



Review article

Cytokines in immunogenic cell death: Applications for cancer immunotherapy



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ABSTRACT

Despite advances in treatments like chemotherapy and radiotherapy, metastatic cancer remains a leading cause of death for cancer patients. While many chemotherapeutic agents can efficiently eliminate cancer cells, long-term protection against cancer is not achieved and many patients experience cancer recurrence. Mobilizing and stimulating the immune system against tumor cells is one of the most effective ways to protect against cancers that recur and/or metastasize. Activated tumor specific cytotoxic T lymphocytes (CTLs) can seek out and destroy metastatic tumor cells and reduce tumor lesions. Natural Killer (NK) cells are a front-line defense against drug-resistant tumors and can provide tumoricidal activity to enhance tumor immune surveillance. Cytokines like IFN- γ or TNF play a crucial role in creating an immunogenic microenvironment and therefore are key players in the fight against metastatic cancer. To this end, a group of anthracyclines or treatments like photodynamic therapy (PDT) exert their effects on cancer cells in a manner that activates the immune system. This process, known as immunogenic cell death (ICD), is characterized by the release of membrane-bound and soluble factors that boost the function of immune cells. This review will explore different types of ICD inducers, some in clinical trials, to demonstrate that optimizing the cytokine response brought about by treatments with ICD-inducing agents is central to promoting anti-cancer immunity that provides long-lasting protection against disease recurrence and metastasis.

1. Introduction

1.1. Cancer and the immune response

In an era of cancer treatment breakthroughs, immunotherapy emerges as a promising approach for cancers that recur and metastasize. Examples of immunotherapy include the use of monoclonal antibodies to block immune checkpoint activity, enabling anti-cancer T cell responses, and adoptive cellular therapy to prime the patient's own lymphocytes to attack cancer cells [1]. The goal of immunotherapy is to generate a robust immune response, stimulating the body's cytotoxic lymphocytes to eradicate tumor cells and ultimately achieve long-term anticancer immunity. In a typical immune response, antigens are captured by dendritic cells (DCs), which then mature and present antigenic peptide in the context of MHC molecules to T cells in lymph nodes, generating effector T cells that migrate towards sites of infection, inflammation or injury. IFN- γ and GM-CSF are central to the process of DC maturation and macrophage activation. DCs in turn release

cytokines like IL-1 β , IL-6, IL-12 or TNF that shape the Natural Killer cell (NK) and T cell responses. CD4⁺ and CD8⁺ T cells, with NK cells, can receive survival signals and stimulation from IL-2, leading to full effector activities, and produce additional IFN- γ . Normal immune regulation involves cytokines like IL-10 and TGF- β to limit the activity of T cells and macrophages and reduce inflammation, terminating immune responses and protecting the host from the immune-mediated damage. However, tumors hijack mechanisms of immunosuppression to evade anti-cancer immune responses, for example, preventing cytotoxic T lymphocytes (CTLs) or NKs from reaching and killing tumor cells [2]. Shifting the balance from inhibitory to activating cytokines in order to generate a protective anti-cancer response, despite tumor immune suppression, remains a major challenge for successful immunotherapy approaches.

One way that cancers evade the immune response is by being poorly immunogenic. Cancer cells can express antigens but these fail to distinguish them from tolerized self-antigens. Frequently such cancers have low mutation rates and produce few *de novo* antigens [3].

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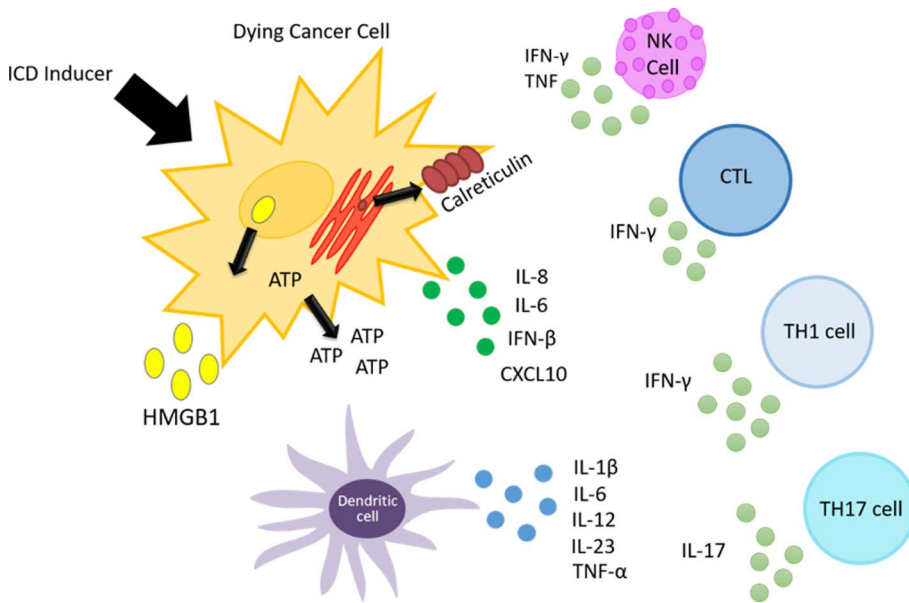


Fig. 1. Cytokines involved in ICD Induction. ICD inducers trigger the release of DAMPs (e.g. HMGB1, Calreticulin, ATP) and inflammatory cytokines from cancer cells. The DAMPs and cytokines signal to dendritic cells and Natural Killer (NK) cells that in turn release effector cytokines. T cells differentiate into cytotoxic lymphocytes (CTLs) or TH1/TH17 cells, releasing additional effector cytokines.

Examples are glioblastoma [4], ovarian cancer [5], and other cancers that lack stimulatory cancer neoantigens and/or promote an immunosuppressive tumor microenvironment by producing anti-inflammatory cytokines [2,6]. This problem is compounded by the fact that some treatments for cancer cause apoptotic cell death that may be immunologically silent and can also weaken the immune system, enabling cancer recurrence [6]. However, in the recent years, a group of chemotherapeutics has emerged that brings about a form of apoptosis known as immunogenic cell death (ICD), alerting the immune system to the presence of dying cancer cells. The induction of ICD could potentially turn these dying cancer cells into “vaccines” to stimulate anti-cancer immunity through the maturation of DCs and activation of CTLs [7] as well as enhancing the cytotoxic activity of NK cells.

