

Phenotypic Analysis of Prostate-Infiltrating Lymphocytes Reveals T_H17 and T_{reg} Skewing

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Abstract Purpose: Pathologic examination of prostate glands removed from patients with prostate cancer commonly reveals infiltrating CD4⁺ and CD8⁺ T cells. Little is known about the phenotype of these cells, despite accumulating evidence suggesting a potential role for chronic inflammation in the etiology of prostate cancer.

Experimental Design: We developed a technique that samples the majority of the peripheral prostate through serial needle aspirates. CD4⁺ prostate-infiltrating lymphocytes (PIL) were isolated using magnetic beads and analyzed for subset skewing using both flow cytometry and quantitative reverse transcription-PCR. The transcriptional profile of fluorescence-activated cell sorted prostate-infiltrating regulatory T cells (CD4⁺, CD25⁺, GITR⁺) was compared with naïve, peripheral blood T cells using microarray analysis.

Results: CD4⁺ PIL showed a paucity of T_H2 (interleukin-4–secreting) cells, a surprising finding given the generally accepted association of these cells with chronic, smoldering inflammation. Instead, CD4⁺ PIL seemed to be skewed towards a regulatory T_{reg} phenotype (FoxP3⁺) as well as towards the T_H17 phenotype (interleukin-17⁺). We also found that a preponderance of T_H17-mediated inflammation was associated with a lower pathologic Gleason score. These protein level data were reflected at the message level, as analyzed by quantitative reverse transcription-PCR. Microarray analysis of pooled prostate-infiltrating T_{reg} revealed expected T_{reg}-associated transcripts (FoxP3, CTLA-4, GITR, LAG-3) as well as a number of unique cell surface markers that may serve as additional T_{reg} markers.

Conclusion: Taken together, these data suggest that T_H17 and/or T_{reg} CD4⁺ T cells (rather than T_H2 T cells) may be involved in the development or progression of prostate cancer.

The presence of CD3⁺ tumor-infiltrating lymphocytes has been shown to correlate with favorable outcome in patients with several types of cancer including ovarian and colorectal carcinomas (1, 2). A similar scenario has been proposed for prostate cancer, yet studies have yielded disparate results (3, 4). Indeed, prostate-infiltrating lymphocytes (PIL) are frequently present both in and around the tumor-burdened areas of the gland. Few studies have investigated the phenotype of CD4⁺ T cells infiltrating the peripheral zone of the prostate, where most adenocarcinomas arise. In addition to the T_H1 and T_H2

subsets, which secrete IFN- γ and interleukin (IL)-4, respectively, a new subset of CD4⁺ (helper) T cells, termed T_H17 cells, has been characterized by the production of IL-17. Whereas all three CD4⁺ T cell subtypes are known to play a role in immunomediated defense against intracellular or extracellular pathogens, T_H17 cells are unique in that they are the key mediators in a number of autoimmune diseases, and may play a role in inflammation-associated cancer (reviewed in refs. 5, 6). Interestingly, increased expression of IL-17 at the mRNA level was shown in tissue from both prostate cancer and benign prostatic hyperplasia before the discovery that T_H17 T cells represent a distinct lineage (7). Whether T_H17 cells comprise a higher proportion of PIL than the peripheral blood lymphocytes of patients with prostate cancer has not been investigated.

Although CD4⁺ T cells are present in the human prostate, it is not yet clear whether these cells mediate an antitumor effector function, or whether they serve to dampen or regulate a CD8⁺ T-cell-mediated antitumor response. Important in this respect are regulatory T cells (T_{reg}), a CD4⁺ T cell lineage involved in the suppression of autoreactive T cells, and thus prevention of autoimmunity. In light of this role in the suppression of self-reactive cells, T_{reg} have recently been investigated as suppressors of antitumor immune responses (reviewed in ref. 8). Increased numbers of T_{reg} have been reported in tumor-infiltrating lymphocytes of several human solid tumors including breast, pancreatic, hepatocellular, and prostate carcinomas (9–11).

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Furthermore, increased percentages of T_{reg} in the peripheral blood of gastric and esophageal cancer patients and in the tumor tissue of ovarian cancer patients correlated with poor prognosis and decreased survival (12, 13). In earlier studies, CD25 was used as a cell surface marker for T_{reg} (8). More recent data have suggested that the forkhead box transcription factor FoxP3 is a more specific marker for T_{reg} (14). Here, we used intracellular staining for FoxP3 to determine the prevalence of T_{reg} among PILs.

In addition to these protein level studies, we also used quantitative reverse transcription-PCR (qRT-PCR) analysis to conduct a comprehensive analysis of the presence of these subsets (T_{H1}, T_{H2}, T_{H17}) in PIL and to determine the relative prevalence of CD4⁺ T_{reg} that are potentially capable of regulating effective antitumor immune responses. Finally, we investigated correlations between T-cell subsets and tumor Gleason score in order to provide data regarding a potential tumor-promoting (or tumor-inhibiting) role for each subset.

