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Report

Immunologic response to cryoablation of breast cancer

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Summary

Purpose. With improvements in breast imaging and image-guided interventions, there is interest in ablative techniques for breast cancer. Cryosurgery initiates inflammation and leaves tumor-specific antigens intact, which may induce an anti-tumor immune response. To help define the mechanisms involved in the cryoimmunologic response, we compared cryosurgery to surgery in a murine model of breast cancer.

Experimental design. BALB/c mice with MT-901 tumors underwent cryoablation or resection. Mice successfully treated were re-challenged with MT-901 or RENCA. Serum cytokine levels were analyzed by ELISA. Tumor draining lymph nodes (TDLN) and spleens were harvested, lymphocytes were activated and assessed for a specific anti-tumor response by both an interferon-γ (IFNγ) release assay and ELISPOT. NK cell activity was assessed by cytotoxicity against YAC-1, an NK-susceptible cell line.

Results. After re-challenge, tumors developed in 86% of mice treated by surgical excision compared to 16% of mice treated by cryosurgery ($p = 0.025$). Cryoablation of MT-901 had no effect on re-challenge with RENCA. Cryoablation led to significantly higher levels of interleukin (IL)-12 (383.6 pg/ml $\pm$ 32.8 versus 251.6 $\pm$ 16.5, $p = 0.025$) and IFN-γ (1564 pg/ml $\pm$ 49 versus 1244 pg/ml $\pm$ 101, $p = 0.009$), but no changes in IL-4 or IL-10. Tumor-specific T-cell responses were evident after cryosurgery in lymphocytes from TDLN but not from spleen. Cryoablation also increased NK activity compared to surgery (24.5% $\pm$ 17.3 versus 16.5% $\pm$ 5.9, $p < 0.001$).

Conclusion. Cryoablation results in the induction of both a tumor-specific T-cell response in the TDLN and increased systemic NK cell activity, which correlates with rejection of tumors upon re-challenge.

Introduction

In 2004 there will be over 217,000 cases of female breast cancer diagnosed in the United States, making it the most common malignant tumor in women [1]. With no difference in survival between breast conservation therapy and mastectomy, there has been a dramatic shift in surgical therapy towards less radical and disfiguring treatments [2]. Improvements in imaging have increased detection of cancers under 1.0 cm [3], and the technology for image-guided interventions has also improved, accelerating intense interest in non-surgical ablative techniques for treating breast cancer. Several methods for the *in situ* ablation of breast cancer are presently being investigated, including radiofrequency ablation (RFA), cryosurgery, laser interstitial therapy, high intensity focused ultrasound, and focused microwave thermotherapy (FMT) [4, 5].

With the exception of cryosurgery, all the above mentioned techniques use hyperthermia, causing melting and fusing of cell membranes and protein denaturation. In contrast, cryosurgery leaves tumor proteins and tumor-associated antigens intact. The presence of residual tumor antigens in an inflammatory microenvironment can stimulate anti-tumor immune responses [6]. Cryosurgery has been successfully used for the ablation of tumors in the liver [7, 8], prostate [9, 10], kidney tumors [11, 12], the palliative treatment of locally advanced breast cancers [13 14], and the treatment of breast fibroadenomas [15]. In several cases there were reports of effects distant from the primary tumor, suggesting a cryoimmunologic benefit to cryosurgery [13, 16–18]. If the cryoablation of breast cancer can induce an anti-tumor immune response capable of reducing both local and distant recurrence, then this approach may not only be superior to other ablative technologies, but perhaps even to surgical excision. To examine this possibility and define the mechanisms involved in the cryoimmunologic response, we compared cryosurgery to surgery in a murine model of breast cancer.

Materials and methods

Animals

Six- to 8-week-old female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, NE, USA)

and maintained in specific pathogen-free conditions at the Animal Maintenance Facility of the University of Michigan Medical Center.

