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Review

Cryo-immunology: A review of the literature and proposed mechanisms for stimulatory versus suppressive immune responses

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ABSTRACT

The use of cryosurgery to ablate tumors is expanding, primarily due to its technical ease and minimal morbidity. A potential secondary advantage to the in situ freezing of malignant disease is the cryo-immunologic response, the generation of an anti-tumor immune response triggered by the natural absorption of the malignant tissue. While initially proposed based on clinical observations of distant disease regressing after cryoablation of a primary tumor, results from preclinical studies have been mixed and the existence of a cryo-immunologic response has been controversial. Recent studies have shed light on the potential mechanism by which cryoablation may modulate the immune system, also reveals that both immunostimulatory and immunosuppressive responses may be triggered. This article reviews the existing evidence regarding tumor cryo-immunology and puts forward hypotheses regarding patient, tumor and technical factors that may influence the resultant immune response and warrant further investigation.

Cryosurgery, the use of extreme cold temperatures to destroy diseased tissue, is increasingly being recognized as a highly efficient, minimally invasive method of treating malignant neoplasms. The idea is not a new one. In England between 1845 and 1851, Dr. James Arnott used iced saline solutions to treat advanced breast and uterine cancers. While his primary goal was anesthesia, he noted the effects the cold temperatures had on the viability of the cancer cells [7,35]. This started the use of freezing techniques as a local treatment of superficial tumors, but the attainable temperatures (between −18 and −24 °C) limited the clinical applicability. This changed in 1877 when the development of adiabatic expansion systems for cooling gases allowed for the liquification of oxygen, air and nitrogen. Liquid air could reach −190 °C and be applied locally to the skin to treat a plethora of diseases, including skin cancers. Modern cryosurgery really gained traction in the 1960s. With the development of systems capable of delivering liquid nitrogen to trocar-type probes, modern cryosurgery became feasible. These cryoprobes had an insulated shaft and a conductive metal tip, allowing for the freezing of tumors within the parenchyma and with minimal trauma to the surrounding tissue.

Over the past few decades, cryosurgery has been used to treat malignancies of the skin, prostate, liver, breast, lung and bone, and more applications are being studied. Compared with surgical extirpation, many potential advantages to cryosurgery have been promoted, particularly the minimally invasive nature of the treatment, less damage to surrounding structures, patient comfort (as freezing has an anesthetic effect), the cost of therapy, and improved cosmetic results. The clinical use of cryosurgery has revealed another potential benefit to freezing tumors and leaving them in situ for the body to absorb; the ability to stimulate an immunologic response to tumor-specific antigens in the frozen tissue. Early in the introduction of cryosurgery to clinical practice were several reports of metastatic foci regressing after ablation of a primary tumor, suggesting a potential systemic benefit to a local therapy [3,23,29,68,70–73,75]. For example, among 80 cases of prostate cancer treated with cryoablation by Ablin and colleagues, there were several cases where metastatic tumors regressed [3]. While it was not absolutely clear that the regression of the metastases was immune-mediated, at least one of the patients had anti-prostatic antibodies detected in their serum after cryosurgery, suggesting a humoral-based response [4].

Unfortunately, immunologic assays at the time of many of these observations were limited and so it was difficult to verify that the isolated cases of regression of distant disease were truly immunologic. The existence of a cryo-immunologic response remained controversial and the mechanisms by which this may occur were unknown. However, the increased interest in the clinical potential of cryosurgery, and a more detailed understanding of the mechanisms by which the immune system recognizes and targets tumor

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antigens has generated a renewed interest in the field of cryo-immunology.

Pre-clinical evidence for a cryo-immunologic response

In an example of "the bedside to the bench", these clinical reports prompted a laboratory examination of cryoablation and a possible immune response. In some of the first publications to document the cryo-immunologic response, Ablin et al. [1,2,5,47] and Shulman et al. [9,49,64,65] documented the humoral response triggered by cryoablation across a variety of models (rabbits, monkeys), documenting the presence of serum antibodies that recognized organ or tumor-specific proteins after cryoablation. These investigators were the first to suggest that cryoablation of tumors may be considered a form of immunotherapy and may be equally effective to tumor vaccines.

At the time (and still today), the most common method for documenting an immune response to a tumor was to treat the primary tumor and then, after an appropriate period of time, re-challenge the animal with a 2nd tumorigenic dose, with the absence of growth of a secondary tumor evidence of an immune response. Tanaka looked at several models, demonstrating a tumor-specific resistance to re-challenge after cryoablation of sarcoma 180 in ICR mice and Vx2 carcinoma in rabbits [71]. Neel et al. [43] used two murine models to compare the immunologic effects of cryosurgery and surgery; adenocarcinomas induced by the mammary tumor virus (MTV) in C3H/HeN mice, and sarcomas induced by 3-methylcholantrene in CDF_1 mice. Tumor-specific immunity, as measured by resistance to re-challenge, was consistently greater with cryosurgery than with surgical excision. Bagley et al. [6] compared surgery to cryosurgery using MCA-10 fibrosarcoma in C57BL/6 mice, harvesting splenic lymphocytes at weekly intervals after treatment for cytotoxicity assays. They did demonstrate that mice undergoing cryoablation had significantly higher cytotoxicity than mice undergoing surgery or untreated mice. Cytotoxicity assays against other tumor types with different antigens showed no effect, demonstrating that the heightened immunity after cryosurgery was tumor-specific.

Blackwood and Cooper also examined the response triggered by cryosurgery in both myosarcoma (MT449A) and carcinosarcoma (Walker 256) in Wistar and Sprague–Dawley rats respectively [8]. In these models, cryosurgery did result in an immune response, preventing re-challenge and causing regression of second tumors. An interesting finding was that the immunologic response was suppressed when the bulk of the frozen tumor tissue was left in the animal. However, if only a small amount of the frozen tissue was left, regression was faster and more complete. These results suggested that there was a threshold of antigenic stimulant and excess antigen might prove detrimental to the immune response. This was one of the first studies to demonstrate that the impact of cryoablation on the immune system was not always positive, but under the right circumstances, could result in immune suppression. A somewhat similar finding was reported by Urano et al. [76]. Two weeks after generating metastatic liver tumors in BALB/c mice by injecting colon-26 cells into the spleen, cryoablation was performed on one of the liver nodules. Two weeks after cryoablation, the mice were sacrificed, the primary tumor in the spleen measured, and the liver tumors enumerated. The authors found that ablation of a single nodule in the liver led to a significant reduction in the number of metastatic deposits. However, cryoablation of multiple nodules actually eradicated this effect, resulting in a greater number of lesions. Although this may have been an effect of increased surgical stress, it is also possible that the increased volume of ablated tissue led to an immunosuppressive effect.

