Biotecnología Aplicada

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REVIEW

Experimental approaches to evaluate the induction of immunogenic cell death

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ABSTRACT

Immunotherapy is considered a promising therapeutic modality in oncology by eliciting a robust antitumor response that aims to re-activate the immune system suppresed by tumor cells. Cancer therapy is most successful when it can induce an immunogenic form of cell death (ICD). ICD implies the activation of a specific immune response against cancer cells that generates a strong and long-lasting anticancer immunity. Its induction is a key determinant of cancer treatment efficacy, combining the ability to eliminate cancer cells with the stimulation of innate and adaptive immune responses and thus the establishment of long-term immune memory. This review is aimed to summarize and discuss the main experimental approaches for the evaluation of a potential ICD-inducing candidate. It is generally accepted that ICD identification and characterization should encompass the performance of an experimental set of *in vitro* and *in vivo* assays, to complementarily assess the ability of tumor cells to undergo ICD in their natural anatomical location. It involves the recruitment of antigen presenting cells and their stimulation to trigger an adaptive immunity response against cancer. ICD induction remains the subject of intense research, given its potential implications for cancer treatment. Unresolved issues remain, such as the relevance of the murine models currently in use and their extrapolation to the oncological context in a clinical setting, as well as the selection of reliable *in vitro* markers for the prognosis of possible ICD elicitation. In summary, not one specific method should be used to assert the occurrence of ICD. Research on this matter requires the parallel execution of *in vitro* and *in vivo* estimations, where the induced regulated cell death is cumulatively evaluated, to unequivocally confirm the elicitation of a specific adaptive antitumor immunity.

Keywords: Immunogenic cell death (ICD), damage-associated molecular patterns, endoplasmic reticulum stress, vaccination assays in ICD, abscopal models

RESUMEN

Aproximaciones experimentales para evaluar la inducción de muerte celular inmunogénica. La inmunoterapia se ha considerado una modalidad terapéutica prometedora en oncología al provocar una sólida respuesta antitumoral que pretende reactivar el sistema inmunitario abortado por las células tumorales. En los últimos años, se ha demostrado que la terapia contra el cáncer tiene más éxito cuando puede inducir una forma inmunogénica de muerte celular. La inducción de una muerte celular inmunogénica (ICD) implica la activación de una respuesta inmunitaria específica contra las células cancerosas que genera una inmunidad anticancerosa fuerte y duradera. La inducción de la ICD en las células cancerosas es un determinante clave de la eficacia del tratamiento del cáncer, ya que combina la capacidad de eliminar las células cancerosas con la estimulación de las respuestas inmunitarias innatas y adaptativas y, por tanto, el establecimiento de una memoria inmunitaria a largo plazo. El objetivo de esta revisión ha sido resumir y discutir los principales enfoques experimentales para la evaluación de un candidato potencial de inducir ICD. En la actualidad, existe consenso en que un estudio de esta naturaleza debe abarcar la realización de un conjunto de ensayos experimentales *in vitro* e que, de forma complementaria, puedan evaluar la capacidad de las células tumorales puedan evaluar la capacidad de las células tumorales de experimentar ICD en su localización anatómica natural, lo que implica el reclutamiento de células presentadoras de antígenos y su estimulación para desencadenar una respuesta de la inmunidad adaptativa contra el cáncer. La inducción de ICD es objeto de una intensa investigación dadas sus potenciales implicaciones para el tratamiento del cáncer, pero hay cuestiones sin resolver como la relevancia de los modelos murinos actualmente en uso y su extrapolación al contexto oncológico en un entorno clínico, así como la selección de marcadores *in vitro* fiables para el pronóstico de la posible inducción de ICD. La investigación en esta área requiere la ejecución paralela de estimaciones *in vitro* e *in vivo*, donde la muerte celular regulada inducida se somete a evaluaciones acumulativas que culminan en la confirmación inequívoca de la generación de inmunidad antitumoral adaptativa específica.

Palabras clave: Muerte celular inmunogénica (ICD), patrones moleculares asociados al daño, estrés del retículo endoplasmático, ensayos de vacunación en ICD, modelos abscopales

How to cite (Vancouver style):
de León-Esperón LM, Llorente Álvarez F, Díaz Navarro O, Soto Febles C, Lanio ME, Sánchez B, *et al*. Experimental approaches to evaluate the induction of immunogenic cell death. Biotecnol Apl. 2023;40(4):4101-4108.

