Microenvironment and Immunology

Potent Induction of Tumor Immunity by Combining Tumor Cryoablation with Anti–CTLA-4 Therapy

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Abstract
Thermal ablation to destroy tumor tissue may help activate tumor-specific T cells by elevating the presentation of tumor antigens to the immune system. However, the antitumor activity of these T cells may be restrained by their expression of the inhibitory T-cell coreceptor CTLA-4, the target of the recently U.S. Food and Drug Administration–approved antibody drug ipilimumab. By relieving this restraint, CTLA-4–blocking antibodies such as ipilimumab can promote tumor rejection, but the full scope of their most suitable applications has yet to be fully determined. In this study, we offer a preclinical proof-of-concept in the TRAMP C2 mouse model of prostate cancer that CTLA-4 blockade cooperates with cryoablation of a primary tumor to prevent the outgrowth of secondary tumors seeded by challenge at a distant site. Although growth of secondary tumors was unaffected by cryoablation alone, the combination treatment was sufficient to slow growth or trigger rejection. In addition, secondary tumors were highly infiltrated by CD4+ T cells and CD8+ T cells, and there was a significant increase in the ratio of intratumoral T effector cells to CD4+ FoxP3+ regulatory cells, compared with monotherapy. These findings documented for the first time an effect of this immunotherapeutic intervention on the intratumoral accumulation and systemic expansion of CD8+ T cells specific for the TRAMP C2–specific antigen SPAS-1. Although cryoablation is currently used to treat a targeted tumor nodule, our results suggest that combination therapy with CTLA-4 blockade will augment antitumor immunity and rejection of tumor metastases in this setting. Cancer Res; 72(2); 430–9. ©2011 AACR.

Introduction
Thermal ablation treatments such as cryoablation have emerged as alternatives to surgical resection to treat many types of inoperable tumors, including prostate, kidney, liver, bone, adrenal, and lung. Cryoablation involves the insertion of a probe into a tumor nodule to administer tissue ablative freezing temperatures (1). Its mechanism of action has been attributed to the mechanical forces of crystallization, the osmotic changes due to crystallization, and the ischemic effects of microvascular injury (2). Furthermore, as an image-guided, needle-based technique, it can be administered percutaneously, making it less invasive than traditional surgery (3, 4). As a result, it is associated with decreased morbidity and mortality and is more cost effective when compared with conventional therapies such as surgical resection (5).

Following ablation, the necrotic tumor lesion remains within the body, and it has been hypothesized that the release of tumor antigens by dying cells could activate a tumor-specific immune response through antigen presentation by antigen-presenting cells (APC) to T cells. This antigen release is potentially significant because, although ablative procedures are very effective in eradicating the targeted tumor nodule, a tumor-specific immune response may facilitate elimination of distant metastases and prevent recurrent disease. Although a few cases of spontaneous remission of metastases following cryoablation have been reported (6), studies in patients and animal models have revealed weak or absent immune responses after ablation (7), despite the massive release of proteins resulting from tumor cell death observed in animal models (8). It has, therefore, been proposed that the immune response could be augmented if cryoablation is combined with immunotherapies that target APCs or modulate T-cell function. A number of tumor studies combining immunomodulation, such as injection of toll-like receptor agonists, with cryoablation have shown a synergistic effect on tumor rejection, and this was attributed to enhanced activation of APC function (9, 10). Here, we investigate how immunotherapies...
that target the inhibitory pathways in T cells can potentially synergize with cryoablation to generate systemic antitumor immunity.

