Cryotherapy with Concurrent CpG Oligonucleotide Treatment Controls Local Tumor Recurrence and Modulates HER2/neu Immunity

Jesse J. Veenstra, Heather M. Gibson, Peter J. Littrup, Joyce D. Reyes, Michael L. Cher, Akira Takashima, and Wei-Zen Wei

Abstract

Percutaneous cryoablation is a minimally invasive procedure for tumor destruction, which can potentially initiate or amplify antitumor immunity through the release of tumor-associated antigens. However, clinically efficacious immunity is lacking and regional recurrences are a limiting factor relative to surgical excision. To understand the mechanism of immune activation by cryoablation, comprehensive analyses of innate immunity and HER2/neu humoral and cellular immunity following cryoablation with or without peritumoral CpG injection were conducted using two HER2/neu+ tumor systems in wild-type (WT), neu-tolerant, and SCID mice. Cryoablation of neu+ TUBO tumor in BALB/c mice resulted in systemic immune priming, but not in neu-tolerant BALB NeuT mice. Cryoablation of human HER2+ D2F2/E2 tumor enabled the functionality of tumor-induced immunity, but secondary tumors were refractory to antitumor immunity if rechallenge occurred during the resolution phase of the cryoablated tumor. A step-wise increase in local recurrence was observed in WT, neu-tolerant, and SCID mice, indicating a role of adaptive immunity in controlling residual tumor foci. Importantly, local recurrences were eliminated or greatly reduced in WT, neu-tolerant BALB NeuT mice. Cryoablation of human HER2+ D2F2/E2 tumor enabled the functionality of tumor-induced immunity, but secondary tumors were refractory to antitumor immunity if rechallenge occurred during the resolution phase of the cryoablated tumor. A step-wise increase in local recurrence was observed in WT, neu-tolerant, and SCID mice, indicating a role of adaptive immunity in controlling residual tumor foci. Importantly, local recurrences were eliminated or greatly reduced in WT, neu-tolerant, and SCID mice when CpG was incorporated in the cryoablation regimen, showing significant local control by innate immunity. For long-term protection, however, adaptive immunity was required because most SCID mice eventually succumbed to local tumor recurrence even with combined cryoablation and CpG treatment. This improved understanding of the mechanisms by which cryoablation affects innate and adaptive immunity will help guide appropriate combination of therapeutic interventions to improve treatment outcomes.

Introduction

As immunotherapy becomes a mainstay in cancer therapy, attention is directed to immune constituents in the tumor microenvironment, particularly the modulation of their activities to enhance treatment outcomes. In parallel with this progress is the advancement in image guided percutaneous cryoablation that utilizes ultra-cold temperatures to precisely destroy cancers of the breast, prostate, kidney, liver, bone, lung, brain, and skin (1). Cryoablation directly induces necrosis by damaging cell membranes and organelles via the formation of ice crystals, and indirectly through osmotic stress and ischemia from thrombosis of the microvasculature (2). Compared with surgical resection, cryoablation is minimally invasive, places less stress on the body, allows for quicker recovery, and is less costly (3). In addition to debulking the tumor, the necrotic tissue becomes a rich reservoir of tumor-associated antigens that are cleared by antigen-presenting cells (APC), creating a unique opportunity to prime or boost systemic antitumor immune responses, which may afford increased survival (4).

Induction of systemic immunity was initially observed in the 1970s when several patients had metastatic lesions regress following cryoablation of primary prostate tumors (5). Further support of “cryoimmunology” was linked to an increase in antibodies against DNA, RNA, and tumor cells in patients receiving palliative cryoablation for advanced cancer (6). More recently, a study following 20 patients with prostate cancer observed elevated levels of circulating inflammatory cytokines and cellular immunity after cryotherapy but found responses were transient and unable to prevent disease relapse (7). In a separate study, cryoablation of metastatic renal cell carcinoma resulted in elevated T cell and antibody (Ab) responses without affecting the growth of untreated foci (8). Although these results stimulated interest in the immunostimulatory potential of cryoablation, mechanisms leading to beneficial immunity have yet to be elucidated.

Although enhanced immune priming after cryoablation has been described in a number of preclinical studies (9–11), others...
indicate that cryoablation does not elicit any change in tumor-specific immunity (12–14), or worse, induces immune suppression and tumor progression (15–17). The inconsistencies in tumor-specific immunity and rejection of distant tumors reflect an inadequate understanding of the mechanisms of immune priming and suppression associated with cryoablation. The discrepancy in findings is, at least in part, due to the wide range of tumor models assessed and their varying immunogenicity in respective hosts.

