# Cryoablation in combination with the checkpoint inhibitor anti-CTLA4 increased T cell activation in a murine breast cancer model

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## Background/Hypothesis

A promising area of breast cancer cryoablation research is its combinational use with checkpoint inhibitors to enhance the antitumor response. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is an inhibitory receptor that acts as a negative regulator to T cells. [1]

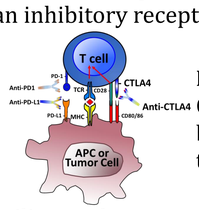


**Figure 1. T cell Modulation/Reactivation.** Checkpoint inhibitors facilitate T cell activation by blocking checkpoint receptors that signal the T cell to "turn off"

Ipilimumab, a monoclonal antibody against CTLA-4, has already been approved for melanoma and is currently being investigated in breast cancer. A pilot study of preoperative single-dose ipilimumab and cryoablation in women with early-stage breast cancer was safe and showed favorable intra-tumoral and systemic immunologic responses. [2] Using a murine cryoablation model for high-risk metastatic breast cancer [3], we investigated cryoablation in conjunction with a CTLA-4 inhibitor to enhance the anti-tumor T cell immune response.

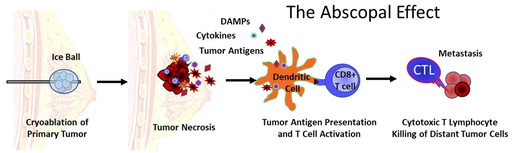


**Figure 2. Schematic of cryoablation induced abscopal effect.**

**Hypothesis:** The checkpoint inhibitor, anti-CTLA4, in conjunction with cryoablation therapy to metastatic cancer increases T cell activation and the abscopal effect.

## Methods

- Balb/C mice were bilaterally transplanted with luciferase expressing metastatic breast cancer cells (4T1-12b-luc) into mammary fat pad.
- At 2 weeks, the mice were separated into two treatment groups. Group 1 (n=5) received cryoablation alone. Group 2 (n=5) received 100 µg anti-CTLA4 intraperitoneal 24-hrs pre-cryoablation as a T cell prime and post-cryoablation as an immune boost. For both groups, cryoablation was only performed on the left tumor, and the right tumor served as proxy for metastatic tumor for abscopal immune readout.
- One-week post-cryoablation, both mouse groups were sacrificed and necropsied for tissue analysis.
- Isolated tissues were homogenized, and lysates were analyzed for systemic anti-CTLA4 antibody distribution by ELISA.
- Peripheral blood (PB) and spleen mononuclear cells were isolated and analyzed for T cell CD4 and CD8 subsets and the activation marker ICOS by flow cytometry.
- Cryoablated (left tumor) and abscopal (right tumor) were analyzed for tumor-infiltrating lymphocyte (TIL) scores by hematoxylin and eosin (H&E) and activated cytotoxic T lymphocytes (CTLs) markers CD8/ICOS and $T_{Reg}$ cell markers CD4/FOXP3 by immunofluorescence.

