BBA - Reviews on Cancer 1871 (2019) 99–108

Contents lists available at ScienceDirect

BBA - Reviews on Cancer

journal homepage: www.elsevier.com/locate/bbacan

Review

An emerging role for nanomaterials in increasing immunogenicity of cancer cell death

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ARTICLE INFO

Keywords:
Nanoparticles
Nanocarriers
Biomaterials
Apoptosis
Necroptosis
Immune response
Immunogenicity
Cell death

ABSTRACT

In the last decade, it has become clear that anti-cancer therapy is more successful when it can also induce an immunogenic form of cancer cell death (ICD). ICD is an umbrella term covering several cell death modalities, including apoptosis and necroptosis. In general, ICD is characterized by the emission of damage-associated molecular patterns (DAMPs) and/or cytokines/chemokines, leading to the induction of strong anti-tumor immune responses. In experimental cancer therapy, new observations indicate that the immunogenicity of dying cancer cells can be improved by the use of biomaterials. In this review, after a brief overview of the basic principles of the concept of ICD and discussion of the potential use of DAMPs as biomarkers of therapy efficacy, we discuss an emerging role of nanomaterials as a promising strategy to modulate the immunogenicity of cancer cell death. We address how nanocarriers can be used to increase the immunogenicity of ICD and then turn our attention to their dual action. Nanocarriers can be used to increase the immunogenicity of dying cancer cells and to reduce the side effects of chemotherapy. Future studies will show whether biomaterials are truly an optimal strategy to modulate the immunogenicity of dying cancer cells and will provide the insights needed for the development of novel treatment strategies for cancer.

1. Introduction

For many years, cancer has remained one of the most serious socially significant concerns for world health due to its association with high mortality and disability. Contemporary anti-tumor treatments consist mainly of surgery (1st strategy), radiotherapy and chemotherapy (2nd strategy) and immunotherapy (3rd strategy). In the treatment of cancer, these strategies are often used in different empirical combinations, and radio- and chemotherapy often precede surgery. Not all patients respond to treatment despite its durable effect; therefore, sometimes it can lead to adverse results. Recent studies indicate that a combination of anti-cancer therapies is most effective when the therapies work in synergy [1,2]. In recent years, it has been reported that the 2nd strategy of therapy (chemotherapy and/or radiotherapy) is more successful when these therapies can also induce an immunogenic form of cell death (ICD), which is associated with induction of an anti-tumor immune response that leads to tumor eradication [3].

One of the main characteristics of cancer cells undergoing ICD is their ability to emit immuno-stimulatory molecules (Fig. 1), among which are damage-associated molecular patterns (DAMPs) [4,5] and cytokines/chemokines, both of which contribute to attraction of antigen presenting cells (*e.g.* dendritic cells, DCs), which engulf dying cancer cells [6–8]. This process culminates in the cross-presentation of antigenic peptides on major histocompatibility complex class I (MHC I) molecules to $CD8^+$ T cells of the adaptive immune system, one of the main driving forces of anti-tumor immune responses (Fig. 1). Thus, triggering ICD-inducing modalities could be a suitable strategy for killing cancer cells while simultaneously eliciting broad antitumor T cell responses. However, it has become clear that not all cancer cell types can die in an immunogenic way, and there is a need to develop novel strategies that enhance the immunogenicity of cancer cell death.

In this review, after a brief overview of the basic principles of the ICD concept, we discuss an emerging role for nanomaterials as a

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https://doi.org/10.1016/j.bbcan.2018.11.004
Received 26 September 2018; Received in revised form 5 November 2018; Accepted 8 November 2018
Available online 06 December 2018
0304-419X/