1.2. The basic principles of ICD

ICD is characterized by the release of molecules with danger-associated molecular patterns (DAMPs). The DAMPs most commonly associated with ICD are membrane-bound calreticulin (CRT) and the secretion of high mobility group box 1 (HMGB1) protein from the nucleus [8]. Heat shock proteins (HSPs) 70 and 90 have also been found on the cell surface during ICD [9]. CRT, and the like, function as “eat me” signals for phagocytes like DCs, enhancing the uptake of antigen and maturation of DCs [8]. Normally, CRT is located in the endoplasmic reticulum (ER) and maintains calcium ion (Ca^{2+}) homeostasis [10,11]. Composed of 3 domains with variable affinities for calcium-binding, CRT also has a segment for retention in the ER lumen. Functions of CRT include chaperoning proteins, calcium release and storage, as well as regulation of cell adhesiveness through integrins [10]. CRT also has important immune functions, such as antigen processing and presentation as well as protection from anoikis [11]. The mechanism of CRT exposure after the induction of ICD is unknown but may involve the loss of a functional ER retention domain. While CRT is a pre-mortem signal, another DAMP triggered by ICD, HMGB1, is released post-mortem from dying or stressed cells. HMGB1 is normally found in the nucleus with some cytoplasmic localization due to shuttling. Macrophages can also secrete HMGB1, which acts in a cytokine-like manner to bind to the surface of APCs, inducing the release of proinflammatory cytokines [8,12]. When released from dying cancer cells, HMGB1 stimulates toll-like receptor (TLR) signaling, leading to protective immunity [13]. Another ICD marker, HSPs, are chaperones involved in protein folding, which can be upregulated when cells undergo stress such as heat shock, as a protective response [14,15]. While there are

several families under the heat shock category, the release of HSP70 and HSP90 is principally associated with ICD. In a process less well understood, HSPs can be exposed on the cell surface and act as signals to attract phagocytes [16] and activate NK cells [17]. Another possible DAMP is the release of ATP from the cell which activates the P2RX_7 receptor on DCs leading to the formation of the NLRP3 inflammasome. This complex activates caspase-1 which cleaves pro IL-1 β into IL-1 β for secretion [18].

ICD is linked to the induction of ER stress, which can trigger a signaling network called the unfolded protein response (UPR). PERK, IRE1, and ATF6 are mediators of three different UPR pathways that are activated by phosphorylation, oligomerization or cleavage during a stress response and either prevent further stress-related damage or, under prolonged stress, cause apoptosis [19]. PERK attenuates protein translation by directly dephosphorylating the initiator of the mRNA translation machinery, eIF2, which can result in cell cycle arrest [20]. ATF6 is a basic leucine zipper transcription factor that upregulates the expression of genes encoding proteins involved in the UPR [21]. IRE1 activates the transcription factor, XBP1 (Xbox binding protein) that further induces UPR “stress” gene expression [22]. While all three pathways involve pro-apoptotic proteins, IRE-1 and PERK are best known for stimulating the JNK pathway and the Bcl-2 inhibitor, CHOP, respectively.

Treatments that *indirectly* initiate an ER stress response are considered to be type I ICD inducers, such as the anthracyclines that target cytosolic or nuclear proteins, causing ER stress as a downstream effect. Treatments that are *directly* linked to ER stress are type II ICD inducers, such as photodynamic therapy (PDT) or oncolytic viruses that target the ER to trigger cell death [8]. Cytokines detected during and after ICD induction (Fig. 1) can be pro-inflammatory, for example TNF, IL-6 and IL-1 β , increasing MHC class I expression on antigen presenting cells (APCs) and promoting T cell differentiation and NK cell activation [23,24]. Dying tumor cells treated with ICD inducers can also release cytokines that modulate the immune response, such as IL-8, IL-6 and others [6,25,26], while critical effector cytokines produced by lymphocytes include IFN- γ made by TH1 cells and CTLs and IL-17 released from TH17 cells [23]. The functionality of NK cells is enhanced by cytokines produced by activated DCs, such as IL-12, and by other innate immune cells, such as IFN- α/β , leading to the secretion of IFN- γ and TNF [27]. This review highlights the immunity-promoting properties of different ICD inducers by demonstrating that the release of cytokines from activated immune cells plays a critical role in the stimulation of protective anti-cancer immune responses. A better understanding of the

role that cytokines play during ICD could provide new insight that would further the development of clinically effective immunotherapy approaches for cancer.

2. Type I ICD induction

2.1. Anthracyclines

Many type I ICD inducers are FDA-approved chemotherapeutics that not only kill cancer cells but also cause the release of immunogenic signals associated with ICD. Multiple studies indicate that the dosage of ICD agents is a key component associated with the release of DAMPs as well as the activation of the immune system [28,29]. The effects of doxorubicin (DOX) and idarubicin (IDA), two well-characterized anthracyclines, were examined using acute lymphoblastic leukemia (REH ALL, HLA-A2+), ovarian cancer (OV90, HLA-A2+), and prostate cancer (LNCap, HLA-A2+) cell lines [9]. After 12 h of treatment with DOX or IDA (100 nM), membrane exposure of HSP70, HSP90, and CRT was detected and remained elevated through 24 h. HMGB1 secretion, a post-mortem event, was maximal 24 h after DOX and IDA treatments. ICD induction was not dependent on cancer lineage, since ICD signals were detected in the different cell lines, including primary ovarian cancer cells [9]. While both DOX and IDA treatments caused apoptosis in the cancer cell lines studied, the dose at which these drugs triggered ICD was generally higher than the dose needed to achieve cytotoxicity [30, 31, 32]. As example, when DOX was used to treat murine neuro-2a neuroblastoma cells ($IC_{50} = 30$ nM [33]) a dose of 1.6 μ M and higher was required to produce immunogenic dying cancer cells [28].

Another therapeutic used in cancer therapy is the platinum-based oxaliplatin (OXA), which interrupts DNA replication to prevent cell proliferation. The ability of OXA to cause ICD was examined in CT26 colon carcinoma cells. After 24 h, CT26 cells treated with 300 μ M OXA underwent apoptosis followed by secondary necrosis [29]. CT26 cells treated with OXA displayed membrane-bound CRT by 4 h, while cisplatin, a platinum-based chemotherapeutic that does not induce ICD, was unable to elicit the same response. While both drugs were able to bring about the release of HMGB1, immunogenicity was reduced if CRT was not concurrently exposed [29]. It is notable that the IC_{50} dose for OXA is 9.2 μ M [34], which is much lower than the dose (300 μ M) needed to produce ICD. To improve the efficiency of OXA at inducing ICD at lower doses, another group encapsulated OXA in PLGA nanoparticles (OXA-NPs). Using the pancreatic cancer cell lines, human Panc-1 and murine Pan02, OXA-NPs (4 μ M) triggered apoptotic cell death after 48 h. The OXA-NPs were more effective than the free OXA at an equivalent dose, and both could cause ICD as measured by CRT exposure and the release of DAMPs: ATP and HMGB1. CRT exposure was detected after 10 h of OXA-NP treatment, while HMGB1 and secreted ATP levels were highest 48 h after treatment [35].