Further evidence that cryosurgery could be either immunostimulatory or immunosuppressive comes from Misao et al. [40]. Using a metastasizing comedo-type breast adenocarcinoma (MRMT-1) in Sprague–Dawley rats to compare surgical excision to cryosurgery, mice were re-challenged after successful local therapy. Although mice treated by surgical excision had a superior rejection rate 1–3 weeks after treatment, mice treated by cryosurgery had a dramatic improvement in tumor rejection compared to surgery by week 10. At this later time point, mice treated by surgery rejected only 18% of tumors compared to 80% in mice treated by cryosurgery. Lymph node metastases were also lower in the cryosurgery treated group. Following up on this data, Maya et al. [39] looked at the immune response within the regional lymph nodes in these animals at varying timepoints. Looking at paracortical hyperplasia and germinal center hyperplasia in the nodes as reflective of T-cell and B-cell activity, both increased by 1 week after treatment and remained high until 10 weeks. Macrophage activity, as measured by sinus histiocytosis, was increased by 3 weeks and also remained high. However, while PHA-induced proliferation of T-cells in the regional lymph nodes increased with cryoablation, it decreased in the peripheral blood at first, recovering to preoperative levels by 6 weeks. Atrophy of the thymus correlated with this as well. The authors concluded that there was an early tumor suppression that took place systemically as a result of cryosurgery, although this eventually reversed, leading to a high resistance to re-challenge with time.

Several animal studies failed to demonstrate a cryo-immunologic response [31,42]. Müller et al. [42], using Dunn osteogenic sarcoma in C3H mice, showed that cryosurgery was superior to surgery in regards to metastases formation. Despite this finding, the authors could not document any differences in immune parameters between the cryosurgery and surgery groups, including NK function, T-cell cytotoxicity or antibody response. Surmising that freezing normal prostate might generate immunity to prostatic antigens shared by both normal and malignant prostate tissue, Friedman et al. [18] found that freezing the normal ventral prostate of Copenhagen rats, combined with intralesional injection of Complete Freund's Adjuvant (CFA) did not confer a protective immunity against a prostate cancer challenge using Dunning R3327 prostate adenocarcinoma. This is in contrast to Lubaroff et al., who found that cryosurgery of Dunning R3327 tumors in rats, combined with BCG, did confer long-term immunity in 50% of the mice. The primary difference between the two studies of course being that the latter group ablated tumors while the former ablated normal prostate. In another study of prostate cancer in Copenhagen rats, this time with a different strain of Dunning tumor, Hoffman et al. [28] examined the effect of cryosurgery on secondary tumor growth after re-challenge, as well as attempted to document an anti-tumor antibody. Although there were anti-tumor antibodies detectable after cryosurgery, there was no significant impact on secondary tumor growth.

Other studies documented only an immunosuppressive effect of cryosurgery [25,26,41,61,63,80]. Hayakawa and colleagues, using a chemically induced fibrosarcoma, found mice treated by cryoablation had a decreased resistance to a secondary tumor challenge, as well as increased growth and metastatic rates of secondary tumors [26]. Shibata et al. [62], examining WKA fibrosarcoma in rats, found that pulmonary metastases established 1 day prior to treatment of a subcutaneous tumor, were enhanced by cryoablation. In contrast, using the same tumor type in a double grafted tumor system, cryosurgery did inhibit the development of contralateral tumors. This effect, however, did not appear to be T-cell dependent, as the anti-tumor activity of splenocytes was decreased in the cryosurgery group.

Examining the mechanisms behind the cryo-immunologic response

As immunologic assays became more sophisticated and a better understanding of the relationships between the innate and adaptive arms of the immune response became known, more detailed studies of the mechanism behind cryo-immunology emerged. Gazzaniga et al. [21] examined the inflammatory changes that take place in the hours and days after cryoablation. Using a human melanoma cell line xenografted in nude mice, the authors excised the tumors at varying time points to determine the presence and nature of the inflammatory cells. Within hours of freezing, PMN leukocytes were densely recruited intravascularly. They eventually infiltrate the peritumoral area, reaching their maximal concentration by day 3. Macrophages became abundant by day 3, peaking at day 7 and persisting through day 15. The authors also found that cryosurgery-induced a significant increase in antibody reactivity to human melanoma cells as determined by ELISA assays of the mouse sera. Sabel et al. [53] looked at MT-901 mammary adenocarcinoma tumors in BALB/c mice treated by cryoablation or surgical resection. After re-challenge, 86% of mice treated by surgery developed second tumors compared with only 16% of mice treated by cryosurgery. This was tumor-specific, as cryosurgery offered no protection against challenge with another cell line. In examining the mechanism behind this observation, cryoablation led to significantly higher levels of serum IL-12 and IFN-γ shortly after freezing, with no corresponding changes in IL-4 and IL-10. NK cell activity was increased significantly in the mice undergoing cryoablation. While a regional response was noted, tumor-specific T-cell responses were evident in the regional lymph nodes, and adoptive immunotherapy with lymphocytes from cryoablated tumor draining lymph nodes (CTDLN) was superior to TDLN from mice treated by surgery in eradicating pulmonary metastases, [52] the authors could not demonstrate a significant systemic T-cell response generated by cryoablation alone.

Den Brok et al. [12] sought to determine whether cryoablation provided an antigen source for dendritic cells. Mice with ovalbumin-transfected B16 (B16/OVA) tumors underwent an i.t. injection of ^{111}Indium-labeled KLH or OVA tracer proteins prior to ablation. Mice treated by cryoablation, as compared to untreated mice, showed a significant uptake of the labeled antigens in the draining lymph nodes. Using magnetic bead sorting for CD11c+ dendritic cells, it was shown that the antigens were primarily within the CD11c+ cells. Cryoablation also induced maturation of the TDLN DC. Compared with radiofrequency ablation (RFA) or a conventional DC vaccine, the accumulation of antigens within the DC was significantly higher with cryoablation. What is not clear from these results are the method by which the DC acquire the antigens; specifically whether the antigens released from the tumor by cryoablation are carried to the nodes via the lymphatics and then engulfed by DC as opposed to DC migrating to the ablated tumor, taking up antigen, and then moving to the lymph nodes. It seems likely, given the presence of antigen within the DC within 1 day of treatment, that the former explanation is more likely.

