Enhanced Functionality of CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> Regulatory T Cells in the Peripheral Blood of Patients with Prostate Cancer

Junko Yokokawa, Vittore Cerdra, Cinzia Remondo, James L. Gulley, Philip M. Arlen, Jeffrey Schlom, and Kwong Y. Tsang

Abstract

**Purpose:** CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> regulatory T cells (Treg) have been shown to inhibit the activation and function of T cells that participate in antigen-specific immune responses. Higher levels of Tregs have been reported in the peripheral blood of patients with several types of tumors. In this study, we investigated the number and functionality of CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> Tregs in patients with prostate cancer (PCa), and their potential role in inhibiting antitumor immune responses.

**Experimental Design:** Levels of Tregs in the peripheral blood of healthy donors and patients with biochemically progressive, localized, and metastatic PCa were each measured by flow cytometry. The functional activity of Tregs was determined by their ability to suppress the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells. Data were analyzed using Wilcoxon rank sum test and unpaired Student’s t test.

**Results:** Although levels of Tregs in the peripheral blood of patients with PCa were not significantly higher than those in healthy donors, Tregs in patients with PCa had significantly greater suppressive functionality than Tregs from healthy donors (P < 0.05). Additionally, there was a direct correlation between the serum levels of prostaglandin E<sub>2</sub> and Treg functionality in patients with localized PCa, using Pearson’s product-moment correlation coefficient (R).

**Conclusions:** These findings further show the potential importance of Tregs in modifying immune responses in patients with PCa. Although longer studies are necessary to confirm these findings, these studies also show for the first time the differences in Treg populations in patients with various stages of PCa, and thus, provide a basis for determining which PCa patient populations are best suited for immunotherapy trials involving the inhibition of Tregs.

Prostate cancer (PCa) is the most common malignancy in men and is a leading cause of cancer death in North America, with ~218,890 new cases and 27,050 deaths expected in 2007 (1). Treatment for localized disease includes radical prostatectomy or radiation therapy. Unfortunately, ~30% of patients will ultimately develop increasing prostate-specific antigen (PSA) levels following these local therapies. Androgen-deprivation therapy and chemotherapy may improve survival and palliate symptoms in patients with metastatic PCa, but there is currently no curative therapy available (2). Although immunotherapy employing several different PCa vaccines has shown recent promise (3), vaccines for PCa have achieved only partial success in the clinical setting, especially in patients with advanced or aggressive disease (4).

It has been shown that CD4<sup>+</sup> regulatory T cells (Treg) that constitutively express high levels of the interleukin 2 receptor α chain (CD25) and specifically express the forkhead/winged helix transcription factor FoxP3 act in a regulatory capacity by inhibiting the activation and function of both self-antigen– and foreign-antigen–reactive T cells (5, 6). The critical physiologic role of Tregs is to control autoimmune diseases (7, 8). However, because most tumor-associated antigens are self-antigens, Tregs may play a critical role in suppressing an effective antitumor immune response.

Increased levels of CD4<sup>+</sup>CD25<sup>high</sup> Tregs have been reported in hematologic malignancies (9–11) and in patients with non–small cell lung carcinoma (12), malignant melanoma (13), gastrointestinal malignancies (14), ovarian cancer (15), squamous cell carcinoma of the head and neck (16), hepatocellular carcinoma (17), breast cancer (18), pancreatic cancer (19), mesothelioma (18), and metastatic renal cell carcinoma (20). This increase has been shown in both the tumor microenvironment and in the peripheral blood. A recent study (2) reported elevated levels of Tregs in the peripheral...
blood of patients with PCa following prostatectomy, and showed in vitro the immunosuppressive function of these Tregs; Tregs were analyzed from patients with early stage PCa.

