

CD4⁺CD25^{high} T Cells Are Enriched in the Tumor and Peripheral Blood of Prostate Cancer Patients¹

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In this study, we investigated whether CD4⁺CD25^{high} regulatory T cells (Treg) are increased in the tumor tissue and peripheral blood of early-stage prostate cancer patients undergoing prostatectomy. We show that the prevalence of CD4⁺CD25^{high} T cells inside the prostate was significantly higher in the tumor compared with benign tissue from the same prostate. Furthermore, the frequency of CD4⁺CD25^{high} T cells in peripheral blood was significantly higher in prostate cancer patients compared with normal donors. A proportion of the CD4⁺CD25^{high} T cells was also shown to be glucocorticoid-induced TNF receptor, ICOS, and FOXP3 positive. Moreover, CD4⁺CD25⁺ T cells from blood and supernatants from cultured prostate tumor tissue samples exhibited immunosuppressive function in vitro. Furthermore, supernatants from cultured prostate tissue samples and prostate cancer ascites fluid induced migration of CD4⁺CD25⁺ T cells and were shown to contain the regulatory T cell chemokine CCL22 by ELISA. Our findings indicate that Tregs are an important cellular component of early-stage prostate tumors, and thus new therapeutic strategies aimed at inhibition or depletion of Tregs may improve prostate cancer immunotherapy. *The Journal of Immunology*, 2006, 177: 7398–7405.

Prostate cancer (PC)³ is currently the most commonly diagnosed cancer in men in Europe and the United States (1, 2), and currently no curative treatment exists for metastatic PC. Recently, immunotherapy with tumor vaccines has emerged as an alternative therapeutic approach (3). However, despite evidence for the induction of tumor-specific T cell responses, significant objective clinical response rates are low (4). The reasons behind the limited success of these approaches in PC patients are still largely unknown. It has been suggested that the presence of CD4⁺CD25⁺ regulatory T cells (Treg) can explain the poor clinical efficacy of immunotherapeutic protocols in human tumors (5), and depletion of these cells improves antitumor immunity (6).

Tregs, originally termed suppressor T cells, are thought to control key aspects of immunological tolerance to self-Ags (7–9). They are broadly identified as a small proportion of CD4⁺ T cells that constitutively express CD25 (IL-2R α -chain) on their surface. Several other markers have been described for Tregs, such as glucocorticoid-induced TNF receptor (GITR), ICOS, CTLA-4, neuropilin-1, OX-40, CD103, Ly6, galectin-1, 4-1BB, and lymphocyte

activation gene-3 (9–13). It has also been shown that Tregs specifically express FOXP3, a forkhead/winged helix transcription factor that is disrupted in the Scurfy mouse and in the human immune dysregulation polyendocrinopathy enteropathy X-linked syndrome. FOXP3 appears to be critical for the development and function of Tregs (14, 15). Although the precise mechanisms of suppression by Tregs remain to be determined, these cells can inhibit immune cell functions either directly through cell-cell contact or indirectly through the secretion of anti-inflammatory mediators, such as IL-10 and TGF- β . The Ags recognized by Tregs appear to be self, tissue-specific Ags (16). It is also becoming clear that most tumor-associated Ags are self-Ags and are expressed either during development or in normal adult tissue (17). Hence, it is possible that by removing tumor-specific Tregs, antitumor immunity could be enhanced (5). Indeed, many studies in mice have now shown that removal or inhibition of this subset of cells can enhance effector T cell antitumor responses in tumors (18, 19).

In humans, high levels of Tregs have now been identified in peripheral blood, lymph node, and ascites from many different types of cancer (20–23), while a few studies have also looked directly at tumor specimens (24–30). Recently, Curiel et al. (27) have shown that tumor cells and microenvironmental macrophages in ovarian carcinoma produce the chemokine CCL22, which mediates Treg trafficking to the tumor. Thus far, there are no studies assessing human prostate tumors for the presence of Tregs, although one study has shown an increase in the frequency of Tregs in draining lymph nodes of a murine transgenic mouse model of prostate dysplasia (31). These studies are difficult to do in human PC for a number of reasons: prostate tumors are relatively small; surgery is only routinely conducted in early-stage disease; the isolation of true tumor-infiltrating lymphocytes (TILs) is problematic due to the infiltrative growth of PC within the prostate gland; low numbers of TILs are seen in PC (32); and the development of malignant ascites in PC is an extremely rare event, with only a few published clinical case reports (33). In the present study, we were able to gain access to blood and fresh prostate tissue samples to examine the presence of CD4⁺CD25^{high} T cells in patients with early stage PC.

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Received for publication February 9, 2006. Accepted for publication September 5, 2006.

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¹ This work was supported partly by grants from the Cancer Society in Stockholm, the Swedish Cancer Society, Karolinska Institute Funds, NordForsk Motility Grant 040226, EU 6-FP “ALLOSTEM” (LSHB-CT-2004-503319), EU 6-FP “ENACT,” and U.S. Department of Defense Prostate Cancer Research Program (PC030958). A.H.B. is supported by the Leukemia Research Fund. A.M.M. is the recipient of a scholarship from Stockholm’s Odd Fellow-Logers.

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³ Abbreviations used in this paper: PC, prostate cancer; Treg, regulatory T cell; GITR, glucocorticoid-induced TNF receptor; TIL, tumor-infiltrating lymphocyte; SDF-1 α , stromal cell-derived factor 1 α .

Table I. Summary of the clinical features of PC patients from whom prostate biopsies were analyzed

Characteristic	Value
Total no.	15
Mean age (years)	62 (50–71) ^a
Mean PSA (ng/ml)	9.9 (3.9–30) ^a
TNM stage	
T1c	7
T2	8
Gleason score	
6	5
7	6
8	2
9	2

^a Numbers in parentheses, range.