Materials and Methods

Patient population and clinical samples. All specimens were acquired under a Johns Hopkins Medicine Institutional Review Board–approved protocol with written informed consent obtained from each patient. PIL samples were obtained from 20 patients (ages 37–66; mean, 56 years old) undergoing radical retropubic prostatectomy for localized adenocarcinoma of the prostate at the Johns Hopkins Hospital in Baltimore, MD (Supplementary Table S1). None of the patients were previously treated with immunosuppressive or radiation therapy. Within 1 h of resection, needle aspirates were taken from the posterior aspect (peripheral zone) of the prostate using a 20-gauge 1.5-inch needle and 5 mL syringe, and collected into RPMI 1640 supplemented with <1% FCS. Because prostate cancer typically presents multiple foci in the peripheral zone of the prostate (15), the aim was to sample T cells from the entire zone as opposed to a single, macroscopically identifiable tumor, as even macroscopically normal-appearing prostate tissue could contain multiple small tumor foci. Cells were resuspended in 1 mL of Dynal Buffer 1 and strained through a 100- μ m strainer. CD4⁺ T cells were positively isolated using the Dynal CD4 Positive Isolation Kit (Invitrogen) and the manufacturer's recommended protocol, including magnetic bead detachment. Positively isolated CD4⁺ T cells were then washed once with CTL medium (RPMI, 1% L-glutamate, 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin, 10% FCS, and 3.47 μ L/L 14.4 mol/L B-mercaptoethanol).

Patient-matched peripheral blood samples were collected by venipuncture into 8.5 mL whole blood tubes with ACD solution A (BD Biosciences Vacutainer Systems) the evening prior to radical retropubic prostatectomy surgery and remained at room temperature with gentle shaking overnight. Multiple control experiments were done to verify that expression of the cytokines examined in this study did not vary over the short overnight incubation period (data not shown). CD4⁺ T cell isolation was done as described for PIL samples, except with 2 mL of whole blood as starting material. Using a directly conjugated monoclonal antibody (mAb) against CD4 (APC, Caltag), ~5,000 cells from each patient were stained to verify the purity of positively isolated CD4⁺ T cells. Samples were typically >95% CD4-positive.

CD4⁺ T cells used for qRT-PCR analyses were isolated from patient-matched peripheral blood and prostate samples as described above, but without bead detachment. These samples were snap-frozen and stored at -80°C until RNA extraction.

CD4 T-cell stimulation. Isolated CD4⁺ T cells from prostate and peripheral blood were resuspended in CTL medium with 0.05 μ g/mL of phorbol 12-myristate 13-acetate, 0.5 μ g/mL of ionomycin, and 1:1,000 GolgiStop (BD Biosciences) and plated at a density of <1 \times 10⁶

in 96-well U-bottomed plates. Cells were stimulated for 4 h at 37°C prior to intracellular staining.

Intracellular antibody staining and flow cytometry. Surface staining with a PE-Cy5 labeled mAb to CD45RO (BD Biosciences) was done prior to cell permeabilization. Intracellular staining with directly conjugated mAbs against IFN- γ (APC) and IL-17 (PE; eBioscience, clones 4S.B3 and eBio64Dec17, respectively) was done using the BD Cytotfix/Cytoperm Fixation/Permeabilization Kit according to the manufacturer's recommended protocol. Intracellular staining with directly conjugated mAbs against FoxP3 (APC, eBioscience, clone PCH101) and IL-4 (PE, BD Biosciences) was done using the eBioscience Human Regulatory T-cell Staining Kit and the manufacturer's recommended protocol. Each of these mAbs (IFN- γ , IL-17, IL-4, and FoxP3) were titrated using naïve CD4⁺ T cells skewed towards the respective subset *in vitro*, and those skewing conditions served as positive controls for staining (Supplementary Materials and Methods). Flow cytometry for all *in vitro* controls as well as human peripheral blood and prostate samples was conducted using FACSCalibur (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Inc.).

qRT-PCR. These studies were done with the assistance of the Human Immunology Core Facility at the Johns Hopkins University School of Medicine (Baltimore, MD). RNA from patient-matched peripheral blood and prostate CD4⁺ T cells was extracted using the Trizol reagent (Invitrogen) with modifications for low cell numbers including the addition of glycogen as an RNA carrier. Synthesis of cDNA was done using random primers and Ready-To-Go beads (GE Life Sciences). The level of gene expression was determined by quantitative PCR done in triplicate with multiplexed target and control gene primer/probe sets using an ABI 7000 prism system (Applied Biosystems). Threshold cycle (C_T) values were calculated as the average of three runs for each gene and were normalized to CD4 using the equation: Ratio = 2^{-(C_T CD4 - C_T target gene)}. The target genes *GAPDH*, *CD4*, *IFN- γ* , *IL-17*, *IL-4*, *FoxP3*, *IL-23R*, *IL-12*, and *IL-10* were analyzed using commercially available primer/probe pairs (Applied Biosystems).

