Immunogenic Cell Death Driven by Radiation—Impact on the Tumor Microenvironment

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10.1 Introduction

For decades, it was believed that apoptosis—defined morphologically as a variant of cell death involving cytoplasmic shrinkage, nuclear condensation (pyknosis) and fragmentation (karyorrhexis), plasma membrane blebbing, and release of small cell corpses (so-called apoptotic bodies) [1]—would invariably be immunologically silent, if not tolerogenic [2]. Conversely, necrosis—defined morphologically as a

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form of cell death lacking the features of apoptosis and autophagic cell death (characterized by cytoplasmic vacuolization) [3, 4]—was widely considered as a pro-inflammatory cell modality [1]. Such an oversimplification originated, at least in part, by the abundant literature on the role of apoptotic cell death in physiological processes (e.g., embryonic development, adult tissue homeostasis), contrasting with the common implication of necrosis in pathological conditions with inflammatory correlates (e.g., burn injuries, neoplastic disorders) [5–7]. It is now clear that the morphological manifestations of cell death, its biochemical features, and its immunological properties can vary independently from each other [8]. Thus, instances of cell death manifesting with an apoptotic morphology can exert robust immunostimulatory effects, while cases of cell death with a necrotic appearance can be potently tolerogenic [9].

The term "immunogenic cell death" (ICD) has been originally introduced by Kroemer and collaborators in 2005 to describe the ability of mouse colorectal carcinoma CT26 cells challenged in vitro with doxorubicin (an anthracycline commonly used for cancer therapy) to provide immunocompetent syngeneic BALB/c mice with long-term immunological protection against the subsequent inoculation of living CT26 cells [10]. More than a decade later, the term ICD is widely employed to indicate cases of cell death that (irrespective of morphology and biochemical correlates) can initiate an adaptive immune response against antigens expressed by dying cells in the absence of any immunological adjuvant [11]. Such a functional definition has several implications, including: (1) irrespective of the existence of several surrogate biomarkers for ICD (see below), *bona fide* ICD can only be assessed in immunocompetent, syngeneic experimental systems [12]; (2) dying cells must express antigens that are not covered by central tolerance in such experimental systems (implying the presence of naïve T cells potentially able to recognize antigenic determinants from dying cells) [13]; and (3) dying cells must release adjuvant-like molecules that promote the recruitment of antigen-presenting cells (APCs) to sites of cell death, the uptake of dead cell corpses and their processing for cross-presentation to $CD8^+$ T cells [14]. These immunostimulatory molecules, which are cumulatively referred to as damage-associated molecular patterns (DAMPs), encompass small metabolites, such as ATP, proteins that are normally sequestered within intact cells, such as calreticulin (CALR) and high mobility group box 1 (HMGB1), as well as cytokines, such as type I interferon (IFN) [9].

Importantly, the presence of specific DAMPs is required for cell death to be perceived as immunogenic, but not sufficient. Indeed, cells lysed by repeated freeze/thawing cycles (which induce cell death with necrotic features) are unable to driven adaptive immunity [15]. In-depth mechanistic explorations revealed that DAMPs must be released in a spatiotemporally defined order (the "key") for the host immune system (the "lock") to correctly interpret such signals and mount the precise cascade of events underpinning adaptive immune responses [16]. Moreover, it became clear that each DAMP is emitted downstream of the activation of specific cellular stress response modules, such as the endoplasmic reticulum (ER) stress response or autophagy [17, 18]. Taken together, these observations explain why

only a few cytotoxic agents can mediate *bona fide* ICD [19–22]. Of note, radiation therapy (RT) is one of such agents, at least when used in specific doses and according to precise fractionation schedules [23–25]. This implies that the immunogenic demise of irradiated cancer cells is associated with the release of DAMPs that contribute to the functional reconfiguration of the tumor microenvironment (TME).