Materials and Methods

Patients and normal donors

For the assessment of Treg levels, we obtained peripheral blood and/or fresh tissue samples, from benign and malignant portions of the prostate after radical prostatectomy, from 15 PC patients (Table I). None of these patients had received any hormonal, immunosuppressive, or radiation therapy before prostatectomy. Three additional T1c/T2 PC patients were leukapheresed to obtain high numbers of blood lymphocytes for functional studies. PC ascites fluid was obtained from a patient with an extremely rare case of PC with abdominal ascites (33). Blood samples were also collected from normal healthy donors for comparison. The study was approved by the local ethics committee, and written informed consent was obtained from all individuals in accordance with the Declaration of Helsinki.

Blood sample preparation

Blood samples were collected in sterile heparinized containers. PBMC were isolated by centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech). The cells were washed twice in PBS without calcium and magnesium (PBS, pH 7.2), and resuspended in X-VIVO 15 medium (BioWhittaker and In Vitro) for further analysis.

Prostate sample preparation

The prostate was kept on ice immediately after operation. All tissue samples were harvested by a single pathologist (L.E.). The prostate was cut into two halves by a horizontal section. Tissue samples were cut from macroscopically visible tumor areas and from grossly normal transition zone tissue and placed in PBS. The cut surfaces were scraped and cytological material was smeared, air-dried, and Giemsa stained for morphological analysis. Sample locations were noted on a specimen map. The remaining prostate was formalin-fixed, sliced horizontally at 4 mm, and totally embedded. The specimens were dehydrated, cut at 4 mm, and stained with H&E. All slides were reviewed by one pathologist (L.E.) verifying that areas where tumor and benign samples were harvested were actually malignant and benign, respectively.

The fresh prostate tissue samples were removed from the PBS, cut into small 2-mm³ pieces with a scalpel blade, then placed into 1 ml of X-VIVO 15 medium containing 10% human serum albumin (Pharmacia) and 20 U/ml IL-2 (Proleukin; Chiron) in a 24-well plate (four pieces per well). After 3 days, T cells that had outgrown from the tissue were collected for further analysis.

Cell lines

The human PC cell line LNCaP was maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and 25 µg/ml gentamicin.

FACS analysis

Four-color flow cytometry was performed on a FACSCalibur (BD Biosciences) with CellQuest Pro software using directly conjugated mAbs against the following markers: CD3-FITC, CD4-PerCP, CD25-allophycocyanin, GITR-PE, or ICOS-PE with corresponding isotype-matched controls (either BD Biosciences or R&D Systems).

FOXP3 staining was conducted using the Human Regulatory T cell staining kit (eBioscience) and run according to the manufacturer's protocol. To deter-

mine the percentage of Tregs, lymphocytes were gated by plotting forward vs side scatter followed by gating on CD3⁺CD4⁺ T cells, and these cells were then analyzed for CD25 expression. For GITR, ICOS, and FOXP3 expression, cells inside the CD4⁺CD25^{high} gate were analyzed.

Immunohistochemistry

FOXP3 expression in prostate tissue sections was analyzed by immunostaining using an anti-human FOXP3 mAb, 236A/E7 (provided by A. H. Banham; Ref. 34). Briefly, 4-µm sections were cut from paraffin blocks and captured on slides. Sections were dewaxed and Ag retrieval was performed by microwave pressure cooking in citric acid buffer. Before staining the sections, endogenous peroxidase was blocked with 0.1% BSA. The slides were then incubated overnight with the FOXP3 Ab, washed with TBS, and the immunodetection was performed with biotinylated anti-mouse secondary Ab (30 min), followed by ABC complex (30 min), and diaminobenzidine chromogen as substrate. Sections were counterstained with Meyer's hematoxylin and mounted.

In vitro immunosuppression assay

CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were purified from PC patient peripheral blood lymphocytes by a CD4⁺CD25⁺ bead selection kit (Miltenyi Biotec) according to the manufacturer's instructions. Enriched cells were used when >90% pure as determined by flow cytometry. CD4⁺CD25⁻ cells were stained with 1 µM CFSE for 10 min at 37°C, quenched for 5 min on ice, washed three times, and resuspended in X-VIVO 15 medium. CFSE-stained CD4⁺CD25⁻ cells were activated polyclonally with Dynabeads CD3/CD28 T cell expander (one bead:three T cells; Dynal) in the presence of varying numbers of CD4⁺CD25⁺ T cells (ratio, 3:1, 1:1, and 1:3). After 5 days samples were washed once, resuspended in FACS buffer (PBS and 0.5% FCS), and proliferation was measured as a decrease in CFSE fluorescence intensity by flow cytometry. Alternatively, purified CD4⁺CD25⁻ T cells were resuspended in supernatant collected from either benign or malignant prostate tissue after 2 days in culture. In these experiments, T cell proliferation was measured after 4 days using a BrdU Proliferation Flow kit with a CD4-FITC Ab (both BD Biosciences).

Multiplex cytokine assay

Supernatants removed from cultured benign or PC biopsies were analyzed in a 9-plex multiple human cytokine assay (Bio-Plex; Bio-Rad) for simultaneous quantification of IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, and IL-17. This assay was run according to the manufacturer's recommended procedure and beads were read on the Bio-Plex suspension array system, and data were analyzed using Bio-Plex manager software with 5PL curve fitting. The limit of detection for this assay is <10 pg/ml (based on detectable signal >2 SD above background).

