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CD4 helper T cells, CD8 cytotoxic T cells, and FOXP3$^+$ regulatory T cells with respect to lethal prostate cancer

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Prostate cancer represents a major contributor to cancer mortality, but the majority of men with prostate cancer will die of other causes. Thus, a challenge is identifying potentially lethal disease at diagnosis. Conflicting results have been reported when investigating the relationship between infiltration of lymphocytes and survival in prostate cancer. One of the mechanisms suggested is the recruitment of regulatory T cells (T_{regs}), a subpopulation of T cells that have a role in promoting tumor growth. T_{regs} counteract tumor rejection through suppressive functions on the anti-immune response but their prognostic significance is still unknown. We report here the results of a conducted case–control study nested in a cohort of men treated with transurethral resection of the prostate and diagnosed incidentally with prostate cancer. Cases are men who died of prostate cancer ($n = 261$) and controls are men who survived >10 years after their diagnosis ($n = 474$). Infiltration of both T_{helper} and $T_{cytotoxic}$ cells was frequently observed and the majority of the T_{regs} were CD4$^+$. T_{helper} or $T_{cytotoxic}$ cells were not associated with lethal prostate cancer. However, we found a nearly twofold increased risk of lethal prostate cancer when comparing the highest with the lowest quartile of CD4$^+$ T_{reg} cells (95% confidence interval: 1.3–2.9). Our conclusion is that men with greater numbers of CD4$^+$ T_{regs} in their prostate tumor environment have an increased risk of dying of prostate cancer. Identification of CD4$^+$ T_{regs} in tumor tissue may predict clinically relevant disease at time of diagnosis independently of other clinical factors.
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Prostate cancer is the most common cancer among European and American men and the second most common cancer diagnosed among men worldwide.[1,2] In the US, it represents the second greatest contributor to cancer mortality behind lung cancer, but the vast majority of men diagnosed with prostate cancer will die of other causes.[2] Thus, a major challenge in the prostate cancer management is identifying potentially lethal disease at time of diagnosis. Gleason score, prostate specific antigen (PSA), and tumor extent are valuable for disease management but they cannot adequately distinguish indolent from aggressive disease. Additional prognostic markers are urgently needed to more effectively guide clinical decision making.

Decades ago, Burnet[3] postulated about the ability of the immune system to detect tumor cells and eliminate them. The various subtypes of T lymphocytes have diverse roles and represent powerful components of this anti-tumor immune response. Several studies have demonstrated that CD8 cytotoxic T cells are capable of recognizing and destroying various tumor cells.[4] Although the main function of CD4 helper T cells is to aid in

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maintaining the expansion of CD8 cytotoxic T cells, CD4 helper T cells are also capable of tumor cell eradication.[5,6]

Despite the potential of inflammatory cells to protect against cancer development, chronic or recurrent inflammation has also been hypothesized to contribute to prostate cancer development, mainly owing to DNA damage caused by reactive molecules released by the inflammatory cells.[7,8] One indicator of previous cellular injury is focal atrophy. In the prostate gland, the presence of chronic inflammation is specifically associated with two subtypes of focal prostatic atrophy, post-atrophic hyperplasia, and simple atrophy.[9,10] These lesions, together referred to as proliferative inflammatory atrophy, have been suggested as regenerative lesions and precursors of prostatic adenocarcinoma, either directly or indirectly via progression to prostatic intraepithelial neoplasia.[10]

