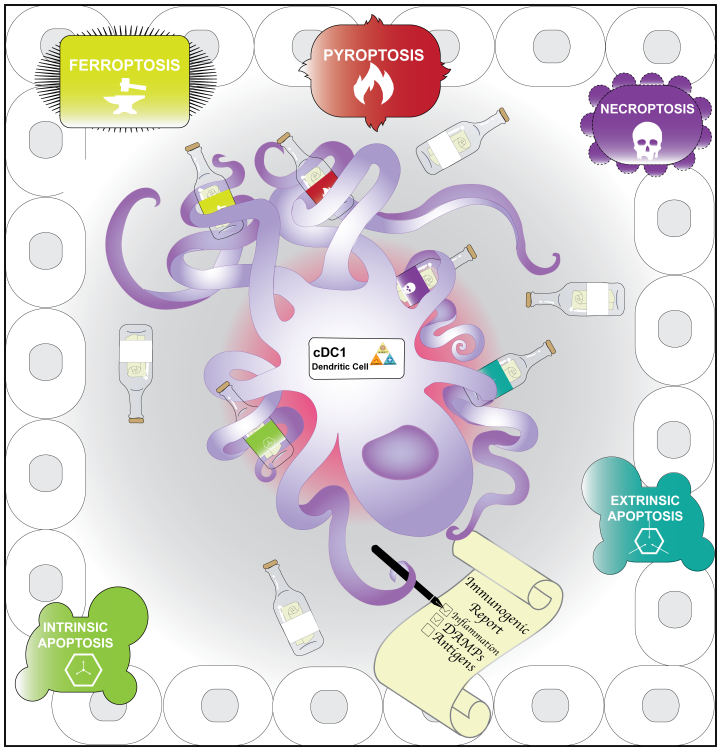
The Diversification of Cell Death and Immunity:

Memento Mori

Graphical Abstract



Authors

Arnaud J. Legrand, Maria Konstantinou,

Emily F. Goode, Pascal Meier

Correspondence

pmeier@icr.ac.uk

In Brief

Legrand et al. provide a timely overview of the different forms of regulated cell deaths and their interconnectivity. They explore how these death events can nucleate innate and adaptive immune responses during pathogen invasion and tumorigenesis, and whether this knowledge can be used to develop more effective cancer immunotherapies.

The Diversification of Cell Death and Immunity: *Memento Mori*

Arnaud J. Legrand[1], Maria Konstantinou[1], Emily F. Goode[1] and Pascal Meier[1*]

[1]Breast Cancer Now Toby Robins Research Centre, The Institute of Cancer Research, London, SW3 6JB, UK

*Correspondence should be addressed to P.M. (email: pmeier@icr.ac.uk)

Summary

Why do cells have so many ways to die? Why does "cellular suicide" exist at all? In the war against pathogens and rogue cells, organisms developed cellular suicide as last resort. Fighting an evolutionary arms race, cell death pathways have adapted and multiplied to cover the complexity of the foes the immune system faces. In this review, we discuss the different types of cell death, the underlying signaling events and their unequal ability to trigger an immune response. We also comment on how to use our knowledge of cell death signaling to improve the efficacy of cancer treatment. We argue that cell death is integral to the immune response and act as a beacon, a second messenger, that guides both immune system and tissue microenvironment to ensure tissue repair and homeostasis. *Memento Mori* "remember you must die", as failure to do so opens the way to chronic infection and cancer.

'Therefore victory in war is not repetitious, but adapts its form endlessly.' Sun Tzu – The Art of War, 5th century BC.

The war against pathogens is immemorial. Its battles, victories and defeats, are deeply rooted in every being's biology. Although ancient (Cooper and Alder, 2006; Kimbrell and Beutler, 2001), our immune strategies are not static. Multicellular organisms and their pathogens are locked in an arms race, where stealth, deception and innovation are key. Amongst the defense against pathogens, the use of cellular suicide or *Regulated Cell Death* (RCD) might appear most extreme. Nevertheless, it can act as a beacon, a message from beyond the veil, giving location, nature, severity and span of the attack (Galluzzi et al., 2017; Stephenson et al., 2016). But why so many types of cell death? Highly specialized pathogens always find a way to stop signaling that relies on a single pathway. Even the ultimate enemy within, cancer, keeps the immune system at bay by denying death and the release of danger signals, much in a similar way to pathogens (Chen and Mellman, 2013; Hanahan and Weinberg, 2011). For these reasons, evolution has shaped intricate, multimodal pathways to trigger multiple types of RCD, luring the attacker into believing their ploy, only to drag them into worse odds. In this review, we stress the importance of the diversity of cell death pathways, and more precisely its impact on the immune response. We summarize the current understanding of the characteristics that control cell death immunogenicity, and why it could have never been simple.

The cell death modalities

How does cell death trigger an immunogenic response? It is a prelude, which encompasses the molecular events between the trigger and the execution of cell death (Chen and Mellman, 2013; Galluzzi et al., 2017; Yatim et al., 2017). Recent classifications of RCDs define cell death modalities by the molecular pathways involved in their process, and not on morphology alone. Following these recommendations, the current scientific consensus describes more than ten different

cell death modalities (Galluzzi et al., 2018). Here, we will mainly focus on cell death modalities commonly encountered in several cell types, and with a known impact on the immune response. The others will only be briefly described (see (Galluzzi et al., 2018) for more details).