Here, we discuss the mechanisms whereby DAMPs emitted by cancer cells undergoing RT-driven ICD reconfigure the TME. Importantly, RT has a multipronged effect on the TME, reflecting its ability to promote ICD as well as its capacity to: (1) favor the release of a variety of immunomodulatory factors beyond DAMPs from cells surviving irradiation, such as transforming growth factor beta (TGF-β) [26, 27]; (2) support the establishment of hypoxia, owing to its elevated cytotoxic potential for endothelial cells [28]. Despite their importance, these and other aspects of the interaction between RT and the TME will not be discussed in detail here.

10.2 Calreticulin

CALR is widely known as an ER chaperone with a major role in protein (re-) folding, and hence in the cellular response to unfolded proteins accumulating as a consequence of viral infection or alterations in intracellular Ca^{2+} homeostasis [29, 30]. Alongside, cells experiencing ER stress expose CALR, as well as other ER chaperones including heat shock protein 90 alpha family class A member 1 (HSP90AA1), heat shock protein family A (Hsp70) member 1A (HSPA1A, best known as HSP70), and protein disulfide isomerase family A member 3 (PDIA3, best known as ERp57) [31], on the outer leaflet of the plasma membrane [15, 32, 33]. In the context of ICD, membrane-exposed CALR operates as a pro-phagocytic signal, de facto boosting the uptake of cell corpses by APCs or their precursors [15, 34]. The precise identity of the APC receptor that underlies such an effect remains elusive. Indeed, while LDL receptor-related protein 1 (LRP1, best known as CD91) has been involved in some settings [35–37], it seems that CD91 is not absolutely required for the pro-phagocytic activity of membrane-exposed CALR [15, 38].

Besides promoting phagocytosis, the interaction between CALR and its receptor delivers immunostimulatory signals to APCs [15, 35], which is at odds with the well-known ability of phosphatidylserine (PS) externalized in the course of apoptosis to mediate robust immunosuppressive activity upon engagement of jumonji domain containing 6, arginine demethylase and lysine hydroxylase (JMJD6) on phagocytes [39, 40]. Importantly, the ICD-associated exposure of CALR on the plasma membrane occurs before the apoptosis-related externalization of PS [41–43], which explains (at least partially) why cells undergoing *bona fide* ICD fail to establish immunological tolerance. Another signal that counteracts the immunostimulatory activity of CALR originates from the interaction of CD47 on cancer cells and signal regulatory protein alpha (SIRPA) on phagocytes [44, 45]. Reflecting a

pathophysiologically relevant role of CARL exposure for human cancer, high levels of total or surface-exposed CALR have been attributed positive prognostic value in patients affected by a variety of malignancies, including acute myeloid leukemia (AML) [46], non-small cell lung carcinoma [47, 48], neuroblastoma [49], and ovarian cancer [48]. Similarly, high levels of CD47 have been correlated with poor clinical outcome in cohorts of patients with AML [50], breast carcinoma [51], as well as esophageal and gastric carcinoma [52, 53].

In line with its ability to drive *bona fide* ICD, RT robustly promotes the exposure of CALR on the membrane of cancer cells [23, 24, 54], as well as an increase in global CALR levels, at least in some cancer types [55]. Thus, these observations indicate that RT-driven ICD is likely to favor the phagocytic activity of tumor-infiltrating myeloid cells along with the delivery or immunostimulatory signals. Of note, soluble CALR has been suggested to mediate immunosuppressive, rather than pro-phagocytic and immunostimulatory, effects, at least in some settings [41, 56], in thus far resembling natural killer (NK) cell activating ligands [57, 58]. That said, how RT affects CALR secretion remains an open conundrum.

10.3 ATP

While the concentration of intracellular ATP is generally quantified in the range of 1–10 mM, extracellular ATP concentration in healthy tissues is very low, at least in part owing to the existence of enzymes that sequentially convert ATP into adenosine [59, 60]. These enzymes include ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1, best known as CD39), which converts ATP into AMP via ADP, and 5′-nucleotidase ecto (NT5E, best known as CD73), which generates adenosine from AMP [61]. As a consequence of plasma membrane breakdown, dead cells release ATP in amounts that (at least temporarily) can saturate the activity of ATP-degrading enzymes, hence resulting in local increments in extracellular ATP concentrations [62]. ATP liberated by dying cells plays a key role in the perception of cell death as immunogenic [63], via at least two mechanisms. First, ATP and other nucleotides released by dying cells operate as chemoattractant for APCs or their precursors upon binding to purinergic receptor P2Y2 (P2YR2) [64, 65]. Second, ATP mediates immunostimulatory activity on myeloid cells via purinergic receptor P2X 7 (P2RX7), which culminates with inflammasome activation and secretion of interleukin 1B (IL1B) [66–68].

