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# Targeting immunogenic cell stress and death for cancer therapy

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## **Abstract**

Immunogenic cell death (ICD), which results from insufficient cellular adaptation to specific stressors, occupies a central position in the development of novel anticancer therapies. Several academic groups and biotechnology companies are pursuing strategies to elicit ICD, either as standalone therapeutic approaches or as means to convert immunologically cold tumors that are insensitive to immunotherapy into hot and immunotherapy-sensitive lesions. The development of ICD-inducing treatments, however, is hindered by various obstacles. Some of these relate to the intrinsic complexity of cancer cell biology, while others arise from the use of conventional therapeutic strategies that were developed according to immune agnostic principles. Moreover, current discovery platforms for the development of novel ICD inducers suffer from limitations that must be addressed to improve bench-to-bedside translational efforts. Finally, an improved appreciation of the conceptual difference among key factors that discriminate distinct forms of cell death will assist the design of clinically viable ICD inducers.

## Introduction

To maintain organismal health, many cell types undergo a constant process of renewal and turnover, meaning that they are continuously generated by cell division and later removed by a combination of cell-intrinsic and -extrinsic mechanisms<sup>1</sup>. Specifically, aging cells that are on the verge of elimination activate cell-autonomous suicidal pathways coupled to the early emission of “come get-me” and “eat-me” signals to the attention of phagocytes, in particular macrophages<sup>2,3</sup>. By this mechanism, dying cells are engulfed and digested before they fragment and their content spills into the extracellular space. Such a silent removal of dying cells, which is referred to as “efferocytosis”, actively prevents potentially damaging inflammatory reactions and (auto)immune responses against dead cell antigens<sup>2,3</sup>.

However, if cell death is induced by infectious pathogens such as viruses and intracellular bacteria, it is in the best interest of the organism to mount innate immune responses connected to the activation of adaptive immune effectors<sup>4</sup>. Innate immunity ensures the prompt destruction of infected cells based on the recognition of rather unspecific signals of stress, such as the exposure of activatory ligands for natural killer (NK) cells<sup>5,6</sup>. Conversely, adaptive immunity provides the host with a mechanism to recall past episodes of infection by same pathogen and hence mobilize a specific response to re-infection in an accelerated fashion<sup>7</sup>. In this context, evolutionary ancient cell-autonomous pathways elicited by infection such as the so-called “integrated stress response” (ISR), encompassing endoplasmic reticulum (ER) stress and autophagy, as well as the secretion of type I interferon (IFN) upon cytosolic nucleic acid sensing are intimately linked to the stimulation of myeloid cells (in particular dendritic cells [DCs], which are the most potent antigen-presenting cells) and lymphoid cells (in particular T cells), thus orchestrating specific immune responses against pathogen-encoded antigens<sup>8</sup>.

Immune responses against malignant cells appear to follow similar rules<sup>4</sup>. Thus, cancer cells – which are antigenically different from normal cells and intrinsically stressed owing to altered metabolic functions - can alert myeloid and lymphoid effectors to clear them in the context of natural immunosurveillance<sup>9</sup>. Moreover, some clinically successful anticancer treatments including conventional chemotherapeutics, focal radiation therapy (at least when used according to specific dose and fractionation schedules) and targeted anticancer agents can stress and kill neoplastic cells in a fashion that mimics the infection by pathogens (so-called “viral mimicry”)<sup>10</sup>, hence eliciting tumor-targeting immune responses<sup>11-14</sup>. Accordingly, the demise of cancer cells succumbing to such “immunogenic cell death” (ICD) inducers can sensitize tumors to immune checkpoint inhibitors (ICIs) and possibly other forms of immunotherapy, as demonstrated in several clinical trials<sup>15-19</sup> (**Box 1**). Of note, it appears that such an indirect, immune-dependent mechanism is even more important than direct debulking of the tumor mass by therapy for determining the long-term outcome of antineoplastic regimens<sup>20</sup>. In line with this notion, the density, composition and functionality of the pre-existing or therapy-induced cancer immune infiltrate (so-called “immune contexture”) predict disease outcome in a variety of oncological settings<sup>21</sup>.

Thus, it is conceptually and practically important to understand the peculiarities of ICD and to distinguish ICD from its non-immunogenic counterpart, be it immunologically silent (as in the setting of physiological development or adult tissue homeostasis) or merely pro-inflammatory (as in the setting of stress-induced cell death not coupled with the activation of adaptive immunity) (**Fig 1**). In a nutshell, the perception of cell death as immunogenic entails 4 key factors. First, cytotoxicity: cells must activate a series of (ultimately unsuccessful) stress responses before they die<sup>4</sup>. Second, antigenicity: cells must express and present antigenic determinants that can be recognized by the mature T cell repertoire, for instance carcinoembryonic antigens, post-translationally modified self antigens, or neoantigens emerging from non-synonymous mutations<sup>22</sup>. Third, adjuvanticity: premortem stress responses and cell

death must be accompanied by the emission of adjuvant-like signals that are usually referred to as danger-associated molecular patterns (DAMPs)<sup>23,24</sup>. DAMPs ensure DC recruitment, the physical interaction between dying cells or their corpses and DCs, the transfer of tumor-associated antigens (TAAs) into DCs, DC maturation and migration to secondary or tertiary lymphoid organs, and finally DC-mediated antigen presentation to T lymphocytes<sup>4</sup>. Fourth, permissive microenvironmental conditions: not only the site of cell death must be (or become) accessible and hospitable to DCs for them to prime adaptive immunity, but also the site of target cells must be (or become) accessible and hospitable to T cells for them to mediate immune effector functions<sup>4,25</sup>. Thus, the activation of a potent tumor-targeting immune response downstream of ICD at a primary tumor site will have no effects at a metastatic site if the latter cannot be infiltrated by T lymphocytes or if malignant cells express high levels of co-inhibitory T cell ligands such as CD274 (best known as PD-L1)<sup>4,26</sup>. Importantly, in oncological settings, the absence of any of these 4 elements (cytotoxicity, antigenicity, adjuvanticity and permissive microenvironmental conditions) is likely to result in primary or secondary resistance to treatment<sup>4</sup>.

In this Review, we summarize the core mechanisms of ICD that can be harnessed for cancer treatment, strategies through which malignant cells subvert ICD induction by therapeutic agents and appropriate countermeasures to reestablish anticancer immunity. Approaches for developing novel pharmacological agents that induce ICD or improve the perception of ICD by immune cells are also discussed.

## Core mechanisms of immunogenic cell stress and death

Stress-driven regulated cell death (RCD) can occur according to different mechanisms including (but not limited to): (1) apoptosis, which is demarcated by caspase activation<sup>27</sup>; (2) ferroptosis, which involves lethal plasma membrane peroxidation<sup>28</sup>; (3) necroptosis, which is driven by the pore-forming protein mixed lineage kinase domain like pseudokinase (MLKL)<sup>29</sup>; and (4) pyroptosis, which involves pore-forming proteins of the gasdermin family<sup>30</sup>. In addition, cells can succumb in a non-regulated, accidental fashion that involves passive cellular lysis.

Although it has been tempting to reductively link the immunogenicity of cell death to specific subroutines (claiming for instance that apoptosis would be intrinsically non-inflammatory, non-immunogenic and even tolerogenic, while necrosis would invariably be pro-inflammatory and immunogenic)<sup>31</sup>, it is now clear that the biology of cell death and its perception by the host is more complex than such an oversimplistic model. In line with this notion, various purely apoptotic instances of ICD have been described<sup>32</sup>. Moreover, both accidental necrosis and ferroptosis are often unable to elicit adaptive immunity<sup>33,34</sup>.

Thus, the accidental release of DAMPs from permeabilized cells can mediate inflammatory responses and is likely to contribute to ICD by interacting with receptors on immune cells (notably DCs), but is not sufficient to optimally stimulate adaptive immunity<sup>4</sup>. This applies to the unregulated release: (1) nucleotides, which can mediate chemotactic and immunostimulatory functions via purinergic receptors; (2) nucleic acids, which exert immunostimulatory effects by binding Toll-like receptors (TLRs); (3) the cytosolic protein annexin A1 (ANXA1), which has a formyl peptide receptor 1 (FPR1)-dependent chemotactic activity; (4) heat shock proteins (HSPs), which are sensed by TLR2 or TLR4 to promote immune cell activation; and (5) the nuclear chromatin-binding protein high mobility group box 1 (HMGB1), which acts on TLR4 and advanced glycation end product receptor (AGER) with

immunostimulatory effects. Altogether, these DAMPs are not sufficient to activate pattern recognition receptors (PRRs) in a way that culminates with T cell priming by DCs<sup>4</sup>. Similarly, the accessibility of F-actin (which is usually shielded by the plasma membrane) on cellular corpses to its DC-associated receptor C-type lectin domain containing 9A (CLEC9A, best known as DNGR-1) may be necessary for cell death to be immunogenic, but it is unlikely to be sufficient<sup>35</sup>.

