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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Rate of Freeze Alters the Immunologic Response After Cryoablation of Breast Cancer

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ABSTRACT

Background. Cryoablation has garnered significant interest as a treatment for solid tumors including breast cancer for both its local effects and potential in stimulating an antitumor immune response. We sought to examine the impact that variances in technique might have on the immune response and examine the mechanism by which cryoablation may stimulate an antitumor immune response.

Materials and Methods. Balb/c mice with established 4T1 mammary carcinomas were treated by cryoablation at either a high rate of freeze or low rate of freeze, or by surgical excision, after spontaneous metastases occurred. Tumor-draining lymph nodes (TDLN) were excised at 1 week for EliSPOT assay and immunophenotyping. Mice were followed after treatment for enumeration of pulmonary metastases and survival.

Results. Compared with surgical excision, cryoablation using a high freeze rate resulted in a significant increase in tumor-specific T cells in the TDLN, a reduction in pulmonary metastases, and improved survival. However, cryoablation using a low freeze rate resulted in an increase in regulatory T cells, a significant increase in pulmonary metastases, and decreased survival.

Conclusions. Cryoablation of breast cancer in mice can generate a tumor-specific immune response that can eradicate systemic micrometastases and improve outcome compared with surgical excision; however, the technique used to freeze the tissue may alter the immune response from stimulatory to suppressive.

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Cryosurgery is increasingly being recognized as a highly efficient, minimally invasive method of treating malignant neoplasms, including malignancies of the skin, prostate, liver, breast, lung, and bone. Compared with surgery, there are several potential advantages including 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. There has also been significant interest in another aspect of 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.[1–9]

These clinical reports prompted a laboratory examination of the relationship between cryoablation and the immune system. Several studies demonstrated an improved resistance to rechallenge among mice treated by cryoablation compared with those treated by surgery and provided evidence of an augmented immune response after cryoablation.[6,10–12] However, other studies demonstrated that cryoablation could lead to immune suppression instead of immune stimulation and that this may depend on the point at which one looked or the volume of tissue ablated.[12–22] Beyond the various animal models being used, these studies varied greatly in the method used to freeze the primary tumor, ranging from the application of liquid cryogens directly to the tumor, contact cryoablation, where a cryoprobe is touched to the periphery of an exposed tumor, to the percutaneous insertion of a cryoprobe to the center of the tumor, propagating the iceball from the center of the tumor outward. Differences in technique result in differences in both the rate of the freeze and the minimum temperature achieved.

Using the most modern approach to cryoablation, we previously reported the generation of a tumor-specific immune response generated by cryosurgery in a murine model of breast cancer.[23] To verify these results, and examine the impact that variances in the method by which the tumor is frozen might have on the subsequent immune response, we compared two methods of in situ cryoablation to surgery in a murine model of metastatic breast cancer.

METHODS AND MATERIALS

Animals and Tumors

Female Balb/c mice 6 to 8 weeks old were purchased from The Jackson Laboratory (Bar Harbor, ME). During the experiments, mice were kept in pathogen-free conditions at the Animal Maintenance Facility of the University of Michigan Medical Center and carefully monitored for signs of toxicity according to the guidelines of the university. The University of Michigan Laboratory of Animal Medicine approved all protocols.

4T1 is a mammary carcinoma cell line that has been studied extensively.[24] RENCA is an immunogenic murine renal cell carcinoma of spontaneous origin in the Balb/c strain.[25] Cell lines were maintained in complete media consisting of RPMI 1640 supplemented with 10% heat-activated fetal bovine serum, 0.1 mM nonessential amino acids, 1μM sodium pyruvate, 100 μg/mL streptomycin, 100 units/mL penicillin, 50 μ g/mL gentamycin, and 0.5 μg/mL fungizone, all from Life Technologies, Inc. (Grand Island, NY).

In vivo generation of tumors was accomplished by injection of 5×10^4 4T1 breast cancer cells suspended in 50 μL of phosphate-buffered saline (PBS) into the mammary fat pads. Tumor size was measured every day in two perpendicular dimensions (a = length; b = width) with a Vernier caliper and the size recorded as a volume (mm^3) as calculated by $a \cdot b^2/2$. When tumors reached 8 to 12 mm in size, mice were treated by cryoablation or surgery as described below. Mice were euthanized when moribund or at the indicated times. For enumeration of pulmonary metastatic nodules, the metastases appeared as discrete white nodules on the black surface of lungs insufflated and stained with a 15% solution of India ink, and then bleached by Fekette's solution.