Once ICD was induced with DOX or IDA as discussed above, these studies showed that DCs undergo maturation by responding to inflammatory mediators or antigens released from the dying cancer cells. To confirm this, treatment with free HMGB1, CRT, and HSP70 also led to the maturation of primary bone marrow-derived DCs (BM-DCs) that had elevated levels of NF κ B [36]. Functional activation of DCs (generated from CD14+ cells) was shown using REH ALL cells treated with 100 nM DOX or IDA for 12 h. These treated tumor cells were phagocytosed by DCs threefold more efficiently than UV irradiated cells, which closely correlated with membrane-CRT exposure on the lymphoma cells. Hence DCs matured when in contact with dying cancer cells, and this was validated by the presence of the maturation markers CD83, CD86, and HLA-DR on the DCs. These matured DCs also had the ability to activate tumor-specific T cells [9]. When DOX (1.6 μ M) was used to treat murine neuro-2a neuroblastoma cells, both CD11b+ spleen cells and primary bone marrow-derived dendritic cells (BM-DCs) phagocytosed the treated neuro-2A cells *in vitro* [28]. The third study, using OXA-NPs (4 μ M) to treat pancreatic cancer cells, assessed the

ability of secreted DAMPs to stimulate DC maturation. Conditioned media from pancreatic cancer cells treated with OXA-NPs was collected and incubated with either primary DCs or DC 2.4 cells, a murine cell line. After the incubation period of 24 h, DCs were measured for maturation markers CD80 and CD83. Both the primary cells and DC 2.4 cell line, cultured in conditioned media from cancer cells treated with OXA-NPs, had increased maturation markers when compared to DCs exposed to untreated cell conditioned media [35]. Taken together, data from these studies indicate that ICD induction, across a range of cancer cell lines and using different type I ICD inducers, resulted in exposure of either membrane-bound or secreted DAMPs (or both), which led to phagocytosis of the dying cancer cells and the robust maturation of antigen-specific DCs.

The hallmark of an active immune response is the production of IFN- γ by immune cells. In a typical response, activated CD8+ T cells can release IFN- γ , which, among other effects, stimulates the classical pathway of macrophage activation [23]. In the studies above of ICD induction with type I inducers like DOX, IDA or OXA, IFN- γ was assessed as a reporter of T cell activity in response to treatments. For example, pulsed monocyte-derived DCs, exposed to DOX or IDA-treated (100 nM) REH ALL cells, matured and promoted the expansion of autologous T cells that produced IFN- γ [9]. When dying DOX-treated (1.6 μ M) neuro-2a cells were co-cultured with both CDb11b+ spleen cells and CD8+ T cells, IFN- γ levels in the culture supernatants were 40,000 pg/mL, which was four fold higher as compared to basal levels of 10,000 pg/mL or less, indicating that effector T cells were generated. The activation of T cells was not solely due to the apoptotic cell death of the cancer cells, since the same cancer cells when treated with cisplatin, which kills cells but does not cause ICD, did not result in more IFN- γ -producing T cells [28].

Tumor regression and protection from tumor rechallenge are critical milestones to show that ICD induction is effective in activating and maintaining an immune response. In another study utilizing DOX to cause ICD in osteosarcoma cells, C3H mice were implanted with murine osteosarcoma cells and after 42 days treated with a combination of DOX and BM-DCs: a contralateral injection of BM-DCs into the gluteal region and intraperitoneal injection of 6 mg/kg of DOX twice weekly. Mice receiving both the BM-DCs and DOX had significantly smaller tumor volumes compared to mice that received single treatments with BM-DCs or DOX alone. Tumor cells from mice that received the combination of DOX and BM-DCs had increased HSP70 and CRT cell surface expression as well as detectable HMGB1 release. Tumors isolated from these mice had infiltrations of both CD11c+ DCs and CD8+ CTLs, which correlated with tumor regression. Importantly, IFN- γ levels in mouse serum increased upon DOX co-treatment with BM-DCs as compared to mice that received BM-DCs alone [36]. Mice bearing CT26 colon carcinoma tumors that were treated with OXA (250 μ g/kg) also produced T cells that secreted IFN- γ , while tumor cells with abrogated or defective CRT, could not [29]. This experiment was important since it linked the induction of a specific ICD marker, CRT, with the activation of T cells. Further, OXA treatment (250 μ g/kg) of mice bearing CT26 tumors was able to significantly slow the growth of tumors compared to no treatment or cisplatin treatment. To test protection from tumor re-challenge, CT26 colon carcinoma cells were first treated with OXA and the dying cancer cells were injected into immune competent mice. Between 7 and 10 days following the injection, mice received a subsequent injection of live CT26 cells and then monitored for an additional 60 days. At the 60-day mark, 80% of mice remained tumor-free [29].

Supportive findings also came from the study using human primary DCs cultured in conditioned media recovered from OXA-NPs-treated (4 μ M) pancreatic cancer cells as described above. These DCs were able to stimulate CD3+ T lymphocytes which secreted IFN- γ . C57BL/6 mice bearing subcutaneous pancreatic tumors treated with OXA-NPs had increased levels of CTLs in tumors and produced measurable levels of IFN- γ in the serum [35]. Next the OXA-NPs-treated Pan02 cells were used to inoculate C57BL/6 mice against live Pan02 cells

injected a week later. Over a course of 60 days, the mice were monitored for tumor growth. Mice treated with OXA-NPs had the lowest incidences of tumors, 40% after 10 days, compared to mice receiving gemcitabine (GEM) (a non-ICD inducer) that had a tumor incidence of 100% in the same timeframe. In immunocompetent mice with established Pan02 tumors, an 18 day treatment with OXA-NPs (6 treatments of 4 mg/kg OXA each) was able to significantly reduce tumor size [35]. This result was mirrored using C57BL/6 mice with established EG7 lymphoma tumors that received peritumoral injections of cyclophosphamide (100 mg/kg) and type I IFN (10^5 IU) for 4 days. Following treatment, 60% of the C57BL/6 mice were cured of their tumors by 40 days and were resistant to subsequent rechallenge by the same cells [37]. Cyclophosphamide, an alkylating agent and immunosuppressant, could induce ICD in EL-4 lymphoma cells implanted in C57BL/6 mice at a non-myeloablative dose (100 mg/kg), allowing DCs to survive and proliferate at a higher rate than untreated mice [37]. Moreover, DCs exposed to both cyclophosphamide and type I IFN were able to cross-prime CD8+ T cells that produced IFN- γ [37]. Hence, the increased IFN- γ levels detected in all of these studies after ICD induction shows that DOX, OXA and similar anthracyclines are all capable of activating cytokine-secreting CTLs that can target and eradicate tumors and protect from tumor rechallenge.

2.2. Other type I ICD inducers

To discover new ICD inducers, a screen of drugs being used in the clinic for cancer therapy was performed [38]. Parameters for the drug screen were the membrane translocation of CRT, ATP release, and HMGB1 secretion. Anthracyclines (mitoxantrone (MTX), daunorubicin, DOX) were among the top inducers of ICD identified, validating the approach. Of interest, two compounds from the cardiac glycoside family were found to cause ICD, digoxin (DIG) and digitoxin (DIGT). Cardiac glycosides are a class of drugs primarily used to restore cardiac rhythm by targeting calcium regulation to force contractions. Cardiac glycosides normally inhibit a plasma membrane Na^+/K^+ ATPase, leading to changes in the Na^+/K^+ balance in cells. A result of the ion imbalance is the accumulation of cellular Ca^{2+} , which is beneficial to cardiac cells but causes cytotoxicity in susceptible cells, such as U2OS cancer cells [39], which undergo ER stress [38]. DIG and DIGT induced the three pathways of ER stress, confirmed by phosphorylation of eIF2 α , translocation of ATF6 to the nucleus, and splicing of XBP1 [38]. Since DIG and DIGT were both able to induce ICD that was equivalent to MTX, these were used for further study. By utilizing about 500 siRNAs targeting 250 genes involved in cell death signaling, it was determined that the ICD pathways for DIG and MTX showed overlap in a cluster of genes associated with CRT exposure, for example BAK1 and FADD, indicating that glycosides and anthracyclines could stimulate common pathways of ICD induction [38].

