Immunogenic cell death (ICD) is a form of regulated cell death (RCD) induced by various stresses and produces antitumor immunity via damage-associated molecular patterns (DAMPs) release or exposure, mainly including high mobility group box 1 (HMGB1), calreticulin (CRT), adenosine triphosphate (ATP), and heat shock proteins (HSPs). Emerging evidence has suggested that ionizing radiation (IR) can induce ICD, and the dose, type, and fractionation of irradiation influence the induction of ICD. At present, IR-induced ICD is mainly verified in vitro in mice and there is few clinical evidence about it. To boost the induction of ICD by IR, some strategies have shown synergy with IR to enhance antitumor immune response, such as hyperthermia, nanoparticles, and chemotherapy. In this review, we focus on the molecular mechanisms of ICD, ICD-promoting factors associated with irradiation, the clinical evidence of ICD, and immunogenic forms of cell death. Finally, we summarize various methods of improving ICD induced by IR.

Keywords: immunogenic cell death, ionizing radiation, ferroptosis, necroptosis, damage-associated molecular patterns, nanoparticles, hyperthermia, chemotherapy

Abbreviations: ASC, Apoptosis-associated speck-like protein containing a caspase recruit domain; ATP, Adenosine triphosphate; Bip, Binding immunoglobulin protein; cDAMPs, Constitutive damage-associated molecular patterns; cGAMP, Cyclic GMP-AMP; cGAS, Cyclic GMP-AMP synthase; CRT, Calreticulin; CTL, Cytotoxic T lymphocyte; CXCL, C-X-C motif chemokine ligand; DAMPs, Damage-associated molecular patterns; DC, Dendritic cell; dsDNA, Double-strand DNA; ER, Endoplasmic reticulum; Fas, Factor related apoptosis; Fei, Factor related apoptosis ligand; HFRT, Hypofractionated radiation therapy; HMGB1, High mobility group box 1; HSPs, Heat shock proteins; HT, Hyperthermia; ICD, Immunogenic cell death; iDAMPs, Inducible damage-associated molecular patterns; IFN-I, Type I interferon; IFN-γ, Type γ interferon; IL-1β, Interleukin-1β; IL-6, Interleukin-6; IR, Ionizing radiation; IRF3, Interferon regulatory factor 3; mFX, Modified dose of FOLFIRINOX; MHC I, Major histocompatibility complex class I; MLKL, Mixed lineage kinase domain-like protein; NLRP3, NOD-like receptor protein 3; NSCLC, Non-small cell lung cancer; P2RX7, P2X purinoceptor 7; P2RY2, Purinergic receptor P2Y2; RBE, Relative biological effectiveness; RCD, Regulated cell death; RCT, Radiochemotherapy; RIPK1, Receptor-interacting serine/threonine-protein kinase 1; ROS, Reactive oxygen species; RT, Radiotherapy; SBRT, Stereotactic body radiotherapy; STING, Stimulator of interferon genes; TBK1, TANK-binding kinase 1; TCR, T-cell receptor; TIL, Tumor-infiltrating lymphocyte; TLR4, Toll-like receptor 4; TNF-α, Tumor necrosis factor-α.
INTRODUCTION

As a major modality in clinical cancer treatment, radiotherapy (RT) cures or palliates cancer in more than 50% of patients (1). However, radiation resistance of some cancers remains a clinical problem (2). Therefore, it is urgent to find effective ways to solve this problem. RT utilizes ionizing radiation (IR) to induce cell death directly through damaging double-strand DNA (dsDNA) (1). Moreover, IR has the potential to produce antitumor immunity, which is suggested by the discovery of the abscopal effect (3). The abscopal effect refers to the retarded growth of distant metastases following irradiation of the primary tumor (4). IR drives an antitumor immune response through a series of mechanisms [such as the upregulation of major histocompatibility complex (MHC) class I molecules, intercellular adhesion molecule-1, and factor-related apoptosis (Fas)], one of which is the induction of immunogenic cell death (ICD) (5).

