Anti-CTLA-4 therapy results in higher CD4<sup>+</sup>ICOS<sup>hi</sup> T cell frequency and IFN-γ levels in both nonmalignant and malignant prostate tissues

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Cytotoxic lymphocyte antigen-4 (CTLA-4) blockade is an active immunotherapeutic strategy that is currently in clinical trials in cancer. There are several ongoing trials of anti-CTLA-4 in the metastatic setting of prostate cancer patients with reported clinical responses consisting of decreases in the prostate specific antigen (PSA) tumor marker for some patients. Immunologic markers that correlate with these clinical responses are necessary to guide further development of anti-CTLA-4 therapy in the treatment of cancer patients. We recently reported that CD4<sup>+</sup> inducible co-stimulator (ICOS)<sup>hi</sup> T cells that produce interferon-γ (IFN-γ) are increased in the peripheral blood and tumor tissues of bladder cancer patients treated with anti-CTLA-4 antibody. Here we present data from the same clinical trial in bladder cancer patients demonstrating a higher frequency of CD4<sup>+</sup>ICOS<sup>hi</sup> T cells and IFN-γ mRNA levels in nonmalignant prostate tissues and incidental prostate tumor tissues removed at the time of radical cystoprostatectomy. Our data suggest immunologic markers that can be used to monitor prostate cancer patients who receive anti-CTLA-4 therapy and indicate that the immunologic impact of anti-CTLA-4 antibody can occur in both tumor and nonmalignant tissues. These data should be taken into consideration for evaluation of efficacy as well as immune-related adverse events associated with anti-CTLA-4 therapy.

Results

Frequency of CD4<sup>+</sup>ICOS<sup>hi</sup> and CD4<sup>+</sup>FOX<sup>+</sup> T Cells in Peripheral Blood of Prostate Cancer Patients. The frequency of CD4<sup>+</sup>ICOS<sup>hi</sup> T cells in the peripheral blood of healthy donors (2–5%) was not different from that of untreated prostate cancer patients (Fig. 1A and B). However, the frequency of CD4<sup>+</sup>FOX<sup>+</sup> regulatory T cells (17), was considerably higher in the peripheral blood of prostate cancer patients than in healthy donors (45% vs. 5%, P < 0.05; Fig. 1A and B). We recently reported variable changes in CD4<sup>+</sup>FOX<sup>+</sup> T cells after treatment of bladder cancer patients with anti-CTLA-4 therapy. In these bladder cancer patients the frequency of CD4<sup>+</sup>ICOS<sup>hi</sup> T cells was as low as shown in Fig. 1 for the prostate cancer patients in pretherapy blood samples; however, there was a significant increase in the frequency of CD4<sup>+</sup>ICOS<sup>hi</sup> T cells in the blood of patients who received anti-CTLA-4 antibody and suggest a broad based systemic impact of anti-CTLA-4 in both malignant and nonmalignant tissues. These findings have implications regarding the immunologic events that may contribute to both clinical efficacy and immune-related adverse events associated with anti-CTLA-4 therapy.
following treatment (12). Similar results would be expected in patients with different tumor types because anti-CTLA-4 antibody targets T cells and is not tumor specific.

