Cancer immunotherapy using tumor cryoablation

Cryoablation is increasingly being used as a primary treatment for localized cancers and as a salvage therapy for metastatic cancers. Anecdotal clinical reports and animal experiments have confirmed an induction of systemic antitumor immune response by tumor cryoablation. To capitalize on the stimulatory effects of cryoablation for cancer immunotherapy, this response must be intensified using other immunomodulatory agents. This article reviews the preclinical and clinical evidence and discusses the mechanism of the antitumor immune response generated by cryoablation. The rationale and evidence behind several immunotherapy approaches that can be combined with cryoablation to devise a cryoimmunotherapeutic strategy with a potential to impact the progression of metastatic disease are described.

KEYWORDS: antitumor immunity ▪ cancer ▪ cancer vaccines ▪ cryoimmunology ▪ cryosurgery ▪ dendritic cells ▪ immune checkpoint ▪ immunomodulators ▪ immunotherapy ▪ Tregs

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Cryoablation, or tissue destruction by extreme cold temperatures, is a widely used treatment modality for both benign and malignant conditions. While cold temperature was used for the management of wounds, fractures and swellings for hundreds of years [1], its role in the treatment of malignancies was first demonstrated only in mid-1800s by Arnott, who used iced saline solutions to treat advanced breast and uterine cancers [2]. For approximately 100 years, liquid air, solid carbon dioxide and liquid oxygen were used as cryogenic agents for superficial application on diseased sites [3,4]. The field of cryosurgery received a huge impetus when Cooper and Lee devised a cryosurgical unit in 1961 capable of delivering liquid nitrogen (-196°) to trocar type probes making deep tissue freezing feasible [5,6]. Gonder *et al.* modified Cooper's unit and used it for ablation of prostates first in dogs and then humans [7,8]. For the next two decades, cryosurgery made slow progress with improvement in designing of liquid nitrogen probes and heating devices [9,10]. In the 1980s, Korpan performed experimental studies to understand the mechanism behind cell damage caused by freezing. His experiments and clinical studies involving cryoablation provided the framework on which the clinical and technical requirements for modern cryosurgery have been formulated [11,12]. His pioneering effort on the use of cryosurgery in the field of oncology involved work on pancreatic cancer, lymph node surgery, liver metastases and advanced breast cancer [1,11].

Advances in the cryoablation technique and equipment, as well as development and refinement of intraoperative ultrasound, have provided the basis for wider application of cryosurgery in the treatment of visceral cancers. In the last two decades, cryoablation has become a popular treatment modality for various cancers with the potential advantages of minimal invasiveness, less damage to surrounding healthy tissue, better patient comfort and reduced cost compared with surgical extirpation. Currently cryosurgery has been used extensively in the management of skin, breast, prostate, renal, lung, liver, bone and soft tissues malignancies [11,13–21].

Mechanism of cell death with cryoablation

Cryoablation turns the tumor into an iceball and induces cell death by damaging the cell membranes and organelles and by causing vascular compromise through thrombosis of small vessels [22–27]. In proximity to the cryoprobe, rapid cooling converts the intracellular fluid into ice crystals that damages cell membranes and organelles through mechanical effects. At the periphery, with slower freezing rates, extracellular fluid is converted into ice, which increases the osmolarity of the remaining fluid. This causes a fluid shift from intracellular to extracellular compartments resulting in cellular dehydration and protein damage, further injuring the membranes and disrupting the enzymatic machinery of the cell.

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Freezing also damages the tumor microvasculature by direct cell damage to vascular endothelial cells and by vessel distention caused by perivascular cellular dehydration. This leads to increased permeability, edema and a coagulation cascade that leads to microthrombi formation in the vessels resulting in tissue ischemia. Reperfusion injury is also proposed to play a role in the cell damage.

Histologically, the aforementioned mechanisms culminate in coagulative necrosis of the tumor. However, at the periphery of the lesion where temperatures are not cold enough, the cells only show signs of apoptosis [27]. Sindelar *et al.* studied the effects of cryosurgery in rat kidney and reported cellular proteins denatured and membranes disrupted within an hour of cryosurgery [28]. At 24 h postcryosurgery, tissue underwent complete coagulative necrosis along with a mild-to-moderate inflammatory response with lymphocytes, plasma cells and macrophages. Thus, tissue freezing resulted in formation of inflammatory debris.

