



Review

Local tumour ablative therapies: Opportunities for maximising immune engagement and activation



Morgan A. O'Brien^{a,*}, Derek G. Power^b, A. James P. Clover^c, Brian Bird^d, Declan M. Soden^a, Patrick F. Forde^{a,*}

^a Cork Cancer Research Centre, Leslie C. Quick Laboratory, BioSciences Institute, University College Cork, Cork, Ireland

^b Department of Medical Oncology, Mercy University Hospital, Grenville Place, Cork

^c Department of Plastic Surgery, Cork University Hospital, Ireland

^d Department of Oncology, Bon Secour Hospital, College Road, Cork, Ireland

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ABSTRACT

The relationship between cancer and the immune system is a complex one. The immune system can prevent tumour growth by eliminating cancer cells but this editing process ultimately results in poorly immunogenic cells remaining allowing for unchallenged tumour growth. In light of this, the focus of cancer treatment should be to maximise cancer elimination and the prevention of escape mechanisms. In this review we will examine current and emerging ablative treatment modalities that induce Immunogenic Cell Death (ICD), a special type of cell death that allows for immune cell involvement and the generation of an anti-tumour specific immune response. When paired with immune modulating agents, capable of potentiating the immune response and reversing the immune-suppressive environment created by tumours, we may be looking at the future of anti-cancer therapy.

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Contents

1.	Introduction	511
2.	Cancer immuno-editing	511
2.1.	Elimination	511
2.2.	Equilibrium	512
2.3.	Escape	512
3.	The abscopal effect & immunogenic cell death	512
3.1.	The abscopal effect	512
3.2.	Immunogenic cell death & DAMPs	512
3.3.	DAMPs	513
3.3.1.	Calreticulin	513
3.3.2.	High Mobility Group Box 1 Protein	514
3.3.3.	Heat shock proteins	514
3.3.4.	Adenosine triphosphate	514
4.	Tumour ablation, ICD and DAMP release	514
4.1.	Radiation	514
4.2.	Radiofrequency ablation	515
4.3.	Cryoablation	515
4.4.	Chemotherapy & electrochemotherapy	516
4.5.	Photodynamic therapy (PDT)	516
5.	Immune modulation & biological intervention	516
5.1.	Immune blockade	516
5.1.1.	Cytotoxic T-Lymphocyte Antigen 4 blockade	516
5.1.2.	Programme Cell Death Protein 1 blockade	517

* Corresponding authors. Tel.: +353 214901395.

E-mail addresses: morgan.obrien@umail.ucc.ie (M.A. O'Brien), p.forde@ucc.ie (P.F. Forde).

5.2. Immune stimulation	517
5.2.1. OX40 agonists	517
5.2.2. CD40 agonists	518
6. Conclusion	518
Acknowledgements	518
References	518

1. Introduction

Cancer can be defined as the rapid and uncontrolled growth of malignant cells in the body and even with recent advances in the areas of detection and treatment, there were 8.2 million cancer related deaths worldwide in 2012 [1]. With an estimated 14.1 million new cases of cancer in 2012 and projections predicting a substantial increase to 19.3 million cases per year by 2025 [1], the need for more effective treatments has never been higher. With this in mind there has been renewed interest in the immune system, its relationship with cancer and the ability to harness its potential for fighting the disease.

The main function of the immune system is to protect us against invading pathogens; it detects these pathogens via a set of pattern recognition receptors (PRRs) that bind pathogen-associated molecular patterns (PAMPs) [2]. PAMPs include viral RNA, the components of bacterial cell walls and, when detected trigger the activation of the innate immune system to protect the host [3]. However not all threats come in the form of invading bacterial/viral organisms and so the immune system has developed the ability to identify and eliminate cancerous/transformed cells. Two landmark murine studies demonstrated the importance of a functional immune system in preventing carcinogenesis; mice lacking IFN- γ responsiveness or specific immune cells (T cells, B cells and NK cells) were more susceptible to chemically induced tumour formation [4,5]. Lung and kidney transplant patients are put on immunosuppressive drugs (cyclosporine A, corticosteroids, azathioprine, etc.) to prevent against transplant rejection, and the fact that these patients have been shown to have a higher chance of developing neoplastic malignancies, reinforces the protective importance of the immune system [6–11]. Human immunodeficiency virus (HIV) and the subsequent acquired immunodeficiency syndrome (AIDs) result in depletion of CD4⁺ T cells and leave patients severely immunocompromised [12]. It is no coincidence that HIV/AIDs sufferers have increased incidences of cancer [13–15] and in fact several cancers (Kaposi's sarcomas, cervical cancer and non-Hodgkin's lymphoma) are now commonly deemed AIDs defining malignancies [16–18]. Subsequent work in the field has shown that 'immuno-surveillance' is only one aspect of the complex relationship between the immune system and cancer [19] and has led to formation of the 'cancer immuno-editing' hypothesis.

2. Cancer immuno-editing

Cancer immuno-editing is a refinement on the original 'immuno-surveillance' idea and suggests that the immune system not only protects the host against cancer, but also shapes tumour immunogenicity (the ability for the tumour to provoke an immune response). Murine studies have shown that tumours that develop in immune-competent mice (deemed 'edited' tumours) often grow more easily than tumours that originate from immunocompromised mice ('unedited'), when transplanted into syngeneic immune-competent mice [5]. Therefore the immune system not only protects the host against tumour formation but also applies selection pressure favouring the development of less immunogenic tumours, which escape recognition by a functioning immune system. Immuno-editing is deemed to have 3 phases, each of which we will examine further.

2.1. Elimination

The innate immune system acts as our body's first line of defence and its main components are dendritic cells (DCs), macrophages and monocytes, neutrophils, natural killer (NK) cells, and natural killer T (NKT) cells. NK cells are important in the early stages of cancer elimination; they have the ability to recognize stress induced ligands such as NKG2D-L, through their NKG2D receptors. The NKG2D ligands can be induced on tumour cells through DNA damage [20] and other stimuli, alerting NK cells to unwanted transformation [21]. NK cells' ability to eliminate tumour cells is dependent on the expression of tumour cell p53, a consequence of the cellular DNA damage response [22], which leads to the secretion of various interleukins and cytokines that recruit NK cells to the tumour site [23]. Tumours lacking p53 expression can evade NK mediated clearance but when p53 activity is restored, the tumour cells are gradually cleared by NK cells and other infiltrating cells [24]. Upon activation, NK cells secrete interferon- γ (IFN- γ), a type II cytokine critical to the initial immune response. IFN- γ up-regulates production of the cytolytic protein perforin [25] as well as the apoptotic inducing Fas ligand [26] and TNF-related apoptosis-inducing ligand (TRAIL) [27]. IFN- γ has also been shown to protect against the growth of transplanted tumours [28], to activate dendritic cells and promote the generation of tumour-specific CD4⁺ T and CD8⁺ T cells [29], and to augment major histocompatibility complex (MHC) expression on tumour cells [30]. These functions are crucial in improving tumour immunogenicity and potentiating the activity of both the innate and adaptive immune responses. Type I interferons (IFN- α/β) have an important role in the immune-editing process; in fact IFN- α is the most used cytokine in patients, used to treat a wide range of cancerous malignancies [31]. IFN- α/β can up-regulate the p53 mediated response of tumour cells to DNA damage [32] but it seems that their major contribution to anti-cancer immunity is their actions on haematopoietic cells. Type I IFNs are important for the *in vivo* proliferation and long-term survival of anti-TAA (tumour specific antigens) specific CD8⁺ T cells [33] and they also enhance the expression of anti-apoptotic genes in human T cells [34]. Dendritic cells (DCs) are often regarded as the most effective of the antigen presenting cells (APCs) and type I IFNs have important effects on DC differentiation and maturation [35,36]. As such type I IFNs are often thought of as an important link between the innate and adaptive arms of the immune system.