**Frequency of CD4+ICOS<sup>hi</sup> and CD4+FOXP3<sup>+</sup> T Cells in Prostate Cancer Tissues.** Prostate adenocarcinoma tumor tissues (Gleason scores 6 and 7) from patients undergoing radical prostatectomy as treatment for their disease were obtained and investigated for phenotypic characteristics of tumor infiltrating CD4 T cells. Similar data were obtained from nonmalignant prostate tissues procured from radical cystoprostatectomy surgeries of bladder cancer patients who had removal of both bladder and prostate organs as treatment for their disease. As shown in Fig. 2, nonmalignant prostate tissues had an average of ~7% CD4<sup>+</sup>ICOS<sup>hi</sup> T cells and ~5% CD4<sup>+</sup>FOXP3<sup>+</sup> T cells (Fig. 2A) while prostate cancer tissues from untreated patients had similar frequencies of CD4<sup>+</sup>ICOS<sup>hi</sup> T cells (~9%) but much higher frequencies of CD4<sup>+</sup>FOXP3<sup>+</sup> T cells (~48%) (Fig. 2B). CD4<sup>+</sup>ICOS<sup>hi</sup> T cells were on average 3-fold higher in tumor tissues (9 ± 5%, Fig. 2B) as compared to peripheral blood (3 ± 3%, Fig. 1B) of prostate cancer patients, which suggests expansion of these cells against specific antigens present within the tumor microenvironment. CD4<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells had a wide range of expression in both peripheral blood (43 ± 19%, Fig. 1B) and tumor tissues (48 ± 21% Fig. 2B) of prostate cancer patients but, was significantly higher (P < 0.05) than observed in healthy donor blood (5 ± 2%, Fig. 1A) and nonmalignant prostate tissues (5 ± 3%, Fig. 2A). The frequency of CD4<sup>+</sup>FOXP3<sup>+</sup> T cells measured in peripheral blood of each prostate cancer patient and healthy donor, and in each prostate tumor tissue and nonmalignant prostate tissue is provided in a graphical format (Fig. 2C). Tumor-infiltrating regulatory T cells have been reported to be similarly high in prostate cancer and other tumor types (18–20). Tumor-infiltrating regulatory T cells were previously reported as a potential mechanism for suppressing effector anti-tumor immune responses and as a correlative marker of poor clinical outcome (21–23). Conversely, increased intratumoral effector T cells and higher ratios of effector to regulatory T cells in tumor tissues have been associated with improved clinical outcome (24, 25).

**Anti-CTLA-4 Therapy Results in a Higher Frequency of CD4+ICOS<sup>hi</sup> T Cells in Both Prostate Cancer and Nonmalignant Prostate Tissues.**

Eight consecutive patients with bladder cancer were treated on a presurgical clinical trial with 2 doses of the anti-CTLA-4 antibody ipilimumab with each dose given 3 weeks apart and radical cystoprostatectomy surgery performed 4 weeks after administration of the last dose of antibody. Seven patients had evaluable prostate tissues consisting of 3 incidental cases of prostate adenocarcinoma (Patients 3, 4, and 8) and 4 cases of nonmalignant prostate tissues (Patients 1, 5, 6 and 7) (Table S1). Patient 2 is not reported here because this patient had extension of the bladder cancer into the prostate. As shown in Fig. 3, both nonmalignant prostate tissues (Fig. 3A) and prostate cancer tissues (Fig. 3B) had increased frequencies of CD4<sup>+</sup>ICOS<sup>hi</sup> T cells after treatment with anti-CTLA-4 antibody, as compared with untreated tissues (Fig. 2).
of CD4 tissues of patients 6 and 7 (Table S1). Patients 3 and 4, who were found to have prostate cancer, had 31% and 22% CD4 T cells of patients 6 and 7 (Table S1). Patients 3 and 4 had 5% and 4% CD4 T cells (8%, Table S1) as compared to untreated prostate cancer tissues. However, CD4 T cells treated with anti-CTLA-4 antibody while FOXP3 levels remained similarly low in both patients (Right) as compared to untreated nonmalignant tissues shown in Fig. 2A. (B) CD4 T cells had a higher frequency of ICOS expression at 31% (patient 3, Left Upper) and 22% (patient 4, Left Lower) in prostate cancer tissues after patients received treatment with anti-CTLA-4 antibody, while frequency of FOXP3 expression was lower at 5% (patient 3, Right Upper) and 4% (patient 4, Right Lower) as compared to untreated prostate cancer tissues shown in Fig. 2B.