Evidence for cryoimmune response

While cryosurgery was used for local treatment of various conditions for centuries, its effect on immune response was not recognized until the late 1960s. Shulman *et al.* first demonstrated the production of tissue and species-specific antibodies against rabbit male reproductive tissues after freezing [29,30]. Around the same time Blackwood *et al.* carried out palliative cryotherapy in 13 patients with advanced or metastatic cancers and found elevated antibodies postfreezing, but did not observe any clinical response [31]. Later, the same group reported a decrease in circulating tumor-specific antibodies in two patients who showed some clinical response postcryotherapy, which increased to pretreatment level after the recurrence [32]. Ablin *et al.* observed a decrease in metastatic burden in prostate cancer patients whose primary tumors were treated with cryoablation and coined the term 'cryoimmunotherapy' [33–35]. They found enhancement of this immune response after repeated cryoablative treatments. This response was speculated to be due to multiple inoculations with the same antigen. Gursel *et al.* demonstrated palliation in bone pain in patients with metastatic prostate cancer. Patients who had decreased pain after cryosurgery also demonstrated an increase in IgM antibodies [36]. These anecdotal reports of regression of metastases in patients where primary tumors were treated by cryosurgery further stirred interest in the immunological aspects of cryoablation. This led to a host of studies in animal models to scientifically establish the cryoimmune response and to understand the underlying mechanism.

Table 1 summarizes some key studies in animal models demonstrating immune-potentiating effects of tumor cryoablation alone. Blackwood *et al.* demonstrated that there is a threshold to antigenic stimulant that can invoke antitumor immunity. They found that regression of tumors was faster when only a small amount of tumor tissue was left in the animals but slower or nonexistent if a bulk of tissue was left [37]. Bagley *et al.*, in 1974, were the first group to demonstrate that cell-mediated immunity is responsible for antitumor effects of cryosurgery [38,39]. Matsumura *et al.* used MRMT-1 in Spague–Dawley rats and demonstrated the initial fall in the antitumor immunity postcryosurgery that gradually increased reaching a peak at approximately 10 weeks postcryosurgery. The initial dip was considered to be due to the activation of suppressor cells via either cryosurgical stress or slow absorption of antigens [40,41].

In the last decade, studies by Sabel *et al.* and den Brok *et al.* have added more evidence to existence of the cryoimmune phenomenon and have further explored the mechanism behind the response. den Brok *et al.* used a melanoma model in mice and demonstrated increased protection from tumor rechallenge in animals treated with tumor cryoablation [42]. They demonstrated increased amounts of tumor antigen-loaded and mature dendritic cells (DCs) in the tumor-draining lymph nodes postcryoablative treatment. Sabel *et al.* examined the response triggered by cryosurgery in breast cancer cells in mice. They found higher levels of Th1 cytokines, IL-2 and IFN-γ, and higher tumor rejection rates in animals treated by cryoablation [43]. In humans, cryoablation of high-risk prostate cancer was shown to increase serum levels of TNF-α and IFN-γ 4 weeks post-tumor ablation [44]. Tumor-specific cytotoxic T lymphocyte (CTL) response and an increased Th1:Th2 ratio was also seen.

While most of the studies demonstrate some degree of antitumor immune response postcryosurgery, a few studies failed to show any response. Studies by Allen *et al.* using hepatoma tumors in Buffalo rats and Hoffmann *et al.* in the Dunning AT-1 prostate cancer model did not demonstrate significant antitumor immune response after cryosurgical ablation [45,46]. Similarly, Zonnevyllee and Zwaveling demonstrated no extra beneficial effects of cryosurgery on metastatic process when compared with surgery [47].

Table 1. Key studies in animal models demonstrating cryoimmune response.

