

# Homeostasis of peripheral FoxP3<sup>+</sup> CD4<sup>+</sup> regulatory T cells in patients with early and late stage breast cancer

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**Abstract** FoxP3<sup>+</sup> CD4<sup>+</sup> regulatory T cells (Tregs) are important mediators of peripheral immune tolerance, acting via multiple mechanisms to suppress cellular immunity including antitumor responses. Although therapeutic strategies have been proposed to deplete Tregs in patients with breast cancer and other malignancies, dynamic changes in the Treg compartment as a function of stage and treatment of breast cancer remain poorly understood. Here, we evaluated peripheral blood CD4<sup>+</sup> T cells and FoxP3<sup>+</sup> CD4<sup>+</sup> T cells from 45 patients with early or late stage breast cancer

and compared percentages, absolute counts, and Treg function to those from healthy volunteers (HV) of comparable age. Patients having completed adjuvant chemotherapy and patients with metastatic cancer exhibited significantly lower absolute CD4 counts and significantly higher percentages of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells. In contrast, the absolute counts of circulating FoxP3<sup>+</sup> CD4<sup>+</sup> T cells did not differ significantly among early stage patients, late stage patients, or HV. Functionally, FoxP3<sup>+</sup> CD4<sup>+</sup> T cells from all donor groups similarly expressed CTLA-4 and failed to secrete IFN- $\gamma$  in response to stimulation. Thus, although Tregs comprise an increased percentage of circulating CD4<sup>+</sup> T cells in patients with metastatic breast cancer and patients in remission after completing the adjuvant chemotherapy, the systemic Treg pool, as measured by absolute counts, appears relatively constant regardless of disease stage or treatment status. Total CD4<sup>+</sup> T cell counts are not constant, however, suggesting that homeostatic mechanisms, or susceptibility to cytotoxic or malignant insults, fundamentally differ for regulatory and non-regulatory CD4<sup>+</sup> T cells.

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## Introduction

Regulatory T cells (Tregs) represent an important CD4<sup>+</sup> T cell subset that contributes to the maintenance of immunological self-tolerance and comprises about 5% of CD4<sup>+</sup> T cells in the peripheral blood of humans [1–4]. Tregs inhibit autoimmune reactions, impede antitumor immunity, and prevent the expansion of other T cells in vivo [1–4]. In particular, human Tregs markedly inhibit CD8 T cell activation [5, 6], and contribute to immune dysfunction in cancer

patients [7–10]. The forkhead/winged-helix transcription factor FoxP3 has been shown to control the development and function of Tregs in mice [4], and mutation of FoxP3 leads to fatal lymphoproliferative disease in mice [11] and profound and life-threatening autoimmunity in humans [12]. FoxP3 expression in humans is not entirely specific for Tregs (e.g., certain activated T cells transiently express FoxP3 [13–15]), but is generally considered superior to CD25 as a marker for Tregs, because some FoxP3<sup>+</sup> CD4<sup>+</sup> T cells in humans are CD25<sup>neg</sup>.

Tregs play a critical role in the inhibition of tumor immune surveillance. In mice, the depletion of Tregs in vivo enhances tumor immunity in mouse models of cancer [16, 17]. Low-dose cyclophosphamide, for example, potentiates the antitumor effects of therapeutic cancer vaccines in mice with breast carcinoma by selectively deleting or inhibiting the cycling population of Tregs [18]. In another strategy, injection of mice with CD25 antibody depletes animals of CD25<sup>+</sup> T cells and markedly enhances response to vaccination [19–21]. In humans, it has been widely reported that there is an increased pool of Tregs in the peripheral blood of cancer patients, prompting attempts to deplete Tregs in cancer patients in vivo to augment immune surveillance or immunotherapy [22–28]. Nevertheless, many of the preclinical studies have been limited by the exclusive use of percentages (rather than absolute counts), the use of CD25 rather than FoxP3 to identify Tregs, or comparisons to healthy volunteers of younger age. Overall, in humans, dynamic changes in the Treg compartment as a function of stage and treatment of disease remain poorly understood, potentially hindering the optimal design of cancer immunotherapy.

In this study, we evaluated peripheral blood Tregs from patients with early or late stage breast cancer and compared the results to healthy volunteers of comparable age. We focused on breast cancer because Tregs exhibit potent

immunosuppressive functions and are known to infiltrate primary tumors and draining lymph nodes [8, 9]. Moreover, it has been demonstrated that within primary breast tumors, higher numbers of Tregs are associated with a poor prognosis [29, 30]. Indeed, the ineffectiveness of numerous immunotherapy strategies in breast cancer may be related in part to the negative effects of Tregs. We, therefore, assessed the percentages, absolute counts, and function of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells in the peripheral blood of 45 patients and identified the dynamic changes in the Treg and non-Treg compartments as a function of stage and treatment.

