

Review

Multimodal immunogenic cancer cell death as a consequence of anticancer cytotoxic treatments

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Apoptotic cell death generally characterized by a morphologically homogenous entity has been considered to be essentially non-immunogenic. However, apoptotic cancer cell death, also known as type 1 programmed cell death (PCD), was recently found to be immunogenic after treatment with several chemotherapeutic agents and oncolytic viruses through the emission of various danger-associated molecular patterns (DAMPs). Extensive studies have revealed that two different types of immunogenic cell death (ICD) inducers, recently classified by their distinct actions in endoplasmic reticulum (ER) stress, can reinstate immune responses suppressed by the tumor microenvironment. Indeed, recent clinical studies have shown that several immunotherapeutic modalities including therapeutic cancer vaccines and oncolytic viruses, but not conventional chemotherapies, culminate in beneficial outcomes, probably because of their different mechanisms of ICD induction. Furthermore, interests in PCD of cancer cells have shifted from its classical form to novel forms involving autophagic cell death (ACD), programmed necrotic cell death (necroptosis), and pyroptosis, some of which entail immunogenicity after anticancer treatments. In this review, we provide a brief outline of the well-characterized DAMPs such as calreticulin (CRT) exposure, high-mobility group protein B1 (HMGB1), and adenosine triphosphate (ATP) release, which are induced by the morphologically distinct types of cell death. In the latter part, our review focuses on how emerging oncolytic viruses induce different forms of cell death and the combinations of oncolytic virotherapies with further immunomodulation by cyclophosphamide and other immunotherapeutic modalities foster dendritic cell (DC)-mediated induction of antitumor immunity. Accordingly, it is increasingly important to fully understand how and which ICD inducers cause multimodal ICD, which should aid the design of reasonably multifaceted anticancer modalities to maximize ICD-triggered antitumor immunity and eliminate residual or metastasized tumors while sparing autoimmune diseases.

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Facts

- Accelerated progresses and discoveries in the field of oncology, immunology, and virology have made it possible to translate several emerging immunostimulatory strategies to treat malignant cancers towards promising clinical benefits.
- Profound understanding of the process of immunogenic cell death (ICD) induction by different ICD inducers such as certain chemotherapeutic agents and oncolytic viruses has highlighted the importance of immunological antitumor effects and proposed novel anticancer therapies.
- The execution of different types of programmed cell death (PCD), including apoptosis, autophagy, necroptosis, and pyroptosis, which are driven by a plethora of stimuli, was recently found to be regulated by orchestrated interactions

among them, and importantly, some of these types of PCD exhibit an ICD property.

- Tumors and cancer cells treated with certain chemotherapeutic agents and oncolytic viruses can undergo ICD and release tumor-associated antigens (TAAs) accompanied by diverse danger-associated molecular patterns (DAMPs) and inflammatory cytokines to restore the tumor microenvironment and incite TAA-specific antitumor immunity.

Open Questions

- What are the recent advances in the development of anticancer immunotherapeutic modalities in clinical settings?

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Abbreviations: ICD, immunogenic cell death; PCD, programmed cell death; PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns; ecto-CRT, calreticulin exposure; HMGB1, high-mobility group protein B1; ATP, adenosine triphosphate; PS, phosphatidylserine; Hsp, heat-shock protein; ACD, autophagic cell death; ER, endoplasmic reticulum; ROS, reactive oxygen species; APCs, antigen-presenting cells; DCs, dendritic cells; TAAs, tumor-associated antigens; GM-CSF, granulocyte-macrophage colony-stimulating factor; PDT, photodynamic therapy; CVB3, coxsackievirus B3; CSCs, cancer stem cells

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- In response to diversified ICD inducers, how are DAMPs such as CRT, high-mobility group protein B1 (HMGB1), and ATP expressed by or released from the dying cancer cells?
- How do the diverse types of PCD differentially induce ICD to mount an efficient antitumor immunity?
- What are the prerequisites for an ideal ICD inducer to obtain an optimum level of ICD for long-lasting antitumor effects?
- It is vital to understand the molecular mechanisms of how ICD inducers, for example, infection with oncolytic viruses and resultant DAMPs, affect the host immune system. Can manipulation of ICD induction and/or combined strategies synergize with current or emerging oncolytic virotherapies?

The concept of immunogenic cell death (ICD) has recently been introduced to describe dying cancer cells that release endogenous danger molecules, the so-called damage-associated molecular patterns (DAMPs), after the exposure to certain cytotoxic agents to be recognized by antigen-presenting cells (APCs) such as dendritic cells (DCs) followed by formation of T-cell-mediated adaptive immunity.¹ Although it has long been considered that apoptotic cell death is tolerogenic, DAMPs have also been found to be released from cells undergoing apoptosis, providing a promising anticancer efficacy.^{2–4} Therefore, comprehension of ICD induction gradually increases its significance, particularly in the field of cancer immunotherapy.

Overall prognosis of advanced cancer patients still remains dismal, thus making it imminent for oncologists to develop novel anticancer strategies. Recently, sipuleucel-T (Provenge, Dendreon, Seattle, WA, USA), indicated for patients with metastatic castration-resistant prostate cancer, received FDA's approval as the first therapeutic cancer vaccine.⁵ In addition, extensive Phase II clinical trials have demonstrated that the oncolytic herpes simplex virus talimogene laherparepvec (T-Vec, Amgen Inc., Thousand Oaks, CA, USA)⁶ and vaccinia virus JX-547 (Pexa-Vec, Jennerex Biotherapeutics, Inc., San Francisco, CA, USA),⁷ both of which carry the gene encoding the immunostimulatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), hold great promise for the treatment of advanced cancer patients. Furthermore, cytotoxic T-cell responses directed against oncolytic virus-infected cancer cells have been identified as an essential factor in the process of destruction of cancer.⁸ Moreover, proinflammatory cytokines generated in the virus-infected cancer cells can restore the immunosuppressive tumor microenvironment.^{9–11} Thus, oncolytic viruses are recently viewed as anticancer immunotherapeutic agents. These backgrounds make it imperative to update the molecular pathways and/or cellular constituents that regulate ICD.