Rather, adaptative premortem responses operating in stressed cells including the protracted secretion of lysosomal ATP in the context of autophagy<sup>36</sup> and the exposure of ER components including HSPs and calreticulin (CALR) on the plasma membrane in the context of the ISR<sup>37</sup> appear to be essential for ICD. ATP is one of the first DAMPs appearing in the microenvironment of stressed cells, where it can be sensed by DC or their precursors via purinergic receptor P2Y2 (P2RY2), which promotes chemotaxis<sup>38,39</sup>, hence attracting DCs to the proximity of dying cells<sup>40</sup>, or purinergic receptor P2X 7 (P2RX7), which promotes DC activation<sup>41</sup>. Specifically, P2RX7 signaling driven by extracellular ATP activates the NLRP3 inflammasome and hence drives caspase 1 (CASP1) maturation, culminating with the secretion of bioactive interleukin 1B (IL1b) and IL18<sup>41</sup>. Of note, extracellular ATP-derived ADP also support ICD by promoting DC micropinocytosis via purinergic receptor P2Y12 (P2RY12)<sup>42</sup>.

Surface-exposed CALR, which translocates to the plasma membrane in complex with protein disulfide isomerase family A member 3 (PDIA3), acts as a potent “eat-me” signal for DCs upon interaction with LDL receptor related protein 1 (LRP1, best known as CD91)<sup>43,44</sup>. Importantly, ICD-associated CALR surface exposure occurs before the apoptotic exposure of phosphatidylserine (PS)<sup>32</sup>, which efficiently redirects dying cells or their corpses from macrophages, mediating immunologically silent efferocytosis<sup>2</sup>, to conventional type 1 DCs (cDC1s), a key DC subset for the activation of tumor-targeting immunity<sup>45</sup>. Surface-exposed CALR also interacts with natural cytotoxicity triggering receptor 1 (NCR1, best known as NKp46) on NK cells, hence promoting their cytotoxic functions<sup>46</sup>.



Interestingly, cancer cell death as mediated by CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) is also a *bona fide* variant of ICD<sup>47,48</sup>, but whether the same applies to NK cell cytotoxicity remains to be formally demonstrated.

Of note, ER stress and autophagy often must be preceded by the ISR for cell death to be perceived as immunogenic. The ISR consists in the phosphorylation of eukaryotic translation initiation factor 2 subunit alpha (EIF2S1, best known as eIF2 $\alpha$ ) by a panel of kinases, namely eukaryotic translation initiation factor 2 alpha kinase 1 (EIF2AK1, best known as HRI), EIF2AK2 (best known as PKR), EIF2AK3 (best known as PERK) and EIF2AK4 (best known as GCN2), leading to a major reshuffling in protein synthesis<sup>49,50</sup>. Experimental ISR induction in cancer cells by inhibition of DNA-to-RNA transcription<sup>51</sup> or microtubular (de)polymerization<sup>52</sup> has been shown to promote *bona fide* ICD instances. Moreover, PKR, PERK and GCN2 have all been mechanistically involved in ICD as driven by various stimuli<sup>53,54</sup>. Finally, not only genetic interventions aimed at preventing eIF2 $\alpha$  phosphorylation have been demonstrated to subvert the immunogenicity of cell death<sup>32</sup>, but eIF2 $\alpha$  phosphorylation also constitutes a biomarker of ICD correlating with autophagy activation and CALR induction at the single cell level<sup>55</sup> as well as with tumor immune infiltration and positive prognostic features in patients with various malignancies<sup>37</sup>.

The active secretion of pro-inflammatory cytokines by dying cells also contributes to the immunogenicity of cell death. For instance, the cell-autonomous activation of PRRs such as the cytosolic double-stranded DNA sensor cyclic GMP-AMP synthase (CGAS), its endosomal counterpart TLR9 or the endosomal RNA sensor TLR3 can stimulate the secretion of type I IFN and cytokines downstream thereof such as C-X-C motif chemokine ligand 9 (CXCL9) and CXCL10 from cancer cells succumbing to immunogenic chemotherapy, which ultimately favor T cell recruitment into the tumor bed<sup>56,57</sup>. Receptor interacting serine/threonine kinase 1 (RIPK1) signaling via the NF- $\kappa$ B axis in

necroptotic cancer cells has also been shown to result in ICD via the secretion of IL6 (Ref. 58), similar to IL1B secretion as elicited in the context of cancer cell pyroptosis<sup>59</sup>.

The leitmotif that emerges is that cancer cells undergoing ICD condition the microenvironment through the active exposure of specific proteins (*e.g.*, CALR, PDIA3 and HSPs), the active secretion of metabolites and cytokines (*e.g.*, ATP, type I IFN, CXCL9, CXCL10, IL1B and IL6), the passive release of soluble macromolecules (*e.g.*, ANXA1, HMGB1, DNA and RNA) and an increased accessibility of cytoskeleton-associated proteins (*e.g.*, F-actin) (**Fig. 2**). These changes in the surfaceome and secretome of stressed or dying cancer cells occur in a way that antigen-presenting cells, in particular cDC1s and their precursors are (1) lured into the tumor microenvironment (TME), (2) attracted into the immediate vicinity of stressed or dying cancer cells, (3) instigated to take up portions of these cells; and (4) driven into maturation for optimal antigen presentation to cytotoxic T lymphocytes<sup>22</sup>. Of note, this cascade of events appears to be particularly efficient if the tumor contains tertiary lymphoid structures, which are complex, spatially organized immune cell aggregates including DCs, B lymphocytes and T cells, to enable local (as compared to nodal) antigen presentation<sup>60,61</sup>.

## Established and innovative strategies for ICD induction

Multiple strategies that promote the demise of malignant cells efficiently drive ICD, as exemplified by drugs including conventional chemotherapeutics and targeted anticancer agents, as well as oncolytic viruses and peptides, and various physical treatment modalities, either employed as standalone regimens or as part of multimodal approaches (**Table 1**).

**Single drugs.** ICD can be triggered by chemotherapeutics that inhibit DNA-to-RNA transcription (e.g., actinomycin D, anthracyclines, lurbinectedin, oxaliplatin) or that interfere with microtubules (e.g., taxanes, vinca alkaloids)<sup>11</sup>. In line with this notion, the clinical response to such chemotherapeutics is associated with an increased infiltration of malignant lesions by DCs and CTLs along with a relative decrease in the intratumoral amount of immunosuppressive CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T (T<sub>REG</sub>) cells<sup>52,62</sup>. Moreover, induction therapy with ICD-inducing chemotherapeutics has been shown to sensitize malignant lesions to ICIs targeting PD-L1 or its receptor programmed cell death 1 (PDCD1, best known as PD-1)<sup>15,18,19</sup>. Bortezomib, a proteasome inhibitor used for the treatment of multiple myeloma, also induces ICD<sup>63,64</sup>. So far, however, no bortezomib-containing regimen has been shown to cooperate with ICIs in this oncological indication. Instead, efforts are being made to target myeloma cells with ICD-inducing drug-antibody conjugates such as the B cell maturation antigen (BCMA)-targeting agent belantamab mafodotin<sup>65</sup>. More than 20 clinical trials are currently testing the safety and therapeutic efficacy of this agent in patient with multiple myeloma (source [www.clinicaltrials.gov](http://www.clinicaltrials.gov)).

Some targeted anticancer agents and notably tyrosine kinase inhibitors (TKIs) also act as *bona fide* ICD inducers, as documented for the broad specificity TKIs crizotinib<sup>66</sup> and cabozantinib<sup>67</sup>. That said, while cabozantinib can be advantageously combined with the PD-1 blocker nivolumab and the cytotoxic T lymphocyte-associated protein 4 (CTLA4) blocker ipilimumab for the treatment of metastatic renal cancer<sup>68</sup>, likely reflecting its ICD-inducing activity, two clinical trials in which crizotinib was

combined with nivolumab or another PD-1 inhibitor (pembrolizumab) for the treatment of ALK-positive non-small cell lung carcinoma (NSCLC) had to be terminated due to severe liver damage<sup>69,70</sup>. Such hepatotoxicity could be mimicked by the simultaneous administration of crizotinib and a monoclonal antibody specific for PD-1 antibody in mice, but it turned out to be avoidable in the context of preserved antitumor activity by adopting a sequential administration schedule (with crizotinib first)<sup>66</sup>. Whether such a revised administration schedule would prevent hepatotoxicity in patients remains to be investigated.

**Galenic formulations.** Major efforts are underway to design novel galenic formulations for ICD-relevant drugs, usually in the form of nanoparticles (including liposomes and solid scaffolds), to pursue one or more of the following goals: (1) selective targeting of malignant lesions, for instance by harnessing tumor-specific antibodies of intrinsic features of the TME (*e.g.*, hypoxia, reduced pH, vascular abnormalities); (2) local release of activation of the drug in response to physical triggers that can be focalized on malignant lesions (*e.g.*,  $\gamma$  irradiation, light, heat, ultrasounds); and (3) the timely (simultaneous or sequential) release of drugs and immunostimulants, including (but not limited to) various PRR agonists and ICIs delivered as recombinant proteins or ICI-coding constructs<sup>71,72</sup>. More than one thousand papers developing such formulations have been published over the past few years, mostly from Chinese research groups. It will be interesting to investigate the clinical utility of the most successful galenic formulations as determined by preclinical tests. Major challenges in this respect are posed by the absence of systematic side-by-side comparisons of distinct galenic formulations in preclinical tumor models, as well as by very choice of rodents as the model of choice, as the selection and characterization of clinically viable galenic approaches may indeed demand the use of larger animals to account for scalability.

