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Dr. Guido Kroemer
Editor-in-Chief, *OncoImmunology*

Paris, July, 31st, 2014

Dear Guido,

enclosed please find the manuscript entitled " **Consensus guidelines for the detection of immunogenic cell death**", by O. Kepp, L. Senovilla *et al.*, which we submit to your attention for publication in *OncoImmunology* as a **Review Article**.

Therein, a committee of more than 90 top-standing experts in the field defines guidelines for the detection of bona fide immunogenic cell death (ICD) in vivo, as well as of surrogate markers of ICD in vitro.

The manuscript includes 2 figures, 1 table, 280 references and is composed of approx. 5,400 words (excluding title page, abstract, acknowledgements, references, tables and their captions).

We hope that you will feel as we do that this Review Article be of interest for the readers of *OncoImmunology* and highly cited

We look very much forward to hearing from you.

Sincerely yours,

Lorenzo Galluzzi

Consensus guidelines for the detection of immunogenic cell death

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Abbreviations: APC, antigen-presenting cell; ATF6, activating transcription factor 6; BAK1, BCL2-antagonist/killer 1; BAX, BCL2-associated X protein; BCL2, B-cell CLL/lymphoma 2 protein; CALR, calreticulin; CTL, cytotoxic T lymphocyte; $\Delta\psi_m$, mitochondrial transmembrane potential; DAMP, damage-associated molecular pattern; DAPI, 4',6-diamidino-2-phenylindole;$DiOC_6(3)$, 3,3'-dihexyloxacarbocyanine iodide; EIF2A, eukaryotic translation initiation factor 2A; ER, endoplasmic reticulum; FLT3LG, *fms*-related tyrosine kinase 3 ligand; G3BP1, GTPase activating protein (SH3 domain) binding protein 1; GFP, green fluorescent protein; H2B, histone 2B; HMGB1, high mobility group box 1; HSP, heat shock protein; HSV-1, herpes simplex virus type I; ICD, immunogenic cell death; IFN, interferon; IL, interleukin; MOMP, mitochondrial outer membrane permeabilization; PDIA3, protein disulfide isomerase family A, member 3; PI, propidium iodide; RFP, red fluorescence protein; TLR, Toll-like receptor; XBP1, X-box binding protein 1.

Abstract

For long, apoptotic cells have been considered as intrinsically tolerogenic or unable to elicit immune responses specific for dead cell-associated antigens. However, multiple stimuli can trigger a functionally peculiar type of apoptotic demise that does not go unnoticed by the adaptive arm of the immune system, which we named "immunogenic cell death" (ICD). ICD is preceded or accompanied by the emission of a series of immunostimulatory damage-associated molecular patterns (DAMPs) in a precise spatiotemporal configuration. Several anticancer agents that have been successfully employed in the clinic for decades, including various chemotherapeutics and radiotherapy, can elicit ICD. Moreover, defects in the components that underlie the capacity of the immune system to perceive cell death as immunogenic negatively influence disease outcome among cancer patients treated with ICD inducers. Thus, ICD has profound clinical and therapeutic implications. Unfortunately, the gold-standard approach to detect ICD relies on vaccination experiments involving immunocompetent murine models and syngeneic cancer cells, an approach that is incompatible with large screening campaigns. Here, we outline strategies conceived to detect surrogate markers of ICD *in vitro* and to screen large chemical libraries for putative ICD inducers, based on a high-content, high-throughput platform that we recently developed. Such platform allows for the detection of multiple DAMPs, like cell surface-exposed calreticulin, extracellular ATP and high mobility group box 1 (HMGB1), and/or the processes that underlie their emission, such as endoplasmic reticulum stress, autophagy and necrotic plasma membrane permeabilization. We surmise that this technology will facilitate the development of next-generation anticancer regimens, which kill malignant cells and simultaneously convert them into a cancer-specific therapeutic vaccine.

Introduction

Cell death can be classified based on several parameters, including morphological manifestations, biochemical features, kinetic considerations and functional outcomes.[1-7] This said, how cell death has been investigated and conceived since its pristine descriptions (dating back to the mid-19th century)[8] has obviously evolved along with the technological advances that have been made throughout the last one and a half centuries.[9,10] Thus, morphology-based classifications postulating the existence of three cell death subroutines (i.e., type I, type II and type III cell death)[2,11-14] have been progressively abandoned in favor of definitions that rely on objectively quantifiable functional features.[3,15-19] Alongside, the long-standing conception according to which distinct types of cell death like apoptosis and necrosis would constitute mutually exclusive and diametrically opposed entities has been refuted. In particular, throughout the past two decades it has become clear that: (1) apoptosis is not the sole type of regulated cell death that contributes to (post-)embryonic development and adult tissue homeostasis;[20] (2) similar to apoptosis, necrosis can occur in a regulated fashion, i.e., it can involve a genetically encoded molecular machinery;[4,5,21] (3) similar to their necrotic counterparts, apoptotic cells can sometimes be detected by the immune system and elicit an adaptive immune response specific for dead cell-associated antigens.[6,7,22,23] Thus, although apoptosis as a physiological process involved in (post-)embryonic development and tissue homeostasis invariably fails to engage the adaptive branch of the immune system,[24,25] specific stimuli can promote an immunogenic variant of regulated cell death that manifests with both morphological and biochemical features of apoptosis.[2,3,6] Of note, defects in the clearance of apoptotic cells by professional phagocytes have been associated with autoimmune conditions such as systemic lupus erythematosus (SLE) and chronic inflammation.[26,27] However, it remains unclear whether this reflects the immunogenic potential of intact apoptotic corpses or the insurgence of secondary necrosis.

Back in 2005, we were the first to report the unexpected finding that murine colorectal carcinoma