Transcriptional analysis. Peripheral blood from four patients undergoing radical retropubic prostatectomy was collected for isolation of naïve CD4 T cells. From each patient, 2 mL of whole blood was lysed with ACK lysing buffer (Quality Biological, Inc.) and PBMC were stained with the following directly conjugated mAbs: CD4 (FITC, Caltag), CD45RA (PE-Cy5, BD Biosciences), and CD25 (PE, Miltenyi Biotec). The CD4⁺CD45RA⁺CD25⁻ population from peripheral blood samples (representing naïve CD4⁺ T cells) was sorted to >95% purity using a FacsVantage instrument (BD Biosciences). Radical prostatectomy specimens from 11 patients were aspirated as described previously and stained with directly conjugated mAbs to CD4 (PC5, Beckman Coulter), CD25 (PE, Miltenyi Biotec), and GITR (FITC, R&D Systems). As above, the CD4⁺CD25^{high}GITR⁺ (T_{reg}) population from prostate samples was sorted to >90% purity using a FacsVantage instrument. Cells were frozen in 1 mL of Trizol and stored at -80°C prior to RNA extraction using the Trizol reagent. The integrity of extracted RNA from both peripheral blood and prostate T cells was analyzed using an Agilent 2100 Bioanalyzer and the RNA 6000 Pico and Nano Kits (Agilent Technologies) and concentrations were determined using a NanoDrop spectrophotometer (NanoDrop Technologies). Transcriptional analysis was done at the Johns Hopkins Microarray Core facility. Per standard protocol, RNA was amplified from 20 ng of starting total RNA with the Nugen Ovation RNA Amplification System V2, following the manufacturer's protocol.⁴ cDNA was synthesized using the Nugen FL-Ovation cDNA Biotin Module V2 kit, following the manufacturer's protocol.⁴ After standard labeling, each sample was hybridized to an Affymetrix U133Plus 2.0 Human Genome array, followed by examination with an Affymetrix GeneChip Scanner 3000.

⁴ http://www.nugeninc.com/pdfs/ov-v2_userguide.pdf

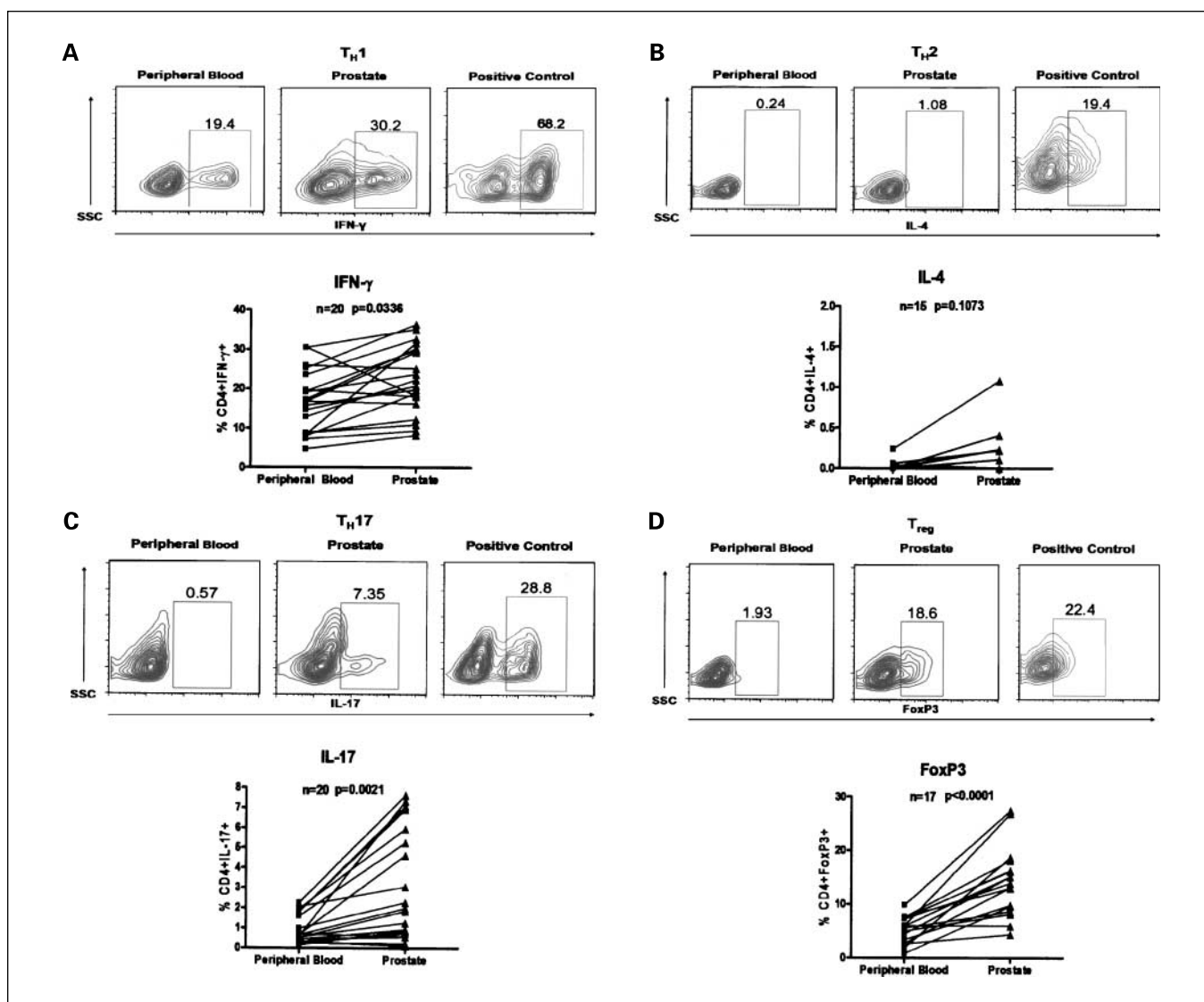


Fig. 1. Frequency of T_{helper} subsets in peripheral blood and prostate tissue of patients with prostate cancer. Positively isolated $CD4^+$ T cells were stimulated for 4 h in the presence of phorbol 12-myristate 13-acetate and ionomycin and analyzed by flow cytometry. Representative fluorescence-activated cell sorting plots of $CD4^+IFN-\gamma^+$ (T_{H1}), $CD4^+IL-4^+$ (T_{H2}), $CD4^+IL-17^+$ (T_{H17}), and $CD4^+FoxP3^+$ (T_{reg}) T cells in peripheral blood and prostate tissue of prostate cancer patients with representative positive controls for each cellular marker from independent *in vitro* skewing experiments as well as a summary of the data. Percentage of $CD4^+$ T cells positive for IFN- γ , IL-4, IL-17, and FoxP3. *P* values were calculated using two-sided Student's *t* test.