Conflicting results have been reported for the relationship between lymphocytic infiltration and survival in prostate cancer patients. Vesalainen *et al*[11] reported that tumors with dense lymphocyte infiltration were associated with higher survival rates compared with those with fewer lymphocytes. On the other hand, greater numbers of CD4 helper T cells have been associated with poor disease outcomes including postoperative biochemical recurrence and cancer-specific death.[12–14] These disparate results could potentially be explained by a more careful examination of the specific subtypes of T lymphocytes, some of which may allow the immune system to act as a promoter for the emergence of primary tumors. Recent studies that have focused on subsets of regulatory T lymphocytes (T_{reg}), including $CD4^+$ T_{reg} cells and $CD8^+$ T_{regs}, have demonstrated immune suppressive function either directly through cell–cell contact or indirectly through the secretion of anti-inflammatory cytokines, such as TGF-β (transforming growth factor beta) and IL-10.[15,16] A recent paper by Miller *et al*[17] revealed an increased prevalence of T_{regs} in prostate cancer tissue compared with normal prostate tissue from the same patient. Although follow-up time was limited, there was also the suggestion of an association between T_{reg} cells and survival among men with prostate cancer.[15] Significant differences in the T_{reg} cell prevalence between the early and advanced gastric cancer and esophageal cancer have also been reported. In both diseases, patients with high proportion of T_{reg} cells showed poorer survival rates compared with those with low proportion.[18]

The majority of previous studies have utilized CD25 as a biomarker for T_{reg} cells. However, the most specific marker presently to identify T_{regs} is FOXP3, a member of the forkhead box family of transcription factors.[19] In this population-based nested case–control study of men diagnosed with localized prostate cancer and extensive follow-up time (up to 30 years) after diagnosis, we have evaluated the role of CD4 helper T (T_{helper}) cells, CD8 cytotoxic T ($T_{cytotoxic}$) cells, $CD4^+FOXP3^+$ regulatory T ($CD4^+T_{reg}$) cells, and $CD8^+FOXP3^+$ regulatory T ($CD8^+T_{reg}$) cells in tumor tissue with respect to acute or chronic inflammation, type of atrophy, prostatic intraepithelial neoplasia, and lethal prostate cancer.

Materials and methods

Tissue Collection and Preparation

The present study is nested within a cohort of men with localized prostate cancer diagnosed in the Örebro and South East Health Care Regions of Sweden between 1977 and 1999, as described in detail previously.[20–22] We initially identified a cohort of 1367 men during the study period. Eligible patients were identified through the population-based prostate cancer quality database in these regions. We included men who were diagnosed with incidental prostate cancer through transurethral resection of the prostate or adenoma enucleation, ie, category T1a-b tumors. In accordance with standard treatment protocols, patients with early-stage/localized prostate cancer were followed expectantly ('watchful waiting').

The study cohort was followed for cancer-specific and all-cause mortality until 1 March 2006, through record linkages to the Swedish Death Register and Migration Register. We obtained information on cause of death for each individual through a complete review of medical records by a study end points committee. Deaths were classified as cancer specific when prostate cancer was the primary cause of death.

Because visual histopathological evaluations and immunohistochemistry (IHC) for archival specimens is both time consuming and expensive, we utilized a novel nested study design that included men who either died from prostate cancer during follow-up (lethal prostate cancer 'cases', $n=261$) or who survived at least 10 years following their diagnosis (indolent prostate cancer 'controls', $n=474$). The study design excluded men with non-informative outcomes who either died from other causes within 10 years after cancer diagnosis, or did not die of prostate cancer and did not have the opportunity to survive 10 years before the end of study follow-up in 2006. We also excluded cases without complete data for both IHC and inflammation/atrophy variables.

Tumor areas marked on H&E slides for the corresponding paraffin-embedded formalin-fixed blocks were re-reviewed by a single pathologist (MF) blinded to disease outcome and other clinical data to confirm cancer status, Gleason score and other notable histopathological features. The Gleason scoring was performed according to the 2005 ISUP recommendations.[23] All slides were assessed for the presence and type of inflammation, either acute or chronic, according to cells of the

inflammatory infiltrate. Chronic inflammation was semi-quantitatively graded as mild, moderate or severe when the area of non-neoplastic prostate tissue covered by inflammatory cells was ≤ 10, ≥ 10–20, and $\geq 20\%$, respectively. Focal prostate atrophy was characterized according to the atrophy classification, proposed in 2006 by the Working Group for Histologic Classification of Prostate Atrophy Lesions with the following subtypes: simple atrophy, simple atrophy with cyst formation, post-atrophic hyperplasia, and partial atrophy. Given that simple atrophy with cyst formation and partial atrophy were rare (6 and 2%, respectively), we did not evaluate these lesions in the present study. A previous publication describes the association between the histopathologically assessed inflammation, atrophy, and prostatic intraepithelial neoplasia variables with lethal prostate cancer.[24]