CT26 cells as well as murine fibrosarcoma MCA205 cells exposed to a lethal dose of doxorubicin *in vitro* are capable of vaccinating syngeneic mice against a subsequent challenge with living cells of the same type.[22] We dubbed such a functionally peculiar variant of cellular demise, manifesting with an apoptotic morphology and depending on the activity of apoptotic caspases, "immunogenic cell death" (ICD).[22] It turned out that the unsuspected ability of doxorubicin (an anthracycline employed for the treatment of various carcinomas) to trigger ICD as a standalone intervention, hence converting dying cancer cells into a vaccine that is efficient in the absence of adjuvants, is shared by a relatively restricted set of lethal triggers.[28-33] These include, but perhaps are not limited to, mitoxantrone and epirubicin (two other anthracyclines currently used in the clinic),[34-37] bleomycin (a glycopeptide antibiotic endowed with antineoplastic properties),[38] oxaliplatin (a platinum derivative generally employed against colorectal carcinoma),[39-42] cyclophosphamide (an alkylating agent approved for the treatment of neoplastic and autoimmune conditions),[43-48] etoposide (a topoisomerase inhibitor currently used for the treatment of several neoplasms) combined with the chemical inhibitor of glycolysis 2-deoxyglucose,[49,50] patupilone (a microtubular inhibitor that has not yet been approved for use in humans),[51-53] septacidin (an antifungal antibiotic produced by *Streptomyces fibriatus*)[54,55] specific forms of radiation therapy,[34,56-64] photodynamic therapy (a clinically approved anticancer intervention that involves the administration of a photosensitizing agent followed by light irradiation),[65-73] high hydrostatic pressure,[74] multiple oncolytic viruses,[75-83] replication-defective viral vectors encoding a potentially cytotoxic product (e.g., thymidine kinase from herpes simplex virus type I, HSV-1) combined with viruses expressing an immunostimulatory molecule (e.g., *fms*-related tyrosine kinase 3 ligand, FLT3LG),[84] the clinically employed proteasomal inhibitor bortezomib,[85-87] shikonin (a component of Chinese herbal medicine),[88] a monoclonal antibody specific for the epidermal growth factor receptor (EGFR),[89] capsaicin (a neurotoxic derivative of homovanillic acid found in chili peppers),[90,91] and perhaps the n3-polyunsaturated fatty acid docosahexaenoic acid,[92] as well as the transgene-driven expression of SMAC mimetics.[93,94] In addition, some interventions are capable of converting non-immunogenic

instances of cell death into *bona fide* ICD. These maneuvers include the administration of cardiac glycosides, which are particularly powerful in this respect as they promote *per se* all major manifestations of ICD (see below),[95-97] or zoledronic acid (a bisphosphonate currently approved to treat osteoporosis and to prevent skeletal fractures in cancer patients with bone metastases),[98,99] as well as the provision of co-stimulatory signals via CD40.[100] This said, it should be kept in mind that the capacity of a given agent to cause ICD or exacerbate the immunogenicity of apoptosis cannot be predicted yet from its structural or chemical properties, since molecules as similar to each other as oxaliplatin and cisplatin do not share this functional profile.[39,40]

The notion that apoptotic cancer cells do not always go undetected by the immune system has profound clinical repercussions.[101] First, it implies that the immune system, at least under specific circumstances, can mount an adaptive immune response against (self) malignant cells, hence mediating antineoplastic effects or contributing to the therapeutic activity of conventional anticancer regimens. This concept represents the theoretical foundation of modern tumor immunology and anticancer immunotherapy.[22,102,103] As a matter of fact, many chemotherapeutics that have been successfully used in the clinic throughout the past century have recently been discovered to mediate off-target immunostimulatory effects, ICD being one of the underlying mechanisms (though not the sole).[104-106] Second, it implies that a large number of parameters reflecting the immunological competence of the host, including the type, quantity and localization of tumor-infiltrating lymphoid and myeloid cells,[107-113] the amount of blood-borne memory T cells that are able to recognize antigens associated with apoptotic cancer cells,[114] the circulating levels of various ICD-associated biomarkers, including the non-histone chromatin-binding protein high mobility group box 1 (HMGB1),[46,115-117] as well as genetic polymorphisms affecting virtually all facets of the immune response,[41,108,118,119] may be endowed with a robust prognostic or predictive value. This notion has already been demonstrated in several ICD-related clinical scenarios. Thus, the relative abundance of tumor-infiltrating $CD8^+$ cytotoxic T lymphocytes (CTLs) and

CD4^{+}CD25^{+}FOXP3^{+} regulatory T cells reportedly predicts the propensity of breast carcinoma patients to benefit from anthracycline- or oxaliplatin-based chemotherapy, respectively.[52,120] Along similar lines, single nucleotide polymorphisms in the genes coding for ICD-relevant receptors such as Toll-like receptor 4 (TLR4) and purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7) have been shown to influence disease outcome among breast carcinoma patients treated with anthracycline-based chemotherapy.[41,119] Taken together, these observations demonstrate that the induction of ICD is a therapeutically relevant objective, calling for the identification of novel ICD inducers and molecules that improve the immunogenicity of conventional variants of apoptosis.

After summarizing the main molecular and cellular determinants that underlie ICD, we discuss the assays that are currently available for the detection of surrogate ICD markers and how these methods can be combined into a platform that is compatible with high-content, high-throughput applications. We surmise that this methodological approach will accelerate the discovery and development of therapeutic regimens that kill malignant cells in an immunogenic fashion.

Immunogenic cell death signaling

According to current models, ICD relies on the ability of specific stimuli to kill cells while provoking the spatiotemporally coordinated emission of immunogenic signals.[7,121-129] Such signals are conveyed by damage-associated molecular patterns (DAMPs), i.e., molecules that are not accessible by the immune system in physiological conditions but are released or exposed on the outer leaflet of the plasma membrane during cytoprotective stress responses or upon cell death.[103,130-133] Similar to their microbial counterparts, many (but not all) DAMPs exert robust immunostimulatory effects upon binding to pattern recognition receptors (PRRs) expressed by immune cells.[121] So far, three DAMPs have been attributed a key role in the immunogenic potential of virtually all ICD inducers: the endoplasmic reticulum (ER) chaperone calreticulin (CALR),[34,65,126,134-136] ATP,[66,124,137-143] and HMGB1.[41,46,115,116,144-147] In addition, many DAMPs have been shown to contribute to the immunogenicity of cell death in a limited amount of experimental scenarios. These include immunostimulatory cytokines like interferon α (IFNα),[148,149] various chaperones of the heat-shock protein (HSP) family, notably heat shock 70kDa protein 1A (HSPA1A, best known as HSP70) and heat shock protein 90kDa alpha (cytosolic), class A member 1 (HSP90AA1, best known as HSP90),[65,71,85,90,145,150-153] sphingomyelin metabolites (e.g., ceramide and sphingosine-1-phosphate),[154] a plethora of mitochondrial products (e.g., mitochondrial DNA, *N*-formylated peptides, cardiolipin),[155-157] cytosolic components like urate and F-actin,[158-161] as well as products of the breakdown of the extracellular matrix (e.g., hyaluronan fragments).[162,163]