Statistical analyses. Differences between peripheral blood and prostate T cell populations determined by flow cytometry were analyzed using a two-sided Student's *t* test and the PRISM package (GraphPad Software, Inc.). Statistical analyses for the microarray experiment are described in the Supplementary Materials and Methods.

Results

T_{H17} skewing in PIL. We first did a subset-specific phenotypic analysis of the $CD4^+$ T cells in PIL from radical prostatectomy specimens. Numerically, the most abundant $CD4^+$ T-cell subset observed in PIL were IFN- γ -secreting T_{H1} cells (Fig. 1A). Interestingly, we found almost no IL-4-secreting cells among these PIL, suggesting a skewing away from a T_{H2} phenotype when compared with a positive *in vitro*-derived T_{H2}

control (Fig. 1B). These data are in contrast with suggestions that prostate cancer arises in the context of T_{H2} -mediated inflammation (16). Remarkably, a significant skewing towards a T_{H17} phenotype was noted in the PIL (Fig. 1C). In some patients, up to 8% of the $CD4^+$ PIL secreted IL-17 upon brief stimulation. A number of factors influence the differentiation of naïve $CD4^+$ T cells towards a T_{H17} phenotype (5). Among these, transforming growth factor- β (TGF- β) and IL-6 have been associated with prostate cancer (17, 18), and might be present in the prostate microenvironment. It should be noted that although TGF- β and IL-6 have been shown to drive the initial lineage commitment of T_{H17} cells in mice (19–22), recent data using human naïve $CD4^+$ T cells suggest that T_{H17} polarization is induced by IL-1 β , possibly enhanced by IL-6, and may be suppressed by TGF- β (23, 24).

A regulatory phenotype in PIL. Because T_{H17} cells were predominant among PIL, and these cells may differentiate under the influence of TGF- β , we queried whether T_{reg} (which also differentiate in response to TGF- β) would be present in the prostate glands of men with cancer. In this regard, previous work had already identified $CD4^+CD25^{high}$ T cells as a component of prostate tumor-infiltrating lymphocytes (11). To extend those studies, we quantified the T_{reg} component of PIL based on intracellular FoxP3 staining, as FoxP3 represents a potentially more specific T_{reg} marker (14). As shown in Fig. 1D, nearly all patients examined showed a relative enrichment of FoxP3 $^+$ T_{reg} with respect to peripheral blood.

Taken together, this phenotypic analysis of PIL suggests that these cells are neither proinflammatory nor consistent with a "smoldering" T_{H2} -mediated process. Rather, the $CD4^+$ T cells in the prostate seem to be skewed towards either the T_{H17} phenotype associated with autoimmunity or the T_{reg} phenotype, which down-regulates $CD8^+$ T cell function (25). We next examined these data in summary, comparing the relative ratio of each T-cell subset in the PIL versus peripheral blood. These data (Fig. 2A) confirm the notion that PIL are generally not skewed towards a T_{H2} phenotype, but rather, seem to be biased towards T_{H17} and T_{reg} . It should be noted that there is a weak skewing of PIL towards a T_{H1} phenotype as well. It is feasible that in some patients, these cells represent potential functional $CD4^+$ effector cells, a point which will be discussed in further detail below.

Due to recent conflicting data regarding the potential influence of TGF- β in the development of T_{reg} and T_{H17} cells, we sought to determine whether these two subsets cosegregated

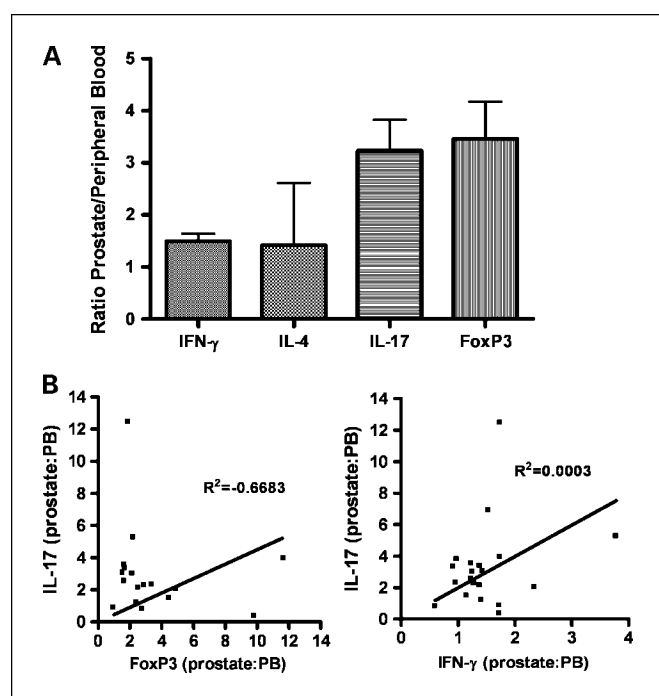


Fig. 2. Prostate-infiltrating T cells are not skewed toward a T_{H2} phenotype. **A**, ratio of prostate-infiltrating T cells positive for each of the markers shown/peripheral blood T cells for that marker. **B**, correlative data for FoxP3 vs. IL-17 and IFN- γ vs. IL-17 (patients were compared by ratio of prostate/peripheral blood for each marker). R^2 value determined by linear least-squares regression.

