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Analysis of circulating regulatory T cells in patients with metastatic prostate cancer pre- versus post-vaccination

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Abstract We have previously shown that the suppressive function of regulatory T cells (Tregs) from peripheral blood mononuclear cells (PBMCs) is enhanced in patients with prostate cancer when compared with healthy individuals. Two phase II studies using the PSA-TRICOM vaccine in patients with metastatic castration-resistant prostate cancer (mCRPC) showed evidence of patient benefit in terms of enhanced survival. The Halabi nomogram has been used to predict survival (HPS) of patients with mCRPC treated with conventional chemotherapy or second-line hormonal therapy. Tregs from PBMCs of patients ($n = 23$) with mCRPC were obtained pre- and post-three monthly vaccinations, and analyzed for number, phenotype, and suppressive function. Changes post- versus pre-vaccination in these parameters were compared with 3-year survival and HPS. No differences in Treg numbers were observed post- versus pre-vaccination. Trends ($P = 0.029$) were observed between overall survival (OS) and a decrease in Treg suppressive function post- versus pre-vaccination. Trends were also observed in analyzing effector:Treg ($CD4^+CD25^+CD127^-$ $FoxP3^+CTLA4^+$) ratio post- versus pre-vaccination with OS versus HPS. These data provide preliminary evidence for a possible association between improved OS and a decrease in Treg function when PBMCs are analyzed after three monthly vaccinations. Patients with an OS > HPS were more likely to have decreased Treg function following vaccine. Larger studies to confirm and extend these findings are warranted.

Keywords Cancer vaccine · Prostate cancer · Immunotherapy · Regulatory T cells · Vaccination

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Introduction

Prostate cancer (PCa) is the most common malignancy in men in USA and a leading cause of cancer death in North America. The American Cancer Society estimates that 217,730 men in USA are diagnosed yearly, resulting in approximately 32,050 deaths [1]. Treatment for localized disease includes radical prostatectomy or radiation therapy, but approximately 30% of patients will subsequently develop rising prostate-specific antigen (PSA). While androgen-deprivation therapy can control the disease temporarily, eventually the majority of patients develop metastatic castration-resistant disease. Docetaxel-based therapy has been shown to improve survival by approximately 2–3 months in this setting [2]. Immunotherapeutic approaches using peptide- or dendritic cell-based vaccines and poxviral-based vaccines have shown evidence of clinical benefit [3]. A recently reported phase III trial of sipuleucel-T vaccine in patients with metastatic castration-resistant PCa (mCRPC) was the first trial with overall survival (OS) as the primary endpoint to demonstrate a statistically significant improvement in OS with a cell-based therapeutic vaccine [4]. This trial has led to the FDA

approval of spiuleucel-T (Provenge®) for the therapy of metastatic prostate cancer. A 43-center phase II randomized controlled trial of a poxviral-based PSA-targeted vaccine (PSA-TRICOM) in mCRPC also demonstrated an improvement in median OS of 8.5 months in this patient population [5]. These data provide additional evidence that therapeutic vaccines can lead to improved OS.

$CD4^+CD25^{high}FoxP3^+$ regulatory T cells (Tregs) play an important role in immune homeostasis because of their ability to suppress the activation of T cells. An increase in the number or functionality of Tregs could thus favor tumor development. Increased levels of $CD4^+CD25^{high}$ $FoxP3^+$ Tregs have been detected in the peripheral blood mononuclear cells (PBMCs), tumor microenvironment, and draining lymph nodes of patients with PCa [6–8] and other solid tumors [9–21], as well as hematologic malignancies [22–24]. Clinical studies have demonstrated that Tregs can inhibit both antigen-specific and non-specific T-cell responses [25, 26], and that an increase in $FoxP3^+$ Tregs is associated with high-risk factors [27–29]. Depletion of human Tregs with a recombinant interleukin (IL)-2 diphtheria toxin conjugated as $DAB_{389}IL-2$ (denileukin diftitox; ONTAK) in carcinoma patients whose tumors expressed carcinoembryonic antigen (CEA) specifically enhanced antigen-specific immune responses to a dendritic cell-based cancer vaccine [30]. Another study in patients with ovarian cancer showed a direct correlation between tumor-infiltrating Tregs and OS. In that study, treatment with denileukin diftitox led to depletion of Tregs and improved antitumor responses [31]. Results of other studies have also suggested that $CD4^+CD25^{high}FoxP3^+$ Tregs could reduce the efficacy of cancer immunotherapy [32], and that depletion of Tregs could augment vaccine-mediated antitumor immune responses [20]. In a recent study [7], we reported that although numbers of $CD4^+CD25^{high}FoxP3^+$ Tregs in the PBMCs of patients with PCa were not significantly increased as compared to healthy donors, their suppressive function was significantly higher than Tregs from healthy donors.