Tumors

MT-901 is a subline of a murine tumor cell line derived from a dimethylbenzantracene-induced mammary carcinoma in the BALB/c strain. This tumor is weakly immunogenic and expresses MHC class I but not MHC class II molecules. RENCA is an immunogenic murine renal cell carcinoma of spontaneous origin in the BALB/c strain. Line-1 is a BALB/c lung alveolar carcinoma cell line. YAC-1 is an NK-susceptible murine lymphoma cell line. 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, 1 μM sodium pyruvate, 100 μg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamycin and 0.5 μg/ml fungizone, all from Life Technologies, Inc. (Grand Island, NY, USA). Tumors were generated *in vivo* s.c. injection of BALB/c mice with 3×10^5 viable MT-901 cells.

Surgical procedures

Mice were placed in sanitized laminar flow hoods and anesthesized with xylazine & ketamine by intraperitoneal injection, then placed in a prone position and the tumor site prepared with alcohol. Procedures were performed using strict aseptic technique. 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.

A table-top Argon-gas-based cryoablation system (Visica Cryoablation System, Sanarus Medical, Pleasanton, CA, USA), designed to create probe temperatures of –160°C was used to perform cryoablation. Anesthesized mice were placed in a prone position on a heating pad to avoid hypothermia and the tumor site prepared with alcohol. The overlying skin was divided and retracted away from the tumor using strict aseptic technique. The freezing tip of the cryoprobe was placed into the tumor mass under direct vision and a 30-s freeze was accomplished by the flow of Argon gas through the probe. The 30-s freeze was repeated if the entire mass was incompletely frozen. The mass and probe were then allowed to passively thaw and the probe was removed. The skin was then closed over the ablated tumor using interrupted Nylon sutures. All mice were placed under a warming lamp during the recovery period.

Tumor-draining lymph node (TDLN) cells and splenocyte activation

At varying time points after either cryoablation or surgery, TDLN and splenocytes were removed aseptically. Lymphoid cell suspensions were prepared by mechanical dissociation with 25-gauge needles and pressed with the blunt end of a 3-ml plastic syringe in RPMI 1640. Spleen cells obtained from BALB/c mice were treated with ammonium chloride–potassium lysis buffer (0.83% ammonium chloride, 0.1% $KHCO_3$ and 0.004% EDTA) for 1 min to deplete erythrocytes and were washed twice with HBSS. The cells were 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 cells were subsequently cultured in recombinant human interlecukin (IL-2) (Chiron Therapeutics, Emeryville, CA, USA) at 60 IU/ml IL 2 for 3 days at 2×10^5 cells/ml.

In vitro *cytokine release assay*

1×10^6 Activated TDLN or splenocytes were co-cultured with 5×10^5 MT-901 tumor cells irradiated to 15,000 cGy in 2 ml of CM per well of a 24-well tissue culture plate. IL-2 (4 IU/ml) was added at the beginning of the cultures. The supernatents were collected and, after centrifugation, analyzed for interferon-γ (IFN-γ) using commercially available ELISA kits from PharMingen. Standard curves were established per manufacturer's instructions with serial two-fold dilutions. Experimental values were computed with the use of regression analysis.

Serum cytokine levels

One hundred micro litres of blood was obtained from mice 1 day after treatment (either cryosurgery or surgery) by a standard tail vein bleed. The specimens were centrifuged and the serum was collected and analyzed for IFN-γ, interleukin (IL)-12 IL-4 and IL-10 using commercially available ELISA kits from PharMingen. A standard curve starting at 1000 U/ml was established with 11 serial two-fold dilutions was performed. Experimental values were computed with the use of regression analysis.