CALR gets exposed on the cell surface early in the course of ICD, i.e., before the apoptosis-associated shuffling of phosphatidylserine between the inner and outer leaflet of the plasma membrane.[34,164,165] The molecular mechanisms underlying this ICD hallmark have been dissected in detail and appear to involve three distinct signaling modules: (1) an ER stress module centered around the phosphorylation of eukaryotic translation initiation factor 2A (EIF2A) and the resultant arrest in protein synthesis; (2) an apoptotic module involving the activation of caspase-8 and the

consequent cleavage of B-cell receptor-associated protein 31 (BCAP31) as well as the pro-apoptotic Bcl-2 family members BCL2-associated X protein (BAX) and BCL2-antagonist/killer 1 (BAK1); and (3) an exocytosis module requiring the actin cytoskeleton as well as vesicle-associated membrane protein 1 (VAMP1) and synaptosomal-associated protein, 25kDa (SNAP25), two proteins involved in intracellular vesicular trafficking.[36] Moreover, in some (but not all) models of ICD,[67] CALR obligatorily translocates to the cell surface together with another ER chaperone, protein disulfide isomerase family A, member 3 (PDIA3).[36,37] Upon binding to low density lipoprotein receptor-related protein 1 (LRP1, also known as CD91), membrane-exposed CALR delivers a major phagocytic signal to professional antigen-presenting cells (APCs) such as dendritic cells, de facto improving their capacity to take up dead cells and their corpses.[66,91,166-173] Interestingly, the phagocytosis-stimulatory effects of CALR is robustly counterbalanced by CD47, which is highly expressed by a large panel of solid and hematopoietic tumors.[166] This latter observation suggests that various neoplasms benefit from avoiding the effects of CALR exposure, perhaps as this prevents the elicitation of an adaptive immune response against the malignant cells that "physiologically" succumb in the course of oncogenesis and tumor progression. Alternatively, the phagocytosis-inhibitory activity of CD47 may confer tumors with an advantage by increasing the local availability of macromolecules derived from the spontaneous demise (and degradation) of some of their cellular constituents. This possibility has not yet experimentally addressed.

The ICD-associated release of ATP proceeds through a complex mechanism that involves (1) the apparent relocalization of vesicular ATP stores from lysosomes to autolysosomes; (2) the redistribution of lysosomal-associated membrane protein 1 (LAMP1) to the plasma membrane; (3) Rho-associated, coiled-coil containing protein kinase 1 (ROCK1)-mediated, myosin II-dependent cellular blebbing; and (4) the opening of pannexin 1 (PANX1) channels, 4 processes that rely on caspases.[140,142,174] In a vast majority of models, the secretion of ATP by cells exposed to ICD inducers requires an intact autophagic machinery.[83,138,139,175] In these settings, the genetic or

pharmacological inhibition of autophagy limits ATP release by cells succumbing to ICD and hence negatively influences their ability to elicit an adaptive immune response upon inoculation in immunocompetent syngeneic mice.[60,138,139] Along similar lines, the chemical inducer of autophagy STF-62247 increases the immunostimulatory potential of ICD as triggered by chlorin-e6-based photodynamic therapy (MK, unpublished observations). However, this does not seem to apply to all ICD inducers.[68] Thus, the ability of hypericin-based photodynamic therapy to induce the secretion of ATP does not appear to change in autophagy-deficient versus autophagy-proficient cells.[68,70,176] Moreover, the former respond to hypericin-based photodynamic therapy by exposing higher amounts of CALR on the plasma membrane than the latter, hence exhibiting a superior immunogenic potential.[68,70,176] Possibly, this reflects the incapacity of autophagy-deficient cells to clear oxidized proteins, resulting in an aggravation of the ER stress response that underlies CALR exposure in the course of ICD.[68,70,176] Irrespective of these variations, extracellular ATP operates as a strong chemoattractant and promotes not only the recruitment of immune cells to sites of ICD, but also their differentiation, an effect that depends on purinergic receptor P2Y, G-protein coupled, 2 (P2RY2).[141,177-179] Moreover, extracellular ATP promotes the activation of the NLR family, pyrin domain containing 3 (NLRP3) inflammasome in APCs, hence stimulating the processing and release of interleukin (IL)-1β and IL-18.[119,180-189] In line with this notion, the immunogenic potential of cells succumbing to ICD can be significantly reduced by pharmacological or genetic interventions that limit the availability of ATP in the pericellular space, such as the administration of recombinant apyrase (an ATP-degrading enzyme) or the transfection-enforced overexpression of ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1, best known as CD39), which converts ATP into ADP and AMP.[190] Intriguingly, CD39 and 5'-nucleotidase, ecto (NT5E, best known as CD73), which transforms AMP into adenosine, are often overexpressed by malignant tissues. This reflects the advantage conferred to cancer cells by the conversion of extracellular ATP, which promotes immunosurveillance, into adenosine, which exerts potent immunosuppressive effects.[191-197] Of note, autophagy is also important for the perception of cell death as immunogenic

because it contributes to several aspects of cellular immune responses, including the differentiation, survival and activation of myeloid and lymphoid cells.[198-200]

The release of HMGB1 from cells succumbing to ICD requires the permeabilization of both the nuclear and plasma membranes, *de facto* constituting a *post-mortem* event.[3,41] Although autophagy has been proposed to contribute to the release of HMGB1 from dying cells, at least under some circumstances,[201] the molecular machinery that underlies this crucial manifestation of ICD has not yet been elucidated in detail. This said, extracellular HMGB1 is well known to mediate robust pro-inflammatory effects upon binding to several receptors on the surface of immune cells, including TLR2, TLR4 and advanced glycosylation end product-specific receptor (AGER, best known as RAGE).[202-210] Moreover, extracellular HMGB1 reportedly exerts a chemotactic activity by forming a complex with chemokine (C-X-C motif) ligand 12 (CXCL12) that signals via chemokine (C-X-C motif) receptor 4 (CXCR4).[211] Finally, at least under some circumstances, endogenous HMGB1 appears to promote autophagy by interfering with the mutually inhibitory interaction between the central autophagic regulator beclin 1 (BECN1) and the anti-apoptotic protein B-cell CLL/lymphoma 2 (BCL2).[212-214] It is therefore tempting to speculate, yet remains to be formally demonstrated, that the nuclear release of HMGB1 may contribute to the autophagic response of cells succumbing to ICD inducers. Of note, the biological activity of extracellular HMGB1 appears to be regulated by its redox state.[215-221] Moreover, HMGB1 binds not only to TLR2, TLR4 and RAGE, but also to hepatitis A virus cellular receptor 2 (HAVCR2, best known as TIM-3), hence mediating immunosuppressive (as opposed to immunostimulatory) effects.[222-224] Taken together, these observations suggest that the biological activity of HMGB1 exhibits a consistent-degree of context-dependency. Nonetheless, HMGB1-deficient malignant cells exposed to ICD inducers fail to elicit adaptive immune responses upon inoculation into immunocompetent syngeneic mice, a defect that can be corrected by the co-administration of synthetic TLR4 ligands.[225-228] Together with the notion that *Tlr4*$^{-/-}$ mice fail to perceive anthracycline-treated syngeneic cells as

immunogenic,[41,229] this observation demonstrates the importance of the HMGB1-TLR4 signaling axis for ICD.