Further *in vivo* experiments with DIG and DIGT were performed to establish their effectiveness at inhibiting tumor growth. Murine fibrosarcoma MCA205 cells were treated *ex vivo* with cisplatin (150 μM), MTX (1 μM), and mitomycin C (200 μM) individually or in combination with DIG (20 μM) or DIGT (40 μM) and the resulting cell death was assessed by annexin V staining. A population of MCA205 cells, in which ~70% had undergone death, were then injected subcutaneously into C57BL/6 mice. After 1 week, mice were subsequently challenged with live MCA205 cells. The percentage of tumor-free mice after injection of DIG-treated MCA205 cells and subsequent rechallenge with live MCA205 cells was approximately 90%. Similar results were obtained using DIGT. These results showed that both DIG and DIGT were comparable to MTX in their ability to induce ICD and prevent tumor formation in mice [38].

To validate that the cardiac glycosides were effective at eliciting anti-cancer immunity, both C57BL/6 mice and athymic nu/nu mice were injected with MCA205 cells and tumors allowed to form. Mice were then treated intraperitoneally with DIG (2.5 mg/kg), mitomycin C

(0.33 mg/kg) and cisplatin (0.22 mg/kg), alone or in combinations. Both mitomycin C and cisplatin combined with DIG reduced tumor growth in the immunocompetent mice, but not in the athymic mice, indicating that the immune response was essential for the effectiveness of DIG. The combination of mitomycin C and DIG promoted tumor infiltration by IL-17 producing γ/δ T cells as well as IFN- γ producing CD4+ and CD8+ T cells. In support of these findings, a screening of 145 carcinoma patients revealed that individuals receiving cardiac glycosides, especially DIG, had a survival rate of 65% after 5 years compared to 52% for patients that did not receive the same treatment. These results indicate that DIG is a potential inducer of ICD, and, while the cytokines stimulated upon DIG treatment remain to fully elucidated, there is promise for its application in immunotherapy due to its history of clinical success [38].

Another chemotherapeutic agent used for cancer therapy is melphalan, an alkylating compound. In a study that tracked the effects of melphalan in antitumor immunity, a microarray/qRT-PCR analysis with melanoma patient biopsy tissues revealed a significant increase in the production of IL-6 as well as IL-8 and IL-1 β in the local tumor area after melphalan treatment. This outcome was mirrored by increases in the protein levels of these cytokines [40]. To evaluate the immune stimulatory effects of melphalan, the metastatic melanoma human A375 and murine B78 cell lines were treated with 300 μM of the chemotherapeutic. Cell viability decreased in a time-dependent manner, and apoptosis was detected as determined by phosphatidylserine (PS) exposure and caspase activation. In the supernatants of dying A375 cancer cells, the pro-inflammatory cytokines, IL-8 and CCL2, were detected. Melphalan treatment caused ER stress by inducing the PERK and IRE1 α pathways, indicated by phosphorylation of eIF2 α and the detection of the splice form of XBP1. However, while melphalan was able to bring about the exposure of HSP90 on the cell surface, it did not trigger CRT exposure or ATP secretion. HMGB1 release was not studied [40]. Hence melphalan caused the apoptotic cell death of melanoma cells that was accompanied with surface expression of HSP90 and led to the release of the proinflammatory cytokines, IL-8 and CCL2.

Melphalan-treated A375 melanoma cells were able to moderately activate and mature DCs, as measured by expression of HLA-DR and CD86, in a manner that was CRT-independent. The DCs also produced IL-8, while the treated A375 melanoma cells had increased levels of IL-6 and IL-1 β and did not release the immunosuppressive cytokine, IL-10. However, *in vitro* co-cultures of melphalan-treated cancer cells with peripheral-blood isolated NK cells, failed to increase expression of NK activating receptors or stimulate NK cell cytotoxic activity, likely due to the lack of robust generation of chemokines and cytokines like IFN- γ . Yet the semi-matured DCs were able to activate and induce the proliferation of both CD4+ and CD8+ T cells, which then secreted a modest amount of IFN- γ into the culture supernatants. To achieve a vaccine effect, murine B78 melanoma cells were treated with melphalan and injected into C57BL/6 mice. Ten days later, the mice were rechallenged with live B78 cells and 40% of mice were able to inhibit the growth of the challenged cells more effectively than the control, brefeldin A, a tolerogenic death inducer. To establish that the adaptive immune response was stimulated by the vaccination with melphalan-treated cancer cells, T cells were depleted and the mice challenged as described above. CD8+ T cell abrogation did prevent the protective immunity caused by the melphalan-treated cancer cells. These results demonstrated that melphalan, while not as robust as other ICD agents, is a still an effective inducer of ICD with the ability to promote a shift to a pro-inflammatory cytokine profile that is protective against cancer and could be used in combinations with other treatments such as recombinant CRT [40].

Drugs are not the only way to induce ICD. Radiation therapy involves two general approaches to kill cancer cells, employing either an external beam generated by a machine that focuses the radiation at a cancer site or placing the source of radiation, either solid or liquid, inside the body near the cancer. To investigate whether radiation

therapy promotes tolerogenic or immunogenic responses, radiation-upregulated proteins were studied to determine if these could cause an inflammatory response leading to ICD. HT-29 cells were treated with varying dose of fractionated radiation up to 8 Gy. A proteomic analysis was subsequently performed, and karyopherin $\alpha 2$ (KPNA2) was identified as a potential modulator of ICD. KPNA2 was also increased in MDA-MB-231 breast cancer cells after a single dose of 10 Gy [41]. The ability of KPNA2 to induce an immune response was determined by incubating BM-DCs with the recombinant protein. After treatment with KPNA2, BM-DCs expressed CD40, CD80, and CD86 costimulatory molecules, MHC class I and II, and CD54 adhesion molecules, indicating maturation of the DCs. Importantly, KPNA2-treated DCs showed increased IL-1 β , IL-6, IL-12, IL-23, and TNF- α , while the immune-modulatory cytokine, TGF- β , was not markedly increased. The matured DCs were able to activate CD4+ T cells as shown by increased detection of intracellular IFN- γ . There was also a slight decrease of regulatory T (Treg) cells. The levels of both IFN- γ and IL-17 were elevated in the supernatants of co-cultured KPNA2-treated DCs and CD4+ T cells, leading to a TH1/TH17 immune shift that was pro-inflammatory rather than anti-inflammatory. The presence of CTLs was also detected with elevated levels of IFN- γ , granzyme B, and perforin. Taken together, these results support that radiation-upregulated KPNA2 functions like an ICD-inducing agent that is able to mediate an immune response that is characterized by a profile of pro-inflammatory cytokines [41].