Immunohistochemistry

Tissue cores with a diameter of 0.6 mm were collected from each transurethral resection of the prostate specimen. Three cores were taken from each patient to address potential tumor heterogeneity. Tissue microarrays were constructed with a Beecher manual arrayer and tissue microarray sections (4 μm) were used for IHC. Deparaffination, rehydration, and antigen retrieval was performed using Target Retrieval Solution (DAKO, Denmark) at pH 9.0. Primary antibodies used for the single staining were mouse monoclonal ready to use antibodies against CD4 (clone 4B12, DAKO), CD8 (clone C8/144, DAKO). Single IHC staining was performed with DAKO autostainer LINK system. The slides were incubated with primary antibodies at room temperature for 20 min and detected with EnVisionTM FLEX$^+$, High pH, (Link) (DAKO). For the triple staining, we used as primary antibodies a mouse monoclonal and rabbit monoclonal ready to use multiplex cocktail against CD4 and CD8 (clone BC/1F6 + SP16, Biocare Medical, Concord, USA) and mouse monoclonal antibody against FOXP3 (clone 236A/E7, eBioscience, San Diego, USA) at 1:100 dilution. After primary antibody incubation for 30 min at room temperature, slides were treated with secondary antibodies and chromogen for detection. To identify CD4 and CD8, Mach 2 doublestain 2 (Biocare Medical) and diaminobenzidin (Biocare Medical) and Warp Red Chromogen kit (Biocare Medical) were used, respectively. To detect FOXP3, Mach 2 mouse HRP-Polymer (Biocare Medical) served as secondary antibody followed by Vina Green Chromogen Kit (Biocare Medical) for visualization. Slides were then counterstained with hematoxylin. Tonsil was used as positive control.

Evaluation of CD4, CD8, and Foxp3 Expression

We quantified T_{helper} cells by CD4 protein expression, $T_{cytotoxic}$ cells by CD8 expression, $CD4^+T_{reg}$ cells by simultaneous CD4 and FOXP3 expression, and $CD8^+T_{reg}$ cells by simultaneous CD8 and FOXP3 expression using a light microscope at $\times 40$ magnification. All T_{reg} cells were counted. For T_{helper} and $T_{cytotoxic}$ cells, up to 50 cells were counted; greater numbers of positive cells were recorded in a single category as >50. The observers (SD and A-LO) were blinded to all the clinical data and conducted evaluations independently.

Statistical Analyses

To evaluate the association between clinical covariates and lethal case *vs* indolent control status, we used χ^2-tests for categorical variables and *t*-tests for continuous variables. We used unconditional logistic regression to estimate odds ratios and 95% confidence intervals for the association between the mean number of T_{helper} lymphocytes, $T_{cytotoxic}$ lymphocytes, and $CD4^+T_{reg}$ cells across an individual patient's three tissue cores with respect to the following outcomes: (1) presence of the histopathological characteristics as assessed visually by the study pathologist (MF); and (2) lethal prostate cancer. The histopathological characteristics included acute inflammation, chronic inflammation (none/mild *vs* moderate/severe), simple atrophy, post-atrophic hyperplasia, and prostatic intraepithelial neoplasia. Statistical significance was determined by a Wald test for the continuous cell count variables. Mixed models were used to assess the association beween T_{helper}, $T_{cytotoxic}$, and T_{reg} cells at the core level. To aid in the interpretation of the odds ratios for lethal prostate cancer, we also estimated odds ratios according to quartiles of the T-cell subtype variables. In addition to univariate analyses, we also ran models adjusted for tumor stage, tumor percent, and primary and secondary Gleason grade. We hypothesized that visually assessed inflammation and atrophy/prostatic intraepithelial neoplasia lesion variables could act synergistically with the specific IHC-assessed T-cell subtypes to predict prostate cancer progression. Thus, we assessed the potential interactions between histopathologically assessed variables and number of T_{helper}, $T_{cytotoxic}$, and T_{reg} cells with respect to lethal prostate cancer. To assess statistical significance of the interactions, we used a Wald χ^2-test to compare unconditional logistic regression models that main effects and the product term of the mean number of positive cells and the dichotomous variable for lesion type, to models without the product term. All statistical analyses were carried out using SAS Statistical Software version 9.2 (Cary, NC). The study was approved by the Ethical Review Boards in Örebro and Linköping, Sweden.