Intrinsic and extrinsic apoptosis

When cell death is necessary, apoptosis appears as a first choice. A well-orchestrated partitioning of the cell into plasma membrane-enclosed apoptotic bodies (blebbing) and the fragmentation of chromosomal DNA offers an orderly departure: efficient, organized and generally immunologically silent. There are many triggers of apoptosis, from homeostasis requirements during development or tissue regression, to diverse cellular challenges such as DNA damage, starvation and mechanical stresses. Apoptosis can be engaged by two different non-exclusive modes: intrinsic and extrinsic (Galluzzi et al., 2018). Both of these processes are tightly regulated and coordinated by a family of specific proteases called caspases. Importantly, caspases are not essential to the conclusion of the intrinsic pathway. Caspases of the intrinsic pathway amplify and accelerate the death signal and, most importantly, silence its immunogenicity (Giampazolias et al., 2017; McArthur and Kile, 2018; McArthur et al., 2018). The crucial event of the intrinsic apoptotic pathway is the *mitochondrial outer membrane permeabilisation* (MOMP) (Galluzzi et al., 2018; Riley et al., 2018; Tait et al., 2010), orchestrated by members of the *B-cell lymphoma-2* (BCL-2) family (Kalkavan and Green, 2018). The pro-apoptotic members *BCL-2 associated X* (BAX), *BCL-2 homologous antagonist killer* (BAK) and *BCL-2 related ovarian killer* (BOK) form pores within the mitochondrial outer membrane, allowing the cytoplasmic release of mitochondrial danger signals such as cytochrome c and *second mitochondria-derived activator of caspase* (SMAC). Conversely, this pro-apoptotic activity is counteracted by anti-apoptotic BCL-2 proteins (Kalkavan and Green, 2018). Thus, the balance between pro- and anti-apoptotic BCL-2 effectors dictates the death threshold. This is shown by BCL-2 inhibitors (e.g. Navitoclax or Venetoclax), which trigger cell death in cancer cells only by removing the anti-apoptotic brake (Cory et al., 2016). Once MOMP occurs, cells are as close as they can be from "a point of no return", although some exceptions have been observed (Colell et al., 2007; Gong et al., 2019; Martinou et al., 1999)). While MOMP is essential for intrinsic cell death, the same cannot be said for caspases as cells typically die following MOMP even in the absence of caspase activity. MOMP-induced activation of caspases merely accelerates cell death processes (Kalkavan and Green, 2018) (Figure 1), allowing the host to quickly and efficiently clear away dead cell corpses without provoking an immune response (Arandjelovic and Ravichandran, 2015). If, however, caspase activity is blocked following MOMP, cell death still occurs but this type of death is now accompanied with a type I interferon (IFN) response and *nuclear factor kappa-light-chain-enhancer of activated B cells* (NF-κB) activation that alerts the immune system (Giampazolias et al., 2017; Rongvaux et al., 2014; White et al., 2014).

Extrinsic apoptosis exists as an additional layer whereby the death signal can be provided by the environment, the immune system or neighboring cells (Annibaldi and Meier, 2018; Galluzzi et al., 2018). The signal comes from death ligands, such as *fas ligand* (FASL), *tumor necrosis factor* (TNF) or *TNF-related apoptosis-inducing ligand* (TRAIL), and are recognized by specific death receptors (FAS, TNFRSF1A and TNFRSF10A/B respectively) (Galluzzi et al., 2018). Following stimulation, cytoplasmic signaling platforms are assembled. For FAS and TNFRSF10A/B, it is called the *death-inducing signaling complex* (DISC) composed of *Fas-*

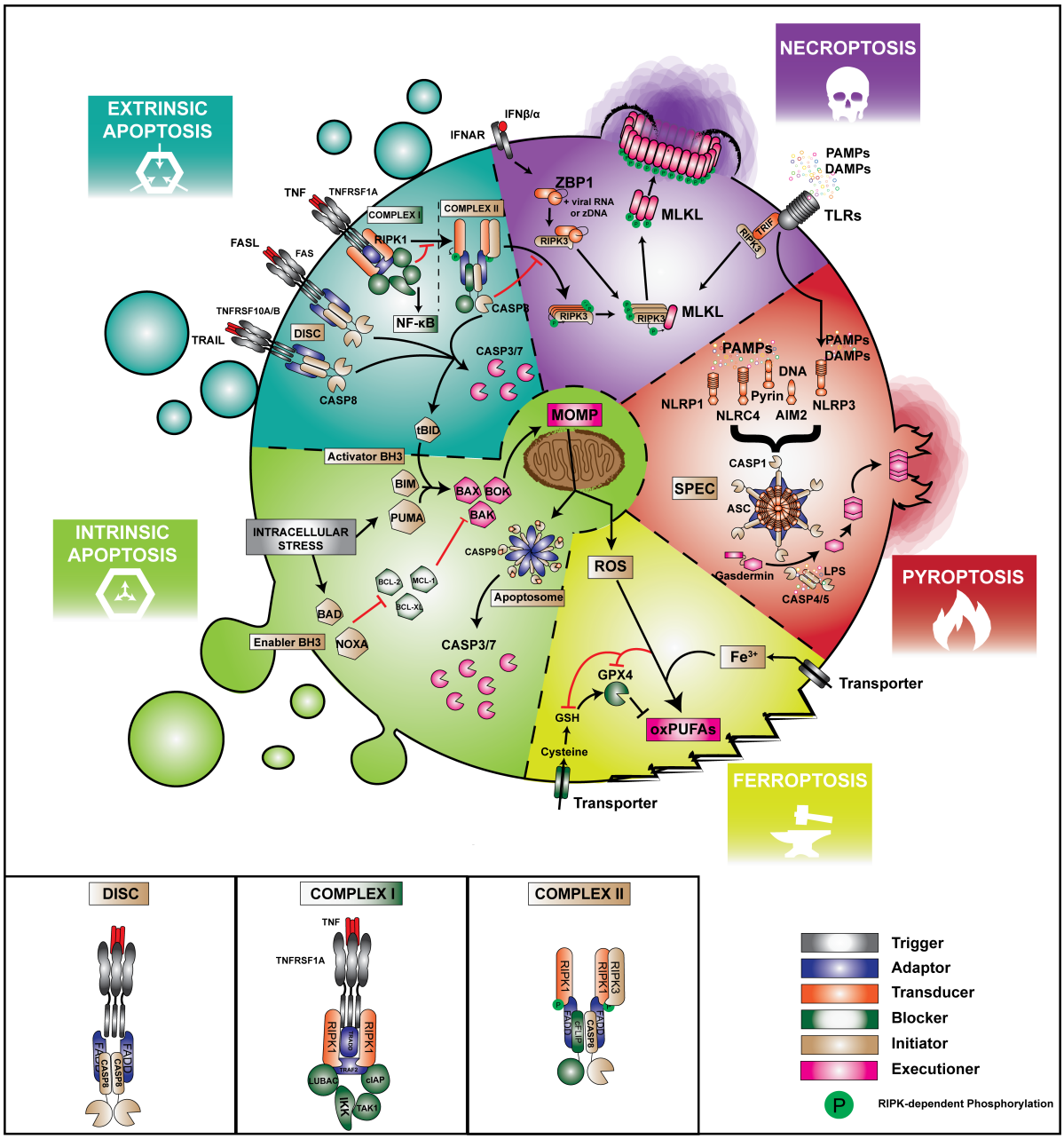


Figure 1: The Wheel of Death. Presented are five of the best characterized cell death modalities. Each modality is summarized from the trigger to the execution. Abbreviations: ROS: Reactive oxygen species; oxPUFAs: oxidized polyunsaturated fatty acids; PUMA: p53 upregulated modulator of apoptosis; BCL-XL: B-cell lymphoma-extra-large; BAD: BCL-2-associated death promoter; LUBAC: linear ubiquitination assembly complex; IKK: IκB kinase complex.

