Immunogenic cell death and DAMPs in cancer therapy

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Abstract | Although it was thought that apoptotic cells, when rapidly phagocytosed, underwent a silent death that did not trigger an immune response, in recent years a new concept of immunogenic cell death (ICD) has emerged. The immunogenic characteristics of ICD are mainly mediated by damage-associated molecular patterns (DAMPs), which include surface-exposed calreticulin (CRT), secreted ATP and released high mobility group protein B1 (HMGB1). Most DAMPs can be recognized by pattern recognition receptors (PRRs). In this Review, we discuss the role of endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) in regulating the immunogenicity of dying cancer cells and the effect of therapy-resistant cancer microevolution on ICD.

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Cell-mediated immunity was first demonstrated more than a century ago by the Nobel Prize Laureate Ilya Metchnikoff. By sticking a rose thorn into starfish larvae, he discovered the process of phagocytosis of foreign material and described a principle mechanism of innate immunity. Although this observation was interpreted for many years as a response to a foreign body, from the current perspective it may also be considered as a host response to injury. In 1994 Polly Matzinger proposed the 'danger theory', which states that the immune system can distinguish between dangerous and innocuous endogenous signals[1]. It became evident that dying, stressed or injured cells release or expose molecules on their surface that can function as either adjuvant or danger signals for the innate immune system[1–3]. These signals were later called damage-associated molecular patterns (DAMPs)[3,4]. Some DAMPs are secreted or released (such as ATP and high mobility group protein B1 (HMGB1; also known as amphoterin)) and others are exposed *de novo* or become enriched on the outer leaflet of the plasma membrane (such as calreticulin (CRT) and heat shock protein 90 (HSP90)). Other DAMPs are produced as end-stage degradation products (such as uric acid) during the course of cell death (TABLE 1). Most of these molecules have predominantly non-immunological functions inside the cell before their exposure on the cell surface or their secretion[1,3]. DAMPs that are released as a result of cellular stress do not always trigger an immune response: some DAMPs, such as HGMB1, can be inactivated by oxidation[5], or by caspase-dependent proteolysis, as occurs with interleukin-33 (IL-33)[6].

The emission of DAMPs was initially connected with necrosis that occurred as a result of physicochemical injury to tissues and cells[4]. However, DAMPs have recently been reported to be actively emitted from dying apoptotic cells and to have a beneficial role in anticancer therapy owing to their interaction with the immune system[7,8]. Chemotherapy and radiotherapy function, at least in part, by inducing apoptosis. As this cell death modality was widely considered immunologically silent or even tolerogenic[9–14], and because the US National Cancer Institute guidelines for drug screening for anticancer therapy require testing with human tumours xenotransplanted into immunocompromised mice[15], the role of the immune system in anticancer therapy has been systematically neglected[16]. However, in the past few years, the concept of immunogenic cell death (ICD) has emerged, which in our opinion underlines the important role of the immune system in the efficacy of cancer therapy not only in mice but also in humans[17–19]. Cancer cell lines treated *ex vivo* with anthracyclines, oxaliplatin, photodynamic therapy (PDT) or γ-irradiation and then implanted subcutaneously into syngeneic immunocompetent mice, function as a cancer vaccine in the absence of any adjuvants or immunostimulatory substances[4,7,8,20]. Moreover, a substantial proportion of these mice is protected against subsequent rechallenges with live cancer cell lines. Further research has shown that DAMPs, such as surface exposed CRT, secreted ATP and passively released HMGB1, and their interactions with phagocytosis receptors, purinergic receptors

Necrosis
A passive process (often called accidental necrosis) characterized by swelling of the organelles (endoplasmic reticulum and mitochondria) and the cytoplasm, as well as by subsequent destruction of the plasma membrane. Often described in negative terms; for example, by the absence of caspase activation and DNA oligonucleosomal fragmentation.

Apoptosis
Characterized by clear morphological criteria such as decreased cellular volume, chromatin condensation and nuclear fragmentation, and blebbing with the formation of apoptotic bodies containing unchanged organelles.

At a glance

- Damage-associated molecular patterns (DAMPs) are molecules that are secreted, released or surface exposed by dying, stressed or injured cells. DAMPs can function as either adjuvant or danger signals for the immune system. DAMPs such as surface-exposed calreticulin (CRT), secreted ATP and passively released high mobility group protein B1 (HMGB1) are vital for the immunogenic cell death (ICD) of cancer cells.
- The pathway by which CRT is surface exposed depends on apoptotic stage: one molecular pathway might exclusively execute the trafficking of surface-exposed CRT, or several signalling pathways might coexist, and depending on the cell death stimulus, one signalling pathway could predominate.
- The trafficking mechanism responsible for the secretion of ATP depends on the apoptotic stage and the type of stress or cell death stimulus that induces it. Moreover, both the mechanisms and the spatiotemporal pattern of ATP secretion from the dying cancer cells might be vital for establishing a suitable extracellular ATP gradient, which is required to engender its chemotactic or DAMP-like functions.
- Extracellular HMGB1 is vital for the immunogenicity of ICD, but it is also associated with tumour progression. Evidence indicates that the multiple functions of extracellular HMGB1 might be attributed to its different redox states in a context-dependent manner. The *in vivo* importance of apoptosis-associated HMGB1 release, especially in the context of ICD in established tumours, needs further research.
- The ability of selected cancer therapies to induce ICD depends on their ability to induce endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) production (either in parallel or in tandem). Both ER stress and ROS production are essential components that instigate the intracellular danger signalling pathways that govern ICD.
- ICD-associated immunogenicity is more effective if it is fostered by focused ROS-based ER stress (induced by type II ICD inducers such as hypericin-based photodynamic therapy (PDT)) rather than by secondary or collateral ER stress effects (as in the case of certain type I ICD inducers such as mitoxantrone and oxaliplatin).
- Pre-existing therapy-resistant variants of tumour cells (formed as a result of cancer microevolution) pose an important problem for the therapeutic use of ICD inducers and ICD-associated danger signalling: ideally, ICD-mediating therapies need to overcome hurdles such as therapy-resistant microevolution in cancer. Future research needs to consider a treatment that is based on combinations of ICD inducers that could be applied simultaneously in order to reduce the probability of resistance arising. Alternatively, an ideal ICD inducer could be developed that targets several pathways. Of the current ICD inducers, those that have most of the ideal properties include mitoxantrone, hypericin-PDT, shikonin, cardiac glycosides and bortezomib.

and pattern-recognition receptors (PRRs), respectively, are required for ICD and that this ultimately leads to the activation of potent anticancer immunity[4,21–24]. That the immune system is involved in the response of a tumour to a number of cancer therapies is not a new concept. Indeed, several drugs have recently been developed to increase the anticancer immune response in cancer patients. Clinical studies using cytotoxic T lymphocyte protein 4 (CTLA4)-blocking antibodies, which release one of the molecular breaks of the adaptive immune response, have shown that this therapy induces a survival benefit in patients with advanced melanoma[25]. Similarly, early stage clinical trials have shown that blocking the programmed cell death protein 1 pathway induces sustained tumour regression in various tumour types[26]. In addition, autologous dendritic cell vaccination for patients with high-grade gliomas improves survival without major toxicity[27]. Developing tumours are also thought to be subject to immunoediting, which results in the outgrowth of less immunogenic tumours[28]. It will be interesting to establish whether such tumours undergo ICD, or whether immunoediting involves DAMPs and leads to ICD resistance (discussed below).

In this Review, we discuss the role of endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) in regulating the immunogenicity of dying cells and the pathways involved in the emission of several vital DAMPs. We also consider how ICD contributes to the microevolution of therapy-resistant tumour cells.

ICD, ER stress and ROS generation

The first systematic screening for agents that can induce ICD in cancer cells identified doxorubicin, mitoxantrone and γ-irradiation as efficient inducers[7]. The ability of these anticancer drugs and treatments to induce ICD[7] was shown to depend on the induction of ER stress (BOX 1). The combined action of ROS and ER stress[29] was shown to activate danger signalling pathways that help to traffic DAMPs to the extracellular space[7,22,30–32]. ROS were proposed to be crucial because the immunogenicity of ICD was found to be diminished in the presence of antioxidants[30,33]. Later, it was shown that cisplatin, which induces changes in redox metabolism[34], was unable to evoke ICD because of its inability to cause ER stress. However, when thapsigargin or tunicamycin were combined with cisplatin, the induced apoptosis was immunogenic[34]. These observations confirmed that ER stress and ROS production are essential components of the intracellular pathways that govern ICD[35,36] and that they should occur at least in parallel to induce ICD. Moreover, the simultaneous presence of ER stress and ROS production increased the number of different DAMPs emitted[8,33,37], which was ultimately crucial for the immunogenicity of the dying cancer cells[33,38,39]. For example, etoposide, which did not induce ROS-based ER stress, only caused surface exposure of HSP70 (REF. 38) and ATP secretion[40] (and thus did not induce ICD[7]); whereas, doxorubicin caused the emission of surface-exposed HSP70, HSP90, CRT[8,33,38] and secreted ATP[40] (and thus induced ICD and accentuated tumour cell-associated immunogenicity[7]).