Monoclonal antibodies (mAb) that block the function of CTLA-4, a transmembrane protein expressed by activated T cells, are a promising new therapy to treat cancer. CTLA-4 inhibits the activation of self-reactive T cells, and it was proposed many years ago that blockade of this pathway, could enhance T-cell responses to tumors. Indeed, in preclinical studies, CTLA-4 blockade led to rejection of immunogenic tumors such as 51Blim10 colon carcinoma and SA/1N fibrosarcoma (11). In additional animal studies, rejection of less immunogenic tumors was achieved when CTLA-4 blockade was combined with a cellular vaccine, or radiation therapy, which likely increase the efficiency of antigen presentation (12–15). Studies in mouse models of prostate cancer have shown decreased metastatic lesions and a reduction of primary tumor incidence when CTLA-4 blockade was combined with surgical resection or a granulocyte macrophage colony-stimulating factor (GM-CSF) secreting tumor vaccine, respectively (16, 17). In addition, CTLA-4 blockade was shown to synergize with thermal ablation in protection of B16 melanoma tumor growth in a prophylactic setting (8, 18).

Clinical trials to validate the efficacy of anti–CTLA-4 mAb (anti–CTLA-4) therapy in humans have been completed or are currently underway for the treatment of various cancers including melanoma, prostate, and renal cancer. Clinical trials in prostate cancer patients have shown improved results when CTLA-4 blockade was combined with a GM-CSF secreting tumor vaccine (GVAX; refs. 19, 20). Furthermore, a phase III trial of unresectable stage III and IV melanoma patients showed anti–CTLA-4 therapy (Ipilimumab; Bristol Meyers-Squibb) to improve the median survival time to 10 months compared with 6.4 months in the control group (21), and this work led to the recent approval of this therapy by the U.S. Food and Drug Administration. This was the first drug of any type to show survival benefit in metastatic melanoma in a blinded, randomized phase III trial. Notably, approximately 25% of the patients had durable responses lasting 2 years and more. Thus, this therapy holds promise for metastatic melanoma patients who have failed the first line therapies and for whom there is currently no other approved treatment. Whereas these results were obtained using anti–CTLA-4 as monotherapy, the current hypothesis is that an even more substantial extension of patient survival in a larger fraction of patients may be achieved when this checkpoint blockade is combined with other treatment modalities that promote tumor destruction and subsequent antigen presentation.

In this article, we test the synergistic effect of CTLA-4 blockade on preventing the development of a secondary tumor when administered in combination with cryoablation of a large primary tumor using the transplantable TRAMP C2 tumor model of metastatic prostate cancer. We report that, in contrast to cryoablation or CTLA-4 blockade alone, the combination therapy slowed or prevented growth of a majority of secondary tumors. Rejection of the second tumor was associated with infiltration or expansion of CD4+ and CD8+ effector T cells, as well as an increase in the T effector (Teff) to T regulatory cell (Treg) ratio within the secondary tumor. Importantly, endogenous CD8+ T cells specific for the TRAMP tumor antigen SPAS-1 (22) were enriched in both secondary tumors and spleens of combination-treated mice compared with controls. Elimination of CD8+ T cells using depleting antibodies showed that CD8+ T cells are important for rejection, implying that SPAS-1–specific T cells contribute to the rejection of TRAMP C2 tumors during immunotherapy. These results suggest that cryoablation, although not effective on its own, can mediate the rejection of metastatic lesions and prevent recurrent disease when combined with CTLA-4 blockade.

Materials and Methods

Cell culture, media, and mice

TRAMP C2 cells were obtained from N. M. Greenburg (Baylor College of Medicine, Houston, TX) in 1999 (23). The cells were periodically authenticated by morphologic inspection and tested negative in 2004 for microbial contamination using the mouse antibody production test. The cells tested negative for Mycoplasma contamination by PCR test in 2008. TRAMP C2 cells were grown in Dulbecco’s modified Eagle’s medium high glucose supplemented with 5% fetal calf serum (FCS; Mediatech, Inc.), 5% Nu Serum IV (BD Biosciences) HEPES, 2-ME, pen/strep, L-glut, 5 μg/mL insulin (Sigma), and 10 mmol/L DHT (Sigma). Seven- to 9-week-old male C57BL/6 mice were purchased from Taconic Farms. Mice were used for experiments no earlier than 1 week after arrival. Mice were bred, housed, and treated according to the approved institutional animal protocols. Mice were injected with 200 μg anti–CTLA-4 (clone 9H10 BioXCell) or hamster IgG isotype control antibodies (BioXCell) in 0.2 mL PBS intraperitoneally on day 1 and 100 μg on days 4, 7, and 10 (Fig. 1A). For CD8+ T-cell depletion 0.5 mg of anti-CD8 (clone 2.43; BioXCell) was injected intraperitoneally 4 days prior to tumor injection and 4 and 14 days after tumor injection.