ELISPOT assay

The number of IFN-γ producing cells was measured using ELISPOT assay at varying time points after either cryoablation or surgical excision. Briefly, 96-well plates were coated with antimouse IFN-γ antibody (Pharmingen). Activated splenocytes (2×10^5 cells/well) were cultured for 48 h at 37 °C in a 5% CO_2 incubator alone or in the presence of 5×10^5 irradiated MT-901 or RENCA tumor cells. After that time, wells were washed and incubated overnight at 4 °C with a different clone of biotinylated anti IFN-γ antibody (PharMingen). Reactions were visualized and counted using anti-biotin-AP.

Cytotoxicity assay

To examine NK cell function, we performed a cytotoxicity assay against YAC-1, an NK-susceptible cell line

[19]. Briefly, activated effectors were plated in 96 well plates, in 10% FBS/DMEM complete media (supplemented with HEPES, Sodium pyruvate, non-essential amino acids, 2-ME, and Pen/Strep) at (1:1) serial dilutions starting with 1×10^6 cells/well. YAC-1 targets were labeled with ^{51}Cr at 0.15mCi/6×10^6 cells/530 μl for 90 min at 37°C under an atmosphere of 7.5% CO_2. Targets were then washed three times with 40 ml media, and plated at effector:target ratios beginning at 40:1. Spontaneous and total ^{51}Cr release were calculated from media plus targets only, and targets plus 1% Triton-X-100, respectively. Plates were centrifuged at 1000 rpm for 3 min, then cultured for 4 h at 37°C under an atmosphere of 7.5% CO_2. At the end of the incubation period, 20 μl of supernatant was collected and applied to a Lumaplate, dried, and counts were read using a TopCount NXT scintillation/luminescence microplate reader. The % Specific Lysis was calculated using the formula {% Specific Lysis = 100% × (Lysis − Average Spontaneous Lysis)/(Average Total Lysis − Lysis)}. The percentages of specific lysis were averaged for each data point (E:T ratio) to obtain standard deviations and graphed using Deltagraph software. The *p* values were determined using *t*-test assuming equal variances.

Results

Cryoablation results in increased protection against re-challenge compared to surgical resection

In order to demonstrate whether mice that underwent cryoablation of subcutaneous MT-901 tumors developed an anti-tumor immune response, successfully treated mice underwent tumor re-challenge. BALB/c mice were inoculated with 3×10^5 MT-901 cells in the right flank. On day 11, all mice with palpable subcutaneous tumors underwent either cryoablation or surgical resection of their tumors. Two weeks later, all tumor-free mice (80%) were re-inoculated on the left flank with an identical tumorigenic dose of MT-901 cells. To gauge the tumor-specific nature of this response, mice treated by cryosurgery were re-inoculated with RENCA cells. By day 10 after re-inoculation, six of seven mice treated by surgical excision had grown tumors in the left flank whereas only one of six mice treated by cryosurgery grew tumors ($p = 0.025$). Four of five mice treated for MT-901 tumors by cryoablation grew RENCA tumors, suggesting this was a tumor-specific immune response (Figure 1).

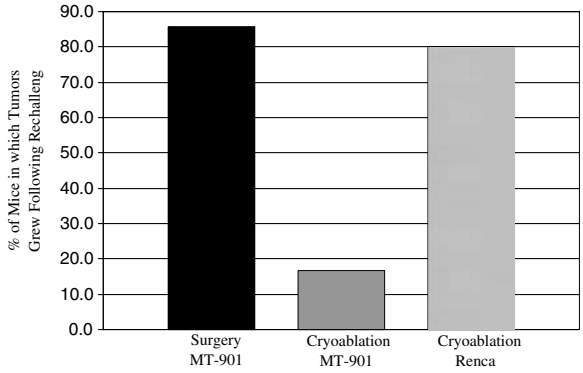

Figure 1. Tumor re-challenge after cryosurgery or surgical excision. BALB/c mice underwent cryoablation or surgical excision of subcutaneous MT-901 tumors. Two weeks later they were re-challenged with tumorigenic doses of either MT-901 or RENCA cells. By day 10 after re-inoculation, six of seven mice treated by surgical excision had grown tumors in the left flank. In mice treated by cryosurgery, only one of six mice grew tumors ($p = 0.025$). Four out of five mice treated for MT-901 tumors by cryoablation grew RENCA tumors, suggesting this was a tumor-specific immune response.