Introduction

Immunotherapy has been regarded as a promising therapeutic modality in oncology, to elicit a robust antitumor response aimed to re-activate the immune system abrogated by tumor cells [1]. Cancer treatment is most effective when it induces an immunogenic form of cell death (ICD). The notion behinds ICD is to trigger a particular immune response against cancer cells that produces specific, enduring and potent

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anticancer immunity [2, 3]. ICD is characterized by the exposure/release of molecular signals, which are commonly referred to as Damage-Associated Molecular Patterns (DAMPs). Cell surface exposure of calreticulin (CRT), the release of HMGB1, ANXA1, and ATP [3–9] are characteristics of this type of cell demise. DAMPs act as adjuvants that promote the activation of antigen-presenting cells (e.g. dendritic cells) which engulf dying cancer cells [3], leading to cross-presentation of antigenic peptides to CD8+ T lymphocytes, key drivers of the anti-tumor immune responses [10-12]. The induction of ICD in cancer cells has recently been recognized as a key determinant of cancer treatment efficacy, combining the ability to eliminate cancer cells with the stimulation of innate and adaptive immune responses and thus the establishment of long-term immune memory. Many ICD inducers, for example, anthracyclines (i.e. doxorrubicin), radiotherapy, and Hypericin-PhotodynamicTherapy (Hyp-PDT), elicit ICD via inducing the surface exposure of CRT and Heat Shock Proteins (HSPs), the secretion of ATP and the High Mobility Group Box 1 (HMGB1) protein, and the release of mtDNA that stimulates the production of type I IFNs [3, 13].

The emergence of new ICD inducers has gained attention, given that circumventing the low immunogenic profile of tumors is therapeutically relevant. DAMPs play a central role in the course of ICD, accounting for the adjuvanticity that drives recruitment and activation of innate immune effectors [9]. However, not all instances of ICD rely on the same DAMPs emission pattern, as the case of chemotherapy-induced ICD in contrast to Hyp-PDT-driven ICD [2]. Moreover, the emission of DAMPs does not necessarily constitute a predictive factor of cell death immunogenicity; the extent of cell death should also be taken into consideration when evaluating ICD. For instance, mouse cancer cells treated with cardiac glycosides expose CRT on their surface, release HMGB1 and ATP, yet the limited cytotoxicity of cardiac glycosides is not sufficient and tumors develop at the vaccination site when such treated tumor cells are inoculated into a syngeneic host [3]. Along similar lines, the ICD-inducing capacity of a certain agent cannot be predicted based on its structural, chemical or physical similarity with another demonstrated ICD inducer, as illustrated by the pair oxaliplatin (ICD inducer)-cisplatin (non-ICD inducer) [4, 5].

All these evidences point to the need for standardized protocols to evaluate the immunogenic potential of cell death. These protocols should comprise the assessment of the extent and mechanism of cell death; stress responses and their connection to DAMPs release; APCs activation and functionality (i.e. their ability to mediate cross-priming *in vitro*); and *in vivo* antitumor immunity generated by dying cells [6].

Importantly, *in vivo* vaccination experiments constitute the gold-standard approach for ICD evaluation in mouse tumor models. The main experimental strategies used for *in vitro* assessment of ICD-associated processes, as well as the main approaches recommended for *in vivo* monitoring of ICD will be outlined in this review (Figure). Remarkably, not only one specific method should be used to assert the occurrence of ICD. In this work, ICD evaluation strategies that encompass all potentially relevant regulated cell death pathways are presented, beyond the classical immunogenic apoptosis' point of view and describing just the essential features of cell death detection and characterization methods for ICD. To draw relevant data in this area, research should be conducted combining the execution of *in vitro* and *in vivo* evaluations, where the induced regulated cell death can be analyzed from cumulative assessments, leading to the unambiguously confirmation of the elicitation of specific adaptive antitumor immunity.

In vitro assays

Even though *in vivo* experiments constitute the most asserted approach to monitor the occurrence of ICD, they are limited by the relatively few syngeneic tumor models available. Hence, they are not compatible with large screening campaigns in the search for ICD inducers [7]. Therefore, a broad panel of ICD-associated processes is used to mechanistically predict the potential of certain therapies to induce tumor immunogenicity. One of the first parameters to be elucidated in these studies should be the cytotoxic effect of the putative ICD inducer in cancer cells, assessed through membrane permeabilization assays, cell viability tests, and/or evaluation of specific cell death-related intracellular pathways. The most common experimental approaches carried out in cancer and immune cells to determine the capacity of a particular intervention to evoke ICD are summarized in tables 1 and 2. Flow cytometry, immunoblotting and immunofluorescence microscopy are among the most employed techniques, among others.