Study (year)	Tumor model	Findings	Ref.
Blackwood *et al.* (1972)	Myosarcoma MT449A and carcinosarcoma Walker 256 in rats	Cryosurgery induced regression of untreated tumor and protected against sc. and ip. tumor rechallenge Immune response attributed to antibodies	[37]
Neel *et al.* (1973)	Mammary adenocarcinoma in C3H/HeN mice and sarcoma in CDF mice	Increased resistance to tumor rechallenge in mice treated with cryosurgery Immune response attributed to antibodies	[82]
Bagley *et al.* (1974)	Fibrosarcoma MCA-10 in C57BL6 mice	Lymphocytes in cryosurgery group demonstrated tumor-specific cytotoxicity	[38]
Faraci *et al.* (1975)	Fibrosarcoma MCA-10 in C57BL6 mice	Both lymphocytes and serum in cryosurgery group demonstrated tumor-specific cytotoxicity Lymphocyte cytotoxicity disappeared in 21 days but the serum cytotoxicity persisted	[39]
Misao *et al.* (1981)	Breast MRMT-1 in Sprague–Dawley rats	Increased resistance to tumor rechallenge in cryosurgery group Fall in the antitumor immunity initially after cryosurgery but increased gradually reaching a peak by 10 weeks	[41]
Muller *et al.* (1985)	Dunn sarcoma in C3H mice	Decreased lung metastasis in cryosurgery group No difference in NK cell activity or resistance to tumor rechallenge compared with excision	[83]
Joosten *et al.* (2001)	Colon-26 and melanoma MV3 in Balb/c mice	Colon-26 model: suppression of contralateral tumor in Cryo group MV3 model: decreased lung metastasis in Cryo group. Increased IL-1α and TNF-α in Cryo group	[84]
Urano *et al.* (2003)	Colon-26 in Balb/c mice	Decrease in liver metastasis after cryoblation of single lesion. Increase in liver metastasis after ablation of three lesions	[85]
Sabel *et al.* (2005, 2006)	Mammary adenocarcinoma MT-901 in BALB/c mice	Higher levels of IL-12 and IFN-γ, NK cell activity and tumor rejection rates in animals treated by Cryo Increased tumor-specific T-cell activity	[43,86]
Den Brok *et al.* (2006)	Melanoma B16-OVA in Balb/c mice	Increased resistance to tumor rechallenge in Cryo group. Increase in amount of antigen-loaded and mature dendritic cell in tumor-draining lymph nodes	[42]
Li *et al.* (2010)	Glioma C6 in Wistar rats	Increase in $CD3^+$ and $CD4^+$ cells after Cryo, increase in $CD4^+$:$CD8^+$ ratio	[87]

Cryo: Cryoablation; ip.: Intraperitoneal; sc.: Subcutaneous.

On the contrary, some groups found tumor cryoablation to have suppressive effects on antitumor immunity. Yamashita *et al.* and Hayakawa *et al.* demonstrated that cryosurgery of 3-methylcholanthrene-induced tumors in WKA/Hok rats resulted in an increased number of deaths due to metastasis compared with surgical excision. The cryosurgically treated mice also had reduced resistance to tumor rechallenge [48,49]. Wing *et al.* used a fibrosarcoma model in hamsters and demonstrated the generation of suppressor cells postcryosurgery that were shown to inhibit T-cell proliferation. Generation of suppressor cells was attributed to inferior prognosis of rats treated by cryosurgery, compared with surgical excision [50,51]. Friedman *et al.* examined the effects of intraprostatic injection of complete Freund's adjuvant and cryosurgery on normal ventral prostate in Copenhagen rats. Cryosurgery in combination with complete Freund's adjuvant injection increased the susceptibility of rats to tumor challenge by Dunning R3327 prostate adenocarcinoma [52].