ICD is a type of regulated cell death (RCD) driven by cellular stressors, including chemotherapy, IR, targeted anticancer agents, photodynamic therapy, and high hydrostatic pressure. It initiates CD8+ T cell-mediated adaptive immune response through damage-associated molecular patterns (DAMPs) emission (6–8). The induction of ICD by IR depends on the type of radiation, radiation dose, fractionation schedule, and tumor types. In addition, ICD such as necroptosis and ferroptosis can improve the radiosensitivity of tumor cells (9, 10). Thus, ICD inducers might be an auxiliary treatment method to produce synergistic antitumor effect combined with RT. In recent years, immunotherapy has been a hotspot in cancer treatment and ICD activation might be a promising cancer therapy modality.

Herein, we mainly discuss the mechanism of IR-driven ICD, factors related IR-induced ICD, and clinical evidence. Lastly, we summarize the methods of enhancing IR-induced ICD.

THE MECHANISM OF IR-DRIVEN ICD

The induction of ICD requires three factors:

1. Inducible damage-associated molecular patterns (iDAMPs): cytokines [such as interleukin-6 (IL-6)] and chemokines [such as C-X-C motif chemokine ligand 1 (CXCL1), CXCL2, CXCL10, and CC chemokine receptor 2];
2. Tumor-associated or tumor-specific antigens;
3. Constitutive damage-associated molecular patterns (cDAMPs): high mobility group box 1 (HMGB1), calreticulin (CRT), adenosine triphosphate (ATP), heat shock proteins (HSPs), and so on (11).

Irradiating tumor cells result in reactive oxygen species (ROS) production and collateral endoplasmic reticulum (ER) stress effects, which are required for cDAMPs release or exposure (12, 13). Specifically, cDAMPs involve HMGB1 and ATP release, CRT relocation, and HSPs exposure (14). cDAMPs act as “find me” signals (ATP and HMGB1) or “eat me” signals (HSPs and CRT) to mobilize antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages, and their precursors to tumor cells with the help of chemokines (15). The activation and maturity of APCs especially DCs depend on the interaction of cDAMPs and pattern recognition receptors (PRRs) on these cells. PRRs include P2RX7 (P2X purinoceptor 7), P2RY2 (purinergic receptor P2Y2), CD91, CD40, and TLR4 (tol-like receptor 4) (16). Belonging to the ionotropic purinergic P2X subfamily, P2RX7 is a 595aa protein expressed on almost all immune cells and promotes IL-18 and IL-1β secretion via the NLRP3/ASC/caspase-1 (NLRP3: NOD-like receptor protein 3; ASC: apoptosis-associated speck-like protein containing a caspase recruit domain) pathway when combined with ATP (17, 18). Interaction of ATP with P2RY2 promotes the recruitment of immature DCs, monocytes or macrophages, and neutrophils (6). CD91, also known as low-density lipoprotein receptor-related protein 1, is an endocytic and cell signaling receptor expressed on the surface of both normal and tumor cells (19). The CRT–CD91 interaction leads to the release of TNF-α (tumor necrosis factor-α) and IL-6 (20). CD91 also combines with HSP90 to facilitate cross-presentation (21). CD40 is a costimulatory molecule belonging to the TNF receptor superfamily and mainly expressed on APCs (22). HSP70 binding to CD40 results in CD8+ cytotoxic T-cell activation (6). As part of the TLR family, TLR4 is mostly expressed on the surface of innate immune cells and recognizes pathogen-associated molecular patterns like lipopolysaccharide and cDAMPs like HMGB1 to elicit immune responses (23). HMGB1-TLR4 stimulates the release of pro-inflammatory cytokines (24). More importantly, combining cDAMPs with PRRs on DCs activates DCs to engulf tumor cells, process tumor antigens, and express these tumor antigens along with MHC-I molecules on the plasma membrane (25). Once activated and maturing, DCs migrate to tumor-draining lymph nodes. Secreted from mature DCs, IL-6, IL-1β, TNF-α, and type γ interferon (IFN-γ) promote the differentiation of T cells into the CD8+ phenotype. Then, CD8+ T cells are activated by antigen cross-presentation from DCs to become cytotoxic T lymphocytes (CTLs) (20). Ultimately, CTLs induce tumor cell apoptosis through the release of perforin and granzyme B, or a combination of Fas ligand (FasL) with Fas (Figure 1) (11, 26).