As opposed to animal models, some studies attempted to document immunologic changes after clinical cryosurgery. In the 1970's and 80's, based on the case reports of metastatic disease regressing after prostate cryoablation, investigators began to look for clinical evidence of an immune response. Several isolated studies at that time documented increases in relatively non-specific markers of immune response among patients undergoing cryoablation of oral cavity cancers, [14,16,17] rectal cancers [34,78] or breast cancer [70]. Osada et al. examined changes in serum cytokine levels (IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ) after cryosurgery of unresectable hepatic tumors (12 metastatic, three primary). There were six patients who had not only local effects but also evidence of necrosis in lesions away from the treated lesions. These patients had higher pre-treatment TNF-α levels, a more significant rise in TNF-α, and an increase in the Th1/Th2 ratio (IFN-γ/IL-4) ratio while the non-immune responders had higher pre-treatment IL-10 levels and a more significant rise in IL-10 levels after cryosurgery. In an interesting study of patients undergoing treatment for colorectal metastases to the liver, Ravindranath et al. [46] measured both the level of serum tumor gangliosides and their antibody titers after cryosurgery, radiofrequency ablation (RFA) or surgical excision. The level of serum gangliosides was significantly increased after cryosurgery but not after RFA or surgery. Likewise, only cryosurgery led to an increase in the IgM titer against tumor gangliosides. The authors concluded that cryosurgery-induced necrosis of the tumor not only released these gangliosides into circulation but also served as an adjuvant to the humoral response as repeated immunization with purified gangliosides failed to elicit an antibody response.

Other studies examined the mechanisms behind the immunosuppressive aspect of cryoablation. In a follow-up to their observation that cryoablation enhanced WKA fibrosarcoma pulmonary metastases in rats, the authors found that the anti-tumor resistance of rats was diminished by the adoptive transfusion of splenocytes from tumor-bearing mice treated by cryoablation, suggesting that the immunosuppression following cryosurgery might be caused by suppressor T-cells (now referred to as regulatory T-cells) [62]. Another animal study of the cryoablation of fibrosarcoma, this time in Sylvian golden hamsters, also suggested an increase in regulatory T-cells following cryosurgery [79].

Two studies offered insight into why cryoablation may alternate between immune enhancement or immune suppression. Hanawa et al. [25] examined anti-tumor immunity in rats following cryoablation of MRMT-1 tumors implanted in the liver. Rats whose tumors had been completely ablated were more susceptible to a subsequent challenge than control mice. However, rats that had the tumors incompletely frozen had increased resistance to re-challenge and a prolonged survival. The authors concluded that the degree of tumor freezing might modulate the systemic immune response. Miya et al. [41] looked at the changes in the local lymphatic and hematogenous vessels around cryoablated tissue, specifically looking at the route and time course of tumor antigens using colloidal carbon perfusion and [^{3}H]thymidine injected intratumorally. Cryonecrotized tumor antigens appeared to be absorbed systemically in the early period (30 min to 6 h) via the peritumoral interstitial space into the regional lymph nodes and lymphatic channels. Hematogenous spread in the early period was obstructed by vascular stasis, presumably secondary to microvessel thrombosis. Ultimately, however, new capillaries formed around the cryonecrotized tissues, leading to blood flow rates near preoperative levels by 120 h. The authors suggest that the large release of tumor antigens via lymphatic routes might act as a blocking factor and participate in depressing anti-tumor immunity in the early postoperative period. They also suggested that different methods of cryoablation might have different effects on antigen release and recovery of peritumoral blood circulation, thus explaining differences in immune responses.

Summarizing the data: how might cryoablation generate an anti-tumor response

The preponderance of data, both clinical and laboratory, suggests that the in situ cryoablation of malignant tissue can have significant effects on the immune system. While the result is often positive, it can also be immunosuppressive. Table 1 summarizes studies of cryoablation alone in preclinical models, either com-

Table 1
Immune response to cryosurgery alone.

Author	Year	Tumor model	Endpoint	Results	Additional observations
Blackwood [8]	1972	Mysoarcoma and carcinosarcoma in rats	Suppression of second tumors and resistance to re-challenge	Significant impact of cryosurgery on regression of 2nd tumors and resistance to re-challenge	Immune response improved with lower volume of residual frozen tissue
Neel [43]	1973	Viral induced mammary adenocarcinoma in C3H/HeN and sarcoma in CDF	Resistance to re-challenge	Superior protection with cryosurgery compared to surgery	
Bagley [6]	1974	MCA-10 fibrosarcoma in C57BL6 mice	Cytotoxicity assays of splenic lymphocytes	Tumor-specific lymphocyte mediated cytotoxicity after cryosurgery	
Javadpour [31]	1979	Intradermal tumors in guinea pigs	Eradiation of microscopic lymph node metastases.	No effect of cryo.	
Misao [40]	1981	MRMT-1 breast in Sprague–Dawley rats	Resistance to re-challenge	Superior protection at 10 weeks compared to surgery (80% vs. 18%, $p < .001$)	
Muller [42]	1985	Dunn sarcoma in the leg of C3H mice	Lung metastases	Decreased lung metastasis with cryo compared to amputation or local resection.	No evidence of increased immune response after cryosurgery, possible immune suppression.
Shibata [61,63]	1998	Fibrosarcoma in WKA rats	Suppression of contralateral tumors and pulmonary metastases	Suppression of contralateral tumors BUT enhancement of early pulmonary metastases	Decrease in antitumor T-cell activity with cryosurgery, possibly due to regulatory T-cells
Gazzaniga [21]	2001	Human IIB-JEL-J melanoma in nude mice	Sera antibody response to melanoma antigens	Significant increase in humoral response of cryo compared with untreated	Early peritumoral PMN infiltrate followed by peritumoral macrophages, peaking at day 7
Hoffman [28]	2001	AT-1 prostate in Copenhagen rats	Resistance to re-challenge	No protection against 2o tumors with cryosurgery	Cryo led to increased anti-tumor antibodies compared to controls, but not to compared with surgical excision
Urano [76]	2003	Colon-26 CA in BALB/c mice	Liver metastases compared to untreated mice	Significant decrease in liver mets after ablation of a single lesion	
Joosten [33]	2003	Colon26 tumors in Balb/c mice	Suppression of contralateral tumors	Significant inhibition of secondary tumor growth with cryo	Inhibition correlated with high plasma levels of TNF-α and IL-1α
Den Brok [12]	2006	B16-OVA melanoma in C57BL6/J mice	Resistance to re-challenge compared to naïve mice	Moderate level of protection with cryo (50% vs. 0%, $p < .005$)	Increased antigen uptake by DC after cryoablation, increased presence of IFN-γ producing tumor-specific T-cells
Udagawa [74]	2006	CT26 colon CA in BALB/c mice	Suppression of contralateral tumors	No suppression with cryo alone	
Sabel [53]	2006	MT-901 mammary adenocarcinoma in Balb/c mice	Resistance to re-challenge	Significant tumor-specific protection after cryoablation	Increased tumor-specific T-cell activation in regional lymph nodes and increased NK function after cryoablation
Machlenkin [37]	2006	Lewis lung carcinoma in C57BL6 mice	Suppression of lung metastases	No change in lung metastases with cryo alone	
Redondo [48]	2007	B16/OVA melanoma in C57BL6/J Mice	Resistance to re-challenge compared to surgery	Low level of protection with cryo (25% vs. 0%, $p < .0001$)	Intense infiltrative PMN response to cryo at day 7

pared with surgical resection or as a control arm of cryoablation combined with other immunotherapies. As variable as the immune response is the number of animal models used and the methods used to freeze the tumor. Looking at the present body of literature, it becomes apparent that the immune response to freezing malignant tissue is dependent on several factors, including (1) tumor type and the inherent immune recognition prior to treatment, (2) the method by which the tumor is frozen, (3) the volume of disease frozen and (4) the time point at which one looks for an immune response. To better understand how cryoablation might stimulate an immune response, it is important to understand (A) how cryoablation kills tumor cells and (B) how an immune response is generated.