In summary, the type I ICD inducers discussed caused apoptosis in various cancer cells that was accompanied by the release of DAMPs 24–72 h after treatment. The DAMPs released were sufficient to mature DCs [40,41] and activate CD4+ and CD8+ T cells [38]. Multiple pro-inflammatory cytokines, associated with both the innate and adaptive immune systems, were produced, indicating that an effective immune response was achieved. Cytokines measured included IL-6, IL-8, IL-12, IL-23, IL-17, IL-1 β , TNF- α , and others [38,40,41]. Of note, IFN- γ or other effector cytokines were not potently produced in response to all type I ICD inducers. Yet, as examples, cardiac glycosides and melphalan were still capable of preventing tumor growth in immunocompetent mice [38,40]. As with KPNA2 [38], type I inducers like DOX could also inhibit immune suppression generated by the tumor microenvironment by reducing the levels of regulatory cytokines such as TGF- β , as shown in one study of adoptive cells transfer [42] and another in combination with cyclo-phosphamide [43]. Overall, as a group, the type I ICD inducers proved effective anti-cancer agents against multiple cancer cell lineages and caused a robust cytokine response with pro-inflammatory potential associated with tumor regression and protection from tumor re-challenge.

3. Type II ICD induction

Unlike most of the type I ICD inducers that are chemotherapeutic drugs, type II ICD inducers rely on physical methods to cause cell death. An example is hypericin-based photodynamic therapy (HYP-PDT). Typically, cells take up a photosensitive drug (e.g. hypericin) and are then treated with light to activate the drug. Treatment produces reactive-oxygen species (ROS) that trigger ER stress and the UPR signaling pathways [44,45]. As was shown with the type I ICD inducers, ER stress is essential for the induction of ICD by type II inducers [8]. However, rather than targeting a specific cellular function with off-target ER stress effects, type II ICD inducers directly cause ER stress.

In one study, HYP-PDT treatment induced ICD in T24 human bladder cancer cells as well as CT26 mouse colon carcinoma cells [45]. Cells were first incubated with 150 nM hypericin for 16 h followed by irradiation with light at a light fluence dose of 2.16 J/cm². Treatment resulted in 87% cell death of the cancer cells as well as enhanced the ability of Mf4/4 phagocytes to engulf the cancer cells. Human immature DCs (hu-iDCs) also matured upon incubation with the treated T24 cells, with detectable increases in MHC class II molecules and costimulatory markers CD80, CD83, and CD86, which was comparable

to exposure to lipopolysaccharide (LPS), a known pathogen-associated molecular pattern (PAMP). The DCs were then evaluated for functional activation by assessing nitric oxide (NO), a marker of respiratory burst, and cytokine production. A pattern emerged showing that human DCs, exposed to HYP-PDT-treated T24 cells, had high levels of NO and minimal IL-10, indicating an active immune response. In contrast, control DCs stimulated with LPS had low levels of NO and IL-10, and DCs incubated with cells that underwent accidental necrosis (freeze/thaw) had high levels of both NO and IL-10, supporting a non-functional response [45].

DAMPs were released from the T24 cells upon treatment with HYP-PDT. Membrane-exposed CRT was detected by immunofluorescence microscopy and confirmed by immunoblotting, while extracellular ATP was examined by detecting ATP bioluminescence. Treated T24 cells released ATP into the supernatant at medium doses of HYP-PDT (150 nM hypericin + 1.35 J/cm² light fluence) and at high doses of HYP-PDT (150 nM hypericin + 2.16 J/cm² light fluence). T24 cells treated with the high dose of HYP-PDT were co-cultured with immature human DCs for 24 h. The conditioned media was examined for IL-1 β production by DCs and the levels were significantly increased as compared to DCs cultured with untreated T24 cells or LPS [45]. In contrast, experiments performed using another photosensitizer, photofrin, which failed to strongly stimulate CRT exposure, showed that photodynamic therapies are not the same in their capacity to cause ICD [45]. The authors hypothesized that the ability of hypericin to induce ICD is linked to its localization to the ER, causing a more robust ER stress response leading to ICD. This idea is supported by showing that plasma membrane trafficking of phosphoinositide 3 kinase (PI3K), PERK, and low-density lipoprotein receptor-related protein 1 (LRP1) were all essential for the release of both membrane-exposed CRT as well as secreted ATP. *In vivo* studies were performed using Balb/c mice that were injected with CT26 murine colon carcinoma cells treated with HYP-PDT before being re-challenged with live CT26 cells. 80% of the mice injected with the HYP-PDT treated cells remained tumor free after 30 days [45].

A follow-up study determined the biological processes that favored ICD induction by HYP-PDT [44]. When T24 cancer cells were pre-treated with L-histidine, an oxygen quencher that inhibits ROS-dependent effects, both CRT exposure and ATP secretion were ablated, suggesting a role for ROS in HYP-PDT-induced ICD. However, induction of autophagy, known to protect from HYP-PDT-induced death [46], was not needed for ATP secretion. A caveat to this is that ATP secretion is cancer cell-type dependent, hence its role in ICD is still under investigation. To test the role of autophagy in ICD, autophagy was ablated in T24 and A375m (melanoma) cancer cell lines by knocking down ATG5 using RNA interference. Loss of ATG5 led to reduced cleavage and lipidation of LC3-I to LC3-II as well as reduced time-dependent LC3-positive puncta accumulation. The autophagy-ablated cells were treated with HYP-PDT and shown to have increased CRT exposure over that observed with autophagy-competent cells [44]. This suggested that HYP-PDT treatment caused the accumulation of oxidatively-damaged proteins that were not cleared when autophagy was inhibited, resulting in enhanced ICD. To show that autophagy inhibition increased the maturation of DCs co-cultured with HYP-PDT treated cancer cells, autophagy-compromised A375m cancer cells were treated with HYP-PDT and incubated with hu-iDCs, which resulted in significant maturation of DCs, as indicated by upregulation of MHC class II molecules and the costimulatory receptor, CD86, over that of DCs cultured with autophagy-competent A375m cells. Conditioned media from A375m-DCs co-cultures contained the cytokines IL-1 β , IL-6, but no IL-10. Importantly, DCs co-cultured with autophagy-compromised A375m cells treated with HYP-PDT secreted more IL-6 than DCs co-cultured with similarly treated autophagy-competent A375m cells. However, this was not dependent on CRT exposure, since use of a CRT blocking antibody did not inhibit the maturation of the hu-iDCs co-cultured with autophagy-compromised cancer cells treated with HYP-PDT [44].