Here, we review the progress of research on ICD, emphasizing how apoptotic, autophagic, and necroptotic cell death, called type 1, 2, and 3 PCD, respectively, are induced by various ICD inducers to achieve successful antitumor immunity. These multiple modes can be categorized by describing initiating events, intermediated changes, terminal cellular events, and their immunological responses, which are summarized in Table 1. In the

later section, we outline the characteristics of anticancer agents and oncolytic viruses and how they induce diversified forms of cell death and interact with host's immune system.

Apoptotic Cell Death as ICD

From ten million to billions of cells die per day as a consequence of normal tissue turnover,¹² which are vital for organisms to retain homeostasis.^{13,14} Therefore, the existence of multiple modes of cell death in nature is not surprising. Apoptosis, type 1 PCD, is a specialized form of cell death, characterized by typical morphological changes, including chromatin condensation, nuclear fragmentation, and membrane blebbing (Table 1).¹⁵ Apoptosis occurs ubiquitously in normal tissues and causes 'quiet' cell death that uses phosphatidylserine (PS) as an 'eat-me' signal to be quickly recognized by peripheral APCs. Although apoptotic cell death has been historically considered to be non-immunogenic,¹⁶ recent studies unraveled that several anti-neoplastic agents, including doxorubicin,^{1,17} oxaliplatin,^{18,19} cisplatin,²⁰ and irradiation,^{21,22,23} can trigger immunogenic apoptosis.² Mechanistically, the immunogenic apoptotic bodies induced by exposure to doxorubicin are sensed by APCs through their TLR-2/TLR-9-MyD88 signaling pathways.¹⁷

DAMPs: as Effectors in ICD

The primary conceptual theory of the pattern recognition of pathogen-associated molecular patterns (PAMPs), such as viral or bacterial components, has failed to fully explain the consequence of immunogenicity. Thus, the secondary concept of DAMPs has been proposed, which could provoke an immune response.²⁴ Released DAMPs as hallmarks of ICD consisted of adenosine triphosphate (ATP), high-mobility group protein B1 (HMGB1), and exposed molecules on the outer membrane of dying cells such as CRT (ecto-CRT), heat-shock proteins (Hsp90 and Hsp70), and endoplasmic reticulum (ER) sessile proteins.^{25,26,27} The excretion of DAMPs was considered to occur during necrosis under inflammatory and/or pathological conditions. However, DAMPs have recently been reported to be produced from apoptotic cancer cells treated with chemotherapy^{1,18} or radiotherapy.²¹

ICD Inducers

ICD inducers include multiple anticancer therapeutic modalities. It has been recently proposed that they can be classified into two categories, type I or II ICD inducers, based on their distinct actions to induce ER stress leading to apoptotic cell death (Tables 2 and 3).^{27,28} The majority of ICD inducers such as chemotherapeutic agents (mitoxantrone,²⁹ anthracyclines,^{2,30,31} oxaliplatin,^{18,19} and cyclophosphamide³²), shikonin,^{33,34} the proteasome inhibitor bortezomib,³⁵ and 7A7 (an epidermal growth factor receptor-specific antibody),³⁶ cardiac glycosides,³⁷ and the histone deacetylase inhibitor (vorinostat)³⁸ are categorized as type I ICD inducers that primarily target cytosolic proteins, plasma membranes, or nucleic proteins. They also induce ER stress via collateral effects. Bortezomib, cardiac glycosides,

Table 1 Comparison of multiple forms of programmed cell death and necrosis

	Apoptosis (type 1 PCD)	Autophagic cell death (type 2 PCD)	Necroptosis (type 3 PCD)	Pyroptosis	Necrosis
Mode of cell death	Programmed	Programmed	Programmed	Programmed	Accidental
Initiators	TNF- α , FasL, or TRAIL, infectious pathogens	Nutrient deprivation, HDAC inhibitors, hypoxia, infectious pathogens	TNF- α , FasL, or TRAIL, microbial infections Ischemic injury	DAMPs, microbial infections	Toxins, infections, inflammation, trauma
Intermediate signalings	Mitochondrial pathway Caspase-3, -6, -7-dependent	Caspase-independent autophagosome formation Lysosomal protease	TNF receptor signaling JNK activation Caspase-independent RIP1/RIP3 necrosome	Nod-like receptors Caspase 1-dependent pyroptosome Inflammasome	-
Terminal cellular events	Non-lytic cell shrinkage DNA fragmentation apoptotic bodies	Non-lytic autophagic bodies	Non-lytic, loss of plasma membrane, swollen cellular organelles	Lytic, rapid loss of plasma membrane, cell swelling, pore formation	Lytic, plasma membrane rupture, leak of content
Inflammation Immunogenicity	Non-inflammatory +	Non-inflammatory +	Proinflammatory ++	Proinflammatory ++	Proinflammatory +++
DAMPs released	Ecto-CRT HMGB1 and ATP release	HMGB1 and ATP release	Long genomic DNA IL-6	HMGB1 and ATP release IL-1 α , IL-1 β , IL-6, IL-18, and TNF- α chemokines	HMGB1 and ATP release IL-1 α , IL-33 mRNA, and genomic DNA
Eat-me signals	Ecto-CRT	LPC secretion PS exposure	LPC secretion PS exposure	PS exposure	PS exposure

Abbreviations: TNF- α , tumor necrosis factor- α ; FasL, Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; HDAC, histone deacetylase; IL-1 α , interleukin-1 α ; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; IL-18, interleukin-18; IL-33, interleukin-33; ICD, immunogenic cell death; LPC, lysophosphatidylcholine; PS, phosphatidylserine; JNK, c-Jun N-terminal kinase.