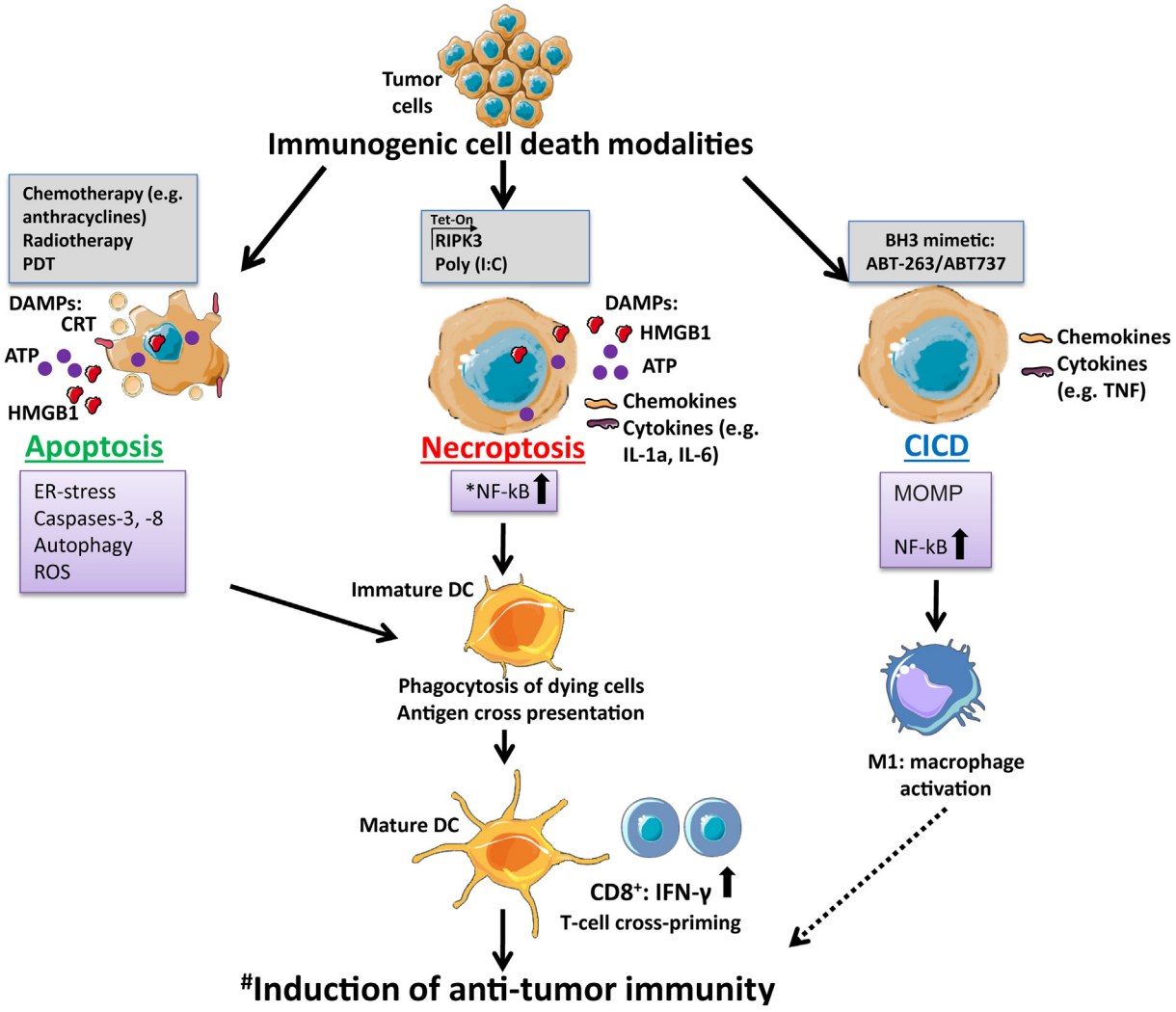

Fig. 1. Immunogenic cancer cell death modalities at a glance.
Immunogenic cell death is an umbrella term covering several cell death modalities, such as apoptosis, necroptosis and caspase-independent cell death. Although these cell death modalities differ from each other morphologically and biochemically (signaling), they are all characterized by the emission of immune-stimulatory molecules (*i.e.*, DAMPs and/or chemokines or cytokines). Immunogenic apoptosis is characterized by ROS-based ER stress and induction of autophagy, and its immunogenicity is caspase-dependent. At the cell surface of immunogenic apoptotic cancer cells, there is exposure of CRT, secretion of ATP, and passive release of HMGB1 due to plasma membrane rupture. Necroptosis can be induced, for example, by Tet-on inducible expression of RIPK3 or other stimuli (*e.g.*, Poly (I:C)) [29,30]. It is characterized by passive release of ATP and HMGB1, and release of chemokines and cytokines in addition to DAMPs [28,83], which might contribute to the immunogenicity.
*Although activation of NF-kB has been described as one of the important prerequisites of necroptosis immunogenicity [28], the immunogenicity of NF-kB independent necroptosis has also been described [29,84]. Recently, it has been reported that BH3 mimetics (ABT-263/ABT-737) can induce a specific form of cell death independently from caspase activation, *i.e.* caspase-independent cell death (CICD) [32]. These BH3 mimetics can cause caspase-independent cell death (CICD), which is more immunogenic than apoptosis and has greater anti-tumor activity. In the presence of CICD, anti-tumor activity is dependent on NF-kB and the emission of cytokines/chemokines (*e.g.* TNF) from dying cancer cells as well as on the preserved immunity of the host. Cancer cells undergoing CICD induce differentiation of macrophages into the M1 phenotype. It is noteworthy that necroptosis is not important for the anti-tumor effects of CICD.
#Cancer cells undergoing ICD induce anti-tumor immunity, which has been validated in tumor prophylactic vaccination mice models and in syngeneic tumor models more closely resembling the therapeutic settings. Induction of ICD promotes DC maturation, which leads to optimal antigen presentation to $CD8^+$ T cells and induction of anti-tumor immunity, resulting in effective suppression of tumor growth and/or regression of neoplasia.
Abbreviations: ATP, adenosine triphosphate; CICD, caspase-independent cell death; CRT, calreticulin; DC, dendritic cell; ER-stress, endoplasmic reticulum stress; HMGB1, high-mobility group Box 1; ICD, immunogenic cell death; IFN, interferon; IL, interleukin; MOMP, mitochondrial outer membrane permeabilization; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; PDT, photodynamic therapy; ROS, reactive oxygen species; TNF, tumor necrosis factor.

promising strategy to modulate the immunogenicity of cell death and how they can be used to increase the immunogenicity of cancer cell death. We also provide a comprehensive and critical perspective on the most recent advances in the use of the DAMPs that are associated with ICD (CRT and HMGB1) as biomarkers of therapy efficacy. Finally, we raise some questions for future research and discuss how future findings could be applied to design novel experimental anti-cancer treatments.

2. ICD concept: in brief

Activation of the immune system in cancer treatment goes back to the 19th century, when the American surgeon William B. Coley used infection with a mixture of killed *Streptococcus pyogenes* and *Serratia marcescens* (nowadays called Coley's toxins) to treat patients with non-operable sarcomas (Box 1). However, because of the rapid development of chemotherapy and radiotherapy, in subsequent years the role of the

Box 1
Cell Death Modalities.

Apoptosis. Morphologically, apoptosis is characterized by chromatin condensation, cleavage of chromosomal DNA into internucleosomal fragments, cell shrinkage, membrane blebbing and formation of apoptotic bodies without plasma membrane breakdown. Biochemically, it involves the activation of caspases, a highly conserved family of cysteine-dependent aspartate-specific proteases that can be inhibited by zVAD-fmk. Immunologically, apoptotic cell death during physiological processes is anti-inflammatory. Importantly, certain types of anti-cancer therapy can induce a specific form of apoptosis – immunogenic apoptosis – which can induce maturation of DCs.

Immunogenic apoptosis. This form of cell death has the same morphological characteristics as apoptosis. At the biochemical level, in addition to caspase activation, the immunogenicity of apoptosis often requires the induction of ER stress and reactive oxygen species (ROS). It is associated with the emission of DAMPs, such as surface exposure of CRT, secretion of ATP, and release of HMGB1. The immunogenicity of a cell line is defined based on whether vaccination of immunocompetent mice with *in vitro* killed cells confers a survival advantage after subsequent challenge with live cells of the same cell type.