## Materials and methods

### Subjects and samples

Peripheral blood was obtained after signed, informed consent using protocols approved by Institutional Review Board of the Hospital of the University of Pennsylvania or the Philadelphia Veterans Affairs Medical Center. Blood was obtained from four groups of donors: (1) healthy volunteers (HV) ( $n = 14$ ); (2) patients with stages I–III breast cancer in remission who had completed initial surgery and were about to begin adjuvant chemotherapy at the time of phlebotomy ( $n = 11$ ); (3) stages I–III patients in remission who had completed adjuvant chemotherapy within the past 3 months ( $n = 11$ ); and (4) stage IV patients with active disease and who had not received chemotherapy for at least 30 days prior to phlebotomy ( $n = 23$ ). Subject characteristics are described in Supplemental Table 1. The mean ( $\pm$ SD) age of all subjects was  $52 \pm 12$  years, and there was no significant difference in age among groups ( $P = 0.18$ ).

All adjuvant patients (groups 2, 3) received combination chemotherapy as determined by their primary oncologist. All but one patient in groups 2 and 3 had stages II or III

**Table 1** Comparative analysis of T cells from healthy volunteers and patients with breast cancer

	Healthy volunteers $N = 14$		Patients with breast cancer						ANOVA	
			Adjuvant pre-chemotherapy $N = 11$		Adjuvant post-chemotherapy $N = 11$		Metastatic disease $N = 23$		$P$	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Absolute lymphocyte count <sup>a</sup>	1,956	521	1,857	1,027	1,073	397	1,173	407	0.0003	
Percent of CD4 <sup>+</sup> among lymphocytes	40.33	13.20	43.60	10.55	37.53	16.64	37.02	12.35	0.55	
Absolute CD4 <sup>+</sup> count	786	372	786	518	407	233	427	195	0.0019	
Percent FoxP3 <sup>+</sup> among CD4 <sup>+</sup> T cells	3.22	1.47	3.24	1.70	5.62	2.04	6.57	1.94	<0.0001	
Absolute FoxP3 <sup>+</sup> CD4 <sup>+</sup> T cell count	22.8	12.8	25.1	17.7	23.8	18.7	27.5	13.4	0.84	
Percent CD25 <sup>+</sup> FoxP3 <sup>+</sup> among CD4 <sup>+</sup> T cells	1.49	0.82	1.20	0.98	1.90	1.49	3.67	1.38	<0.0001	
Absolute CD25 <sup>+</sup> FoxP3 <sup>+</sup> CD4 <sup>+</sup> T cell count	11.2	5.2	9.7	10.6	10.4	11.1	16.0	9.6	0.21	

<sup>a</sup> Absolute counts expressed as cells/ $\mu$ l

breast cancer. The remaining patient had a 1.7 cm primary tumor that was negative for hormone receptor and HER2/neu expression without lymph node involvement. Adjuvant chemotherapy was not homogenous, but all patients received regimens that are standard of care. In the post-chemotherapy group (group 3), a majority of these patients (7/11, 64%) received doxorubicin plus cyclophosphamide followed by a taxane [31] while the others (4/11, 36%) received taxane-based regimens without anthracyclines [32]. In group 3, phlebotomy was performed at a median of 29 days after the completion of chemotherapy (range 0–90 days); two of these patients had initiated radiation therapy prior to the time of phlebotomy. Absolute lymphocyte count (ALC) was obtained from a complete blood count and differential. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden).

#### Monoclonal antibodies

Fluorochrome-conjugated mAb used were PE-Cy7-CD3 clone SK7, FITC- or APC-CD4 clone RPA-T4, PerCP-CD4 clone SK3, PE-CD25 clone M-A251 or 4E3, PerCP- or APC- or APC-Cy7-CD14 clone M $\phi$ P9, PerCP-CD19 clone 4G7, PE-CD27 clone M-T271, PE-CD45RA clone HI100, PE-CD45RO clone UCHL1, and PE-CD152 clone BNI3 (BD Biosciences, San Jose, CA); Alexa fluor 488-anti-FoxP3 clone 259D and Alexa fluor 488-conjugated isotype control clone MOPC-21 (Biolegend, San Diego, CA); APC-anti-FoxP3 clone PCH101, APC-anti-IFN- $\gamma$  clone 4S.B3, and APC-conjugated isotype control clone MOPC-21 (eBioscience, San Diego, CA); and PE-CCR7 clone 150503 (R&D Systems, Minneapolis, NM).

