

# Increased peripheral CD4<sup>+</sup>CD25<sup>high</sup> Treg in prostate cancer patients is correlated with PSA

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## ABSTRACT

**الأهداف:** التحقق من النسب الهامشية لخلايا تي التنظيمية (CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup>) لدى المرضى المصابين بسرطان البروستاتا، ومعرفة ما إذا كان هناك علاقة بين هذه النسب والمستويات الكلية لمستضد البروستاتا النوعي لدى مرضى سرطان البروستاتا.

**الطريقة:** أُجريت هذه الدراسة في معمل الأمراض المناعية بجامعة أكدينيز، وقسم العيادات الخارجية للمسالك البولية، أنطاليا، تركيا وذلك خلال الفترة من ديسمبر 2008م إلى يناير 2010م. لقد قمنا بجمع عينات الخلايا من 56 مريض، وقد أخذ من هؤلاء المرضى خزعات البروستاتا (مستويات المستضد النوعي للبروستاتا أكثر من أو تساوي 2.5 نانوغرام/مل). ومن ثم قمنا بتقصي العلاقة بين مستويات خلايا تي التنظيمية والمستضد النوعي للبروستاتا على مرحلتين وهي المرحلة الأولى التي ركزت على عينة الدراسة ككل وتشمل الأصحاء، ومرضى سرطان البروستاتا، ومرضى تضخم البروستاتا الحميد، والمرحلة الثانية التي شملت 9 مرضى مصابين بسرطان البروستاتا. وخلال الدراسة قمنا بعمل مقارنة بين هذه المجموعات الثلاث.

**النتائج:** أشارت نتائج الدراسة إلى أن النسب الهامشية لخلايا تي التنظيمية قد زادت بشكل واضح لدى مجموعة المرضى المصابين بسرطان البروستاتا (العدد: 19، 3.23±1.59) وذلك بالمقارنة مع مرضى تضخم البروستاتا (العدد: 27، 1.08±0.43) ومجموعة الأصحاء (العدد: 10، 1.08±0.43) (p=0.007). كما كانت نسب خلايا تي التنظيمية لدى مرضى تضخم البروستاتا أعلى بكثير من الأصحاء (p=0.007)، وقد ارتبطت زيادة هذه الخلايا لدى مرضى تضخم البروستاتا الحميد ومرضى سرطان البروستاتا بعلاقة طردية مع المستويات الكلية لمستضد البروستاتا النوعي (r=0.75, p=0.007).

**خاتمة:** أظهرت هذه الدراسة وجود العلاقة بين تركيز النسب الهامشية لخلايا تي التنظيمية ومستويات مستضد البروستاتا النوعي لدى مرضى تضخم البروستاتا الحميد ومرضى سرطان البروستاتا، وهذا قد يكون بسبب دور مستضد البروستاتا النوعي في تخفيض نسب خلايا تي التنظيمية أو المحافظة عليها.

**Objectives:** To determine peripheral frequencies of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> regulatory T cells (Treg) in prostate cancer (PCa) patients, and to investigate if there is a correlation between peripheral Treg and total serum prostate specific antigen (PSA) levels in PCa patients.

**Methods:** Peripheral blood mononuclear cells from 56 subjects undergoing diagnostic prostate biopsies (PSA≥2.5ng/ml) were analyzed for Treg numbers. Association between the peripheral Treg and serum PSA values was first determined in the entire population, including people with no prostate pathology, PCa, and benign prostate hyperplasia (BPH) patients, and second, in 9 PCa patients before and after curative prostatectomy. In this study, the 3 groups were compared. This project was performed in the Akdeniz University Immunology laboratory, and the Urology outpatient clinic, Antalya, Turkey from December 2008 to January 2010.

**Results:** Peripheral Treg frequencies were significantly increased in the PCa patients (n=19, 3.23±1.59) compared with BPH patients (n=27, 1.66±0.80), and healthy subjects (n=10, 1.08±0.43) (p=0.007). The percentage of Treg in BPH patients was also significantly higher than healthy subjects (p=0.007). The increase of Treg in BPH and PCa patients was positively correlated with total serum PSA levels (r=0.75; p=0.007).