Accidental necrosis. This is an unregulated cell death modality that can be caused by extreme physicochemical stress (*e.g.*, heat shock and freeze-thawing). It is associated with the release of DAMPs. This cell death type was initially contrasted to apoptosis.

Regulated necrosis. This is an umbrella term covering several necrotic cell death modalities (*e.g.* necroptosis, ferroptrosis and pyroptosis) sharing common morphological features, such as cell and organelle swelling and rupture of the plasma membrane. But these necrotic cell death modalities are biochemically regulated by different mechanisms.

Necroptosis. This cell death mode can be blocked by inhibitors of RIPK1 (*e.g.* Nec-1 s), RIPK3 (*e.g.* GSK'872) and MLKL (*e.g.* necrosulfonamide for human cells only). It is characterized by the absence of caspase activation and thus insensitivity to pan-caspase blockers such as zVAD-fmk. It often occurs when caspase-8 is downregulated or inhibited [25,71]. Necroptosis is associated with emission of DAMPs (*e.g.* HMGB1 and ATP) and cytokines/chemokines and is usually immunogenic.

Ferroptosis. This is characterized by iron-dependent ROS production and lipid peroxidation and is executed *via* oxygenation of polyunsaturated phosphatidylethanolamines (PE) by 15-lipoxygenases (15-LO), which normally use free polyunsaturated fatty acids as substrates. It can be induced by reduction of GSH level by blocking the x_c^- system, the glutamate/cysteine antiporter, or GPX4 activity. PEBP1 is an emerging master regulator of ferroptosis. It can be blocked by blockers of lipid peroxidation (*e.g.* Fer-1, vitamin E and liproxstatins).

Pyroptosis. This cell death type is initiated by inflammasomes, which drive activation of caspase-1 or caspase-11, −4 and − 5, leading to the cleavage of gasdermin D. It is associated with the release of IL-1β and IL-18.

Entosis. It is a form of epithelial cell cannibalism involving the engulfment of viable cells by non-phagocytic cells of the same (homotypic) or a different (heterotypic) type and leading to formation of a so-called 'cell-in-cell' structure. Often (but not always), engulfment is followed by the death of internalized cells. Entosis can be triggered by matrix deadhesion, mitosis and glucose starvation [72,73].

immune system as one of the main players in anti-cancer therapy was mainly neglected.

After the first morphological description of apoptotic cell death [9], it was firmly believed that cancer cells undergoing apoptosis steer the extracellular environment to an anti-inflammatory state and thereby contribute to an immunosuppressive network at the primary tumor site, promoting further tumorigenesis [10–15]. However, the revival of interest in cancer cell death as a potential activator of the immune system, which is one of the key elements of successful anti-cancer therapy, started mainly from the concepts of the "danger theory" by Polly Matzinger in 1994. This theory states that the immune system can discriminate self from non-self and distinguish dangerous from innocuous signals [4,5]. The current concept of ICD stands on the shoulders of the "danger theory." Although this theory was purely theoretical at that time, it proposed that antigen presenting cells (APC) can be activated by danger/alarm signals derived from injured cells, such as those exposed to pathogens, toxins, or mechanical damage. Since then, many danger signals and damage associated molecular patterns (DAMPs) have been proposed. They can be constitutive or inducible, passively or actively secreted, and derived from the cytosol, nucleus, mitochondria, or endoplasmic reticulum, or even be part of the extracellular matrix. They are either proteins that are often modified by proteolysis and/or oxidation, or non-proteins, *e.g.* nucleotides. One important prerequisite for an immuno-stimulatory molecule to be considered a DAMP is that it has a mainly non-immunological function inside cells but acquires immuno-stimulatory properties once it is exposed outside the cells.

In the late nineties, several reports appeared on the immuno-stimulatory role of cells undergoing apoptosis (Box 2) and their ability to induce the maturation of APC [16]. The ICD concept states that certain types of anti-cancer treatments, such as chemotherapeutics (*e.g.* anthracyclines and oxaliplatin) [3], gamma-irradiation [17,18] and photodynamic therapy [19] induce a specific form of apoptosis, namely immunogenic apoptosis (Box 2). This form of cell death is associated with the emission of DAMPs (*e.g.* surface exposure of CRT, secretion of ATP and release of HMGB-1). DAMPs function as adjuvants that stimulate the innate and adaptive immune systems by activating membrane-bound or cytoplasmic pattern recognition receptors (PRRs, *e.g.* Toll-like receptor, TLR-4), phagocytic or scavenger receptors (*e.g.* LDL-receptor-related protein, LRP1/CD91), and purinergic receptors (*e.g.*, P2RX7/P2RY2). Induction of the maturation of DCs by DAMPs mediates the induction of the anticancer immune response. Apoptosis is the first potentially immunostimulatory cell death modality to be described [3], in marked contrast to the initial notion that it is a silent or anti-inflammatory mode of cell death [10–15].

The immunogenicity of apoptosis at the molecular level is highly dependent on the induction of endoplasmic reticulum stress [19–21], autophagy [22–24] and production of reactive oxygen species [24]. It has become clear that immunogenicity is linked not only to apoptosis but also to other cancer cell death modalities, including necroptosis (Box 2), a regulated from of necrosis [25–27]. Indeed, the recently described immunogenicity of necroptosis in experimental cancer models has attracted substantial interest as an alternative strategy for killing cancerous cells to induce anti-tumor immunity [28–31]. In this context, triggering other cell death modalities in cancer cells (*i.e.* necroptosis, a caspase independent cell death [32]) represents an attractive alternative for overcoming apoptosis resistance, which often occurs during cancer therapy and is one of the hallmarks of cancer [33]. These data also suggest that these immunogenic properties are independent of the type of cell death cancer cells undergo but are dependent on the type of cancer cells and on cell death stimuli. Therefore, ICD is an umbrella term covering all cell death types that have immunogenic

Box 2
Historical background: from Coley's toxins to the immunogenic cell death concept.

