Phenotypic Analysis of Prostate-Infiltrating Lymphocytes Reveals TH17 and Treg 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 naive, peripheral blood T cells using microarray analysis. Results: CD4+ PIL showed a paucity of Th2 (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 Treg phenotype (FoxP3+) as well as towards the Th17 phenotype (interleukin-17+). We also found that a preponderance of Th17-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 Treg revealed expected Treg-associated transcripts (FoxP3, CTLA-4, GITR, LAG-3) as well as a number of unique cell surface markers that may serve as additional Treg markers. Conclusion: Taken together, these data suggest that Th17 and/or Treg CD4+ T cells (rather than Th1,2 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 Th1 and Th2 subsets, which secrete IFN-γ and interleukin (IL)-4, respectively, a new subset of CD4+ (helper) T cells, termed Th17 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, Th17 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 Th17 cells represent a distinct lineage (7). Whether Th17 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 (Treg), 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, Treg have recently been investigated as suppressors of antitumor immune responses (reviewed in ref. 8). Increased numbers of Treg have been reported in tumor-infiltrating lymphocytes of several human solid tumors including breast, pancreatic, hepatocellular, and prostate carcinomas (9–11).

References:

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Furthermore, increased percentages of T<sub>reg</sub> 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<sub>reg</sub> (8). More recent data have suggested that the forkhead box transcription factor FoxP3 is a more specific marker for T<sub>reg</sub> (14). Here, we used intracellular staining for FoxP3 to determine the prevalence of T<sub>reg</sub> 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<sub>1</sub>, T<sub>2</sub>, T<sub>17</sub>) in PIL and to determine the relative prevalence of CD<sup>4+</sup> T<sub>reg</sub> 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 stained through a 100-μm strainer. CD<sup>4+</sup> 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<sup>+</sup> 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 μL/L 1.44 mol/L B-mercaptoethanol).

Patient-matched peripheral blood samples were collected by venipuncture into 8.5 mL whole blood tubes with ACDF solution A (BD Biosciences Vacutainer Systems) the evening prior to radical retropubic prostatectomy surgery and remained at room temperature under a Johns Hopkins Medicine Institutional Review Board–approved protocol. RNA from patient-matched peripheral blood and prostate CD4<sup>+</sup> 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<sub>T</sub>) values were calculated as the average of three runs for each gene and were normalized to CD4 using the equation: Ratio = 2<sup>-ΔΔC(T)</sup> (CT<sub>Target</sub> - CT<sub>Control</sub>). 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 naive CD4<sup>+</sup> 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<sup>+</sup>CD45RA<sup>-</sup>CD25<sup>+</sup> population from peripheral blood samples (representing naïve CD4<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup> 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 conducted 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. cDNA was synthesized using the Nugen FL-Ovation cDNA Biotin Module V2 kit, following the manufacturer’s protocol. 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

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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

TH17 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 TH1 cells (Fig. 1A). Interestingly, we found almost no IL-4-secreting cells among these PIL, suggesting a skewing away from a TH2 phenotype when compared with a positive in vitro-derived TH12 control (Fig. 1B). These data are in contrast with suggestions that prostate cancer arises in the context of TH2-mediated inflammation (16). Remarkably, a significant skewing towards a TH17 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 TH17 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 TH17 cells in mice (19–22), recent data using human naïve CD4⁺ T cells suggest that TH17 polarization is induced by IL-1β, possibly enhanced by IL-6, and may be suppressed by TGF-β (23, 24).