associated protein with death domain (FADD), initiator caspase-8 (CASP8) (sometimes CASP10 in human) and *FLICE-like inhibitory protein* (c-FLIP) (Galluzzi et al., 2018) (Figure 1). In the case of TNFRSF1A, the complex (I) formed at the receptor is more convoluted and centered on the function of the kinase *receptor-interacting serine/threonine-protein kinase 1* (RIPK1) (Annibaldi and Meier, 2018). Indeed, in contrast to FAS and TNFRSF10A/B, the primary output of TNFRSF1A is not death but NF-κB signaling, a crucial pathway for inflammation and anti-pathogen response (Annibaldi and Meier, 2018) (Figure 1). To switch to death, multiple checkpoints of TNF-induced cell death signal, operating at both transcriptional and post-translational levels, must be

unfastened (for review see (Annibaldi and Meier, 2018)). Once these brakes are released, RIPK1 leaves complex I and forms a new, cytosolic complex (II) with FADD, CASP8 and cFLIP. Depending on the levels of cFLIP, this complex is either rapidly degraded or accumulates to drive cell death (Annibaldi and Meier, 2018; Galluzzi et al., 2018) (Figure 1). RIPK1 is not always essential for TNF-induced apoptosis but CASP8 is (Annibaldi and Meier, 2018; Galluzzi et al., 2018). After CASP8 activation, executioner caspases CASP3 and CASP7 can be turned on in some cells (type I), such as lymphocytes (Figure 1). Interestingly, the extrinsic pathway is not always sufficient to trigger cell death. In type II cells, such as hepatocytes, an amplification loop via MOMP is necessary for death, highlighting the importance of a crosstalk between extrinsic and intrinsic death pathways (Kalkavan and Green, 2018). Indeed, CASP8 cleaves BCL-2 family protein *BH3 interacting-domain death agonist* (BID). The truncated form of BID (tBID) then activates BAX and BAK and effectively triggers MOMP (Haudek et al., 2007; Jost et al., 2009) (Figure 1).

It is by looking at apoptotic triggers that we better understand the essential role of cellular suicide as the safeguard of host survival. The primary defense against pathogens are inflammatory responses caused by signaling pathways such as NF-κB (Rahman and McFadden, 2011) and type I interferon (IFN) (Schneider et al., 2014). However, because pathogens can inhibit these pathways and thus silence the immune response, it became essential to detect pathogen with this ability and sound the appropriate alarm, cell suicide (Annibaldi and Meier, 2018; Orzalli and Kagan, 2017; Rahman and McFadden, 2011). A perfect example resides in the ability of bacteria *Yersinia enterocolitica* to disable NF-κB signaling by inhibiting the kinase *TGF-beta activated kinase 1* (TAK1) (Menon et al., 2017; Mukherjee et al., 2006). TNFRSF1A activation without TAK1 and NF-κB outputs immediately cause RIPK1 transition from complex I to complex II, and thus apoptosis (Menon et al., 2017; Peterson et al., 2017).

Is apoptosis the final answer to all attacks against the organism? Unfortunately, no. Pathogens and cancer cells designed their own solution to the problem. Viruses, most notably, mastered the control of apoptotic signals. Multiple strategies include inhibiting caspases (e.g. herpesviruses, poxviruses) or blocking MOMP (e.g. Kaposi, EBV) (Mocarski et al., 2011; Neumann et al., 2015). In the case of cancers, the ability of executing apoptosis is simply often blocked (Hanahan and Weinberg, 2011). Although apoptosis is frequently under pathogen control, not all is lost. Our immune system has still several tricks up its sleeves.

Necroptosis

A pathogen infects a cell. Interferences with NF-κB signal causes RIPK1 to form a pro-apoptotic complex II. However, when CASP8 activity is blocked then apoptosis is prevented. What is left? Well, if this happens in a cell type that expresses *receptor interacting serine/threonine kinase 3* (RIPK3), the culprit pathogen has just activated an immunological landmine called necroptosis (Cho et al., 2009; Degterev et al., 2005; Yatim et al., 2015). The first substrate of CASP8 is RIPK1. In the absence of CASP8 cleavage, RIPK1 activity booms and mobilizes RIPK3, which causes the phosphorylation of the pseudokinase *mixed lineage kinase domain like* (MLKL) (Sun et al., 2012; Weinlich and Green, 2014). The consequence of MLKL phosphorylation are, at cellular level, cataclysmic. MLKL oligomerizes and relocate to the membrane where it forms pores, disrupting the integrity of the plasma membrane (Petrie et al., 2019) (Figure 1). Other than during TNF signaling, necroptosis can also be triggered following type I or type II IFN stimulation, more particularly in absence of RIPK1 (Dillon et al., 2014; Lin et al., 2016; Newton et al., 2016). This is due to an interferon inducible protein called *Z-DNA-binding protein 1* (ZBP1) that can

recruit RIPK3 directly and cause necroptosis (Dillon et al., 2014; Kuriakose and Kanneganti, 2018). Interestingly, MLKL is also interferon-inducible (Knuth et al., 2019; Sarhan et al., 2019). Finally, *pattern recognition receptors* (PRRs) can as well trigger necroptosis. These signaling pathways depend on RIPK1, ZBP1 or *TIR-domain-containing adapter-inducing interferon-β* (TRIF) to recruit RIPK3. It has been observed with RNA sensors *toll-like receptor 3* (TLR3) (He et al., 2011; Kaiser et al., 2013) and *retinoic acid-inducible gene I* (RIG-I) (Brault et al., 2018; Di Paolo et al., 2013; Schock et al., 2017), DNA sensor *cyclic GMP-AMP synthase* (cGAS)/ *stimulator of interferon genes* (STING) (Brault et al., 2018; Chen et al., 2018; Di Paolo et al., 2013; Schock et al., 2017) and LPS receptor TLR4 (He et al., 2011). Thus, necroptosis can be considered as a second line of defense. Primed by the interferon response, by direct detection of replicating viruses or bacterial components, and by the failure of apoptosis, necroptosis ensures that a powerful message of alert is sent to the immune system: There has been a major breach in the defenses.