The Endoplasmic Reticulum (ER) stress is crucial for DAMPs release from cancer cells subject to most ICD stimuli. The response to such phenomenon can be assessed by monitoring the main arms of the unfolded protein response (UPR): the ER to nucleus signaling 1 (ERN1, also known as IRE1α) branch, the activating transcription factor 6 (ATF6) branch, and the Protein Kinase RNA-activated (PKR)-like ER Kinase (PERK) branch. The activation of IRE1α can be monitored by the splicing status of X-box binding protein 1 (XBP1) mRNA, through real time RT-PCR, or using cell lines expressing fluorescent versions of XBP1 [8-10]. Similarly, the nuclear redistribution of ATF6 can be assessed by immunofluorescence microscopy in cell lines expressing fluorescently-tagged variants of ATF6 [11]. Otherwise, the inactivating phosphorylation of eIF2α, which occurs downstream of PERK activation, constitutes a sole biomarker of ICD and correlates with CRT exposure [32]. The eIF2α phosphorylation can be assessed with phospho-neo-epitope-specific antibodies, via immunoblotting, flow cytometry, or fluorescence microscopy [33].

Nevertheless, several studies point to the crucial role played by Reactive Oxygen Species (ROS) production and its connection to ER stress and ICD, as the immunogenicity of cell death diminished in the presence of antioxidants [3, 22] or a $^1O_2$ quencher [22]. Based on how ICD inducers engage ER stress for cell death and danger signaling, ICD inducers are classified as Type I or Type II. Type I inducers are defined as those agents that act on non-ER proteins for the induction of cell death, but promote collateral ER stress for danger signaling, thereby operating on

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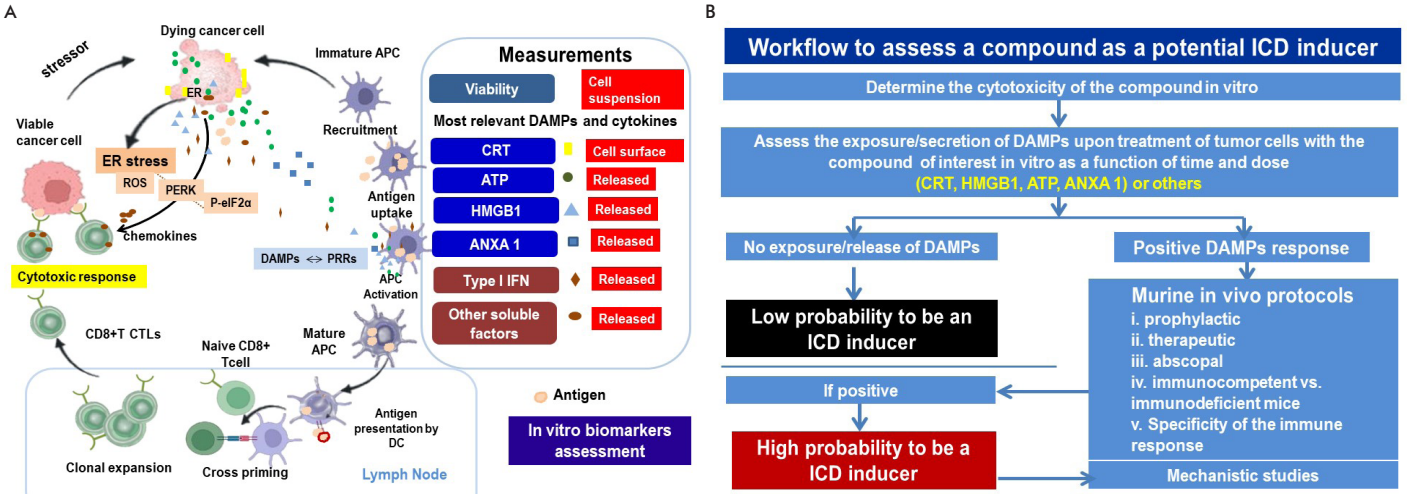