The central role of Tregs in suppressing antitumor immune responses has been shown by numerous preclinical and clinical studies. In mice injected with syngeneic tumor cells, removal of CD4+CD25+ Tregs induced antitumor immunity (21). In another murine study, administration of anti-CD25 monoclonal antibody abrogated immune tolerance and induced the spontaneous development of tumor-specific T cells and natural killer cells (22). Clinical studies in patients with melanoma have shown that Tregs can inhibit both antigen-specific and nonspecific T cell responses (23, 24). In patients with ovarian cancer, a direct correlation has been shown between tumor-infiltrating Tregs and overall survival (15). In these patients, treatment with the recombinant interleukin 2 diphtheria toxin conjugate DAB201 (27, 28) and/or anti-CD25 monoclonal antibody (29) led to the depletion of Tregs and improved antitumor responses (25). Denileukin diftitox has also been shown to significantly reduce the number of Tregs in the peripheral blood of patients with metastatic renal cell carcinoma, and to abrogate Treg-mediated immunosuppression in vitro (20). In summary, several studies have indicated that, in some tumor types, CD4+CD25high Tregs could reduce the efficacy of immunotherapeutic protocols (26), and that depletion of these cells could enhance vaccine-mediated antitumor immune responses (20).

Studies of the pathways controlling the activity of Tregs have shown that tumor-derived prostaglandin E2 (PGE2) enhances in vitro inhibitory function and up-regulates Foxp3 expression in purified human Tregs (27). Many investigators have reported that cyclooxygenase-2 (COX-2) and PGE2 are overexpressed in Tregs (28, 29). COX-2 is an enzyme that converts arachidonic acid to prostaglandin H2, which is further metabolized to other prostaglandins, including PGE2 (30). COX-2 has been shown to be overexpressed in PCa tissue—with consistently high levels found in lymph node metastases—and to promote the progression of PCa (31).

In a clinical trial at the National Cancer Institute, PCa patients treated with a recombinant poxviral vaccine encoding PSA developed PSA-specific T-cell responses (32); in spite of this immune response, many patients had subsequent tumor progression, possibly due to the inhibitory function of Tregs. To further elucidate the critical role of Tregs in inhibiting antitumor immune responses, we analyzed the levels of CD4+CD25highFoxp3+ Tregs in the peripheral blood of patients with biochemically progressive PCa, localized PCa, and metastatic PCa and compared them with levels in healthy donors. We also analyzed the functional activity of CD4+CD25highFoxp3+ Tregs in patients with biochemically progressive PCa, localized PCa, and metastatic PCa and compared it to the functional activity of Tregs in healthy donors. Tregs in the patients with PCa showed increased functionality compared with the healthy donors, which may be an important factor in the suppression of tumor-specific immune responses in these patients. We also analyzed the effect of PGE2 on the suppressive function of Tregs in patients with PCa. This is the first qualitative and quantitative analysis of Treg populations from patients with different stages of PCa.

Materials and Methods

Patients and healthy donors. Peripheral blood mononuclear cells (PBMCs) were collected from patients with biochemically progressive PCa (n = 10), localized PCa (n = 24), and metastatic PCa (n = 24). PBMCs from 25 healthy donors were collected and used as a control. To be eligible for this study, patients with biochemically progressive PCa had to have a locally recurrent prostate tumor, confirmed either by biopsy or by biochemical failure, defined by the ASTRO consensus criteria as three consecutive increases in PSA levels. No patient in this group had clear metastatic disease. Patients with localized PCa had to have a histologically confirmed diagnosis of adenocarcinoma of the prostate and no prior local therapy. All patients with metastatic PCa had to have metastatic androgen-independent PCa, with evidence of disease progression based on (a) increasing serum PSA as measured by PSA consensus criteria (33); (b) a new metastatic finding on bone scan; and/or (c) disease progression on computerized tomography scan. Patients involved in the study could not have received any radiation therapy or chemotherapy within 6 months prior to the blood draw. All patients on antiandrogen therapy had to have evidence of increasing PSA following antiandrogen withdrawal. All patients signed a consent form approved by the National Cancer Institute Institutional Review Board.

PBMC collection. PBMCs were isolated by Ficoll (Amersham Biosciences) density gradient separation, washed twice, and cryopreserved in liquid nitrogen at a concentration of 1 × 107 cells/mL until assayed.