The table gives a schematic overview of the multiple forms of cell death including apoptotic cell death (type 1 PCD), autophagic cell death (type 2 PCD), cell death induced by necroptosis (type 3 PCD), pyroptosis and necrosis. The extent of immunogenicity in each cell death subsection is scored as +, ++, and +++, according to the expression levels of 'eat-me' signals and DAMPs emission. ICD in cancer can display different 'eat-me' signals, including ecto-CRT and LPC, on the cell membrane, as well as emission of DAMPs, ATP, and HMGB1. This peculiar ecto-CRT, which facilitates engulfment of TAAs from cancer cells by DCs, can only be found on cells that succumb to immunogenic apoptosis, whereas it is not present on cells dying in an immunologically silent manner. LPC secretion, PS exposure, and ATP release require autophagy induction. Numerous exquisite expression patterns shaped by the constituents of DAMPs and the interactive status of the immune system will predominantly determine the fate of subsequent immune responses, namely, immune tolerance or antitumor immunity

and shikonin effectively impede protumorigenic cytokine signaling.²⁷ Shikonin has been found to induce type 1 or 3 PCD, which is determined by caspase-8 activation as the 'decision-making switch'.³⁹ On the other hand, type II ICD inducers, which preferentially target the ER, include hypericin-based photodynamic therapy (PDT)^{40,41,42} and oncolytic coxsackievirus B3 (CVB3).⁹ Hypericin-based PDT is an anticancer therapy that utilizes hypericin to induce reactive oxygen species (ROS) in the vicinity of the ER.⁴³ Cancer cell infection with oncolytic viruses produce large amounts of viral proteins, which inevitably cause ER stress and ROS production to promote viral replication.^{44,45} The quality and/or quantity of ER stress linking ROS triggered by ICD inducers may determine the ICD properties. Indeed, the previous finding that rigorous ROS-mediated ER stress augmented the release of DAMPs revealed an unrecognized role of RNA-dependent protein kinase (PKR)-like ER kinase (PERK) as a constituent of mitochondria-associated ER membranes to exert ROS-mediated mitochondrial apoptosis.^{40,41,46} These observations indicate the superiority of type II ICD inducers with respect to immunological antitumor efficacies. However, further investigations to elucidate the precise interconnection between the ER stress and ROS production will be required to optimize antitumor immune responses.

Calreticulin Exposure

In response to specific chemotherapeutic agents, oncolytic viruses, and vorinostat, ecto-CRT has been found only on cells succumbing to immunogenic apoptosis.^{2,9,38} This 'eat-me' signal promotes phagocytosis by DCs, thereby facilitating their tumor antigen presentation and incitement of TAA-specific cytotoxic T cells.^{2,47} It has been shown that blockade of CRT inhibits phagocytosis of anthracycline-treated tumor cells by DCs and impairs their immunogenicity in mice.^{2,47} In general, CRT exposure during ICD is an earlier process occurring within a few hours than PS externalization.⁴⁸ The ecto-CRT induction capacity of ICD inducers has been shown to depend on the properties of ER stress and ROS production.^{2,37,49} Cancer cells can induce ecto-CRT followed by disturbance of the ER structure with GADD34 activation and PERK phosphorylation. It has been shown that depletion of PERK abolishes anthracycline-driven ecto-CRT and immunogenicity of cellular death (ER stress module),¹⁹ and that caspase-8 acts upstream of apoptotic proteins Bax and Bak, and subsequent cleavage of its substrate Bap31 (apoptotic module) is indispensable for ecto-CRT induction.¹⁹ Furthermore, a direct interaction between ecto-CRT and ERp57 was shown to be required for their cotranslocation to the cell surface (Figure 1).²⁹ Unlike the release of HMGB1 and ATP,

Table 2 Classification of type I ICD inducers determined by their major targets to provoke antitumor responses

Anticancer agents	Type of cell death induced	DAMPs	Major targets by ICD inducers	Preclinical observations for inciting antitumor immunity
Cytotoxic agents (mitoxantrone, oxaliplatin, anthracyclines)	Apoptosis, autophagic cell death, necroptosis	Ecto-CRT, ERp57, HMGB1, and ATP release	Nucleus (DNA or DNA-related proteins for cell mitosis)	<i>In vivo</i> antitumor effect is mitigated by depletion of CD8 ⁺ T cells. Immunogenicity requires ecto-CRT in prophylactic tumor vaccination mouse models.
Cyclophosphamide (CTX)	Apoptosis	Ecto-CRT, HMGB1 release	Nucleus (DNA)	Metronomic doses of CTX deplete Treg from bed and tumors, CTX modulates DCs to produce IL-12
Shikonin	Apoptosis, necroptosis	Ecto-CRT, ecto-Hsp70	Cytosol (pyruvate kinase-M2 protein)	DCs incubated with shikonin increase Th1 cells but decrease Treg cells
Bortezomib	Apoptosis, autophagic cell death	Ecto-Hsp90	Cytosol (26S proteasome)	Cytotoxicity of NK cells against bortezomib-treated cells increased
7A7 (EGFR-specific antibody)	Apoptosis	Ecto-CRT, ERp57, ecto-Hsp70, ectp-Hsp90	Cell surface receptor (EGFR)	Contribution of CD4 ⁺ T and CD8 ⁺ T to 7A7-triggered suppression of metastasis in mice model
Cardiac glycosides	Apoptosis	Ecto-CRT HMGB1 and ATP release	Cell surface (Na ⁺ /K ⁺ -ATPase, enzyme)	Prophylactic antitumor immunity is partially dependent on CD8 ⁺ T cells accompanied with Th17 cells
UVC irradiation	Apoptosis, necroptosis, necrosis	Ecto-CRT and ERp57, HMGB1 and ATP release	Nucleus (DNA)	UVC-treated cells increase susceptibility to attack by NK cells and total splenocytes
Vorinostat (HDAC inhibitor)	Apoptosis Autophagic cell death	Ecto-CRT	Nucleus (chromatin structure)	Promote the differentiation of CD8 ⁺ T cells to memory cells