Several investigators have reported that CTLA-4 plays an essential role in the function of Tregs [33, 34]. In a recent report, Miyara et al. [35] demonstrated that human $FoxP3^+$ $CD4^+$ T cells can be separated into three populations: $FoxP3^{low}CD45RA^+$, $FoxP3^{high}CD45RA^-CD25^{+++}$, and $FoxP3^{low}CD45RA^-CD25^{++}$. $FoxP3^{high}CD45RA^-CD25^{+++}$ cells express higher levels of intracellular CTLA-4, HLA-DR, and inducible co-stimulator (ICOS). CTLA-4 is a pivotal negative regulator of T-cell signaling in conventional T cells, but not much is known about how CTLA-4 modulates T-cell signaling in Tregs. A recent report demonstrated that treatment with anti-CTLA-4 antibody does not deplete $CD4^+FoxP3$ Tregs, but expands effector T cells as well as

functional Tregs in humans [36]. These results suggest that, similar to its action on effector T cells, CTLA-4 may inhibit Treg proliferation [36, 37].

The Halabi nomogram [38] has been shown to predict the survival (HPS) of over 1,100 patients with mCRPC who have been treated with chemotherapy or second-line hormonal therapy; the nomogram is based on seven baseline disease characteristics: visceral disease, Gleason score, performance status, baseline PSA, LDH, alkaline phosphatase, and hemoglobin. In a clinical trial at the National Cancer Institute (NCI), mCRPC patients were treated with a poxviral-based vaccine containing the transgenes for PSA and three costimulatory molecules (PSA-TRICOM) [39]. Vaccinated patients with a HPS >18 months had a significant improvement in OS above the HPS. Twelve of 15 patients with an HPS ≥18 months had an OS > HPS ($P = 0.035$); median OS for this group has not been reached, with 8/15 patients still alive at 37.3 months, an improvement of ≥16.4 months over the median HPS of 20.9 months. In contrast, of patients with an HPS of <18 months, an improvement of only 2.3 months over the HPS of 12.3 months was observed. In the study reported here, we evaluated the number, phenotype and function of Tregs pre- and post-vaccination, and analyzed Treg phenotype in relation to suppressive function. Results showed a correlation between improved OS over that predicted by the Halabi nomogram and a decrease in Treg function from PBMCs obtained after three PSA-TRICOM vaccinations. More comprehensive studies on larger populations will be required to validate this finding. To our knowledge, however, this is the first qualitative and quantitative analysis of Treg populations in patients with mCRPC pre- and post-vaccination.

Patients and methods

Patients

This clinical trial [39] enrolled 32 patients with mCRPC who had not received any radiotherapy or chemotherapy within 6 months prior to blood draw and 23 patients were evaluable in this analysis. Patients were given a primary vaccination with recombinant vaccinia (rV)-PSA-TRICOM, and then received monthly boosts of recombinant fowlpox (rF)-PSA-TRICOM until progression [39]. At enrollment all patients had evidence of disease progression as defined by (a) new metastatic findings on bone scan and/or (b) disease progression on computerized tomography scan, or (c) increased serum PSA as determined by PSA consensus criteria [40]. All patients signed a consent form approved by the NCI Institutional Review Board.

Collection of PBMCs

Apheresis was performed prior to vaccination and after three vaccinations (around day 85). PBMCs were isolated by Ficoll (Amersham Biosciences; Piscataway, NJ) density gradient separation, washed three times, and cryopreserved in liquid nitrogen at a concentration of $1-2 \times 10^7$ cells/mL until assayed.

Flow cytometry analysis

Cryopreserved PBMCs were analyzed by 5-color flow cytometry for phenotypic characterization of Tregs. Cells were resuspended in staining buffer (PBS containing 3% fetal bovine serum) and stained for 30 min at 4°C with FITC-conjugated anti-CD4, PECy7-conjugated anti-CD25, PE-conjugated or PerCp-Cy5.5-conjugated anti-CD127, and PE-conjugated anti-CTLA-4 (BD Biosciences; San Jose, CA, USA). FoxP3 intracellular staining was performed on cells stained with anti-CD4, anti-CD25, and anti-CD127. Cells were fixed and permeabilized using a fix/perm kit (eBioscience; San Diego, CA, USA) according to the manufacturer's instructions, then labeled with APC-conjugated anti-FoxP3 antibody (236A/E7 clone) (eBioscience) or its isotype control antibody (eBioscience), as a negative control. Flow cytometry was done on a Becton–Dickinson LSRII (BD Biosciences); 1×10^5 cells were acquired and data were analyzed using DiVa software (BD Biosciences). To determine the percentage of Tregs, lymphocytes were gated by plotting forward versus side scatter. The $CD4^+$ population was gated first, followed by the $CD25^+CD127^-$ and $FoxP3^+$ populations.

$CD4^+CD25^{high}$ T-cell enrichment

$CD4^+CD25^{high}$ T cells were enriched using a $CD4^+CD25^+$ Treg isolation kit (Miltenyi Biotec; Auburn, CA, USA), with modifications to the manufacturer's instructions. $CD4^+CD25^{high}$ T cells were enriched by a method described by Yokokawa et al. [7]. $CD4^+$ T cells were negatively enriched by LD column, and positive selection for $CD25^+$ T cells was done on the negatively selected $CD4^+$ T cells. The $CD25^+$ fraction was collected by eluting the cells twice through a magnetic separation column for further enrichment of $CD4^+CD25^{high}$ T cells. In a representative experiment, 92.3% of Tregs isolated by this method were $CD4^+CD25^{high}$. By comparison, 93.0% of Tregs obtained by a cell-sorting procedure from PBMCs of the same donor were $CD4^+CD25^{high}$ (data not shown), showing that Tregs isolated by MicroBeads had the same phenotypic profile as Tregs isolated by cell sorting.

