Chapter 6
Immunogenic Apoptotic Cell Death and Anticancer Immunity

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Abstract For many years it has been thought that apoptotic cells rapidly cleared by phagocytic cells do not trigger an immune response but rather have anti-inflammatory properties. However, accumulating experimental data indicate that certain anticancer therapies can induce an immunogenic form of apoptosis associated with the emission of damage-associated molecular patterns (DAMPs), which function as adjuvants to activate host antitumor immune responses. In this review, we will first discuss recent advances and the significance of danger signaling pathways involved in the emission of DAMPs, including calreticulin, ATP, and HMGB1. We will also emphasize that switching on a particular signaling pathway depends on the immunogenic cell death stimulus. Further, we address the role of ER stress in danger signaling and the classification of immunogenic cell death inducers in relation to how ER stress is triggered. In the final part, we discuss the role of radiotherapy-induced immunogenic apoptosis and the relationship of its immunogenicity to the fraction dose and concomitant chemotherapy.

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6.1 Introduction

In the human body close to 500 billion cells die each day by apoptosis, and they are continuously recognized and removed by the phagocytic system without causing inflammation or scars. The process of clearing—dying cells play a critical role in development, maintenance of tissue homeostasis, control of immune responses, and resolution of inflammation. Immunological responses elicited by apoptotic cells have been studied extensively in the last two decades. Back in the nineties it was shown that uptake of apoptotic neutrophils or eosinophils by human monocyte-derived macrophages does not induce secretion of granulocyte macrophage colony stimulating factor (GM-CSF) or thromboxane B2 [1, 2]. In later studies it was shown that apoptotic cells actually inhibit the production of many proinflammatory cytokines by antigen-presenting cells (Fig. 6.1) [1, 3–10]. Cells undergoing apoptosis are known

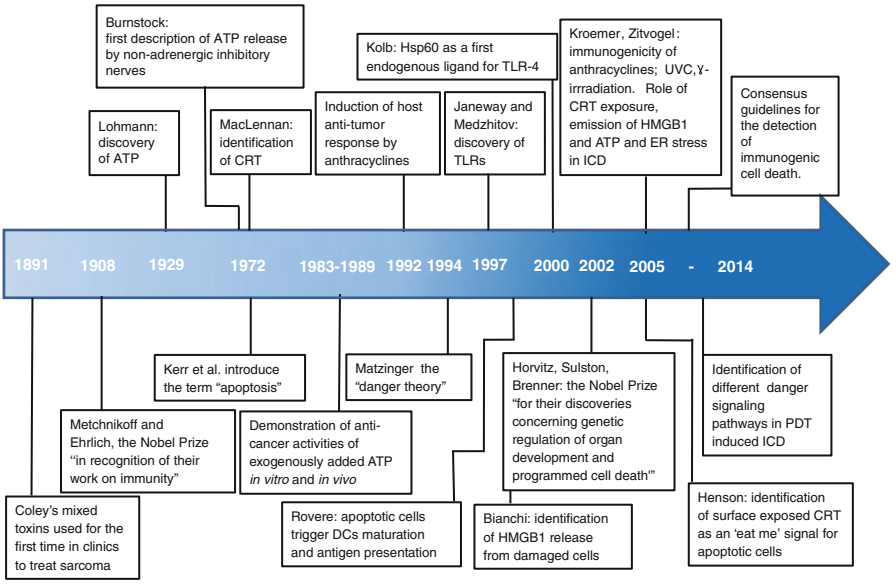

Fig. 6.1 Timeline of the key milestones in the development of the immunogenic cell death concept. Of note that immunotherapy in the treatment of cancer was first successfully used in 1891 by William B. Coley, who injected streptococcal products into patients with inoperable cancer. These products became known as Coley's Toxins. The following references are used to make this figure: [14–16, 20, 25, 32, 33, 42, 43, 46, 92–104]