To begin elucidating and exploiting the immunologic mechanisms of cryotherapy, we evaluated antitumor immunity following cryoablation of BALB/c mouse mammary adenocarcinomas TUBO and D2F2/E2, which respectively express rat neu and human HER2 and exhibit well-characterized immunogenicity in wild-type (WT) and neu-tolerant transgenic mice. To further amplify and modulate cryoablation-induced immunity, we also tested a Toll-like receptor (TLR) 9 agonist, CpG oligodeoxynucleotides. Dendritic cells (DC) and B cells are the primary cell types that express TLR9, although mice have additional expression on monocytes and macrophages (18). Activation of these cells by CpG initiates stimulatory pathways that result in the indirect maturation, differentiation, and expansion of additional DCs, T cells, natural killer (NK) cells, and macrophages (19–21). These cells subsequently secrete cytokines that generate a proinflammatory and strongly Th1 biased environment (21–23). These conditions enhance cytokine-mediated T-cell responses and inhibit Th2-mediated suppression, which is associated with more efficacious antitumor immunity (24). Previous work by den Brok and colleagues initially found that peritumoral injection of CpG immediately following tumor cryoablation results in more robust systemic tumor protection via increased DC maturation and cross-presentation of the model tumor antigen OVA (25, 26). The study focused on DC activation but did not assess antigen-specific Ab responses or other innate immune activation after treatment. In this study, we provide a comprehensive evaluation of innate immunity and α-HER2/neu adaptive immunity following cryoablation with or without CpG as well as tumor excision using two tumor systems in WT, immune-tolerant, and SCID mice. Importantly, we assessed the impact of peritumoral CpG injection on local recurrence, which is a potential clinical limitation for cryoablation of locally aggressive or high-risk tumors (27, 28).

Materials and Methods

Mice

Female BALB/c and SCID/NCR (BALB/c background) mice (6–8-week-old) were purchased from Charles River Laboratory. Heterozygous C57BL/6 pL1-β-DsRed transgenic mice have previously been described (29). Heterozygous IL-1β-DsRed (BALB/cxC57BL/6) F1 mice were generated by crossing heterozygous C57BL/6 DsRed males with WT BALB/c mice and screened for transgene expression. Heterozygous BALB/NeuT female mice, which express a transforming rat neu, develop atypical ductal hyperplasia at 3 weeks of age that progresses to carcinoma in situ and then palpable tumors between 16 weeks and 18 weeks of age (30, 31). All animal procedures were approved by and performed in accordance with the regulation of Wayne State University Animal Investigation Committee (Detroit, MI).

Cell lines

The neu TUBO line cloned from a spontaneous mammary tumor in a female BALB NeuT mouse was obtained through Dr. Guido Forni (University of Torino, Torino, Italy; ref. 32). The D2F2 line was established in our group from a spontaneous mammary tumor that arose from the prolactin-induced hyperplastic alveolar nodule line, D2 (33). The mouse origin of TUBO and D2F2 was verified by spectral karyotyping (34) and aliquots of frozen stock were thawed for short-term culture in each experiment. Each individual culture was verified by flow cytometry using mAb M1/42 to BALB/c MHC I BALB/c mice inoculated with TUBO or D2F2 cells develop progressive tumors to validate their BALB/c origin. D2F2 cells transfected with human HER2 (35) were passaged through BALB/c mice to select D2F2/E2 that maintains HER2 expression in vivo. APCs 3T3/EBK and 3T3/NKB were generated by transfecting NIH3T3 cells (American Type Culture Collection) with Kd, B7.1 (CD80), and HER2 (EBK) or neu (NKB) as we described (36). Expression of these molecules is monitored monthly by flow cytometry.

Tumor inoculation and DNA vaccination

Mice were inoculated with 2.5 × 10^6 tumor cells in mammary fat pad #4 (left) or #9 (right). Tumor growth was monitored by palpation and caliper measurement. Tumor volume was calculated as v = (l × w^2) / 2. Recurrent tumors were defined as new growth at the primary site after treatment completion.

