IMMUNOGENIC CELL DEATH IN CANCER: FROM BENCHSIDE RESEARCH TO BEDSIDE REALITY

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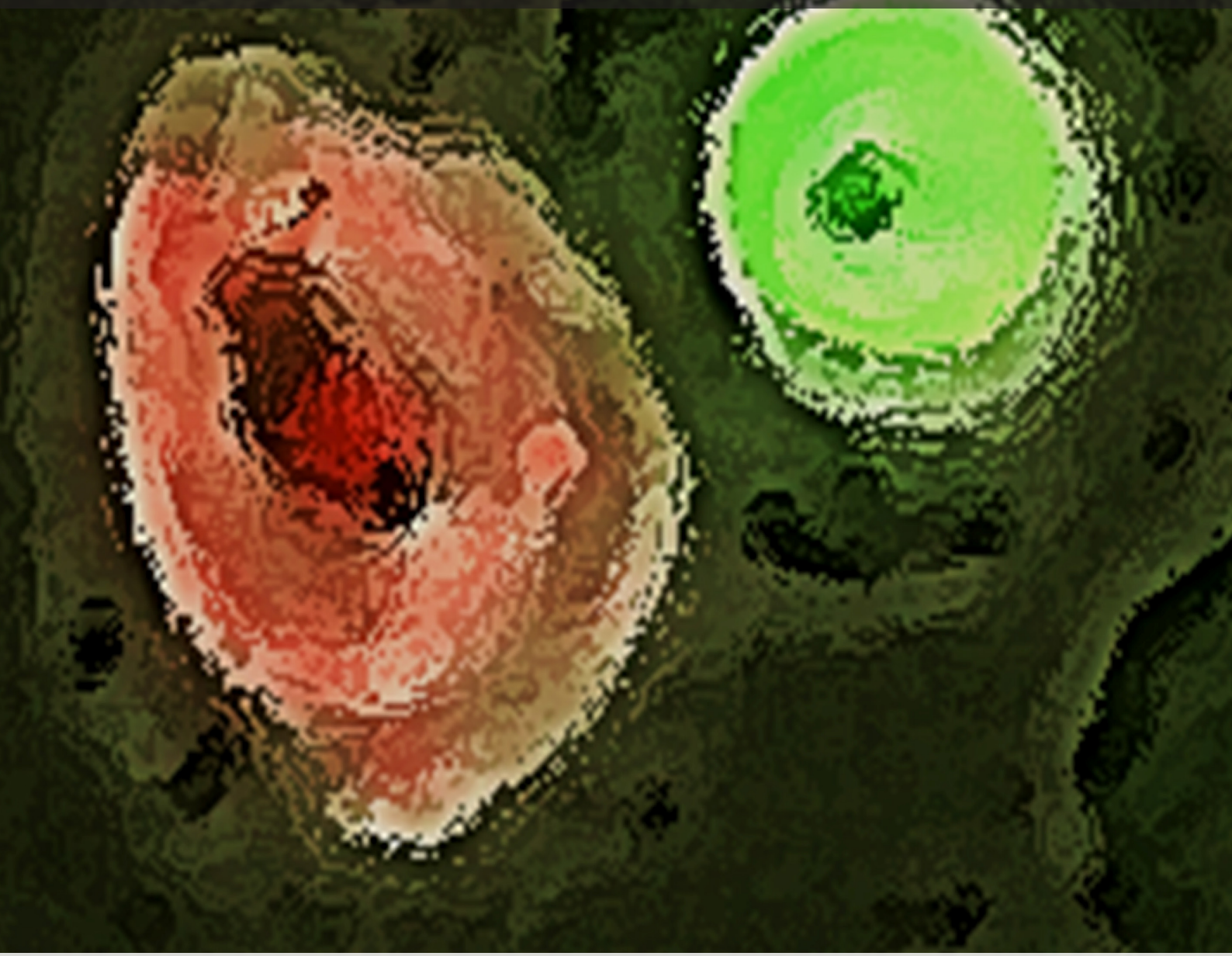

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IMMUNOGENIC CELL DEATH IN CANCER: FROM BENCHSIDE RESEARCH TO BEDSIDE REALITY

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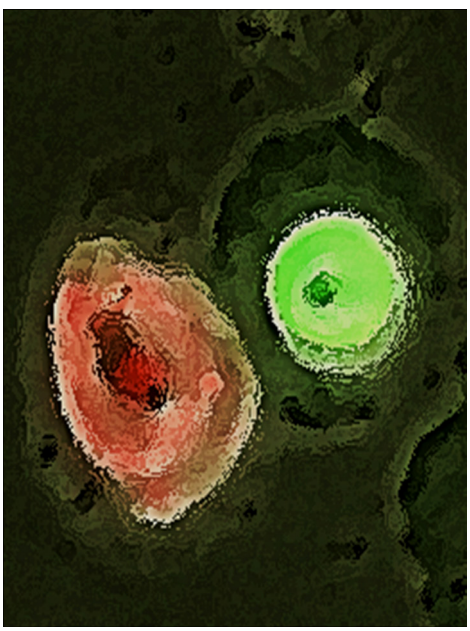

Glow-diffused rendition of a fluorescence microscopy image depicting a human dendritic cell (green) interacting with a human cancer cell (T24 bladder carcinoma cells; red) undergoing immunogenic cell death (ICD) induced by Hypericin-based Photodynamic Therapy (Hyp-PDT).

Image by Abhishek D. Garg and Patrizia Agostinis.

Classically, anti-cancer therapies have always been applied with the primary aim of tumor debulking achieved through widespread induction of cancer cell death. While the role of host immune system is frequently considered as host protective in various (antigen-bearing) pathologies or infections yet in case of cancer overtime it was proposed that the host immune system either plays no role in therapeutic efficacy or plays a limited role that is therapeutically unemployable. The concept that the immune system is dispensable for the efficacy of anticancer therapies lingered on for a substantial amount of time; not only because evidence supporting the claim that anti-cancer immunity played a role were mainly contradictory, but also largely because it was considered acceptable (and sometimes still is) to test anticancer therapies in immunodeficient mice (i.e. SCID/athymic mice lacking adaptive immune system). This latter practice played a detrimental role in appreciating the role of anticancer immunity in cancer therapy. This scenario is epitomized by the fact that for a long time the very existence of cancer-associated antigens or cancer-associated 'danger signaling' remained controversial.

However, over last several years this dogmatic view has been considerably modified. The existence of cancer-associated antigens and 'danger signaling' has been proven to be incontrovertible. These developments have together paved way for the establishment of the attractive concept of "immunogenic cell death" (ICD). It has been established that a restricted class of chemotherapeutics/targeted therapeutics, radiotherapy, photodynamic therapy and certain oncolytic viruses can induce a form of cancer cell death called ICD which is accompanied by spatiotemporally defined emission of danger signals. These danger signals along with other factors help cancer cells undergoing ICD to activate host innate immune cells, which in turn activate T cell-based immunity that helps eradicate live (or residual) surviving cancer cells.

The emergence of ICD has been marred by some controversy. ICD has been criticized to be either experimental model or setting-specific or mostly a concept based on rodent studies that may have very limited implications for clinical application. However, in recent times it has emerged (through mainly retrospective or prognostic studies) that ICD can work in various human clinical settings hinting towards clinical applicability of ICD. However a widespread consensus on this issue is still transitional.

In the current Research Topic we aimed to organize and intensify a discussion that strives to bring together the academic and clinical research community in order to provide a background to the current state-of-the-art in ICD associated bench-side research and to initiate fruitful discussions on present and future prospects of ICD translating towards the clinical, bedside reality.

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EDITORIAL
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Editorial: Immunogenic Cell Death in Cancer: From Benchside Research to Bedside Reality

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The Editorial on the Research Topic

Immunogenic Cell Death in Cancer: From Benchside Research to Bedside Reality

Immunogenic cell death (ICD) has emerged as a cornerstone of therapy-induced antitumor immunity (1–3). ICD is distinguished by spatiotemporally defined emission of danger signals or damage-associated molecular patterns (DAMPs) that elevate the immunogenic potential of dying cells [Garg et al.; (4)]. The important role played by DAMPs in immunity, tissue remodeling, and inflammation is discussed in details by Venereau et al. (Marco E. Bianchi lab).

Most potent ICD inducers, characterized so far, elicit danger signaling through oxidative-endoplasmic reticulum stress (5). Several ICD inducers have been characterized, e.g., some chemotherapies, some physicochemical therapies (e.g., radiotherapy or photodynamic therapy/PDT), and oncolytic viruses (2, 6). Here, radiotherapy is among the first recognized immunogenic therapies [on account of "abscopal-effect" (7)]. The immunogenic potential of radiotherapy and possibilities for its combination with immune checkpoint blockers is discussed by Derer et al. (Udo S. Gaipl lab). It is noteworthy that ICD can also be achieved by various "smart" combinatorial strategies – an important point for clinically applied non-ICD inducers, discussed in details by Bezu et al. (Guido Kroemer lab).

Several lines of experimental evidence have established the validity of ICD. However, the overreliance on usage of prophylactic vaccination in transplantable (heterotopic) tumor models has attracted some criticism (8). While these criticisms are valid, the field is already moving toward tumors produced orthotopically (curative/therapeutic) or in genetically engineered mouse models (GEMM) (at least for few ICD inducers, e.g., hypericin-PDT, Newcastle disease virotherapy and anthracyclines) (9–12). Moreover, the clinical existence of ICD has been proven through retrospective analysis involving cancer patient's survival/therapy-responsiveness data (13–17). These observations have encouraged the increased usage of ICD-associated DAMPs as predictive/prognostic biomarkers – a point discussed in detail by Fucikova et al. (Radek Spisek lab). The promising results generated by systemically administered ICD inducers have also paved way for application of ICD-based dendritic cell (DC) vaccines (12). This important development has been discussed from the preclinical/clinical vantage points of various solid tumors by Vandenberk et al. (Stefaan W. van Gool lab) and lymphoma by Zappasodi et al. (Massimo Di Nicola lab). In the latter case, it is clear that the field is moving toward chimeric antigen receptor (CAR)-T cell's application, and it will be interesting to see its combination with ICD in near future.

Nevertheless, the insurmountable complexity of cancer makes it inevitable that in certain contexts, ICD may fail. This failure may stem from various factors, e.g., tumor heterogeneity (8), MHC-level heterogeneity (12), pre-established niches enriched in immunosuppressive factors or immune-checkpoints (1), stem cell-based immune-evasion (12), low mutational load, inactivating

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mutations/polymorphisms in certain immune-receptors (1), general ablation of danger signaling (14), and other genetic or even epigenetic causes. Several of these pro-cancerous immune-evasive mechanisms and immunotherapeutic strategies required for overcoming them are discussed in detail by Kersten et al. (Karin E. de Visser lab). The strategies for targeting epigenetic processes to improve immunotherapy are further discussed by Wachowska et al. (Jakub Golab lab).

We believe that the valuable contributions of key researchers/clinicians toward this research topic/special edition have largely fulfilled its primary aim, i.e., to foster a critical discussion on experimental and clinical relevance of ICD. In fact, to further summarize and organize the fields of ICD and DAMPs, we have produced a multi-author consensus paper within this research topic that attempts to classify DAMPs and ICD inducers with an eye on translational potential of ICD (Garg et al.). This classification paper brings together >50 authors from the fields of ICD and DAMPs, and tries to reach a comprehensive accord on various terminologies related to DAMPs/ICD, the historical background of these concepts, ICD classification system (Type I vs. Type II inducers), and the relevant preclinical/clinical criteria crucial for the field(s) (Garg et al.). We hope that this consensus paper will be a useful literature resource for various researchers/clinicians. These contributions, while summarizing the *status quo*, have also exposed a set of major questions and challenges that still need to be addressed.

MAJOR QUESTIONS TO RESOLVE

1. *Which danger signaling module is most specific to ICD?* Ecto-CRT seems to have remarkable exclusivity to ICD (10, 18–20) yet certain ICD inducers do not induce secreted-ATP (10), released-HMGB1 (19), or Type I IFN-responses (21). Alternatively, many non-ICD inducers induce secreted-ATP (22), released-HMGB1 (23), or Type I IFN-response (21). In fact, Type I IFN-responses can neutralize oncolytic viruses through antiviral signaling (24).
2. *Are ICD-associated DAMPs interchangeable?* Ecto-HSP90 was proposed to be interchangeable with ecto-CRT (25, 26), but this was recently invalidated in another set-up (21).
3. *Could ICD-associated DAMPs act as bystanders in certain contexts?* Induction of ICD-associated DAMPs may not always translate into a relevant functional outcome, e.g., Bleomycin induces all ICD-associated DAMPs yet elicits Tregs induction (27).
4. *What is the full extent of "plasticity" of ICD-associated danger/immunogenic signaling?*
5. *What is the exact role of cellular catabolic processes in regulating ICD?* Current results are highly variable; while macroautophagy positively regulates secreted-ATP (28), yet it can also negatively regulate ecto-CRT (29–31). Also, the exact roles of chaperone-mediated autophagy/CMA [CMA-essential gene *Lamp2a* regulates ecto-CRT (29)] or proteasome activity remains unresolved (Bortezomib induces ICD but not MG132, yet both inhibit the proteasome) (5).
6. *What are the common molecular determinants of ICD across various cell death pathways?* ICD-profile is largely associated with caspase-dependent apoptosis (18) but association with necroptosis is also emerging (10).
7. *How does ICD counter-act the (innately) apoptosis-associated immunosuppressive processes?*
8. *Does the role of ROS in ICD extend beyond a proximal stressor?* e.g., ROS-elicited oxidation-associated molecular patterns/OAMPs have been shown to mediate immunogenic potential (11).
9. *Why ICD fails in certain (GEMM) cancer mice models* (8) *but works in others* (9, 32)?
10. *Can epigenetic events* [*e.g., Long non-coding/micro-RNA* (33)] *regulate ICD and how?*

TRANSLATIONAL/CLINICAL CHALLENGES

1. *Can ICD's clinical translation withstand the "adverse effects" of mice-to-human immune differences?*
2. *Confirming ICD's existence in a prospective (high-powered/supervised) clinical trial.*
3. *Can ICD withstand the (clinical-)operational/regulatory (GLP/GMP/GCP) hurdles associated with anticancer vaccines-production?* [indications for which are emerging (12)]
4. *Characterizing ICD-resistance mechanisms in the clinic.*
5. *Characterizing reliable ICD-biomarker(s) detectable in patient tumor/sera-samples.*
6. *Investigating ICD as a source of robust prognostic/predictive/mechanistic biomarkers* [a point investigated recently in some studies (13, 34)].

We believe that the operational function of ICD (i.e., a dying cancer cell eliciting heightened immunogenicity-driven antitumor immunity) is incontrovertibly valid; but, owing to the incomprehensible complexity of cancer, the "specifics of ICD" (i.e., its molecular, signaling, and immunological determinants) will always remain open to amenability and variations. We envisage that overtime various "variants" of ICD may emerge that differ from each other in a manner dependent upon, the type of anticancer therapy, cancer cell death pathways, cancer-types, tumor antigen make-up, the *in vivo*/*in situ* location, and the location-dependent immune-contexture.

AUTHOR CONTRIBUTIONS

ADG wrote the manuscript. PA provided senior supervision and critically revised the manuscript.

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REVIEW
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DAMPs from cell death to new life

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Our body handles tissue damage by activating the immune system in response to intracellular molecules released by injured tissues [damage-associated molecular patterns (DAMPs)], in a similar way as it detects molecular motifs conserved in pathogens (pathogen-associated molecular patterns). DAMPs are molecules that have a physiological role inside the cell, but acquire additional functions when they are exposed to the extracellular environment: they alert the body about danger, stimulate an inflammatory response, and finally promote the regeneration process. Beside their passive release by dead cells, some DAMPs can be secreted or exposed by living cells undergoing a life-threatening stress. DAMPs have been linked to inflammation and related disorders: hence, inhibition of DAMP-mediated inflammatory responses is a promising strategy to improve the clinical management of infection- and injury-elicited inflammatory diseases. However, it is important to consider that DAMPs are not only danger signals but also central players in tissue repair. Indeed, some DAMPs have been studied for their role in tissue healing after sterile or infection-associated inflammation. This review is focused on two exemplary DAMPs, HMGB1 and adenosine triphosphate, and their contribution to both inflammation and tissue repair.

Keywords: DAMP, tissue repair, HMGB1, ATP, inflammation

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Introduction

Our body evolved mechanisms to detect pathogens through the recognition of conserved molecular motifs, called pathogen-associated molecular patterns (PAMPs). The binding of these molecules to pattern recognition receptors (PRR), such as Toll-like receptors (TLR), triggers the response of the immune system against the intruder (1). However, this "Stranger Theory" could not explain why strong immune responses are elicited in sterile conditions such as ischemic injuries, trauma, tumors, tissue transplants, and autoimmune diseases. By symmetry to the PAMP concept, Polly Matzinger proposed the "Danger Theory" in which the injured tissues were postulated to release intracellular molecules [damage-associated molecular patterns (DAMPs)] that activate the immune system (2). This concept has roots in a clinical trial on kidney transplantation, in which the oxygen free radical scavenger superoxide dismutase was exploited to avoid reperfusion injury (3). However, for many years the "Danger Theory" remained a theoretical model, until High Mobility Group Box 1 (HMGB1) and uric acid crystals were recognized as DAMPs (4, 5). Since then, many more DAMPs were identified and their roles in health and disease are now partially understood (**Table 1**).

Damage-associated molecular patterns are molecules that have a physiological "day-time job" inside the cell, and have the additional job of signaling cell damage when they are outside the cell. Location, inside vs. outside the cells, is critical: DAMPs are invisible to the immune system when performing their day-time job, and become visible only when exposed to the extracellular environment.

Timing is also important. Initially, DAMPs were expected to attest cell death, and therefore to be released passively from dead cells. Indeed, HMGB1 was identified as a DAMP because it is

TABLE 1 | List of putative DAMPs and role in inflammation and tissue repair.

	DAMPs	Receptors	Release	Role in inflammation/immunity	Role in tissue repair	Reference
Nucleus	Histones	TLR2, TLR4 and TLR9	P, S and A	TLR- and inflammasome-dependent inflammatory response	N.D.	(6)
	Genomic DNA	TLR9	P	TLR9- and NALP3-mediated innate immune response, DC maturation	N.D.	(6)
	HMGB1	TLR2, TLR4, RAGE and TIM3	P and A	Recruitment/activation of immune cells	Migration/proliferation of stem cells, pro-angiogenic mediator.	(7)
	IL1a	IL-1R	P	Strong pro-inflammatory activity	Protective during early phase of inflammation	(7)
	IL33	ST2	P	Secretion of pro-inflammatory and Th2 cytokines	Epithelial cells proliferation and mucus production in the gut	(8)
Cytosol	ATP	P2Y2 and P2X7	P and A	Macrophages recruitment, IL-1β production by DC, antitumor immunity	Migration/proliferation of epithelial and endothelial cells, pro-angiogenic role	(9)
	F-actin	DNGR1	P	Contribution in recognition of necrotic cells by DC	N.D.	(10)
	Cyclophilin A	CD147	A	Inflammatory cells recruitment, inflammatory mediators release	N.D.	(10)
	HSPs	CD91, TLR2, TLR4, SREC1 and FEEL1	P, S and A	Recruitment of immune cells DC maturation, T cell-based antitumor immunity	Wound debris clearance, cell migration/proliferation and collagen synthesis in skin	(7)
	Uric acid crystals	NLRP3	P	DC maturation and neutrophil recruitment	N.D.	(7)
	S100s	TLR2, TLR4, RAGE	P	Potent immunostimulatory activity, monocytes and neutrophils recruitment	Myoblast proliferation/differentiation	(7)
Mitochondria	Mitochondrial DNA	TLR9	P	Macrophages and neutrophils activation	N.D.	(11)
	Mitochondrial trascription factor A	RAGE and TLR9	P	DC activation, type I interferon release	N.D.	(11)
ER	Calreticulin	CD91	P and S	Potent "eat me" signal, mediator of tumor immunogenicity	Cell migration/proliferation, extracellular matrix production	(10)

P, passive release; A, active secretion; S, surface exposure; ER, endoplasmic reticulum; N.D. not described.

passively released by necrotic cells, which undergo an untimely death, but not by apoptotic cells, which eliminate themselves in an elaborately programed way (4). However, an important addition to the DAMP concept is that DAMPs do not necessarily originate from dead cells: DAMPs can be secreted or exposed by living cells undergoing a life-threatening stress. Indeed, alerting the immune system as soon as possible can bring advantages. HMGB1 can be secreted by stressed cells via a private secretion pathway, not involving the endoplasmic reticulum (12, 13). Adenosine triphosphate (ATP) can be actively released via vesicles and connexin or pannexin hemichannels (14). Other DAMPs, such as calreticulin and heat shock protein 90 (HSP90), are exposed *de novo* or become enriched on the outer leaflet of the plasma membrane (15).

It is now time to recognize another essential feature of DAMPs: they are essential for tissue healing after inflammation, both sterile and infection-associated. This review will focus on two exemplary DAMPs, HMGB1 and ATP, and their contribution to both inflammation and tissue repair.

HMGB1 and ATP as Exemplary DAMPs

HMGB1, a Redox-Sensitive DAMP

HMGB1 is a mobile chromatin protein that acts as a DNA chaperone, by binding DNA transiently and bending it reversibly. As a DNA chaperone, it facilitates nucleosome formation, contributes to the binding of proteins, including transcription factors that distort DNA upon binding, and participates in transcription, replication, and DNA repair (16). HMGB1 is constitutively expressed in almost all cell types, and to act as a DAMP it must relocate into the external environment: it is passively released following traumatic cell death (but not apoptosis) and is secreted during severe stress (4, 17).

HMGB1 secretion is not completely understood. Drawing a comparison with another leaderless protein, IL-1β, a "two-step model" for HMGB1 secretion was proposed, which involves a first trigger to induce HMGB1 acetylation and cytoplasmic translocation and a second trigger to elicit its extracellular transport (18) Indeed, secreted HMGB1 (as opposed to HMGB1 passively released by dead cells) is hyperacetylated (19). In accordance with the two-step model, Lu et al. (20) have demonstrated that the inflammasome, in particular NLRP3, is involved in the release of HMGB1. Inflammasomes are large caspase-1-activating complexes, composed by the assembly of proteins that are ultimately activated by both PAMPs and DAMPs (21). There are multiple inflammasome complexes, and among them the one containing NLRP3 (also known as NALP3 and cryopyrin) is the most studied. Since the synthesis of NLRP3 is triggered by TLR signaling, it has recently been proposed that HMGB1 itself could "prime" the inflammasome through its binding to TLR2/TLR4 (22). Indeed, the role of HMGB1 in inflammasome activation has been demonstrated in a model of heatstroke-induced liver injury (23).

Once in the extracellular milieu, HMGB1 signals danger to the surrounding cells, triggers inflammation, and activates innate and adaptive immunity by interacting with multiple receptors (24).

The first receptor described for HMGB1 is the receptor for advanced glycation endproducts (RAGE), a multifunctional transmembrane protein of the immunoglobulin superfamily (25). Under physiological conditions, RAGE is expressed at low levels in the majority of tissues and, interestingly, at high levels in the lung. In pathophysiological conditions such as chronic inflammation, RAGE expression is considerably increased in different tissues, in particular activated endothelium and leukocytes (26). HMGB1 signaling through RAGE leads to activation of the nuclear factor-κB (NF-κB) pathway, as well as to signal transduction through JNK, and p38 (27). In addition, HMGB1/RAGE interactions lead to the activation of the ERK MAP kinase pathway, which is important in cell migration, tumor proliferation and invasion, and expression of matrix metalloproteinases. The HMGB1/RAGE axis is mainly involved in the recruitment and migration of cells, directly by inducing expression of adhesion molecules, such as VCAM-1 and ICAM-1 (28), or indirectly by inducing secretion of chemokines, in particular CXCL12, which in turn forms a heterocomplex with HMGB1 (29).

HMGB1 also binds to TLRs. In complex with CpG-ODNs, HMGB1 binds to TLR9 and enhances cytokine production in plasmacytoid dendritic cells (DCs) (30). When HMGB1 is bound to nucleosomes, it activates macrophages and DCs through TLR2 (31). However, most studies focused on the HMGB1/TLR4 axis. TLR4 mediates cell responses to lipopolysaccharide (LPS), but responds to several DAMPs as well. The contribution of the HMGB1/TLR4 axis to inflammation and immune regulation has been demonstrated in a wide range of experimental models, such as liver and lung damage, cancer, and epilepsy (32–35). Recently, a large body of evidence demonstrated that the redox state of cysteines modulates the binding of HMGB1 to its receptors, and consequently its activities.

HMGB1 contains three cysteines: C23 and C45 can form a disulfide bond, and C106 is unpaired. These cysteines are modified by redox reactions, giving rise to three isoforms named "fully reduced HMGB1" for the all-thiol form, "disulfide HMGB1" for the partially oxidized one, and "sulfonyl HMGB1" for the terminally oxidized form (36). Fully reduced HMGB1 forms a heterocomplex with the chemokine CXCL12, which binds with increased affinity to its CXCR4 receptor (29). Conversely, the extracellular TLR4 adaptor myeloid differentiation factor 2 (MD-2) binds specifically to disulfide HMGB1, and not to the other redox forms, triggering the expression of chemokines and cytokines (37). Notably, interaction with MD-2 also requires the third cysteine, in the fully reduced form. Thus, the disulfide bond between C23 and C45 makes HMGB1 a proinflammatory cytokine, whereas further cysteine oxidation to sulfonates abrogates both the chemoattractant and proinflammatory activities of HMGB1 (38). Several studies demonstrated a correlation between the presence of the disulfide HMGB1 and the onset of pathologies such as brain injury, liver damage, myositis, and juvenile idiopathic arthritis (19, 39–41). Moreover, disulfide HMGB1, and not the reduced form, contributes to nociceptive signal transmission via activation of TLR4 (42) (**Figure 1**).

The HMGB1 inside the cell (nucleus or cytosol) is completely reduced, and early prevalence of fully reduced HMGB1 and subsequenct appearance of disulfide HMGB1 were observed in models of brain, muscle, or liver injuries and in patients with Juvenile Idiopathic Arthritis (19, 39, 41). Supernatants from LPS-activated THP-1 monocytic cells contain both fully reduced and disulfide HMGB1 (38), suggesting that activated monocytes/macrophages contribute to inflammation by producing disulfide HMGB1. Tandem mass-spectrometric analysis showed that systemic levels of the disulfide HMGB1 isoform dramatically increased during early Macrophages Activation Syndrome (43). Similarly, a study revealed that cells undergoing unprimed pyroptosis release a reduced HMGB1 redox isoform, whereas priming with TLRs ligands results in the conversion to disulfide HMGB1 (44).

In conclusion, it is now essential to identify the redox state of HMGB1 in each specific condition and locale *in vivo*.

ATP, a Time-Resolved DAMP

Nucleotides as well, particularly ATP, have both intra- and extracellular roles. They are well known for their function as a universal energy source in cell reactions and metabolism. The multiple functions of extracellular ATP have been known since the late 1940s, when its vasoactive property and its release in shock were discovered [reviewed by Gordon (45)]. Later, ATP was found to be released at nerve terminals, affecting smooth muscle tone. Moreover, ATP and adenosine are involved in the mechanisms underlying local control of vessel tone, while ADP induces platelet aggregation and is released, together with ATP, from platelet granules (45). Several cell types release ATP during inflammatory, ischemic, and hypoxic conditions. ATP release can occur in a passive fashion, for example during necrosis, but many molecular pathways have been described for active release, as ATP-containing lysosome exocytosis from astrocytes, pannexin-mediated ATP release during apoptosis, and connexin- or pannexin-mediated ATP release from inflammatory cells, such as neutrophils (46). Moreover, it has been recently demonstrated that ATP can also be secreted by dying cancer cells through the classical endoplasmic reticulum/Golgi secretory pathway (47).

In the extracellular compartment, nucleotide signaling is intrinsically short-lived. Signaling is terminated in the timescale from seconds to minutes by the enzymatic conversion of ATP to adenosine through the ecto-nucleoside triphosphate diphosphohydrolase CD39 (from ATP/ADP to AMP) and the ecto-5′-nucleotidase CD73 (from AMP to adenosine) (48). ATP acts as a signaling molecule through the activation of purinergic P2 receptors (9). These receptors have a widespread expression throughout different tissues and are involved in innate and adaptive immune responses (46, 48). P2 receptors can be further subdivided into metabotropic P2Y receptors (P2YRs), which are G-protein-coupled, and ionotropic P2X receptors (P2XRs), which are nucleotide-gated ion channels.

P2YR signaling has been linked with chronic inflammation, and one of the most studied receptor of this class is P2Y2R, which is activated by UTP or ATP. P2Y2R agonists promote mucociliary clearance and wound healing [reviewed by Idzko et al. (9)]. For these reasons, P2YR agonists were exploited for the treatment of cystic fibrosis (49). Apoptotic cells release ATP

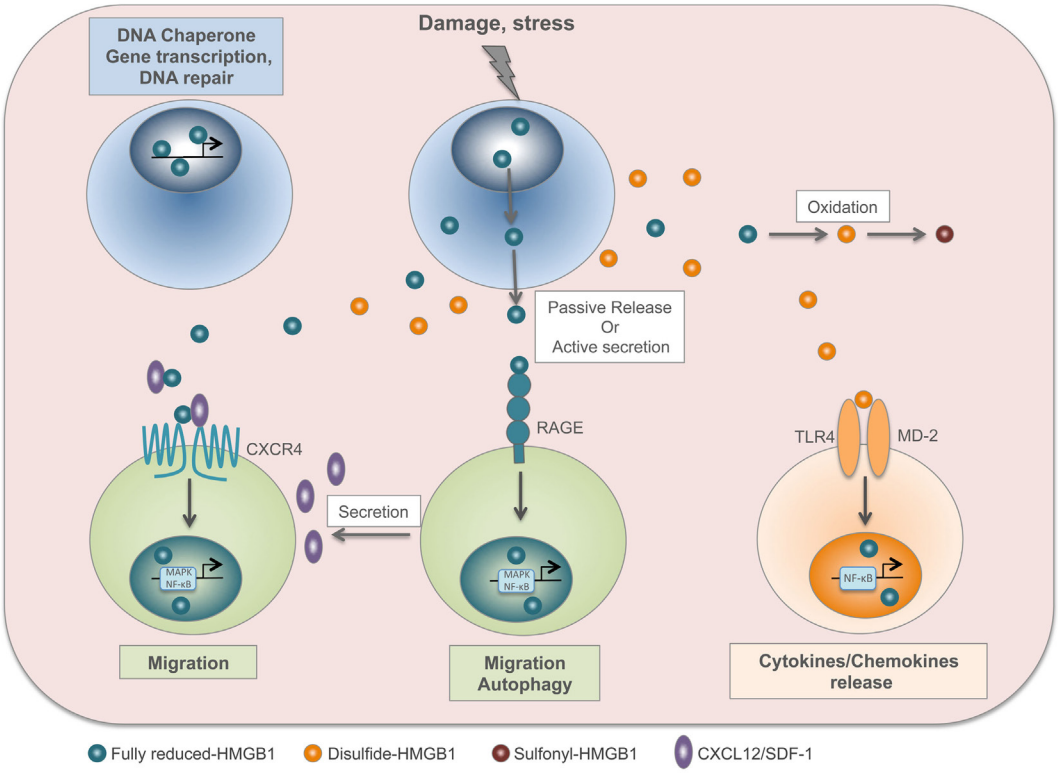

FIGURE 1 | HMGB1 is a redox-sensitive DAMP. In the nucleus, fully reduced HMGB1 acts as a DNA chaperone and contributes to gene transcription and DNA repair. Upon injury or stress, HMGB1 is passively released by dead cells or actively secreted by stressed cells. The fully reduced HMGB1 binds to CXCL12 chemokine to form a heterocomplex, which in turn binds to CXCR4 and induces cell migration. In addition, HMGB1 interacts with RAGE to induce CXCL12 secretion and autophagy. In the extracellular compartment, disulfide HMGB1 derives from the active secretion and/or the conversion of fully reduced HMGB1 by oxidation. Disulfide HMGB1 binds to TLR4/MD-2 complex and induces cytokine/chemokine release. Finally, HMGB1 cysteines are terminally oxidized to sulfonates; sulfonyl-HMGB1 is neither chemoattractant nor has cytokine-inducing activity.

as a "find-me" signal that binds P2Y2R on macrophages, stimulating their phagocytic activity and the clearance of apoptotic cells (50). During pneumonia, neutrophil-dependent ATP release and autocrine activation of P2Y2R contribute to purinergic chemotaxis, thereby enhancing bacterial clearance (51). However, ATP-elicited P2Y2R signaling can lead to uncontrolled inflammation and chronic inflammatory diseases. On alveolar epithelial cells or eosinophils, P2Y2R signaling causes production of pro-allergic mediators (for example, IL-33, IL-8, eosinophil cationic protein) during allergic airway disease (52). Similarly, P2Y2R signaling on DC has a role during the induction and self-perpetuation of asthma (53). In general, P2Y2R antagonists can evolve into useful drugs for chronic inflammatory diseases.

P2XR channels are opened by the binding of ATP, allowing sodium and calcium influx and potassium efflux. The increased level of intracellular calcium activates p38 MAPK or phospholipase A2 signaling, while potassium efflux activates the inflammasome (9). Then, P2XR channels gradually dilate into pores permeable to larger organic cations and small hydrophilic molecules with a molecular mass below 900 dalton (including ATP) (54). Among P2XRs, P2X7R is predominantly expressed on immune cells such as mast cells, macrophages, microglia, and DCs, and its signaling has been linked to inflammatory and infectious disorders (46). P2X7R is required for appropriate inflammatory defense mechanism against invading pathogens and cancer cells. For instance, it is important during intracellular killing of *Mycobacterium tuberculosis* by macrophages (55). Dying tumor cells release ATP that activates P2X7R on DCs, which in turn promote the priming of IFN-γ-producing cytotoxic $CD8^+$ T cells that kill cancer cells (56). On the other hand, P2X7R signaling contributes to the induction and maintenance of chronic inflammation. Indeed, P2X7R signaling on DCs is involved in the sensitization phase of allergic disorders such as contact hypersensitivity (through CD81 T-cell priming) (57) and asthma (through CD41 T-cells, TH2 response) (58), and contributes to transplant rejection (through CD41 T cells, TH1 response) (59). Furthermore, P2X7R signaling on enteric neurons or mast cells has been implicated in promoting intestinal inflammation during inflammatory bowel disease (60) (**Figure 2**).

As already mentioned, the binding of extracellular ATP to P2X7R elicits NLRP3 activation (21). The contribution of the ATP/P2X7 receptor axis to inflammasome activation in pathogenic conditions has been shown in a bleomycin model of pulmonary inflammation in mice. This leads to IL-1β maturation and secretion, causing lung inflammation that evolves to fibrosis (61). Moreover, P2X7R upregulation in atherosclerotic lesions in mice

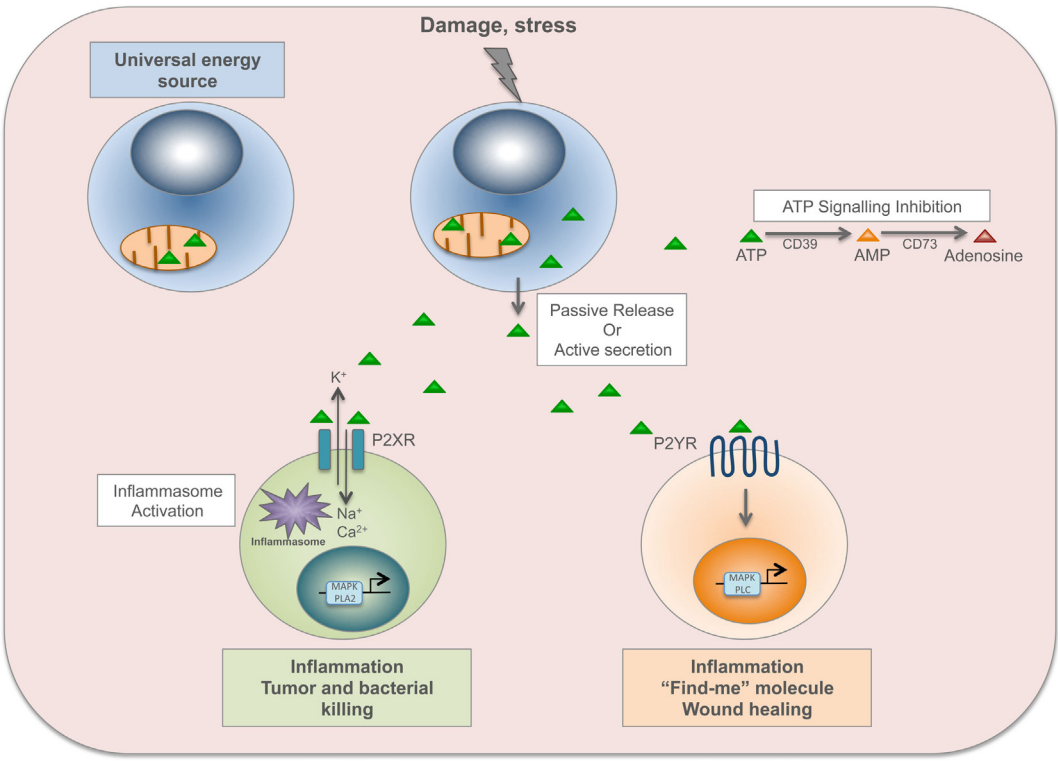

FIGURE 2 | ATP is a time-resolved DAMP. In the cell, ATP derived from mitochondria is a universal energy source in cell reactions and metabolism. Upon damage or stress, ATP, and other nucleotides, are passively released by dead cells or actively secreted by stressed cells. ATP binds to ionotropic P2X receptors (P2XR), which are nucleotide-gated ion channels, allowing sodium (Na^+) and calcium (Ca^{2+}) influx and potassium (K^+) efflux. The increased level of intracellular calcium activates p38 MAPK or phospholipase A2 signaling, while potassium efflux activates the inflammasome. P2XR signaling is involved in inflammation, tumor and bacterial killing. ATP also binds to metabotropic P2Y receptors (P2YR), which are G-protein-coupled, and induces activation of MAPK and phospholipase C (PLC). P2YR signaling is implicated in inflammation and wound healing, and ATP released by apoptotic cells acts as a "find-me" signal to recruit macrophages to the site of damage and to promote clearance of apoptotic cells. ATP signaling is abolished by the enzymatic conversion of ATP to adenosine through the ecto-nucleoside triphosphate diphosphohydrolase CD39 (from ATP to AMP) and the ecto-5′-nucleotidase CD73 (from AMP to adenosine).

modulates NLRP3 inflammasome activation, and is involved in the progression and development of atherosclerosis (62).

As we reviewed in this chapter, DAMPs, in particular HMGB1 and ATP, have been linked to inflammation and related disorders. Hence, inhibition of DAMP-mediated inflammatory responses might appear as a promising strategy to improve the clinical management of infection- and injury-elicited inflammatory diseases. However, it is important to keep in mind that these sophisticated molecules are danger signals important not only for the inflammatory response but also for tissue repair. Here, we review the latest findings on the regenerative properties of HMGB1 and ATP.

HMGB1 and ATP in Tissue Repair

The functions of DAMPs consist in alerting the body about danger, stimulating the immune system in order to initiate the immune response, and finally promoting the regeneration process. This last property of DAMPs has been particularly investigated for two members of the family: HMGB1 and ATP (**Figure 3**).

HMGB1, a Chemotactic and Proangiogenic DAMP

HMGB1 plays an important role in promoting tissue regeneration after acute inflammation. Locally released HMGB1 recruits bone-marrow derived mesenchymal stem cells (MSCs), and promotes the proliferation and differentiation of tissue-associated resident stem cells, such as dental pulp stem cells, mesoangioblasts, and MSCs (63). Adult MSCs have attracted intense interest because they can be isolated from the bone marrow and can be expanded in culture while maintaining their multipotency, and thus may be used for the repair of bone, cartilage, muscle, bone marrow stroma, tendon, fat, and other connective tissues. HMGB1 induces migration of MSC (64–66) and their differentiation into osteoblasts (64). Moreover, intravenous administration of HMGB1 in mice induces MSC accumulation in skin grafts, promoting inflammatory suppression in the grafts, and subsequent tissue regeneration (67). However, a recent study showed that HMGB1 induces migration of monocytes but not of MSCs (68). Further experiments are necessary in order to understand these discrepancies, in particular, the culture conditions that

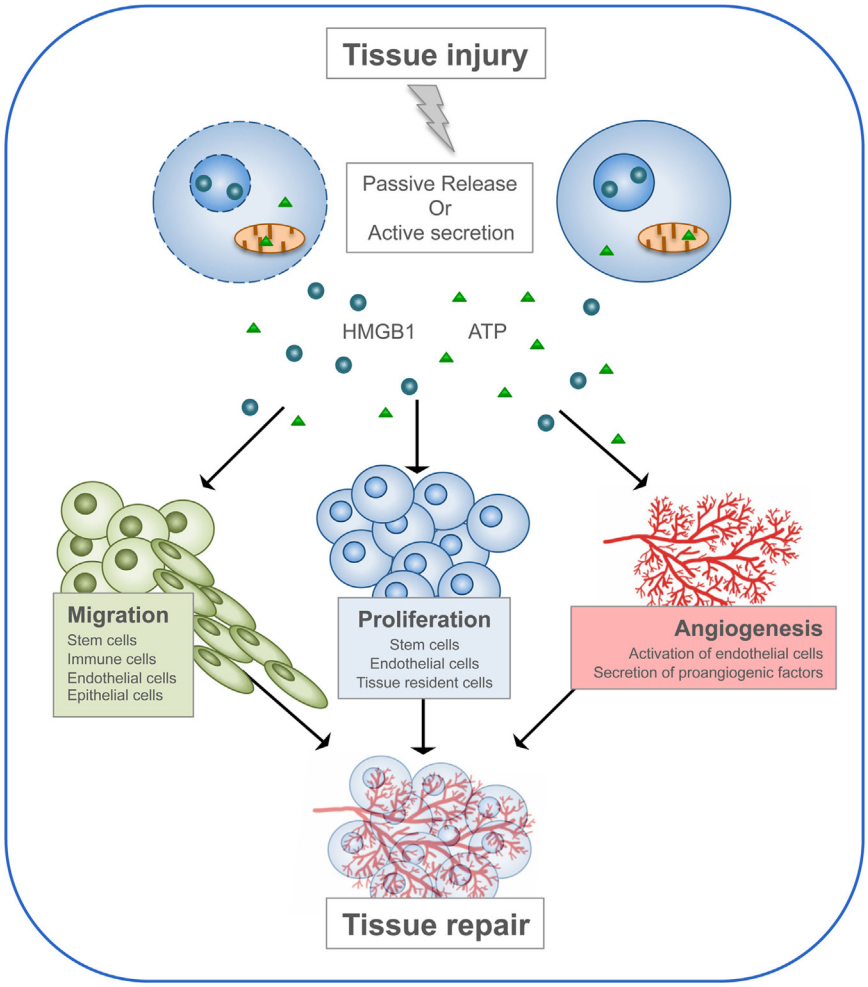

FIGURE 3 | HMGB1 and ATP in tissue repair. Following tissue injury, HMGB1 and ATP are passively released by dead cells or actively secreted by stressed cells. Then, they recruit to the site of damage the cell types required to heal the wound. First, immune cells are needed to clean the wound by engulfing dead cells and cellular debris. Then, stem cells and neighboring cells are induced to proliferate and build new tissue, together with its extracellular matrix. Endothelial cells are activated to form new blood vessels.

could modulate the redox state of HMGB1 and consequently its chemotactic activity.

Tissue repair requires angiogenesis, and numerous studies have identified HMGB1 as a proangiogenic factor [recently reviewed by Yang et al. (69)]. Briefly, HMGB1 plays an important role in neovascularization of ischemic areas by recruiting endothelial progenitor cells through activation of integrins and inducing the migration and sprouting of endothelial cells in a RAGE-dependent manner (70, 71). In addition, HMGB1 stimulates endothelial cells and macrophages to release proangiogenic cytokines, such as VEGF, TNF-α, and IL-8 (72). HMGB1 secreted by leukocytes is important for the skeletal muscle to react to hypoxia and to initiate angiogenesis in response to injury (73).

The regenerative properties of HMGB1 have been studied in different models of tissue injury, including spinal cord, skin, muscle, and heart. In a model of spinal cord injury in zebrafish, the authors observed that HMGB1 expression increases after injury in both motoneurons and endothelial cells. Moreover, inhibition of HMGB1 decreases locomotor recovery and axonal formation (74). In a model of spontaneous spinal cord regeneration in the gecko, HMGB1 does not mediate the inflammatory response but promotes regeneration. Here, HMGB1 induces migration of oligodendrocytes by interacting with RAGE, but not TLRs (75).

In skin, HMGB1 was identified as a chemoattractant for bone marrow-derived epithelial progenitors, which contribute to epithelial regeneration (67). A well-known consequence of diabetes is impaired skin wound repair, and topical treatment with recombinant fully reduced HMGB1 accelerated wound healing in diabetic mice (76). Accordingly, HMGB1 levels are low in diabetic human and mouse skin, and inhibition of endogenous HMGB1 impaired wound healing in non-diabetic mice but had no effect in diabetic mice. Conversely, HMGB1 also plays a role in scar formation in fetal skin (77). Interestingly, the authors used a

recombinant HMGB1 described to induce TNF release, suggesting that it corresponds to the disulfide form.

In a mouse model of acute myocardial infarction, overexpression of HMGB1 in cardiac cells or local administration of HMGB1 induced myocardial regeneration, restored cardiac function, and improved survival (78, 79). These effects were due to proliferation and differentiation of cardiac stem cells and induction of angiogenesis. Moreover, HMGB1 stimulates primary cardiac fibroblasts to exert a paracrine action on cardiac stem cells (80), and intramyocardial injection of HMGB1 improved global cardiac function by reducing fibrosis and cardiomyocyte hypertrophy (81). HMGB1 activates a number of genes involved in cardiac protection and regeneration, and Notch1 signaling plays a key role in HMGB1 ability to activate cardiac stem cells (82). Interestingly, beneficial effects of HMGB1 were also observed in models of heart failure (81–83). Conversely, HMGB1 blockade caused an expansion of the infarct scar and marked hypertrophy of the non-infarcted area (84).

Finally, HMGB1 is important in skeletal muscle regeneration. The presence of only half of the normal amount of HMGB1 results in defective myogenesis both during development and after acute injury (85). In particular, the absence of HMGB1 in leukocytes results in defective angiogenesis and a delay in muscle regeneration (73). HMGB1 levels are increased in regenerating skeletal muscle after ischemia/reperfusion, and intramuscular administration of HMGB1 enhances both vascularization and myofiber formation (86). Besides angiogenesis, HMGB1 also promotes myogenesis by stimulating migration and proliferation of mesoangioblasts and migration of skeletal myoblasts and smooth muscle cells, and by accelerating myogenic differentiation (86–89).

In conclusion, HMGB1 released by injured tissues promotes tissue repair by inducing migration and proliferation of stem cells, and by promoting angiogenesis. However, several studies have demonstrated the beneficial effect of blocking HMGB1 in animal models of spinal cord, liver, brain, and myocardial damage after ischemia/reperfusion injury (24). Indeed, HMGB1 also activates fibroblasts and astrocytes, which might induce fibrosis as a program of tissue consolidation if successful regeneration is not achieved. The discrepancy between these results might be due to the fact that the redox state of HMGB1 has not been rigorously identified in most of the studies reported. Indeed, even if the fully reduced HMGB1 is the most used recombinant form, the reduced and disulfide forms can easily interconvert both *in vitro* and *in vivo*.

Nucleotides as "Find-Me" Signals in Tissue Repair

The interplay between nucleotides and the immune system is essential for regenerative processes in the body (46, 90). During tissue regeneration, the organism needs to remove dead cells and debris to recruit various types of cells and to stimulate their proliferation in order to achieve wound closure. Nucleotides participate actively to these three phases by interacting with purinergic receptors on different cell types.

The two families of P2Rs appear to have separate roles: P2XRs are involved in defense mechanisms and cell death, and P2YRs in wound healing (9). Indeed, prevalently P2YRs have been studied in different models of tissue regeneration. Both ATP and UTP released by apoptotic cells in a caspase-1-dependent manner act as "find-me" signals that recruit macrophages through P2Y2R, and stimulate their phagocytic activity (50). Neutrophils release ATP that in turn recruits neutrophils, in a feed forward loop (51). In addition, both ATP and UTP promote migration of vascular smooth muscle cells through binding of P2Y2R to filamin A (91).

Stimulation of P2Y receptors has a mitogenic effect on multiple cell types, including brain capillary endothelial cells (92), cardiac endothelial cells (93), and fibroblasts (94). Non-hydrolyzable nucleotide analogs (e.g., ATPγS, ADPβS) strongly promote proliferation of HUVEC cells and of mammalian vascular smooth muscular cells (95). These observations strongly suggest that nucleotide might be proangiogenic factors important for tissue repair.

Nucleotide release from dying cells after acute kidney injury induces proliferation of neighboring tubular cells, thus promoting wound closure via the downstream activation of Akt (96). In the liver, ATP released after partial hepatectomy, both from hepatocytes and from Kupffer cells, contributes to liver regeneration by activating cell cycle progression in hepatocytes (97). Calcium waves elicited by ATP released from damaged cells are important in the developing brain of *Xenopus laevis*, where neural progenitor cells reorganize their cytoskeleton and activate the actomyosin contractile machinery to drive the expulsion of damaged cells into the brain ventricle. This represents a mechanism for rapid wound healing in the developing brain (98).

Shockwave treatment is a new technology used to treat chronic painful conditions of the musculoskeletal system. Shockwaves induce ATP release, which leads to Erk1/2 and p38 MAPK activation and cell proliferation, and increased wound healing in a rat model (99). During skin wound healing, extracellular nucleotides have a dual function: they inhibit keratinocyte motility and facilitate migration of other cell types (e.g., endothelial cells) (100, 101). Treatment of mouse ear wounds with Mg-ATP encapsulated in lipid vesicles (ATP-vesicles) induced macrophage accumulation, *in situ* proliferation and new tissue growth (102). ATP release from HaCaT keratinocytes caused the propagation of intercellular calcium waves from cells at the frontier facing the scar toward the cells in the rear, in a P2Y-receptor-dependent manner (103). Finally, the most striking evidence of P2YR signaling in tissue repair is the delay of wound healing observed in *P2y2r*$^{-/-}$ mice (94).

In zebrafish larvae, when the tail fin is wounded, osmolarity differences between the interstitial fluid and the ambient water trigger ATP release, which initiates rapid wound closure through long-range activation of basal epithelial cell motility. In this case, P2Y2R is probably irrelevant, since the P2Y2R inhibitor suramin had little effect, even at high concentrations (104). Indeed, wound healing is known to involve other purinergic receptors. In cystic fibrosis, ATP release from epithelial cells activates P2RY11 on nearby epithelial cells, stimulating proliferation, migration and wound repair (105). P2X7 activation participates in angiogenesis and wound repair by promoting VEGF release from human monocytes (106). Moreover, P2X7 is necessary for timely healing of abrasion wounds and normal stromal collagen structure (107).

Acute UV irradiation of keratinocytes causes ATP release that triggers P2X7R on skin-resident T cells and participates to DNA repair response essential for skin regeneration (108). Thus, even P2XRs might switch from their killing activity, opening pores on the plasma membrane that cause the cell to collapse, to a pro-regenerative function, helping tissue repair.

Conclusion and Future Directions

Nature is remarkably conservative, in that it uses the same molecule over and over again to attain related goals (109). DAMPs are exemplary from this point of view, as they are (generally abundant) molecules that are involved in the everyday functioning of the cell, and double up as signals of cell damage when they are present outside of the cell. As it happens, this simple invention that allowed to discern damage (DAMP-out) from normality (DAMP-in), could be used further to better describe the nature of the damage, and to record its occurrence for future memory. Thus, after being released (either passively or actively), DAMPs act to:

(1) convey the message of danger to other cells,
(2) trigger inflammation and activate innate immunity to stop the damage,
(3) participate in cell–cell communication that instructs adaptive immunity, to help establish immunological memory,
(4) orchestrate tissue repair and healing.

Points (1) and (2) have been widely described (7, 110). The cooptation of DAMPs into the process of immunological memory (point 3) and the related process of Immunogenic Cell Death are the subject of other reviews in this Frontiers collection. Immunogenic cell death is a perfect example of interplay between several DAMPs to alert and activate the immune system.

Here, we have focused on the role played by ATP and HMGB1 in wound repair and tissue reconstruction. ATP and other nucleotides, and their purinergic receptors, have been known to participate in tissue repair since the late 1990s, even before they were recognized as DAMPs. Examples involving HMGB1 are now as numerous. However, a fundamental problem must be acknowledged: how can the organism use the same DAMPs to trigger inflammation and to orchestrate tissue repair, which should occur *after* resolution of inflammation? Here, we can only speculate, and perhaps suggest future avenues of research. Usually, a signal with two possible meanings must be disambiguated by either the state of the receiver or the context of the signaling. Thus, to disambiguate the DAMP in inflammation and tissue repair, cells would need two different receptors, on different cells or on the same cell but at different times. Perhaps relevant here is that RAGE, a receptor for HMGB1, is low at the beginning of inflammation and induced by it.

Context in signaling is easy to picture: contextuality is paramount in everyday human communication. In the case of inflammation and tissue repair, context is the co-presence of other ligand-receptor pairs in different situations, in addition to the DAMP and its receptor, so that cells are differently activated or polarized. In fact, inflammation creates a microenvironment that is acidic, oxidizing (rich in oxygen and ROS), and where the metabolism of inflammatory cells is shifted toward glycolysis, whereas tissue repair occurs in a microenvironment which is neutral, reducing, and where macrophage metabolism is shifted towards oxidative phosphorylation and fatty acid oxidation (111, 112).

Also notable is that tissue reconstruction and inflammation, or at least some aspects of both, occur simultaneously in chronically inflamed tissue. In situations like rheumatoid arthritis, where inflammation is rampant and the synovia grows exuberantly into a pannus, DAMPs might not be disambiguated, and might actually activate both programs at the same time. Not surprisingly, targeting DAMPs or their receptors during chronic inflammation is often beneficial. However, finely tuning might be better than blocking them altogether. Thus, better understanding of the activity and the interaction of cells in inflammation and tissue reconstruction, and of DAMP signaling, is key to control excessive inflammation, resolve chronic inflammation, and promote tissue repair and healing.

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Radio-immunotherapy-induced immunogenic cancer cells as basis for induction of systemic anti-tumor immune responses – pre-clinical evidence and ongoing clinical applications

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Radiotherapy (RT) primarily aims to locally destroy the tumor via the induction of DNA damage in the tumor cells. However, the so-called *abscopal*, namely systemic and immune–mediated, effects of RT move over more and more in the focus of scientists and clinicians since combinations of local irradiation with immune therapy have been demonstrated to induce anti-tumor immunity. We here summarize changes of the phenotype and microenvironment of tumor cells after exposure to irradiation, chemotherapeutic agents, and immune modulating agents rendering the tumor more immunogenic. The impact of therapy-modified tumor cells and damage-associated molecular patterns on local and systemic control of the primary tumor, recurrent tumors, and metastases will be outlined. Finally, clinical studies affirming the bench-side findings of interactions and synergies of radiation therapy and immunotherapy will be discussed. Focus is set on combination of radio(chemo)therapy (RCT) with immune checkpoint inhibitors, growth factor inhibitors, and chimeric antigen receptor T-cell therapy. Well-deliberated combination of RCT with selected immune therapies and growth factor inhibitors bear the great potential to further improve anti-cancer therapies.

Keywords: radiotherapy, abscopal effect, immune therapy, checkpoint inhibitors, PD-L1, DAMP, EGFR, anti-tumor immunity

Radiotherapy (RT) is an integral part of multimodal cancer treatments (1). Besides its local mode of action on tumor cell DNA, it can induce systemic and immune-mediated anti-tumor responses, especially in combination with additional immune activation (2). The current review focuses on induction of immunogenic cancer cell death by RT and on interactions of RT with selected immune therapies to induce a long-lasting, local, and systemic tumor control.

According to the WHO, cancer incidences are expected to increase by over 50% until 2020 (3). With this in mind, the clinical management of treatment modalities is a big challenge for scientists and clinicians alike. Consequently, improving the understanding of cellular and molecular processes occurring in the patients during therapies will help to optimize the design of clinical trials and ultimately that of patient treatment as well. Common therapy options comprise surgery,

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RT, chemotherapy (CT), immunotherapy (IT), targeted therapy, hyperthermia (HT), and hormonal therapy, all of which are either administered as a stand-alone therapy or in various combinations. Out of all these options, over 50% of all cancer patients receive RT (4).

RT-Induced DNA Damage

The clear-cut aim of RT is the deposition of a maximal dose of ionizing radiation (IR) in the tumor while simultaneously sparing healthy tissue. A significant amount of damage within the malignant cells ultimately leads to the loss of clonogenicity, the induction of cell death and finally in the reduction of tumor size. This is achieved either directly or indirectly: radiation induces DNA lesions and creates highly reactive radicals that then also damage DNA. The accumulation of DNA lesions can jeopardize the genomic stability of the cell, especially when the DNA damage response (DDR) system is impaired. Individuals with germ-line mutations in DDR genes show a higher predisposition for cancer. Errors in this highly regulated process of DDR can result in accumulation of genomic mutations and malignant transformation. On the other hand, DDR also acts as a negative saboteur to resist CT and RT (5). Forms of IR-induced DNA damage that endanger chromatin integrity are single-strand breaks (SSBs) and double-strand breaks (DSBs) of the DNA, whereas SSBs (~1000/Gy) (6) are way more frequent than DSBs (~40–50/Gy) (7). However, the time to repair DSBs takes much longer. Cells have developed several DNA repair pathways, such as homologous recombination (HR), non-homologous end-joining (NHEJ), nucleotide excision repair (NER), and base excision repair (BER) as well as mismatch repair (MMR) dependent on size and modality of the DNA damage (for further interest on this topic refer to Ref. (8)). Still, if the cell is no longer able to compensate the damage, cell death is the final consequence.

RT-Induced Cell Death

Mitotic catastrophe, apoptosis, autophagy, and senescence have been the most prominent observed forms of cell death induced by RT (9). Within the last years, it has become evident that tumor cell necrosis can be induced in a programed manner besides occurring through a more or less unregulated process (10).