Antibodies, tetramer, and flow cytometry

Cells were blocked with 50 μg/mL 24.G2 (MSKCC mAb core) and stained with surface antibodies or tetramer for 60 minutes on ice. Antibodies were used at a 1:100 dilution, whereas the tetramer was used at a final concentration of 1.1 μg/mL. Antibodies detecting the following surface proteins were used: CD4 PerCP (clone RM4-5; BD Pharmingen), CD4 APC (Clone GK1; eBioscience) CD8 pacific blue or PE (clone 53-6.7; eBioscience), CD44 APC (clone IM7; eBioscience). The SPAS-1 H-2 Db tetramer PE was generated by the MSKCC tetramer core facility. The FoxP3 staining set (eBioscience) was used according to the manufacturer’s instructions using anti-FoxP3 APC (clone FJK-16s; eBioscience) 1:100 in perm/wash buffer for 60 minutes on ice. The cells were analyzed on an LSR II (BD Biosciences) flow cytometer. Flow cytometry data were further analyzed with FlowJo (Treestar, Inc.)

ELISPOT

IFNy ELISPOT kits (BD Biosciences) containing plates, coating antibody, detection antibody, and streptavidin–horse radish peroxidase enzyme were used according to the manufacturer’s
Cryopreservation and anti-CTLA-4 combination therapy synergize to mediate rejection of a second TRAMP C2 tumor challenge. A, scheme to study the immune response to cryoablation and anti-CTLA-4 combination therapy. C57BL/6 male mice were challenged with TRAMP C2 prostate tumor cells on the left flank. Cryoablation was administered when the tumors reached about 5 to 8 mm in diameter. Mice were challenged the following day after cryoablation with a second injection of TRAMP C2 on the right flank and some of the mice were also treated with CTLA-4 blocking antibodies (clone 9fH10) or hamster IgG isotype control antibodies. Calipers were used to monitor the growth of the second tumor. T-cell infiltrates into the second tumor were analyzed on day 15. B, mean and individual tumor growth. Error bars, SEM. C, tumor-free survival. Data shown is representative of at least 3 independent experiments. Tumor-free survival was plotted as a Kaplan–Meier curve and the log-rank test was used for statistical analysis. Number of mice that rejected the right flank tumor challenge: No Treatment: 0/5; Anti-CTLA-4 only 0/5; Cryo + IgG: 0/5; Cryo + Anti-CTLA-4: 4/9. One mouse from the Cryo + Anti-CTLA-4 group was omitted from the analysis due to partial ablation of the primary tumor.

**Tumor Inoculation and Measurement of Growth**

TRAMP C2 cells were grown to about 90% confluency in 15-cm plates and harvested with trypsin. After washing 3 times with HBSS, cell suspensions were counted and resuspended in the appropriate volume of PBS. Mice were briefly anesthetized by isoflurane inhalation. For tumor measurement experiments, 0.2 to 0.5 x 10^6 cells were injected and for tumor harvest experiments, 1 x 10^6 cells were injected. For tumor implantation, mice were injected with cells in 0.1 mL PBS intradermally on day 0. Tumors were measured every 3 to 4 days using Vernier calipers (Fisher Scientific), and tumor volume was calculated as the product of length, width, and height.