Results

The clinical characteristics of the lethal cases and indolent controls are presented in Table 1. When

Table 1 Clinical and histopathological characteristics in the Swedish Watchful Waiting Cohort ($N=735$)

	Indolent PCa controls (N = *474*)	*Lethal PCa cases* (N = *261*)	P-*value*
Age at Dx, Mean (Std)[a]	72 (6)	75 (7)	<0.0001
Calendar year of Dx[b]			
1977–1982	15 (3)	7 (3)	0.005
1982–1986	28 (6)	10 (4)	
1987–1991	174 (37)	131 (50)	
1992–1999	257 (54)	113 (43)	
Tumor stage[b]			
T1a	221 (47)	78 (30)	<0.0001
T1b	251 (53)	183 (70)	
Gleason score[c]			
2–6	277 (58)	42 (16)	<0.0001
3 + 4	113 (24)	38 (15)	
4 + 3	61 (13)	52 (20)	
8–10	23 (5)	129 (49)	
Tumor percent[c] *(72 missing)*			
Q1: ≤2	172 (39)	37 (16)	<0.0001
Q2: >2–≤10	131 (30)	65 (29)	
Q3: >10–≤60	111 (25)	86 (38)	
Q4: >60	23 (5)	38 (17)	

[a]*P*-value from a *t*-test.
[b]*P*-value from a χ^2-test.
[c]*P*-value from a χ^2-test of trend.

IHC was used for visualization, FOXP3 expression was localized in the nuclei of the T cells, whereas CD4 and CD8 expression was localized in the cell membrane. The lymphocytes were predominantly located in the stroma surrounding the tumor, either as single cells or as aggregates. Intraepithelial immune cells were also seen but only as scattered cells (Figures 1a and b). The majority of the FOXP3$^+$ cells were also CD4$^+$ (Figures 1c and d). The mean counts of T_{helper} cells, $T_{cytotoxic}$ cells, and CD4$^+$ T_{reg} cells in an individual patient were 18, 27, and 2, respectively. Only three patients had CD8$^+$ T_{reg} cells; these cells were not evaluated further.

Simultaneous infiltration of both T_{helper} and $T_{cytotoxic}$ cells was frequently observed. T_{helper} cells and CD4$^+$ T_{reg} cells were positively moderately correlated within an individual patient ($r=0.38$) and within a single tissue core (β estimate $=0.08$, $P<0.0001$). Similarly, a positive correlation was also seen between $T_{cytotoxic}$ cells and CD4$^+$ T_{regs} at both the patient and tissue core levels ($r=0.26$; and β estimate $=0.06$, $P<0.0001$). T_{helper}, $T_{cytotoxic}$, and CD4$^+$ T_{reg} cells were not associated with Gleason score or tumor stage. However, percent of tumor in the specimen was significantly inversely correlated with number of T_{helper} cells ($r=-0.11$; $P=0.004$) and $T_{cytotoxic}$ cells ($r=-0.14$; $P=0.0002$).