Table 1 | **An overview of DAMPs associated with various types of cell death and their immunomodulatory functions**

DAMPs	Receptor	Type of cell death (and mode of emergence)	Immunomodulatory functions	Refs
ATP	P_2Y_2 and P_2X_7	Accidental necrosis (passively released) and immunogenic apoptosis (either pre-apoptotic or early apoptotic active secretion)	Can act as a 'find me' signal, causes NLRP3-inflammasome-based IL-1β production from dendritic cells and mediates mitoxantrone- and oxaliplatin-induced antitumour immunity	4,8,36, 39,146
BCL-2	TLR2	Secondary necrosis?	Reduces reperfusion injury of skeletal or cardiac muscle when injected extracellularly	147
Calreticulin	CD91	Secondary necrosis (passively released) and immunogenic apoptosis (either pre-apoptotic or early or mid apoptotic surface exposure)	A potent 'eat me' signal and mediator of tumour immunogenicity crucial for antitumour immunity. Possesses homologues of prominent phagocytosis motifs (NPxY and KGE)	7,8, 62,71
Cyclophilin A	CD147	Necrosis (passively released)	Highly pro-inflammatory and mediates acetaminophen-induced liver injury	148
F-actin	DNGR1	Accidental necrosis and secondary necrosis (exposure following cell membrane permeabilization)	Helps in recognition of necrotic cells by $CD8\alpha^+$ dendritic cells	149
HSP70, HSP90, HSP60, HSP72, GRP78 and GP96	CD91, TLR2, TLR4, SREC1 and FEEL1	Necrosis (passively released) and immunogenic apoptosis (either pre-apoptotic or early or mid-apoptotic surface exposure)	Can attract monocytes and neutrophils. Can cause NK cell activation and dendritic cell maturation. Surface-exposed HSP90 can mediate T cell-based antitumour immunity. Secreted HSP90β can inhibit the activation of TGFβ1	3,4,8, 62,150, 151
Hepatoma-derived growth factor	Unknown	Secondary necrosis (passively released)?	Resembles HMGB1 in terms of immunostimulation	152
Histones	TLR9	Accidental necrosis	Their release can cause initiation of TLR9–MyD88-mediated inflammation such that histone neutralization protects against injury	153
HMGB1	TLR2, TLR4, RAGE and TIM3	Accidental necrosis and immunogenic apoptosis (secondary necrosis, passively released). Cell death accompanied by autophagy (early or mid apoptotic active secretion)	Can act as a strong cytokine and attract various immune cells. Can cause dendritic cell maturation. Immunostimulatory activity of HMGB1 might be inactivated during apoptosis	3–5,95, 101,107, 113,154
HMGN1	TLR4	Secondary necrosis (passively released)?	Induces dendritic cell maturation, recruitment of APCs and antigen-specific immune responses	155

Since the initial screening study[7] many agents and modalities have been shown to induce ICD (TABLE 2). The inducers of ICD are diverse both in terms of their biology (oncolytic virus and epidermal growth factor receptor (EGFR)-specific antibodies, for example) and their chemistry (chemotherapeutic drugs, ionizing radiation and light-activated drugs all induce ICD) (TABLE 2). Even the drug-based ICD inducers belong to distinct and often dissimilar chemical classes: anthracyclines (doxorubicin and idarubicin)[41,42]; platinum-based compounds (oxaliplatin)[33]; oxazophorines (cyclophosphamide)[43]; anthracenediones or anthraquinones (mitoxantrone[44,45] and hypericin[46]); and dipeptides (bortezomib)[47]. Thus, this rules out the existence of any simple structure–function relationship that could account for the ability of these agents to induce ICD[33]. However, the diversity of these ICD inducers further advocates a need to classify them in a manner that reflects the relevance and context of their ICD-inducing capabilities. To this end, we propose a classification system that is based on whether an ICD inducer triggers apoptotic cell death as a consequence of a direct action at the ER or whether it instigates both ER stress and apoptosis through convergent, but separate, mechanisms (TABLE 2).

Interestingly, most of the known ICD inducers target cytosolic proteins[33,48–51], plasma membrane channels or proteins[33,52], or DNA-replication proteins[37,53–55], rather than primarily targeting the ER[33] (FIG. 1; TABLE 2). Indeed, doxorubicin and mitoxantrone mostly localize in the nucleus[37,54], and only a small proportion is found in extranuclear compartments such as the ER[37,41,53]. These agents can be classified as type I ICD inducers (FIG. 1; TABLE 2); that is, agents that induce apoptotic cell death through targets that are not associated with the ER and that stimulate ICD-associated immunogenicity through secondary or 'collateral' ER stress effects. Conversely, type II ICD inducers selectively target the ER and can induce immunogenic apoptosis by directly altering ER homeostasis and triggering ER stress (FIG. 1; TABLE 2). Thus, ER stress induced by type I ICD inducers might be qualitatively different from the ER stress induced by type II inducers because it could be milder and might instigate pro-survival signalling. This has been shown in response to anthracyclines, which reduce the levels of activating transcription factor 4 (ATF4) and CCAAT/-enhancer-binding protein homologous protein (CHOP; also known as DDIT3), which abrogate classical ER stress-induced apoptosis[56]. It is notable that strong or prolonged primary or focused ER stress

Table 1 (cont.) | **An overview of DAMPs associated with various types of cell death and their immunomodulatory functions**

DAMPs	Receptor	Type of cell death (and mode of emergence)	Immunomodulatory functions	Refs
IL-1α	IL-1R	Accidental necrosis (passively released)	This is a cell type-specific endokine DAMP with strong pro-inflammatory activity	156
IL-33	ST2	Accidental necrosis (passively released)	Can bind ST2 on mast cells and T_H2 cells and trigger secretion of pro-inflammatory and T_H2 cytokines. The immunostimulatory activity of IL-33 might be inactivated during apoptosis	6
IL-6	IL-6R and GP130	Necroptosis	This is a cell type-specific endokine DAMP with strong pro-inflammatory activity	157
Mitochondrial DNA	TLR9	Accidental necrosis (passively released)	Can activate macrophages and neutrophils	24,158
Mitochondrial transcription factor A	RAGE and TLR9	Accidental necrosis (passively released)	A mitochondrial 'danger' signal functionally and structurally homologous to HMGB1. It causes potent dendritic cell activation, leading to type I interferon release	159
Monosodium urate	Unknown	Accidental necrosis (passively released)	Derived from uric acid and possesses pro-inflammatory properties: it can cause dendritic cell maturation and neutrophil attraction	160,161
N-formyl peptides	FPR1	Necrosis (passively released)?	Can act as a find me signal and chemoattractant for platelets, monocytes and neutrophils	24,158, 162,163
Reactive carbonyls and oxidation-specific epitopes	CD36, SRA, TLR2, TLR4 and CD14	Apoptosis or necrosis induced by ROS-producing agents or cell death associated with (internal) ROS production (passively released or surface exposed)	Reactive carbonyls can enhance antigen presentation accompanied by induction of T_H2 cell polarization	164,165
Ribonucleoproteins, mRNA and genomic DNA	TLR3	Accidental and secondary necrosis (passively released)	These DAMPs possess potent pro-inflammatory activity and can interact with various innate immune cell receptors	4,160, 166,167
S100A8, S100A9 and S100A12	RAGE	Accidental necrosis (passively released)	These DAMPs possess potent immunostimulatory activity and can attract monocytes and neutrophils. However, they might be susceptible to inactivation by the highly oxidizing extracellular matrix environment	3,160, 168

APC, antigen presenting cell; CD, cluster of differentiation; DAMPs, damage-associated molecular patterns; DC, dendritic cell; DNGR1, dendritic cell NK lectin group receptor- 1; FEEL1, fasciclin EGF-like; FPR1, formyl peptide receptor 1; HMGB1, high mobility group protein B1; HMGN1, high mobility group nucleosome binding domain 1; HSP, heat shock protein; IL, interleukin; NK, natural killer; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; SRA, scavenger receptor A; SREC1, scavenger receptor class F member 1; TGF, transforming growth factor; TLR, Toll-like receptor.

instigates pro-death signalling[57–60]. This point is further supported by the observation that anthracyclines that do not reach the nucleus owing to defective subcellular localization cannot cause cell death, even if they are present in extranuclear compartments such as the ER[54].

We recently showed that ICD-associated immunogenicity is more effective if it is fostered by focused ROS-based ER stress[37,59,61] (as induced by hypericin-based PDT, which is a type II ICD inducer) rather than simply induced by secondary or collateral ER stress effects (as is the case for mitoxantrone or doxorubicin, which are type I ICD inducers) (FIG. 1; TABLE 2). Specifically, focused ROS-based ER stress[59] was found to increase the number of DAMPs that can be emitted in the pre-apoptotic stage (before phosphatidylserine externalization)[8,37,62]; reduce the trafficking of DAMPs complexed with non-DAMP molecules[62]; increase the relative amounts of emitted DAMPs[8,62]; and reduce the number of molecular components involved in mediating the danger signalling pathways and thereby simplifying DAMP trafficking[8,37]. This supports the hypothesis that type II ICD inducers should have better efficiency, effectiveness and robustness in inducing tumour immunogenecity compared with type I ICD inducers (FIG. 1; TABLE 2). We also found that danger signalling in cancer cells has two types of molecular components: core components, which are commonly engaged for danger signalling by several ICD inducers; and particular components, which are engaged for danger signalling in a stimulus-dependent manner[37,61]. The core components predominantly mediate housekeeping functions, such as secretory trafficking and ER to Golgi transport, whereas the particular components have more limited roles, which include proteins involved in caspase signalling and the unfolded protein response (UPR)[8,37,61]. Hypericin-based PDT is the only type II ICD inducer that is known to induce ICD directly through ROS-based ER stress[8,59] (TABLE 2). This is because hypericin is an ER-localizing drug or photosensitizer[8,37,59] that causes massive production of ROS at the ER when excited by light of a specific wavelength[3,63], thereby leading to targeted or focused ROS-based ER stress[37,59,64].