**Immunofluorescence and confocal microscopy**

Tumors were dissected from the mice and snap frozen in optimal cutting medium (O.C.T. TissueTek). Seven micrometer sections were cut using a cryotome and mounted on slides. Sections were fixed in ice-cold acetone for 10 minutes prior to rehydration with PBS. After blocking in a solution of 50 μg/mL 24.G2 with 5% normal goat, rabbit and rat serum and 3% FCS, sections were stained with primary antibodies overnight at 4°C. The following directly conjugated primary antibodies at a dilution of 1:50 in blocking solution were used: anti-CD4 Alexa Fluor 488 (Clone GK1; eBioscience), anti-CD8 Pacific blue (clone 53–6.7; eBioscience), and anti-CD31 APC (clone 390; eBioscience). Slides were mounted in SlowFade Gold anti-fade reagent (Invitrogen) and analyzed on an inverted confocal microscope (Leica) with a 20 x water immersion objective.
Preparation of single-cell suspensions of tumor infiltrates
Mice were sacrificed by CO₂ inhalation and the inside of the skin containing the tumor was exposed. Tumors were removed using forceps and surgical scissors and weighed. Tumors from each group were pooled and minced with scissors prior to incubation with 1.67 Wünsch U/mL Liberase and 0.2 mg/mL DNase for 30 minutes at 37°C. Tumors were homogenized by repeated pipetting and filtered through a 70-μm nylon filter. Remaining tumor pieces were pushed through the filter using the end of a 1-mL syringe. Cell suspensions were washed once with complete RPMI and the end of a 1-mL syringe. Cell suspensions were washed once with complete RPMI and ficolled. Live cells at the interface were collected and counted for further analysis.

Cryoablation
Mice were inoculated with 1 × 10⁶ TRAMP C2 cells on the left flank as described. Tumors were cryoablated when they reached 5 to 8 mm in diameter, about 28 days after tumor inoculation. The mice were anesthetized with a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine. The mice were surgically prepped by shaving the targeted area and cleansing it with alternating povidone iodine scrub and 70% alcohol rinse. A cryoablation probe (Endocare 1.7-mm PerCryo Cryoprobe) was inserted into the tumor and freezing was administered for 1 to 2 minutes to less than −100°C at the needle hub. After the ablation, the site was monitored for bleeding or any other post-procedural complications. Standard recovery procedures were implemented.

Statistical analysis
Statistical significance was calculated using Prism 5.0 (GraphPad Software, Inc.).

Results

Cryoablation and CTLA-4 blockade synergize to mediate the rejection of a subsequent TRAMP C2 tumor challenge
We developed a model system to test the capacity of cryoablation and anti-CTLA-4 combination therapy to generate sufficient immunity to slow or reject a second tumor challenge. To this end, we injected TRAMP C2 tumor cells on the left flank of male B6 mice and administered cryoablation when it reached 5 to 8 mm in diameter, about 28 days after injection (Fig. 1A). To model recurrent disease due to the outgrowth of a micrometastatic lesion, the following day, mice were challenged with a second injection of TRAMP C2 on the opposite flank. We monitored the growth of secondary tumors in mice treated with cryoablation and anti–CTLA-4 combination therapy (Cryo + Anti–CTLA-4), mice treated with cryoablation plus hamster IgG isotype control antibodies (Cryo + IgG), mice that received only the secondary tumor implant and anti–CTLA-4 monotherapy (Anti–CTLA-4 Only), and mice that received only the secondary tumor implant with no further treatment (no treatment). Mice treated with only anti–CTLA-4 or cryoablation plus IgG grew secondary tumors at a similar rate compared with untreated controls (Fig. 1B). In contrast, cryoablation and anti–CTLA-4 combination therapy led to slower outgrowth or rejection of the second tumor (Fig. 1B). Similarly, although 100% of the untreated, anti–CTLA-4 only and cryoablation plus IgG-treated mice succumbed to progressive growth of the second tumor, combination treatment led to rejection by 44% of the mice (Fig. 1C). Our results indicate that, although cryoablation alone does not influence the growth of a second tumor challenge, when it is combined with CTLA-4 blockade, induction of sufficient immunity leads to tumor rejection.