Immunosuppression assay

A modified version of the protocol described by Yokokawa et al. [7] was used for the immunosuppression assay. $CD4^+CD25^-$ T cells (1×10^4 cells/well) were cultured alone or with $CD4^+CD25^{high}$ T cells in three different ratios with 1 µg/mL of anti-CD3 antibody (OKT3; eBioscience) in the presence of irradiated (3,500 rad) T-depleted PBMCs (1×10^5 cells/well) in a 96-well flat-bottomed plate at 37°C and 5% CO_2. Cells were cultured in RPMI 1640 (Mediatech; Manassas, VA, USA) supplemented with 10% heat-inactivated human AB serum (Gemini Bio-Products; West Sacramento, CA, USA), 100 units/mL of penicillin, 100 µg/mL of streptomycin (Mediatech), and 2 mmol/L of L-glutamine (Mediatech). Proliferation was measured by [3H]thymidine (PerkinElmer; Waltham, MA, USA) incorporation at 1 µCi (0.037 MBq) per well. Cells were pulsed on day 4 and quantified 18 h later using a liquid scintillation counter (PerkinElmer). All experiments were done in triplicate. Proliferation of $CD4^+CD25^-$ T cells without co-culturing with Tregs constituted 100% proliferation.

Statistical analysis

Differences among groups were assessed using the paired Student's t test. $P < 0.05$ was considered statistically significant. Spearman's rank correlation coefficient (r) (–1.00 to +1.00) was used to calculate the correlation between two variables.

Results

Treg levels in peripheral blood

We investigated levels of Tregs in the peripheral blood of 23 patients pre- and post-vaccination with PSA-TRICOM. CD4 cells were first gated for expression of $CD25^+$ and $CD127^-$ cells. The $CD4^+CD25^+CD127^-$ population was then gated for $FoxP3^+$ cells (Fig. 1a). No significant differences in levels of $CD4^+CD25^+CD127^-$ $FoxP3^+$ Tregs pre- and post-vaccination were seen among patients enrolled on study (4.6 and 4.1%, respectively; $n = 23$; $P = 0.1$) (Fig. 1b). In those patients with OS > HPS, there were no significant differences in levels of Tregs pre- and post-vaccination (4.9 and 4.3%, respectively; $n = 16$; $P = 0.107$) (Fig. 1c). In patients with OS < HPS, there were also no significant differences in levels of Tregs pre- and post-vaccination (3.8 and 3.7%, respectively; $n = 7$; $P = 0.745$) (Fig. 1d). In addition, the percentages of $CD4^+CD25^-$ T cells pre- and post-vaccination were also similar between patients with OS > HPS (73.7 and 75.1%,
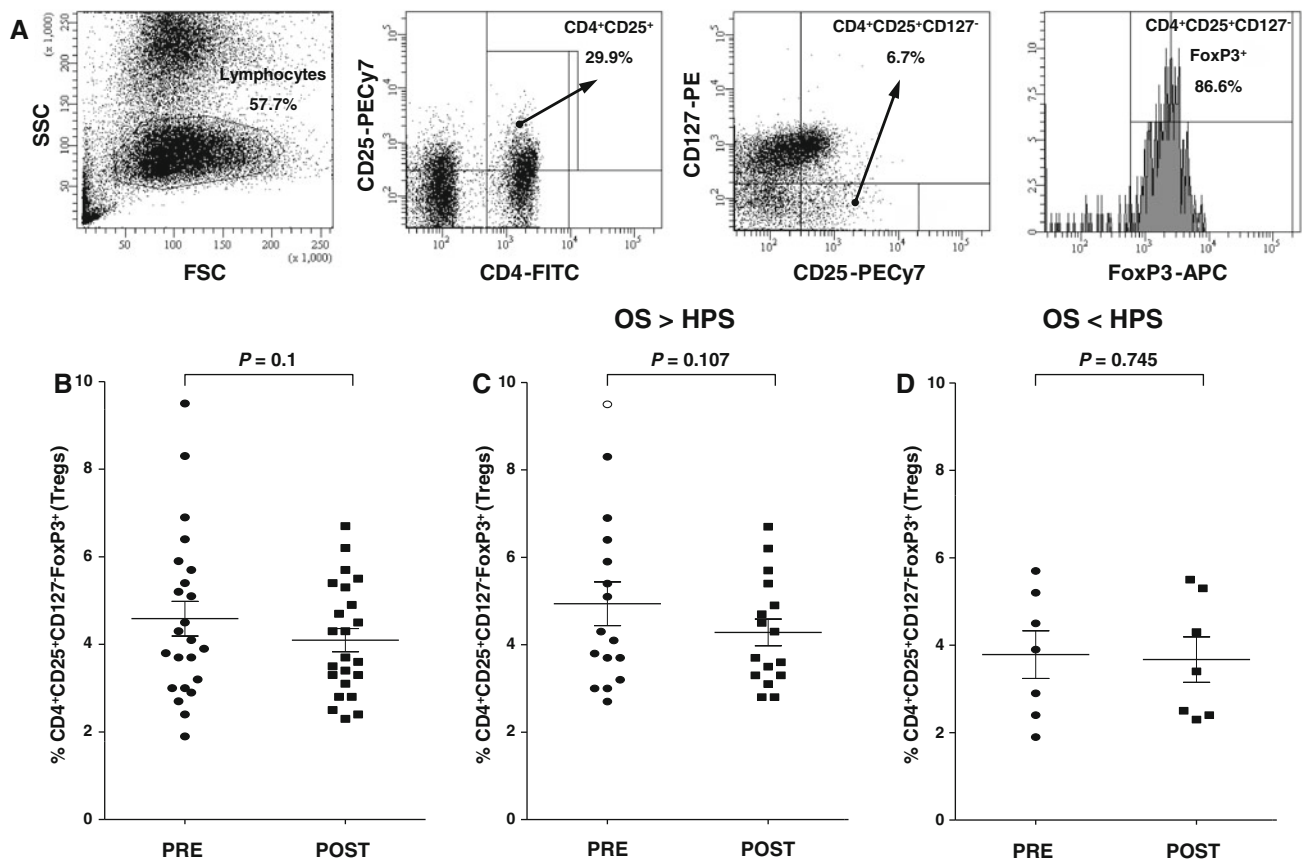
Fig. 1 Expression of FoxP3 in CD4$^+$CD25$^+$CD127$^-$ Tregs in patients receiving the PSA-TRICOM vaccine. **a** Expression of FoxP3 in CD4$^+$CD25$^+$CD127$^-$ Tregs. PBMCs from a patient were analyzed by flow cytometry after cell surface staining with FITC-conjugated anti-CD4, phycoerythrin-conjugated anti-CD127, PECy7-conjugated anti-CD25, and intracellular staining with APC-conjugated anti-FoxP3. **b** Percentage of CD4$^+$CD25$^+$CD127$^-$Foxp3$^+$ Tregs in CD4 population pre- and post-vaccination in the peripheral blood of all patients enrolled on study. **c** Percentage of CD4$^+$CD25$^+$ CD127$^-$Foxp3$^+$ Tregs in CD4 population pre- and post-vaccination in the peripheral blood of patients with OS > HPS. **d** Percentage of CD4$^+$CD25$^+$CD127$^-$Foxp3$^+$ Tregs in CD4 population pre- and post-vaccination in the peripheral blood of patients with OS < HPS. There were no significant differences in levels of Tregs pre- and post-vaccination among patients on study, patients with OS > HPS, and patients with OS < HPS ($P = 0.1$, $P = 0.107$, and $P = 0.745$, respectively)