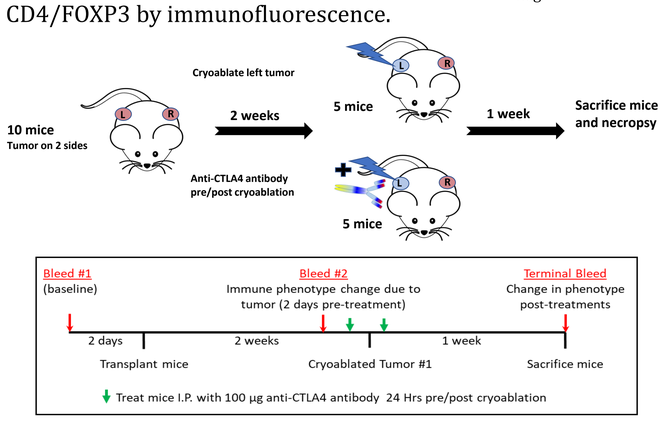


**Figure 3. Schematic of experimental approach and timeline.**

## Results

- Cryoablation completely killed the left tumors for all mice as demonstrated by IVIS analysis.
- Mouse necropsies showed cryoablated tumors undergoing coagulative necrosis.
- Tissue analysis for anti-CTLA4 antibody demonstrated systemic distribution with the highest levels detected in peripheral blood.
- Mice treated with both cryoablation and anti-CTLA4 antibody had an increased percentage of CD4 and CD8 T cells expressing $ICOS^{hi}$ in peripheral blood and spleen compared to cryoablation alone.
- Mice treated with combination of cryoablation and anti-CTLA4 antibody had a significant increase in TILs in the cryoablated tumors.
- Immunofluorescence analysis revealed significant increase of CD4 and CD8 T cells in the cryoablated tumors compared to the abscopal tumors.
- Mice treated with combinational therapy had increased T cells in abscopal tumors with a significant increase in CD4+ T cells compared to cryoablation alone.

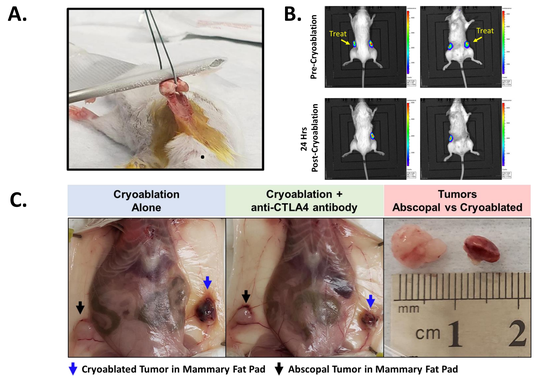


**Figure 4. Representative mouse demonstrating cryoablation technical procedures. A)** Cryoablation of the left mammary 4T1-12b-luc tumor – tumor is completely frozen. **B)** In vivo imaging by IVIS before and 24 Hrs post-cryoablation. **C)** Necropsy of mice for tissue analysis: cryoablated tumor (left) vs. the abscopal tumor (right).

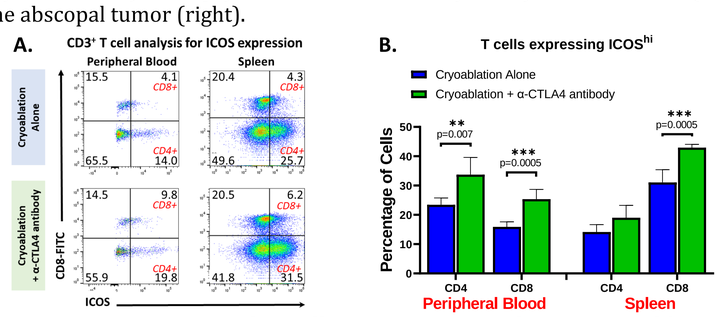


**Figure 6. Comparison of T cell activation 1 week post cryoablation vs. cryoablation + anti-CTLA4. A**) CD4 and CD8 T cell subsets were analyzed for activation by $ICOS^{hi}$ expression. **B**) Percentage of $ICOS^{hi}$ expressing CD4 and CD8 T cells in peripheral blood (n=5) and spleen (n=5). Student T test with $p<0.05$ being statistically significant.

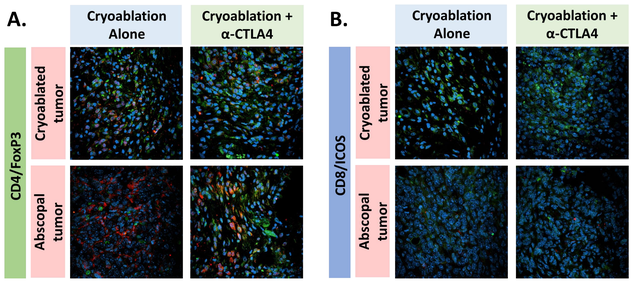


**Figure 8. Tumor analysis for TIL T cell markers by immunofluorescence.** Tumor tissues were stained for $T_{Regulatory}$ cells (CD4/FOXP3) and activated cytotoxic T cells (CD8/ICOS). CD4/CD8 = Green; FOXP3/ICOS = Red; Cell nuclei = blue. **A**) CD4/FoxP3 **B**) CD8/ICOS