The molecular links between ER stress and ROS production for other type I or type II ICD inducers (TABLE 2) need to be revealed in order to fully understand the

Hypericin-based PDT
(Hypericin-based photodynamic therapy). An anticancer therapeutic method that uses hypericin, which associates with the endoplasmic reticulum (ER). When activated by light of a suitable wavelength, it causes massive production of reactive oxygen species at the ER. This ultimately culminates in ER stress-mediated, BAX and BAK-based mitochondrial apoptosis.

Box 1 | **ER stress: an introduction**

The endoplasmic reticulum (ER) is a eukaryotic organelle that accomplishes vital sensing, biosynthetic and signalling functions[143] and is responsible for the synthesis, folding and post-translational modifications of a large of number of proteins[58,144]. Different physiopathological situations, such as ER Ca^{2+} depletion, hypoglycaemia, hypoxia, viral infections and injury owing to reactive oxygen species (ROS) production, can disturb ER homeostasis by causing an imbalance between protein folding load and capacity: this is termed ER stress[145]. The ER responds to stress by activating a complex signalling pathway, called the unfolded protein response (UPR)[58,143]. The UPR consists of three main signalling branches originating from the ER-sessile proteins, PERK, IRE1α and activating transcription factor 6 (ATF6). The main aim of the signalling pathways that originate from these three branches of the UPR is to re-establish ER homeostasis and promote survival[60], but when ER stress is too severe the UPR turns from a pro-survival pathway into a pro-death pathway[60,144,145]. This generally culminates in intrinsic mitochondrial apoptosis[57,58]. The biochemistry and signalling biology of ER stress and the UPR has been discussed extensively in recent reviews[29,57,58,60,144,145].

relevance of these processes and the pathways that they regulate. For example, although γ-irradiation and cyclophosphamide are capable of generating ROS[65,66], their ER stress-inducing capabilities are mostly unexplored. Conversely, bortezomib, a 26S proteasome inhibitor, is a potent indirect ER stressor[58,67] that can also cause ROS production in treated cells[68], but whether it leads to the local generation of ROS at the ER is unknown.

ICD-associated DAMPs and antitumour immunity

Molecular mechanisms of CRT surface translocation in ICD. CRT (TABLE 1) was identified in 1974 as a soluble protein in the lumen of the ER[69]. This highly conserved, 46 kDa Ca^{2+}-binding protein has three domains (a lectin-like globular N domain, a proline-rich P domain and a Ca^{2+}-binding C domain) followed by a four-amino acid ER retention sequence (KDEL) at the carboxyl terminus. In the ER, CRT has several functions, which include chaperone activity and the regulation of Ca^{2+} homeostasis and signalling. CRT also assists in the proper assembly of major histocompatibility complex (MHC) class I molecules and the loading of antigen. CRT has other functions outside the ER, such as regulation of nuclear transport (both import and export), and cell proliferation and migration[4,70]. A proportion of CRT that is found on the plasma membrane of viable cells (ecto-CRT)[71] serves various non-immunological functions. Ecto-CRT is an important signal that enables phagocytes to efficiently engulf dead cells[71]. However, Obeid *et al.*[7] have placed CRT-mediated phagocytosis in a different context. They reported that the exposure of CRT on the surface of cancer cell lines undergoing ICD in response to certain chemotherapeutics (such as anthracyclines) also facilities their engulfment by dendritic cells, which leads to tumour antigen presentation and tumour-specific cytotoxic T lymphocyte (CTL) responses. In addition, a recent study has shown that the immunomodulatory functions of ecto-CRT reside in the amino terminal lectin domain, which can bind various glycosylated protein molecules with a fairly high affinity[72]. These authors showed that a recombinant N-terminal fragment of CRT (39-272) is a potent inducer of activation and maturation of B cells and macrophages and can trigger Ig class switching in B cells without T cell help *in vitro* and *in vivo*[72].

Ig class switching
A process during which a subset of B cells undergoes class switch recombination, in which the heavy chain constant region is changed to a different immunoglobulin isotype without the introduction of variable region mutations.

Proximal secretory pathway
Denotes the events in the early phase of the secretory pathway, which include packaging of suitable cargo in COPII-coated vesicles, their exit from the endoplasmic reticulum and subsequent fusion of these vesicles with the Golgi complex.

The pathway by which ecto-CRT is exposed depends on the apoptotic stage during which the exposure takes place (FIG. 2a–c). In other words, it depends on whether the cell is in the pre-apoptotic stage (no phosphatidylserine externalization and no plasma membrane permeabilization), the early apoptotic stage (phosphatidylserine externalization but no plasma membrane permeabilization) or the mid to late apoptotic stage (plasma membrane permeabilization). Depending on the apoptotic stage, one molecular pathway might exclusively execute the trafficking of ecto-CRT, or several signalling pathways might coexist, and depending on the cell death stimulus, one signalling pathway could predominate. In any case, the kinetics of ecto-CRT compared with phosphatidylserine exposure depend on the type of cell death stimulus. For example, CRT exposure in ICD is an active process that in many cases precedes phosphatidylserine exposure and the morphological signs of apoptosis[7,73] (FIG. 2a). Panaretakis *et al.*[30] showed that the chemotherapy-induced CRT translocation pathway is dependent on PERK (also known as EIF2AK3)-mediated eIF2α phosphorylation, the secretory pathway and caspase 8-mediated B cell receptor associated protein 31 (BCAP31)-dependent activation of BAX and BAK proteins (FIG. 3). By contrast, we have found that only PERK, BAX, BAK, and the secretory pathway are required for hypericin-PDT-induced translocation of CRT to the surface[8] (FIG. 3). In this case, PERK governed the trafficking of ecto-CRT by regulating the proximal secretory pathway[8]. The apparent dispensability of phosphorylated eIF2α and caspase 8 (or of caspase signalling in general) indicates that induction of CRT surface exposure by hypericin-PDT-induced ER stress is coordinated by a pathway that is different from the one induced by chemotherapeutics[37] (FIG. 3), which might have important consequences for cancer therapy (discussed below). We have also found that induction of CRT translocation by chemotherapeutics and hypericin-PDT is dependent on the PI3K-regulated distal secretory pathway[8] (FIG. 3). It is noteworthy that, in the model of hypericin-PDT-induced CRT translocation[8], the KDEL sequence of ecto-CRT was not proteolytically removed but instead was carried with it to the surface, suggesting that CRT translocation occurs despite the presence of the KDEL sequence. In addition, in this model[8], ERp57 (also known as PDIA3), an ER luminal thiol-disulphide oxidoreductase[74], was not found to be associated with ecto-CRT, as has been reported for the chemotherapy-mediated translocation pathway[7,30]. Moreover, we demonstrated that pre-apoptotic ecto-CRT might dock with pro-low-density lipoprotein receptor-related protein 1 (LRP1; also known as CD91) on the surface of cancer cells undergoing ICD[8] (FIG. 3). All these findings indicate that the ER stress pathway plays an important part in inducing ICD by enabling pre-apoptotic surface exposure of CRT.

Thus, pre-apoptotically induced ecto-CRT exposure depends on the ER to Golgi transport, PERK-governed proximal and a PI3K-mediated distal secretory pathway for its trafficking (FIG. 2a). However, in the case of early apoptotic cells, it has been reported that ecto-CRT

Table 2 | **A classification of ICD inducers based on their ability to target the ER in a focused manner to induce antitumour immunity**

Immunogenic cell death inducer	DAMPs released	*In vivo* model used as evidence for antitumour immunity	Site of focused effects (and specific targets)	Refs
Type I inducers				
Mitoxantrone, oxaliplatin, UVC irradiation, γ-irradiation and anthracyclines	Pre-apoptotic ecto-CRT and ERp57; early apoptotic secreted ATP; mid to late apoptotic ecto-HSP70; late apoptotic passively released HMGB1	Prophylactic tumour vaccination model with transplantable tumours; for mitoxantrone — curative tumour model tested with subsequent tumour rechallenge	Nucleus (DNA or the DNA replication machinery proteins)	7,33,36, 38,39,95
Shikonin	Early to mid apoptotic ecto-CRT; early to mid apoptotic ecto-HSP70; early to mid apoptotic ecto-GRP78	Prophylactic autologous dendritic cell vaccination model with transplantable tumours	Cytosol (tumour-specific pyruvate kinase-M2 protein)	51,169
7A7 (EGFR-specific antibody)	Pre-apoptotic ecto-CRT and ERp57; early to mid apoptotic ecto-HSP70; early to mid apoptotic ecto-HSP90	Prophylactic tumour vaccination model with transplantable tumours	Cell surface (EGFR)	52
Cyclophosphamide	Pre-apoptotic ecto-CRT; late apoptotic passively released HMGB1	Curative tumour model accompanied by transplantable tumour rechallenge	Nucleus (DNA)	55
Bortezomib	Early to mid apoptotic ecto-HSP90	*In vitro* validation of T cell-based antitumour immunity. Exact *in vivo* immunogenicity data are seldom available	Cytosol (26S proteasome or ERAD machinery; CIP2A)	49,50,67
Cardiac glycosides	Pre-apoptotic ecto-CRT; early to mid apoptotic ATP release; late apoptotic passively released HMGB1	Prophylactic tumour vaccination model with transplantable tumours	Cell surface (Na^+,K^+-ATPase)	33
Type II inducers				
Hypericin-based PDT	Pre-apoptotic ecto-CRT; pre-apoptotic secreted ATP; pre-apoptotic ecto-HSP70; late apoptotic passively released HSP70, HSP90 and CRT	Prophylactic tumour vaccination model with transplantable tumour; curative tumour model tested with subsequent tumour rechallenge	ER (ROS-based damage at the ER membrane)	3,8,37, 62,64,170
Coxsackievirus B3	Early apoptotic ecto-CRT; early apoptotic secreted ATP; late apoptotic passively released HMGB1	Intra-tumoural infiltration of phenotypically mature dendritic cells and NK cell-mediated lytic processes in CVB3-infected tumours	ER (ER membranes and lumen)	171–174