The ability to stimulate T cells is an important function of DCs. To determine whether attenuation of autophagy in HYP-PDT treated cancers could produce DCs capable of activating T cells that made IFN- γ , autophagy-competent or compromised A375m cancer cells were incubated with hu-iDCs for 24 h and then exposed to T cells simultaneously isolated from allogeneic donors and labelled with a fluorescent proliferation tracking dye [44]. DCs cultured with HYP-PDT-treated, autophagy-competent cancer cells caused a statistically significant increase in T cell proliferation as compared to DCs cultured with untreated cancer cells. Interestingly, untreated, autophagy-compromised cancer cells were also able to mature DCs and stimulate CD4+/CD8+ T cells beyond that achieved with treated, autophagy-competent cancer cells. But it was the combination of HYP-PDT treatment and autophagy ablation in cancer cells that resulted in the most robust maturation of DCs and activation of T cells [44]. Since the presence of IFN- γ producing cytotoxic T cells is known to improve the outcome of cancer after treatment [35,44], IFN- γ released from T cells after incubation with DCs cultured with cancer cells was measured. Conditioned media from A375m-DC-T cell co-cultures contained IFN- γ , with the highest levels achieved using HYP-PDT treated cancer cells. Co-culture with autophagy-compromised, treated cancer cells did not suppress IFN- γ production by T cells, as in one report of chemotherapy-induced ICD [47], and IFN- γ production was comparable if not slightly higher than that observed upon co-culture with autophagy-competent, treated cancer cells. Taken together, the data suggests that ICD induction after HYP-PDT treatment is partly ROS-dependent and could be impaired by the autophagy that is associated with development of cancer [44], indicating that the use of autophagy inhibitors could improve immune stimulation when employing this approach.

Another photosensitive drug, rose-bengal acetate (RBAC), works like hypericin to produce ROS in cancer cells. RBAC-PDT, at a dose of 10^{-5} M followed by 1.6 J/cm^2 light fluence, caused autophagy and apoptotic cell death in HeLa cells, rearranging sugar molecules on the plasma membrane in a manner that is distinct from other cell death inducers such as puromycin [48]. Photo-killed HeLa cells were phagocytosed by human and murine macrophages more efficiently when compared to cells undergoing apoptosis or necrosis induced by drugs like puromycin or serum starvation. Upon injection of photo-killed HeLa cells into male Wistar rats via hepatic circulation, up to 80% of the dead cells were removed by the hepatic sinusoidal epithelial cells (HSEs) and Kupffer cells (KCs). *In vitro*, macrophages were able to migrate and engulf photo-killed HeLa cells, being more attracted to the autophagic rather than the apoptotic cells [48]. The percent of migrating macrophages correlated to the amount of the chemokine, fractalkine (FKN), released by the dying cancer cells. To show that RBAC-PDT treatment causes ICD, HSP70 was detected by immunoblotting and shown to translocate to the cell surface within hours of treatment and was also detected in soluble form in the culture media. Macrophages incubated with RBAC-PDT-treated HeLa cells, at the dose described above, released anti-inflammatory TGF- β but not IL-10. TNF- α was also produced by macrophages cultured with cells undergoing photosensitization, as well as serum starvation (which promotes necrosis), but not after puromycin treatment, which causes apoptosis but not ICD. These results showed that RBAC-PDT's mechanism of action was through the induction of ICD. Further studies were performed by injecting photo-killed HeLa cells into the flank of male Wistar rats to show that matured macrophages (CD68) were recruited to the site of injection over a course of 15 days. Cells producing TGF- β RII were also found in the same area over the same time period. This held true when conditioned media from RBAC-induced autophagic HeLa cells was used instead of whole cells. It should be noted that TGF- β can have positive or negative effects on cancer cells that are context dependent, suppressing malignant transformation but also modulating tumor growth and invasion. These results indicate that RBAC-PDT is a potential ICD inducer that releases HSP70 and stimulates and attracts macrophages by promoting the release of FKN from treated cells, causing the secretion of TNF- α and TGF-

β but not IL-10 by macrophages and other immune cells at tumor sites [48].

Other type II ICD inducers include physical stressors such as high hydrostatic pressure (HHP). Using acute lymphoblastic leukemia, ovarian cancer, and prostate cancer cells as well as primary tumor cells, various amounts of pressure ranging from 150 to 250 MPa over 24 h was applied and cells checked for apoptosis and DAMP release [49]. When cancer cells were exposed to HHP at 200 MPa and higher, at least 80% of cells were dead after 24 h, as measured by annexin-FITC/PI staining. Surface exposure of CRT, HSP70, and HSP90 on cancer cells was detected after 6 h at pressure levels above 150 MPa and was maximal between 12 and 24 h. HMGB1 release was detected after HHP with the strongest response at 48 h post-treatment that was almost 2-fold higher in the ovarian and prostate cancer lines as compared to the other cancer types tested. Within an hour of treatment, HHP also caused significant release of ATP from cancer cells into the extracellular space compared to roughly 6 h needed for IDA to achieve the same [49].

Ovarian and prostate cancer cells treated with HHP were phagocytosed by DCs at a much higher rate and to a greater extent than cells treated with UV-B radiation. DCs (generated from CD14+ cells) displayed maturation markers, such as CD83, CD86, and MHC class II, and released cytokines like IL-6, IL-12p70, and TNF- α but not IL-10 or IFN- α . Within two weeks, matured DCs were able to stimulate IFN- γ producing CD4+ and CD8+ T cells. T cell activation upon DC interaction was confirmed by increased levels of CD28 and CD62 and down-regulation of CD57. Further, the capacity to induce the proliferation of Treg cells was reduced in DCs pulsed with cancer cells that were killed using HHP, as compared to immature DCs or LPS-pulsed DCs. Importantly, the apoptotic pathway triggered by HHP included rapid phosphorylation of eIF2 α and activation of caspases 3, 8, and 9, with caspase-8 being shown essential for HHP induced ICD. Overall, HHP is potent inducer of ICD that has the added benefit of being easily standardized, and therefore the translational potential for clinical use is high [49].

Viruses as ICD inducers are being evaluated for cancer treatment in human clinical trials. The Edmonston vaccine strain of the measles virus (EMV) has tropism for CD46, a cofactor found on the membrane of nucleated cells. Tumor cells can upregulate CD46, hence such cells are ideal targets for infection and killing by EMV. This was supported by data showing that EMV can invade, infect and replicate in human cancer cell lines. [50]. Immunogenicity of cell death caused by EMV was assessed through the production of inflammatory cytokines, immune system activation, and HMGB1 release using melanoma cancer cells. In this study, IL-6 was increased in 3 of the 4 melanoma cell lines tested (Mel888, Mel624, MeWo, and SkMel28) in a dose-dependent manner, while IL-8 was increased in all 4 melanoma cell lines. IFN- α was upregulated in the MeWO cell line and IFN- β was upregulated in the Mel624 line. IFN- λ was increased in the Mel888 line. These results demonstrate that each melanoma cell line infected with EMV produced different cytokines. As example, RANTES was found elevated in 3 of the 4 melanoma cell lines and correlated with increased HMGB1 release. Primary melanoma lines were also susceptible to EMV treatment and secreted IL-6, IL-8, and RANTES by 72 h of treatment [50].