For DNA vaccination, an admix of 30 μg each of pGM-CSF and pNeu-E2TM encoding a rat neu and human HER2 fusion protein or pVax1 (control) in 50 μL PBS was injected intra-muscularly in the left gastrocnemius followed immediately by application of electrode gel and square wave electroporation using a BTX830 (BTX Harvard Apparatus; ref. 35).

Cryoablation and surgical procedures

Cryoablation was performed on tumors approximately 4 × 7 mm (~60 mm^3) in size, using the argon-based CryoCare system with the 1.7-mm diameter PERC-15 Percryo CryoProbe—round ice (Endocare). Briefly, an ellipse of skin over the tumor was removed, and the tumor was retracted without interrupting tumor vasculature. The cryoprobe was longitudinally inserted through the tumor and freezing was initiated at 100% power for 1 minute reaching −150°C, followed by the thawing cycle, which lasted approximately 1 minute. After two freeze–thaw cycles were completed, the skin was closed over the tumor. Sham surgery was identically performed without freezing and thawing. Surgical excision was performed using electrocautery to remove the tumor and adjacent mammary tissue. Incisions were closed using surgical staples.

CpG ODN mm2395

The murine-specific class C CpG sequence: 5′-TCGACG-TTTTTCCGCCGCGGCGCGG-3′ with a phosphorothioated backbone (Integrated DNA Technologies) was designed by
antigen stimulation and multiplexing

Y follows day X. The sum of values for all time points makes up the AUC.

Dickinson; MICR core), using HER2-expressing SKOV3 cells (day X).

was measured for each mouse using the equation ((day Y) −

chem) equivalent for HER2 and neu-binding Ab, respectively,

plate (8

of

ImageJ densitometry software. Hematoxylin and eosin (H&E)

tissue relative to tumor (10

mammary tissue relative to tumor (10

m10

were incubated for 15 minutes on ice in PBS) with anti-mouse CD16/CD32 (2.4G2; BD Pharmingen).

plates were collected with white light images. Mean DsRed fluorescence of each tumor slice was quantified using ImageJ densitometry software. Hematoxylin and eosin (H&E) staining was performed in the Animal Model and Therapeutics Evaluation Core. Histology images were captured using the SCN-400 slide scanner and software (Leica Microsystems).

Subcutaneous melanoma (B16F10) xenografts were established in BALB/cxC57BL/6 F1 mice and imaged immediately in the In Vivo MS FX PRO (Carestream) in the Microscopy, Imaging, and Cytometry Resources (MICR) core. Fluorescent spectra collected for 30 seconds were merged with white light images. Mean DsRed fluorescence of each tumor slice was quantified using ImageJ densitometry software. Hematoxylin and eosin (H&E) staining was performed in the Animal Model and Therapeutics Evaluation Core. Histology images were captured using the SCN-400 slide scanner and software (Leica Microsystems).

substituting the human hexamer motif (5'-GTGTTT-3') for

the optimal mouse motif (5'-GAGATT-3') in the C-Class ODN 2395 (37). Of note, 100 μg of CpG was administered percutaneously over three injection sites: lateral, caudal, and rostral mammary tissue relative to tumor (10 μL/injection site).

was calculated by regression analysis. AUC for Ab levels

was measured for each mouse using the equation ((day Y)

Antibody measurement

HER2 and neu-specific IgG levels in the serum were quantified by flow cytometry with a BD FACSCanto II (Becton Dickinson; MICR core), using HER2-expressing SKOV3 cells or neu-transfected 3T3/NKB cells as previously described (38). Normal mouse serum was a negative control. An Ab5 (α-HER2 mAb TA-1, Calbiochem) or Ab4 (α-neu mAb 7.16.4, Calbiochem) equivalent for HER2 and neu-binding Ab, respectively, was calculated by regression analysis. AUC for Ab levels was measured for each mouse using the equation ((day Y) −

In vitro antigen stimulation and multiplexing

Lymph node cells (LN) or splenocytes were enumerated using the Cellometer Vision (Nexcelom) and added to a 24-well plate (8 × 10^5 cells/well). 3T3/EKB or 3T3/KB was treated with 10 μg/mL mitomycin C for 3 hours before coincubation with lymph nodes or splenocytes (8 × 10^6 cells/well). Supernatants were collected after 48 hours.

were incubated with lymph nodes or splenocytes for 48 hours. Spots were enumerated with the ImmunoSpot analyzer (CTL). Results were expressed as spot forming units per 10^6 cells.