In summary, the spatiotemporally coordinated emission of specific DAMPs promotes the recruitment of APCs to sites of ongoing ICD, their ability to take up dead cell-derived particulate material, as well as their capacity to prime an adaptive immune response.[6] This generally proceeds in two phases, involving the sequential recruitment and activation of IL-17-secreting γδ T cells and αβ CTLs.[31,230] The latter not only mediate direct antineoplastic effects, mostly by secreting interferon γ (IFNγ) and via the granzyme-perforin pathway, but also underlie the establishment of protective immunological memory (**Figure 1**).[231]

Gold-standard methods to monitor ICD

As it stands, the gold-standard approach to evaluate the ability of a specific stimulus to cause *bona fide* ICD relies on vaccination assays.[6,22,30] In this setting, malignant cells are exposed *in vitro* to the lethal stimulus of choice, thoroughly washed (to remove the stimulus), resuspended in an adequate volume of PBS, and then inoculated subcutaneously into the flank of immunocompetent syngeneic mice. One week later, living cells of the same type are introduced subcutaneously into the opposite flank, and mice are routinely monitored for the appearance of a palpable neoplastic lesion (**Figure 2A**). The proportion of mice that do not develop subcutaneous tumors reflects the degree of immunogenicity of cell death as induced by the lethal trigger under evaluation. As a note, murine cells succumbing to prototypic inducers of ICD such as doxorubicin and mitoxantrone effectively vaccinate 80% of mice.[34,95,232]

As a confirmatory assay, putative ICD inducers can be assessed for their ability to mediate immune system-dependent therapeutic effects against established neoplastic lesions.[6,34,233] In this scenario, grafted, genetically-driven or chemically-induced subcutaneous or orthotopic tumors are established in both immunocompetent and immunodeficient mice. Malignant lesions are then allowed to progress until a pre-determined size or time point, beyond which tumor-bearing mice are treated with the compound under evaluation (**Figure 2B**). In this experimental setup, *bona fide* ICD inducers mediate optimal therapeutic effects in immunocompetent, but not in immunodeficient, mice.[34,41,95,119,233] Importantly, this latter approach is suitable to validate the results of vaccination experiments but cannot be employed alone to determine the capacity of a specific intervention to cause ICD. Indeed, even the activity of antineoplastic regimens that fail to render dying cells immunogenic but induce other immunostimulatory effects is negatively affected by the absence of a functional immune system.[104,105] Among other molecules, this applies to the microtubular inhibitor paclitaxel and the nucleoside analogue gemcitabine.[104,105]

The main drawbacks of these types of assay relate to the use of rodents and syngeneic tumor models: the need for a tightly controlled sterile facility (which is mandatory for working with immunodeficient animals), prolonged times for the establishment/growth of neoplastic lesions, and significant costs. Moreover, vaccination and therapeutic tests for the detection of ICD are limited by the relatively restricted number of syngeneic tumor models that are currently available. Thus, although they constitute the gold-standard approach for the detection of ICD, vaccination assays relying on immunocompetent mice and syngeneic cancer cells are intrinsically incompatible with large screening campaigns. To circumvent this issue, various techniques that allow for the detection of one or more ICD manifestations *in vitro* and *in vivo* have been developed.[6,234] Monitoring the immunostimulatory activity of lead compounds (be it linked to the induction of ICD or reflecting other mechanisms) early in the drug discovery pipeline may indeed speed up significantly the development of novel anticancer agents.[104]

Detection of surrogate ICD biomarkers

A relatively ample panel of ICD-associated phenomena can be monitored *in vitro* to obtain insights into the ability of a specific intervention to provoke ICD (**Table 1**).

Cell death. By definition, ICD inducers must be cytotoxic and provoke cell death above a minimal threshold level. Cancer cells emit indeed a wide panel of DAMPs in response to non-lethal perturbations of homeostasis. However, such DAMPs differ in both qualitative and quantitative terms from those emitted by cells of the same type dying in response to the same stimulus applied with a lethal intensity/duration. Living cells are less likely to be taken up by APCs and ignite an adaptive immune response than their dying counterparts. Moreover, if the fraction of dying cells is excessively low, neoplastic lesions develop at the vaccination site and protective immunity cannot be established.[34,95] Thus, agents that stimulate all the key manifestations of ICD including CALR exposure, ATP secretion and HMGB1 release, but fail to exert robust cytotoxic effects cannot be considered as authentic ICD inducers. This is the case of cardiac glycosides including digoxin and digitoxin, which nonetheless are powerful at converting non-immunogenic instances of cell death into *bona fide* ICD, hence operating as potent immune adjuvant.[95-97,235]

Several assays are commercially available to monitor cell death-associated parameters, the most reliable indicator of cell death being end-stage plasma membrane permeabilization.[9,236] This can be conveniently monitored by so-called exclusion dyes like the DNA-binding chemicals propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI), which only accumulate in cells with permeabilized plasma membranes. PI and DAPI can be conveniently detected by flow cytometry or fluorescence microscopy (absorption/emission peaks: 535/617 and 358/461 nm, respectively). On flow cytometry, both PI and DAPI can be combined with fluorescence variants of the protein annexin A5 (ANXA5), permitting the detection of phosphatidylserine exposure,[9,237,238] as well as with 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, absorption/emission peaks: 482/504 nm),

allowing for the quantification of mitochondrial transmembrane potential ($\Delta\psi_m$).[239-241] The externalization of phosphatidylserine (a phospholipid normally restricted to the inner leaflet of the plasma membrane) accompanies indeed multiple (though not all) instances of apoptotic cell death,[16,242-245] while the permanent dissipation of the $\Delta\psi_m$ as a result of mitochondrial outer membrane permeabilization (MOMP) constitutes one of the major hallmarks of mitochondrial apoptosis.[17,18,246,247] Of note, $DiOC_6(3)$ is not compatible with fixation, but other $\Delta\psi_m$-sensitive probes that exist are, including chloromethyltetramethylrosamine (absorption/emission peaks: 554/576 nm).[248] MOMP is accompanied by the massive activation of caspase-9 and -3, while caspase-8 is required for ICD upstream of MOMP. The activation of caspases can be documented by flow cytometry or fluorescence microscopy, either upon the immunostaining of cells with monoclonal antibodies specific for active caspase fragments, or with cell-permeant caspase substrates that become fluorescent upon cleavage.[9,249,250] Alternatively, caspase activation can be detected in a semi-quantitative manner by immunoblotting, with antibodies specific for caspases (which are themselves activated by cleavage) or their substrates.[250,251]