Secondary tumors from cryoablation/anti–CTLA-4 combination-treated mice are infiltrated by CD8⁺ and CD4⁺ T cells
To identify the T cell types that mediate rejection of the secondary tumors in our system, we isolated the tumors and assayed them for T-cell infiltration by immunofluorescence staining and flow cytometry. T-cell infiltration into contralateral tumors was assessed 15 days after implantation of TRAMP C2 tumors into naïve or cryoablation-treated mice. Cryosections of secondary tumors were stained with anti-CD4 and anti-CD8 antibodies to determine the types of T cells that infiltrated the tumor. In addition, anti-CD31, a marker of endothelial cells was used to stain the tumor vasculature. Immunofluorescence staining of untreated tumors revealed limited T-cell infiltration (Fig. 1A, left panel). In contrast, secondary tumors from combination-treated mice were highly infiltrated by both CD8⁺ and CD4⁺ T cells (Fig. 1A, right panel).

To better quantify the T cells that we observed by immunofluorescence, we evaluated T-cell infiltration by flow cytometry. On day 15, TRAMP C2 tumors were very small due to the slow growth of this tumor model. Therefore, to obtain enough tumor material to analyze by flow cytometry, we increased the tumor inoculum to 1 × 10⁶ TRAMP C2 cells and pooled 6 tumors from each group. Tumors from untreated, anti–CTLA-4 only, or cryoablation plus IgG-treated mice contained smaller percentages of CD4⁺ T cells, compared with combination-treated tumors (Fig. 2B). Anti–CTLA-4 treatment alone, or when combined with cryoablation resulted in an increased percentage CD8⁺ T cells within the secondary tumor (Fig. 2B). When absolute cell numbers and the mass of the pooled tumors were accounted for in the analysis, the total CD8⁺ T cells per mg of tumor in combination-treated mice increased 2-fold over anti–CTLA-4 alone and 10-fold over no treatment and cryoablation plus IgG (Fig. 2C). Likewise, CD4⁺FoxP3⁺ T cells were increased in combination-treated tumors 8-fold and 4-fold compared with both untreated and cryoablation plus IgG-treated tumors and anti–CTLA-4–treated tumors, respectively (Fig. 2C). The increased T-cell infiltration into tumors observed in the combination-treated mice correlated with tumor rejection and suggests that these T cell types are important for tumor eradication.

CD8⁺ T cells are important for tumor rejection and combination treatment produces an immune response to the immunodominant CD8⁺ TRAMP C2 antigen SPAS-1
Having shown the efficacy of combination treatment in mediating tumor infiltration by T cells, we next focused on the specificities of these infiltrates. The data from Fig. 2
suggested that CD8⁺ T cells are important for tumor rejection because we observed a correlation between CD8⁺ T-cell infiltration and tumor rejection. To determine the necessity of CD8⁺ T cells in controlling tumor growth after combination treatment, we depleted CD8⁺ T cells prior to therapy by in vivo administration of CD8-depleting antibodies. The antibody was successful in depleting most of the CD8⁺ T cells, as assessed by staining of peripheral blood leukocytes prior to cryoablation (Supplementary Fig. S1). A significant difference in survival between combination-treated mice with and without depletion was observed, indicating that CD8⁺ T cells contribute to tumor eradication in our system (Fig. 3).