Apoptosis

Apoptosis is a programed cell suicide and the best characterized form of cell death. It is of particular importance during development and aging to maintain a homeostatic balance in tissues. A dysfunctional regulation can result in autoimmune diseases, viral infections, or cancer. In cancer therapy, apoptosis can be induced in tumor cells by the use of IR and/or cytotoxic drugs (11). In general, two main pathways exist: the intrinsic mitochondrial pathway is mainly regulated by proteins of the B-cell lymphoma-2 (Bcl-2) family, which includes pro- and anti-apoptotic proteins, whereas the extrinsic pathway is induced by cell death receptors on the cell surface. In early stages of apoptosis, the cell maintains its organelle integrity and the cell membrane remains intact. In later stages, various morphological changes of the cell are visible: cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing, and formation of apoptotic bodies (12). Under normal conditions, apoptotic cells are engulfed by neighboring "non-professional" phagocytic cells, such as mesenchymal and epithelial cells (13). However, if the number of apoptotic cells exceeds a certain level, professional phagocytes are attracted to the site by the so-called "find me" signals that are released by dying cells (14, 15). These signals include factors such as nucleotides, proteins, and phospholipids (14, 15). The uptake of apoptotic cells by other cells is facilitated by changes within the outer membrane of the dying cell, the so-called "eat me" signals. One of the most prominent of these signals is phosphatidylserine (PS) that, under normal conditions, is located on the inner plasma membrane leaflet. During apoptosis, however, it translocates to the outer side of the lipid layer where it can be recognized by adaptor proteins and specific PS receptors on phagocytes (16). The detection and ingestion of apoptotic material by macrophages predominantly induces the release of anti-inflammatory cytokines while simultaneously inhibiting the production of pro-inflammatory cytokines (**Figure 1**). By contrast, the uptake of apoptotic cells by immature dendritic cells (DCs) inhibits their maturation and induces tolerance (17). Therefore, apoptosis subserves several pro-tumor functions (18). Strategies have emerged to increase the immunogenicity of apoptotic cells by blocking their clearance by macrophages with the PS-binding protein AnnexinA5 (AnxA5) (19). Pre-clinical experiments revealed a long-lasting immune memory against tumor cells and a delayed tumor growth mediated by AnxA5 when given in combination with IR (20).

Nevertheless, apoptosis often plays a subordinate role in solid tumors, as tumor cells acquire resistance to apoptosis through several mechanisms; e.g., the tumor suppressor gene p53 is mutated in more than 50% of human malignancies (9). Other resistance mechanisms are overexpression of anti-apoptotic proteins, inactivation of pro-apoptotic genes, as well as interference with the death cell receptor and perforin/granzyme pathway (21).

Necrosis

In contrast to apoptosis, necrosis has often been defined as an uncontrolled or pathological cell death, which can be induced by extreme cellular stress such as trauma, infections, detergents, toxic agents, or heat. Morphologically, it is characterized by cellular swelling, rupture of the plasma membrane, and loss of intracellular content (22). It is considered to be a pro-inflammatory form of cell death due to its release of damage-associated molecular patterns (DAMPs) such as heat shock proteins (HSP), high mobility group box 1 (HMGB1), nucleotides, or uric acid leading to an activation of both, the innate and the adaptive immune system (22, 23) (**Figure 1**). In the last few years, it has become clear that there is a second form of necrosis, the so-called necroptosis, which is dependent on the receptor-interacting protein (RIP) kinases RIP1 and RIP3 (24, 25). It can be induced by factors such as tumor necrosis factor (TNF), Fas Ligand, or TNF-related apoptosis-inducing ligand (TRAIL) and utilizes the same initial signaling cascade as cell death receptor-induced apoptosis (25, 26). In addition, necroptosis can be manipulated by inhibitors such as Necrostatin 1, which blocks RIP1 kinase activity (27).

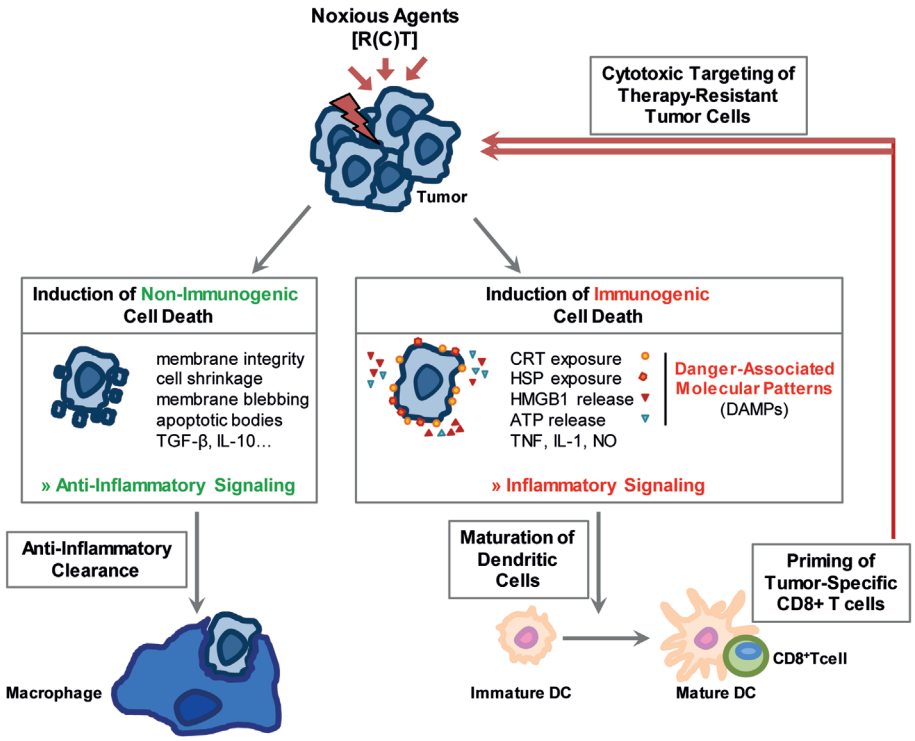

FIGURE 1 | Noxious agents may induce non-immunogenic and immunogenic cell death. Stressed cells can either undergo a non-immunogenic cell death resulting in their anti-inflammatory clearance. The stress-resulting damage might, however, also foster immune reactions. Immunogenic forms of cell death, main characteristics of which are displayed in the figure, stimulate the immune system especially through the release of damage associated molecular patterns (DAMPs). Dendritic cells mature, are activated and initiate a cytotoxic T-cell response against the tumor cells.

Therapeutic applications of RT and CT, either as stand-alone therapies or in combination with targeted therapies or IT, should stimulate local and systemic tumor control through the induction of immunogenic forms of cell death, which in turn can initiate persistent anti-tumor immune response. Particularly, both forms of necrosis are considered to be more immunogenic than the apoptosis and can therefore be useful tools to shift the tumor microenvironment toward an immunostimulatory rather than an immunosuppressive one (28) (**Figure 1**).

Impact of the Fractionation of Radiation on Anti-Tumor Responses

It has become obvious that radiation-induced non-(DNA) targeted, systemic effects are immune mediated and therefore also in part dependent on the primary cell death induction in the irradiated area (29, 30). With the emerging development of accelerators that has made it possible to deliver precisely higher single doses into the tumor area, one should focus on the immunological consequences of the different forms of radiation treatment as current pre-clinical data are not conclusive (2). RT can be administered in conventional fractionation schemes (1.8–2.2 Gy/fraction; 1 fraction/day, 5 days/week for 3–7 weeks), hyperfractionation (0.5–2.2 Gy/fraction, two fractions/day, 2–5 fractions/week for 2–4 weeks) or hypofractionation (3–20 Gy/fraction, 1 fraction/day) (31) using various therapeutic systems, including stereotactic radiosurgery. In the latter, the external radiation procedure utilizes multiple convergent beams to deliver high single doses to a small volume while sparing adjacent normal tissue. Currently, three different main modalities, namely LINAC, Gamma Knife, and protons are used for stereotactic radiosurgery, especially for the treatment of brain tumors with limited size that cannot be removed surgically (32).

Pre-clinically, Rubner et al. showed that fractionated RT is the main stimulus for cell death induction and HSP70 release in p53 mutated and O6-methylguanine methyltransferase, a DNA repair protein, negative glioblastoma cell lines (33). Tsai et al. investigated whether single high dose vs. multiple small doses with a total dose of 10 Gy differentially alters gene expression. They found out, amongst others, that there are significant differences in the gene response depending on the fractionation of radiation: 10 Gy delivered in fractions lead to a more stable induction of genes (34). Multhoff et al. hypothesized that conventional fraction schemes over several weeks are thought to be rather negative for radiation-induced anti-tumor immune responses as tumor-infiltrating immune lymphocytes might be killed by the repeating irradiation (35). Dewan et al. investigated the effects of RT with immune modulatory anti-CTLA4-antibodies on induction of anti-tumor immune responses. In his model system 3 × 8 Gy was superior

to that of 5 × 6 Gy in induction of a T-cell-dependent abscopal anti-tumor effect (36). This indicates that a higher single dose applied in hypofractionated schemes is advantageous to boost the immune system.

While it has been known that cellular effects of stereotactic radiosurgery include the induction of necrotic cell death and endothelial proliferation with luminal narrowing and thrombosis, Witham et al. used a rat glioma model to investigate whether gamma knife radiosurgery also induces apoptosis. They found tumor apoptosis to be statistically higher in treated animals at 6, 24, and 48 h after radiosurgery (37). Taken together, all these data show that more pre-clinical and clinical research are needed to define the best single dose and the respective fractionation scheme for induction of immunogenic cancer cell death and consecutive anti-tumor immunity.

Induction of Anti-Tumor Immunity

The Role of DCs in Anti-Tumor Immune Response

The activation of the immune system is vital to promote a long-lasting anti-tumor response. An essential asset for creating a potent anti-tumor immunity is the activation of DCs and consecutively cytotoxic $CD8^+$ T lymphocytes (CTL) alongside with $CD4^+$ T lymphocytes. Being the most efficient antigen-presenting cells, DCs play an important role in the initiation of the adaptive immunity. However, before DCs can stimulate any other cell type, they have to be activated properly. Such activating signals are not only foreign substances or infected cells, but can also be derived endogenously from stressed cells or cells dying by necrosis (38), as it is the case in tumor therapy. Immature DCs can then acquire and process tumor material, migrate to lymph nodes, and present or cross-present peptides of tumor-associated antigens (TAA) to naïve T cells in a MHC-II- or MHC-I-dependent manner, respectively (39). Aside from stimulating T-cell responses via the expression of co-stimulatory molecules such as CD80, CD86, as well as members of the TNF family (e.g., CD40, CD137 (4-1BB) L, OX40L) that can interact with the corresponding receptors on T cells, mature DCs secrete a wide range of pro-inflammatory cytokines (40). They therefore favor T-cell activation, survival, and differentiation and thus specific anti-tumor immune responses (41, 42).

DAMPs as Mediators of DC Activation

Other important factors for DC activation and maturation are secreted or exposed danger signals by dying cells, the so-called DAMPs (43). The surface exposure or release of DAMPs can be induced by IR or certain immunogenic chemotherapeutics, which are therefore capable of initiating a solid anti-tumor immune response (44). One of these signals is the early pre-apoptotic exposure of the endoplasmic reticulum (ER) protein calreticulin (CRT) on the plasma membrane surface. This can be induced by IR or substrates such as anthracyclines or oxaliplatin and triggers the uptake of tumor cells by DCs. In the presence of later DAMPs, such as HMGB1, the internalized tumor antigens get processed and cross-presented finally resulting in stimulation of tumor-specific CTLs (45, 46).

High mobility group box 1 is a chromatin-associated nuclear protein functioning as a DAMP when being expressed extracellularly. It is passively released by necrotic or damaged cells and secreted by immune cells such as macrophages, natural killer (NK) cells, neutrophils, mature DCs, and T cells and binds with high affinity to the receptor for advanced glycation end-products (RAGE) as well as the toll-like receptors (TLR)2, TLR4, and TLR9 (47). Its release from tumor cells can be induced by various stimuli, such as RT (33) and especially after combinatory treatment of RT with further immune stimulation, e.g., HT (48). Chemotherapeutic agents like temozolomide, melphalan, and paclitaxel might also foster its release (49, 50). HMGB1 interaction with a functional TLR4 on DCs is required for an efficient cross-presentation of tumor-antigens to T cells (51) and the priming of a tumor-specific T-cell response. The importance of TLR4 activation via danger signals can be seen in patients suffering from breast cancer, head and neck squamous cell carcinomas (HNSCC), or colorectal cancer carrying a loss of function single-nucleotide polymorphism (SNP) in the *Tlr-4* locus that have a predicted worsened outcome after immunogenic CT with anthracyclines or oxaliplatin (51, 52). However, HMGB1 also shows pro-tumorigenic properties. Thus, overexpression of HMGB1 and its receptor RAGE is observed in several cancers and is associated with tumor growth and metastasis (53). A possible explanation for the contradictory effects of HMGB1 might be a change of its redox state. Reducible HMGB1 binds to RAGE but not to TLR4 and promotes resistance to melphalan, paclitaxel, doxorubicin, and oxaliplatin, oxidized HMGB1, on the other hand, increases the cytotoxicity of these agents (54). One might speculate that RT-induced mitochondrial ROS production contributes to oxidation of HMGB1 and thereby to immunogenicity (55).

Another example for a DAMP that can be either passively released or actively secreted by dying or stressed cells is adenosine-5-triphosphate (ATP). It acts on purinergic P2RX7 receptors on DCs that in turn activate the NLRP3/ASC/caspase-1 inflammasome, finally resulting in the secretion of interleukin (IL-) IL-18 and IL-1β (56). IL-1β is required for efficient priming of $CD4^+$ T cells and interferone-γ (IFN-γ) producing tumor antigen-specific $CD8^+$ CTLs (57) and therefore for the generation of an anti-tumor immune response. Furthermore, ATP release from tumor cells also contributes to tumor growth and modulates immunosuppressive properties of myeloid-derived suppressor cells (MDSC) via a P2 × 7 receptor dependent mechanism (58).

HSP70 released from stressed cancer cells can also serve as a danger signal. HSPs are among the most abundant proteins in cells. Intracellular HSPs function as chaperons ensuring the correct folding or degradation of misfolded proteins. Under stress-induced conditions such as oxidative stress, HT, irradiation, or chemotherapeutics, intracellularly located HSPs are overexpressed and can be translocated to the plasma membrane or be released into the extracellular compartment, thereby acting as danger signals. In this way, HSP70 and HSP90 in particular play a dual role in cancer. Intracellularly, they protect tumor cells from programed cell death by interfering with apoptotic processes (59). However, if they are bound to the plasma membrane or released they contribute to the activation of the innate and adaptive immune system (60, 61). HSP70 promotes DC maturation as

well as NK cell migration, activation, and cytolytic activity. Also HSP70 is thought to be associated with tumor antigens triggering their cross-presentation via MHC-I on DCs and stimulating a $CD8^+$ T-cell response (62). Relevance of exposed HSP70 as a tumor-specific recognition structure is given by the group of Multhoff et al. who found that HSP70 is expressed on the plasma membrane of 40 (colon), 37 (gastric), 43 (lower rectal), and 42% (squamous cell) tumor specimens, but never on healthy cells. However, during the investigation, it became clear that the tumor entity is of major importance for clinical outcome. They therefore suggest the usage of HSP70 as a potential prognostic marker for overall survival (OS) (63).

To sum up, danger signals such as CRT, HMGB1, ATP, and HSPs are inducible by several chemotherapeutic drugs or irradiation. They play important roles in the priming of anti-tumor immune responses, but, depending on their location, concentration, and redox state, can also promote tumor development and progression.

Therapy-Dependent Modulation of the Tumor Microenvironment

Tumors have developed several molecular and cellular mechanisms to evade immune surveillance. These strategies include the secretion of immunosuppressive factors such as TGF-β, IL-10, or indoleamine 2,3-dioxygenase (IDO) (64–68), the alteration of antigen-presentation (69, 70), disruption of T-cell activation (71), apoptosis promotion of activated T cells (72), as well as the recruitment of regulatory cells or in general the inhibition of immune cells (73–75).

However, given that the immune system provides a possible strategy to create an efficient and long-lasting anti-tumor response, it is necessary to find treatment strategies that overcome the protective immunosuppressive microenvironment created by the tumor. Lately, it has become clear that standard treatments, namely RT and CT, can already render tumors and their microenvironment more immunogenic (76). As outlined above, RT and CT are able to induce both apoptotic and necrotic tumor cell death resulting in surface exposure and release of danger signals or TAAs. Aside from inducing tumor cell death, various chemotherapeutics, even or especially at low concentrations, stimulate, e.g., the expression of components of the antigen-processing machinery together with co-stimulatory molecules (e.g., CD40, CD80, CD86, MHC-II) on DCs thus promoting the stimulation of tumor-specific T cells, resulting in an anti-tumor immune response.

Immunogenicity of Radiotherapy

While low doses of IR have anti-inflammatory effects (77), higher doses (>1 Gy) applied in tumor therapy are capable of stimulating the immune system in several ways: RT can enhance the expression of MHC-I on the surface of tumor cells alongside with cell death receptors Fas/CD95 and NKG2D ligand, thus boosting the recognition and killing of irradiated tumor cells through T cells and NK cells (78–80). IR also has the ability to induce the production and release of CXCL16 in tumor cells. CXCL16 is a chemokine binding to its receptor CXCR6 on activated T cells therefore enhancing their recruitment to the tumor site (81). In addition, it also increases IFN-γ production that alters expression of adhesion molecules on vasculature, chemokines, and MHC-I expression, thus creating a microenvironment beneficial for T-cell infiltration and recognition of tumor cells by CTLs (82). Both, fractionated, hypofractionated, and ablative regimes bear the potential to stimulate immune responses (83, 84). However, which fractionation scheme and single dose of RT is the most immunogenic is under current intensive investigation and discussion (15, 42).

Taking all these factors into account, it becomes clear that CT and RT aside from their initial purpose to stop the proliferation of tumor cells and kill them are useful tools to shift an immunosuppressive tumor microenvironment to a more beneficial immune stimulatory one. A detailed understanding of the molecular mechanisms underlying these effects is therefore essential toward an optimized treatment.

Systemic Effects of Radiotherapy

As mentioned before, radiation, together with surgery and chemotherapeutics, is one of the most important tools in cancer treatment with the primary goal to achieve local control of tumor growth. Furthermore, it also enhances the tumors immunogenicity through the induction of distinct tumor cell death forms and the release of pro-inflammatory cytokines, chemokines, as well as danger signals. It therefore bears the potential to induce adaptive and innate immune responses, resulting in systemic anti-tumorigenic effects even outside of the field of irradiation (85). The phenomenon of regression of distant tumors or metastases outside the irradiation field is called *abscopal effect* of RT and its connection with immune events was first described by Nobler in 1969 (86). Since abscopal sounds a bit mystic, one should rather term it *systemic immune-mediated* effects of RT nowadays. Such reactions have been observed in many pre-clinical studies as well as in clinical settings for several tumor entities, including melanoma, hepatocellular, renal-cell, and mammary carcinomas, chronic lymphocytic leukemia (CLL), or malignant lymphomas [for further reading, see Ref. (42, 87)].

On a cellular level, it was demonstrated that the adaptive immune systems contributes to these systemic reactions and that NK cells are also involved (88, 89). In addition, the release of danger signals or cytokines such as TNF-α and IFN-γ by radiation-damaged tumor cells promote DC maturation and cross-presentation resulting in the regression of more distant tumor masses through activation of tumor-specific T cells (36, 88, 90).

However, in most tumor entities RT alone is not sufficient to induce such systemic immune reactions (89). Therefore, combination with IT might be the solution. A combined treatment of RT with the DC growth factor Flt-3 induced immune-mediated anti-tumor responses outside the irradiation field (89). Shiraishi et al. observed such effects after combined treatment of colon26 adenocarcinoma-bearing Balb/c mice with fractionated RT and the macrophage inflammatory protein-1 alpha variant ECI301 (88). A better local control and regression of the not irradiated tumor was observed by Jurgenliemk-Schulz and colleagues after additional rIL-2 treatment to RT in SL2 lymphoma or M8013

mammary carcinoma inoculated mice (91). Another approach is the modification of tumor cells or DCs with genetically engineered viruses expressing various cytokines, including IL-2 (92, 93), IL-12, IL-18 (94, 95), GM-CSF (96), or IFN-β (97) to enhance anti-tumor immunity and protect against tumor re-challenge. Just recently, Golden et al. reported about immune-mediated systemic tumor responses when combing RT with GM-CSF for the treatment of patients with metastatic solid tumors (98).

A further encouraging strategy to improve the effectiveness of standard therapies is the usage of monoclonal antibodies (mAb) targeting immune cells or tumors. In this matter, therapeutic Ab (summarized in **Table 1**) that can be used either alone or in combination with RT, CT, or IT are involved in depletion of Tregs (anti-CD25) (99) or target (i) co-stimulatory molecules such as CD40 (100, 101), OX40 (CD134) (102), and 4-1BB (CD137) (103, 104) on immune cells; (ii) checkpoint inhibitors PD-1, PD-L1 (103, 105) and CTLA-4 (99, 104); and (iii) cell growth factors or their receptors, e.g., epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and VEGFR (106, 107), all of which will be discussed in the following paragraph.

Co-stimulatory Molecules as Target to Improve RT and CT-Induced Systemic Immune Responses

CD40

CD40, a member of the TNF receptor (TNF-R) family, is expressed on APCs such as DCs, B cells, and macrophages. Interaction with its ligand (CD40L) on activated T cells promotes their activation and subsequently the induction of adaptive immune responses. Furthermore, the interaction between CD40 and its natural ligand (CD40L, CD154) was shown to modulate the growth of malignant B cells, thus CD40-related therapies have been considered for a range of cancer entities, including B-cell malignancies, leukemia, and multiple myelomas (MMs) (108), making it an attractive target structure. CD40 agonists mediate tumor cell death and in combination with DC activation anti-tumor immune responses. Pre-clinical models showed that anti-CD40 therapy in combination with RT results in a CD8 T-cell-dependent immunity to B-cell lymphoma (101). Currently, there are several anti-CD40 antibodies such as CP-870,893, dacetuzumab, and lucatumumab either as stand-alone treatments or in various combinations under investigation, such as a phase 1A/II study (NTC00670592) of patients with advanced non-Hodgkin lymphoma (NHL) or Hodgkin lymphoma (HL), which demonstrated a modest lucatumumab activity (109). However, there is still a lack of clinical data assessing the efficacy of targeting CD40 especially in combinatory therapy regimens with RT, CT, and other ITs, which is why further investigations are necessary.

OX40

OX40 (CD134), a co-stimulatory molecule expressed on activated T cells, is also part of the TNF-R superfamily. A phase I trial (NCT01644968), focusing on anti-OX40 monotherapy with a murine agonistic anti-human Ox40 mAb (9B12) in patients with metastatic solid malignancies showed an increased proliferation of $CD4^+$/FoxP3- and $CD8^+$ T cells as well as CD3-/NK cells. While anti-OX40 treatment was well tolerated with mild to mediate side effects, 12 out of 30 patients showed regression of at least one metastatic lesion (110). In order to increase this effect, a variety of combinatory therapy strategies of anti-OX40 treatment with CT, RT, or other IT are currently under investigation. For instance, a murine model of stereotactic body radiation therapy (SBRT) of lung cancer showed significant enhancement of tumor-free survival through intensified tumor antigen-specific $CD8^+$ T-cell responses under RT combined with adjuvant anti-OX40 therapy (111). Furthermore, a phase I/II trial with SBRT plus anti-OX40 in patients suffering from metastatic breast cancer (NCT01862900) and a phase Ib trial with cyclophosphamide, RT, and anti-OX40 in patients with progressive metastatic prostate cancer (NCT01303705) are currently ongoing with results not yet published.

CD137 (4-1BB)

CD137, expressed on activated $CD4^+$ and $CD8^+$ T cells, as well as on several APCs, including DCs, activated B cells, and macrophages, co-stimulates T-cell activation and clonal expansion after T-cell receptor (TCR) engagement through interactions with CD137-ligand. Importantly, the therapeutic use of 4-1BB agonists *in vivo* leads to a biased $CD8^+$ T-cell activation with a concomitant decline of B cells, NK, and $CD4^+$ T cells in an IFN-, TNF-, TGF-β, and IDO-dependent fashion (112). Furthermore, stimulation of CD137 on tumor endothelial cells via an agonistic antibody upregulates ICAM1, VCAM1, and E-selectin and thereby enhances T-cell recruitment into tumor tissue (113). In murine lung (M109) and breast carcinoma (EMT6) models, the efficiency of BMS-469492, another agonistic CD137 mAb, in combination with RT was evaluated. In the case of lung carcinoma treatment only a combination of the antibody with RT administered as a high radiation dose of 15 Gy resulted in an enhanced tumor response. In the breast cancer model, the CD137 agonist alone already led to significant tumor growth inhibition that could even be potentiated by using high single doses or fractionated radiation. The authors concluded that the different responses in the two models could result from differences in intrinsic immunogenicity of the different tumor entities and that anti-CD137 antibodies may not only be used as a stand-alone therapy but in combination with conventional anti-cancer treatments, e.g., RT (114). Furthermore, the combination of RT and anti-CD137 in an intracranial glioma model resulted in complete tumor elimination and prolonged survival in 67% of the mice. The combination therapy highly increased the numbers of tumor-infiltrating $CD4^+$ and $CD8^+$ lymphocytes as well as IFN-γ production (115). Thus, based on promising pre-clinical data of combining anti-CD137 and RT/CT, two currently ongoing clinical phase I studies have been initiated. While NCT00461110 investigates agonistic anti-CD137 (BMS-663513) treatment in combination with chemo-radiation (RT, paclitaxel, carboplatin) in non-small cell lung carcinoma (NSCLC) patients, NTC00351325 focuses on a combination therapy of BMS-663513 with CT (paclitaxel, carboplatin) in patients suffering from recurrent ovarian carcinoma.

TABLE 1 | Selected monoclonal antibodies and tyrosine kinase inhibitors against co-stimulatory and checkpoint molecules and growth factors that are in clinical phase I–III trials either alone or in combination with RT, CT or immunotherapy.

Target	Drug	Developer	Target disease (not all listed)
Co-stimulatory molecules			
CD40	CP-870,893 Dacetuzumab Lucatumumab	Pfizer Seattle Genetics, Inc. Novartis	Melanoma; pancreatic carcinoma; *renal*-cell carcinoma; breast cancer Diffuse large B-cell lymphoma (DLBCL); chronic lymphocytic leukemia (CLL); non-hodgkin's lymphoma (NHL); multiple myeloma (MM) CLL; NHL; MM
CD134 (OX40)	MEDI6469	AstraZeneca	Advanced solid tumors; aggressive B-cell lymphomas; HNC; metastatic prostate cancer
CD137	BM-663513	Bristol-Myers Squibb (BMS)	Melanoma; advanced solid malignancies; B-cell malignancies
Checkpoint inhibitors			
CTLA-4	Tremelimumab Ipilimumab	Pfizer BMS	Metastatic melanoma; HNSCC; NSCLC; advanced solid malignancies Yervoy™ approved for unresectable or metastatic melanoma[a]; lymphoma; NSCLC; HNC; prostate, pancreatic, liver, lung, kidney and renal-cell cancer; melanoma
PD-1	Nivolumab Pembrolizumab Pidilizumab	BMS Merck CureTech Ltd	Obvido® approved for unresectable or metastatic melanoma and NSCLC[a]; MM; NHL Renal-cell carcinoma (RCC); advanced solid tumors; melanoma; NSCLC Keytruda® approved for advanced or unresectable melanoma[a]; NSCLC; HNSCC; lymphoma; breast cancer; malignant glioma; melanoma MM; gliomas; lymphomas
PD-L1	BMS-936559 MEDI4736	BMS AstraZeneca	Recurrent solid tumors Advanced solid tumors; NSCLC; HNSCC; GBM
Growth factor inhibitors			
EGFR	Cetuximab Panitumumab Gefitinib Erlotinib	BMS Amgen AstraZeneca Genentech/Roche	Erbitux® approved for *K-ras* wild-type, EGFR-expressing metastatic colorectal cancer and recurrent/metastatic HNSCC[a]; NSCLC; HNSCC; colorectal cancer Vectibix™ approved for colorectal cancer[a]; HNSCC; colorectal cancer Iressa® approved for NSCLC[a]; HNC; skin, breast, colorectal cancer; GBM; NSCLC Tarceva® approved for NSCLC and pancreatic cancer[a]; HNC; prostate, breast, esophageal, colorectal cancer; NSCLC; pancreatic cancer
HER2/neu receptor	Trastuzumab	Genentech/Roche	Herceptin® approved for HER2-overexpressing breast cancer and HER2-overexpressing metastatic gastric or gastroesophageal (GE) junction adenocarcinoma[a]; breast cancer; NSCLC
VEGFRs, PDGFRs, FLT-3, c-Kit, RET; CSF-1R	Sunitinib	Pfizer	Sutent® approved for pancreatic neuroendocrine tumors (pNET); kidney cancer and gastrointestinal stromal tumor (GIST)[a]; pNET; kidney cancer; GIST; RCC, pancreatic and bladder cancer
VEGFRs, PDGFRs, RAF, FLT-3, c-Kit, RET	Sorafenib	Bayer	Nexavar® approved for recurrent or metastatic, progressive differentiated thyroid carcinoma (DTC), unresectable hepatocellular carcinoma (HCC) and advanced RCC[a]; HCC; RCC, bladder cancer; brain neoplasms; advanced solid tumors
VEGFRs	Axitinib	Pfizer	Inlyta® approved for advanced RCC[a]; advanced gastric cancer; hepatocellular and colorectal carcinoma; prostate cancer; GBM; RCC; NSCLC
VEGFRs, PDGFRs, c-Kit	Pazopanib	GlaxoSmithKline	Votrient® approved for advanced soft tissue sarcoma and RCC[a]; ovarian cancer; fallopian tube cancer; peritoneal carcinoma; NSCLC; RCC
VEGF-A	Bevacizumab	Genentech/Roche	Avastin® approved for recurrent epithelial ovarian, fallopian tube, or primary peritoneal cancer, recurrent/metastatic cervical cancer, metastatic HER2 negative breast cancer, RCC, GBM, NSCLC[a]; advanced cancers

[a]*FDA-approved drugs.*

Checkpoint Inhibitors as Targets to Improve RT-Induced Systemic Immune Responses

In order to ensure peripheral tolerance and to avoid overshooting immune reactions, endogenous mechanisms to dampen T cells have been evolved. Cytotoxic T lymphocyte-antigen-4 (CTLA-4) and PD-1 are major negative co-stimulatory molecules expressed on activated T cells (116–118). While CTLA-4 regulates early stages of T-cell activation, PD-1 limits the activity of T cells in peripheral tissues during inflammatory response and is therefore a major immune resistance mechanism in the tumor microenvironment.

CTLA-4

T-cell activation and survival are dependent on positive signaling from the TCR as well as co-stimulatory molecules such as CD28. CTLA-4 is an inhibitory molecule that is upregulated on the surface of effector T cells and competes with CD28 for the binding to CD80/86 (B7.1 and B7.2). Under physiological

conditions, this limits the T-cell response and helps to maintain T-cell homeostasis (119). However, with regard to cancer treatment, the down-regulation of a tumor-specific T-cell response is an unwanted scenario, thus favoring an antagonistic CTLA-4 therapy. Indeed, various pre-clinical and clinical studies have already proven the efficiency of anti-CTLA-4 therapy, especially for melanoma in multimodal settings. This tumor entity has a high prevalence for somatic mutations (120) and is therefore suitable for being specifically targeted by activated immune cells.

Pre-clinical melanoma models showed that tumors do not always respond to an anti-CTLA-4 mAb alone, while additive treatments with GM-CSF (121) or activation of the T-cell co-stimulatory receptor 4-1BB (122) are able to promote an anti-tumor response. Furthermore, a combination of RT and CTLA-4 mAb treatment prolonged the OS in an orthotopic GL261 glioma model, whereas CTLA-4 mAb alone was not able to extend the survival in comparison to untreated controls. A triple combination of RT, anti-CTLA-4, and anti-CD137 further improved survival in this pre-clinical model through a $CD4^+$ T-cell-dependent manner and created a glioma-specific protective memory response (104). Dewan and colleagues reported an abscopal effect in breast (TSA) and colon cancer (MCA38) models: An increased frequency of $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes along with tumor-specific IFN-γ production was observed after a combined administration of anti-CTLA-4 mAb (9H10) and fractionated (3×8 Gy or 5×6 Gy fractions in consecutive days), but not single-dose RT with 20 Gy. Furthermore, three doses of 8 Gy in combination with anti-CTLA-4 was able to induce a more potent systemic effect and higher frequency of tumor-specific T cells than five doses of 6 Gy plus anti-CTLA-4 (36), suggesting that fractionation influences the induction of anti-tumor immune responses with further immune stimulation (76).

Two fully humanized anti-CTLA-4 antibodies, tremelimumab and ipilimumab, advanced for testing in clinical trials. Most studies so far are focused on melanoma, where treatment-related adverse effects were found to be manageable (123–125). Various phase I and II studies evaluated anti-CTLA-4 therapy in a stand-alone therapy setting or in various combinations such as tumor antigen-loaded DCs (126, 127), the TLR9 agonist PF-3512676 (128), IFN-α-2b (129) or in combination with various chemotherapeutics (for further reading, refer to NCT00257205 (130), NCT02262741, NCT02319044, NCT02369874, NCT02352948, NCT02040064).

In summary, current study results show the importance of investigating the optimal dose, schedule, and combination of anti-CTLA-4 antibodies with other therapy options to ensure high patient safety and efficacy in selected cancer entities.

As approximately 50% of cancer patients receive RT with the primary goal of local tumor control (4), combinatory therapies of RT with immune checkpoint inhibitors targeting T cells might be a good synergistic option to induce additional systemic anti-tumor immune responses, as it has already been shown in many mouse models **(Table 2)** (36, 131–134). A tremelimumab/SBRT pilot study for patients suffering from unresectable pancreatic cancer (NCT02311361) is currently recruiting patients.

Another anti-CTLA-4 mAb is ipilimumab, as with tremelimumab, patients with advanced metastatic cancer can benefit from it. Adverse side effects such as strong autoimmune reactions have been observed in a dose-dependent manner in various phase I/II trials (163–165). An increase of the ipilimumab-induced response rate might be achieved through a combination with immunogenic RT. Postow et al. described the first case of a systemic immune-mediated effect in a patient suffering from metastatic melanoma that has been treated with ipilimumab and a concomitant palliative RT (28.5 Gy in three fractions) that correlated with beneficial immune changes in the peripheral blood when RT was added (136). Five months after RT and an additional administered ipilimumab dose, RT-treated and non-RT-treated lesions had regressed and remained stable with minimal disease progression after 10 months, as confirmed by computed tomography scans. A second case of complete systemic response after a combined treatment of ipilimumab followed by high-dose stereotactic RT of 54 Gy in three fractions to two out of seven metastatic liver lesions was reported in a patient with advanced melanoma (137). Several studies have also provided evidence of ipilimumab effectiveness in cases of melanoma with brain metastases (165, 166). Hence, in a retrospective study of 21 patients suffering from advanced melanoma and brain metastases, Grimaldi and colleagues (138) reported abscopal responses in 52% of patients receiving an initial ipilimumab therapy followed by localized RT. Furthermore, this systemic response was correlated with prolonged OS.

These promising results of combined ipilimumab and RT treatment spiked the interest and led to the initiation of studies for other cancer entities than melanoma. Likewise, a phase I/II study in patients with metastatic castration-resistant prostate cancer (mCRPC) suggests the induction of clinical anti-tumor activity with disease control and manageable adverse effects after 10 mg/kg of ipilimumab and RT with 8 Gy per lesion (167). A phase III trial (NCT00861614) evaluating ipilimumab administration (10 mg/kg) vs. placebo after RT in patients suffering from mCRPC with disease progress after docetaxel reported an improvement of median OS within the ipilimumab group (11.2 vs. 10 months in the placebo group). Conversely, most of the common adverse effects (26 vs. 3%) and four deaths occurred in patients receiving ipilimumab treatment vs. placebo (139). Just recently, a systemic response was reported in a patient suffering from metastatic NSCLC 2.5 months after the start of a combined ipilimumab and fractioned RT (140). This suggests that a combination of local RT alongside IT could be a useful approach to further improve clinical outcome of patients with metastatic disease (2). Therefore, various phase I/II studies of combined RT and ipilimumab administration for metastatic NSCLC (NCT02221739), advanced cervical cancer (NCT01711515), metastatic cancers of liver and lungs (NCT02239900), and patients with melanoma and brain metastases (NCT02115139) have been initiated and are currently recruiting patients.

PD-1/PD-L1 (B7-H1)

PD-1, another negative regulator of TCR signaling, and its ligand PD-L1 play an important role in modulating T-cell activity not only in physiological conditions but also in the tumor microenvironment of various cancer entities. Thus, blockage of PD-1 and PD-L1 interaction through mAb is a promising strategy to

TABLE 2 | Systemic effects observed in pre-clinical and clinical studies after multimodal treatment of RT, CT, and immunotherapy.

Checkpoint	Tumor type	Treatment	Systemic effects + key mediator	Reference
PRECLINICAL MOUSE-MODELS				
CTLA-4	Metastatic mammary carcinoma (4T1)	RT (2 × 12 Gy) of primary tumor + anti-CTLA-4 mAb i.p. (3×)	Inhibition of lung metastases, ↑**CD8⁺ CTLs**	(131)
	Metastatic mammary carcinoma (4T1)	RT (2 × 12 Gy) of primary tumor + anti-CTLA-4 (9H10) mAb i.p. (3×)	Inhibition of lung metastases, increased survival, ↑**CD8⁺ CTLs**	(132)
	Mammary carcinoma (TSA), colon carcinoma (MCA38)	RT of primary tumor (20 Gy, 3 × 8 Gy, 5 × 6 Gy) + anti-CTLA-4 (9H10) mAb i.p. (3×)	Growth-inhibition of irradiated and non-irradiated tumor, ↑**CD8⁺ CTLs and CD4⁺ Th-cells, IFNγ**	(36)
PD-1	Melanoma (B16), renal cortical adenocarcinoma (RENCA)	SABR (15 Gy) + anti-PD-1 mAb (5×)	Near-complete regression of primary tumor, 66% size reduction of non-irradiated tumor,↑**CD8⁺ CTLs**	(133)
	Glioma (GL261)	RT (10 Gy) + anti-PD-1 mAb i.p.	Tumor regression and long-term survival,↓ **Tregs,** ↑ **CD8⁺ CTLs, IFNγ**	(105)
	Melanoma (B16), breast carcinoma (4T1-HA)	RT (12 Gy) + anti-PD-1 mAb i.p. (3×)	Tumor regression and tumor control, ↓ **Tregs,** ↑ **CD8⁺ CTLs**	(135)
PD-L1	Mammary carcinoma (TUBO)	SABR (12 Gy) + anti-PD-L1 mAb (4×)	Size reduction of primary and abscopal tumors, ↓ **MDSC,** ↑ **CD8⁺ T-cells**	(134)
CD137 (4-1BB)	Lung carcinoma (M109)	RT (5, 10 or 15Gy) + anti-CD137 (BMS-469492) mAb i.v. (3×)	Tumor growth retardation at a dose of 15 Gy	(114)
	Breast carcinoma (EMT6)	RT (5, 10, 15Gy, 11 × 4 Gy) + anti-CD137 (BMS-469492) mAb i.v. (3×)	Enhanced tumor growth retardation at all radiation doses	(114)
	Glioma (GL261)	RT (2 × 4 Gy) + anti-CD137 mAb i.p. (3×)	Tumor eradication, prolonged survival (6/9), rejection of challenging tumors (5/6), ↑**CD8⁺ CTLs and CD4⁺ Th-cells, IFNγ**	(115)
CTLA-4 + CD137	Glioma (GL261)	RT (10 Gy) + anti-CD137 and anti-CTLA-4 mAb i.p. (3×)	Prolonged survival, ↑**CD8⁺ CTLs and CD4⁺ Th-cells**	(104)
CLINICAL STUDIES				
Checkpoint inhibitors				
CTLA-4	Metastatic melanoma (n = 1)	RT (28.5 Gy in 3 fractions) + ipilimumab	Regression of irradiated and non-irradiated tumor lesions, stable lesions and minimal disease 10 months after RT	(136)
	Metastatic melanoma (n = 1)	RT (54 Gy in 3 fractions) + 4 cycles of ipilimumab	Regression of irradiated and non-irradiated tumor lesions, CR, no evidence of disease 12 months after RT	(137)
	Melanoma with brain metastasis (n = 21)	Four cycles of ipilimumab + loco-regional RT	13/21 LR, 11/21 with LR abscopal effect and 2/21 stable disease	(138)
	mCRPC (n = 799) [NCT00861614]	RT (1 × 8 Gy) per lesion + 1–4 doses of ipilimumab (n = 399) or placebo (n = 400)	Improved median OS	(139)
	Metastatic NSCLC (n = 1)	RT (5 × 6 Gy) + four cycles of ipilimumab	Regression of irradiated and non-irradiated tumor lesions	(140)
PD-1	Melanoma, NSCLC, mCRPC, colorectal cancer, and renal cancer (n = 236)	nivolumab	Cumulative response rates in 14/76 among NSCLC patients, in 26/94 of melanoma patients and in 9/33 renal-cell cancer patients	(141)
	Advanced melanoma	Pembrolizumab (lambrolizumab; MK-3475)	52% response rate drug-related adverse effects were reported by 79% of patients, with 13% reporting grades 3 and 4 secondary effects	(142)
	Patients with DLBCL undergoing AHSCT [NCT00532259]	AHSCT + 3 doses pidilizumab	At 16 months, PFS was 0.72, among the 35 patients with measurable disease after AHSCT, overall response rate was 51%, ↑ **circulating lymphocyte subsets including PD-L1-bearing lymphocytes**	(143)
PD-L1	Dose-escalation study in patients with NSCLC, melanoma, colorectal, renal-cell, ovarian, pancreatic, gastric, and breast cancer (n = 207) [NCT00729664]	Administration of BMS-936559 in 6-week cycles; up to 16 cycles	Objective response rate in 9/52 in melanoma, in 2/17 in renal-cell cancer, in 5/49 in NSCLC, and in 1/17 in ovarian cancer	(144)

(Continued)

TABLE 2 | Continued

Checkpoint	Tumor type	Treatment	Systemic effects + key mediator	Reference
Growth factor inhibitors				
VEGF-A	Advanced nasopharyngeal carcinoma (n = 44) [NCT00408694]	IMRT (50–70 Gy) + CT + concurrent and adjuvant BEV	Localregional PFS (83.7%) and distant metastasis-free interval (90.8%), PFS (74.7%), OS (90.9%) within 2 years median followup	(145)
	Advanced colorectal carcinoma (n = 19)	RT (15x–3.4 Gy) + concurrent and adjuvant BEV + CT	CR (68.5%) and PR (21.1%) within 2 years median follow	(146)
	Newly diagnosed GBM [NCT00943826]	RT (60 Gy) + concurrent and adjuvant TMZ + BEV (n = 458) or placebo (n = 463)	Improved PFS	(147)
	Newly diagnosed GBM (n = 621) [NCT00884741]	RT (60Gy) + concurrent and adjuvant TMZ + BEV or placebo	Improved PFS	(148)
EGFR	LA-HNC [NCT00004227]	RT with concurrent cetuximab (n = 211) or RT alone (n = 213)	Improved median OS	(149)
	Unresectable LA-SCCHN (n = 60) [NCT00096174]	RCT with concurrent and adjuvant cetuximab	Improved median OS in HPV(+) tumors	(150)
	Esophageal cancer *[ISRCTN47718479]*	RCT with cetuximab (n = 129) or RCT alone (n = 129)	↓ Survival in cetuximab group	(151)
	Unresectable NSCLC [SWOG 0023]	RCT with adjuvant gefintinib (n = 118) or placebo (n = 125)	↓ Survival in gefinitib group	(152)
	LA-HNC (n = 66)	CRT + concurrent and adjuvant gefintinib	CR (90%), PFS (72%), OS (74%) within 3.5 years median followup	(153)
	Metastatic NSCLC (n = 24)	SBRT + CT with neoadjuvant, concurrent and adjuvant erlotinib	Improved PFS and OS	(154)
	Advanced cervical cancer (n = 36)	RCT with neoadjuvant, concurrent erlotinib	Improved PFS and OS	(155)
	Lung adenocarcinoma with brain metastases	WBRT with concurrent and adjuvant erlotinib (n = 23) or WBRT alone (n = 21)	Median local PFS 6.8 vs. 10.6 month (mOS: 6.8 vs. 10.6 month, response rate 54.84 vs. 95.65%) in RT vs. RT + E	(156)
	Newly diagnosed GBM (n = 65)	RCT with concurrent and adjuvant erlotinib	Median PFS 8.2 vs. 4.9 month (mS: 19.3 vs. 14.1 month) RCT + E vs. historical controls (only RCT)	(157)
EGFR + VEGF-A	LA-HNC (n = 27) [NCT00140556]	Neoadjuvant BEV and/or erlotinib concurrent CRT + BEV and erlotinib	CR (96%), local control (85%) and distant metastasis-free survival rate (93%), PFS (82%), OS (86%) within 3 years median followup	(158)
VEGFR, PDGFR, KIT, RAF	Advanced hepatocellular carcinoma (n = 40)	RT with concurrent and adjuvant Sorafenib (S)	No improved efficacy of RT + S compared to RT alone	(159)
	Newly diagnosed GBM (n = 47)	RCT with adjuvant sorafenib (S)	No improved efficacy of RCT + S compared to RCT alone	(160)
RTK inhibitor	Patients with oligometastases (n = 25) [NCT00463060]	Sunitinib + IGRT (10 × 5 Gy)	Local (75%) and distant (52%) tumor control, PFS (56%), OS (71%) within 18-month median followup	(161)
	Patients with oligometastases (n = 46)	Sunitinib + SBRT (10 × 5 Gy)	Local (75%) and distant (40%) tumor control, PFS (34%), OS (29%) within 4-year median followup	(162)
Co-stimulatory molecules				
CD40	Advanced NHL (n = 74)or HL (n = 37) [NTC00670592]	Escalating doses of lucatumumab (once weekly for 4 weeks of an 8-week cycle)	Modest activity in relapsed/refractory patients with advanced lymphoma	(109)

↑, increase; ↓, decrease; NSCLC, non-small cell lung carcinoma; mCRPC, metastatic castration-resistant prostate cancer; GBM, glioblastoma multiforme; LA-HNC, locally advanced head and neck cancer; LA-SCCHN, locally advanced squamous cell head and neck cancer; DLBCL, diffuse large B-cell lymphoma; NHL, non-Hodgkin lymphoma; HL, Hodgkin lymphoma; SBRT, stereotactic body radiation therapy; SABR, stereotactic ablative RT; IMRT, intensity modulated radiation therapy; IGRT, image-guided radiotherapy; WBRT, whole brain radiotherapy; AHSCT, autologous hematopoietic stem-cell transplantation; OS, overall survival; PFS, progression-free survival; CR, complete response; PR, partial response; LR, local response; BEV, bevacizumab; R-ICE, rituximab, ifosfamide, carboplatin and etopside; MDSCs, myeloid-derived suppressor cells.

overcome tumor-escape from a tumor-specific immune response (168, 169).

Pre-clinical studies have demonstrated an enhancement of anti-tumor immunity through a combination of RT together with antibody-mediated PD-1 blockade (133–135). For instance, the effectiveness of treatment of mouse melanoma and renal-cell tumors with stereotactic ablative radiotherapy (SABR) was dependent on PD-1 expression. Only 20% of PD-1 KO mice and none of the wild-type mice survived beyond 40 days. The combination of SABR with PD-1 blockade resulted not only in an almost complete regression of the irradiated primary tumor, but also in a 66% size reduction of the non-irradiated secondary tumors. Park et al. therefore suggest a SABR-induced systemic tumor-specific immune response also targeting the secondary non-irradiated tumors that can further be increased by PD-1 blockade (133). Of note is that optimal timing of RT in combination with a checkpoint blockade is mandatory: since IR temporarily increases the expression of PD-1L on tumor cells (134), a concurrent application is suggested. Addition of anti-PD-L1 mAb after RT does not result in prolonged survival of tumor-bearing mice (170, 171).

Single-agent trials have already been initiated using the anti-PD-1 mAbs nivolumab, pembrolizumab, and pidilizumab, as recently summarized by Philips and Atkins (172). Those studies include planned or ongoing phase I–II trials of anti-PD-1 mAb monotherapy for various cancer entities, such as lymphoma (NCT02038946, NCT02038933, NCT01953692), NSCLC (NCT02066636, NCT01840579), hepatocellular carcinoma (HCC) (NCT01658878), HNSCC (NCT02105636), melanoma (NCT02374242, NCT01844505, NCT02306850), and glioma (NCT02337686, NCT02359565, NCT01952769), respectively, either alone or in comparison to CT or IT. A phase I trial investigating safety and reactivity of nivolumab in 236 patients with melanoma, NSCLC, mCRPC, colorectal cancer, and renal cancer concluded cumulative response rates in 14 of 76 among NSCLC patients, in 26 of 94 of patients suffering from melanoma and in 9 of 33 renal-cell cancer patients. Anti-PD-1 treatment-related toxic effects (grades 3 and 4) occurred in 14% of the patients (141). A phase I trial with BMS-936559, a PD-L1-specific Ab in NSCLC, melanoma, colorectal, renal-cell, ovarian, pancreatic, gastric, and breast cancer (NCT00729664) patients resulted in an objective response rate in 9 of 52 in melanoma, in 2 of 17 in renal-cell cancer, in 5 of 49 in NSCLC, and in 1 of 17 in ovarian cancer, while drug-related adverse effects of grades 3 and 4 occurred in 9% of treated patients (144). A clinical investigation of lambrolizumab (MK-3475) in patients with advanced melanoma showed a 52% response rate. However, during the treatment drug-related adverse effects were reported by 79% of patients, with 13% reporting grades 3 and 4 secondary effects (142). These investigations lead to a FDA approval of pembrolizumab (formerly MK-3475 and lambrolizumab) in patients suffering from advanced or non-resectable melanoma that are no longer responsive to standard medications.

As it has been shown by Ansell et al., cells within the microenvironment in lymphomas express PD-L1, and with intratumorally found T cells also expressing PD-1, this discovery provides the possibility to successfully target this immune checkpoint also in malignancies of hematopoietic origin (173). In the case of pidilizumab, an international phase II study was conducted in patients with diffuse large B-cell lymphoma (DLBCL) that are undergoing autologous hematopoietic stem-cell transplantation (AHSCT). The investigators discovered that among 35 out of 66 patients with measurable disease after AHSCT, the overall response rate after pidilizumab was 51%. In addition to that, an increase of activated $CD4^+$ helper and central memory T cells along with circulating $CD8^+$ peripheral and central memory T cells was found, which was the first reported clinical activity of PD-1 blockage in DLBCL (143). Recently, a study examining nivolumab in relapsed or refractory Hodgkin's lymphoma revealed a substantial therapeutic activity with an objective response rate of 87% and an acceptable safety profile in the evaluated cases (NCT01592370) (174).

In summary, PD-1 or PD-1L antagonistic mAb are able to promote a positive anti-tumor immune response in patients, while the response rate depends on the tumor entity. Thus, a combination therapy of anti-PD-1 mAb with RT could further improve the outcome and especially be an efficient strategy in the management of metastatic disease. The interactions of multiple co-stimulatory and inhibitory molecules regulating T-cell responses that can be targeted to strongly enhance radio(chemo) therapy (RCT)-induced anti-tumor immune responses are summarized in **Figure 2**.

Growth Factors as Targets for Cancer Therapeutics

The activation of receptors by growth factors such as EGF, VEGF, transforming growth factor-α (TGF-α), and basic fibroblast growth factor (bFGF) triggers various cellular processes, including proliferation, differentiation, apoptosis, migration, adhesion, invasion, vascular permeability, or angiogenesis. As EGF and VEGF signaling pathways are a key feature in the development, progression, and metastatic formation in a wide range of cancer entities, they function as important targets for therapeutic Ab (175). In addition, pre-clinical models demonstrated a broad efficacy for anti-EGFR and anti-VEGF Abs alone (176–178) and in combination with RT (179). As in the case of checkpoint inhibitors, concurrent application should be most effective, since, e.g., VEGF-C expression is enhanced after irradiation (180). While many inhibitors are currently undergoing clinical evaluation, several others are already used in cancer therapy (**Figure 2**). Some of the FDA-approved inhibitors are anti-EGFR mAb that either work via binding the extracellular domain of EGFR (cetuximab, panitumumab, and trastuzumab) or target the intracellular EGFR domain such as the tyrosine kinase inhibitors gefitinib and erlotinib (181). FDA-approved anti-VEGFR mAb, on the other hand, inhibit angiogenesis through VEGF-A blocking [e.g., bevacizumab (BEV)] or also act as VEGFR tyrosine kinase inhibitors such as sunitinib, sorafenib, axitinib, and pazopanib (182, 183). They are approved for a variety of tumor entities, including metastatic colorectal cancer, gastric or gastro-esophageal carcinoma, renal-cell carcinoma (RCC), advanced soft tissue sarcoma, pancreatic neuroendocrine tumors (pNET), breast cancer, NSCLC, HNSCC, and glioblastoma. Several

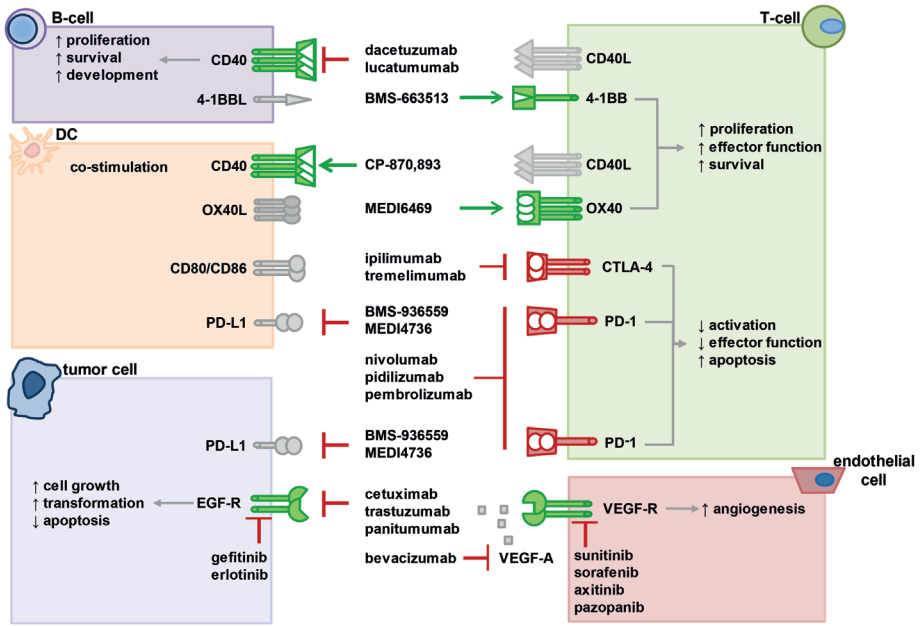

FIGURE 2 | Interactions of various co-stimulatory and inhibitory molecules regulate T-cell responses, tumor cell behavior, and vascularization. Immunotherapies with agonistic or antagonistic monoclonal antibodies have been developed to modulate these interactions by stimulating or blocking their activity. In the figure, a selection of important molecular interactions, their most relevant cellular source (not exclusive), and examples of antagonistic (red lines) or agonistic (green arrow) monoclonal antibodies as well as inhibitors are displayed. Activating receptors are depicted in green, suppressive receptors are shown in red, ligands are gray. For further information, please refer to the main text.

reports about the safety and efficacy of growth factor inhibitors either in the form of monotherapy or as a combinatory therapy paired with IT, CT, or RT have been released. However, targeting VEGF or EGFR alone does not always provide adequate tumor control in the clinic. In the next section, we will therefore focus on FDA-approved inhibitors in combinatory therapy settings together with RT or RCT.

Growth Factor Inhibitors and RT

Patients suffering from loco-regional advanced squamous-cell carcinoma of the head and neck cancer (HNC) being treated in a phase III trial with a high-dose RT in combination with weekly cetuximab administration showed an increased loco-regional tumor control (24.4 vs. 14.9 months), along with increased median OS (49.0 vs. 29.3 months), increased median progression-free survival (PFS,17.1 vs. 12.4 months), and reduced mortality in comparison to high-dose RT monotherapy (149). A combination of erlotinib with R(C)T is also able to enhance OS as well as PFS in patients with metastatic NSCLC (154), cervical cancer (155), lung adonocarcinoma (184), or GBM (157). Tong et al. demonstrated a protective effect of a combination of sunitinib with RT on oligometastases (161). Their results were confirmed by Kao et al. who found a 75% local control and 40% distant control of oligometastases, a PFS of 34%, and an OS of 29% over a 4-year period in patients with HNC, liver, lung, kidneys, and prostate cancers that have been treated with SBRT and concomitant sunitinib therapy (162). However, a combination therapy of RT and sorafenib in comparison with standard therapy was not able to enhance the efficacy in GBM and hepatocellular carcinoma (159, 160, 185).

Growth Factor Inhibitors and R(C)T

A phase II study demonstrated a near-complete or complete tumor response in 53% of patients treated with a combination of panitumumab and RCT vs. 32% of patients treated with standard RCT in patients with advanced rectal cancer with wild-type *KRAS* (186). *In vitro* studies on this matter also demonstrated an elevated level of radiosensitivity (187), while the clinical relevance of a combination of RT and adjunctive trastuzumab therapy is still under investigation. A phase II study investigating the effects of gefitinib with concomitant RCT in locally advanced HNC found a 4-year enhanced OS (74%), PFS (72%) and disease-specific survival rates (89%), respectively (153). BEV is the first approved angiogenesis inhibitor and is used in metastatic colorectal cancer, NSCLC, and breast cancer. As a result of the poor prognosis of patients with GBM and thus a need for new therapy modalities, the combination of standard RCT and anti-angiogenic antibodies such as BEV might be a promising approach in the treatment of this tumor entity. Therefore, various clinical trials dealt with this notion and revealed an extended PFS and improved life quality in newly diagnosed GBM patients that have been treated with standard RCT and BEV, whereas no change in OS was observed (147, 148, 188). BEV has also been examined in various other entities: an addition of BEV to RCT in pancreatic adenocarcinoma resulted in survival rates similar to those of standard approaches (189), in cases of nasopharyngeal carcinoma it was able to promote OS and has been linked to a delayed progression of distant metastases (145). Furthermore, an application of BEV in metastatic colorectal cancer resulted in high rates of long-term complete responses (CRs) (146). A simultaneous VEGF-A and

EGFR blockade (BEV + erlotinib) in locally advanced head and neck cancer (LA-HNC) together with concurrent RCT are favorable when being compared to historical controls (158).