Figure 1. Overview of the generation of major immunostimulatory DAMPs mechanistically linked to immunogenic cell death (ICD) in cancer and their assessment. A) Tumor cells experiencing ICD in response to certain stressors can prompt an adaptive immune response specific for antigens associated with the dead cells. Dying cells emit a panel of DAMPs, immunostimulators and cytokines that, following a precise spatiotemporal pattern, favor the recruitment, phagocytic activity and maturation of antigen-presenting cells (APCs), enabling them to engulf antigens, migrate to lymph nodes and prime a specific cytotoxic T-lymphocyte (CTL)-dependent immune response. These events result in the elicitation of tumor-targeting immune responses associated with the elimination of residual cancer cells, and the establishment of immunological memory (not represented). Tumor microenvironment (TME) is generally characterized by an immunosuppressive profile that may hamper the initiation or execution of ICD-driven anticancer immunity (not represented). Thus, the ultimate ability of the ICD to drive adaptive immunity depends not only on the initiating stimulus and the dying cell, but also on intrinsic host characteristics. Panel A shows a selection of DAMPs/cytokines exposure/release, which confer adjuvanticity to dying cancer cells. They comprise cell surface-exposed calreticulin (CRT) as well as secreted: ATP, high-mobility group box 1 (HMGB1) protein, annexin A1 (ANXA 1), and type I interferon (Type I IFN). Additional hallmarks of ICD include the phosphorylated form of eukaryotic translation initiation factor 2 subunit-α (P-eIF2α), among others. These signals are decoded by pattern recognition receptors (PRRs) expressed by immune cells. CRT exposure on cell surface occurs at an early stage of ICD promotes the uptake of dying cells and Type I IFN secretion by APCs. Type I IFN stimulates APC maturation, cross-presentation and T cell recruitment. ATP in tumor microenvironment boosts the recruitment, maturation and cross-presentation activity of APCs. The detection of the phosphorylated form of eIF2α typically in cell homogenates is associated with Endoplasmic Reticulum stress and ICD. P-eIF2α elicits the maturation and cross-presentation activity of APCs. The protein annexin 1 (ANXA) directs the interaction of APCs to dying cells or their corpses. B) Once there is reasonable *in vitro* evidence it is recommendable to give a further step towards a more robust in vivo assay. A summarized diagram of a possible workflow to determine the potentialities of a compound to be an ICD inducer is depicted in the figure.

multiple targets. In turn, Type II ICD inducers are anticancer agents that target the ER for both cell death induction and danger signaling [34]. Type II ICD agents like Hypericin-based Photodynamic Therapy, have a ROS-based ER stress effect which dictates their ICD-inducing ability [22]. Thus, the assessment of ROS production with the use of antioxidants, $^1O_2$ quenchers, or ROS detection commercial kits can shed light on the intracellular mechanisms driving ICD.

The exposure of ER chaperones on the cell membrane constitutes a relevant event for the immunogenicity associated to cell demise. The presence of proteins like CRT, ERp57, HSP70, and HSP90 on the cell surface can be monitored with the simultaneous use of specific antibodies (or dedicated constructs that allow tracking) and vital dyes for the exclusion of dead cells, like propidium iodide (PI), 7- aminoactinomycin D (7- AAD), or 4′,6- diamidino-2- phenylindole (DAPI), by flow cytometry [5, 18, 19]. Moreover, the use of surface protein biotinylation, subsequently followed by precipitation with streptavidin beads and immunoblotting, has been described as an efficient method to detect ER chaperones on the plasma membrane of cancer cells exposed to ICD inducers [23, 35]. Finally, fluorescence microscopy permits the use of paraformaldehyde for the sequential fixation and detection with specific antibodies [20, 29, 36], or tracking ER chaperones cell localization by expressing its fluorescently-tagged variants within genetically engineered cell lines [3, 5, 37].

There are several methods for the detection of soluble mediators of ICD. ATP release can be evaluated by direct assessment of this molecule on culture supernatants, or by measuring the residual intracellular ATP in cell lysates, with commercial luminometric assays based on the ability of eukaryotic luciferases to produce light in an ATP-dependent fashion [38, 39]. Additionally, intracellular ATP can be quantified with the use of the ATP-binding fluorochrome quinacrine [40], or by fluorescence resonance energy transfer (FRET) assays, using ATP-sensitive fluorescent probes [27].

The presence of the relevant DAMP HMGB1 on culture supernatants can be detected with enzyme-linked immunosorbent assay (ELISA) commercial kits, or by immunoblotting with specific antibodies, while its intracellular counterpart can be traced with an HMGB1-fluorescent variant by fluorescence microscopy, upon measure of residual fluorescence [29-31]. Along similar lines, annexin A1 (ANXA1) secretion can be evaluated with immunoblotting on culture supernatants [41], while type I IFN secretion can be assessed with commercial ELISA [16].