**Oncolytic viruses and peptides.** Reflecting its evolutionary origin, ICD can be induced by viruses including oncolytic viruses<sup>73,74</sup>. The first oncolytic virus approved by the US Food and Drug Administration (FDA) was talimogene laherparepvec (best known as T-Vec), a genetically modified human herpes simplex virus 1 (HSV-1)<sup>75</sup>. Of note, one of these modifications consisted in the deletion of the neurovirulence factor ICP34.5, which promotes eIF2 $\alpha$  dephosphorylation and hence blocks the ISR<sup>76,77</sup>. Thus, the ability of T-Vec to trigger ICD has been improved by genetic engineering to drive an exacerbated ISR. Several other experimental oncolytic viruses have also been shown to induce ICD, which now is invoked as a central mechanism of their antineoplastic action<sup>78</sup>. Logically, numerous attempts are underway to equip oncolytic viruses with transgenes encoding immunostimulatory factors that enhance DC- and T cell-mediated immune responses, as well as to combine oncolytic viruses with ICIs<sup>73,79</sup>. While likely, whether these next generation oncolytic virotherapies will provide a therapeutic benefit to patients with cancer remains to be investigated in properly controlled clinical studies. Finally, oncolytic peptides may also induce ICD<sup>80</sup>, as documented for LTX-315/VPX-315 (Refs. 81,82). This agent, which mediates ICD by permeabilizing plasma and mitochondrial membranes<sup>81</sup>, has been shown to efficiently synergize with CTLA (but not PD-1) blockers, as well as with other immunostimulatory agents such as radiation therapy, in multiple preclinical tumor models<sup>82,83</sup>. Recently, LTX-315/VPX-315 has demonstrated promising single agent activity against basal cell carcinoma in a Phase II clinical trial (source <https://newsweb.oslobors.no/message/596578>), further exemplifying the potential of ICD inducers for clinical cancer management.

**Physical stimuli.** Cancer cell stress and death as elicited by a variety of physical stimuli including (but not limited to) focal radiation therapy, microwave ablation and high hydrostatic pressure has been shown to result in the activation of tumor-targeting immune responses that can synergize with ICIs or other forms of immunotherapy in a variety of preclinical cancer models<sup>84-87</sup>. However, the translatability of these findings to a broad panel of oncological indications in humans still presents

multiple obstacles. For instance, while focal radiotherapy has been successfully combined with ICIs in patients with NSCLC<sup>16,88,89</sup> and esophageal or gastroesophageal junction cancer who received preoperative (neoadjuvant) chemoradiotherapy<sup>90</sup>, the same degree of cooperativity could not be documented in patients with glioblastoma<sup>91,92</sup> and head and neck squamous cell carcinoma<sup>93</sup>. At least in part, this reflects the use of conventional radiotherapy schedules and target volumes that are associated with considerable immunosuppression locally (e.g., when tumor-draining lymph nodes are in the radiation field) or systemically (e.g., when large volumes of blood are irradiated) and hence are intrinsically incompatible with ICI-driven immunostimulation<sup>94</sup>. However, at the frontier between pharmacology and biophysics, both photodynamic therapy (PDT) and extracorporeal photochemotherapy (ECP) have been successfully developed novel strategies for ICD induction in patients<sup>53,95,96</sup>. In both settings, photosensitizers are administered systemically and then activated by light, either *in situ* (for PDT, which is used for skin tumors), or within specialized apheresis devices that expose circulating lymphocytes (for ECP, which is used for cutaneous T cell lymphoma)<sup>97,98</sup>. Supporting a role for ICD induction in the therapeutic activity of these approaches, clinical responses to PDT correlate with enhanced immune infiltration of treated lesions<sup>99</sup>, while the peripheral blood mononuclear cells (PBMCs) of patients responding to ECP manifest increased secretion of pro-inflammatory cytokines including tumor necrosis factor (TNF) as compared to PBMCs from non-responders<sup>100</sup>. Whether PDT and ECP can be safely and effectively combined with ICIs or other immunotherapeutics in patients, however, has not yet been formally evaluated.

**Combinatorial strategies.** ICD can be caused by the combination of agents that *per se* do not operate as *bona fide* ICD inducers. One example is provided by the combination of cisplatin, which is not an optimal ICD driver as it generally fails to elicit the ISR, with an agent capable of correcting such signaling defect, such as cardiac glycosides or crizotinib<sup>66,101</sup>. Along similar lines, gemcitabine elicits all the immunostimulatory ICD-relevant DAMPs, yet fails to stimulate adaptive anticancer immunity

due to its ability to stimulate the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which has powerful tumor-repopulating and immunosuppressive effects<sup>102,103</sup>. Thus, combining gemcitabine with the prostaglandin-endoperoxide synthase 2 (PTGS2, best known as COX2) inhibitor celecoxib robustly triggers ICD in preclinical tumor models<sup>104</sup>, a possibility that is currently being investigated in at least two clinical trials enrolling patients with pancreatic carcinoma (NCT03498326) or bladder cancer (NCT02885974; source [www.clinicaltrial.gov](http://www.clinicaltrial.gov)). In addition, it is possible to boost the efficacy of ICD-inducing chemotherapeutics by combination regimens. For example, *bona fide* ICD inducers such as mitoxantrone or oxaliplatin can be advantageously combined with autophagy-inducing agents (e.g., aspirin, hydroxycitrate or spermidine) to obtain improved tumor control and therapeutic cooperativity with PD-1 blockade<sup>105-107</sup>. Such a positive interaction likely involves the favorable effects of autophagy inducers on ATP secretion by stressed cancer cells<sup>36,62</sup>, as well as an improvement of the stem cell-like properties of memory T cells<sup>108</sup>. At least one clinical trial testing the ability of aspirin to improve the immune contexture of patients with ovarian carcinoma is currently ongoing (NCT05080946; source [www.clinicaltrial.gov](http://www.clinicaltrial.gov)). To the best of our knowledge, instead, hydroxycitrate and spermidine are not being tested for the ability to promote ICD in the clinic.

## ICD suppression by cancer cells

Pathogenic viruses often encode proteins that suppress: (1) cell death, for instance by inhibiting caspases or mitochondrial permeabilization; (2) the ISR, for instance by blocking PKR or promoting eIF2 $\alpha$  dephosphorylation; (3) autophagy, for instance by interfering with the core autophagy regulator beclin 1 (BECN1); (4) type I IFN responses, for instance by inhibiting master transcription factors such as interferon regulatory factor 3 (IRF3) or limiting the expression of type I IFN receptors; (5) phagocytosis, for instance by promoting the expression of the CALR antagonist CD47<sup>4</sup>. All such proteins act as virulence factors that limit ICD by interfering with cytotoxicity and adjuvanticity. In addition, viruses can limit the antigenicity of infected cells, for instance by suppressing MHC Class I expression (as documented for ICP47, which is a virulence factor expressed by HSV-1 but deleted from T-Vec)<sup>109</sup>. Finally, viruses can encode immunosuppressive factors (for instance by blocking TNF signaling) or inhibitors of T cell effector molecules such as granzyme B (GZMB) to avoid the recognition of infected cells by the immune system<sup>110,111</sup>.

Similar to viruses, malignant cells are subjected to a constant Darwinian selection in favor of the fittest, resulting in the progressive selection of cells with increased replicative potential, resistance to cell death and escape from (ICD-dependent) immune recognition<sup>112</sup> (**Table 2**). Interestingly, cancer cells often exhibit one or more defects in ICD-relevant pathways before treatment with an ICD inducer (primary resistance), a fact that likely reflects the importance of spontaneous ICD for immunosurveillance. Indeed, as they acquire increasingly malignant features, cancer cells experience considerable perturbations of intracellular homeostasis that result in the activation of ICD-relevant response pathways and in a relatively high propensity for spontaneous death, resulting in their detection by immune effectors<sup>113,114</sup>. Alternatively, cancers may initially respond to therapeutic ICD inducers, but subsequently develop secondary resistance to the treatment. Fortunately, each strategy of ICD



subversion by cancer cells can be addressed by appropriate countermeasures, at least at the preclinical level, as discussed below.

**Cell death resistance.** Alteration in a number of oncoproteins and tumor suppressor proteins confer cancer cells with an increased resistance to cell death induction, as exemplified by alterations in the tumor protein p53 (TP53, best known as p53) system, which are the most common somatic alterations across all cancer types<sup>115,116</sup>. However, the inactivation of one RCD variant (*e.g.*, apoptosis) does not necessarily imply resistance to another RCD variant (*e.g.*, ferroptosis, necroptosis), pointing to the possibility to use rather distinct drugs (or combinations thereof) to enforce the demise of malignant cells<sup>117</sup>. For this reason, efforts are underway to develop pharmacological ferroptosis inducers that would kill apoptosis- or necroptosis- resistant cells in an immunogenic fashion<sup>118,119</sup>. However, this strategy has not yet reached clinical development.