Taken together, all these data suggest a synergized effect of combination treatment of R(C)T with VEGF and/or EGFR inhibitors as seen in NSCLC cells reported by Kriegs et al. (190). Most of the approved mAb used in cancer IT are generally well tolerated in humans (191). Conversely, mAb application can also be associated with an increased risk of unwanted and possibly even fatal adverse effects (191–194), including cytokine release syndrome, induced autoimmunity, organ toxicity, opportunistic infections, and even cancer as a result of immune suppression. This shows how, despite the success of therapeutic Ab, their clinical efficacy greatly depends on tumor type, treatment duration, administered dose, and combination-therapy options. With this in mind, a new and promising approach in IT, the adoptive cell transfer, might be another useful therapy option to be combined with RT. Here, autologous T cells that are either tumor-specific or genetically engineered are expanded *ex vivo* before being infused back into the patient. In this article, we will focus on genetically engineered T cells only.

Chimeric Antigen Receptors as Tool to Recognize Specific Tumor-Associated Antigens

As mentioned above, tumors are able to establish an immunosuppressive microenvironment resulting, amongst other effects, in the inhibition of an anti-tumor-specific T-cell response. This state is achieved through release of immunosuppressive cytokines, altered MHC expression, recruitment of regulatory T cells, and/or the up-regulation of immune suppressing molecules such as CTLA-4, PD-1, and PD-L1 [reviewed in Ref. (195)]. Genetically engineered T cells, possessing a cloned tumor-specific TCR or chimeric antigen receptor (CAR) and thus the ability to recognize specific TAAs, might provide a new, promising immunotherapeutic strategy for cancer treatment. CARs are constructed from an antigen-binding domain [i.e., single chain antibody variable fragment (scFv)] that is derived from the variable region (Fab-fragment) of a mAb which is linked to a transmembrane motif as well as an intracellular signaling domain of one or more co-stimulatory molecules such as CD28, Ox40, or CD137 (196).

Currently about 70 clinical trials investigating CAR T cell ITs are registered in *ClinicalTrials.gov*, with most of these studies exploring B-cell malignancies targeting CD19. One of these studies, a phase I trial of CD19-CAR T cells used in refractory B-cell malignancies, reported a CR in 70% of patients with acute lymphoblastic leukemia (B-ALL) as well as an OS at a median followup of 10 months with 51.6% at 9.7 months (197). A second study evaluating the effects of CD19-directed CAR (CTL019) T-cell therapy in relapsed or refractory ALL reported a 90% rate of complete remission (198). Along with other clinical trials (199, 200), these findings suggest a high beneficial effect of adoptive cell transfer with anti-CD19 CAR T cells in patients suffering from B-cell malignancies with manageable toxicities. These results give rise for cautious optimism in the treatment of solid tumors, including advanced Her2-positive malignancies, GBM, neuroblastoma, sarcomas, melanoma, metastatic pancreatic cancer, and metastatic breast cancer. In order to enhance anti-tumor effects of CAR T cell therapy, it can also be combined with other therapy options or the so-called bi-specific CARs recognizing two antigens that are composed of two tandem-scFv fragments separated by a linker (201). The lymphodepletive and tumoricidal effects of standard-of-care CT and RT might potentiate the expansion and function of adoptively transferred CAR T cells, as suggested by Riccione et al. (202). However, more data of combination of RT with CAR T cells are first needed to allow for definite conclusions whether this treatment induces enhanced anti-tumor responses, locally and systemically.

Summary

A tumor is much more than just an accumulation of tumor cells. The cell death resistance of the malignant cells to anti-cancer therapies is one massive problem in the clinic. One of the challenges for researchers and clinicians is to identify treatments that will overcome or bypass the cell death resistance mechanisms established by the tumor cells, but also those of the microenvironment (68). Nowadays, the involvement of the immune system as a vital player in the recognition and eradication of malignant cells is generally accepted (203). While RT and CT are crucial for curative and palliative treatments, they do not only display cytotoxic or cytostatic effects and target the tumor directly, but are also involved in the activation of the immune system through the induction of immunogenic cell death or immunostimulatory mechanisms (29). In general, the modulation of the immune system via modifications of either tumor or immune cells with methods such as mAbs or small molecule inhibitors provides a great potential in the improvement of cancer therapies and numerous pre-clinical and clinical studies are ongoing. Even though these approaches often induce only modest and transient clinical responses in distinct malignancies, a combination with RT, and/or immunogenic CT and additional immune therapies such as vaccination might result in an improved clinical benefit. Thus, additional multi-center large-scaled randomized studies further evaluating the safety, efficacy, and clinical local and systemic outcome of monotherapy and combinatorial strategies are urgently needed. A more personalized treatment of patients through integration of predictive and prognostic biomarkers and considering individual radiosensitivity together with time and dose adaptions should be in the mind of clinicians and scientist alike. However, both have to keep in mind: it is crucial to first gain knowledge about the mechanisms and mode of action of the treatments to then be able to design multimodal therapies with respect to combinations and chronology. And if it does not work in the first try, go back to the lab and find out what can be optimized.

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Combinatorial strategies for the induction of immunogenic cell death

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The term "immunogenic cell death" (ICD) is commonly employed to indicate a peculiar instance of regulated cell death (RCD) that engages the adaptive arm of the immune system. The inoculation of cancer cells undergoing ICD into immunocompetent animals elicits a specific immune response associated with the establishment of immunological memory. Only a few agents are intrinsically endowed with the ability to trigger ICD. These include a few chemotherapeutics that are routinely employed in the clinic, like doxorubicin, mitoxantrone, oxaliplatin, and cyclophosphamide, as well as some agents that have not yet been approved for use in humans. Accumulating clinical data indicate that the activation of adaptive immune responses against dying cancer cells is associated with improved disease outcome in patients affected by various neoplasms. Thus, novel therapeutic regimens that trigger ICD are urgently awaited. Here, we discuss current combinatorial approaches to convert otherwise non-immunogenic instances of RCD into *bona fide* ICD.

Keywords: ATP, autophagy, calreticulin, endoplasmic reticulum stress, HMGB1, type I interferon

Introduction

The expression "immunogenic cell death" (ICD) generally refers to a functionally peculiar case of regulated cell death (RCD) that – in immunocompetent hosts – is capable of activating an adaptive immune response against dead cell-associated antigens (1–5). Of note, ICD generally (but not obligatorily) manifests with apoptotic morphological features, and at least some of its manifestations depend on components of the apoptotic apparatus (6–8). Irrespective of these morphological and biochemical considerations, immunocompetent mice injected s.c. with cancer cells succumbing to

Abbreviations: APC, antigen-presenting cell; CALR, calreticulin; CTLA4, cytotoxic T lymphocyte-associated protein 4; CXCL10, chemokine (C–X–C motif) ligand 10; DAMP, damage-associated molecular pattern; EIF2A, eukaryotic translation initiation factor 2A, 65 kDa; ER, endoplasmic reticulum; HMGB1, high-mobility group box 1; ICD, immunogenic cell death; IFN, interferon; IFNAR, interferon (alpha, beta, and omega) receptor; mAb, monoclonal antibody; P2RX7, purinergic receptor P2X, ligand gated ion channel, 7; P2RY2, purinergic receptor P2Y, G-protein coupled, 2; RCD, regulated cell death; siRNA, small-interfering RNA; TLR, toll-like receptor.

bona fide ICD (in the absence of any adjuvant) develop a cellular immune response associated with the establishment of immunological memory that protects them from a subsequent challenge with living cells of the same type (1–3). Importantly, vaccination experiments of this type, involving murine cells and syngeneic mice, remain the gold-standard method to identify *bona fide* ICD, though several tests have been developed to detect some of its cellular manifestations (see below) (2, 3, 9, 10).

Only a few lethal stimuli are intrinsically endowed with the ability to trigger ICD (9, 11–14). These include some chemotherapeutic agents that are employed in the clinic, including (1) various anthracyclines (i.e., doxorubicin, epirubicin, and idarubicin), which are commonly used against a wide panel of malignant conditions (15–17); (2) mitoxantrone, an anthracenedione generally used for the treatment of acute myeloid leukemia, breast carcinoma, non-Hodgkin's lymphoma, and prostate carcinoma (15, 16); (3) oxaliplatin, a platinum derivative approved for use in combination with 5-fluorouracil to treat advanced colorectal carcinoma (18, 19); (4) cyclophosphamide, an alkylating agent that is employed against various neoplastic and autoimmune conditions (20–23); and (5) bortezomib, a proteasomal inhibitor approved for the therapy of multiple myeloma and mantle cell lymphoma (24–26). Specific forms of irradiation as well as photodynamic therapy, both of which are habitually employed for the treatment of various neoplasms, have also been shown to trigger *bona fide* ICD (27–34). Finally, a bunch of hitherto experimental agents is intrinsically endowed with the capacity to initiate ICD, including (but not limited to) some oncolytic viruses (35–39), the microtubular inhibitor patupilone (40–42), and elevated hydrostatic pressures (43).

According to accepted models, ICD relies on the establishment of adaptive stress responses that promote the spatiotemporally coordinated emission of endogenous danger signals from dying cells (44, 45). The endogenous molecules that dispatch danger signals in response to stress are cumulatively known as "damage-associated molecular patterns" (DAMPs) and operate upon binding to receptors expressed by bystander cells, including cellular components of both the innate and adaptive immune system (2, 46–49). As it stands, four DAMPs have been shown to be required for RCD as induced by anthracyclines to be perceived as immunogenic, namely, (1) the exposure of the endoplasmic reticulum (ER) chaperone calreticulin (CALR) on the outer surface of the plasma membrane (16); (2) the secretion of ATP (50); (3) the production of type I interferon (IFN) (51); and (4) the release of the non-histone chromatin-binding protein high-mobility group box 1 (HMGB1) into the extracellular space (52). This said, it cannot be formally excluded that other hitherto undiscovered DAMPs are required for anthracycline-elicited RCD to promote an adaptive immune response. Along similar lines, not all these DAMPs may be required for RCD as induced by agents other than anthracyclines to be perceived as immunogenic (53–55).

In this context, i.e., anthracycline-induced ICD, CALR exposure obligatorily relies on the establishment of a pre-mortem ER stress response centered around the phosphorylation of eukaryotic translation initiation factor 2A, 65 kDa (EIF2A) (7, 56), ATP secretion requires the induction of autophagy (57), and type I IFN production stems from toll-like receptor 3 (TLR3) signaling (51). The molecular mechanisms underlying the ability of anthracyclines and other ICD inducers to promote HMGB1 release remain obscure (2, 3). Cumulatively, these DAMPs recruit antigen-presenting cells (APCs) to sites of active ICD and stimulate the uptake, processing, and presentation of dead cell-associated antigens, eventually resulting in the priming of an adaptive immune response (2, 3). In particular, CALR promotes antigen uptake by APCs by binding to low density lipoprotein receptor-related protein 1 (LRP1, best known as CD91) (58); ATP stimulates the recruitment of APCs and their activation upon binding to purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) and purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7), respectively (50, 59, 60); type I IFNs exert immunostimulatory effects via IFN (alpha, beta, and omega) receptors (IFNARs) (51); and HMGB1 does so through TLR4 and advanced glycosylation end product-specific receptor (AGER, best known as RAGE) (52, 61).

A detailed discussion of the molecular and cellular mechanisms involved in the detection of ICD-associated DAMPs goes beyond the scope of this review and can be found in Ref. (2, 3). However, it is important to note that the failure of cancer cells to emit one (or more) of these DAMPs completely compromises the immunogenicity of RCD (2, 3). Thus, at odds with their wild-type counterparts, $Calr^{-/-}$ murine CT26 colorectal cells exposed to anthracyclines are unable to vaccinate mice against a subsequent inoculation with malignant cells of the same type (16). The same holds true in several other situations in which adaptive responses cannot proceed normally, including the genetic inhibition of autophagy (e.g., upon the expression of short-hairpin RNAs targeting the essential autophagy proteins Atg5 or Atg7) or the unfolded protein response (e.g., upon the expression of a non-phosphorylatable variant of EIF2A) (7, 57, 62, 63).

Accumulating clinical evidence indicates that the (re-)activation of a proficient immune response against malignant cells is associated with improved disease outcome in patients affected by a wide panel of neoplasms (64–68), in particular when malignant lesions are highly infiltrated by immune effector cells prior to therapy (69). Considerable efforts are therefore being devoted to the development of clinically implementable strategies that (re-)instate anticancer immunosurveillance (70, 71). So far, the most successful of these approaches involves the administration of monoclonal antibodies (mAbs) that block immunosuppressive receptors expressed by activated T cells, such as cytotoxic T lymphocyte-associated protein 4 (CTLA4) and programed cell death 1 (PDCD1, best known as PD-1) (72, 73). Three distinct checkpoint blockers of this type, namely, the CTLA4-targeting mAb ipilimumab and the PD-1-targeting mAbs nivolumab and pembrolizumab, are approved by the US Food and Drug Administration and other regulatory agencies worldwide for use as standalone immunotherapeutic interventions in melanoma patients (74–77). In addition, the administration of checkpoint blockers has been shown to improve the clinical profile of various chemotherapeutic and immunotherapeutic agents (78). Along similar lines, various combinatorial immuno(chemo)therapeutic regimens are being investigated in clinical trials for their ability to mediate superior antineoplastic effects as compared to monotherapies based on their constituents (79, 80). In this framework, various attempts are

being made to render immunogenic otherwise non-immunogenic instances of therapy-induced RCD, thereby converting them into *bona fide* ICD (79, 81–84). This can be due to molecular defects that prevent cancer cells from emitting DAMPs appropriately, as mentioned above, as well as to the intrinsic features of the therapeutic agent under consideration (**Table 1**). For instance, at odds with its derivative oxaliplatin, cisplatin is intrinsically unable to trigger ICD since it does not stimulate the exposure of CALR on the outer surface of the plasma membrane (18, 19, 85).

Here, we discuss strategies to convert non-immunogenic instances of RCD into *bona fide* ICD. In particular, we will review approaches for (1) correcting the incapacity of some therapeutic agents to kill cancer cells while provoking the emission of one or more DAMP(s); or (2) complementing the missing DAMP(s) with exogenous interventions. On the contrary, we will not dwell on strategies that boost the immunogenicity of RCD by operating downstream of DAMP-sensing receptors.

Combinatorial Strategies to Restore CALR Exposure

Some anticancer therapeutics efficiently kill cancer cells (hence promoting the release of HMGB1) and stimulate the secretion of both ATP and type I IFNs, but selectively fail to promote CALR exposure. Most often, such a defect originates from the inability of these agents to trigger an ER stress response resulting in EIF2A phosphorylation (56, 114), and hence can be corrected by the co-administration of an ER stressors. As mentioned above, cisplatin is one of the antineoplastic agents that fail to trigger *bona fide* ICD as it does not drive a robust ER stress response (18, 19, 85). The ER-stressing agents that have been shown to correct this defect, hence rendering cisplatin-induced RCD immunogenic, include thapsigargin, an inhibitor of various members of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (19, 114); tunicamycin, an inhibitor of *N*-glycosylation (19, 94, 114); pyridoxine, a cell-permeant precursor of bioactive vitamin B6 (90, 91, 115); and $ZnCl_2$ (92). Similar results have been obtained by establishing an ER stress response through the enforced overexpression of reticulon 1 (RTN1), an ER protein involved in vesicular trafficking and secretion (116, 117). The latter approach is obviously incompatible with clinical applications. Nonetheless, these data reinforce the notion that the immunogenicity of cisplatin-induced RCD can be restored by various interventions that induce an ER stress (94).

Another strategy that successfully restores CALR exposure in cells succumbing to chemicals that *per se* do not enable this phenomenon consists in the co-administration of inhibitors of the EIF2A phosphatase composed of protein phosphatase 1, regulatory subunit 15A (PPP1R15A, best known as GADD34), and pyrophosphatase (inorganic) 1 (PPA1, best known as PP1), resulting in accrued EIF2A phosphorylation even in the absence of overt ER stress (16). Thus, whereas CT26 cells treated with etoposide (a topoisomerase II inhibitor currently approved for the treatment of various malignancies) (118, 119) do not expose CALR as they die, and hence fail to vaccinate mice against a subsequent challenge with neoplastic cells of the same type, they efficiently do so in the presence of tautomycin, calyculin A, and salubrinal (three distinct GADD34/PP1 inhibitors) (16). Similar results have been obtained with the small-interfering RNA (siRNA)-mediated downregulation of PP1 or GADD34 (16), as well as with short cell-permeant peptides that disrupt the physical interaction between these two proteins (102). Although siRNA- and peptide-based strategies may not be easily implemented in clinical settings, these results corroborate the specificity of tautomycin, calyculin A, and salubrinal, and lend further support to the notion that interventions that stimulate EIF2A phosphorylation efficiently promote CALR exposure even in the absence of overt ER stress (120).

At least theoretically, the co-administration of ER stressors or molecules that promote EIF2A phosphorylation can be harnessed to reconstitute the immunogenicity of RCD induced by all anticancer agents that *per se* do not stimulate CALR exposure on the cell surface but provoke ATP secretion, type I IFN production, and HMGB1 release. In addition, the inability of some anticancer agents to cause the translocation of CALR to the outer leaflet of the plasma membrane can be corrected, at least in some settings, by the co-administration of exogenous, recombinant CALR (7, 16, 106). CALR is indeed relatively "sticky" and its absorption on malignant cells succumbing to non-immunogenic RCD *in vitro* has been shown to fully restore the ability of these cells to vaccinate syngeneic mice against a subsequent neoplastic challenge (16). To the best of our knowledge, however, whether the systemic or intratumoral administration of recombinant CALR to tumor-bearing mice treated with non-immunogenic therapeutics is able to convert them into *bona fide* ICD inducers has not been tested yet. As compared to administration of small molecules that establish an ER stress response or promote EIF2A phosphorylation, the use of recombinant CALR appears advantageous in that (at least theoretically) it would complement the lack of CALR exposure in all scenarios, irrespective of the underlying molecular defects (including the downregulation or loss of CALR itself). However, such an approach may not be implementable in the clinic, owing to pharmacodynamic and pharmacokinetic issues (e.g., distribution of the recombinant protein, serum half-life, etc. . .) as well as economic considerations. Current efforts are therefore being focused on the identification of novel (and the refinement of existing) small molecule-based strategies to stimulate CALR exposure upon the establishment of an ER stress or the induction of EIF2A phosphorylation.

Combinatorial Strategies to Boost ATP Secretion

In some settings, anticancer agents kill malignant cells in an efficient fashion (which corresponds to a consistent release of HMGB1), while stimulating the exposure of CALR and the production of type I IFN, but this is not accompanied by the accumulation of extracellular ATP (57, 121), a defect that can stem from at least three different causes. First, some therapeutic agents are unable to stimulate (or even inhibit) autophagic responses, which are required for dying cells to secrete ATP in sufficient amount for signaling via P2RY2 and P2RX7 receptors (57). Second, some malignant cells bear genetic or epigenetic defects that affect the molecular machinery for autophagy (122, 123). These cells are intrinsically unable to preserve the intracellular ATP pool in the

TABLE 1 | Immunogenicity of chemotherapy-induced regulated cell death (examples).

Drug	CALR exposure	ATP secretion	Type I IFN production	HMGB1 release	[a]*Bona fide* ICD inducer	Restoration of ICD	Reference
5-Fluorouracil	Debated	No	n.d.	Yes	n.d.	RT	(16) (86) (87)
Bleomycin	Yes	Yes	Yes	Yes	Yes	n.a.	(88)
Bortezomib	Yes	n.d.	Yes	Yes	Yes	n.a.	(24) (25) (26) (89)
Camptothecin	Debated	No	n.d.	Yes	No	n.d.	(16) (87)
Carboplatin	Partial	Yes	n.d.	Partial	No	RT	(16) (86)
Cisplatin	No	Yes	n.d.	Yes	No	Pyridoxine Thapsigargin Tunicamycin $ZnCl_2$	(19) (90) (91) (92) (93) (94)
Cyclophosphamide	Yes	Yes	Yes	Yes	Yes	n.a.	(20) (21) (95)
Digitoxin Digoxin	Yes	Yes	n.d.	Partial	No	Cytotoxic agents	(81) (83)
Docetaxel	Yes	No	n.d.	No	No	n.d.	(96) (97)
Doxorubicin	Yes	Yes	Yes	Yes	Yes	n.a.	(15) (16) (17) (51) (98) (99)
Epirubicin	Yes	Yes	n.d.	Yes	Yes	n.a.	(16) (17)
Etoposide	No	Yes	n.d.	Yes	No	Calyculin A Salubrinal Tautomycin PP1/GADD34-targeting peptides 2-deoxyglucose	(16) (17) (93) (100) (101) (102)
Gemcitabine	No	Partial	n.d.	Yes	No	PX-478	(103)
Idarubicin	Yes	n.d.	n.d.	Yes	Yes	n.a.	(17) (16) (104)
Irinotecan	n.d.	n.d.	n.d.	Yes	n.d.	n.d.	(105)
Mafosfamide	Yes	n.d.	n.d.	Yes	Yes	n.d.	(20)

(Continued)

TABLE 1 | Continued

Drug	CALR exposure	ATP secretion	Type I IFN production	HMGB1 release	[a]*Bona fide* ICD inducer	Restoration of ICD	Reference
Melphalan	Debated	n.d.	n.d.	Yes	n.d.	n.d.	(106) (107) (108)
Mitomycin C	Debated	No	n.d.	Yes	No	n.d.	(16) (87)
Mitoxantrone	Yes	Yes	Yes	Yes	Yes	n.a.	(7) (16) (17) (51) (57) (93) (109)
Oxaliplatin	Yes	Yes	Yes	n.d.	Yes	n.a.	(7) (18) (52) (57) (93) (110)
Patupilone	Yes[b]	n.d.	Yes	Yes[b]	Yes[b]	n.a.	(41) (42)
Temozolomide	No	Yes	n.d.	Yes	n.d.	Oncolytic virotherapy Cyclophosphamide	(111) (112)
Vemurafenib	Yes	n.d.	n.d.	Yes	n.d.	n.d.	(103) (113)

CALR, calreticulin; HMGB1, high-mobility group box 1; ICD, immunogenic cell death; IFN, interferon; n.a., not applicable; n.d., not determined; RT, radiation therapy.
[a]*As determined in gold-standard vaccination experiments.*
[b]*Unpublished observations from our group.*

course of stress responses, resulting in limited ATP secretion during death (124). Third, some neoplastic cells express high levels of either ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1, best known as CD39) or 5′-nucleotidase, ecto (NT5E, best known as CD73), two membrane-bound nucleotidases that degrade extracellular ATP (125).

So far, one general strategy has been shown to restore extracellular ATP concentrations to levels that are compatible with the efficient recruitment and activation of APCs, namely, the pharmacological inhibition of CD39. Thus, CT26 cells lacking essential components of the autophagic machinery, such as Atg5, Atg7, or Beclin 1 (Becn1), secrete limited amounts of ATP as they succumb to anthracyclines, and hence are incapable of vaccinating syngeneic mice against a subsequent challenge with malignant cells of the same type (57). Such a functional defect can be corrected by the co-administration of ARL67156, a broad spectrum inhibitor of extracellular nucleotidases (57). Further confirming these findings, CT26 engineered to overexpress CD39 and exposed to anthracyclines are unable to protect syngeneic mice against a subsequent injection with neoplastic cells of the same type (57, 125). This defect can be corrected by the co-administration of ARL67156, along with the restoration of RCD-associated ATP secretion (57, 125). Taken together, these results indicate that inhibitors of extracellular nucleotidases may constitute a convenient manner to boost the immunogenicity of RCD instances that are normally not associated with ATP secretion.

Importantly, the pharmacological activation of autophagy does not suffice for cancer cells to become immunogenic (16, 57). Nonetheless, combining anticancer agents that *per se* are unable to trigger ATP secretion with molecules that upregulate the autophagic flux, such as inhibitors of mechanistic target of rapamycin (MTOR) complex I (MTORCI), may efficiently convert non-immunogenic RCD instances into *bona fide* ICD. This hypothesis awaits formal experimental confirmation. Indeed, while other inducers of autophagy such as the glycolytic inhibitor 2-deoxyglucose (126) have been shown to reinstate the immunogenicity of etoposide-elicited RCD, such an effect was ascribed to the restoration of CALR exposure (indeed, etoposide kills malignant cells while promoting ATP secretion) (100). Finally, it should be noted that the establishment of an ATP gradient around dying cells may not constitute a general requirement for the perception of RCD as immunogenic (127). Moreover, at least in some settings, autophagy may actually inhibit ICD by limiting the production of reactive oxygen species in the course of adaptive stress responses, hence counteracting the establishment of ER stress and consequent CALR exposure (54, 55). Thus, further work is required to precisely identify malignancies in which autophagy

supports ICD. Only in these scenarios, the co-administration of autophagy inducers may constitute a proper approach to reinstate the immunogenicity of RCD.

Combinatorial Strategies to Promote Type I IFN Production

Whereas the role of type I IFN in the regulation of innate and adaptive immune responses is well known (128, 129), type I IFN signaling in malignant cells has been identified as a requirement for (anthracycline-induced) ICD only recently (51). Thus, cancer cells respond to various anthracyclines by activating a TLR3-elicited signal transduction cascade resulting in type I IFN release, autocrine/paracrine type I IFN signaling, and chemokine (C–X–C motif) ligand 10 (CXCL10) secretion, two phenomena that underlie their vaccinating potential. At odds with their wild-type counterparts, *Tlr3*$^{-/-}$ and *Ifnar1*$^{-/-}$ murine cancer cells exposed to anthracyclines fail to vaccinate syngeneic mice against a subsequent injection of living cells of the same type (51). It has already been demonstrated that the inability of *Tlr3*$^{-/-}$ cells to undergo ICD can be corrected by the co-administration of recombinant type I IFNs or recombinant CXCL10. Similarly, *Ifnar1*$^{-/-}$ cells succumbing to anthracyclines turn immunogenic in the presence of recombinant CXCL10 (but not type I IFNs) (51).

Various synthetic TLR3 agonists are available and some of them, including polyinosinic:polycytidylic acid (polyI:C) and its clinical grade analog polyI:polyC12U (also known as rintatolimod and Ampligen™), have been extensively tested as immunostimulants in cancer patients (130, 131). It is therefore tempting to speculate that the co-administration of TLR3 agonists may restore the ability of anticancer agents that *per se* do not promote type I IFN release to trigger *bona fide* ICD. This hypothesis awaits urgent experimental confirmation. For the considerations presented above, small molecules that trigger TLR3 signaling would indeed be more convenient as clinical tools to restore type I IFN signaling than recombinant type I IFN or CXCL10 themselves.

Combinatorial Strategies to Substitute for HMGB1 Release

HMGB1 release occurs upon (nuclear and) plasma membrane permeabilization, i.e., it constitutes a post-mortem event (5, 132). Thus, all antineoplastic agents that efficiently kill malignant cells (as opposed to molecules that exert cytostatic effects or induce cell senescence) (133) promote HMGB1 release, perhaps with different kinetics (5, 132). However, the expression levels of HMGB1 vary in different tumor types and evolve along with tumor progression, implying that some malignant cells may express HMGB1 to levels that are not compatible with the activation of TLR4 and RAGE in immune cells upon release (134, 135). Importantly, the immunogenicity of anthracycline-induced RCD is compromised in these cells, as well in cells artificially depleted of HMGB1 by means of specific siRNAs (135). Recent results indicate that this defect can be efficiently corrected by the exogenous supply of a synthetic TLR4 agonist, i.e., dendrophilin, at least in experimental models (135). Since dendrophilin has not yet entered clinical development (130, 131), it will be interesting to see whether TLR4 agonists that are already licensed by regulatory agencies for use in humans, such as the Bacillus Calmette–Guérin (BCG) (80) and monophosphoryl lipid A (MPL) (136), are also able to restore the immunogenicity of HMGB1-deficient cells succumbing to ICD.

In this context, it is worth noting that cancer cells exposing CALR, secreting ATP, producing type I IFNs but releasing limited amounts of HMGB1 as they respond to a lethal stimulus in a suboptimal manner fail to elicit adaptive immune responses (137). Upon inoculation into immunocompetent mice, these cells actually form tumors at the vaccination site (as a significant fraction of them is not dying) and the animals are unable to control a subsequent challenge with cell of the same type (3). We have observed this to occur in murine cancer cells treated with digoxin or digitoxin, two glycosides approved in many countries for the treatment of cardiac conditions (81). These molecules efficiently inhibit the human Na^+/K^+ ATPase, which explains their pharmacological properties and their ability to kill some neoplastic cells of human origin, but not its murine counterpart (83). Thus, cardiac glycosides *per se* are unable to trigger ICD, at least in the murine system. However, clinical data indicate that they may convert non-immunogenic RCD as elicited by a very large panel of chemotherapeutics into *bona fide* ICD (83). From another standpoint, any anticancer agent that efficiently kills malignant cells could be considered as a means to restore the immunogenicity of cells responding to cardiac glycosides. We have recently initiated a clinical trial to prospectively test this hypothesis in head and neck squamous carcinoma patients.

Concluding Remarks

In spite of old beliefs, cancer cells continuously interact with the immune system: first, as they are generated by healthy cells upon malignant transformation; second, as they evolve and acquire additional neoplastic features; and third, when they are challenged with therapeutic interventions. During the last decade, such a conceptual revolution, i.e., considering tumors as entities that can be detected and destroyed by the immune system, has paved the way toward the development of novel therapeutic agents conceived to re(instate) anticancer immunity, and some of these interventions have already been licensed for use in humans by international regulatory agencies. In addition, it has become clear that many therapeutics that had been used for decades in the clinic are efficient (for the most part) because they engage the host immune system against malignant cells. ICD is one of the several mechanisms through which cytotoxic chemotherapeutics, targeted anticancer agents as well as some forms of radiotherapy can elicit tumor-targeting immune responses. Identifying novel ICD inducers as well as measures that convert non-immunogenic RCD into *bona fide* ICD is of primordial importance. Promising preclinical results and preliminary clinical findings suggest, indeed, that agents that promote CALR exposure, ATP secretion, type I IFN production, HMGB1 release or stimulate the downstream signal transduction pathway may considerably improve the clinical profile of conventional therapeutic regimens (**Figure 1**). A systematic investigation of the ability of currently available anticancer agents to elicit the abovementioned ICD-associated processes in human cancer

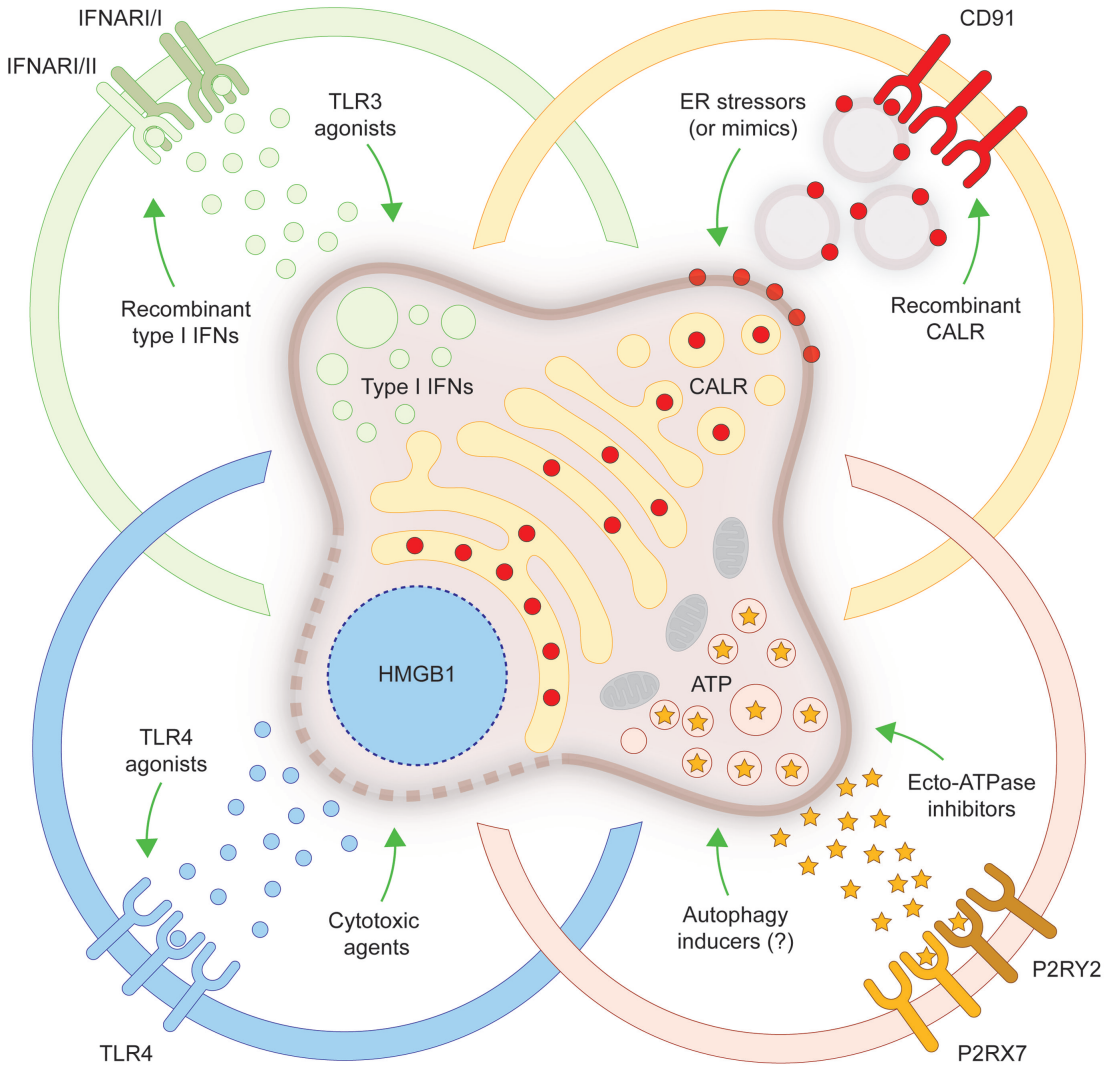

FIGURE 1 | Strategies to convert non-immunogenic RCD into *bona fide* ICD. Upon inoculation into immunocompetent syngeneic hosts, cancer cells responding to a panel of lethal stimuli trigger an adaptive immune response against dead cell-associated antigens. Such an immunogenic variant of regulated cell death (RCD), commonly known as immunogenic cell death (ICD), relies on the exposure of calreticulin (CALR) on the cell surface, on the secretion of ATP, on the production of type I interferons (IFNs) and on the release of high-mobility group box 1 (HMGB1, which accompanies cell death). When any of these damage-associated molecular patterns cannot be emitted (in the appropriate spatiotemporal order), dying cancer cells cannot be perceived anymore as immunogenic by the host immune system. Several strategies have been conceived to correct these defects, hence converting non-immunogenic RCD into *bona fide* ICD. ER, endoplasmic reticulum; IFNAR, interferon (alpha, beta, and omega) receptor; P2RX7, purinergic receptor P2X, ligand gated ion channel, 7; P2RY2, purinergic receptor P2Y, G-protein coupled, 2; TLR, toll-like receptor.

cells of distinct histological origin is urgently awaited. These data may pave the way to the clinical implementation of combinatorial immuno(chemo)regimens that efficiently promote ICD and hence mediate complete tumor regression in a high proportion of patients.

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Corrigendum: "Combinatorial strategies for the induction of immunogenic cell death"

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We realized that the family name of one of the authors was misspelled in the original version of the article. Indeed, the name of the co-first author is not Ligia C. Gomes-de-Silva, but Ligia C. Gomes-da-Silva. We apologize to this author and to the readers of *Frontiers in Immunology* for the inconvenience this may have caused.

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REVIEW
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Prognostic and predictive value of DAMPs and DAMP-associated processes in cancer

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It is now clear that human neoplasms form, progress, and respond to therapy in the context of an intimate crosstalk with the host immune system. In particular, accumulating evidence demonstrates that the efficacy of most, if not all, chemo- and radiotherapeutic agents commonly employed in the clinic critically depends on the (re)activation of tumor-targeting immune responses. One of the mechanisms whereby conventional chemotherapeutics, targeted anticancer agents, and radiotherapy can provoke a therapeutically relevant, adaptive immune response against malignant cells is commonly known as "immunogenic cell death." Importantly, dying cancer cells are perceived as immunogenic only when they emit a set of immunostimulatory signals upon the activation of intracellular stress response pathways. The emission of these signals, which are generally referred to as "damage-associated molecular patterns" (DAMPs), may therefore predict whether patients will respond to chemotherapy or not, at least in some settings. Here, we review clinical data indicating that DAMPs and DAMP-associated stress responses might have prognostic or predictive value for cancer patients.

Keywords: ATP, autophagy, calreticulin, ER stress response, HSPs, type I interferon

Abbreviations: AGER, advanced glycosylation end product-specific receptor; AML, acute myeloid leukemia; APC, antigen-presenting cell; ATF6, activating transcription factor 6; BECN1, beclin 1; C1q, complement component 1, q subcomponent; CALR, calreticulin; CLEC9A, C-type lectin domain family 9, member A; CLL, chronic lymphocytic leukemia; CRC, colorectal carcinoma; CXCL10, chemokine (C-X-C motif) ligand 10; CXCR4, chemokine (C-X-C motif) receptor 4; DAMP, damage-associated molecular pattern; EIF2A, eukaryotic translation initiation factor 2A; EIF2AK2, eukaryotic translation initiation factor 2-alpha kinase 2; ENTPD1, ectonucleoside triphosphate diphosphohydrolase 1; FPR1, formyl peptide receptor 1; HCC, hepatocellular carcinoma; HMGB1, high mobility group box 1; HSP, heat-shock protein; HSP90AA1, heat shock protein 90 kDa alpha (cytosolic), class A member 1; HSPA1A, heat shock 70 kDa protein 1A; HSPA5, heat shock 70 kDa protein 5; HSP90B1, heat shock protein 90 kDa beta (Grp94), member 1; ICD, immunogenic cell death; IFN, interferon; IFNA8, interferon, alpha 8; IFNAR1, interferon (alpha, beta and omega) receptor 1; IL-6, interleukin-6; KLRD1, killer cell lectin-like receptor subfamily D; LMAN1, lectin, mannose-binding, 1; MAP1LC3, microtubule-associated protein 1 light chain 3; MX1, MX dynamin-like GTPase 1; MYD88, myeloid differentiation primary response gene 88; NSCLC, non-small cell lung carcinoma; NK, natural killer; NT5E, ecto 5′-nucleotidase; P2RY2, purinergic receptor P2Y, G-protein coupled, 2; PLSCR1, phospholipid scramblase 1; PS, phosphatidylserine; TAA, tumor-associated antigen; THBS1, thrombospondin 1; TICAM1, Toll-like receptor adaptor molecule 1; TLR, Toll-like receptor; TM173, transmembrane protein 173; TNFα, tumor necrosis factor α; UPR, unfolded protein response; XBP1, X-box binding protein 1.

Introduction

For a long time, tumors were considered as highly homogenous entities resulting from the clonal expansion of a single cell with specific genetic or epigenetic defects (1). Now, it is clear that both hematopoietic and solid neoplasms are highly heterogenous, not only because malignant cells with distinct phenotypic and behavioral features generally co-exist, but also because multiple non-transformed cells are co-opted by growing cancers to support their needs. This is especially true for solid tumors, which contain an abundant non-malignant cellular compartment encompassing stromal, endothelial, and immune components (2, 3). The immune compartment of the tumor mass is *per se* very heterogenous, varying not only with tumor type, stage, and therapeutic regimen, but also on an inter-individual basis (4). Evidence accumulating over the last decade indicates indeed that human tumors form, progress, and respond to therapy in the context of an intimate, bidirectional interaction with the immune system (5, 6). Thus, clinically manifest neoplasms can develop only when they are able to escape immunosurveillance (7, 8), and they do so by evolving under the selective pressure imposed by the immune system (6, 9). Moreover, the composition, density, and intratumoral localization of the immune infiltrate have been ascribed with a robust prognostic or predictive value in several cohorts of cancer patients (10–12). Finally, the efficacy of most, if not all, therapeutic regimens commonly employed in cancer patients has been etiologically linked to the (re)elicitation of an adaptive immune response targeting malignant cells (13, 14).

Conventional chemotherapeutics and targeted anticancer agents can favor the (re)elicitation of anticancer immune responses through several mechanisms (13–15). A precise description of all these immunostimulatory pathways goes largely beyond the scope of this review, and can be found in Ref. (13, 14). However, it is useful to note that anticancer therapy can boost immunosurveillance by either of two mechanisms. First, it can directly modulate the functions of immune cells, including dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), $CD8^+$ cytotoxic T lymphocytes (CTLs), and $CD4^+CD25^+FOXP3^+$ regulatory T (T_{REG}) cells (14). Second, it can promote the immunogenicity or adjuvanticity of cancer cells as it subjects them to a state of stress (which sometimes leads to their death) (14, 16). In particular, some chemotherapeutic agents like anthracyclines, oxaliplatin, and bortezomib, as well as specific forms of radiation therapy and photodynamic therapy, are able to trigger a functionally peculiar variant of caspase-dependent cell death that *per se* is perceived as immunogenic by the immune system (17–21). This means that, upon inoculation in immunocompetent hosts, cells succumbing to such an immunogenic form of cell death are sufficient to elicit an adaptive immune response against dead cell-associated antigens associated with the establishment of immunological memory (22, 23).

Mechanistically, immunogenic cell death (ICD) relies on the pre-mortem activation of several stress response pathways that are associated with the emission of a well-defined set of danger signals by dying cancer cells (24–26). When delivered in the correct spatiotemporal order, such damage-associated molecular patterns (DAMPs) recruit specific cellular components of the innate and adaptive immune system to the tumor bed and activate them, ultimately resulting in the elicitation of a tumor-targeting immune response (22, 26). Conversely, in physiological conditions DAMPs are generally inaccessible to the immune system, and serve metabolic, structural, or enzymatic functions (26–28). Of note, DAMPs are not only involved in ICD-associated anticancer immunosurveillance, but also play a key role in the etiology of shock conditions triggered by trauma and other non-microbial stimuli (29, 30).

So far, four DAMPs have been ascribed a non-redundant, essential function in the context of anthracycline-induced ICD, namely (1) the pre-apoptotic exposure of the endoplasmic reticulum chaperone calreticulin (CALR) and various heat-shock proteins (HSPs) on the outer leaflet of the plasma membrane, which ensues the activation of an ER stress response orchestrated around the phosphorylation of eukaryotic translation initiation factor 2A, 65 kDa (EIF2A) and the overgeneration of reactive oxygen species (ROS) (31–36); (2) the production of type I interferons (IFNs), which depends on Toll-like receptor 3 (TLR3) signaling (37–40); (3) the secretion of ATP, which relies on the activation of autophagy (41, 42); and (4) the release of the non-histone chromatin-binding protein high mobility group box 1 (HMGB1) into the extracellular space, which correlates with cell death induction (43, 44). The role of other DAMPs such as mitochondrial DNA (mtDNA), *N*-formylated peptides, cardiolipin, and filamentous (F)-actin in ICD signaling has not yet been investigated in detail (30, 45).

Accumulating preclinical evidence indicates that monitoring DAMPs or DAMP-associated stress responses in cancer patients may have prognostic or predictive value. Here, we review clinical data lending further support to this hypothesis.

Calreticulin, HSPs, and the ER Stress Response

Cancer cells undergoing ICD exhibit several manifestations of the so-called unfolded protein response (UPR) (34, 46), i.e., the ensemble of mechanisms aimed at the re-establishment of intracellular homeostasis following the accumulation of unfolded proteins within the ER lumen (47). In particular, ICD is etiologically associated with the phosphorylation of EIF2A on S51 (48), and this appears to be required for the exposure of CALR and HSPs on the surface of dying cells (34). On the cell surface, CALR, heat shock 70 kDa protein 1A (HSPA1A, best known as HSP70) and heat shock protein 90 kDa alpha (cytosolic), class A member 1 (HSP90AA1, best known as HSP90) play partially overlapping (but not identical) immunostimulatory functions. Indeed, CALR, HSP70 and HSP90 all bind to low density lipoprotein receptor-related protein 1 (LRP1, best known as CD91) on antigen-presenting cells (APCs), hence stimulating the uptake of dead cell-associated antigens in the form of apoptotic bodies (32, 33). HSP70 and HSP90 favor CTL cross-priming by APCs upon interaction with Toll-like receptor 4 (TLR4) and CD14 (33, 49–51). In some settings, soluble HSPs and CALR operate as cytokines, stimulating the NF-κB-dependent secretion of pro-inflammatory mediators like interleukin-6 (IL-6) and

tumor necrosis factor α (TNFα) (52, 53). HSP70 boosts the cytotoxic functions of natural killer (NK) cells by binding to killer cell lectin-like receptor subfamily D, member 1 (KLRD1, best known as CD94) (54, 55). Moreover, ecto-HSP70 binds to phosphatidylserine (PS), a phospholipid that is exposed in the course of regulated cell death owing to the caspase-dependent activation of phospholipid scramblase 1 (PLSCR1) (56). The actual relevance of this interaction for ICD, however, has not been determined yet. Along similar lines, it remains obscure whether additional CALR receptors such as CD69; thrombospondin 1 (THBS1); complement component 1, q subcomponent (C1q); lectin, mannose-binding, 1 (LMAN1); and various integrins of the CD49 family are etiologically implicated in the perception of ICD (57). Of note, ecto-CALR has been suggested to act as a DC receptor for the tumor-associated antigen (TAA) NY-ESO-1, hence facilitating the interaction between DCs and malignant cells (58). To the best of our knowledge, however, this finding has not been confirmed by independent investigators.

Accumulating clinical evidence indicates that various parameters linked to ICD-associated CALR and HSP signaling may have prognostic or predictive value for cancer patients (**Table 1**). In addition, the results of multiple clinical trials suggest that HSPs can be harnessed as a means to boost the efficacy of anticancer vaccines. High CALR levels in malignant cells have been shown to correlate with favorable disease outcome in a cohort of 68 neuroblastoma patients (irrespective of treatment) (59), and in a

TABLE 1 | Clinical studies assessing the prognostic and predictive value of ICD-associated CALR and HSP signaling in cancer patients.

Parameter	Cancer	Treatment	No	Note(s)	Reference
CALR	AML	Anthracyclines-based chemotherapy	20	CALR exposure on blasts correlated with improved RFS	(63)
	Bladder carcinoma	Surgery	195	High CALR levels correlated with poor disease outcome	(67)
	Breast carcinoma	Surgery	23	High CALR levels correlated with poor MFS	(68)
	CRC	Surgical resection and chemotherapy	68	High CALR levels correlated with improved 5-y survival rate	(61)
	Gastric carcinoma	Gastrectomy and lymphadenectomy	79	High CALR levels correlated with poor disease outcome	(69)
	Lung carcinoma	n.a.	58	High CALR levels correlated with malignancy and tumor grade	(64)
		Radiotherapy	23	High CALR levels correlated with prolonged OS	(60)
	Mantle cell lymphoma	Surgery	163	High CALR levels correlated with poor disease outcome	(67)
	Neuroblastoma	Surgery alone or combined with chemotherapy	729	High CALR levels correlated with poor disease outcome	(67)
			68	High CALR levels correlated with favorable disease outcome	(59)
	Non–Hodgkin's lymphoma	Autologous cancer cell-based vaccine	18	CALR exposure was associated to clinical responses	(62)
	Ovarian carcinoma	Paclitaxel-based chemotherapy	220	High CALR levels correlated with prolonged DFS and OS	(60)
CD47	AML	n.a.	137	High CD47 levels correlated with shortened OS	(70)
	Esophageal carcinoma	Surgery	102	High CD47 levels correlated with shortened OS	(71)
	Ovarian carcinoma	Surgery	86	Low CD47 levels correlated with improved disease outcome	(72)
CD91	Melanoma	n.a.	16	High CD91 levels were associated with slow progression	(73)
ER stress	AML	Anthracycline-based chemotherapy	105	*XBP1* splicing correlated with prolonged DFS and OS	(74)
	Breast carcinoma	Anthracycline-based chemotherapy	60	Cancer cells from non-responders had high phosphorylation of EIF2A	(75)
		Surgical resection and/or hormonotherapy	100	*XBP1* splicing correlated with poor disease outcome	(76)
	DLBCL	Bortezomib	119	High HSPA5 levels correlated with worsened OS	(77)
	HNC	Surgery	79	High HSPA5 levels correlated with improved OS	(78)
	Lung cancer	Surgery	132	High HSPA5 levels correlated with improved disease outcome	(79)
	NSCLC	Surgery	193	PKR activation and EIF2A phosphorylation correlated with improved OS	(80)
HSP90	CRC	n.a.	182	Increased serum levels were associated with oncogenesis	(65)
	Non–Hodgkin's lymphoma	Autologous cancer cell-based vaccine	18	CALR exposure was associated to clinical responses	(62)
HSPA1A	Gastric carcinoma	n.a.	39 patients 186 controls	SNPs in *HSPA1A* affected disease incidence	(81)
LMAN1	Ovarian carcinoma	n.a.	289 patients 126 controls	SNPs in *LMAN1* affected disease incidence	(82)
THBS1	Gastric carcinoma	n.a.	275 patients 275 controls	SNPs in *THBS1* affected disease incidence	(83)

AML, acute myeloid leukemia; CRC, colorectal carcinoma; DFS, disease-free survival; DLBCL, diffuse large B-cell lymphoma; ER, endoplasmic reticulum; HNC, head and neck cancer; ICD, immunogenic cell death; MFS, metastasis-free survival; NSCLC, non-small cell lung carcinoma; n.a., not applicable or not available; OS; overall survival; RFS, relapse-free survival; SNP, single nucleotide polymorphism.

cohort of 23 lung cancer patients and 220 ovarian cancer patients treated with ICD inducers (*i.e.*, radiotherapy and paclitaxel, respectively) (60). Moreover, increased CALR expression by cancer cells has been associated with tumor infiltration by $CD45RO^+$ memory T cells and improved 5-year overall survival amongst 68 subjects with Stage IIIB colorectal carcinoma (CRC) (61). Elevated levels of HSP90 and CALR on the surface of neoplastic cells have been associated with clinical responses amongst 18 patients with relapsed indolent non-Hodgkin's lymphoma treated with an autologous cancer cell-based vaccine (62). Moreover, CALR exposure by malignant blasts has been linked to prolonged relapse-free (but not overall) survival in a cohort of 20 individuals with acute myeloid leukemia (AML) (63). Of note, the blasts of some of these patients exposed CALR spontaneously, and this correlated not only with the degree of EIF2A phosphorylation in malignant cells, but also with the ability of autologous T cells to secrete IFNγ on stimulation (63). Along similar lines, healthy individuals have been shown to differ from lung carcinoma patients with respect to the circulating levels of soluble CALR, as well as to the amount of CALR expressed on the surface of pulmonary (normal *versus* malignant) cells (64). Moreover, increased concentrations of soluble HSP90 have been detected in the serum of CRC patients ($n = 172$) as compared to healthy individuals ($n = 10$) (65). Interestingly, soluble HSP90 appears to activate cancer cell-intrinsic signaling pathways that promote disease progression (65, 66). These data indicate that cancer cells expose and/or shed CALR as well as HSPs even in the absence of chemotherapy (at least to some degree), possibly as a result of oncogenic stress and/or adverse microenvironmental conditions. Moreover, they suggest that membrane-bound CALR and HSPs have a different biological activity than their soluble counterparts.

Apparently at odds with the abovementioned clinical findings, total CALR levels have been positively associated with accelerated disease progression and poor outcome in a cohort of 79 gastric cancer patients (69), in 23 women with breast carcinoma upon surgery (68), as well in large cohorts of neuroblastoma ($n = 729$), bladder carcinoma ($n = 195$) and mantle cell lymphoma ($n = 163$) patients, irrespective of treatment type (67). Moreover, CALR expression by malignant cells failed to affect overall survival in 88 patients with esophageal squamous cell carcinoma treated with neo-adjuvant chemoradiotherapy and surgical resection (84). These results may reflect the intracellular functions of CALR in the preservation of reticular homeostasis, which is particularly important for malignant cells owing to their highly accelerated anabolic metabolism (85), or the fact that CALR exposure is generally associated with an increased expression of CD47, a very potent anti-phagocytic signal (67).

The phosphorylation of EIF2A as well as the activation of eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2, best known as PKR) have been associated with favorable disease outcome in a cohort of 193 non-small cell lung carcinoma (NSCLC) patients (80). On the contrary, elevated degrees of EIF2A phosphorylation in neoplastic cells have been correlated with nuclear size (a surrogate marker of DNA content), preferential tumor infiltration by T_{REG} cells, and poor disease outcome in a cohort of 60 breast carcinoma patients treated with anthracycline-based chemotherapy and tested longitudinally (75). Other manifestations on an ongoing UPR have been ascribed with prognostic or predictive value, including (but not limited to): (1) the expression levels of the ER chaperone heat shock 70 kDa protein 5 (HSPA5, best known as GRP78), as demonstrated in cohorts of 132 lung carcinoma patients (79), 79 individuals with head and neck cancer (78) and 119 patients with diffuse large B-cell lymphoma treated with the proteasome inhibitor bortezomib (which is a *bona fide* ICD inducer) (77); and (2) the splicing of X-box binding protein 1 (*XBP1*) (48), as demonstrated in a cohort of 105 AML patients tested at diagnosis (74). Of note, both CALR and GRP78 expression levels are also indirect manifestations of the activation of another branch of the ER stress response, *i.e.,* the derepression of activating transcription factor 6 (ATF6) (74, 86). Finally, some studies have associated markers of an ongoing UPR with dismal disease outcome. For instance, Davies and colleagues have linked low levels of unspliced *XBP1* as well as a high spliced/unspliced *XBP1* ratio with poor disease outcome in 100 primary breast carcinoma patients treated with adjuvant hormonal therapy (76). The apparent discrepancy in these observations may reflect the differential reliance of distinct tumor types (or similar tumors at distinct stages of progression) on the ER stress response for survival in adverse microenvironment conditions (87).

Other processes and parameters linked to CALR and/or HSP exposure and their immunostimulatory effects have been shown to influence disease outcome in cancer patients. For instance, high CD47 levels have been reported to constitute an independent negative prognostic factor in cohorts of 86 patients with ovarian clear cell carcinoma (72), 102 individuals with esophageal squamous cell carcinoma (71), and 137 subjects with karyotypically normal AML (70). Along similar lines, the monocytes of 8 advanced melanoma patients progressing in an unusually slow fashion have been found to express increased amounts of CD91 as compared to those of 8 patients progressing normally (73). Moreover, single nucleotide polymorphisms (SNPs) affecting *HSPA1A* have been linked to an increased incidence of gastric carcinoma (as determined in a cohort of 39 patients and 186 controls) (81), a SNP affecting *THBS1* has been correlated with gastric cancer occurrence and progression in a cohort of 275 patients and 275 healthy individuals (83), while a SNP in *LMAN1* as well as the consequent decrease in LMAN1 levels appear to be associated with an increased risk for ovarian carcinoma (as determined in a cohort of 289 women seen in gynecologic oncology practice and 126 healthy volunteers) (82).

The robust immunostimulatory activity of HSPs has been harnessed to develop various anticancer vaccines that are nowadays in clinical development. These preparations generally consist in HSP-enriched (autologous or heterologous) cancer cell lysates that are administered directly to patients, in the presence of adequate immunological adjuvants (88, 89). The most common of these approaches relies on heat shock protein 90 kDa beta (Grp94), member 1 (HSP90B1, best known as GP96) and is often referred to as HSPPC-96 (Oncophage® or Vitespen®) (90). So far, the safety and clinical profile of HSPPC-96 have been tested in cohorts of patients with metastatic melanoma ($n = 36$–322) (91–94), CRC ($n = 29$) (95), non-Hodgkin's lymphoma ($n = 20$) (96); pancreatic adenocarcinoma ($n = 10$) (97), metastatic renal cell carcinoma ($n = 84$–409) (98, 99), glioma ($n = 12$) (100), recurrent

glioblastoma ($n = 41$) (101), and assorted advanced malignancies ($n = 16$) (102). These studies demonstrate that the administration of HSPPC-96 to cancer patients is safe and is generally associated with markers of immunostimulation. However, most often such effects are weak and unable to mediate long-term therapeutic activity (99). Thus, further studies are required for translating the well-established ability of HSPs to stimulate the priming of TAA-specific immune responses into a therapeutic reality.

Taken together, these clinical observations suggest that CALR, HSPs and various processes associated with their exposure, secretion and signaling functions may have prognostic, predictive and therapeutic value.

Type I IFN and TLR3 Signaling

Cancer cells responding to anthracyclines secrete type I IFNs as a consequence of TLR3 activation (39), and this is required for cell death to initiate adaptive immunity (39). By binding to homodimeric or heterodimeric receptors expressed on several immune effector cells, type I IFNs mediate multipronged immunostimulatory effects (40). In particular, type I IFNs promote cross-priming (103), boost the cytotoxic functions of CTLs and NK cells (104), and increase the survival of memory CTLs (105). Moreover, type I IFNs can protect antigen-activated $CD8^+$ CTLs from elimination by NK cells (106, 107), trigger the secretion of pro-inflammatory mediators by macrophages (108), and counteract the immunosuppressive functions of T_{REG} cells (109). Besides such immunostimulatory effects, type I IFNs can ignite a cancer cell-intrinsic signal transduction pathway leading, amongst various effects, to the synthesis of the chemotactic factor chemokine (C–X–C motif) ligand 10 (CXCL10) (39). Indeed, at odds with their wild-type counterparts, *Ifnar1*$^{-/-}$ cancer cells succumbing to anthracyclines are unable to prime adaptive immune responses, even upon inoculation in wild-type hosts (39). Thus, type I IFN signaling in cancer cells appears to be critical for anthracycline-induced cell death to be perceived as immunogenic (39). Conversely, the efficacy of other immunotherapeutic agents such as the TLR7 agonist imiquimod requires type I IFN signaling in the host (110).