Cryoablation results in an increase of Th1-type cytokines but not Th2-type cytokines compared to surgical excision

In order to evaluate the mechanisms contributing to this response, blood draws were performed 24 h after either cryoablation or surgical excision, and serum levels of Th1 (IL-12, IFN-γ) and Th2 (IL-4, IL-10) cytokines were measured using commercially available ELISA kits. Groups of five mice were compared by unpaired Student's *t*-test. The results are summarized in Figure 2. Cryosurgery was associated with significantly higher levels of both IL-12 (383.6 pg/ml ± 32.8 versus 251.6 ± 16.5, $p = 0.025$) and IFN-γ (1564 pg/ml ± 49 versus 1244 pg/ml ± 101, $p = 0.009$) compared to mice undergoing surgical excision. Serum levels of IL-4 and IL-10 were not significantly different.

Cryoablation induces tumor-specific pre-effector cells regionally but not systemically

Mice with MT-901 tumors treated by either cryoablation or surgical excision were sacrificed at varying time points, and lymphocytes from both TDLN and spleens were evaluated *in vitro*. Lymphocytes from mice treated by cryoablation secreted dramatically higher release of IFN-γ in response to MT-901 tumor cells than to Line-1 cells (an irrelevant tumor) after treatment (Figure 3). Mice treated by surgical excision exhibited similar responses to MT-901 and Line-1.

The tumor specific response observed in TDLN was not seen in splenocytes. As shown in Figure 4, lymphocytes secreted equivalent amounts of IFN gamma in both treatment groups. Similar responses were observed to MT-901 or Line-1 cells whether mice had been treated by cryosurgery or surgical excision. Similar results were seen on day 19. To evaluate further the systemic T-cell response, IFN-γ producing cells that specifically recognized MT-901 cells were counted by ELISPOT assay. Cryoablation and surgical excision resulted in similar responses to MT-901 cells (Figure 5).

Cryoablation leads to increased NK cell cytotoxicity compared to surgical excision

Mice treated were killed 1 week after treatment, and the splenocytes harvested and cultured overnight with IL-2.