Abbreviations: Ecto-CRT, calreticulin exposure; DAMPs, damage-associated molecular patterns; ICD, immunogenic cell death, HMGB1; high-mobility group protein B1; Hsp, heat-shock protein; Treg, regulatory T cells; DCs, dendritic cells; IL-12, interleukin-12; NK, natural killer; EGFR, epidermal growth factor receptor; ATP, adenosine triphosphate; UVC, ultraviolet C

Table 3 Classification of type II ICD inducers determined by their major targets to provoke antitumor responses

Anticancer agents	Type of cell death induced	DAMPs	Major targets by ICD inducers	Preclinical observations for inciting antitumor immunity
PDT with hypericin	Apoptosis, autophagic cell death dependent on Bax/Bak, necroptosis	Ecto-CRT, ecto-Hsp70, ectp-Hsp90, HMGB1, and ATP release	ER (ROS generation)	PDT-hypericin therapy provokes antitumor immunity in both prophylactic and therapeutic murine tumor models. Same therapy-treated tumor cells result in phenotypic maturation of DCs and robust CD4 ⁺ T and CD8 ⁺ T cell expansion
CVB3	Apoptosis	Ecto-CRT, HMGB1 translocation, ATP release	ER (ROS generation)	Intratumoral CVB3 administration markedly recruited NK cells and granulocytes, both of which contribute to the antitumor effects as shown by depletion assays, macrophages, and mature DCs into tumor tissues
Ad5/3-hTERT-E1A-hCD40L: chimeric Ad5/3 capsid, an hTERT promoter and human CD40L	Apoptosis	Ecto-CRT, HMGB1 release, ATP release	ER (ROS generation)	In two syngeneic mouse models, murine CD40L induced activation of APCs, leading to increased IL-12 production in splenocytes, associated with induction of the Th1 cytokines IFN- γ , RANTES, and TNF- α . Tumors treated with Ad5/3-CMV-mCD40L displayed an enhanced presence of macrophages and cytotoxic CD8 ⁺ T cells
Edmonston strain MV	Apoptosis	IL-6 production, HMGB1 release	ER (ROS generation)	Coculture of MV-infected melanoma cells with human DCs led to both CD80 and CD86 upregulation on them. CD8 ⁺ T cells cocultured with tumor cell-loaded and MV-infected DCs degranulated CD107a to target tumor cells with functional killing activity

Abbreviations: PDT, photodynamic therapy; CVB3, coxsackievirus B3; MV, measles virus; ROS, reactive oxygen species; ER, endoplasmic reticulum; hTERT, telomerase reverse transcriptase; hCD40L, human CD40 ligand; Th1, T helper type 1; RANTES, regulated and normal T cell expressed and secreted; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6

ecto-CRT could be one of the determinants that distinguishes between immunogenic and non-immunogenic cell death.⁴⁷

HMGB1

HMGB1, one of the DAMPs, is a DNA-binding protein originally known as a nuclear non-histone chromatin-binding protein.⁵⁰ Although extracellular HMGB1 had been deemed to be released mainly from the nucleus during necrosis,⁴² it was found to be excreted from cells undergoing late stage of apoptosis and autophagy.^{30,51} HMGB1 inhibition in cancers undergoing immunogenic apoptosis impaired their ability to incite antitumor immunity in a prophylactic vaccination model.³⁰ HMGB1 initiates potent inflammation by stimulating the production of proinflammatory cytokines⁵² from APCs via its binding to different surface receptors including receptor for advanced glycation end-products (RAGE), TLR2, TLR4, TLR9, and TIM3 (Figure 1).^{53,54} Importantly, the binding of HMGB1 to TLR4 on APCs was required to suppress tumor development, which is consistent with clinical study showing that breast cancer patients harboring a single-nucleotide polymorphism (Asp299Gly) in the *TLR4* gene undergo an early relapse after anthracycline treatment.^{30,55,56} In contrast, secreted HMGB1 could induce a protumor inflammation to facilitate tumor progression.⁵⁷ In addition, HMGB1 expression

is significantly associated with overall survival of patients with bladder cancer.⁵⁸ As HMGB1 is an intrinsic sensor of oxidative stress,⁵⁹ the immunomodulatory properties of HMGB1 might be determined by its redox status.^{60,61} Indeed, reduced HMGB1 production from dying cells was shown to trigger the immunogenic DCs, whereas oxidized HMGB1 during apoptosis fails.⁵¹ As the extracellular space is usually oxidative under physiological conditions but is unpredictably variable under pathogenic conditions,⁶² the unstable redox status of the tumor microenvironment might account for these inconsistent findings. However, the observation that the tumor microenvironment tends to be pro-oxidative⁶³ implies that a therapeutic approach using antioxidants to decrease ROS production would be favorable to stimulate antitumor immunity. Importantly, many anticancer agents, including chemotherapy,³⁰ radiation,²² or oncolytic viruses,^{9,64,65} have been shown to induce HMGB1 release from cancer cells, highlighting the significance of further addressing the mechanism of how these modalities affect the redox status of HMGB1.