**Apoptotic caspase activation.** Importantly, most cancer cells are resistant to the induction of apoptosis<sup>120</sup>, but often retain an intact apparatus that operates downstream of mitochondrial outer membrane permeabilization (MOMP) to precipitate cell death in the absence of immunostimulation, *i.e.*, “executioner caspases” (Ref. 27). Executioner caspases and notably caspase 3 (CASP3) have indeed been shown to actively operate in support of immunological tolerance, most likely as an organismal defense from autoimmune reactions elicited by physiological cellular turnover, which largely proceeds through apoptotic mechanisms<sup>27</sup>. More specifically, CASP3 as well as its activator CASP9 actively suppress type I IFN secretion as elicited by mitochondrial DNA (mtDNA) exposed to the cytosol upon MOMP<sup>121-124</sup>, at least in part owing to the ability of CASP3 to proteolytically inactivate CGAS<sup>125</sup>. CASP3 activation also promotes immunosuppression by favoring the synthesis and secretion of PGE<sub>2</sub> (Ref. 103), as well as by stimulating the rapid exposure of PS on the plasma membrane of dying cells<sup>126</sup>, which elicits the immunogenically silent clearance of dying cells by

macrophages. Finally, caspase-independent variants of cancer cell death are more prone to elicit inflammatory NF- $\kappa$ B signaling in support of anticancer immunity than their caspase-dependent counterparts<sup>127</sup>. Taken together, these observations point to the inhibition of executioner caspases in the context of preserved or accrued MOMP as promising strategy to improve the immunogenicity of cancer cell death.

**Reduced extracellular ATP accumulation.** Autophagy is often inactivated during early oncogenesis, hence curtailing ICD-associated autophagy-dependent ATP secretion<sup>128</sup>. In line with this notion, autophagy inhibition downstream of insulin like growth factor 1 receptor (IGF1R) hyperactivation is a poor prognostic marker coupled with signs of deficient immunosurveillance in breast cancer<sup>129</sup>. At least theoretically, this opens the possibility to reactivate autophagy by inhibitors of IGF1R or its signal transducers, notably mechanistic target of rapamycin (MTOR). The latter is indeed hyperactivated in a number of malignancies (often through IGF1R-independent mechanisms), resulting in a robust suppression of autophagic flux that may be relieved with the MTOR complex 1 (MTORC1) inhibitor rapamycin<sup>130</sup>. In addition, malignant cells as well as immunosuppressive cells populating the TME can enzymatically degrade extracellular ATP by the sequential activity of ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1, best known as CD39), which catalyzes the dephosphorylation of ATP to ADP and AMP, and 5'-nucleotidase ecto (NT5E, best known as CD73) then converts AMP into adenosine, which has potent immunosuppressive effects<sup>36,131</sup>. Elevated levels of CD39, CD73 and adenosinergic receptors have a poor prognostic impact in a number of oncological settings, suggesting that they may constitute useful pharmacological targets<sup>132</sup>. Various Phase I clinical trials have evaluated adenosine A2a receptor (ADORA2A) antagonists with encouraging results<sup>133,134</sup>. Moreover, two parallel Phase II studies reported promising activity for a monoclonal antibody neutralizing CD73 (*i.e.*, oleclumab) in combination with the PD-L1 blocker durvalumab delivered as neoadjuvant

interventions to patients with operable NSCLC<sup>135</sup> or as part of the management of unresectable NSCLC<sup>136</sup>.

**Inhibited phagocytosis.** Neoplastic cells can inactivate the ISR at the apex of the CALR exposure pathway by overexpressing V-set domain containing T cell activation inhibitor 1 (VTCN1, best known as B7-H4)<sup>137</sup> or endoplasmic reticulum oxidoreductase 1 alpha (ERO1A)<sup>138</sup>. In addition, cancer cells can limit CALR expression<sup>37</sup>, retain CALR intracellularly by overexpressing stanniocalcin 1 (STC1)<sup>139</sup>, minimize CALR binding sites on the cell surface<sup>140</sup>, secrete a truncated version of CALR that masks CD91<sup>141</sup>, or upregulate CD47, which neutralizes the pro-phagocytic function of CALR<sup>140,142</sup>. Accordingly, reduced eIF2 $\alpha$  phosphorylation or CALR expression, as well as high STC1 and CD47 expression, are poor prognostic markers in several distinct malignancies<sup>37,139,140</sup>. A number of experimental strategies have been developed to enforce CALR exposure on the cancer cell surface or to block CD47 functions<sup>37</sup>. In this context, promising results have been obtained in Phase I clinical trials testing CD47-blocking antibodies in patients with hematological cancers<sup>143-145</sup>. Conversely, approaches aimed at blocking the CD47 receptor signal regulatory protein alpha (SIRPA) have not reached clinical development yet<sup>146,147</sup>.

**Other mechanisms.** In addition to the aforementioned mechanisms, malignant cells can avoid ICD by abundantly secreting gelsolin (GSN), which competitively inhibits the interaction between DNGR-1 and F-actin, and high GSN mRNA levels are indeed associated with poor prognosis in multiple oncological indications<sup>148</sup>. Moreover, cancer cells can interfere with type I IFN responses in multiple ways, including: (1) the downregulation of CGAS, its main signal transducer stimulator of interferon response cGAMP interactor 1 (STING1), or its main transcriptional effectors IRF3 and IRF7 (Refs. 149,150); (2) the autophagy-dependent degradation of cellular sources of CGAS-activatory DNA species, including permeabilized mitochondria<sup>151</sup> and micronuclei<sup>152,153</sup>; and (3) defects in the signal

transducers of type I IFN receptors, such as Janus kinase 1 (JAK1)<sup>154</sup>. While the clinical translation of currently available autophagy inhibitors still present multiple obstacles<sup>155</sup>, it is plausible that maximizing the release of CGAS-activating DNA from mitochondria by FDA approved agents such as the selective BCL2 apoptosis regulator (BCL2) inhibitor venetoclax (which is currently used for the management of multiple hematological tumors)<sup>120</sup> may considerably boost the immunogenicity of cell death.

During tumor progression, neoplastic cells can also lose the expression of some DAMPs, including ANXA1 and HMGB1<sup>156,157</sup>. The immunological defect caused by HMGB1 loss can be successfully repaired – at least in preclinical tumor models – by the intratumoral injection of synthetic TLR4 agonists<sup>82</sup>. The loss of ANXA1 phenocopies loss-of-function mutations of *FPR1* (which encodes the ANXA1 receptor). In preclinical models, the resistance to immunogenic chemotherapy caused by ANXA1 or FPR1 defects can be abolished by the systemic administration of TLR3 agonists<sup>158</sup>. Of note, the loss of FPR1 in mice accelerates carcinogen-induced breast and colon carcinogenesis<sup>158,159</sup>, in line with the observation that a frequent *FPR1* loss-of-function polymorphism (rs867228, allelic frequency 20%) is linked to premature diagnosis of various carcinomas including luminal B breast cancer, gastrointestinal carcinomas as well as head and neck cancer<sup>158,160</sup>. It remains to be determined whether TLR3 agonists can be used to postpone the development of cancers developing in the context of *FPR1* deficiency.

In sum, although ICD signaling can be perturbed at multiple levels in tumors that escape from natural or therapy-induced immunosurveillance, there are effective strategies for overcoming such perturbation, at least at preclinical level of development. We propose to refer to such strategies as “ICD correctors”.



## **Therapeutic obstacles against ICD-driven anticancer immunity**

Although ICD-inducing drugs have demonstrated substantial clinical activity, there are several obstacles to their therapeutic efficacy. As discussed above, cancer cells can subvert ICD signaling to escape from immunosurveillance as a primary or secondary mechanism of resistance. Moreover, the clinical activity of ICD inducers can be limited by including inappropriate dosing and scheduling, inefficient combination regimens, as well as suboptimal timing of the treatment with respect to surgery.

**Dosing and scheduling.** A plethora of established chemotherapeutic agents induce anticancer immunity through their capacity to kill cancer cells via ICD. Speculatively, it appears plausible that such ICD-inducing agents have been empirically selected during clinical development over other drugs that are unable to evoke anticancer immunity. Moreover, clinical optimization has likely favored doses and schedules that avoid the inhibition of immune responses, for instance as a consequence of myelosuppression or lymphodepletion. Indeed, in routine clinical praxis, established chemotherapeutics are generally used at doses that are well below the maximum tolerated dose (MTD) and are not administered in a constant fashion, but rather in cycles, most likely because such treatment schedules are compatible with antitumor immunity. Further supporting this contention, biomarkers of immunosuppression such as an elevated neutrophil-to-lymphocyte ratio are associated with poor disease outcome in the vast majority of oncological indications. Moreover, the poor cooperativity of specific ICD inducers with ICIs in at least some patient cohorts may reflect local or systemic immunosuppressive effects that are unavoidable with conventional doses and schedules<sup>94</sup>. These considerations should be taken into account for the design of clinical trials involving ICD inducers, both as standalone therapeutics and as part of combinatorial regimens involving immunotherapy.