Adaptive immunity consists of T and B lymphocytes and their respective mediators (cytokines and antibodies) and its ability to generate an immune 'memory'. It is the interplay and communication between both arms of the immune system that make it effective against cancer functional cytotoxic CD8⁺ (CTL) and helper CD4⁺ T (T_H1) cells are critical for the eradication of cancerous cells. The T cell receptor (TCR) of cytotoxic CD8⁺ T cells is capable of binding with the MHC-1 molecule of harmful/cancerous cells or antigen presenting cells (APC) and causes subsequent cellular lysis through the release of perforin, granzymes and granzysin. Once activated, the CD8⁺ T cells undergo rapid clonal expansion, aided by the MHC-II/TCR mediated secretion of IL-2 from the CD4⁺ T helper cells, which is a potent growth and differentiating factor. Higher numbers of total circulating and tumour infiltrating CD8⁺ and CD4⁺ T cells are associated with improved prognosis/survival in patients with various cancer types [37–39]. T

cells displaying $\gamma\delta$ TCRs ($\gamma\delta$ T cells) represent a very important subset of the T cell population and have cytotoxic activity against a wide range of cancers [40]. More importantly, $\gamma\delta$ T cells secrete IFN- γ and attract activated lymphocytes and APCs through the secretion of a number of chemokines [41,42]. Mice lacking $\gamma\delta$ T cells are more susceptible to chemically-induced tumour formation than wild-type mice [43]. The generation of a robust anti-cancer immune response following chemotherapy and effectiveness of the chemotherapy itself has been found to be dependent on $\gamma\delta$ T cells and their secretion of the effector cytokine; IL-17 [44].

2.2. Equilibrium

Tumour cell variants that survive the elimination phase are then proposed to enter the equilibrium phase. This phase is the least well understood of the three but the generally accepted theory is that the intense pressure endured by the cancer cells during the elimination phase leads to genetic selectivity in the surviving cells, resulting in a surviving population with reduced immunogenicity. This 'dormant' population may be held in check by the immune system for years before returning as recurrent disease at the site of the primary tumour or possibly as distal metastases [45]. Experimental evidence for the existence of equilibrium is mostly based on murine studies. In one such study, mAb mediated T cell/ IFN- γ depletion in immuno-competent mice with chemically induced, stable tumours, resulted in rapid tumour growth [46]. In another similar study it was shown that the balance between IL-12 (a cytokine with anti-tumour properties) and IL-23 (a cytokine with tumour promoting properties) was found to hold chemically induced tumours in a state of equilibrium [47]. Examining the microenvironment of tumours deemed to be in this state of equilibrium or dormancy, one study found high numbers of CD8 + T cells, NK cells, and $\gamma\delta$ T cells and low proportions of FOXP3 + Treg cells, and MDSCs, indicating a balance between anti-cancer and pro-cancer immune cells [48].

2.3. Escape

Tumour cells that have survived the elimination phase and passed through the equilibrium phase of the cancer immuno-editing process will have undergone intense selectivity and possess the tools necessary to escape the control of the immune system. One method used by cancer cells to create an immunosuppressive environment is the downregulation of MHC-1; CD8 + cytotoxic T cells scan the MHC-1 molecule for peptides which may be related to TAAs and induce apoptosis to prevent the spread of the disease [49]. Downregulation of MHC-1 has been shown in many cancer cell types [50] and has also been linked to poor prognosis survival rates in patients with ovarian, renal cell and other types of cancer [51–53]. One of the functions of NK cells is to identify cells lacking MHC-1 expression and subject them to NK-mediated cell death [54]. For NK cells to be effective, they require the presence of activation receptors and ligands, of which the NKG2D receptor and its ligand (NKG2D-L) are the most vital. However NKG2D and NKG2D-L are down-regulated in many cancer types and this too is an indicator of poor prognosis [55–57]. In contrast, higher numbers of circulating and tumour infiltrating NK cells correlate with better prognosis and survival in pancreatic, lung and gastric cancers [58–60].

Immune inhibitory receptors such as Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and Programmed Death 1 (PD-1), which are expressed on the surface of activated T cells, can effectively shut down T cell activity upon binding to their ligands CD80/CD86 and PD-L1/PD-L2 respectively. The complex interplay between these stimulatory and inhibitory mechanisms has been shown to be crucial in the immune mediated elimination of cancer [61]. For example increased expression of PD-L1 is associated with decreased T cell activity, infiltration and poorer prognosis in many cancers [62–65]. Similar correlations have been found in melanoma patients expressing higher levels of CTLA-4

receptor and its ligands [66]. Regulatory T cells (Tregs), a type of T cell that suppresses exaggerated and prolonged immune responses, have a very negative impact on tumour elimination by promoting an immunosuppressive environment [67,68]. Increased Tregs at the tumour site correlates with more advanced disease and poorer prognosis [69–72] while low Treg percentage correlates with improved survival [72,73].

3. The abscopal effect & immunogenic cell death

In light of this complex relationship between the host immune system and cancer, emphasis should be placed on maximising 'Elimination' and preventing 'Escape' mechanisms when applying interventions or developing new treatments. While most conventional treatment modalities (radiation, chemotherapy) were once thought to induce cancer cell death in an immune independent manner, however this is not the case.

3.1. The abscopal effect

There are a number of physical ablative modalities and chemotherapeutics which have caused regression in tumours that are distal to the primary site of treatment [74]. This has been observed in more than one type of cancer [75–77] and strongly indicates that immune mechanisms are involved. This phenomenon has been termed the 'Abscopal Effect' and has been a topic of interest since the 1950s; however the mechanism of action still remains unclear. A number of theories have been put forward, including the importance of certain cytokines [78, 79], NK cells [80,81] and functional T cell populations [82] in the generation of an abscopal effect, all of which point towards a link between the immune system and the abscopal effect. Murine data has also highlighted the importance of the transcription factor, p53, and its role in apoptosis in the abscopal effect [83]. In addition to those mentioned above, another postulated mechanism of the abscopal effect is the release of 'tumour associated antigens' (TAAs) caused by treatment at the primary site and the subsequent activation of the immune system due to 'danger signals' [84]. This concept is closely related to the "danger" model of immunity originally proposed by Matzinger in 1994 [85]. While the understanding of the abscopal effect is still incomplete at best, it does have interesting parallels with immunogenic cell death, a type of cell death that induces a positive immune response through the release of damage associated molecular proteins (DAMPs) [86]. Whether ICD plays a direct role in mediating the abscopal effect is yet to be determined but it could go a long way to explaining the phenomenon.