Surgery and Cryoablation

Mice were placed in a sanitized laminar flow hood and anesthetized with xylazine and ketamine by intraperitoneal injection, then placed in a prone position and the tumor site prepared with alcohol. Procedures were performed using strict aseptic technique. For mice in the surgery group, wide excision surgical resections were performed with grossly negative surgical margins. After control of self-limited hemorrhage was obtained, the wound was closed with interrupted nylon sutures.

Cryoablation was performed using a tabletop argon gas based cryoablation system (Visica Cryoablation System, Sanarus Medical, Pleasanton, CA). Anesthetized mice were placed prone on a heating pad to avoid hypothermia and the tumor site prepared with alcohol. A small cut in the skin was made to allow the freezing tip of the cryoprobe to be placed into the tumor mass. At that point, cryoablation was accomplished by the flow of argon gas through the probe. Cryoablation was performed with either a "high" rate of freeze (HRF) or a "low" rate of freeze (LRF). A high rate of freeze is defined as a rapid freeze accomplished through a 100% argon duty cycle to create the desired iceball. The high rate of freeze was maintained until the entire tumor mass was encompassed in the iceball (approximately 30 s). A low rate of freeze involves using a 10% argon duty cycle, where the gas flows for 1 second and is then off for 9 seconds for every 10-second period. This was also maintained until the entire tumor mass was frozen, which often took a few minutes. After completion of cryoablative procedure, the tumor was allowed to thaw passively, the probe was removed, and the skin over the ablated tumor was closed. Mice were placed under a warming lamp during the recovery period.

ELISPOT Assay

Balb/c mice with 4T1 tumors were treated by cryoablation (low and high freeze rate) and surgery and sacrificed 7 days after therapy. ELISPOT assay was performed to quantify tumor-specific IFN-γ producing cells. Lymphocytes from the tumor-draining lymph nodes (TDLN) were surgically excised from the ipsilateral inguinal basin, pooled, and then activated with 1 μg/mL anti-CD3 monoclonal antibody (mAb) immobilized in 24-well plates (4×10^6 cells/2 mL/well) for 2 days. The LN cells were subsequently cultured in 60 IU/mL IL-2 for 3 days at 2×10^5 cells/mL. The number of IFN-γ producing cells was measured using ELISPOT assay. Briefly, 96-well plates were coated with anti-mouse IFN-γ antibody (BD Biosciences Pharmingen, San Diego, CA). Activated lymphocytes (1×10^6 cells/well) were cultured for 48 hours at 37°C in a 5% CO_2 incubator alone or in the presence of 4×10^5 irradiated 4T1 or RENCA tumor cells (as a control for tumor specificity). After that time, wells were washed and incubated overnight at 4°C with a different clone of biotinylated anti-IFN-γ antibody (BD Biosciences Pharmingen, San Diego, CA). Reactions were visualized and counted using anti-biotin-AP.

FACS Analysis

Immunophenotyping of lymphoid cells was carried out by direct immunofluorescence. Analysis was performed using a FACScan flow microfluorometer (Becton Dickenson, Mountain View, CA). Lymphoid cells were stained with the following antibodies: PE labeled anti-CD4 mAb, FITC labeled anti-CD8a, APC labeled anti-CD3e, FITC labeled anti-CD25, PE labeled anti CD11c, PerCP-Cy5 labeled anti-CD11b, FITC labeled anti-CD80 (B7.1), and FITC labeled anti-CD86 (B7-2) per manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA). Fluorescence profiles were generated by analyzing 10,000 cells and displayed as logarithmically increasing fluorescence intensity versus cell numbers. Number of cells was calculated by multiplying the fraction of a particular subset by the total number of cells within the TDLN.

Statistical Analysis

Statistical significance between groups was calculated using an unpaired t test. Survival studies were assessed using the Kaplan–Meier product limit method. A P value less than .05 considered statistically significant.