**Combinatorial regimens.** The empiric optimization of chemotherapeutic regimens achieved in the past (which was completely immune agnostic) has yielded drug cocktails that are relatively efficient

such as doxorubicin plus cyclophosphamide or taxanes plus cyclophosphamide for the management of breast cancer, the FOLFOX regimen (folinic acid plus 5-fluorouracil plus oxaliplatin) for the treatment of colorectal cancer, and cisplatin plus gemcitabine for patients with NSCLC. A posteriori, it appears that the therapeutic effects of ICD induction by doxorubicin and taxanes may be amplified by cyclophosphamide due to its capacity to deplete T<sub>REG</sub> cells<sup>161</sup>. Along similar lines, oxaliplatin-induced ICD may benefit from the depletion of immunosuppressive myeloid-derived suppressor cells (MDSCs) by 5-fluorouracil<sup>162</sup>. Moreover, although imperfect at eliciting ICD, gemcitabine may enhance the effects of cisplatin, which per se fails to induce ICD<sup>104</sup>. These examples highlight the possibility to design optimal combinatorial regimens involving ICD inducers and “ICD boosters” in a data-driven fashion. Obviously, there is a growing rationale to combine ICD-stimulatory therapies with ICIs<sup>20</sup>. However, while most ICD inducers show favorable interactions with ICIs specific for PD-1 or PD-L1 in preclinical tumor models, this is not fully generalizable. For example, tumor lysis by LTX-315/VX-315 drives an anticancer immune response that is improved by CTLA4, but not PD-1, blockade<sup>82</sup>. The mechanistic underpinnings of this discrepancy remain elusive.

**Adjuvancy versus neoadjuvancy.** A long-lasting paradigm of clinical cancer care has been to propose, whenever possible, the surgical removal of the tumor together with one (“sentinel”) or multiple tumor-draining lymph nodes, followed by adjuvant treatment with chemotherapy, radiotherapy, targeted therapy and/or immunotherapy. Conversely, neoadjuvant therapies have classically been reserved to patients with inoperable (e.g., highly metastatic) cancers. However, recent studies strongly suggest that, at least in some types of cancer such as microsatellite-unstable (MSI) colorectal cancer and melanoma, immunotherapy with ICIs can be administered in a neoadjuvant setting at an operable stage (as true for MSI colorectal cancer), or without lymph node dissection (as true for melanoma) with a highly favorable treatment outcome<sup>163,164</sup>. Mechanistically, this may be explained by the fact that the presence of the primary tumor or the tumor-draining lymph nodes offers

superior chances to ICIs to (re)establish a therapeutically relevant immune response as compared to a post-surgical scenario in which only (mostly dormant) micrometastases are present<sup>20,165</sup>. In this context, it appears tempting to speculate that immunostimulation by chemotherapy or targeted agents would also be more efficient in the neoadjuvant than in the adjuvant setting. Indeed, patients with operable NSCLC have been advantageously treated by neoadjuvant chemoimmunotherapy<sup>166</sup>. That said, whether conventional anticancer agents inducing ICD should also be systematically used in a preoperative fashion when they must be administered alone, for instance in underdeveloped countries, still remains elusive.



## ICD assays for drug discovery

Since the capacity of a given approach to elicit ICD appears to mark the therapeutic success of anticancer therapy, considerable efforts have been devoted to the discovery of novel ICD inducers. Interestingly, ICD inducers, and in particular those that inhibit transcription, appear to exhibit specific physicochemical properties that distinguish them from non-ICD inducers<sup>11</sup>. However, medium- and high-throughput screening campaigns followed by validation experiments in preclinical tumor models remain required for the identification of *bona fide* ICD-inducing drugs. Alongside, attempts have been launched to identify drugs that enhance the capacity of DCs to sense ICD and to cross-present tumor-associated antigens to T lymphocytes, which we refer to as “ICD enhancers”. The ability of a given molecule to act as an ICD enhancer must also be validated *in vivo*, in suitable preclinical tumor models (Fig. 3).

***In vitro* assays for ICD inducers.** In a classical approach, human cancer cells of choice can be engineered with fluorescent biosensors that allow for the measurement of: (1) autophagy, based on the exogenous expression of a fluorescent variant of microtubule associated protein 1 light chain 3 beta (MAP1LC3B, best known as LC3B) that forms quantifiable puncta during the autophagic process; (2) ATP release, based on the ATP-detecting dye quinacrine staining; (3) CALR exposure on the cell surface, based on the exogenous expression of CALR fused to a fluorescent tag; (4) HMGB1 release, based on a similar principle, and (5) type I IFN signaling, based on the expression of a reporter gene under the control of a type I IFN-sensitive promoter, such as the *MXI* promoter. Such biosensors are then exposed to various concentrations of each drug candidate in a robotized platform, followed by the acquisition of fluorescence signals by fluorescence microscopy or flow cytometry, and their automated analyses. Candidates eliciting all these ICD-associated processes must then undergo manual validation with alternative low-throughput approaches (ideally with genetically unmodified cancer cells), for

instance the assessment of ATP release by luminescence assays, the secretion of HMGB1 and type I IFN by ELISA, and the exposure of CALR on the cell surface by flow cytometry<sup>167</sup>.

***In vivo* assays for ICD inducers.** Drugs that have been shortlisted as potential ICD inducers must be subjected to further validation steps *in vivo*. One common approach involves the treatment of mouse cancer cells with the drug of choice *in vitro*, followed by a washout step and their subcutaneous administration (generally in a total number of  $1-3 \times 10^6$  cells, of which 50-70% are dying) into syngeneic, fully immunocompetent mice, in the absence of any exogenous adjuvant, with the expectation that no tumor develops at the injection site. One-two weeks later, these mice are challenged by the contralateral subcutaneous injection of healthy cancer cells of the same type (generally in a number that is 100% efficient at generating developing tumor in naïve mice). This latter “index” site is then assessed for the appearance of developing tumors, the absence of which will be interpreted as a sign of a successful vaccination downstream of *bona fide* ICD induction<sup>4</sup>. Another common approach involves the standard establishment of subcutaneous or orthotopic mouse tumors in syngeneic immunocompetent vs syngeneic immunodeficient (for instance upon the antibody-mediated depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells or the deletion of *Rag2*), followed by the treatment of mice with the drug candidate. In this setting, the therapeutic activity of *bona fide* ICD will be reduced in immunodeficient mice. Along similar lines, immunocompetent mice bearing subcutaneous or orthotopic tumors can be treated with syngeneic DCs exposed *ex vivo* to cancer cells succumbing to a potential ICD inducer<sup>168-170</sup>. Of note, also in this model treatment efficacy is expected to depend in large part on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Finally, the immunogenicity of a drug candidate can be assessed in immunocompetent mice subcutaneously bearing two syngeneic tumors (one on each flank), only one of which will receive the drug intratumorally (or by other localized approaches, for instance with focal radiotherapy). In this case, the control of the contralateral untreated tumors will inform on the immunogenicity of treatment. While such therapeutic assays are closer to the clinical reality than prophylactic vaccination assays,

they are intrinsically unable to discriminate between *bona fide* ICD induction versus ICD-independent immunostimulation, as demonstrated for a number of commonly employed anticancer agents<sup>171</sup>. Obviously, the switch from in vitro assays (on human cancer cells) to in vivo experiments (involving mouse cancer cells and their syngeneic hosts) constitutes a potential problem. At least theoretically, this problem might be circumvented by the use of “humanized” mice bearing a human immune system<sup>172</sup>, but this technology still requires considerable refinements.

***In vitro* assays for ICD enhancers.** DCs are the first-line immune effectors contacting cells that undergo ICD<sup>173</sup>. Unfortunately, DCs are terminally differentiated cells and rather rare (less than 1% of tumor-infiltrating leukocytes), rendering screening campaigns based on primary DCs impractical. However, it is possible to conditionally immortalize DC precursor cells by the transgene enforced expression of a viral oncoprotein that inactivates two cellular tumor suppressors, namely p53 and retinoblastoma 1 (RB1) under the control of a doxycycline (DOX) and dexamethasone (DEX)-inducible system<sup>174</sup>. This genetic modification enables not only the indefinite expansion of immortalized DC precursors in the presence of DOX/DEX, but also (1) the implementation of additional genetic manipulations (for instance with the CRISPR/Cas9 technology); and (2) the de-immortalization of DC precursors by DOX/DEX withdrawal, which culminates with cDC1 differentiation<sup>175</sup>. In summary, this system generates high numbers of terminally differentiated cDC1s that can be employed to assess DC functions as elicited by cancer cells potentially undergoing ICD, such as phagocytosis, expression of activation markers, and/or T cell priming<sup>175</sup>, a strategy that has been successfully employed for identifying venetoclax, navitoclax – a dual inhibitor of BCL2 and BCL2 like 1 (BCL2L1, best known as BCL-X<sub>L</sub>) – and the hexokinase 2 (HK2) inhibitor ikarugamycin as *bona fide* ICD enhancers<sup>176,177</sup>.

***In vivo* assays for ICD enhancers.** De-immortalized DCs generated as described above can be intravenously injected into wild-type mice, *Batf3*<sup>-/-</sup> or *Wdfy4*<sup>-/-</sup> mice (both of which exhibit defects in cDC1-dependent cross-presentation)<sup>45,178</sup> to test the ability of potential ICD enhancers to boost the therapeutic activity of ICD inducers<sup>177</sup>. The capacity of these cells to migrate into tumor lesions and elicit T cell-dependent anticancer immunity can also be investigated in preclinical tumor models, adding a layer of preclinical validation to the *in vitro* screening assays described above. Another strategy for preclinical validation consists in the comparison of the therapeutic activity of candidate ICD enhancers in mice containing vs lacking cDC1-mediated cross-presentation due to the knockout of *Batf3* or *Wdfy4*, or following repeated intravenous injections of recombinant cytochrome c, somatic (CYCS), which depletes cDC1s. In these two latter settings, bona fide ICD enhancers are indeed expected to mediate suboptimal anticancer effects<sup>177</sup>. On theoretical grounds, ICD enhancers should have broad therapeutic effects across multiple cancer types as they act on DCs rather than on malignant cells themselves. However, this conjecture requires further preclinical validation. Similarly, the possibility that ICD enhancers might be used as an alternative to, and perhaps in combination with, ICIs (which mostly act on T lymphocytes) awaits experimental confirmation. As a major caveat, present DC-based drug development efforts involve murine, not human DCs. Hence, it will be important to create human cDC1 precursor cell lines for high-throughput screening purposes or, at least, to design human cDC1-based systems that can be subjected to reliable low-throughput validation assays.