3.2. Immunogenic cell death & DAMPs

The term Immunogenic Cell Death (ICD) was coined to describe cell death that stimulates immune cells in the tumour environment and results in a favourable anti-cancer host immune response. Whether a dying cell can be described as undergoing ICD, depends on a number of factors; the activation state of the cell is one such criteria [87]. The type of cellular stress is another differentiator; the DNA-damage-response and endoplasmic reticulum (ER) stress are two specific examples known to elicit immunogenic responses [20,88]. Caspase activation is also important, as inhibiting caspase activity can prevent the mobilisation of immuno-epitopes within the cell also inhibits certain signals, key to the ICD response [89]. However the release of DAMPs from dying cells is probably the most important factor characterizing ICD as DAMPs are the key mediator in causing the ICD related immune response generated by stressed or dying cancerous cells [90].

DAMPs are intracellular molecules that when exposed on the cell surface or secreted extracellularly, induce a potent pro-inflammatory immune response. These molecules can exert various beneficial effects on APCs, including activation, maturation and antigen processing/presentation. The emission of DAMPs was initially thought to be exclusively related to necrosis that occurred due to physical or chemical

damage to cancer cells [91]. Necrosis is caused as a result of massive acute cellular injury and involves the rupture of the cell's plasma membrane and the destruction of the intracellular organelles [92]. More recently however, DAMPs have been shown to be secreted from apoptotic cells too [93]. Apoptosis can be thought of as a programmed or controlled type of cell death, commonly characterized by the caspase activation and the permeabilization of the mitochondrial membrane [94,95].

Many different DAMPs have been identified and their presentation/exposure to the immune system depends on the cancer treatment used and the mechanism of cancer cell death involved. Some of the key DAMPs involved in immunogenic cell death and how they interact with immune cells, are detailed below and visually outlined in Fig. 1.

3.3. DAMPs

3.3.1. Calreticulin

Calreticulin (CRT) is a highly conserved, Ca²⁺ binding chaperone protein, mainly located in the lumen of endoplasmic reticulum (ER) and plays a key role in the activity and regulation of Ca²⁺ homeostasis/signalling and through the interaction with the isomerase ERp57, CRT facilitates proper folding of ER-chaperoned proteins [96]. CRT also aids in the correct assembly of MHC-1 molecules and insures effective loading of antigens [97]. CRT that is found on the plasma membrane cells, plays an important role in ICD by signalling phagocytes to engulf dying cells [98]. CRT translocates to the cell surface via a number of

mechanisms, depending on the stage of cell death. Pre-apoptotic ecto-CRT expression relies on PERK mediated translocation and the PI3K distal secretory pathway, while later stages of apoptosis involve co-exposure with phosphatidylserine and binding with lipid rafts on the plasma membrane [98–100].

Ecto-CRT is upregulated during cellular apoptosis and several conventional cancer treatments including radiotherapy, anthracyclines and platinum based chemotherapeutics, have the ability to increase ecto-CRT exposure [101–103]. This is favourable as the ecto-CRT has been shown to promote the engulfment of apoptotic cells by professional phagocytes (e.g. macrophages and DCs), through binding and activation of the CD91 receptor (also called the LDL-receptor related protein or LRP) on the phagocytes [98]. This allows APCs to process and present TAAs from the engulfed cells to CD8⁺ and CD4⁺ T cells through their MHC-1 molecules. CRT exposed on the surface of dying cancer cells is essential for the immunogenicity of apoptosis, without which the resulting cell death would go un-noticed by the immune system and is effectively 'silent'. Anti-cancer vaccines that rely on dying tumour cells for their efficacy have been shown to be ineffective when ecto-CRT function is neutralized through CRT-specific antibodies or siRNA-mediated CRT, when exogenous CRT was then re-administered the effects of the vaccination were restored [101,104]. When all of this is taken into account, ecto-CRT can effectively be thought of as an 'eat me' signal for professional phagocytes. Increased CRT expression is deemed to be a positive prognostic factor in patients with gastric cancer and neuroblastoma [105,106].