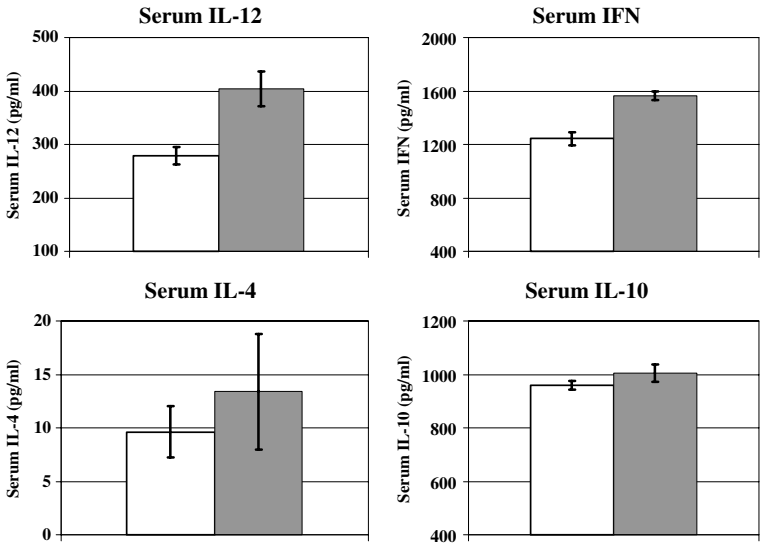

Figure 2. Serum cytokine levels after cryoablation or surgical excision. Blood was drawn 24 h after BALB/c mice underwent either cryoablation or surgical excision of subcutaneous MT-901 tumors. ELISA was used to measure the presence of Th1 (IL-12, IFN-γ) and Th2 (IL-4, IL-10) cytokines. Groups were compared by unpaired student's *t*-test. A significant higher level of both IL-12 (383.6 pg/ml $\pm$ 32.8 versus 251.6 $\pm$ 16.5, $p = 0.025$) and IFN-γ (1564 pg/ml $\pm$ 49 versus 1244 pg/ml $\pm$ 101, $p = 0.009$) was seen in mice who underwent cryosurgery as compared to mice who underwent surgical excision. There was no significant difference noted for IL-4 or IL-10. Error bars represent standard deviation (SD).

NK function was measured in a chromium release assay against YAC-1 cells. Statistical analysis was performed using unpaired Student's *t*-test. The results are shown in Figure 6. Mice treated by cryoablation demonstrated increased NK activity at an E:T ratio of 40:1 (24.5%) versus mice treated by surgery (16.5%, $p < 0.001$) or naïve BALB/c mice (9.6%, $p < 0.001$).

Discussion

The use of freezing temperatures to treat breast cancer is not a new concept. Over a century ago, irrigation devices were designed to bring iced saline solutions in contact with advanced breast tumors, resulting in the amelioration of pain and bleeding, and a reduction in the size of the tumor [20–22] The clinical applicability of cryosurgery expanded with the introduction of automated cryosurgical units that pump liquid nitrogen through a metal probe. Tanaka treated 49 patients with advanced or recurrent breast cancer with cryosurgery, reporting not only alleviation of pain, control of hemorrhage, and reduction of tumor bulk, but also a 5-year survival of 44.4% in this group of 'incurable' patients [14]. Suzuki described eight patients with stage IV breast cancer who had advanced primary tumors treated by cryosurgery. In addition to palliation of the primary tumor, two of the eight patients

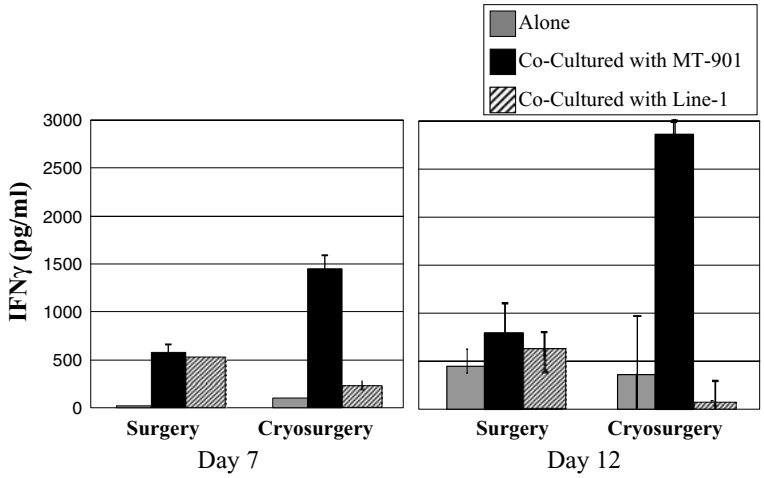

Figure 3. Response in tumor draining lymph nodes after cryoablation or surgical excision. Mice with MT-901 tumors treated by either cryoablation or surgical excision were sacrificed 7 and 12 days after treatment, and TDLN were harvested for CD3/IL-2 activation. 1×10^6 activated TDLN were co-cultured with irradiated MT-901 or Line-1 tumor cells in the presence of IL-2 (4 IU/ml). The supernatents were collected and analyzed for IFN-γ by ELISA. Mice treated by cryoablation showed a dramatically higher release of IFN-γ in response to MT-901 tumor cells than to Line-1 cells at both time points.