Patients 1 and 5, who were found to have nonmalignant prostate tissues, had 29% and 23% CD4 T cells, respectively, which were about 2–3 times higher as compared to untreated nonmalignant prostate tissues (7 ± 4%, Fig. 2A). Similar data regarding ICOS expression was obtained from the nonmalignant prostate tissues of patients 6 and 7 (Table S1). Patients 3 and 4, who were found to have prostate cancer, had 31% and 22% CD4 T cells, respectively, which were also 2–3 times higher in frequency as compared to untreated prostate cancer tissues (9 ± 5%, Fig. 2B). Similar data regarding ICOS expression was observed in prostate cancer tissues from patient 8 (Table S1). CD4 FOXP3+ T cells were lower in prostate cancer tissues (Fig. 3B) after treatment with anti-CTLA-4 antibody as compared to untreated prostate cancer tissues (Fig. 2B). Patients 3 and 4 had 5% and 4% CD4 FOXP3+ T cells, respectively, as compared to untreated prostate cancer tissues (48 ± 21%, Fig. 2B). Patient 8 also had a lower frequency of CD4 FOXP3+ T cells (8%, Table S1) as compared to untreated prostate cancer.

**Anti-CTLA-4 Therapy Results in Higher Levels of IFN-γ and T-bet mRNA in Both Prostate Cancer and Nonmalignant Tissues.** Prostate tissues were analyzed for differences in mRNA levels for several genes related to T cell function (Fig. 4). FOXP3 mRNA levels were found to be lower in prostate cancer tissues from patients treated with anti-CTLA-4 antibody as compared to untreated prostate cancer tissues, which was consistent with data presented in Figs. 2 and 3. Both prostate cancer and nonmalignant tissues had higher mRNA levels for IFN-γ, a Th1 cytokine (26), and T-bet, a transcription factor responsible for regulating IFN-γ production (27), as compared to untreated tissues. IL-2 mRNA levels were also slightly increased in treated tissues as compared to untreated tissues but this increase was not as pronounced as the increase seen for IFN-γ. However, mRNA levels for IL-10, a Th2 cytokine (28), did not differ significantly between groups. Similarly, mRNA levels for GATA-3, a transcription factor associated with IL-10 production (29), did not differ between groups. The average ratio of Th1/Th2 cytokine inferred from the ratio of IFN-γ/IL-10 was notably increased from 0.2/L in untreated tissues to ~1.5/L in treated tissues, regardless of tumor status. Therefore, CTLA-4 blockade alters the Th1 to Th2 ratio in both prostate cancer and nonmalignant tissues.

**Prostate Tumor-infiltrating CD4 T Cells from an Anti-CTLA-4 Treated Patient Recognize the NY-ESO-1 Tumor Antigen.** Prostate tissues from anti-CTLA-4-treated patients were infiltrated with CD4 T cells (Fig. 5A, representative sample, patient 4). We obtained tumor infiltrating CD4 T cells in sufficient quantity for functional analysis from only one patient’s prostate sample (patient 4). The prostate tumor from patient 4 expressed the NY-ESO-1 tumor antigen (Fig. 5B). CD4 tumor infiltrating lymphocytes from this patient’s prostate sample recognized the NY-ESO-1 tumor antigen with subsequent production of IFN-γ (Fig. 5B). As shown in Fig. 5C, these contained a population of ICOS+ cells that produced additional cytokines (TNF-α) and chemokines (MIP-1β).

**Fig. 3.** Expression of ICOS and FOXP3 by CD4 T cells in normal and malignant prostate tissues from patients treated with anti–CTLA-4. (A) CD4 T cells had a higher frequency of ICOS expression at 29% (patient 1, Left Upper) and 23% (patient 5, Left Lower) in nonmalignant prostate tissues after patients received treatment with anti–CTLA-4 antibody while FOXP3 levels remained similarly low in both patients (Right) as compared to untreated nonmalignant tissues shown in Fig. 2A. (B) CD4 T cells had a higher frequency of ICOS expression at 31% (patient 3, Left Upper) and 22% (patient 4, Left Lower) in prostate cancer tissues after patients received treatment with anti–CTLA-4 antibody, while frequency of FOXP3 expression was lower at 5% (patient 3, Right Upper) and 4% (patient 4, Right Lower) as compared to untreated prostate cancer tissues shown in Fig. 2B.