**Conclusion:** Peripheral Treg densities are correlated with PSA in BPH and PCa patients, suggesting that PSA may have a role in Treg induction and/or maintenance.

*Saudi Med J 2011; Vol. 32 (10): 1003-1008*

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*Received 25th May 2011. Accepted 22nd August 2011.*

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Prostate cancer (PCa) is the most commonly diagnosed cancer among men in the world.<sup>1</sup> Approximately two-thirds of PCa cases are confined to the prostate, and can be treated by radical prostate removal or radiotherapy.<sup>2</sup> In addition, approximately 25-55% of treated, locally confined tumors reappear within 10 years, and may progress as either a local recurrence or distant metastases.<sup>3</sup> In the quest for effective prevention and treatment modalities for metastatic PCa,<sup>4</sup> immunotherapy attempts using several different methods have shown very limited success.<sup>5-9</sup> Various immune evasion mechanisms, such as defects in antigen presentation, secretion of immunosuppressive agents by the tumor cells, and T cell receptor defects, are thought to limit the success of these trials. Recently, natural regulatory T cells (CD4<sup>+</sup>CD25<sup>high</sup>; Treg), one of the key regulators of self-tolerance, have also been implicated in immune evasion by PCa.<sup>10,11</sup> The CD4<sup>+</sup>CD25<sup>high</sup> Treg cells, which are known to control self-tolerance in the periphery, derive from the thymus,<sup>12,13</sup> and constitute 1-2% of peripheral lymphocytes in adult humans.<sup>14</sup> The importance of these cells in tumor immunity was first demonstrated 3 decades ago.<sup>15</sup> Since then, especially after the introduction of Foxp3<sup>16</sup> as a specific marker, studies of numerous mouse tumor models have demonstrated that they can interfere with the anti-tumor immune response at either the induction or effector phase. Increased peripheral and intratumoral Treg densities have been reported in lung, ovarian, colorectal, esophageal and gastric, melanoma, head and neck, prostate, and pancreatic cancers.<sup>17-20</sup> Evidence for Treg in PCa patients, however, has been limited to a few recent studies with significant controversy.<sup>21</sup> Studies from experimental models have demonstrated that Tregs may either be induced or activated within the tumor draining lymph nodes by tumor-derived factors, including tumor-associated antigens (TAA). Then, these cells prevent tumor-specific immune responses either at the induction phase in the tumor draining lymph nodes, or at the effector phase within the tumor milieu.<sup>22,23</sup> The first evidence that TAA-specific Tregs may be involved in suppression of tumor-specific immune responses in humans has recently been reported.<sup>24,25</sup> Prostate-specific antigen (PSA), a serine protease produced by the prostate gland, is the best known TAA of the prostate. Large amounts of PSA are produced and released into the circulation in PCa and benign prostate hyperplasia (BPH) patients, as well as in people with prostate inflammation. The varying results concerning peripheral Treg densities in PCa patients and the lack of information regarding a correlation between PSA levels and Treg cell frequency led us to investigate the number of peripheral CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs and their association with total serum PSA levels in PCa and BPH patients.