Flow cytometry analysis. Cryopreserved PBMCs were analyzed by three-color flow cytometry for phenotypic characterization of Tregs. Cells were resuspended in staining buffer (PBS containing 3% fetal bovine serum) and stained for 30 min at 4°C with PerCP Cy5.5–conjugated anti-CD4 and phycoerythrin-conjugated anti-CD25 (both from BD Biosciences). Foxp3 intracellular staining was done on the cells stained with anti-CD4 and anti-CD25. Cells were fixed and permeabilized using a fix/perm kit (eBioscience) according to the manufacturer’s instructions, then labeled with FITC-conjugated anti-Foxp3 antibody (PCH101 clone), or its isotype control antibody (eBioscience), as a negative control. Flow cytometry was done on a Becton Dickinson LSRII (BD Biosciences); 1 × 106 cells were acquired and data were analyzed using FlowJo software (BD Biosciences). To determine the percentage of Tregs, lymphocytes were gated by plotting forward versus side scatter, followed by gating of the CD4+ population. Then the CD25high and Foxp3+ populations were gated. The CD25high population was separated from the CD25low population on the basis of the level of CD25 expression in CD4+ T cells, as previously described (34, 35).

CD4+CD25high T-cell enrichment. CD4+CD25high T cell-enriched T cells were isolated using a CD4+CD25 T-cell isolation kit (Miltenyi Biotec), with modifications to the manufacturer’s instructions. In order to achieve a consistently high CD4+ purity rate, the amount of CD4 antibodies, Anti-Biotin MicroBeads, and incubation time were increased by 25%, as previously described (36). After CD4+ T cells were negatively enriched, positive selection for CD25+ T cells was done on the negatively selected CD4+ T cells. The CD25high fraction was collected by eluting the cells twice through a magnetic separation (LS) column to further enrich for CD4+CD25high T cells. In a representative experiment, 92.3% of Tregs isolated by this method were CD4+CD25high. By comparison, 93.0% of Tregs obtained by a cell-sorting procedure from PBMCs of the same donor were CD4+CD25high (data not shown), showing that Tregs isolated by MicroBeads had the same phenotypic profile as Tregs isolated by cell sorting.

Immunosuppression assay. CD4+CD25 T cells (5 × 104 cells/well) were cultured alone or cocultured with CD4+CD25high T cells (5 × 104 cells/well) in three different ratios with 1 ng/mL of anti-CD3 antibody (OKT3; eBioscience) in the presence of irradiated (3,500 rad) T-depleted PBMCs (2.5 × 104 cells/well) in a 96-well flat-bottomed plate at 37°C and 5% CO2. Cells were cultured in RPMI 1640 (Mediatech, Inc.) supplemented with 10% heat-inactivated human AB serum (Gemini Bio-Products). 100 units/mL of penicillin, 100 μg/mL of streptomycin

www.aacrjournals.org
(Mediatech), and 2 mmol/L of l-glutamine (Mediatech). Proliferation was measured by [3H]thymidine [1 μCi (0.037 MBq) per well; Perkin-Elmer] incorporation pulsed on day 4 and quantified 18 h later using a liquid scintillation counter (Wallac). All experiments were done in triplicate wells. One hundred percent proliferation was defined as the proliferation of CD4+CD25- T cells without coculturing with Tregs.

Measurement of serum PGE2. Serum samples collected from patients with biochemically progressive PCa (n = 10), localized PCa (n = 22) and metastatic PCa (n = 29), as well as from 15 healthy donors, were measured for PGE2 using a Prostaglandin E Metabolite EIA Kit (Cayman Chemical). PGE2 is rapidly converted in serum by 15-OH prostaglandin dehydrogenase to its 13,14-dihydro-15-keto metabolite. Thus, PGE2 metabolite measurement was employed to provide a reliable estimate of actual PGE2 concentrations in the serum samples.

Suppression assay with PGE2-treated CD4+CD25^{high} Tregs. CD4+CD25^{high} or CD4+CD25^{-} T cells (5 × 10^4/well) isolated by Anti-Biotin MicroBeads (Miltenyi) were treated with increasing concentrations (0, 13, 26, 52, 100 μmol/L) of dimethyl PGE2 (Cayman Chemical) for 24 h in a total volume of 200 mL in a round-bottomed 96-well plate (37). Following treatment, cells were washed twice in PBS and a suppression assay was done, as previously described.

Statistical analysis. Statistical analysis was done with the assistance of the Biostatistics and Data Management Section, Center for Cancer Research, National Cancer Institute, using the Wilcoxon rank sum test and unpaired Student’s t test to assess differences among the four study groups. P < 0.05 was considered statistically significant. Pearson’s product-moment correlation coefficient (R), which ranges between -1.00 and +1.00, was used to calculate the correlation between two variables.