Yet again, the foes find a way. Herpesviruses are able to block interaction between RIPK3 and RIPK1, ZBP1 and TRIF. This is due to the fact that these three proteins contact RIPK3 through a conserved *RIP homotypic interaction motif* (RHIM) targeted by the virus (Mocarski et al., 2015). Additionally, poxviruses, like *Vaccinia*, produce viral proteins that can interfere with ZBP1's ability to recruit and activate RIPK3 (Koehler et al., 2017). In several cancer types, RIPK3 expression is often lost, by hypermethylation of its promoter rendering the execution of necroptosis close to impossible (Koo et al., 2015).

Pyroptosis

Pyroptosis, like necroptosis, is a form of lytic RCD that culminates with the perforation of the plasma membrane and the release of intracellular content (Galluzzi et al., 2018). It is mostly observed in monocytes and macrophages, but has also been characterized in certain epithelial cells (Shi et al., 2014). Pyroptosis is defined by the processing of gasdermin D or E (GSDMD or GSDME) by caspases (Kayagaki et al., 2015; Shi et al., 2015; Wang et al., 2017). The cleavage of GSDMD can be ensured by CASP1, CASP4/5 (CASP11 in mouse) or CASP8 (Kayagaki et al., 2015; Orning et al., 2018; Sarhan et al., 2018a; Shi et al., 2015), whereas GSDME is cleaved by CASP3 and CASP8 (Sarhan et al., 2018a; Wang et al., 2017). Once processed, gasdermins form large pores in the plasma membrane, leading to cell death (Ding et al., 2016) (Figure 1). In parallel, in monocytes and macrophages, active CASP1 or CASP8 processes two cytokines from the interleukin (IL)-1 family, IL-1ß and IL-18, into their mature form. Once matured, IL-1ß and IL-18 are released via GSDMD or GSDME pores, together with other alarmins (Kayagaki et al., 2015; Monteleone et al., 2018; Shi et al., 2015), with great consequences on the immune response (Mantovani et al., 2019).

What is upstream of caspase activation? What are the signals that trigger pyroptosis? For CASP8 and CASP3, the upstream signals are similar to the ones described earlier during apoptosis. Interestingly, TAK1 inhibition by *Yersinia enterocolitica* triggers generally apoptosis but can also cause pyroptosis in proficient cells such as macrophages. In this setting, both apoptosis and pyroptosis are orchestrated through CASP8 (Orning et al., 2018; Sarhan et al., 2018a). CASP4/5/11 can directly be activated after LPS detection (Kayagaki et al., 2015). CASP1 activation, however, requires the formation of a very large, spec-like, multimeric inflammasome complex that consist of a PRR and the *apoptosis-associated speck-like protein containing a CARD* (ASC) and CASP1 (Van Gorp and Lamkanfi, 2019)(Figure 1). Inflammasomes are key signaling platforms that detect pathogenic and sterile stressors. Scientific consensus describes five major

inflammasome sensor molecules (Van Gorp and Lamkanfi, 2019) (Figure 1), although more exist (e.g. NLRP6, IFI16). Three of them are very specialized: *NACHT, LRR, FIIND, CARD domain and PYD domains-containing protein 1* (NLRP1), *NLR family CARD domain-containing protein 4* (NLRC4) and Pyrin. They sense *Bacillus anthracis* lethal toxin, bacterial flagellin/T3SS and Rho GTPase modifying toxins respectively (Van Gorp and Lamkanfi, 2019). *Absent in melanoma 2* (AIM2) inflammasome recognized double-stranded DNA from bacteria, viruses, mitochondria or nuclear leakage. Finally, the most common inflammasome, NLRP3, presents far less specificity, sensing a whole array of *pathogen-associated molecular patterns* (PAMPs), *danger-associated molecular patterns* (DAMPs), toxins, mitochondrial DNA, cAMP or even ATP or K^+ efflux (Lee et al., 2012; Mariathasan et al., 2006; Van Gorp and Lamkanfi, 2019; Zhong et al., 2018). At present, there is no evidence of direct ligand binding by NLRP3, which led to the hypothesis that NLRP3 senses changes in the cellular milieu. NLRP3 activation occurs as a two-step event. First, TLR-mediated NF-κB signaling induces priming, triggering the transcriptional expression of NLRP3 and IL-1β. Additionally, a non-transcriptional priming of NLRP3 can occur by stimulating its deubiquitylation and desumoylation (Barry et al., 2018; Juliana et al., 2012). In the second step, the now primed NLRP3 can be activated by one of the plethora of activators and insults it can detect (He et al., 2016).

The rapid death kinetics, inflammasome diversity and the hyper-inflammatory nature of pyroptosis through IL-1 secretion and DAMP release make it very challenging for pathogens to bypass it. However, only a very selective portion of cells can engage pyroptosis. Moreover, the complex long-term consequences of IL-1 secretion can be counterproductive, ranging from autoimmune diseases to promoting cancer initiation and progression (Mantovani et al., 2019; Van Gorp and Lamkanfi, 2019).

Ferroptosis

Apoptosis, necroptosis and pyroptosis are the only forms of cellular "suicide" known to date. Other cell death modalities, if not restricted to one specific cell type (e.g. NETosis, neutrophils), are often passive processes caused by "system overdrive" (e.g. Parthanatos [PARP1 over-activation]; *Mitochondrial permeability transition* (MPT)-driven necrosis [severe oxidative stress; cytosolic Ca^{2+} overload]; Lysosome-dependent cell death; Autophagy-dependent cell death) or cell to cell "cannibalism" (e.g. Entotic cell death).