We assessed the relationship between the various lymphocytes and the presence of inflammation, atrophy, and prostatic intraepithelial neoplasia lesions apparent by visual histopathological evaluation (Table 2). The mean numbers of all three T-cell subtypes were significantly and positively associated with the presence of moderate/severe chronic inflammation as assessed semi-quantitatively by the study pathologist, with the strongest association being apparent for CD4$^+$ T_{regs} (odds ratio: 1.07; 95% confidence interval: 1.02–1.13). We also observed significant associations with acute inflammation for both the mean number of T_{helper} cells (odds ratio: 1.02; 95% confidence interval: 1.00–1.04) and the mean number of CD4$^+$ T_{regs} (odds ratio: 1.06; 95% confidence interval: 1.01–1.12). We found that the association between the mean number of CD4$^+$ T_{reg} cells was statistically significantly and inversely associated with the odds of having simple atrophy visible in the tumor specimen (odds ratio: 0.94; 95% confidence interval: 0.89–0.99). The mean number of $T_{cytotoxic}$ cells was inversely associated with the presence of prostatic intraepithelial neoplasia (odds ratio: 0.98; 95% confidence interval: 0.96–0.99).

We investigated whether T_{helper} cells, $T_{cytotoxic}$ cells, or CD4$^+$ T_{reg} cells were associated with a lethal prostate cancer (Table 3). Using the mean cell count value across the three cores, we observed no association between the infiltration of T_{helper} cells or $T_{cytotoxic}$ cells in the univariate or multivariate analyses. By contrast, every additional CD4$^+$ T_{reg} cell was associated with a 12% increase in odds of dying of prostate cancer (odds ratio:1.12; 95% confidence interval: 1.02–1.23) after adjustment for tumor stage, tumor percent, and primary and secondary Gleason score. When the mean number of cell counts across cores was categorized into quartiles, we observed a nearly twofold increase in the odds of dying of prostate cancer when comparing the highest with the lowest quartile of CD4$^+$ T_{reg} cells (odds ratio: 1.98; 95% confidence interval: 1.15–3.40).

We also explored interactions between acute and chronic inflammation, simple atrophy, post-atrophic hyperplasia and mean number of T_{helper}, $T_{cytotoxic}$, and CD4$^+$ T_{reg} cells (data not shown). The association between T_{helper} cells and lethal prostate cancer was modified by the presence of post-atrophic hyperplasia in the multivariate model (*P*-interaction $=0.05$). Among men without post-atrophic hyperplasia present, an increase in T_{helper} cells was not significantly associated with the odds of dying of prostate cancer (odds ratio: 1.00; 95% confidence interval: 0.98–1.02). Among men with post-atrophic hyperplasia, every one-unit increase in T_{helper} cells was associated with a 5% increase in the odds of lethal prostate cancer (odds ratio: 1.05; 95% confidence interval: 1.01–1.09). No other statistically significant interactions between lymphocyte types or between the lymphocytes and inflammation/atrophy/prostatic intraepithelial neoplasia were observed.