Activation of the autophagic machinery is required for the preservation of lysosomal ATP stores in several ICD scenarios. A relevant autophagic event that can be readily monitored is the accumulation of Microtubule-associated protein 1A/1B-light chain 3 (MAP1LC3B, also known as LC3) into autophagosomes upon its conjugation to phosphatidylethanolamine. Using fluorescent variants of LC3, its distribution, from a diffuse

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Table 1. Methods to assess immunogenic cell death-related processes *in vitro* in cancer cells

Event	Parameter	Method	Methodological requirements	References
ER stress	Phosphorylation of elf2α or elf2α kinases	Immunoblotting	Specific phosphoepitopes antibodies	[14]
	XBP 1 splicing	IF microscopy Flow cytometry Fluorescence microscopy qReal Time-PCR	Cells that express a fluorescently-tagged version of XBP1	[8,10]
	ATF6 nuclear translocation	Fluorescence microscopy	Cells that express a fluorescently-tagged version of ATF6	[11]
	ROS production	Incubation with antioxidants or quenchers	Commercial kits available. Fluorescent dyes. Determined in whole cell lysates or with ER-targeted probes (ER-fractionation)	[15]
Autophagy	Autophagosome formation	Fluorescence microscopy	Cells that express a fluorescently-tagged version of LC3	[15]
PRR activation	IRF3 phosphorylation	Immunoblotting	Specific phosphoepitopes antibodies	[16]
	Ectopic accumulation of nucleic acids	Fluorescence microscopy	Dedicated antibodies	
		Subcellular fractionation	Enzymatic degradation of nucleic acids and absorbance-based quantification	[17, 18]
ER chaperones exposure	Surface exposure of CRT, ERp57, HSP70, HSP90	Flow cytometry + vital dyes	Vital dyes allow exclusion of dead cells	[19, 20]
		Fluorescence microscopy	Specific ER chaperones antibodies	[21, 22]
		Immunoprecipitation + Immunoblotting	Protein biotinylation+streptavidin mediated precipitation	[23, 24]
ATP release	Extracellular ATP	Luminometry Mass spectrometry	D (-)-luciferin that must be added exogenously; assessed in culture supernatants	[25, 26]
	Intracellular ATP	Flow cytometry Fluorescence microscopy	Staining with the ATP-specific dye quinacrine	[27, 28]
		FRET Luminometry	YFP-CFP fusion protein containing an ATP-sensitive domain	
HMGB1 release	Extracellular HMGB1	ELISA	Commercial kits available	[29, 30]
		Immunoblotting	Specific antibody; assessed in culture supernatants	
	Intracellular HMGB1	Fluorescence microscopy	Cells that express a fluorescent version of HMGB1	[31]
Type I IFNs release	Extracellular Type I IFN	ELISA	Commercial kits available	[19]
ANXA1 exposure/rel	Extracellular ANXA1	Immunoblotting	Assessed in culture supernatants	[32]

to a punctate pattern indicating the activation of an autophagic response, can be examined by fluorescence microscopy [14, 42]. Activation of pattern recognition receptor (PRR) in cancer cells in the course of ICD can be assessed with antibodies specific for key phosphorylated transducers like IFN regulatory factor 3 (IRF3) by immunoblotting, or by qRT-PCR-mediated evaluation of IFN stimulated-genes (ISG) transcription, including chemokines genes like *cxcl10*, *ccl2* and *cxcl1* [16]. In addition, the ectopic localization of nucleic acids in cancer cells after a putative ICD inducing treatment has been monitored by subcellular fractioning or fluorescence microscopy [16, 17].

In cancer cells, all these aforementioned techniques can be implemented to expose the abilities of certain agents to induce ER stress, comprising DAMPs release/exposure, and/or autonomous PRR activation in the context of ICD stimulation. Nevertheless, several common procedures can be performed *in vitro* on immune cells to evaluate their possible participation in the immune response orchestrated upon ICD induction in cancer cells (Table 2). Such evaluations cannot be performed *in vivo* in human tumor models, therefore *in vitro* studies in immune cells allow a functional estimation of immunogenicity of cell death. In this context, dendritic cells (DCs) are the main focus of attention in terms of pahgocytosis, maturation, and cross- priming potential evaluation [6]. The engulfment capacity of DCs can be investigated by co-culturing dying cancer cells and DCs, or their precursors, followed by flow cytometry or fluorescence microscopy, using individual pre-labeling with non-toxic cytoplasmic fluorescent dyes [28, 36, 43]. The up-regulation of CD40, CD80, CD86, CD83 and MHC-II molecules in the surface of DCs as a sign of maturation can be also monitored with specific antibodies via flow cytometry [20, 36].