To examine the capacity of EMV infection to elicit anti-cancer immunity, peripheral blood mononuclear cells (PBMCs) were incubated with melanoma cancer cells exposed to EMV. Enhanced killing of the melanoma cells by immune cells within the PBMC populations was observed. The killing was not melanoma specific, since an ovarian cell line used for comparison was also killed. As the most likely subset of immune cells with tumor-killing activity, NK cell activity was assessed. CD69, an early activation marker, was upregulated on NK cells after co-culture with EMV-treated melanoma cells. Degranulated NK cells were identified by surface expression of CD107, which suggested that the NK cells were killing the EMV-treated melanoma cells. Maturation of DCs also resulted as a consequence of EMV infection. Importantly, both healthy human DCs and melanoma patient DCs expressed costimulatory

molecules CD80 and CD86 after being exposed to EMV-treated melanoma cells. To confirm that the treated cells, and not the virus, caused maturation of the DCs, tumor-conditioned media (previously filtered to remove viruses) was used to culture the DCs and maturation markers measured. The tumor-conditioned media enabled upregulation of CD80 and CD86 on DCs. However, cytokine release by DCs was not increased, as with the viral infection, suggesting that EMV was needed to induce cytokine secretion. To show that DCs exposed to EMV-infected melanoma cells could stimulate T cells, DCs were incubated with the Mel888 cell line that was infected with EMV and then cultured with PBMCs, containing T lymphocytes. The DCs were able to promote the cytotoxic activity of CD8+ T cells against melanoma cells. The activated CD8+ T cells recognized a melanoma specific antigen and produced IFN- γ . Overall, these studies support that EMV treatment is an effective ICD inducer against melanoma and can be used to elicit protective immunity [50].

The type II ICD inducers vary widely in their mechanisms of action, but all share a common target in the ER. ER stress caused by the various treatments leads to robust induction of ICD with stimulation of both the innate and adaptive immune system. *In vitro* studies utilizing the type II ICD inducers are promising and have laid the groundwork for the pre-clinical *in vivo* studies that are necessary to demonstrate clinical relevance.

4. Other ICD inducers

While most ICD inducers can be categorized into type I or type II, a group of ICD inducers act in ways that are dissimilar from type I or II inducers or work in combination therapies that may have multiple effects. One example is the combination therapy of oncolytic parvovirus H-1PV infection and GEM, a non-ICD inducing chemotherapeutic. These treatments have different effects when used alone but synergize when combined. For example, when pancreatic ductal carcinoma cells (PDACs) were infected with H-1PV only HMGB1 release was detected after 72 h, not CRT exposure or ATP release. Killing of cancer cells by H-1PV infection showed a pattern of annexin V+ and PI+ staining, indicating apoptosis was occurring. IL-1 β accumulated in one of the PDAC culture supernatant (T3M4 cells) after infection with H-1PV. GEM was also able to cause IL-1 β release by the same cells, but not HMGB1, which showed that H-1PV infection was likely causing active secretion of HMGB1 [51]. When used alone, GEM was unable to induce ICD and blocked the release of HMGB1 [51]. However, when used in combination therapy with H-1PV infection, GEM enabled HMGB1 release as well as triggered significant ATP release in 2 of the 4 PDAC lines tested. While, CRT was not exposed in any of the cell lines, the ATP released bound to P2RX₇ and induced secretion of IL-1 β , likely via NLRP3 inflammasome signaling [18,51]. The virus was also able to replicate in GEM-treated cells, meaning that introduction of GEM prior to H-1PV infection was a feasible approach. These results suggest that, while each treatment alone could not cause ICD, in a combination approach, GEM and H-1PV infection could together produce an effective immunogenic death response [51].

Retinoic acid-inducible gene I (RIG-I) and melanoma-differentiated associated antigen 5 (MDA5) are Rig-like helicases (RLHs) that function as pattern recognition receptors (PRRs) and recognize viral double-stranded RNA (dsRNA). Synthetic ligands are 5'-triphosphate RNA (ppp-RNA) for RIG-I and polyinosinic:polycytidylic acid (poly(I:C)) for MDA5. When the RLHs are bound by their target ligands, a signaling cascade initiates an antiviral immune response characterized by release of type I IFN and other cytokines. These synthetic ligands can also activate an immune response protective against cancer cells. Panc02 cells were treated with either ppp-RNA or poly(I:C) and, prior to undergoing cell death, released IFN- β , CXCL10, and IL-6 [52]. Silencing the receptors, RIG-I or MDA5, prevented the synthetic ligands from causing cell death. As evidence that the synthetic ligands were inducing ICD, CRT exposure, HSP70 exposure, and HMGB1 release were all found

elevated after treatment of PDAC cells with ppp-RNA or poly(I:C) [52].

Co-cultures with RLH-treated Panc02 cells and murine-derived DCs resulted in upregulation of both CD80 and CD86, indicating maturation of DCs [52]. The DCs also produced IL-6 and CXCL10, however TNF- α , IL-1 β , and IL-12p70 were not detected. Maturation of DCs was mediated by a soluble factor in the supernatant that was later identified to be IFN- β . The matured DCs were also able to phagocytose the RLH-treated Panc02 cells. To show that treatment could inhibit tumor growth, mice with palpable Panc02 tumors received an intratumoral injection of poly(I:C), which resulted in prolonged survival and decreased tumor burden. Confirmation that the RLH treatment elicited protective immunity was demonstrated when Panc02 cells, treated with poly(I:C), were injected into C57BL/6 mice. One week later, mice were challenged with live Panc02 cells, and 6 out of 8 mice remained tumor-free. Cytokine release in mice was measured by qRT-PCR using homogenized tumor tissue and elevated levels of IFN- β , CXCL10, IL-12p40, IL-6, IL-5, and IFN- γ were detected. Mature DCs were also found in splenic tissue. Overall, RLH synthetic ligands were capable of inducing ICD both *in vitro* and *in vivo* situations, with significant cytokine release occurring suggestive of lasting anti-cancer immunity [52].

5. Conclusion

Most agents that cause ICD are capable of triggering cytokine release from immune cells, although the types of cytokines vary and may depend on the assays performed (Table 1). A general trend observed was that most ICD-inducing agents brought about the release of IFN- γ , which is a potent stimulator of TH1 responses and cytotoxic lymphocytes that are beneficial for eliciting ant-tumor immunity. Other cytokines produced in response to ICD included pro-inflammatory mediators like IL-1 β , TNF and IL-6. The type I ICD inducers caused both cancer cells and immune cells to release pro-inflammatory cytokines, including IFN- γ and IL-1 β [9,28,29,35,36,38,40,41]. By causing a shift in the tumor microenvironment toward immune activation rather than suppression, type I ICD inducers enabled the immune system to act effectively against tumors. Type II ICD inducers shared a similar cytokine release profile with the type I inducers. Other ICD inducers that shared characteristics with both types I and II also released inflammatory cytokines [43–45,48–52]. However, one difference noted between the inducers was the effect on TGF- β production. Studies using KPNA2 [38] or the type I ICD inducer DOX, in a single low dose or in combination with cyclophosphamide, noted a decrease in TGF- β levels after treatment [42,43], while another study, based on the type II ICD inducer RBAC, showed an increase in TGF- β [48]. These results suggest that cytokine secretion during ICD induction may be context dependent and based on the inducer used.