Adenosine Triphosphate

Extracellular ATP released from apoptotic cells is another important factor in ICD induction. ATP signaling recognized by P2Y2 receptors on phagocytes as a 'find-me' signal enables

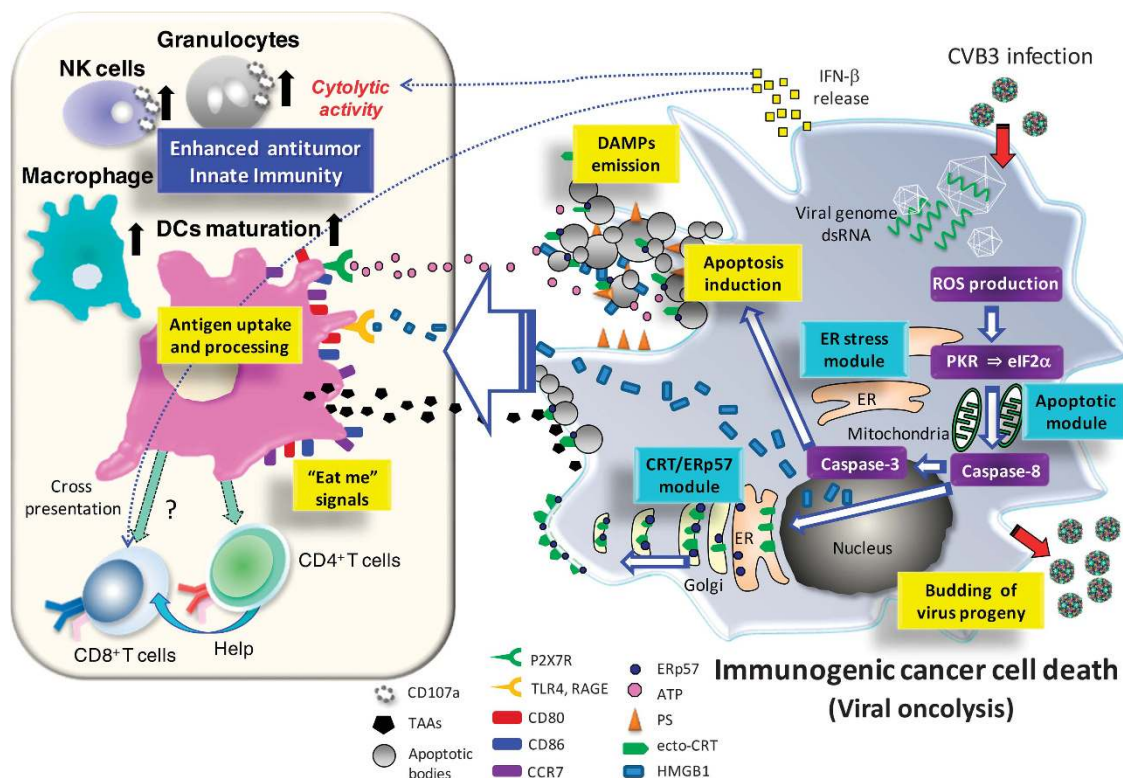


Figure 1 Oncolytic virus (CVB3) infection-triggered cancer cell death induces innate immune cell-mediated antitumor immunity. Intratumoral CVB3 infection-activated natural killer (NK) cells and granulocytes with enhanced expression of CD107a, a cytolytic degranulation marker, have been found to contribute to substantial antitumor effects as evidenced by NK cell and granulocyte depletion assays. Upon CVB3 infection, tumor cells can partially induce ecto-CRT on human tumor cells during early apoptosis, whereas majority of other viruses subvert ICD by circumventing ecto-CRT induction, and followed by robust release of DAMPs, including ATP and HMGB1, during later stages of cell death, which facilitates maturation of DCs via binding to Toll-like receptor 4 (TLR4)/RAGE and $P_2 \times 7R$, respectively. Viral genomes and/or viral progenies also stimulate DCs for their activation. Mature DCs may then efficiently phagocytose TAAs simultaneously released from dying cells and ultimately cross-present them to CD8⁺ T cells with the support with CD4⁺ T cells to elicit substantial antitumor immunity. Although ATP secretion relies on autophagic machinery, the other forms of cancer cell death, such as autophagic cell death and necroptosis, triggered by CVB3 infection have not yet been fully investigated

them to migrate into inflamed sites.⁶⁶ Indeed, ATP released from cancer cells treated with chemotherapeutic agents is essential for effective antitumor immune responses.⁶⁷ In addition, small interfering RNA-mediated inhibition of autophagic machinery abolishes ATP release from chemotherapy-treated tumor cells and mitigates the antitumor response.⁶⁸ Radiotherapy triggers ATP release from dying tumor cells through its interaction with the P2 × 7 purinergic receptor,⁶⁹ possibly resulting in the activation of the NLRP3–ASC–inflammasome axis and subsequent secretion of IL-1 β .⁷⁰

We and others recently showed that oncolytic viruses induce secretion of extracellular ATP from human cancer cells (Figure 1).^{9,65} Unlike ecto-CRT induction, the release of ATP and HMGB1 is triggered by a range of death-inducing stimuli, and is not restricted to induction in apoptotic cell death.⁴⁷ Although ATP production is required for efficient vaccinia virus production⁷¹ and facilitates HIV infection through its interaction with P2Y2 receptors,⁷² there is little knowledge of how oncolytic viruses provoke ATP release.