RESULTS

Impact of Cryoablation and Surgery on Pulmonary Metastases and Survival

The 4T1 tumor bearing Balb/c mice were treated by cryoablation at either a high freeze rate or low freeze rate, or surgical excision, 2 weeks after tumor inoculation. In addition, a control group was left untreated. At 2 weeks following treatment, mice were euthanized and the lungs harvested for enumeration of pulmonary metastases. A second group of mice, treated in an identical manner, were followed for long-term survival.

Untreated mice developed an average of 47.2 pulmonary nodules/mouse. As demonstrated in Fig. 1, cryoablation using a high freeze rate led to the most dramatic decrease in pulmonary disease, with 4.89 nodules/mouse, significantly fewer than surgery (9 nodules/mouse, $P = .05$) and cryosurgery at a low freeze rate (17 nodules/mouse, $P = .016$). The difference in pulmonary disease between a low freeze rate and surgery did not reach statistical significance ($P = .08$).

Figure 2 demonstrates the Kaplan–Meier survival curve for 4T1 bearing mice treated by cryosurgery (high and low freeze rate), surgery, or mice left untreated. Overall, the log rank P value is <.0001, and all three treatment modalities were an improvement compared with untreated mice. Specifically looking at the two types of cryoablation versus surgery, cryosurgery at a high rate of freeze did lead to a significant improvement in survival compared with surgical excision ($P = .013$). However, there was no significant difference between using a low freeze rate and surgery ($P = .18$).

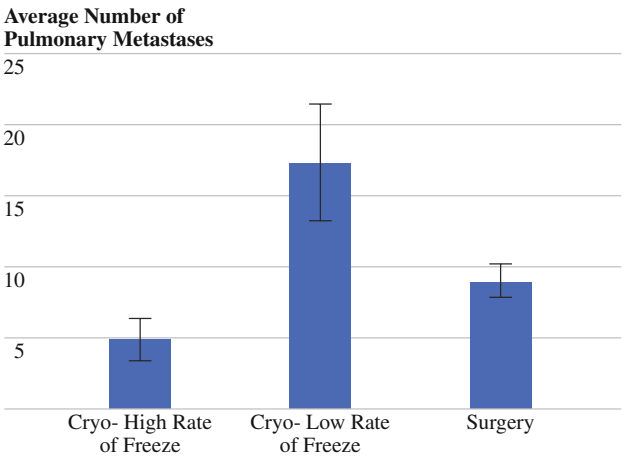

FIG. 1 Pulmonary metastases after cryoablation or surgery. In vivo generation of tumors was accomplished by injection of 5×10^4 4T1 breast cancer cells suspended in 50 μL of PBS into the mammary fat pads. When tumors reached 8–10 mm in size, mice were treated by cryoablation (HRF or LRF) or surgery. Mice were euthanized 2 weeks after treatment. Lungs were harvested, insufflated, and stained with a 15% solution of India ink, then bleached by Fekette's solution for enumeration of pulmonary metastases. HRF cryoablation resulted in an average of 4.89 nodules/mouse, significantly fewer than surgery (9 nodules/mouse, $P = .05$) and cryosurgery at a low freeze rate (17 nodules/mouse, $P = .016$). *Error bars* = standard error

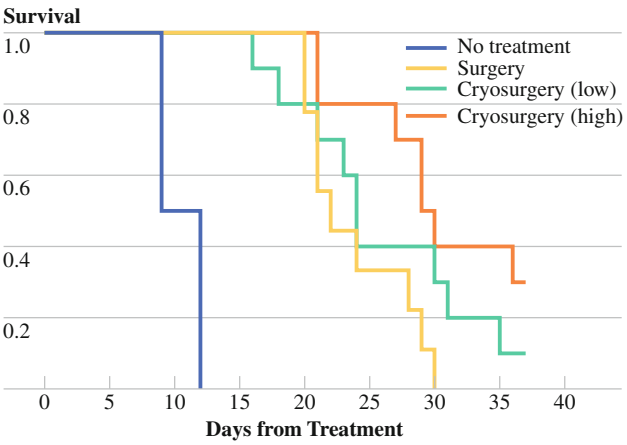

FIG. 2 Kaplan–Meier survival curve after cryoablation or surgery. When 4T1 tumors reached 8–10 mm in size, mice were treated by cryoablation (HRF or LRF) or surgery. Control mice were untreated. Overall, the log rank P value is <.0001. All three treatment modalities were an improvement compared with untreated mice (log rank P value <.0001). Cryosurgery at a high rate of freeze did lead to a significant improvement in survival compared with surgical excision ($P = .013$). However, there was no significant difference between using a low freeze rate and surgery ($P = .18$)