Cell phenotyping

Peripheral blood mononuclear cell (PBMC) phenotyping was performed with flow cytometry using a BD FACSCanto II (Becton Dickinson; MICR core). Approximately 2 × 10^6 PBMCs were incubated for 15 minutes on ice in flow buffer (0.25% FBS in 1× PBS) with anti-mouse CD16/CD32 (2.4G2; BD Pharmingen).

Cryotherapy with CpG in Local Recurrence and Tumor Immunity

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Cryotherapy of TUBO mammary adenocarcinoma induces α-neu IgG and systemic tumor protection

To test whether cryoablation induces systemic tumor immunity, BALB/c mice inoculated with neu^+ TUBO adenocarcinoma were treated with cryoablation and tumors were removed 1, 3, 9, and 29 days later for H&E histology (Fig. 1). Complete coagulative necrosis in the ablated tissue was evident by the absence of nuclear staining. Consistent with the classical wound healing process, polymorphonucleocytes (PMN) in the peripheral and perivascular regions of the tumor were apparent 1 day after cryoablation (Fig. 1A) and dissipated by day 3 (40). Macrophage and fibroblast infiltration was evident by day 9 (Fig. 1A). Over the next 4 weeks, fibroblasts continued to expand and produce collagen, indicative of tissue remodeling.

To detect functional inflammatory infiltrates in cryoablated tumors, D2F2/E2 mammary tumor was inoculated into pL1β-DsRed transgenic (BALB/cxCS7BL/6 F1) mice, which utilize IL1β promoter to drive the fluorescent marker gene DsRed. Mice received cryoablation or sham surgery when tumors reached approximately 60 mm^3. On day 15 post-treatment, tissues were removed for ex vivo imaging on a Carestream MS FX Pro in vivo imager (Fig. 1B). Mean densitometry of tumor slices showed significantly greater DsRed fluorescence in cryoablated tumors relative to sham-treated tumors (Fig. 1C). This finding provides direct evidence of IL1β activation, consistent with inflammatory infiltrates in ablated tissues.

IFNγ ELISPOT

Antigen-specific IFNγ production was measured by ELISPOT assay as previously described (38). Engineered APCs were incubated with lymph nodes or splenocytes for 48 hours. Spots were enumerated with the ImmunoSpot analyzer (CTL). Results were expressed as spot forming units per 10^6 cells.

Results

Necrosis and inflammatory infiltration following tumor cryoablation

To evaluate cellular responses to cryoablation, BALB/c mice inoculated with neu^+ TUBO adenocarcinoma were treated with cryoablation and tumors were removed 1, 3, 9, and 29 days later for H&E histology (Fig. 1). Complete coagulative necrosis in the ablated tissue was evident by the absence of nuclear staining. Consistent with the classical wound healing process, polymorphonucleocytes (PMN) in the peripheral and perivascular regions of the tumor were apparent 1 day after cryoablation (Fig. 1A) and dissipated by day 3 (40). Macrophage and fibroblast infiltration was evident by day 9 (Fig. 1A). Over the next 4 weeks, fibroblasts continued to expand and produce collagen, indicative of tissue remodeling.

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Cells were stained with the eFluor 780 viability dye (eBioscience) and the following: α-TCRβ (H57-597), α-CD11c (N418), α-CD49b (DX5). All antibodies were from eBioscience. Data were analyzed using FlowJo software (Tree Star). All populations enumerated as percentage of viable singlets.

Statistical analyses

Statistical analyses were conducted using GraphPad Prism 6. Error bars shown represent SEM unless otherwise noted. Survival percentages were calculated using the Kaplan–Meier method and significance determined by log-rank test (39). For Kaplan–Meier curves, symbols indicate censored subjects due to experimental endpoint. All tests use one-way ANOVA with Tukey’s posttest unless otherwise noted. P values less than 0.05, 0.01, and 0.001 are noted as *, **, and ***, respectively.

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Once cryoablated tumors had fully resolved (~8 weeks), tumor-free mice received a secondary TUBO inoculation on the contralateral side to simulate outgrowth of a distant tumor. Cryoablation of the primary tumor protected 6 of 11 (55%) mice from secondary inoculation, whereas addition of CpG to cryoablation protected 15 of 16 (94%) mice (Fig. 2C and D). Thus, cryoablation of TUBO induced systemic antitumor immunity that was significantly enhanced by concurrent TLR9 stimulation via CpG. In contrast, surgical excision eliminated the primary tumor without triggering immune priming and only 1 of 6 mice rejected the secondary inoculation. These results suggest that cryoablation, but not surgical excision released tumor-associated antigens to prime tumor-specific adaptive immunity.