respectively) and patients with OS < HPS (71.9 and 72.9%, respectively).

Treg suppressive function

We analyzed the functionality of CD4$^+$CD25^{high}FoxP3$^+$ Tregs from 23 patients pre- and post-vaccination by investigating their ability to suppress autologous CD4$^+$CD25$^-$ T-cell proliferation upon stimulation with anti-CD3 monoclonal antibody. Figure 2 shows the functional activity of Tregs from a representative patient with OS > HPS. The suppressive activity of Tregs decreased in 12/15 patients (80%) in the OS > HPS group pre- and post-vaccination (Table 1), but only in 2/8 patients (25%) in the OS < HPS group (Table 1). For all 23 patients on study, there was a correlation between decreased suppressive activity of Tregs in PBMCs pre- and post-vaccination and OS (Spearman's

$P = 0.029$; $r = -0.45$). The suppressive activity of Tregs decreased in 9/10 patients in the longer than 30-month survival group, as compared to 5/13 patients in the shorter than 30-month survival group.

We performed additional experiments to determine whether the levels of suppressive function seen in Tregs obtained from PCa patients pre- and post-vaccination were influenced by the patients' effector T cells. Effector cells were obtained from a patient pre- and post-vaccination, then cocultured with autologous Tregs isolated post-vaccination and stimulated with anti-CD3 monoclonal antibody and autologous APCs. Suppression of proliferation of effector cells by post-vaccination Tregs was similar in the pre- and post-vaccination samples (19.2 and 20.6%, respectively, at a 1:1 effector:Treg ratio) (Fig. 3a, b). Next, effector cells from a patient pre- and post-vaccination were cocultured with Tregs isolated pre-vaccination from the
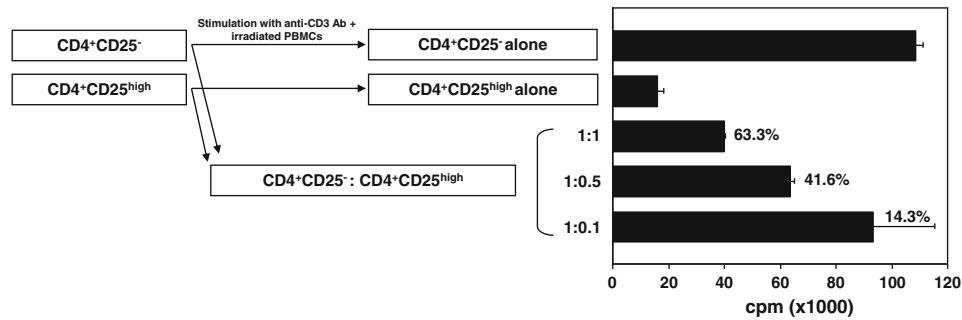
Fig. 2 Suppression of CD4$^+$CD25$^-$ T-cell proliferation by CD4$^+$CD25^{high} Tregs in a patient with PCa. Isolated effectors (CD4$^+$CD25$^-$) and Tregs (CD4$^+$CD25^{high}) were cultured alone or at three different ratios (1:1, 1:0.5, and 1:0.1) upon stimulation with anti-CD3 and irradiated T cell-depleted PBMCs. Experiments were performed in triplicate. *Columns* mean (cpm), *bars* SD. Percentages indicate level of inhibition

**Table 1** Treg suppressive activity pre- versus post-vaccination

Patients	Patients with OS > HPS			Patients	Patients with OS < HPS		