Results

A greater percentage of patients with metastatic PCa have increased levels of CD4+CD25^{high}FoxP3+ Tregs in peripheral blood compared with patients with localized PCa or biochemically progressive PCa and healthy donors. We first investigated

![Image](fig1.jpg)

Fig. 1. Expression of FoxP3 in CD4+CD25^{high} Tregs from a healthy donor. Human FBMCs were analyzed by flow cytometry after cell surface labeling with PerCP Cy5.5-conjugated anti-CD4, phycoerythrin-conjugated anti-CD25, and intracellular staining with FITC-conjugated anti-Foxp3. A, CD4+CD25^{high} Tcells were gated as CD25^{high} and CD25^{low} relative to the level of CD25 expression in CD4 T cells. B, expression of FoxP3 in CD4+CD25^{high}, CD4+CD25^{low}, and CD4+CD25^{-} Tcell populations.

![Image](fig2.jpg)

Fig. 2. Percentage of CD4+CD25^{high}FoxP3^{+} Tregs in the peripheral blood of patients with PCa and healthy donors. A, levels of CD4+CD25^{high}FoxP3^{+} Tregs in the peripheral blood of healthy donors (HD; n = 25) and patients with biochemical progression PCa (n = 10), localized PCa (n = 24), and metastatic PCa (n = 24). Individual levels of CD4+CD25^{high}FoxP3^{+} Tregs are presented as a percentage of total CD4+ Tcells; no statistically significant differences were detected in the levels of CD4+CD25^{high}FoxP3^{+} Tregs among the four groups (P > 0.05). A level of >5.24% is considered higher than normal, based on the 95th percentile of CD4+CD25^{high}FoxP3^{+} Tregs obtained from 25 healthy donors. B, individual frequencies of CD4+ Tcells are presented as a percentage of total lymphocytes. P values were calculated using unpaired Student’s t test. No statistically significant differences were detected in the percentage of CD4+ Tcells in healthy donors or in patients with biochemical progression, localized, or metastatic PCa (P > 0.05).
the percentage of FoxP3+ cells in the CD4+CD25+ Treg population. Figure 1 is a representative histogram of a sample from a healthy donor, demonstrating Tregs as CD4+CD25high FoxP3+ T cells. CD4+ cells were designated as (a) CD25- if CD25 expression fell within the background staining using an isotype control monoclonal antibody; (b) CD25high if CD25 expression exceeded that seen in CD4- cells; and (c) CD25low if CD25 expression fell between the areas designated CD25- and CD25high. Figure 1A shows the percentages of PBMCs that were CD25high (1.76%), CD25low (12.3%), and CD25- (6.67%). As shown in Fig. 1B, 86.79% of CD4+CD25high Tregs were FoxP3+, whereas only 14.78% of CD4+CD25low Tregs were FoxP3+.

Studies were then initiated to determine the percentages of Tregs in the peripheral blood of patients with biochemically progressive PCa (n = 10), localized PCa (n = 24) and metastatic PCa (n = 24), and in 25 healthy donors. To detect this cell population, first CD4+ cells were gated, then CD25high cells (designated on the basis of the level of CD25 expression in non-CD4 T cells) and FoxP3+ cells were gated. These studies revealed no significant differences in the levels of CD4+CD25highFoxP3+ Tregs among the four groups (P > 0.05). However, a greater percentage of patients with metastatic PCa had high levels of Tregs (5 of 24), compared with healthy donors (0 of 25) or patients with localized PCa (1 of 24) and biochemically progressive PCa (1 of 10; Fig. 2A). Treg levels >5.24% were designated as higher than normal, based on the 95th percentile of CD4+CD25highFoxP3+ T cells obtained from 25 healthy donors. Levels of CD4+CD25highFoxP3+ T cells per CD4+ T cells were as follows: healthy donors, 3.28 ± 1.16% (range, 1.46-5.25%; n = 25); patients with biochemically progressive PCa, 3.78 ± 1.46% (range, 2.18-7.5%; n = 10); patients with localized PCa, 3.25 ± 1.29% (range, 0.89-5.81%; n = 24); and patients with metastatic PCa, 4.10 ± 1.93% (range, 2.22-10.09%; n = 24). To confirm that the higher level of Tregs among patients with metastatic PCa was not due to the higher percentage of CD4+ T cells in this group, the percentage of CD4+ T cells in each group was analyzed. Results showed that individuals from all four groups studied had similar levels of CD4+ T cells in the peripheral blood (Fig. 2B).