## Concluding remarks

Progress in immunopharmacology and tumor immunology achieved over the past 2 decades suggests that the therapeutic success of nonsurgical cancer treatments including classical chemotherapies can be largely attributed to the elicitation of anticancer immune responses, often due to the induction of ICD. Spurred by this realization, considerable efforts have been made to identify new ICD-inducing drugs, some of which already been approved by the US FDA for use in patients<sup>179,180</sup> or are under currently clinical evaluation (*e.g.*, LTX-315/VC-315), and to combine them with immunotherapies, especially ICIs.

Notwithstanding such an undeniable progress, considerable obstacles exist in the preclinical and clinical development of ICD inducers, which can be summarized as follows:

- ICD inducers, which are often cytotoxic inhibitors of DNA-to-RNA transcription or microtubular poisons, have considerable non-specific side effects, including non-negligible detrimental effects on healthy (including immune) cells. More targeted ICD inducers including TKIs or proteasome inhibitors also exhibit a similar toxicity profile. Thus, more specific strategies to induce ICD signaling events in cancer cells including the activation of the ISR or type I IFN responses are warranted. To achieve this goal, the drug development pipeline required for large-scale identification and validation of ICD inducers requires further optimization.
- The conventional approach to evaluate anticancer drugs at doses close to MTD that are administered in a regular fashion to maintain drug concentrations within a stable range may cause immunosuppressive side effects that ultimately reduce therapeutic efficacy. Hence, clinical trials investigating the safety and efficacy of old and new ICD inducers should be designed to avoid non-specific immunosuppression by reducing doses and/or implementing

intermittent treatment cycles. Moreover, the neoadjuvant therapy of operable tumors should be explored at a large scale, for instance in the form of window-of-opportunity trials.

- By virtue of their genetic and epigenetic plasticity and heterogeneity, cancer cells can avoid efficient ICD signaling, which calls for specific countermeasures to restore ICD in a personalized fashion. This will require not only a careful molecular diagnosis of such ICD defects, but also the development of novel ICD correctors with an elevated potential to translate into clinical cancer care.
- Several tumors also develop strategies to exclude, exhaust or inactivate immune effector cells and/or to recruit immunosuppressive cells, two situations that can be addressed with an arsenal of immunotherapeutic countermeasures. It remains to be seen whether novel galenic formulations that specifically enable ICD signaling at tumor sites combined with local immunotherapy will help in overcoming these hurdles or whether systemic immunotherapy with ICIs will demonstrate superiority in this respect.
- The propensity of a patient to mount therapeutically relevant anticancer immune responses is not only dictated by the tumor and its microenvironment but also depends on the bodywide ecosystem (encompassing, among other factors, general metabolic status and the composition of the gut microbiome). Thus, co-morbidities such as the metabolic syndrome or intestinal dysbiosis as well as an unhealthy lifestyle may constitute additional obstacles to the successful implementation of ICD-based therapies (**Box 2**). These issues can only be managed by a proactive role from internal medicine physicians coupled with a holistic management of the patient.

Irrespective of the obstacles enumerated above, it appears that the concept of ICD has significantly contributed to the development of anticancer drugs and will continue to do so. Indeed, multiple

academic institutions and biotech companies are identifying, characterizing and optimizing new ICD inducers. We anticipate that the oncological armamentarium of ICD inducers will be completed by additional compounds including ICD correctors (to restore defective ICD signaling in malignant cells), ICD boosters (to counteract compensatory immunosuppressive mechanisms driven by ICD) and ICD enhancers (to improve the communication between malignant cells experiencing immunogenic stress and DCs), ultimately providing patients with cancer with personalized regimens that maximize the therapeutic potential of ICD across a variety of malignancies.

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## Highlighted References

Ref. 33

This is the first demonstration that ferroptosis is not always immunogenic *per se*, and actively inhibits the immunogenicity of apoptotic cell death.

Ref. 46

This paper provides an elegant demonstration that besides mediating pro-phagocytic effects, CALR can directly bind NKp46 on the surface of NK cells to promote their cytotoxicity.

Refs. 47,48

These two articles were the first to show that the death of cancer cells as mediated by CD8<sup>+</sup> CTLs is a bona fide instance of ICD.

Ref. 62

This paper documents the importance of proficient autophagic responses in cancer cells to support optimal ICD-associated ATP release.

Refs. 121,122

These back-to-back articles were the first to demonstrate that the activation of executioner caspases robustly suppresses type I IFN responses elicited by MOMP.

Ref. 127

This original report provided compelling evidence in support of the notion that caspase-independent cancer cell death is more immunogenic than its caspase-dependent counterpart.

Ref. 148

This article elegantly demonstrates that secreted GSN inhibits the binding of F-actin to DNGR-1 hence suppressing antigen cross-presentation by cDC1s.

Ref. 151

This report was the first to connect MOMP-dependent mtDNA-driven CGAS signaling in malignant cells with the ability of radiotherapy to elicit anticancer immune responses.

Ref. 177

This article provides abundant evidence supporting the notion that BCL2 inhibit antigen cross-presentation by cDC1s by preventing mtDNA-driven CGAS signaling.

Ref. 181

This paper elegantly demonstrates that efficient autophagic responses in malignant cells limit anticancer immunity by degrading MHC Class I molecules.

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**Competing Interests.** LG is/has been holding research contracts with Lytix Biopharma, Promontory and Onxeo, has received consulting/advisory honoraria from Boehringer Ingelheim, AstraZeneca, OmniSEQ, Onxeo, The Longevity Labs, Inzen, Imvax, Sotio, Promontory, Noxopharm, EduCom, and the Luke Heller TECPR2 Foundation, and holds Promontory stock options. GK has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Tollys, and Vascage. GK is on the Board of Directors of the Bristol Myers Squibb Foundation France. DS is a full-time employee of Violet Therapeutics. GK is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara Therapeutics and Therafast Bio. GK is in the scientific advisory boards of Hevolution, Institut Servier, Longevity Vision Funds and Rejuveron Life Sciences. GK is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis and metabolic disorders. GK's wife, Laurence Zitvogel, has held research contracts with Glaxo Smyth Kline, Incyte, Lytix, Kaleido, Innovate Pharma, Daiichi Sankyo, Pilege, Merus, Transgene, 9 m, Tusk and Roche, was on the on the Board of Directors of Transgene, is a cofounder of everImmune, and holds patents covering the treatment of cancer and the therapeutic manipulation of the microbiota. GK's brother, Romano Kroemer, was an employee of Sanofi and now consults for Boehringer-Ingelheim. The funders had no role in the design of the study; in the writing of the manuscript, or in the decision to publish the results. FMM is a full-time employee of Sonata Therapeutics.

**Author contributions.** LG, DS, GK and FMM wrote the first version of the article, with constructive input from EG. EG build display item under direct supervision from LG. All authors approve the submitted version of the article.

**Table 1. Major strategies for ICD induction in cancer.**

Strategy	Examples	Pros	Cons	Notes	Ref.
Chemotherapy	Doxorubicin Oxaliplatin Paclitaxel	Widely available for clinical use	Generally associated with non-negligible toxicity	Thoroughly characterized in preclinical models	11
CTL-mediated cytotoxicity	N/A	Highly targeted to malignant cells	Difficult to control in clinical settings	May contribute to the efficacy of CAR T cells	47 48
ECP	8-MOP-based ECP	Well-defined toxicity profile	Largely restricted to hematological cancers	Associated with robust DC maturation	95 96
High hydrostatic pressure	N/A	Broad antigenic spectrum	Restricted to DC-based vaccine development	Under clinical evaluation	86
Microwave thermal ablation	N/A	Well-defined toxicity profile	Limited to a few oncological indications	Scarce preclinical characterization	87
Oncolytic peptides	LTX-315/VPX-315	Minimally invasive	Restricted to injectable (skin) tumors	Promising clinical activity in BCC	81
Oncolytic virotherapy	T-vec	Minimally invasive	Restricted to injectable (skin) tumors	Approved for use against melanoma	74
PDT	Hypericin-based PDT	Not invasive and associated with limited toxicity	Restricted to skin cancers	Immunogenicity varies with photosensitizer	53
Radiotherapy	N/A	Not invasive and associated with limited toxicity	Potentially limited by conventional approaches	Thoroughly characterized in preclinical models	84
Targeted anticancer therapy	Cabozantinib Cetuximab Crizotinib	More selective than chemotherapy	Generally associated with non-negligible toxicity	Potent synergy with immunotherapeutics	66 67 182

**Abbreviations:** 8-MOP; 8-methoxypsoralen; BCC, basal cell carcinoma; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ECP, extracorporeal photochemotherapy; ICD, immunogenic cell death; N/A, not applicable; PDT, photodynamic therapy; T-vec, talimogene laherparepvec.