CRT, calreticulin; DAMP, damage-associated molecular pattern; DCs, dendritic cells; Ecto, cell surface exposed; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; GRP, glucose-regulated protein; HMGB1, high mobility group protein B1; HSP, heat shock protein; ICD, immunogenic cell death; NK, natural killer; PDT, photodynamic therapy; phox, photo-oxidative; ROS, reactive oxygen species; UVC, ultraviolet C.

induction might also rely on phosphatidylserine exposure[75] (FIG. 2b). The association of ecto-CRT with externalized phosphatidylserine has been reported for ultraviolet (UV)-induced apoptosis[71]. Finally, certain types of apoptotic cells might exhibit ecto-CRT as a result of general exposure of ER chaperones, as well as ER and Golgi membranes on the cell surface[76] (FIG. 2c). It is important to note that most of the ecto-CRT studies reported for cancer cells showed that anticancer treatments engaged the pathway either in the pre-apoptotic stage or in the mid to late apoptotic stage[8,30,37–39]. To this end, it is clear from FIG. 2a–c that the secretory pathway has an important role in danger signalling-based trafficking of ecto-CRT, and probably other DAMPs of ER origin.

Extracellular ATP: more than a 'find me' signal? The extracellular release of ATP (TABLE 1) is a ubiquitous means of modulating different cellular functions, such as survival, death, adhesion, proliferation, differentiation and mobility. Extracellular ATP is also a well known 'find me' signal that is released from apoptotic cells. ATP released from apoptotic cells is sensed by P_2Y_2 receptors on monocytes and induces their recruitment to the site of apoptosis[77]. It was recently shown that cells dying by different cell death modalities release or secrete ATP (Supplementary information S1 (Table)). In fact, Rapaport and Fontaine[78] showed in 1989 that intraperitoneal injection of ATP results in an antitumour response[78]. It was shown 20 years later that ATP in the extracellular space is required for the generation of an effective chemotherapy-elicited anticancer immune response[40]. Cancer cell lines exposed to various chemotherapeutic agents release extracellular ATP during the phase of phosphatidylserine exposure on the plasma membrane[40]. Recently, several mechanisms have been proposed to explain the secretion of ATP from dying apoptotic cells. Similar to ecto-CRT, recent data indicate that the trafficking mechanism responsible for the secretion of ATP strongly depends on the apoptotic stage and the type of stress or cell death stimulus that induces ATP secretion (FIG. 2d–f). We found that pre-apoptotic secretion of ATP in the absence of plasma membrane permeabilization (FIG. 2d) was dependent on the classical, as well as the PERK-regulated, proximal secretory pathway and PI3K-dependent exocytosis, but was independent of BAX or BAK[8] (FIG. 3). In the case of cells in the early phases of apoptosis, it has recently been shown that suppression of autophagy by knockdown of essential autophagy-related genes

Autophagy
A primary survival mechanism activated in cells subjected to chemical or biological stress and/or nutrient or obligate growth factor deprivation. However, if cellular stress continues, autophagy often becomes associated with features of apoptotic or necrotic cell death.

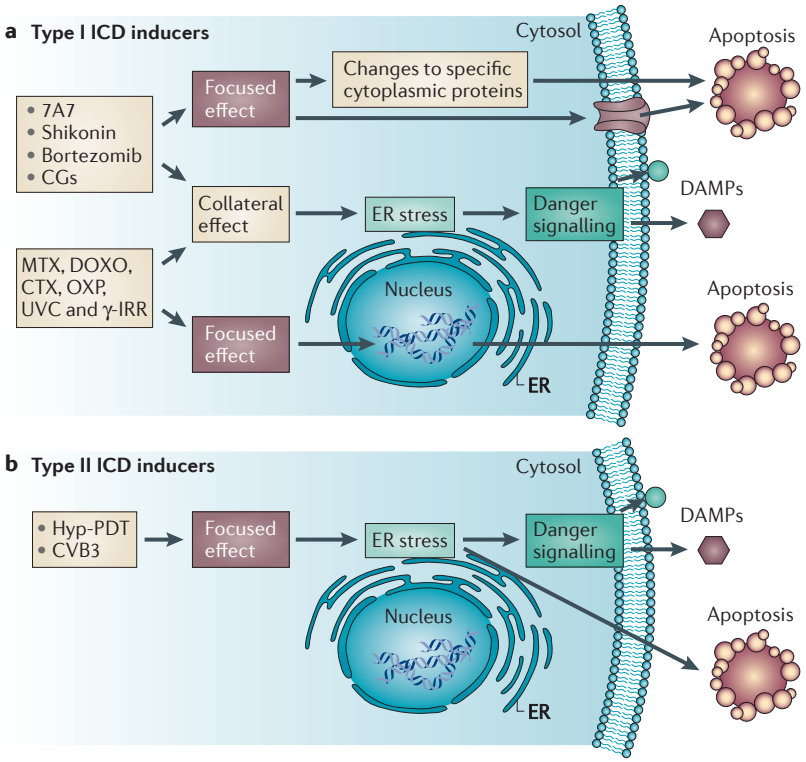

Figure 1 | **The origins of ICD pathways induced by type I and type II ICD inducers.** **a** | The site of primary or focused effects for most type I immunogenic cell death (ICD) inducers is the nucleus (proteins involved in DNA replication and repair), the cytosol (certain cytosolic proteins) and the plasma membrane (certain transmembrane proteins or channels) (TABLE 2). Their ability to induce cell death or apoptosis predominantly stems from these focused effects. These agents can also induce endoplasmic reticulum (ER) stress via secondary or collateral effects. Their ability to induce the emission of damage-associated molecular patterns (DAMPs) comes from these collateral effects. **b** | For type II ICD inducers, the ER is the site of focused effects in the form of primary ER stress. Their ability to induce apoptosis, as well as danger signalling and DAMP emission, stems from their primary ER stress-inducing capabilities. CG, cardiac glycoside; CTX, cyclophosphamide; CVB3, coxsackievirus B3; DOXO, doxorubicin; Hyp-PDT, hypericin-based photodynamic therapy; MTX, mitoxantrone; OXP, oxaliplatin; UVC, ultraviolet C radiation; γ-IRR, γ-irradiation.

(*ATG5*, *ATG7* and *BECN1*) inhibited the secretion of ATP from dying cells that were killed by mitoxanthrone and oxaliplatin (FIG. 2e; FIG. 3) and compromised the tumour-specific immune response *in vivo*[39,79]. Conversely, UV and CD95 (also known as FAS)-specific antibodies might also induce ATP secretion from cells in the early phases of apoptosis, but in a pannexin 1 hemichannels-dependent manner[80] (FIG. 2e). Importantly, for both pannexin 1-dependent and autophagy-dependent ATP secretion pathways, caspases were found to be important for ATP secretion[39,77] (FIG. 2e). Finally, for mid to late apoptotic cells, ATP might also be secreted passively owing to a defective plasma membrane (FIG. 2f). The immense complexity and diversity of danger signalling pathways that exist in cancer cells with respect to different DAMPs is important to appreciate (FIG. 2). Given that responses are different based on the cancer therapeutic used to induce ICD, there is little if any opportunity for generalization between the type of cancer therapy and the danger signalling pathways engaged.

NALP3–ASC–inflammasome
A multimeric danger-sensing platform that promotes autocatalytic activation of the cysteine protease caspase 1 and mediates the cleavage of inactive pro-interleukin (IL)-1β and IL-18, among other proteins, into their active forms.

ATP that is released from dying cells activates purinergic P_2X_7 receptors on dendritic cells, thereby activating the NALP3–ASC–inflammasome and driving the secretion of IL-1β. This cytokine, together with antigen presentation, is required for the polarization of interferon-γ (IFNγ)-producing $CD8^+$ T cells and for an adaptive immune response to cancer cells[36]. It should be noted that stimulation of P_2Y_2 receptors is required for monocyte attraction, whereas P_2X_7 receptors are required for NALP3 inflammasome activation and immunogenicity. It has been proposed that the consequence of the ATP action on these receptors depends on the amount of extracellular ATP. For P_2X_7 activation, ATP is required in the dose of EC_{50} >100 μM, and for P_2Y_2 ATP is needed at a dose of EC_{50} <1 μM[81]. Therefore, it may be speculated that both the mechanisms and the spatiotemporal pattern of ATP secretion from the dying cancer cells are vital to establish a suitable extracellular ATP gradient, which is required to engender its chemotactic or DAMP-like functions.

ATP can be hydrolysed within seconds[82,83] to immunosuppressive adenosine (FIG. 4) by the action of surface-expressed ectonucleotidases, such as CD39 (an ectonucleoside triphosphate diphosphohydrolase 1 (NTPDase 1; also known as ENTPD1)) and CD73 (an ecto-5′-nucleotidase)[84,85]. Indeed, a high concentration of extracellular ATP at tumour sites[86] could lead to a higher concentration of adenosine[87]. Unlike ATP, adenosine suppresses immune responses by activating G protein-coupled receptors and could thereby contribute to a tumour-promoting microenvironment that reduces the effectiveness of antitumour immune responses[85]. Another angle of the complexity to the role of ATP in immunogenicity has been added by a recent observation that targeted inhibition of CD73 can reduce tumorigenesis and metastasis[88,89]. Notably, it has been shown that overexpression of CD39 abolishes the immunogenicity of cell death[90]. This study suggests that ATP is required for immunogenicity. However, one could also interpret these findings as showing that adenosine is a signal that might compromise the immunogenicity of ICD. Mice that are genetically deficient in A2A receptor have an increased rejection capacity of established tumours compared with wild-type mice[87]. Interestingly, adenosine could be released by macrophages that contribute to the engulfment-dependent apoptotic cell suppression of inflammation[91]. All these studies suggest that adenosine could modulate ICD, but future studies are needed to address this issue. Therefore, the ATP–ectonucleotidase–NTPDase–adenosine system might determine the final outcome of the antitumour response (FIG. 4).