In vitro migration assay

CD4⁺CD25⁺ T cells were purified from mononuclear cell fractions obtained from normal donor peripheral blood using the CD4⁺CD25⁺ bead selection kit described above. Migration of CD4⁺CD25⁺ T cells was then assessed in 96-well chemotaxis chambers (NeuroProbe). Briefly, lower chambers of plates were filled with 420 µl of cell supernatant, plasma, ascites supernatant, or migration medium (phenol red-free RPMI 1640 plus 0.1% human serum albumin) with or without chemokines (100–100,000 pg/ml). Chemokines tested included human stromal cell-derived factor 1α (SDF-1α) or CCL22 (PeproTech). A 5-µm pore polyvinylpyrrolidone-free polycarbonate filter (NeuroProbe) was then placed over the plate, 5 × 10⁴ cells in 100 µl were added to the top chamber, and migration was allowed to proceed for 2 h at 37°C. The number of cells in the bottom chamber was detected by the addition of MTT reagent (Cell Proliferation kit; Roche) and incubation at 37°C. After 4 h, a solubilization solution (Cell Proliferation kit) was added to dissolve the MTT crystals and cells were incubated overnight. The plate was then read on a spectrophotometer at 550 nm, and cell numbers were calculated from a standard curve of known cell number.

CCL22 ELISA

The levels of CCL22 protein in plasma, ascites, and cell culture supernatants were measured by ELISA (R&D Systems). Cell-free tumor ascites and cell culture supernatants were prepared by centrifuging at 300 × g for 10 min and removing supernatant. High protein-binding ELISA plates (96-well, Corning; VWR) were coated overnight at +4°C with an anti-human CCL22 capture Ab. After blocking with PBS containing 0.05% Tween 20 for 2 h, diluted samples and standards were added to the plate and incubated at room temperature for 2 h. Biotinylated anti-human CCL22 detection Ab was then added to each well and incubated at room temperature for

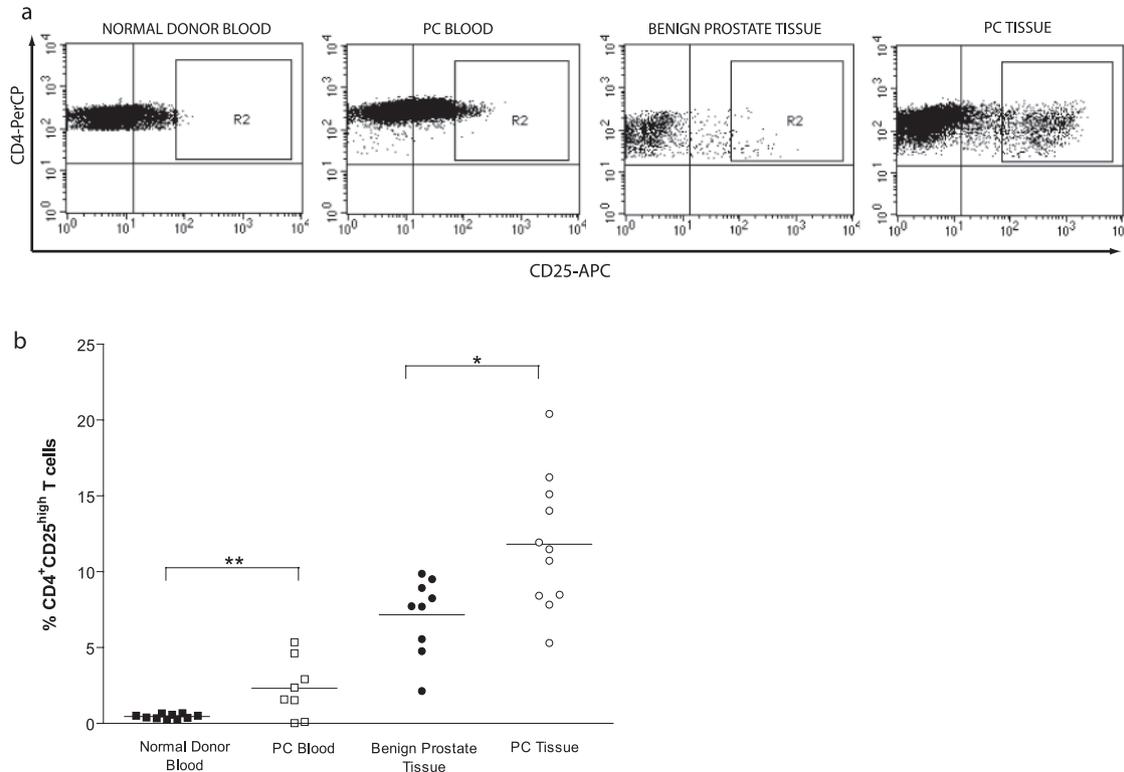


FIGURE 1. Prevalence of $CD4^+CD25^{high}$ T cells is elevated in peripheral blood and tissue samples from PC patients. In the case of tissue biopsy samples, T cells were outgrown by culture in IL-2 (20 U/ml) for 3 days, then analyzed by FACS. Representative flow cytometry plots of $CD4^+CD25^{high}$ T cells (region R2) in peripheral blood from a normal donor and PC patient, and in benign and cancer tissue samples of the prostate (a); scatter plot showing the mean percent $CD25^{high}$ cells of total $CD4^+$ T cells in each sample group (b). The prevalence of $CD4^+CD25^{high}$ T cells in peripheral blood from PC patients was significantly higher than in normal donors (Student's unpaired *t* test; **, $p < 0.01$), and significantly higher in PC tissue vs benign prostate tissue samples (Student's unpaired *t* test; *, $p < 0.05$).

2 h. Assay was developed using phosphatase substrate tablets (S0942; Sigma-Aldrich) and measured spectrophotometrically at 405 nm. Samples were analyzed in duplicate.