Cryoablation results in tumor death by several mechanisms; (1) solution effects, (2) intracellular ice formation, (3) microvascular thrombosis, and (4) apoptosis. Close to the cryoprobe, the freezing rates are high enough to induce freezing of the intracellular fluid. This is a lethal event associated with irreversible membrane damage. Freezing the tumor as quickly as possible will maximize the formation of intracellular ice as well as the cryogenic lesion. However, further from the probe, freezing rates are slower. Here, the extracellular fluid will freeze but the intracellular fluid has better protection by the lipid membrane. However, as ice is essentially pure water, an osmotic imbalance occurs. The high concentration of solutes in the remaining extracellular fluid leads to fluid shifting from the intracellular compartment to the extracellular compartment and cellular dehydration. The cell shrinkage results in damage to the membrane.

For the ablation of cancer, at least two freeze–thaw cycles is typically recommended to maximize cell killing. Thawing the thawing of the tissue may be just as damaging to the cells as the freezing. During the thaw, which should be as long as possible, the solute effects are maximized. The intracellular compartment is now hypertonic and as the ice melts, fluid rushes into the damaged membranes and the cells burst. In addition to this, large ice crystals form during recrystallizatoin in the warming period, and these create direct shearing forces which further disrupt the tissues. When the freezing is repeated, the damaged tissue conducts the cold much more efficiently, increasing the area of necrosis beyond the first cycle.

All cells, however, are not killed by direct cryo-injury. The same direct mechanisms that destroy tumor cells also destroy endothelial cells of the microvasculature. This results in post-thaw platelet aggregation and vascular stasis. Thrombosis and ultimately ischemia occur within the treated area, leading to necrosis of the frozen tumor. At the peripheral zone of the cryogenic lesion, where the

temperature may not have been cold enough to kill all the cells, many of the cells show signs of apoptosis. Thus, in the central zone, there is a coagulation necrosis while in the periphery, there may be apoptotic cells. In this outermost area, in contact with the still viable tissue, is where wound repair begins. Inflammatory cells infiltrate and new blood vessels grow into the injured tissue. Ultimately, fibroblasts and new collagen formation will occur.

The relative contributions of these various mechanisms, and their success in completely ablating the tumor, is dependent upon several factors including cell structure and surrounding anatomy (large vessels may act as 'heat sources'), the lowest temperature reached, the hold time at that temperature, the number of freeze–thaw cycles and the freezing and thawing rates. This combination of effects not only impacts the ability of cryosurgery to completely ablate the tumor, but also trigger those steps necessary to generate an immune response. It must therefore be recognized that the clinical aspects of cryosurgery that may be optimal for complete tumor ablation, may or may not be optimal for the cryo-immune response.

So does cryoablation provide the necessary stimuli to generate an anti-tumor response? In a normal immune response to pathogens, local tissue damage induces the synthesis of pro-inflammatory cytokines. These mediators induce the synthesis of vascular adhesion receptors and chemokines that, in turn, initiate recruitment of circulating leukocytes. Early recruits from the innate immune system (granulocytes, monocytes and macrophages, NK cells) not only have direct effects, but elaborate additional soluble mediators that further modify the local environment. The acquired immune response begins when antigen presenting cells (APCs) take up antigen. Ultimately this will lead to a humoral response (the generation of antigen-specific antibodies) or a cellular response (T-cells). There are several types of T-cells that may become activated. The two main subsets of T-cells include helper T-cells (T_h cells, also known as CD4+ T-cells) which help guide the subsequent immune response through the secretion of cytokines, and cytotoxic T-cells (Tc cells, or CD8+ T-cells) which destroy virally infected cells or tumor cells. However, another subset of T-cells that can be activated are regulatory T-cells (Treg cells, previously known as suppressor T-cells). These cells serve to shut down T-cell mediated immunity and provide immunologic balance. Generation of Treg cells can lead to immune suppression.

The two primary APCs are macrophages and dendritic cells (DC). DC are bone-marrow derived mononuclear cells found in both the blood and in the periphery. They are extremely efficient at capturing antigens by phagocytosis, macropinocytosis and adsorptive endocytosis. Antigen sources can include infectious agents, apoptotic cells, necrotic cells, immune complexes, opsonized tumor cells, and heat shock proteins. The exogenous antigens are processed into peptides, loaded onto major histocompatibility complex class I and II (MHC Class I and II) molecules and transported to the cell surface for recognition by antigen-specific T-cells. Dendritic cells efficiently capture antigens in their "immature" state, and effectively present antigens in their "mature" state. One of the major differences between macrophages and DC is cross-presentation. Typically, exogenous antigens processed by APC are presented on MHC Class II molecules, whereas only endogenous antigens (from self-components or viral infections) are presented on MHC Class I (Fig. 1). MHC Class I expression is crucial to generating a cytotoxic T-cell response. Cross-presentation is the process by which exogenous antigens enter the MHC Class I processing pathways to generate cytotoxic T-ells. Macrophages fail to cross-present antigenic material to the degree that DC can, and thus are not very effective at promoting T-cell priming.

The type of acquired response generated is dependent upon the cytokines released by the APC, the helper T-cells and other cells within the microenvironment. In the presence of co-stimulatory molecules on the APC, and cytokines being released from the Th1 helper T-cell (IL-2, IFN-γ, TNF-α, GM–CSF), the cytolytic T-cell is activated. However, a second cascade can occur when a Th2 helper T-cell is activated and the secretion of the B-cell stimulatory cytokines (IL-4, IL-5, IL-10), which stimulate the B-cell to proliferate and differentiate into plasma cells. It is therefore evident that the ability of cryoablation to generate an anti-tumor response, and the nature of that response, will be dependent upon:

(1) the cytokine profile triggered by cryoablation,
(2) the availability of antigens in a form that can be processed by antigen presenting cells,
(3) the mechanism of cell death (apoptosis vs. necrosis),
(4) the subsets of phagocytic cells responsible for clearing the ablated cells (DC vs. macrophages).

Depending on these variables, cryoablation could potentially trigger a humoral response only, a cellular response, a combined response, no adaptive response or perhaps even immune suppression. Several changes induced by cryoablation may impact the immune response either positively or negatively.

Apoptosis, necrosis or both?