Fig. 1. Frequency of TH17 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-γ⁺ (TH1), CD4⁺ IL-4⁺ (TH2), CD4⁺ IL-17⁺ (TH17), and CD4⁺ FoxP3⁺ (Treg) 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.
A regulatory phenotype in PIL. Because T\(_{117}\) cells were predominant among PIL, and these cells may differentiate under the influence of TGF-\(\beta\), we queried whether T\(_{reg}\) (which also differentiate in response to TGF-\(\beta\)) 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\(_{112}\)-mediated process. Rather, the CD4\(^+\) T cells in the prostate seem to be skewed towards either the T\(_{117}\) 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\(_{112}\) phenotype, but rather, seem to be biased towards T\(_{117}\) and T\(_{reg}\). It should be noted that there is a weak skewing of PIL towards a T\(_{111}\) 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-\(\beta\) in the development of T\(_{reg}\) and T\(_{117}\) cells, we sought to determine whether these two subsets cosegregated in patients, i.e., whether patients with a relative up-regulation of T\(_{117}\) 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\(_{117}\) skewing seemed to show less of a T\(_{reg}\) bias. Because one major difference between T\(_{117}\) and T\(_{reg}\) development is the presence of IL-6 during the differentiation process (5), it might be that patients with relative T\(_{117}\) 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\(_{117}\)
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 $T_{17}/T_{reg}$ 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 $T_{17}$ and $T_{1}$ skewing (Fig. 2B). As expected, no such relationship was found ($R^2 = 0.0003$).

$T_{17}$ 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 ($T_{1}$) might be associated with a relatively lower tumor grade, as in some systems, $T_{1}$-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 $T_{17}$ skewing in men with a higher Gleason grade. Finally, we examined the relationship between $T_{17}$ skewing and Gleason grade, testing the hypothesis that a more pronounced $T_{17}$ 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 $T_{17}$ skewing and tumor grade (Fig. 3). This finding is surprising and suggests the interesting possibility that $T_{17}$ T cells in the prostate might potentially mediate an antitumor effect. An alternative hypothesis is that $T_{17}$ skewing is an independent effect of locally produced cytokines, i.e., as tumors progress, the local milieu progresses from one that favors $T_{17}$ skewing (TGF-$\beta$, IL-6, etc.) to one that favors $T_{1}$ skewing (IL-12 and IL-18). We are currently in the process of investigating a more comprehensive picture of cytokine expression in the CD4$^+$ T cells that infiltrate the prostate gland of men with benign prostatic hyperplasia has been studied, but little is known about the specific cytokine profile in these cells. We have recently developed a qRT-PCR analysis of CD4$^+$ T cells to determine whether the increased frequency of CD4$^+$ T cells producing cytokines indicative of $T_{17}$ or $T_{reg}$ skewing in prostate cancer, confirming these results with a larger patient set.

**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_{12} phenotype has been associated with inflammation, the $T_{17}$ subset has been described as proinflammatory as well. In addition, $T_{17}$ 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 $T_{17}$ phenotype. This result is not in of itself surprising, as $T_{17}$ cells most likely develop under the influence of the cytokines TGF-$\beta$ and IL-6, and both of these cytokines have been associated with advanced prostate cancer (17, 18). Indeed, the presence of $T_{17}$ 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 TH17 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 TH17 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).
prostate cancer progresses. They might also reflect a differential role for T<sub>H17</sub> cells in tumors of different grades. Most intriguingly, these data also raise the interesting possibility that TH17 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 PIL population.

Our data further confirm recent studies demonstrating that T<sub>Treg</sub> 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<sub>Treg</sub> in the prostate; however, it should be noted that FoxP3 is certainly not a perfect or exclusive T<sub>Treg</sub> 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<sub>Treg</sub>-associated markers CTLA-4, 4-1BB, and LAG-3 seem to be highly up-regulated in prostate-infiltrating CD4<sup>+</sup> T cells, suggesting potential targets for immunotherapeutic intervention. In addition, our data reveal a number of cell surface markers not previously associated with T<sub>Treg</sub>. 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<sub>H17</sub>-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<sub>H17</sub>-skewed CD4<sup>+</sup> 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 TH2 cells (GATA-3) in prostate tissue microarray collections. In contrast, T<sub>H11</sub> cells were found to be the most predominantly enriched T<sub>Treg</sub>-associated 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<sub>Treg</sub>-associated markers CTLA-4, 4-1BB, and LAG-3 seem to be highly up-regulated in prostate-infiltrating CD4<sup>+</sup> T cells, suggesting potential targets for immunotherapeutic intervention. In addition, our data reveal a number of cell surface markers not previously associated with T<sub>Treg</sub>. We are currently evaluating the specificity as well as the functional role of a number of these markers.

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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_{reg}, 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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References


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