Autophagic Cell Death

Autophagy physiologically has catabolic roles, particularly in cell survival.⁷³ However, persistent autophagy causes a caspase-independent form of cell death that is, morphologically defined as autophagic cell death (ACD), termed as type 2 PCD, through lysosomal proteinase-regulated elimination of cellular organelles.^{74,75} Autophagy sometimes directs itself to cellular death, either in cooperation with apoptosis or as a back-up system, and thus is deemed as a cellular program with a 'double-faced' role.

Interestingly, the key molecules of autophagy and apoptosis pathways are intricately intertwined with shared several molecules including regulatory genes such as *p53* and *p19ARF*.⁷⁶ This crosstalk therefore can be viewed as a significant clue to understand the fate of dying cancer cells from therapeutic view points. Although ACD occurs without chromatin condensation but with massive autophagic vacuolization,⁷⁷ autophagy, often disabled in cancer, has been shown to be required for induction of immunogenicity.⁶⁸ First, dying cells in embryoid bodies that lack autophagy-related gene are unable to express the 'eat-me' signals and secrete lower levels of the 'come-get-me' signal, lysophosphatidylcholine.⁷⁸ Second, autophagy deficiency hinders ATP secretion from dying cancer cells, resulting in the impairment of DC recruitment and formation of adaptive immunity responses (Table 1).⁶⁸ Third, the inability of autophagy-deficient cancer cells to provoke antitumor immunity after chemotherapy can be reverted by suppression of extracellular ATP-degrading enzymes.^{68,79} Therefore, immunogenicity of ACD could be mediated by subtle spatiotemporal alterations in the treated cancer cells.

Novel strategy of autophagy inhibition is therapeutically effective for eliminating apoptosis-resistant cancer cells based on the rationale that growing tumors may harness autophagy as an adaptation to resist therapeutic stresses.^{80,81,82} Hence, more efforts should be made to elucidate the intricate interaction between autophagy inhibition and resulting effects on the immunogenicity.

Necrotic Cell Death and Necroptosis

Necrotic cell death is induced by external factors such as toxins, cancer, infections, and trauma, and is morphologically characterized by cellular swelling, rupture of the plasma membrane, and loss of cytoplasmic contents.⁸³ Understanding the immunogenicity of necrotic cell death is becoming important because it frequently induces robust inflammatory reactions to mount protective immune responses (Table 1).^{84,85,86} Although necrosis has long been viewed as non-PCD, its execution was shown to be controlled by specific signal-transduction pathways and catabolic mechanism.^{87,88,89} This alternative form of necrotic PCD, aptly termed necroptosis (type 3 PCD), is induced by tumor necrosis factor (TNF) receptor signaling that involves activation of the receptor-interacting protein (RIP) family. Upon inhibition of apoptotic pathway by the caspase inhibitor, activation of RIP1 and RIP3 kinase leads to mitochondrial instability and cell death.^{90,91} Phosphorylated RIP1 and RIP3 generate a molecular complex called the necrosome, which initiates necroptosis. ROS production under necroptosis has been shown to facilitate TNF- α -induced cell death by sustaining c-Jun N-terminal kinase activation.⁹² Intriguingly, necroptosis can also be executed via stimulation by apoptosis-inducible ligands such as TNF- α , FasL, or TRAIL (Table 1). Notably, cytotoxic agents are shown to induce necrotic cell death in apoptosis-defective cancer cells,⁹³ probably because necroptosis is principally induced when a cell cannot die via apoptotic pathways.⁹⁴ On the other hand, conventional therapy-resistant cancer stem cells (CSCs) have a higher antiapoptotic activity than that of their counterparts.^{95,96} Therefore, it would be vital to clarify the key machinery of not only the necroptosis induction in cancer cells for CSC-directed therapeutic application but also the resultant immunogenicity to modulate antitumor immunity.

Pyroptosis

Pyroptosis is a recently identified form of PCD stimulated by microbial infections and non-infectious stimuli such as myocardial infarction and cancer. In contrast to apoptosis, pyroptosis is uniquely mediated by caspase-1 activity triggered by the formation of a cytosolic complex termed the 'inflammasome', resulting in highly inflammatory outcomes (Table 1). Pyroptotic cells represent morphological characteristics, some of which are shared with apoptosis and necrosis.⁹⁷ The function of activated caspase-1 is to cleave proteolytically the proforms of the proinflammatory cytokines, IL-1 β and IL-18, to their active forms.⁹⁷ Although pyroptosis has been intensively studied in the context of bacteria-infected macrophages,⁹⁸ it can also be triggered in human cancer cells infected with recombinant herpes simplex virus 2 (HSV-2) (Table 4).⁹⁹ Pyroptotic cancer cells induced by microbial infection have been recently shown to facilitate phagocytosis by macrophages, presumably through their PS exposure and ATP release.¹⁰⁰ Accordingly, the caspase-1-dependent generation of proinflammatory cytokines and other DAMPs could be essential factors to provide a suitable inflammation for ICD induction.