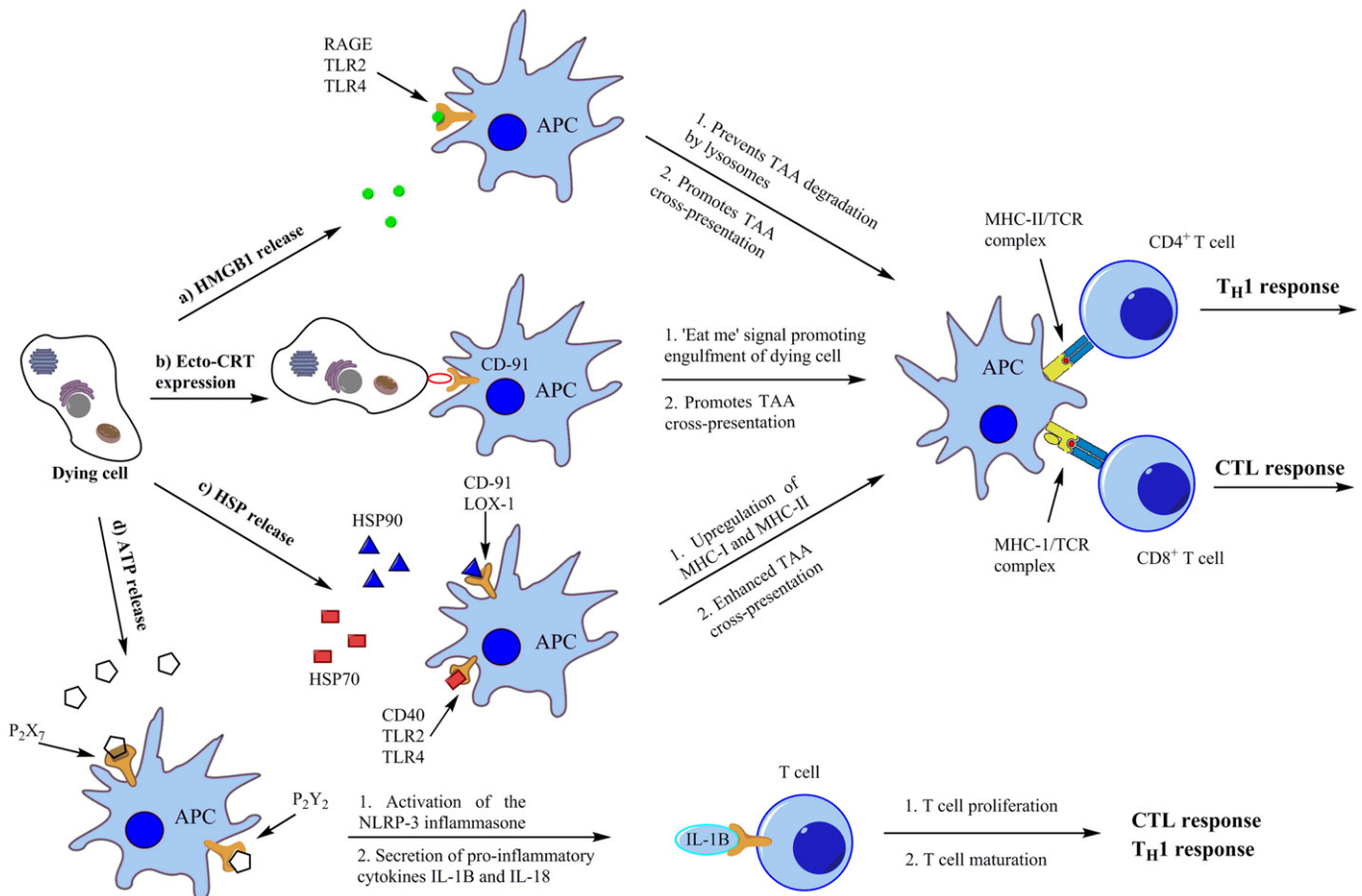


Fig. 1. Interaction between the cells of the immune system and DAMPs released from dying tumour cells. a) HMGB-1 released from dying cell has the ability to bind to RAGE, TLR2 and TLR4 receptors on APCs, which increases the amount of TAAs processed and presented to T cells by preventing the degradation of TAAs in the lysosomes of APCs. b) The expression of ecto-CRT on the surface of dying cells, via various pathways, effectively marks them for engulfment by APCs, and the contents of the tumour cell (including TAAs) are then processed and presented to the T cells. c) HSPs can bind to many surface receptors on APCs, including endocytic receptors and immune signalling receptors. This leads to increased tumour cell uptake and up-regulation of the antigen presenting molecules MHC-1 and MHC-II. d) Extracellular ATP binds to purigenic receptors on APCs, activating the NLRP-3 inflammasome and causing the secretion of pro-inflammatory cytokines to promote T cell activity.

3.3.2. High Mobility Group Box 1 Protein

In healthy cells High Mobility Group Box 1 Protein (HMGB1) is localized in the nucleus and binds to chromatin and influences transcription and other nuclear functions [107]. While CRT is exposed on the plasma membrane early in the apoptosis process, HMGB1 is secreted extracellularly during 1° necrosis and late apoptosis (2° necrosis) [108–110]. Within the cell, HMGB1 has several important 'pro-survival' functions, such as mediating autophagy, aiding with transcription and the assembly of protein complexes within the nucleus [110,111]. Outside of the cell, the function of HMGB1 is drastically different and can be thought to act like a pro-inflammatory 'cytokine'. Studies have shown the importance of HMGB1 in chemotherapy and radiotherapy mediated cell death, implicating it as one of the molecules involved in ICD and the abscopal effect [112,113]. HMGB1 has been shown to play a powerful role in the activation of APCs and is known to bind to at least three different pattern recognition receptors (PRRs) expressed on APCs; namely the receptor for advanced glycosylation (RAGE), TLR2 and TLR4 [114,115]. TLR4 (Toll like receptor 4) controls antigen presentation in APCs by preventing the fusion of phagosomes with lysosomes, allowing TAAs within the phagosome to form into tumour antigen peptides that can be presented to the other cells of the immune system via the APC's MHC molecules [116]. Without TLR4 activation through HMGB1 binding, the TAAs engulfed by the APCs would be degraded by fusion with lysosomes, rendering the cell death non-immunogenic [117]. The significance of the HMGB1/TLR4 relationship in chemotherapeutic or radiotherapy induced ICD is further emphasised by several studies across multiple cancer types. Neutralization or knockdown of HMGB1 and/or knockout of TLR4 inhibits the initiation of a pro-inflammatory immune response and the priming of T cells [112,113,118]. However the picture of HMGB1 as a potent anti-cancer DAMP is being challenged by recent studies showing that in certain circumstances it can promote tumour angiogenesis, as well as invasion and metastasis [115,119]. Therefore HMGB1 can be viewed as having context dependent functions, with intracellular HMGB1 being pro-survival and even pro-tumourigenic, while extracellular HMGB1 has anti-tumourigenic functions.

3.3.3. Heat shock proteins

Heat shock proteins (HSPs) are a family of highly conserved chaperone proteins that play an important role in folding of newly synthesized proteins and also in the refolding of proteins in response to cellular stress [120]. Similarly to CRT, HSPs are expressed intracellularly in healthy cells but can be induced to translocate to the plasma membrane (ecto-HSP) under stressful conditions. This has been observed with platinum based chemotherapeutic drugs [102,121] as well as several ablative modalities, including; radiofrequency ablation, thermal ablation and ultrasound ablation [122–124]. While intracellular overexpression of HSPs causes cyto-protection and inhibits apoptosis [125], extracellular expression of HSPs during late apoptosis and necrosis can elicit immune-stimulatory effects [126].

Ecto-hsp70, ecto-hsp90 and gp96 may play a role in ICD due to their ability to interact with a number of endocytic promoting surface receptors on APCs (CD91 and LOX1) [127,128] and also several signalling receptors (CD40, TLR2 and TLR4) [129,130]. This causes the up-regulation of many maturation markers such as CD86 and CD40 [129,131,132] and consequently improves the cross-presentation of TAAs to CD8 + and CD4 + T cells through up-regulation of the MHC-I and MHC-II molecules [133]. Activation at these receptors results in the secretion of many pro-inflammatory cytokines which are key for an optimum immune response; these include tumour necrosis factor- α (TNF- α), IL-1 β , IL-12 and GM-CSF [129,134]. The translocation of NF- κ B transcription factor to the nucleus of APCs and its subsequent activation due to the above stimuli, further drive the transcription of other pro-inflammatory cytokines and growth factors required for immune cell activation and differentiation [129,135]. Vaccination with HSPs, isolated from tumour stressed tumour lysates has shown the ability to induce a specific anti-

tumour immune response [136]. The fact that HSPs derived from damaged/stressed tumour tissue are more immune-stimulatory than exogenous recombinant HSPs, suggests that HSPs may actually form complexes with TAAs and it is this complex that accounts for their immune-stimulating abilities [137].