Nevertheless, one last very intriguing type of cell death worth noting is ferroptosis (Friedmann Angeli et al., 2019), a form of cellular "sabotage" caused by oxidative shattering of lipids in the plasma membrane (Green, 2019). It is difficult to define a "trigger" for ferroptosis. Two elements are needed for the perfect storm: 1) excessive oxidative modification of *polyunsaturated fatty acids* (PUFAs), 2) inhibition of glutathione peroxidases, more particularly glutathione peroxidase 4 (GPX4) (Hassannia et al., 2019), the only enzyme able to detoxify PUFAs (Figure 1). The first is caused by a disproportionate reliance on iron metabolism or excess in reactive oxygen species and the second, usually, by the exhaustion of the intracellular pool of glutathione (Hassannia et al., 2019). What makes ferroptosis particularly interesting is that tumors do meet these two requirements, and certain cancer types could potentially be targeted by drug-triggered ferroptosis (Hassannia et al., 2019). Even more intriguing is the recent observation that cytotoxic $CD8^+$ T cells, unleashed by immunotherapy, can kill cancer cells through IFNγ-induced downregulation of antioxidant import, showing an additional relevance for ferroptosis in cancer treatment (Wang et al., 2019).

Unifying cell death pathways

It is easy to see the different cell death modalities as separated entities. Indeed, the way we define "a pathway" leads us to assume of their exclusivity. However, in a cell where several death cascades coexist, crosstalk is both unavoidable and essential. All the pathways we described above should be seen as a global cellular military strategy, which *adapts its form endlessly* (to again quote Sun Tzu). It sets a plan A and a plan B, lays booby traps and instructs the actions to take as a last resort. Extrinsic apoptosis communicates with the intrinsic by CASP8's cleavage of BID (Luo et al., 1998), both extrinsic and intrinsic pathways can engage necroptosis through RIPK3 (Annibaldi and Meier, 2018; Giampazolias et al., 2017) and CASP8 as well as CASP3 can trigger pyroptosis via activation of gasdermin pores (Orning et al., 2018; Wang et al., 2017). Finally, loss of GPX4 activity, a crucial event of ferroptosis, can engage necroptosis in mouse erythroid precursors (Canli et al., 2016). Therefore, we need to integrate all incoming signals to understand the final output. In the case of cell death, the final output goes far beyond cell intrinsic events, and very much involves cells of the tissue micro-environment and immune system. As such, death is not an endpoint, but the beginning of an immune response (Yatim et al., 2017).

Ars moriendi: The art of immunogenic death

Defining Immunogenic Cell Death

Why and how does cell death become immunogenic? An answer to these questions is far from trivial. Formerly, *immunogenic cell death* (ICD) was exclusively seen as a protective mechanism against pathogen infection, whereby PAMPs are recognized by infected cells and/or immune cells, triggering the production and release of a potent cocktail of danger signals that alert the immune system, and ultimately leads to the RCD of the infected cells. Combined with antigens from pathogens, such danger signals provide both adjuvanticity and antigenicity, as well as a potent inflammatory response: The three essential components of a *bona fide* ICD that mobilizes the adaptive immunity (Yatim et al., 2017) (Figure 2).

However, it is now clear that ICD can happen in sterile conditions. This was first observed in response to chemotherapy or radiotherapy treatments (Galluzzi et al., 2017). There, dying cells release the sterile equivalent of PAMPs, a pro-inflammatory cocktail of molecules, DAMPs. Similar to PAMPs, they alert the immune system (Yatim et al., 2017). In cancer, the antigenicity is provided by neo-epitopes caused by mutations, genomic instability, post-translational modifications and cellular stress (Lee et al., 2018; Matsushita et al., 2012; Tureci et al., 2016). Thus, ICD can be considered as a defense mechanism against both pathogens and rogue cells.

In an attempt to further characterize the nature of sterile ICD, a distinction was made between apoptosis, a clean programmed non-immunogenic death, *versus* necrosis, an accidental and messy death, therefore immunogenic. Yet, our perception of cell death has nowadays radically changed. As described above, several lytic forms of RCD, thus non-accidental, have been discovered (Galluzzi et al., 2018). In fact, accidental necrosis, if inflammatory, is not immunogenic as it lacks the production of inducible DAMPs (Aaes et al., 2016; Goldszmid et al., 2003; Sarhan et al., 2018b; Yatim et al., 2017; Yatim et al., 2015). Finally, even apoptosis, the paragon of non-immunogenic death, has been shown to trigger immune responses under the right circumstances (Galluzzi et al., 2017; Yatim et al., 2017). Consequently, the failure to associate ICD to a specific type of RCD has resulted in classifying it as a separate, poorly known entity (Galluzzi et al., 2018).

We propose a different approach to the definition of ICD. We integrate it into the wider context of tissue repair, and not only as a cellular death event. The nature of ICD lies in the complex cellular

communication between dying and immune cells (Yatim et al., 2017). Depending on its quality, this interaction can change the immunogenic outcome as much as would the type of RCD (Galluzzi et al., 2017; Yatim et al., 2017). Altogether, we argue that ICD is not a distinct type of RCD but a subtle successful dialogue between a dying cell and a rightly disposed immune system.

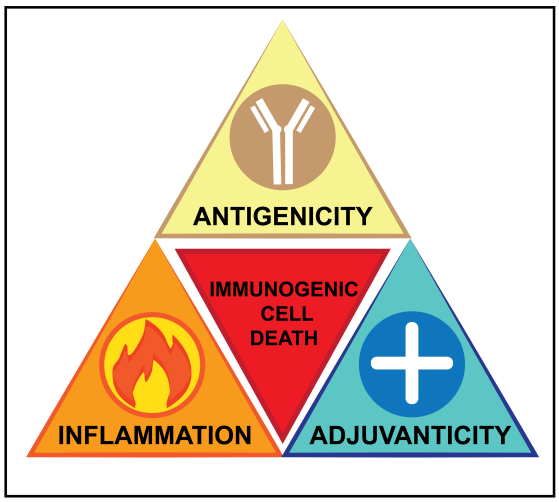


Figure 2: The Triforce of immunogenic cell death. Key parameters that determine cell death immunogenicity: Antigenicity, Inflammation and Adjuvanticity. While they can be produced by different cells in the tissue-microenvironment, the very same APC must encounter, at the same time, neo-antigens, inducible and constitutive DAMPs.