to modulate their tissue microenvironments either by acting on phagocytes and thereby inhibiting immunological and inflammatory responses and promoting "healing" signaling pathways and/or by releasing immunomodulatory signals. Indeed, in the context of anticancer therapy it is generally accepted that most chemotherapeutic drugs elicit apoptotic cell death. Phagocytosis of apoptotic cells maintains an anti-inflammatory state in the extracellular environment and thereby contributes to an immunosuppressive network in a primary tumor site to promote further tumorigenesis [11]. Several studies have confirmed this notion. It has been shown that apoptotic tumor cells promote coordinated tumor growth, angiogenesis, and accumulation of tumor-associated macrophages (TAMs) in aggressive B cell lymphomas [12]. It has also been demonstrated that radiotherapy induces caspase-3-dependent release from apoptotic cells of arachidonic acid and prostaglandin E_2, which then promote the growth of the tumor cells that survive radiation activation [13]. This correlates with observations in cancer patients that tumors with elevated levels of activated caspase-3 are associated with a poor disease outcome [13]. All these studies indeed demonstrate that cancer cells undergoing apoptosis can promote tumor progression. However, in the late nineties it was reported that dendritic cells (DCs) internalize apoptotic cells and process them for presentation to both MHC class I- and class II-restricted T cells with an efficiency that is dependent on the number of apoptotic cells [14]. Later, it was discovered that certain types of anticancer treatments, such as chemotherapeutics (e.g., anthracyclines) [15], γ-irradiation [16, 17], and photodynamic therapy [18–21] (Table 6.1) can induce a specific form of apoptosis, which was named immunogenic apoptosis (IA) due to its immunostimulatory or adjuvant-like properties (Fig. 6.1). When cancer cell lines exposed to lethal doses of inducers of immunogenic apoptosis in vitro are used to vaccinate syngenic mice, they protect them against a subsequent challenge with live cancer cells of the same type. The immunogenicity of apoptotic cancerous cells relies on the spatiotemporal emission of specific signals called danger-associated molecular patterns (DAMPs), such as calreticulin (CRT), ATP, and HMGB-1. Most of these molecules have predominantly nonimmunological functions inside the cell but they become immunogenic after they are emitted extracellularly. DAMPs are derived from different subcellular compartments, including the plasma membrane, nucleus, ER, and cytosol, and they can often be modified by the proteolysis and/or oxidation associated with cell death mechanisms [22, 23]. DAMPs exert their immunostimulatory effects upon their recognition by membrane-bound or cytoplasmic pattern-recognition receptors (PRRs, e.g., Toll-like Receptor-4, TLR4), phagocytic receptors or scavenger receptors (e.g., LDL-receptor-related protein, LRP1/CD91), and purinergic receptors (e.g., P_2RX_7/P_2RY_2). These danger signals, in combination with cancer antigens, induce maturation of dendritic cells (DCs) and can lead to an adaptive immune response against tumor cells, thereby mediating anticancer immunity. This review covers recent advances in our understanding of the molecular mechanisms involved in danger signaling, DAMPs emission, the role of ER stress, and classification of immunogenic cell death inducers in relation to the way ER stress is triggered. In the final part, we discuss the role of radiotherapy-induced immunogenic apoptosis and the relationship of its immunogenicity to the fraction dose and concomitant chemotherapy.

Table 6.1 An overview of immunogenic cell death inducers and emission of DAMPs related to the stage of cell death