IR also induces ICD via the IFN-1 response. Nucleic dsDNA is damaged by IR and released from the nucleus to aggregate in the cytoplasm. Cytosolic dsDNA is sensed by cyclic GMP-AMP (cGAMP) synthase (cGAS), resulting in cGAMP generation. Consequently, the STING–TBK1–IRF3 pathway (STING: stimulator of interferon genes; TBK1: TANK-binding kinase 1; IRF3: interferon regulatory factor 3) is activated, causing IFN-1 production and its release from inside to outside the cell (27). The interaction between IFN-1 and its receptor on DCs promotes DCs maturation, increases the expression of costimulatory molecules on DCs, and enhances the migration ability of DCs to tumor-draining lymph nodes, eventually promoting antitumor immunity mediated by CD8+ T cells (28). IFN-1 also stimulates macrophages to secrete inflammatory factors and restrains the immunosuppressive ability of CD4+CD25+FOXP3+ Tregs (29).
IR-ASSOCIATED FACTORS ABOUT ICD ACTIVATION

IR-driven ICD is not only correlated with tumor genetic background and tumor microenvironement but also linked with post-irradiation time, and the type, dose, and fractionation schedule of radiation (2, 30, 31).

Within a certain range, IR induces ICD in a time-dependent manner. Three radical beams (proton, photon, and carbon ion) drive CRT exposure in four human carcinoma cell lines (CNE-2, A549, U251, and Tca8113), and CRT exposure raises with an increase in post-irradiation time (12, 24, and 48 h) (32). In addition, a study found that the increase in HSP70 and HMGB1 started 24 and 48 h, respectively, in MC-38 cells following irradiation with $^{213}$Bi (33).

To compare ICD induction by different types of IR (carbon-ion, proton, and photon irradiation), the relative biological effectiveness (RBE) must be taken into consideration. RBE is defined as the ratio of physical doses that lead to the same biological effect (34). The RBE value of proton and photon is regarded as 1.1, but carbon ion is 2–3 (34, 35). Certainly, the RBE value is related with the type of tumor cells. Therefore, the equivalent biological dose (the product of physical dose and RBE) of carbon ion is more than twice that of proton and photon at the same physical dose in the CNE-2 cell line. Yangle Huang et al. confirmed that carbon-ion induced more CRT exposure.
In the clinical setting, it is difficult to prove the induction of ICD in patients treated with RT. Some clinical studies combined RT with immunotherapy to verify ICD indirectly according to enhanced systemic tumor responses (2). In fact, RT–driven antitumor immunity is not completely attributed to ICD, as mentioned at the beginning of the review. The current studies might only indirectly verify ICD by detecting cDAMPs in serum or tumor tissue resected from patients after RT (44). One study confirmed that elevated tumor cell surface expression of CRT was detected in tumor samples of patients with renal clear cell carcinoma treated with stereotactic body radiotherapy (SBRT) (45). Takashi Murakami et al. suggested that neoadjuvant chemoradiotherapy induces the overexpression of MHC I-related chain A/B, CRT, and HSP70, and creates a favorable immunogenic tumor microenvironment with a low Treg/tumor infiltrating lymphocyte (TIL) ratio in patients with pancreatic cancer (46). Yoshiyuki Suzuki et al. indicated that chemoradiotherapy induced tumor antigen–specific T-cell responses in patients with esophageal squamous cell carcinoma along with increased HMGB1 release in serum (47).