In the early 1700s, it was observed in clinical practice that some cancer patients who developed a bacterial infection had a remission from cancer [74]. About 100 years later, in 1881, the American surgeon William B. Coley (the father of immunotherapy) tested this observation by using erysipelas infection to treat patients with non-operable sarcoma [75]. Of interest, Coley was not the first to undertake such a trial. In 1868, W. Busch [76] made a similar attempt but on a smaller scale, even before the streptococcal etiology of erysipelas was known, and similar work was repeated by F. Fehleisen in 1882, who discovered that streptococcus is the etiologic agent of erysipelas [74]. However, at that time it was extremely problematic to control the process of infection. Some patients required re-infection while in others the infection did not develop at all. After unsuccessful attempts, in 1892 Coley began to use a vaccine composed of heat-killed Gram-positive *Streptococcus pyogenes* and Gram-negative *Serratia marcescens* (formerly known as *Bacillus prodigiosus*). By 1909, this combination (now known as Coley's toxins) yielded some positive results in clinical practice [77] However, at that time there was no understanding of the mechanisms of the positive effects of infection on the reduction of tumor growth. Moreover, after Coley's death in 1936 and because of the emergence of chemotherapy and radiotherapy, interest in such vaccines decreased sharply. Nevertheless, Coley's work is one of the first reports of the positive effect of induction of inflammation in the tumor bed on tumor treatment. In the 1960s, when it became evident that long-term control of cancer was not possible with the available treatments, interest in the clinical use of vaccines reemerged [78–80]. In the late 1980s, it was shown that immune cells can recognize pathogen-infected cells through recognition of pathogen-associated molecular patterns (PAMPs), which are of viral or bacterial origin or are certain nucleic acid sequences of their genomes [81,82]. The current ICD concept [3] is based on the "danger theory," which was proposed in 1994 by Polly Matzinger [4,5].

properties.

3. DAMPs as biomarkers to assess the efficacy of anti-cancer therapy

In parallel with the development of the ICD concept, it has become clear in several cohorts of cancer patients that several DAMPs and DAMP-associated factors (*e.g.*, DAMP and PRR expression levels, genetic polymorphisms in genes encoding DAMPs or PRR) could have a prognostic or predictive value. In this section, we focus mainly on CRT and HMGB1 (Table 1). For discussions of the role of PRR expression levels in cancer, we direct the reader to recently published reviews [34,35].

3.1. CRT

Several research groups considered the changes in CRT expression as a useful indicator in cancer diagnosis and treatment planning (Table 1). A study on 68 patients with neuroblastoma showed that increased CRT is positively associated with tumor differentiation and a favorable outcome. In that study [36], it was shown that increased CRT expression predicts longer survival of patients with advanced-stage neuroblastoma. Moreover, the increased CRT levels in urinary samples could serve as a diagnostic marker of bladder carcinoma [37]. Detection of the level of CRT expression can also be considered as a powerful prognostic tool for overall survival of patients. It has been shown in two independent cohorts of patients with non-small cell lung cancer (receiving neoadjuvant chemotherapy or not) totaling > 350 patients that regardless of the type of treatment, high levels of CRT were associated with longer survival. One of the reasons for the positive dynamics was activation of adaptive immune responses in the tumor microenvironment and infiltration of mature DCs and effector T-cell subsets, which was possibly triggered by CRT overexpression [38]. That study indicates that CRT can be used as a prognostic biomarker that reflects the state of local anti-tumor immune responses in the lungs. Similar clinical observations were made on patients with acute myeloid leukemia (AML). Determination of CRT in the peripheral blood of 50 AML patients established a positive correlation between CRT exposure on the plasma membrane of malignant blasts and the frequency of circulating T cells specific for leukemia-associated antigens. This means that surface exposure of CRT favors the initiation of anticancer immunity and increases overall survival of patients with AML [39]. Although all these studies point to increased expression of CRT as a favorable prognostic marker, it is necessary to mention the other face of CRT. It has been shown in 79 patients with gastric cancer that increased expression of CRT in tumor tissue was associated with unfavorable prognosis and increased risk of early cancer-related death. In combination with experimental work on cell cultures, the authors showed that CRT overexpression increases cell proliferation and migration and upregulates the expression and secretion of proangiogenic factors (*e.g.* VEGF, PlGF)

Table 1
Prognostic and diagnostic aspects of DAMPs (CRT and HMGB1) in several types of cancer.

Type of cancer	DAMPs level and prognosis	Type of biomaterials	Refs
Neuroblastoma	Increased CRT: positive prognosis.	Tissue sections of tumor (immunohistochemical study)	[36]
Gastric cancer	Increased CRT: high risk for early cancer-related death.	Tumor tissue	[40]
Bladder urothelial carcinoma	Increased urinary CRT: diagnostic criterion of bladder tumor.	Urine specimens	[37]
Lung carcinoma	CRT: correlates with malignancy and tumor grade.	Serum samples	[85]
Non-small cell lung cancer (NSCLC)	Increased CRT: positive prognosis.	Tumor tissue	[38]
Acute myeloid leukemia (AML)	Increased CRT: positive prognosis.	Peripheral blood[serum, peripheral blood mononuclear cells (PBMCs)]	[39]
Esophageal squamous cell carcinoma (ESCC)	HMGB1: expression increases overall survival. CRT-strong and CRT-weak patients: no survival difference.	Peripheral blood lymphocytes (PBL). Formalin-embedded tumor samples.	[44]
Localized breast cancer	Increased nuclear HMGB1 in combination with LC3B: positive life expectancy.	Breast cancer surgical specimens.	[86]
Early breast cancer	Immediate increasing HMGB1: improved outcome.	Peripheral blood (plasma samples).	[42]
Melanoma	HMGB1: no significant correlation between its expression and patient survival.	Metastatic melanoma samples.	[46]
Breast cancer	HMGB1 and CRT expression: no effect on pathological response and overall survival.	Pre-treatment biopsy specimens and surgically resected specimens.	[45]

in gastric cancer cells, and also regulates the cancer progression-related gene CTGF [40]. Therefore, additional studies are needed to resolve this controversy and to determine whether these differences are cancer-type dependent.