The immune response to cryoablated tissue will depend upon the mechanism of cell death, a phenomenon known as the *danger theory*. The concept of "self" versus "non-self" has often been used to describe how the immune system recognizes which antigens to target and which to ignore. However, rejection may not be soley due to the "foreignness" of the antigen, but also the "danger signals" associated with the antigen. The danger theory proposed by Matzinger suggests that it is not simply a matter of self and non-self, but also dangerous and not dangerous [19,38]. The generation of a cytotoxic T-cell response is often thought of as requiring two signals; signal 1 being the recognition of the peptide antigen with the T-cell receptor and signal 2 being the interaction of adhesion and co-stimulatory molecules on the APC cell surface and T-cell. (Fig. 1). However, a 3rd signal is necessary, one which activates the APC. In the absence of this 3rd signal, the naïve T-cell will receive signal 1 without signal 2, leading to the T-cell being down-regulated or deleted. This "3rd signal" consists of "danger signals" and their presence is often related to the nature of the invading organism (exogenous danger signals) or the mechanism by which cells in the body die (endogenous danger signals) (Table 2).

These endogenous danger signals are most relevant to cryoablation. (Fig. 2) Apoptosis and necrosis are the primary mechanisms of tumor cell death and have a significantly different impact on the immune response [77]. Necrosis occurs with mechanical tissue damage, such as cryoablation, and is characterized by cellular breakdown and release of intracellular contents. Many of these intracellular contents can be immunostimulatory. This includes not only pro-inflammatory cytokines, but also heat shock proteins (HSP), DNA and RNA which are recognized by Toll-like receptors or "danger signals" such as uric acid or the chromosomal protein HMGB1 (high mobility group box chromosomal protein 1), which can further activate the innate immune response [66]. The immune system may also be alerted to massive cell death not only by factors emanating from not only from dying cells, but also from disruption of tissue architecture, such as fibrinogen, oligosaccharides of hyaluronan, extra domain A (EDA)-containing fibronectin and heparin sulfate proteoglycan [10,11,44,67]. Several studies have demonstrated that necrotic cells will lead to increased DC maturation and macrophage activation [20,54].

Apoptosis, or programmed cell death, results in several steps that allow the uptake of cellular debris by both macrophages and

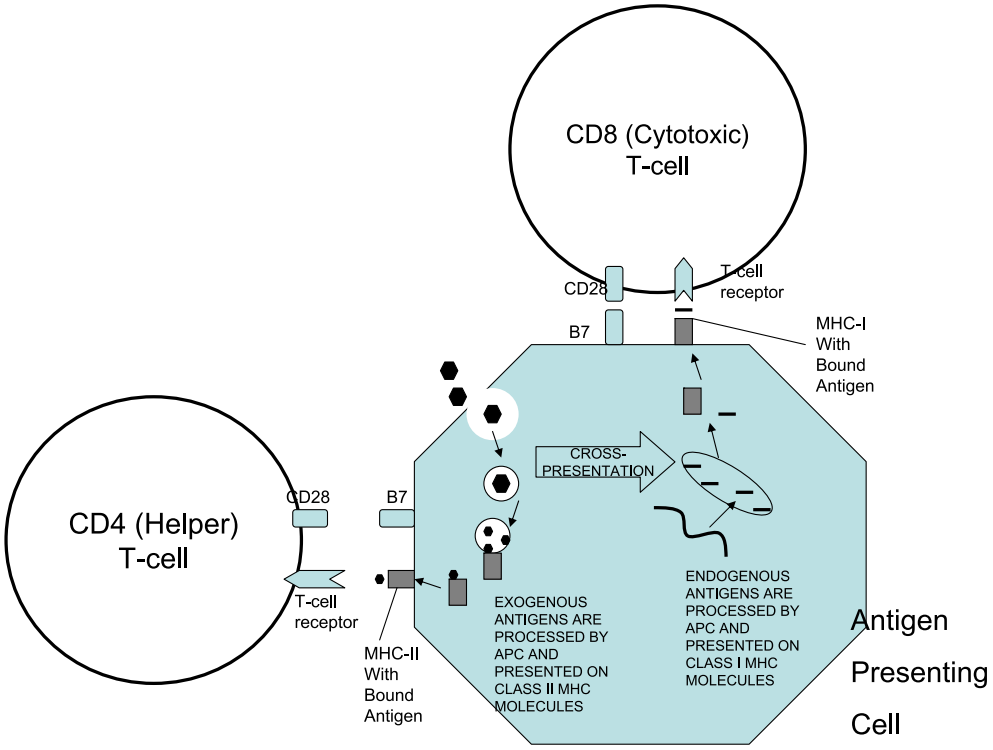

Fig. 1. Activation of T-cells by dendritic cells (DC). Exogenous antigens are typically processed into peptides, loaded onto major histocompatibility complex class II (MHC Class II) molecules and transported to the cell surface for recognition by naïve antigen-specific CD4+ T-cells. Activation of these T-cells are dependent upon not only recognition of the antigen by the T-cell receptor (TCR) but also co-stimulatory signals, such as CD28 on the T-cell recognizing B7.1 (CD80) or B7.2 (CD86) on the DC. As endogenous proteins (self-components or viral proteins) are degraded, peptides are bound to MHC Class I molecules and expressed on the surface. Naïve CD8+ T-cells can be activated by recognition of the antigen by the TCR in the presence of co-stimulation. For cytotoxic T-cells to be activated against exogenous antigens (as must take place for an anti-tumor CD8 response), cross-presentation must take place. Cross-presentation is the process by which exogenous antigens enter the MHC Class I processing pathways to generate cytotoxic T-cells.

Table 2
Endogenous danger signals.

Danger signals	Endogenous danger signals
Lipopolysaccharide	Cytokines (TNF-α, IL-6, IL-1β, IFN-α)
Lipoteichoic acid	ATP and UTP
Lipoarabinomannan	Heat shock proteins
Lipopeptides	Long unmethylated CpG sequences
Peptidoglycan	Breakdown products of hyaluronan
Mannans and mannoproteins	DNA and RNA
Viral capsids	Uric acid
Unmethylated CpG and dsRNA	HMGB1

dendritic cells, but without causing inflammation and thus stimulate an immune response. Apoptotic cells do not release their contents (HSP, DNA, RNA, HMGB1) as do necrotic cells. In fact, several studies have shown that apoptosis not only does not stimulate immune recognition, but quite the opposite [45,77]. This makes some sense as apoptosis occurs physiologically in many tissues, and their uptake may be one mechanism by which the body maintains "self" versus "non-self". The continual transport of apoptotic "self" cells and presentation of self-antigen may relate to peripheral tolerance [30,57]. The recognition and phagocytosis of apoptotic cells is mediated by a large number of receptors and opsonins which bind to cellular ligands exposed on the surface of apoptotic cells. This not only prevents the release of the intracellular contents, but modulates phagocyte function, inhibiting pro-inflammatory cytokine release and increasing TGF-B1 production [15,55]. Dendritic cells that take up apoptotic cells have suppressed cytokine production and do not mature [36,69]. These non-mature DC not only do not stimulate an immune response, but can trigger clonal deletion and anergy [45]. Defects in the manner by which apoptotic cells are cleared have been associated with the development of autoimmune diseases [77].