Role of HMGB1 in anticancer treatment. HMGB1 (TABLE 1) is an abundant nuclear non-histone chromatin-binding protein[4]. HMGB1 modulates the transcriptional activity of various proteins, including steroid hormone receptors, p53 and nuclear factor-κB (NF-κB), facilitates VD(J) recombination and participates in chromatin-level transcriptional regulation[92]. Interestingly, various cytosolic and extracellular functions of HMGB1 have recently been reported. In the cytosol, HMGB1 can

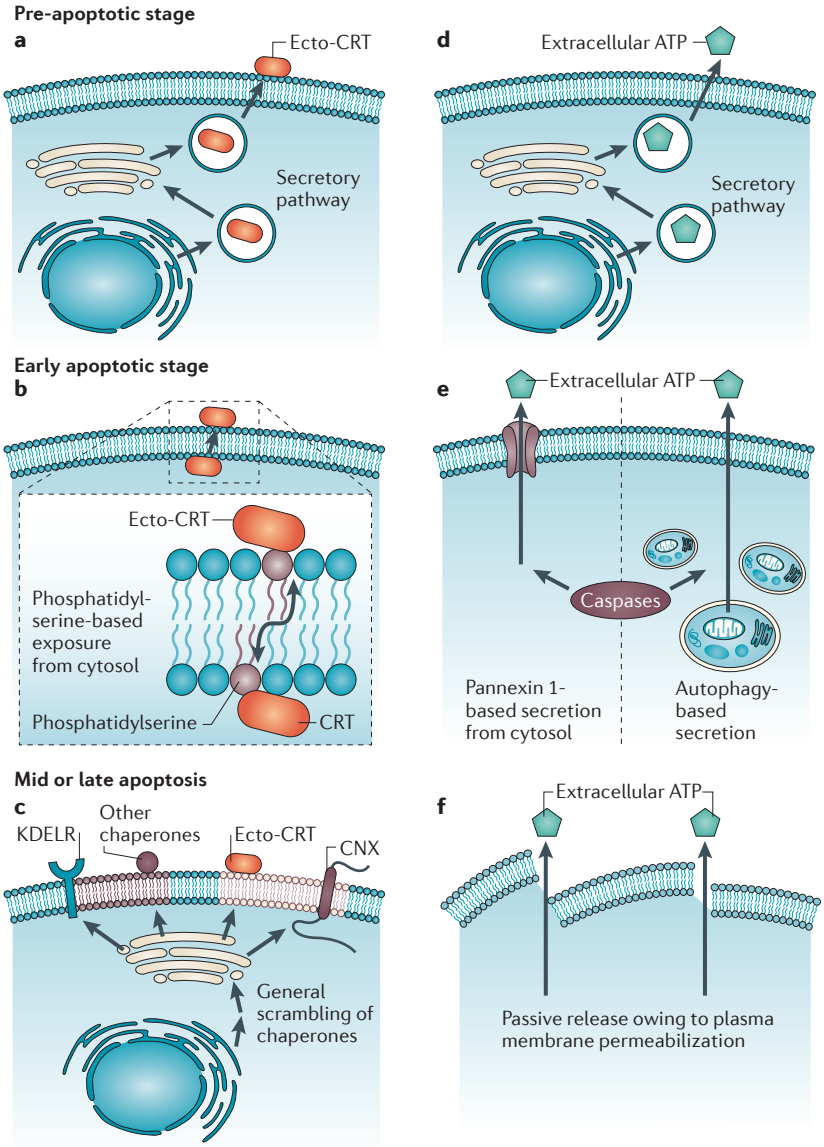

Figure 2 | **The molecular trafficking pathways for surface-exposed calreticulin and secreted ATP during the different apoptotic stages.** The pre-apoptotic surface exposure of calreticulin (CRT) has been reported to be predominantly dependent on the secretory pathway (part **a**). However, in certain cases early apoptotic surface-exposed CRT (ecto-CRT) could occur in association with phosphatidylserine exposure (part **b**). Moreover, early apoptotic cells that are part of a population predominantly undergoing late apoptosis might exhibit ecto-CRT as a result of general exposure of chaperones from the endoplasmic reticulum (ER) accompanied by general trafficking of ER and Golgi membranes towards the surface; this is evident from the surface emergence of ER or Golgi sessile proteins such as calnexin (CNX) and KDEL receptor (KDELR) (part **c**). Similarly, the secretory pathway has been reported to be at the core of trafficking mechanisms for pre-apoptotic secreted ATP (part **d**). However, in the early apoptotic stage, depending on the cell death stimulus, ATP secretion might be dependent either on the pannexin 1 channel (for anti-CD95 and ultraviolet C (UVC) treatments) or on autophagy (for mitoxantrone (MTX) and oxaliplatin (OXP) treatments) (part **e**). Nevertheless, in both of these cases, caspases have an important role in mediating ATP secretion. Finally, the bulk of the secreted ATP in the mid to late apoptotic stage has been attributed to the defective plasma membrane, which is damaged in secondary necrotic stages (part **f**).

mediate autophagy[93] by interacting with beclin 1 (REF. 94). HMGB1 can also be secreted as a cytokine (not as a DAMP) through the secretory route by macrophages and monocytes that have been activated by IL-1β, tumour necrosis factor (TNF) or lipopolysaccharide (LPS)[4,92]. Interestingly, extracellular HMGB1 has been found to be vital for the immunogenicity of ICD. Using a prophylactic tumour vaccination model (TABLE 1) based on the CT26 murine colon adenocarcinoma cell line, it was observed that immunization with HMGB1-depleted CT26 cancer cells or co-injection of HMGB1-specific antibody compromised the ability of mice to resist rechallenge with live CT26 tumour cells[95]. These authors showed that the eradication of tumours by chemotherapy requires the binding of HMGB1 (released from cells undergoing ICD) to Toll-like receptor 4 (TLR4)[95]. This is in line with retrospective clinical analysis in patients with breast cancer, which showed that a single nucleotide polymorphism (SNP; Asp299Gly) in the *TLR4* gene that prevents the binding of HMGB1 to TLR4 correlated with early relapse after anthracycline treatment[95,96]. Conversely, however, HMGB1 has also been shown to be associated with tumour progression in several models[97–100].

It has been known for some time that necrotic cells can passively release large amounts of HMGB1 as a DAMP[4,101] (TABLE 1). This extracellularly released HMGB1 can induce intense inflammation[101]. For example, it stimulates the production of pro-inflammatory cytokines (such as TNF, IL-1, IL-6 and IL-8)[102] from innate immune cells, including neutrophils, macrophages and monocytes[103]. To carry out these activities, extracellular HMGB1 binds to various receptors, including TLR2, TLR4 and the receptor for advanced glycosylation end products (RAGE)[103–105] (TABLE 1). It was recently shown that HMGB1 might also be released by cells undergoing secondary necrosis[95,106]. HMGB1 can also be released by cancer cell lines undergoing ICD: its release during the later stages of ICD involves a pathway that can be blocked by Z-VAD-FMK, a pan-caspase inhibitor that delays the induction of secondary necrosis[95]. Intriguingly, it was recently shown that early apoptotic epithelial and glioblastoma cancer cells treated with EGFR-targeted diphtheria toxin (DT-EGF) can secrete HMGB1 actively in an autophagy-dependent manner[107]. It is unknown whether this kind of autophagy-mediated HMGB1 secretion is specific to DT-EGF or whether it also applies to other agents that are capable of inducing cell death accompanied by autophagy.

However, as is clear from the discussion above, the precise role of HMGB1 in ICD is unclear. Indeed, depending on the study under consideration, HMGB1 seems to cause different effects. This clearly raises a need for a proper unification theory that reconciles the multiple behaviours of HMGB1. Recently, a couple of studies[108,109] have presented evidence that the activity of HMGB1 might be context-dependent with respect to redox modification of HMGB1 induced by the cell or the extracellular environment. It has been reported that these HMGB1 redox states have their own distinct non-overlapping immunomodulatory characteristics[108]. Redox changes switch the activity of HMGB1 between chemoattractant DAMP (fully reduced HMGB1), pro-inflammatory cytokine-inducing DAMP (disulphide-bond possessing HMGB1) and inactivated DAMP (fully oxidized HMGB1)[108,109]. In the case of inactivated DAMP, when HMGB1 was converted into a non-oxidizable form, it retained its chemoattractant properties