To show that these CD8⁺ T cells were specific for the TRAMP tumor, we stained both tumors and spleens with SPAS-1/MHC class I tetramers. We have previously shown that the SPAS-1 TRAMP tumor antigen is an immunodominant CD8⁺ T cell target on TRAMP cells (22). Of the CD8⁺ T cells infiltrating TRAMP C2 tumors, 10.8%, 65%, and 16.9% contained for both tetramer and the activation marker CD44 in untreated, anti-CTLA-4, cryoablation + IgG and cryoablation + anti-CTLA-4 combination-treated tumors, respectively (Fig. 4A). This increase in percentage translated to a 58-fold increase in total SPAS-1-specific CD8⁺ T cells in combination therapy–treated tumors (Fig. 4A). Interestingly, the percentage of CD4⁺ SPAS-1–specific CD8⁺ T cells increased to 80.6% in combination therapy–treated tumors (Fig. 4A). This increase in percentage translated to a 58-fold increase in total SPAS-1–specific T cells per mg of tumor over no treatment and cryoablation plus IgG and a 2.5-fold increase over anti-CTLA-4 monotherapy treatment, indicating that tumors from treated mice are highly infiltrated by activated tumor-specific CD8⁺ T cells (Fig. 4B). Finally, we determined the frequency of SPAS-1–specific T cells in the spleen. We found that CD4⁺ SPAS-1–specific CD8⁺ T cells were significantly increased in the spleens of combination-treated mice compared with controls, suggesting that the combination treatment induced systemic expansion of tumor-specific CD8⁺ T cells (Fig. 5A and B).
and without depletion of CD8 T cells contributed to tumor rejection in our model (Fig. 3) and based on our previous data, showing that SPAS-1 is the major target in the anti-TRAMP tumor response (22), it is likely that these SPAS-1-specific T cells are playing a major role in the immune response induced by combination therapy.

**Cryoablation/CTLA-4 blockade combination therapy increases the intratumoral effector to Treg ratio**

CD4+FoxP3+ Tregs have been shown to play a role in dampening the immune response against tumors (24). Several studies from our laboratory using the B16 melanoma tumor model reported that the efficiency of different immunotherapeutic regimens in combination with CTLA-4 blockade (GVAX, Treg depletion, adoptive cell therapy, and FLT3 ligand expressing vaccine) directly correlates with a shift in the intratumoral balance from Tregs to Teffs (25–28). To assess the role of Tregs in cryoablation/anti–CTLA-4 therapy, we evaluated secondary tumors 15 days after cryoablation for the presence of CD4+FoxP3+ Tregs by flow cytometry. Twenty-nine to 44% of the CD4+ T cells inside untreated, anti–CTLA-4 and cryoablation plus IgG-treated tumors were FoxP3+ (Fig. 6A). In contrast, a much lower 6.22% of the intratumoral CD4+ T cells from the combination-treated tumors stained for FoxP3, indicating that a relatively suppressive environment exists within progressively growing TRAMP C2 tumors (Fig. 6A). This decrease in percentage of Tregs was not due to fewer Tregs infiltrating the combination-treated tumors, as the total number of CD4+FoxP3+ Tregs per mg of tumor was similar among the 4 groups (Fig. 6B). Rather, the decrease was due to the elevated expansion or infiltration of CD4+FoxP3+ T cells, T cells.
which are likely to be CD4⁺ effector cells in treated tumors. This result is consistent with the relative increase in the CD4⁺ Teff to Treg ratio compared with control tumors (Fig. 6C). In addition, an increase in the intratumoral CD8⁺ T cell to Treg ratio was observed in the tumor reflecting the increased numbers of CD8⁺ T cells observed in the treated tumors depicted in Fig. 2C (Fig. 6C). Thus, consistent with results obtained previously in our laboratory in the GVAX/anti–CTLA-4 B16 system (27), cryoablation and CTLA-4 blockade therapy dramatically shifts the intratumoral balance, increasing the ratio of Teff to Tregs, and this shift correlates with tumor immunity.

Discussion

We have shown here that cryoablation and anti–CTLA-4 combination therapy produces systemic immunity and tumor rejection in the TRAMP C2 mouse prostate model. Our model system involved the cryoablation of a large primary tumor followed one day later by anti–CTLA-4 administration and a secondary tumor challenge on the opposite flank. We injected the second tumor the day following cryoablation to model the outgrowth of micrometastases that become established after cryoablation and lead to recurrent disease. A study in the B16 model showed that proteins are released from the tumor into the draining lymph nodes and presented on dendritic cells (DC) up to 3 days following cryoablation (18). Thus, the injection of a second tumor one day after cryoablation in our model represents a nascent micrometastasis that develops during this antigen release.