So far, only a few studies addressed the prognostic or predictive value of parameters reflecting the proficiency or activation status of TLR3 or type I IFN signaling (**Table 2**). High expression levels of TLR3 and/or toll-like receptor adaptor molecule 1 (TICAM1, a component of the TLR3 signaling apparatus best known as TRIF) have been associated with improved disease outcome in two cohorts of 85 and 172 subjects with hepatocellular carcinoma (HCC) (111, 112), as well as amongst 99 patients with neuroblastoma (113). Along similar lines, TLR3 expression levels have been shown to predict the response of 194 breast carcinoma patients treated with adjuvant radiotherapy plus a TLR3 agonist (114). SNPs affecting *TLR3* have been shown to influence prognosis in cohorts of 582 patients with CRC, especially among untreated individuals

TABLE 2 | Clinical studies assessing the prognostic and predictive value of TLR3 status and type I IFN signaling in cancer patients.

Parameter	Cancer	Treatment	No	Note(s)	Reference
IFNAR1	CRC	n.a.	1327 patients 758 controls	A SNP in *IFNAR1* was linked to increased risk for oncogenesis	(122)
	Glioma	n.a.	304	A SNP in *IFNAR1* was shown to affect patient OS	(123)
TLR3	Breast carcinoma	n.a.	102 patients 72 controls	A SNP in *TLR3* was linked to increased risk for oncogenesis	(118)
		polyA:U plus radiotherapy	194	High TLR3 levels predicted clinical responses to therapy	(114)
	Cervical carcinoma	n.a.	130 patients 200 controls	A SNP in *TLR3* was linked to increased risk for oncogenesis	(117)
	CRC	n.a.	582	SNPs in *TLR3* were shown to influence disease outcome	(115)
			2309 patients 2915 controls	SNPs in *TLR3* were linked to increased disease incidence	(121)
	HCC	n.a.	466 patients 482 controls	A SNP in *TLR3* was linked to increased risk for oncogenesis	(120)
			172	High TLR3 levels correlated with prolonged OS	(111)
		Surgery	85	High TLR3 levels correlated with prolonged OS	(112)
	Neuroblastoma	n.a.	99	High TLR3 levels correlated with favorable disease outcome	(113)
	NSCLC	Surgery	568	SNPs in *TLR3* were shown to influence disease outcome	(116)
	Oral squamous cell carcinoma	n.a.	93 patients 104 controls	SNPs in *TLR3* were linked to increased risk for oncogenesis	(119)
			240 patients 223 controls	A SNP in *TLR4* was linked to increased risk for oncogenesis	(124)
TRIF	HCC	Surgery	85	High TRIF levels correlated with prolonged OS	(112)
Type I IFN	Breast carcinoma	Anthracycline-based chemotherapy	50	A type I IFN-related signature predicted improved disease outcome	(39)
	CRC	n.a.	483	A SNP in *IFNA7* was shown to affect patient OS	(122)
	Glioma	n.a.	304	A SNP in *IFNA8* was shown to affect patient OS	(123)

CRC, colorectal carcinoma; HCC, hepatocellular carcinoma; NSCLC, non-small cell lung carcinoma; n.a., not applicable or not available; OS; overall survival; SNP, single nucleotide polymorphism.

with Stage II disease (115) and 568 NSCLC patients (116). Along similar lines, *TLR3* SNPs have been associated with an altered risk for cervical cancer amongst 330 Tunisian women (117), breast carcinoma amongst 174 African-American women (118), oral squamous cell carcinoma amongst 197 individuals (119) HCC amongst 948 subjects (120), and CRC amongst more than 5,000 individuals (121). A type I IFN-related transcription signature centered around the expression of MX dynamin-like GTPase 1 (*MX1*) has been shown to predict the likelihood of 50 breast carcinoma patients to respond to neo-adjuvant anthracycline-based chemotherapy (39). Moreover, SNPs affecting interferon (alpha, beta and omega) receptor 1 (*IFNAR1*) have been associated with an increased risk for the development of CRC amongst 2085 individuals (122), as well as with significantly reduced overall survival in a cohort of 304 glioma patients (123). Similar results have been obtained for SNPs affecting the genes coding for two variants of IFNα (*i.e.,* IFNA7 and IFNA8) (122, 123).

The results of these studies suggest that monitoring biomarkers of TLR3 and type I IFN signaling may not only have prognostic/predictive relevance for cancer patients, but also inform on the risk for cancer development in healthy subjects. Of note, recombinant IFN-α2a (Roferon-A®) is approved by the US Food and Drug Administration and other regulatory agencies worldwide for use in subjects with hairy cell leukemia and Philadelphia chromosome-positive chronic myelogenous leukemia upon minimal pretreatment, while recombinant IFN-α2b (Intron A®) is currently employed for the treatment of hairy cell leukemia, AIDS-related Kaposi's sarcoma, follicular lymphoma, multiple myeloma, melanoma, condyloma acuminata and cervical intraepithelial neoplasms.(125, 126) It remains to be determined to which extent, if any, the therapeutic efficacy of type I IFNs reflects their ability to promote the initiation of adaptive immune responses against dying cancer cells.

Extracellular ATP and Autophagy

ATP is secreted during ICD through a mechanism that involves pannexin 1 (PANX1) channels and lysosomal exocytosis (127, 128). Importantly, autophagy is required for cancer cells succumbing to anthracyclines to release ATP in immunostimulatory amounts (42, 129, 130). Thus, the ability of anthracyclines to cause *bona fide* ICD is lost when cancer cells are rendered autophagy-deficient by genetic manipulations or engineered to overexpress ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1, best known as CD39), an enzyme that degrades extracellular ATP (42, 129). In line with this notion, the administration of CD39 inhibitors or CD39-neutralizing monoclonal antibodies reportedly relieves tumor-mediated immunosuppression (131), and (at least in some models) allows autophagy-deficient cells treated with anthracyclines to elicit normal immune responses upon inoculation in immunocompetent mice (42, 129). Extracellular ATP exerts immunostimulatory functions via at least three mechanistically distinct pathways: (1) by promoting the recruitment of APCs or APC precursors to sites of cell death, upon binding to purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) (132–134); (2) by activating the so-called NLRP3 inflammasome and hence triggering the secretion of pro-inflammatory IL-1β (135, 136), an effect that relies on purinergic receptor P2X, ligand gated ion channel, 7 (41); and (3) by boosting the proliferation and cytotoxic activity of NK cells (26). Notably, extracellular ATP is sequentially metabolized by CD39 and 5′-nucleotidase, ecto (NT5E, best known as CD73) into ADP, AMP and adenosine, the latter of which has robust immunosuppressive effects (137).

Accumulating clinical evidence ascribes to parameters linked to the capacity of cancer cells to recruit and activate immune effectors (through extracellular ATP) a prognostic or predictive value for cancer patients (**Table 3**). A SNP compromising the function of P2RX7 has been associated with decreased time-to-metastasis in a cohort of 225 breast carcinoma patients treated with adjuvant anthracycline-based chemotherapy (41), with worsened clinicopathological parameters amongst 121 subjects with papillary thyroid cancer (138), and with an increased risk for the development of chronic lymphocytic leukemia (CLL), as determined in a cohort of 40 patients and 46 age-matched healthy individuals (139). Contrasting with these latter findings, however, the same SNP has been associated with increased overall survival in a cohort of 170 subjects with CLL (140), or found to have no correlation with disease incidence and/or outcome in independent cohorts of 144 CLL patients and 348 healthy controls (141), 121 individuals with CLL (142) 111 CLL patients and 97 controls (143), and 136 subjects with multiple myeloma (144). These apparently discrepant observations may reflect the cancer cell-intrinsic functions of P2RX7, which is known to control proliferation and regulated cell death (145). Of note, increased *P2RY2* mRNA levels have also been detected in gastric cancer biopsies from 14 patients (as compared to the adjacent healthy mucosa) (146), but these findings do not allow to determine whether gastric neoplasms were infiltrated by $P2RY2^+$ immune cells or whether they overexpressed P2RY2.

Further corroborating the advantage conferred to malignant cells by an increased ability to convert immunostimulatory extracellular ATP into immunosuppressive AMP and adenosine, several studies ascribed a negative prognostic or predictive value to increased CD39 or CD73 levels. For instance, elevated amounts of CD39 and CD73 have been detected in 29 endometrial tumor samples as compared to the adjacent non-malignant tissues, and expression levels correlated with tumor grade (152). Along similar lines, CD39 (but not CD73) levels on the surface of $CD4^+$ and $CD8^+$ T cells have been shown to positively correlate with disease stage in two independent cohorts of 34 and 62 patients with CLL (150, 151), while CD73 downregulation has been associated with prolonged disease-free survival amongst 500 individuals with glioblastoma (154). At stark contrast with these findings, high levels of *CD39* mRNA have been linked to improved disease outcome in a cohort of 28 pancreatic cancer patients treated with surgery (153). The reasons underlying this discrepancy have not yet been clarified.

Of note, quantifying functional autophagy in tissue biopsies is rather complex, because most autophagic markers accumulate both when the autophagic flux is increased and when lysosomal degradation is blocked (155). Moreover, autophagy often serves a dual role in the course of tumor progression: (1) on the one hand it favors the survival of cancer cells exposed to adverse microenvironmental conditions (including nutritional, metabolic and therapeutic cues); (2) on the other hand, it is required for

TABLE 3 | Clinical studies assessing the prognostic and predictive value of ATP release and extracellular ATP signaling in cancer patients.

Parameter	Cancer	Treatment	No	Note(s)	Reference
Autophagy	Breast carcinoma	n.a.	1067 patients 1992 patients	Low BECN1 levels correlated with worsened disease outcome	(147)
	HCC	Surgery	190	High LC3 levels correlated with prolonged OS	(148)
	HNC	Surgery	79	High LC3 levels correlated with node involvement and TNM score	(78)
	Pancreatic carcinoma	Surgery	73	High levels of BECN1 and other autophagy-related proteins correlated with poor outcome	(149)
CD39	CLL	n.a.	34 patients 31 controls	High CD39 levels on T cells correlated with late disease	(150)
			62	High CD39 levels on T cells correlated with late disease	(151)
	Endometrial cancer	Surgery	29	High CD39 levels correlated with tumor grade	(152)
	Pancreatic carcinoma	Surgery	28	High CD39 levels were linked to improved disease outcome	(153)
CD73	Endometrial cancer	Surgery	29	High CD73 levels correlated with tumor grade	(152)
	Glioblastoma	n.a.	500	CD73 downregulation was associated with improved DFS	(154)
P2RX7	Breast carcinoma	Anthracycline-based chemotherapy	225	A SNP in *P2RX7* was linked to shortened MFS	(41)
	CLL	n.a.	40 patients 46 controls	A SNP in *P2RX7* was linked to increased risk for oncogenesis	(139)
			144 patients 348 controls	Lack of correlation between *P2RX7* status and disease incidence	(141)
			111 patients 97 controls	Lack of correlation between *P2RX7* status and disease incidence	(143)
			170	A SNP in *P2RX7* was associated to increased OS	(140)
			121	Lack of correlation between *P2RX7* status and pathological features	(142)
	Multiple myeloma	n.a.	136 patients 95 controls	Lack of correlation between *P2RX7* status and disease incidence	(144)
	Papillary thyroid cancer	n.a.	121	A SNP in *P2RX7* was linked to poor clinicopathological features	(138)
P2RY2	Gastric cancer	n.a.	14 patients	Increased expression of P2RY2 in malignant cells	(146)

CLL, chronic lymphocytic leukemia; DFS, disease-free survival; HCC, hepatocellular carcinoma; HNC, head and neck cancer; MFS, metastasis-free survival; n.a., not applicable or not available; OS; overall survival; SNP, single nucleotide polymorphism.

ICD-associated ATP secretion and for the elicitation of robust TAA-targeting immune responses (130, 156, 157). Notwithstanding these caveats, immunohistochemistry has been employed to study the prognostic or predictive value of autophagic markers such as the expression and lipidation of microtubule-associated protein 1 light chain 3 (MAP1LC3, best known as LC3) (158), with mixed results. For instance, LC3 expression has been associated with prolonged overall survival in a cohort of 190 HCC patients (148), but with lymph node involvement and high TNM score amongst 79 individuals with head and neck cancer (78). Along similar lines, reduced expression of beclin 1 (BECN1), a key component of the molecular machinery for autophagy, has been associated with poor prognosis in two independent cohorts of 1067 and 1992 breast carcinoma patients (147), but with improved disease outcome in a cohort of 73 patients with pancreatic cancer (149). These are only two examples of an abundant scientific literature correlating the expression of autophagy proteins in biopsies from patients affected with virtually all types of malignancies to clinicopathological features and/or markers of disease progression. The development of assays to monitor the functionality of the autophagic apparatus in clinical samples is urgently awaited to properly assess the prognostic and predictive value of autophagy for cancer patients.

HMGB1 and Cell Death

According to current models, HMGB1 gets released in the course of cell death passively, upon the breakdown of the nuclear and plasma membrane (145, 159). Thus, besides differences in expression level, the extent of HMGB1 release generally correlates with the degree of cell death (160). However, changes in the oxidation status of extracellular HMGB1 have been suggested to dramatically alter its biological activity (161–163). Indeed, while reduced HMGB1 efficiently dimerizes with CXCL12 and mediate potent chemotactic functions upon binding to chemokine (C–X–C motif) receptor 4 (CXCR4) (164, 165), its oxidized counterpart fails to do so (162). Rather, oxidized HMGB1 signal via TLR2, TLR4 and advanced glycosylation end product-specific receptor (AGER, best known as RAGE) to stimulate the production of pro-inflammatory cytokines (162, 166–168). In addition, TLR4 signaling promotes cross-priming by inhibiting the fusion of antigen-containing endosomes with lysosomes (169). Interestingly, HMGB1 also binds to TLR9 (170) and hepatitis A virus cellular receptor 2 (HAVCR2, best known as TIM-3) (171), in particular when complexed with DNA. However, while TLR9 promotes cytokine secretion by plasmacytoid DCs and B cells (170), TIM-3 signaling blunts the ability of DCs to respond efficiently to inflammatory stimuli (171). Thus, extracellular HMGB1 mediates multipronged and context-dependent immunomodulatory functions.

Various clinical studies indicate that monitoring parameters linked to HMGB1 release and signaling may convey prognostic or predictive information for cancer patients (**Table 4**). High expression levels of HMGB1 in malignant cells have been shown to correlate with improved overall survival in 88 patients with

TABLE 4 | Clinical studies assessing the prognostic and predictive value of HMGB1 release and extracellular HMGB1 signaling in cancer patients.

Parameter	Cancer	Treatment	No	Note(s)	Reference
CASP3	Endometrial carcinoma	n.a.	1028 patients 1003 controls	A SNP in *CASP3* was linked to increased risk for oncogenesis	(182)
CASP7	Endometrial carcinoma	n.a.	1028 patients 1003 controls	SNPs in *CASP7* were linked to increased risk for oncogenesis	(182)
CASP9	CRC	n.a.	402 patients 480 controls	SNPs in *CASP9* were linked to decreased risk for oncogenesis and improved disease outcome	(183)
HMGB1	Bladder carcinoma	n.a.	164	High HMGB1 levels correlated to worsened disease outcome	(175)
	Breast carcinoma	Anthracycline-based chemotherapy	232	Loss of nuclear HMGB1 positively correlated with tumor size	(173)
			41	Increases in circulating HMGB1 were linked to clinical response	(184)
	CRC	n.a.	219 patients 75 controls	High levels of serum HMGB1 correlated with disease incidence	(185)
		n.a.	192	High HMGB1 levels correlated with worsened disease outcome	(177)
		Radioembolization therapy	49	High levels of serum HMGB1 correlated with decreased OS	(186)
		Surgery	72	Co-expression of HMGB1 in the nucleus and in the cytoplasm of malignant cells was linked to worsened 5-year survival rate	(174)
	Esophageal carcinoma	Chemoradiotherapy and surgery	88	High HMGB1 levels correlated with improved OS	(84)
	Gastric adenocarcinoma	Surgery	76	High HMGB1 levels in malignant cells correlated with improved OS	(172)
	HCC	n.a.	208	High HMGB1 levels correlated with worsened disease outcome	(179)
			161	High HMGB1 levels correlated with worsened disease outcome	(178)
	HNC	n.a.	71 patients 50 controls	High levels of serum HMGB1 correlated with disease progression	(187)
			103	High HMGB1 levels correlated with worsened disease outcome	(180)
	Malignant mesothelioma	n.a.	61 patients 45 controls	High levels of serum HMGB1 correlated with disease incidence	(188)
	Nasopharyngeal carcinoma	n.a.	166	High HMGB1 levels correlated with worsened disease outcome	(176)
	Pancreatic carcinoma	Multicomponent chemotherapy	78	High circulating HMGB1 correlated with poor therapy response	(189)
		n.a.	70	High levels of serum HMGB1 correlated with decreased OS	(190)
	Prostate carcinoma	n.a.	85	High HMGB1 levels correlated with worsened disease outcome	(181)
	Solid tumors	Virotherapy	17	Increases in circulating HMGB1 levels were linked to clinical response	(191)
			202	Increases in circulating HMGB1 levels were linked to clinical response	(192)
MYD88	CRC	Surgery	108	High MYD88 levels correlated with shortened DFS and OS	(193)
	Lymphoma	Conventional chemotherapy	29	MYD88 mutations were involved in the pathogenesis of the disease	(194)
	Ovarian carcinoma	Surgery	123	High MYD88 levels correlated with worsened disease outcome	(195)
			109	High MYD88 levels correlated with shortened DFS and OS	(196)
RAGE	Breast carcinoma	n.a.	509 patients 504 controls	A SNP in *AGER* was linked to increased risk for oncogenesis	(197)
			120 patients 92 controls	High levels of circulating RAGE correlated with advanced disease stage but improved outcome	(198)
	Gastric carcinoma	Surgery	180	High RAGE levels were associated with worsened disease outcome	(199)
	HCC	Transarterial chemoembolization	71	High levels of circulating RAGE correlated with clinical response	(200)
	NSCLC	Platinum-based chemotherapy	562 patients 764 controls	SNPs in *AGER* were linked to increased risk for oncogenesis and differential clinical response	(201)
	Ovarian carcinoma	n.a.	190 patients 210 controls	A SNP in *AGER* was linked to increased risk for oncogenesis	(202)
TLR2	CRC	n.a.	2309 patients 2915 controls	SNPs in *TLR2* were associated with decreased 5-year survival rate	(121)
	Gastric carcinoma	n.a.	289 patients 400 controls	A SNP in *TLR2* was linked to increased risk for oncogenesis	(203)
	HCC	n.a.	211 patients 232 controls	SNPs in *TLR2* were linked to increased risk for oncogenesis	(204)
	Lymphoma	n.a.	710 patients 710 controls	A SNP in *TLR2* was linked to increased risk for oncogenesis	(205)
	Prostate carcinoma	n.a.	195 patients 250 controls	A SNP in *TLR2* was linked to increased risk for oncogenesis	(206)

(*Continued*)

TABLE 4 | Continued

Parameter	Cancer	Treatment	No	Note(s)	Reference
TLR4	Breast carcinoma	Anthracycline-based chemotherapy	280	A SNP in *TLR4* was linked to shortened MFS	(43)
	CRC	n.a.	2309 patients 2915 controls	SNPs in *TLR4* were associated with risk variations and increased OS	(121)
		Surgery	108	High TLR4 levels were associated with shortened DFS and OS	(193)
	HNC	Adjuvant systemic chemotherapy	188	A SNP in *TLR4* was linked to shortened DFS and OS	(207)
	Melanoma	Allogenic cancer cell-based vaccine	72	A SNP in *TLR4* was linked to shortened DFS and OS	(208)
		Various	622	A SNP in *TLR4* was linked to shortened DFS and OS	(209)
	Ovarian carcinoma	Surgery	123	High TLR4 levels were associated with worsened disease outcome	(195)
	Prostate carcinoma	n.a.	700 patients 700 controls	A SNP in *TLR4* was linked to increased risk for oncogenesis	(210)
			258 patients 258 controls	A SNP in *TLR4* was linked to increased risk for oncogenesis	(211)
			157 patients 143 controls	A SNP in *TLR4* was linked to increased risk for oncogenesis	(212)
			240 patients 223 controls	A SNP in *TLR4* was linked to increased risk for oncogenesis	(124)

CRC, colorectal carcinoma; DFS, disease-free survival; HCC, hepatocellular carcinoma; HNC, head and neck cancer; MFS, metastasis-free survival; NSCLC, non-small cell lung carcinoma; n.a., not applicable or not available; OS; overall survival; RFS, relapse-free survival; SNP, single nucleotide polymorphism.

esophageal squamous cell carcinoma subjected to neo-adjuvant chemoradiotherapy and surgical resection (84), as well as in 76 subjects with reseactable gastric adenocarcinoma (172). In a cohort of 232 breast carcinoma patients treated with anthracycline-based adjuvant chemotherapy, loss of nuclear HMGB1 has been positively associated with tumor size (173). Along similar lines, the co-expression of HMGB1 in the nucleus and in the cytoplasm of malignant cells has been shown to inversely correlate with tumor infiltration by $CD45RO^+$ memory T cells and 5-year survival rate in 72 individuals with Stage IIIB CRC (174). Finally, HMGB1 overexpression has been shown to correlate with advanced clinical stage or decreased disease-free and/or overall survival amongst 164 patients with bladder carcinoma (175), 166 individuals with nasopharyngeal carcinoma (176), 192 CRC patients (177), 208 and 161 individuals with HCC (178, 179), 103 subjets with head and neck squamous cell carcinoma (180), as well as 85 patients with prostate cancer (181).

Notably, circulating HMGB1 and RAGE levels have been intensively investigated for their predictive or prognostic value. Elevations of HMGB1 in the serum have been correlated with incidence, progression or unfavorable disease outcome in cohorts of 49 individuals with CRC, or 219 CRC patients and 75 healthy controls (185, 186), 70 individuals with pancreatic adenocarcinoma (190), 71 laryngeal squamous cell carcinoma patients and 50 healthy controls (187), 61 subjects with malignant pleural mesothelioma (188), and 78 pancreatic carcinoma patients (189). Conversely, a treatment-related increase in the circulating levels of HGMB1 has been associated with pathological complete response or partial remission amongst 41 breast carcinoma patients treated with neo-adjuvant chemotherapy based on epirubicin (an ICD inducer) (184), as well as amongst 17 and 202 subjects with chemotherapy-refractory tumors treated with oncolytic virotherapy (191, 192). High levels of RAGE in the serum have been linked to advanced tumor stage but improved clinical outcome amongst 120 patients with breast carcinoma (198). Along similar lines, serum RAGE concentrations were significantly higher in 32 individuals with HCC who favorably responded to transarterial chemoembolization therapy than in 39 patients who progressed upon treatment (200).

Thus, in many (but not all) clinical settings high intratumoral and circulating levels of HMGB1 have a negative prognostic or predictive value. These findings may reflect the ability of some tumors to retain HMGB1 in the course of stress response, the intrinsic resistance of such tumors to the induction of cell death, or the cancer cell-intrinsic functions of HMGB1 (213). In other settings, however, circulating HMGB1 and RAGE levels appear to reflect well the death of cancer cells exposed to immunogenic treatment modalities (184, 191, 192). Possibly, the timing of detection plays a critical role in this setting, calling for the development of optimized monitoring procedures.

SNPs in *TLR2*, *TLR4* and *AGER*, as well as the circulating levels of a soluble RAGE variant have been shown to affect cancer susceptibility as well as disease outcome in several studies. In particular, *TLR2* polymorphisms have been linked to an increased risk for lymphoma (as determined in 710 patients and as many healthy subjects) (205), gastric carcinoma (as assessed in 289 patients and more than 400 controls) (203), prostate carcinoma (as investigated in 195 patients and 250 healthy individuals) (206), HCC (as tested in 211 patients and 232 controls) (204), and CRC (as assessed in 2,309 patients and 2,915 healthy individuals) (121). Loss-of-function variants of TLR4 have been associated with decreased time-to-metastasis amongst 280 women with non-metastatic breast carcinoma treated with surgery followed by anthracycline-based chemotherapy and local irradiation (43), with reduced disease-free and overall survival amongst 188 head and neck cancer patients receiving adjuvant systemic therapy (207), amongst 72 melanoma patients vaccinated with a heat-shocked allogeneic melanoma cell line (208), and amongst 622 melanoma patients subjected to various treatment modalities (209). Along similar lines, SNPs affecting *TLR4* or *AGER* have

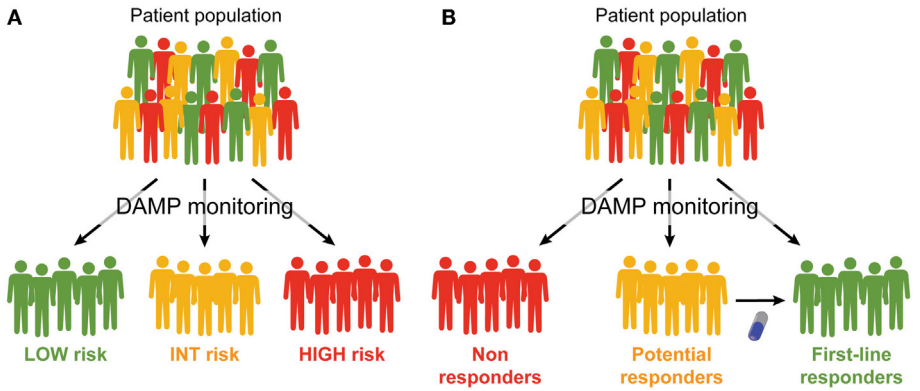

FIGURE 1 | Prognostic and predictive value of DAMPs and DAMP-associated processes. (A,B). Monitoring the emission of damage-associated molecular patterns (DAMPs) or DAMP-associated processes may have a multifaceted impact on the clinical management of cancer patients. First, it may allow for a prognostic assessment and permit the stratification of patients in different risk groups **(A)**. Second, it may allow for the identification of patients who are intrinsically capable or uncapable to respond to a specific treatment, and amongst the latter, those who may benefit from combinatorial therapeutic approaches aimed at restoring normal DAMP signaling **(B)**.

been linked to an increased risk for prostate cancer (as determined in multiple studies collectively testing more than 1,000 patients and as many age-matched controls) (124, 210–212), ovarian cancer (as assessed in a study testing 190 patients and 210 controls) (202), breast carcinoma (as investigated in 509 patients and 504 healthy women) (197), CRC (as determined in a large cohort encompassing 2,309 patients and 2,915 healthy individuals) (121), and NSCLC (as tested in 562 patients and 764 controls) (201). Notably, this latter study also identified a specific *AGER* SNP associated with a differential response of NSCLC patients to chemotherapy (201).

Conversely, elevated expression levels of RAGE, TLR4 and/or components of the TLR signaling machinery like myeloid differentiation primary response gene 88 (MYD88) by malignant tissues have been correlated with shortened disease-free and overall survival in 2 cohorts of 109 and 123 ovarian carcinoma patients subjected to surgery (195, 196), in a cohort 108 subjects with CRC (193), and amongst 180 individuals with gastric carcinoma (199). Along similar lines, activating mutations in *MYD88* have been linked to the pathogenesis of primary central nervous system lymphomas (194). Most likely, these findings reflect the advantage conferred to malignant cells by the expression of RAGE and TLR4, which can activate robust pro-survival pathways via NF-κB (214).

Finally, distinct SNPs affecting caspase-7 (*CASP7*) and one affecting caspase-3 (*CASP3*) have been associated with an altered risk for endometrial carcinoma (as investigated in a cohort of 1,028 patients and 1,003 healthy women) (182), whereas SNPs affecting caspase-9 (*CASP9*) have been linked to reduced CRC incidence or improved disease outcome (as determined in a cohort of 402 patients and 480 healthy controls) (183). It remains to be determined whether these SNPs truly compromise the ability of cancer cells to emit DAMPs (and hence trigger immunosurveillance mechanisms).

Other DAMPs

The abovementioned molecules and processes may constitute only the tip of an iceberg, meaning that several other DAMPs may contribute to the immunogenicity of cell death, at least in some circumstances. These DAMPs include (but are not limited to) various mitochondrial products like mtDNA, cardiolipin and *N*-formylated peptides (30) as well as cytosolic proteins like filamentous F-actin (45). Robust preclinical evidence implicates mtDNA in the etiology of septic and non-septic shock as well as in heart failure (29, 215). Cytosolic, extra-cytosolic and extracellular mtDNA molecules have indeed robust pro-inflammatory effects as they trigger type I IFN synthesis via transmembrane protein 173 (TM173, best known as STING) (216) or TLR9 activation (215). In line with this notion, circulating mtDNA levels have been shown to reflect the degree of inflammation and the extent of tissue damage in patients under maintenance hemodialysis (217). Moreover, mtDNA concentrations in the plasma of severe sepsis patients admitted to the emergency room have been ascribed robust predictive value on disease outcome (218). Upon binding to formyl peptide receptor 1 (FPR1), *N*-formylated peptides reportedly attract neutrophils, stimulate their degranulation, activate monocytes and favor the production of pro-inflammatory cytokines (219–223). Cardiolipin, a lipid that is specifically contained in the inner mitochondrial membrane, binds CD1D on the surface of APC, thus endowing them with the ability of priming CD1D-restricted γδ T cells (224). Finally, F-actin becomes accessible upon disruption of the plasma membrane and promotes the elicitation of adaptive immune responses against dead cell-associated antigens by binding to C-type lectin domain family 9, member A (CLEC9A, best known as DNGR1) on the surface of DCs (45). Studies elucidating the actual contribution of these DAMPs to ICD are urgently awaited.

Concluding Remarks

It is now clear that the emission of DAMPs according to a specific spatiotemporal pattern is an absolute requirement for the elicitation of immune responses against malignant cells succumbing to treatment, and that such responses are necessary for the full-blown efficacy of most (if not all) anticancer therapeutic regimens. In many settings, however, neoplastic cells exposed to conventional chemotherapeutics, radiotherapy or targeted anticancer agents fail to emit DAMPs in a manner compatible with the activation of the immune system, calling for the development of complementation strategies (16). Several approaches are being conceived to address this issue, including the implementation of combinatorial therapeutic regimens including (1) ER stressors, recombinant CALR or recombinant HSPs, to complement for defects in the CALR or HSP exposure pathway; (2) TLR3 agonists or recombinant type I IFNs, to correct problems in the secretion of type I IFN; (3) autophagy inducers or inhibitors of extracellular ATP-degrading enzymes, to maximize the amount of ATP secreted in the course of cell death; and (4) recombinant HMGB1, TLR4 agonists or cytotoxic agents, to restore HMGB1-dependent immunostimulation (225). Besides, consistent efforts are being devoted to the identification of additional strategies that *per se* induce ICD, *in vivo* (with direct therapeutic purposes), and *in vitro* (for instance, for the development of anticancer vaccines) (20). Monitoring DAMPs and DAMP-associated processes may therefore have a dual clinical relevance (**Figure 1**). First, it may improve patient stratification by allowing for the identification of individuals with different prognosis and/or subjects who are likely to respond (or are responding) to a particular therapeutic regimen. Second, it may instruct therapeutic choices by spotting specific molecular or cellular defects that may be corrected pharmacologically. We surmise that the prognostic and/or predictive value of DAMPs and DAMP-associated processes will have a significant impact on the clinical management of cancer patients.

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REVIEW
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Exploiting the Immunogenic Potential of Cancer Cells for Improved Dendritic Cell Vaccines

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Cancer immunotherapy is currently the hottest topic in the oncology field, owing predominantly to the discovery of immune checkpoint blockers. These promising antibodies and their attractive combinatorial features have initiated the revival of other effective immunotherapies, such as dendritic cell (DC) vaccinations. Although DC-based immunotherapy can induce objective clinical and immunological responses in several tumor types, the immunogenic potential of this monotherapy is still considered suboptimal. Hence, focus should be directed on potentiating its immunogenicity by making step-by-step protocol innovations to obtain next-generation Th1-driving DC vaccines. We review some of the latest developments in the DC vaccination field, with a special emphasis on strategies that are applied to obtain a highly immunogenic tumor cell cargo to load and to activate the DCs. To this end, we discuss the effects of three immunogenic treatment modalities (ultraviolet light, oxidizing treatments, and heat shock) and five potent inducers of immunogenic cell death [radiotherapy, shikonin, high-hydrostatic pressure, oncolytic viruses, and (hypericin-based) photodynamic therapy] on DC biology and their application in DC-based immunotherapy in preclinical as well as clinical settings.

Keywords: immunotherapy, dendritic cell vaccines, immunogenic cell death, antitumor immunity, tumor lysate, immunogenicity

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INTRODUCTION

Cancer immunotherapy has gained considerable momentum over the past 5 years, owing predominantly to the discovery of immune checkpoint inhibitors. These inhibitors are designed to release the brakes of the immune system that under physiological conditions prevent auto-immunity by negatively regulating cytotoxic T lymphocyte (CTL) function. Following the FDA approval of the anti-cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) monoclonal antibody (mAb)

Abbreviations: CD, cluster of differentiation; CRT, calreticulin; CVB3, coxsacievirus B3; DAMP, damage-associated molecular pattern; DC, dendritic cell; ER, endoplasmic reticulum; HHP, high-hydrostatic pressure; HMGB1, high-mobility group box 1; HSP, heat shock protein; Hyp, hypericin; ICD, immunogenic cell death; NDV, Newcastle disease virus; OAMP, oxidation-associated molecular pattern; PDT, photodynamic therapy; ROS, reactive oxygen species; TLR, Toll-like receptor; UV, ultraviolet.

ipilimumab (Yervoy) in 2011 for the treatment of metastatic melanoma patients (1), two mAbs targeting programed death (PD)-1 receptor signaling (nivolumab and pembrolizumab) have very recently joined the list of FDA-approved checkpoint blockers (respectively, for the treatment of metastatic squamous non-small cell lung cancer and relapsed/refractory melanoma patients) (2, 3).

However, the primary goal of cancer immunotherapy is to activate the immune system in cancer patients. This requires the induction of tumor-specific T-cell-mediated antitumor immunity. Checkpoint blockers are only able to abrogate the brakes of a functioning antitumoral immune response, implying that only patients who have pre-existing tumor-specific T cells will benefit most from checkpoint blockade. This is evidenced by the observation that ipilimumab may be more effective in patients who have pre-existing, albeit ineffective, antitumor immune responses (4). Hence, combining immune checkpoint blockade with immunotherapeutic strategies that prime tumor-specific T cell responses might be an attractive and even synergistic approach. This relatively new paradigm has lead to the revival of existing, and to date disappointing (as monotherapies), active immunotherapeutic treatment modalities. One promising strategy to induce priming of tumor-specific T cells is dendritic cell (DC)-based immunotherapy.

Dendritic cells are positioned at the crucial interface between the innate and adaptive immune system as powerful antigen-presenting cells capable of inducing antigen-specific T cell responses (5). Therefore, they are the most frequently used cellular adjuvant in clinical trials. Since the publication of the first DC vaccination trial in melanoma patients in 1995, the promise of DC immunotherapy is underlined by numerous clinical trials, frequently showing survival benefit in comparison to non-DC control groups (6–8). Despite the fact that most DC vaccination trials differ in several vaccine parameters (i.e., site and frequency of injection, nature of the DCs, choice of antigen), DC vaccination as a monotherapy is considered safe and rarely associates with immune-related toxicity. This is in sharp contrast with the use of mAbs or cytokine therapies. Ipilumumab has, for instance, been shown to induce immune-related serious adverse events in up to one-third of treated melanoma patients (1). The FDA approval of Sipuleucel-T (Provenge), an autologous DC-enriched vaccine for hormone-resistant metastatic prostate cancer, in 2010 is really considered as a milestone in the vaccination community (9). After 15 years of extensive clinical research, Sipileucel-T became the first cellular immunotherapy ever that received FDA approval, providing compelling evidence for the substantial socio-economic impact of DC-based immunotherapy. DC vaccinations have most often been applied in patients with melanoma, prostate cancer, high-grade glioma, and renal cell cancer. Although promising objective responses and tumor-specific T cell responses have been observed in all these cancer-types (providing proof-of-principle for DC-based immunotherapy), the clinical success of this treatment is still considered suboptimal (6). This poor clinical efficacy can in part be attributed to the severe tumor-induced immune suppression and the selection of patients with advanced disease status and poor survival prognostics (6, 10–12).

There is a consensus in the field that step-by-step optimization and standardization of the production process of DC vaccines, to obtain a Th1-driven immune response, might enhance their clinical efficacy (13). In this review, we address some recent DC vaccine adaptations that impact DC biology. Combining these novel insights might bring us closer to an ideal DC vaccine product that can trigger potent CTL- and Th1-driven antitumor immunity.

One factor requiring more attention in this production process is the immunogenicity of the dying or dead cancer cells used to load the DCs. It has been shown in multiple preclinical cancer models that the methodology used to prepare the tumor cell cargo can influence the *in vivo* immunogenic potential of loaded DC vaccines (14–19). Different treatment modalities have been described to enhance the immunogenicity of cancer cells in the context of DC vaccines. These treatments can potentiate antitumor immunity by inducing immune responses against tumor neo-antigens and/or by selectively increasing the exposure/release of particular damage-associated molecular patterns (DAMPs) that can trigger the innate immune system (14, 17–19). The emergence of the concept of immunogenic cell death (ICD) might even further improve the immunogenic potential of DC vaccines. Cancer cells undergoing ICD have been shown to exhibit excellent immunostimulatory capacity owing to the spatiotemporally defined emission of a series of critical DAMPs acting as potent danger signals (20, 21). Thus far, three DAMPs have been attributed a crucial role in the immunogenic potential of nearly all ICD inducers: the surface-exposed "eat me" signal calreticulin (ecto-CRT), the "find me" signal ATP and passively released high-mobility group box 1 (HMGB1) (21). Moreover, ICD-experiencing cancer cells have been shown in various mouse models to act as very potent Th1-driving anticancer vaccines, already in the absence of any adjuvants (21, 22). The ability to reject tumors in syngeneic mice after vaccination with cancer cells (of the same type) undergoing ICD is a crucial hallmark of ICD, in addition to the molecular DAMP signature (21).

Here, we review the effects of three frequently used immunogenic modalities and four potent ICD inducers on DC biology and their application in DC vaccines in preclinical as well as clinical settings (**Tables 1** and **2**). Moreover, we discuss the rationale for combining different cell death-inducing regimens to enhance the immunogenic potential of DC vaccines and to ensure the clinical relevance of the vaccine product.

THE IMPACT OF DC BIOLOGY ON THE EFFICACY OF DC VACCINES

Over the past years, different DC vaccine parameters have been shown to impact the clinical effectiveness of DC vaccinations. In the next section, we will elaborate on some promising adaptations of the DC preparation protocol.

Given the labor-intensive *ex vivo* culturing protocol of monocyte-derived DCs and inspired by the results of the Provenge study, several groups are currently exploiting the use of blood-isolated naturally circulating DCs (76–78). In this context, De Vries et al. evaluated the use of antigen-loaded purified plasmacytoid DCs

TABLE 1 | A list of prominent enhancers of immunogenicity and ICD inducers applied in DC vaccine setups and their associations with DAMPs and DC biology.

Treatment modality	Associated DAMPs	Effect on DC biology
Immunogenic treatment modality		
UV irradiation	Pre-apoptotic ecto-CRT (23); post-apoptotic passive release of HSP70 and HMGB1 (24); mutation-induced neo-antigens (25)	Efficient engulfment; phenotypic maturation; increased IL-12 secretion; stimulate the polarization of T cells toward CTLs (19, 24, 26, 27)
Oxidation-inducing modalities ($HOCl/H_2O_2$ treatment or freeze–thaw cycles followed by X-ray irradiation)	OAMPs (reactive protein carbonyls, peroxidized phospholipids, oxidized low-density lipoprotein) (14, 18, 28–30); carbonylated protein products presented as neo-antigens (30, 31)	Efficient antigen uptake and presentation; induction of IL-12; increased *in vivo* induction of tumor-reactive T cells (14); induction of Th1- and CTL-driven antitumor immunity (18)
Heat shock	Passive release of heat shock proteins like HSP60/70/90 (17, 32); passive release of HMGB1 (33); increased expression of tumor-specific antigens (34)	Upregulation of DC maturation markers (CD40, CD80, and CD86) and induction of IL-12 (32); enhanced priming of CTL responses (17, 34)
Inducers of immunogenic cell death		
Radiotherapy	Pre-apoptotic exposure of ecto-CRT (23, 24, 35); early/mid-apoptotic exposure of ecto-HSP70 (36); post-apoptotic passive release of HMGB1 (33, 35); mutation-induced neo-antigens (25)	Efficient phagocytosis and enhanced phenotypic maturation (37); increased infiltration in the tumor environment (38, 39); enhanced stimulation of antigen-specific CTL responses (40)
Shikonin	Early/mid-apoptotic induction of ecto-HSP70, ecto-CRT and ecto-GRP78 (an inducer of pro-tumorigenic effects) (41)	Increased phenotypic ($CD40^{high}$, $CD80^{high}$, $CD86^{high}$) and functional maturation ($IL\text{-}12p70^{high}$, $TGF\text{-}\beta^{high}$, $IL\text{-}6^{high}$, $IL\text{-}23^{low}$) but only in combination with LPS; increased capacity to induce Th1 and Th17 differentiation (41)
High-hydrostatic pressure	Early/mid-apoptotic exposure of ecto-HSP70, ecto-HSP90, ecto-CRT; pre-apoptotic ATP release; post-apoptotic passive release of HMGB1, HSP70/90, and CRT (42)	Efficient phagocytosis; enhanced phenotypic and functional maturation; induction of antigen-specific T cells without inducing Tregs (42)
Oncolytic viruses	CVB3 and oncolytic adenovirus: (early-apoptotic) exposure of ecto-CRT; (early/mid-apoptotic) secretion of ATP and (post-apoptotic) release of HMGB1 (43, 44) NDV: early/mid-necroptotic exposure of ecto-CRT and post-necroptotic release of HMGB1 (45)	Enhanced expression of CD80/CD86 (44, 46, 47) and CCR7 (44); more efficient priming of tumor-specific $CD8^+$ CTL responses (43, 46, 47) and Th1 responses (43); increased accumulation in tumor microenvironment (43, 44)
Hypericin-based PDT	Pre-apoptotic ecto-CRT, ecto-HSP70 and secreted ATP; late apoptotic passive release of HSP70/90, CRT and HMGB1; accumulation of OAMPs like protein carbonyls (48–50)	Enhanced phagocytosis; phenotypic maturation ($CD80^{high}$ $CD86^{high}$ $CD83^{high}$ $MHC\text{-}II^{high}$) and immunogenic functional stimulation (NO^{high} $IL\text{-}10^{absent}$ $IL\text{-}6^{high}$ $IL\text{-}1\beta^{high}$ $IL\text{-}12p70^{medium}$); clonal expansion of human IFN-γ producing $CD4^+$ and $CD8^+$ T cells (49, 53, 54)
Photofrin-based PDT	early/mid-apoptotic exposure of CRT, HSP60/70, ceramide and S1P; post-apoptotic release of HMGB1 (51, 52)	Increased phenotypic maturation ($CD86^{high}$, $MHC\text{-}II^{high}$) and enhanced IL-12 production (55); increased infiltration in tumor draining lymph nodes after peritumoral vaccination (56)

CRT,calreticulin; CTL, cytotoxic T lymphocyte; CVB3, coxsackievirus B3; DAMPs, damage-associated molecular patterns; HMGB1, high-mobility group box-1 protein; HSP, heat shock protein; ICD, immunogenic cell death; IFN, interferon; LPS, lipopolysaccharide; NDV, Newcastle disease virus; NO, nitric oxide; OAMPs, oxidation-associated molecular patterns; PDT, photodynamic therapy; TGF, transforming growth factor; Treg, regulatory T cell.

for intranodal injection in melanoma patients (79). This strategy was feasible and induced only very mild side effects. In addition, the overall survival of vaccinated patients was greatly enhanced as compared to historical control patients. However, it still remains to be determined whether this strategy is more efficacious than monocyte-derived DC vaccine approaches (78). By contrast, experiments in the preclinical GL261 high-grade glioma model recently showed that vaccination with tumor antigen-loaded myeloid DCs resulted in more robust Th1 responses and a stronger survival benefit as compared to mice vaccinated with their plasmacytoid counterparts (80).

In view of their strong potential to stimulate cytotoxic T cell responses, several groups are currently exploring the use of Langerhans cell-like DCs as sources for DC vaccines (81–83). These so-called IL-15 DCs can be derived from $CD14^+$ monocytes by culturing them with IL-15 (instead of the standard IL-4). Recently, it has been shown that in comparison to IL-4 DCs, these cells have an increased capacity to stimulate antitumor natural killer (NK) cell cytotoxicity in a contact- and IL-15-dependent manner (84). NK cells are increasingly being recognized as crucial contributors to antitumor immunity, especially in DC vaccination setups (85, 86). Three clinical trials are currently evaluating these Langerhans cell-type DCs in melanoma patients (NCT00700167, NCT 01456104, and NCT01189383).

Targeting cancer stem cells is another promising development, particularly in the setting of glioma (87). Glioma stem cells can foster tumor growth, radio- and chemotherapy-resistance, and local immunosuppression in the tumor microenvironment (87, 88). Furthermore, glioma stem cells may express higher levels of tumor-associated antigens and MHC complex molecules as compared to non-stem cells (89, 90). A preclinical study in a rodent orthotopic glioblastoma model has shown that DC vaccines loaded with neuropspheres enriched in cancer stem cells could induce more immunoreactivity and survival benefit as compared

TABLE 2 | A list of preclinical tumor models and clinical studies for evaluation of the *in vivo* potency of DC vaccines loaded with immunogenically killed tumor cells.

Treatment modality	Preclinical experience in DC vaccine settings	Clinical experience in DC vaccine settings
Immunogenic treatment modalities		
UV irradiation	B16 melanoma in C57BL/6 – curative immunizations (19); ID8-ova ovarian carcinoma model in C57BL/6 mice – weekly curative immunizations (14)	Only in combination with γ-irradiation and heat shock in B-cell lymphoma patients (57)
Oxidation-inducing modalities ($HOCl/H_2O_2$ treatment or freeze–thaw cycles followed by X-ray irradiation)	ID8-ova ovarian carcinoma model in C57BL/6 mice – weekly curative immunizations (14); orthotopic GL261 high-grade glioma model in C57BL/6 mice – both prophylactic and curative vaccination settings induced a pro-inflammatory shift in the brain-infiltrating immune cells and the protein carbonyl content in the tumor lysate positively correlated with tumor rejection (18)	Freeze–thaw cycles in combination with high-dose irradiation: often reported in clinical trials involving high-grade glioma and melanoma patients (8, 58–66) HOCl: pilot study in five recurrent ovarian cancer patients demonstrated potent T cell responses against tumor antigens, decreased circulating Treg levels, and serum IL-10 levels and two patients experienced durable PFS responses of ≥24 months (14)
Heat shock	PANCO2 pancreatic cancer model in C57BL/6 mice – curative vaccinations (17); in combination with 30 Gy irradiation in B16-ova model in C57BL/6 mice – prophylactic vaccinations (16)	Non-randomized trial in newly diagnosed glioblastoma patients (67): significantly improved tumor control rates and survival rates in DC vaccine group than in control group; increased proportions of peripheral $CD4^+$ and $CD8^+$ T cells post vaccination compared to control group; in combination with other cell killing modalities in B-cell lymphoma and melanoma patients (57, 68)
Inducers of immunogenic cell death		
Radiotherapy	B16 melanoma in C57BL/6 – prophylactic immunization model with critical involvement of $CD4^+$ and $CD8^+$ T cells (15, 37); E.G7 (SCCVII) in C57BL/6 – curative vaccination model (40)	Radiotherapy as a single intervention: multiple clinical trials in melanoma patients (8) and two clinical trials in high-grade glioma patients (69, 70). This study by Cho and colleagues reported a survival advantage of more than 15 months in the vaccinated glioblastoma patients in comparison to the control group (receiving conventional treatment) Radiotherapy as part of an ICD-inducing cell death protocol in B-cell lymphoma patients (57)
Shikonin	B16 melanoma in C57BL/6 – curative immunization model with strong induction of CTL responses (41)	Not available
High-hydrostatic pressure	Preclinical experiments are currently ongoing (71)	Multiple clinical trials are initiated involving prostate and ovarian cancer patients (71)
Oncolytic viruses	Not applied as ICD-based DC vaccines yet; curative combination of intratumoral oncolytic virus treatment and peripheral DC vaccination in B16 melanoma (C57BL/6) (72) and in subcutaneous CMT64 or KNL205 tumors (in C57BL/6 mice and DBA/2 DREG mice, respectively) (73)	Case report of breast cancer patient treated with combination of local hyperthermia, intravenously administered NDV and intradermal DC vaccines loaded with NDV-oncolysate (74)
Hypericin-based PDT	Not available	Not available
Photofrin-based PDT	*In vivo* photofrin-PDT treatment in combination with curative DC vaccination in C-26 colon carcinoma (BALB/c) (75); curative vaccinations with DCs charged with PDT-induced tumor lysate in EMT6, Renca and 4T1 non-orthotopic tumor modes (BALB/c), induction of CTL and Th1 responses	Not available

CRT, calreticulin; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ICD, immunogenic cell death; NDV, Newcastle disease virus; PDT, photodynamic therapy; PFS, progression-free survival.

to DCs loaded with GL261 cells grown under standard conditions (91). Currently there are four clinical trials ongoing in high-grade glioma patients evaluating this approach (NCT00890032, NCT00846456, NCT01171469, and NCT01567202).

With regard to the DC maturation status of the vaccine product, a phase I/II clinical trial in metastatic melanoma patients has confirmed the superiority of mature antigen-loaded DCs to elicit immunological responses as compared to their immature counterparts (92). This finding was further substantiated in patients diagnosed with prostate cancer and recurrent high-grade glioma (93, 94). Hence, DCs need to express potent costimulatory molecules and lymph node homing receptors in order to generate a strong T cell response. In view of this finding, the route of administration is another vaccine parameter that can influence the homing of the injected DCs to the lymph nodes. In the context of prostate cancer and renal cell carcinoma it has been shown that vaccination routes with access to the draining lymph nodes (intradermal/intranodal/intralymphatic/subcutaneous) resulted in better clinical response rates as compared to intravenous injection (93). In melanoma patients, a direct comparison between intradermal vaccination and intranodal vaccination concluded that, although more DCs reached the lymph nodes after intranodal vaccination, the melanoma-specific T cells induced by intradermal vaccination were more functional

(95). Furthermore, the frequency of vaccination can also influence the vaccine's immunogenicity. Our group has shown in a cohort-comparison trial involving relapsed high-grade glioma patients that shortening the interval between the four inducer DC vaccines improved the progression-free survival curves (58, 96).

Another variable that has been systematically studied is the cytokine cocktail that is applied to mature the DCs. The current gold standard cocktail for DC maturation contains TNF-α, IL-1β, IL-6, and PGE_2 (97, 98). Although this cocktail upregulates DC maturation markers and the lymph node homing receptor CCR7, IL-12 production by DCs could not be evoked (97, 98). Nevertheless, IL-12 is a critical Th1-driving cytokine and DC-derived IL-12 has been shown to associate with improved survival in DC vaccinated high-grade glioma and melanoma patients (99, 100). Recently, a novel cytokine cocktail, including TNF-α, IL-1β, poly-I:C, IFN-α, and IFN-γ, was introduced (101, 102). The type 1-polarized DCs obtained with this cocktail produced high levels of IL-12 and could induce strong tumor-antigen-specific CTL responses through enhanced induction of CXCL10 (99). In addition, CD40-ligand (CD40L) stimulation of DCs has been used to mature DCs in clinical trials (100, 103). Binding of CD40 on DCs to CD40L on $CD4^+$ helper T cells licenses DCs and enables them to prime $CD8^+$ effector T cells.

A final major determinant of the vaccine immunogenicity is the choice of antigen to load the DCs. Two main approaches can be applied: loading with selected tumor antigens (tumor-associated antigens or tumor-specific antigens) and loading with whole tumor cell preparations (13). The former strategy enables easier immune monitoring, has a lower risk of inducing auto-immunity, and can provide "off-the-shelf" availability of the antigenic cargo. Whole tumor cell-based DC vaccines, on the other hand, are not HLA-type dependent, have a reduced risk of inducing immune-escape variants, and can elicit immunity against multiple tumor antigens. Meta-analytical data provided by Neller et al. have demonstrated enhanced clinical efficacy in several tumor types of DCs loaded with whole tumor lysate as compared to DCs pulsed with defined tumor antigens (104). This finding was recently also substantiated in high-grade glioma patients, although this study was not set-up to compare survival parameters (105).

TOWARD A MORE IMMUNOGENIC TUMOR CELL CARGO

The majority of clinical trials that apply autologous whole tumor lysate to load DC vaccines report the straightforward use of multiple freeze–thaw cycles to induce primary necrosis of cancer cells (8, 93). Freeze–thaw induced necrosis is, however, considered non-immunogenic and has even been shown to inhibit toll-like receptor (TLR)-induced maturation and function of DCs (16). To this end, many research groups have focused on tackling this roadblock by applying immunogenic modalities to induce cell death.

Immunogenic Treatment Modalities

Tables 1 and **2** list some frequently applied treatment methods to enhance the immunogenic potential of the tumor cell cargo that is used to load DC vaccines in an ICD-independent manner (i.e., these treatments do not meet the molecular and/or cellular determinants of ICD). Immunogenic treatment modalities can positively impact DC biology by inducing particular DAMPs in the dying cancer cells (**Table 1**). **Table 2** lists the preclinical and clinical studies that investigated their *in vivo* potential. **Figure 1** schematically represents the application and the putative modes of action of these immunogenic enhancers in the setting of DC vaccines.

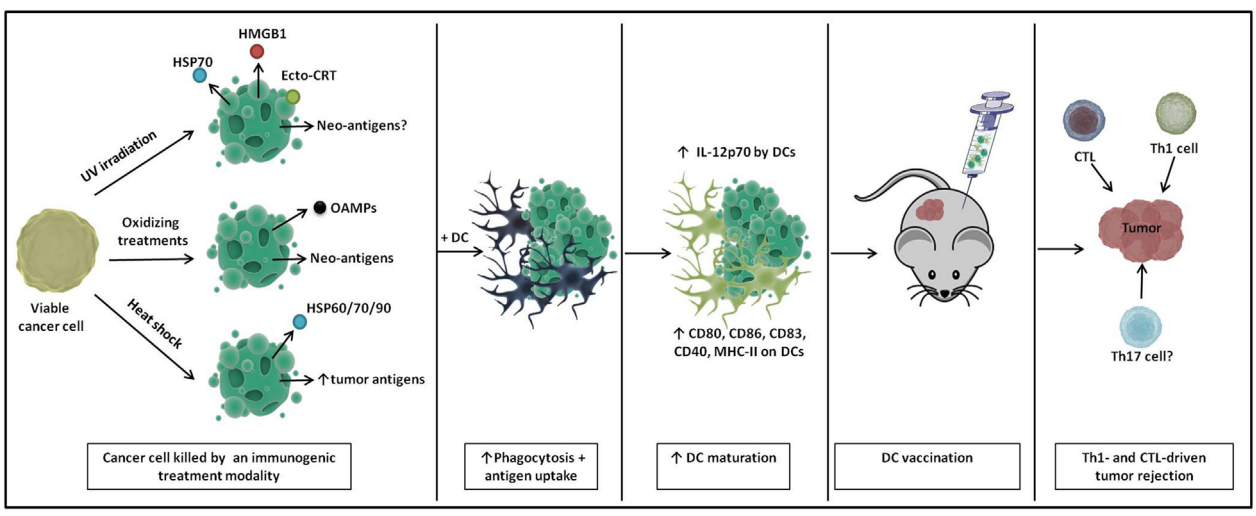

FIGURE 1 | A schematic representation of immunogenic DC vaccines. Cancer cells show enhanced immunogenicity upon treatment with UV irradiation, oxidizing treaments, and heat shock, characterized by the release of particular danger signals and the (increased) production of tumor (neo-)antigens. Upon loading onto DCs, DCs undergo enhanced phagocytosis and antigen uptake and show phenotypic and partial functional maturation. Upon *in vivo* immunization, these DC vaccines elicit Th1- and cytotoxic T lymphocyte (CTL)-driven tumor rejection.

Ultraviolet Irradiation

Ultraviolet (UV) light is considered an electromagnetic non-ionizing radiation with a wavelength between 100 and 400 nm. Its immunogenic potential was discovered in 1991 when Begovic et al. demonstrated that vaccination of immunocompetent mice (but not immunodeficient nude mice) with UV-irradiated cancer cells could induce resistance to subsequent rechallenge with live tumor cells (23, 106, 107). This antitumor effect was crucially mediated by NK cells and $CD8^+$ T cells. UV-treated cancer cells are efficiently engulfed by DCs, leading to phenotypic maturation and increased IL-12 production (19, 24, 26) (**Table 1**). Moreover, these matured DCs in turn stimulated the polarization of T cells toward IFN-γ producing $CD8^+$ T cells (24, 26). Of note, human DCs that had ingested UV-irradiated apoptotic tumor cells were shown to be more effective in generating $CD8^+$ CTLs than DCs pulsed with freeze–thaw lysates (27). In addition, immunization with DCs loaded with UV-treated tumor cells could elicit effective antitumor therapeutic efficacy in a B16 mouse melanoma model, albeit non-superior to DCs loaded with necrotic freeze–thaw lysate (19) (**Table 2**). The induction of specific DAMPs, such as ecto-CRT, and the release of heat shock protein 70 (HSP70) and HMGB1 determines the immunogenicity of UV irradiation (23, 24, 33) (**Table 1**). Moreover, as UV light is known to affect mainly DNA, mutation-induced tumor neo-antigens might also contribute to increasing the host antitumor immune response (108). T cells reactive against mutated neo-antigens are theoretically less susceptible to central and peripheral tolerance. Vaccination with UV-induced tumor neo-antigens might be particularly useful in UV-induced tumors (e.g., cutaneous and uveal melanoma) that might share the *ex vivo* UV-induced tumor neo-antigens. Besides, it has previously been shown that immunization of tumor-bearing mice with mutated melanoma-derived self-antigens can elicit efficient cross-reactive $CD8^+$ T cell responses against multiple non-mutated epitopes of the tumor protein and against the melanoma cells (109). This led to the rejection of established poorly immunogenic B16 melanoma tumors (109). To the best of our knowledge, there are no reports of clinical trials that used UV irradiation as a single treatment for obtaining an antigen source to pulse DC vaccines (**Table 2**). This is probably related to the fact that UV light as a single treatment is not able to induce high levels of cancer cell death in the vaccine, an absolute requirement for clinical translation.

Oxidation-Inducing Modalities

In recent years, an increasing number of data were published concerning the ability of oxidative stress to induce oxidation-associate molecular patterns (OAMPs), such as reactive protein carbonyls and peroxidized phospholipids, which can act as DAMPs (28, 29) (**Table 1**). Protein carbonylation, a surrogate indicator of irreversible protein oxidation, has for instance been shown to improve cancer cell immunogenicity and to facilitate the formation of immunogenic neo-antigens (30, 31).