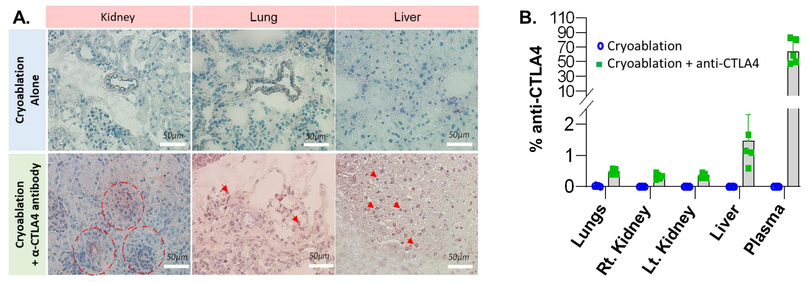


**Figure 5. Biodistribution of anti-CTLA4 antibody in treated mice.** **A**) Immunohistochemistry of tissue sections stained for anti-CTLA4 with DAB stain (brown color). Red arrows depict anti-CTLA4 bound to cells expressing CTLA4. **B**) Quantification of anti-CTLA4 in homogenized tissues.

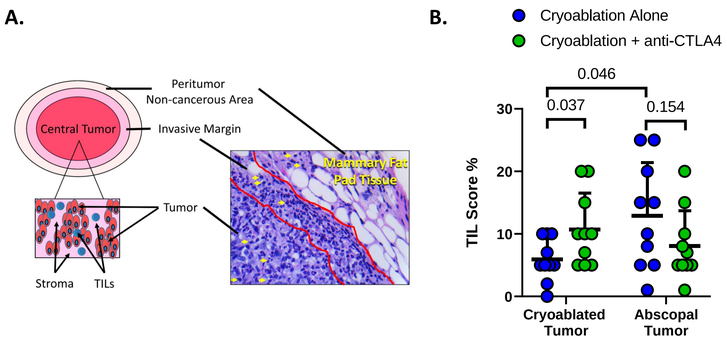


**Figure 7. TIL Scores for cryoablated vs. abscopal tumors.** **A**) Schematic and H&E histopathology demonstrating TILs in each region of the tumor used to score TILs.[3] **B**) Quantitative TIL analysis comparing cryoablated vs cryoablated + anti-CTLA4 treated mice. Student T test with $p<0.05$ being statistically significant.

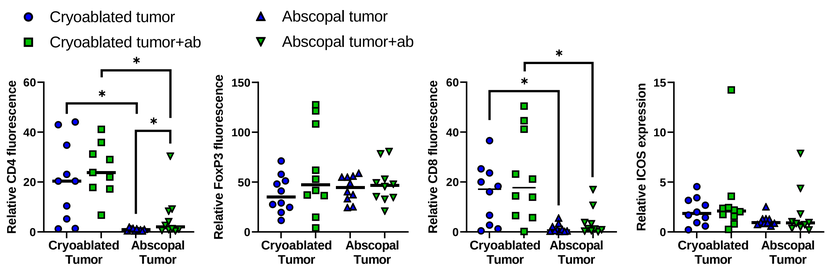


**Figure 9. Quantitative analysis of immunofluorescence for TIL T cell markers.** Immunofluorescence for each marker was quantitated using Fiji-ImageJ software.[4] Student T test with $p<0.05$ being statistically significant.

## Conclusions

**Cryoablation in combination with anti-CTLA4 antibodies increased T cell activation compared to cryoablation treatment alone. The next step is to evaluate whether combinational therapy increases the abscopal effect in controlling metastasis in long-term survival studies in our breast cancer murine model before proceeding to clinical trials.**

**Acknowledgements**

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