**Fig. 4.** Changes in expression of specific gene transcripts in prostate tissues following treatment with anti–CTLA-4. Fold induction of CD3ζ, IFN-γ, IL-10, IL-2, FOXP3, T-bet, and GATA-3 mRNA levels in untreated prostate cancer tissues, untreated nonmalignant prostate tissues, anti–CTLA-4 antibody treated prostate cancer tissues, and anti–CTLA-4 antibody treated nonmalignant prostate tissues as compared to GAPDH mRNA levels as assessed by real-time PCR. Anti–CTLA-4 treatment induced higher IFN-γ and T-bet mRNA levels in both cancer and nonmalignant tissues as well as lower FOXP3 mRNA levels in cancer tissues as compared to untreated cancer tissues.
triplicate and data were plotted as mean ± standard deviation. (C) CD4+ICOShi T cells produce TNF-α (Right) and MIP-1β (Left) in the presence of APCs pulsed with NY-ESO-1 overlapping peptides.

Fig. 5. NY-ESO-1 is recognized by CD4 tumor-infiltrating T cells from an anti-CTLA-4 treated patient sample. (A) H & E staining of patient 4 prostate tissues showing CD4 T cell infiltration. (B) NY-ESO-1 expression on prostate tumor cells. (C) An ELISPOT assay demonstrating CD4 T cell recognition of antigen-presenting cells (APCs) pulsed with NY-ESO-1 overlapping peptides as compared to unpulsed APCs (no peptides). The assay was done in triplicate and data were plotted as mean ± standard deviation. (C) CD4+ICOShi T cells produce TNF-α (Right) and MIP-1β (Left) in the presence of APCs pulsed with NY-ESO-1 overlapping peptides.

Discussion

Anti-tumor responses and toxicities that occur as a result of anti-CTLA-4 therapy are both likely to be mediated by immunologic events. Here we present data showing that both nonmalignant prostate tissues and prostate cancer tissues are similarly affected following treatment of patients with anti-CTLA-4 antibody. Both nonmalignant prostate tissues and prostate cancer tissues from treated patients had a higher frequency of CD4+ICOShi T cells. CD4+ICOShi T cells contain a population of effector T cells leading to a higher ratio of effector to regulatory T cells in tumor tissues after treatment. Similarly, anti-CTLA-4 therapy also leads to a higher ratio of Th1 to Th2 cytokines in tumor tissues. Although these changes are expected to be beneficial in the setting of tumor tissues, and have been correlated with tumor rejection in animal models (30), it remains questionable as to whether they are detrimental in nonmalignant tissues. It is likely that many factors contribute to the development of irAEs as a result of anti-CTLA-4 therapy. We previously reported that decreased concentrations of plasma IL-10 appear to be associated with irAEs (31). In our small cohort of patients, we did not observe irAEs in all patients who demonstrated a higher frequency of CD4+ICOShi T cells or a higher ratio of Th1 to Th2 cytokines in nonmalignant tissues.