However, the absolute amount of extracellular ATP does not appear as the major factor in this setting, as demonstrated by the fact that cells subjected to repeated freeze/thawing cycles (which release all their ATP as plasma membrane breaks down) fail to vaccinate syngeneic immunocompetent mice against a challenge with living cancer cells of the same type [15]. In this setting, it appears indeed that ATP must be released by cells that are still physical intact, in a premortem process that (1) involves the exocytosis of vesicular ATP pools, cellular blebbing, and opening of ATP-permeant pannexin 1 (PANX1) channels [69], and (2) is dependent on

autophagy [70]. In line with a key role for ATP release downstream of functional autophagic responses in the perception of cell death as immunogenic, the ability of CT26 cells undergoing chemotherapy-driven ICD to provide immunological protection to BALB/c mice is lost when CT26 cells overexpress CD39 or are depleted of key autophagy factors including ATG5, ATG7, and beclin 1 (BECN1) [70, 71]. Along similar lines, ATG5-depleted CT26 cells growing in immunocompetent BALB/c mice lost (entirely or partially) their ability to respond to mitoxantrone (a chemotherapeutic agent that induces *bona fide* ICD) [70] and RT [72], which is known to cause ATP release [24]. That said, proficient autophagic responses have also been linked to limited CALR exposure, and hence poor immunogenicity, at least in the context of photodynamic therapy-initiated ICD [73, 74]. Thus, the precise impact of autophagy and downstream ATP release on the immunogenicity of cell death may vary, at least to some degree, with context-dependent variables. In line with this notion, unpublished results from our laboratory demonstrate that *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ mouse mammary carcinoma TSA cells exhibit increased (not decreased) responses to RT when established in immunocompetent BALB/c mice (as compared to wild-type cells), and preserve complete immunostimulatory potential when used as vaccine upon irradiation (Yamazaki et al., unpublished observations).

Despite these apparently controversial and hitherto unresolved observations (which may reflect the differential importance of specific DAMPs in the immunogenicity of cell death driven by different stimuli or in different cell type), several lines of evidence support the notion that ATP released by dying cancer cells and the consequent engagement of P2R2Y and P2RX7 on immune cells have therapeutic implications for cancer patients [75]. For instance, loss-of-functions polymorphisms in P2RX7 have been associated with poor disease outcome in cohorts of patients with breast carcinoma receiving neoadjuvant anthracycline-based chemotherapy [66], and individuals with papillary thyroid cancer [76]. Moreover, CD39 and/or CD73 are upregulated on malignant or immune cells in a variety of human neoplasms, generally correlating with disease progression [77–79] and/or poor clinical outcome [80].

Thus, ATP released in the context of RT-driven ICD may support tumor infiltration by APCs or their precursors, as well as the establishment of a pro-inflammatory TME characterized by robust IL1B secretion, at least theoretically. However, RT is known to initiate several immunosuppressive pathways that strongly counteract these therapeutically beneficial processes, such as increased TGF-β bioavailability [26, 27]. Moreover, inflammasome activation downstream of spontaneous ATP release and consequent P2RY2 and P2RX7 signaling has been linked with radioresistance in human models of breast cancer [81], and chemoresistance in human and mouse models of melanoma [82]. These findings suggest that predicting the impact of purinergic signaling associated with RT-driven ICD on the TME is challenging, awaiting urgent experimental verification.