As MOMP ensues the assembly of BAX/BAK1-containing oligomers across the outer mitochondrial membrane, the process can also be monitored by means of green fluorescent protein (GFP)-BAX chimeras (GFP absorption/emission peaks: 395/509 nm). In this setting, the relocalization of BAX to mitochondria can be followed by fluorescence microscopy as a shift in GFP fluorescence from a diffuse to a punctate or network-like pattern.[40,252] Finally, one of the major morphological modifications of apoptosis (and hence of ICD) is nuclear condensation (pyknosis).[1,2,95] Also this process can be conveniently monitored by fluorescence microscopy, either in cells that constitutively express a GFP- or red fluorescent protein (RFP)-tagged variant of histone 2B (RFP-H2B, absorption/emission peaks: 584/607 nm) or upon fixation and staining with the chromatinophilic dye Hoechst 33342 (absorption/emission peaks: 361/461 nm).[40,95,235]

CALR exposure. Several assays are available to directly monitor the ICD-associated translocation of CALR on the outer leaflet of the plasma membrane. For instance, this can be achieved on flow cytometry, by staining non-permeabilized cells with a CALR-specific antibody, or in cells that stably express a CALR-HaloTag™ fusion protein.[40,95] In the latter scenario, the HaloTag™ label can be visualized by a cell-impermeant fluorescent chemical, resulting in the specific detection of the CALR molecules that are effectively accessible for ligand binding from the extracellular microenvironment.[40,95] In both cases, it is imperative to remove from the analysis dead (PI^+ or $DAPI^+$) cells, as the permeabilized plasma membrane allows both the CALR-specific antibody and the normally cell-impermeant HaloTag™ ligand to access intracellular CALR.[34,40] Alternatively, CALR exposure can be monitored upon the biotinylation of cell surface proteins (which must be performed in pre-apoptotic conditions, when plasma membranes are intact, to avoid false-positive results owing to intracellular CALR), followed by streptavidin-mediated precipitation, and detection by immunoblotting,[34,66,253] or by fluorescence microscopy, in cells that constitutively express a CALR-GFP fusion construct. For the sake of precision, it should be noted that the latter system does not detect CALR-GFP exposure in itself, but the ER perinuclear clustering that invariably accompanies exposure.[20,232] We have also successfully employed a PDIA3-specific antibody and flow cytometry as well as PDIA3-GFP-expressing cells and fluorescence microscopy to (indirectly) assess CALR exposure in the course of ICD, as in our models PDIA3 invariably co-translocates with CALR on the surface of cells exposed to ICD inducers.[36,37,95] However, this does not apply to all experimental settings,[66,67] implying that the PDIA3-GFP fusion is a useful confirmatory tool but cannot be employed as a standalone means to identify all instances of ICD.

In some instances, it may be important to monitor CALR exposure along with the proficiency of the ER stress response. This may indeed allow for the identification of defects in the signaling pathway that leads to the translocation of CALR to the outer leaflet of the plasma membrane. Several assays are currently available for the detection of the different arms of the ER stress response.[136,254-256] For

instance, the phosphorylation state of EIF2A and/or of the major EIF2A kinases, including EIF2A kinase 1 (EIF2AK1, best known as HRI),[257] EIF2AK2 (best known as PKR),[258] and EIF2AK3 (best known as PERK),[259-261] can be assessed by immunoblotting, flow cytometry or immunofluorescence microscopy with phosphoneoepitope-specific antibodies.[260] The splicing status of X-box binding protein 1 (*XBP1*) mRNA, reflecting the activation of the ER stress sensor endoplasmic reticulum to nucleus signaling 1 (ERN1, best known as IRE1α), can be monitored by quantitative real-time RT-PCR,[262] as well as by flow cytometry or fluorescence microscopy, either in cells that express a fluorescently-tagged version of XBP1[263] or upon the administration of a self-quenched RNA probe that can be cleaved by IRE1α.[264] Finally, the nuclear redistribution of activating transcription factor 6 (ATF6) can be easily evaluated by fluorescence microscopy in cells that constitutively express GFP- or RFP-tagged variants of ATF6.[52] As an alternative, ER stress can be indirectly monitored upon the formation of GTPase activating protein (SH3 domain) binding protein 1 (G3BP1)-containing granules in cells genetically modified to express a G3BP1-GFP fusion.[40,265] This said, G3BP1 appears to redistribute to granules in response to a wide panel of stressful conditions that are not limited to specific perturbations of reticular homeostasis. Thus, monitoring G3BP1 aggregation can be useful to determine whether cells mount a stress response to a putative inducer of ICD, yet cannot be employed to formally imply the ER in this process.

ATP secretion. The ICD-associated secretion of ATP can be monitored by two complementary approaches: directly, by quantification of extracellular ATP,[137,180] or indirectly, by the assessment of residual intracellular ATP.[137,139] The most employed method currently available for the quantification of ATP levels relies on the ability of eukaryotic luciferases to produce light while oxidizing *D*-(-)-luciferin (which must be added exogenously) in a ATP-dependent manner.[266,267] This can be applied to culture supernatants as well as to cell lysates, and hence is compatible with both the direct and indirect assessment of ATP secretion in the course of ICD. The vesicular pool of

ATP can also be visualized by fluorescence microscopy upon staining cells with the ATP-binding fluorochrome quinacrine (absorption/emission peaks: 436/525 nm).[268] Alternatively, intracellular ATP can be monitored in living cells by a fluorescence resonance energy transfer (FRET)-based assay involving a yellow fluorescent protein-cyan fluorescent protein (YFP-CFP) fusion containing a domain that changes its conformation upon ATP binding, hence shifting the spectral properties of the probe.[269]

In some settings, it may be relevant to monitor the autophagic response that generally precedes and is required for ICD-associated ATP release. This can be achieved by a wide panel of techniques, whose detailed discussion goes largely beyond the scope of this set of recommendations.[15,270,271] This said, one of the most convenient approaches to obtain insights into the autophagic response of cells exposed to homeostatic perturbations relies on the use of a GFP- or RFP-tagged variant of microtubule-associated protein 1 light chain 3 (MAP1LC3, best known as LC3).[272] In the course of autophagy, LC3 gets conjugated to phosphatidylethanolamine, hence acquiring the ability to accumulate into forming autophagosomes.[273,274] In line with this notion, GFP-LC3 redistributes from a diffuse to a punctate pattern in cells mounting an autophagic response, a phenomenon that can readily be monitored by fluorescence microscopy.