#### Flow-cytometric analysis

Peripheral blood mononuclear cells in PBS with 5% heat-inactivated fetal calf serum were then stained for the surface markers CD3 (PE-Cy7), CD4 (APC), CD14 (PerCP), and CD25 (PE), followed by intracellular staining for FoxP3 (clones PCH101 or 259D) using a fixation/permeabilization kit (eBioscience), according to the manufacturer's instructions. Cells were washed twice in staining buffer and analyzed immediately using a FACSCanto cytometer and FACSDiva software (BD Biosciences). Quadrants and box gates were set in reference to isotype controls, and percentages of the parent CD4<sup>+</sup> population were calculated.

#### Intracellular cytokine assay

Peripheral blood mononuclear cells in complete media comprising RPMI 1640 (Invitrogen) supplemented with

10% heat-inactivated human AB serum, 20 mM HEPES (Sigma-Aldrich), and 2 mM L-glutamine (Invitrogen) were stimulated with or without phorbol 12-myristate 13-acetate (PMA) (5 ng/ml) and ionomycin (1  $\mu$ M) (Sigma, St. Louis, MO) for 4 h in the presence of 1  $\mu$ M monensin (BD Biosciences). PBMC were then washed twice, re-suspended in staining buffer and stained for the surface markers CD3, CD4, CD14, and CD25 followed by intracellular staining for FoxP3 and IFN- $\gamma$  using a fixation/permeabilization kit (eBioscience), according to the manufacturer's instructions. PBMC were washed twice in staining buffer and analyzed immediately by flow cytometry.

#### Treg suppressive assay

CD25<sup>high</sup> CD4<sup>+</sup> T cells were isolated using a Treg isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) from peripheral blood of patients or healthy volunteers. The number of Tregs quantified for the immunosuppressive assay was based on the total number of isolated cells, 70–80% of which expressed FOXP3 as determined by post-sort flow cytometry. The responder cells were total PBMC isolated from a healthy volunteer. Tregs (1.0  $\times$  10<sup>6</sup> per ml) were co-cultured with responder PBMC (1.0  $\times$  10<sup>6</sup> per ml) labeled with 5  $\mu$ M carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Carlsbad, CA) in the presence of 1.3 CD3-conjugated M-450 tosylactivated Dynabeads (DynaL Biotech, Hamburg, Germany) per PBMC for 4 days, as previously described [33]. CD8<sup>+</sup> T cell proliferation was determined by dilution of CFSE among labeled cells compared with control PBMCs cultured without Tregs.

#### Statistical analysis

Analysis of variance (ANOVA) was employed to compare the mean values of a variable among the four blood donor groups (e.g., healthy volunteers, stages I–III breast cancer patients prior to adjuvant chemotherapy, stages I–III breast cancer patients after adjuvant chemotherapy, and breast cancer patients with metastatic disease). Once a significant difference among the donor groups was established, pairwise post hoc comparisons were performed using Scheffé's test at a  $P < 0.05$  significance level, which controls for multiple paired testing. The magnitude of linear correlation between two variables was assessed by Pearson's correlation coefficient. Prior to testing, the normality assumption was assessed for each variable using a histogram and normal probability plot. If a variable was not considered to be normally distributed, then a natural log transformation was applied and the normality assessment was repeated. To control the overall type-I error rate, the Benjamini–Hochberg

procedure [34] for multiple testing was employed. Taking into consideration the total number of ANOVA tests performed,  $P < 0.03$  was considered to be statistically significant. All statistical tests were two-sided and were performed using STATA v10.0 software (StataCorp).