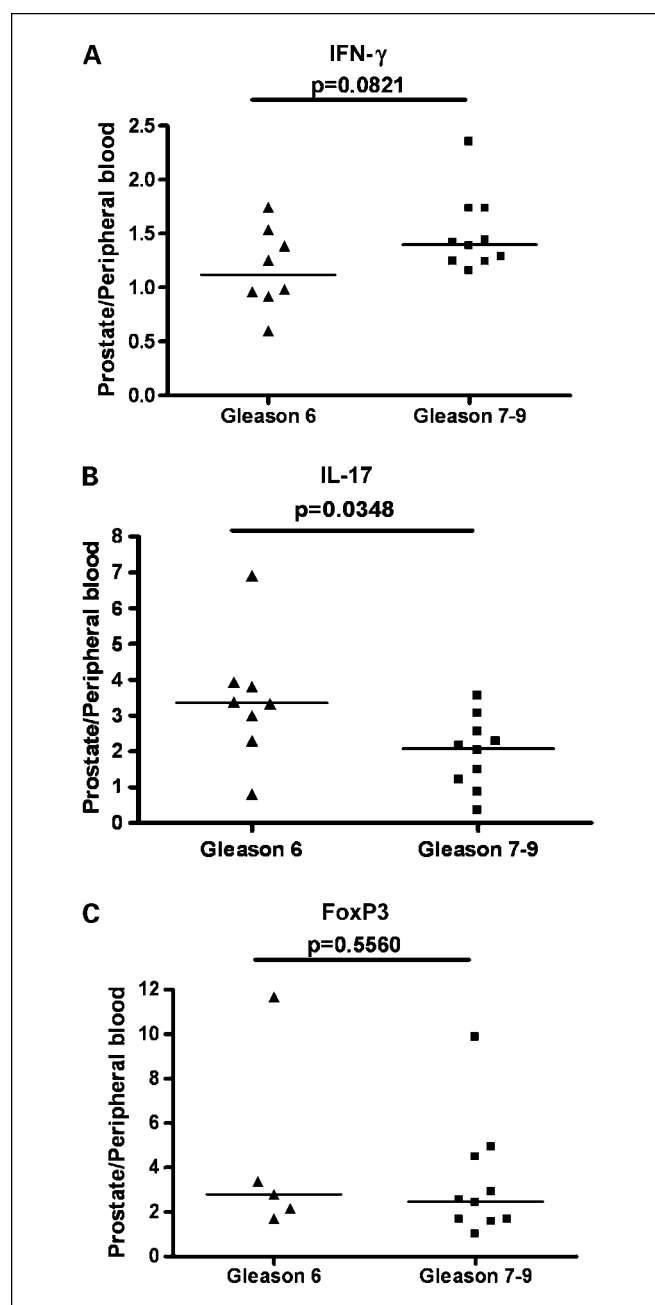


Fig. 3. T_{H17} skewing of prostate-infiltrating $CD4^+$ T cells inversely correlates with Gleason grade. Y-axis, ratio of prostate-infiltrating $CD4^+$ T cells positive for a given cytokine or marker/peripheral blood $CD4^+$ T cells positive for that cytokine or marker. Bar, median; P values were calculated by two-sided Student's t test.

in patients, i.e., whether patients with a relative up-regulation of T_{H17} cells in the prostate also showed a relative up-regulation of T_{reg} . As shown in Fig. 2B, this was not the case, patients with a relative T_{H17} skewing seemed to show less of a T_{reg} bias. Because one major difference between T_{H17} and T_{reg} development is the presence of IL-6 during the differentiation process (5), it might be that patients with relative T_{H17} skewing had higher levels of IL-6. We tested this hypothesis by determining serum IL-6 levels in these patients using ELISA analysis (data not shown). Those results did not show a significant correlation between serum IL-6 levels and T_{H17}

skewing; however, it is far more likely that local (i.e., intraprostatic) IL-6 levels would need to be examined to fully determine a role for IL-6 in this process. Recent data also indicate that IL-2 might play a role in regulating the T_{H17}/T_{reg} balance in the tumor microenvironment (26), but once again, those data would need to be determined at the tissue level rather than at the serum level. We also examined a potential relationship between T_{H17} and T_{H1} skewing (Fig. 2B). As expected, no such relationship was found ($R^2 = 0.0003$).

T_{H17} skewing of prostate-infiltrating $CD4^+$ T cells inversely correlates with Gleason grade. As there is accumulating evidence for an association between increased numbers of tumor-infiltrating T_{reg} and high-grade or high-risk disease for several types of cancer (12, 13, 27), we next sought to determine whether T_{reg} skewing (as assayed at the protein level by intracellular FoxP3 staining) associated with disease grade in this group of men with prostate cancer. As shown in Fig. 3, such an association was not observed. We further hypothesized that an effector phenotype (T_{H1}) might be associated with a relatively lower tumor grade, as in some systems, T_{H1} -skewed T cells seemed to mediate an antitumor effect (28). This was also not observed; in these samples, there was a nonsignificant trend towards a more pronounced intraprostatic T_{H1} skewing in men with a higher Gleason grade. Finally, we examined the relationship between T_{H17} skewing and Gleason grade, testing the hypothesis that a more pronounced T_{H17} skewing would be associated with a higher tumor grade as suggested by the data of Langowski et al. (28). Interestingly, a statistically significant inverse correlation was found between T_{H17} skewing and tumor grade (Fig. 3). This finding is surprising and suggests the interesting possibility that T_{H17} T cells in the prostate might potentially mediate an antitumor effect. An alternative hypothesis is that T_{H17} skewing is an independent result of locally produced cytokines, i.e., as tumors progress, the local milieu progresses from one that favors T_{H17} skewing (TGF- β , IL-6, etc.) to one that favors T_{H1} skewing (IL-12 and IL-18). We are currently in the process of investigating a comprehensive series of tissue microarrays from patients with prostate cancer to confirm these results with a larger patient set.