Methods to assess ICD-related processes *in vitro* in cancer cells

The secretion of cytokines is usually evaluated by flow cytometry with intracellular staining or by ELISA, to further assess its functional maturation. Thus, an increase in the levels of pro-inflammatory cytokines like IL-1β, IL-18, IL-12, IL-6, and IL-23 can be used as a sign of DCs phenotypic maturation [19, 22, 44]. Importantly, DCs pre-labeling with fluorescent dyes also allows for the evaluation of their migratory capacity driven by chemotactic factors secreted by dying cancer cells [45, 46]. DCs mediate T cell functions by cross-presenting antigenic material derived from dying cells to cytotoxic lymphocytes (CTLs). Once exposed to dying cancer cells, the ability of DCs to mediate cross-priming can be measured by co-culturing them with syngeneic, naive T cells, and the posterior evaluation of T cell proliferation by flow cytometry [47]. In turn, T cell activation can be measured by monitoring up-regulation of CD69, LAMP1 or PD-1 [44, 48], and T cell effector function, by flow cytometry with intracellular antibodies specific for interferon (IFN)-δ, Gran-

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Table 2. Methods to assess ICD-related processes *in vitro* with immune cells

Event	Parameter	Method	Methodological requirements	References
Dying cells engulfment by APCs	Phagocytosis rate	Flow cytometry Fluorescence microscopy	Pre-labeling of APCs and cancer cells with different fluorescent dyes	[28, 36]
DC maturation	Up regulation of CD80, CD86, CD83 and MHC-II	Flow cytometry	Staining with specific antibodies; involves co-culture of DCs and dying cancer cells	[20, 44]
	Cytokine secretion (IL-1β, IL-6, IL-12, IL-23)	ELISA Flow cytometry	Detection of IL-1β, IL-18,IL-6, IL-12, IL-23 secreted to the supernatant or intracellular staining for flow cytometry	[19, 22, 45]
	DC migration	Video microscopy	Pre-labeling with fluorescent dyes	[31, 41]
DC cross-priming potential/T cell function	T cell proliferation	Flow cytometry	Co-culture of DCs and naïve T cells, and previous labeling with carboxyfluorescein succinimidyl ester	[45, 46]
	T cell activation	Flow cytometry	Staining with CD69, LAMP1 or PD-1 specific antibodies	[47, 48]
	T cell effector function	Flow cytometry	Intracellular staining with IFN-δ, PRF-1 and GZMB specific antibodies	[19, 20]
		ELISPOT	Quantification of extracellular IFN-δ	[47, 49]
	Cytotoxic potential of CTLs	Cytotoxicity assays Flow cytometry	Colorimetry, flow cytometry or other techniques to assess cell lysis	[50, 51]

zyme B, or perforin-1 [19, 48]. Alternatively, T cells release of IFN-δ can be detected by ELISPOT [49]. The cytotoxic potential of cross-primed CTLs is generally evaluated by measuring lysis of live cancer cells [50, 51], or by analyzing the effective adaptive antitumor immune response generated by T cells, upon the interaction with specific tumor-associated antigens (TAAs), cross-presented by dendritic cells to the T cells [52, 53].

Experimental *in vivo* ICD models

The level of antigenicity of malignant cells is a direct consequence of the tumor mutational burden (TMB) shaping the development of neoplastic lesions, largely reflecting the potential to generate TAAs [51-54]. Thus, TMB can be highly heterogeneous across dissimilar types of tumors, between different stages of malignant progression, and even in different anatomical areas within the same tumor [55-58]. Furthermore, some tumors benefit from defects that compromise the antigen presentation machinery, as a consequence of mutations or the down regulated expression of a few key factors. Furthermore, tumors with high TMB preferentially expand cancer cell clones that do not express antigens subject to active immunity [59, 60]. The capacity to recognize and mount an immune response against TAAs depends on the presence of naïve T cells specific for epitopes that were not covered by central tolerance, indicating that a strong component of antigenicity is host-related. Similarly, adjuvanticity relies on immunostimulatory DAMPs exposed/released from cancer cells upon a stressful stimulus and DAMPs recognition by cognate receptors present on the host's immune cells [64-67]. This process communicates a state of danger and activates the specific responses [68]. In fact, knockdown or knockout mice for genes encoding critical DAMPs receptors are usually unable to fully respond to ICD inducing therapies. Consequently, loss-of-function polymorphisms in genes encoding DAMPs receptors like *P2rx7*, *Fpr1* or *Tlr4* represent a negative prognosis in breast cancer or colorectal carcinoma patients treated with ICD-inducing chemotherapeutics [4, 41, 44].