One prototypical enhancer of oxidation-based immunogenicity is radiotherapy (21, 23). In certain tumor types, such as high-grade glioma and melanoma, clinical trials that apply autologous whole tumor lysate to load DC vaccines report the random use of freeze–thaw cycles (to induce necrosis of cancer cells) or a combination of freeze–thaw cycles and subsequent high-dose γ-irradiation (8, 18) (**Table 2**). However, from the available clinical evidence, it is unclear which of both methodologies has superior immunogenic potential. In light of the oxidation-based immunogenicity that is associated with radiotherapy, we recently demonstrated the superiority of DC vaccines loaded with irradiated freeze–thaw lysate (in comparison to freeze–thaw lysate) in terms of survival advantage in a preclinical high-grade glioma model (18) (**Table 2**). This survival advantage was associated with an increased tumor infiltration of Th1 cells and CTLs and accompanied by a reduced invasion of regulatory cells (Tregs), macrophages, and myeloid-derived suppressor cells. Moreover, this study revealed a significant positive correlation between the level of protein carbonylation – as a measure of the total oxidative content – in the tumor lysates used to load the DCs and the percentage of mice able to reject the aggressive intracranial tumors. Treatment of the tumor lysate with hydrogen peroxide (H_2O_2, a strong oxidant) even induced higher tumor protection than irradiated freeze–thaw lysate, warranting the preclinical investigation of other strong oxidizing modalities to further potentiate the immunogenicity of whole tumor antigen-pulsed DC vaccinations.

In line with these results and through a series of elegant *ex vivo* an *in vivo* mouse experiments, Chiang et al. recently selected hypochlorous acid (HOCl)-based oxidation (to induce primary necrosis of tumor cells) as the method of choice (as compared to UVB irradiation and freeze–thaw cycles) for preparing whole tumor lysate-loaded DC vaccines in the preclinical ID8 ovarian cancer model (14) (**Table 2**). Interestingly, T cells stimulated by DCs loaded with HOCl-induced oxidatively modified tumor cells were still able to recognize non-modified tumor cells, an essential requirement if the cells are to exert antitumor activity (30). In a pilot study containing five recurrent ovarian cancer patients, these autologous DCs loaded with HOCl-oxidized autologous tumor lysate could produce high levels of IL-12, elicited strong antigen-specific T cell responses and reduced the levels of circulating Tregs and serum IL-10 (14). Moreover, two patients experienced durable progression-free survival intervals of more than 24 months after vaccination (**Table 2**).

Heat Shock Treatment

Heat shock is a term that is applied when a cell is subjected to a temperature that is higher than that of the ideal body temperature of the organisms of which the cell is derived. Heat shock can induce apoptosis (41–43°C) or necrosis (>43°C) depending on the temperature that is applied (110). The immunogenicity of heat shock treated cancer cells largely resides within their ability to produce HSPs, such as HSP60, HSP70, and HSP90 (17, 32) (**Table 1**). These HSPs can function as chaperones for tumor antigens, facilitating their cross-presentation (17). Moreover, after recognition by their receptors (CD91, TLR2/4), these HSPs can instigate the attraction of neutrophils and monocytes and the activation of NK cells and DCs (111). These events are crucial for the initiation of tumor-specific immune responses. Independent of the induction of HSPs, heat shock treatment

has also been shown to upregulate the transcription of specific tumor-associated antigens (34).

Co-incubation of heat-stressed apoptotic cancer cells with immature DCs resulted in the upregulation of DC maturation markers (CD40, CD80, and CD86) and higher IL-12 levels (32) (**Table 1**). Interestingly, splenocytes from mice immunized with heat-stressed apoptotic cancer cells got polarized toward a Th1 cytokine profile. Furthermore, DCs loaded with heat shock stressed melanoma cells can efficiently cross-prime tumor-antigen-specific CTLs both *in vitro* and *in vivo* (34). Of note, direct comparison of heat shock treated tumor lysate with freeze–thaw tumor lysate in a DC vaccine setup demonstrated a stronger tumor regression in favor of heat shock lysate in a mouse model for pancreatic cancer (**Table 2**). Again, this was associated with a stronger priming of tumor-specific CTL responses (17).

Dendritic cells loaded with heat shocked cancer cells have already been successfully applied in clinical practice in high-grade glioma patients (**Table 2**). Jie et al. recently published an open labeled non-randomized clinical trial in which 12 newly diagnosed glioblastoma patients received conventional therapy and 13 patients received additional DC vaccines loaded with heat shock treated autologous glioblastoma cells (67). The vaccinated patients had a significantly improved overall survival and progression-free survival. Interestingly, the proportions of peripheral $CD4^+$ T cells, $CD8^+$ T cells, and NK cells were significantly higher after DC vaccination in comparison to the control group. Moreover, increased levels of IFN-γ, IL-2, and IL-12 were measured in the sera of DC vaccinated patients.

All together, these data suggest that an immunogenic treatment of cancer cells can positively impact the potency of DCs interacting with them (**Figure 1**). In light of this finding, the relatively new concept of ICD of cancer cells can be considered a promising strategy for loading DC-based anticancer vaccines, potentially giving rise to a next generation of potent Th1-driving DC vaccines (111, 112) (**Figure 2**).

Inducers of Immunogenic Cell Death

Immunogenic cell death is a cell death regimen that is associated with the spatiotemporally defined emission of immunogenic DAMPs that can trigger the immune system (20, 21, 113). ICD has been found to depend on the concomitant induction of reactive oxygen species (ROS) and activation of endoplasmatic reticulum (ER) stress (111). Besides the three DAMPs that are most crucial for ICD (ecto-CRT, ATP, and HMGB1), other DAMPs such as surface-exposed or released HSPs (notably HSP70 and HSP90) have also been shown to contribute to the immunogenic capacity of ICD inducers (20, 21). The binding of these DAMPs to their respective immune receptors (CD91 for HSPs/CRT, P2RX7/P2RY2 for ATP, and TLR2/4 for HMGB1/HSP70) leads to the recruitment and/or activation of innate immune cells and facilitates the uptake of tumor antigens by antigen-presenting cells and their cross-presentation to T cells eventually leading to IL-1β-, IL-17-, and IFN-γ-dependent tumor eradiation (22). This *in vivo* tumor rejecting capacity induced by dying cancer cells in the absence of any adjuvant, is considered as a prerequisite for an agent to be termed an ICD inducer. Recently, a classification system for ICD inducers was proposed based on whether an ICD inducer triggers apoptotic cell death as a consequence of direct action at the ER (Type II ICD inducer), or whether it initiates both ER stress-dependent danger signaling and apoptosis through divergent mechanisms (Type I ICD inducer) (111).

Although the list of ICD inducers is constantly growing (113), only few of these immunogenic modalities have been tested in order to generate an immunogenic tumor cell cargo to load DC vaccines (**Tables 1** and **2**). **Figure 2** schematically represents the preparation of ICD-based DC vaccines and their putative modes of action.

Radiotherapy

Ionizing X-ray or γ-ray irradiation exerts its anticancer effect predominantly via its capacity to induce DNA double-strand breaks leading to intrinsic cancer cell apoptosis (114). The idea

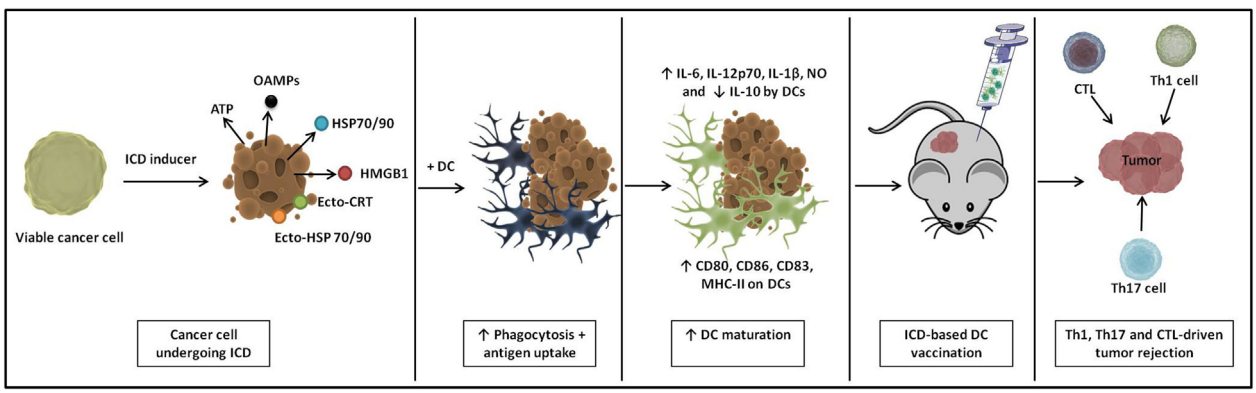

FIGURE 2 | A schematic representation of immunogenic cell death (ICD)-based DC vaccines. ICD causes cancer cells to emit a spatiotemporally defined pattern of danger signals. Upon loading of these ICD-undergoing cancer cells onto DCs, they induce extensive phagocytosis and antigen uptake. Loaded DCs show enhanced phenotypic and functional maturation and immunization with these ICD-based DC vaccines instigates Th1-, Th17-, and cytotoxic T lymphocyte (CTL)-driven antitumor immunity *in vivo*.

that radiotherapy could also impact the immune system was derived from the observation that radiotherapy could induce T-cell-mediated delay of tumor growth in a non-irradiated lesion (115). This abscopal (ab-scopus, away from the target) effect of radiotherapy was later explained by the ICD-inducing capacity (116). Together with anthracyclines, γ-irradiation was one of the first treatment modalities identified to induce ICD. Although this type I ICD inducer is known to induce ROS, its ER stress-inducing capability remains largely unexplored (111). The DAMPs that are induced following radiotherapy treatment of cancer cells include the exposure of ecto-CRT (23, 24, 35) and ecto-HSP70 (36), and the release of HMGB1 (33, 35) (**Table 1**). Irradiated B16 melanoma cells have been shown to be efficiently phagocytosed by DCs and to induce phenotypic DC maturation (15, 37). In addition, human DCs pulsed with irradiated tumor cells could efficiently stimulate antigen-specific CTL responses (40) (**Table 1**). Furthermore, mice immunized with DCs loaded with irradiated cancer cells could efficiently suppress tumor growth following inoculation with live syngeneic tumor cells in multiple preclinical cancer models (15, 40). In this setting, splenocytes from vaccinated animals could efficiently prime $CD4^+$ and $CD8^+$ T cells and exerted antigen-specific cytolytic activity (15) (**Table 2**).

Dendritic cell vaccines exposed to irradiated cancer cells have also been successfully implemented in clinical practice in melanoma and HGG patients (8, 69, 70) (**Table 2**). Cho et al. have shown that the implementation of DC vaccines loaded with irradiated autologous tumor cells in the conventional treatment regimen of newly diagnosed glioblastoma patients could significantly prolong the median overall survival (by more than 15 months) as compared to a control group receiving solely conventional treatment (69). Interestingly, the group of Di Nicola reported that vaccination with DCs loaded with dying autologous tumor cells after exposure to a cell death protocol consisting of heat shock, γ-ray, and UV ray could elicit clinical responses in 6 out of 18 relapsed B-cell lymphoma patients (117). Later, they showed the impaired ability of the neoplastic cells used to vaccinate non-responders to undergo ICD upon exposure to the cell death protocol (57). Importantly, they revealed a positive association between the extent of CRT and HSP90 surface expression in the DC antigenic cargo and the clinical and immunological responses achieved (57).

Shikonin

The phytochemical shikonin, a major component of Chinese herbal medicine, is known to inhibit proteasome activity. It serves multiple biological roles and can be applied as an antibacterial, antiviral, anti-inflammatory, and anticancer treatment. The latter application has been shown to yield responsiveness in late-stage lung cancer patients (118). Apoptotic cell death elicited by this type I ICD inducer can be inhibited by anti-oxidants, suggesting a role of shikonin-induced ROS (119, 120). The link between shikonin treatment and ER stress is not evidenced yet. The ICD that is induced in shikonin-treated cancer cells is characterized by the early induction of HSP70, HSP90, GRP78, and HMGB1 (41) (**Table 1**). Importantly, shikonin treatment could significantly improve the survival of mice bearing P388 leukemia and this antitumor effect of shikonin was less pronounced in immunodeficient mice (120). Moreover, the tumor lysate from shikonin-treated B16 cells could enhance phenotypic and functional DC maturation and differentiation of Th1 and Th17 cells, two important features of ICD-associated antitumor immunity (41) (**Table 1**). Additionally, curative vaccination of B16 melanoma-inoculated mice with shikonin-lysate-loaded DCs could delay tumor growth (41). This was associated with increased cytolytic activity of splenocytes on target tumor cells (**Table 2**). Although shikonin is administered to breast cancer patients for observational application (NCT01287468), clinical experience evaluating shikonin-lysate-loaded DC vaccines is unfortunately still lacking (**Table 2**).

High-Hydrostatic Pressure

High-hydrostatic pressure (HHP) is an established method to sterilize pharmaceuticals, human transplants, and food. HHP between 100 and 250 megapascal (MPa) has been shown to induce apoptosis of murine and human (cancer) cells (121–123). While DNA damage does not seem to be induced by HHP <1000 MPa, HHP can inhibit enzymatic functions and the synthesis of cellular proteins (122). Increased ROS production was detected in HHP-treated cancer cell lines and ER stress was evidenced by the rapid phosphorylation of eIF2α (42).

The anticancer activity of HHP was already demonstrated more than four decades ago in bladder cancer patients (124). Later, preclinical experiments demonstrated *in vivo* immunogenicity of HHP-treated cancer cells in the B16 melanoma model and the 3LL-D122 lung metastasis model (125, 126). Subsequently, it was shown that HHP-treated mammalian cancer cell lines undergoing apoptosis can release HSP70 and HMGB1, while retaining their immunogenicity *in vivo* (127). Very recently, Fucikova and colleagues have shown the ability of HHP to induce prototypical ICD in human prostate and ovarian cancer cell lines and in acute leukemia cells (42). HHP treatment induced the rapid expression of ecto-HSP70, ecto-HSP90, and ecto-CRT and the release of HMGB1 and ATP (**Table 1**). Interestingly, HHP-treated cancer cells were rapidly phagocytosed by DCs and induced the upregulation of CD83, CD86, and HLA-DR, and the release of pro-inflammatory cytokines (**Table 1**). This led to the stimulation of high numbers of tumor-specific T cells without inducing Tregs. Hence, all ICD-associated molecular criteria are fulfilled for HHP. This group is currently testing the *in vivo* immunogenicity of HHP killed tumor cells in prophylactic and curative murine vaccination settings (**Table 2**). Moreover, they have initiated multiple clinical trials to evaluate the potential of DC vaccines loaded with HHP-treated cancer cells in ovarian and prostate cancer patients (71).

Oncolytic Viruses

Oncolytic viruses are self-replicating, tumor selective virus strains that can directly lyse tumor cells. Over the past few years, a new oncolytic paradigm has risen; entailing that, rather than utilizing oncolytic viruses solely for direct tumor eradication, the cell death they induce should be accompanied by the elicitation of antitumor immune responses to maximize their therapeutic efficacy (128). One way in which these oncolytic viruses can fulfill this oncolytic paradigm is by inducing ICD (128).

Thus far, three oncolytic virus strains can meet the molecular requirements of ICD; coxsackievirus B3 (CVB3), oncolytic adenovirus and Newcastle disease virus (NDV) (**Table 1**) (113). Infection of tumor cells with these viruses causes the production of viral envelop proteins that induce ER stress by overloading the ER. Hence, all three virus strains can be considered type II ICD inducers (113). While CVB3 and oncolytic adenoviruses induce the surface expression of CRT, followed by the release of ATP and the passive release of HMGB1 in apoptotic tumor cells (in non-small cell lung carcinoma and adenocarcinoma cells, respectively) (43, 44), NDV induces necroptosis accompanied by the surface exposure of ATP and the post-necroptotic release of HMGB1 in GL261 glioma cells, with no contribution of ATP (**Table 1**) (45). In addition, NDV-infected GL261 cells upregulated the expression of the PMEL17 tumor antigen (45).

Intratumoral administration of CVB3 in nude mice resulted in the marked infiltration of NK cells, macrophages, granulocytes, and mature DCs into the tumor tissue (**Table 1**) (44). Tumor-infiltrating DCs expressed significantly higher levels of costimulatory molecules CD80 and CD86, as well as the lymph node homing receptor CCR7 (44). CD40-ligand encoding oncolytic adenoviruses have also been shown to facilitate the recruitment of DCs to the tumor tissue, this way entailing efficient Th1 and $CD8^+$ CTL responses (**Table 1**) (43). Measles virus is another oncolytic virus that requires further investigation. Although extensive analysis of *in vitro* ICD determinants is lacking for this virus (only the release of HMGB1 has been documented), DCs exposed *in vitro* to measles-virus treated melanoma cells showed increased CD80 and CD86 expression levels (**Table 1**) (46). This resulted in the efficient priming of melanoma-specific cell killing by IFN-γ producing $CD8^+$ T cells. Moreover, in terms of priming these melanoma-specific CTL responses, measles virus-infected melanoma cells constituted more effective tumor lysates (also termed oncolysates) for loading of DCs than uninfected melanoma cell lysates (46). The DC stimulatory capacity of NDV-derived oncolysates has already been demonstrated more than a decade ago by Schirrmacher et al. (47). DCs derived from breast cancer patients pulsed with NDV-oncolysates showed increased expression of costimulatory molecules in comparison to DCs loaded with tumor lysate from non-infected breast carcinoma cells (**Table 1**) (47). In addition, NDV-oncolysate-loaded DCs were more effective in stimulating bone-marrow-derived reactive memory T cells *in vitro* (47).

Oncolytic viruses hold great potential for application in ICD-based DC vaccines given their potential to elicit several ICD-related DAMPs. Furthermore, these viruses might directly affect DC maturation and activation through interaction with pathogen recognition receptors on the tumor cells. This way, biological oncolysates may render the use of an artificial maturation cocktail otiose. Unfortunately, there are no preclinical *in vivo* data available yet to evince the efficacy of DC vaccines loaded with immunogenic oncolysates (**Table 2**). Nevertheless, several studies have documented the beneficial effect of intratumoral application of oncolytic viruses in combination with tumor-directed systemic DC vaccinations (72, 73). Very recently, Schirrmacher et al. disclosed a case report of a breast cancer patient with liver metastasis that was treated with local hyperthermia, intravenously administered NDV, and subcutaneous vaccination with DCs loaded with NDV-infected breast cancer cells (oncolysate) (74). This combination therapy led to long-lasting tumor-specific memory T cell responses and stable disease for more than 66 months in this particular patient. The use of autologous DCs loaded with NDV-mediated oncolysate is licensed by the Paul Ehrlich Institute to the Immunologic-Oncologic Centre Cologne (IOZK) since May 2015.

Of note, in October 2015, the FDA approved the first oncolytic virus, Imlygic (a genetically modified live oncolytic herpes virus) for the treatment of melanoma lesions in the skin and lymph nodes. This FDA approval should facilitate the approval of other oncolytic viruses as well as the application of oncolysates in DC vaccine settings.

Photodynamic Therapy

Photodynamic therapy (PDT) is an established, minimally invasive anticancer treatment modality. It has a two-step mode of action involving the selective uptake of a photosensitizer by the tumor tissue, followed by its activation by light of a specific wavelength. This activation results in the photochemical production of ROS in the presence of oxygen (129–131). One attractive feature of PDT is that the ROS-based oxidative stress originates in the particular subcellular location where the photosensitizer tends to accumulate, ultimately leading to the destruction of the tumor cell (132). PDT-based antitumor effects are multifactorial and depend on its abilities to damage the tumor vasculature, directly kill tumor cells, exert cytotoxic effects toward tumor-infiltrating immune cells, and recruit and activate immune cells that can initiate adaptive antitumor immune responses (131).

Increasing preclinical information is available regarding the impact of PDT on the immune system. Recent studies have demonstrated that PDT can effectively generate several DAMPs. HSP70, the best studied DAMP associated with PDT, is exposed on the surface of cancer cells treated with photofrin-PDT, 5-Aminolevulinic acid (5-ALA)-PDT, and Foscan-PDT (51, 133, 134). Of note, the uptake of tumor antigens and DC maturation induced by 5-ALA-PDT treated GBM spheroids were inhibited when HSP70 was blocked (133). Later, it was reported that photofrin-PDT also promotes the early/mid-apoptotic surface expression of CRT and the post-apoptotic release of HMGB1 (52) (**Table 1**). Very recently, the DAMPs profile induced by Rose Bengal Acetate (RBAc)-based PDT was unraveled. RBAc-photosensitized apoptotic/autophagic Hela cells were found to expose and/or release ATP, HSP70/90, HMGB1, and CRT (135). In terms of its immunogenicity, hypericin can be considered the best studied photosensitizer. Recently, hypericin-PDT became the first PDT modality capable of inducing prototypical ICD in cancer cells (20, 48, 49, 111). Hypericin localizes predominantly in the ER and upon irradiation it causes photo-oxidative ER stress, making hypericin-PDT the only known modality able to induce ICD through focused ROS-based ER stress (Type II ICD inducer), eventually culminating in mitochondrial apoptosis (49, 136). In the pre-apoptotic stage, it induces the active emission of three crucial ICD-associated DAMPs, i.e., ecto-CRT, ecto-HSP, and secreted ATP (at a faster rate than what was previously published for these DAMPs), followed by the passive release of

HSP70 and HMGB1 (48, 49) (**Table 1**). Interestingly, this ICD-subroutine was more effective in comparison to chemotherapy- or radiotherapy-induced ICD (48, 49).

The immunogenic features of Hyp-PDT–treated cancer cells have also been confirmed by *ex vivo* and *in vivo* experiments (**Tables 1** and **2**). Hyp-PDT-treated cancer cells form a productive interface with DCs in terms of phagocytosis (CRT-dependent) and maturation (49) (**Table 1**). More specifically, the interacting DCs exhibit functional stimulation (NO^{high}, $IL\text{-}10^{absent}$, $IL\text{-}6^{high}$, $IL\text{-}1\beta^{high}$, and $IL\text{-}12p70^{median}$) and phenotypic maturation ($CD80^{high}$, $CD83^{high}$, $CD86^{high}$, and $MHC\text{-}II^{high}$) (49, 53). Moreover, these immunogenic and fully mature DCs induce the clonal expansion of human IFN-γ producing $CD4^+$ and $CD8^+$ T cells (53, 54). Consequently, this *in vitro* antitumor immunity induced by Hyp-PDT-induced ICD led to the efficient rejection of murine tumors *in vivo* in the absence of any adjuvants (both in prophylactic and curative vaccination models) (49, 137). Besides hypericin-based PDT, photofrin-based PDT is to date the only PDT modality that is capable to fulfill this critical *in vivo* requirement for ICD characterization. Here, curative immunization with benzoporphyrin-based PDT-treated squamous cell carcinoma cells constituted a potent anticancer vaccine in this poorly immunogenic model (56).

Importantly, inoculation of mature DCs in PDT-treated tumors resulted in the cytolytic activation of T cells and NK cells, leading to effective tumor eradication (75). Moreover, DC vaccines loaded with PDT-induced tumor lysates have been shown to cure fully established solid non-orthotopic tumors. This was associated with enhanced CTL responses and Th1 immunity (138) (**Table 2**). These data already suggest the clinical potential of PDT-based DC vaccines. In this regard, Hyp-PDT-induced ICD-based DC vaccines are currently being tested in a preclinical model for ovarian cancer by Baert et al. (personal communication). Unfortunately, there are no clinical data available yet reporting the use of PDT-based DC vaccines.

Combinatorial Regimens

In DC vaccine settings, cancer cells are often not killed by a single treatment strategy but rather by a combination of treatments. In some cases, the underlying rationale lies within the additive or even synergistic value of combining several moderately immunogenic modalities. The combination of radiotherapy and heat shock has, for instance, been shown to induce higher levels of HSP70 in B16 melanoma cells than either therapy alone (16). In addition, a combination therapy consisting of heat shock, γ-irradiation, and UV irradiation has been shown to induce higher levels of ecto-CRT, ecto-HSP90, HMGB1, and ATP in comparison to either therapy alone or doxorubicin, a well-recognized inducer of ICD (57). Besides, the sequence of the applied methodologies seems to matter. The application of radiotherapy prior to freeze–thaw cycles was recently shown to negatively impacted the survival of high-grade glioma-bearing mice (in comparison to freeze–thaw cycles followed by X-ray irradiation) in the context of DC-based immunotherapy (18). A second rationale for combining several cell killing methodologies is to meet the clinical requirement of reaching 100% cancer cell death (14). Subcutaneous injection of irradiated tumor cells has, for instance, induced subcutaneous tumor growth in one glioblastoma patient (139). In general, most single treatment modalities discussed in this review cannot meet this requirement, postulating their combination with other (potentially less immunogenic) cell death modalities. In view of this, preclinical testing should always consider the most clinically relevant version of the vaccine.

CONCLUDING REMARKS

Triggering antitumor immune responses is an absolute requirement to tackle metastatic and diffusely infiltrating cancer cells that are resistant to standard-of-care therapeutic regimens. ICD-inducing modalities, such as PDT and radiotherapy, have been shown to be able to act as *in situ* vaccines capable of inducing immune responses that caused regression of distal untreated tumors. Exploiting these ICD inducers and other immunogenic modalities to obtain a highly immunogenic antigenic tumor cell cargo for loading DC vaccines is a highly promising application. In case of the two prominent ICD inducers, Hyp-PDT and HHP, preclinical studies evaluating this relatively new approach are underway and HHP-based DC vaccines are already undergoing clinical testing. In the preclinical testing phase, more attention should be paid to some clinically driven considerations. First, one should consider the requirement of 100% mortality of the tumor cells before *in vivo* application. A second consideration from clinical practice (especially in multi-center clinical trials) is the fact that most tumor specimens arrive in the lab in a frozen state. This implies that a significant number of cells have already undergone non-immunogenic necrosis before the experimental cell killing strategies are applied. In case of ICD inducers, this could potentially hamper the immunogenicity of the tumor cells as these modalities mainly rely on active danger signaling pathways. Finally, for a more clinically relevant evaluation of the effect of immunogenic DC vaccines on tumor cell stromal interactions, orthotopic tumor inoculation should be applied. As tumor cells are implanted in the anatomically appropriate location, orthotopic tumors reflect the clinical situation (e.g., the tumor microenvironment) much better than conventional subcutaneous non-orthotopic models.

Even the most potent active immunotherapeutic strategies such as (ICD-based) DC vaccines will, however, be hampered by the presence of immunomodulatory immune checkpoint molecules (such as PD-1 and CTLA-4) that inhibit cytotoxic immune responses or even induce immune tolerance. The development of drugs that can unleash these inhibitory molecules has become one of the most active areas in oncology. This creates the opportunity to combine checkpoint inhibitors with DC-based immunotherapy. The synergistic action of a CTLA-4 blocking Ab (tremelimumab) in combination with DC therapy has already been demonstrated in advanced melanoma patients and several other trials evaluating this approach are on the horizon (6, 140, 141).

We believe that the specialty of DC-based immunotherapy is considerably moving forward by focusing on developing more immunogenic Th1-driving vaccines, such as ICD-based DC

vaccines. Moreover, the combination of ICD-based DC vaccines with checkpoint inhibitors or other drugs that can inhibit the severe tumor-induced immune suppression might be able to reveal the full efficacy of DC-based immunotherapy for cancer.

AUTHOR CONTRIBUTIONS

LV did the literature study, data collection, and wrote the manuscript. SV provided senior supervision, helped in writing, and critically revised the manuscript. All the other co-authors have substantially contributed to the design of the work. All co-authors critically revised the manuscript and approved the final manuscript. All co-authors can be considered accountable for all aspects of the work.

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Lymphoma immunotherapy: current status

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The rationale to treat lymphomas with immunotherapy comes from long-standing evidence on their distinctive immune responsiveness. Indolent B-cell non-Hodgkin lymphomas, in particular, establish key interactions with the immune microenvironment to ensure prosurvival signals and prevent antitumor immune activation. However, reports of spontaneous regressions indicate that, under certain circumstances, patients develop therapeutic antitumor immunity. Several immunotherapeutic approaches have been thus developed to boost these effects in all patients. To date, targeting CD20 on malignant B cells with the antibody rituximab has been the most clinically effective strategy. However, relapse and resistance prevent to cure approximately half of B-NHL patients, underscoring the need of more effective therapies. The recognition of B-cell receptor variable regions as B-NHL unique antigens promoted the development of specific vaccines to immunize patients against their own tumor. Despite initial promising results, this strategy has not yet demonstrated a sufficient clinical benefit to reach the regulatory approval. Several novel agents are now available to stimulate immune effector functions or counteract immunosuppressive mechanisms, such as engineered antitumor T cells, co-stimulatory receptor agonist, and immune checkpoint-blocking antibodies. Thus, multiple elements can now be exploited in more effective combinations to break the barriers for the induction of anti-lymphoma immunity.

Keywords: B-cell lymphoma, immunotherapy, anticancer vaccines, tumor-associated antigens, dendritic cells, adaptive immune response

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Introduction

Lymphomas are a clinically and biologically heterogeneous group of malignancies that arise from mature T- or B-lymphocytes in secondary lymphoid organs. Hodgkin's lymphomas (HLs) account for ~10% of all lymphomas and comprise two major disease categories based on their clinical and histological characteristics: classical HLs, which represent the majority of the cases, and nodular lymphocyte predominant HLs. Non-Hodgkin lymphomas (NHLs) instead are much more frequent diseases, representing the fifth most common cancer in the United States. Their incidence has progressively increased in the past three decades for non-completely certain reasons (1). About 85% of NHLs are of B-cell origin (B-NHLs) and includes a wide spectrum of malignancies with different clinical and biological courses, ranging from indolent [such as chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL), follicular lymphoma (FL), and marginal-zone lymphoma (MZL)] to aggressive [such as diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma (BL), and mantle cell lymphoma (MCL)].

These tumors, in particular the aggressive forms, are highly sensitive to both chemotherapy and radiotherapy (2); however, relapse and resistance prevent the ultimate goal of achieving a cure in all patients. In the last few decades, the introduction of improved chemotherapy regimens, monoclonal antibodies (mAbs), radioimmunotherapy, and targeted therapies against pro-lymphoma pathways have provided significant advances in the management of these patients, in particular those with B-NHLs. The chimeric anti-CD20 mAb rituximab has been the most valuable addition to the B-NHL treatment armamentarium. Its combination with poly-chemotherapy still represents the standard therapy for both indolent and aggressive B-cell lymphomas (3, 4). However, difficulties in the management of relapse and resistance to rituximab (5, 6) and the late toxicities associated with its administration (7) still pose significant challenges. Alternative approaches are thus continuously sought to ameliorate the management and the clinical outcome of the many patients that become resistant to rituximab.

In the past 20 years, the understanding of the molecular basis of B-cell lymphomagenesis and the role of the lymphoma microenvironment has significantly progressed, thus underscoring multiple novel rational therapeutic modalities for B-cell malignancies.

B-cell maturation is dictated by a series of steps that drive the development of a functional B-cell receptor (BCR) with the same antigen specificity as the secreted Abs that B cells will eventually produce. A BCR is composed of two clonally variable antigen-binding chains (heavy and light chains) codified by several different gene segments (V, variable; D, diversity; J, joining; C, constant), which need to be properly rearranged to produce a functional antigen-binding receptor. This occurs via an error-prone process involving the combinatorial rearrangement of the V, D, and J gene segments in the heavy (H) chain locus and the V and J gene segments in the light (L) chain loci. Mature (naïve) B cells carry a BCR composed of two identical heavy chain and two identical light-chain immunoglobulin (Ig) polypeptides covalently linked (8). Antigen recognition by naïve B cells favors their recruitment into lymphoid follicles where they undergo somatic hypermutation of V genes, to increase the affinity for the targeted antigenic epitopes, and class switch recombination at the IgH locus, for the production of different classes of Ab (from IgM to IgG, IgA, or IgE). These processes form the germinal center (GC) reactions, whereby new B-cell clones expressing Abs with improved antigen specificity and suitable class are positively selected by receiving the proper survival signals from follicular dendritic cells (DCs) presenting the pathogenic antigens and helper T cells (9). If, on one hand, these events are required to increase the probability of generating a specific B-cell response able to clear infecting pathogens, on the other, they pose at risk of developing oncogenic mutations. DNA rearrangement, induction of somatic mutation, and provision of anti-apoptotic/pro-survival signals from the microenvironment during the B-cell maturation process may all favor the generation of a malignant B-cell clone if not tightly regulated. Reciprocal chromosomal translocations involving one of the Ig loci and a proto-oncogene, which may occur as by-products of the extensive DNA rearrangement during the GC reactions, constitute the hallmarks, and thus diagnostic markers, of many types of B-cell lymphoma (10, 11) (**Table 1**).

Mutations in pro-apoptotic genes (CD95), tumor-suppressor genes (TP53, PTEN), BCR downstream signaling pathways (CD79B/A, IκBα, CARD11, API2–MALT1 translocation) and other oncogenes (EZH2, Jak2, genomic amplifications REL) are associated to specific subtypes of B-cell lymphomas, indicating a role of these events in their pathogenesis (**Table 1**).

Finally, viruses may also be involved in lymphoma transformation, in particular Epstein–Barr virus (EBV), Kaposi's sarcoma herpesvirus (KSHV), human immunodeficiency virus type 1 (HIV-1), and human hepatitis C virus (HCV). They can directly infect and transform B or T cells (EBV and KSHV), or induce lymphocyte transformation as a consequence of chronic inflammation (HIV, EBV, and hepatitis viruses), or, more indirectly, promote the onset of neoplastic clones by causing immunodeficiency (HIV-1) (12). The most obvious example in this regard is EBV, which is found in nearly all the endemic BLs and in many post-transplant and primary effusion lymphomas (13, 14).

The possibility to interfere with ongenic pathways activated in the different subtypes of B-cell lymphomas has been an area of intense investigation, with two molecular inhibitors targeting bruton tyrosine kinase (ibrutininb) (15–17) or PI3K (idelalisib) (18) receiving the FDA approval for the treatment of relapsed/resistant B-NHLs in the last 2 years. However, since these therapies target oncogenic events associated to specific molecular lymphoma subtypes, they are unlikely to be available for all rituximab-resistant patients, and imply the requirement of an up-front extensive molecular characterization. In addition, being directed against a single molecular target, these drugs may induce the selection of resistant clones. This indicates the need to integrate anti-lymphoma treatments in multicombinatorial therapeutic approaches, which employ different strategies to reach the desired improvement in clinical benefit.

Immunotherapy seems one of the best candidates because of the easy accessibility of lymphomas by the immune system as they grow in secondary lymphoid organs and the availability of unique targetable tumor-specific antigens. The major advantage of immunotherapy is the possibility to induce an adaptive immune response against the tumor, with the potential to generate a long-lasting immunological memory able to prevent further relapses. Carrying the same BCR on the surface, B-cell lymphomas are distinguished by the unique antigenic determinants of BCR hypervariable regions, termed idiotype (Id), which constitutes a prototype immunotherapeutic target to specifically redirect immune responses against the malignant clone. The clonotypic Id of B-cell malignancies was indeed the first identified tumor-specific antigen able to elicit a T-cell response (19, 20). The crucial interactions between lymphoma cells and the immune microenvironment for their maintenance and progression, in particular in the case of HL and indolent B-NHLs (21), have underscored other potential immunotherapeutic targets. Tumor-infiltrating immune cells, including T lymphocytes, macrophages and DCs, can provide survival signals for malignant B cells (22, 23). As an example, FL growth strictly depends on stromal cells, such as follicular DCs, which provide anti-apoptotic signals through CD40 (24, 25). On the other hand, T regulatory cells (Tregs) (26–28)

TABLE 1 | B-cell lymphoma classification.

Lymphoma	Frequency among lymphoma (%)	Proposed cellular origin	Chromosome translocation (frequency)	Tumor-suppressor gene mutation (frequency)	Viruses (frequency)	Other alterations (frequency)
cHL	9	GC B cells	–	SOCS1 (40), NFKBIA and NFKBIE (10–20), A20 (40)	EBV (40)	Mutation of multiple oncogenes, including REL (30), JAK2 (20), NIK (25)
NLPHL	1	GC B cells	–		EBV	
B-CLL	7	CD5+ small memory, naive, or marginal-zone B cells	–	ATM (30), TP53 (15)	–	Deletion on 13q14 (60)
MCL	5	CD5+ mantle-zone B cells	CCND1-IgH (95)	ATM (40)	–	Deletion on 13q14 (50–70)
FL	20	GC B cells	BCL2-IgH (90)	–	–	–
MALT	7	Marginal-zone B cells	API2-MALT1 (30), BCL10-IgH (5), MALT1-IgH (15–20), FOXP1-IgH (10)	CD95 (5–80)	Indirect role of Helicobacter Pylori in gastric MALT lymphomas	–
MZL	2	Marginal-zone or monocytoid B cells	–	–	–	–
Splenic MZL	1	Small IgD+ naive marginal-zone B cells	–	–	–	Deletion on 7q22–36 (40)
BL	2	GC B cells	MYC-IgH or MYC-IgL (100)	TP53 (40), RB (20–80)	EBV (endemic, 95; sporadic, 30)	–
DLBCL	30–40	Post-GC B cells	BCL6–various (35) BCL2-IgH (15–30) MYC-IgH or MYC-IgL (15)	CD95 (10–20), ATM (15), TP53 (25)	–	Aberrant hypermutation of multiple proto-oncogenes (50)
Primary mediastinal B-cell lymphoma	2	Thymic B cells	–	SOCS1 (40)	–	Mutation of multiple proto-oncogenes (40)
Post-transplant lymphoma	<1	GC B cells	–	–	EBV (90)	–
Primary effusion lymphoma	<0.5	(Post) GC B cells	–	–	HHV8 (95), EBV (70)	–
LPL; Waldenstrom's disease	1	(Post) GC B cells	PAX5-IgH (50)	–	–	–

cHL, classical Hodgkin's lymphoma; NLPHL, nodular lymphocyte predominant Hodgkin's lymphoma; B-CLL, B-cell chronic lymphocytic leukemia; MCL, mantle-cell lymphoma; FL, follicular lymphoma; MALT, mucosa associated lymphatic tissue lymphoma; MZL, marginal zone lymphoma; BL, Burkitt's lymphoma; DLBCL, diffuse large B-cell lymphoma; LPL, lymphoplasmacytic lymphoma; GC, germinal center.

and immunosuppressive lymphoma-associated macrophages (29–31) can contribute to lymphoma growth by dampening the immune system attack. Therefore, acting on the immune microenvironment can also be exploited as a rational anti-lymphoma immunotherapeutic treatment.

The following sections review the most important and recent advances in anti-lymphoma immunotherapy, with a particular focus on strategies exploiting the T-cell arm of the immune response against B-NHLs.

Anti-Lymphoma Immunotherapy

Anticancer immunotherapy is aimed at eradicating tumor cells by conferring either a passive or an active specific immunity with less toxic effects than using conventional anticancer agents. Passive immunotherapy is meant to supply the immune response through the infusion of tumor-specific mAbs or cytotoxic T cells (CTLs), with the major limitation that it may be short-lived. Active immunotherapy instead is thought to stimulate an endogenous immune response to clear neoplastic cells and induce a specific immunological memory that controls disease recurrence, and thus represents an ideal immunotherapeutic modality. More recently, thanks to the development of immunomodulatory agents, a new area of immunotherapy has started to be explored with the aim to induce and/or sustain endogenous antitumor immune responses, providing substantial clinical results.

B-NHLs, in particular the indolent forms, represent one of the most suitable settings for immunotherapeutic interventions. They have long been regarded as highly immune sensitive diseases, based on the detection of lymphoma-specific CTLs in B-NHL patients (32) and reports of spontaneous regressions in 10–20% of the low-grade cases. Moreover, the course of indolent lymphomas leaves an optimal therapeutic window to study immunotherapy without affecting the standard of care of these

patients, given that immunotherapy, relying on endogenous immune system functions, may require longer periods of time to induce a therapeutic effect.

In the last two decades, a number of immune-based treatments have been developed and tested in B-NHL patients. To date, the use of mAbs directed against B-NHL antigens has produced the most convincing results, with rituximab being the prototype example in this treatment category. The introduction of rituximab-based chemoimmunotherapy has improved the overall survival (OS) of indolent lymphoma patients, providing a change in the natural history of these diseases (33, 34). However, resistance to rituximab remains a problem (35) and more effective regimens are still needed. MAbs for new lymphoma targets as well as new generation Abs are thus being developed with the aim to further ameliorate patients' outcome.

Patient-specific vaccines targeting the clonally derived Ig-Id protein or the whole antigenic tumor repertoires have been largely tested against B-NHLs, with certain degrees of success also in severely pretreated patients (36). Furthermore, on the basis of the high sensitivity of these diseases to graft-versus-tumor effects after allogeneic bone marrow transplantation/donor lymphocytes infusions (DLIs), adoptive transfer of tumor-specific CTLs has been also used in lymphoma patients (37, 38). Building upon these findings, lymphoma-specific chimeric antigen receptor (CAR)-engineered T cells are now being explored for the treatment of lymphoma patients with very promising results (39). Finally, the availability of immune checkpoint-blocking agents (40, 41) now allows the opportunity to counteract immune tolerant mechanisms, which are considered the major obstacle to the efficacy of anticancer immunotherapy, and to explore potentially more effective immunotherapeutic combinations against B-NHLs.

Active Immunotherapy for B-Cell Lymphomas

The availability of a tumor-specific antigen in B-NHLs enabled the development of specific vaccines. Id immunodominant peptides or the whole Id determinants have been extensively used to vaccinate patients as protein- or DNA-based vaccines or loaded into DCs (**Figure 1**) (36). Different types of carriers and immune adjuvants have been combined with these vaccines to potentiate the activation of an immune response against a self-antigen. As an alternative strategy to reduce the complexity of the production of patient-specific Id and widen the spectrum of targeted tumor-associated antigens (TAAs), vaccines based on the whole lymphoma proteome have been investigated. Whereas protein- and DNA-based vaccines are designed to target DCs *in vivo*, whole tumor cell antigens have been usually loaded into DCs *ex vivo*, with the advantage to select/generate the most suitable source of DCs able to efficiently present TAAs and activate an immune response *in vivo* upon injection (**Figure 1**).

Protein-Based Vaccines

Anti-Id vaccines have used Id proteins produced by either somatic hybridization of tumor cells with a myeloma cell line (hybridoma), or recombinant technology, by cloning Ig genes into stable cell lines (36). The latter strategy is faster, taking 1 month, but in contrast to the hybridoma technology, the Id glycosylation pattern, and in turn immunogenicity, is strictly dependent on the origin of the cell line used (42). The capability of the Id vaccination to induce tumor protection was extensively demonstrated in plasmacytoma, myeloma, B-cell lymphoma, and leukemia preclinical models (36). Being *per se* a weakly immunogenic protein, the Id was conjugated to the carrier protein keyhole limpet hemocyanin (KLH) and co-administrated with low-dose granulocyte-macrophage colony-stimulating factor (GM-CSF). This strategy demonstrated to promote anti-Id B- and T-cell responses associated with therapeutic effects in animals with low tumor burden (36), and paved the way for the clinical evaluation of anti-Id vaccination.

Early-phase clinical studies were performed in indolent B-NHL patients in clinical remission after standard chemotherapy regimens, using Id proteins produced either by hybridoma or recombinant technology, conjugated with KLH and co-administered with low-dose GM-CFS or Syntex adjuvant formulation (43). These studies demonstrated the feasibility of producing patient-specific Id-vaccines, and the safety and efficacy of this strategy to induce anti-lymphoma immune responses, eventually associated with an improved clinical outcome (43). In line with the preclinical results, the co-administration of low-dose GM-CSF with Id-KLH showed to promote anti-Id T-cell responses and molecular remissions in patients with minimal residual disease after prednisone, doxorubicin, cyclophosphamide, and etoposide (PACE) induction therapy (44). In a following trial, anti-Id vaccination after cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP)-like second-line induction therapy resulted in longer clinical remissions compared to those achieved in the same patients by the front-line standard therapy (45). Interestingly, patients mounting either an Ab or a T-cell anti-Id response after vaccination experienced the longest second complete remission, providing the first in-human evidence of the association between vaccine-specific immune responses and clinical efficacy. A more recent retrospective study demonstrated that achieving a complete response/complete response unconfirmed (CR/CRu) to induction chemotherapy and developing anti-Id Abs were two independent factors that each correlated with longer OS at 10 years after vaccination (46). This study included FL patients who received vaccines produced by either the hybridoma or recombinant technology in both mammalian cells and in tobacco plants. Interestingly, the probability of developing an anti-Id immunity was not influenced by the method of vaccine generation, although in patients vaccinated with hybridoma-derived Id, the rate of specific T-cell responses trended to be higher and the correlation between anti-Id Ab responses and OS resulted particularly significant (46). This is probably due to the presence of a more physiological glycosylation pattern in the hybridoma-derived Id, which may improve the immunogenicity of the Id. Given the critical role of the induction of anti-Id immune responses for the therapeutic efficacy of Id vaccination, two clinical trials with Id-KLH + GM-CSF explored the impact of B-cell depletion by rituximab as part of the induction therapy before vaccination. Importantly, they showed that, even if delayed, Id-specific Ab responses could be equally achieved, whereas the induction of antitumor T-cell immunity was not affected (47, 48). Remarkably,

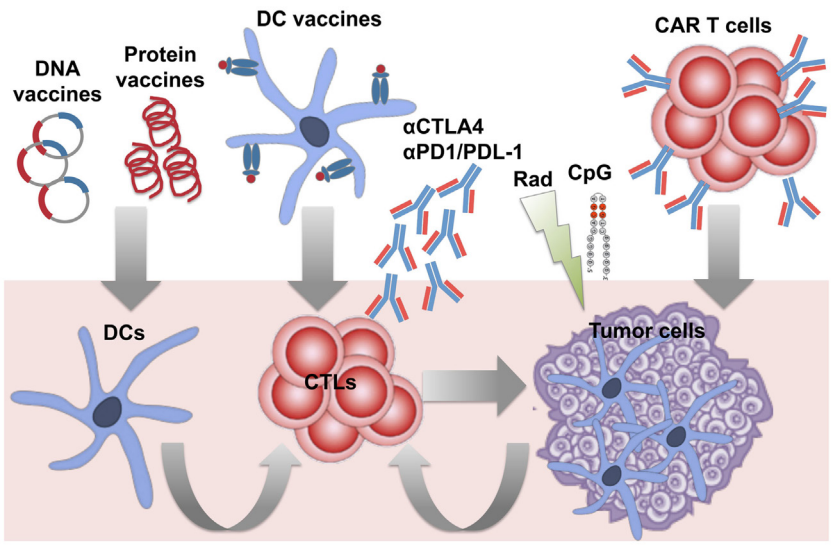

FIGURE 1 | Immunotherapeutic strategies under investigation against B-cell lymphomas. Several approaches have been developed to induce therapeutic anti-lymphoma T-cell responses, by either targeting dendritic cells (DCs) *in vivo* or *ex vivo*, or adoptive transfer of specific cytotoxic T cells (CTLs), and/or appropriate modulation of T-cell functions *in vivo*. Active immunization with patient-specific Id proteins or DNA plasmids encoding for the Id have been exploited to target DCs *in vivo* and activate T cell against B-cell lymphomas. DCs optimally pulsed *ex vivo* with lymphoma antigens (Id or whole tumor antigens) have been employed as vaccines to improve the stimulation of specific T cells *in vivo*. To bypass *in vitro* manipulation, the strategy to induce *in vivo* immunogenic lymphoma cell death (with radiation therapy) and activation of DCs (with the TLR agonist CpG) has been studied to favor the occurrence of a vaccinal effect *in vivo* (*in situ* vaccination). To overcome the difficulties of generating endogenous T-cell responses able to eradicate tumors in pluritreated lymphoma patients, adoptive transfer of activated tumor-specific T cells (such as anti-lymphoma CAR-engineered T cells) has been also investigated. Finally, the availability of several immunomodulatory agents offers the opportunity to target the tumor immune microenvironment from multiple sides. Blocking Abs against the immune checkpoints PD-1 and CTLA-4 are among the first therapies in the pipeline to be tested with the aim to boost T-cell functions and counteract immunosuppression in lymphoma patients.

an improved time to progression (TTP) was reported for patients receiving vaccination after rituximab compared to the historical controls treated with rituximab alone, suggesting a potential clinical benefit of active immunotherapy also in the setting of B-cell recovery after rituximab therapy.

The feasibility, tolerability, and efficacy of Id vaccines demonstrated in early-stage clinical trials led to the initiation of three large-scale randomized phase-III studies aimed at demonstrating a clear-cut survival improvement in vaccinated patients. They tested either recombinant Id (MyVax, Genitope Corporation (49); FavId, Favrille) (50) or hybridoma-derived Id (BiovaxId, Biovest International Inc.) (51) in grades 1–3 FL patients that achieved at least disease stabilization (50), partial (49) or complete (51) remission after induction with a standard course of rituximab or cyclophosphamide, vincristine and prednisone (CVP) or PACE, respectively (**Table 2**).

The Genitope trial enrolled 287 previously untreated patients with the aim to show a significant increase in disease-free survival (DFS) in the vaccinated cohort as its principal endpoint. Even though this was not achieved, among the vaccinated patients, those who mounted an anti-Id immune response experienced a significantly improved PFS, further strengthening the correlation between the induction of vaccine-specific immune effects and the clinical benefit (49). The Favrille study compared TTP between the vaccine and control cohorts, who included 349 patients in total, with ~80% being treatment-naïve, but failed to demonstrate any clinical improvement in the experimental arm (50). Unfortunately, immune responses were not monitored in these patients and the association between immunological and clinical effects could not be verified. The Biovest trial enrolled 234 previously untreated patients: 177 achieved a CR/CRu after induction chemotherapy and were thus randomized, but 60 of these patients did not receive the vaccine because of relapse or other reasons, thus missing the expected intention to treat (ITT) endpoint. However, among the treated patients, those who received the vaccine ($n = 76$) experienced a prolongation of the DFS by 13.6 months compared to those treated with the placebo ($n = 41$) (44.2 versus 30.6, $p = 0.045$), but without any increase in OS (51). In particular, treatment with Id of the IgM class, but not IgG, showed to significantly improve DFS compared to the isotype-matched control (52.9 versus 28.7 months; $p = 0.001$). Although results from the Biovest study are not definitive because of the non-met ITT and the low statistical significance level of the difference in DFS between the two cohorts, they granted BiovaxId the orphan drug status by the FDA. For a proper interpretation of this study, it is important to consider that patients who received the vaccine had to remain in remission during the period of the vaccine preparation. Since the average time of vaccine production was 8 months, it is possible that vaccinated patients had less aggressive and/or less chemoresistant diseases, thus explaining a longer-lasting complete response. Alternatively, these results may simply reflect the concept that complete tumor

TABLE 2 | Main features and interpretation of phase-III clinical trials with anti-Id vaccination.

	Genitope	Favrille	NCI/Biovest
Vaccine	MyVax	FavId	BiovaxId
Patients	FL, untreated	FL, 80% untreated	FL, untreated
Source of tumor	FNA/core biopsy	FNA/core biopsy	Excisional biopsy
Idiotype	Recombinant	Recombinant	Hybridoma
Induction therapy	CVP (8 cycles every 3 weeks)	Rituximab (weekly ×4)	PACE/R-CHOP (6–8 cycles every 4 weeks)
Type of comparison (experimental/control)	2/1 randomization	1/1 randomization	2/1 randomization
Patient status before vaccination	First CR or PR	First CR, PR, or SD	First CR or CRu
Vaccination	Id-KLH + GM-CFSE or KLH + GM-CSF (sc, 7 doses)	Id-KLH + GM-CFSE or placebo + GM-CSF (sc, until PD)	Id-KLH + GM-CFSE or KLH + GM-CSF (sc, 5 doses)
Number of patients (actual/expected)	Vaccine: 192/240; control: 95/120	Vaccine: 174/171; control: 175/171	Vaccine: 76/250; control: 41/125
Primary end point	PFS ($p < 0.01$)	TTP ($p < 0.01$)	DFS ($p < 0.01$)
Results	Median PFS, 19.1 (experimental) versus 23.3 (control) mos ($p = 0.297$)	Median TTP, 9 (experimental) versus 12.6 (control) mos ($p = 0.019$)	Median DFS, 44.2 (experimental) versus 30.6 (control) mos ($p = 0.045$)
Reference	(49)	(50)	(51)

CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; CNOP, cyclophosphamide, mitoxantrone, vincristine, and prednisone; RTX, Rituximab; CRu, complete response unconfirmed; CVP, cyclophosphamide, vincristine, and prednisone; DFS, disease-free survival; GM-CSF, granulocyte-macrophage colony-stimulating factor; ITT, intent to treat; KLH, keyhole limpet hemocyanin; n.s, not significant; PACE, prednisone, doxorubicin, cyclophosphamide, and etoposide; PFS, progression-free survival; pts, patients; CR, complete response; PR, partial response; SD, stable disease; TTP, time to progression; mos, months.

eradication predisposes to the achievement of a clinical benefit after vaccination.

Although the outcome of these phase-III clinical studies did not meet the high expectation, they have provided important information to improve the design of future trials. They indeed confirmed in significantly larger cohorts of patients (1) the safety and tolerability of Id-KLH produced either by recombinant or hybridoma technology; (2) the potential advantage of the latter method for the generation of more immunogenic and effective Id vaccines; and (3) the importance of inducing complete remission before vaccination in order to increase the probability of a clinical success. Moreover, results from these studies point to patients' selection and vaccine formulation as the areas with the highest room for potential improvement, in particular in view of the better definition of the molecular prerequisites to achieve an effective antitumor immune response. Importantly, these findings may be useful to optimize the design of anti-lymphoma active immunotherapy across different types of vaccines.

DNA-Based Vaccines

As an additional option to target DC *in vivo* and immunize cancer patients, viral vectors and plasmid DNA encoding TAAs have been exploited. This strategy requires *in vivo* transfection and antigen production. The optimized gene sequence is delivered intradermally, subcutaneously, or to the muscle, which allows, respectively, the *in vivo* transfection of professional APCs (epidermal keratinocytes and Langherans DCs) or myocytes and secondary cross-presentation of tumor antigens by the recruited DCs. The advantages of DNA-based vaccines over other immunization strategies include (1) the possibility of incorporating multiple epitope-encoding DNA regions to target several antigens in a single vaccine formulation, (2) no need to know the human leukocyte antigen (HLA) type because the protein products are processed *in vivo* by host APCs, (3) low production costs, and (4) the easy procedure required for their generation. However, as a drawback, it is possible that antigen expression, processing, and presentation take place in the improper cell subsets without the adequate stimuli, thus resulting in tolerance or an unwanted type of immunity rather than in the priming of an antitumor adaptive immune response (52).

Initial clinical trials demonstrated the feasibility and safety of vaccination with Id-encoding plasmid DNA, with no relevant levels of integration into host cellular DNA, or development of autoimmune reactions. However, due to the limited biological efficacy and no clinical benefit of Id-encoding naked DNA plasmid (53), more potent Id-DNA vaccines were generated by fusing the Id sequence to virus-derived immune stimulatory sequences (such as the fragment C of the tetanus toxin) (54) or cytokine-encoding genes (55), to favor DC chemotaxis, antigen uptake and presentation. In *in vivo* lymphoma models, these formulations showed prophylactic and therapeutic antitumor effects that relied on the induction of a specific T-cell response. As an additional strategy, pretreatment of the vaccination sites with low-dose cardiotoxin was found to generate a favorable immune microenvironment, which facilitated antigen-specific T-cell priming toward a long-term antitumor immunological memory (43).

The availability of more and more accurate mathematical algorithms for a better prediction of the most immunogenic peptides within TAAs will probably favor improving the design of DNA-based vaccines in the near future (56).

DC-Based Vaccines

To overcome the limitation of producing a custom-made protein for each patient, targeting a single antigen, and relying on the host's antigen processing machinery, presentation, and T-cell co-stimulation, loading DCs *ex vivo* with TAAs in the presence of the proper activation stimuli has also been exploited. In this case, DCs are properly differentiated from $CD34^+$ hematopoietic progenitors or, more commonly, from peripheral blood monocytes in the presence of the proper DC differentiation and maturation cytokine cocktails, and pulsed with TAAs as to recapitulate *ex vivo* the early phase of DC activation. The source of DCs, TAAs, the antigen-engulfing strategy, cytokine cocktails, and the route of vaccine administration can be multiple and require precise consideration to optimize the therapeutic efficacy of DC-based vaccines (52).

Clinical efficacy of DC-based vaccines seems to be superior compared to that achieved by Id-protein vaccines against lymphoma (57), confirming observations in different tumor settings of the advantages of this strategy over protein-based vaccines (58). Interestingly, when a DC-vaccine was used to immunize against the single antigenic Id protein, FL patients with relapsed or residual diseases after induction therapy developed anti-Id T-cell and Ab responses associated with durable tumor regressions, in particular when Id was conjugated with KLH (59). DCs loaded with tumor cell lysates showed to elicit significant anti-lymphoma immunity in preclinical models (60) and in small clinical trials (61). In a pilot study, we showed that vaccination with autologous DCs loaded with apoptotic and necrotic autologous tumor cells increased natural killer (NK) cell activation, reduced Treg frequency and induced both T- and B-cell antitumor responses associated with clinical efficacy in 6 of 18 heavily pretreated indolent B-NHL patients with measurable disease (61). Interestingly, in responder patients, the humoral responses induced by vaccination were directed against common lymphoma antigens (62). Of note, we showed that the levels of immunogenic stimuli in dying lymphoma cells used to pulse DCs positively correlated with the probability of a clinical success of the vaccine (63). Therefore, favoring the occurrence of this process, namely immunogenic cell death (64), by exogenously supplying antigenic/proinflammatory signals to boost DC engulfing, cross-presentation, and maturation, may increase the efficacy of DC-based vaccines.

As additional modalities to load DCs *ex vivo* with the full lymphoma antigenic repertoire, fusion of DCs with tumor cells (65) and transduction of DCs with tumor-derived mRNA have started to be investigated in the preclinical setting (66–68). The latter is a promising technique in light of the minimal sample size required for the amplification of total tumor RNA, which considerably decreases the cost of vaccine production. However, it has to be considered that DC transduction channels TAAs primarily into the MHC-I presentation pathway, thus limiting the activation $CD4^+$ T cells (69), which are crucial to sustain both Ab and CTL responses.