As discussed, both necrosis and apoptosis play a role in tumor cell death after cryoablation. Therefore, the relative contribution of necrosis and apoptosis in the death of the tumor cells may shift the immune response from stimulatory to suppressive. As described, the amount of necrosis versus the amount of apoptosis may vary depending on the rate of freezing, number of freeze–thaw cycles and size of the cryolesion. Cryoablative techniques that result in large areas of apoptotic cell death, as opposed to necrosis, may result in immunosuppression.

This picture is not completely clear, however, and some studies have suggested that apoptotic tumor cells may be superior to necrotic cells in stimulating an anti-tumor immune response [27,50,56,58]. This is likely secondary to superior phagocytosis by dendritic cells of tumor cell-derived apoptotic bodies, as compared with necrotic cells, and thus cross-presentation of antigens to CD8+ T-cells [27,32]. Failure to clear apoptotic cells may lead to a secondary necrosis of uncleared cells, and thus the necessary pro-inflammatory signals [51]. It has been hypothesized that while the uptake of apoptotic cells is normally immunologically silent (or suppressive), the uptake of apoptotic cells by DC in the presence of inflammatory or danger signals from necrosis is the ideal situation for cross-presentation of antigen and priming of effector T-cells. Therefore, death primarily by necrosis may generate a humoral response, death primarily by apoptosis may generate immune tolerance, while death by a combination of necrosis and apoptosis may lead to a combined humoral and cellular response.

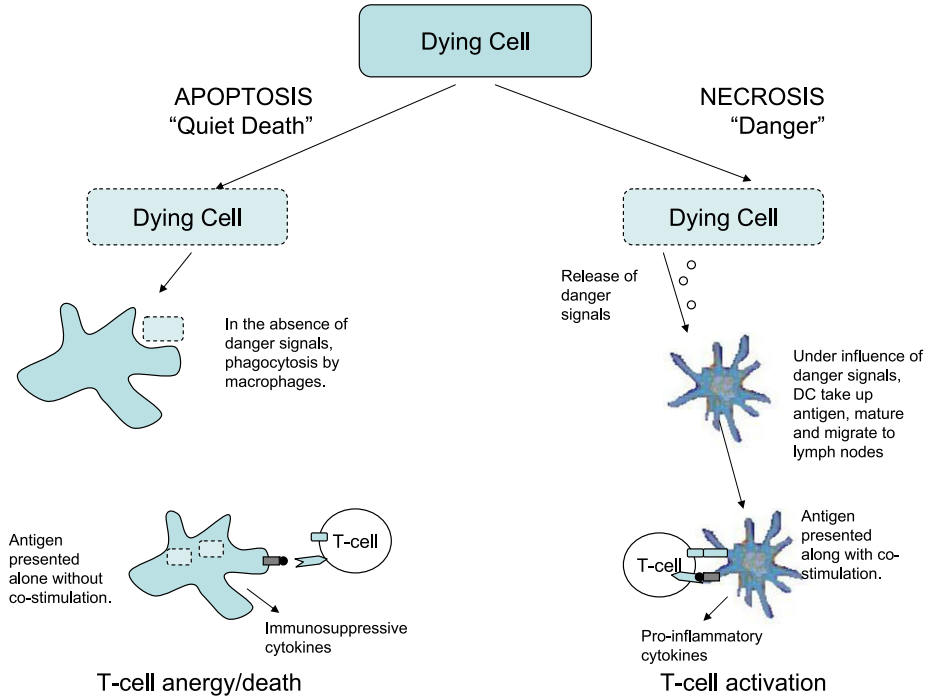

Fig. 2. The danger theory and tumor cells. The immune response to cell death is dependent upon the presence of danger signals. The recognition and phagocytosis of apoptotic cells are largely mediated by receptors and opsonins which bind to cellular ligands exposed on the surface of apoptotic cells. Thus, apoptosis results in the uptake of cellular debris without causing inflammation or releasing the intracellular contents. The antigen presenting cells that take up the apoptotic cells not only do not generate an immune response, but can lead to clonal deletion and anergy. In contrast, necrotic cell death is characterized by cellular breakdown and release of intracellular contents, many of which are danger signals. These signals promote cross-presentation, maturation of the DC and ultimately the activation of antigen-specific T-cells.

Cytokine release after cryoablation

As the generation of an immune response, and the nature of that response, is highly dependent upon the release of cytokines, one important question is whether cryoablation induces the right mix of cytokines to initiate a response. Cytokine release after cryoablation can come from two sources. The first is direct release from the ablated tissue itself. Cytokines can also be released from tumor cells, stromal cells or immune cells within the cryolesion. Cryoablation, by causing membrane damage but leaving the proteins intact, can lead to a release of intracellular proteins, including cytokines, immediately after treatment. In the case of freezing large tumors, this can lead to a phenomenon known as "cryoshock", a syndrome of coagulopathy, disseminated intravascular coagulation and multiorgan failure [24,59]. While described for several tumor types, it appears rare after cryoablation of renal or prostate tumors, but a more common complication of hepatic cryoablation. Cryoshock is believed to be due to the systemic release of cytokines after cryoablation, and serum levels of IL-1β, IL-6 and TNF-α have been shown to rise after the cryoablation of large hepatic metastases [22,60].

Although serum levels of cytokines necessary to induce a systemic response such as cryoshock appears rare, and related to the size of the area frozen, it is clear that ablation of a smaller tumor may release enough cytokine to impact cells within the regional draining lymph nodes. Depending on the cytokines released regionally, this may lead to increased proliferation of lymphocytes or maturation of antigen presenting cells. The nature of the cytokines released, however, would depend upon the presence and composition of lymphocytes within the tumor microenvironment, which may not only vary greatly among tumor types, but also from patient to patient with similar tumors.

Another immediate source of cytokines may come from the tumor cells themselves. It has been shown repeatedly that a variety tumor cells are capable of producing cytokines that can locally impact immune function. In many cases, these cytokines are immunosuppressive, such as IL-10 and TGF-β and act to diminish the anti-tumor immune response. This is one mechanism by which tumors escape immune recognition. Release of these cytokines from the ablated tumor cells may act to increase the proliferation of regulatory T-cells, down-regulate antigen presentation and possibly lead to further or enhanced tolerance to tumor antigens. This is one manner by which cryoablation may potentially lead to immune suppression, particularly in the early period. Again, the immunologic impact of cryoablation may vary depending on the tumor type and their innate production of immunoregulatory cytokines.