Statistical analysis

All data are mean \pm SEM unless stated otherwise. Statistical analysis was performed using paired or unpaired Student's *t* tests. All analyses were performed using GraphPad Prism software (GraphPad).

Results

$CD4^+CD25^{high}$ T cells are enriched in blood and tumor tissue of PC patients

Samples of peripheral blood from normal donors vs PC patients and benign vs malignant prostate tissue samples were screened by flow cytometry for the presence of $CD4^+CD25^{high}$ T cells using stringent gating criteria (Fig. 1a). In peripheral blood, the mean frequency of $CD3^+CD4^+$ T cells that were also $CD25^{high}$ was $0.5 \pm 0.1\%$ from normal donors vs $2.3 \pm 0.7\%$ in PC patients ($p < 0.01$; Fig. 1b). The mean frequency of $CD4^+CD25^{high}$ T cells in benign tissue was significantly lower than that in PC tissue ($6.9 \pm 0.8\%$ vs $11.3 \pm 1.3\%$, respectively, $p < 0.05$; Fig. 1b).

Phenotype of the $CD4^+CD25^{high}$ T cells

By gating on the $CD4^+CD25^{high}$ T cells (region R2; Fig. 1a), three additional cell markers could be studied: GITR, ICOS, and FOXP3. Surface expression of GITR and ICOS on $CD4^+CD25^{high}$ T cells was lower in those from blood compared with those from tissue, with similar levels of expression between T cells in peripheral blood of normal donors vs PC patients and between T cells from benign prostate vs prostate tumor tissue (Fig. 2, a and b).

High levels of FOXP3 expression were detected in $CD4^+CD25^{high}$ T cells in all samples (Fig. 2c).

Immunohistochemical staining of prostate tissue sections was used to visualize the presence of FOXP3-positive cells in prostate tumors. Ten sections of prostate tissue were used from tumors matched for Gleason score and zonal origin (L.E.) to those tumors used for the Treg FACS analysis. All 10 cases contained FOXP3-positive cells. Benign glands were surrounded by fewer inflammatory cells and fewer FOXP3-positive cells (Fig. 3a) when compared with malignant glands of the prostate (Fig. 3b).

Peripheral blood $CD4^+CD25^+$ T cells and PC tissue supernatants are immunosuppressive in vitro

To determine whether the $CD4^+CD25^+$ T cells in blood from PC patients are functional Tregs, we used an in vitro suppression assay. When activated with CD3/CD28 beads $CD4^+CD25^-$ T cells, but not $CD4^+CD25^+$ T cells, proliferate strongly. In the presence of increasing numbers of $CD4^+CD25^+$ T cells, this proliferation is suppressed in a dose-dependent manner (Fig. 4a).

Because of the small size of prostate tumors, very low numbers of $CD3^+$ T cells were obtained from the tissue ($<200,000$) and hence it was not possible to carry out functional assays directly using these cells. Instead, we looked at the effect of supernatant, taken from either benign or PC tissue in culture, on proliferation of $CD4^+CD25^-$ cells. Proliferation was markedly suppressed in the presence of PC supernatant but not in the presence of benign supernatant (Fig. 4b). Cytokines in supernatants taken from the benign and PC biopsies were measured after 2 days in culture. The