A number of ICD-inducing agents are in clinical trials (representative examples shown in Table 2), most are in phase I or II, indicating a growing interest in this class of anti-cancer agents. IDA is being tested alone [53] or in combination with Cytarabine [54] in the treatment of Acute Myeloid Leukemia (AML). Ongoing trials with EMV are being conducted with ovarian cancer patients [55] and, in combination with cyclophosphamide, with myeloma patients [56]. OXA is being evaluated for the treatment of metastatic breast cancer [57] and DIG for the treatment of head and neck cancers [58]. A topical approach using HYP-PDT for the treatment of lesions caused by T cell lymphomas is ongoing [59], and H-1PV trials are examining therapeutic approaches for patients with glioblastoma [60] and pancreatic cancer with hepatic metastasis [61]. While many of these trials are in the early stages of testing for toxicity and maximum tolerated dosages, initial results are encouraging. Further, incorporating ICD induction in combination with immunotherapy, such as CTLA-4 blockage [62] could be a promising approach with established agents that already have FDA approval.

A significant challenge that remains is that many ICD-inducing agents (e.g. anthracyclines) must be used at high doses (when

Table 1
Examples of the immune response and cytokines induced by ICD agents.

Inducer	Cytokines Released	Immune Response	References
<i>Type I ICD inducers</i>			
Cardiac glycosides	IL-17, IFN- γ	γ/δ and CD4+ and CD8+ T cells	[38]
Oxaliplatin	IFN- γ	T cells, primary DCs	[29,35]
Doxorubicin	IFN- γ	CD4+ and CD8+ T cells, fewer Tregs, human primary DCs	[9,28,36]
Idarubicin	IFN- γ	CD4+ and CD8+ T cells, fewer Tregs	[9]
Radiation-upregulated karyopherin alpha 2	IL-1 β , IL-6, IL-12, IL-23, TNF- α , IFN- γ , IL-17	Dendritic cells, CD4+ T cells, CD8+ T cells	[41]
Melphalan	IL-6, IL-8, IL-1 β , CCL2, IFN- γ	DCs, CD4+ and CD8+ T cells	[40]
Doxorubicin and cyclophosphamide combination	IFN- γ , TNF- α Reduced Foxp3 and TGF- β	Tumor-draining lymph node cells	[43]
<i>Type II ICD inducers</i>			
Hypericin-based photodynamic therapy	IL-1 β , IL-6, no increased IL-10, IFN- γ	DCs, CD4+, CD8+ T cells	[44,45]
Measles virus	IL-6, IL-8, IFN- α or β , IFN- λ , RANTES	NK cells, DCs, CD8+ T cells	[50]
High hydrostatic pressure	IL-6, IL-12p70, TNF- α , IFN- γ	DCs, CD4+ and CD8+ T cells, reduced Treg cells	[49]
Rose-bengal acetate photodynamic therapy	TGF- β , TNF- α	Macrophages	[48]
<i>Other ICD inducers</i>			
Rig-like helicase ligands	IL-6, CXCL10, IFN- β , CXCL10, IL-12p40, IL-5	DCs, CD8+ T cells	[52]
Oncolytic parvovirus H-1PV and Gemcitabine Combination	IL-1 β , IL-8	Unknown	[51]

compared to *in vitro* IC₅₀ doses) to cause ICD and promote immune responses, such as maturing DCs that can stimulate CTLs. While the ICD-inducing dose could be cell type specific, the use of targeted delivery systems to reduce systemic side effects produced by agents like DOX or OXA would improve clinical applicability. One success was the use of nanoparticles to encapsulate OXA and deliver the ICD inducing agent, which was effective at 75-fold lower doses than the free drug [35]. Other challenges are elucidating how ICD stimulates the activation and expansion of NK cells. ICD inducers like OXA, for example, could be used alongside NK cell-based therapy for maximal patient benefit [63]. Studies to improve the ICD-inducing capacity of known agents and identification of new agents are needed as well as elucidating the importance of ICD markers, like CRT, as prognostic indicators of immune cell activity [64]. Autophagy is another key factor that needs further study in the context of ICD. While one study mentioned that autophagy inhibition created a robust response to a type II ICD inducer, HYP-PDT [44], another study utilizing a type I ICD inducer, OXA, found that autophagic ATP release was essential for an *in vivo* anti-tumor immune response [47]. A better understanding of the role of autophagy in ICD induction could lead to new combination approaches with autophagy inhibitors like chloroquine [65] as well as

possible autophagy enhancers depending upon the type of ICD being induced [47]. When successfully induced, ICD could provide long-term protection against both recurrence and metastasis of cancer due to the activation of protective immunity. In addition to the type I ICD inducers, further studies on type II ICD inducers, like HHP, would provide viable options for ICD to be used in the treatment of different cancers.

One aspect that needs to be considered when therapeutically stimulating the immune response is the potential to trigger immune regulatory mechanisms like checkpoints. Treatments with anthracyclines, such as DOX or OXA, achieved better efficacy when combined with blockade of immune suppression. Examples are the inhibition of CD73, which enhanced the response of anthracyclines in breast cancer therapy [66], and the synergy with anti-PD-1 or anti-CTLA-4 checkpoint inhibitors that sensitized tumors to ICD inducers [67,68]. Hence the possibility exists that patients with poorly immunogenic tumors could be made responsive to checkpoint inhibitors when used in combination with ICD induction. This is supported by data that IFN- γ secreted from lymphocytes may promote PD-L1 expression on tumors [69,70] in a manner that correlates with TH1 and TH2 responses [71]. Therefore, cytokines play a pivotal role in the activation of the immune system and a better understanding of the cytokine profile associated with ICD as

Table 2
Representative clinical trials for various ICD inducers.

ICD inducers	Therapeutic use	Clinical phase	References
<i>Type I ICD inducers</i>			
Idarubicin	Acute Myeloid Leukemia	Phase I/II	[53]
	Acute Myeloid Leukemia	Phase Ib	[54]
Cyclophosphamide	Metastatic Breast Cancer	Phase I	[72]
	Malignant Melanoma	Phase II	[62]
Doxorubicin	Recurrent Ovarian Cancer	Phase I/II	[73]
Oxaliplatin	Metastatic Breast Cancer	Phase I	[57]
Digoxin	Head and Neck Cancer	Phase I/II	[58]
Melphalan	Acute Myelogenous Leukemia	Pilot Study	[74]
<i>Type II ICD inducers</i>			
Hypericin Photodynamic Therapy	Cutaneous T Cell Lymphoma	Phase III	[59]
Edmonston Measles Virus	Refractory Ovarian Epithelial Cancer/Primary Peritoneal Cancer	Phase I	[55]
	Relapsed/Refractory Myeloma	Phase II	[56]
<i>Other ICD inducers</i>			
H-1 Parvovirus	Glioblastoma Multiforme	Phase I/II	[60]
	Pancreatic Cancer	Phase I/II	[61]

well as immune regulation will benefit both researchers and clinicians with goal of developing effective immunotherapies for recurrent, refractory and incurable cancers.

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