ICD inducers	Cellular target for ICD inducers	Surface exposed DAMPs and the stage of apoptosis	Secreted or released DAMPs and the stage of apoptosis	Refs
Type I				
Mitoxantrone, doxorubicin, idarubicin, oxaliplatin, UVC, γ-irradiation	Nucleus (DNA or DNA proteins related to cell mitosis	Preapoptotic: CRT/ERp57	Early apoptotic secreted: ATP	[33, 42, 46]
		Mid to late apoptotic: HSP-70	Late apoptotic passive release: HMGB1	
Cyclophosphamide	Nucleus (DNA)	Preapoptotic: CRT	Late apoptotic passive release: HMGB1	[105]
Bortezomib	Cytosol (26S proteasome, CIP2A and ERAD machinery)	Early to mid apoptotic: HSP90	Late apoptotic passive release: HMGB1	[106–110]
Cardiac glycosides	Cell surface (Na^+/K^+ ATPase)	Preapoptotic: CRT	Early to mid apoptotic ATP	[111]
			Late apoptotic passive release HMGB1	
Shikonin	Cytosol (tumor-specific pyruvate kinase-M2 protein)	Early to mid apoptotic: CRT, HSP90, GRP78	ND	[112, 113]
7A7 (EFR-specific antibody)	Cell surface receptor (EGFR)	Preapoptotic: CRT and ERp57	ND	[114]
		Early to mid apoptotic: HSP70 and HSP90		
Wogonin	Mitochondria	Early apoptotic: CRT	Late passive release ATP and HMGB1	[115]
High hydrostatic pressure	Cellular proteins	Preapoptotic (?): CRT, HSP70, HSP90	Late passive release ATP and HMGB1	[116]
Vorinostat (histone deacetylase inhibitor)	Nucleus (chromatin structure))	Early to mid apoptotic: CRT	Late passive release ATP and HMGB1	[117–119]
Bleomycin	Nucleus (DNA)	Early to mid apoptotic: CRT and ERp57	Early apoptotic secreted: ATP	[120]
			Late apoptotic passive release: HMGB1	

(continued)

Table 6.1 (continued)

ICD inducers	Cellular target for ICD inducers	Surface exposed DAMPs and the stage of apoptosis	Secreted or released DAMPs and the stage of apoptosis	Refs
Electrical pulses[a]	Cellular proteins	Early to mid apoptotic: CRT	Early to mid apoptotic ATP	[121]
			Late apoptotic passive release: HMGB1	
Septacidin	Cellular proteins	Early to mid apoptotic: CRT	Early to mid apoptotic ATP	[122]
			Late apoptotic passive release: HMGB1	
Honokiol	Cellular proteins (possibly)	CRT (stage is ND)	ND	[123]
Type II				
Hypericin-based PDT	Endoplasmic reticulum	Preapoptotic: CRT, HSP70	Preapoptotic secreted ATP	[19, 20, 43, 124]
			Late apoptotic passive release HSP70, HSP90, and CRT	
Oncolytic viruses (e.g., CVB3)	Endoplasmic reticulum	Early apoptotic: CRT	Early apoptotic secreted ATP late apoptotic passive release HMGB1	[125, 126]

CRT calreticulin, *DAMP* damage-associated molecular pattern, *ND* not determined, *EGFR* epidermal growth factor receptor, *ERAD* endoplasmic reticulum-associated degradation, *GRP* glucose-regulated protein, *HMGB1* high mobility group protein B1, *HSP* heat shock protein, *ICD* immunogenic cell death, *PDT* photodynamic therapy, *UVC* ultraviolet C, *CVB3* coxsackievirus B
[a]Combining electric pulses with the chemotherapeutic agent bleomycin was required for HMGB1 release

6.2 ER Stress and ROS: Crucial Players in Danger Signaling

Immunogenic anticancer drugs and treatments can trigger IA in dying cancer cells via the combined action of ER stress and ROS production, which activate danger signaling pathways and mediate the trafficking of DAMPs to the extracellular space [20, 24, 25]. ER stress was proposed to be a crucial component because the emission of DAMPs (e.g., calreticulin and ATP) and subsequent immunogenicity of cell death in vivo was found to be diminished when molecular effectors of the ER stress pathway were silenced [20, 25]. Anticancer drugs that do not induce ER stress (e.g., cisplatin) are poor inducers of IA [26]. Notably, the immunogenicity of drugs such as cisplatin could be restored by combining it with thapsigargin or tunicamycin [26]