3.3.4. Adenosine triphosphate

Adenosine Triphosphate (ATP) is a ubiquitous intracellular metabolite; it has a key role in cellular energy production and can also act as a paracrine/autocrine messenger molecule [138]. ATP can be released passively into the extracellular space during necrosis or via various mechanisms during apoptosis [139,140]. This release of ATP from dying cells has been shown to be caused by a number of common chemotherapeutic agents (etoposide, mitomycin C, oxaliplatin, cisplatin and doxorubicin) [141] and ablative methods such as radiotherapy and electroporation [142,143]. Once released from dying cells, ATP can bind the P₂Y₂/P₂X₇ purinergic receptors on macrophages and DCs, activating the NLRP3 inflammasome [144,145]. NLRP3 mediates the production of the protease' caspase-1, which in turn drives the production of pro-inflammatory cytokines IL-1 β and IL-18 [145]. IL-1 β in particular, is crucial in the recruitment and functional maturation of both innate $\gamma\delta$ T cells and TAA primed CD8 + T cells [145,146]. NLRP3 function is critical for the efficacy of many chemotherapeutics and the generation of an immunogenic response. One study that highlighted this compared the tumour growth of WT mice treated with oxaliplatin and mice with deficient NLRP3 inflammasomes or lacking IL-1 β , and results showed that both were essential for the efficacy of the chemotherapy [147]. The crucial importance of IL-1 β in the antitumour effects of oxaliplatin was shown in chemically induced tumours [145].

4. Tumour ablation, ICD and DAMP release

With recent research highlighting the importance of DAMP release and ICD in immune mediated clearance of cancer, the focus now needs to shift towards developing clinical interventions that harness this potential. Fig. 2 illustrates that the types of DAMPs released from dying cells depends on the cause of cell death and below we will look at some of the established and emerging ablative technologies known to induce ICD and/or DAMP release.

4.1. Radiation

Ultraviolet C (UVC) and ionizing radiation (radiotherapy) have both been proven to cause DAMP release following treatment. UVC induced ICD relies on caspase 8 signalling [99] and has been shown to cause pre-apoptotic ecto-CRT release, as well as ATP release during early apoptosis [101,145]. Ionizing radiation is more relevant as a treatment modality as it is one of the cornerstones of cancer therapy, long known for its ability to induce cancer cell death through apoptotic and necrotic mechanisms. Its ability to enhance tumour immunogenicity and generate an abscopal effect was realised following numerous cases, across different cancer types, where distant metastases spontaneously regressed after radiation therapy at a primary site [75,79,148,149].

Subsequent research has shown that radiation therapy causes the release of DAMPs and pro-inflammatory cytokines from dying cells, effectively priming the immune response. Ecto-CRT, the potent 'eat me' signal causing macrophage engulfment, is expressed on the surface of dying irradiated tumour cells, facilitating better TAA cross-presentation to other immune cells [93,101]. Other DAMPs released following radiation mediated cell death include HMGB1 and ATP, inflammatory molecules with the ability to bind to TLRs and purinergic receptors on APCs, which also causes TAA processing and T-cell priming following the release of IL-1 β [113,150,151]. Radiation therapy also up-regulates the expression of several proteins with anti-cancer properties including; the Fas death receptor (CD95) [152], MHC-1 [153] and the chemokine CXCL16, responsible for the recruitment of T cells to the tumour site [154]. However, while

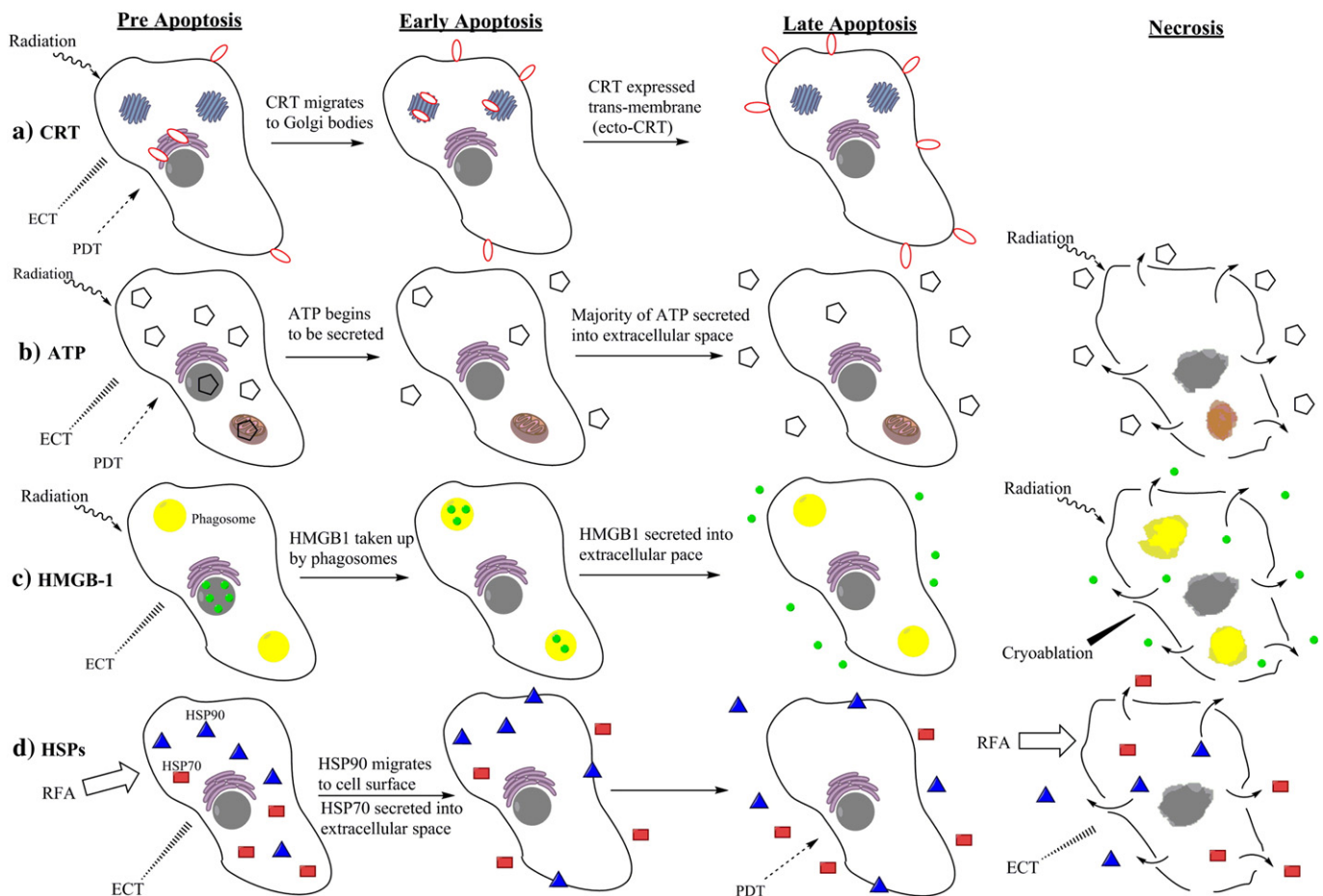


Fig. 2. Immunogenic Cell Death (ICD) caused by ablative modalities and subsequent DAMP release. a) Cellular stress causes CRT to migrate from ER to Golgi bodies and as apoptosis progresses; CRT is expressed on the plasma membrane (ecto-CRT). This can be induced by radiation and ECT. b) During cellular apoptosis, ATP is actively secreted from dying cells. The loss of membrane integrity during necrosis allows leakage of ATP into the extracellular space. Radiation and ECT have been proven to cause this ATP release. c) HMGB-1 is typically located within the nucleus but during apoptosis it relocates to phagosomes in the cytoplasm, late stages of apoptosis and necrosis sees it expelled in the extracellular space. Ablative techniques that cause this include cryoablation, radiation and ECT. d) Heat shock proteins move from intracellular compartments to the extracellular space and form complexes on the plasma membrane as the stages of apoptosis advance. Cellular necrosis, as caused by RFA and in some cases ECT, leads to passive diffusion of HSPs into the extracellular space.

radiation therapy may seem like the ideal ablative treatment option, it is severely limited by its adverse side effects profile. It has been known to cause neutropenia (a condition characterized by severely low neutrophil count) [155], myelo-suppression (reduced production of oxygen carrying red blood cells, immune precursor lymphocytes and platelets in the bone marrow) [156] and production of the inhibitory cytokines IL-6, IL-10 and TGF- β [157,158].