It has been reported that some cDAMPs might have the potential to predict clinical outcomes of patients following RT (44). For example, HMGB1 was significantly increased in the serum of patients with esophageal cancer after chemoradiation, and its level was positively correlated with patient survival (47, 48). However, cDAMPs as double-edged swords can also promote tumor growth, so elevated cDAMPs expression might be associated with decreased survival (21). In general, whether high HSP70 levels predict positive clinical outcomes depends on its position in cells, tumor types, and its durable upregulation time following RT. It has been confirmed that intracellular HSP70 suppresses tumor cell apoptosis whereas membrane-bound and extracellular HSP70 stimulates the innate and adaptive antitumor immunity to retard tumor growth (49). Stefan Stangl et al. suggested that high intracellular HSP70 expression and low NK cell infiltration in patients with squamous-cell carcinoma of the head and neck were linked with unfavorable outcomes after surgery and radiochemotherapy (RCT) (49). In contrast, progression-free survival and overall survival were significantly better after RCT in patients with primary glioblastomas and having high intracellular HSP70 expression levels (50). Another study demonstrated that the level of free HSP70 was upregulated in the serum of patients with breast cancer up to 6 weeks after RT, which might predict an unfavorable prognosis as a result of a chronic inflammatory response (51).

### IMMUNOGENIC FORMS OF CELL DEATH

The types of tumor cell death induced by IR include mitotic catastrophe and mitotic death, apoptosis, necrosis, senescence, necroptosis, and ferroptosis (2). Among them, necroptosis and caspase-independent apoptosis elicited by high levels of radiation are regarded as immunogenic forms of cell death (52). A common feature of these cell death types is the release or exposure of cDAMPs; therefore, the latter are the key molecules of initiating antitumor immunity (52). In addition, it is proved that the gold standard for ICD evaluation is to vaccinate immunocompetent mice with dying syngeneic cancer cells and then rechallenge them with live cancer cells. An absence or slowdown of tumor growth would indicate the induction of an adaptive antitumor immune response (53).

As a form of RCD, necroptosis is mediated by receptor-interacting serine/threonine-protein kinase 1 (RIPK1), RIPK3, and mixed lineage kinase domain-like protein (MLKL), and leads to cellular swelling, membrane disruption, and intracellular contents release (54, 55). The successful induction of necroptosis with IR in non-small cell lung cancer (NSCLC) cells depends on irradiation dose, fractionation schedule, and RIPK3 expression. In NSCLC cells with low RIPK3 expression, apoptosis and necroptosis are activated dose-dependently both with <10 Gy/ fraction RT and ≥10 Gy/fraction ablative hypofractionated radiation therapy (HFRT). In NSCLC cells with high RIPK3 expression, necroptosis is preferentially stimulated by ≥10 Gy/ fraction ablative HFRT. The induction and activation of MLKL and HMGB1 release are involved both in vitro and in vivo (37). The release of HMGB1 from necrotic tumor cells that underwent ablative HFRT suggests that necroptosis induced by ablative HFRT is immunogenic and has the potential to activate immune response although there is very little direct evidence.
available about it. Indeed, necroptosis has been confirmed as ICD in that tumor-bearing mice injected with necroptotic fibroblasts initiate a systemic immune response (56). The activation of the RIPK1/RIPK3/NF-κb pathway in necroptotic cells results in the release of cytokines that promote DC activation and stimulate antitumor immunity mediated by CTLs (56).

Ferroptosis is a form of iron-dependent RCD that is regulated by lipid, iron, and cysteine metabolism with lipid peroxidation and lethal ROS accumulation (10, 57). Like necroptosis, ferroptosis also belongs to regulated necrosis, which can release cDAMPs to activate antitumor immune response (58, 59). A study demonstrated that irradiated tumor cells released microparticles to induce ferroptosis, which promotes cell surface exposure of CRT, ATP secretion, and macrophage phagocytosis (60). Although IR induces ferroptosis indirectly to activate antitumor immune response, there is growing evidence regarding the induction of ferroptosis by IR in tumor cells and the immunogenicity of ferroptosis (42, 61, 62). The connection between IR-induced ferroptosis and antitumor immunity might be linked to many factors such as radiation dose and type. The previous studies have indicated that IR induces tumor cell ferroptosis and ferroptosis improves the radiosensitivity of tumor cells (10, 63, 64). Based on this, RT combined with ferroptosis inducers may improve the therapeutic effect of RT by increasing lipid peroxidation and promoting immune response rather than DNA damage or caspase activation (65). Besides cDAMPs, ferroptotic tumor cells may release oxidized lipids such as arachidonic acid-derived oxidation products and oxidized phospholipids to regulate antitumor immunity, but further studies are needed to confirm this (66).