Suppressive function of CD4+CD25high Tregs is enhanced in the peripheral blood of patients with PCa compared with healthy donors. We analyzed the functional activity of CD4+CD25high Tregs from patients with biochemically progressive PCa (n = 9), localized PCa (n = 7), and metastatic PCa (n = 8), as well as...
from 13 healthy donors, by investigating their ability to suppress autologous CD4+CD25- T-cell proliferation upon stimulation with anti-CD3 monoclonal antibody. Figure 3A shows the functional activity of Tregs derived from a healthy donor, a patient with biochemically progressive PCa, a patient with localized PCa, and a patient with metastatic PCa. All three patients with PCa had a higher level of suppressive activity at three different effector/suppressor ratios compared with the healthy donors. Figure 3B shows individual data on the suppressive function of Tregs from healthy donors (n = 13) and patients with biochemically progressive PCa (n = 9), localized PCa (n = 7), and metastatic PCa (n = 8) at a CD4+CD25-/CD4+CD25high ratio of 1:1. Interestingly, the suppressive function of CD4+CD25high Tregs was higher in all three groups of patients with PCa compared with healthy donors, and significantly higher in patients with localized PCa and metastatic PCa compared with patients with biochemically progressive PCa (Fig. 3B). The percentages of suppression at a CD4+CD25-/CD4+CD25high ratio of 1:1 were as follows: healthy donors, 31.2 ± 6.55% (range, 14.6-40.1%); patients with biochemically progressive PCa, 42.5 ± 7.8% (range, 57.2-31.0%); patients with localized PCa, 64.85 ± 24.75% (range, 30.8-98.9%); and patients with metastatic PCa, 62.5 ± 14.89% (range, 37.8-84.0%). A comparison between the combined PCa patient groups (n = 24) and the healthy donors (n = 13) showed a statistically significant difference in the suppressive activity of CD4+CD25high Tregs (PCa group, 55.7 ± 18.92%; healthy donors, 31.2 ± 6.55%; P < 0.0001). The proliferation of effector T cells in all four groups was analyzed to confirm that the increased suppressive activity of CD4+CD25high Tregs among patients in the three PCa groups was not a result of fluctuations in CD4+CD25- effector T-cell proliferation. Results showed that individuals in all four groups had similar levels of CD4+CD25- effector T-cell proliferation (Fig. 3C).

We undertook further studies to confirm that the levels of suppressive function seen in CD4+CD25high Tregs obtained from patients with PCa were not influenced by the patients’ effector T cells. CD4+CD25- T cells from a healthy donor were cocultured with CD4+CD25high Tregs from either a patient with PCa or a healthy donor, and stimulated with anti-CD3 monoclonal antibody and autologous antigen-presenting cells. In this allogeneic setting, proliferation of CD4+CD25 T cells from the healthy donor was suppressed by CD4+CD25high T cells from the patient with PCa at three different effector/Treg ratios (Fig. 4A), and CD4+CD25high Tregs from the patient with PCa showed increased suppressive activity compared with Tregs from the healthy donor (Fig. 4A). The outcome was similar when the CD4+CD25- T cells used in the experiment were obtained from two different healthy donors (HD2 and HD3; Fig. 4B).

**Increased PGE2 expression in the peripheral blood of patients with localized PCa enhances the inhibitory function of Tregs.** It has been reported that PGE2 enhances the in vitro inhibitory function of purified human Tregs and up-regulates FoxP3 expression in these cells (27). The same study suggests that PGE2 may contribute to tumor-induced immunosuppression by modulating Treg function and differentiation. In this study, we sought to determine whether PGE2 could affect the functional activity of CD4+CD25high Tregs in patients with PCa by analyzing the levels of serum PGE2 in patients with PCa and healthy donors. Serum PGE2 levels (Fig. 5A) were significantly higher in patients with localized PCa (n = 22) than in patients with metastatic PCa (n = 29) and biochemically progressive PCa (n = 10) or in healthy donors (n = 15). In addition, in patients with localized PCa, there was a direct correlation (R = 0.814) between the level of Treg functionality and the level of serum PGE2 (Fig. 5B). No correlation between these two variables was observed in patients with metastatic PCa (R = 0.218) or biochemically progressive PCa (R = 0.05).