**Methods.** This project was performed in the Akdeniz University Immunology Laboratory and Urology outpatient clinic, Antalya, Turkey from December 2008 to January 2010. All patients signed patient consent forms, and the study was conducted in accordance with the principles of the Helsinki Declaration. Ethical approval was obtained from the Akdeniz University Ethical Committee. A total of 56 patients with total serum PSA values of >2.5 ng/ml, referred for transrectal ultrasound (TRUS)-guided sextant prostate biopsy at Akdeniz University Urology outpatient clinic were recruited. For Treg screening, 8 ml peripheral blood samples were drawn from patients into heparinized tubes just before the biopsy. The exclusion criteria were any known current infections, taking any immunomodulatory medications, and a history of cancer. Of the total number of 56 patients analyzed, 19 were diagnosed with PCa, 27 with BPH, and 10 with no apparent prostate pathology (Table 1). Of the 19 PCa patients, 17 had initial stage, locally confined cancer, while 2 had radiologically detected bony metastasis. The Gleason score was used to grade prostate cancer; where a high score is associated with advanced disease and poorer prognosis. Of the 17 locally confined cancer patients, one patient scored 5, 5 patients scored 6, 3 patients scored 7, and 10 patients scored 9. Of the 17 with locally confined PCa, 9 were treated by laparoscopic radical prostatectomy at our hospital and were re-analyzed for both total serum PSA and peripheral Treg levels one month following the surgery. Ten who were negative for BPH, PCa, and prostatitis through pathological assessment were used as healthy controls.

**Phenotypic and quantitative analysis of lymphocytes.** Peripheral blood mononuclear cells (PBMCs) were isolated using a Fycoll-Hypaque density gradient. Surface staining with anti-human CD4-FITC (biomedical kit (BD); 555346), CD25-PE (BD; 555432) and intracellular staining with Foxp3-APC (e-Bioscience; 17-4776-73) antibodies was performed as previously described (eBioscience Inc, San Diego, CA, USA).<sup>26</sup> Briefly, PBMCs were washed 3 times with Dulbecco's phosphate-buffered saline (D-PBS) and stained for

**Table 1** - Summary of patients' characteristics.

Parameter	Healthy	BPH	PCa	Total subjects
Mean age	55 (43-68)	60 (44-70)	62 (41-78)	60 (43-78)
PSA	3.3 (2.5-6)	20.4 (2.7-51)	45.4 (4.8-48)	25.3 (2.5-51)
Number	10	27	19	56

\*Numbers in parentheses show range, PSA - prostate specific antigen, PCa - prostate cancer, BPH - benign prostate hyperplasia

surface CD4 and CD25 markers for 30 minutes at room temperature. Finally, the cells were washed twice with saponin buffer and once with washing buffer and analyzed using a BD FACSCalibur Flow Cytometer (BD, New Jersey, USA). Flow-Jo software (Tree Star Inc., San Carlos, CA, USA) was used to analyze the samples and determine the frequencies of Treg cells. Absolute lymphocyte counts were determined by using an automated hematological analyzer (Sysmex XT-2000iV, Sysmex, EU, Hamburg).

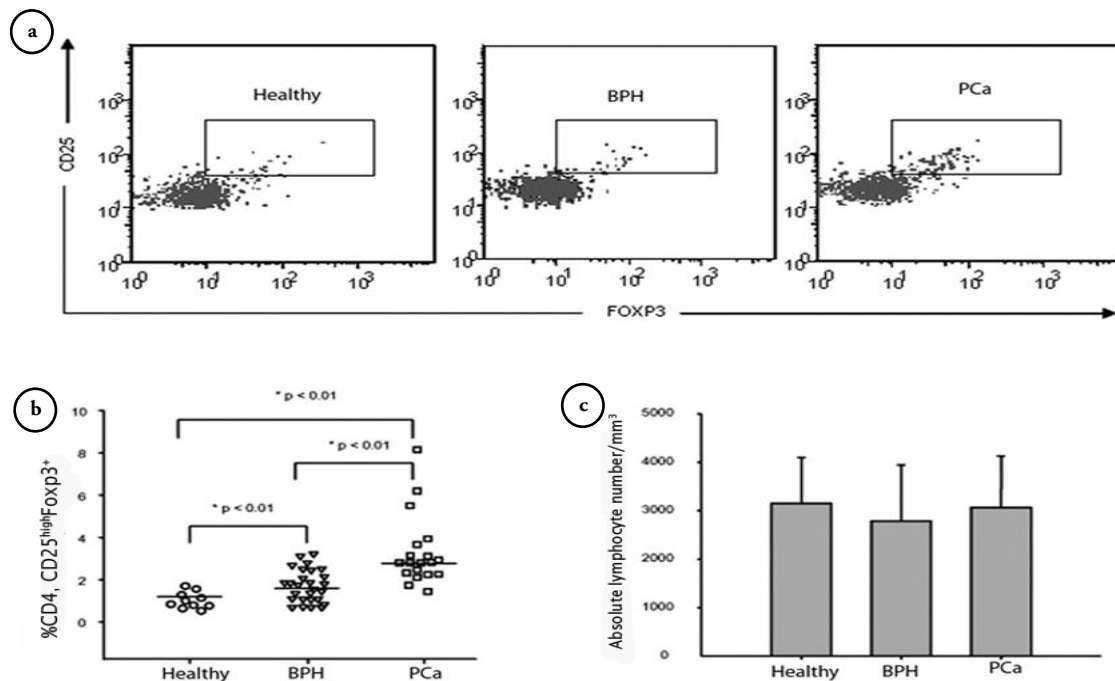
**Serum PSA quantification.** Total PSA (free + complexed) from serum samples was measured using an electro chemiluminescence immunoassay (ECLIA) with a Roche Elecsys Modular Analytics E170 immunoassay analyzer according to manufacturer's instructions. (Roche Ltd, Basel, Switzerland).