**Table 2. Main strategies for ICD evasion by cancer cells and potential countermeasures.**

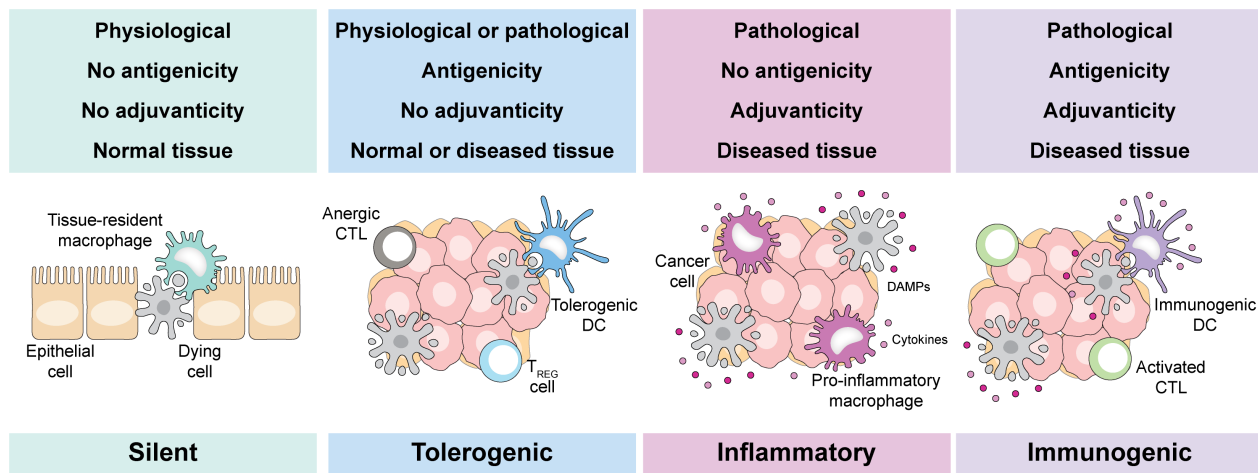
Strategy	Consequence(s)	Mechanism(s)*	Countermeasure(s)	Ref.			
Evading antigenicity	Limited recognition by tumor-targeting T cells	Antigen loss	Activation of antigen-independent cytotoxicity	183 184			
		Decreased MHC Class I expression	Restoration of MHC Class I presentation	22			
		Autophagy activation	Autophagy inhibition	181			
Evading cytotoxicity	Limited release of antigenic material for DC uptake	Active RCD suppression	Engagement of alternative RCD pathways	118 119			
			ANXA1 downregulation	Recombinant CALR administration	157		
Evading adjuvanticity	Limited uptake of tumor-derived material by DCs		Autophagy inhibition	Autophagy activation	107 106 105		
			B7-H4 expression	Inhibition of B7-H4 glycosylation	137		
			CALR downregulation	Recombinant CALR administration	34		
			tCALR secretion	tCALR neutralization	141		
			CD47 upregulation	CD47 blockage SIRPA blockage	142 140		
			ERO1A expression	ERO1A inhibition	138		
			STC1 expression	STC1 inhibition	139		
			Autophagy activation	Autophagy inhibition	151		
			<i>FPRI</i> loss	TLR3 agonism	158		
			GSN secretion	GSN neutralization	148		
			HGMB1 downregulation	TL4 agonism	82		
Limited DC activation and antigen cross-presentation to cytotoxic T lymphocytes			Accrued MOMP	151			
			Type I IFN inhibition	CASP3 inhibition	121 122 127		
			Conditioning the TME	Active suppression of DC and/or T cell functions	Immunosuppressive cell recruitment	MDSC and T <sub>REG</sub> cell depletion	161 162
					Immunosuppressive cytokine secretion	Cytokine neutralization	185
					PD-L1 expression	PD-1 or PD-L1 blockage	82

**Abbreviations:** ANXA1, annexin A1; B7-H4 (official name: VTCN1), V-set domain containing T cell activation inhibitor 1; CALR, calreticulin; CASP3, caspase 3; CD39 (official name: ENTPD1), ectonucleoside triphosphate diphosphohydrolase



1; CD73 (official name: NT5E), 5'-nucleotidase ecto; DC, dendritic cell; ERO1A, endoplasmic reticulum oxidoreductase 1 alpha; GSN, gelsolin; HMGB1, high mobility group box 1; ICD, immunogenic cell death; IFN, interferon; ISR, integrated stress response; FPR1, formyl peptide receptor 1; MDSC, myeloid-derived suppressor cell; MOMP, mitochondrial outer membrane permeabilization; PD-1 (best official name: PDCD1), programmed cell death 1; PD-L1 (official name: CD274); RCD, regulated cell death; SIRPA, signal regulatory protein alpha; STC1, stanniocalcin 1; tCALR, truncated calreticulin; TME, tumor microenvironment; TLR, toll-like receptor; T<sub>REG</sub>, regulatory T. \*main examples.

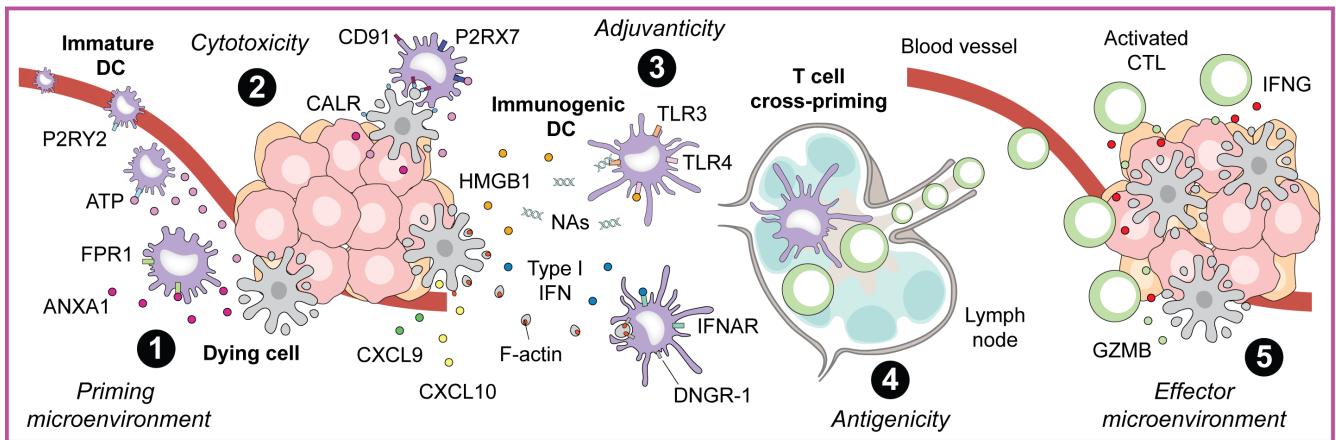
## Figures



**Figure 1**

**Figure 1. Conceptual differences between immunogenic and non-immunogenic cell death.**

Depending on a number of parameters, dying cells can: (1) be largely ignored by the innate and adaptive immune system, a physiological scenario generally reflecting the rapid uptake of dying cells or their corpses by macrophages; (2) be actively tolerogenic, *i.e.*, promote the establishment of antigen-specific peripheral tolerance upon the activation of regulatory T (T<sub>REG</sub>) cells that generally results from antigenicity in the absence of proper adjuvant signals; (3) activate innate (but not adaptive) immune mechanisms, notably inflammatory responses, as a result of abundant adjuvanticity in the absence of antigens that can be recognized by the mature T cell repertoire; or (4) overtly immunogenic, when cell death occurs in the context of sufficient adjuvanticity, failing adaptation to stress coupled with the emission of damage-associated molecular patterns (DAMPs) and pro-inflammatory cytokines, and under microenvironmental conditions that are permissive for dendritic cell recruitment, activation and consequent T cell priming (at cell death sites) as well as for T cell infiltration and effector functions (where the cellular targets of adaptive immunity reside). CTL, cytotoxic T lymphocyte; DC, dendritic cell.



**Figure 2**

**Figure 2. Core mechanisms of immunogenic cell stress and death.** The perception of cell stress and death as overtly immunogenic (*i.e.*, resulting in the activation of adaptive immune responses specific for dead cell-associated antigens) mechanistically relies on the following core components: (1) the site of cell death must contain or be permissive for the recruitment dendritic cells (DCs) or their precursors, and must not be dominated by immunosuppressive signals that may prevent DC activation (*microenvironmental conditions for priming*); (2) cell death must occur in the context of unsuccessful responses to stress (*cytotoxicity*) that are associated with the spatiotemporally regulated emission of damage-associated molecular patterns (DAMPs) and cytokines that: (a) unless the site of cell death is abundantly infiltrated by immune cells *a priori*, recruit DC precursors, (b) attract DC precursors to the close proximity of dying cells, (c) promote the phagocytic uptake of dying cells or material thereof by DC precursors, (d) provide robust immunostimulatory signals to DC precursors for their maturation and functional licensing, and (e) enable mature DCs to reach secondary or tertiary lymphoid structures and to cross-prime antigen-specific T lymphocytes (*adjuvanticity*); (3) dying cells must express antigenic determinants that can be recognized by the mature T cell repertoire (*antigenicity*); and (4) the microenvironment of cells targeted by such an adaptive immune responses is permissive for infiltration by antigen-specific T lymphocytes and the activation of their effector functions (*microenvironmental*

conditions for effecting). ANXA1, annexin A1; CALR, calreticulin; CD91 (official name: LRP1), LDL receptor related protein 1; C-X-C motif chemokine ligand, CXCL; DNGR-1 (official name: CLEC9A), C-type lectin domain containing 9A; FPR1, formyl peptide receptor 1; GZMB, granzyme B; HMGB1, high-mobility group box 1; IFN, interferon; IFNAR, IFN interferon alpha and beta receptor; IFNG, interferon gamma; P2RX7, purinergic receptor P2X 7; P2RY2, purinergic receptor P2Y2; TLR, Toll-like receptor.