or by expression of the ER resident protein reticulon-1 [27]. ROS was also proposed to be required for immunogenicity of cell death because antioxidants (*N*-Acetyl cysteine, glutathione ethyl ester, and L-histidine) decrease its immunogenicity [20, 25]. As many immunogenic cell death inducers are diverse both biologically and chemically (reviewed in detail in [24, 28]), there seems to be no simple structure–function relationship that could explain the ability of these agents to induce IA. Therefore, we proposed that immunogenic cell death inducers can be classified into two categories (Type I and Type II) based on their distinct mode of action in the induction of ER stress and apoptosis [24]. Most of immunogenic cell death inducers (Table 6.1) are categorized as type I immunogenic cell death inducers that primarily trigger cell death via targeting cytosolic proteins, plasma membranes, or nucleic proteins rather than primary targeting ER mechanisms [24, 29, 30]. The type II immunogenic cell death inducers preferentially target the ER and include hypericin-based PDT and oncolytic coxsackievirus B3 (CVB3, Table 6.1). Although ER stress and ROS are essential in the immunogenicity of cell death, it is still not clear how these two signaling modules cooperate to efficiently induce immunogenic cell death. Therefore, further studies to elucidate the precise interplay between the ER stress and ROS is required to modulate antitumor immune responses.

6.3 Main Effectors of Immunogenic Cell Death: CRT, ATP, and HMGB1

Calreticulin (CRT) is an ER chaperone and its function is usually linked with Ca^{2+} homeostasis [31]. The role of CRT in the clearance of apoptotic cells was first described by Gardai et al. [32], who showed that CRT acts as a recognition ligand ("eat me" signal) on the surface of apoptotic cells by binding and activating LRP1/CD91 on the engulfing cell (Fig. 6.1). However, a new life was given to CRT by studies showing that CRT exposure is a key determinant of immunogenicity of dying cells and anticancer immune responses [33]. In that study, the authors found that anthracyclines induce rapid preapoptotic translocation of CRT to the cell surface and that blockade or knockdown of CRT suppresses the immunogenicity of apoptotic cancerous cells in mice. Several signaling pathways triggered by immunogenic cell death inducers have been described (Fig. 6.2). One pathway is induced by anthracyclines and relies on the phosphorylation of eukaryotic initiation factor 2a (eIF2a) by the ER stress-sensing kinase, PKR-related ER kinase (PERK), the activation of caspase-8, BAX and BAK, the transport of ER-derived vesicles through the Golgi apparatus, and the SNAP receptor (SNARE)-dependent exocytosis of these vesicles [25]. It has also been shown that paracrine signals that involve the chemokine CXCL8 contribute to CRT exposure on the cell surface [34]. The second pathway for CRT exposure is more rapid and relies on PERK-mediated trafficking of ecto-CRT by regulation of the proximal secretory pathway [20]. In this signaling pathway, eIF2a phosphorylation and caspase-8 signaling were not required for CRT exposure. Vaccination of mice with cells deficient in any of the proteins