Mechanism behind cryoimmune response

The danger model of immune response states that the immune system has evolved not to discriminate self from nonself but to detect and respond to situations of tissue distress or abnormal cell death [53]. The key prediction of the danger model is that cells dying pathologically (e.g., by necrosis) liberate 'danger signals', which result ultimately in the activation of a destructive T-cell response against the dangerous entity, exogenous or endogenous. Cryoablation of tumor tissue leads to the coagulative necrosis of cells. Upon thawing, necrotic tumor cells within the iceball release intact tumor antigens, and 'danger signals', such as proinflammatory cytokines, nuclear proteins and HMGB1, a molecule that promotes antitumor immunity through interactions with Toll-like receptors (TLRs). These 'danger signals' serve as a stimulus for the innate immune response and attract granulocytes, macrophages and NK cells. These cells further release

cytokines and chemokines. DCs, the professional APCs, then reach the damaged tissue and take up tumor antigens in a background of inflammation and abundant cytokines [42]. The activated and mature DCs display tumor antigens in context of MHC molecules, migrate to the nearest lymph nodes and present the antigens to T cells leading to their activation. When the acquired immune system becomes activated, $CD8^+$ T cells (cytotoxic T cells) affect the lysis of tumor cells, while $CD4^+$ T cells (helper T cells) help them in the process. However, cryoablation does not always lead to immune stimulation and there are multiple explanations for this. First, cells at the periphery of the iceball die through apoptosis. Recognition and phagocytosis of apoptotic cells prevents release of intracellular contents, inhibiting proinflammatory cytokine release. Antigens are presented in absence of danger signals leading to immune tolerance. The ratio of necrosis to apoptosis might play a critical role in determining the stimulating or suppressive nature of the immune response to cryoablation [27]. The rate of freezing could also play a role in determining immune-stimulating versus immunosuppressive effects of cryoablation [54]. While high-rate freezing causes necrosis and activates the immune response, low-rate freezing leads to an increase in Tregs and a decreased necrosis:apoptosis ratio resulting in immune suppression. In addition, when a large amount of tumor is frozen, large quantities of immune complexes generated may cause 'high zone tolerance', a phenomenon where antigen overloading may lead to immunosuppression.

By contrast to cryoablation, other thermal ablation modalities such as radiofrequency ablation and high-intensity focused ultrasound are used clinically to kill tumor cells by high temperatures. These modalities, too, have demonstrated immune-stimulating effect by providing tumor antigens in inflammatory medium. In contrast to freezing of tissue, which preserves the tertiary structure of proteins, burning tissue induces protein denaturation, which may compromise immunogenicity and limit antitumor response.

Cancer immunotherapy using tumor cryoablation

There is tremendous interest in cryosurgery and cryoimmunology for the management of malignancies, as it has the potential to control the disease both locally and systemically. While a plethora of studies in animals and humans provide evidence of immune response generated by cryoablating tumor, the response has not been shown to be consistently immune-stimulating and strong enough to control established systemic disease. Advanced or metastatic cancer is associated with several mechanisms inhibiting antitumor immunity [55]. One mechanism by which tumors inhibit immune surveillance occurs through the recruitment of suppressive cells. Tregs are a subset of CD4 cells expressing the IL-2 receptor α-chain (CD25) and Foxp3, transcription factor. Tregs are actively recruited and induced by tumors to block the priming and/or effector function of antitumor T cells. They have a potent ability to suppress immunity by inhibiting both cytotoxic T lymphocytes and NK cells, and are thought to play a role in tolerance to tumor and self-antigens [56]. Myeloid suppressor cells that accumulate in tumors are bone marrow-derived immature DCs that suppress the antitumor immune response by interfering with antigen presentation and blocking T-cell functions through production of arginase I and activation of inducible nitric oxide synthase [55]. Other mechanisms by which tumors evade immune surveillance, is by decreasing expression of costimulatory molecules on tumor cells and APC so that antigens are presented by tolerogenic APCs resulting in failure to stimulate the immune response. Dysfunction of DCs in the tumor microenvironment, alteration in T-cell receptor signaling and expression of inhibitory coreceptors (e.g., B7-H1), further hamper generation of an antitumor immune response [57]. Against these headwinds, the effect of cryoimmune response on metatstatic cancer will be modest. To capitalize on the immune potentiating effects of cryoablation in patients, this response has to be intensified by using other immunomodulatory agents to counter inhibitory influences of metastatic cancer. Tanaka, while reporting his experiments with cryoimmune response in animals, first suggested the necessity of augmenting this response for achieving a reliable clinical response [58]. Multiple animal studies have demonstrated augmented response using a combination of cryoablation with other immunomodulators (Table 2). Since cryoablation is already being extensively used in the management of various advanced cancers, intensification of the cryoimmune response will give us an immunotherapeutic strategy against advanced and metastatic cancers. One or more of the following immunomodulatory approaches can be combined with tumor cryoablation to strengthen the cryoimmune response.