qRT-PCR analysis of $CD4^+$ PIL. To determine whether the increased frequency of $CD4^+$ T cells producing cytokines indicative of T_{H17} or T_{reg} skewing in PIL by flow cytometry corresponds to increased transcription at the RNA level, we did qRT-PCR analyses. This technique also allows us to develop a more comprehensive picture of cytokine expression in the $CD4^+$ T cells that infiltrate the prostate gland of an individual cancer patient, as multiple messages could be simultaneously analyzed from relatively small cell numbers. As shown in Fig. 4, the profiles suggested by fluorescence-activated cell sorting analysis were largely reflected at the message level. Three of four patients analyzed showed a relative up-regulation of IL-17 in the prostate. This was accompanied by an increase in IL-23 receptor message level. These data are interesting, as IL-23 signaling is required for T_{H17} maintenance (5). The FoxP3 message was also up-regulated in three of the four patients analyzed, again confirming the fluorescence-activated cell sorting data (Figs. 1 and 2). These data also address a potential correlation between T_{reg} and T_{H17} infiltration (Fig. 2B); in two of the patients (patients 3 and 4) messages for FoxP3 and IL-17 seemed to correlate, but in the other two patients studied (patients 1 and 2), they seemed to digress. Overall, qRT-PCR

analyses generally confirms the flow cytometry data, suggesting that the protein level cytokine changes are mediated at the message level; but these data also suggest that the situation is complex because, in some patients, the $CD4^+$ T cell phenotype may be differentially skewed towards either a T_{reg} or T_{H17} phenotype.

Microarray analysis of prostate-infiltrating T_{reg} . Although several studies have explored the transcriptional phenotype of regulatory T cells, the profile of human tissue-resident T_{reg} has yet to be reported (29–31). To address this question, we enriched T_{reg} from the prostate gland by sorting $CD4^+CD25^{high}$ -GITR $^+$ cells. Sorted cells were >90% pure, but because the isolation procedure was stringent, we could not obtain sufficient cells for microarray analysis from a single patient. Therefore, sorted T_{reg} were pooled from a number of patients. Although pooling T_{reg} samples may introduce some “noise,” pooling also has the potential advantage of dampening low-level message level changes introduced by individual patient variability. As a reference $CD4^+$ T cell population, we used naïve $CD4^+$ T cells from the patients’ peripheral blood. Thus, differentially regulated transcripts will consist of T_{reg} -specific messages, prostate homing markers as well as T cell activation markers associated with the $CD25^+$ phenotype. The complete results of this analysis are presented in Supplementary Tables S2 and S3, and selected transcripts are highlighted in Table 1. As expected, we noted the up-regulation of T_{reg} -associated transcripts such as FoxP3, providing a broad verification of our sorting and analysis procedures. The T_{reg} in the prostate also seem to up-regulate a number of additional inhibitory markers, including CTLA-4 and LAG-3. In addition, we noted the increased expression of several T_{H17} -associated transcripts, reflecting the notion that our sorting procedure also captures a prostate-infiltrating population of activated $CD4^+$ T cells ($CD25^+$), and providing further verification of both our flow cytometry data (Fig. 1) and our qRT-PCR data (Fig. 4). Interestingly, the CCR homing molecule most up-regulated in these cells is CCR2, consistent with recent data describing CCR2 as a marker for human T_{H17} T cells (32). Finally, these prostate-infiltrating T cells also seem to display a relative up-regulation of TLR-3 and TLR-7. These receptors recognize double-stranded and single-stranded RNA, respectively; thus, these data are especially interesting in light of recent data demonstrating a novel retrovirus in some patients with prostate cancer (33).

Discussion

Recent studies have suggested that chronic or recurrent inflammation may play a role in the development of many types of cancer—including prostate cancer (34). Although a T_{H2} phenotype has been associated with inflammation, the T_{H17} subset has been described as proinflammatory as well. In addition, T_{H17} cells have been determined to have a distinct lineage from T_{reg} (5). Our data show that, in men with prostate cancer, a significant portion of the cells that infiltrate the gland are skewed towards a T_{H17} phenotype. This result is not in of itself surprising, as T_{H17} cells most likely develop under the influence of the cytokines TGF- β and IL-6, and both of these cytokines have been associated with advanced prostate cancer (17, 18). Indeed, the presence of T_{H17} cells in the prostate gland of men with benign prostatic hyperplasia has been

previously reported (7), before these cells were known to represent a distinct lineage. What is surprising, however, is that a relative skewing of PIL with respect to peripheral blood seems to correlate inversely with Gleason grade. A seminal study by Langowski et al. (28) showed that T_H17 cells seemed to be

required for the development of cancer in an animal model, and a number of additional studies suggest that IL-23/IL-17-mediated immunity thwarts active tumor surveillance (35). Our results may reflect a bystander role, wherein the tumor microenvironment shifts away from a T_H17 skewing once