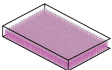
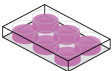
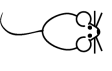
	ICD inducers or correctors	ICD enhancers	ICD boosters
 <b>Robotized platforms</b>	<b>THROUGHPUT</b> Incubation with cancer cell-based biosensors for surrogate ICD biomarkers -ISR activation -CALR exposure -ATP release -HMGB1 release -Type I IFN secretion	Incubation with cancer cell + de-immortalized DC precursor cell co-cultures and high-throughput assays -Phagocytosis -DC activation -T cell cross-priming	Incubation with T <sub>REG</sub> cells or MDSCs and high-throughput assays -Viability -Immunosuppressive markers -Immunosuppressive functions
 <b>In vitro models</b>	Incubation with cancer cells and low-throughput/alternative validation of surrogate ICD biomarkers -ISR activation -CALR exposure -ATP release -HMGB1 release -Type I IFN secretion	Incubation with cancer cell + bone marrow-derived DCs and low-throughput/alternative validation -Phagocytosis -DC activation -T cell cross-priming	Incubation with T <sub>REG</sub> cells or MDSCs and low-throughput/alternative validation -Viability -Immunosuppressive markers -Immunosuppressive functions
 <b>In vivo models</b>	<b>VALIDATION</b> In vivo assays to assess the development of systemic immune reactivity and immunological memory -Prophylactic vaccination -Bilateral tumor models -Therapeutic assays in immunocompetent vs immunodeficient hosts	In vivo assays with WT vs <i>Ba1t3<sup>-/-</sup></i> , <i>Wdly4<sup>-/-</sup></i> or CYCS-exposed mice -Therapeutic vaccination with DCs previously exposed to ICD enhancer -Therapeutic assays with ICD inducer(s)	In vivo assays with WT mice or mice subjected to T <sub>REG</sub> cell or MDSC depletion - Therapeutic assays with ICD inducer(s) +/- ICD boosters(s)

Figure 3

**Figure 3. Potential pipeline for preclinical development of novel ICD-relevant drugs.** Both immunogenic cell death (ICD)-inducing (a) and ICD-enhancing (b) drug candidates can be identified *in vitro* by medium-to-high screening efforts based on: (a) human cancer cell lines expressing biosensors or treated with chemical dyes that enable the assessment of autophagy activation, calreticulin (CALR)

exposure on the plasma membrane, ATP secretion and high mobility group box 1 (HMGB1) loss; or (b) a conditionally immortalized mouse dendritic cell (DC) precursor cell line that can be de-immortalized *ad hoc* and exposed to mouse cancer cells undergoing ICD in the presence of potential ICD enhancers, followed by the assessment of ICD-related parameters including phagocytic capacity, expression of DC maturation markers, and T cell cross-priming proficiency. In the case of ICD inducers, low throughput *in vitro* validation with alternative technologies should be followed by *in vivo* assays based on prophylactic vaccination, therapeutic tests in immunocompetent vs immunodeficient hosts, and/or therapeutic assays in bilateral tumor models (when ICD induction can be achieved by local interventions). In the case of ICD enhancers, low throughput *in vitro* validation can involve human DCs or mouse bone-marrow derived DCs, followed by *in vivo* validation based on therapeutic DCs vaccination assays upon *ex vivo* DC exposure to candidate drugs, or ICD inducer plus enhancer treatment of mouse tumors established in syngeneic immunocompetent mice or mice lacking conventional type 1 DCs (cDC1s), such as *Batf3*<sup>-/-</sup> mice or mice administered with recombinant cytochrome c, somatic (CYCS). The pipeline for the discovery of agents restoring deficient ICD signaling in cancer cells, so-called ICD correctors, strictly resembles the one for ICD inducer discovery, except for the fact that combinatorial strategies are tested (*i.e.*, suboptimal ICD inducer plus potential ICD corrector). Along similar lines, candidate ICD boosters be identified *in vitro* by high-throughput strategies testing regulatory T (T<sub>REG</sub>) cell or myeloid-derived suppressor cell (MDSC) viability and immunosuppressive functions, but can only be validated by low throughput *in vivo* assays. IFN, interferon.

## **Box 1. Immunotherapeutic agents benefiting from ICD induction.**

During the past two decades, immunotherapy not only has become a major player in the clinical management of cancer, largely driven by the approval of immune checkpoint inhibitors (ICIs) for an ever increasing number of oncological indications, but has also diversified to encompass a number of approaches beyond ICIs<sup>20</sup>. Many of these immunotherapeutic interventions, some of which have already been licensed by various regulatory agencies worldwide for use in cancer patients, may benefit (directly or indirectly) from immunogenic cell death (ICD) induction, at least on theoretical grounds. For instance, while chimeric antigen receptor (CAR)-expressing T cells, which are currently employed in the clinical management of various hematological malignancies<sup>186</sup>, kill malignant cells based on the expression of a specific surface marker independent of antigen presentation on MHC Class I molecules<sup>187</sup>, cancer cell death as elicited by T cells is a *bona fide* variant of ICD<sup>47,48</sup>. This suggests that at least part of the efficacy of CAR T cells may originate from an endogenous T cell response to CAR-unrelated antigens (so-called “antigen spreading”) downstream of initial ICD induction by CAR T cells. Such a component of the response would be particularly important for patients that develop secondary resistance to CAR T cells upon the selection of malignant cell clones that no longer express the antigenic CAR target<sup>188</sup>. Along similar lines, multiple tumor-targeted monoclonal antibodies are believed to operate by blocking the delivery of trophic signaling to malignant cells and by favoring the activation of innate immune effector cells that express Fc gamma receptors like natural killer (NK) cells<sup>189</sup>. However, the fact that some of them, including epidermal growth factor receptor (EGFR)-targeting antibodies like cetuximab robustly drive ICD<sup>182</sup> argue in favor of an at least supportive role for ICD in the therapeutic effects of these agents. Finally, an abundant preclinical literature suggests that ICD inducers represent promising combinatorial partners for a wide panel of immunomodulatory agents including (but not limited to): (1) agonists for immunostimulatory receptors such as TNF

receptor superfamily member 9 (TNFRSF9, best known as 4-1BB)<sup>190</sup> or CD40 (Ref. <sup>191</sup>); (2) immunostimulatory cytokines<sup>192</sup>; (3) immunomodulatory agents including (IDO1) inhibitors<sup>193</sup>; and (4) therapeutic cancer vaccines<sup>194</sup>. Taken together, these observations lend additional support to the critical position occupied by ICD in modern cancer (immuno)therapy.

## **Box 2. Emerging obstacles against anticancer immunity driven by ICD.**

Tumor-targeting immune responses as elicited by immunogenic cell death (ICD) inducers can be impaired not only by issues affecting ICD signaling in malignant cells, the perception of cell death as immunogenic by the immune system or the microenvironment of dying or target cells, but also by hitherto underappreciated alterations of the bodywide ecosystem that directly or indirectly affect the immunological fitness of the patient<sup>195</sup>. Some of such alterations that (at least in preclinical tumor models) have been characterized include: (1) changes in the abundance and composition of the gut microbiome, which appear to have a direct effect on systemic immune tone<sup>196</sup>; (2) local alterations of the tumor microenvironment (TME) as imposed by the (generally unsupervised) usage of over-the-counter drugs such as aspirin, which has been associated with a potentially detrimental inhibition of cyclic GMP-AMP synthase (CGAS) signaling<sup>197</sup>; (3) detrimental alterations of immune function caused by drugs that are commonly employed for clinical cancer management but often overlooked for their (in some instances well known) immunosuppressive effects, such as glucocorticoids<sup>198,199</sup>; (4) considerably detrimental shifts of the local or systemic TME that result from inappropriate dietary habits<sup>200-202</sup>; and (5) deteriorations of intratumoral or systemic immune tone elicited by a sedentary lifestyle<sup>203</sup> and/or psychological distress<sup>199</sup>. It is likely that multiple other over-the-counter products including common medications and nutritional supplements may impact the ability of ICD to elicit tumor-targeting immune responses. Along similar lines, it is more than plausible that additional lifestyle habits with powerful biological effects (*e.g.*, sleep duration and quality) may influence intratumoral or systemic immune tone to alter the ultimate immunogenicity of cancer cell death. Additional work is required to assess such impact and characterize the underlying molecular and cellular mechanisms in preclinical tumor models. In the meantime, we believe that the implementation of ever more comprehensive anamnestic questionnaires by internal medicine physicians may provide



an invaluable source of data for correlatively assessing the impact of the aforementioned factors on disease progression in patients with cancer treated with ICD inducers (or other drugs).