Increasing antigen uptake & presentation by APCs

DCs are professional APCs and are crucially important in the capture, processing and

Table 2. Studies demonstrating augmented immune response using combination of cryoablation with other immunomodulators.

Study (year)	Tumor model	Findings	Ref.
Lubaroff *et al.* (1981)	Prostate Ca Dunning R3327 in Copenhagen rats	Cryo + BCG, but not Cryo alone was protective against tumor rechallenge	[64]
Tanaka *et al.* (1982)	Sarcoma 180 in ICR mice Melanoma B16 in BDF_1 mice	Augmentation of cryoimmune response with EA6 (protein polysaccharide derived from a mushroom), levamisole and BCG providing protection against tumor rechallenge	[58]
Machlenkin *et al.* (2005)	Lewis lung tumor D122 and melanoma B16-MO5 in C57/Bl6 mice	D122: Cryo alone had no immune-stimulating effects, Cryo + *in situ* immature DCs induced a significant CTL response, decreased lung metastases and increased survival B16-MO5: Cryo + *in situ* DC increased proliferation of tumor-specific CD8 cells and resistance to rechallenge	[72]
Udagawa *et al.* (2006)	Colon CT26 in Balb/c mice	Cryo + intratumoral DCs cocultured with BCG (TLR2/4 agonist) induced greater suppression of untreated tumor compared with either modality alone Combination induced greater tumor-specific CTL response	[73]
den Brok *et al.* (2006)	Melanoma B16/OVA in C57BL/6n mice	Cryo + peritumoral CpG (TLR9 agonist) provided superior protection against tumor rechallenge, increased regression of contralateral tumors, increased DC maturation and antigen crosspresentation	[67]
Redondo *et al.* (2007)	Melanoma B16/OVA in C57BL6/J mice	Cryo + topical imiquimod (TLR7 agonist) provided superior protection against tumor rechallenge, increased *in vitro* IFN-γ production and T-cell proliferation	[68]
Levy *et al.* (2009)	Colon CT-26 in Balb/c mice	Cryo + Cy cured 50% of mice with metastatic disease and provided resistance to tumor rechallenge. Combination induced tumor-specific CTL response and lowered the regulatory to effector T cell ratio	[81]
Zou *et al.* (2010)	Colon CT26 in Balb/c mice	Cryo + CpG ODN provided superior protection against tumor rechallenge, higher serum IL-12 and IFN-γ	[88]
Waitz *et al.* (2011)	Prostate Tramp C2 in C57BL/6 mice	Cryo + anti-CTLA-4 Ab led to slower growth or rejection of secondary tumor Cryo alone had no effect	[89]
den Brok *et al.* (2012)	Melanoma B16-F10 in C57BL/6n mice	Cryo + peritumoral saponin-based adjuvants provided superior protection against tumor rechallenge than Cryo alone and Cryo + peritumoral CpG Cryo + saponin-based adjuvants increased antigen loading of DCs	[90]

Ab: Antibody; Cryo: Cryoablation; CTL: Cytotoxic T lymphocyte; Cy: Cyclophosphamide; DC: Dendritic cell; ODN: Oligodeoxynucleotide; TLR: Toll-like receptor.

presentation of tumor antigens to tumor-specific T cells [59]. Activating DCs can potentially augment a weak immune response. Cytokines like GM-CSF promotes DC activation and migration [60] and forms the basis of cell-based vaccines using tumor cells transduced with GM-CSF. Irradiated tumor cells transduced with GM-CSF are injected into the patients, where the cells undergo death, and in the presence of GM-CSF tumor antigens are more efficiently taken up by the DCs [61,62]. Adding cytokines such as GM-CSF to tumor cryoablation can serve as an *in situ* tumor vaccine, promoting DC activation and maturation. Thakur *et al.* studied a cryoimmunotherapy protocol involving cryoablation of lung metastasis along with aerolized GM-CSF in six patients with metastatic renal cell carcinoma [63]. They were able to demonstrate enhanced tumor-specific CTL response and cytokine production in responders compared with nonresponders.