![Fig. 4. CD4+CD25high Tregs from patients with PCa inhibit the proliferation of autologous CD4+CD25- T cells from healthy donors.](image-url)
The effect of PGE\(_2\) on the suppressive function of Tregs was evaluated in a suppression assay in which purified CD\(4^+\)CD25\(^{high}\) Tregs were cultured with or without various concentrations of PGE\(_2\) for 24 h. As shown in Fig. 6A, the suppressive function of Tregs from a healthy donor increased as the concentration of PGE\(_2\) added to the cultures increased (range, 1.6-fold increase in suppressive function at 13 \(\mu\)g/mL PGE\(_2\) to 2.4-fold increase at 100 \(\mu\)g/mL PGE\(_2\)). Addition of PGE\(_2\) to the culture medium increased the suppressive function of Tregs from patients with localized PCs and biochemically progressive PCs, but at lower levels than those seen in healthy donors. PGE\(_2\) did not affect the inhibitory function of Tregs in patients with metastatic PCs.

We did functional suppressive assays to confirm that the levels of suppressive function seen in PGE\(_2\)-cultured CD\(4^+\)CD25\(^{high}\) Tregs from patients with metastatic PCs were not influenced by the nature of the patients’ effector T cells. CD\(4^+\)CD25\(^{high}\) T cells from a healthy donor were cocultured with either autologous CD\(4^+\)CD25\(^{high}\) Tregs or allogeneic CD\(4^+\)CD25\(^{high}\) Tregs from a patient with metastatic PCa, then stimulated with anti-CD3 monoclonal antibody. As shown in Fig. 6B, the addition of PGE\(_2\) had no effect on the suppressive activity of CD\(4^+\)CD25\(^{high}\) Tregs isolated from a patient with metastatic PCa (75% suppression at various concentrations of PGE\(_2\)). On the other hand, the addition of PGE\(_2\) enhanced the suppressive activity of Tregs obtained from a healthy donor (18% suppression at 0 \(\mu\)g/mL, 25% at 13 \(\mu\)g/mL, and 36% at 52 \(\mu\)g/mL of PGE\(_2\)). These results show that the effect of PGE\(_2\) on Tregs from patients with metastatic PCa is not influenced by the nature of the patient’s effector T cells.

**Discussion**

It has been suggested that CD4 Tregs could be subdivided into two major subsets: natural and adaptive. Natural CD4 Tregs are differentiated in the thymus and migrate to the periphery (38). In humans, natural CD4 Tregs express high levels of CD25 and FoxP3 (36, 39–41). Several other surface markers such as CTLA-4, a negative costimulatory molecule, and glucocorticoid-induced tumor necrosis factor receptor–related protein have also been described in human natural CD4 Tregs. Adaptive CD4 Tregs (Tr1, Th3, and inducible CD4\(^+\)CD25\(^{high}\)FoxP3\(^{+}\)) are generated in vitro and in vivo from mature cells in the periphery, in defined conditions such as in the presence of interleukin 10 and transforming growth factor \(\beta\), two privileged cytokines that induce the differentiation of adaptive Tregs (42, 43). In particular, human adaptive Tr1 Tregs are CD4\(^+\)CD25\(^{+}\) and can express FoxP3 when they exhibit suppressive activity. The present study investigated for the first time the levels and function of CD4\(^+\)CD25\(^{high}\)FoxP3\(^{+}\) Tregs in the peripheral blood of patients with biochemically progressive, localized, and metastatic PCa. Healthy donors were used as controls. Using the gating variables described in Materials and Methods, CD4 Tregs were identified as CD4\(^+\)CD25\(^{high}\) FoxP3\(^{+}\) (Fig. 1A). Approximately 86.8% of cells in the CD4\(^+\)CD25\(^{high}\) population were FoxP3\(^{+}\) (Fig. 1A), confirming that FoxP3 expression is mostly confined to CD25\(^{high}\) cells in humans (20).