10.4 HMGB1

HMGB1 is a non-histone chromatin-binding protein that—according to current models—gets passively released by cells as they die, consequent to the breakdown of the nuclear envelope and plasma membrane [8, 83]. Thus, the amount of HMGB1 released by a cell population undergoing ICD generally correlates with the degree of cell death, at least when such population express HMGB1 at homogeneous levels [84]. The biological activity of extracellular HMGB1 appears to depend on its oxidation state. In particular, reduced HMGB1 efficiently partners with CXCL12 to exert robust chemotactic functions via chemokine (C-X-C motif) receptor 4 (CXCR4) [85, 86]. Conversely, oxidized HMGB1—which is unable to dimerize with CXCL12—stimulates cytokine synthesis upon binding to advanced glycosylation end product-specific receptor (AGER, best known as RAGE), Toll-like receptor 2 (TLR2) and TLR4 [87, 88], a transcriptional activity depending on NF-κB and interferon regulatory factor 3 (IRF3) [89, 90]. Among other, these cytokines (and chemokines) include: IL1B, IL6, tumor necrosis factor (TNF), C-X-C motif chemokine ligand 10 (CXCL10), as well as type I IFN (see below) [18]. Furthermore, HMGB1 signaling via TLR4 facilitates cross-priming by inhibiting the fusion of antigen-containing endosomes with lysosomes [91].

Supporting a central role for HMGB1 release in the perception of cell death as immunogenic, the knockdown of HMGB1 by short-hairpin RNAs (shRNAs) as well as its neutralization with specific antibodies compromise the ability of cancer cells responding to anthracyclines in vitro to confer long-term immunological protection to syngeneic mice when used as a vaccine [92]. Consistent with this, both *Tlr4*$^{-/-}$ mice and *Myd88*$^{-/-}$ mice (which lack a transducer of TLR4 signaling) lose the ability to mount a protective immune response against syngeneic cancer cells undergoing chemotherapy-driven ICD [92, 93]. The same does not hold true for *Tlr2*$^{-/-}$ and *Ager*$^{-/-}$ mice [92, 93], suggesting that TLR4 is the key receptor for HMBG1 in this setting. In line with this notion, the TLR4 agonist dendrophilin has been successfully employed to restore the immunogenicity of HMGB1-deficient mouse tumors [94].

Elevated levels of HMGB1 in malignant cells have been correlated with improved disease outcome in patients with esophageal squamous cell carcinoma [95], and gastric adenocarcinoma [96]. Moreover, loss of nuclear HMGB1 has been positively associated with tumor size in patients with breast carcinoma undergoing anthracycline-based adjuvant chemotherapy [94]. Conversely, high HMGB1 levels have been linked with advanced disease or poor outcome in cohorts of patients with bladder [97], nasopharyngeal [98], colorectal [99], hepatocellular [100, 101], head and neck [102], and prostate carcinoma [103]. These apparently contradictory observations may reflect the intracellular functions of nuclear and cytoplasmic HMGB1, the latter being capable of promoting cytoprotective autophagic responses [104, 105].

TLR4 loss-of-functions variants have been linked with poor disease outcome in patients with breast carcinoma [92], head and neck cancer [106], and melanoma [107, 108], comforting the notion that TLR4 signaling supports anticancer immunity in a variety of clinical settings. Conversely, elevated levels of TLR4 or MYD88 in cancer biopsies have been correlated with shortened survival in patients with ovarian [109] and colorectal carcinoma [110]. Most likely, these findings reflect the evolutionary advantage provided to neoplastic cells by TLR4 expression, which can initiate robust pro-survival signaling pathways via NF-κB [89]. Of note, whether NF-κB signaling downstream of TLR4 activation is mechanistically involved in the perception of cell death as immunogenic remains an open conundrum, as (at least apparently) contradictory reports exist on this aspect of ICD [111, 112].