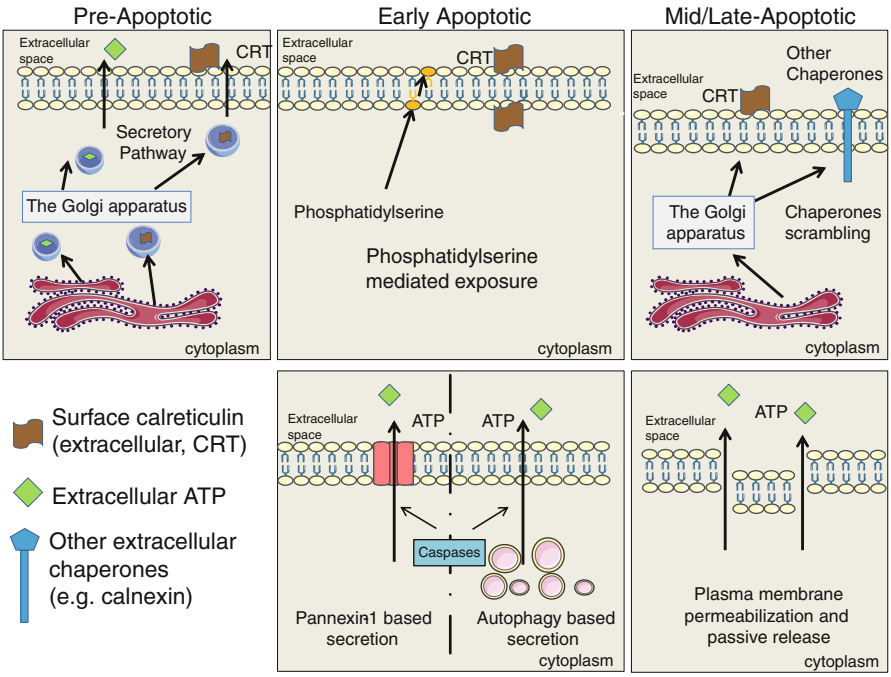

Fig. 6.2 An overview of the danger signaling pathways involved in surface CRT exposure and ATP secretion and their relation to different apoptotic stages. Signaling pathways responsible for surface exposure of CRT and secretion of ATP depend on immunogenic cell death stimuli [24]

required for CRT exposure or with cells in which CRT was knocked down reduced the immunogenicity of the cancer cells [20, 33]. All these results underline the key role of CRT exposure on the cell surface to the efficacy of anticancer therapy.

ATP is involved in various cellular metabolic processes and intracellular responses. However, it has become clear that APT is also actively secreted or passively released from dying cancerous cells, and that it is modulating the immunogenicity of dying cancerous cells (Fig. 6.1) [22, 23, 35, 36] via activation of purinergic P_2X_7 and P_2X_2 receptors [37]. The mechanisms of ATP secretion are strongly dependent on the type of immunogenic cell death inducer. Anthracyclines induce ATP secretion by a mechanism involving the caspase-dependent activation of pannexin 1 channels, lysosomal exocytosis, and plasma membrane blebbing [36, 38, 39]. Moreover, cancer cells undergoing IA in response to anthracycline secrete ATP in an autophagy-dependent manner [40–42]. Autophagy-deficient tumors exposed to chemotherapy cannot attract tumor-infiltrating leukocytes and therefore do not induce therapeutic anticancer immune responses [42]. However, in contrast to anthracyclines, hypericin-based PDT-induced ATP secretion is independent of autophagy [43] and involves the classical and PERK-regulated proximal secretory pathway, as well as PI3K-dependent exocytosis [20]. All these studies suggest that the mechanisms of ATP secretion might vary from one immunogenic cancer cell death inducer to another (Fig. 6.2).

HMGB1 is a broadly expressed and highly abundant nonhistone chromatin-binding protein expressed constitutively by all eukaryotic cells, and it has various cytosolic and extracellular functions [44, 45]. It was found that the immunogenicity of IA also depends on the passive release of HMGB1 from cells undergoing immunogenic death and on its binding to TLR-4 [46]. Nevertheless, the role of HMGB1 in anticancer immunity is complex, and the diversity of HMGB1 extracellular functions can also be partially explained by the posttranslation modifications, including different redox states and cell death types [23, 47, 48].