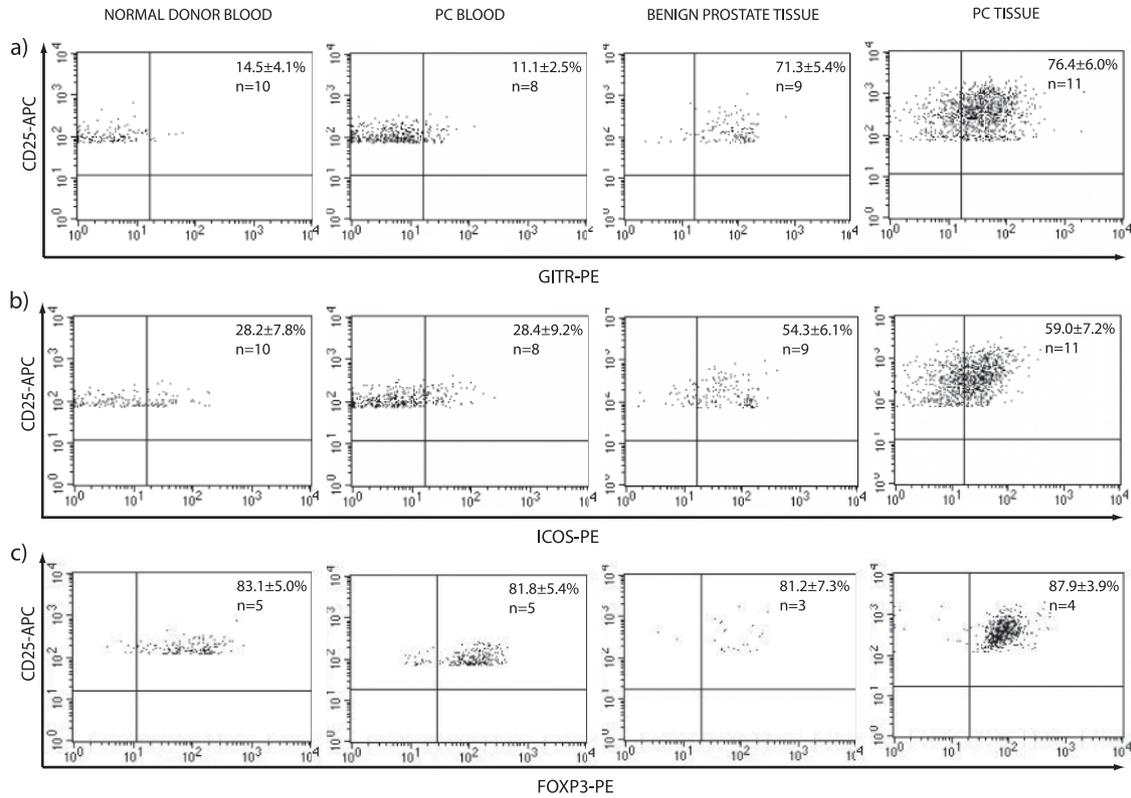


FIGURE 2. CD4⁺CD25^{high} T cells from PC patients demonstrate Treg phenotype by coexpression of GITR, ICOS, and FOXP3 markers. In the case of tissue biopsy samples, T cells were outgrown by culture in IL-2 (20 U/ml) for 3 days, then analyzed by FACS. Representative flow cytometry plots showing mean percentage of CD25^{high} T cells that coexpress GITR (a), ICOS (b), and FOXP3 (c) in peripheral blood from a normal donor and PC patient and in benign prostate and PC tissue samples.

cytokines IFN- γ , IL-4, and very high levels of IL-6 were detected (Fig. 4c). All other cytokines measured were below the limit of detection.

CD4⁺CD25⁺ T cells migrate toward PC tissue supernatant and tumor ascites

We analyzed the migration of purified populations of CD4⁺CD25⁺ T cells from peripheral blood of normal donors to recombinant chemokines alone or culture supernatants from PC tissue or cells and malignant ascites. Both SDF-1 α and CCL22 induced migration of CD4⁺CD25⁺ T cells (Fig. 5a), and the typical chemokine-induced bell-shaped curve was obtained. Supernatants taken from LNCaP cells and both benign and malignant tissue in culture were able to induce migration of CD4⁺CD25⁺ T cells (Fig. 5b). Also, tumor ascites supernatant obtained from a patient with rare PC exhibited powerful chemotactic activity. Plasma from the same patient was also able to chemoattract these cells but to a lesser extent than the ascites supernatant (Fig. 5b).

Supernatants were analyzed for the presence of the chemokine CCL22 using ELISA (Fig. 5c). Low levels of CCL22 were detected in culture supernatants taken from the LNCaP cell line (0.12 ± 0.05 ng/ml). More CCL22 was detected in supernatants taken from both benign and malignant tissue in culture (9.04 ± 3.75 vs 7.76 ± 3.21 ng/ml), although no significant difference was seen in the levels of CCL22 despite the fact that the tumor supernatant induced more cell migration than benign supernatant (Fig. 5b). Because of the rarity of ascites in PC patients, we only had one ascites sample available for testing. Tumor ascites had higher levels of CCL22 than plasma taken from the same patient (3.79 ± 0.07 and 1.12 ± 0.01 ng/ml, respectively), although levels were

lower than those seen in biopsy supernatants, despite ascites inducing a higher migration of cells.

Discussion

There is accumulating evidence that CD4⁺CD25⁺ T cells are recruited to human carcinomas and their abundance may predict for reduced survival (20–29). In the current article, we present the first detailed study demonstrating that the frequency of CD4⁺CD25^{high} T cells is clearly increased in both tumor tissue and peripheral blood of patients with early-stage PC.

It has been demonstrated by Baecher-Allan et al. (35) that T cells with regulatory properties mainly reside in the CD4⁺ T cell fraction expressing CD25 at a high level (CD25^{high}). In addition, Kuniyasu et al. (36) have demonstrated that Tregs express higher levels of CD25 than activated cells. Therefore, in this study the gate for CD25 was deliberately set high for isolation of Tregs as opposed to activated T cells expressing CD25. Using this method, we were able to demonstrate that blood from PC patients contains a higher frequency of CD4⁺CD25^{high} T cells than that from normal donors (Fig. 1b). Malignant prostate tissue samples had a significantly higher frequency of CD4⁺CD25^{high} T cells than benign tissue from the same prostate (Fig. 1b). The phenotype of the CD4⁺CD25^{high} cells was confirmed by demonstrating coexpression of the markers GITR, ICOS, and FOXP3 (Fig. 2). Both GITR and ICOS were more highly expressed in Tregs from tissue than those in blood. This may be reflective of the IL-2 used in tissue culture conditions (see below). Alternatively, McHugh et al. (10) have shown that GITR expression is induced upon activation of CD4⁺CD25⁺ T cells. Hence, it is possible that the Tregs in the tissue are activated or induced by their environment in vivo to

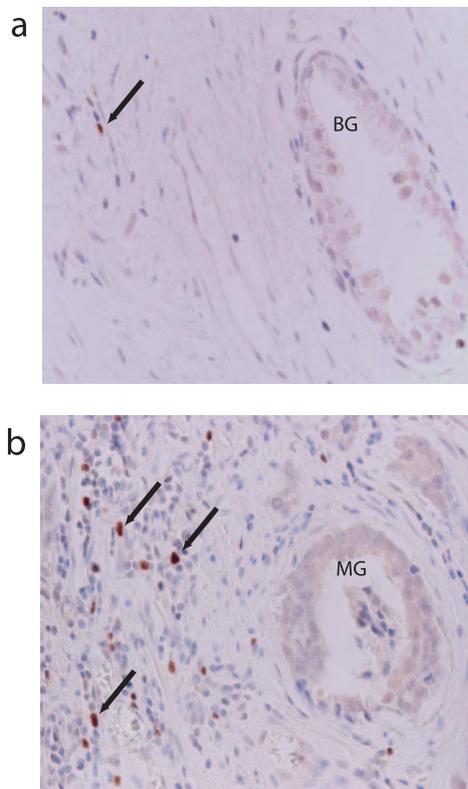


FIGURE 3. FOXP3 expression in prostate tissue. Representative sections of prostate tissue, showing benign (*a*, BG) or malignant gland (*b*, MG; Gleason score, 3 + 3 = 6) from the same prostate. Arrows indicate FOXP3-positive cells (brown nuclear staining; original magnification, $\times 40$).

express more GITR and ICOS, while the Tregs in blood are resting.