We tracked the antigen-specific CD8⁺ T-cell response to TRAMP C2 cells using tetramer and ELISPot assays to detect the recently identified, immunodominant TRAMP C2 antigen SPAS-1 (22). We report that, after combination therapy, SPAS-1–reactive T cells made up more than 80% of the intratumoral CD8⁺ T cells and increased in absolute numbers in these treated mice, showing the ability of the therapy to enhance the tumor-specific CD8 response. The combination therapy also resulted in an enhanced frequency of SPAS-1–reactive CD8⁺ T cells in the spleen, which is evidence of a systemic response with the potential to eradicate disseminated disease. Depleting CD8⁺ T cells diminished the therapeutic effect and because most of the CD8⁺ T cells in the tumors of treated mice were specific for SPAS-1, it is likely that SPAS-1–specific T cells are important for tumor rejection.

TRAMP C2 cells were derived from a primary prostate tumor of a TRAMP mouse, which develop tumors due to prostate-specific expression of SV40 T antigen (29). The mutation in SPAS-1 likely arose in the prostate tumor of the mouse from which it was derived, and therefore, is only present in the cell line making it unique to the TRAMP C2 cells. The antigenic epitope of SPAS-1 was found to be highly immunogenic compared with its nonmutated counterpart, and peptide-pulsed DCs conferred protection against a TRAMP C2 tumor challenge (22). In human tumors, mutated tumor antigens may be important targets of the immune response generated by immunotherapies. A study by Segal and colleagues identified mutations in breast and colorectal tumors that resulted in neoepitopes that could bind HLA-A‘0201 (30). They found that breast tumors contained an average of 10 neoepitopes whereas colorectal tumors had an average of 7 neoepitopes. The extent by which these neoantigens elicit immune responses is currently unknown, but because of their proposed higher immunogenicity relative to
tetramer assays for SPAS-1 described for the first time herein will likely be useful tools to show a favorable immunologic response.

We addressed the cellular mechanisms mediating tumor rejection in our system by assessing both the effector and Treg responses. We showed that both CD8− and CD4−FoxP3+ T cells are increased in tumors as a result of cryoablation/anti-CTLA-4 combination therapy. Importantly, the intratumor ratio of Teff to Treg was also increased as a result of our treatment, which has been correlated with tumor rejection in the B16 GVAX/anti–CTLA-4 model (27). This has also been observed in clinical studies of ovarian cancer, in which a high Teff to Treg ratio in tumors was prognostic of improved survival (31) and associated with increased tumor necrosis in patients treated with anti–CTLA-4 (32). These studies further support the use of this ratio as a marker of a favorable immunologic response to immunotherapy. Moreover, tumor immunity was enhanced when Treg depletion, through anti-CD25–depleting antibodies or cyclophosphamide treatment was combined with cryoablation (8, 33), indicating that Tregs are important modulators of tumor immunity in response to cryoablation.

Since the regression of metastases after cryoablation was first observed in patients, investigators have examined the immune response in animal models in hopes of showing a “cryoimmunologic” effect. The results of these studies are mixed, preventing a consensus with regard to the effect of cryoablation as a monotherapy to treat metastatic disease. Numerous studies report immunologic benefit resulting in the rejection of secondary tumor challenges following cryoa- blation (34–37). However, others have reported no effect (38) or an enhancement of secondary tumor growth after cryoa- blation (39–41). The results of our cryoablation study indicate that in our system, cryoablation alone had no effect on secondary tumor growth or T-cell infiltration into secondary tumors. Despite the lack of a response from cryoablation alone, we observed a synergistic effect when cryoablation was combined with anti–CTLA-4. Although the immune response to cryoablation alone may vary with each patient, combination with CTLA-4 blockade has the potential to create a robust antitumor immune response that controls the growth of metastases and prevents the recurrence of disease. This work has led to the generation of proposals for clinical trials in which cryoablation will be combined with CTLA-4 blockade to treat cancer.

**Disclosure of Potential Conflicts of Interest**

J.P. Allison is the inventor of intellectual property licensed by Bristol Myers Squibb, and is a consultant and is on the advisory board of Medarex. The other authors disclosed no potential conflicts of interest.

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