**Statistical analyses.** All of the statistical analyses were performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) version 16. Statistical differences between groups were evaluated by using Kruskal-Wallis analysis. The differences between 2 groups were determined by the Mann-Whitney test with Bonferroni correction. Correlation was tested by the non-parametric Spearman method. The statistical significance ( $p$ -value) was set at  $<0.05$ .

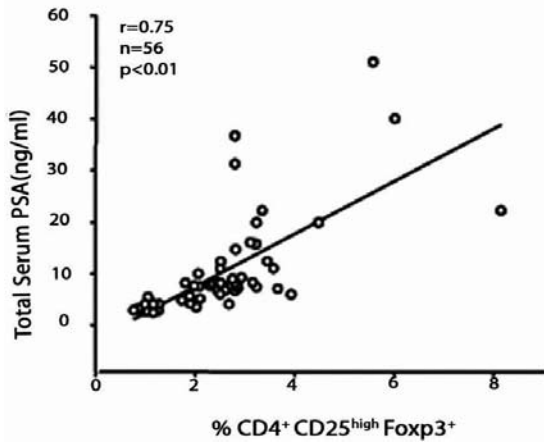
**Results.** The mean age of the patients was 62 years (range 41-78 years).

**Phenotypic analysis of Tregs in PCa patients.** Peripheral blood samples were analyzed by flow cytometry using CD4, CD25, and Foxp3 antibodies. The gating strategy used for selecting CD25<sup>high</sup> cells was very stringent (Figure 1a). The mean frequencies of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Treg cells as percentages of peripheral lymphocytes were determined as 3.23%±1.59% (n=19) in PCa patients, 1.66%±0.80% (n=27) in BPH patients, and 1.08%±0.43%, (n=10) in healthy controls (Figure 1b), showing a significant increase through out the PCa and BPH patients when compared with healthy controls. To determine whether the differences in the Treg mean frequencies resulted from the differences in absolute lymphocyte numbers, we next compared these values among the 3 groups. There was no difference in the mean absolute lymphocyte numbers among healthy controls (3150.4±950.6), BPH patients (2786.3±1152), and PCa patients (3068.2±1063.2) ( $p>0.05$ , Figure 1c).