Another method of DC activation is using TLR agonists in combination with cryoablation. TLRs expressed on APCs, such as DCs and macrophages, serve as main pattern recognition receptors with key roles in induction of innate immune responses, as well as subsequent development of adaptive immune responses through initiation of the DC maturation process and induction of cytokines. In addition, TLRs on T cells play a role in T-cell activation and function. Lubaroff *et al.* studied the immunologic aspects of prostate cancer using Dunning R3327 tumors in Copenhagen rats. In their experiments, cryosurgery alone was not effective in protecting animals against tumor rechallenge, but its combination with BCG (TLR2/4 agonist) enhanced antitumor immunity adequately to protect 50% of the animals against rechallenge [64]. Another method of TLR activation is intratumoral injection of unmethylated CpG that interacts with TLR9 on DCs. Multiple studies in animal models have shown efficacy of CpGs in potentiating the antitumor immune response when used alone or in combination with other immune-stimulating

agents [65,66]. den Brok *et al.* demonstrated that *in situ* destruction of tumor by cryoablation combined with DC activation by CpG can constitute an '*in situ* DC vaccine' that can augment antitumor immunity for metastasis control [67]. In their experiments, combination of cryoablation with CpG-ODN provided superior protection against tumor rechallenge through increased DC maturation and antigen cross presentation. Similar results were reported in melanoma model using imiquimod, a TLR7 agonist [68].

Antigen uptake, maturation and presentation by DCs are markedly enhanced when the antigen is coated with antibody [69]. This mechanism has been implicated in the efficacy of rituximab [70]. Using antibodies specific for tumor antigens, the cryoimmune response can be augmented against metastatic cancer. Tumor antibodies, such as humanized antiprostate-specific membrane antigen [71], in combination with tumor cryoablation, can form an immunotherapeutic strategy for control of advanced disease.

DCs activated and pulsed with tumor-associated antigens or tumor lysate-derived proteins have been the backbone of various cancer vaccines. DC-based vaccines increase tumor antigen presentation to T cells, thus activating adaptive immunity and generating cytotoxic T cells in cancer patients. Combination of DC injections along with cryoablation can serve as a potent tumor vaccine obviating the need to prime DCs *ex vivo*. Machlenkin *et al.* studied combination of intratumoral immature DC injections with cryoablation in Lewis lung carcinoma D122 and melanoma B16-MO5 models [72]. In their model, cryoablation alone failed to achieve any significant antitumor immune response. Combination modality demonstrated significant tumor-specific CTL response, diminished lung metastasis, increased survival and increased resistance to tumor rechallenge. Udagawa *et al.* used two tumor models to evaluate the antitumor response generated by combination of intratumoral DCs and cryoablation. They cocultured immature DCs with BCG cell wall skeleton to induce DC maturation through TLR2/4 stimulating [73]. Combination of tumor cryoablation and BCG treated DCs provided the greatest suppression of untreated tumor compared with cryoablation alone or in combination with immature DCs, thus demonstrating the immune-potentiating effects of activated DCs.

Increasing T-cell activation

Once APCs uptake antigens and mature, they migrate to tumor-draining lymphnodes where they present antigens to T cells. T-cell activation requires two signals. The first signal is transmitted when the unique T-cell receptor binds to the antigen presented by an APC. The second signal is delivered by interaction of costimulatory molecules on APCs with T-cell costimulatory receptor. Immune checkpoints are inhibitory signals that oppose T-cell activation and serve to keep the magnitude of immune response in limits. One such immune checkpoint is interaction of an inhibitory T-cell coreceptor CTLA-4 with B7 ligand on APCs [74]. Antibody-mediated blockade of CTLA-4 has been shown to abrogate restrictive T-cell signaling so as to facilitate the host's ability to mount an effective antitumor immune response [75,76]. Anti-CTLA-4 antibody can be used to augment the antitumor immune response generated by tumor cryoablation. Using prostate cancer model in mice, Waitz *et al.* demonstrated that CTLA-4 blockade synergizes with cryoablation of a primary tumor to prevent the growth of secondary tumors seeded by challenge at a distant site. With combination therapy, secondary tumors were highly infiltrated by T cells with a significant increase in the ratio of intratumoral T effector cells to Tregs. den Brok *et al.* had also found CTLA-4 blockade to augment the weak immune response generated by cryoablation alone [42].