3.2. HMGB1

It has been reported that increased HMGB1 in the peripheral blood during the first few days of combined chemotherapy with epirubicin and docetaxel can indicate early response to chemotherapy (Table 1). Indeed, a significant increase in HMGB1 was detected only in patients who eventually showed pathological tumor regression, and no change in HMGB1 was detected in the non-responder group [41]. Furthermore, studies on 36 patients with early breast cancer showed that an early increase in HMGB1 levels in the peripheral blood in response to chemotherapy correlates with long-term survival [42]. Therefore, the level of HMGB1 could be used as a valuable complementary biomarker for early estimation of prognosis. Immunohistochemical analysis of tumor specimens obtained from 232 patients with breast carcinoma revealed that increased HMGB1 staining was associated with a smaller tumor size. The loss of nuclear staining for HMGB1 was observed more frequently in larger tumors [43]. A positive correlation between the level of HMGB1 and survival of patients with esophageal squamous cell carcinoma was also shown [44]. Yet, it is important to mention another face of HMGB1. It has been shown that conventional chemotherapy alone significantly induced the upregulation of HMGB1 in breast cancer and esophageal squamous cell carcinoma, indicating that some degree of ICD can be significantly induced after chemotherapy. However, there was no significant correlation between the level of HMGB1 and pathological response after neoadjuvant chemotherapy, or between the level of HMGB1 and survival [45]. It is important to mention that in another study no significant prognostic value was seen in any of the tested biomarkers of immunogenic cell stress and death in melanoma: microtubule-associated proteins 1A/1B light chain 3B (MAP-LC3B, best known as LC3B), presence of nuclear HMGB1, phosphorylation of eIF2α, and increase in cancer cell ploidy [46].

Taking all these clinical studies into consideration (Table 1), it is clear that determination of the levels of DAMPs (CRT and HMGB1) in biological materials can be used to assess the response to therapy and to predict the course of the disease. However, these markers are not universal and can only be used for certain types of cancer or certain cohorts of patients. The main question for future research is to understand why the same DAMPs in one type of cancer are associated with a better prognosis, while in other types they are associated with a worse prognosis. To answer that question, it will be essential to characterize the antigenic repertoire of tumors and to perform spectral analysis of DAMPs and their modification states in different types of cancer.

4. Biomaterials and immunogenicity of cancer cell death

Delivery of different chemotherapeutics *via* nanomaterials, including liposomes, synthetic polymers, micelles, and inorganic nanostructures, has been intensively investigated. In this section, we will focus mainly on the chemotherapeutics belonging to the class of ICD inducers and discuss how nanomaterials can be used to modulate the anti-tumor immune responses induced by immunogenic cell death (Table 2, Fig. 2).

4.1. Nano and micro-particles: an emerging strategy to increase the immunogenicity of dying cancer cells

It has been shown that the delivery of ICD inducers packed into nanoparticles significantly increases their ICD potential (Table 2). Zhao et al. [47] have shown that packaging into polymeric nanoparticles [monomethoxy-poly(ethylene glycol)-poly(d,L-lactide-co-glycolide)] increases the *in vitro* and *in vivo* efficacy of oxaliplatin, a well-known ICD inducer [48]. These results have shown that treatment of tumor cells with nanoparticles containing oxaliplatin induced more release of DAMPs and stronger immune responses of DCs and T lymphocytes *in vitro* than oxaliplatin without nanoparticles. Moreover, the authors showed that oxaliplatin incorporated in nanoparticles exhibited stronger therapeutic effects than free oxaliplatin in the murine tumor prophylactic vaccination model [47]. It is noteworthy that loading a non-immunogenic drug (5-fluorouracil or gemcitabine) in the same nanoparticles did not grant an immunogenic potential to the non-immunogenic chemotherapeutics, indicating that the immunogenic potential is inducer-dependent [47]. It is important to mention that there are also dual delivery carriers for chemotherapeutics for ICD induction that can be co-delivered in the soft and hard nanocarrier platforms together with lipid-conjugated immune-stimulatory compounds (Table 2). This approach indeed might lead to more effective anti-tumor immune responses. In this regard, the immunogenic chemotherapeutic oxaliplatin was co-delivered with an indoleamine 2,3 dioxygenase inhibitor (indoximod) conjugated to a phospholipid [49]. This strategy first allows the self-assembly of the prodrug indoximod into nanovesicles or its incorporation into a lipid bilayer that encapsulates mesoporous silica nanoparticles. The core in these porous silica nanoparticles allows concurrent delivery of oxaliplatin. The authors showed that nanovesicles plus free oxaliplatin or oxaliplatin together with indoximod nanoparticles induce effective innate and adaptive immunity against pancreatic ductal adenocarcinoma when used as a vaccine by direct injection in the tumor or by intravenous biodistribution to an orthotopic pancreatic ductal adenocarcinoma site [49]. The anti-tumor effects were associated mainly with recruitment of cytotoxic T lymphocytes and downregulation of $Foxp3^+$ T cells. It is also possible to induce robust antitumor immunity with nanoparticles loaded with ICD-chemotherapy that can also sensitize tumors to immune checkpoint blockade. To this end, synthetic high-density lipoprotein (sHDL)-like nanodiscs have recently been developed [50]. The nanodiscs are composed of an apolipoprotein A1 mimetic peptide and phospholipids to provide stimuli-responsive delivery of doxorubicin [50]. In this study, the authors demonstrated that doxorubicin formulation in sHDL nanodiscs markedly potentiated the antitumor T cell responses and the therapeutic efficacy of αPD-1 immunotherapy, leading to elimination of established CT26 and MC38 colon carcinoma in 80 to 88% of the mice, inhibition of CT26 liver metastasis, and induction of long-term immunity against re-challenge with tumor cells. The authors showed that doxorubicin-carrying nanodiscs led to robust antitumor $CD8^+$ T cell responses while broadening their epitope recognition to tumor-associated antigens, neoantigens, and intact whole tumor cells [50].