Are caspases the master regulators of ICD?

With respect to the intrinsic pathway of apoptosis, our new understanding of caspases' function as non-essential enactors of cell death, could tempt us to undervalue caspases' contribution. While caspases are not essential for the death following MOMP, they are crucial to modulate how this death is perceived. However, there are no easy answers as caspases can both potentiate as well as silence the immunogenicity of a dying cell.

Indeed, studies of immunogenic apoptosis in sterile conditions have shown that the proteolytic activity of caspases has the potential of exposing neo-epitopes and DAMPs, which can then be used for antigen cross-presentation (Green et al., 2009; Yatim et al., 2017). Several cancer treatments including anthracyclines, oxaliplatin or radiotherapy generate immunogenic apoptosis potentially through that mechanism (Galluzzi et al., 2017). In mice and humans, these treatments contribute to the therapy-induced tumor clearance. Strikingly, cancer cells pre-treated and killed by anthracyclines can be used as a vaccination vehicle in mice to protect them from future tumor challenge. Studies on the action of anthracyclines have demonstrated that caspases were not necessary for cell death but essential for chemotherapy-driven immunogenicity (Casares et al., 2005). Additionally, the role of CASP1 in processing IL-1ß, a major DAMP, as well as the involvement of CASP1/3/4/5/8 in activating gasdermin pores and pyroptosis further demonstrate how caspases can engage the immune system.

In complete contrast, caspase activity can also be essential for the non-inflammatory nature of cell death (Martin et al., 2012). It is context dependent. As described above, viruses have developed strategies to inhibit caspases' activity. In reaction, cells have engineered safeguarding mechanisms to proceed with cell death under these circumstances. Studies have shown that cell death events can become highly immunogenic by default if caspase activity is blocked. For example, following MOMP a type I interferon response and NF-κB activation is triggered if CASP9 and CASP3 are blocked (Rongvaux et al., 2014; White et al., 2014). This is because apoptotic caspases also shut down type I IFN responses by cleaving, notably, *cyclic GMP-AMP synthase* (cGAS) and *interferon regulatory factor 3* (IRF3) (Ning et al., 2019).

Further, caspase-independent cell death by necroptosis is immunogenic (Aaes et al., 2016; Snyder et al., 2019; Yatim et al., 2015). The combination of RIPK1-dependent NF-κB signalling and MLKL membrane perforation allow necroptotic cells to release multiple

DAMPs (Yatim et al., 2017). Because of this immunogenic potential, necroptotic cells have been used successfully to vaccinate mice to drive anti-tumour immunity (Aaes et al., 2016; Yatim et al., 2015).

How to conciliate the fact that caspases can positively as well as negatively influence the immunogenicity of death? As pointed out before, death pathways are not exclusive, and instead we need to integrate fully the different actors of cell death. Cells that express RIPK1, RIPK3 and MLKL, for example, are designed to react violently to RIPK1 activation if caspases are blocked, whereas other cells that lack RIPK3 will not. Future research is needed to gain a better understanding how the immunogenicity of dying cells is influenced. The only thing certain is that all deaths are not immunologically equivalent, indiscriminately of the cell death modality.

DAMPs: The messengers of immunogenicity

The immunogenicity of RCD is critically dependent on the production of DAMPs, generated during the process of dying. In the following paragraphs, we define the different sorts of DAMPs, and other molecules involved in the second step of immune activation.

Coordination of cell death and NF-κB signalling

The NF-κB pathway controls diverse cellular processes, from proliferation and cell survival to cytokine production, anti-pathogen response and inflammation. Under certain circumstances, NF-κB is tightly connected to decisions of life and death, notably during extrinsic apoptosis and necroptosis (Annibaldi and Meier, 2018). In the context of ICD, uncoupling cell death from NF-κB signaling has a negative impact on the immunogenicity of death (Yatim et al., 2015). By triggering a "pure" form of extrinsic apoptosis or necroptosis, Matthew Albert and colleagues demonstrated that apoptosis, by artificial dimerization of CASP8, cannot activate NF-κB signaling and ICD. In a similar fashion, direct activation of necroptosis through oligomerization of RIPK3$^{\Delta RHIM}$, which is unable to recruit RIPK1, is nearly as inefficient (Yatim et al., 2015). Only when RIPK3 co-engages RIPK1, and RIPK1 drives activation of NF-κB signaling, cell death by necroptosis becomes immunogenic. Clearly, RIPK1 and RIPK3 have additional functions to cell death regulation, and it has been suggested that they play key roles in the production of inflammatory cytokines (Kang et al., 2013; Kearney and Martin, 2017; Lawlor et al., 2015; Vince et al., 2012). This was based on the observation that, whilst *ripk3*$^{-/-}$ and *mlkl*$^{-/-}$ null mice both show deficiency in necroptosis, *mlkl*$^{-/-}$ null mice exhibit a severe inflammatory phenotype absent in their *ripk3*$^{-/-}$ counterparts (Alvarez-Diaz et al., 2016). Additionally, a recent study showed that intra-tumoral injection of necroptotic non-tumor cells can increase dendritic cell cross-priming and CD8^{+} activation *in vivo*, providing a lasting and systemic anti-tumor response. Intriguingly, this phenomenon does not require antigen provision by the dying cell itself (Snyder et al., 2019). The injected bystander cell instead activates resident DCs to uptake antigens from neighboring tumor cells. What is most surprising is that the anti-tumor immune effect of the injected bystander cells is purely dependent on RIPK3-driven and RIPK1-mediated activation of NF-κB. Necroptosis, or CASP8-mediated apoptosis, is completely dispensable, at least in this system (Snyder et al., 2019). However, another study reports that the immunogenicity of necroptotic cells does not correlate with the extent of NF-κB activation, and instead relies on the release of DAMPs (Aaes et al., 2016). Therefore, while it is clear that RIP kinases function as sentinels to alert the immune system of stress and danger, the underlying immunogenic processes remain to be elucidated.