It is possible that the immunologic changes that we observed within prostate tissues are due to the anatomical proximity of the prostate and bladder and are simply an extension of the immunologic changes that are ongoing within the bladder tumor tissues of our patients. Additional tissue samples from anti-CTLA-4-treated patients would need to be studied to determine whether the immunologic changes that we observed are ongoing in other nonmalignant tissues. However, in the only other reported study to analyze tissues after anti-CTLA-4 therapy, the authors demonstrated by immunohistochemical methods that T cell infiltration in nonmalignant and malignant tissues was comparable after patients were treated, thus making it less likely that our data are related to anatomical proximity and supporting the concept of a systemic response induced by therapy. In a previous report it was shown that anti-CTLA-4 therapy led to the development of skin rash in a subset of melanoma patients and biopsies from these nonmalignant areas revealed CD4 and CD8 T cell infiltrates (32). The authors also demonstrated that biopsies from enlarged lymph nodes of treated patients revealed CD3 T cell infiltration without evidence of HMB-45 and MART-1 expressing melanoma tumor cells. Similarly, in 2 ovarian carcinoma patients who developed diarrhea after treatment with anti-CTLA-4 antibody, biopsies from the gastrointestinal tract revealed CD4 and CD8 T cell infiltration (32). These findings of dense T cell infiltration in nonmalignant tissues were also seen in biopsies of metastatic melanoma lesions after patients were treated with anti-CTLA-4 antibody (32). Therefore, it is feasible that anti-CTLA-4 therapy leads to a broad-based systemic activation of T cell responses, which can then lead to irAEs or anti-tumor responses depending on the T cell repertoire and/or additional immunological events that are ongoing in select patients. Interestingly, we could not expand CD4 T cells from tumor tissues that did not express the NY-ESO-1 antigen with our current in vitro culture techniques using the NY-ESO-1 overlapping peptides, which suggests that appropriate antigenic stimuli are likely to be necessary to expand tumor-infiltrating T cells that are specific for other tumor antigens. Similarly, CD4 T cells from nonmalignant prostate tissues did not survive and could not be expanded in vitro as was possible for CD4 T cells from the patient whose prostate cancer expressed the NY-ESO-1 tumor antigen (Fig. 5). This finding suggests that T cells within nonmalignant tissues may require additional signals before subsequent immunologic responses could occur as compared to T cells from tumor tissues.

Previous studies have also documented induction of NY-ESO-1-specific antibody and T-cell responses (12, 32, 33) in patients treated with anti-CTLA-4 therapy. These antigen-specific T cells suggest that only particular subsets of T cells will be able to expand and perpetuate a tissue-destructive immune response, thereby preventing similar immune responses from occurring in nonmalignant tissues. We previously reported that NY-ESO-1-specific T cell responses can be induced in the systemic circulation of prostate cancer patients vaccinated with the NY-ESO-1 DNA vaccine but these responses were suppressed in a subset of patients as a result of regulatory T cell function (34). Here we show that NY-ESO-1-specific T cells also exist within incidental prostate adenocarcinoma samples obtained from patients treated with anti-CTLA-4 antibody. CD4+FOXP3+ T cells were less prevalent in tumor tissues after anti-CTLA-4 therapy, which suggests that the FOXP3+ regulatory T cells were necessary to expand tumor-infiltrating T cells that are specific for other tumor antigens. Similarly, CD4 T cells from nonmalignant prostate tissues did not survive and could not be expanded in vitro as was possible for CD4 T cells from the patient whose prostate cancer expressed the NY-ESO-1 tumor antigen (Fig. 5). This finding suggests that T cells within nonmalignant tissues may require additional signals before subsequent immunologic responses could occur as compared to T cells from tumor tissues.

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marker that can be used to monitor patients, including prostate cancer patients, who receive anti-CTLA-4 therapy. Additional studies are warranted to investigate the role of CD4+ ICOS+ T cells and cytokine levels in anti-tumor responses and irAEs.