In a fraction of cryoablation-treated mice, it was noted that primary tumors recurred but not if CpG was concurrently used. To further investigate this finding, recurrences were compiled from four independent experiments to include mice treated with either cryoablation alone or cryoablation + CpG. All mice were treated when tumors were approximately 60 mm³ and monitored for 30 to 90 days (Fig. 2E). Cryoablation alone had a recurrence rate of approximately 26%, occurring between 34 days and 60 days. When CpG was combined with cryoablation, the recurrence rate fell to 0%. Surgical excision of similar size tumors produced no detectable recurrences (n = 24; not shown). Therefore, equivalent long-term recurrence rates as surgical resection can be achieved with cryoablation if CpG is used concurrently in WT mice.

To determine the mechanism of tumor rejection by cryoablation α-neu humoral and cellular immune responses were measured. Several reports have shown that α-neu Ab is sufficient for rejection of TUBO tumor (32, 36, 41). We found inoculation and growth of TUBO in naive mice did not induce α-neu IgG (Supplementary Fig. S1A), suggesting a block or lack of antigen presentation by the untreated tumor. Using DNA electrovaccination as previously described (35), a hybrid rat neu/human HER2 DNA construct (pNeuE2) induced α-neu IgG to correlate with TUBO regression (Supplementary Fig. S1B), suggesting tumor regression by α-neu Ab. Thus, TUBO may resemble Herceptin-sensitive breast cancer, which the immune system can potentially recognize but does not without exogenous manipulation (42).

Mice treated with cryoablation produced α-neu IgG beginning 14 days postoperatively (16 ± 7 µg/mL) and plateaued thereafter (Fig. 3A). Injection of CpG without cryoablation also induced α-neu IgG (22 ± 7 µg/mL), although tumors failed to regress (Fig. 2B). When CpG was used in combination with cryoablation, α-neu IgG levels increased to 58 ± 16 µg/mL at day 41, and remained elevated to at least day 70, indicating immune synergy between cryoablation and CpG injection. AUC analysis found significant differences occurring between cryoablation + CpG and cryoablation groups, as well as between cryoablation and excision groups. Mice undergoing surgical excision produced very low levels of α-neu IgG (1.5 ± 0.03 µg/mL) similar to untreated tumor-bearing mice (Fig. 3A). In addition, cryoablation + CpG induced both IgG1 and IgG2a, whereas cryoablation alone induced primarily IgG1 (Fig. 3B).
These results indicate that cryoablation triggers a Th2-biased response that can be shifted toward a Th1 response with addition of CpG. However, CpG treatment alone was unable to mediate tumor regression despite elevated Ab levels, which argues for concurrent tumor debulking.

To test whether α-neu Ab mediates tumor rejection, we performed an adoptive serum transfer experiment. A single injection of serum from naïve mice, mice treated with cryoablation + CpG or pNeuE2 vaccination, or monoclonal Ab4, were injected into mice inoculated 3 days earlier with TUBO. Cryoablation + CpG serum greatly reduced TUBO growth relative to control serum ($P < 0.01$), and had equivalent activity relative to pNeuE2 vaccination serum and monoclonal Ab4 (Supplementary Fig. S2). Therefore, α-neu Ab induced by cryoablation + CpG plays a significant role in controlling tumor growth.

Tumor-specific cellular activity was assessed from tumor draining lymph node cells (TDLN) and splenocytes after in vitro neu stimulation. IFNγ ELISPOT and 10-plex cytokine analyses of mice treated with cryoablation or cryoablation + CpG showed minimal cytokine production relative to untreated tumor-bearing controls (not shown) despite the significant changes in α-neu IgG. Together these results suggest negligible T-cell activation by cryoablation + CpG of TUBO.