**Peripheral Treg frequency is correlated with serum PSA.** Correlation between peripheral Treg frequencies and serum PSA levels was determined by using the



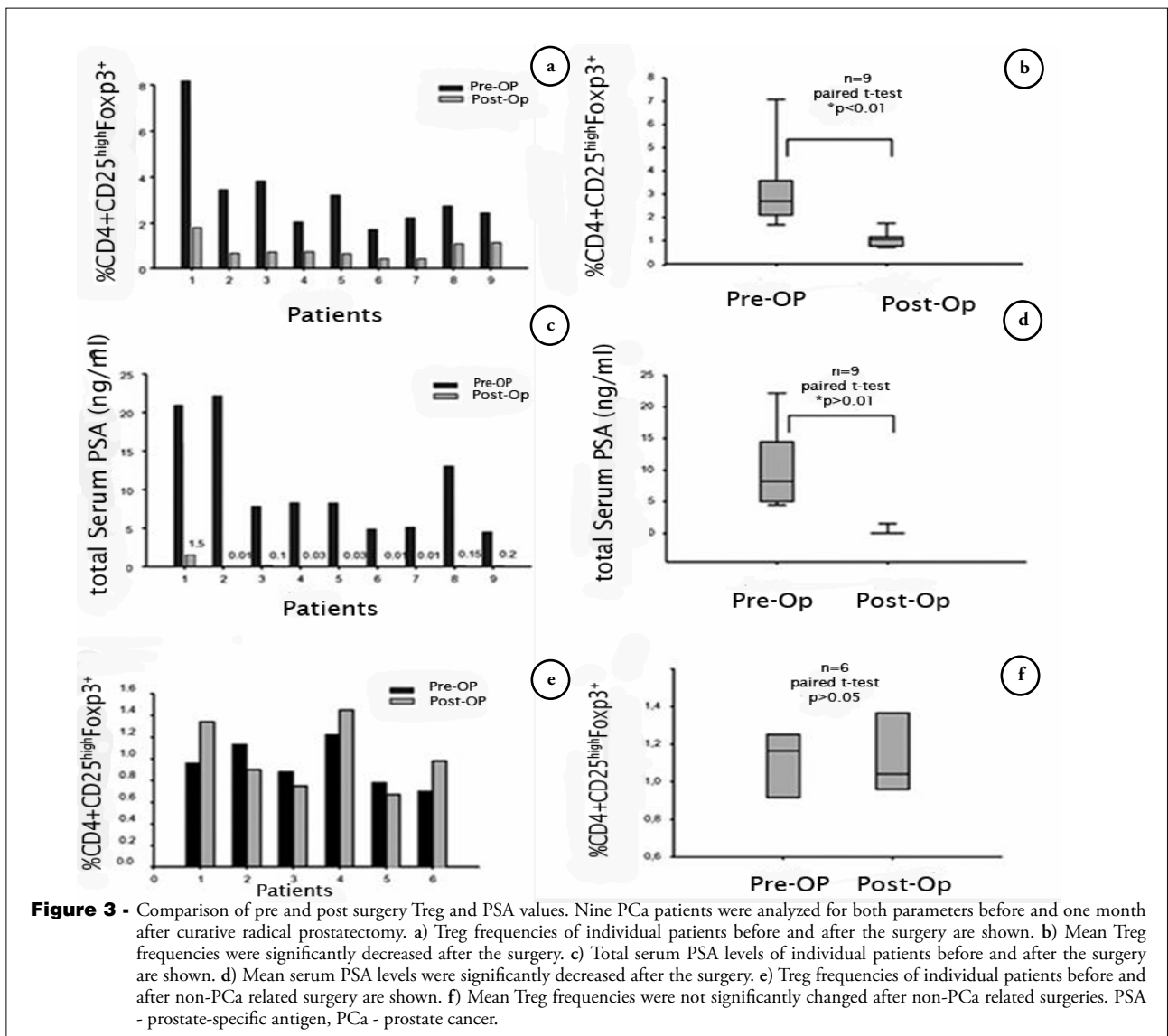
**Figure 1** - Increased frequency of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Treg cells in PBMC of prostate cancer and BPH patients: a) representative flow cytometric data from PCa and BPH patients plus healthy donors are shown. Flow cytometric analysis was performed by 3-color-staining. The first gate was put on CD4<sup>+</sup>CD25<sup>+</sup> T cells (not shown). Second gates, shown as the rectangular boxes, were placed on CD25<sup>high</sup>Foxp3<sup>+</sup> cells. b) The mean percentage of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> T cells of the total lymphocytes in PCa patients were significantly higher than those of BPH patients and healthy controls. The mean percentage of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Treg cells in BPH patients was also significantly higher than that of healthy subjects. Horizontal bars show the means for each group. c) Mean absolute lymphocyte numbers of healthy controls, BPH patients and PCa patients are not statistically different.