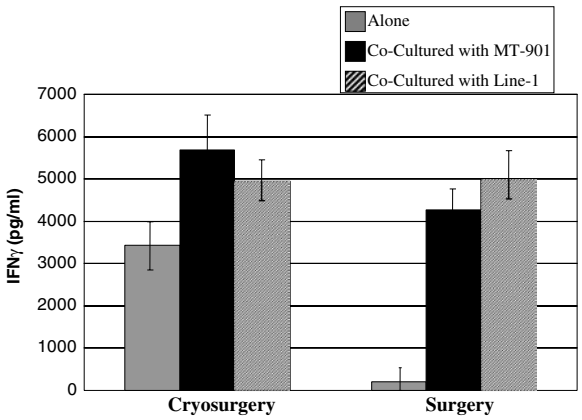

Figure 4. Response in splenocytes after cryoablation or surgical excision by IFN-γ release assay. Mice with MT-901 tumors treated by either cryoablation or surgical excision were sacrificed 7 days later, and spleens were harvested for CD3/IL-2 activation. 1×10^6 activated splenocytes were co-cultured with irradiated MT-901 or Line-1 tumor. The supernatents were collected and analyzed for interferon-γ (IFNγ) by ELISA. Although there appeared to be a non-specific activation of lymphocytes, there was no significant difference in the tumor-specific response between cryoablation and surgery. These results were identical up to 3 weeks after treatment.

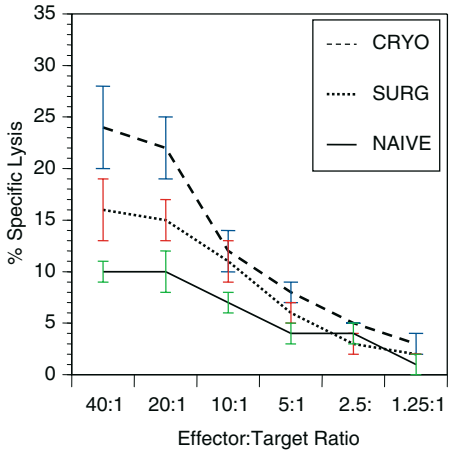

Figure 6. Natural killer (NK) cell function after cryoablation or surgical excision. Mice with MT-901 tumors treated by either cryoablation or surgical excision were sacrificed 7 days later, and spleens were harvested for a cytotoxicity assay against YAC-1, an NK-susceptible cell line. The % Specific Lysis was calculated using the formula {% Specific Lysis = 100% × (Lysis − Average Spontaneous Lysis)/(Average Total Lysis − Lysis)}. The p values were determined using t-test assuming equal variances. Mice treated by cryoablation demonstrated increased YAC-1 cytotoxicity (24.5% ± 17.3) versus both mice treated by surgery (16.5% ± 5.9, $p < 0.001$) or naïve BALB/c mice (9.6% ± 1.3, $p < 0.001$). The difference between mice with MT-901 tumors surgically excised was significantly increased over naïve mice ($p = 0.002$).

had resolution of distant disease, including regional adenopathy, contralateral metastases and pleural effusion [13]. Clinical observations of sites distant from the regions treated by cryosurgery have also been described in other tumor types [23, 24].

Clinical interest in the *in situ* ablation of breast cancer has resurfaced with improvements in breast imaging and ablative technologies. The majority of these methods are based on thermal destruction of the tumor, most of which (RFA, laser, microwave, focused ultrasound) are hyperthermic. Heating above 50 °C causes cell membranes to melt and fuse, and higher temperatures lead to protein denaturation and irreversible cell death [25, 26]. Cryoablation uses freezing temperatures which exert a much different damaging effect on tumor cells, including the disruption of metabolic pathways, conversion of the lipid layers of the cell membrane into semisolids, breakdown of the cytoskeletal microtubular networks, exposure of the cells to a hyperosmotic environment and the grinding action of irregular ice formations.