	% Treg suppression		Δ Suppression		% Treg suppression		Δ Suppression
	Pre	Post	Post − pre		Pre	Post	Post − pre
Patients with decreased suppression				*Patients with decreased suppression*			
#1	72.1	56.4	**−15.7**	#2	65.0	45.0	**−20.0**
#3	83.9	73.0	**−10.9**	#11	55.8	30.8	**−25.0**
#6	25.7	24.4	**−1.3**				
#14	38.7	29.1	**−9.6**				
#15	76.9	64.2	**−12.7**				
#17	23.8	14.1	**−9.7**				
#19	50.6	40.1	**−10.5**				
#20	48.3	23.8	**−24.5**				
#23	66.3	43.0	**−23.3**				
#24	93.3	85.0	**−8.3**				
#25	52.9	22.3	**−30.6**				
#32	33.5	15.1	**−18.4**				
Mean	**55.5**	**40.9**	**−14.6**	**Mean**	**60.4**	**37.9**	**−22.5**
Patients with increased suppression				*Patients with increased suppression*			
#5	37.8	55.3	**17.5**	#4	37.0	55.3	**18.3**
#13	39.0	59.0	**20.0**	#8	40.2	63.0	**22.8**
#26	63.3	90.6	**27.3**	#12	28.4	39.3	**10.9**
				#18	51.7	64.6	**12.9**
				#21	50.4	74.3	**23.9**
				#28	61.9	75.0	**13.1**
Mean	**46.7**	**68.3**	**21.6**	**Mean**	**44.9**	**61.9**	**17.0**

Results are expressed as % of Treg suppression of CD4$^+$CD25$^-$ effector cell proliferation

same patient and stimulated with anti-CD3 monoclonal antibody and autologous APCs, with a similar outcome (40.2 and 39.4%, respectively, at a 1:1 effector:Treg ratio) (Fig. 3c, d). These results suggest that the difference in suppressive function pre- and post-vaccination was due to the difference in Treg function, not to the functional activity of effector cells.

CTLA-4-expressing Tregs

Measuring suppressive function of Tregs is labor intensive and, more importantly, requires large-volume blood draws or leukapheresis to obtain enough cells for assay. We thus investigated if a more defined phenotype of Tregs would correlate with suppressive function and possibly OS of
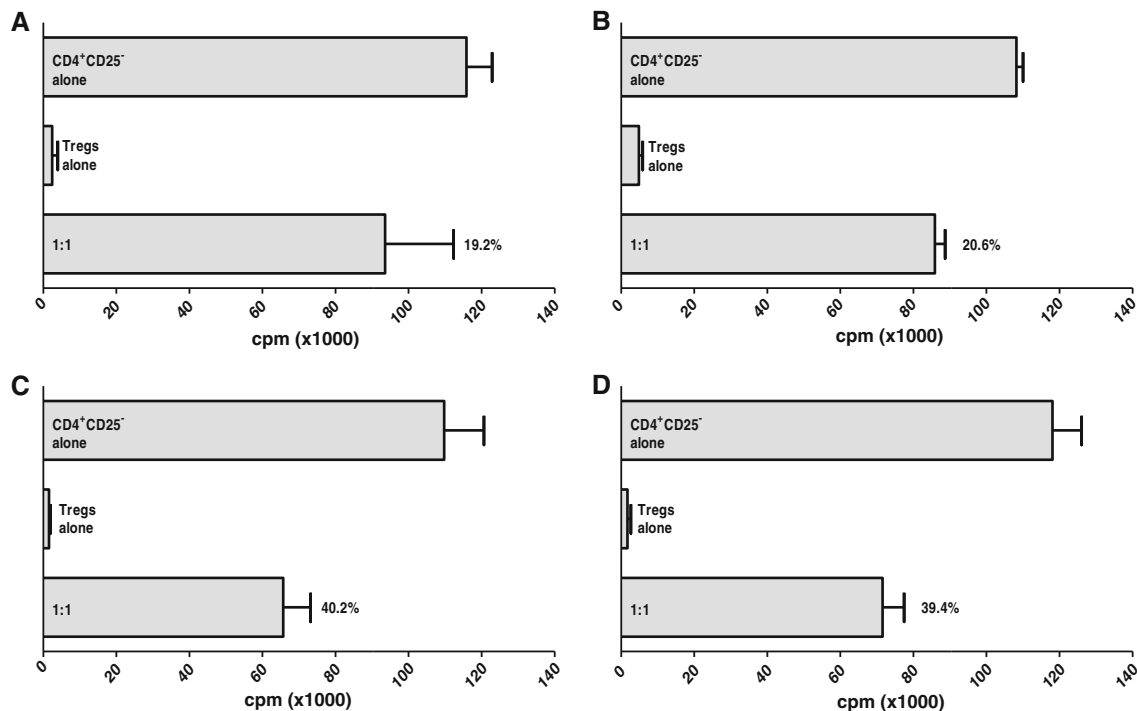
Fig. 3 Treg suppressive function was not influenced by effector T cells. CD4$^+$CD25$^-$ T cells from a PCa patient pre-vaccination (**a**) and post-vaccination (**b**) were cultured alone or at a 1:1 ratio with Tregs isolated from the same PCa patient post-vaccination, upon stimulation with anti-CD3 and irradiated T cell-depleted PBMCs. CD4$^+$CD25$^-$ T cells from a PCa patient pre-vaccination (**c**) and post-vaccination