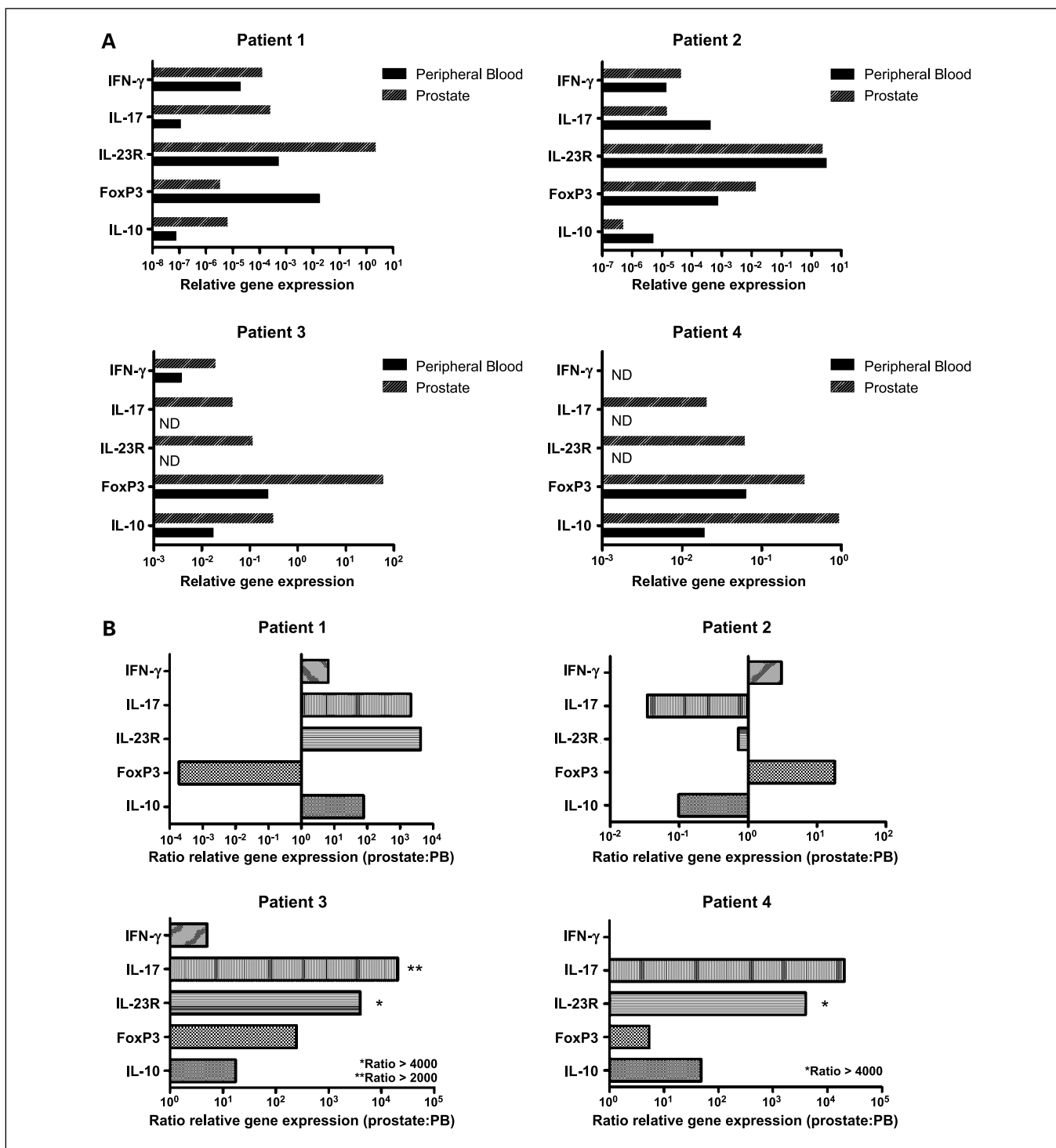


Fig. 4. Prostate-infiltrating CD4⁺ T cells have heterogeneous cytokine gene expression profiles. *A*, qRT-PCR results normalized to CD4 message levels. *B*, ratio of message levels in prostate versus peripheral blood. IL-4 and IL-12 expression were not shown because message levels were undetectable in all patients. ND, not detectable. The Gleason scores for patients 1 to 4 were as follows: 3 + 3 = 6; 3 + 4 = 7; 3 + 4 = 7; 4 + 3 = 7 (tertiary 5).