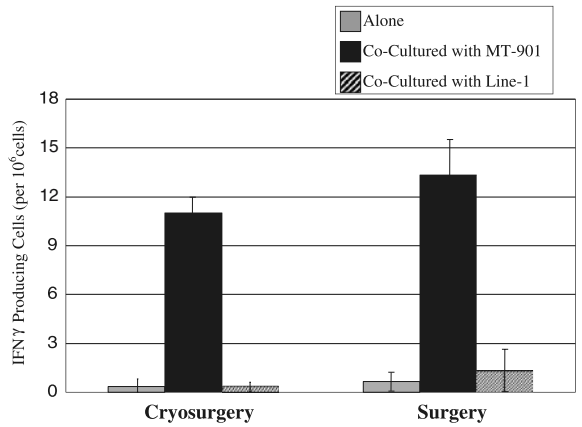

Figure 5. Response in splenocytes after cryoablation or surgical excision by ELISPOT assay. The number of IFN-γ producing cells was measured using ELISPOT assay at varying time points after either cryoablation or surgical excision. Activated splenocytes (2×10^5 cells/well) were cultured for 48 h alone or in the presence of irradiated MT-901 or Line-1 tumor cells in plates coated with anti-mouse IFN-γ antibody. After that time, wells were washed and incubated overnight with a different clone of biotinylated anti-IFN-γ antibody (PharMingen). Reactions were visualized and counted using anti-biotin-AP. ELISPOT assay demonstrated no difference in the systemic T-cell response between cyroablation and surgical excision.

Cryoablation of small breast cancers has recently been demonstrated to be feasible for tumors < 1 cm in size, and invasive ductal carcinomas < 1.5 cm [27, 28] Radiofrequency ablation and laser interstitial therapy have also been described to be successful for tumors < 1.5 cm size [29, 30]. There are advantages and disadvantages to all of these approaches, including potential side effects, clinical applicability, or anesthesia requirements. However, if cryosurgery can induce an anti-tumor immune response capable of preventing recurrence, it may prove to be superior not only to other ablative technologies, but to lumpectomy as well.

To elicit the mechanisms behind any cryo-induced immune response, we utilized a weakly immunogenic mammary adenocarcinoma cell line, MT-901, in BALB/c mice. In all experiments, we compared cryosurgery (freezing the tumor and leaving it in place) to surgical resection of the tumor. In previous reports, results have been mixed as to whether cryoablation of breast tumors, as compared to surgical excision, stimulates an immune response capable of preventing re-challenge. Some

studies showed no difference between cryoablation and surgery [31]. Most studies, however, have demonstrated a benefit to cryosurgery, although in some of these cases the response may have been non-specific [32–36]. Our results not only confirm that cryosurgery, can prevent re-challenge but also that this response is tumor-specific, as it did not prevent a secondary non-mammary adenocarcinoma cell line.

More importantly, it is imperative to understand the mechanisms behind this response if it to be utilized clinically. The tissue destruction that occurs during *in situ* freezing is accompanied by an inflammatory response in which many different cytokines may be produced [37]. In fact, this has been of interest in hepatic cryoablation as the release of pro-inflammatory cytokines has been associated with distant organ injury after freezing of large liver tumors ('cryoshock') [38, 39]. The types of cytokines that increase after cryosurgery would influence the type of immune response generated. Therefore, we examined both Th1 and Th2 cytokines in the serum of mice treated by cryoablation or surgical resection. Levels of IL-12 and IFN-γ were significantly increased 24 h after cryoablation compared to surgery, while there were no differences in IL-4 and IL-10. These results suggest that cryosurgery promotes the inflammatory cytokines involved in cell-mediated immunity.

We next examined both T-cell function and NK-cell function after cryosurgery compared to surgery. To look for the presence of a T-cell response, we utilized an IFN-γ release assay of lymphocytes from the tumor draining lymph nodes (TDLN) and spleens. In the TDLN of mice treated with cryoablation, lymphocytes demonstrated a dramatic increase in IFN-γ when cultured in the presence of irradiated MT-901. This was a tumor-specific response, as lymphocytes cultured with Line-1 did not stimulate IFN-γ production. This response was not seen in mice undergoing surgical excision.