cDAMPs and iDAMPs

DAMPs are recognized by cell surface or intracellular PRRs, such as TLRs (Broz and Monack, 2013). They encompass constitutive DAMPs (cDAMPs) and inducible DAMPs (iDAMPs).

cDAMPs are immunogenic cellular components, present inside healthy cells, that are released following plasma membrane rupture (for review see (Galluzzi et al., 2017; Yatim et al., 2017)). They include "find me" and "eat me" signals, such as calreticulin, ATP, F-actin, high mobility group box 1 (HMGB1) and many more (Galluzzi et al., 2017; Galluzzi et al., 2018). They are released by dying cells or activated immune cells to attract phagocytes and enhance antigen presentation efficiency (Blachere et al., 2005; Elliott et al., 2009; Elliott and Ravichandran, 2016; Sandilos et al., 2012). Because of their constitutive nature, they can also be released during accidental necrosis. However, it is noteworthy that accidental necrosis, for example induced by repeated cycles of freeze/thawing, is not immunogenic. Therefore, the release of cDAMPs alone is insufficient to fully unleash an immunogenic response.

In contrast to cDAMPs, iDAMPs are not present in healthy cells but are induced and/or altered upon cell death (Galluzzi et al., 2017; Yatim et al., 2017). iDAMPs encompass a wide range of molecules, mostly NF-κB-induced cytokines (TNF, IL-6), type I IFNs and IL-1 family. Type I IFNs, such as IFNα and β, are cytokines secreted in response to environmental stresses, PAMPs and TLR activation. They function in numerous ways in host-defense mechanisms (e.g. infections, anti-tumor functions and immune modulation) (Lasfar et al., 2014), and can act both as iDAMPs and inducers of cell death (Papageorgiou et al., 2007; Thyrell et al., 2007). Particularly, type I IFN signaling in dendritic cells is crucial for an anti-tumor immune response. Accordingly, animals that lack IFNα/β receptor 1 (IFNAR1) in dendritic cells are unable to reject cancer cells (Diamond et al., 2011). Finally, the IL-1 family encompasses powerful iDAMPs (Martin, 2016). Many cell types including immune, stromal and epithelial cells can produce and secrete IL-1 cytokines in response to infection (PAMPs), trauma (DAMPs) or NF-κB activation (Akdis et al., 2016), where they stimulate various innate and adaptive immune responses (e.g. T cell polarization) (Martin, 2016). Hence, the IL-1 family appears to be one of the last signals that 'informs' the immune system for the presence of dying cells.

La Danse Macabre: The antigen cross priming

For cell death to directly activate an immune response the dying cell needs to trigger a total of five steps. The cell death event itself is considered as **Signal -1**, while the coordinated release of DAMPs constitutes **Signal 0**. In the following paragraph, we shortly describe **Signal 1**, **2** and **3** that ultimately lead to T cell activation (Figure 3).

Following **Signal 0**-mediated attraction of phagocytic cells, dying cells are engulfed and processed for their antigens to be presented on the cell surface of antigen presenting cells (APC). In the presence of the right adjuvant, this ultimately leads to APC activation (Yatim et al., 2017). The migration of activated APC to lymphatic drainage organs induces their maturation and allows them to encounter lymphocytes (Martin-Fontecha et al., 2009). Self-antigens and cancer cell-derived neo-antigens are indistinguishably loaded on MHC receptors. **Signal 1** is the antigen recognition event that is mediated through the T cell receptor (TCR), and triggered by MHC class I-associated or MHC class-II associated peptides processed from the antigen after the phagocytosis of dying cells by APCs (Galluzzi et al., 2017). Loading on MHC class I requires cross-presentation, which means antigens must leave the phagosome and be processed by the proteasome before being imported in the ER for further processing in order to fit the MHC class I pocket (Figure 3). MHC class II is less restrictive and therefore

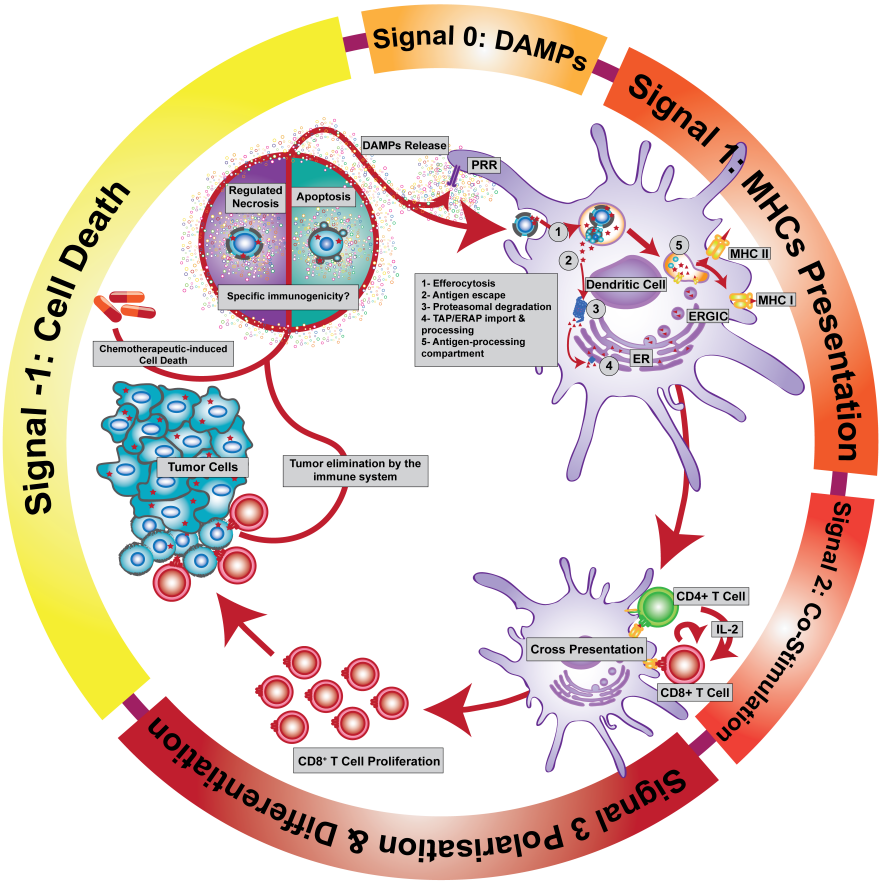


Figure 3: La Danse Macabre. Presented are the signals that are required for anti-tumor immune responses, from -1 to 3 as well as interactions between immune cells and their modality. DAMPs: danger-associated molecular patterns; PRR: pattern recognition receptor; TAP: antigen transporters; ERAP: ER aminopeptidase; ER: endoplasmic reticulum; ERGIC: ER-Golgi intermediate compartment; MHC: major histocompatibility complex.