Another inhibitory pathway is the interaction of PD-1 expressed on activated T cells with its ligand PD-L1 or B7H1 on APCs. PD-1 is an immunoinhibitory receptor belonging to the CD28 family. Antibodies against PD-1 or its ligand potentiates any antitumor immune response by inhibiting the inhibitory influences of PD-1–PD-L1 signaling [77]. Thus, blockade of any of the inhibitory checkpoints could potentially enhance any pre-existing antitumor immunity generated by cryoablation.

Inhibiting suppressive cells

Tregs are potent suppressors of antitumor immunity. When Treg number or function was reduced in experimental models, a surge in antitumor response was seen [78,79]. Thus, Treg depletion has the potential to strengthen the weak antitumor immune response generated by another modality. When cryoablation was combined with Treg depletion using anti-CD25 antibody, an enhanced antitumor immune response was demonstrated in mice models [42].

Cyclophosphamide, an alkylating agent used to treat cancer, has been shown to mitigate suppression of antitumor immunity through effects on Tregs. treatment with cyclophosphamide enhances the apoptosis and decreases homeostatic proliferation of these cells [80]. Thus, combining cryoablation

with cyclophosphamide could potentially increase the antitumor immune response. Our experiments studying the combination of cryoablation and cyclophosphamide in a murine metastatic model yielded 50% cure rates against established metastatic disease [81]. Combination provided complete protection against tumor rechallenge in cured animals, generated tumor-specific CTL response and suppressed Tregs.

Conclusion

Cryoablation of a tumor has the potential to stimulate antitumor immunity, however, clinically this potential has not yet been realized. Achieving enhanced DC function in combination with blockade of immune inhibitory checkpoints and inhibition of Tregs may prove the most efficacious method of augmenting antitumor immunity generated by tumor cryoablaton. This 'cryoimmunotherapy' strategy has the potential to markedly impact on the control of multiple metastatic cancers.

Future perspective

Cryoablation represents a promising component of cancer immunotherapy in treatment of advanced and metastatic cancer. Tumor cryotherapy serves as a source of intact tumor antigens in the inflammatory environment that can initiate an antitumor immune response. In coming years, a multimodal approach, using cryotherapy in combination with other immunotherapeutic agents, will serve as a potent weapon against advanced malignancies.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants ovr patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Cryosurgery is extensively used in the management of skin, breast, prostate, renal, lung, liver, and bone and soft tissue malignancies.
- Cryoablation induces cell death by damaging the cell membranes and organelles and by causing vascular compromise through thrombosis of small vessels.
- Anecdotal clinical reports of regression of metastatic lesions after tumor cryoablation stimulated interest in immune-potentiating effects of cryoablation.
- A plethora of animal experiments has confirmed an induction of systemic antitumor immune response by tumor cryoablation. Cell-mediated immunity was found to be responsible for this immune response.
- Lack of strong and consistent immune response limits utility of cryoablation alone in clinical settings for control of systemic disease.
- The cryoimmune response needs to be augmented with a combination of other immunomodulators to overcome immunological tolerance of metastatic malignancy.
- Approaches which increase antigen uptake and presentation by dendritic cells (cytokines and Toll-like receptor agonists) can strengthen a weak immune response.
- Immune checkpoint blockade with anti-CTLA-4 and anti-PD-1 antibodies can augment T-cell activation and function.
- Ultimately, removing inhibitory influences by depleting Tregs can provide further boost to antitumor immune response.
- Cryosurgery in combination with immune modulation agents can offer an immunotherapeutic strategy to control metastatic cancer.

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▪▪ **Discusses evidence for cryoimmune response and proposes mechanisms to explain it.**

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