In Situ Vaccination

The understanding that certain anticancer treatments, including antracyclines and radiation, can favor the induction of an immunogenic tumor cell death (64), supported the possibility to combine them with proper immune adjuvants to achieve *in vivo* a vaccinal antitumor effect (**Figure 1**). To facilitate *in vivo* TAA processing and T-cell cross-priming, toll-like receptor (TLR) agonists are particularly suitable as they can activate and bridge the innate and adaptive immunity (70). The preclinical observation that intratumoral injection of the TLR9 agonist CpG oligodeoxynucleotides plus systemic chemotherapy eradicated large tumors inspired the clinical evaluation of low-dose locoregional radiation plus intratumor CpG injection in low-grade B-NHL patients (71). This approach achieved clinical responses at distal tumor sites (abscopal effect) in association with the induction of tumor-reactive $CD8^+$ T cells and reduction of intratumor Tregs (71). This study underscored the feasibility, safety, and efficacy to provide the conditions for the *in vivo* generation of an antitumor vaccine, thus overcoming the limitations for the manufacture of patient-specific products. Following these promising results, *in situ* vaccination with GpC and local radiation therapy was evaluated in resistant/refractory cutaneous-T-cell-lymphoma patients in a phase I/II study (72). Also in this case, treatment resulted safe and achieved systemic clinical responses, somehow associated with a positive immune modulation, in one-third of the patients. These findings point to the availability in the near future of a non-customized vaccine approach widely applicable with no requirement of any *ex vivo* cellular manipulation.

As a complementary modality to *in situ* vaccination, adoptive transfer of vaccine-primed autologous T cells after *in vitro* expansion, namely immunotransplant, has been exploited. Upon the achievement of the proof-of-concept in preclinical models (73), patients with newly diagnosed MCL were subjected to this procedure. In this case, the vaccine was made of autologous tumor cells that were treated *in vitro* with CpG and irradiated before administration into patients previously exposed to cytoreductive standard chemotherapy (74). Vaccine-primed T cells were then harvested, expanded *in vitro*, and reinfused after standard autologous stem cell transplantation. Preliminary results showed the feasibility of this approach in aggressive lymphoma patients and its efficacy in boosting antitumor T-cell responses. This provides the proof-of-principle for further investigations of the sequential combination of active and adoptive immunotherapy in cancer patients.

T-Cell Therapies

The ultimate objective of active immunotherapy is to induce an endogenous immune response able to activate T cells against the tumor. The clinical experience with anti-lymphoma vaccines has clearly shown a limited efficacy of this strategy to consistently expand a sufficient number of activated antitumor T cells able to clear established tumors in pluritreated patients. With the same rationale of the use of immunotransplant, lymphoma patients can be adoptively transferred with an adequate amount of tumor-specific T cells optimized *ex vivo* to recognize and kill cancer cells, in order to maximize the probability to achieve a therapeutic effect (**Figure 1**). Two main T-cell therapeutic strategies have shown considerable success against B-cell lymphomas: transfer of EBV-specific T cells for the treatment of EBV-associated lymphomas

and CAR T cells engineered to target B-cell lineage markers that continue to be expressed in the malignant clones.

Post-transplant lymphoproliferative diseases (PTLDs), caused by the reactivation of EBV infection in B cells of donor or recipient origin after allogeneic hematopietic stem cell transplantation (HSCT) or solid organ transplants (SOTs) respectively, continue to be a significant clinical problem (75). Management of heavily immunosuppressed patients with anticancer treatment poses several limitations, and standard treatment with rituximab eventually associated with less-intensive chemotherapy regimens often fail to cure PTLDs (76). EBV-infected B-cells do not actively produce virus, and, as such, are not sensitive to antiviral agents (77), but express viral latency-associated proteins, which may represent effective targets for immunotherapy. Depending on the type of latency of EBV, malignant B cells express more or less immunogenic EBV antigens. Tumors that arise in severely immunocompromised patients, such as in the early phases after allogenic HSCT or in SOT recipients, are usually highly immunogenic and express all the 10 EBV latency-associated proteins. Expressing the same 10 viral antigens and high levels of class-I and class-II HLA as well as co-stimulatory molecules, EBV-transformed B cells are optimal APCs for the activation of HLA-matched EBV-specific T cells to be used in this setting. With this strategy, polyclonal anti-EBV CTLs have been rapidly and abundantly generated from healthy EBV-seropositive donors, and proved safe and effective in preventing or treating PTLDs in recipients of allogenic HSCT (78, 79). Based on these encouraging results, a similar strategy has been attempted to treat post-SOT PTLDs. In this case, anti-EBV CTLs have been generated from the organ recipients and demonstrated some success in patients with either elevated EBV viral load or active disease (80–82). The constant immunosuppression status and the fact that SOT patients do not receive any lymphodepleting pre-conditioning treatment, which instead favors T-cell expansion in HSCT recipients, may account for the reduced persistence and efficacy of the transferred anti-EBV CTLs in this setting. However, these results have been crucial to demonstrate the feasibility of anti-EBV T-cell therapy in SOT recipients and the absence of any risk to induce rejection of the transplanted organ.

Interestingly, efficient control of PTLD was also achieved when "off-the-shelf" EBV-specific T cells derived from partially matched third-party donors were used in the context of both SOT and HSCT (79, 83, 84). This represented a dramatic improvement in the management of PTLD patients as anti-EBV CTLs of different HLA specificities may be generated and banked in advance in order to be readily available when needed. Very recently, anti-EBV CTLs derived from either patient's transplant or third-party donors have shown similar substantial efficacy in producing long-lasting remissions in patients with aggressive rituximab-resistant post-HSCT PTLDs [(85), AACR Annual Meeting 2015, abstract CT107]. These results granted breakthrough therapy designation to anti-EBV CTLs generated from third-party donors for the treatment of patients with rituximab-refractory PTDLs.

Since EBV-related HLs and B-NHLs express only the weakly immunogenic EBV latency proteins (type II EBV latency proteins, LMP1, LMP2, and EBNA-1) (86), T cells specific for these antigens rather than polyclonal anti-EBV T cells need to be infused in order to achieve a clinical effect. However, the time required for their generation makes the procedure not suitable for the treatment of patients with active disease (87). For the same reason, T-cell therapy has not been developed for the treatment of the type-I EBV latency BL, which express only the poorly immunogenic protein EBNA1.

To broaden the specificity of T cells against multiple TAAs, transduction of high-affinity TCRs or CARs into mature or precursor T cells have been accomplished to make adoptive immunotherapy more easily available for patients with different tumor types (88). This latter option has found relatively wide application for the treatment of B-cell malignancies. CARs contain an extracellular domain with the Ab variable regions recognizing the target TAA genetically fused to the intracellular CD3ζchain (89). T cells transduced with CARs are therefore redirected toward the target antigen via the Ab regions, which, once engaged, trigger the CD3ζchain-downstream signaling cascade for T-cell activation. The activity of CAR-T cells thus becomes independent from HLA recognition, and this constitutes a major advantage of this strategy. The consistent expression of the B-cell lineage markers CD19 and CD20 across most B-cell malignancies and the reported safety/efficacy of anti-CD19/-CD20 mAbs in these diseases made them the preferred targets for CAR-T cells. In the preclinical setting, first generation CAR-T cells against CD19 or CD20 (CD19-/CD20-ζ) showed adequate engraftment and anti-lymphoma activity in either mice xenografted with patients' tumors and autologous CAR-T cells or in syngeneic murine models following lymphodepletion with cyclophosphamide or radiation (90–93). However, the limited persistence of these CAR T-cells, partially driven by the presence of endogenous normal B cells expressing the target antigens (92, 94), led to the development of second-generation CARs, where the CD3ζ region was fused to the intracellular signaling domains of T-cell co-stimulatory molecules, such as CD28 or CD137 (4-1BB). CD19·CD28-ζ and CD19·CD137-ζCAR-T cells demonstrated enhanced functions, proliferation and survival, and resistance to Treg suppression, which resulted in increased persistence and antitumor activity in xenografted mice (95–99). This strategy seemed to be particularly effective when the tumor cells expressed low levels of ligands for co-stimulatory molecules (95, 97), because, being transduced with co-stimulatory domains, second-generation CAR-T cells did not depend anymore on physiologic co-stimulation signals. Third generation CAR-T cells with all the three T-cell signaling domains fused together (CD3ζ, CD28, and 4-1BB) have not definitely proven to exert a better antitumor activity (98, 100). Another approach studied with the aim to increase CAR-T cell *in vivo* persistence has been to engineer T cells specific for common viruses, such as EBV. Transduced virus-specific lymphocytes maintain the capability to become physiologically activated *in vivo* through their natural T-cell receptor and to persist in the memory compartment, offering the advantage to control their expansion by vaccination with the cognate viral antigens (101, 102).

Based on these preclinical findings, clinical studies mainly investigated second-generation CARs, either with CD28 or 4-1BB signaling domains, alone or in combination with lymphodepleting conditioning regimens. Experience accumulated so far in patients with B-cell malignancies indicates (1) the feasibility of

generating and using CARs in the clinical setting, (2) the advantage of retro/lentiviral gene transduction methods over plasmid transfection technology to generate more functional CAR-T cells (no resistance selection genes, shorter culture periods), and (3) the importance of lymphodepleting pre-conditioning treatments to facilitate engraftment and in turn the therapeutic effects of CAR-T cells, with no specific restriction to the regimen to be applied. These observations, made initially in early small clinical trials with refractory/resistant B-NHL and acute lymphoid leukemia patients, are being confirmed in larger studies (39, 103–106). Persistent clinical responses and relatively manageable toxicities were induced by autologous CD19 CAR-T cells in patients relapsing after multiple lines of treatments. Interestingly, this approach proved effective also when donor-derived allogeneic CAR-T cells were administered in B-NHL patients who relapsed or were at high risk to relapse after allogeneic HSCT (107). Redirecting allogenic T cells against a TAA with CARs appeared an effective strategy to uncouple graft-versus-host-disease (GVHD) and graft-versus-leukemia (GVL) effect in patients who failed HSCT and DLI. In this context, the use of virus-specific T cells for the generation of donor-derived CD19 CAR-T cells showed promising results in controlling both the disease and viremia in patients with viral reactivation after allogeneic HSCT (108).

Altogether these findings indicate the substantial therapeutic potential of CAR-T cells against B-cell malignancies; however, this approach has still a wide margin for improvement, which mainly relies on the need for a better understanding of the biology of CAR-T cells, more robust biomarkers of clinical response and methods to reduce toxicity. Cytokine release syndrome and neurologic toxicities are not uncommon side effects of CAR-T cells. Therefore, there is a huge effort toward the understanding of how to control the functions of these T cells. Preclinical studies have investigated the potential to eliminate CAR-T cells in case of toxicity by co-transducing chemically inducible apoptosis-promoting fusion proteins, such as Fas and Caspase 9 (109), or targets of cell-depleting antibodies, such as CD20 or truncated epidermal growth factor receptor (110, 111). By eliminating CAR-T cells themselves, however, such strategies abolish both their side effects and therapeutic potential. As an alternative option, already tested in patients, blocking IL-6 receptor with the specific mAb tocilizumab has shown promising results in reversing cytokine release syndrome while sparing expansion and therapeutic effects of CAR-T cells (112, 113).

Finally, in light of the potential ability of tumor cells to escape CAR-T cell therapy, for example, by downregulating the expression of the targeted antigen (112), it is important to study strategies for counteracting such mechanisms. Toward this goal, CAR-T cells engineered to target multiple and/or alternative (114, 115) lymphoma antigens or their combination with other immunotherapeutic modalities are under investigation.

Targeting the Immune Microenvironment in Lymphoma

In order to grow and progress in lymphoid organs, lymphomas need to subvert immunosurveillance while preserving the pro-lymphomagenic functions of nearby immune cells, thus becoming real parasites of the immune system. The prototype example of the role of the crosstalk with immune cells in the lymphoma microenvironment is HL, where the Hodgkin and Reed–Sternberg (HRS) tumor cells account only for 1% of the affected tissue, being the rest all inflammatory cells, which provide crucial interactions through CD80 and CD40/CD40L for HRS cell survival. In this case, mechanisms of immune evasion include polarization of infiltrating T cells toward a T helper 2/Treg phenotype through the release of IL-10 and TGF-beta, and inhibition of NK cells and CTLs via overexpression of FAS ligand and the ligands of the immune checkpoint receptor programed-death 1 (PD-1) (116). As an additional demonstration of the importance of the immune infiltrate in lymphoma development and progression, genetic and immunohistochemical signatures of non-tumor cells in the neoplastic tissue currently represent the best predictors for B-NHL patients' prognosis (29, 117–120). These studies showed that a reduced survival and the risk of transformation of indolent B-NHLs are associated with the infiltration of specific immune cell subsets. In particular, lymphoma-associated macrophages (29), $CD4^+CD25^+FOXP3^+$ Tregs (121) monocytic myeloid-derived suppressor cells (bearing a $CD14^+HLA\text{-}DR^{low/-}$ phenotype) (122, 123), and exhausted T cells expressing intermediate levels of PD-1 (124) have been all associated with a negative clinical impact in FL patients. The fact that immune cells are not usually targeted by conventional treatments may explain why, despite major therapeutic advances, indolent B-NHLs still remain incurable, underscoring the importance of modulating the microenvironment as a part of the lymphoma treatment.

Lately, several strategies able to modulate T-cell functions have become available, allowing preclinical and in some cases clinical evaluation of the anti-lymphoma effects of Tregs inhibition, promotion of T-cell co-stimulation, and inhibition of immune checkpoints. The IL-2-diptheria toxin fusion protein denileukin diftitox (ontak), the anti-CD25 mAb daclizumab, and anti-folate receptor 4 (FR4) mAbs have been studied to deplete Tregs. Agonist mAbs directed against the co-stimulatory molecules, OX40 (CD134), glucocorticoid-induced TNF-related protein (GITR), and 4-1BB (CD137), have been used to boost antitumor T-cell functions, whereas blocking mAbs for the co-inhibitory molecules cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and PD-1 have been employed to prevent a negative regulation of tumor-specific T cells (**Figure 1**). In lymphoma preclinical models, T-cell modulation by anti-OX40, -GITR, -CD137, -CTLA-4, or -FR4 mAbs has shown to significantly improve the therapeutic efficacy of several immunotherapeutic modalities, including anti-tumor vaccination and mAb therapy (125–127). This evidence has led to the clinical evaluation of T-cell modulating agents for the treatment of these diseases. CTLA-4 or PD-1 blockade with the mAbs ipilimumab or pidilizumab, respectively resulted in safe and induced modest but occasionally long-lasting clinical responses in replapsed/refractory B-NHL patients evaluated in early-phase trials (128–130). Interestingly the combination of pidilizumab and rituximab was well tolerated and active in patients with rituximab-sensitive FL relapsed after 1–4 previous therapies (131), underscoring the importance of further investigating this strategy in B-NHL patients. An unexpected therapeutic activity of single-agent anti-PD-1 mAb nivolumab was

instead found in heavily pretreated HL patients (40), which may thus provide a real therapeutic option for this patient category with an otherwise very unfavorable prognosis. The basis for the substantial clinical effects observed in this study probably relies on the high frequency of copy-number gain in PD-1 ligand loci in the enrolled patients (40). This points to a genetically defined sensitivity to PD-1 blockade in this disease. Given that genetic alterations of PD-1 ligands were not reported to be as frequent in newly diagnosed HL patients, it is possible that they define a subset of HLs with a particularly adverse prognosis.

Another straightforward way to redirect immune cells against lymphoma clones within the tumor microenvironment has been to modulate NK cell activity to enhance the effector functions of mAb therapy. As one of the major mechanisms of action of therapeutic mAbs is Ab-dependent cell mediated cytotoxicity (ADCC), whereby NK cells and phagocytes are redirected to the targeted tumor cells through Ab Fc receptors, the possibility to further co-stimulate ADCC cellular mediators via immunomodulatory mAbs was hypothesized to synergize with antitumor mAbs. Since the co-stimulatory molecule 4-1BB is upregulated on NK cells upon Fc receptor engagement (132), agonist anti-4-1BB mAbs have been investigated in combination with anti-lymphoma mAbs with the aim to increase antitumor ADCC. According to this hypothesis, agonist anti-4-1BB mAbs significantly improved the anti-lymphoma effects of anti-CD20 mAbs in preclinical models (127). In addition, human NK cells were found to consistently up-regulate 4-1BB when exposed to rituximab-coated autologous lymphoma cells (127), providing the rationale to explore the combination of anti-4-1BB and -CD20 mAbs in the clinical setting. Based on these findings, a phase-Ib study of the anti-4-1BB mAb urelumab and rituximab in relapsed/refractory B-NHL patients has recently started (NCT01775631).

Finally, because of their immunomodulatory properties, thalidomide and its derivatives have been also exploited to target the microenvironment in B-NHLs. Besides their potential to directly interfere with tumor growth and induce apoptosis in tumor cells, these agents promote antitumor immunity, including mAb-mediated ADCC, and antiangiogenic effects. Lenalidomide has been the most widely investigated drug in this category, showing significant single-agent anti-lymphoma activity in phase-II trials (133–136), in particular against aggressive B-NHLs. Building upon these results, a larger phase-II study was initiated to test safety and efficacy of lenalidomide in MCL patients relapsed after a second-line therapy with bortezomib, for whom no therapeutic options were available (137). Based on the tolerability and durable clinical responses induced by lenalidomide in this patient population, in June 2013, the FDA approved this drug for the treatment of MCL patients relapsed or progressed after two prior therapies including bortezomib. Lenalidomide has also been explored in combination with rituximab in relapsed/refractory indolent and aggressive B-NHLs showing significant and consistent clinical efficacy across different phase-II trials (138, 139). Interestingly, this combination compared favorably with single-agent rituximab in historical controls (5, 140). In light of the activity of lenalidomide against aggressive B-NHL, its combination with rituximab-based chemotherapy (CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone) has been investigated as front-line therapy for these diseases in phase-II studies, proving to be highly effective and safe also in this contest (141–143). A phase-III randomized, double blind, placebo-controlled, and multicenter study to compare the efficacy and safety of lenalidomide with R-CHOP versus placebo with R-CHOP in patients with previously untreated DLBCL is underway (NCT02285062).

Conclusion

New curative treatments are needed for B-cell lymphomas. The availability of specific antigens and the easy accessibility of the immune system to these diseases have supported the extensive study of immunotherapy in the attempt of improving the management of B-cell lymphoma patients. Even though active immunotherapy through antitumor vaccination theoretically represents the ideal immunotherapeutic modality to induce antitumor immunity and control disease recurrences, the possibility to activate effective endogenous immune responses has proven challenging even in lymphoma patients. Alternative approaches to promote tumor targeting by T cells have more recently been investigated with promising results, with T-cell therapy regaining considerable attention thanks to the recent clinical successes of CAR-T cells. However, with the increasing use of anticancer immunotherapy, we are becoming aware of the advantages and limitations of the different strategies now available to activate/modulate antitumor immunity. It seems clear that if active and adoptive immunotherapy as well as immunomodulatory mAbs may not reach the desired activity as single agents, they can be exploited in rational combinations to maximize the probability of a clinical benefit (57). In conclusion, the significant advancements in the development and application of immunotherapy against B-cell lymphomas hold promise for a better definition of curative options for these diseases in the near future.

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REVIEW
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Exploiting the immunomodulatory properties of chemotherapeutic drugs to improve the success of cancer immunotherapy

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Cancer immunotherapy is gaining momentum in the clinic. The current challenge is to understand why a proportion of cancer patients do not respond to cancer immunotherapy, and how this can be translated into the rational design of combinatorial cancer immunotherapy strategies aimed at maximizing success of immunotherapy. Here, we discuss how tumors orchestrate an immunosuppressive microenvironment, which contributes to their escape from immune attack. Relieving the immunosuppressive networks in cancer patients is an attractive strategy to extend the clinical success of cancer immunotherapy. Since the clinical availability of drugs specifically targeting immunosuppressive cells or mediators is still limited, an alternative strategy is to use conventional chemotherapy drugs with immunomodulatory properties to improve cancer immunotherapy. We summarize the preclinical and clinical studies that illustrate how the anti-tumor T cell response can be enhanced by chemotherapy-induced relief of immunosuppressive networks. Treatment strategies aimed at combining chemotherapy-induced relief of immunosuppression and T cell-boosting checkpoint inhibitors provide an attractive and clinically feasible approach to overcome intrinsic and acquired resistance to cancer immunotherapy, and to extend the clinical success of cancer immunotherapy.

Keywords: cancer immunotherapy, immune checkpoint blockade, chemotherapy, tumor microenvironment, immunosuppression, anti-tumor immunity

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Introduction

Cancer immunotherapy – harnessing the patient's immune system against cancer – is currently gaining momentum in the clinic. Clinical trials with immune checkpoint inhibitors show remarkable success in patients with advanced metastatic melanoma, non-small cell lung cancer, renal cancer, bladder cancer, and Hodgkin's lymphoma (1–6). As a result, the journal *Science* proclaimed cancer immunotherapy as the breakthrough of 2013 (7). Furthermore, these encouraging results led to FDA approval of the immune checkpoint inhibitors ipilimumab (anti-CTLA-4), nivolumab, and pembrolizumab (anti-PD-1) in the past few years. Although cancer immunotherapy was proclaimed a breakthrough, a significant proportion of cancer patients do not show clinical benefit. There are various cancer cell-intrinsic and cancer cell-extrinsic processes that regulate intrinsic or acquired resistance to cancer immunotherapy. Cancer cell-intrinsic characteristics like the mutational load have been reported to affect responsiveness to immunotherapy (8, 9). In terms of cancer cell-extrinsic processes, tumors exploit different strategies to induce immune escape by hampering the recruitment and activation

of effector T cells, and by creating a local immunosuppressive environment through recruitment of suppressive myeloid and regulatory T cells that dampen T cell effector functions. Which of these immune escape mechanisms are active in a certain tumor depends on the tumor type, tumor stage, and therapy history. A deeper understanding of the molecular mechanisms underlying these processes will contribute to the identification of biomarkers that can predict therapeutic efficacy of immunotherapy and to the design of combinatorial strategies aimed at maximizing the success of immunotherapy.

In this review, we discuss how tumor-induced immunosuppressive networks counteract efficacious anti-tumor immune responses, and how disruption of these networks can increase the anti-cancer efficacy of cancer immunotherapy with immune checkpoint inhibitors. Development and clinical testing of novel drugs specifically targeting immunosuppressive networks are ongoing and preliminary results are promising (10). An alternative strategy to relieve tumor-induced immunosuppressive states is to use conventional, and more easily accessible, anti-cancer treatment strategies with known immunomodulatory properties, such as chemotherapy, radiotherapy, and targeted therapy (11–15). Here, we focus on the immunomodulatory properties of conventional chemotherapy, and how these properties can be exploited to improve the anti-cancer efficacy of immune checkpoint inhibitors.

Cancer Immunotherapy: Opportunities and Challenges

Tumor-Induced Mechanisms of Immune Escape

Cancers do not merely consist of tumor cells, but comprise a variety of cell types that together form the tumor microenvironment (TME) (**Figures 1** and **2**). Infiltrating immune cells are of special interest because of their paradoxical role in cancer progression. While some immune cell populations have pro-tumorigenic properties, others counteract tumorigenesis (16–18). Many tumors are characterized by an immunosuppressive TME, which makes it unfavorable for anti-tumor immunity. To mount effective anti-tumor immunity, tumor-associated antigens need to be sampled and processed by antigen-presenting cells (APCs). After receiving specific maturation signals, these APCs migrate to tumor-draining lymphoid organs where antigens are presented to T cells. Upon activation and proliferation, tumor antigen-specific T cells migrate to the tumor bed where they exert their cytotoxic function. At every step of this T cell priming and effector process, tumors employ strategies to hamper anti-cancer immunity.

Tumors often show dysfunctional recruitment and activation of dendritic cells (DCs), which are the most potent APCs for initiating immune responses. Several studies show that tumor-infiltrating DCs display an immature phenotype (20, 21). Tumor-derived factors like IL10, IL6, CSF1, and VEGF interfere with DC maturation, causing failure to migrate to the tumor-draining lymphoid organs, and to provide the appropriate co-stimulatory signals required to stimulate T cells (21). Although a thorough analysis of the antigen-presenting myeloid immune cell compartment in the *MMTV-PyMT* mammary tumor model showed that intratumoral DCs are able to ingest and present tumor antigens to T cells, they fail to activate them (22). Nevertheless, even in these immunoevasive tumors, a rare population of IL12-expressing $CD103^+$ DCs exists that is able to prime tumor antigen-specific T cells (23). Besides hampered T cell priming, the recruitment of activated T cells and their access into the tumor bed is often disrupted by the disorganized tumor vasculature and impaired expression of adhesion molecules on endothelial cells (24, 25). Some studies suggest that tumor-derived chemokines may cause selective trapping of T cells in the tumor stroma preventing access into the tumor bed (26). When tumor-specific T cells do succeed to reach the tumor, downregulation of MHC class I expression on tumor cells renders them invisible to T cell attack (27). Additionally, T cells face systemic and local tumor-induced immunosuppression, which limits their activation and function (28). Tumor-associated immunosuppression can be caused by tumor-infiltrating or systemically expanded myeloid cells or regulatory T cells (T_{regs}) that – directly or indirectly via secretion of soluble mediators – hamper T cell priming and effector function or even induce T cell death (28). These mechanisms will be discussed in more detail later.

Enhancing Anti-Tumor Immunity by Immune Checkpoint Inhibitors

To improve anti-tumor T cell immunity, different types of cancer immunotherapy approaches exist. While passive immunotherapy is based on adoptive transfer of (genetically engineered) autologous T cells, active immunotherapy boosts the endogenous immune response via cancer vaccines or inhibitors of immune checkpoints. The therapeutic effect of the latter is aimed at inhibition of negative immune regulatory pathways including cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and the programed cell death protein-1 (PD-1) receptor and one of its ligands, PD-L1 (B7-H1; CD274) (29). CTLA-4 is a member of the CD28 immunoglobulin superfamily and is expressed mainly on the surface of activated $CD4^+$ T cells and T_{regs}, while absent on naïve T cells (30). CTLA-4 plays a central role in maintaining immune tolerance by competing with CD28 to bind the ligands CD80 and CD86 present on activated APCs to inhibit T cell co-stimulation. The PD-1/PD-L1 axis shows similarities to that of CTLA-4. PD-1 is mainly expressed on activated T cells upon T cell receptor (TCR) engagement and on T_{regs}, while naïve and memory T cells do not usually express this surface marker. Recent studies suggest that PD-1, rather than being a marker of activated T cells, identifies exhausted T cells (31). PD-L1 is expressed on multiple cell types, whereas expression of PD-L2 (B7-DC; CD273) seems to be restricted to APCs (32, 33). Like CTLA-4, binding of PD-L1/PD-L2 to its receptor results in an inhibitory signal that prevents T cell activation. While CTLA-4 blockade is hypothesized to act mainly in secondary lymphoid organs during the T cell priming phase, it is believed that blockade of PD-1 or PD-L1 targets the TME during the T cell effector phase (34). However, PD-1 can also play a role in the early T cell response as a regulator of $CD8^+$ T cell expansion upon antigen recognition (35). In addition to its role in T cell priming, CTLA-4 also regulates the suppressive function of tumor-infiltrating T_{regs} (36, 37). In line with this, blockade of CTLA-4 in the B16 melanoma model acts locally

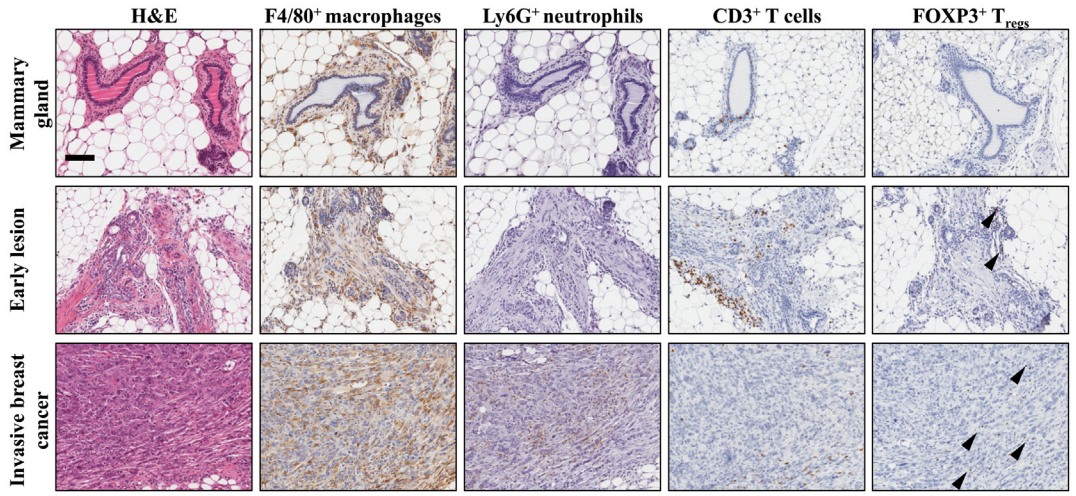

FIGURE 1 | Establishment of the immune microenvironment during breast cancer progression in a conditional mouse model for mammary tumorigenesis. Female *K14Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice develop *de novo* invasive mammary tumors that closely resemble human invasive lobular carcinoma (19). Immunohistochemical staining on mammary tissue from *K14Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice obtained during different stages of mammary tumor progression. From top to bottom are represented wild-type mammary gland (top), early lesion (middle), established mammary tumor (bottom). From left to right, identification of different immune cell populations by H&E, F4/80 (macrophages), Ly6G (neutrophils), CD3 (total T cells), and FOXP3 (regulatory T cells) staining showing the dynamics of the tumor microenvironment. Arrowheads indicate FOXP3+ nuclei. Scale bar 100 μm.

in the TME by inactivating T_{regs} in an Fc-dependent manner resulting in a favorable shift in the effector T cell/T_{reg} ratio (38). The exact mechanisms of action of anti-CTLA-4 and anti-PD-1/PD-L1 are not completely clear. Just recently, the combination of anti-CTLA-4 and anti-PD-1 was reported to significantly increase the fraction of melanoma patients responding to immunotherapy compared to anti-CTLA-4 monotherapy-treated patients (39), emphasizing the different modes of action of CTLA-4 and PD-1.

The rational of using CTLA-4 blockade in cancer therapy is to release the brake on pre-existing tumor-reactive T cells and to generate new T cell responses. Ipilimumab (anti-CTLA-4) was the first immune checkpoint inhibitor that yielded a significant increase in survival of patients with metastatic melanoma, for which all conventional therapeutic options had failed (1). Interestingly, a broadening of the tumor-reactive T cell repertoire was reported upon ipilimumab treatment (40). In a second clinical study, ipilimumab was combined with dacarbazine in metastatic melanoma patients resulting in prolonged survival compared to dacarbazine alone (41). In both studies, a fraction of patients showed long-term durable responses (42). Similarly, clinical trials with anti-PD-1 have shown tumor regression in a substantial fraction of cancer patients (3). These initial results lead to an immense increase in clinical trials with drugs targeting the PD-1/PD-L1 axis in different cancer types, and many report anti-tumor efficacy (3–6, 43). Recent clinical observations show that the combination of anti-CTLA-4 and anti-PD-1 is more effective than either monotherapy (39). Although very successful and promising, a significant proportion of cancer patients do not show long-term benefit of immune checkpoint inhibitors. Therefore, it is of utmost importance to mechanistically understand intrinsic and acquired resistance to cancer immune checkpoint inhibitors, in order to identify biomarkers that can be used to pre-select those patients that will or will not benefit from cancer immunotherapy and to develop therapeutic strategies to overcome or bypass resistance mechanisms.

What are the Requirements for Therapeutic Response to Checkpoint Inhibitors?

To predict the response to immunotherapy per patient and tumor type, several variables should be taken into account. For successful activation of a T cell-mediated anti-tumor immune response, T cells need to "see" the cancer cells with their TCR. In general, there are three classes of tumor antigens that can potentially be recognized by T cells: viral antigens, self-antigens, and neo-antigens. Our T cell repertoire is basically built to recognize and respond to viral antigens because these antigens are perceived as foreign or non-self. However, only a subset of established human cancers expresses viral antigens. During the T cell maturation process, thymic selection eliminates maturing lymphocytes that display a high avidity for self-antigens. As a consequence, only low-avidity self-specific T cells can be found in the peripheral T cell repertoire, which may not be ideal for cancer immunotherapy. Non-synonymous somatic mutations can give rise to neo-antigens toward which no central T cell tolerance is present. Recently, neo-antigen-specific T cell responses have been reported in melanoma patients (44–46), indicating that these mutations can be recognized by T cells and induce tumor-specific T cell responses. In line with this, the number of predicted neo-antigens is linked with a metric for immune cytolytic activity based on gene expression in a large panel of cancer types (47). Thus, the extent of the mutational load of a certain tumor would serve – albeit at a low resolution – as a predictor of response to cancer immunotherapy. Indeed, a growing body of data supports this hypothesis (48). Whole-exome sequencing analyses revealed

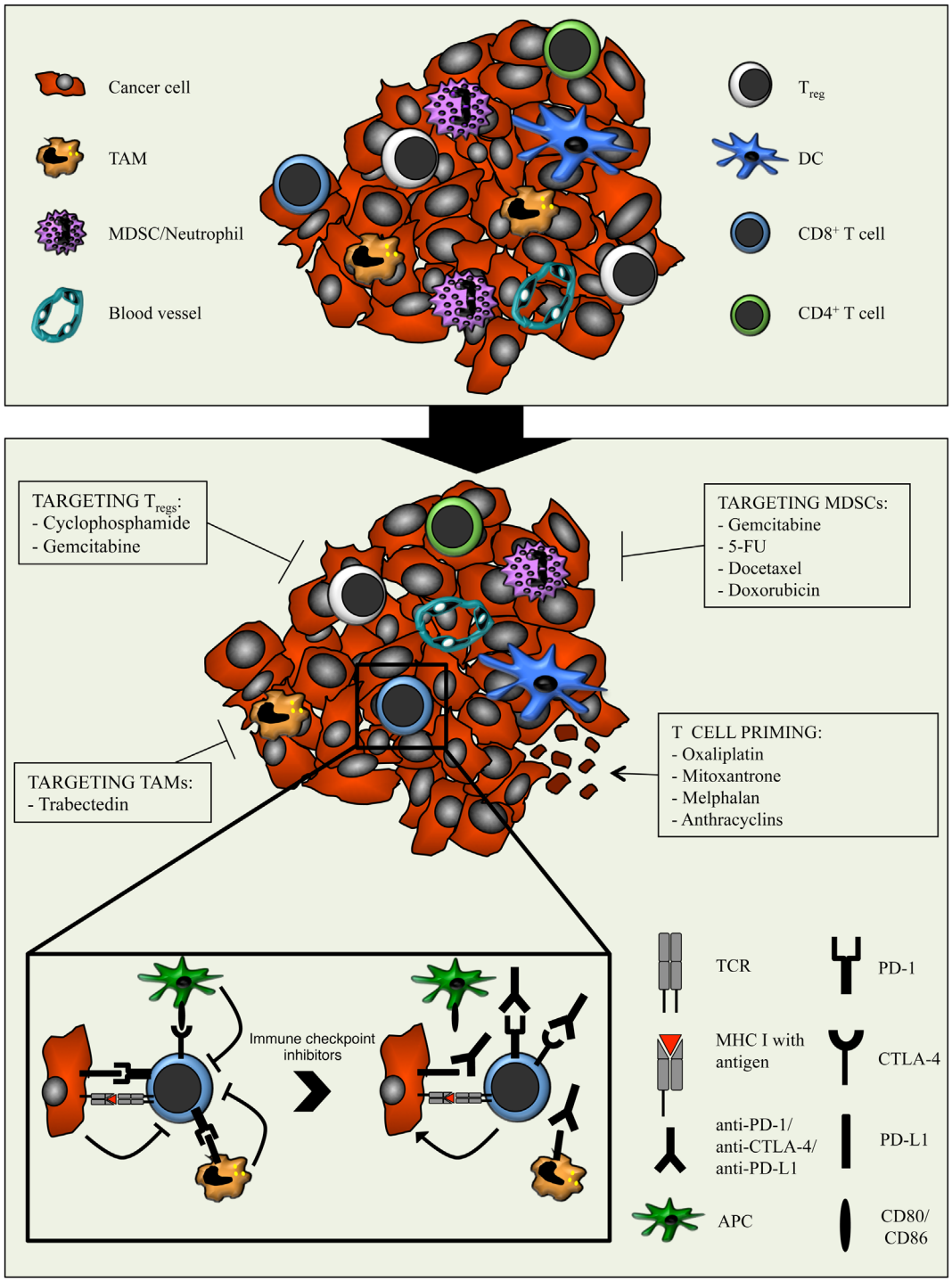

FIGURE 2 | Combination strategies aimed at relieving the immunosuppressive tumor microenvironment with chemotherapy and potentiating cytotoxic T cells with immune checkpoint inhibitors. The tumor microenvironment is characterized by the presence of various immune cell types, including different subsets of adaptive immune cells and TAMs, MDSCs, and T_{regs}. The latter dampens the anti-cancer activity of T cells through several mechanisms. Moreover, cancer cells and myeloid cells express PD-L1/PD-L2 and APCs express CD80/CD86. Binding of these molecules to PD-1 and CTLA-4 respectively, expressed on T cells, results in inhibitory signals that counteract T cell activation and function. The immunomodulatory properties of different types of chemotherapeutic drugs can be exploited to enhance anti-tumor immunity. By optimally matching the immunomodulatory features of specific chemotherapeutic drugs with the T cell-boosting effect of immune checkpoint inhibitors, the efficacy of immunotherapy might be improved.

that melanoma and lung cancer – the two cancer types that show promising responses to immunotherapy – bear relatively high mutational loads compared to other types of cancer due to their exposure to DNA damaging insults like UV radiation and tobacco smoke, respectively (49). Recent studies uncovered that a high mutational load is associated with long-term clinical benefit to checkpoint inhibitors (8, 9). However, not all cancer patients with tumors bearing a high mutational load respond to checkpoint inhibitors, and some patients bearing tumors with low mutational load do (8, 9). Together, these results suggest that the mutational load of tumors is correlated with response to immune checkpoint inhibitors, but it cannot solely be used to predict response.

A growing body of clinical observations suggests that the intratumoral presence of pre-existing T cells is required for clinical benefit of immunotherapy (50). PD-1 expression on tumor-infiltrating $CD8^+$ T cells has been suggested to identify the repertoire of clonally expanded tumor-reactive T cells (51). In addition, T cell infiltration correlates with PD-L1 expression in tumors and is associated with increased responsiveness to drugs targeting the PD-1/PD-L1 axis in melanoma patients (50, 52, 53). Expression of PD-L1 in tumors is one of the main characteristics pursued as a potential biomarker for response to PD-1/PD-L1 blockade. However, there are examples of tumors with high expression of PD-L1 that do not respond to PD-1 blockade, and PD-L1 negative tumors that do respond (53). Why certain tumors express PD-L1 and others do not remains to be elucidated.

Interestingly, expression of PD-L1 and responsiveness to immune checkpoint blockade is associated with genomic instability in different tumor types (54). Patients bearing mismatch-repair-deficient colorectal cancer (CRC) respond better to anti-PD-1 therapy than mismatch-repair-proficient CRC patients (54). In line with this, a microsatellite instable (MSI) subset of CRC patients shows high T cell influx (55). However, this is counterbalanced by simultaneous upregulation of checkpoint molecules including PD-1, PD-L1, and CTLA-4 leaving T cells dysfunctional (55). Moreover, in breast cancer, the expression of PD-L1 is correlated with TIL infiltration, and is mostly prevalent in basal-like, hormone-receptor-negative, and triple-negative tumors (56, 57). Furthermore, in glioma patients increased expression of PD-L1 in tumors was correlated with PTEN loss (58), suggesting that patients bearing genetically unstable cancer types might benefit from treatment with checkpoint inhibitors. Intriguingly, not only cancer cells, but also tumor-infiltrating myeloid cells express PD-L1, and counteract anti-tumor immunity in ovarian carcinoma and MSI-CRC (55, 59). Actually, PD-L1 expression on tumor-infiltrating immune cells has been suggested to be a better predictor of clinical response to anti-PD-L1 therapy than PD-L1 expression on cancer cells (52). It will be interesting to explore, which other cancer types are characterized by the influx of PD-L1-expressing myeloid cells.

In conclusion, to increase the efficacy of immunotherapy in different types of cancer, we could consider manipulating the many variables that determine intrinsic and acquired resistance. While altering cancer cell-intrinsic characteristics, such as mutational load or genomic instability, might be challenging, cancer cell-extrinsic characteristics, like an immunosuppressive TME, are easier to manipulate.

Evasion from Cancer Immunotherapy: Relieving Immunosuppression as an Attractive Strategy to Improve the Efficacy of Immune Checkpoint Blockade

Established tumors are characterized by an abundant influx of a variety of immune cells with immunosuppressive activity, including T_{regs}, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs) (**Figures 1** and **2**). There is accumulating evidence that interference with these immunosuppressive networks can improve anti-tumor immunity. Here, we discuss the different types of immunosuppressive immune cells present in the TME, and how blockade or reprograming of these cells or their downstream effects can enhance anti-tumor immunity and the efficacy of immune checkpoint blockade.

Regulatory T Cells

Regulatory T cells play an important role in maintaining homeostasis during infections and in preventing the development of autoimmune diseases by blocking proliferation and cytotoxic activity of effector T cells. The history of T_{regs} goes back to the 1970s, when it was discovered that a subpopulation of thymocytes induced tolerance to certain antigens in mice (60). A turning point in the research of these "suppressor cells" came in 1995. T_{regs}, phenotyped as $CD4^+CD25^+$ cells, were shown to be important for self-tolerance in mice, as inoculation of $CD4^+$ cells depleted of $CD4^+CD25^+$ cells resulted in autoimmunity in nude mice (61). Another big step forward in the characterization of T_{regs} was the identification of FOXP3, a member of the fork-head/winged-helix family of transcription factors and a key regulator of T_{reg} development and function (62). In the following years, the knowledge of T_{regs} expanded enormously. Two subpopulations of T_{regs} were identified: natural T_{regs} and induced T_{regs} (or adaptive T_{regs}), which are formed in the thymus and in the periphery, respectively. Regardless of their origin, both natural and induced T_{regs} inhibit effector T cells (63).

In 1980, it was hypothesized that a T cell population in tumors suppresses anti-tumor immune responses (64). Indeed, many experimental studies support the notion that tumor-associated T_{regs} contribute to immune escape via suppression of anti-tumor $CD8^+$ T cells. For example, elimination of T_{regs} in MO4 melanoma cell line-bearing mice results in T cell-dependent tumor rejection (65). Moreover, in a xenotransplant model for $HER2^+$ ovarian cancer, adoptive transfer of autologous $CD3^+CD25^-$ T cells and DCs loaded with $HER2^+$ antigen results in T cell-mediated tumor regression, whereas concomitant transfer of T_{regs} blocks this antigen-specific immune response (66). T_{regs} not only suppress $CD8^+$ T cells, but also $CD4^+$ T cells, NK, NKT, and B cells (67). T_{regs} exert their immunosuppressive function either by direct suppression of effector cells, or indirectly by affecting the activation state of APCs. Importantly, in order to exert their functions, T_{regs} need to be activated via their TCR, but once activated their suppressive function is non-specific (68, 69). The direct T cell-suppressive functions are mediated by release of cytokines, serine proteases and the expression of enzymes that catabolize ATP. For example, T_{regs} inhibit T cells via secretion of cytokines like TGFβ,

IL10, and IL35 (70–72) or even induce T cell apoptosis by the release of granzyme B (GRZMB) or perforin (73–75). In addition, T_{regs} express CD39 and CD73, two ectoenzymes that generate the immunosuppressive molecule adenosine from extracellular ATP (76). It has been shown that T_{regs} from CD39 knock-out mice fail to inhibit $CD4^+CD25^-$ cell proliferation (76). Finally, CTLA-4^+ T_{regs} can indirectly impair T cells by reducing the CD80/CD86 levels on APCs (36).

Supporting these data, increased numbers of intratumoral T_{regs} correlate with worse overall survival in patients with ovarian cancer, breast cancer, gastric cancer, and hepatocellular carcinoma (66, 77–81). Interestingly, this is not true for CRC in which a high number of $CD8^+$ cells and $FOXP3^+$ cells correlates with a good prognosis (82). This may be explained by the fact that T_{regs} in CRC attenuate inflammation against gut microbiota that would otherwise enhance tumor growth (82). These findings illustrate that the tumor context dictates the function of associated immune cells. Although strategies targeting CD25 (like the neutralizing monoclonal antibody daclizumab and the recombinant interleukin 2/diphtheria toxin conjugate Ontak) showed transient depletion of peripheral T_{regs} and increased activity of $CD8^+$ T cells, these approaches only result in a modest clinical benefit in cancer patients (83, 84). This might be explained by the fact that CD25 is also expressed on active effector T cells, so the lack of specificity for T_{regs} might complicate their clinical applicability. Therefore, a mechanistic understanding of the role of T_{regs} in different tumor contexts will be important for the design of therapeutic strategies aimed at suppressing the downstream effects of T_{regs}.

Myeloid-Derived Suppressor Cells

The first report describing the existence of MDSCs showed that bone marrow-derived cells were able to suppress the killing activity of splenocytes *in vitro* (85). These cells were called "natural suppressor cells" or "null cells" because they did not express markers of B, T, or NK cells or macrophages (86). Subsequently, these cells were found to expand in inflammatory conditions and in tumor-bearing hosts (85, 87). In tumor-bearing mice, tumor-derived growth factors trigger the accumulation of T cell suppressive myeloid cells in the bone marrow and spleen (87, 88). The identification of these cells was hampered by the lack of clear markers, which caused variation in terminology and ambiguity among researchers. In order to bring some clarity into the field, Gabrilovich and colleagues published a consensus paper in 2007 in which they coined the term "MDSC" to refer to a heterogeneous population of myeloid cells with the ability to suppress T cell activity (89). MDSCs consist of a group of immature and mature myeloid cells that are defined by their immunosuppressive function. Within the MDSC population, two subpopulations can be distinguished based on the expression of Ly6G and Ly6C: $Ly6C^{high}Ly6G^-$ monocytic-MDSC and $Ly6C^{low}Ly6G^+$ granulocytic-MDSC. In humans, MDSCs are defined as $CD11b^+CD33^+HLA\text{-}DR^-Lin^-$ cells with the addition of CD14 or CD15 to discriminate between monocytic- or granulocytic-MDSCs, respectively (90).

In patients with various cancer types, including melanoma, gastric, breast, and CRC, increased numbers of MDSCs in the circulation correlate with poor survival (91–93). Numerous cytokines have been implicated in the expansion of MDSCs during cancer progression, including G-CSF, GM-CSF, and stem-cell factor (SCF or KIT ligand) (94–96).

Myeloid-derived suppressor cells exert their immunosuppressive function by different mechanisms, one of which is the consumption of essential amino acids from the environment. MDSCs frequently express high levels of arginase I, which catabolizes arginine, thereby depriving T cells from arginine, which is essential for their metabolism and function (97, 98). L-Arginine is also the substrate of another enzyme highly expressed in MDSCs, called iNOS. The release of reactive oxygen species (ROS) and nitric oxide (NO) by iNOS can lead to the inhibition of MHC class II expression on APCs causing impaired antigen presentation to $CD4^+$ T cells (99). Moreover, NO can cause apoptosis of $CD8^+$ T cells (100). Another amino acid is tryptophan, whose breakdown by the enzyme IDO suppresses T cell proliferation. MDSCs isolated from human breast cancer tissues inhibit T cell proliferation and induce T cell apoptosis in an IDO-dependent manner (101). Moreover, IDO inhibitors enhance the therapeutic efficacy of anti-CTLA-4 treatment leading to intratumoral accumulation of T cells and improved survival in the B16 melanoma model (102). Additionally, the amino acid cysteine is also important for T cell activation and function. T cells depend on other cells (macrophages and DCs) for cysteine metabolism. MDSCs internalize cystine (formed of two cysteines linked via a disulfide bond), catabolize it to cysteine and, unlike macrophages and DCs, do not release it into the environment. Therefore, MDSCs limit the amount of cystine that macrophages and DCs can metabolize to activate T cells (103). Finally, MDSCs contribute to an immunosuppressive TME by inducing the development of T_{regs} in tumor-bearing mice, as adoptive transfer of MDSCs and $CD4^+$ T cells in MCA26 colon carcinoma cell line-bearing irradiated mice, induces expression of FOXP3 in transferred T cells (104). Thus, these data suggest that MDSCs play an important role in creating an immunosuppressive network in tumors, supporting the idea that reprograming or depletion of MDSCs could benefit immunotherapy strategies. Strategies to inhibit MDSCs include blocking their development or recruitment, targeting their immunosuppressive molecules or depleting them.

Tumor-Induced Neutrophils

In various cancer patients, a high neutrophil to T lymphocyte ratio in blood is associated with poor disease outcome (105, 106). Recent studies have reported that neutrophils also expand in experimental mouse tumor models, and that they exert immunosuppressive activity. A distinguishing feature of murine neutrophils is the expression of Ly6G, a surface marker shared with granulocytic-MDSC. When the T cell suppressive ability of neutrophils is confirmed, they can be categorized into the granulocytic-MDSC population (107). We recently showed in a mouse model for *de novo* breast cancer metastasis that neutrophils have a pro-metastatic phenotype and exert their function through suppression of $CD8^+$ T cells. While depletion of $Ly6G^+$ neutrophils results in decreased multi-organ metastasis, double depletion of neutrophils and $CD8^+$ T cells reverses this phenotype (108). In line with this, chemotherapy-induced neutropenia correlates with improved overall survival in breast cancer patients (109).

The metastasis-promoting role of neutrophils has also been demonstrated in UV-induced melanoma and in tumor inoculation models (110, 111). It would be interesting to study whether – as in the experimental tumor models – T cells in neutropenic cancer patients are more active. Interestingly, in 4T1-tumor-bearing mice, neutrophils inhibit the seeding of metastatic cells in the lung by the release of hydrogen peroxide (112). These data indicate a controversial role of neutrophils in metastasis that might be explained by the differences in tumor subtype or tumor model.

We and others have shown that T cell-suppressive neutrophils accumulate systemically during cancer progression in a G-CSF-dependent fashion (108, 113). In the transgenic *MMTV-PyMT* mammary tumor mouse model, tumor-derived G-CSF skews hematopoietic cell differentiation toward the granulocytic lineage in the bone marrow, resulting in increased numbers of immunosuppressive neutrophils in the circulation (113). In 4T1 mammary tumor-bearing mice, TGFβ polarizes mature neutrophils from cytotoxic anti-tumor activity toward pro-tumor immature immunosuppressive neutrophils (114). This is in line with previous findings identifying TGFβ as one of the drivers of pro-tumor polarized neutrophils (115). As such, it is tempting to speculate that for those tumors characterized by pro-metastatic neutrophils, inhibition of these cells – either by targeting upstream or downstream molecules – may be an interesting strategy for therapeutic intervention, in particular when combined with cancer immunotherapy.

Tumor-Associated Macrophages

Macrophages are frequently the most predominant immune cell type in tumors. In the past, macrophages were subdivided into classically activated macrophages (M1) exerting microbicidal and anti-tumor activity, or alternatively activated macrophages (M2) exerting pro-tumoral, immunosuppressive, and tissue repair functions (116, 117). TAMs are frequently classified as M2 macrophages. However, there is a growing realization that this black and white distinction of macrophage subsets is too simplistic and does not accurately reflect the heterogeneity, plasticity, and versatility of macrophages (118). Transcriptome and bioinformatic analyses of cultured macrophages exposed to different stimuli revealed a spectrum of activation programs for each stimulus that goes beyond the M1 and M2 model (119). Based on these data, it is to be expected that TAMs will also change their phenotype and function according to the cytokine milieu present in a specific tumor type. In the vast majority of cancers, high intratumoral macrophage density correlates with poor prognosis (120, 121). However, macrophages in CRC are associated with good prognosis, and in other types of cancers, like prostate and lung cancer, their role is still controversial (122). Depletion of macrophages by genetic ablation of CSF-1 in the *MMTV-PyMT* mammary tumor model reduces metastasis formation without affecting primary tumor growth (123). Likewise, several other experimental studies have reported a pro-metastatic role of macrophages (124, 125). TAMs produce a variety of factors that foster tumor growth and invasiveness, angiogenesis, and immunosuppression (120, 124, 126).

Tumor-associated macrophages exert their immunosuppressive activity in a similar fashion as that of MDSCs. TAMs can express various enzymes like arginase 1, IDO (127–129), and cytokines like IL10 (130). Another mechanism by which TAMs suppress T cells is the upregulation of PD-L1. In hepatocellular carcinoma, high density of peritumoral macrophages that express PD-L1 correlates with worse overall survival (131). Co-culture experiments showed that PD-L1$^+$ macrophages suppress T cell activity unless anti-PD-L1 antibody is added in the culture (131). Based on these immunosuppressive properties, it is tempting to speculate that interference with TAMs will unleash anti-tumor immunity. Indeed, this idea has recently been supported by experimental studies in mouse models for glioblastoma and pancreatic cancer showing that CSF-1/CSF-1R pathway blockade can shift TAM polarization toward an anti-tumor phenotype, resulting in enhanced CD8$^+$ T cell-mediated anti-tumor immunity (132, 133). Similarly, targeting the CCL2/CCR2 chemokine pathway – involved in recruitment of monocytes and macrophages – relieves the immunosuppressive phenotype of TAMs and enhances anti-tumor CD8$^+$ T cell responses (134, 135). Based on these encouraging results, clinical trials are ongoing in which compounds targeting TAMs are being tested in cancer patients. Preliminary results of a clinical trial with anti-CSF-1R in patients with various types of solid malignancies showed a decrease in TAMs and an increase in intratumoral CD8/CD4 ratio (10).

Blocking the Suppressors to Release Anti-Tumor T Cells

As discussed above, many immunosuppressive cells and mediators can be identified in the TME that dampen anti-tumor T cell responses and may contribute to immune escape upon cancer immunotherapy. The combination of compounds that relieve immunosuppression with T cell-boosting therapy seems attractive to overcome immune tolerance toward the tumor.

Regulatory T cells seem to be interesting targets, since, as discussed earlier in this review, these cells suppress the functionality of CD4$^+$ and CD8$^+$ effector cells. In line with this, in the transgenic TRAMP prostate cancer model – engineered to express prostate-specific antigen (PSA) – T_{reg}-depletion enhances IFNγ production by PSA-specific CD8$^+$ T cells (136). This augmented effect of anti-tumor immunity is further enhanced by CTLA-4 blockade, and results in delayed tumor growth. Interestingly, the same experiments performed in the parental TRAMP model show only a modest activation of PSA-specific T cells upon anti-CD25 and anti-CTLA-4, and no survival benefit, suggesting the requirement of a tumor-specific antigen for this anti-tumor response (136). In the ID8 ovarian cancer model, tumor-infiltrating T_{regs} – which express both CTLA-4 and PD-1 – are reduced upon CTLA-4 and PD-1 dual blockade coinciding with increased tumor-infiltrating CD8$^+$ T cells (137). However, additional depletion of T_{regs} does not further enhance this effect. In the same model, blockade of PD-L1, expressed on tumor cells and tumor-infiltrating immune cells, reduces the number of MDSCs and T_{regs} and enhances the frequency of effector T cells, resulting in prolonged survival (138). Furthermore, in a mouse model for rhabdomyosarcoma, PD-1 blockade increases the number of tumor-infiltrating CD8$^+$ T cells, but does not change their activation status. Upon interference with the chemokine receptor CXCR2, which prevents MDSC trafficking into the tumor, enhanced activation of CD8$^+$ T cells

is observed (139). Blockade of CXCR2 improves the therapeutic efficacy of anti-PD-1 treatment resulting in a significant survival benefit (139). Moreover, in a mouse model of pancreatic ductal adenocarcinoma, blockade of CSF-1/CSF-1R signaling results in macrophage reprograming to support anti-tumor immune function and modestly delays tumor growth (133). TAMs obtained from anti-CSF1 treated mice are impaired in suppressing $CD8^+$ T cell proliferation compared to control TAMs. The induction of CTLA-4 expression on $CD8^+$ T cells and PD-L1 expression on tumor cells suggests the onset of acquired resistance to effective anti-tumor immune responses. Combining anti-CTLA-4 and anti-PD-1 with a CSF-1R inhibitor shows profound synergy with a significant reduction in tumor burden (133). Thus, together these results indicate that alleviation of immunosuppression reactivates anti-tumor immunity, which can be further enhanced by checkpoint inhibition.

Immunomodulatory Properties of Chemotherapeutic Drugs

Although various novel compounds targeting tumor-associated myeloid cells and their immunosuppressive mediators are being developed and tested, their clinical availability is still limited. An alternative and clinically available strategy is to relieve immunosuppression by exploiting the immunomodulatory effects of conventional anti-cancer strategies like chemotherapy (**Figure 2**). The impact of chemotherapeutic drugs on the proportion and phenotypic and functional characteristics of immune cells is to a great extent dictated by the type of drug and the dosing scheme: while high-dose chemotherapy usually results in lympho- or myelodepletion, low-dose (metronomic) treatment has more subtle anti-angiogenic and immunomodulatory effects (140, 141). In this section, we discuss the effects of chemotherapy on the immunosuppressive TME.

The Impact of Chemotherapy on T Cell Priming

Optimal T cell priming is dependent on antigen processing, presentation, and co-stimulation by properly matured and activated DCs. As discussed, impaired DC function and T cell priming are important mechanisms of immune escape by tumors. Certain chemotherapeutics induce anti-cancer immune responses by improving the recruitment and functionality of intratumoral DCs (142, 143). For example, low-dose cyclophosphamide promotes DC maturation (144). Besides the enhanced release of tumor antigens through induction of cancer cell death, chemotherapeutics, including oxaliplatin, doxorubicin, mitoxantrone, and melphalan, induce HMGB1 release and calreticulin translocation in cancer cells, facilitating antigen uptake by DCs and subsequent T cell stimulation (145–147). In addition, in the MCA205 fibrosarcoma model, anthracyclins induce the differentiation of myeloid cells in the tumor bed toward a DC-like phenotype in an ATP-dependent manner (142). In these relatively high immunogenic tumor models, the activated T cells subsequently enhance the anti-cancer efficacy of chemotherapy (142, 143, 145).