Of interest, cancer cells dying by an ICD modality can be converted into a versatile platform for cancer vaccination, at least in murine tumor models. Fan et al. [51] used dying colon carcinoma CT26 and melanoma B16 cells undergoing ICD as the source of both tumor antigens and “danger” signals and amplified their potency by surface-modification of dying tumor cells with nano-multilayered particles containing CpG (Table 2). CpG is an adjuvant and a potent TLR-9 agonist known to promote antigen cross-presentation and cross-priming of CD8+ T-cell responses. The authors coupled nanoparticles loaded with CpG onto the surfaces of dying tumor cells *via* sulfhydryl-maleimide chemistry. This strategy was very efficient because immunogenically dying tumor colon carcinoma CT26 cells (*i.e.* killed by mitoxantrone *in vitro*) and decorated with CpG-nanoparticles promoted DC maturation as well as antigen cross-presentation, leading to robust antigen-specific T-cell responses with antitumor activity *in vivo* [51]. Although this study shows for the first time that surface modification of cancer cells undergoing ICD with nanocarriers containing a potent adjuvant is a promising novel experimental “personalized” therapy, additional research is needed to adapt this strategy to co-deliver, for example, ICD inducers together with adjuvants to tumors as vaccines without *ex vivo* manipulations.

The efficiency of nanoparticles as nanocarriers of

Table 2
Representative examples of nanomaterials used to increase the immunogenicity of cancer cell death.

Type of particles	Features of chemical synthesis	Average size and unique characteristics	Active substances	Refs
Nanoparticles				
Polymeric nanoparticles	Monomethoxy-poly(ethylene glycol)-poly(D,L-lactide-*co*-glycolide) (mPEG-PLGA) polymeric nanoparticles prepared by the double emulsion (W/O/W) method	Individual particles, with a well-defined spherical structure and about 50–60 nm in size.	Oxaliplatin 5-fluorouracil Gemcitabine	[47]
Lipid-bilayer coated MSNP dual-delivery carrier	Oxaliplatin is trapped in the mesoporous interior of a MSNP; Core is trapped by a LB that contains the indoximod	A particle size of the MSNP core - ~70 nm; LB-coated particles - ~83 nm (including a 6.5 nm thick lipid bilayer).	Oxaliplatin Indoximod	[49]
Chimeric polypeptide-doxorubicin (CP-Dox)	Based on conjugation of multiple copies of doxorubicin to one end of the chimeric recombinant polypeptide *via* an acid-labile bond	Drug conjugation triggers the self-assembly of near monodisperse nanoparticles into sub-100 nm in size.	Doxorubicin	[60].
Nanodiscs				
Synthetic sHDL–like nanodiscs	Compose of an ApoA1 mimetic 37-mer peptide and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and prepared by a thermal-cycling method	Hydrodynamic size of ~10 nm, pH-responsive release of doxorubicin in the endosomes/lysosomes	Doxorubicin	[50]
Micelles				
DSPE-PEG micelles with changeable particle sizes	Based on the "click"-type reactions Cu(I)-catalyzed azide/alkyne cycloaddition enables the surface modification of peptides, nucleic acids, supramoleculars and a large variety of other ligands.	DSPE-PEG micelles modified with azide or alkyne group had small sizes of ~ 25 nm. In the tumor tissues, cycloaddition occurred between the groups, leading to significant increase in micelle size (~120 nm)	Doxorubicin Monophosphoryl lipid A	[54]
Liposomes				
Nanodepot platform	The lipid-polymer hybrid nano-depot platform constructed by complexation between cationic liposomes and thiolated hyaluronic acid, an anionic biopolymer, followed by crosslink-mediated stabilization	Size ~ 250 nm with their surface charge converted to −16 mV with 81% of loading efficiency	CpG oligonucleotide, a potent Toll-like receptor-9 (TLR9) agonist	[51]
Cationic lipid-protamine-DNA nanoparticles	The liposomes prepared by a hydration-extrusion method and consist of DOTAP and cholesterol (1:1, mol/mol). LPD cores are self-assembled as adding protamine in DI water to Wnt5a trap plasmid	Doxorubicin is intercalated with DNA and used to construct PEGylated LPD nanoparticle for siRNA delivery	Doxorubicin; Trimeric trap protein with the extracellular domain of Fizzled 7 receptor that binds Wnt5a	[65]
Nanostructured lipid carriers (NLCs)	NLCs are prepared by the thin-film hydration method from THP, oleic acid, E80, HS15, α-tocopherol, and structured triglyceride	A hydrodynamic diameter of THP-NLC ~112 nm. Average zeta potential of THP-NLC is −17.5 mV. Drug encapsulation efficiency 90.01%, loading efficiency of THP-NLC - 2.78%	Pirarubicin; iRGD: a tumor-penetrating peptide (CRGDKGPDC)	[56]
PEGylated liposome	Containing polyethylene glycol-derivatized phospholipids	Size ~150 nm	Doxorubicin, Doxil, anti-PD-1, CTLA-4 mAbs	[62]

Abbreviations: CTLA-4, cytotoxic T-lymphocyte-associated protein 4; Doxil, liposomal doxorubicin; LB, lipid bilayer; LPD, liposome-polycation-DNA; mAbs, monoclonal antibodies. MSNP, mesoporous spherical silica nanoparticles; NLCs, nanostructured lipid carriers; PD-1, programmed cell death protein 1; sHDL, synthetic high-density lipoprotein; THP, pirarubicin.