### THE METHODS OF ENHANCING IR-INDUCED ICD

Many ICD inducers have been developed to promote ICD and augment antitumor immunity, including cytotoxic drugs like anthracyclines or again oncolytic viruses (7). There are some therapies that boost IR-activated ICD, such as nanoparticles, hyperthermia, or chemotherapy. Table 1 summarizes the methods that further stimulate ICD upon IR.

As drug delivery carriers, nanoparticles have high tumor-targeting, permeability and retention effect, and allow to decrease toxicity by reducing drug dosing (79). As a result, the development of nanoparticles has become a research hotspot currently. Nanoparticles are classified into synthetic polymers, biomimetic materials, and inorganic materials according to their physical and chemical properties (80). Some smart nanoparticles are activated by specific conditions such as PH, light, and hyperthermia to release drugs (80). Accumulating studies have combined nanoparticles with immunoadjuvants, chemotherapeutics, photodynamic therapy, or again photothermal therapy in order to reshape the tumor hypoxic microenvironment to elicit or enhance ICD (81, 82). These novel nanoparticles synergized with IR and enhanced antitumor immune function (83–87). Qian Chen et al. confirmed that PLGA-R837@Cat nanoparticle-based x-ray radiation inhibited primary tumors significantly in comparison with RT alone. Moreover, this method generated strong antitumor immune responses with enhanced ICD induction; DC activation and maturation; the secretion of IL-2, IFN-γ, and TNF-α; and CTL infiltration. RT enhanced by PLGA-R837@Cat combined with

| Table 1 | Methods promoting RT-induced ICD. |
|----------|-----------------------------------|
| Methods  | Tumor cells | ICD hallmarks | References |
| Nanoparticles | | | |
| AuNPs | MDA MB 231 cells | CRT exposure | (67) |
| H@Gd-NCPs | CT26 cells | CRT exposure | (68) |
| PLGA-R837@Cat | CT26 cells | CRT exposure | (69) |
| UCNP-DOX | H460 cells | ATP secretion | (70) |
| W02.9-WSe2-PEG | 4T1 cells | CRT exposure | (71) |
| S-AuNC | Tramp C1 cells | CRT expression | (72) |
| ASTX660 | MOC1 cells | CRT/HSF70 exposure | (73) |
| Hyperthermia | | | |
| mFX | B16-F10 cells | HSP70/HMG1 release | (74) |
| platinum | HCT15 cells | HSP70/HMG1 release | (75, 76) |
| Chemotherapy | | | |
| Bip inhibition | Gloma stem cells | CRT exposure | (77) |
| | | ATP secretion | (78) |

Zhu et al. Ionizing Radiation and Immunogenic Cell Death

Frontiers in Immunology | www.frontiersin.org August 2021 | Volume 12 | Article 705361

5
anti-CTLA4 checkpoint blockade not only led to complete elimination of primary tumors but also further retarded the growth of metastatic tumors with long-term immune memory protection (69).

Hyperthermia (HT) is a cancer treatment strategy by raising tissue temperature to 39–45°C for a certain period of time and it has been reported in many phase II and phase III trials combined with radiotherapy and chemotherapy (88, 89). HT can not only cause protein denaturation and aggregation to activate downstream pathways such as the DNA damage response to kill tumor cells directly but also induce ICD to reverse tumor immunosuppressive microenvironment and produce antitumor immunity (90). HSPs are the most important cDAMPs to initiate tumor-specific immune responses via TLR4 signaling (16, 90). One research treated CT26 tumor cells with RT and/or HT, then injected them subcutaneously into Balb/c, and monitored tumor growth. Cell treatment with RT at 2 or 5 Gy plus HT delayed tumor growth whereas irradiation with 2 Gy alone did not significantly inhibit tumor growth. In an in vitro experiment, x-radiation in combination with HT promoted the increase of HSP70 release and the activation, maturation, and cross-presentation of DCs (75). Fractionated RT (3x2 Gy) retards tumor growth significantly in B16-F10 tumor-bearing mice when combined with HT, in comparison with fractionated RT alone. RT with 2 Gy plus HT increases HSP70 and HMGB1 release significantly in tumor cells; RT with 3x2 Gy plus HT facilitates CD8+ T cell, DC, and NK cell infiltration, but inhibits Treg and MDSC infiltration in the tumors (74).