Our findings show that a greater number of patients with metastatic PCa (5 of 24) had increased levels of CD4\(^+\)CD25\(^{high}\)FoxP3\(^{+}\) Tregs in peripheral blood compared with patients with biochemically progressive or localized PCa or to healthy donors (0 of 25; Fig. 2A). Studies of many different types of cancer have shown elevated levels of Tregs in peripheral blood (12, 14–16, 19, 44), but very few studies have assessed Tregs in patients with PCa. A previous study by Miller et al. reported increased levels of CD4\(^+\)CD25\(^{high}\) Tregs in the tissue and peripheral blood of patients with early stage PCa who had undergone prostatectomy (2). Our study showed that there were no significant differences in the levels of CD4\(^+\)CD25\(^{high}\)FoxP3\(^{+}\) Tregs in patients with biochemically progressive PCa and localized PCa (\(n = 34\)) compared with healthy donors.
These differences may be due in part to the fact that the levels of Tregs per CD4+ T cells in healthy donors reported by Miller et al. were relatively lower (0.5 ± 0.1%) than the levels of Tregs per CD4+ T cells in healthy donors reported in other studies (~1% to 3%), including our investigation. As a consequence, relatively higher levels of Tregs in patients with PCa were reported in their study. Other possible reasons for the reported differences in Treg levels may be that (a) Miller et al. defined Tregs as CD4+CD25high T cells, whereas our study defined Tregs as CD4+CD25highFoxP3+ T cells, and (b) our study enrolled a greater number of patients with PCa and from different PCa stages.

Currently, little is known about the cause of this increase in peripheral blood Tregs in patients with cancer. It was previously suggested that the increase was due to active proliferation rather than redistribution from other compartments such as secondary lymphoid organs or bone marrow (45). However, it now seems likely that tumor tissue establishes a concentration gradient of antigens and other factors (transforming growth factor β, chemokines, etc.), resulting in elevated Tregs that are detectable in peripheral blood because of the convergence of lymphatics and blood vessels (46). In fact, a recent study showed that resection of gastric cancers restored peripheral blood Tregs to nearly normal levels, suggesting that tumor-related factors directly induce an increase in peripheral Tregs (47). It has also been proposed that Tregs are attracted to tumor via CCL22/CCR4, and that this is a possible mechanism for the increase in Tregs in peripheral blood (48). In patients with metastatic PCa, the increase in Tregs may be partially related to more aggressive tumor phenotype.

Although most human CD4+CD25high Tregs are FoxP3+, in the absence of more specific cell surface markers, it is essential to characterize human CD4+CD25high Tregs by analyzing both phenotype and inhibitory function. In this study, the functional activity of CD4+CD25high Tregs was significantly higher in patients with PCa than in healthy donors, and was higher in patients with localized and metastatic PCa than in patients with biochemically progressive PCa. To confirm that the increased functionality of Tregs in patients with PCa was not a result of decreased proliferation of their effector T cells, Tregs from patients with PCa were cocultured with allogeneic CD4+CD25- effector T cells isolated from healthy donors and stimulated with anti-CD3 in the presence of autologous antigen-presenting cells. Even in this allogeneic setting, Tregs isolated from the patients with PCa showed increased suppressive activity, as also reported by others (49). Our study found no correlation...
between the frequency and functional activity of Tregs in patients with PCa. It is possible, but unlikely, that the similarity in Treg levels and the difference in functionality in Tregs from healthy donors may be due to the small number and/or the nature of the healthy donors used in this investigation. A study involving a larger number of healthy donors is in progress to confirm the present findings. The cause of the increased suppressive activity of Tregs in patients with localized and metastatic PCa compared with patients with biochemically progressive PCa is not known, but increased tumor burden, and a consequent increase in secretion of immunosuppressive factors such as PGE2 by tumor cells, may be partially responsible (44). In future studies, it would be interesting to analyze the serum levels of transforming growth factor β and interleukin 10 in these patients to better understand the mechanism of Treg modulation in PCa.

Many investigators have reported that COX-2 is overexpressed in a variety of cancers and is associated with decreased apoptosis, increased tumor invasiveness, and angiogenesis (28, 29). It has also been shown that COX-2 expression is increased in PCa tissue, with consistently high levels in lymph node metastasis, suggesting that COX-2 may act early in tumor promotion and progression of PCa (31). The COX-2 enzyme converts arachidonic acid to prostaglandin H2, which is further metabolized to other prostaglandins, including PGE2 (30).