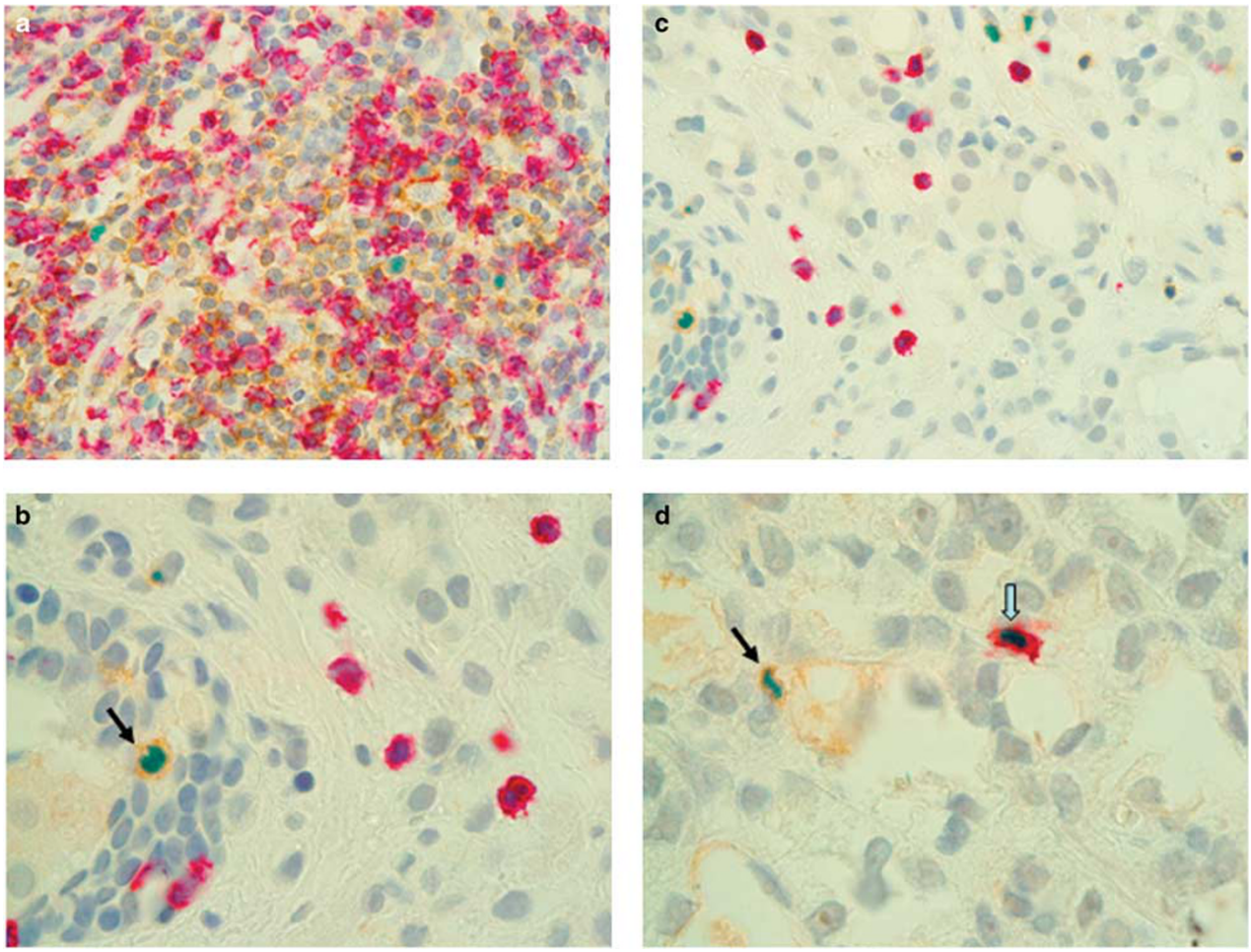

Figure 1 CD4 (brown), CD8 (red), and FOXP3 (green) expression in prostate tissue. (**a**) (magnification ×60) Immune cells located in the stroma as a aggregate. (**b**) (magnification ×100) Immune cells located in the epithelia and in the stroma. (**c**) (magnification ×60) Arrows indicate $CD4^+FOXP3^+$ T cells. (**d**) (magnification ×100). Solid arrow indicate a $CD4^+FOXP3^+$ T cell and open arrow indicate a $CD8^+FOXP3^+$ T cell.