The majority of CD25^{high} T cells were FOXP3-positive in all samples. This transcription factor thought to be specifically expressed by and currently the most accurate marker to identify Tregs (14, 34). In addition, we analyzed FOXP3 expression in prostate tumors by immunohistochemistry. Malignant glands were surrounded by a dense inflammatory cell infiltrate and many FOXP3-positive cells, when compared with benign glands where fewer FOXP3-positive cells were seen (Fig. 3).

As described by others previously, we cultured the prostate tissue samples in low-dose IL-2 (20 U/ml) for 3 days to allow the outgrowth of TILs (37). It is possible that the IL-2 altered expression of CD25 (38), GITR, or ICOS, all of which were higher in the tissue T cells vs the blood T cells; however, the tumor and benign tissue were cultured in exactly the same way and yet tumor tissue had a significantly higher percentage of CD25^{high} cells. Furthermore, we cultured PBMC from normal donors in the same concentration of IL-2 for 3 days and saw no change in CD25, GITR, or ICOS expression (data not shown), and the concentration of IL-2 used here is very low in comparison to other studies (37).

Tregs from the peripheral blood of PC patients suppressed the proliferation of CD4⁺CD25⁻ T cells (Fig. 4*a*). This suppression was perhaps at a slightly lower level than seen with Tregs from other tumor types (27), but this may be a reflection of the assay conditions used since CD3/CD28 beads are known to deliver an extremely strong proliferation stimulus (39).

Surgery for PC is performed only in early-stage localized disease with a small tumor. Therefore, the current study could only be conducted in very small PC biopsies, with a limited

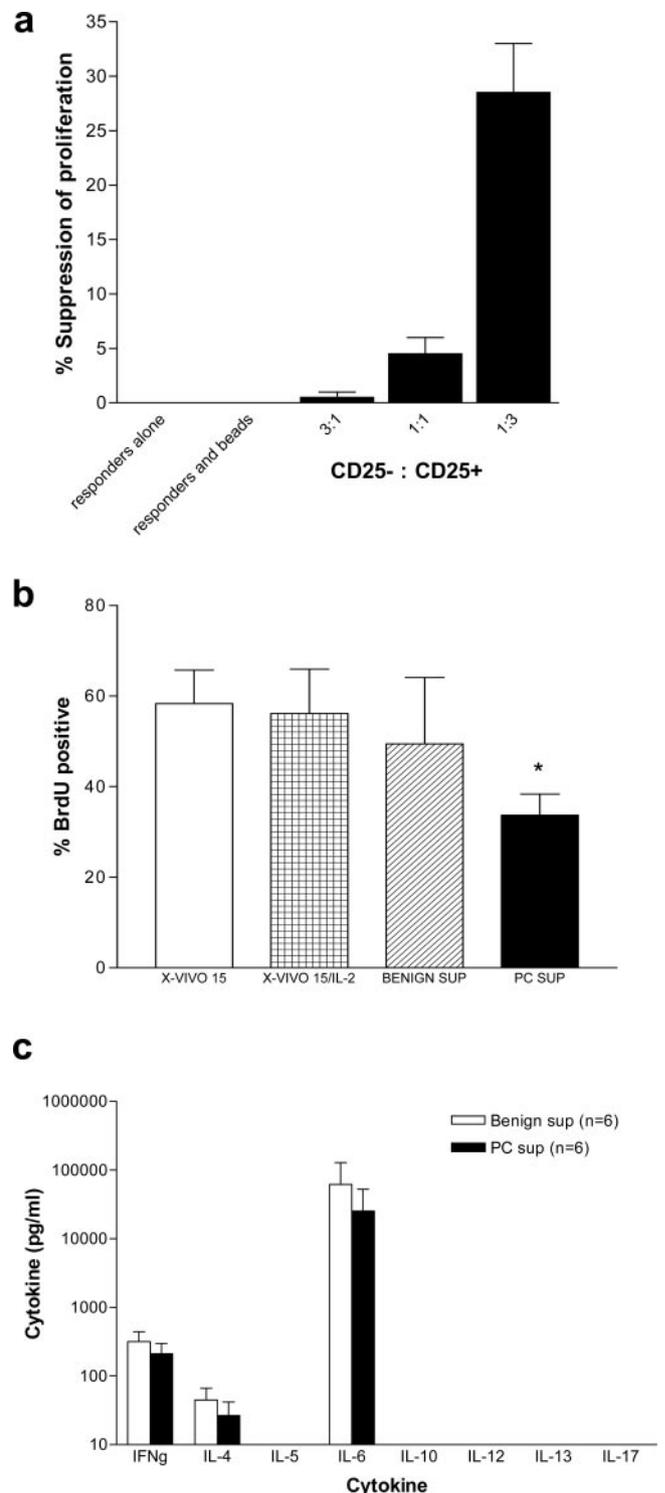


FIGURE 4. CD4⁺CD25⁺ T cells isolated from the peripheral blood of PC patients and PC tissue culture supernatants suppress T cell proliferation in vitro. *a*, CD4⁺CD25⁻ T cells were stained with 1 μ M CFSE, stimulated with CD3/CD28 beads, and cultured in the presence of the indicated ratio of CD4⁺CD25⁺ T cells ($n = 2$). Suppression of proliferation was calculated as percentage of the decrease in CFSE mean fluorescence intensity produced by the responders plus beads sample. *b*, CD4⁺CD25⁻ T cells were stimulated with CD3/CD28 beads, cultured in tissue culture supernatants from benign or PC tissue ($n = 4$), and proliferation was assessed by measuring BrdU incorporation. Proliferation of CD4⁺CD25⁻ T cells in the presence of PC supernatant was significantly inhibited compared with control (X-VIVO medium with 20 U/ml IL-2) (Student's paired *t* test; *, $p < 0.05$). *c*, Bio-Plex assay for the measurement of multiple cytokines present in benign (\square) and tumor (\blacksquare) culture supernatant ($n = 6$).