Table 4 DNA oncolytic viruses and their differential properties to induce either multiple forms of cell death or antitumor immunity

Oncolytic viruses	Type of cancer cells	Type of cell death induced	DAMPs	Possible mechanism of antitumor immunity
hTERT-Ad: CRAds regulated by human hTERT promoter	Human glioma, cervical and prostate cancer	Autophagy	NA	NA
hTERT-Ad	Human lung cancer	Autophagy via E2F1-miR-7-EGFR	NA	NA
OBP-702: p53-armed hTERT-Ad	Human osteosarcoma	Apoptosis Autophagy	NA	NA
CRAd-S-RGD: Ad5 carrying the RGD motif and survivin promoter	Human glioma cells	Autophagy	NA	NA
Ad5/3-hTERT-E1A-hCD40L: chimeric Ad5/3 capsid with hTERT promoter	Murine urothelial carcinoma, melanoma	Apoptosis	Ecto-CRT, ATP and HMGB1	Enhanced recruitment of macrophages and CD8 ⁺ T cells
ZD55-IFN- β : Oncolytic adenovirus carrying IFN- β	Human hepatoma, breast cancer	Apoptosis Necroptosis	NA	NA
Vaccinia virus	Human colon, breast, ovarian cancer	Not apoptosis Possibly necrosis	HMGB1 release	NA
vSP: antiapoptosis genes, SPI-1- and SPI-2-deleted vaccinia virus	Murine colon adenocarcinoma	Apoptosis Necrosis	HMGB1 release	NA
HSV2: Human simplex virus 2	Human endometrial cancer	Apoptosis Necrosis	HMGB1 release	NA
HSV-1716: a replication-restricted mutant herpes simplex virus	Murine ovarian cancer	NA	NA	Intratumoral injection induced IFN- γ , CXCL9 and CXCL10 with increase in NK and CD8 ⁺ T cells
HSV-2 mutant Δ PK: ICP10PK-deleted HSV-2 virus	Human melanoma cells	Apoptosis Pyroptosis	NA	Dominant induction of CD4 ⁺ Th1 cells

Abbreviations: hTERT, telomerase reverse transcriptase; CRAds, conditionally replicative adenoviruses; miR-7, microRNA-7, EGFR, epidermal growth factor receptor, ecto-CRT, calreticulin exposure; DAMPs, damage-associated molecular patterns; ICD, immunogenic cell death, HMGB1, high-mobility group protein B1; ATP, adenosine triphosphate; IFN- β , interferon- β ; HSV, herpes simplex virus; IFN- γ , interferon- γ ; CXCL9, chemokine (C-X-C motif) ligand 9; CXCL10, chemokine (C-X-C motif) ligand 10; NK, natural killer cells; NA, not assessed; Th1, T helper type 1

DAMPs Induced by Infection with Oncolytic Viruses

Because oncolytic viral infection can produce abundant PAMPs, including viral proteins and nucleic acids, followed by the release of DAMPs and the entire repertoire of TAAs from treated tumors,¹⁰¹ oncolytic virus-triggered ICD may be more effective for induction of antitumor immunity. As viruses have developed sophisticated machineries to evade apoptotic cell death and interfere with ER stress and autophagy responses for their survival,^{102,103} ICD may have played an essential role in the everlasting war between viruses and their hosts. We and other groups have found that many oncolytic viruses can induce apoptotic cell death and/or necrosis in cancer cells,^{9,104,105,106} supporting their immunostimulatory potential to augment antitumor efficacy (Tables 4 and 5).^{107,108} CVB3 infection induces multiple DAMPs including ecto-CRT, HMGB1 translocation from nuclei, and ATP release from human lung cancer cells. Importantly, intratumoral CVB3 administration can prominently recruit cytolytic degranulation marker CD107a-mobilized NK cells and granulocytes, and mature DCs into the tumor bed (Figure 1).^{9,27} As pathogenic viruses have developed their strategies to subvert ecto-CRT and circumvent ICD induction,¹⁰⁹ it is noteworthy that CVB3 infection can induce

ecto-CRT accompanied by other DAMPs.⁹ Furthermore, we demonstrated that both NK cells and granulocytes substantially contributed to the CVB3-mediated antitumor efficacy as evidenced by *in vivo* depletion assays.⁹

Upon intratumoral replication of oncolytic viruses, resultant alterations in tumor microenvironment may restore the compromised antitumor immunity, presumably through induction of IFNs and/or cytokines that activate NK cells and APCs.^{110,111} Although tumor-infiltrating DCs were impaired at maturation by immunosuppressive IL-10, PGE₂, and transforming growth factor β produced from tumor cells,¹¹² unidentified components in the culture media from reovirus-infected cancer cells facilitated maturation of DCs.¹¹³

Recent studies delineated that oncolytic viruses such as vaccinia, measles, HSV-2, and adenovirus cause the release of HMGB1.^{64,65,114,115,116,117} Although HMGB1 interacts with viral components and may modulate viral replication,¹¹⁷ the molecular mechanisms of how each oncolytic virus differentially produces these DAMPs remain largely elusive.

Multimodal PCD Induced by Oncolytic Viruses

We showed that approximately 20% of CVB3-mediated cytotoxicity of A549 cells resulted from apoptotic cell death.⁹

Table 5 RNA oncolytic viruses and their differential properties to induce either multiple forms of cell death or antitumor immunity

Oncolytic viruses	Type of cancer cells	Type of cell death induced	DAMPs	Possible mechanism of antitumor immunity
Edmonston vaccine strain of MV	Human melanoma	NA	IL-6 HMGB1 release	Human DC maturation Priming an adaptive T-cell response
MV-NPL: genetically engineered MV	Human renal cell carcinoma	Apoptosis	NA	NA
MV-CEA:Edmonston vaccine MV genetically engineered to produce CEA antigen	Human breast cancer	Apoptosis	NA	NA
CVB3	Human non-small-cell lung cancer	Apoptosis	Preapoptotic ecto-CRT, HMGB1 translocation, ATP release	Phenotypic activation of immature DCs and lytic NK cells in tumors. Deletion of NK and granulocytes abrogated the CVB3-induced <i>in vivo</i> antitumor immunity
NDV	Human glioma	Autophagy	NA	NA
Reovirus	Human multiple myeloma	Apoptosis Autophagy	NA	NA
Live-attenuated poliovirus	Human neuroblastoma	Apoptosis	NA	NA
M51R: M protein mutant VSV	Human glioblastoma multiforme	Apoptosis	NA	NA
Interferon-sensitive VSV (AV3 strain)	Human prostate cancer	Apoptosis	NA	NA