Another point is that, during tumor development, the immune system plays an important role by immunoediting, as malignant cells are subjected to an increased immunological pressure that selects the most aggressive and less immunogenic tumor variants [69]. This decreased immunogenicity is supported by subversion of DAMPs release and sensing at expenses of adjuvanticity, mediated by genetic or epigenetic silencing of specific DAMPs [70-72]. Hampering intracellular stress response pathways associated with DAMPs, cytokines and chemokine release, including UPR, autophagy and cell death-precipitating mediators also decrease the expected immunogenicity of DAMPs [73]. In addition, there are several microenvironmental factors influencing the cell death outcome, just adding more layers of complexity to the elicitation of an immune response. Robust tumor-mediated immunosuppressive circuitries can largely disrupt ICD-associated antitumor immunity by several mechanisms, including, but not limited to:

1) Recruitment of immunosuppressive immune cells to the tumor microenvironment, like CD4+CD25+FOXP3+ regulatory (Treg) cells, myeloid-derived suppressor cells (MDSCs), and/or M2-polarized tumor-associated macrophages (TAMs). This process generally occurs at the expense of effector or immunostimulatory cells including mature DCs, CD8+ T cells and M1 macrophages. Moreover, such immunosuppressive immune populations infiltrating the tumor microenvironment express high levels of ectonucleotidases CD39 and CD73, which reduce extracellular levels of ATP by converting it to adenosine, further reinforcing immunosuppression and directly antagonizing ICD occurrence [74-76].

2) Persistent release of immunosuppressive and tumor progression-associated cytokines, such as IL-10 and TGF-β, by tumor cells and Treg, MDSCs and M2-TAMs [77-79].

3) Elevated expression of co-inhibitory receptors like CTLA4, PD-1 and TIM-3 by T cells that cause dysfunction of immune-infiltrating T cells and immune exhaustion [80-82].

4) Vascular exclusion and a dense stroma can limit tumor infiltration of primed CTLs in tumor-draining lymph nodes [83-85].

All these factors lead to tumor evasion of immunosurveillance, and explain why the same cancer cells exposed to a certain therapeutic agent do not respond equally *in vitro* and *in vivo*.

These factors highlight the importance of the latter assessment for accurate predictions regarding a

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functional ICD response *in vivo* [6]. Five main scenarios are used for the assessment of ICD elicitation in cancer. The gold-standard approach employed almost indispensably consists on vaccination assays with immunocompetent, syngeneic mice [7, 86].

Prophylactic scenario

Tumor cells previously treated *in vitro* with a potential ICD inducer are injected into one lateral flank of immunocompetent syngeneic mice in the absence of any adjuvant. After a latency of one to two weeks, mice are challenged with untreated (living) cancer cells of the same type, injected into the opposite flank. Tumor growth and incidence are monitored at both sides as well as the overall survival. If the evaluated inducer of cell death is immunogenic, it will generate a protective anticancer immunity, with a partial or total reduction of the tumor growth and incidence. An increase of overall survival, as compared to the negative control groups, *i.e.* inoculated with cells treated with non-ICD inducers, will be also observed. This is the only scenario capable of reducing tumor incidence. Noteworthy, this vaccination assay can be performed not only with pre-treated cells, but also with DCs pre-loaded with dead cells antigens *in vitro* after co-culture with dying cancer cells [87]. Prophylactic interventions, however, have one main disadvantage: the ability of anticancer agents to induce ICD is usually reduced in settings where tumors are previously established, reflecting the potent immunosuppressive networks promoted by developing tumors; vaccination experiments cannot bring into the equation this critical factor [88].

Therapeutic scenario

Firstly, untreated cancer cells are inoculated in immunocompetent syngeneic mice, and once tumors are palpable, they are treated i) with autologous dying cancer cells-exposed DCs or ii) with autologous CD8+ T cells, exposed *in vitro* to the same DCs [89]. This latter intervention is generally carried out by combining the inducer with cytokines that support T cell expansion *in vivo* [28, 90]. The tumor is monitored and, if the evaluated inducer of cell death is indeed immunogenic, a partial or total reduction of the tumor growth and an increase of overall survival will occur, compared to a negative control group treated with a non-ICD inducer.

Additionally, the systemic outreach of ICD-related antitumor immunity can be evaluated by abscopal models, or intracranial/extracranial models.

Abscopal models

These models typically rely on the s.c. generation of two slightly asynchronous, anatomically distant tumors, in immunocompetent syngeneic mice either artificially or via inoculation of pro-metastatic cancer cells. Then, only one of these tumors is treated with the potential ICD inducer [91-93]. Either way, this setting is only compatible with focalized irradiation therapy, or with intratumoral delivery of drugs [92, 94, 95]. Global survival, and non-treated tumor progress, or metastatic load, are monitored as a sign of an abscopal response, consisting on the regression of tumor lesions outside of the treated field and an increase of overall survival compared to negative control groups treated with non-ICD inducers.