We analyzed the level of serum PGE2 in the peripheral blood of healthy donors and patients with biochemically progressive, localized, and metastatic PCa and found that serum PGE2 levels were significantly higher in patients with localized PCa than in the other groups. In addition, there was a direct correlation (R = 0.814) between the level of Treg suppressive activity and PGE2 serum levels in patients with localized PCa, suggesting the possibility that, in these patients, increased levels of serum PGE2 are partially responsible for the increased suppressive function of Tregs. However, no direct correlation was detected between PGE2 serum levels and the suppressive activity of Tregs in patients with metastatic PCa (R = 0.218) and biochemically progressive PCa (R = 0.05).

Further studies are in progress to elucidate the correlation or lack of correlation among these three groups of patients with PCa.

In vitro studies have shown that tumor-derived PGE2 enhances the inhibitory function of human Tregs and up-regulates FoxP3 expression in Tregs, at both mRNA and protein levels (27). COX-2 inhibition has been shown to reduce the frequency and suppressive activity of Tregs, attenuate FoxP3 expression in tumor-infiltrating lymphocytes, and decrease tumor burden (37). Based on these preclinical findings, we investigated the effects of PGE2 on the suppressive function of Tregs in patients with three types of PCa and in healthy donors. Results showed that treating Tregs with PGE2 increased their inhibitory activity in a dose-dependent manner in healthy donors (n = 3) and in patients with biochemically progressive (n = 3) and localized PCa (n = 3), but not in patients with metastatic PCa (n = 3; Fig. 6A). This observation was confirmed in an allogeneic setting, using CD4+CD25hi T cells from patients with metastatic PCa as Tregs and CD4+CD25 T cells from healthy donors as effectors (Fig. 6B). Our findings suggest that the high functional activity of Tregs from patients with localized PCa may be caused by the presence of high tumor-derived PGE2 serum levels, whereas the functional activity of Tregs from patients with metastatic and biochemically progressive PCa may depend on other factors in addition to the levels of PGE2. Further studies will be required to determine the reason(s) for these findings.

The results of the study reported here on the level and functional activity of Tregs in patients with PCa help to form the basis for new immunotherapeutic strategies directed toward Tregs. In addition, measurement of Treg function may prove to be a useful parameter for monitoring patients before and after vaccination. Moreover, a report indicating that vaccination of tumor-bearing mice expands Tregs, thus blocking the execution of effector function in vitro and in vivo (50), suggests that strategies which combine Treg depletion with cancer vaccines may be an effective means of improving immunotherapeutic outcomes for patients with cancer.

Acknowledgments

The authors thank Sandra Doren for her coordination of the blood samples, and Bonnie L. Casey and Debra Weingarten for their editorial assistance in the preparation of this manuscript.

References

Mukherji B, Guha A, Chakraborty NG, et al. Clonal...

Shimizu J, Yamazaki S, Sakaguchi S. Induction of...

Chakraborty NG, Twardzik DR, Sivanandham M,...

Liyanage UK, Moore TT, Joo HG, et al. Prevalence...

DeLong P, Carroll RG, Henry AC, et al. Regulatory...

Sheng H, Shao J, Kirkland SC, et al. Inhibition of...

Antony PA, Restifo NP. Do CD4+ CD25+ immuno-


Antony PA, Restifo NP. Do CD4+ CD25+ immuno-

Barstelli F, Lin Y, Zhu L, et al. Prostaglandin E2 indu-
ces FOXP3 gene expression and Tregulatory cell func-

Sheng H, Shao J, Kirkland SC, et al. Inhibition of human colon cancer cell growth by selective inhibi-

Uefuji K, Ichikura T, Mochizuki H. Cyclooxygenase-


Sharma S, Yang SC, Zhu L, et al. Tumor cyclooxy-


Hori S, Nomura T, Sakaguchi S. Control of regulato-


Vigier M, Lemaître F, Verlo O, et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepres-


Enhanced Functionality of CD4+CD25^{high}FoxP3^{+} Regulatory T Cells in the Peripheral Blood of Patients with Prostate Cancer

Junko Yokokawa, Vittore Cereda, Cinzia Remondo, et al.