6.4 Immunostimulatory Effects of Chemotherapeutics Not Related to DAMPs

In addition to the induction of danger signaling and modulation of DAMPs emission in cancer cells (discussed earlier), many chemotherapeutics can induce immunostimulation by targeting other elements of anticancer immunity [36]. Chemotherapeutic drugs can increase the expression or presentation of tumor-associated antigens (TAA) on the surface of cancer cells and increase their so-called antigenicity by inducing antigen presentation of both dominant and subdominant epitopes. It has been shown that the variety of TAA eliciting cytotoxic T lymphocytes (CTL) can be increased by cisplatin and gemcitabine [49]. The authors showed that chemotherapy reveals weaker tumor antigens to the immune system, resulting in the induction of specific CTLs. The antigenicity of cancer cells can be enhanced by increasing the expression of MHC class I molecules (e.g., cyclophosphamide, gemcitabine, oxaliplatin, paclitaxel, and γ-irradiation) [36, 50, 51]. In addition, some anticancer drugs can increase the expression of TAA, including carcinoembryonic antigen (induced by 5-fluorouracil), multiple cancer testis antigens (increased by 5-aza-20deoxycytidine and γ-irradiation), and melanoma-associated antigens (increased by vemurafenib) [36, 50, 52, 53]. It is of interest that subtoxic doses of paclitaxel and doxorubicin increased the expression of components of the MHC class I antigen processing machinery (calmodulin, LMP2, LMP7, TAP1, and tapasin) in cancer cells [54]. Chemotherapeutic agents also cause immunopotentiation by directly stimulating immune cells. It has been shown that low doses of paclitaxel, doxorubicin, mitomycin C, and methotrexate that do not cause cell death up-regulate the ability of DCs to present antigens to antigen-specific T cells [55]. Recently, we demonstrated that intraperitoneal injection of doxorubicin in mice triggers the signs of acute inflammatory response (accumulation of neutrophils and increased levels of IL6, TNF, and MCP-1) [56–58]. Of interest is that the inflammatory response was significantly reduced in mice deficient in myeloid differentiation primary response gene 88 (MyD88), TLR-2 or TLR-9 [58], or tumor necrosis factor receptor-1 (TNFR1) [57]. These studies provide important new insights into how the innate immune system is modulated by immunogenic drugs such as doxorubicin (Table 6.1). It was also shown that the percentage of regulatory T cells among the $CD4^+$ lymphocytes was decreased by cyclophosphamide, which allowed a whole tumor cell vaccine or costimulatory

receptor OX40 (OX86) immunotherapy to eradicate established tumors in colon carcinoma or melanoma models [59, 60]. The number of myeloid-derived suppressor cells (MDSCs) was reduced by gemcitabine in the spleen of mice bearing large tumors but did not affect CD4 and CD8 T cells, NK cells, macrophages, and B cells [61–63]. The bisphosphonate zoledronate, a drug that has been approved by the FDA for the treatment of bone metastases, was shown to induce caspase-1 activation in DC-like cells, which then provide mature IL-18 and IL-1β for the activation of IL-2-primed NK cells [64]. All these data suggest that some chemotherapeutics can directly stimulate immune cell functions and that their therapeutic efficacy could be at least partly explained by their ability to modulate the host immune system.

6.5 Radiotherapy-Induced Immunogenic Cell Death: Fraction Dose and Concomitant Chemotherapy

Together with surgery and chemotherapy, gamma-irradiation (RT) is important in the treatment of cancer. For decades, its main antitumor activity was believed to result from a direct and local cytotoxic effect on malignant cells within the irradiated area [65]. Nowadays, there is growing evidence for the occurrence of immune-mediated systemic effects resulting from local RT. Clinical proof of principle for such abscopal effects is provided by regression of distant metastases after local RT. Abscopal effects have been observed with various dose and fractionation regimens in melanoma (3×8 Gy to 3×18 Gy) [66–68] and lung adenocarcinoma (5×6 Gy) [69]. The necessity of combining RT with immunotherapy (in these cases CTLA4 blockade) to achieve these abscopal effects indicates that proimmunogenic effects are often dampened by the immune-suppressive microenvironment that characterizes cancer [70–73].

As for other immunogenic agents [74], radiation-induced immunogenic cell death is characterized, in cell cultures, by preapoptotic exposure on the extracellular surface of the “eat-me” signal CRT [25, 75, 76] and emission of ATP [75, 77, 78], and by late-apoptotic release of the “find-me” signal HMGB-1 [46, 75, 77, 79]. Animal and clinical experimental evidence supporting the ability of RT to induce immunogenic cell death remains scarce [77], and the clinical relevance of these pathways to the therapeutic efficacy of RT has yet to be validated.