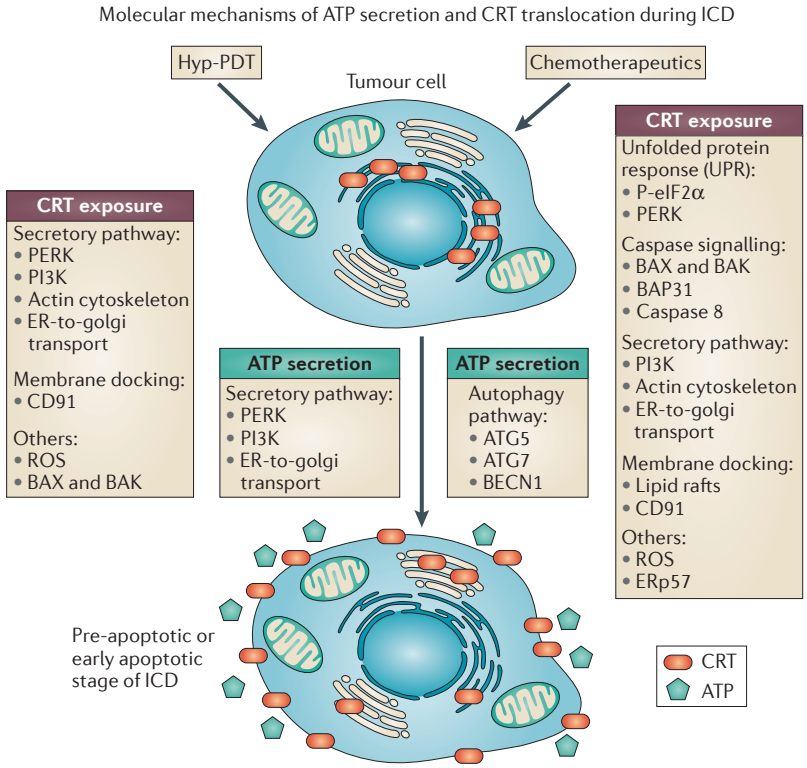

Figure 3 | **Comparative overview of the molecular mechanisms responsible for the trafficking of the immunogenic signals, ecto-CRT and ATP.** The signalling pathways induced by hypericin-photodynamic therapy (hyp-PDT) or various chemotherapeutics treatments overlap, but they are not identical. For example, the chemotherapy-induced calreticulin (CRT) translocation pathway seems to be dependent on PERK-mediated eIF2α phosphorylation (P-eIF2α), the secretory pathway, caspase 8 and activation of BAX and BAK proteins. However, only PERK, BAX, BAK and the secretory pathway are required for hypericin-PDT-induced translocation of CRT to the surface[8]. The induction of CRT translocation by chemotherapeutics and hypericin-PDT is dependent on the PI3K-regulated distal secretory pathway[8], but ERp57, an endoplasmic reticulum (ER) luminal thiol-disulphide oxidoreductase[74], has not been found to be associated with hypericin-PDT-mediated expression of surface-exposed CRT (ecto-CRT), although this has been reported for the chemotherapy-mediated translocation pathway[7,30]. Pre-apoptotic ecto-CRT might dock with low-density lipoprotein receptor-related protein 1 (LRP1) on the surface of cancer cells undergoing immunogenic cell death (ICD) in response to chemotherapy[8]. Similar to ecto-CRT, recent data indicate that the trafficking mechanism responsible for the secretion of ATP depends on the apoptotic stage and the type of stress or cell death stimulus that induces it. The pre-apoptotic secretion of ATP in the absence of plasma membrane permeabilization might be dependent on the classical, as well as PERK-regulated, proximal secretory pathway and PI3K-dependent exocytosis, but is independent of BAX and BAK[8]. Suppression of autophagy by knockdown of essential autophagy-related genes (*ATG5*, *ATG7* and *BECN1*) inhibits the secretion of ATP from dying cells that are killed by mitoxanthrone and oxaliplatin[39,79]. ROS, reactive oxygen species.

but lost its ability to induce pro-inflammatory cytokine production[108]. Thus, depending on which redox state of HMGB1 predominates in a particular *in vitro* or *in vivo* experimental set-up or pathological condition, HMGB1 might exhibit one or the other immunomodulatory property. For example, in physiological conditions or in normal cells *in vitro*, the extracellular space is predominantly oxidative[110], which might favour HMGB1 inactivation. However, the scenario is more complex in cancer cells, at least *in vitro*. Evidence indicates that the redox state of the extracellular medium of the cultured cancer cell lines is highly variable[110]. This implies that different cancer cell types or different stages of progression of the same cancer cell type might generate unique extracellular redox states[110]. This disorganization and plasticity of cancer cell-associated extracellular redox state may explain to a certain extent the diversity of contradictory *in vitro* evidence derived for the role of HMGB1 in antitumour immunity.

The *in vivo* importance of HMGB1 release in antitumour immunity or apoptosis is still a matter of some debate[5,111–113]. Activation of caspases during apoptosis can amplify mitochondrial ROS production by targeting the permeabilized mitochondria and cleaving the 75 kDa subunit of respiratory complex I (p75 NDUSF1), a component of the electron transfer chain[5]. This caspase-dependent mechanism leads to the oxidation of HMGB1, which neutralizes its immunostimulatory activity and thereby promotes apoptosis-associated tolerance[5]. However, it is worth pointing out that this study was carried out predominantly in non-cancerous cells, limiting the conclusions that can be drawn for antitumour immunity. However, it is vital to keep in mind that apoptotic cells (cancerous or non-cancerous) are cleared rapidly *in vivo*[112], and that this process could be further accentuated during ICD in cancer cells owing to the pre-apoptotic surface exposure of CRT[7,62,71]. This minimizes the probability of secondary necrotic cells or late-apoptotic cells persisting in the tissues long enough to release substantial amounts of DAMPs such as HMGB1 (REF. 112). This reduces the *in vivo* relevance of HMGB1 released during secondary necrosis and inactivated by ROS, or for that matter, any DAMP associated with secondary necrosis[114] (TABLE 1).

Nevertheless, a recent study suggests that HMGB1 can in fact hinder antitumour immunity *in vivo*. This study showed that, in an established tumour microenvironment, HMGB1 may bind to a TIM3 receptor that is highly expressed by dendritic cells that have infiltrated the tumour[113]. Through this mechanism HMGB1 can interfere with and diminish the immunogenicity of nucleic acids released from cancer cells as danger signals[113] (TABLE 1) and thereby suppress nucleic acid-mediated antitumour immunity[113]. These results for HMGB1 in mice with established tumours[113] might be more relevant to overall antitumour immunity than the results obtained for ICD in tumour cell vaccinated mice[33,95] as this does not recapitulate the behaviour of an actual tumour mass. Furthermore, it has been found that an established tumour microenvironment *in vivo* tends to be highly pro-oxidative in nature[115]. This means that HMGB1 is likely to be oxidized, thereby minimizing its immunological effect. However, it is imperative in the near future to completely characterize the actual immunological effect of HMGB1 on antitumour immunity *in vivo* in established tumours, with reconciliation for various redox states of HMGB1.

ICD and therapy-resistant cancer microevolution

Cancer progression is a process of microevolution[116] that consists of a series of changes and adaptations, including cell death resistance, increased cellular proliferation, increased invasiveness, metabolic reprogramming,

Secondary necrosis
A late stage of apoptosis characterized by the loss of plasma membrane integrity. Secondary necrotic cells are often observed *in vitro* in the absence of phagocytosis, or in some cases *in vivo* when apoptotic cells cannot be cleared rapidly enough.

Tolerance
A state that involves (active) hypo- or non-responsiveness of innate and adaptive immune cells to a particular biological or chemical entity.

neovascularization and inflammation[117,118]. During this microevolution, it seems that different mutations can confer a selective advantage depending on the changes in the tumour microenvironment[119]. During tumour initiation, the immune system exerts a strong selection pressure on the tumour cells as a part of tumour immunosurvelliance[4,120]. Most initial neoplastic lesions are thought to be eliminated by the immune system before they can form clinically relevant tumours. However, in certain cases, immunoevasive variants of tumour cells are formed. These cancer cells undergo expansion and escape despite a constant selection pressure (so-called cancer immunoediting) that is exerted by the immune system, which eliminates all the other immunosusceptible tumour cells[4,120]. These immunoevasive tumour cells further undergo several rounds of natural selection under diverse selection pressures that are exerted by various factors, including the disturbed and hypoxic tumour microenvironment, acidosis, tumour stromal cells, nutrients and growth factor deprivation, and cytokine-induced hyperplasia[118,120,121]. Thereafter, radiotherapy and/or chemotherapy exert a strong selection pressure on tumour cells, which might lead to therapy-resistant microevolution[117].

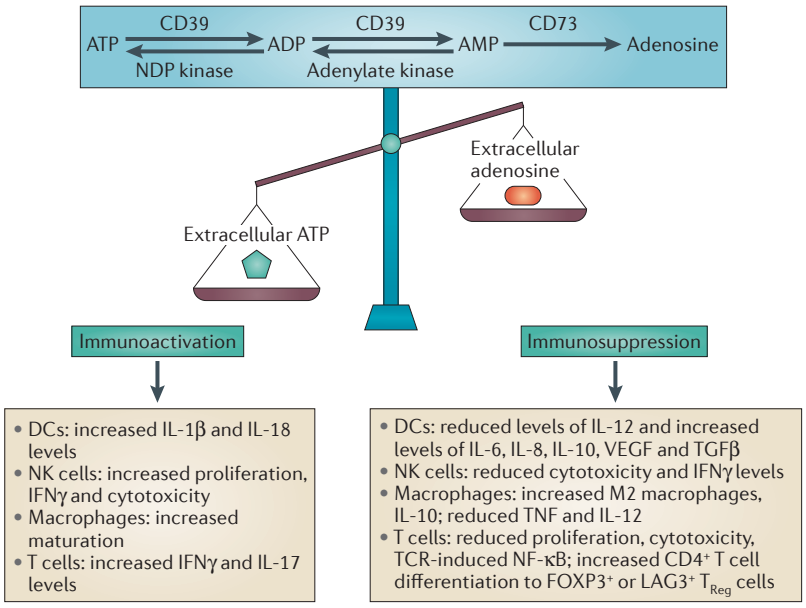

Figure 4 | **Immunomodulatory effects of extracellular ATP and extracellular adenosine.** ATP released during the course of immunogenic cell death (ICD) (as a result of treatment with anthracyclines, for example) could activate the immune system. However, as a negative-feedback mechanism, extracellular ATP could be converted to immunosuppressive adenosine. The conversion of ATP into AMP is mostly catalysed by CD39, and only small amounts of ADP are released. Further conversion of AMP into adenosine is catalysed by CD73. Importantly, conversion of ATP to AMP by CD39 is reversible by the actions of the extracellularly located kinases NDP kinase and adenylate kinase. However, CD73 converts AMP into adenosine reversibly only following intracellular transport of adenosine, where it can be converted to AMP by adenosine kinase[85]. In the tumour microenvironment, a shift of the balance towards ATP might be crucial in mediating an effective antitumour response. DC, dendritic cell; FOXP3, forkhead box protein P3; IFNγ, interferon-γ; IL, interleukin; NF-κB, nuclear factor-κB; NK, natural killer; TGFβ, transforming growth factor-β; T_{Reg}, T regulatory; VEGF, vascular endothelial growth factor.