**Figure 2** - Correlation of serum PSA values with peripheral Treg frequencies. Correlation between the 2 parameters was determined using the non-parametric spearman method. The correlation coefficient (r) was 0.75 (n=56, p<0.01). PSA - prostate-specific antigen

data obtained from all of the patients (PSA range 2.5-51 ng/ml, n=56). There was a strong positive correlation between the 2 parameters with a correlation coefficient (r) of 0.75 ( $p=0.00763$ , Figure 2).

*Peripheral Treg frequencies and total serum PSA levels decrease concomitantly after prostatectomy in PCa patients.* Nine PCa patients, who were treated by laparoscopic radical prostatectomy, were re-analyzed one-month post surgery for peripheral PSA and Treg levels (Figure 3). The mean level of total serum PSA was  $10.00\pm5.97$  and the mean frequency of peripheral Treg was  $3.20\pm1.61$  before the surgery. Mean Treg frequencies in these patients decreased significantly ( $1.09\pm0.32$ ; paired t-test  $p<0.01$ , Figures 3a & 3b), paralleling the reduction in serum PSA levels of all the patients as expected ( $0.20\pm0.49$ ; paired t-test  $p<0.01$ , Figures 3c & 3d). This result suggests that PSA alone or in combination with other tumor-derived factors may



**Figure 3** - Comparison of pre and post surgery Treg and PSA values. Nine PCa patients were analyzed for both parameters before and one month after curative radical prostatectomy. a) Treg frequencies of individual patients before and after the surgery are shown. b) Mean Treg frequencies were significantly decreased after the surgery. c) Total serum PSA levels of individual patients before and after the surgery are shown. d) Mean serum PSA levels were significantly decreased after the surgery. e) Treg frequencies of individual patients before and after non-PCa related surgery are shown. f) Mean Treg frequencies were not significantly changed after non-PCa related surgeries. PSA - prostate-specific antigen, PCa - prostate cancer.

be required for the increased presence of Treg in the periphery. In order to rule out a possible post-surgery stress effect on Treg frequencies, we recruited 6 more patients in addition to our study group described above that had previously planned to undergo non-PCa related prostate surgeries at our hospital. These people were screened for Treg frequencies before and one month after the surgery. In these patients, the frequencies of Treg did not change significantly after the surgery ( $1.11 \pm 0.20$  to  $1.13 \pm 0.22$ ; paired t-test  $p > 0.05$ ), demonstrating that surgery by itself does not cause a decrease in Treg frequency (Figures 3e & 3f).

**Discussion.** This study demonstrates that the increased frequency of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Treg in PCa and BPH patients correlates with PSA levels. Our results suggest that PSA may have a role in Treg induction or maintenance in these patients.

The purpose of this study was twofold. First, we sought to verify whether the peripheral frequencies of CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Treg cells are elevated in PCa patients; second, we aimed to determine the direct correlation between the peripheral Treg and total serum PSA levels in these patients. We first demonstrated that CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Treg densities are increased in PCa patients compared with BPH patients and healthy controls. In addition to this observation, which is consistent with the findings of Miller et al<sup>10</sup> we also recorded a significant enhancement in Treg frequency in the periphery of BPH patients compared with healthy controls. Even though Miller et al<sup>10</sup> also clearly showed increased levels of Treg infiltration in prostate tissue samples of BPH patients, these authors did not analyze those patients for peripheral Treg densities. Our results and those of Miller et al,<sup>10</sup> however, contradict the findings of Yokokawa et al,<sup>11</sup> who observed no change in the frequencies of Treg cells in PCa patients except in those with metastatic cancers. Seventeen of 19 patients in the current study, and all of the patients by Miller et al<sup>10</sup> were reported to have locally confined cancer. Furthermore, despite the fact that the mean frequency of Tregs in PCa patients was comparable in all 3 studies, the mean frequency reported for healthy subjects by Yokokawa et al<sup>11</sup> was considerably higher than the recorded results from our study as well as others, including Miller et al<sup>10</sup> This discrepancy might have resulted from the age of healthy controls used, which was 55 years (range 41-78 years) in our study. Furthermore, our analysis clearly demonstrates that Tregs from PCa patients have significant suppressive activity.<sup>27</sup>