HMGB1 release. Similar to the secretion of ATP, the release of HMGB1 in the supernatant of cells undergoing ICD can be monitored directly or indirectly, as a function of residual intracellular HMGB1.[41,207,275] The former approach relies on the immunoblotting-based assessment of HGMB1 in concentrated cell supernatants, or (most often) on commercially available enzyme-linked immunosorbent assay (ELISA) kits specific for human or murine HMGB1. These kits generally allow for the precise quantification of HMGB1 concentrations in a wide panel of biological specimens, including culture supernatants, serum samples and interstitial fluids, yet may be relatively expensive for use in large-scale screening campaigns.[95,147,275] Alternatively, HMGB1

release can be assessed by fluorescence microscopy in cells expressing a GFP-tagged variant of HMGB1, as the loss of colocalization between the GFP signal and a nuclear staining (e.g., Hoechst 33342, H2B-RFP).[275] This said, the precise quantification of HMGB1 variants exhibiting differential redox states requires mass spectroscopy.[276]

High-content, high-throughput platform. Cell death that is not accompanied by CALR exposure, ATP secretion and HMGB1 release is generally not perceived as immunogenic.[34,41,119] In other words, the absence of only one such ICD-associated events often entails a consistent decrease in the immunogenicity of cell death, if not its total loss. This implies that the ability of a given intervention to promote ICD can be inferred *in vitro* only upon the concurrent evaluation of all ICD hallmarks. Indeed, cells succumbing to homeostatic perturbations that stimulate ATP secretion and HMGB1 release but not CALR exposure, such as the administration of cisplatin, fail to elicit adaptive immune responses upon inoculation into immunocompetent mice.[34,39,40] This said, a platform that would allow for the simultaneous detection of cell death, CALR exposure, ATP secretion and HMGB1 release in the context of large screening campaigns was missing. To circumvent this obstacle to the identification of novel, perhaps clinically relevant *bona fide* inducers of ICD, we recently developed a robotized cell biology platform that allows for entirely automated compound handling and multiplex read-out capability (including fluorescence microscopy, flow cytometry and bioluminescence detection) in sterile conditions. We then designed fully automated workflows based on various combinations of the assays described above and including appropriate procedures for data handling/normalization and statistical analysis. This approach is compatible with the high-content, high-throughput screening of large chemical libraries, returning a cumulative score that represents the ability of a specific compound to promote the four tenets of ICD. Importantly, this integrated platform does not abolish the need to evaluate putative ICD inducers for their capacity to elicit protective anticancer immune responses in gold-standard vaccination assays.

Nonetheless, it allows for the relatively straightforward identification of candidate molecules. By means of this approach, septacidin has been identified as a *bona fide* ICD inducer.[232] Moreover, cardiac glycosides were found to robustly improve the immunogenic potential of cell death.[95-97,235] We expect this platform not only to allow for the discovery of other ICD inducers, but also to facilitate the understanding of the molecular pathways that underlie ICD and how these can be modulated for therapeutic purposes.

Concluding remarks and future directions

As described above, the simultaneous detection of cell death, CALR exposure, ATP secretion and HMGB1 release by means of a high-content-, high-throughput-compatible platform is useful for the identification of candidate ICD inducers among large chemical libraries. Nonetheless, vaccination assays involving immunocompetent mice and syngeneic cancer cells do not cease to constitute the gold-standard approach to formally identify *bona fide* triggers of ICD.

Paradoxically, the major obstacle to the identification and development of clinically relevant ICD inducers appears to be represented by the murine system itself, as rodent and human cells do not necessarily respond to a specific stimulus in a comparable fashion. As a standalone example, mouse cells are highly resistant to the cytotoxic activity of cardiac glycosides, owing to the expression of a mutated subunit of their target, the Na^{2+}/K^{+} ATPase.[95,277] This implies that formally determining whether a given intervention provokes ICD in the human system is complicated. Humanized rodent models, i.e., immunodeficient mice reconstituted with a human immune system,[278] may partially circumvent this issue. However, the interaction between human immune cells and the murine microenvironment may be negatively influenced by inter-species molecular variations that compromise the ability of the former to mount an appropriate immune response.[279,280] Thus, although attempts are being made to limit such variations,[281] experimental models that allow for the proper evaluation of ICD in the human system require further improvements. Finally, the procedure outlined above for the identification of novel ICD inducers assesses the biochemical processes that are required for the immunogenicity of anthracycline-induced cell death. However, ICD might exist in functionally distinct variants, implying that hitherto uncharacterized mechanisms might render cell death immunogenic. This possibility should be actively investigated in future studies.

Irrespective of these caveats, we are confident that the screening of large chemical or small-interfering RNA libraries combined with vaccination assays in the murine model will allow for the

identification of novel, therapeutically relevant interventions for the induction or modulation of ICD. Moreover, the immunohistochemical detection of ICD-associated biomarkers in bioptic specimens from cancer patients may convey robust predictive or prognostic indications, at least under some circumstances.[282,283] The implementation of well-designed, longitudinal immunomonitoring procedures into the clinical development of antineoplastic agents is required to ascertain the actual prognostic or predictive value of ICD-associated processes among oncological patients.[284-286] Of note, a phase I clinical study has recently been launched to investigate the safety and preliminary therapeutic efficacy of adenoviral vectors genetically modified to trigger ICD, in subjects with malignant glioma and glioblastoma multiforme (NCT01811992). In this setting, serotype 5, replication-defective, first-generation adenoviruses encoding the HSV-1 thymidine kinase and similar vectors coding for FLT3LG are co-infused at the time of surgical tumor resection, followed by valacyclovir (a gancylovir-like prodrug converted by the viral thymidine kinase and cellular kinases into its triphosphate cytotoxic variant)[287,288] in the context of current standard-of-care therapy (source https://clinicaltrials.gov/). The results of such a first-in-man study relying on the genetic induction of ICD in cancer patients are urgently awaited.

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Legends to Figures

Figure 1. **Molecular and cellular mechanisms of immunogenic cell death.** Cancer cells succumb to specific stimuli (e.g., anthracyclines, oxaliplatin, some forms of radiation therapy, photodynamic therapy) while emitting a spatiotemporally ordered combination of damage-associated molecular patterns (DAMPs). These signals include (but are not limited to) the pre-apoptotic exposure of the endoplasmic reticulum chaperone calreticulin (CALR) on the surface of dying cells, the secretion of ATP during the blebbing phase of apoptosis, and the release of the nuclear protein high mobility group box 1 (HMGB1) upon plasma membrane permeabilization. Upon binding to specific receptors, immunogenic cell death (ICD)-associated DAMPs promote the recruitment of antigen-presenting cells (APCs) and stimulate their ability to take up particulate material and cross-present exogenous antigens to $CD8^+$ cytotoxic T lymphocytes (CTLs) while secreting interleukin (IL)-1β. The consequent adaptive immune response also involves γδ T lymphocytes that produce IL-17. Both γδ T cells and αβ CTLs mediate direct antineoplastic effects by secreting interferon γ (IFNγ) and via the granzyme-perforin pathway. In addition, some CTLs acquire a memory phenotype, underlying the establishment of long-term immunological protection.