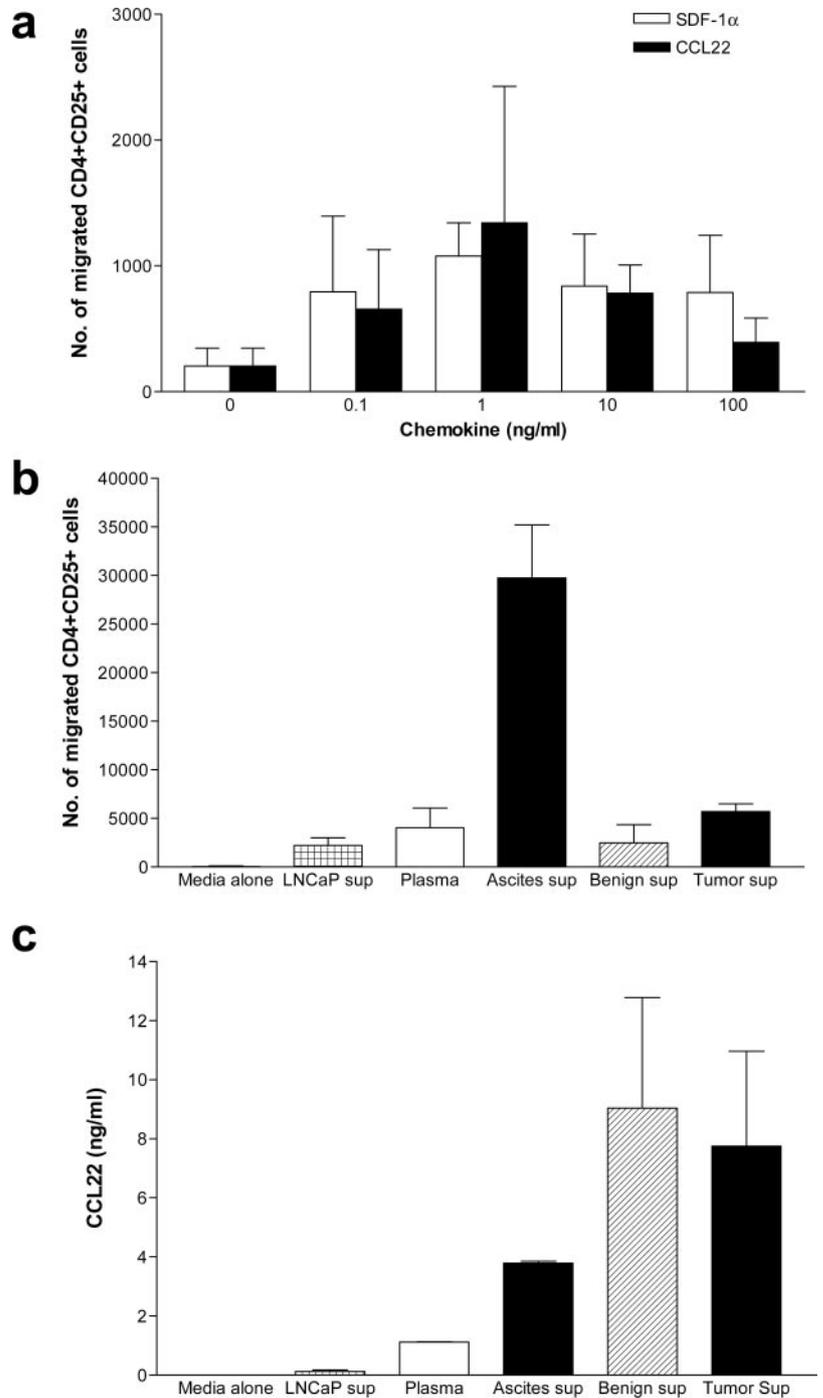


FIGURE 5. Prostate tumors secrete factors that induce migration of Tregs: CD4⁺CD25⁺ T cells isolated normal donors migrate to (a) chemokines CCL22 and SDF-1 (*n* = 4); (b) supernatants removed from LNCaP cells (*n* = 5), plasma, or ascites (*n* = 6), and benign prostate or PC tissue samples after 2 days in culture (*n* = 4). CCL22 levels (nanograms per milliliter) were measured by ELISA in tissue culture supernatants from LNCaP cells (*n* = 1), plasma or ascites (*n* = 1), and benign prostate or PC tissue (*n* = 4) (c). All data are shown as mean \pm SEM, except *n* = 1 values where the mean of duplicate measurements \pm SD are shown.