**Cryoablation and α-neu immunity in neu-tolerant BALB/NeuT and immunodeficient SCID mice.**

Cryoablation of neu+ TUBO was further tested in BALB/NeuT mice, which express a transforming rat neu and exhibit immune tolerance to neu (30, 31). Initial studies found cryoablation, with or without CpG injection, insufficient to induce α-neu Ab, consistent with immune tolerance to neu (not...
shown). As in patients with breast cancer, moderate \( \alpha \)-neu immunity can be induced in NeuT mice by DNA vaccination (43, 44). Therefore, we tested whether cryoablation impacts vaccine-induced immunity in these mice. To establish \( \alpha \)-neu immunity, NeuT males were inoculated with TUBO and subsequently electrovaccinated using pNeuE2 at 2 and 4 days after TUBO inoculation (Fig. 4A). When tumors were approximately 60 mm\(^3\) in size, mice were treated with cryoablation \pm \text{CpG} or excision. The majority of tumors treated with cryoablation alone recurred after initial regression, whereas only 1 of 8 mice treated with cryoablation \pm \text{CpG} developed local recurrence (Fig. 4B). Recurrences were compiled from two independent experiments using NeuT males treated with cryoablation alone, cryoablation \pm \text{CpG}, or excision. All mice were treated when tumors were approximately 60 mm\(^3\) and monitored for 40 to 60 days (Fig. 4C). Cryoablation alone had a recurrence rate of approximately 71%, with recurrences detected between 30 days and 43 days after cryoablation. When \text{CpG} was combined with cryoablation, the recurrence rate fell to 12%. No recurrences were detected with excision.

To simulate undetected systemic disease, mice received a secondary TUBO inoculation on the contralateral side 15 days after cryoablation. Neither cryoablation, with or without \text{CpG}, altered vaccine-induced \( \alpha \)-neu IgG (Fig. 4D) or protected mice from secondary tumor growth (Fig. 4E and F). Despite the lack of adaptive immune modulation, addition of \text{CpG} significantly reduced recurrences to a similar level as that of excision. To determine whether this protective effect was mediated by innate immunity, recurrence rates of cryoablation alone and cryoablation \pm \text{CpG} were compared in TUBO-bearing SCID mice (Fig. 4G). All mice were treated when tumors were approximately 60 mm\(^3\) and monitored for 65 days. Treatment with cryoablation \pm \text{CpG} significantly delayed tumor recurrence by >2 weeks, although all but one tumor eventually recurred, indicating partial protection by innate immunity and the importance of adaptive immunity for long-term protection.

**Cryoablation of D2F2/E2 mammary adenocarcinoma**

The impact of cryoablation was further tested in D2F2/E2 mammary adenocarcinoma, which, unlike TUBO, induces significant levels of \( \alpha \)-HER2 IgG1 and splenocyte IFN\(\gamma\) response without exogenous intervention (Supplementary Fig. S3). However, this endogenous \( \alpha \)-HER2 immunity is insufficient to prevent progressive tumor growth. Thus, D2F2/E2 may be representative of HER2\(^+\) tumors, which are immunogenic but refractory to HER2-targeted therapy (34, 45). BALB/c mice were inoculated with D2F2/E2 cells and when tumors reached approximately 60 mm\(^3\), they were treated with cryoablation \pm \text{CpG}, \text{CpG} injection alone, tumor excision, or sham surgery (Fig. 5A). All tumors treated with cryoablation \pm \text{CpG} or surgical excision completely regressed by day 41 with the exception of one mouse in the cryoablation alone group that developed a recurrence on day 29. \text{CpG} treatment alone did not significantly change tumor growth relative to untreated mice (Fig. 5B).

Once cryoablated tumors had fully resolved (~7 weeks), tumor-free mice received a secondary D2F2/E2 inoculation on the contralateral side to simulate outgrowth of a distant tumor. Treatment of the primary D2F2/E2 tumor with excision or cryoablation \pm \text{CpG} resulted in similar protection from the secondary inoculation (~70%–85%; Fig. 5C), suggesting that \( \alpha \)-HER2 immunity induced by D2F2/E2 tumor growth renders systemic protection once the primary tumor is ablated, regardless of the treatment modality. By day 59, mice treated with cryoablation \pm \text{CpG} or tumor excision produced comparable levels of IFN\(\gamma\) after in vitro HER2 stimulation of secondary TDLNs, which was significantly elevated relative to naïve mice, illustrating host reactivity to D2F2/E2 tumor growth (Fig. 5D). Similar to TUBO, AUC analysis of \( \alpha \)-HER2 IgG1 found no significant difference between treatment groups, but IgG2a levels were significantly elevated when cryoablation and \text{CpG} were used concurrently, further supporting the notion of a Th1 shifted response (Fig. 5E). These results indicate that \( \alpha \)-HER2
immunity is induced by D2F2/E2 tumor growth and exerts systemic protection after tumor resolution.