Pre-existing therapy-resistant variants of tumour cells also pose an important problem for ICD inducers and ICD-associated danger signalling. As discussed above, ICD depends on the DAMPs and the danger signalling that mediates their emission[8,30,37]. Thus, pre-existing tumour cell variants in which the gene for a protein or signalling molecule required for danger signalling has undergone a loss-of-function mutation or has been deleted would not be able to emit the DAMPs or immunogenic signals crucial for ICD. Thus, an ICD inducer would end up acting as a selection force for such variants, ultimately leading to the elimination of ICD-susceptible tumour cells and the expansion of ICD-resistant tumour cells. In TABLE 3 we present an analysis in which the data available in the Tumorscape[119] and COSMIC[122] databases (see Further information), as well as in the published literature, have been used to evaluate which proteins involved in mediating danger signalling during type I or type II agents-induced ICD[8,30,37,61] might be targeted by tumour cells to promote resistance to ICD. Caspase 8 and BAX are two such proteins: their ablation could potentially result in resistance to ICD (TABLE 3). Caspase 8 ablation is more widely reported in different cancer types than BAX ablation[119,122–124]. Indeed, according to the predictions in the Tumorscape database[119], the probability of caspase 8 ablation is higher in all types of cancers (TABLE 3). As type I inducers such as mitoxantrone, oxaliplatin, UVC and doxorubicin depend on caspase 8 and BAX for ICD induction[8,30,61] (TABLE 3), the probability of ICD-resistant cancer variants forming increases. Conversely, ICD induced by hypericin-PDT is caspase 8 independent (TABLE 3), and the associated ATP secretion is BAX and BAK independent and ecto-CRT induction is BAX and BAK dependent[8]. Moreover, the dependence of mitoxantrone, oxaliplatin and UVC (but not hypericin-PDT[8,37]) on eIF2α phosphorylation[30,61] might represent another Achilles' heel of these agents because it has been reported that abrogation of eIF2α phosphorylation might be a part of malignant transformation in some cases[125]. In fact, attenuation of eIF2α phosphorylation may be a chemotherapy-resistant phenotype in certain cancer types[126]. However, it is worth mentioning that as genetic alterations in these genes drive many other pro-tumorigenic processes (such as resistance to apoptosis[123,124,127], neoplastic transformation[128], increased tumour growth[123,124,127], and increased tumour differentiation and malignancy[129]) (TABLE 3), it is unlikely that these alterations are selected only because of their function in implementing ICD. More research is required to ascertain whether certain somatic mutations mentioned in TABLE 3 ablate the ability of human cancer cells to emit the respective DAMPs. Last, the above discussion raises the pertinent point that, in order to be effective, a particular anticancer agent should not only be endowed with the ability to incite ICD but also to overcome cancer cell-autonomous hurdles, such as therapy-resistant cancer microevolution, which might dampen the emission of danger signals, thereby promoting chemotherapy-induced tolerogenic cancer cell death. In this context, a type II ICD inducer, such as hypericin-PDT, may be favoured over type I ICD inducers, such as mitoxantrone and doxorubicin.

Table 3 | **Somatic copy number alterations and mutations in pathway components that mediate the emission of DAMPs**

ICD inducers	Pathway component involved in DAMP emission	SCNA data in Tumorscape database[119] (Q value)*	Somatic mutation data in COSMIC database[122] (cancer type)	Possible effects on cancer progression
MTX[39] and OXP[39]	ATG5 and ATG7	None	• ATG5: one NSS in a rectal adenocarcinoma and two MSS in colon adenocarcinomas • ATG7: one MSS in a colon adenocarcinoma and one MSS in a squamous cell carcinoma of the scalp	Somatic mutations in *ATG5*, although rare[175], cause reduction of ATG5 protein levels in human tumours[175,176]. This has been suggested to be beneficial for primary neoplasms[175,176]. Not much is known about *ATG7* mutations
MTX[30]	BAP31 (encoded by *BCAP31*)	Amplification[177] (1.29×10^{-13})	None	Increased BAP31 expression in primary colorectal cancer was found to correlate with better overall survival of patients[178]
MTX[8,30], UVC[30] and OXP[30]	Caspase 8	Deletion[123] (3.64×10^{-9})	Four NSS and three MSS in squamous cell carcinoma of the mouth; two NSS and five MSS in breast carcinomas; two NSS and eight MSS in adenocarcinomas of the caecum; two NSS and four MSS in colon adenocarcinomas; one MSS in head and neck squamous cell carcinoma; and one MSS in melanoma	Inactivation of caspase 8 by epigenetic or genetic means, alternative splicing or post-translational modifications help cancer cells to evade apoptosis[123]. Mutated caspase 8 acts in a dominant-negative manner and blocks death receptor-mediated apoptosis[123]
MTX[8,30], UVC[30] and OXP[30]	Phosphorylated eIF2α	None	None	Downregulation of phosphorylated eIF2α has been reported to correlate with a resistant phenotype in certain cancer types[125,126]
MTX[62,74], UVC[74] and DIG[33]	ERp57 (encoded by *PDIA3*)	None	One MSS in melanoma; one MSS in breast carcinoma; two MSS in ovarian serous carcinoma; and one MSS in colon adenocarcinoma	Somatic mutations in *PDIA3*, although not frequent, have been reported to compromise the antigen-processing machinery in cancer cells, thereby assisting in loss of HLA class I expression and cancer immunoevasion[179,180]
MTX[30], UVC[30], DIG[33], DOXO[8] and Hyp-PDT[8]	BAK (encoded by *BAK1*)	None	None	*BAK1* ablative mutations or deletions are rarely reported, but low levels of BAK expression are associated with cancer cell resistance to immunotoxins[127]
MTX[30], UVC[30], DOXO[8] and Hyp-PDT[8]	BAX	Deletion[124] (1.08×10^{-8})	One NSS in lung adenocarcinoma and one MSS in glioblastoma	Certain cancers (especially those with microsatellite mutator phenotype[181,182]) tend to show loss-of-function mutations or deletions in *BAX*, thereby contributing to resistance to apoptotic cell death[124,181,182]
MTX[30], UVC[30], OXP[30], DOXO[8] and Hyp-PDT[8]	PERK (encoded by *EIF2AK3*)	None	One NSS and two MSS in ovarian serous carcinoma	Unknown
MTX[8] and Hyp-PDT[8]	PIK3CA	Amplification[183] (1.19×10^{-17})	Thousands of mutations reported across many cancer types	PI3K has been found to be amplified or mutated in a broad range of cancers[183]. Such mutations confer constitutive kinase activity on PI3K and can drive tumorigenesis[183]

ATG, autophagy-related gene; BAK, Bcl-2 homologous antagonist killer; BAP31, B cell receptor-associated protein 31; BAX, Bcl-2-associated X; COSMIC, catalogue of somatic mutations in cancer; DAMP, damage-associated molecular pattern; DIG, digoxin; DOXO, doxorubicin; eIF2α-P, phosphorylated eukaryotic initiation factor 2α; ERp57, endoplasmic reticulum protein 57; Hyp-PDT, hypericin-based photodynamic therapy; ICD, immunogenic cell death; MSS, missense substitution mutation; MTX, mitoxantrone; NSS, nonsense substitution mutation; OXP, oxaliplatin; SCC, squamous cell carcinoma; SCNA, somatic copy-number alteration; UVC, ultraviolet C. *A Q-value of lower than 0.25 suggests that the amplifications and deletions at the respective gene locus are enriched by selective pressures[119]. This value describes the probability of this alteration happening in all types of cancers.

Future research on experimental therapy could consider a treatment based on combinations of ICD inducers that could be applied simultaneously in order to lower the probability of the formation of a tumour cell containing all of the therapy-resistant genetic variations[117], in an analogy to the avoidance of escape mutants in HIV therapy.

Conclusions and future challenges

Substantial progress has been made over the past few years in identifying a growing list of the DAMPs that are exposed during immunogenic cell death (TABLE 1) and in understanding the peculiarities of the molecular mechanisms of their emission[7,8,30,95]. For example, the molecular pathway responsible for CRT translocation

by hypericin-PDT-induced ICD is subjected to fewer molecular checkpoints[8,62] (FIG. 3; TABLE 3), and so it is less likely to be subverted in cancer cells than the pathway that is initiated by chemotherapeutics[30], because it does not require caspase 8, ERp57 and phosphorylated eIF2α[8,37,62] (TABLE 3). This illustrates that we are just beginning to unravel the molecular mechanisms dictating ICD. Depending on the stimulus, different molecular processes may converge on the immunogenic pathways that lead to the emission of DAMPs.