Table 1. Selected transcripts up-regulated in prostate-infiltrating T_{reg}

Probe ID	Fold increase	Gene definition	Gene symbol	Cellular component
T _{reg} associated				
211786_at	122	Tumor necrosis factor receptor superfamily, member 9 (4-1BB)	<i>TNFRSF9</i>	Membrane
234895_at	95	CTL-associated protein 4	<i>CTLA4</i>	Membrane
206486_at	86	Lymphocyte-activation gene 3	<i>LAG3</i>	Membrane
211269_s_at	58	IL-2 receptor- α (CD25)	<i>IL2RA</i>	Membrane
223851_s_at	31	Tumor necrosis factor receptor superfamily, member 18 (GITR)	<i>TNFRSF18</i>	Membrane
224211_at	17	Forkhead box P3	<i>FOXP3</i>	Nucleus
Cytokines				
216876_s_at	262	IL-17A	<i>IL17A</i>	Extracellular
210354_at	148	IFN γ	<i>IFNG</i>	Extracellular
207433_at	116	IL-10	<i>IL10</i>	Extracellular
205207_at	29	IL-6 (IFN β 2)	<i>IL6</i>	Extracellular
Cytokine receptors				
202948_at	254	IL-1 receptor, type I	<i>IL1R1</i>	Membrane
1552912_a_at	39	IL-23 receptor	<i>IL23R</i>	Membrane
Chemokines				
205476_at	291	Chemokine (C-C motif) ligand 20	<i>CCL20</i>	Extracellular
204103_at	189	Chemokine (C-C motif) ligand 4	<i>CCL4</i>	Extracellular
216598_s_at	138	Chemokine (C-C motif) ligand 2	<i>CCL2</i>	Extracellular
205114_s_at	52	Chemokine (C-C motif) ligand 3	<i>CCL3</i>	Extracellular
Chemokine receptors				
206978_at	111	Chemokine (C-C motif) receptor 2	<i>CCR2</i>	Membrane
206991_s_at	45	Chemokine (C-C motif) receptor 5	<i>CCR5</i>	Membrane
206983_at	17	Chemokine (C-C motif) receptor 6	<i>CCR6</i>	Membrane
Toll-like receptors				
206271_at	310	Toll-like receptor 3	<i>TLR3</i>	Membrane
220146_at	26	Toll-like receptor 7	<i>TLR7</i>	Membrane

NOTE: See Supplementary Tables S2 and S3 for complete data set.

prostate cancer progresses. They might also reflect a differential role for T_H17 T cells in tumors of different grades. Most intriguingly, these data also raise the interesting possibility that T_H17 T cells might, in some cancers, mediate an antitumor effect, a hypothesis best evaluated using animal models of tumor immunity. A major limitation of the present study is the lack of prostate tissue samples from patients with no evidence of cancer. The difficulties in obtaining such tissues are considerable. First, as radical retropubic prostatectomies are restricted to men with prostate cancer, this makes an identical isolation procedure difficult. Whereas in the present study, we were able to obtain lymphocyte samples within 1 hour of organ resection, autopsy samples would be challenging, if not impossible, to obtain in an identical time frame. Likewise, autopsy studies show that the incidence of occult prostate cancer is fairly high (~50% in men ages 50, and 70% in men >70 years of age; ref. 36). Thus, we would have to obtain tissue from an additional 40 to 50 age-matched men for such an analysis. We have explored the possibility of obtaining samples from men undergoing radical cystoprostatectomy for bladder cancer. Such studies are in the planning stages, however, even these are compromised by the possibility that cancer in an adjacent organ may alter the systemic cytokine profile and the PHL population.

Our data further confirm recent studies demonstrating that T_{reg} infiltrate the prostate gland (11). As those earlier studies were done without FoxP3 staining, our data most likely represent a more specific evaluation of T_{reg} in the prostate; however, it should be noted that FoxP3 is certainly not a perfect

or exclusive T_{reg} marker (37), and may mark a population of activated T cells in humans. These data were confirmed at both the protein and transcriptional level, using qRT-PCR as well as microarray analysis. The T_{reg}-associated markers CTLA-4, 4-1BB, and LAG-3 seem to be highly up-regulated in prostate-infiltrating CD4⁺ T cells, suggesting potential targets for immunotherapeutic intervention. In addition, our data reveal a number of cell surface markers not previously associated with T_{reg}. We are currently evaluating the specificity as well as the functional role of a number of these markers.

Several groups have suggested that cancers which arise in the context of inflammation arise out of a chronic, T_H2-mediated inflammatory milieu (16), and such data are well supported by experimental studies. In the prostate, we were unable to find significant numbers of T_H2-skewed CD4⁺ T cells either at the message or protein level. One possibility to explain these data would be that the cells are present, but nonfunctional—we are currently developing techniques to perform immunohistochemical staining for the major transcription factor of T_H2 cells (GATA-3) in prostate tissue microarray collections. In contrast, T_H1 cells were found to be the most predominantly enriched CD4⁺ T-cell subset in the prostate glands of these patients. This observation is particularly intriguing in light of recent evidence which supports the notion that at least a subset of tumors may exist in an “equilibrium state” specifically maintained by the adaptive immune system (38). However, the presence of these cells was not associated with a lower Gleason grade, as might be predicted. Indeed, our data suggested the opposing possibility, that T_H1-mediated inflammation might be associated with a

higher Gleason grade, again, a possibility best addressed through a comprehensive tissue microarray analysis.

In summary, these data provide the first comprehensive, protein level analyses of the phenotype of CD4⁺ T cells in the glands of men with prostate cancer. They document a relative skewing towards T_H17, and raise important questions regarding a potential causal versus antitumor role for these cells in the development of human prostate cancer. We also document the presence of regulatory T cells, but the lack of correlation between T_{reg} skewing and Gleason grade also raises interesting questions regarding the relative role of these cells in the etiology of prostate cancer versus other tumor types. Finally, we report the first microarray analyses of CD4⁺ T_{reg} from the prostate glands of men with cancer—those data confirm several

known cell surface markers which are targets for antitumor immunotherapy approaches and also suggest a number of additional cell surface markers and homing receptors potentially important in either the etiology or in the immunotherapeutic treatment of this common disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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