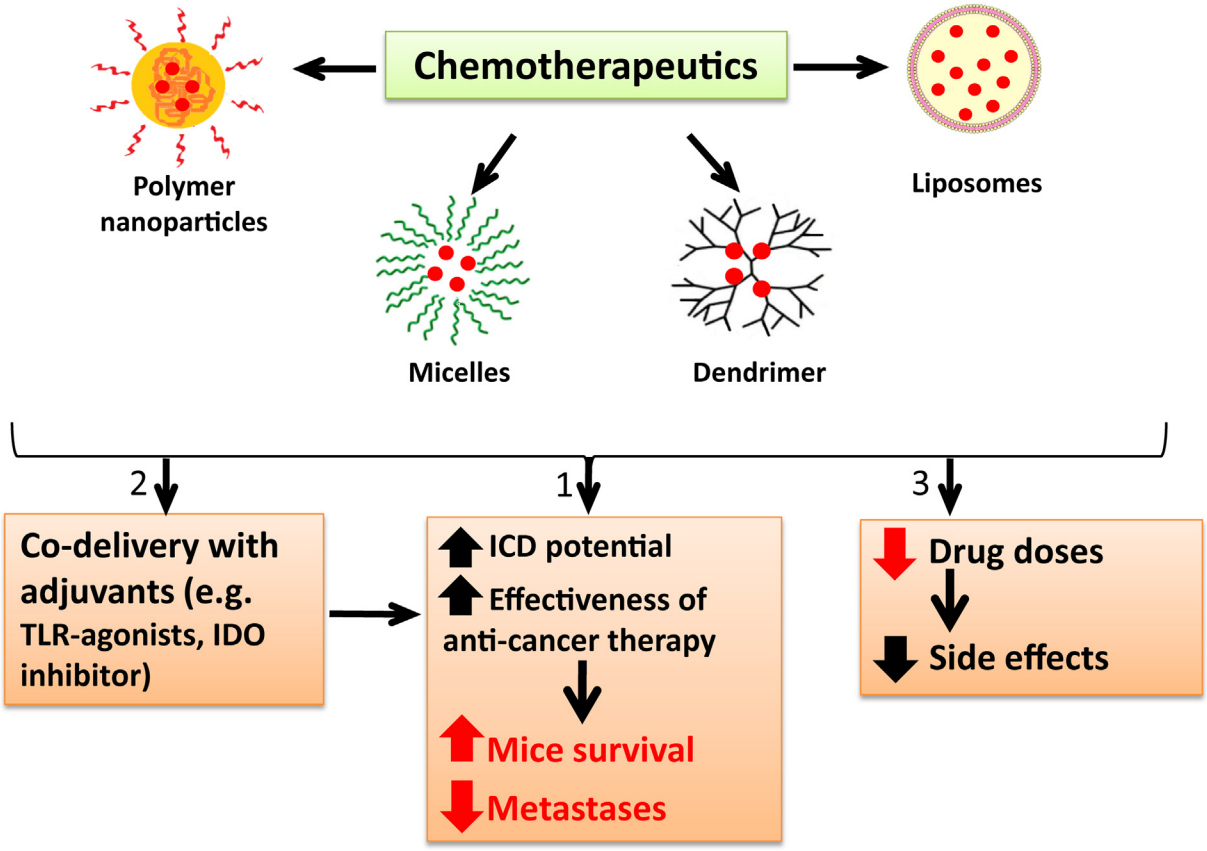

Fig. 2. Nano-particles and micro-particles: an emerging strategy to modulate the immunogenicity of immunogenic cell death.
Biomaterials are widely used to modulate the effectiveness of chemotherapeutics in experimental anti-cancer therapy. (1) Delivery of chemotherapeutics *via* biomaterials (*e.g.* polymer nanoparticles, micelles, dendrimers and liposomes) increases their immunogenicity (Table 2) and effectiveness in anti-cancer therapy, leading to decreased metastasis and longer survival. (2) Immunogenicity can often be amplified by co-delivery of TLR agonists or IDO inhibitors by using dual delivery carriers to obtain simultaneous delivery of chemotherapeutics and adjuvants. The use of adjuvants with the main chemotherapeutics can enhance the engagement of immune responses towards ICD. (3) Another benefit of the delivery of chemotherapeutics in nanocarriers is the ability to reduce the drug dose. Encapsulation of the drugs in nanoparticles significantly reduces their toxicity to the host cells. Nanoparticle delivery systems also avoid drug accumulation in critical organs (*e.g.* heart, intestine) and permit the administration of larger doses, which is not possible with a free drug. All these strategies are being tested in experimental mouse models, and other studies are needed to translate the findings to the clinical settings.
Abbreviations: ICD, immunogenic cell death; IDO inhibitor, indoleamine 2,3 dioxygenase inhibitor TLR, Toll like receptors.

chemotherapeutics for modulation of anti-tumor-immune responses is greatly dependent on their size, which in fact is a double-edged sword. On the one hand, smaller nanoparticles increase penetration into the tumors, mainly *via* the leaky tumor vasculature [52]. On the other hand, larger nanoparticles have significantly larger retention capacity [53]. Therefore, it is clear that nanoparticles of fixed size are not optimal. One way to cope with this dilemma is to design nanoparticles with changeable size. Mei et al. [54] developed DSPE-PEG micelles with sizes that can changed by using chemical reactions. First, the micelles modified with azide or alkyne group are 25 nm in size, which optimizes their penetration into the tumor bed. But once they reach the tumor bed, micelles increase in size to ~120 nm due to a chemical reaction, and this increases their retention and accumulation. The authors used this strategy to co-deliver doxorubicin and monophosphoryl lipid A, an adjuvant that is an agonist of TLR-4, to induce ICD and thereby further promote the maturity and antigen presentation of DCs in order to induce strong effector T cells *in vivo* that can suppress tumor growth and prevent tumor metastasis. This study demonstrates a technological platform for changing the size of nano-particles to improve their penetration and accumulation in the tumors.

4.2. Nano-carries to trigger ICD in order to reduce serious complications of cancer therapy

Another positive aspect of using nano-carriers to trigger ICD is reduction of the serious complications of cancer therapy [55], which frequently compromise treatment and dramatically reduce the quality of life of patients. Furthermore, these strategies can increase the immunogenicity of dying cancer cells, enabling the development of effective anti-tumor immune responses (Table 2, Fig. 2). Nanoparticle delivery can reduce the side effects by redistributing drug accumulation away from critical organs such as the heart and intestine. They also permit the administration of larger doses than it is possible with a free drug. In this context, it has been shown that when the cytotoxic drug pirarubicin, a commonly used anthracycline and ICD inducer, was entrapped into nanostructured lipid carriers, it led to reduced myelosuppression but at the same time it enhanced anti-tumor effects in mice bearing 4 T1 breast tumors [56]. Another immunogenic anthracycline, doxorubicin [57,58], was loaded into a nanoparticle delivery system based on conjugation of multiple copies of doxorubicin to one end of the chimeric recombinant polypeptide *via* an acid-labile bond [59]. In this recent work, the authors showed that $CD8^+$ cells and IFN-γ are necessary for the full efficacy of the chimeric polypeptide-doxorubicin formulation, whereas their depletion had no effect in mice bearing 4 T1 mammary carcinoma and treated with freely dissolved doxorubicin