4.2. Radiofrequency ablation

Radiofrequency ablation (RFA) is another physical ablative method that causes cellular necrosis by using a high frequency current to heat and coagulate tissues, causing protein denaturation [159]. While RFA does not cause classical ICD, the necrotic cell death can lead to a positive immune response due to the passive release of DAMPs into the micro-environment [122,160,161].

RFA has been used successfully to treat many cancer types including hepatocellular carcinoma, renal cell carcinoma and lung cancers. RFA has been shown to cause an abscopal effect and the regression of distal pulmonary metastases in renal cancer [82]. Other studies have highlighted the ability of RFA to increase the activity and immune stimulating abilities of APCs [162], generate tumour specific CD8⁺ and CD4⁺ T cell responses [163] and increase the numbers of NK cells in the tumour environment [163,164]. When coupled with a TAA derived vaccine, RFA has been shown to be very effective at causing local and distal tumour regression [165]. The mechanism by which RFA is able to

cause tumour specific systemic immune response is likely due to the release of HSPs following RFA treatment. One of the drawbacks of RFA is that given its method of administration, the types of tumours that can be treated are currently limited and the margin of treatment is also quite small. Other serious adverse effects are lethal portal vein thrombosis, haemorrhages, liver abscesses, pleural effusion and pneumothorax [166,167]. Another major concern when using RFA is tumour seeding along the track of the needles used to deliver the current, a highly undesirable consequence [168,169].

4.3. Cryoablation

Cryoablation uses extreme cold to cause tumour destruction. Freeze-thaw cycles cause cellular injury by disrupting cellular metabolism, and cell dehydration leads to protein damage and subsequent disruption of the cell membranes. Intracellular ice crystal formation causes further mechanical damage to organelles and cell membranes, leading to necrotic cell death [170,171]. The ability of cryoablation to induce an anti-tumour specific immune response was first postulated in the 1960s, with the thinking being that cryoablation caused the release of TAAs [172,173]. More recent research has provided a clearer insight into its immune modulating abilities. Increased numbers of PMN leukocytes and macrophages are recruited to the tumour microenvironment following cryoablation [174]. In a murine breast cancer model, cryoablation was shown to protect mice from tumour re-challenge following treatment of the primary tumour, indicating the generation of anti-tumour

specific immune response [175]. A more in-depth look into this mechanism showed increased NK cell activity and increased production of IL-12 and IFN- γ after treatment. Another study using radioactively labelled TAAs showed increased uptake of the antigens by DCs in the lymph nodes following cryoablation, indicating that cryoablation facilitates improved antigen presentation and T cell priming [176]. The exact mechanism by which cryoablation induces a favourable immune response may be explained by the results of a recent study that showed that cryoablation causes the extracellular release of HMGB 1 and nucleotides from dying cells [177]. As we know HMGB 1 has the ability to bind with RAGE and TLRs on APCs, preventing the degradation of TAAs by lysosomes and increasing TAA presentation via MHC-I and MHC-II molecules. Depending on the site of treatment, the drawbacks of cryoablation include incontinence and erectile dysfunction (prostate) [178], pleural effusion and pneumothorax (lung) [179].

4.4. Chemotherapy & electrochemotherapy

A number of commonly used chemotherapeutic agents have been shown to cause ICD. Platinum based drugs, such as oxaliplatin, and various anthracyclines (doxorubicin) have been shown to induce the release of many DAMPs, including; ecto-CRT, HSPs, ATP and HMGB1 [93,113,145,146,180,181]. These DAMPs, as previously discussed are critical to inducing ICD. In fact the anti-cancer abilities of oxaliplatin and anthracyclines are better in immune-competent mice, than in immune-deficient athymic mice, highlighting the importance the immune system plays in their mechanisms [93,112,113]. As well as interfering with DNA replication, cyclophosphamide can also cause dendritic cell activation through the release of HMGB1 and ecto-CRT, which has knock-on effects in terms of pro-inflammatory cytokine production and T cell proliferation [182,183].

Mitoxantrone (MTX) is a type-II topoisomerase inhibitor that is commonly used to treat breast cancer, leukaemia and prostate cancer [184–186]. By disrupting DNA-replication proteins, MTX can indirectly cause ER stress [93]. ER stress is crucial for the activation of danger signalling pathways that help to traffic DAMPs to the extracellular space [187]. Amongst the DAMPs released by MTX treatment are ecto-CRT, ecto-HSP70, ATP and HMGB-1 [113,145,146].

Bortezomib, a proteasome inhibitor, can cause an immunological response following treatment by activating DCs and subsequently inducing an anti-tumour specific T cell response [188]. This anti-tumour response was shown to be dependent on contact between HSP-90 exposed on the surface of dying cells and DCs [188]. The ability of bortezomib to cause ICD and DAMP release is thought to be related to its secondary ability to generate reactive oxygen species (ROS), causing potent cellular stress [189–191].

These capabilities become even more valuable when adding the potential of electrochemotherapy (ECT) to the picture. ECT involves the application of an electric field to tumour tissue, resulting in temporary formation of pores in the cell membrane (electroporation or EP) with subsequent administration of a chemotherapeutic agent [192,193]. The cell membrane generally acts as a physical barrier and prevents the entry of hydrophilic drugs, molecules and peptides into the cell; the ability to render this barrier 'permeable' allows for the influx of otherwise non-permeable or poorly permeable anti-cancer molecules into the cytosol of cancer cells. This effectively enhances the cytotoxicity of the drug used, with platinum based chemotherapeutics (cisplatin) exhibiting a 70 fold increase in efficacy when used as part of electrochemotherapy treatment [194]. Given the ability of platinum based drugs to induce ICD through the release of DAMPs, this increase in cytotoxicity is very desirable. Another compound frequently used as part of ECT, bleomycin, has displayed the ability to induce ICD in vitro. Bleomycin is a glycopeptide that causes DNA strand breaks, inhibits replication and generates ROS in the process, resulting in oxidative cellular stress [195,196]. Bleomycin as a stand-alone agent has caused ICD in a murine model [197], however when combined with EP, as part of an ECT regime, the activity of

bleomycin is increased 700 fold [198]. ECT with bleomycin causes the release of HMGB1 from dying cells [199], and interestingly, the same study showed that application of electric pulses alone was enough to cause ATP release and extracellular CRT expression on treated cells [199]. Therefore ECT can be thought of as a tool to enhance the effect of other ICD inducers, facilitating their access into tumour cells in much higher concentrations. Unlike other ablative methods, ECT is non-destructive to healthy tissues surrounding treated tumour sites and because it requires much lower doses of chemotherapy drugs to achieve greater results it has an incredibly low side effect profile, making it a very attractive method of causing ICD [200].