In less immunogenic models, such as *de novo* tumorigenesis models, an important role for T cells in chemotherapy efficacy is lacking (120, 148, 149). One possible explanation is that spontaneously arising tumors are characterized by local and systemic immunosuppression, which may overrule any chemotherapy-induced T cell responses. Indeed, in the *MMTV-PyMT* mammary tumor model, TAM-derived IL10 indirectly blocks anti-tumor $CD8^+$ T cell activity by suppressing IL12 expression by intratumoral DCs upon paclitaxel treatment (149). These results apply to human breast cancer patients since low $CD68^+$ macrophage over $CD8^+$ T cell ratio prior to neo-adjuvant chemotherapy correlates with a better pathologic response (120). Moreover, high levels of *IL12A* mRNA in human breast cancer samples correlates with expression of DC-related transcription factors and *GRZMB*, *CD8A*, and *IFN*γ expression, suggesting an active anti-tumor T cell response (149). However, the role of TAMs and their potential suppressive function in cancer patients was not evaluated. Together, these results suggest that therapeutic targeting of TAMs could enhance the functionality of intratumoral DCs and anti-tumor T cell responses in chemotherapy treatment.

Impact of Chemotherapy on T_{regs}

With the knowledge that T_{regs} play an important role in suppressing effector T cell responses, a lot of effort has been put into the identification of chemotherapeutic drugs that target these cells. The best studied is cyclophosphamide, an alkylating agent, which crosslinks DNA, thus interfering with replication. Cyclophosphamide is known for its dose-dependent effect on the immune system. High doses of cyclophosphamide result in immunosuppression by reducing T cell proliferation and inducing apoptosis, thus making it useful for the prevention of graft-versus-host disease or rejection of transplanted organs (150, 151). In contrast, low doses selectively ablate T_{regs} and dampen their T cell suppressive ability (152). While the anti-tumor effect of high-dose cyclophosphamide is mainly due to its cytotoxic activity against cancer cells, the anti-tumor effect of low-dose cyclophosphamide depends on its immune-modulatory effects (153). Indeed, studies in T cell-deficient mice bearing inoculated tumors show loss of the anti-cancer activity of low-dose cyclophosphamide (153, 154). Moreover, reinfusion of $CD4^+CD25^+$ T cells in tumor-bearing mice, pre-treated with low-dose cyclophosphamide, abrogated the anti-tumor effect of the drug, emphasizing that T_{regs} counteract the therapeutic efficacy of the drug (153). In line with this, patients with different types of metastasized solid tumors receiving low-dose metronomic cyclophosphamide show a specific decrease of T_{regs} in the periphery with concomitant enhancement of NK lytic activity and T cell proliferation (155). In cancer patients receiving higher doses of metronomic cyclophosphamide, all lymphocyte populations were depleted, emphasizing the importance of accurate drug dosing to achieve selective T_{reg} depletion (155). It has been proposed that the increased sensitivity of T_{regs} for cyclophosphamide is linked to their low ATP levels. Low levels of ATP result in decreased synthesis of glutathione, which is important for cyclophosphamide detoxification (156).

Another chemotherapeutic drug affecting T_{regs} is gemcitabine, a nucleoside analog interfering with DNA replication. In an orthotopic pancreatic cancer model, gemcitabine reduces the percentage of T_{regs} in the tumor resulting in a small but significant survival benefit (157). Whether this also results in improved $CD8^+$ and $CD4^+$ T cell activity remains unknown. A study performed in

cancer patients showed that the percentage of T_{regs} in blood was decreased after gemcitabine treatment (158). Among the $CD4^+$ cells, T_{regs} were identified as the most proliferative cells, which may explain the selectivity of gemcitabine for these cells. However, the effect of gemcitabine on other T cell populations was not assessed in this study (158). Also, other (combinations of) chemotherapy drugs have been reported to influence the presence or function of T_{regs} (159, 160).

Chemotherapeutics with Inhibitory Activity Toward Tumor-Associated Myeloid Cells

Several chemotherapy drugs have been implicated in the selective reduction of MDSCs in the tumor and spleen of tumor-bearing mice (161, 162). In an EL4 inoculation tumor model, a set of chemotherapy drugs was tested for their influence on the number of splenic and intratumoral MDSCs (161). This study showed that high-dose gemcitabine and 5-fluorouracil (5-FU), two anti-metabolite drugs that interfere with DNA replication, reduce MDSC accumulation (161). Consequently, 5-FU-mediated MDSC depletion results in increased IFNγ-producing intratumoral $CD8^+$ T cells. This effect is reverted by adoptive transfer of MDSCs, suggesting that the effect of 5-FU is exerted through MDSCs (161). Similar results were obtained in the MCA203 cell line inoculation sarcoma model combined with cytotoxic T cell transfer (163), highlighting the critical role of MDSCs in dampening T cell activity upon 5-FU treatment. While the exact mechanisms underlying the selectivity of 5-FU for MDSCs are unknown, it has been proposed that 5-FU inhibits the enzyme thymidylate synthase and that the resistance to 5-FU is due to insufficient inhibition of this enzyme (164). Indeed, low levels of thymidylate synthase are found in MDSCs compared to splenocytes and EL4 tumor cells, suggesting that 5-FU selectivity for MDSCs could be due to this low enzymatic expression (161).

High-dose gemcitabine induces similar effects on MDSCs as 5-FU (162). *In vitro* analyses of splenocytes from TC-1 lung cancer-bearing mice showed the cytotoxic specificity of gemcitabine for MDSCs, while $CD4^+$, $CD8^+$ T cells, and B cells are unaffected (162). Although the exact mechanism underlying this specificity has not been identified, it has been hypothesized that gemcitabine induces apoptosis in MDSCs (162). Yet, a thorough mechanistic analysis of gemcitabine-induced apoptotic cell death in various immune cell populations has not been performed. In the 4T1 breast cancer mouse model, gemcitabine treatment also reduces splenic MDSC accumulation, which results in increased proliferation and IFNγ production by splenic lymphocytes upon antigen stimulation compared to untreated mice (165). However, no difference in anti-cancer efficacy of gemcitabine was observed between immunocompetent and nude mice, indicating a T cell-independent mechanism of 4T1 tumor control by gemcitabine (165). Perhaps, this observation might be explained by the presence of other immunosuppressive cells in the TME, like T_{regs} or macrophages.

The beneficial effect of chemotherapeutic drugs on the immunosuppressive TME is not only a direct result of reduced MDSC numbers, but also a result of a more favorable phenotype of the remaining MDSCs. For example, in the 4T1-Neu mammary tumor model, docetaxel reduces splenic granulocytic-MDSCs and enhances $CD8^+$ and $CD4^+$ cytotoxic activity (166). The remaining MDSCs exhibit a different phenotypic profile compared to MDSCs from untreated mice. In line with these *in vivo* findings, MDSCs pre-treated with docetaxel induce the proliferation of OVA-exposed OT-II $CD4^+$ T cells compared to untreated MDSCs *in vitro*, suggesting that docetaxel treatment induces a phenotypical switch to a more favorable state (166). Likewise, doxorubicin selectively decreases the proportion of MDSCs in the 4T1 breast tumor model via apoptosis and subdues the immunosuppressive phenotype of the remaining MDSCs. The remaining MDSCs have a lower expression of immunosuppressive molecules like ROS, ARG-1, and IDO (167). This less suppressive environment caused by doxorubicin enhanced the activity of adoptively transferred T helper cells (167). Interestingly, some subpopulations of MDSCs may be more susceptible to chemotherapy than others. Whether chemotherapy selectively depletes pro-tumorigenic MDSCs or skews them toward an anti-tumor phenotype is unknown. Future studies using lineage tracing methodologies would provide more insight into this topic.

Besides the favorable immunomodulatory "off-target" effects of various chemotherapeutic drugs, these drugs can at the same time exert less desirable functions. For instance, in addition to its inhibitory effect on T_{regs}, cyclophosphamide increases the number of $CD11b^+Gr1^+$ MDSCs. In a transgenic mouse model for melanoma, a single injection of low-dose cyclophosphamide increases the accumulation of MDSCs in the tumor and spleen, stimulates their immunosuppressive ability by inducing NO and ROS production, and reduces splenocyte proliferation (168). In line with these findings, MDSCs accumulate in the blood of breast cancer patients after treatment with doxorubicin or cyclophosphamide (169). This may be due to IFNγ release by $CD4^+$ and $CD8^+$ T cells that promotes survival of MDSCs (170). Based on these data, a combination of cyclophosphamide and cancer immunotherapy might not work; however, additional studies in other tumor models should be performed to test this.

Another study underscoring the complex impact of chemotherapy on myeloid cells shows that in EL4-tumor-bearing mice 5-FU induces IL1β secretion in MDSCs in an Nlrp3 inflammasome-dependent manner (171). Using depletion experiments and knock-out mice, it was shown that the MDSC-derived IL1β triggers IL17 production by $CD4^+$ T cells, which limits the anti-cancer efficacy of 5-FU (171). These data highlight that the effect of certain chemotherapy drugs is not simply limited to depletion of immunosuppressive cells but these drugs also change the functionality of cells that may impair their efficacy. These results suggest that the combination of chemotherapeutic and immunomodulatory compounds must be chosen carefully to increase their anti-cancer efficacy (172).

While several chemotherapy drugs have been reported to target MDSCs, thus far only one drug seems to strongly affect TAMs. Trabectedin, a drug that binds DNA and affects transcription and DNA repair pathways, depletes macrophages, and suppresses the differentiation of monocytes in the tumor bed in the transplantable MN/MCA1 fibrosarcoma tumor model through a TRAIL-dependent mechanism (173). Importantly, this macrophage selectivity is also observed in sarcoma patients after

trabectedin neo-adjuvant treatment (173). It would be interesting to assess whether the anti-cancer activity of trabectedin is $CD8^+$ T cell mediated. The macrophage-depleting effect of trabectedin makes it an interesting candidate for combination strategies with immunotherapy.

As discussed before, many studies illustrate the complexity of immunomodulation by conventional chemotherapeutics, which is highly context-dependent. The differential effect on specific immune cells of different types of chemotherapeutics is to a large extent dependent on the timing and dosing schedule. While high-dose chemotherapy often depletes immune cell subsets, low-dose metronomic chemotherapy exerts a more subtle anti-angiogenic and immunomodulatory mode of action (140, 141). It will be interesting to perform a side-by-side comparison of various types of chemotherapies administered at high versus low (metronomic) dose and evaluate their immunomodulatory effects, followed by more mechanistic studies. Ideally, these types of experiments would be performed in clinically relevant mouse models that faithfully recapitulate human cancer (**Box 1**) to facilitate clinical translation.

BOX 1 | Experimental mouse models to study the anti-tumor immune response.

Understanding the complex crosstalk between innate and adaptive immune cells and (disseminated) cancer cells requires the use of preclinical mouse models that faithfully recapitulate human cancer. The most widely used experimental mouse models are carcinogen-induced cancer models and cell line inoculation models. The latter is based on inoculation of large numbers of (genetically modified) homogenous cancer cells grown in 2D conditions. Implantation of these cells often results in massive cell death, thereby priming an effective anti-tumor immune response. Shaping of the tumor immune microenvironment during cancer progression in these models can hardly take place in the short amount of time that it takes for transplanted tumors to grow to their maximum tolerated size. Of notice, when implanting human cancer cells, either patient-derived tumor material or established human cancer cell lines, immunocompromised mice are used, thereby excluding the important role of the adaptive immune system.

While cell line inoculation models proved useful to decipher some aspects of the anti-tumor immune response, we should keep in mind that these models do not reflect physiological processes as they occur in human patients. Genetically engineered mouse (GEM) models, which develop *de novo* cancers, generally mimic human cancer genetically – because of the introduction of specific driver mutations – and histopathologically (180). In addition, tumor progression occurs in a multi-step nature in their natural microenvironment shaping the local immune responses (**Figure 1**), therefore mimicking the human setting. In contrast to inoculation models expressing known tumor antigens, the anti-tumor immune response in GEM models can be considered a black box. Due to their cellular and genetic heterogeneity, GEM models induce a variety of T cell responses directed against multiple unknown tumor neo-antigens, which faithfully reflects human cancer. Interestingly, comparative studies have shown that inoculation models greatly differ from GEM models in terms of response to anti-cancer therapies and endogenous T cell responses (181, 182). The advantages and disadvantages of different experimental mouse models in studying responsiveness to anti-cancer therapy have been recently discussed (14, 183).

Future Perspectives: Exploiting the Immunomodulatory Properties of Chemotherapeutic Drugs to Improve Cancer Immunotherapy

Given their immunomodulatory properties, conventional chemotherapy drugs are interesting candidates to combine with T cell-boosting immunotherapy – a concept termed chemo-immunotherapy (174). Clinical trials report enhanced anti-tumor T cell responses in cancer patients treated with chemotherapy in combination with cancer vaccines (13). Moreover, clinical testing of chemotherapy combined with other immunotherapy approaches like adoptive transfer of (genetically engineered) autologous T cells or toll-like receptor (TLR) agonists are likely to be explored in the near future. Indeed, various experimental studies support the concept that chemotherapy-induced relief of immunosuppression could improve cancer immunotherapy. In a passive immunotherapy setting, in the MC203 fibrosarcoma and TC-1 lung cancer cell line inoculation models, low-dose gemcitabine and 5-FU reduced the splenic population of $CD11b^+Gr1^+$ MDSCs, resulting in enhanced anti-tumor activity of adoptively transferred tumor-specific CTL (163).

The results obtained in preclinical models combining chemotherapeutics with immune checkpoint inhibitors are promising. The immunomodulatory effects of melphalan – administered in a subtherapeutic dose – synergizes with CTLA-4 blockade in a plasmacytoma model (175). *In vitro* assays revealed that splenocytes obtained from melphalan-treated mice co-cultured with anti-CTLA-4 induced tumor cell cytotoxicity, while splenocytes from non-treated mice – irrespective of CTLA-4 blockade – did not (175). Furthermore, in the poorly immunogenic AB-1 malignant mesothelioma and Lewis lung cancer (LLC) inoculation tumor models, a combination therapy of gemcitabine and CTLA-4 blockade synergizes, inducing potent anti-tumor immune responses and subsequent regression of tumors in a CD4- and CD8-dependent manner (176). In addition, in a subcutaneous murine mesothelioma model, synergy is observed between cisplatin and CTLA-4 blockade, resulting in a profound anti-tumor effect that is characterized by increased influx and activation of $CD4^+$ and $CD8^+$ T cells in the tumor (177). Moreover, preclinical studies in mice show that doxorubicin, cisplatin, and paclitaxel in addition to their immunomodulatory role, can sensitize tumor cells for CTL attack in a direct manner (178). Here, chemotherapy causes increased permeability of tumor cell membranes to GRZMB, which sensitizes cancer cells to the cytotoxic effects of T cells and improved different cancer immunotherapy strategies (178). Together, these preclinical studies – albeit limited numbers – show the potential to exploit immunomodulatory chemotherapeutic drugs to improve the efficacy of checkpoint blockade.

Clinical trials that evaluate the combination of chemotherapeutic drugs and checkpoint inhibitors in cancer patients are still limited. Some studies in melanoma and lung cancer have used chemotherapeutics in combination with checkpoint blockade resulting in improved survival compared to chemotherapy alone (41, 179). However, the rational of these studies was not to evaluate the effect of treatment on the immunosuppressive microenvironment. Moreover, the design of clinical trials makes it impossible to perform a structural comparison in patients to study the effect of the immunosuppressive microenvironment on immunotherapy efficacy and whether this efficacy can

be enhanced by adding chemotherapeutics to the treatment regimen. Therefore, we need to rely on preclinical research in mouse tumor models that faithfully recapitulate human cancer in terms of the genetic composition, anti-tumor immunity, and the immunosuppressive TME (**Box 1**). Results obtained in mouse models that mimic human cancer might shape the design of clinical trials and guide toward interesting treatment strategies. There are still various important questions that need to be addressed to maximally exploit the therapeutic efficacy of chemotherapy and immunotherapy combinations, like the determination of the most optimal combinations. Based on preclinical findings, different cancer types will likely require different combinations of therapy. In addition, despite the devastating effects of metastatic disease, mechanistic insights into the site-specific therapeutic response profiles and resistance mechanisms of cancer immunotherapy are completely lacking. Moreover, it is critical to gain insights into the mechanisms underlying intrinsic and acquired resistance to cancer immunotherapy. To answer these questions within the next decade, it is critical that basic researchers and clinicians intensify their efforts to join forces, so that results from preclinical research can guide the design of clinical trials, and the results from clinical trials, in turn, can guide mechanistic studies in mouse models. Together, these efforts will improve treatment strategies using chemotherapeutics to alleviate immunosuppression and enhance cancer immunotherapy.

Author Contributions

KK, CS, and KV reviewed relevant literature and drafted the manuscript. KV revised the manuscript and supervised KK and CS. All authors read and approved the final manuscript.

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REVIEW
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Targeting epigenetic processes in photodynamic therapy-induced anticancer immunity

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Photodynamic therapy (PDT) of cancer is an approved therapeutic procedure that generates oxidative stress leading to cell death of tumor and stromal cells. Cell death resulting from oxidative damage to intracellular components leads to the release of damage-associated molecular patterns (DAMPs) that trigger robust inflammatory response and creates local conditions for effective sampling of tumor-associated antigens (TAA) by antigen-presenting cells. The latter can trigger development of TAA-specific adaptive immune response. However, due to a number of mechanisms, including epigenetic regulation of TAA expression, tumor cells evade immune recognition. Therefore, numerous approaches are being developed to combine PDT with immunotherapies to allow development of systemic immunity. In this review, we describe immunoregulatory mechanisms of epigenetic treatments that were shown to restore the expression of epigenetically silenced or down-regulated major histocompatibility complex molecules as well as TAA. We also discuss the results of our recent studies showing that epigenetic treatments based on administration of methyltransferase inhibitors in combination with PDT can release effective mechanisms leading to development of antitumor immunity and potentiated antitumor effects.

Keywords: photodynamic therapy, cancer, immunotherapy, epigenetic mechanisms, histone deacetylase, methyltransferase

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Introduction

Photodynamic therapy (PDT) is a light-based therapeutic approach used for the treatment of various solid tumors and non-malignant diseases. Its mechanism of action involves three non-toxic and harmless components: photosensitizer (PS), light, and oxygen. Their spatiotemporal encounter triggers photochemical reaction leading to formation of singlet oxygen and ensuing photodamage in tumor tissue (1). PS can be applied topically or administered systemically. After a period allowing for PS accumulation within the tumor, light of appropriate wavelength is precisely delivered to tumor site, usually from laser sources. Light activates PS from its ground state to the excited triplet state. Activated PS transfers its energy to molecular oxygen, leading to generation of highly reactive singlet oxygen, or reacts directly with biomolecules forming free radicals such as superoxide ion, hydroxyl radical, or hydrogen peroxide. These reactive oxygen species (ROS) mediate oxidative damage of intracellular macromolecules causing tumor cell death (2, 3).

Photodynamic Therapy and Anticancer Immunity

Direct cytotoxic effects induced by PDT do not explain strong antitumor activity of this treatment observed in experimental animals. For example, cells from the tumors excised immediately after curative PDT are still clonogenic indicating that there must be other, indirect mechanisms triggered by PDT that contribute to the efficacy of the treatment (4). These indirect mechanisms include disruption of tumor vasculature, leading to oxygen and nutrient deprivation, and induction of robust inflammatory reaction, which can further stimulate development of antitumor immune responses (5, 6). A critical role in the therapeutic outcome of PDT is played by the immune system. Studies in immunodeficient mice or in mice inoculated with lymphocyte-depleting antibodies revealed that the presence of effector immune cells is necessary for maximum therapeutic response (7–9).

Innate Immune Response in PDT

Massive PDT-induced photooxidative damage occurs mainly in the membranes and cytoplasm of tumor cells, tumor vasculature, and other stromal elements. This substantial PDT-mediated local injury threatens the host tissue integrity and homeostasis. Therefore, a host response develops as acute local inflammation in order to eliminate dead and injured cells, heal lesion, and restore tissue function as well as maintain its homeostasis (8). This response is induced almost instantaneously following PDT. Triggered by massive cell death, release of cytoplasmic components, vasoactive substances, as well as activation of the complement cascade, it leads to the secretion of leukocyte chemoattractants, cytokines, growth factors, and other immunoregulators that lead to a robust infiltration of the tissue with neutrophils, mast cells, macrophages, and NK cells (10).

A number of cytokines have been detected both within the tumor and in the plasma of mice undergoing PDT. Among a wide range of cytokines (IL-1β, IL-6, IL-10, TNF, and G-CSF) and chemokines (KC and MIP2) induced after PDT, a very important role is assigned to IL-1β and IL-6 (11). Neutralization of IL-1β reduces the cure rates of PDT-treated tumors, whereas no significant effects are observed with anti-TNF-alpha and anti-IL-6 antibodies (12). Also, recombinant cytokines such as G-CSF, GM-CSF, and TNF combined with PDT enhance antitumor response (13–15), whereas blocking anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) improves the cure rates of PDT-treated tumors (8).

Neutrophils are the first cells invading PDT-treated tumor sites showing remarkable impact on PDT-mediated tumor damage. It was shown that neutrophils depletion in tumor bearing mice and rats attenuates the efficacy of antitumor PDT (16, 17). Monocytes/macrophages infiltrating tumor bed also seem to participate in regulating the outcomes of PDT. Inactivation of macrophages with silica particles decreases cure rates in mice, whereas treatment with macrophage-activating factor Vitamin D3-binding protein or GM-CSF potentiates antitumor effects of PDT (13, 18). Role of innate immune response in antitumor PDT is also associated with activity of NK cells. Depletion of NK cells significantly inhibits the response to PDT at suboptimal dose (19).

Innate immune cells encounter released tumor antigens (including oxidatively modified ones) together with molecules known as damage-associated molecular patterns (DAMPs) or cell death-associated molecular patterns (CDAMPs) (9). DAMPs play an analogous role to pathogen-associated molecular patterns (PAMPs), serving as warning signals. Recognition of PAMPs leads to initiation of the pathogen-induced responses, whereas DAMPs promote inflammatory responses to cell stress, injury, or death. DAMPs bind to pattern recognition receptors (PRRs) on the surface of infiltrating leukocytes and activate antigen-presenting cells (APCs) to stimulate innate and adaptive immunity. Therefore, DAMPs released from PDT-treated tumor cells are believed to be the key players in the immunogenicity of tumor cells (20, 21). The best known and frequently reported examples of PDT-induced DAMPs include heat-shock protein (HSP) family (HSP70 and HSP90), high mobility group box-1 (HMGB-1), adenosine triphosphate (ATP), and calreticulin (CRT).

Adaptive Immune Response in PDT

It was demonstrated that the degree of PDT-mediated inflammation influences adaptive immune response and generation of antitumor immunity (22). The link between innate and adaptive immune response is dendritic cells (DCs), the most potent APCs, capable of migrating to secondary lymphoid tissues to prime T cells (23). PDT that triggers necrotic and apoptotic tumor cell death, accompanied by oxidative stress and induction of HSPs, is believed to shape a unique environment with tumor antigens and "danger" signals activating DC maturation (24, 25). It was shown that PDT-elicited local and systemic inflammation results in attraction of DCs to the tumor site, their activation, and maturation (24, 26, 27). DCs that have captured tumor-derived proteins and encounter DAMPs undergo activation and functional maturation, migrate to the tumor-draining lymph nodes, where they present tumor-associated antigens (TAA) in association with major histocompatibility complex (MHC) class I and II molecules to T lymphocytes. This allows selection, proliferation, and differentiation of the rare antigen-specific T lymphocytes into effector T cells (28). During effective adaptive antitumor immune response, activated T cells return to the circulation in order to home to the tumor site to carry out their effector functions (29). There are several independent studies underscoring the role of effector $CD8^+$ cytotoxic T cells (CTLs) in PDT outcome. Long-term tumor control after PDT treatment is possible only in immunocompetent mice, whereas in immunodeficient SCID or nude mice the long-term effects are abrogated. However, adoptive transfer of T cells from normal mice that underwent successful PDT restores antitumor PDT efficacy in immunodeficient animals. Importantly, T-cell depletion studies revealed that the $CD8^+$ T-cell population is critical for a successful PDT response whereas $CD4^+$ T cell population plays only a supportive role (19).

Several reports describe the essential role of $CD8^+$ T cells also in clinical PDT efficacy. Tumors lacking MHC class I on their surface are resistant to specific antitumor immune response since recognition of MHC I is necessary for $CD8^+$ T cell activation (30). Moreover, PDT of multifocal angiosarcoma resulted in spontaneous regression of untreated distant tumors accompanied by increased infiltration with $CD8^+$ T cells (31).

Tumor Escape Mechanisms from Immune Surveillance

There is a strong evidence from mouse and human studies for the existence of antitumor immune response. However, tumor cells engage diverse mechanisms to modulate the immune response and to evade recognition and elimination by effector lymphocytes (32). "Cancer immunoediting" concept was proposed to describe the interactions between tumors and the immune system. According to this paradigm tumors are kept under surveillance of the immune system that either eliminates nascent tumor cells or keeps them at check in the so called equilibrium. But this protective response also shapes transformed cells in the "immunoediting" process to select for variants that develop escape mechanisms mitigating development of an effective antitumor immune response (33). A variety of mechanisms develop in tumors to avoid recognition by cells of the immune system. These mechanisms can be either inherent to tumor cells themselves or develop in stromal compartment.

Defective Antigen Presentation

Presentation of TAA to lymphocytes is critically dependent on the multiple components of the antigen processing machinery (APM). It consists of four major steps: (1) peptide processing, (2) peptide transport, (3) MHC class I assembly, and (4) antigen presentation (34). A fundamental mechanism resulting from immunoediting and allowing tumor cells to evade immune surveillance is associated with down-modulation of APM. The loss of MHC class I antigens is one of the most frequent way to evade immune recognition (35, 36). Total loss of MHC I may be a result of structural changes in the β2-microglobulin gene resulting from mutations, deletion or loss of heterozygosity. Whereas decreased expression of these molecules largely depends on the regulation of transcriptional processes, involving epigenetic modulation (see below). Impaired APM can also be caused by decreased expression of proteasome subunits LMP-2 LMP-7, and LMP-10 down-regulation of proteasome activator PA28, peptide transporters TAP-1, and TAP-2 as well as chaperones tapasin and calnexin. These phenomena are observed in various types of tumors both in mice and humans, often in metastases (35, 37–39).

Another mechanism of insufficient antigen presentation involves loss or down-regulation of potentially immunogenic TAA expression. In melanoma, tumor development is frequently related to low level of TAA (32). Similarly, reduced expression of MUC-1 antigen is observed in human breast cancer cells. $CD8^+$ T lymphocytes isolated from patients with low expression of MUC1 do not react to autologous tumors (40). Molecular mechanisms responsible for changes in MHC expression on tumor cells include several types of gene modifications. However, *in vitro* studies show that loss of one allele or haplotype occurs very rarely (41). Therefore, it is suggested that tumor cells engage different strategies affecting antigen presentation in order to escape from immune recognition. Recent studies emphasize the role of epigenetic changes not only in tumor development and progression but also in tumor evasion (42, 43). It seems that epigenetic modifications play a key role in regulation of MHC, APM, and TAA expression level in tumor cells.

Tolerance, Deviation, and Adaptation

Although tumor cells, with rare exception of hematologic malignancies, do not express co-stimulatory molecules, they can express inhibitory molecules, such as PD-L1, PD-L2, LAG-3, TIM3, BTLA-4, or VISTA that induce deletion or anergy of tumor-reactive T cells. Some of these molecules as well as tumor-expressed FasL (CD95L/Apo1L) can also induce cell death in both T and NK cells. Another related mechanism is associated with surface expression of non-classical MHC class I molecules HLA-G and HLA-E that inhibit cytotoxic activity of effector CTLs and NK cells (44–46). Circulating MICA and MICB molecules, ligands for NKG2D receptor attenuate effector capacity of T lymphocytes and NK cells (47).

Suppressed antitumor immune response may be a result of tumor-induced changes in the function of DCs. Human and mouse tumors release cholesterol metabolites down-regulating the expression of CCR7 receptor on maturing DCs. This inhibits CCR7-dependent DC migration to lymphoid organs (48). Moreover, tumor cells produce vascular endothelial growth factor (VEGF) responsible not only for tumor angiogenesis, but also for impairment of DC maturation. Treatment with monoclonal antibodies against VEGF improves DC function *in vivo* and the efficacy of cancer immunotherapies (49). TGF-β negatively influences the activity of T lymphocytes and NK cells, inhibits maturation of DCs, and facilitates the recruitment of regulatory T cells (50). Likewise, immunosuppressive IL-10 is known to inhibit the function of APCs and generation of CTLs as well as suppress the activity and/or migration of CTLs (51).

Moreover, tumor cells can release enzymes that metabolize amino acids regulating activity of immune cells. One of such enzymes is indoleamine 2,3-dioxygenase (IDO), responsible for tryptophan catabolism. Enhanced expression of IDO in some types of tumors causes local shortage of tryptophan, leading to disturbances in proliferation of alloreactive T lymphocytes and their cell cycle arrest (52). Additionally, some tryptophan metabolites induce apoptosis in $CD4^+$ T lymphocytes whereas kynurenine, a product of IDO-mediated tryptophan catabolism, leads to their differentiation into T regulatory cells (Tregs) that down-regulate immune response (53, 54).

Tumor cells that are unable to escape from immune recognition using the above mechanisms develop adaptation mechanisms to evade effector CTL-induced death. They can up-regulate expression of antiapoptotic molecules such as FLIP or $BCL\text{-}X_L$ (55, 56). Otherwise, in order to avoid cell death, tumors can express inactive death receptors such as TRAIL-R1, TRAIL-R2, or FAS (57, 58).

Immunosuppressive Cells

Together with tumor-intrinsic immune escape mechanisms described above, tumors may also highjack parts of the immune system to evade immune attack. To achieve this goal, they induce or recruit immune-suppressive Tregs as well as myeloid-derived suppressor cells (MDSC), which under normal conditions serve as safeguards against overwhelming inflammation. In this way, tumors turn the immune system against itself, and exercise a powerful arsenal of mechanisms unavailable to tumor cells themselves to mitigate anti-tumor immune activity. Tregs inhibit

activation and expansion of antigen-specific $CD8^+$ T lymphocytes, through high expression of immune-inhibitory receptors cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and PD-L1, secretion of immunosuppressive cytokines such as IL-10 and TGF-β, and by consuming IL-2 (59). There are also other regulatory populations of lymphocytes that can be found among subsets of B cells and NKT cells inhibiting antitumor effector cell responses (60). MDSCs are heterogeneous population of cells originating from bone marrow including progenitor and immature myeloid cells of granulocytic or monocytic lineages (61). MDSCs engage several diverse strategies to suppress tumor growth by inhibiting tumor cell cytotoxicity mediated by NK cells and by blocking the activation of tumor-reactive $CD4^+$ and $CD8^+$ T cells (62, 63). These mechanisms include production of immunosuppressive cytokines such as TGF-β and IL-10, production of reactive nitrogen and oxygen species, interference with T cell homing, and contribution to tumor angiogenesis (61, 63, 64). Moreover, MDSCs prevent antigen/MHC peptide recognition by nitrosylation of T cell receptor (TCR) and deplete amino acids such as tryptophan (IDO) or arginine (arginase-1) that are required for activation and proliferation of T cells (65). Additionally, MDSCs induce accumulation of Tregs, which in turn down-regulate cell-mediated immunity and promote a Th2 type response that favors tumor progression (66).

The Role of Epigenetic Changes in Immune Escape

Epigenetic mechanisms include post-translational modifications of histone proteins affecting chromatin remodeling, DNA methylation, and regulation of gene expression by non-coding RNAs. A number of epigenetic events seem to play a pivotal role both in tumor progression and in avoiding immune recognition (67, 68). The most widely studied and best understood in terms of modulating immunity are DNA methylation and histone modifications (**Figure 1**).

Methylation occurs predominantly in CpG-rich regions called "CpG islands." A characteristic feature of tumor cells is global hypomethylation of their genome, and hypermethylation of CpG island in promoter regions of various genes (69). Methylation of DNA involves covalent addition of methyl group to C5 of cytosine ring leading to generation of 5-methylcytosine (70). Methylation of promoter regions leads to recruitment of methyl-CpG-binding proteins that form chromatin-remodeling co-repressor complexes resulting in gene silencing (67). Methylation pattern in every cell is established and maintained by a family of proteins called DNA methyltransferases (DNMTs).

Histones acetylation is a reversible process of adding an acetyl moiety to lysine residues on histone proteins resulting in neutralization of their positive charge and impairing their interaction with negatively charged DNA. Therefore, acetylation increases the accessibility of regulatory proteins to DNA, enabling activation of various genes expression (71). This process may be reversed by the opposite activities of histones deacetylases (HDACs) that remove acetyl groups from histone proteins leading to recovery of N-terminal tail affinity to DNA strand. This results in chromatin condensation and suppression of transcription process.

Influence of Epigenetic Therapy on Tumor Antigen Expression

DNA methylation is one of the most important epigenetic mechanism regulating expression of genes responsible for recognition of tumor cells by host immune system. Particularly, this is relevant to methylation of gene promoters, which leads to silencing of TAA and APM proteins, enabling escape from tumor immune-surveillance (72).

The presence of TAA in cancer cells is a mandatory requirement for activation of effector CTLs. TAA can be divided into four different groups: (i) differentiation antigens, which are lineage-specific and expressed in tumor as well as in normal cells from which the tumors arise; (ii) overexpressed antigens, which are broadly expressed in many normal tissues, but present in tumor cells at higher levels; (iii) tumor-specific antigens, usually typical for individual tumors, resulting from genetic alterations; (iv) cancer–testis antigens (CTA) that are expressed in various types of malignant human tumors and are restricted in normal tissues to germ cells of the testis, with occasional expression in female reproductive organs (73). CTA are particularly susceptible to epigenetic regulation. They include melanoma-associated antigen (MAGE), NY-ESO-1, and SSX gene families as well as the GAGE/PAGE/XAGE superfamily. MAGE, GAGE, BAGE, SSX, and LAGE-1/NY-ESO-1 are frequently methylated and down-regulated in tumor cells. The CTA family also includes P1A antigen, one of the best known murine TAA, which is a homolog of human MAGE (74). P1A is an endogenous protein, initially identified in chemically induced mast cell-mastocytoma 815 (75). As a classical CTA, P1A is not expressed in normal tissues, but expressed only in placenta and testis. P1A epitopes are presented to T lymphocytes through MHC H2-k2d and may induce strong specific response of CTLs (76). Similar to human MAGE gene family, in several murine tumors P1A is not expressed as a result of methylation of the promoter regions (77).

Immunoregulatory Effects of Drugs Targeting Epigenetic Mechanisms

Drugs targeting epigenetic mechanisms can modulate expression of multiple genes including tumor suppressor genes, oncogenes, tumor associated antigens, as well as molecules involved in antigen presentation, co-stimulatory signaling and cytokines. Among different classes of genes described as epigenetically regulated, these encoding TAA are undoubtedly essential for T cell activation and tumor recognition by immune response. The expression of CTA can be restored by a number of hypomethylating agents (78, 79). Over 20 years ago, it was demonstrated that 5-aza-2′-deoxicitidyne (5-aza-dC) up-regulates MAGE-1 expression in tumor, but not in normal cells, and leads to HLA-A1-restricted lysis of tumor cells by CTLs (78). Further studies revealed that a variety of other CTA can be induced by either 5-aza-dC or other inhibitors of DNMTs (80–82). Methyltransferase inhibitors can also induce expression of MHC class I molecules. These effects result from the impact on both MHC genes as well as from regulation of virtually all components of the APM, including TAP1 and 2, proteasome subunits (81, 83). Moreover, antigen presentation can be augmented by up-regulation of type I and II interferons, their receptors, and components of the IFN-signaling pathways (84). Importantly, the effects of methyltransferase inhibitors are long-lasting as CTA are

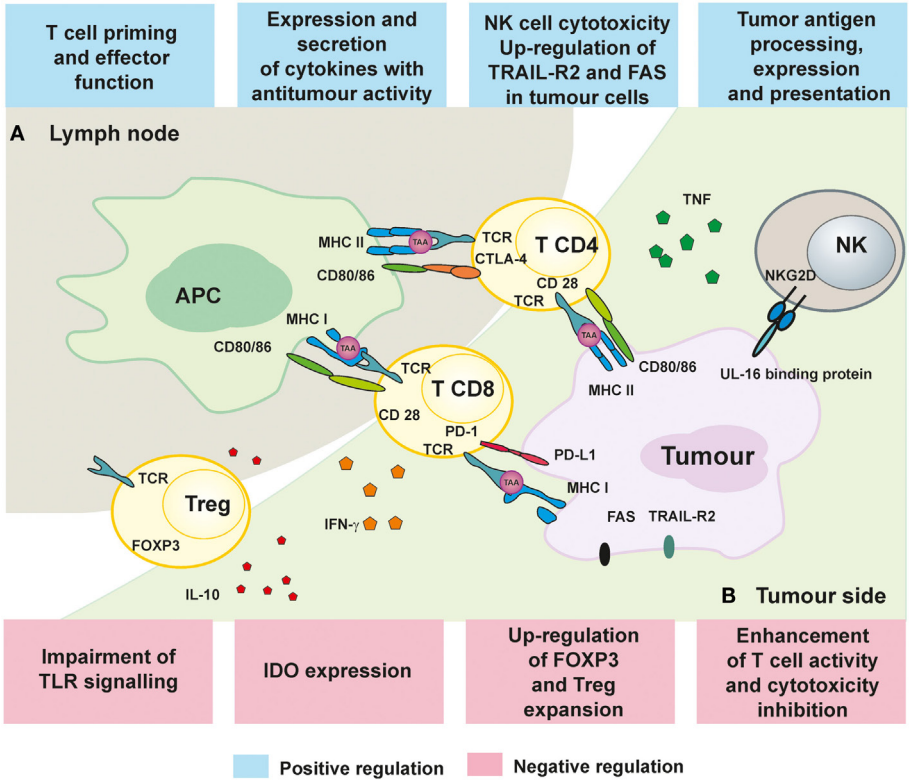

FIGURE 1 | Immunoregulatory mechanisms of epigenetic treatment. **(A)** The influence of HDACi and hypomethylating drugs on APC presentation of TAA as well as activation and proliferation of T cell in lymph nodes. **(B)** Immune response in the tumors can be improved by epigenetic treatment by augmenting T and NK cell cytotoxicity and secretion of TNF and IFN-γ.

detectable for next several weeks after treatment and they are recognized by antigen-specific $CD8^+$ T cells (77, 85, 86). Also histone deacetylase inhibitors (HDACi) may affect expression of TAA, but mostly by increasing or restoring expression level of proteins involved in antigen presentation. For example, trichostatin A (TCA) was shown to up-regulate or induce expression of TAP-1, TAP-2, tapasin, and LMP-2 in murine tumor cell lines (87, 88). Moreover, the TCA-mediated increase in MHC levels results in activation of adaptive immune response and inhibition of tumor growth in mice (88). Additionally, HDACi increase the expression of MHC class II and co-stimulatory molecules in human and mouse melanoma and trophoblast cell lines (89, 90). TCA, by means of activation of the pIII-CIITA promoter in neoplastic cells and induction of MHC class II expression, was also found to augment $CD4^+$ T cells proliferation (91). However, the result of their action is complex, since they down-regulate one antigen, Muc1, and up-regulate another, NY-ESO-1, at the same time (92). Based on these findings, a great deal of interest has been generated in investigation of epigenetic therapy influence on antitumor immune response.

It was also demonstrated that HDACi can affect polarization of naive $CD4^+$ T cells toward Th1 and Th2 subsets. Vorinostat by inhibiting STAT6 and TARC may impair the functions of $CD4^+$ T cells, shifting the balance toward Th1 response (93). Also hypomethylating agents increase production of cytokines, including IL-2, TNF, and IFN-γ (94, 95). Epigenetic treatment facilitates killing of tumor cells by NK cells or CTLs through up-regulation of TRAIL-R2 and FAS in transformed cells (96, 97). Furthermore, enhancement of NK-mediated tumor cell death was also induced by TCA by up-regulation of UL-16-binding protein expression (a ligand for cytotoxicity NKG2D receptor) (98).

Tumor Cell Recognition by Immune System After Photodynamic Therapy Enhanced by Epigenetic Treatment

The still elusive goal for effective cancer immunotherapy is to overcome tumor escape mechanisms and to trigger development of systemic adaptive antitumor immune response allowing for the control of distant metastases. As described above, PDT is an effective local treatment that induces acute local inflammatory response. However, development of systemic adaptive immune response after PDT strongly depends on the efficacy of presentation and recognition of tumor antigens by the immune cells (1). Various approaches have been examined to accelerate the priming phase of immune response after PDT. Induction of antitumor activity depends on activation of $CD8^+$ T cells and administration of immature DCs into the PDT-treated tumors resulted in effective activation of T and NK cells (24). Also, the PDT effectiveness was improved by administration of adjuvants, such as glycated chitosan (99). Additionally, the

role of expression level of MHC class I in PDT was evaluated in the treatment of patients with vulval intraepithelial neoplasia (VIN). In VIN lesions that respond to PDT, significant increase of $CD8^+$ infiltration after treatment was observed when compared to non-responders. Interestingly, none of responding VIN patients showed any evidence of MHC class I down-regulation, whereas all of the cases of VIN lacking of MHC I failed to respond to PDT (100).

One of the approaches to increase immunogenicity of tumor cells focused on their genetic modification to enhance activation of CTLs by DCs. Introduction of foreign antigen, such as green fluorescence protein (GFP) to radiation-induced fibrosarcoma cells was observed to induce strong tumor-specific immune response allowing for long-term tumor control. Re-challenge experiments revealed that survived mice developed resistance to GFP-positive cells (101). These data are in line with another study demonstrating that the presence of β-galactosidase antigen in tumor cells is able to increase immunogenicity allowing PDT to elicit strong antitumor effects and long-term immunity to re-inoculated tumor cells (102). In this vein, the same authors have transfected tumor cells with a gene encoding P1A, a model CTA in the mouse. The presence of this antigen led to effective antitumor immune response that only developed when PDT was used and was found sufficient to prevent tumor growth when tumor cells were re-inoculated (74). Moreover, PDT was shown to enhance systemic immune responses to tumors in patients with Basal cell carcinoma. The immune recognition of cancer cell antigen – Hip 1 – was improved by PDT treatment (103).

Considering that 5-aza-dC restores expression of CTA including P1A as well as MHC class I molecules (77), we sought to evaluate the antitumor effects of the combined treatment involving PDT and administration of 5-aza-dC. We have observed that treatment with 5-aza-dC alone restores expression of MHC class I molecules as well as induces expression of P1A antigen in four different murine tumor models and two strains of mice (104). Antitumor effects of 5-aza-dC were rather insignificant when used alone. However, when we combined 5-aza-dC with PDT, we observed prolonged complete antitumor responses in mice with EMT6 mammary tumors and CT26 colon adenocarcinomas and significant prolongation of survival in mice bearing 4T1 mammary tumors and LLC lung carcinomas. The antitumor effects of the combination treatment were strongly dependent on the presence of $CD8^+$ CTLs as their depletion with monoclonal antibodies almost completely abrogated antitumor effects. On the other hand, $CD4^+$ T cells played only a supportive role. We have also observed that the combined treatment led to expansion of IL-17-producing $CD4^+$ T cells, which are known to stimulate $CD8^+$. Intriguingly, pentamer staining for P1A-specific $CD8^+$ T cell population revealed no significant changes in draining LNs and spleens between experimental groups. Moreover, all mice treated with PDT and 5-aza-dC that remained long-term tumor-free have rejected re-inoculated tumor cells even if the cells were P1A-negative. These findings suggest that the presence of P1A is not essential for the maintenance of long-lasting antitumor immunity. It is possible that 5-aza-dC combined with PDT may lead to increased expression and release of TAA in the PDT-treated microenvironment. Together with PDT-induced inflammation and the release of DAMPs, the combination treatment would confer better antigen presentation of P1A. Improved tumor recognition by immune cells can further expand the repertoire of antigen-specific T cells thereby increasing the

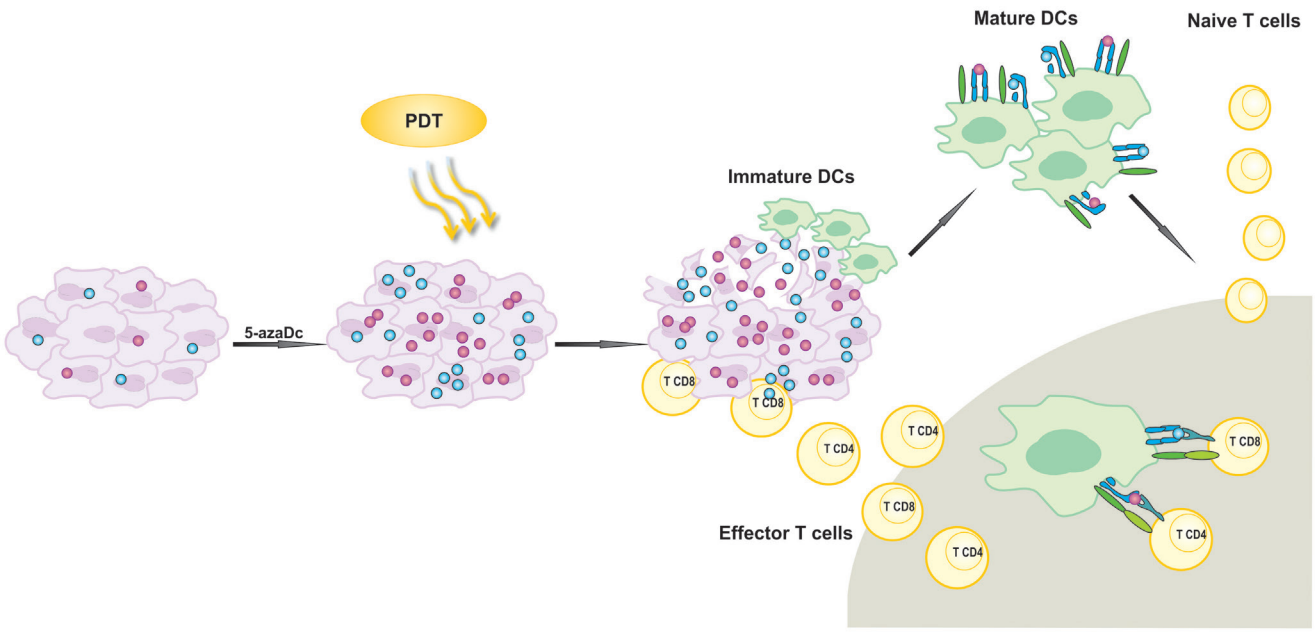

FIGURE 2 | Activation of antigen-specific antitumor immune response by photodynamic therapy (PDT). 5-aza-2′-deoxycitidine (5-aza-dC) up-regulates expression of silenced tumor-associated antigens (TAA). PDT leads to the release of TAA that are further phagocytosed by attracted to the tumor lesion of immature dendritic cells (DCs). Activated DCs migrate to local lymph nodes and present TAA-derived peptides in association with MHC molecules to T lymphocytes. T cells are activated and subsequently differentiate into effector cells homing to the tumor site in order to destroy residual tumor cells.

immunogenic outcome of PDT. Thus, it can be hypothesized that PDT of tumors with TAAs up-regulated by 5-aza-dC can induce concomitant immunity to other subdominant and possibly weakly immunogenic antigens and facilitate epitope spreading. Immunity to the latter can sustain antitumor activity in mice. To summarize, our findings demonstrate that treatment combining 5-aza-dC with PDT leads to local cytotoxic effects accompanied by the release of induced TAAs allowing for development of antitumor immune response and long-term survival (**Figure 2**). Based on these findings, we hypothesize that inhibition of DNA methylation could unleash stronger immune response against TAA in tumor-bearing mice undergoing PDT.

Author Contributions

MW, AM, and JG reviewed relevant literature. MW, AM, and JG drafted the manuscript. JG revised the manuscript and supervised MW and AM. MW and AM designed the figure. All authors read and approved the final manuscript.

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Molecular and Translational Classifications of DAMPs in Immunogenic Cell Death

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Abbreviations: DAMP, damage-associated molecular pattern; DC, dendritic cell; ER, endoplasmic reticulum; GEMM, genetically engineered murine model; HSP, heat shock protein; Hyp, hypericin; ICD, immunogenic cell death; NDV, Newcastle disease virotherapy; PDT, photodynamic therapy; ROS, reactive oxygen species.

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The immunogenicity of malignant cells has recently been acknowledged as a critical determinant of efficacy in cancer therapy. Thus, besides developing direct immunostimulatory regimens, including dendritic cell-based vaccines, checkpoint-blocking therapies, and adoptive T-cell transfer, researchers have started to focus on the overall immunobiology of neoplastic cells. It is now clear that cancer cells can succumb to some anticancer therapies by undergoing a peculiar form of cell death that is characterized by an increased immunogenic potential, owing to the emission of the so-called "damage-associated molecular patterns" (DAMPs). The emission of DAMPs and other immunostimulatory factors by cells succumbing to immunogenic cell death (ICD) favors the establishment of a productive interface with the immune system. This results in the elicitation of tumor-targeting immune responses associated with the elimination of residual, treatment-resistant cancer cells, as well as with the establishment of immunological memory. Although ICD has been characterized with increased precision since its discovery, several questions remain to be addressed. Here, we summarize and tabulate the main molecular, immunological, preclinical, and clinical aspects of ICD, in an attempt to capture the essence of this phenomenon, and identify future challenges for this rapidly expanding field of investigation.

Keywords: anti-tumor immunity, immunogenicity, immunotherapy, molecular medicine, oncoimmunology, patient prognosis, translational medicine

INTRODUCTION AND HISTORICAL BACKGROUND

Augmenting the immunogenicity of cancer cells to improve the efficacy of cancer therapy is a paradigm that has gained significant momentum over the past 5 years (1–5). Researchers have realized that besides therapeutically exploiting innate or adaptive immune cells directly (e.g., through dendritic cell (DC)-based vaccines or adoptive T-cell transfer) and/or improving the effector functions of T cells (through checkpoint-blocking therapies), cancer cells also need to be made immunogenic (1, 4, 6, 7). This has diverted attention toward studying the interface between stressed or dying cancer cells and the immune system, in the hope of efficiently exploiting it for therapeutic purposes (1).

Early indications regarding immune system-driven tumor control emerged in the eighteenth century, when feverish infections in cancer patients were circumstantially associated with tumor remission (8). The first evidence that immunotherapy can be applied to achieve tumor regression emerged from the work of William Coley, who in the 1890s achieved tumor regression in some sarcoma/lymphoma patients upon the intra-tumoral injection of streptococcal cultures (provided by Robert Koch) (8, 9). In the following 43 years, Coley injected nearly 900 (mostly sarcoma) patients with his bacterial preparation (achieving a cure rate >10%), which later became known as "Coley's toxin" (8, 10). However, the Coley's toxin came under intense scrutiny owing to an elevated toxicity and some difficulties in reproducing remission rates (8). Eventually, the first experimental evidence that virus-unrelated tumors can indeed be recognized by the host immune system emerged in the 1940s, and by the 1960s, coupled with the discovery of T cells, it was proposed that the human immune system may also react against tumors (11). The ability

of anticancer therapies to enhance the immunogenic potential of malignant cells gained some appreciation by the 1970s (12–14). It was recognized that if specific treatments are applied (e.g., radiotherapy, the bacillus Calmette–Guerin, or some chemotherapeutics), the immunogenicity of malignant cells increases enough to induce durable anti-tumor immunity (12–14). By the 1980s, researchers started to report more specific observations regarding the therapeutic impact of cancer cell immunogenicity, e.g., the ability of curative hyperthermia to cause the (heat-shock based) generation of circumstantial anti-tumor immunity (15), the fact that the immunogenicity of cancer cells influences patient prognosis after radiotherapy (16), and the increase in tumor immunogenicity due to hydrostatic pressure (17). However, these early studies (especially those published before the 1980s) had several issues linked to a lack in consensus. For instance, due to early controversies on the existence of tumor-associated antigens (TAAs) (11), the target of tumor-specific immune responses was unclear, and the mechanism of action of some therapies came under scrutiny. Moreover, such therapies could operate by directly modulating immune effector cells rather than improving the immunogenic potential of tumors (18). In particular, the death of cancer cells exposed to therapy was never suspected to drive anti-tumor immunity, since it was considered to be a relatively "silent" process in terms of immunogenicity (19). Moreover, the classical "self/non-self" theory was unable to explain the possibility that dying cancer cells could elicit an immune response (20).

By the early 1990s, the molecular characterization of mice and human TAAs clarified the entities targeted by anti-tumor immune responses (11). Similarly, the so-called "danger theory" started to emerge, challenging the classical model of "self/non-self" immune recognition, especially in a diseased or damaged tissue (20, 21). This model proposed that the immune recognition is not restricted to "non-self" entities, but rather discriminates between "dangerous" and "safe" entities, irrespective of source (20–22). Indeed, "dangerous" entities include pathogens as well as injured, infected, diseased and necrotic tissues, or cells undergoing non-physiological cell death which emit danger signals (or alarmins) with pro-inflammatory activity (21, 22). These danger signals are now collectively referred to as "damage-associated molecular patterns" (DAMPs) (23). DAMPs are endogenous molecules that are concealed intracellularly in normal conditions, but are exposed or released upon stress, injury, cell death, thereby becoming able to bind cognate receptors on immune cells (3, 24–27). **Table 1** summarizes the most prominent DAMPs characterized to date and their mode of emission, the cell death pathway they are associated with, and their known cognate receptors. It is important to consider that not all DAMPs may act as immunogenic danger signals. Several DAMPs exist that are crucial for the maintenance of tissue homeostasis, and the avoidance of auto-immune responses, as they exert immunosuppressive effects, including phosphatidylserine (PS), annexin A1 (ANXA1), death domain 1α (DD1α), B-cell CLL/lymphoma 2 (BCL2) and some extracellular matrix-derived molecules (**Table 1**). Accordingly, the blockade of these anti-inflammatory DAMPs accentuates the immunogenic potential of dying cells, or renders immunogenic otherwise tolerogenic forms of cell death (28, 29). Moreover, some danger signals are not always involved in the immunogenicity of cell death, but act as "bystanders." This is the case for heat shock protein 90 kDa alpha (cytosolic), class A member 1 (HSP90AA1, best known as HSP90) exposed on the cell surface after melphalan treatment (30). Last (but not least), several DAMPs may be subjected to post-translational modifications (e.g., oxidation, reduction, citrullination) that may potentially neutralize, increase, or change their immunogenic properties (31, 32) – a process that is still incompletely understood.

Despite these advances, the overall role of regulated cell death (RCD) (97) in augmenting cancer immunogenicity remained obscure. Initial observations involving the immunogenicity of cell death in the efficacy of cancer therapy were published between 1998 and 2004, when it was proposed that the non-apoptotic demise of malignant cells (within the context of the so-called "immunogenic death") could be associated with the emission of the danger signal heat shock 70 kDa protein 1A (HSPA1A, best known as HSP70) (**Table 1**), enhancing the immunogenic potential of dying cancer cells *in vivo* (98, 99). The dogmatic view that only necrotic or non-apoptotic (as postulated by the "immunogenic death" concept) cancer cells are characterized by an elevated immunogenic potential started to be questioned by a series of studies published between 2005 and 2007 (41, 70, 100, 101). These publications outlined that cancer cells undergoing apoptosis in response to specific anticancer therapies are immunogenic [a subroutine termed immunogenic cell death (ICD)], as long as they emit precise DAMPs in a spatiotemporally defined fashion (26, 102, 103). Cells succumbing to ICD are sufficient for the elicitation of durable anti-tumor immune responses (1, 26, 53, 102, 104). ICD is indeed paralleled by the redirection and emission of DAMPs, owing to the stimulation of distinct danger signaling pathways occurring in synchrony with cell death signaling (103). **Table 2** summarizes the main signaling pathways that play a role in the trafficking and emission of DAMPs. ICD-associated DAMPs and other immunostimulatory factors released by cells destined to undergo ICD favor the establishment of a productive interface between dying cancer cells and innate immune cells (like DCs or macrophages), thereby leading to the initiation of a therapeutically relevant adaptive immune response (**Figure 1**) (102, 105). In some contexts, DAMPs may regulate the function of specific innate immune cell subsets, e.g., following anthracycline treatment, extracellular adenosine triphosphate (ATP) assists in recruitment and differentiation of $CD11c^+Cd11b^+Ly6C^{high}$ cells into $CD11c^+CD86^+MHCII^+$ DCs (106); similarly, necrosis associated F-actin exposure activates an immune response by directing the dead cell debris to specifically $CD8\alpha^+$ DCs (59, 107). Indeed, DCs and other antigen-presenting cells exposed to cancer cells succumbing to ICD can then prime $CD4^+$ T cells (and polarize them into T_H1, T_H17, or T_H1/T_H17-like phenotype), $CD8^+$ cytotoxic T lymphocytes (CTLs) and γδ T lymphocytes against one or several TAAs (**Figure 1**) (102). Of note, residual cancer cells that survive ICD inducers can also show some enduring immunogenic characteristics that make them susceptible to immunological control by CTLs (108–110).