Similar to TUBO, primary D2F2/E2 tumors recurred after cryoablation in a fraction of mice, and these results were compiled from four independent experiments to include mice treated with either cryoablation alone or cryoablation + CpG. All mice were treated when tumors were approximately 60 mm³ and monitored for 30 to 60 days (Fig. 5F). Cryoablation alone had a recurrence rate of approximately 29%, with recurrences detected between 24 days and 58 days. When combined with CpG, the recurrence rate fell to 0%, consistent with activation of innate immunity and manifestation of endogenous adaptive immunity.

**Transient immune refractory period after cryoablation**

Although cryoablation of D2F2/E2 protected mice from a second inoculation once the ablated tumor had completely resolved, the presence of resolving necrotic tumor along with wound healing and inflammation may affect the growth of a coexisting tumor. Thus, we tested the level of tumor protection and growth during the wound healing phase. D2F2/E2 tumor-bearing mice were treated as previously described and a second inoculation was administered on day 13 during the wound healing phase (Fig. 6A). With cryoablation, 1 of 9 (12%) mice were protected from the second inoculation (Fig. 6B), compared with 4 of 5 (80%) mice when inoculated after tumor resolution (day 41; Fig. 5C). Addition of CpG in combination with cryoablation resulted in improved protection with 8 of 16 (50%) mice rejecting the second inoculation, compared with 5 of 6 (80%) after tumor resolution. Secondary tumors that grew in cryoablation/CpG treated mice grew at comparable rates to untreated mice, whereas tumors in the excision group grew significantly slower relative to cryoablation alone.

**Figure 4.** Cryoablation recurrences in tolerant NeuT and immunodeficient SCID mice are significantly reduced with CpG treatment. A, experimental scheme in male BALB/NeuT mice. B, primary TUBO tumor growth (tumor eliminated/total). C, NeuT recurrence-free survival, pooled from two independent experiments. D, total α-neu IgG. E, secondary tumor volume. F, protection from growth of secondary TUBO inoculation. G, SCID experimental scheme and recurrence-free survival. *P < 0.01; **P < 0.001.
All groups had significantly delayed secondary tumor growth relative to naive mice, indicating partial tumor inhibition by endogenous α-HER2 immunity (Fig. 6C). As expected, all tumor-experienced mice produced elevated α-HER2 splenocyte IFN-γ responses relative to naive mice; however, there was no significant difference between treatment groups (Fig. 6D). Therefore, resolving necrotic tumor left by cryoablation results in a transient immune refractory period relative to tumor excision, which can partially be abrogated with CpG.

**Peritumoral CpG treatment activates innate and adaptive immunity**

Peritumoral CpG injection reduces cryoablation recurrences independent of adaptive immunity as observed with NeuT mice (Fig. 4). To evaluate systemic effects from CpG treatment, cytokine levels were examined in plasma on days 2 and 12 after treatment with 10-plex cytokine analyses (Fig. 7A). On day 2, plasma mice treated with CpG, either alone or in combination with cryoablation, showed significant increases in IL1β, IL6, IL12, IFN-γ, and TNF-α relative to all other groups, indicating acute inflammation (Fig. 7B). By day 12, most cytokine levels had subsided, with the exception of IL12, which remained significantly elevated after CpG treatment.

Local and systemic tumor-specific immunity was assessed from TDLNs and splenocytes harvested on day 12 with in vitro HER2 stimulation. IFN-γ production from TDLNs with CpG treatment, with or without cryoablation, was significantly elevated, but no significant differences were found between treatment groups in splenocytes, suggesting CpG effects on adaptive immunity are regionalized to the injection site (Fig. 7C). There was a 3-fold increase in circulating DCs (CD11c+) 2 days after CpG treatment with or without cryoablation (Fig. 7D). NK cells (CD49b+) also significantly increased after treatment with cryoablation + CpG. Similar to plasma cytokine levels, this increase in both DCs and NK cells was transient and dissipated by day 12 (not shown). Therefore, peritumoral CpG injection strongly activates innate immunity and enhances regional adaptive responses with D2F2/E2.