ASTX660 is an inhibitor of apoptosis protein antagonist, resulting in cellular Inhibitor of Apoptosis Proteins 1/2 degradation and X-linked inhibitor of apoptosis protein inhibition (91). Previous research has demonstrated that ASTX660 can potentiate antitumor immunity by enhancing the sensitivity of tumor cells to induction of TNFR superfamily ligation-related apoptosis downstream (92). The combination of ASTX660 and RT significantly boosts tumor regression through the function of TNFα, CD8+ T cells, and NK cells (92). Wenda Ye et al. indicated that ASTX660 further facilitated CRT and HSP70 exposure and HMGB1 release in MOG1 cells when combined with IR as compared to ASTX660 alone. In in vivo experiments, ASTX660 heightened IR-induced ICD modestly to inhibit tumor generation in 72% of mice in contrast with 50% of mice treated with IR alone. In addition, ASTX660 combined with IR promoted clonal expansion of TIL by enhancing DCs activation and MHC I expression (73).

As a traditional therapy, chemotherapy is widely used in advanced cancer treatment and functions as the auxiliary therapy following surgery. Consistent with RT, chemotherapy induces ICD characterized by CRT exposure, ATP, and HMGB1 secretion through collateral ER stress effects (93). Nevertheless, not all chemotherapeutics can activate ICD, and chemotherapeutics certified to have the ability of ICD induction include idarubicin, epirubicin, doxorubicin, mitoxantrone, oxaliplatin, bortezomib, and cyclophosphamide (94). Concurrent treatment with a modified dose of FOLFIRINOX (mFX) and SBRT enhanced antitumor efficacy and produced long-term systemic antitumor immunity. KCKO cells treated with mFX and SBRT were subcutaneously injected into mice. The results showed that percentage of tumor-free mice was 100% and enhanced ICD was verified in tumor tissue featured by increased HMGB1 release and CRT exposure compared with single treatment. Treatment with mFX and SBRT boosted the phagocytosis, maturation, and cross-presentation of DCs and intratumoral infiltration of CD8+ T cells (77).

Known as a molecular chaperone in the ER, binding immunoglobulin protein (Bip) plays a key role in the unfolded protein response to protect cells from denatured proteins and to suppress apoptosis (95). It has also been reported that Bip expression is associated with increased tumor progression (96). Accordingly, Bip inhibition can inhibit tumor growth by enhancing ER stress to elicit cDAMPs release or exposure to activate antitumor immunity (78). The combination of Bip inhibition and 10 Gy IR enhanced IR-induced ICD with increased CRT exposure, ATP secretion, and HMGB1 release and promoted the phagocytosis and maturation of DCs. As a vaccine, glioma stem cells treated with Bip inhibition and IR could delay tumor generation efficiently, facilitate the proliferation of CD4+ and CD8+ T cells, and decrease Treg cell infiltration in vivo (78).

CONCLUSION AND PERSPECTIVES

IR can induce ICD in tumor cells to produce an adaptive immune response mediated by CD8+ T cells. The irradiation dosage and fractionation schedule for ICD induction have not been determined, so future research should focus on proper dose and fractionation to activate ICD efficiently, while keeping side effects tolerable. Currently, there are few clinical studies on IR-induced ICD and ICD verification remains indirect. Thus, the number and function of immune cells activated by cDAMP should be further detected. In addition, necroptosis and ferroptosis have dual effects for regulating immune response; thus, it is pivotal to induce these cell death forms to a beneficial direction for cancer treatment.

AUTHOR CONTRIBUTIONS

MZ wrote and edited the manuscript. MY, JZ, SQ, HZ, and FY participated in the conception and design of the study. YY, XF, and YZ collected the literature and data. MY, JZ, SQ, HZ, and FY modified grammatical errors and provided intellectual guidance. All authors contributed to the article and approved the submitted version.

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