[60]. These studies underline an interesting and important aspect of the immunomodulatory effects of doxorubicin in mice, that it can be enhanced by a nanoparticle delivery system even in a poorly immunogenic model system such as 4 T1 mammary carcinoma. This is very relevant clinically because weakly immunogenic tumors are one of the main problems of current anti-cancer therapy [61]. Indeed, the study by Mastria et al. [62] is distinct from the studies in which the immunogenicity of doxorubicin loaded into PEGylated liposomes (Doxil) was validated in the CT26 and MCA205 cancer models, which are immunogenic cancer cell lines. These authors demonstrated that the Doxil formulation was more effective in immunocompetent mice and synergized with checkpoint blockade (*i.e.* anti-PD-1 and CTLA-4 mAbs) in CT26 and MCA205 tumor models [62]. Of note, that these checkpoint inhibitors have been approved by the Food and Drug Administration, USA, as a therapy for several types of cancer, including melanoma and non-small cell lung cancer [63]. For this discovery, James P. Allison and Tasuku Honjo have been awarded the Nobel Prize in Physiology or Medicine in 2018. Interestingly, several studies have indicated that the anti-tumor immunity induced by ICD may be reinforced by anti-PD-1 and anti-CTLA-4 drugs. For example, it has been shown that Dinaciclib induces immunogenic cell death and enhances anti-PD1-mediated tumor suppression [64].

Another approach to reduce the side effects of chemotherapy is to reduce the dose. However, due to the strong immunosuppressive environment of certain tumors (*i.e.* melanoma driven by B-Raf proto-oncogene (BRAF) mutation), low doses of doxorubicin often lead only to a partial response [65]. Of note, the immunosuppressive environment of BRAF mutant melanoma cells is highly dependent on Wnt family member 5A (Wnt5a), which contributes to the induction of DC tolerance and tumor fibrosis [66], thereby hindering effective antigen presentation. In order to reduce the immunosuppressive effects, which are mediated by Wnt5a signaling, the authors validated the extracellular domain of Fizzled 7 receptor, which as a trimeric trap protein binds Wnt5a with a Kd of ~ 278 nM [65]. Moreover, plasmid DNA packed into cationic lipid-protamine-DNA nanoparticles was used to deliver the Wnt5a trap. This strategy was very effective in significantly inhibiting tumor growth and increasing host survival, suggesting that local Wnt5a trapping significantly remodels the immunosuppressive tumor microenvironment to facilitate immunogenic cell-death-mediated immunotherapy at low doses of ICD inducer. Future studies will show whether nanomaterials are truly a better strategy for increasing the immunogenicity of dying cancer cells while reducing the severe side effects. Future studies will also provide the insights needed for the development of novel treatment strategies for cancer.

5. Concluding remarks and future perspectives

Only about one decade ago, apoptosis was considered as immunologically neutral or even anti-inflammatory and immuno-suppressive [10–12]. However, insights from the last decade increasingly support the view that certain anti-cancer treatment modalities can induce ICD (*i.e.* immunogenic apoptosis), which contributes to anti-tumor immune responses. Moreover, it is clear that ICD is an umbrella term that covers a broad range of different types of cell death, including apoptosis, necroptosis, and other immunogenic cell modalities (Box 2).

On one hand, recent studies suggest that nanomaterials can be used to amplify the immunogenicity of cancer cell death (Table 2) and to reduce the severe side effects of anti-cancer therapy. These findings should be validated in clinically relevant tumor models and *ex vivo*. However, intriguing questions remain, such as how feasible these formulations are in the clinical setting, when large-scale manufacturing and proven human safety are required. For example, nanoparticles typically require PEGylation to obtain sufficient circulation half-life and compound accumulation in the tumor bed, but this could lead to chronic-foot syndrome [67] and generation of anti-PEG (polyethylene glycol) antibodies [68], which can delay their clinical application. Further research is also needed to develop nanomaterials with catalytic properties that can be used for sequential induction of ICD, ER stress and ROS and at the same time induce the release of the DAMPs and cytokines/chemokines required for the immunogenicity of dying cancer cells.

On the other hand, current interest is focused on the development of nanoparticles as targeted delivery systems for induction of ICD, *i.e.* immunogenic apoptosis. But cancer cells often develop resistance to apoptosis [30], so novel strategies are needed to trigger alternative forms of death in cancer cells. Recently, it has been shown that drugs can be successfully nanotargeted to induce ferroptosis (Box 1) in cancer cells [69]. It is noteworthy that nanomaterials can even be provided with direct cell death inducing capabilities, which might turn out to be a promising therapeutic option. It has been shown that ultrasmall silica nanoparticles (< 10 nm in diameter) coated with poly(ethylene glycol) (PEG) and functionalized with melanoma-targeting peptides can induce ferroptosis (Box 1) [70]. This study opens possibilities to develop strategies that allow the design of nanoparticles with dual properties: targeting delivery of ICD inducers to the tumor bed and possession of intrinsic capacities to trigger different cancer cell death modes (apoptosis, necroptosis, or ferroptosis). This may lead to synchronous and selective killing of cancer cells, which are most susceptible to specific types of cell death. Future studies will provide the necessary insights needed for the development of novel treatment strategies to increase the immunogenicity of cancer cell death by using nanomaterials and to validate these strategies in clinical settings. That is an exciting area for upcoming research.

Author's contributions

TM, EM IB MV: drafted the manuscript and the figures. OK: revised the article and the figures. DVK: designed, supervised the study and revised the manuscript.

Acknowledgments

The study was supported by a grant from Russian Science Foundation (RSF, project No.18-15-00279). The authors thank Servier for the figures, which were produced using Servier Medical Art (www.servier.com).

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