**Discussion**

We show that cryoablation of neu+ TUBO in BALB/c mice resulted in systemic immune priming and tumor protection,
but had little impact in neu-tolerant NeuT mice. Cryoablation of HER2⁺ D2F2/E2 enabled the functionality of tumor-induced immunity but secondary tumors were refractory if rechallenge occurred during the resolution phase of the cryoablated tumor. CpG following cryoablation significantly eliminated, reduced, or delayed tumor recurrences in WT, neu-tolerant, and SCID mice, respectively. Therefore, tumor antigens released by cryoablation induce varying levels of innate and adaptive immunity in different host environments, which is enhanced by CpG. Importantly, innate immunity induced by CpG plays a critical role in controlling local tumor recurrences.

Cryoablation of TUBO in WT mice resulted in α-neu IgG production and systemic protection in the majority of mice. In contrast with previous reports (4), resulting immunity was not Th1 biased but favored a Th2 response as evident by a dominant IgG1 Ab response, and little T-cell activation. To reverse Th2 biased immunity, the Th1 promoting TLR9 agonist CpG was tested in combination with cryoablation. Although CpG monotherapy of TUBO was not capable of mediating tumor regression, cryoablation + CpG resulted in a dramatic increase in α-neu IgG and shifted the response toward Th1, as evident by increased IgG2a levels. A similar, albeit less dramatic enhancement in IgG2a was also observed with D2F2/E2. This response protected nearly 100% of TUBO-bearing mice from secondary inoculation. These findings are further corroborated by Nierkens and den Brok, who reported similar enhanced protection with combined CpG treatment (10, 25).

Notably, tumor recurrences of both TUBO and D2F2/E2 dropped to 0% in WT mice and to 12% in NeuT mice when CpG was concurrently used. Innate immunity significantly contributes to this effect as evident by the delay in tumor recurrence seen in SCID mice. However, long-term recurrence-free survival is dependent on the capacity of adaptive immunity, which is shown by the step-wise increase in recurrences observed in WT, tolerant NeuT, and SCID mice. Increased levels of cytokines, including IFNγ and IL12, correlated with increased levels of circulating DCs and NK cells 2 days after CpG treatment, consistent with innate immunity activation (24, 42). In addition, CpG may also promote the tumoricidal properties of infiltrating macrophages (23) following cryoablation (Fig. 1). For high-risk tumors and oligometastatic disease, residual tumor microfoci and subsequent recurrence are significant clinical consideration following cryoablation (46, 47). The added protection provided by CpG may expand the utility of cryoablation to successfully treat more patients. Furthermore, local CpG injection may enable cryoablation to completely ablate lesions without requiring standard freeze...
Cryoablation of D2F2/E2 uncovered a transient immune response to cryoablation. Although not tested in this study, the use of therapeutics directly targeting immunosuppressive cells, such as Tregs (11, 49, 50), has enhanced cryoablation immune responses, and may further improve responses using cryoablation + Cpg treatment, especially in tolerant hosts. Other means of redirecting tissue inflammation toward a Th1 response and promoting CD8 T-cell activation following cryoablation may also improve outcomes with cryoablation. As new immunotherapeutic options emerge, it is essential to understand the mechanisms by which cryoablation affects antitumor immunity so an appropriate combination of therapeutic interventions can be used to improve clinical outcomes.

**Disclosure of Potential Conflicts of Interest**

P.J. Littrup received a commercial research grant from Galil and Endocare. No potential conflicts of interest were disclosed by the other authors.

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**Authors’ Contributions**

Conception and design: J.J. Veenstra, P.J. Littrup, W.-Z. Wei

Development of methodology: J.J. Veenstra, H.M. Gibson, P.J. Littrup, J.D. Reyes, A. Takashima, W.-Z. Wei

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.J. Veenstra, H.M. Gibson, J.D. Reyes, A. Takashima, W.-Z. Wei

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.J. Veenstra, P.J. Littrup, W.-Z. Wei

Writing, review, and/or revision of the manuscript: J.J. Veenstra, H.M. Gibson, P.J. Littrup, M.L. Cher, W.-Z. Wei

No potential conflicts of interest were disclosed by the other authors.
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.-Z. Wei
Study supervision: P.J. Littrup, W.-Z. Wei

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Cryotherapy with CpG in Local Recurrence and Tumor Immunity

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Cryotherapy with Concurrent CpG Oligonucleotide Treatment Controls Local Tumor Recurrence and Modulates HER2/neu Immunity

Jesse J. Veenstra, Heather M. Gibson, Peter J. Littrup, et al.

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