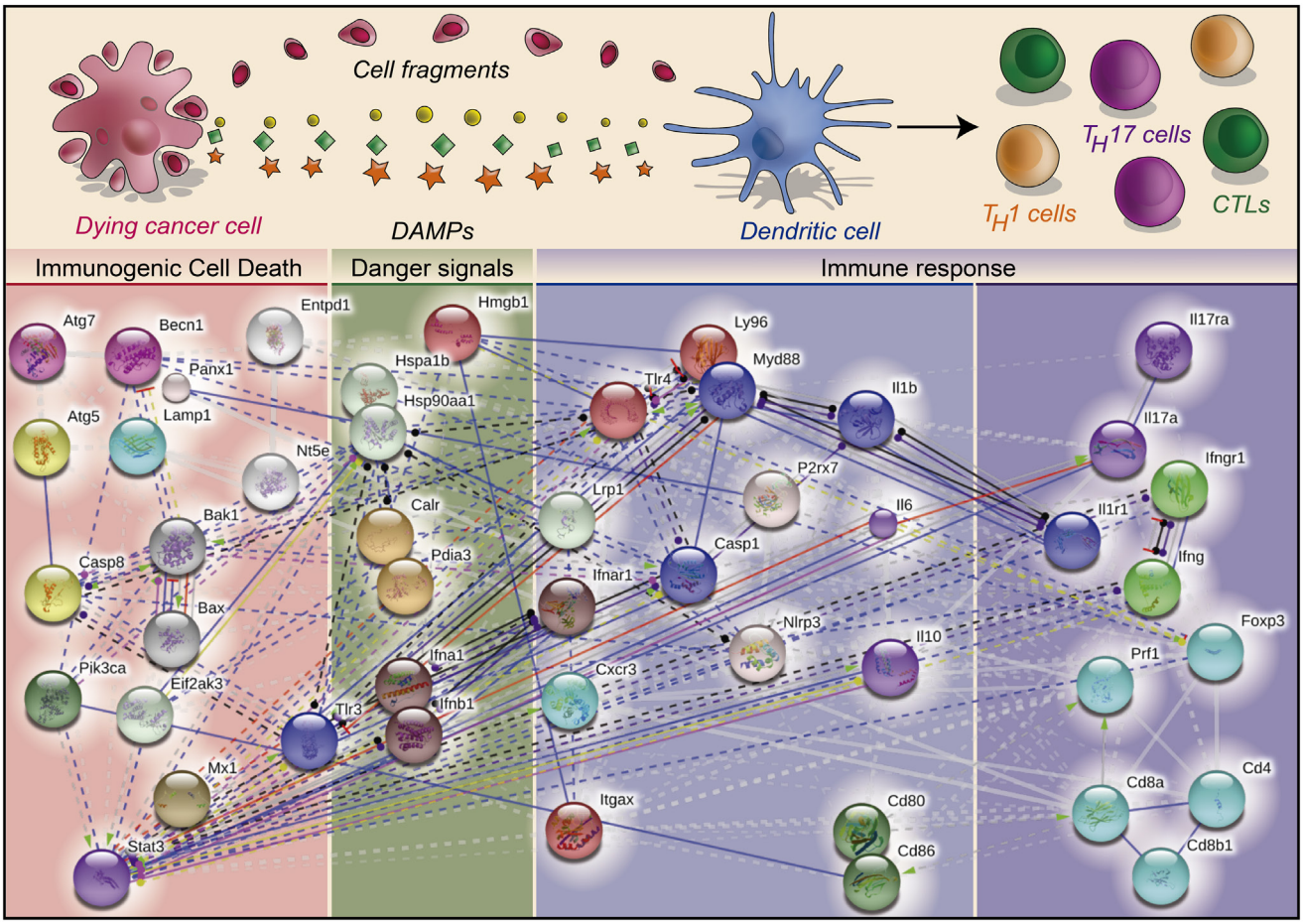

FIGURE 1 | The molecular complexity of immunogenic cell death in cancer. Cancer cells undergoing immunogenic cell death (ICD) emit danger signals for establishing a productive interface with components of the host immune system, including dendritic cells (DCs). DCs exposed to cancer cells succumbing to ICD "prime" the adaptive arm of the immune system, consisting of various effector T-cell populations, which in turn targets therapy-resistant cancer cells. Various molecules are critical for the execution of these processes. The molecular network of ICD-relevant proteins was build using the STRING modeling database (http://string-db.org/) (126).

IMMUNOGENIC CELL DEATH INDUCERS

Over the past few years, a number of single-agent ICD inducers have been discovered, encompassing conventional chemotherapeutics, targeted anticancer agents and various other biological and physicochemical therapies (18, 102, 104, 127). **Table 3** summarizes single-agent ICD inducers characterized so far, as per consensus guidelines (104), and the spectra of DAMPs and other immunostimulatory signals associated with them. For combinatorial therapeutic strategies capable of achieving ICD, readers may want to refer to other recent publications (18, 128, 129). It is clear that a general structure–function relationship capable of clustering all existing ICD inducers and predicting new ones does not exist (130), an issue that makes discovering new ICD-inducing therapies based on cheminformatic analyses challenging, if not impossible. A peculiar characteristic of most, if not all, ICD inducers is their ability to induce reactive oxygen species (ROS)-based/associated endoplasmic reticulum (ER) stress, as first delineated for anthracyclines (30, 34, 35, 42, 123, 131–133). This peculiarity was exploited for the targeted discovery of hypericin-based photodynamic therapy (Hyp-PDT) – a therapeutic modality that can trigger ICD through the induction of ROS that target the ER (35, 116, 134). Along with an ever more precise characterization of the links between ROS, ER stress, and ICD induction (135, 136), it became clear that the more "focused" ER stress is, the higher the probability of inducing ICD (3, 26, 53, 137). These observations paved way for a classification system based on how ICD inducers engage ER stress for cell death and danger signaling (3, 26, 53, 138). Based on this classification, Type I ICD inducers are defined as anticancer agents that act on non-ER proteins for the induction of cell death, but promote collateral ER stress for danger signaling, thereby operating on multiple targets (3, 26, 53), while Type II ICD inducers are anticancer agents that target the ER for both cell death induction and danger signaling (3, 26, 53). **Table 4** summarizes the classification of current ICD inducers into Type I and Type II, and their cell death/danger signaling targets. Such a classification suggest that while Type I ICD inducers can be discovered through various approaches (e.g., DAMP-based drug

screening platforms) (130, 139), putative Type II ICD inducers can be characterized rapidly on the basis of their ability to selectively or predominantly target the ER. Recent findings comforted the purpose and usefulness of this classification system, as two novel Type II ICD inducers [i.e., Pt^{II} N-heterocyclic carbene complex (140) and Newcastle disease virotherapy (NDV) (43)] were identified based on the notion that they induce predominant ROS-based ER stress (138). Nevertheless, as more ICD inducers and features are discovered, this classification system is expected to evolve or be substituted by a more refined one.

Since its discovery, a plethora of molecular and immunological components responsible for ICD have been discovered (**Figure 1**) (26, 102, 188). **Table 5** summarizes the molecular and immunological determinants of ICD characterized so far, as well as the models of ICD in which they operate (in a positive, negative or dispensable manner). Anthracyclines and oxaliplatin are the most common ICD inducers employed in experimental settings, followed by Hyp-PDT. According to current understanding, cancer cell-associated determinants of ICD can be subdivided into those that are common to all ICD inducers (i.e., "core" signaling components), and those that operate in an ICD inducer-dependent manner (i.e., "private" signaling components) (26, 189). Thus, eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3, best known as PERK) and the ER-to-Golgi secretory machinery are considered "core" signaling components on the cancer cell side (26, 102). Similarly, from the immune system side, a general role for (IFNγ-producing) $CD4^+$ and $CD8^+$ T cells has been confirmed for most, if not all, ICD inducers (**Table 5**). Interestingly, some components that are required for ICD induction by some agents (like autophagy for anthracyclines and oxaliplatin) (190) might be either dispensable for ICD induction by other agents, e.g., autophagy for NDV (43) and phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), caspase-8 (CASP8) activation or cytosolic Ca^{2+} levels for Hyp-PDT (35); or even negatively regulate ICD in some settings, e.g., autophagy in case of Hyp-PDT (34) (**Table 5**). Thus, it will be important to expand our molecular knowledge of ICD to as many experimental settings as possible.

IMMUNOGENIC CELL DEATH FROM BENCH TO BEDSIDE

The relevance of ICD has been verified in a number of rodent models, with a variety of chemical and physicochemical ICD inducers (26, 102). **Table 6** summarizes the most prominent mouse or rat models used so far for the characterization and study of ICD. For the moment, ICD has been mostly investigated in heterotopic syngeneic subcutaneous models (195). Within such models, inter-species differences (mouse *versus* rats), inter-strain differences (among BALB/c, C57BL/6, C3H and KMF mice), and inter-cell line differences, as well as differences in therapeutic setups (prophylactic *versus* curative) have been amply accounted for (**Table 6**). Nevertheless, there is predominance in the use of cancer cells derived from carcinogen-induced tumors and transplanted subcutaneously (**Table 6**). In very few cases, ICD has been characterized in either orthotopic (for NDV) or spontaneous (for anthracyclines) tumor murine models (**Table 6**). This has been questioned as a prominent Achilles' heel of ICD research (195). While this criticism is valid, it has to be recognized that no rodent model is perfect at all immunological levels (196).

As a recent systematic review summarized (196), heterotopic murine models suffer from a number of caveats, including the inability to recapitulate the early interaction between transformed cells and the immune system and the incompatibility between the cancer type and the site-of-transplantation (196). Orthotopic murine models are useful as they overcome the cancer cell-tissue type incompatibility issue (196). While genetically engineered tumor murine models (GEMMs) overcome most of the issues mentioned above, they come with their own set of shortcomings, including a limited genetic mosaicism, a low tumor heterogeneity, a lack of well-defined immunogenic TAAs, the presence of unintended "passenger" genetic modifications, and a reduced mutational spectrum (196). Many of these parameters are critical for responses to immunotherapy/ICD. For instance, the lack of well-defined immunogenic TAAs was the reason why preliminary results obtained in spontaneously developing murine tumors disputed the very existence of TAAs (11). Similarly, a high mutational spectrum (which produces considerable amounts of neo-antigens) has been found to be mandatory for the clinical efficacy of checkpoint blockers (209). Last (but not least), laboratory rodent models in general are associated with some critical issues, including the fact that a high level of inbreeding (which produces a number of shortcomings e.g., homozygous recessive defects) reduces the general immunological fitness, responsiveness and diversity in these models (196, 210, 211). Moreover, numerous immunological differences between mouse and humans tend to affect the translational relevance of the findings obtained (26, 211, 212). Also, the time frames of tumor growth rates between rodent models and humans are relatively divergent (196, 213, 214). This further complicates clinical translation of immunotherapeutic paradigms since the level of immunosurveillance and immunoediting experienced by human tumors can be much higher than any rodent tumor model.

In summary, it would be ideal to test ICD across as many different rodent models as possible, in order to determine the features that can be exploited for therapeutic purposes in humans. Moreover, if ICD fails in a specific experimental model, active effort should be made to characterize the mechanisms behind such failure, since resistance phenotypes can have profound clinical implications. This emerges from various studies summarized in **Table 7**. Indeed, several ICD resistance mechanisms exist operating at both the cancer cell and the immune system level, which have been characterized in different experimental models. Several of these resistance mechanisms have also been identified in cancer patients, thereby justifying further studies along these lines **Table 7**.

A considerable amounts of clinical findings support the relevance of ICD or ICD-related signatures in (at least subsets of) cancer patients. As summarized in **Table 8**, various ICD-linked (specific) parameters have been associated with the prognosis of cancer patients treated with clinically relevant ICD inducers (like anthracyclines, oxaliplatin, paclitaxel, or radiotherapy). Moreover, it is becoming clear that ICD-related or ICD-derived (immunological) genetic signatures (e.g., a *MX1*-centered

metagene, a *CXCR3-PRF1-CASP1*-centered metagene, an *ASAH1*-centered metagene) can be positively associated with good prognosis in patients affected by various neoplasms, including breast, lung, and ovarian malignancies (141, 188, 220). These observations indicate that ICD or ICD-relevant parameters may have prognostic or predictive relevance in at least a subset of cancer patients. It will be important to characterize new and more specific ICD-associated parameters linked to patient prognosis as well as biomarkers that may predict improved disease outcome in cancer patient treated with ICD inducers. Of note, considering the current clinical experience with immunotherapies (209, 221), the patients with an increased likelihood to benefit from ICD inducers are probably those that display pre-existing (baseline) immune reactivity against cancer cells (220, 222, 223). This may depend on the ability of ICD to reboot and/or revive pre-existing TAA-directed immunity rather to prime *de novo* immune reactivity (5, 191, 224). In future, it would be crucial to characterize biomarkers that allow clinicians to delineate patients with reduced baseline immune reactivity against malignant cells so that proper combinatorial therapies involving ICD inducers can be implemented.

CONFRONTING THE CLINICAL REALITIES OF ANTI-TUMOR IMMUNITY

It is well-established that the response of cancer patients to immunotherapy relies on the activity of effector T cells [that employ their T-cell receptors (TCRs) for recognizing TAAs]. However, these TAA-targeting T cells may also constitute obstacles for effective anti-tumor immunity (234). As opposed to T lymphocytes recognizing pathogen-associated antigens (PAAs) (**Figure 2**), indeed, T cells directed against some TAAs (derived from non-mutated proteins that are source of self or near-to-self antigens) are developmentally subjected to negative selection in the thymus and peripheral lymphoid organs (234, 235) (**Figure 2**). As a result, T cells bearing TCRs with high affinity for self antigens (including some TAAs) are clonally deleted to avoid auto-immunity (234–237) (**Figure 2**). However, some "leakiness" in this process allows TAA-specific T cells possessing TCRs with low affinity to escape deletion (234, 236, 237) and persist, although at low precursor frequencies (238) (**Figure 2**). Unfortunately, as compared to PAA-specific T cells, which bear high-affinity TCRs (**Figure 2**), TAA-specific T cells exhibit limited effector and memory functions (234, 239). Coupled with the tendency of progressing tumors to generate a highly immunosuppressive microenvironment, this renders the insurgence of lifelong protective immunity nearly impossible (234). Of note, central and peripheral tolerance may not affect T cells reactive toward neo-tumor-specific antigens (neo-TSAs) e.g., tumor-specific neo-antigens that are generated *de novo* in the course of tumor progression because of mutational events (240, 241). However, the extent to which such neo-TSAs can elicit consistent "immunodominant" T cell reactivity is still a matter of investigation (240, 241). Nevertheless, in this context, inefficient T-cell stimulation can be overcome through the ICD-based improvement of effector T-cell functions (102). ICD can be further combined with checkpoint-blocking therapies, which

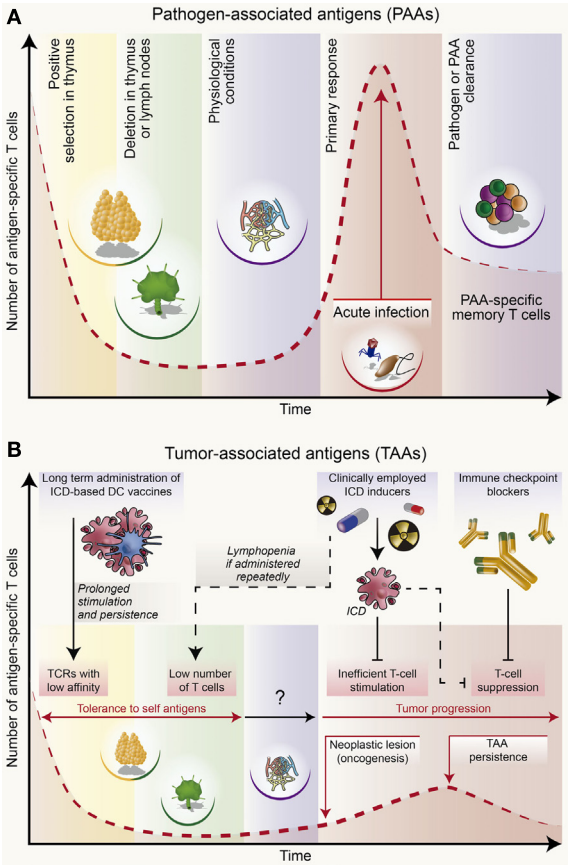

FIGURE 2 | Population dynamics of antigen-specific T cells during an immune response to infection or cancer. **(A)** T cells capable of putatively recognizing non-self, pathogen-associated antigens (PAAs) are not exposed to negative selection in the thymus or peripheral organs like lymph nodes. This allows for the constitutive presence of T lymphocytes bearing high-affinity T-cell receptor (TCR) in naïve conditions. Upon infection, these cells undergo robust expansion and acquire potent effector functions, hence driving an immune response that clears the pathogen and PAAs. Finally, PAA-specific T cells undergo contraction along with the establishment of immunological memory. To a limited extent, T cells reacting against PAAs expressed by virus-induced tumors may exhibit similar (although not identical) responses. **(B)** T cells that may recognize self or close-to-self antigens expressed by virus-unrelated malignancies undergo robust negative selection in the thymus and lymph nodes. Thus, all putative T lymphocytes bearing a high-affinity TCR against tumor-associated antigens (TAAs) are eliminated. However, some leakiness in this process allows for the persistence of TAA-specific T lymphocytes with low-affinity TCR, although at very low precursor frequencies. This is one of the reasons why in some individuals immunosurveillance at some stage fails to impede tumor progression. As malignant lesions progress, the amount of TAAs increases, causing a weak rise in TAA-specific T cells. However, tumor progression is generally coupled with the establishment of robust immunosuppressive networks that potently inhibit such TAA-targeting T cells. In this context, the administration of immunogenic cell death (ICD) according to a schedule that does not lead to lymphodepletion can favor the stimulation of TAA-targeting T cells and (re) instate immunosurveillance. Combining ICD inducers with checkpoint-blocking agents may further boost TAA-targeting immune responses. However, these treatments may not ensure the lifelong persistence of TAA-recognizing T cells, some of which are susceptible to elimination through tolerance mechanisms. Anticancer vaccines may counteract, at least to some extent, such loss. The figure was partly inspired from Baitsch et al. (234).

potently reverse immunosuppression (209, 242). However, the lifelong maintenance of anti-tumor T cells remains a particularly hard challenge.

In the clinical reality, anticancer agents are administered to patients in a limited number of cycles. Even if these therapeutic regimens may attain optimal efficacy in terms of ICD induction, they are unlikely to ensure the lifelong persistence of TAA-directed T cells with low-affinity TCR (234, 243). This probably reflects the contraction of TAA-targeting T cells occurring once the immunostimulatory stimulus provided by ICD ceases, owing to peripheral tolerance mechanisms (234). Clinically, it may not be feasible to administer ICD inducers repeatedly over time, since many of them can cause lymphopenia (which negatively affects disease outcome), or are associated with other side effects (244). It has been proposed that active immunization with ICD-based anticancer vaccines (which are associated with robust immunogenicity) given in a repetitive manner may achieve this goal (**Figure 2**) (234, 243, 245). Thus, it will be important to test whether the long-term administration of ICD-based anticancer vaccines can sustain the effector function of TAA-specific T cells bearing low-affinity TCRs, hence, ensuring lifelong disease-free survival. Of note, in the case of hematological malignancies, this issue could be overcome upon the adoptive transfer of CTLs expressing chimeric antigen receptors (CARs) (1). However, whether CAR-expressing CTLs generate protective immunological memory in the absence of considerable side effects remains to be determined. Moreover, the use of this therapeutic strategy against solid malignancies is relatively challenging owing to lack of well-defined "unique" TAAs (1, 246).

CONCLUSION

The model of ICD has been considerably refined since the initial identification of a cell death modality manifesting apoptotic features but able to induce an adaptive immune response. This model strives to integrate several phenomena observed throughout the second half of the twentieth century in one therapeutically relevant platform. However, as discussed above, several challenges still need to be addressed. First, comprehensive testing should be performed in advanced experimental settings like GEMMs or orthotopic tumor models. Second, ICD resistance mechanisms should be characterized with precision. Third, various issues linked to the successful translation of ICD to cancer therapy will have to be resolved, including (but not limited to) treatment schedules, dosages, and combinatorial strategies. This translational drive also needs to be coupled with effective strategies for the discovery of new and effective ICD inducers. Drug screening programs are often complicated by the possibility of false-positive (due to bystander presence of DAMPs) (30) or false-negative (due to limited number of biomarkers used for screening) hits. This issue can only be ironed out by discovering new and common regulators of ICD, and integrating them into existing screening platforms. Last, but not least, it will be important to identify new ICD-related/derived biomarkers that can be used to improve current protocols of patient stratification and clinical decision making. We are positive that all these objectives are at reach.

AUTHOR CONTRIBUTIONS

ADG did the literature study, data collection, as well as conceived and wrote the manuscript. PA provided senior supervision and guidance, conceived the paper, helped in writing, and critically revised the manuscript. LG improved and edited the manuscript. JMBSP helped with the preparation of figures. All authors participated in the critical reading of the manuscript (wherever applicable), approved content and conclusions, as well as helped in ensuring the accuracy of cited literature.

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TABLES

TABLE 1 | A list of prominent damage-associated molecular patterns (DAMPs) associated with cell death pathways or extracellular matrix.

DAMPs	Localization and mode-of-emission	Relevant cell death pathway	Receptors	Reference
Annexin A1	Surface exposed or actively/passively released?	Apoptosis	FPR-1 receptor	(33)
Adenosine triphosphate	Actively or passively released	ICD, apoptosis/secondary necrosis and necrosis	P_2Y_2 and P_2X_7	(34–37)
B-cell CLL/lymphoma 2	Passive release	Necrosis	TLR2	(38)
Biglycan	Extracellular matrix	–	TLR2, TLR4, P_2X_4, and P_2X_7	(39, 40)
Calreticulin	Mostly surface exposed; sometimes passively released	ICD	CD91	(35, 41–44)
Cardiolipin	Surface exposed?	Apoptosis	?	(45, 46)
Ceramide and sphingosine-1-phosphate	Surface exposed	Apoptosis	?	(47)
Covalent/cross-linked dimer of ribosomal protein S19	Passively released?	Apoptosis	CD88	(48–51)
Carbamoyl-phosphate synthase 1	?	?	?	(52)
Cyclophilin A	Passive release	Necrosis	CD147	(53)
Cytochrome *c*	Passively released?	Secondary necrosis and necrosis?	LPG?	(54, 55)
Death domain 1α	Surface exposed	Apoptosis	DD1α	(56)
Endothelial monocyte-activating polypeptide II	Passively released?	Apoptosis	CXCR3?	(50, 57, 58)
F-actin	Passive release	Necrosis	DNGR-1/Clec9a	(59)
Fibrinogen	Extracellular matrix	–	TLR4	(40)
Fibronectin extra domain A	Extracellular matrix	–	TLR4?	(40)
Fragments of human tyrosyl tRNA synthetase	Passively released?	Apoptosis	?	(50)
Genomic DNA, mRNA, snRNPs	Passive release	Necrosis	TLR3	(3, 60, 61)
GRP78/BiP	Passive release	Necrosis, apoptosis?	?	(31)
H_20_2	?	Apoptosis	?	(62)
Heat shock proteins (HSP70, HSP90, HSP60, HSP72, and GP96)	Surface exposure, active secretion, or passive release	ICD, apoptosis/secondary necrosis, necrosis	CD91, TLR2, TLR4, SREC-1 and FEEL-1	(63–67)
Heparan sulfate fragments	Extracellular matrix	–	TLR4	(40)
Hepatoma-derived growth factor	Passively released	Necrosis	?	(68)
Histones	Passively released	Necrosis	TLR-9	(69)
High-mobility group box 1	Mostly passively released; sometimes actively released	ICD, secondary necrosis and necrosis	TLR2, TLR4, RAGE and TIM3	(70–73)
High-mobility group nucleosome binding domain 1	Passive release	Necrosis	TLR4	(74)
Hyaluronan	Extracellular matrix	–	TLR2 and TLR4	(40)
IL-1α	Passive release	Necrosis	IL-1R	(75)
IL-33	Passive release	Necrosis	ST2	(3, 61)
IL-6	Passive release	Necrosis	IL-6R and GP130	(76)
Lysophosphatidylcholine	Passively released?	Apoptosis	G2A	(50, 77)
Mit DNA	Passively released	Necrosis	TLR-9	(78–80)
Monosodium urate or uric acid	Passively released	Necrosis	Purinergic receptors	(50, 81)
N-formylated peptides	Passively released	Necrosis	FPR-1	(78, 82–84)
Oxidation-associated molecular patterns (reactive protein carbonyls, per-oxidized phospholipids, oxidized low-density lipoprotein)	Passively released	Necrosis, Secondary necrosis	CD36, SR-A, TLR-2/4, CD14	(85–87)
Peroxiredoxin 1	Actively secreted or passively released	Apoptosis, necrosis	TLR4	(88)

(Continued)

TABLE 1 | Continued

DAMPs	Localization and mode-of-emission	Relevant cell death pathway	Receptors	Reference
Phosphatidylserine	Actively externalized on the surface	Apoptosis	TIM-1/-3/-4, BAI1, Stabilin-2, MFG-E8, C1q	(56, 89–93)
S100/calgranulin protein family members (S100A8, S100A9, S100A12/EN-RAGE)	Passively released	Necrosis	RAGE	(50, 94)
Tenascin-C	Extracellular matrix	–	TLR4?	(95)
Thrombospondin 1 and its heparin-binding domain	Passively released or surface associated	Apoptosis	$\alpha_v\beta_3$ integrin	(50, 96)
Versican	Extracellular matrix	–	TLR2, TLR6, and CD14	(40)

CD, cluster of differentiation; CLEC9A, C-type lectin domain family 9, member A; CPS-1, carbamoyl-phosphate synthase 1, mitochondrial; CXCR3, C-X-C motif receptor 3; FEEL-1/CLEVER-1, fasciclin EGF-like/common lymphatic endothelial and vascular endothelial receptor-1; FPR-1, formyl peptides receptor-1; G2A, G2 accumulation; HMGB1, high-mobility group box 1; HSP, heat shock proteins; ICD, immunogenic cell death; IL, interleukin; LPG, leucine-rich alpha-2-glycoprotein-1; MFG-E8, milk fat globule-egf factor 8 protein; Mit DNA, mitochondrial DNA; P2XR, P2X receptor; P2YR, P2Y receptor; RAGE, receptor for advanced glycation endproducts; SREC-1, scavenger receptor class f member 1; TFAM, mitochondrial transcription factor A; TIM, transmembrane immunoglobulin and mucin domain; TLR, toll-like receptor(s).

Glossary (5, 19, 97): (1) Necrosis: primary necrosis is a form of cell death that can occur in a regulated or accidental manner, characterized by cellular swelling and rapid breakdown of the plasma membrane; (2) Necroptosis: necroptosis is a form of regulated cell death (RCD) manifesting with necrotic morphology and controlled by a signaling cascade involving (among other proteins) RIPK1, RIPK3, and MLKL; (3) Apoptosis: apoptosis is a form of RCD largely dependent on caspases activity and morphologically characterized by cell shrinkage, membrane blebbing, formation of apoptotic bodies, chromatin condensation, and systematic DNA fragmentation; (4) Secondary Necrosis: Secondary necrosis is a terminal process experienced by late-apoptotic cells if they are not cleared by phagocytes in time, and is characterized by general spill-over of apoptotic cellular contents.

"?" Unclear or not determined yet.

TABLE 2 | Danger signaling pathways characterized as traffickers of DAMPs.

DAMPs	Role of ROS	Role of ER stress	Role of autophagy	Role of chaperone-mediated autophagy	Role of secretory pathway	Caspase activity	Role of lysosomes	Comments	Reference
Secreted ATP	+	+/0	+/0	0	+/0	+	+/0	Underlying pathway is highly inducer dependent	(34, 35, 111–113)
Released HMGB1	0	0	+	?	0	–	?	Mostly released passively on account of necrosis; only DT-EGF reported to cause active secretion so far	(73, 114, 115)
Secreted or surface HSP70	?	?	?	?	?	+	+	ABC transporters help in endolysosomal-secretion; HSP70 has also been reported to be secreted in an exosome surface-bound format	(116–122)
Surface CRT	+	+	–/0	+	+	+/0	?	LRP1/lipid rafts mediate surface tethering; components that positively regulate surface-CRT in an inducer-dependent fashion: ERp57, PI3K p110α, BAX/BAK, cytosolic ER-Ca^{2+}, BAP31; of note, anthracycline-induced pathway of surface CRT induction has been found to be conserved from yeast to mammals	(34, 35, 111, 112, 116, 123, 124)
Surface HSP90	+	+	–	?	+	+	?	–	(30, 125)

"+" denotes ability to positively regulate trafficking; "–" denotes ability to negatively regulate trafficking; "0" denotes confirmation of no role in regulation of trafficking and "?" denotes that the role in regulating the trafficking is unknown; "+/0" denotes positive or no role in regulation of trafficking in an inducer-dependent fashion; "–/0" denotes negative or no role in regulation of trafficking in an inducer-dependent fashion.

ATP, adenosine triphosphate; CRT, calreticulin; DT-EGF, epidermal growth factor receptor-targeted diphtheria toxin; ER, endoplasmic reticulum; HMGB1, high-mobility group box 1 protein; HSP, heat shock protein; LRP1, low-density lipoprotein receptor-related protein 1; ROS, reactive oxygen species.

TABLE 3 | A list of prominent single-agent immunogenic cell death (ICD) inducers in cancer and their specific associations with danger signaling and other immunostimulatory signaling.

ICD inducers	Associated ICD-relevant DAMPs		Other immunostimulatory activities or danger signals and other comments on immunomodulatory activity	Reference
	DAMP	Stage of cell death		
Anthracyclines (epirubicin, doxorubicin, idarubicin, mitoxantrone), oxaliplatin, UVC radiation and radiotherapy	Surface CRT Surface HSP70 Secreted ATP Released HMGB1	Pre-apoptotic Mid-apoptotic Early/mid-apoptotic Post-apoptotic	Activation of Type I IFN response comprising MX-1 centered signature, consisting of IFN-α/β and CXCL10; surface exposure of mannose-6-phopshate receptor, which enables better interface with CTLs and facilitates GZMB-mediated cell death; radiotherapy is known to increase expression levels of various antigens in number of cancer models as well as induce "abscopal effect" in both preclinical and clinical models; overall *CALR* levels were predictive of prolonged OS in radiotherapy-treated lung cancer patients	(26, 42, 102, 127, 141–144)
Anti-EGFR antibody – 7A7	Surface CRT Surface HSP70 Surface HSP90	Pre-apoptotic Early/mid-apoptotic Early/mid-apoptotic	–	(145)
Bleomycin	Surface CRT Secreted ATP Released HMGB1	Mid/post-apoptotic Mid/post-apoptotic Post-apoptotic	Induces ambivalent immune response, i.e., all valid ICD markers but also increased Treg differentiation and, thus, a good candidate for anti-Treg combinatorial therapy	(146)
Bortezomib	Surface HSP90 Surface CRT Surface HSP70	Early/mid-apoptotic Early/mid-apoptotic Early/mid-apoptotic	–	(26, 66, 100, 127)
Oncolytic Adenovirus	Surface CRT Released ATP Released HMGB1	?	Immunogenicity of these viruses can be further increased by producing transgenic versions producing CD40L or GM-CSF	(147, 148)
Clostridium difficile toxin B	Surface CRT Released ATP Released HMGB1 Released HSP70/90	Early/mid-apoptotic Post-apoptotic Post-apoptotic Post-apoptotic	–	(149)
Coxsackievirus B3 (CVB3)[#]	Surface CRT Secreted ATP Released HMGB1	Early-apoptotic Early/mid-apoptotic Post-apoptotic	–	(150, 151)
Cyclophosphamide	Surface CRT Released HMGB1	Pre-apoptotic Post-apoptotic	Facilitates an interface between gut microbiota (leaked due to gut perforation) and host immune system thereby allowing Th17 cells-dependent anti-tumor immune responses; cyclophosphamide's effects on anti-tumor immunity are strongly dose dependent. High doses of this chemotherapeutic can be immunosuppressive yet low or metronomic doses facilitate anti-tumor immunity through targeted depletion of Tregs/MDSCs. In ICD set-up, a low dose (100 mg/kg in mice) of cyclophosphamide was shown to exert anti-tumor immunity	(18, 152, 153)
High hydrostatic pressure	Surface CRT Surface HSP70 Surface HSP90 Secreted ATP Released HMGB1	Early/mid-apoptotic Early/mid-apoptotic Early/mid-apoptotic Mid/post-apoptotic Mid/post-apoptotic	–	(154–156)
Hypericin-based PDT	Surface CRT Surface HSP70 Surface HSP90 Secreted ATP Released HMGB1 Released HSP70/90 Released CRT	Pre-apoptotic Pre-apoptotic Pre-apoptotic Pre-apoptotic Post-apoptotic Post-apoptotic Post-apoptotic	High accumulation of OAMPs like protein carbonyls; down-regulates CD47; induces up-regulation of various molecules associated with Type I IFN response (*IRF7*, *IRF1*, *OASL, IL18, CXCL2, IL15, IL8*) but not IFN-α secretion	(26, 30, 34, 35, 112, 116, 157)
Microwave thermal ablation	Surface CRT Secreted ATP Released HMGB1	?	–	(158)
Newcastle disease virus (NDV)	Surface CRT Released HMGB1	Early/mid-necroptotic Post-necroptotic	Increases expression levels of PMEL17 antigen in glioma cells; NDV treatment has also been shown to induce "abscopal effect" in a murine melanoma model	(43, 159)
Paclitaxel	Surface CRT Released HMGB1	Early/mid-apoptotic Post-apoptotic	Overall *CALR* levels were predictive of prolonged OS or PFS in paclitaxel-treated ovarian cancer patients thereby establishing clinical validity of ICD in paclitaxel treatment set-up; paclitaxel has also been reported to enhance overall antigen levels	(42, 144, 160)

(Continued)

TABLE 3 | Continued

ICD inducers	Associated ICD-relevant DAMPs		Other immunostimulatory activities or danger signals and other comments on immunomodulatory activity	Reference
	DAMP	Stage of cell death		
Patupilone	Surface CRT	Early/mid-apoptotic	–	(128)
Photofrin-based PDT	Surface CRT Surface HSP70/60 Released HMGB1 Surface ceramide Surface S1P	Early/mid-apoptotic Early/mid-apoptotic Post-apoptotic Early/mid-apoptotic Early/mid-apoptotic	The only anticancer modality for which a comparison between DAMPs induced by *in vitro* versus *in vivo* treatment was carried out – however, none of ICD-related DAMPs were tested	(47, 161–164)
Pt^{II} N-heterocyclic carbene complex	Surface CRT Released ATP Released HMGB1	Pre-apoptotic Post-apoptotic Post-apoptotic	–	(140)
RIG-I-like helicases (RLH) ligand	Surface CRT Released HMGB1 Released HSP70	Early-apoptotic Post-apoptotic Post-apoptotic	Induces Type I IFN response	(165)
Septacidin	Surface CRT Secreted ATP Released HMGB1	Pre-apoptotic Early/mid-apoptotic Post-apoptotic	–	(139)
Shikonin	Surface CRT Surface HSP70	Early/mid-apoptotic Early/mid-apoptotic	Also, causes surface exposure of GRP78 a prominent inducer of pro-tumorigenic effects; enhances overall cancer antigen levels	(160)
Vorinostat	Surface CRT Secreted ATP Released HMGB1	Early/mid-apoptotic Post-apoptotic Post-apoptotic	–	(166)
Wogonin	Surface CRT Released ATP Released HMGB1	Early-apoptotic Post-apoptotic Post-apoptotic	Surface-Annexin A1 is also induced by wogonin. In an ICD set-up, the role of Annexin A1 is not clear since it is a noted anti-inflammatory factor	(167)

CRT or CALR, calreticulin; CTLs, cytotoxic T lymphocytes; DAMPs, damage-associated molecular patterns; EGFR, epidermal growth factor receptor; GZMB, granzyme B; HMGB1, high-mobility group box-1 protein; HSP, heat shock protein; ICD, immunogenic cell death; IFN, interferon; MDSC, myeloid-derived suppressor cells; OAMPs, oxidation-associated molecular patterns; OS, overall survival; PFS, progression-free survival.

Important note: It is worth noting that recently various promising candidate therapies have emerged that induce in vitro DAMPs relevant for ICD, e.g., Rose Bengal-based PDT (168), Docosahexaenoic acid (169), and Capsaicin (170, 171). Such agents may emerge as potent inducers of ICD in future, however, in order to establish them as inducers of ICD-like immunogenicity, it is imperative to confirm their (i.e., cancer cells treated with these agents) ability to stimulate T cells (in vitro or in vivo) and/or induce anti-cancer vaccination effect, in vivo, as per the consensus guidelines (104).

Glossary: In the current setting, it is crucial to differentiate between the meanings of the words, "immunogenic" and "immunogenicity" as they are not supposed to have interchangeable meanings. Immunogenic, derives from the word immunogen, which refers to any substance that can elicit an immune response; this includes, whole cells or organisms (eukaryotic or prokaryotic), specific cellular entities or specific proteins (e.g., antigens) (172). On the other hand, immunogenicity is a much more specific terms that is closer to antigenicity in operational sense, since it refers to the ability of a specific entity (e.g., an antigen or an epitope) to be recognized by the immune system through binding interactions with T or B cells, which may or may not result in an overt immunological response (4, 11).

"?" Unclear or not determined yet.

"#" Unconfirmed anti-tumour immune responses in adaptive immune system-competent.

TABLE 4 | Classification of ICD inducers into Type I and Type II based on their ER or non-ER-targeting *modus operandi*.

ICD inducer	Site of Cell-death inducing effects	Site of danger signaling induction	Reference
Type I inducers – agents that induce icd through a "collateral" er stress effect			
Anthracyclines (epirubicin, doxorubicin, idarubicin, mitoxantrone), oxaliplatin, UVC radiation and radiotherapy	Nucleus (DNA or the DNA replication machinery proteins)	ER, autophagy, pannexin channels, lysosomes	(36, 41, 70, 111, 130, 173, 174)
Anti-EGFR antibody – 7A7	Cell surface (epidermal growth factor receptor or EGFR)	ER	(145)
Bleomycin	Nucleus (causes DNA strand-breaks)	ER?	(146)
Bortezomib	Cytosol (26S proteasome or ERAD machinery; CIP2A/cancerous inhibitor of protein phosphatase 2A)	ER	(100, 175, 176)
Clostridium difficile toxin B	Cytoskeleton (causes cytoskeletal disruption by targeting RhoA, CDC42 and Rac1)	ER	(149, 177)
Cyclophosphamide	Nucleus (DNA)	ER	(152)
High hydrostatic pressure	Broad disrupting/denaturing effects on membranes, and proteins	ER (mitochondria?)	(154, 178)
Microwave thermal ablation	Hyperthermic ablation of cellular components	ER?	(158)
Paclitaxel, patupilone	Cytoskeleton (target microtubules thereby disrupting cytoskeletal functions)	ER	(42, 104, 179)
Photofrin-based PDT	Cellular membranes (ROS-based damage of membranes)	ER?	(180, 181)
RIG-I-like helicases (RLH) ligand	Cytosol (targets RIG-I-like helicases)	ER?	(165)
Septacidin	?	ER	(139)
Shikonin	Cytosol (tumor-specific pyruvate kinase-M2 protein)	ER	(160, 182)
Vorinostat	Nucleus/Cytosol (targets histone deacetylase)	ER?	(166)
Wogonin	Mitochondria (generates mitochondria-derived ROS)	ER	(167, 183)
Type II inducers – agents that induce icd through a "focused" er stress effect			
Hypericin-based PDT	ER (ROS-based damage at the ER membrane)	ER	(35, 63, 116, 181, 184, 185)
Oncolytic adenovirus	ER (ER membranes and lumen)	ER	(104, 147)
Oncolytic coxsackievirus B3 (CVB3)	ER (ER membranes and lumen)	ER	(150, 186)
Oncolytic Newcastle disease virus (NDV)	ER (ER membranes and lumen)	ER	(43, 159, 187)
Pt^{II} N-heterocyclic carbene complex	Predominantly targets ER (generates ER-directed ROS)	ER	(140)

EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ICD, immunogenic cell death; PDT, photodynamic therapy; ROS, reactive oxygen species.
"?" Unclear or not determined yet.

TABLE 5 | A list of molecular and immunological components crucial for regulation of ICD.

Molecular or immunological components	Acting on the level of?	Role in regulating ICD or ICD-related determinants for various therapies/inducers			Confirmed by which experimental intervention?	Reference
		Positive regulation	Negative regulation	No role in regulation		
Actin cytoskeleton	Cancer cells	Anthracyclines, hypericin-PDT	–	–	Pharmacological inhibitors of actin polymerization	(35, 123)
ATG5, ATG7, or BECN1	Cancer cells	Anthracyclines, oxaliplatin	Hypericin-PDT	Newcastle disease virotherapy	ATG5, ATG7 or BECN1 si/shRNA, ATG5 KO MEFs, or transgenic mice model of spontaneous melanoma with *Atg7*$^{-/-}$ phenotype or pharmacological inhibitors of macroautophagy	(34, 43, 112)
BAX/BAK	Cancer cells	Anthracyclines, hypericin-PDT	–	–	BAX/BAK KO MEFs or Bax/Bak si/shRNA	(35, 123)
Calreticulin	Cancer cells	Anthracyclines, radiotherapy, oxaliplatin, hypericin-PDT	–	–	CRT si/shRNA	(35, 41, 116, 123)
Caspase 1	Host immune system	Anthracyclines and/or oxaliplatin	–	–	*Casp1*$^{-/-}$ mice	(36)
Caspase-8	Cancer cells	Anthracyclines	–	Hypericin-PDT	Caspase-8 si/shRNA or HeLa cancer cells expressing CrmA (a caspase-8 inhibitory protein)	(35, 123)
$CD4^+/CD8^+$ T cells	Host immune system	Anthracyclines and/or oxaliplatin, hypericin-PDT, high hydrostatic pressure, bortezomib, vorinostat, photofrin-PDT, Newcastle disease virotherapy, cyclophosphamide	–	–	Antibody-based depletion; *Ex vivo* co-culture experiments	(34, 43, 100, 102, 152, 161, 162, 166, 191)
CXCL10	Host immune system	Anthracyclines and/or oxaliplatin	–	–	Recombinant protein	(102, 141)
CXCR3	Host immune system	Anthracyclines and/or oxaliplatin	–	–	*Cxcr3*$^{-/-}$ mice or antibody-based blockade	(141)
eIF2α-P	Cancer cells	Anthracyclines	–	Hypericin-PDT	MEFs expressing non-phosphorylable version of eIF2α-P, salubrinal or pharmacological inhibitors of GADD34	(35, 123)
ER-Ca^{2+}	Cancer cells	Anthracyclines	–	Hypericin-PDT	BAPTA, a Ca^{2+} chelator or Reticulon-1C overexpression;	(35)
ERp57	Cancer cells	Anthracyclines	–	Hypericin-PDT	ERp57 si/shRNA or ERp57 KO MEFs	(35, 116)
ER-to-Golgi transport	Cancer cells	Anthracyclines, hypericin-PDT	–	–	Brefeldin A, a secretory pathway inhibitor	(35, 123)
HMGB1	Cancer cells	Anthracyclines	–	–	HMGB1 si/shRNA	(70)
HSP90	Cancer cells	Bortezomib	–	–	Pharmacological HSP90 inhibitors	(66, 67, 100)
HSP70	Cancer cells	Shikonin	–	–	Antibody-mediated protein depletion	(192)
IFN-α/β or IFN-α-receptor	Cancer cells	Anthracyclines, cyclophosphamide, and/or oxaliplatin	–	–	Antibody-based blockade or recombinant proteins (wherever applicable)	(141, 152)
IFN-γ and IFN-γ-receptor	Host immune system	Anthracyclines and/or oxaliplatin	–	–	*Ifng*$^{-/-}$ or *Ifngr1*$^{-/-}$ mice	(70, 102)
IL17A or IL17A-receptor	Host immune system	Anthracyclines and/or oxaliplatin	–	–	*Il17a*$^{-/-}$ or *Il17ra*$^{-/-}$ mice	(36, 193)
IL1-receptor	Host immune system	Anthracyclines and/or oxaliplatin	–	–	*Il1r1*$^{-/-}$ mice	(36)
IL-1β	Host immune system	Anthracyclines and/or oxaliplatin	–	–	Antibody-based blockade	(36)
Lipid rafts	Cancer cells	Mitoxantrone	–	Hypericin-PDT	MBC, a cholesterol-chelator that disrupts lipid rafts	(35)

(Continued)

TABLE 5 | Continued

Molecular or immunological components	Acting on the level of?	Role in regulating ICD or ICD-related determinants for various therapies/inducers			Confirmed by which experimental intervention?	Reference
		Positive regulation	Negative regulation	No role in regulation		
LRP1	Cancer cells	Mitoxantrone, hypericin-PDT	–	–	LRP1 shRNA, LRP1 KO MEFs, LRP1 KO CHO cells and LRP1 overexpression in CHO cells	(35)
LY96 and MyD88 (TLR-adaptors)	Host immune system	Anthracyclines and/or oxaliplatin	–	–	*Ly96*$^{-/-}$ or *Myd88*$^{-/-}$ mice	(102)
NLRP3	Host immune system	Anthracyclines and/or oxaliplatin	–	–	*Nlrp3*$^{-/-}$ mice	(36)
P2 × 7 receptor	Host immune system	Anthracyclines and/or oxaliplatin	–	–	*P2rx7*$^{-/-}$ mice	(36)
Perforin	Host immune system	Anthracyclines and/or oxaliplatin	–	–	*Prf1*$^{-/-}$ mice	(36, 70, 102)
PERK	Cancer cells	Anthracyclines, hypericin-PDT, wogonin	–	–	PERK si/shRNA, PERK KO MEFs	(35, 123, 167)
PI3K p110α	Cancer cells	Anthracyclines, hypericin-PDT, wogonin	–	–	PI3K p110α shRNA or wortmannin, a pharmacological inhibitor	(35, 167)
Rag2	Host immune system	Anthracyclines and/or oxaliplatin, vorinostat, cyclophosphamide, photofrin-PDT, Newcastle disease virotherapy	–	–	*Rag2*$^{-/-}$ mice	(43, 70, 102, 152, 161, 162, 166)
STAT3	Cancer cells	Anthracyclines and/or oxaliplatin	–	–	*Stat3*$^{-/-}$ cancer cells	(194)
TLR3	Cancer cells	Anthracyclines and/or oxaliplatin	–	–	TLR3 si/shRNA or *Tlr3*$^{-/-}$ cancer cells	(141)
TLR4	Host immune system	Anthracyclines and/or oxaliplatin	–	–	*Tlr4*$^{-/-}$ mice	(70, 102)
TNF or TNF-receptor	Host immune system	Anthracyclines and/or oxaliplatin	–	–	*Tnf*$^{-/-}$ or *Tnfr1*$^{-/-}$ mice	(102)
LAMP2A	Cancer cells?	Mitoxantrone and hypericin-PDT	–	–	LAMP2A KO MEFs	(112)

ATG, autophagy-related protein; BECN1, beclin-1; CD, cluster of differentiation; CRT, calreticulin; CXCL, C-X-C ligand; CXCR, C-X-C motif receptor; eIF2, eukaryotic initiation factor 2; ER, endoplasmic reticulum; ERp57, endoplasmic reticulum protein 57; HMGB1, high-mobility group box 1; HSP, heat shock protein; Hyp-PDT, hypericin-based photodynamic therapy; ICD, immunogenic cell death; IFN, interferon; IL, interleukin; KO MEFs, knock-out murine embryonic fibroblasts; LAMP, lysosome-associated membrane glycoprotein; LRP1, low-density lipoprotein receptor-related protein 1; MBC, methyl-β-cyclodextrin; NLRP3, NOD-like receptor family, pyrin domain containing 3; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PI3K, phosphoinositide 3-kinase; PRF, perforin; TLR, toll-like receptor; TNF, tumor necrosis factor.

TABLE 6 | A list of prominent preclinical mice or rat models used for analysis of ICD.

ICD inducer	Mice tumor models utilized for positive ICD characterization or ICD "restoration/rescue" analysis			
	Heterotopic subcutaneous mice or rat models	Orthotopic mice models	Spontaneous tumor mice models	Carcinogen-induced tumor models
Anthracyclines	CT26 cells in BALB/c mice – prophylactic immunization model (41, 70, 111, 123, 197) and curative tumor model (41, 70, 111, 197); MCA205 cells in C57BL/6 mice – prophylactic immunization and curative tumor model (36, 70, 111, 130); MCA-2/-4 cells in C57BL/6 mice – curative tumor model (36); D122 cells in C57BL/6 mice – prophylactic immunization model (145); AY27 cells in Fischer 344 rats – prophylactic immunization model (42)	–	MMTV-*Neu*T breast cancer mice model – curative set-up (198); *Braf*$^{Ca/+}$; *Pten*$^{fl/fl}$-melanoma mice model – curative set-up (199)	–
Anti-EGFR antibody (7A7)	D122 cells in C57BL/6 mice – curative tumor model and prophylactic immunization model (145)	–	–	–
Bleomycin	CT26 cells in BALB/c mice – curative tumor model (146)	–	–	–

(Continued)

TABLE 6 | Continued

ICD inducer	Mice tumor models utilized for positive ICD characterization or ICD "restoration/rescue" analysis			
	Heterotopic subcutaneous mice or rat models	**Orthotopic mice models**	**Spontaneous tumor mice models**	**Carcinogen-induced tumor models**
Bortezomib	67NR cells in BALB/c mice – prophylactic immunization model with use of stimulated DCs (200); B16 cells in C57BL/6 mice – curative tumor model, combination treatment with AdVMART1/DC and bortezomib is significantly better than bortezomib alone (201); HM-1 cells in C57BL/6 x C3/He F_1 origin mice – prophylactic immunization model (202)	–	–	–
CD40L-encoding Oncolytic Adenovirus	MB49 cells in C57BL/6 mice – curative tumor model (147)	–	–	–
Clostridium difficile toxin B	CT26 cells in BALB/c mice – prophylactic immunization model (149)	–	–	–
Coxsackievirus B3	A549 and EBC-1 cells in nude BALB/c mice – curative tumor model (150)	–	–	–
Cyclophosphamide	EG7 cells in C57BL/6 mice (152); AB1-HA cells in BALB/c mice – curative tumor model followed by resistance to challenge with live cells (203)	–	–	–
Hypericin-based PDT	CT26 cells in BALB/c mice – prophylactic immunization model (35); – curative tumor model (184); AY27 cells in Fischer 344 rats – prophylactic immunization model (42); B78 cells in C57BL/6 mice – prophylactic immunization model (30)	–	–	–
Microwave thermal ablation	K7M2 cells in BALB/c mice or UMR106 cells in SD rats – prophylactic immunization model (158)	–	–	–
Newcastle disease virus (NDV)	B16 cells in C57BL/6 mice – curative tumor model (159)	GL261 cells in C57BL/6 mice – curative tumor model (43)	–	–
Oxaliplatin	CT26 cells in BALB/c mice – prophylactic immunization model (123, 197); – curative tumor model (197); EL4 cells in C57BL/6 mice – curative tumor model (36); EG7 cells in C57BL/6 mice – curative tumor model (36); EG7 cells in C3H mice – prophylactic immunization model (70)	–	–	–
Photofrin-based PDT	EMT6 cells in BALB/c mice – curative tumor model (161); SCCVII cells in C3H/HeN mice – curative tumor model (162, 163)	–	–	–
Radiotherapy	CT26 cells in BALB/c – prophylactic immunization model (204); 410.4 cells in BALB/c mice – prophylactic immunization model (205); EG7 cells in C57BL/6 mice and SCC VII cells in C3H mice – prophylactic immunization model (206); B16F10 cells in C57BL/6 mice – prophylactic immunization model with the use of irradiated cancer cells, as well as DCs stimulated with irradiated cancer cells (207)	–	–	–
RIG-I-like helicases (RLH) ligand	Panc02 cells in C57BL/6 mice – prophylactic immunization and curative tumor model (165)	–	–	–
Septacidin	MCA205 cells in BALB/c mice – prophylactic set-up (139);	–	–	–
Shikonin	B16 cells in C57BL/6 mice – prophylactic immunization model (160); P388 cells in KMF mice – curative tumor model (208)	4T1 cells in BALB/c mice – curative tumor model (192);	–	–
UVC irradiation	CT26 cells in BALB/c mice – prophylactic immunization model (204); EG7 cells in C57BL/6 mice – curative tumor model (152)	–	–	–
Vorinostat	MC38 or Eμ-myc 4242/299 lymphoma in C57BL/6 mice – curative tumor set-up (166)	–	–	–
High hydrostatic pressure	No mice or rat based preclinical data available to support their ICD-functions			
Pt^{II} N-heterocyclic carbene complex				

DC, dendritic cell; ICD, immunogenic cell death; PDT, photodynamic therapy.

TABLE 7 | Existence of intrinsic or naturally occurring resistance to ICD in experimental cancer models.

ICD inducer(s)	Experimental set-up where resistance was observed	Reason behind Resistance	Rescued by?	Clinical applicability verified?	Reference
***In vivo* preclinical setting (cancer cell or host immune system-level resistance)**					
Anthracyclines or anthracycline plus oxaliplatin	C3H mice with naturally occurring *tlr4* mutation	Host immune system-level resistance**:** defective *TLR4* in C3H mice causes failure of HMGB1-mediated immunity thereby leading to resistance to anti-cancer vaccination effect associated with anthracyclines treatment	Adoptive transfer of TLR4-expressing DCs loaded with dying tumor cells	Yes; breast cancer, colon cancer, and lung cancer patients carrying TLR4 gene mutation that ablates its ability to bind its ligands is associated with worse prognosis post-treatment	(215)
Doxorubicin	AT-3 or 4T1.2 breast cancer cells in C57BL/6 or BALB/c mice, respectively	Cancer cell-level resistance: CD73 overexpression confers chemo-resistance to doxorubicin by suppressing anti-tumor immunity through A2A adenosine receptors	Blockade of CD73	Yes; in triple-negative breast cancer patients, high CD73 in anthracycline-treatment set-up associated with lower rate of complete responses	(216)
Mitoxantrone and Hypericin-PDT	AY27 rat bladder cancer cells in Fischer 344 rats	Cancer cell-level resistance: low endogenous CRT levels, resulted in severely reduced surface-CRT upon treatment with mitoxantrone or Hyp-PDT; this in turn compromised immunogenic phagocytic clearance and anti-cancer vaccination effect	Exogenous addition of recombinant CRT	Yes; high tumoral *CALR* levels correlated with high expression of phagocytosis-associated genes and predicted for prolonged survival after RT or PTX treatment of lung or ovarian cancer patients respectively	(42)
Oxaliplatin	Autochthonous transgenic adenocarcinoma of the mouse prostate (TRAMP) model of metastatic prostate cancer	Host immune system-level resistance**:** immunosuppressive B cells expressing IgA, IL10 and PD-L1 cause resistance to anti-tumorigenic effects of oxaliplatin	Genetic or pharmacological depletion of B cells	Not directly, but possible validity is supported by human patient data showing that IL-10 expressing IgA+ cells are abundant in therapy-resistant prostate cancer and are negative prognostic indicators	(217)
***In vitro* preclinical setting (cancer cell-level resistance)**					
Anthracycline	SH-SY5Y neuroblastoma cell line	Anthracycline treatment of these cells failed to induce surface-CRT due to reduced capacity to efflux ER-Ca^{2+} into cytosol	Overexpression of reticulon-1C	–	(132)
Doxorubicin	HT29-dx and HT29 iNOS-cells (human colon cancer cells)	Doxorubicin failed to induce NO synthesis, which resulted in reduced toxicity, reduced surface-CRT and subsequently compromised immunogenic phagocytic clearance and DC stimulation	Addition of sodium nitroprusside or a NO donor	–	(218)
Doxorubicin	MDR+ human cancer cells (HT29-dx, A549-dx and MCF-7-dx)	Increased MDR levels caused increased P-glycoprotein expression which caused resistance to doxorubicin-induced ICD by affecting immunogenic phagocytic removal	Addition of zoledronic acid	Not directly	(219)

CD, cluster of differentiation; CRT or CALR, calreticulin; DC, dendritic cells; ER, endoplasmic reticulum; HMGB1, high-mobility group box-1 protein; HSP, heat shock protein; Hyp-PDT, hypericin-photodynamic therapy; ICD, immunogenic cell death; IL, interleukin; MDR, multiple drug-resistance; NO, nitric oxide; NOS, nitric oxide synthase; PD-L1, programed cell death protein ligand 1; PTX, paclitaxel; RT, radiotherapy; TLR, toll-like receptor.

TABLE 8 | A list of clinical observations supporting the existence of ICD in cancer patients.

ICD inducer	Standard-of-care therapy or regularly applied palliative therapy in clinic?	ICD-related characteristics regulating clinical patient prognosis or treatment-responsiveness
Anthracyclines	Yes	*P2RX7* loss-of-function mutation that compromises ICD also negatively affects MFS in breast cancer patients treated with adjuvant anthracyclines (36); breast cancer patients possessing a wild-type *TLR4* benefited more from the anthracyclines than those who possessed a mutated *TLR4* that compromises ICD (70); an *MX1*-centered Type I IFN signature in anthracycline-treated breast cancer patients predicts for improved disease outcome (141); combined positivity for cytoplasmic LC3B+ puncta and nuclear HMGB1 is a positive predictor of improved survival following adjuvant anthracycline-based chemotherapy (225)
High hydrostatic pressure	No; but HHP-based anticancer DC vaccines are currently being applied in clinical trials against prostate cancer and ovarian cancer (155)	No data are available
Hypericin-based PDT	No; but few clinical trials have been carried out for non-melanoma skin cancer (226), cutaneous T-cell lymphoma (227), mesothelioma (228), and basal or squamous cell carcinoma (229)	No data are available
Oncolytic adenoviruses	No; but oncolytic adenoviruses are currently being applied in various clinical trials in cancer patients	Serum HMGB1 levels and the temporal change in their levels during treatment was identified as a prognostic and predictive biomarker in cancer patients (230)
Oxaliplatin	Yes	Similar to anthracyclines, cancer patients possessing wild-type *TLR4* exhibited prolonged PFS and OS in comparison to patients bearing the loss-of-function allele of *TLR4* (197)
Paclitaxel	Yes	High tumoral *CALR* levels in paclitaxel-treated ovarian cancer patients associated with prolonged OS/PFS as well as increased expression levels of various phagocytosis-associated genes (42)
Photofrin-based PDT	Yes; FDA-approved for application in esophageal and lung cancer (231)	No data available
Radiotherapy	Yes	In patients of eosophageal squamous cell carcinoma (ESCC) receiving chemo-radiotherapy significant increase in serum HMGB1-levels and increased intra-tumoral staining of HMGB1 correlated with better patient survival (232); high tumoral *CALR* levels in radiotherapy-treated lung cancer patients associated with prolonged OS as well as increased expression levels of various phagocytosis-associated genes (42)
Shikonin	No; but shikonin is currently being applied in an observational clinical study of breast cancer patients (NCT01287468)	No data are available
UVC irradiation	No; but UV treatment is sometimes applied for the preparation of clinical cell-based anticancer vaccines (233)	No data are available
Bortezomib, Anti-EGFR antibody (7A7), bleomycin, cyclophosphamide, microwave thermal ablation, vorinostat	Yes	No data are available
Coxsackievirus B3; *Clostridium difficile* toxin B; Microwave thermal ablation; Newcastle disease virus (NDV); RIG-I-like helicases (RLH) ligand; Septacidin; Pt^{II} N-heterocyclic carbene complex; Patupilone	No	No data are available

CRT or CALR, calreticulin; HMGB1, high-mobility group box-1 protein; Hyp-PDT, hypericin-photodynamic therapy; ICD, immunogenic cell death; IFN, interferon; OS, overall survival; PFS, progression-free survival; TLR, toll-like receptor.

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