Impact of Cryoablation and Surgery on the Tumor-Draining Lymph Nodes

To better understand differences in the immune response between different freeze rates, FACS immunophenotyping and EliSPOT assay of the TDLN were used. FACS staining was performed 7 days after treatment for cytotoxic T cells ($CD45^+CD3^+CD8^+$), helper T cells ($CD45^+CD3^+CD4^+$), regulatory T cells ($CD45^+CD3^+CD4^+CD25^{high}$), macrophages ($CD45^+CD3^-CD11b^+$), and DC ($CD45^+CD3^-$ CD11b-$CD11c^+$). Relative fractions of the total number of cells harvested from the TDLN/mouse are expressed as the absolute number of cells/mouse within the regional nodes.

The change in T-cell populations induced by intratumoral cytokine release is demonstrated in Fig. 3. Cryoablation and surgery had minimal effect on the absolute number of CD8+ T cells within the TDLN compared with untreated mice. However, CD4+ T cells were dramatically higher in both the low freeze rate cryoablation and surgery groups. We further analyzed the CD4+ population for expression of CD25, a marker of regulatory T cells (Fig. 4). Mice treated by high freeze rate cryoablation had a lower number of regulatory T cells compared with surgery or untreated mice, while mice treated by a low rate of freeze had a higher number of $CD4^+CD25^{high}$ cells.

Despite no increase in T-cell number, the number of tumor-specific T cells within the TDLN was significantly increased by cryoablation with a high freeze rate as evidenced by EliSPOT assay. In the TDLN (Fig. 4), a significant response was obtained with cryoablation using a high freeze rate. There was no significant difference between cryoablation using a low freeze rate or surgery compared with untreated mice. These responses were all highly tumor specific as cryoablation and surgery had no effect on T cells recognizing RENCA.

DISCUSSION

The mechanism by which cryoablation results in tumor cell death is complex and related to the method by which freezing is initiated. Close to the cryoprobe, the rate of freezing is high, inducing rapid freezing of the intracellular fluid, a lethal event associated with irreversible membrane damage. When freezing rates are slower, which typically occur farther from the probe, extracellular fluid may freeze but intracellular fluid is better protected. This leads to an osmotic imbalance. 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. When the tumor thaws, the intracellular compartment is now hypertonic, and as the ice melts fluid rushes into the damaged membranes and the cells burst. Beyond direct cryo-injury, cells are also killed by thrombosis and ischemia resulting from the destruction of endothelial cells, postthaw platelet aggregation, and vascular stasis.

There are several factors that can impact the resultant cryo-induced cell death. These include nonalterable factors such as cell structure and surrounding anatomy and factors