Intracranial/extracranial models

These models are useful for evaluating ICD in brain metastasis and chemotherapeutics that do not cross the blood-brain barrier (BBB) [95]. Two tumors are generated into immunocompetent syngeneic mice, one extracranial and one intracranial. Only one of them is treated, the former with a systemic agent BBB-impermeant, or the latter with radiation therapy. In this scenario, the presence of extracranial lesions stimulates CTL trafficking and improve effectiveness of immunotherapy [96, 97]. If the treatment is in fact immunogenic, a regression of both tumor lesions will be observed, and an increase of general survival compared to negative control groups treated with non-ICD inducers [96, 97].

Immunocompetent versus immunodeficient mice

To ensure that the therapeutic efficacy of ICD inducers depends on the immune system, antitumoral assays can be performed comparing their effect on mouse cancer cells growing in immunocompetent versus immunodeficient mice [87]. In most of these scenarios, the durability of the antitumor response can be assessed later on as follows. In mice that showed long-term disease eradication after the treatments, by re-challenging with the same living cancer cell type. Similarly, specificity of the antitumor immunity can be evaluated by re-challenge with syngeneic cancer cells, but different to the initial ones used [91, 98, 99].

The vaccination model is advantageous in that it offers a better control on the experimental conditions of cell death induction, along with increased sensitivity, as hosts are tumor-naïve and hence they lack cancer-driven immunosuppression. Nevertheless, its clinical relevance is limited, precisely because a very high degree of sensitivity differs from the scenario of established tumors. On the contrary, the abscopal model is highly relevant from a clinical perspective as it mimics established metastatic disease in humans, but is limited in that requires local delivery, and hence cannot be employed for systemic therapies [6, 90]. Another obvious drawback associated with *in vivo* models for ICD is that only murine systems are currently available, and this could limit the translation of such findings to human settings since both systems differ at least in some degree in molecules and cell populations involved [100, 101]. Despite many efforts are being directed to the development of humanized mice models, they also have limitations. For instance, they lack thymic selection, and a poorly understood possible cross-talk occurs between the engrafted functional human immune cells and the residual components of the mice immune system [102, 103].

Concluding remarks and perspectives

Much effort is now invested in restoring the immunogenicity of cancer cells, and the induction of ICD emerges as a clinically relevant target. These protocols should comprise assessment of cell death extent and mechanism; stress responses and their connection to DAMPs release; APCs activation and functionality (i.e. their ability to mediate cross-priming *in vitro*); and *in vivo* antitumor immunity generated by dying cells.

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There is consensus that a study of this nature should incorporate the performance of an experimental combined set of *in vitro* and *in vivo* assays. They complementarily assess the ability of tumor cells to undergo ICD in their natural anatomical location, involving the recruitment of APCs and their stimulation to trigger an adaptive immunity response against cancer. Growing evidence demonstrates that activation of ICD is emerging as an important therapeutic target for cancer treatment. It is well established that the ability of various agents to promote ICD in cancer is hampered by immunosuppressive circuits established in the tumor microenvironment during tumor-host coevolution. Given the current clinical success of immune checkpoint blockers (ICBs), their management may be critical for inactivation of such circuits and in concert with ICD inducers achieve immunotherapeutic success [6].

Considering this setting, the results obtained in the *in vitro* evaluation is a necessary but not definitive preliminary response to define an ICD inducer candidate. The *in vivo* approach in a murine model becomes the primary evidence for efficacy, to characterize a given candidate as an ICD inducer. Nevertheless, since most of the ICD research is performed in cancer cells and murine models of tumor vaccination, it would be of paramount importance to test ICD in more advanced tumor models, such as orthotropic and genetically engineered mice, as to reproduce the complexity of human disease. Remarkably, although ICD has been demonstrated in several preclinical models, the evidence for ICD in human patients is less convincing [104-106]. Therefore, further research in human patients is needed to investigate the clinical potential of ICD [13].

Another aspect comprises the identification of biomarkers aiding to stratify patients according to the demonstrated benefit from ICD immunotherapy. Given that certain danger signaling markers have been found in both treated and untreated patients, more research is needed to unravel the actual impact of the exposure to therapy-driven ICD DAMPs and oncogenesis-driven DAMPs [104].

It is tempting to speculate that administration of one or more ICD inducers, in addition to harnessing innate immunity and/or immune checkpoint inhibition, is likely to potentiate antitumor immune responses. This would result an abscopal effect further conferring long-term systemic protection against cancer development. All these hypotheses remain to be tested, as the ICD field is actively expanding and dosage and treatment regimens need to be considered to avoid toxicities and the emergence of tumor resistance [13].

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Received in July, 2023.
Accepted in December, 2023.