This tumor specific response raises the possibility that cryoablation may be a beneficial adjunct to adoptive immunotherapy; the passive administration of cells with anti-tumor activity to the tumor-bearing host. The primary obstacle to adoptive immunotherapy has been generating sufficient numbers of tumor-specific cells for transfer, as there are few among the peripheral blood lymphocytes and the harvesting of tumor infiltrating lymphocytes from breast tumors is technically challenging. In some breast cancer patients, tumor-reactive T-lymphocytes expanded from regional lymph nodes demonstrate tumor-specific responses [40, 41]. Cryoablation of tumor prior to harvest of the regional lymph nodes may provide a richer source of tumor-specific T-cells for eventual transfer back to the patient.

Systemically, although there appeared to be an increased non-specific *in vivo* stimulation of T-cells with cryosurgery, there was no significant increase in tumor recognition as measured by cytokine release assay of the splenocytes. IFN-γ ELISPOT assay confirmed no change in tumor-specific T-cells between mice that underwent cryoablation or surgical resection at either 2 or 3 weeks after treatment. In contrast to this finding, systemic NK cell responses increased after cryoablation. Cryoablation of MT-901 in BALB/c mice also led to an increase in both serum IL-12 and IFN-γ, both of which have been shown to augment NK cell proliferation and activation [42]. These results are consistent with Bayjoo et al. [43]. who demonstrated an increase in NK cell cytotoxicity after cryosurgery. Although NK cells are not considered to be tumor-specific, a subpopulation called NKT cells, which express both NK cell receptors and a T-cell receptor, demonstrate antigen specific cytotoxicity [44, 45]. NKT cells are activated by IL-12 [46], and may be responsible for the tumor-specific prevention of re-challenge after cryoablation seen in this model.

The majority of the evidence for cryoimmunologic responses comes from reports of distant disease resolving after ablation of a primary tumor. Several isolated studies from the 1980's attempted to characterize the immunologic changes in patients undergoing cryoablation of oral cavity cancers [47, 48] rectal cancer [49, 50] or breast cancer [13]. Although they demonstrated increases in non-specific markers of immune response, they were limited by the available immunologic assays. More recently, Ravindranath et al. [51] demonstrated that cryoablation of colorectal metastases to the liver led to the augmentation of serum antibodies against cancer-associated gangliosides. This rise in anti-tumor antibodies was not seen with RFA or surgical resection. The authors suggest that cryosurgery is similar to autologous vaccines because it releases autologous tumor-antigens, thereby augmenting the immune response to the tumor itself. The clinical feasibility of autologous tumor vaccines for breast cancer patients is extremely limited given the small amount of tumor available and the difficulty in establishing breast cancer cell lines. Cryoablation of breast cancer, even if it needs to be followed by lumpectomy for local control, may therefore be an attractive form of immunotherapy that can help prevent recurrence.

Additional information is needed on the mechanisms by which cryoablation may stimulate tumor immunity in order to amplify the response. The results of these studies demonstrate that cryoablation is superior to surgical excision in terms of stimulating T-cell and NK cell activity and in preventing tumor growth after re-challenge. This immunologic response is clearly limited, however, because a brisk T-cell response was present in the tumor draining lymph nodes, but not in the splenocytes. As with autologous vaccines, it may be necessary to augment this immunologic response with an adjuvant. Several studies have demonstrated that the immune response initiated by cryoablation can be amplified by injecting BCG into the lesion [52, 53]. One could envision a combination of cryoablation and intralesional cytokines [54] or systemic cytokines to achieve this goal. Further research on the optimum methods for increasing the cryoimmunologic response and realizing the clinical benefit in the treatment of breast cancer is warranted.

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