In line with its prominent cytotoxic effects, RT efficiently promotes the release of HMGB1 from dying cancer cells [24], which might impact the TME in a dual manner. On the one hand, RT-driven ICD favors tumor infiltration by $CCR4^+$ monocytes downstream of HGMB1-bound CXCL12. On the other hand, the cytotoxic activity of RT promotes the establishment of an immunostimulatory milieu as a consequence of the HMGB1-dependent activation of TLR4 in tumor-infiltrating myeloid cells, which culminates with the secretion of multiple cytokines and chemokines. That said, the response of mouse colorectal carcinoma MC38 cells to a single RT dose of 20 Gy is not influenced by the deletion of *Myd88* from the host or by the administration of HMGB1-neutralizing antibodies [113]. Thus, the actual relevance of TLR4 signaling downstream of the ICD-associated release of HMGB1 for therapeutic responses remains to be clarified. Additional experiments are required to elucidate this unknown. Along similar lines, whether HMGB1 released by cancer cells succumbing to chemotherapy-driven *versus* RT-driven occurs via different kinetics calls for urgent experimental verification. Intriguingly, ultraviolet light has recently been suggested to favor HMGB1 release by melanocytes and keratinocytes, culminating with the expression of the immunosuppressive molecule CD274 (best known as PD-L1) downstream of AGER signaling [114]. Whether a similar pathway can be initiated by RT remains obscure.

10.5 Type I IFN

Best known for its key role in viral interference (the process whereby virally infected cells establish local resistance to infection via paracrine circuitries) [115, 116], type I IFN is also abundantly produced by cancer cells undergoing chemotherapy-driven [117] and RT-driven ICD [25]. Type I IFN signals via homodimeric interferon (alpha, beta, and omega) receptor 1 (IFNAR1), which has a particularly high affinity for IFN-β, or via IFNAR1/IFNAR2 heterodimers, which bind all type I IFNs, culminating with the activation of immunostimulatory transcriptional programs dependent on signal transducer and activator of transcription 1 (STAT1) and STAT2 [118, 119]. In particular, type I IFN promotes cross-priming

[120], boosts the cytotoxic functions of $CD8^+$ cytotoxic T lymphocytes and natural killer cells [121], increases the survival of memory T cells [122], and drives the expression of CXCL10, a potent chemotactic factor for effector T cells [123]. Thus, type I IFN secretion by dying cancer cells not only delivers robust immunostimulatory signals to tumor-infiltrating cells, but also favors the recruitment of effector T cells to the TME [119].

Importantly, in the course of chemotherapy-driven ICD type I IFN is secreted downstream of TLR3 activation by endogenous RNA species, and largely acts by driving CXCL10 production in cancer cells [117]. Thus, neither *Tlr3*$^{-/-}$ nor *Ifnar1*$^{-/-}$ mouse cancer cells succumbing to anthracyclines in vitro preserve their ability to vaccinate immunocompetent syngeneic mice against a subsequent challenge with cancer cells of the same type, while the immunogenicity of wild-type cancer cells is preserved in *Ifnar1*$^{-/-}$ mice [117]. Conversely, irradiated cells produce type I IFN upon the accumulation of cytosolic DNA [25, 124–126], a process that is under negative regulation by the RT-responsive nuclease three prime repair exonuclease 1 (TREX1) [25]. This explains the existence of RT dose thresholds above which type I IFN secretion by irradiated cancer cells becomes inefficient [25]. Cytosolic DNA favors cyclic GMP-AMP synthase (CGAS) activation and downstream signaling via transmembrane protein 173 (TMEM173, best known as STING) [127, 128]. Importantly, irradiated cancer cells can also trigger type I IFN secretion by dendritic cells (DCs), largely upon the exosomal transfer of DNA species [129]. In this setting, IFNAR1 expression by the host (not by cancer cells) appears to play a major role [130–133]. Of note, although nuclear DNA is currently viewed as the main source of cytosolic DNA driving CGAS-STING signaling in irradiated cells [124, 125], our unpublished preliminary data indicate that mitochondrial DNA may play an equal or even superior role in this setting (Yamazaki et al., unpublished observations). At least in part, this explains why *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ TSA cells exhibit superior (not compromised, as expected per their limited capacity to secrete ATP as they die) responsiveness to RT when growing in immunocompetent BALB/c mice (see above).