A second source of pro-inflammatory cytokines would then come from the response to the local tissue damage. This would include the vascular adhesion receptors and chemokines that initiate the healing process, and ultimately recruit polymorphonuclear cells, macrophages and dendritic cells to the site of the ablated tumor. These cells then also produce and release cytokines that would further modify the local immune response. The nature of this response will depend upon the composition and time course of infiltrating cells after cryoablation, as described below.

Dendritic cells versus macrophages

Histologic studies have shown a rapid infiltration of macrophages to the site of a cryoablated tumor. However, if macrophages are responsible for most of the uptake of necrotic tumors, this may tilt the immune response towards away from a cellular response and towards a humoral response, seeing that macrophages do not cross-present antigen as dendritic cells do. Macrophages may also release IL-10 or TGF-β, which can further impair a T-cell response.

A cellular response is dependent upon the cross-presentation of antigens by dendritic cells. It remains unclear to what degree dendritic cells infiltrate and take up either necrotic or apoptotic cells at the ablated tumor site. The microvessel damage that cryoablation induces may delay the infiltration of blood-borne dendritic cells and the rapid clearance by macrophages may leave little cellular material available to dendritic cells when they do arrive. This may depend on the organ being treated and the size of the lesion treated. However, an apparent lack of infiltration of the ablated tumor by immature DC has prompted many investigators to combine cryoablation with methods of attracting DC, including TLR agonists or the direct injection of immature DC [13,37,48].

Antigen release and immune complexes

While the uptake of antigen by DC and their maturation en route to the regional lymph nodes is the classic picture of antigen presentation, there is an alternate method by which cryoablation may lead to DC presentation of antigen and T-cell stimulation. In addition to the release of cytokines and other inflammatory mediators, soluble antigen is released after cryoablation. As shown by Den Brok et al. [12] mice treated by cryoablation, as compared to untreated mice, demonstrated a significant amount of antigen within the DC within the draining lymph nodes. It is possible that immature DC in the regional nodes take up antigen released by the ablated tumor, and in the presence of the cytokines and inflammatory signals, mature and activate naïve T-cells, thus generating a T-cell response. The release of heat shock proteins from the ablated tumor cells may also facilitate DC uptake of antigen, as may the presence of immune complexes.

Another method by which DC may take up antigen without infiltrating the site of the ablated tumor is through antigen–antibody complexes or immune complexes (IC). Antigen, released by cryoablation, can bind to serum antibodies forming immune complexes. The FcγR, which binds the Fc domain of IgG, is expressed on most cells of the hemopoietic lineages, including DC. Uptake of immune complexes by DC appears to be a superior method of stimulating cross-presentation, CD8+ CTL responses and cellular tumor immunity. This is one method by which the humoral and cellular responses are linked. The release of soluble antigen by cryoablation, and the humoral response that develops, may lead to a significant quantity of IC that lead to FcγR internalization by DC and CD8+ T-cell activation. However, large quantities of IC have also been associated with "high zone tolerance", a phenomenon by which antigen overloading may lead to immunosuppression. Although the mechanisms behind high zone tolerance have not been fully elicited, this has been suggested as another possible mechanism by which cryoablation may be immunosuppressive.

Future directions

A review of the literature strongly supports the notion that cryoablation can be immunogenic, resulting in the immune recognition of tumor-specific antigens and the eradication of distant disease. Evidence ranges from anecdotal observations in clinical cryosurgery, a variety of animal models and correlative immune studies in patients undergoing cryoablation. It is not surprising that there is tremendous interest in cryosurgery and cryo-immunology, as cryoablation has the potential to be both a local and systemic therapy, directly ablating the primary tumor and reducing distant recurrences by eradicating micrometastases through the immune system. However, the generation of an anti-tumor immune response is complex, and several factors can not only preclude the development of a positive response, but tilt that response towards immunosuppression (Figs. 3–5). As the clinical use of cryosurgery expands, it becomes increasingly imperative that we better understand the means by which cryoablation modulates the immune system, as any potential for further suppressing

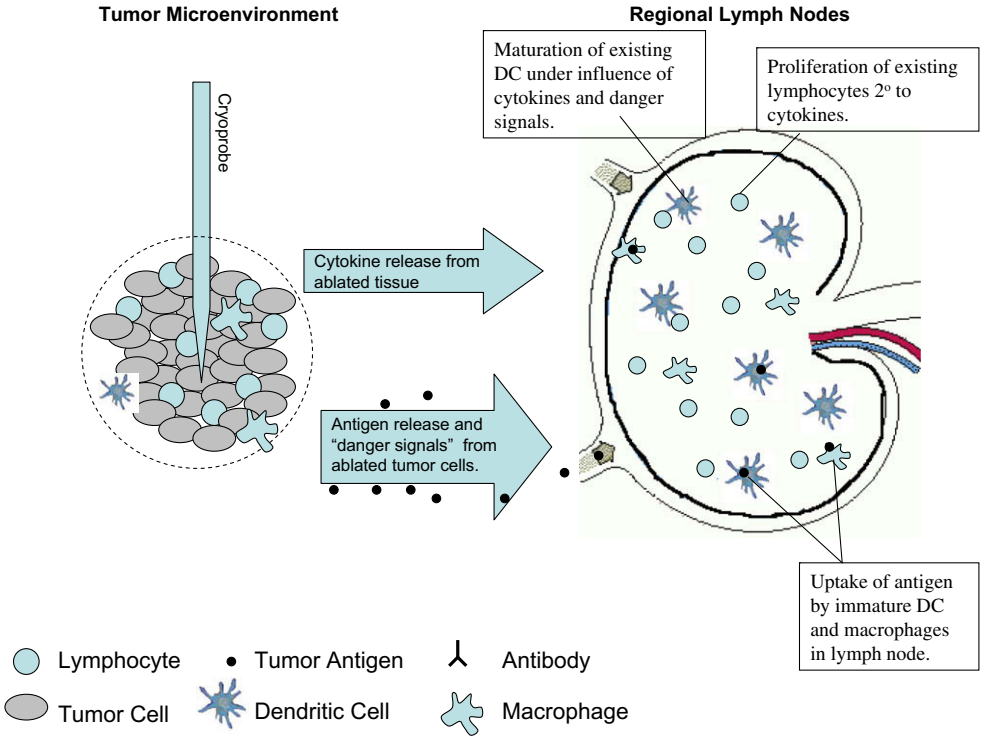

Fig. 3. Possible immediate immunologic effects of cryoablation. Cytokines are initially released from ablated tissue, including tumor cells and tumor infiltrating lymphocytes (TIL). The composition of the TIL and the production of cytokines by the tumor cells can have varying effects on the antigen presenting cells (APC) and lymphocytes within the tumor draining lymph nodes. Pre-existing tumor-specific T-cells may proliferate in response to pro-inflammatory cytokines. Antigen is released which can be taken up by APC within the lymph node. "Danger signals" from necrotic cells may stimulate maturation of dendritic cells (DC) and increase antigen presentation and activation of naïve T-cells.