(**d**) were cultured alone or at a 1:1 ratio with Tregs isolated from the same PCa patient pre-vaccination, upon stimulation with anti-CD3 and irradiated T cell-depleted PBMCs. Experiments were performed in triplicate. *Columns* means (cpm), *bars* SD. Percentages indicate level of inhibition

patients in this trial. We thus measured expression of CTLA-4 in CD4$^+$CD25$^+$CD127$^-$FoxP3$^+$ Tregs from patients pre- and post-vaccination with PSA-TRICOM. Results showed no differences in levels of CTLA-4 expression in Tregs pre- and post-vaccination among patients enrolled on study ($n = 22$, $P = 0.380$) (Fig. 4a). Results also showed no difference in the level of CTLA-4 expression in Tregs in patients with OS > HPS pre- and post-vaccination ($P = 0.184$) (Fig. 4b), but an apparent increase in CTLA-4 expression in Tregs from patients with OS < HPS post-vaccination ($P = 0.019$) (Fig. 4c). Moreover, only 4 of 11 patients (36%) with decreased suppressive functional activity had increased levels of CTLA-4$^+$ Tregs, as compared to 7 of 8 patients (87.5%) with increased suppressive functional activity who had increased levels of CTLA-4$^+$ Tregs (Table 2).

We then analyzed the effector:CTLA-4$^+$ Treg ratio in patients pre- and post-vaccination and found no differences in the effector:CTLA-4$^+$ Treg ratio among patients enrolled on study pre- and post-vaccination ($P = 0.894$) (Fig. 5a). However, we found an increase in the post-versus pre-vaccination ratio in patients with OS > HPS ($P = 0.029$) (Fig. 5b) and a decrease in the post-vaccination ratio in patients with OS < HPS ($P = 0.027$) (Fig. 5c).

Discussion

The analyses of Tregs and effector:Treg ratios reported here were carried out using PBMCs obtained from patients after three monthly vaccinations versus pre-vaccination, and results were compared with OS (median 26.6 months). While this is a retrospective analysis, and a relatively small trial, we believe the results are hypothesis generating and merit similar analyses in larger trials. If validated in such trials, one could employ these analyses to help define, early in the vaccination regimen (i.e., at 3 months), which patients are likely to benefit from further vaccination and, perhaps more importantly, which patients will most likely not benefit. These latter patients can thus go on to receive other therapies.