**Materials and Methods**

**Patients.** Tissues were collected from surgical samples including prostate samples of all male patients who were treated by radical cystoprostatectomy for bladder cancer or radical prostatectomy for prostate cancer. Surgical samples from patients who were treated with anti-CTLA-4 therapy were obtained from an ongoing clinical trial wherein bladder cancer patients receive 2 doses of ipilimumab anti-CTLA-4 antibody before surgery, which is performed 4 weeks after the last dose of antibody. Ipilimumab was administered at a dose of 3 mg/kg or 10 mg/kg each time, with a 3-week interval between doses. All patients were monitored for safety. This is an ongoing clinical trial. The results reported in prostate tissues reflect data obtained from 7 treated patients (4 patients with nonmalignant prostate tissues and 3 patients with prostate adenocarcinoma). Patient 2 had urothelial carcinoma of the bladder extending into the prostate and his prostate sample was not included in this reported data set. Nonmalignant prostate tissues (n = 5) were obtained from prostate samples of male bladder cancer patients who were treated by radical cystoprostatectomy and who did not participate in the anti-CTLA-4 antibody trial. These patients were consented onto a separate IRB-approved sample acquisition laboratory protocol. Nonmalignant prostate tissues were found to have no evidence of cancer within the tissues by pathology review. Untreated prostate cancer tissues (n = 10) were obtained from patients undergoing surgery as treatment for localized prostate cancer. These patients were also consented onto a separate IRB-approved sample acquisition laboratory protocol. Blood samples from untreated prostate cancer patients with localized disease (n = 10) were acquired from patients undergoing radical prostatectomy. Healthy donor blood was obtained from volunteers (n = 10).

**Blood and Tissue Processing.** Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density centrifugation using Lymphocyte Separation Medium (Mediatech) and Leucosep tubes (Greiner Bio-one). Cells recovered from the gradient interface were washed twice in RPMI medium 1640 (Mediatech), counted, and immediately used for cytokine analysis, staining, and flow cytometry analysis. Fine needle aspirations (PNAs) of prostate tissues were washed twice with cold PBS supplemented with 2% BSA and 2 mM EDTA before performing cell surface and intracellular staining for ex vivo flow cytometric analysis, as previously reported (12).

**Flow Cytometry.** Antibodies used for flow cytometry consisted of: CD3-FITC and CD4-PerCP-Cy5.5 (BD Pharmingen), FOXP3-PE (ebioscience, clone PHC101), ICOS-biotinylated (ebioscience) conjugated with streptavidin-APC-Cy7 (BD Biosciences). Intracellular staining for FOXP3, TNF-α, and MIP-1β were conducted according to the manufacturer’s guidelines. Samples were analyzed using the FACS Canto II (Becton Dickinson). Data were analyzed using BD FACS Diva software. Gates were set according to appropriate isotype controls.

**Real-time PCR.** Total RNA samples from tissues were isolated with the RNeasy kit (Qiagen). Reverse transcription was performed using the SuperScript III Reverse Transcriptase kit (Invitrogen). Real-time quantitative PCR was performed with the 7500 Fast Real-time PCR system (Applied Biosystems) according to the manufacturer’s instructions. Samples were used as templates in reactions to obtain the threshold cycle (Ct) that were normalized with the Ct of GAPDH from the same sample (ΔCt). To compare the relative levels of gene expression in different tissues, ΔΔCt values were calculated with the ΔCt values associated with the lowest expression levels as 1. Fold induction was calculated using 2^ΔΔCt. CD3ε-IFN-γ, T-bet, and FOXP3 probes were synthesized with the Taqman Gene Expression Assay (Applied Biosystems). Other primers used were synthesized by Integrated DNA Technologies (IDT) and were used with the SYBR Green PCR Master Mix System (Applied Biosystems). Primers used for PCR included GAPDH sense: TCACCCCAACTGATCG; anti-sense: GCGATGCAGGTGCATGAG; IL-2 sense: AAATTGATGATTTTGGTTGAC; IL-10 sense: TGGGGGAAACC TGAAGAC; anti-sense: ACAGGAGAAAGATCCTAGA; GATA-3 sense:GCT- TCGGTAGCAGTTCCCA; anti-sense: GCCCCACAGTCTCACACCT.


Supporting Information
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Table S1. Characteristics of prostate tissues and frequency of infiltrating CD4 T cell subsets

<table>
<thead>
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<th>Patients</th>
<th>Prostate tissues</th>
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<th>% CD4⁺FOXP3⁺ cells</th>
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