Figure 2. **Assays for the evaluation of immunogenic cell death *in vivo*. A.** *Vaccination assays.* Murine cancer cells of choice are exposed *in vitro* to a putative inducer of immunogenic cell death (ICD), 1 μM mitoxantrone (positive control) or 50 μM cisplatin (negative control) for a predetermined time (normally 6-24 hours), then washed, resuspended in PBS, and eventually injected s.c. into one flank (vaccination site) of immunocompetent syngeneic mice (ideally 5-10 per group). One week later, mice are challenged with living cancer cells of the same type, which are inoculated s.c. into the contralateral flank (challenge site). Tumor incidence and growth are routinely monitored at both injection sites over a 1-2 months period. The development of neoplastic lesions at the vaccination site indicates that the stimulus under investigation is unable to cause cell death (under the circumstances under investigation) to a degree that is compatible with the

elicitation of adaptive immunity. Conversely, in the absence of tumors at the vaccination site, the ability of the experimental maneuver under evaluation to promote *bona fide* ICD inversely correlates with the number of neoplastic lesions developed at the challenge site. As an indication, neoplastic cells exposed *in vitro* to 1 μM mitoxantrone for 6 hours and maintained in culture for additional 18 hours vaccinate approximately 80% of mice against a challenge with living cells of the same type. **B.** *Therapeutic assays.* Immunocompetent and immunodeficient syngeneic mice bearing grafted, genetically-driven or chemically-induced subcutaneous or orthotopic tumors are treated with a putative ICD inducer, mitoxantrone (positive control) or cisplatin (negative control) at therapeutic doses, followed by the monitoring of tumor size over a 1-3 weeks period. In this setting, *bona fide* ICD inducers mediate optimal antineoplastic effects in immunocompetent, but not in immunodeficient, mice. Since this is also the case of therapeutic interventions that exert off-target immunostimulatory effects, this assay cannot be employed alone to discriminate between ICD and non-immunogenic cell death (nICD). Please note that all curves represented in this figure do not depict primary data but have been created for the sake of exemplification.

Table 1. **Assays for the detection of immunogenic cell death-associated processes *in vitro*.**

Process	Parameter	Platform	Main advantage	Main disadvantage	Notes
Cell death	BAX activation	Flow cytometry Fluorescence microscopy Immunoblotting	Compatible with real-time detection	Real-time detection requires transgenic cell lines	Based on conformation-specific antibodies or cell lines expressing GFP-tagged BAX
	$\Delta\psi_m$ dissipation	Flow cytometry Fluorescence microscopy Fluorometry	Early process in the cascade of events leading to cell death	The $\Delta\psi_m$ can be dissipated in the course of cell death-unrelated processes	Several $\Delta\psi_m$-sensitive probes with different spectral and biochemical properties are available, including DiOC(3) and CMTMRos
	Caspase activation	Flow cytometry IF microscopy Fluorescence microscopy Fluorometry Immunoblotting	Directly involved in CALR exposure	Some caspases get activated in the course of cell death-unrelated processes	Antibodies specific for active caspases or their substrates, as well as self-quenched peptides that emit upon cleavage are available
	Nuclear pyknosis	Fluorescence microscopy	Compatible with simultaneous assessments	Prone to underestimation, owing to the detachment of cells from the substrate	Based on chromatinophilic dyes like Hoechst 33342 or cell lines expressing RFP-tagged variants of H2B
	PMP	Flow cytometry Fluorescence microscopy Light microscopy	Straightforward and very reliable indicator of cell death	End-stage measurement	Several exclusion dyes with different spectral properties are available, including trypan blue, DAPI and PI
	Surface-exposed PS	Flow cytometry Fluorescence microscopy	Compatible with simultaneous assessments	PS exposure does not always accompany cell death	Based on fluorochrome-tagged variants of the protein annexin A5
CALR exposure	Surface-exposed CALR	Flow cytometry Fluorescence microscopy Native gels	Compatible with real-time detection and simultaneous assessments	Real-time detection requires transgenic cell lines	Based on CALR-specific antibodies, cell lines expressing HaloTag™-tagged CALR variants, GFP-tagged CALR variants, or GFP-tagged PDIA3 variants, or the quantification of cell surface proteins upon biotinylation
ER stress	Phosphorylation of EIF2A or EIF2A kinases	IF microscopy Immunoblotting	EIF2A phosphorylation is required in CALR exposure	Incompatible with high-throughput platforms	Based on phosphoneoepitope-specific antibodies
	XBP1 splicing	Fluorescence microscopy	Compatible with real-time detection	Incomplete assessment of the ER stress response	Based on cell lines expressing a fluorescent variant of XBP1

	ATF6 activation	Fluorescence microscopy	Compatible with real-time detection	Incomplete assessment of the ER stress response	Based on cell lines expressing a fluorescent variant of ATF6
	Formation of stress granules	Fluorescence microscopy	Compatible with real-time detection	Not specific for ER stress	Based on cell lines stably expressing a GFP-tagged variant of G3BP1
ATP secretion	Extracellular ATP	Luminometry HPLC-MS	Very sensitive and compatible with real-time detection	Extracellular ATP is exposed to several ectonucleotidases	Extracellular ATP can be monitored in culture supernatants or in cells stably expressing luciferase on their surface
	Cytosolic ATP	Fluorescence microscopy Luminometry HPLC-MS	Cytosolic ATP is more stable than its extracellular counterpart	Indirect indication of ATP secretion	Residual cytosolic ATP can be monitored upon cell lysis or in cells expressing ATP-sensitive FRET-based probes
	Vesicular ATP	Flow cytometry Fluorescence microscopy	Compatible with real-time detection	Indirect indication of ATP secretion	Based on the fluorescent probe quinacrine
Autophagy	Autophagosome formation	Fluorescence microscopy Immunoblotting Other techniques	Can be monitored with a large panel of techniques	Autophagy is not always required for the secretion of ATP in the course of ICD	Cell lines stably expressing GFP-LC3 offer a means to monitor the formation of autophagic vacuoles in real-time
HMGB1 release	Extracellular HMGB1	ELISA Immunoblotting Mass spectroscopy	Very sensitive and compatible with real-time detection	Relatively expensive	ELISA kits for the detection of HMGB1 are available from commercial providers
	Intracellular HMGB1	Fluorescence microscopy Immunoblotting	Compatible with real-time detection	Indirect indication of HMGB1 release	Based on HGMB1-specific antibodies or cell lines expressing fluorescent variants of HMGB1