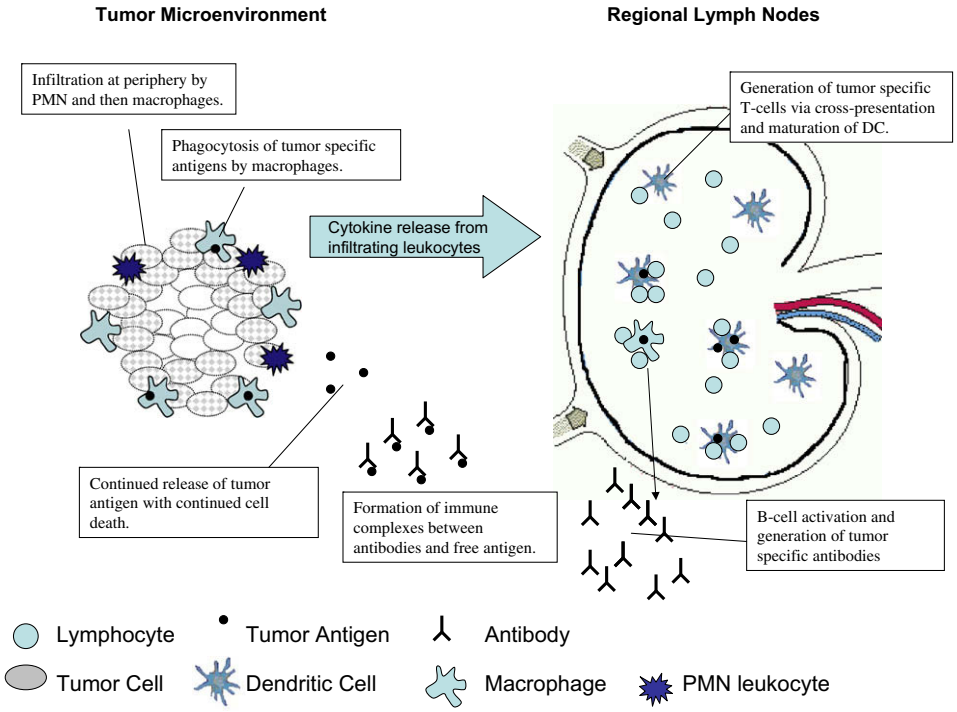

Fig. 4. Possible early and intermediate immunologic effects of cryoablation. Infiltration of the ablated tumor by PMN (polymorphonuclear cells) and macrophages release secondary cytokines and uptake antigen for presentation, primarily stimulating a humoral response. Antibodies recognize soluble antigen, forming immune complexes which may be taken up by DC or in large quantities may be immunosuppressive (high zone tolerance). New tumor-specific T-cells may be generated by mature DC expressing tumor antigens.

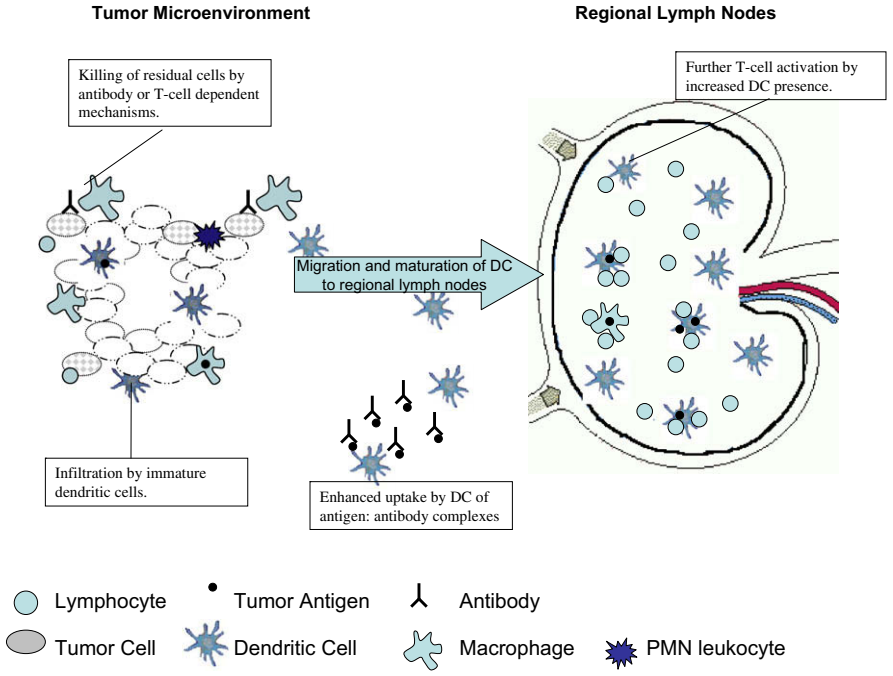

Fig. 5. Possible later immunologic effects of cryoablation. Immature DC potentially infiltrate the ablated tissue and take up antigen (or immune complexes), maturing as they migrate to the regional lymph nodes and stimulating T-cell activation and proliferation. This would be dependent on vascular stasis and the efficiency by which macrophages clear the ablated tissue. Apoptotic cells may be taken up by DC in an immunologically silent method and may induce tolerance.

the anti-tumor immune response in a cancer patient could have untoward effects.

Several important questions need to be addressed, and while some of these factors have been hypothesized, most remain unknown or unproven. Significant questions include (but are not limited to):

- What are the specific danger signals released from cryoablated tumor cells?
- What is the ideal ratio of necrosis to apoptosis to generate an immune response, and what is the ideal cryoablative technique to accomplish this?

- What are the cytokines released after cryoablation, either from the necrotic tumor microenvironment or the subsequent innate immune response, and their time course?
- What is the nature and time course of the antigen presenting cells responsible for resorbtion of the ablated tissue, and how can this be altered?
- What aspects of cryoablation and subsequent resorbtion of the tissue activates the immunosuppressive arm of the immune response (immunosuppressive cytokines, regulatory T-cells, immune complexes), and how can this be minimized?

In addition to better understanding how variations in the cryoablation technique itself may impact the immune response, answering these questions will guide the proper selection of methods to augment the cryo-immunologic response, tilting it away from suppression and towards stimulations, are needed. It is increasingly apparent that cryoablation alone may not be sufficient to generate a clinically relevant immune response or consistently favor stimulation versus suppression, and that cryoablation may best be incorporated into strategies combining freezing with immune adjuvants. Several authors have examined potential strategies, including following cryosurgery with immunostimulants, [76] combining cryoablation with anti-CTLA-4 blockade [12] or TLR stimulation [13,48] or following cryoablation with the intratumoral injection of immature DC [37] [74]. Additional approaches are necessary, balancing immunogenicity with clinical feasibility. Considerable research in this field is warranted, as the potential reward—a single therapeutic approach that can both destroy the primary cancer with minimal complexity or side effects AND eradicate distant micrometastases with little toxicity represents the holy grail of cancer treatment.

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