availability of cells (<200,000 TILs outgrow the tumor and even fewer from benign tissue). This makes any functional analysis of the TILs or the subpopulations of Tregs from prostate biopsies technically very difficult. Therefore, although we were able to demonstrate some suppressive activity of Tregs from the peripheral blood of the PC patients this was not possible with the tumor Tregs. Instead, we show that supernatant from prostate tumors is immunosuppressive (Fig. 4b). We conducted a multiplex cytokine analysis of the supernatants to investigate which cytokines may be mediating the suppressive effects. We were able to demonstrate presence of IFN- γ , IL-4, and extremely high levels of IL-6 in supernatants taken from both benign and PC biopsies in culture (Fig. 4c). Both IL-4 and IL-6 have been shown to have some immunosuppressive properties

(40, 41); however, because they appear in similar levels in both benign and PC supernatants this cannot account for the higher suppression seen with the PC supernatant (Fig. 4b). IL-10 was not detected in any of the samples. Although it has been shown in some studies that Tregs may mediate immune suppression through the secretion of IL-10 (42), other studies have shown that TGF- β or suppression by direct cell-cell contact may be as important (43). Indeed, prostate tumors have been shown in previous studies to secrete many substances capable of suppressing T cell activation, including TGF- β and others such as prostate-specific Ag mucin 1, cyclooxygenase 2, and L-arginine metabolites (44–47). Hence, these other tumor-derived factors may be responsible for the increased suppression seen with PC supernatant.

The presence of Tregs in prostate tumors emphasizes their potential role to down-regulate the functions of effector T cell subsets, and future studies should be directed at examining their Ag specificity and function. Indeed, recent work has shown EBV-encoded nuclear Ag 1-specific Tregs in normal donors and LAGE1- or ARTC1-specific Treg cells in TIL lines of melanoma patients, providing evidence that tumor-specific ligands may play a critical role in inducing tumor-specific immune tolerance (48–50).

There are several possible explanations for increased numbers of Tregs inside tumors. First, it is possible that the tumor cells or other cells inside the tumor secrete chemokines that attract Tregs to migrate into the tumor. Treg cells have been shown to express a variety of chemokine receptors, including CCR4, CCR7, CCR8, CXCR4, and CXCR5 depending on their activation status and tissue locality (51, 52). Recently, Curiel et al. (27) have shown that tumor cells and microenvironmental macrophages produce the chemokine CCL22, which mediates Treg trafficking to the tumor. In this study, we demonstrate that some PC cell lines, malignant ascites fluid from a PC patient, and prostate tumor biopsies in culture contain or secrete CCL22 and can chemoattract Tregs in an *in vitro* migration assay (Fig. 5). However, it is clear that this is not the only chemokine secreted by the tumor. Benign and tumor supernatants contained equivalent levels of CCL22, but tumor supernatant induced more cells to migrate. Furthermore, ascites fluid was strongly chemotactic for Tregs, but contained lower levels of CCL22 than both benign and tumor supernatants. The chemokine receptors expressed by Tregs have a variety of ligands, such as CCL22, CCL17, CCL1, and vMIP-I (CCR4 and CCR8); CCL19 (CCR7); SDF-1 α (CXCR4); and CXCL13 (CXCR5). Thus, further studies will be necessary to assess which chemokines prostate tumors secrete and which of those are responsible for the increased presence of Tregs inside these tumors.

An additional explanation for the increased numbers of CD4⁺CD25^{high} T cells within tumor biopsies may reflect that locally produced proinflammatory cytokines can up-regulate CD25 expression or induce and expand Treg pools. Other studies have shown that activation of CD4⁺ T cells to a CD25⁺ state in itself is insufficient for conferring a regulatory phenotype on T cells (36). It has also been suggested that in tumors there may be a difference in sensitivity of CD4⁺ T cells to clonal depletion or apoptosis, in which CD4⁺CD25⁺ Tregs are resistant and that tumor-derived factors can induce apoptosis selectively in CD4⁺CD25⁻ T cells (53). Alternatively, it was also shown that repetitive stimulation with immature dendritic cells induced the development of Tregs (54). Hence, it is possible that dendritic cells, which have been shown to be inside prostate tumors (55), may induce the formation of Tregs.

In this study, we were able to demonstrate that Tregs are already present in relatively small localized tumors at an early stage. Although we were unable to study any correlation between elevated Tregs and prostate-specific Ag levels, tumor stage, or Gleason score (due to low patient numbers), it is hypothesized that the presence of increased numbers of Tregs may predict for reduced survival. This has been shown to be true for advanced ovarian cancer (27), but in PC the relationship between lymphocytic infiltration and survival is still unclear. Vesalainen et al. (56) reported that tumors with a dense tumor lymphocyte infiltration were associated with higher survival rates than tumors with absent or decreased infiltrates (56). In contrast, Irani et al. (57) reported that an increased inflammatory cell infiltrate within the tumor was associated with an increased risk of tumor recurrence (57). More recently, McArdle et al. (58) have reported that the presence of CD4⁺ T cell infiltrate was associated with poor cancer survival in patients with PC (58). One interpretation may be that patients who lack intratumoral T cells fail to mount an immune response to the

tumor, whereas patients with intratumoral T cells are in the process of mounting an immune response, the success of which depends on the relative proportion of effector and Tregs (59).

In conclusion, this study demonstrates that Tregs appear to be increased in patients with early-stage PC. These cells may play a role in modulation of effector T cell responses against prostate tumors. New therapeutic strategies aimed at inhibiting Tregs, such as the IL-2 diphtheria toxin conjugate recently shown to enhance vaccine-mediated immunity in renal cancer patients (6), may also improve tumor-specific immunotherapy in PC patients.

Acknowledgments

We thank Christina Trollmo and Vivianne Malmström for helpful discussions during the project, Liss Garberg and Margareta Rodensjö for assistance with immunohistochemistry, and the patients and personnel at the Urology Clinic for providing samples.

Disclosures

The authors have no financial conflict of interest.

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