Abbreviations: ATF6, activating transcription factor 6; BAX, BCL2-associated X protein; CALR, calreticulin; CMTMRos, chloromethyltetramethylrosamine; $\Delta\psi_m$, mitochondrial transmembrane potential; DAPI, 4',6-diamidino-2-phenylindole; DiOC(3), 3,3'-dihexyloxacarbocyanine iodide; EIF2A, eukaryotic translation initiation factor 2A; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; G3BP1, GTPase activating protein (SH3 domain) binding protein 1; GFP, green fluorescence protein; H2B, histone 2B; HGMB1, high mobility group box 1; HPLC, high-performance liquid chromatography; ICD, immunogenic cell death; IF, immunofluorescence; MS, mass spectrometry; PDIA3, protein disulfide isomerase family A, member 3; PI, propidium iodide; PMP, plasma membrane permeabilization; PS, phosphatidylserine; RFP, red fluorescent protein; XBP1, X-box binding protein 1.

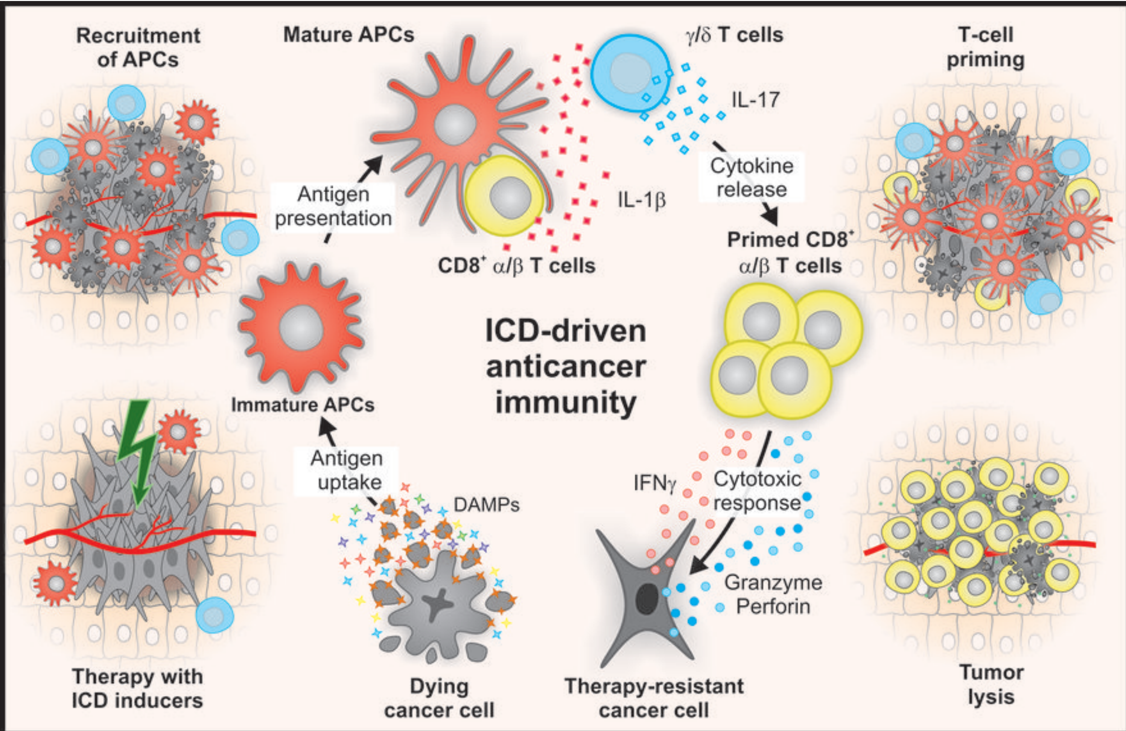
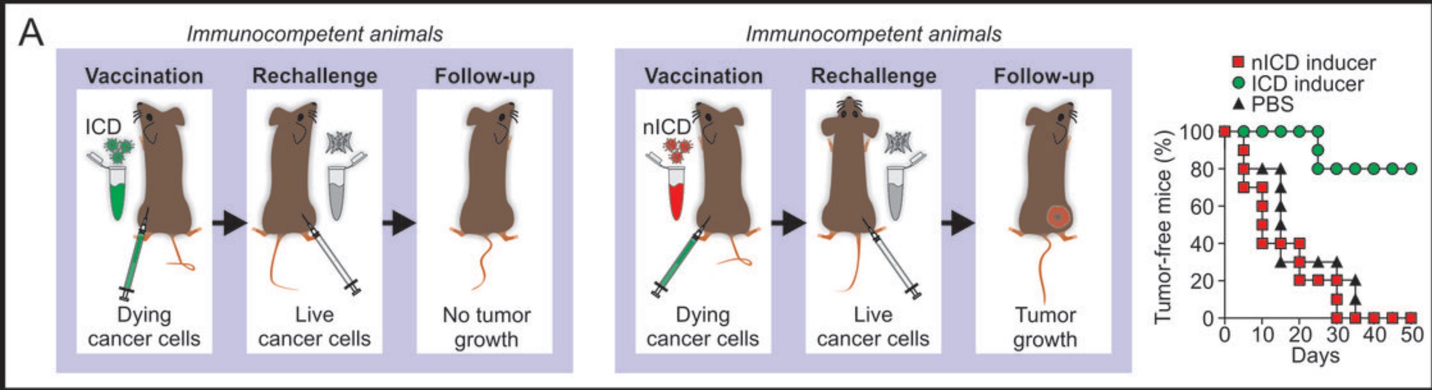


Figure 1



B

Immunocompetent animals

Induction
Treatment
Follow-up
ICD inducer
Tumor establishment
In vivo chemotherapy
Optimal efficacy

Immunocompetent animals

Induction
Treatment
Follow-up
nICD inducer
Tumor establishment
In vivo chemotherapy
Suboptimal efficacy

nICD inducer
ICD inducer
PBS
Tumor volume (cm^3)
5 4 3 2 1 0
0 10 20 30
Days

Immunodeficient animals

Induction
Treatment
Follow-up
ICD inducer
Tumor establishment
In vivo chemotherapy
Suboptimal efficacy

Immunodeficient animals

Induction
Treatment
Follow-up
ICD inducer
Tumor establishment
In vivo chemotherapy
Suboptimal efficacy

nICD inducer
ICD inducer
PBS
Tumor volume (mm^3)
5 4 3 2 1 0
0 10 20 30
Days

Figure 2