However, this new knowledge has also raised new questions and challenges. A major limitation of the prophylactic tumour vaccination model used in ICD-related studies is that it does not fully recapitulate tumour formation in patients with co-evolving tumour–host interactions and an immunosuppressive microenvironment. Recent research using spontaneous mammary tumour models has shown that the adaptive immune system is not needed for the therapeutic efficacy of immunogenic chemotherapeutics such as oxaliplatin and doxorubicin[130]. Therefore, it is crucial to understand why the concept of immunogenic cell death is not applicable to certain spontaneous tumour models, why the adaptive immune system sometimes does not contribute to chemoresponsiveness and how the concept of immunogenic cell death should be modified in order to develop efficient treatments for these spontaneous tumour models. Moreover, it has been reported that the DAMP spectra can change for the same cancer cell line depending on whether it is treated *in vitro* or *in vivo*[3,131]. Thus, as most of the ICD parameters are predominantly tested *in vitro*[8,30,33], it would be crucial to test their presence in spontaneous tumours *in vivo*. At the very least, ICD parameters and DAMP analyses in response to the treatment of spontaneously formed tumours *in vivo,* or their derived primary cultures, should be preferred over the use of cultured cancer cell lines, which may not accurately recapitulate the genetic profile of the treated cancer, as they have not been through a process of microevolution in order to adapt to the microenvironment and to escape the immune surveillance system. Therefore, an ideal ICD inducer will probably have more limitations when used on *ex vivo* tumour cells than when used on *in vitro* cancer cell lines that have not been counterselected by the immune system.

Taking into account the various trends in the fields of ICD, cancer immunology, cancer immunosurveillance, cancer inflammation and cancer therapy over the past decade[4,22,31,37,120,121,132], we propose a list of properties of an ideal ICD inducer. First, an ideal ICD inducer should be an efficient instigator of apoptosis or other types of programmed cell death (at doses that can be used preclinically or clinically without substantial toxicities or side effects[133]). This would allow the emission of DAMPs in the absence of plasma membrane permeabilization[8,22,120]. Second, an ideal ICD inducer should be capable of inducing emission of multiple types of DAMPs, TLR agonists and immunogenic signals[3,37], preferably in the pre-apoptotic or early apoptotic stages before tolerance to cells dying through apoptosis[112] is induced. This would ensure the early sensitization of the immune system to the dying cancer cells, thereby assisting in robust antitumour immunity[4,31,32,37]. Third, an ideal ICD inducer should not be subject to drug-efflux pathways[134,135] or to an altered subcellular localization[136]. Moreover, as severe and focused ER stress is vital for ICD[7,8,32,37] and can activate robust pro-death signalling[58,60], an ideal ICD inducer should be capable of inducing ER stress as a focused effect[3,37] (FIG. 1), which would make it possible to improve DAMP trafficking and to increase their emission[8,37]. As discussed above, during cancer microevolution, cancer cells can acquire genetic ablations that could cripple the danger signalling pathways (TABLE 3). Thus, an ideal ICD inducer should be able to overcome as many of these immunogenicity-impeding mutations as possible. Activation of pro-inflammatory transcription factors such as NF-κB in cancer cells often correlates with increased tumour growth and a negative prognosis[29,121,137], as these pathways are associated with resistance to cell death and pro-tumorigenic inflammation[58,121,132]. Thus, an ideal ICD inducer should be capable of inhibiting or strongly dampening the activation of pro-inflammatory transcription factors in order to be able to induce tumour regression[121]. However, at the same time an ideal ICD inducer should have negligible suppressive or inhibitory effects (topically or systemically) on immune cells such as mature dendritic cells, natural killer cells, $CD3^+CD4^+$ T cells (mainly T_H1 phenotype), cytotoxic $CD3^+CD8^+$ T cells, memory $CD3^+CD4^+$ or $CD3^+CD8^+$ $CD45RO^+$ T cells and B cells that infiltrate a tumour site following treatment, as they are likely to be required for immune reactions that are centred on the tumour[64,138]. Conversely, it is crucial for an ideal ICD inducer to be able to inhibit immune-suppressive responses, such as those mediated by tumour-associated macrophages (TAMs; mainly M2 phenotype), myeloid-derived suppressor cells (MDSCs), regulatory T cells and $CD3^+CD4^+$ T cells (mainly T_H2 phenotype)[64,121,138]. Finally, an ideal ICD inducer should be capable of directly targeting not only the primary tumour but also metastases[132].

In TABLE 4 we present a literature-based analysis of the known clinically applied or experimental anticancer ICD inducers to understand which possess all of the properties of an ideal ICD inducer. Evidently, no ideal ICD inducer exists, but it is vital to find a multitasking agent or to develop combinatorial therapies of multiple drugs that could achieve these ideal properties. Of the currently known clinically or preclinically relevant ICD inducers, those that have most of these properties include mitoxantrone, hypericin-PDT, shikonin, cardiac glycosides and bortezomib. However, improved ICD inducers could be developed as we learn more about the ICD pathways.

Another aspect that should be considered is the complex interaction of DAMPs with their cognate PRRs. Indeed, binding of DAMPs to specific receptors is required for antigen presentation and immune response generation. However, DAMPs can also bind to TLRs expressed on tumour cells, and it has been reported that triggering of TLR7 and TLR8 on human cancer cells can promote chemoresistance and cell survival through the activation of NF-κB and the upregulation of the anti-apoptotic protein BCL-2 (REF. 139). In addition, activation of TLR9 on human breast, prostate and lung cancer cells induces tumour invasion and metastasis[140,141]. These

Table 4 | **Which ICD inducers possess most of the properties of an ideal inducer?**

Property of an ideal inducer of ICD	Agents that have this property	Agents that lack this property	Not yet characterized
Efficient inducer of apoptosis or other programmed cell death subroutines	MTX[7,39], OXP[39], DOXO[7] γ-irradiation[7], shikonin[169], 7A7 (REF. 52), CTX[55], Hyp-PDT[8], CGs[33] and bortezomib[67]	CVB3 (REF. 172)	
Capable of inducing strong immunogenicity that mediates antitumour immunity	MTX[7,39], OXP[39], DOXO[7], γ-irradiation[7], Hyp-PDT[8,37,62], CVB3 (REF. 172) and CGs[33]	Shikonin[169], 7A7 (REF. 52), CTX[55] and bortezomib[67]	
Not susceptible to drug-efflux channels	Hyp-PDT[184], γ-irradiation, CVB3 (REF. 185), 7A7, shikonin[186] and bortezomib[187]	MTX[188], OXP[189], DOXO[134], CGs[190] and CTX[135]	
Capable of inducing severe focused ER stress	Hyp-PDT, CVB3 and bortezomib	MTX, OXP, DOXO, CTX, γ-irradiation, CGs, 7A7 and shikonin	
Capable of overcoming loss-of-function mutations that cripple danger signalling during cancer micro-evolution	Hyp-PDT[8,37,62,185]	MTX, OXP, DOXO, CGs and γ-irradiation[8,33,37,61]	CVB3, shikonin, 7A7, CTX and bortezomib
Capable of downregulating cancer-based induction of pro-inflammatory transcription factors	Hyp-PDT[191,192], shikonin[193], CGs[194] and bortezomib[195]	MTX[196], OXP[197], DOXO[198], γ-irradiation[199,200] and CVB3 (REF. 201)	7A7 and CTX
Negligible inhibitory effects on infiltrating anti-tumorigenic immune cells	Hyp-PDT[170], DOXO[202], MTX[203], γ-irradiation (local)[204], CTX (low dose)[205], shikonin[206], CGs[207] and 7A7 (REF. 208)	OXP[209], CTX (high dose)[202], bortezomib[202] and γ-irradiation (whole body)[210]	CVB3
Inhibitory effects on tumour-associated pro-tumorigenic immune cells	CTX (low dose)[202], OXP[202], bortezomib[202] and MTX[211]		γ-irradiation, shikonin, Hyp-PDT, 7A7, CGs, CVB3 and DOXO
Capable of directly targeting metastasized cells	MTX, OXP, DOXO, shikonin, 7A7, CTX, bortezomib, CGs and CVB3	γ-irradiation and Hyp-PDT	

CG, cardiac glycoside; CTX, cyclophosphamide; CVB3, Coxsackievirus B3; DAMP, damage-associated molecular pattern; DOXO, doxorubicin; ER, endoplasmic reticulum; ICD, immunogenic cell death; Hyp-PDT, hypericin-based photodynamic therapy; MTX, mitoxantrone; NF-κB, nuclear factor-κB; OXP, oxaliplatin.

data indicate that not only do the interactions between DAMPs and PRRs contribute to immunogenic outcome but so does their location (immune cells versus cancer cells). Another complexity of the DAMP–TLR interaction is that certain SNPs in TLR genes reduce their functions and so might compromise the immunostimulatory activity of danger signals and interfere with the elimination of tumour cells. Chemotherapy with anthracyclines is less effective in patients with breast cancer bearing loss-of-function alleles of *P2RX7* (Glu496Ala) or *TLR4* (Asp299Gly)[95,142]. Loss-of-function alleles in TLR4 also reduce the therapeutic efficacy of oxaliplatin in patients with colorectal cancer [35]. Therefore, investigating the underlying mechanism of signalling mediated by TLR–DAMP interactions will also contribute to the development of new strategies for cancer therapy.

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Competing interests statement

The authors declare no competing financial interests.

FURTHER INFORMATION

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