Induction of immunogenic cell death is most likely highly dependent on total dose and fractionation. Golden et al. showed, in cell cultures, that the clinically used single doses between 2 and 20 Gy (1×2–20 Gy) effectively induce the signals for each individual component of immunogenic cell death in a dose-dependent manner [75]. Gameiro et al. showed the same, albeit with a clinically irrelevant single dose of 100 Gy [77]. Demaria et al. overviewed the literature and found immunogenic cell death to be often detected in tumor cell cultures exposed to mid-to-high doses of RT (1×>5–10 Gy) [80]. They initiated animal experiments using three RT regimens (1×20 Gy, 3×8 Gy and 5×6 Gy) combined with CTLA-4 antibody treatment in syngeneic mice with breast and colorectal carcinoma. While anti-CTLA-4 treatment

on its own and its combination with a single-dose RT were not able to induce an abscopal effect, the fractioned regimens did [81]. This could explain why a single 8-Gy fraction treatment of bone metastases in prostate cancer patients failed to induce an abscopal effect when combined with anti-CTLA-4 treatment [82], whereas the above described clinical trials succeeded [66–69].

In addition to the induction of immunogenic cell death, other components up- or downregulated in response to RT are involved in antitumor immunity [71]. Tumor cell surface expression of MHC Class I molecules increases and CD47 (a "don't eat-me" signal for DCs) decreases in a dose-dependent manner in cell cultures [83–85]. Additionally, it was shown in a murine model that RT (2×12 Gy) increases the expression on tumor cell surface of RAE-1, a ligand for natural killer cell group 2D [86]. Distinct radiation fraction doses also have a direct effect on the irradiated tumor microenvironment. Clinical observations showed that immune-suppressing Treg cells are more radioresistant than $CD8^+$ T cells [87, 88]. In a xenotransplant mouse model, a lower RT dose (1×2 Gy) reprograms macrophages toward an iNOS+/M1 phenotype, allowing them to recruit tumor-specific T cells [89].

The above-mentioned data support the growing consensus that hypofractionated regimens (a limited number but >1 fraction high doses per fraction) are more effective at inducing the proimmunogenic effects of RT than single high doses or normofractionation (2 Gy per fraction or "×" times×2 Gy) [90]. The hypofractionated regimens are mostly used to treat small (often oligo-) metastatic lesions, whereas for treatment of the primary tumor, normofractionation combined with chemotherapy is often the standard treatment. Concomitant use of both treatments has been shown to be superior to sequential chemo-RT in numerous clinical trials. It should be considered that concomitant chemo-RT causes a tumor cell death that is both qualitatively and quantitatively different from that achieved by each therapy alone [83]. Frey et al. showed that combining 5-FU, oxaliplatin, and irinotecan with RT could induce immunogenic cell death in colorectal cancer cells [91]. Golden et al. designed a cell culture assay to examine the effect on immunogenic cell death when combining RT (1×2 Gy) with paclitaxel and found that all three components of immunogenic cell death (i.e., CRT, ATP, and HMGB1; discussed earlier) to be increased significantly when chemotherapy and RT were used together as compared to separate treatments [75, 83]. Animal and clinical experiments are awaited to validate these interesting findings.

6.6 Conclusions

Only one decade ago, apoptotic cell death was presented as anti-inflammatory and tolerogenic, or even as a silent mode of cell death. However, insights over the last decade increasingly support the view that under specific conditions certain types and regimens of anticancer therapy can induce an immunogenic form of apoptosis that can be beneficial for the induction of anticancer immunity and long-lasting remission in cancer patients. Many questions remain regarding what determines the

difference between immunogenic aspects of apoptosis and the danger signaling subroutines in the various types of cancers. Deeper insight into the molecular mechanisms of immunogenicity of apoptotic cells will lead to novel experimental immunotherapies for cancer, and is therefore a challenging research area. This work highlights the need for careful preclinical testing of the immunological effects of chemotherapies, alone and in combination with partner cytotoxic agents and immunotherapies, before proceeding to clinical investigations.

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