antigens do not require these steps (Figure 3). For effective T-cell activation, other co-stimulatory receptors on dendritic cells (e.g. CD80 or CD86) must engage with T-cells (**Signal 2**). Additional polarization and differentiation signals, such as IL-12 or Type I IFNs, are crucial for T-cell differentiation into a T-cell effector (**Signal 3**). This is the last step required to induce adaptive immunity (Yatim et al., 2017). All five signals (-1, 0, 1, 2 and 3) are required for efficient T-cell priming (Figure 3).

ICD in cancer treatment

Drugs that mobilize the immune system against cancer are dramatically improving care for many people. The goal of immunotherapy is to generate a robust immune response, stimulating the body's cytotoxic lymphocytes to eradicate tumor cells and ultimately

achieve long-term anticancer immunity. Key to this approach is the successful induction of immunogenic cell death (Yatim et al., 2017). There are two issues with this approach. First, evasion of cell death is a characteristic 'hallmark' of human cancers (Hanahan and Weinberg, 2011). Second, emerging cancers evade the immune response by being poorly immunogenic, either by having low mutation rates and few de novo antigens, and/or by producing anti-inflammatory cytokines.

To transform a tumor immune profile from "cold" to "hot", knowing the cause of the absence of immune infiltration/activation is paramount. Without a sufficient pool of antigens, or under immune suppression, a strategy based solely on ICD would be powerless in activating the immune system as it would lack essential signals. Because tumors might have already been immune edited during their ontogenesis, the aim in cancer therapy needs to focus on enhancing the visibility/accessibility of cancer antigens to the immune system, as well as re-activating it.

Oncolytic viruses

Oncolytic viruses are therapeutic vehicles that are designed to preferentially flag cancer cells to the immune system. Since the first FDA approval for the herpes virus type 1-derived laherparepvec (Imlygic) in metastatic melanoma (Andtbacka et al., 2015; Ott and Hodi, 2016), an increasing amount of clinical trials have been launched to use oncolytic viruses against diverse types of cancers (Twumasi-Boateng et al., 2018). Such viruses preferentially infect and cause lysis of cancer cells. Virotherapy is highly immunogenic as it not only provides neo-epitopes but also potent adjuvant properties. This produces the perfect immunogenic cocktail, potentiating the vaccination against tumor cells (Twumasi-Boateng et al., 2018).

Combining ICD and Immune Checkpoint Blockade

Immunosuppression is a major barrier to cancer treatment. To reactivate the anti-tumor response, it is necessary to remove immunosuppressive checkpoints that restrain cytotoxic lymphocytes from attacking cancer cells. Recently, new immunotherapies have revolutionized cancer research, and offer hope of improved outcome for many cancer patients (Ribas and Wolchok, 2018). Immunotherapies boost tumor elimination by manipulating the anti-tumor immune response (Burugu et al., 2017; Hu et al., 2018; June et al., 2018; Ribas and Wolchok, 2018). So far, successful and durable response depends on patient stratification (Copier et al., 2009; Hu et al., 2018; Ribas and Wolchok, 2018). Indeed, the removal of immune "brakes" does not guarantee an anti-tumor response, and intrinsic tumor immunogenicity is an equal contributor. Moreover, patient stratification may also include a patient's microbiome as modification of the immune response by the microbiome also plays an important role in therapy response (Pouncey et al., 2018).

While ICD of cancer cells can boost the availability of neo-antigens to the immune system, ICD can also trigger the production of IFNγ by immune cells (Aaes et al., 2016; Inoue et al., 2014). Tumors exposed to high quantities of IFNγ, released by infiltrating lymphocytes, react by expressing increased amount of PD-L1. This ligand binds PD1 receptors on the lymphocyte surface, and strongly impedes activation, cytotoxicity and proliferation of lymphocytes, *de facto* neutralizing the immune response. Thus, induction of ICD and the use of *immune checkpoint blockade* (ICB) represent two sides of the same therapeutic coin. While ICD accelerates the immune response, ICB removes the brakes. However, the success of this combined approach will critically depend on the tolerability by the patient, and the presence of strong immunogenic antigens in tumor cells.

Concluding remarks

How can we take advantage of the wide diversity of cell death pathways? Our improved understanding of apoptosis, necroptosis, pyroptosis and ferroptosis, and their interconnectivity with innate and adaptive immunity, has unearthed new opportunities to guide our immune system to bring down cancer. The ultimate goal will be to use pharmacological means to replicate some aspects of the signaling outputs triggered by pathogens so that their combination with standard-of-care therapies causes a response that mimics an infection (**'pathogen mimicry'**). This might cause dying cancer cells to release 'danger signals' that make them more detectable by the patient's own immune system. Currently only a handful of chemotherapeutics induce ICD (Galluzzi et al., 2017). They act through the release of DAMPs as well as by increasing the expression of death receptors and co-stimulatory ligands that activate both the innate and adaptive immune response (Bezu et al., 2015). Manipulating PRR and cytokine receptor-signaling checkpoints (Annibaldi and Meier, 2018) might provide additional cues into ICD. Small-molecule *inhibitor of apoptosis* (IAP) antagonists (Smac mimetics (SM)) that bind and induce degradation of cellular IAPs provide an attractive entry point into sensitizing cells to death ligands (Varfolomeev et al., 2007; Vince et al., 2007). Many challenges are still to come in our understanding of cell death. This is particularly true for immunogenic cell death (Riddle #4 (Green, 2019)). For the past decades, the field of cell death has predominantly focused on the cell intrinsic events of death. Yet, so little is understood about the diversity of cell death outputs, and how they coordinate immune-mediated tissue homeostasis.

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