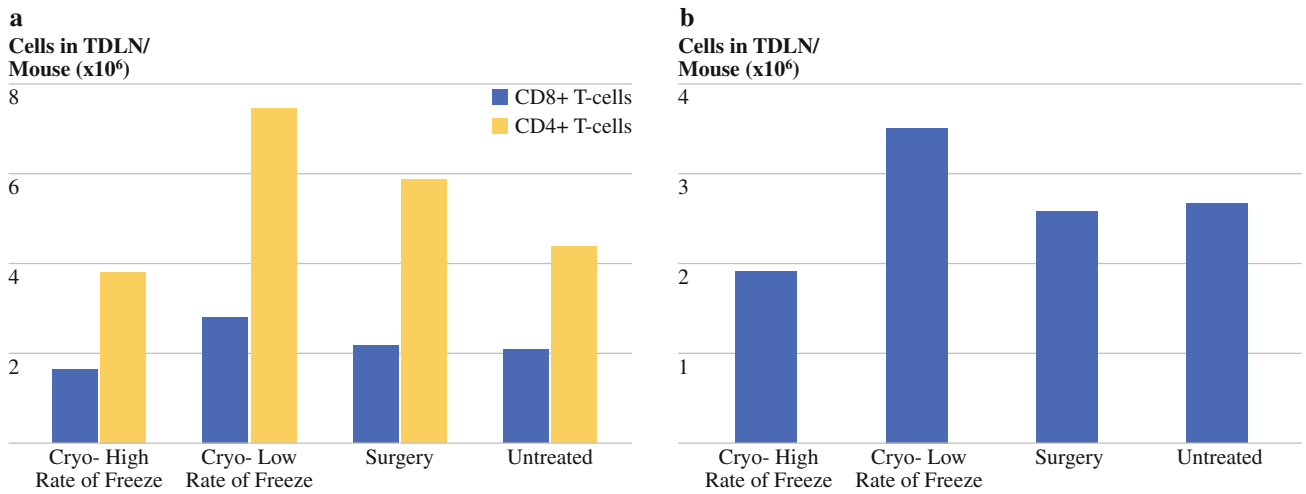

FIG. 3 T-cell populations in TDLN after cryoablation or surgery. Seven days after intratumoral injection of cytokine loaded PLAM. FACS staining was performed for cytotoxic T cells (CD45+CD3+CD8+), helper T cells (CD45+CD3+CD4+) and regulatory T cells ($CD45+CD3+CD4+CD25^{high}$). **a** Cryoablation and surgery had minimal effect on the absolute number of CD8+ T cells within the TDLN compared with untreated mice. However, CD4+ T cells were dramatically higher in both the low freeze rate cryoablation and surgery groups. **b** Mice treated by high freeze rate cryoablation had a lower number of regulatory T cells compared with surgery or untreated mice, while mice treated by a low rate of freeze had a higher number of $CD4^+CD25^{high}$ cells

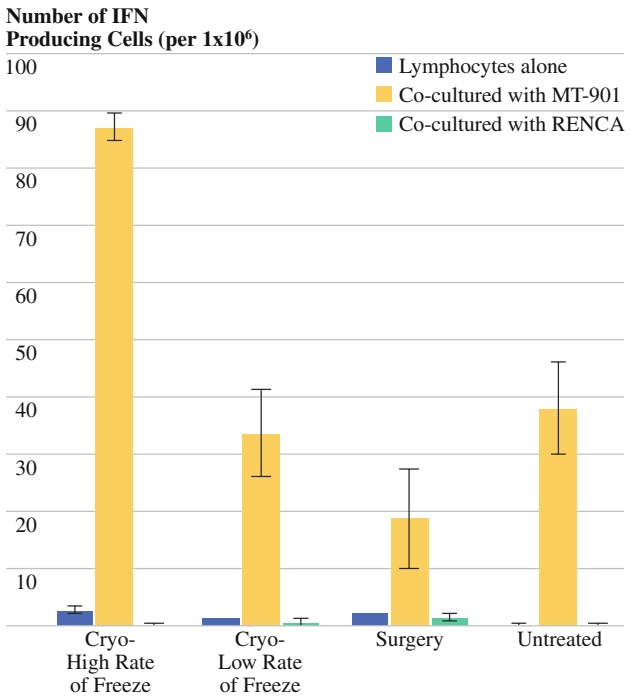

FIG. 4 Regional tumor-specific response after treatment with cytokine-loaded PLAM. Lymphocytes from TDLN were activated with 1 mg/mL anti-CD3 monoclonal antibody (mAb) immobilized in 24-well plates (4×10^6 cells/2 mL/well) for 2 days. The LN cells were subsequently cultured in 60 IU/mL IL-2 for 3 days at 2×10^5 cells/mL. The number of IFN-γ producing cells was measured using ELISPOT assay after intratumoral injection of cytokine-loaded PLAM. Lymphocytes were also co-cultured with RENCA as a control for tumor specificity. HRF cryoablation led to a significant response compared with LRF cryoablation or surgery. These responses were all highly tumor specific as cryoablation and surgery had no effect on T cells recognizing RENCA

that can be manipulated, including the lowest temperature reached, the hold time at that temperature, the number of freeze-thaw cycles, and the freezing and thawing rates. Given the conflicting data regarding cryoablation both stimulating and suppressing an antitumor immune response, we sought to examine whether varying the cryoablation protocol would impact the immunologic response, recognizing that clinical aspects of cryosurgery that may be optimal for complete tumor ablation and may or may not be optimal for the cryo-immune response.