Table 2 Odds ratios and 95% confidence intervals for number of $CD4^+$, $CD8^+$, and $CD4^+/FOXP3^+$ cells with respect to presence of histopathological variables

	Overall, N *(%)*	*$CD4^+$ cells*	*$CD8^+$ cells*	*$CD4^+/FOXP3^+$ cells*
Acute inflammation	101 (14)	**1.02 (1.00–1.04)**	1.02 (1.00–1.03)	**1.06 (1.01–1.12)**
Moderate/severe chronic inflammation	193 (26)	**1.02 (1.00–1.03)**	**1.02 (1.01–1.04)**	**1.07 (1.02–1.13)**
Simple atrophy	438 (60)	1.00 (0.98–1.01)	1.00 (0.99–1.01)	**0.94 (0.89–0.99)**
Post-atrophic hyperplasia	155 (21)	1.00 (0.98–1.01)	1.01 (0.99–1.02)	0.94 (0.87–1.02)
Prostatic intraepithelial neoplasia	94 (13)	0.99 (0.98–1.01)	**0.98 (0.96–0.99)**	0.98 (0.91–1.06)

Table 3 Odds ratios and 95% confidence intervals of lethal prostate cancer, Swedish Watchful Waiting Cohort, 1977–2006

Marker	*Odds ratio*[a] *(95% confidence interval)*	*Odds ratio*[b] *(95% confidence interval)*
Mean $CD4^+$	1.01 (1.00–1.02)	1.01 (0.99–1.03)
Mean $CD8^+$	1.00 (0.98–1.01)	1.00 (0.98–1.02)
Mean $CD4^+/FOXP3^+$	**1.13 (1.06–1.20)**	**1.12 (1.02–1.23)**

[a]Unadjusted odds ratio among 261 lethal cases and 474 indolent controls.
[b]Odds ratio controlling for tumor stage (T1a *vs* T1b), tumor percent, and primary and secondary Gleason among 226 lethal cases and 437 indolent controls.

Discussion

Our data suggest at least two distinct pathways leading to lethal prostate cancer: (1) post-atrophic hyperplasia combined with evidence of moderate/severe chronic inflammation and a shift in the CD4/CD8 T-cell ratio; and (2) inflammation-independent, tumor-assisted increase of the $CD4^+$ T_{reg} population. These findings may explain why previous studies of lymphocytic infiltration that did not characterize specific T-lymphocyte subtypes produced apparently inconsistent associations with prostate cancer outcomes.[11–13]

In a previous study, in the same study population, chronic inflammation in the presence of post-atrophic hyperplasia was associated with greater likelihood of prostate cancer death.[24] The results obtained in the present study shows that the T_{helper} cells, specifically interact with post-atrophic hyperplasia to influence cancer progression. Among men with post-atrophic hyperplasia, every additional CD4 helper T cell increases the risk of dying of prostate cancer. It has been suggested that inactivity of a substantial proportion of T-lymphocytes found in carcinoma leads to an ineffective anti-tumor immune response.[25] Our data do not address the function of infiltrating CD4 helper T cells or the impact they may have on the prostate tissue as this will require a more thorough phenotypic analysis of the lymphocytes.

Accumulating evidence suggests that T_{reg} cells are recruited to a variety of human carcinomas where they suppress the anti-tumor immune response performed mainly by T_{helper} and $T_{cytotoxic}$ lymphocytes, and that infiltration may predict poor survival.[18] Although neither infiltration of total CD4 helper T cells nor CD8 cytotoxic T cells was associated with lethal prostate cancer, a more detailed evaluation of the T-cell subpopulations in the tumor environment revealed that it is in fact the number of $CD4^+$ T_{regs} cells that predict worse outcome in prostate cancer. We found that every additional $CD4^+$ T_{reg} cell was associated with a statistically significant 12% increase in prostate cancer death independently of other clinical factors. We also found a moderate positive correlation between the presence of $CD4^+$ T_{reg} cells and both T_{helper} cells and $T_{cytotoxic}$ cells, indicating that the T cell populations involved in tumor eradication may be more important than the total burden of T cells.