4.5. Photodynamic therapy (PDT)

PDT is an anticancer therapeutic method that uses the photo-sensitive drug (eg. hypericin), which localizes in the ER [201]. When activated by light of a suitable wavelength, it causes massive production of ROS at the ER, resulting in ER membrane damage and BAX- and BAK-based mitochondrial apoptosis [202–204]. Because of its ability to directly damage the ER through ROS production, PDT is classed as a type II ICD inducer. Other agents such as oxaliplatin, cyclophosphamide, mitoxantrone and ionizing radiation are therefore type I ICD inducers as the resulting ER stress is secondary to their primary mechanisms of action [88,99]. Type II ICD inducers are thought to be more favourable due to the primary ROS damage caused, making it harder for tumour cells to initiate 'protective' cellular mechanisms as may be the case with type I inducers. PDT causes the release of a number of DAMPs as the stages of cell death progress; pre-apoptotic ecto-CRT; pre-apoptotic ATP; pre-apoptotic ecto-HSP70; and late apoptotic passively released HSP70, HSP90 and CRT [99,203,205,206]. In a clinical setting, PDT was shown to increase the immune recognition of TAAs and promote a better immunological response than surgery [207]. In addition PDT lysates can cause DC maturation, IL-12 production and the activation of macrophages [208,209]. While PDT may seem to be the ICD inducer of choice it has one glaring drawback; its application is limited to easily accessible malignancies on the skin.

5. Immune modulation & biological intervention

While ablative technologies are certainly effective at causing ICD and the generation of anti-tumour specific immune response, they are often not enough to cause total cancer elimination on their own, especially in cases of metastatic disease. To potentiate their effects, the combination of an immune modulating agent with an ablative modality, is the logical step forward. A summary of current and ongoing clinical trials combining ablative methods with immune modulators can be found in Table 1. Broadly speaking immune modulation can be split into 2 categories; immune blockade and immune stimulation.

5.1. Immune blockade

The immune inhibitory receptors that have generated the most interest in cancer therapy are CTLA-4 and PD-1, and the development of checkpoint blocking antibodies has been largely focused on these two receptors.

5.1.1. Cytotoxic T-Lymphocyte Antigen 4 blockade

Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) binds to two ligands, CD80 (B7-1) and CD86 (B7-2), which it shares with the powerful co-stimulatory molecule, CD28. Binding of CD28 causes increased production of IL-2 and other pro-inflammatory molecules, leading to increased T cell proliferation [210,211] and prolonging T cell activity [212]. Conversely, CTLA-4 binding decreases the production of IL-2 [213], increases the production of immunosuppressive cytokines such as TGF- β [214] and causes T cell anergy by disrupting cell cycle progression [213,215]. Given that CTLA-4 has much greater affinity for the CD80/CD86 receptors than CD28 it effectively out-competes with it for ligand

Table 1

Table of all current clinical trials combining immune modulators and ablative modalities.

Biological agent	Ablative intervention	Chemical agent	Cancer type	Study size	Outcome	Ref.
Ipilimumab	–	–	Metastatic melanoma	Phase III – 167 patients	10.1 month median overall survival	[219]
Ipilimumab	–	Carboplatin, Dacarbazine	Small Cell Lung Cancer (SCLC)	Phase II – 130 patients	irPFS of 6.2 month and median overall survival of 12.9 months	[220]
Ipilimumab	–	W/wo dacarbazine	Advanced melanoma	Phase II – 72 patients	14.3 and 11.4 month median overall survival respectively	[221]
Ipilimumab	–	Dicarbazine	Metastatic melanoma	Phase III – 251 patients	11.2 month median overall survival	[222]
Ipilimumab	Radiotherapy	–	Prostate Cancer	Phase I/II – 33 patients	PSA decline $\geq$ 50% and 21% positive response	[223]
Ipilimumab	Radiotherapy	–	Prostate cancer	Phase II – 45 patients	PSA decline $\geq$ 50% and median positive response of 23 weeks	[224,225]
Ipilimumab	Cryoablation	–	Breast cancer	Pilot – 18 patients	Study ongoing	[228]
Ipilimumab	ECT	Bleomycin	Advanced melanoma	Pilot – 15 patients	67% local regression, 47% distal regression and median survival of 12.4 months	[229]
Nivolumab	–	–	NSCLC, melanoma, renal cell carcinoma	Phase I – 296 patients	NSCLC: 18% Melanoma: 28% Renal cell carcinoma: 27% Positive durable response rate lasting over 12 months	[244]
Nivolumab	–	–	Advanced melanoma	Phase I – 107 patients	31% response rate and improved median survival of 17 months	[245]
CT-011	–	Gemcitabine	Pancreatic cancer	Phase II – 39 patients	Study ongoing	NCT01313416
CP-870,893	–	–	Advanced solid malignancies	Phase I – 29 patients	14% overall positive response; 27% positive response in melanoma patient sub-group	[277]
CP-870,893	–	Carboplatin and Paclitaxel	Advanced solid malignancies	Phase I – 32 patients	20% positive partial responses observed, with another 40% displaying stable disease	[282]
SGN-40	–	–	Non-Hodgkin's lymphoma	Phase I – 35 patients	37.5% positive objective response rate and overall survival monitoring still ongoing	[280]

binding [216]. CTLA-4 is not expressed on naïve CD4+ or CD8+ T cells but is hugely upregulated on their cell surfaces following TCR activation [217]; this can be beneficial in preventing runaway immune responses like those associated with autoimmune diseases [218] but is undesirable in terms of tumour elimination.

In the clinic, anti-CTLA-4 monoclonal antibodies (mAb) such as Ipilimumab are showing promise as viable biological interventions. In a large trial in patients with previously treated advanced metastatic melanoma, treatment with Ipilimumab alone led to increased survival in patients (10.1 months compared to untreated) [219]. Ipilimumab has also been paired with traditional chemotherapy and results from numerous studies have shown that Ipilimumab plus chemotherapy is more effective than chemotherapy alone [220–222]. When combined with radiation therapy, Ipilimumab also improves patient survival when compared to Ipilimumab or radiation therapy alone [223–225] and there are a number of ongoing larger scale clinical trials currently ongoing using this examination. Cryoablation plus Ipilimumab has shown promise in murine models by inducing potent anti-tumour immunity [226,227] and there is an ongoing pilot study in human patients [228]. Other ablative technologies that display improved efficacy when combined with Ipilimumab include ECT [229] and there is an ongoing trial in the Netherlands examining the benefit of RFA plus Ipilimumab treatment (NTR3488). One drawback to the use of anti-CTLA-4 antibodies is that a number of studies have reported quite severe, dose dependent, autoimmune side effects in small percentages of patients, including; high grade enterocolitis [230], hypophysitis [231], and hepatitis [232].