Tregs are heterogeneous, but are predominantly composed of two main subsets of CD4 cells: (a) thymus-derived natural CD4 Tregs (nTregs) that migrate to the periphery and (b) adaptive CD4$^+$ Tregs (Tr1, Th3, and inducible CD4$^+$CD25$^+$FoxP3$^+$) [41]. Inducible CD4$^+$CD25$^+$FoxP3$^+$ Tregs (iTregs) are induced from CD4$^+$CD25$^-$ precursors in the peripheral lymphoid organs [42]. In humans, nTregs express high levels of CD25 and the FoxP3 transcription factor [43–45]. iTregs are induced with IL-2 and transforming growth factor-β [46]. The present study investigated
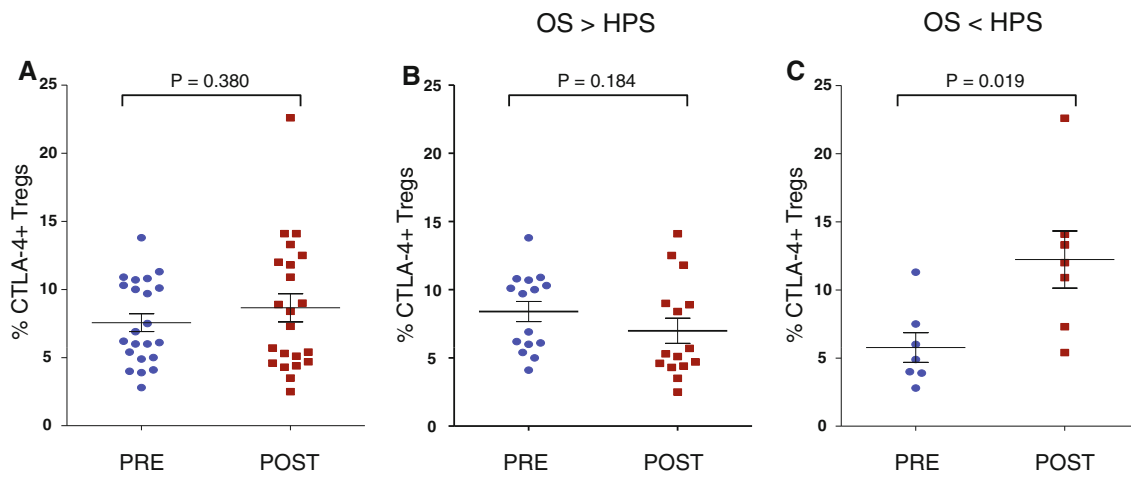
Fig. 4 Expression of CTLA-4 on Tregs. **a** Levels of CTLA-4-expressing $CD4^+CD25^+CD127^-FoxP3^+$ Tregs in PBMCs of all PCa patients on study. No statistically significant differences were detected pre- and post-vaccination ($P = 0.380$). **b** Levels of CTLA-4-expressing $CD4^+CD25^+CD127^-FoxP3^+$ Tregs in PBMCs of PCa patients with OS > HPS. No statistically significant differences were detected pre- and post-vaccination ($P = 0.184$). **c** Levels of CTLA-4-expressing $CD4^+CD25^+CD127^-FoxP3^+$ Tregs in PBMCs of PCa patients with OS < HPS. Statistically significant differences were detected pre- and post-vaccination ($P = 0.019$)

Table 2 Correlation between Treg suppressive activity pre- and post-vaccination and CTLA-4 expression on Tregs

Patients with decreased Treg suppression				Patients with increased Treg suppression			
Patients	% CTLA-4$^+$ Tregs		Δ CTLA-4$^+$ Tregs	Patients	% CTLA-4$^+$ Tregs		Δ CTLA-4$^+$ Tregs
	Pre	Post	Post − pre		Pre	Post	Post − pre
Patients with decreased CTLA-4$^+$ Tregs				*Patients with decreased CTLA-4$^+$ Tregs*			
#6	10.0	2.5	**−7.5**	#5	10.9	4.4	**−6.5**
#14	6.0	5.7	**−0.3**				
#15	9.7	8.9	**−0.8**				
#20	6.9	4.6	**−2.3**				
#23	10.7	5.3	**−5.4**				
#24	10.3	4.7	**−5.6**				
#32	5.0	3.5	**−1.5**				
Mean	**8.4**	**5.0**	**−3.3**	**Mean**	**10.9**	**4.4**	**−6.5**
Patients with increased CTLA-4$^+$ Tregs				*Patients with increased CTLA-4$^+$ Tregs*			
#1	6.1	12.5	**6.4**	#4	6.0	10.9	**4.9**
#2	7.5	13.3	**5.8**	#12	4.9	22.6	**17.7**
#11	2.8	7.3	**4.5**	#13	13.8	14.1	**0.3**
#17	4.1	9.0	**4.9**	#18	4.0	12.0	**8.0**
				#21	3.9	5.4	**1.5**
				#26	10.1	11.8	**1.7**
				#28	11.3	14.1	**2.8**
Mean	**5.1**	**10.5**	**5.4**	**Mean**	**7.7**	**13.0**	**5.3**

Results are expressed as % of CTLA-4$^+$ Tregs in $CD4^+CD25^+CD127^-FoxP3^+$ Tregs