Abbreviations: MV, measles virus; CVB3, coxsackievirus B3; NDV, New castle disease virus; CEA, carcinoembryonic antigen; VSV, vesicular stomatitis virus

This induction was presumably due to the capacity of CVB3 infection to induce PKR-mediated phosphorylation of eIF2 and caspase-8-mediated activation of proapoptotic mediator, caspase-3 (Figure 1).^{118,119} Other DNA and RNA oncolytic viruses have been reported to induce apoptotic cancer cell death (Tables 4 and 5). However, there are only two reports showing that virus-induced ecto-CRT was correlated with enhanced intratumoral infiltrations of immune subpopulations, which accounted for the '*in vivo*' remarkable antitumor immunity.^{9,65}

Several studies showed that recombinant oncolytic adenoviruses induced ACD in human malignant glioma cells,¹²⁰ brain tumor stem cells,¹²¹ osteosarcoma cells,¹⁰⁵ and lung cancer cells.¹²² Newcastle disease virus also triggered autophagy in glioma cells to promote its viral replication.¹²³ Reovirus-mediated oncolysis of multiple myeloma was reported to be orchestrated via upregulation of autophagy.¹²⁴ Because cancer cells are largely refractory to apoptotic inducers but vulnerable to necroptosis,³⁹ overcoming anticancer drug resistance may be achieved by activation of necroptotic rather than apoptotic pathways, where the former might be the intrinsic 'Achilles heel' of cancers.¹²⁵ So far only recombinant adenovirus has been shown to facilitate both necroptotic and apoptotic cell death with a synergistic effect on cancer cells when combined with doxorubicin (Tables 4 and 5).¹²⁶ In addition, most oncolytic viruses may induce pyroptotic cancer cell death accompanied by abundant proinflammatory cytokines and DAMPs. Accordingly, some oncolytic viruses may induce multimodal

ICD, allowing them to be a plausible modality as promising agents of immunotherapy.

Strategies to Enhance the Potentials of ICD Induced by Oncolytic Viruses

Besides DAMPs, massive production of type I IFNs (IFN- α/β) upon oncolytic viral infection can be a potent immunomodulator through their indirect immunostimulatory effects on neutrophils and T cells,^{127,128} as well as through their direct antiproliferative effects.¹²⁹ Despite a creation of multimodal ICD by oncolytic viruses to facilitate antitumor immunity, much attention should be paid to the preferential antiviral immunity that might impede direct viral oncolysis-mediated tumor destruction. To avoid this, cyclophosphamide is shown to retard immune removal of oncolytic viruses, enhancing the persistence of viral infection.¹³⁰ Another promising strategy to overcome antiviral immunity could be potentiating immune responses by gene modification of oncolytic viruses to arm them with immunostimulatory cytokines, such as GM-CSF, IL-2, IL-12, and IL-15. Indeed, the results of clinical trials of the GM-CSF gene-harboring oncolytic vaccinia virus JX-594 and the GM-CSF gene-harboring oncolytic herpes virus talimogene laherparepvec demonstrated that a clinical benefit can be accomplished by combined respective oncolytic activity with the recruitment of immune cells.^{6,7,131} The combination of adoptive T-cell therapy with oncolytic viruses is shown to elicit an increased antitumor effect.^{131,132} Collectively, the design of combinatorial therapies of oncolytic viruses with immunotherapeutic modalities may

hold the key to mount maximally a multifaceted attack against cancers.

Conclusions

Although mechanism of ICD induction is a very complicated process, we need to elucidate how dying cells become much more stimulatory in shaping antitumor immune responses than was ever expected. Notably, intermediate death processes, including caspase activation, mitochondrial degradation by autophagy, ROS production, and oxidative modification of DAMPs, have been found to fine-tune the balance between antitumor tolerance and immunity, providing implications in manipulation of ICD.

Four forms of PCD, apoptosis, autophagy, necroptosis, and pyroptosis, may jointly decide the fate of cells of malignant cells. However, in terms of immunogenicity, investigations of only apoptotic cell death in cancer cells have just begun. Therefore, further elucidation of determinants of respective PCD-inducing pathway and characterization of resultant ICD should aid to develop novel anticancer strategies. A recent review advocates a list of characteristics for an ideal ICD inducer,²⁷ as follows: (1) efficiently activates apoptosis or necrosis leading to emission of multiple DAMPs and TLR agonists;^{133,134} (2) irrelevant in drug-efflux pathways;¹³⁵ (3) can induce ER stress;¹³⁴ (4) has negligible suppressive or inhibitory effects on immune cells;¹³⁶ (5) counteracts immunosuppressive responses;^{136,137} and (6) directly targets not only the primary tumor but also metastases.¹³⁸ No ideal ICD inducer exists, but it is important to seek for ideal combinatorial therapies that could achieve these properties. Of the currently known relevant ICD inducers, those that meet most of these properties include mitoxantrone, hypericin-PDT, and shikonin. However, diverse oncolytic viruses could be the promising ICD inducer as we gain more knowledge about the properties yet to be investigated. Evidently, they can destroy conventional therapy-resistant CSCs,¹³⁹ possibly through their ability to induce distinctive PCD and/or modification to express genes that target CSC-specific signaling pathways underpinning their cell survival.¹⁴⁰

Gaining more detailed insights into the mechanisms of ICD induction, to be perceived by the immune system, will not only ameliorate the development of promising anticancer agents or combinatorial therapies but also offer useful knowledge in various life science fields including virology, immunology, and clinical medicine.

Conflict of Interest

The authors declare no conflict of interest.

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