5.1.2. Programme Cell Death Protein 1 blockade

Programme Cell Death Protein 1 (PD-1) is another important immune inhibitory checkpoint receptor that is expressed on CD4+/CD8+ T cells, natural killer T cells, B cells and activated monocytes [233,234]. It is part of the same super family as CD28/CTLA-4 and shares structural homology with both. As with CTLA-4 it has two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC) [235,236]. PD-L1 is expressed on most cells and is greatly upregulated in many cancer types [65,237–239], suggesting its role in the 'escape' of immune surveillance. PD-L2 is only known to be expressed on activated macrophages, DCs, B cells and mast cells [240–242]. Upon

binding to either of its ligands, PD-1 can inhibit T cell proliferation in the periphery and promote T cell anergy. While the exact mechanism behind this action is not fully understood, some studies suggest that it is related to inhibition of pro-inflammatory cytokines such as IFN- γ [236, 243].

Nivolumab is a mAb developed to target the PD-1 receptor and has shown exciting results in the clinical setting. Its efficacy was evaluated in a large study; 296 patients with various cancer types, with a positive response rate of 20–25% across all cancer types [244]. Out of this group, a cohort of 107 patients with advanced melanoma was chosen for further follow-up. An objective response rate of 31% was observed for this group, with an overall median improved survival of 17 months [245]. To date anti-PD-1 mAb therapy has only been combined with one ablative technology, radiotherapy. A murine study using an intracranial glioma model showed that mice receiving a combination of anti-PD-1 blockade and localized radiotherapy had a median survival more than double mice receiving either treatment alone [246]. An ongoing phase 2 study looking at the potential of a chemotherapy/anti-PD-1 blockade combination using gemcitabine as the chemical intervention is currently ongoing (NCT01313416) and suggests that anti-PD-1 therapy should be combined with ECT in the future.

5.2. Immune stimulation

5.2.1. OX40 agonists

OX40 (CD137) is a member of the tumour necrosis factor receptor (TNFR) family [247] and acts as a powerful co-stimulatory molecule for CD4+ and CD8+ T cells [247,248]. Its ligand (OX40-L) is expressed on a wide range of immune cells, such as activated B cells, dendritic cells, and macrophages [249,250]. Ligation of OX40 has shown to be critical for effective T cell activation and proliferation [251] and is especially important in the generation of CD4+ memory T cell populations [252,253]. Anti-OX40 agonist mAbs have been used in a number of animal models and displayed the ability to cause complete tumour regression by inducing anti-tumour immune responses [254–256]. Anti-OX40 agonists have been used in combination with the chemotherapeutic agent cyclophosphamide in a melanoma mouse model. Neither treatment alone provided much protection against tumour growth but

significantly improved the survival of animals that received the combination. This result was shown to be achieved through the generation of potent anti-tumour immune response [257]. In this respect anti-OX40 agonists should be used in conjunction with ECT in the future. When used in combination with radiation therapy, anti-OX40 agonists significantly boost tumour free survival in a CD8 + T cell dependant manner [258,259]. Anti-OX40 agonists have not been used in human trials as of yet but could represent the future of anti-cancer immune stimulation.

5.2.2. CD40 agonists

Another member of the TNFR family that is being looked at for its ability to overcome and reverse the immunosuppressive environment generated by tumours is the CD40 receptor. CD40 is a transmembrane protein that is expressed on a number of immune cells, including; B cells, monocytes and APCs (DCs and macrophages) [260–263]. Its ligand, CD40-L (CD154), is primarily expressed on activated T cells [264]. CD40/CD40-L binding on the surface of APCs plays a critical role in generating a tumour specific immune response, upregulating the expression of co-stimulatory molecules CD80 and CD86 on APCs [261, 265]. It causes the increased production of the T-cell stimulatory cytokine IL-12 [266], promoting the maturation of naïve T cells into CD4 + T cells [267], which in turn drives the secretion of IFN- γ and TNF- α [268,269]. Interestingly, CD40 is also expressed in all B cell malignancies as well as on the surface of many solid tumour types and exogenous activation leads to the inhibition of tumour growth by activating apoptotic pathways and improving the cytotoxicity of CD8 + T cells [270–274].

Given this potential, a lot of effort is being put into developing anti-CD40 agonistic mAbs. CP-870,893, a fully human anti-CD40 mAb, has shown single agent activity in a wide range of tumour types, including colorectal, melanoma and leukaemia [275–277]. CP-870,893 anti-tumour activity was shown to include both non-immune and immune mediated pathways; more detailed analysis revealed that the immune mechanism involved the rapid expansion of B cell populations and subsequent up-regulation of co-stimulatory molecule CD86 on their surface [277]. SGN-40, a humanized anti-CD40 mAb, has exhibited efficacy in inhibiting tumour growth in preclinical myeloma and lymphoma models [278,279]. A phase 1 trial, using SGN-40 in the treatment of non-Hodgkin's lymphoma, showed positive objective response in 15% of patients [280].

CP-870,893 in combination with radiation therapy has been used in murine lymphoma models. Radiation or antibody only was ineffective but together they caused significantly increased survival, with this result being accredited to the generation of CD8 + T cell response, that was not observed in non-tumour bearing mouse receiving the same treatment [281]. CP-870,893 has been paired with a chemotherapy regimen (carboplatin and paclitaxel) in a phase 1 study; 20% of patients receiving the combination had partial responses and a further 40% had stable disease following treatment. These results correlated with an increase in serum IL-6 and TNF- α levels [282]. With this in mind, anti-CD40 agonists seem synergistic with chemotherapy, possibly due to the chemo-induced release of DAMPs into the tumour environment. Combining with ECT could potentiate this efficacy even further.

6. Conclusion

Significant strides have been made in recent years in understanding the intricate relationship between the immune system and cancer. We now have a better picture of the mechanisms used by the immune system to detect and eliminate cancer, as well as the ways in which cancer cells can evade and manipulate it. The emergence of ICD and DAMPs has opened our eyes to the fact that causing cancer cell death is simply not enough; the type of cell death and the method by which it is achieved are crucial if we are to get the best responses. Conventional cancer therapies tend to focus on either causing maximal cancer cell death or modulating the immunological response of the body to the cancer. Going forward, these two strategies must be married together; developing

treatment regimens that maximise cancer cell death in an immunogenic manner and harnessing the formidable power of the immune system will lead to powerful results. Initial data from current clinical trials, combining these two modalities, is very positive and shows that we are already on the right path. Emerging ablative technologies such as ECT show great promise given its low side effect profile and its ability to generate ICD; further work combining ECT and immune modulators could yield very positive outcomes in the clinic.

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