Supporting the central role of type I IFN signaling in the perception of cell death as immunogenic, high levels of TLR3 or its signal transducer TLR3 and/or toll-like receptor adaptor molecule 1 (TICAM1, best known as TRIF) have been associated with improved disease outcome in patients with hepatocellular carcinoma [134, 135], neuroblastoma [136], and breast carcinoma [137]. Of note, in this latter setting women with breast cancer were treated with RT plus a TLR3 agonist [137], lending further support to the importance of type I IFN signaling for radiosensitivity. Along similar lines, a type I IFN-related transcriptional signature has been shown to predict the likelihood of breast carcinoma patients to obtain clinical benefits from neoadjuvant anthracycline-based chemotherapy [117], and polymorphic IFNAR1 variants with reduced functions have been linked to poor disease outcome in patients with colorectal carcinoma [138]. Furthermore, the metastatic dissemination of human breast cancers to the bone is often linked to deficient type I IFN secretion by carcinoma cells, generally consequent of IRF7 downregulation [139]. That said, type I IFN-related transcriptional signatures have also been correlated with poor disease

outcome in patients with breast carcinoma [140, 141] and melanoma [142]. Most likely, these apparently contradictory findings reflect the opposed biological outcome of robust, acute *vs* mild, chronic type I IFN secretion [18].

Altogether, these observations suggest that type I IFN secretion in the context of RT-driven ICD is instrumental for the TME to acquire a robust T_H1 polarization and to recruit BATF3-dependent conventional DCs (cDC1) and naïve T cells [25], key processes that are required for the initiation of anticancer immunity [123]. However, several of the immunosuppressive effects of RT, including increased TGF-β bioavailability [26, 27] and some degree of vascular disruption [28] may offset the ability of type I IFN to polarize the TME toward a robustly immunostimulatory state with anticancer activity. In line with this notion, TGF-β blockade enhances the priming of tumor-specific T cells in multiple mouse models of mammary carcinomas [26].

10.6 Concluding Remarks

In summary, DAMPs emitted by malignant cells succumbing to RT (Table 10.1) are able (at least hypothetically) to establish optimal conditions for the activation of potent innate and adaptive anticancer immunity. Of note, several other ICD-associated DAMPs have been characterized, including DNA of both nuclear and mitochondrial origin [143, 144], as well as the endogenous protein annexin A1 [145]. However, the ability of RT to drive danger signaling through these DAMPs remains unexplored. Moreover, RT has also multipronged immunosuppressive effects that often compromise, at least to some degree, the ability of cancer cells undergoing ICD to initiate therapeutically relevant immune responses. In this

Table 10.1 Main effects of danger signals emitted in the course of RT-driven ICD

DAMP	Stress response	Receptor	Target cells	Effect
ATP	Autophagy	P2RX7	Myeloid cells	Cytokine secretion
ATP	Autophagy	P2RY2	Myeloid cells	Recruitment
CALR	ER stress	CD91 (?)	Myeloid cells	Phagocytosis
CXCL10	Cytokine signaling	CXCR3	Effector T cells	Recruitment
HMGB1 (oxidized)	Cell death	TLR4	Myeloid cells	Cytokine secretion
HMGB1 (reduced)	Cell death	CXCR4	Myeloid cells	Recruitment
Type I IFN	Nucleic acid stress	IFNARs	Cancer cells	Cytokine secretion
Type I IFN	Nucleic acid stress	IFNARs	$CD8^+$ T cells	Cytotoxicity and memory
Type I IFN	Nucleic acid stress	IFNARs	Dendritic cells	Cross-presentation
Type I IFN	Nucleic acid stress	IFNARs	Myeloid cells	Cytokine secretion
Type I IFN	Nucleic acid stress	IFNARs	NK cells	Cytotoxicity

DAMP damage-associated molecular pattern; *ER* endoplasmic reticulum; *ICD* immunogenic cell death; *NK* natural killer; *RT* radiation therapy

scenario, the balance between immunostimulation and immunosuppression is a major determinant for the clinical benefits that patients receiving RT can experience. Thus, efforts should be dedicated to the identification of optimal RT doses and fractionation schedules [146, 147] as well as to the identification of combinatorial partner that boost RT-driven immunostimulation [148, 149]. We surmise that moving down these avenues will provide important insights into the interactions between RT-driven ICD and the TME, and hence will generate new therapeutic paradigms for preclinical and clinical testing.

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