Our data support the hypothesis that prostate cancer tumors recruit T_{regs} that suppress antitumor immunity and thereby aid tumor growth. A study of 20 prostate cancer patients undergoing prostatectomy for localized prostate adenocarcinoma by Sfanos *et al*[26] found enrichment of T_{reg} cells in almost all cases. Fox *et al.*[15] identified FOXP3-positive cells in 126 of 146 tissue specimens obtained from prostate cancer patients. They were, however, unable to demonstrate a correlation between numbers of T_{regs} and PSA rise, as an indicator of survival, due to a limited follow-up time. Other recent studies show an enhanced frequency of T_{reg} cells with a suppressive function in lymphocytes obtained both from prostate tissue and peripheral blood of patients with prostate cancer.[16,27] In addition, Sotosek *et al*[28] found statistically significant higher levels of T_{reg} cells in peripheral blood lymphocytes of prostate cancer patients when compared with benign prostatic hyperplasia patients and healthy volunteers. Further they suggest, in line with our study, that upregulation of T_{reg} cells could be essential for tumor progression, as a positive correlation between PSA values and frequency of T_{reg} cells was detected.

There are several possible explanations for the increased infiltration of T_{reg} cells in the tumor area. First, tumor cells or macrophages inside the tumor are able to secrete the chemokine CCL22, which has affinity for the receptor CCR4, expressed on T_{reg} cells.[29] Second, cytokines secreted by the prostate tumors, such as TGF-β, can upregulate FOXP3 expression and expand the T_{reg} population.[30] TGF-β is a multi-functional cytokine that increases survival and proliferation of transformed cells, including transformed prostate epithelial cells, and it has been found in elevated levels in patients with metastatic disease.[31]

To our knowledge, it is the first study to investigate the role of T_{reg} cells with respect to prostate cancer mortality. We assessed mortality from prostate cancer using >30 years of follow-up time after diagnosis among a cohort of men diagnosed with localized prostate cancer. We quantified the T_{regs} based on FOXP3 staining, as FOXP3 currently is considered to be the best single marker of T_{reg} using a monoclonal antibody (236A/E7) found to be the most specific clone and recommended for IHC on paraffin-embedded tissue.[32] To further strengthen the evaluation, we quantified FOXP3-positive cells based on a triple staining (CD4, CD8, and FOXP3) following recent studies that identified FOXP3 expression in prostate tumor cells.[33] However, all prostate cancer patients included in the present study were diagnosed by transurethral resection of the prostate or adenoma enucleation before the PSA era. Given that PSA testing and needle biopsies are more often utilized for prostate cancer diagnosis in contemporary practice, further studies in PSA screened population and biopsy specimens will be required to verify the generalizability of our findings.

Chronic inflammation is common in tumor specimens from prostatectomy, transurethral resection of the prostate, and biopsy samples.[7,10,24] In line with previous reports,[12,14,34] our study revealed a high degree of infiltration of both T_{helper} cells as determined by CD4 expression and $T_{cytotoxic}$ cells as determined by CD8 expression. The present study also demonstrates that IHC markers of inflammation are positively related to histopathological classification of inflammation, as all of the three T-cell

subtypes were positively associated with the presence of moderate/severe chronic inflammation. In future studies, it will also be important to consider the potential sources of the common occurrence of prostatic inflammation. Bacteria are well-known to trigger infection and subsequently inflammatory response. Recent reports have identified *Propionibacterium acnes* as the most frequently observed bacterium in prostate tissue from men with prostate cancer.[35,36] The results obtained in the present study support the importance of continued investigation regarding the role of infectious agents and other modifiable contributors of prostatic inflammation in prostate cancer development and progression.

In summary, our data provide evidence that men with greater numbers of $CD4^+$ T_{regs} in their prostate tumor environment have an increased risk of dying of their disease. In addition, the outcome of the disease in men with evidence of post-atrophic hyperplasia varies according to the number of CD4 helper T cells present. Our findings suggest that identification of $CD4^+$ T_{regs} in tumor tissue predicts clinically relevant prostate cancer at time of diagnosis independently of other clinical factors.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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