Using the 4T1 model of metastatic breast cancer, we therefore compared two different cryosurgical techniques to surgical excision, a single freeze-thaw cycle at a high rate of freeze (HRF-cryoablation) and a single freeze-thaw cycle at a low rate of freeze (LRF-cryoablation). Our results confirm our previous findings that cryoablation of breast cancer (using a high rate of freeze) can induce a tumor-specific immune response capable of eradicating distant disease and improving outcome. Mice treated by HRF-cryoablation both had a significant decrease in pulmonary metastases compared with mice undergoing surgical excision and a significant improvement in survival. EliSPOT assay demonstrates a dramatic increase in tumor-specific T cells with the TDLN of mice treated with cryoablation.

However, this result is not only absent, but possibly reversed, when the cryoablation is performed at a lower rate of freezing. LRF-cryoablation failed to generate an increase in tumor-specific T cells compared with surgery and ultimately resulted in an increase in pulmonary metastases compared with surgical excision (although this did not reach statistical significance). While HRF-cryoablation significantly improved survival compared with surgery, LRF-cryoablation did not.

Immunophenotyping of the tumor-draining lymph nodes highlights several differences in the immunologic consequences of freezing at a high rate compared with a low rate. A significantly higher number of T cells within the TDLN demonstrated tumor specificity (producing IFN-γ after co-culture with tumor cells) after HRF-cryoablation. LRF-cryoablation did not have a similar response, and while LRF-cryoablation did increase the number of T cells, this was primarily by increasing the number of CD4+ cells. This resulted in an increase in CD4+ CD25+ T cells compared with surgery or HRF-cryoablation. It is therefore conceivable that while HRF-cryoablation led to the generation of tumor-specific CD8 T cells and a subsequent eradication of distant disease and improved outcome, LRF-cryoablation led instead to an increase in regulatory T cells, suppressing CD8 T-cell stimulation, and ultimately resulting in increased metastatic disease. LRF-cryoablation did lead to a dramatic increase in macrophages within the TDLN compared with HRF-cryoablation or surgery.

Based on these results, one may hypothesize that the immunologic consequences of cryoablation may be related to the method of cell death induced by the freeze. The danger theory proposed by Matzinger suggests that the generation of an immune response is not simply a matter of self and non-self, but also dangerous and not dangerous, and requires a third signal beyond recognition of the peptide antigen with the T-cell receptor and the interaction of co-stimulatory molecules on the APC cell surface and T cell.[26,27] Also known as "danger signals," their release is often dependent on the mechanism by which cells in the body die. Cryoablation-induced necrosis is characterized by cellular breakdown, disruption of tissue architecture, and release of several immunostimulatory factors, including heat shock proteins (HSP), DNA, and RNA, which are recognized by Toll-like receptors uric acid, the chromosomal protein HMGB1 (high-mobility group box chromosomal protein 1), fibrinogen, oligosaccharides of hyaluronan, extra domain A (EDA)-containing fibronectin, and heparin sulfate proteoglycan.[28–32] In contrast, the

recognition and phagocytosis of apoptotic cells is mediated by a large number of receptors and opsonins that 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 proinflammatory cytokine release and increasing TGF-B1 production.[33,34] Several studies have shown that apoptosis may suppress a resultant immune response, although some studies have suggested that apoptotic tumor cells may be superior or a combination of apoptosis and necrosis is the ideal scenario.[35–46] As the extent of necrosis is related to the rate of freeze, changing the freeze rate might alter the relative numbers of necrotic and apoptotic cells within the tumor microenvironment and thus alter the immune response to tumor antigens.

In summary, cryoablation of breast cancer can generate a tumor-specific immune response that can eradicate systemic micrometastases and improve outcome compared with surgical excision. Cryoablation of breast cancer shows strong potential as a treatment for breast cancer as it may not only treat the primary tumor in a cosmetically appealing manner, but may stimulate an immune response capable of eradicating distant micrometastatic disease and reducing local and distant recurrence. However, the technique used to freeze the tissue may alter the immune response. This may, in part, explain why some experimental data suggest a proinflammatory response to cryosurgery while other reports document immune suppression. Further research into the mechanisms by which cryoablation induces cell death in vivo, how this can be manipulated by changing the method of freezing, and the impact of those changes on the immune system is necessary to identify the optimal cryoablative protocol for both destroying the primary tumor and generating an antitumor immune response while avoiding immune suppression.

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