Secondly and more importantly, we demonstrated that a strong association exists between the high density of Tregs and total serum PSA in both BPH and PCa patients, suggesting that the increased frequency of Tregs may not be a result of malignancy, but may rather be

caused by the excessive amounts of PSA accumulation in these patients. We obtained the first line of evidence for this assumption during the diagnostic process, by showing that Treg frequencies were strongly correlated with serum PSA in patients with serum PSA levels of 2.5-51 ng/ml, regardless of the disease status ( $r=0.75$ ,  $p=0.01$ ).

The evidence for the strong association of PSA with Tregs was obtained from the analysis of 9 patients with locally confined cancers, who were treated by radical prostatectomy. Treg frequencies of all these patients subsided remarkably one month following surgery. Interestingly, although 3 of these patients had Treg levels near the threshold Treg frequency of 2.1% (95% CI), these levels further declined. This suggests that tumor-specific Tregs might still exist and be depleted upon antigen removal, even in patients whose Treg levels are not elevated. Our result is in agreement with Kono et al,<sup>28</sup> who recently observed a similar reduction of peripheral Treg levels in gastric cancer patients that underwent curative surgeries. Taken together, our results strongly suggest that PSA, either alone or with other prostate derived factors, is involved in either the induction and/or maintenance of peripheral Tregs in BPH and PCa patients. The patient number limited this study, as our clinic is not a urologic cancer clinic and also, studies in literature have similar patient numbers.

The reason for the enhanced number of Tregs in human cancers is not clear. It is hypothesized that TAA in the presence of soluble mediators, such as TGF- $\beta$  and chemokines may be required for peripheral induction and/or expansion of Tregs within the tumor-draining lymph nodes.<sup>29</sup> We also do not know whether other factors, either tumor-derived or tumor-induced, are also required in these processes. Previous in vitro studies demonstrated that PSA is able to induce TGF- $\beta$  production and impair dendritic cell maturation.<sup>30</sup> Both of these pathways are known to induce Tregs, in vivo in experimental models and in vitro in human PBMC cultures and thus, it is reasonable to assume that excessive amounts of PSA in both BPH and PCa patients may invoke one or both of the above pathways to either induce or expand PSA-specific Tregs. It is also likely that other mechanisms, such as IDO (Indoleamine 2-3 dioxygenase) or PGE-2 production by the cells within the tumor milieu might contribute to the process.<sup>11</sup> Further studies addressing these questions will be important for our understanding of the biology of the Treg in human cancer.

Restoring peripheral Treg levels upon removal of cancerous prostates also has important implications for immunotherapy for PCa. Many prostate-specific vaccine trials using PSA peptides to stimulate tumor-specific immune responses before the surgical removal of tumors have not yielded desired results.<sup>5-7,9</sup>

In conclusion, in this study we present evidence that increased frequency of circulating CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs in PCa and BPH patients is correlated with PSA levels, and we also demonstrate a strong dynamic association between a TAA and Tregs in cancer bearing humans. In the light of this findings, we may expect better response of treatment of tumour specific immunotherapy after removal of prostate gland, which includes adeno carcinoma cells. In the light of this study, more comprehensive studies on immunotherapy may provide new forms of treatments for Pca.

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