the levels and function of $CD4^+CD25^+CD127^-FoxP3^+$ Tregs in the peripheral blood of patients with mCRPC pre- and post-vaccination with a vector-driven PSA-specific vaccine. Tregs were identified as $CD4^+CD25^+CD127^-FoxP3^+$ (Fig. 1a). Approximately 86.6% of cells in the $CD4^+CD25^+CD127^-$ population were FoxP3$^+$, which is consistent with FoxP3 expression largely confined to this population [47–49]. Studies of many different types of cancer have shown elevated levels of Tregs in PBMCs [6, 13, 16, 19, 50]. Previous studies have shown that the number of $CD4^+CD25^+FoxP3^+$ Tregs correlates inversely with outcome in various types of cancers [15, 27, 51–53]. However,
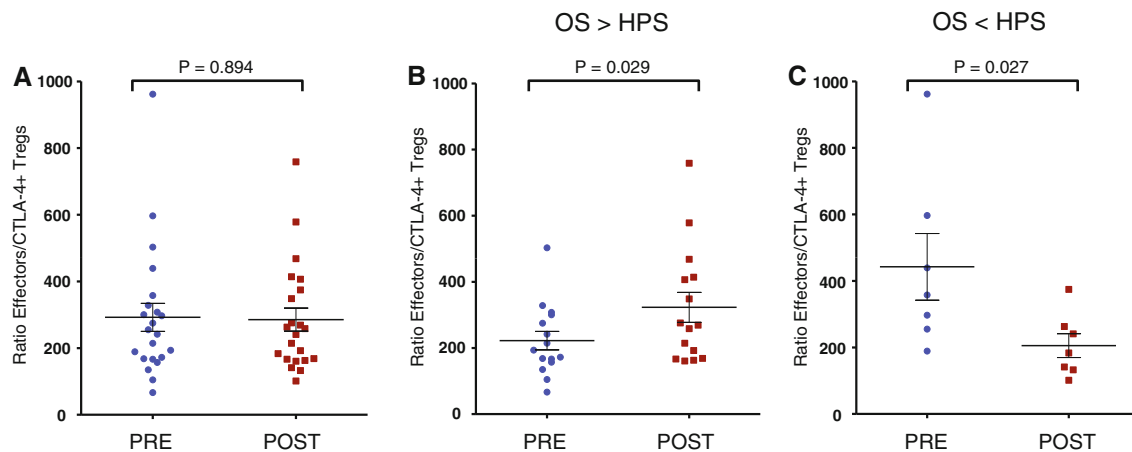
Fig. 5 Effector:CTLA4$^+$ Treg ratios post- versus pre-vaccination. **a** There were no significant differences in effector:CTLA-4$^+$ Treg ratio pre- and post-vaccination among PCa patients enrolled on study ($P = 0.894$). **b** There was a statistically significant increase in effector:CTLA-4$^+$ Treg ratio post-vaccination in PCa patients with OS > HPS ($P = 0.029$). **c** There was a statistically significant decrease in effector:CTLA-4$^+$ Treg ratio post-vaccination in PCa patients with OS < HPS ($P = 0.027$)

in most of these studies, the functional activity of Tregs was not evaluated. Furthermore, CD127 was not used as a negative surface marker for Tregs, and not all FoxP3$^+$ T cells are functionally suppressive in humans [54]. In a previous study, we demonstrated that although levels of CD4$^+$CD25^{high} FoxP3$^+$ Tregs in PBMCs of patients with PCa were not significantly higher than levels in healthy donors, Tregs in PCa patients had significantly greater suppressive functionality than Tregs from healthy donors [7]. In the absence of more specific cell surface markers for human Tregs, it is thus important to characterize human Tregs by analyzing both phenotype and inhibitory function.

In the study reported here, the functional activity of Tregs was significantly decreased in patients with OS > HPS after vaccination with PSA-TRICOM vaccine, indicating a correlation between decreased suppressive functional activity of Tregs post-vaccination and OS of these patients. To our knowledge, a trend in the correlation between Treg levels and functionality and disease outcome in patients with PCa has not been reported.

We analyzed the frequency of CTLA-4$^+$ Tregs in PCa patients pre- and post-vaccination with PSA-TRICOM and found a significant correlation between the percentage of CTLA-4$^+$ Tregs and functional activity. This finding agrees with other studies that have observed that CTLA-4 expression is linked to enhanced suppressive activity and higher expression of FoxP3 when compared with CTLA-4$^-$ Tregs in humans. Blockade of CTLA-4 in this subset of Tregs resulted in a significant, but not complete loss of Treg suppressive functional activity [55]. CTLA-4 may also mediate Treg suppressive activity through a direct interaction between CTLA-4 on Tregs and CD80 molecules on dendritic cells to induce tryptophan catabolism [56–58]. We investigated the

CD4$^+$CD25$^-$ effector:CTLA-4$^+$ Treg ratio in PCa patients pre- and post-vaccination with PSA-TRICOM. Results indicate that a high effector:CTLA-4$^+$ Treg ratio post-vaccination is associated with longer survival in most patients, and that a low effector:CTLA-4$^+$ Treg ratio post-vaccination is associated with shorter survival.

Conclusion

This study investigated the level and functional activity of Tregs in mCRPC patients treated with PSA-TRICOM vaccine. It is the first report of a trend in correlation of Treg function and OS in mCRPC patients treated with a PCa vaccine. Results suggest that measuring Treg function may provide a useful parameter for monitoring PCa patients pre- and post-vaccination, and that measuring the frequency of CTLA-4-expressing Tregs may help to further characterize Tregs. Additional vaccine trials will be required to correlate expression of CTLA-4 in Tregs with patient survival. Overall, results suggest that protocols that combine Treg depletion strategies and vaccines may provide effective immunotherapy for PCa. These hypothesis-generating findings provide the impetus for larger validation studies looking at the prospective correlation of Treg function and clinical outcomes with PSA-TRICOM and other vaccines.

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