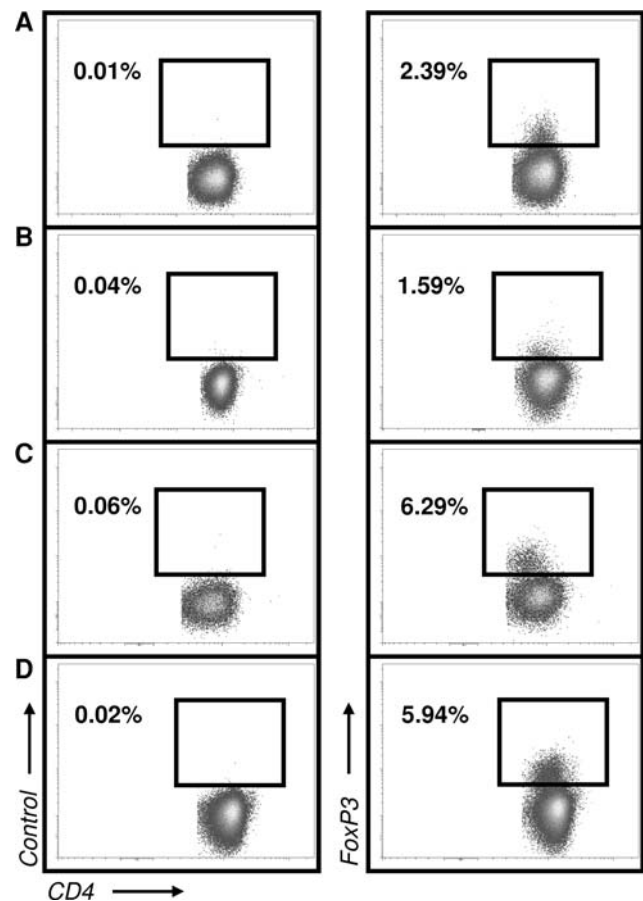
## Results

### FoxP3<sup>+</sup> CD4<sup>+</sup> T cells in healthy volunteers and patients with breast cancer

To explore the hypothesis that Treg homeostasis is altered in patients with breast cancer, we used multiparameter flow cytometry to evaluate T cells in unmanipulated PBMC from donors in each of four donor groups: (1) healthy volunteers (HV) ( $n = 14$ ); (2) patients with stages I–III breast cancer immediately prior to the initiation of adjuvant chemotherapy ( $n = 11$ ); (3) stages I–III patients in remission who had completed adjuvant chemotherapy ( $n = 11$ ); and (4) stage IV patients with active disease. Subject characteristics are described in Supplemental Table 1 and further detailed in “Materials and methods”. Peripheral blood FoxP3<sup>+</sup> CD4<sup>+</sup> T cells were identified for all donors in each group (Fig. 1). Two monoclonal antibodies (mAb) were used to evaluate FoxP3 expression in light of recent controversy in this area [35, 36], but the pattern of FoxP3 expression and the percent positivity was nearly identical when comparing the anti-FoxP3 mAb clone PCH101 versus 259D (data not shown).

To further characterize and compare FoxP3<sup>+</sup> CD4<sup>+</sup> T cells in healthy volunteers and patients, we then analyzed FoxP3<sup>+</sup> CD4<sup>+</sup> T cells for expression of CTLA4, CD45RO, CD27, CD45RA, and CCR7. We found no significant differences in the phenotype of CD4<sup>+</sup> FoxP3<sup>+</sup> T cells among the four donor types (Fig. 2). In particular, the expression of CTLA4, which has been implicated in mediating function of Tregs in humans [37], was similarly positive in each case.

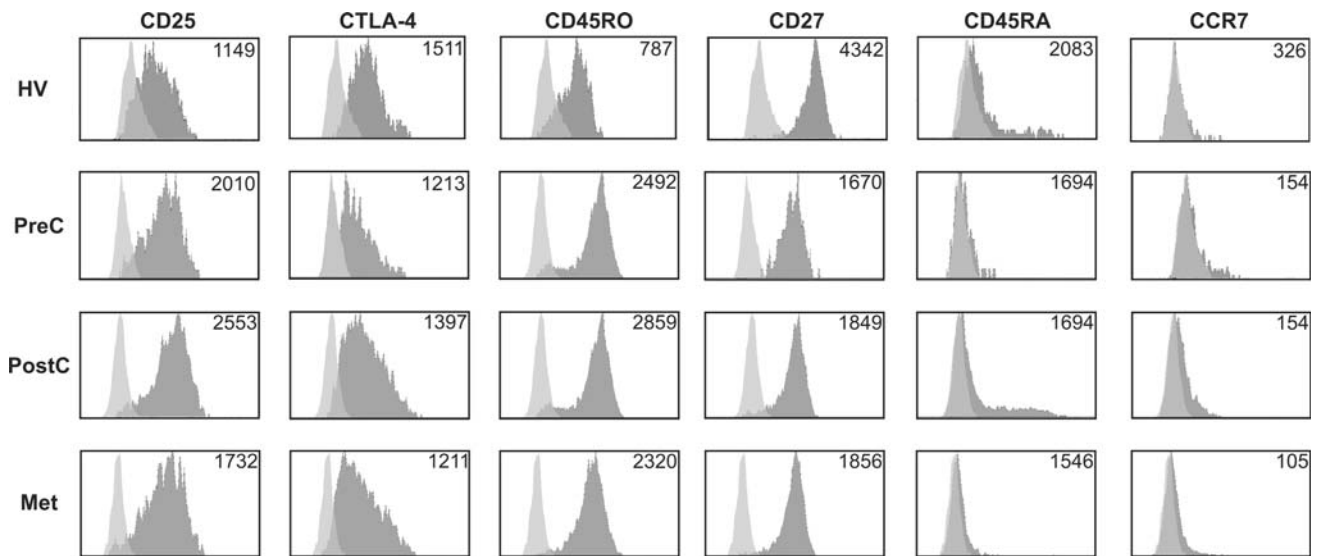
To evaluate the functional status of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells, we first measured IFN- $\gamma$  secretion from FoxP3<sup>+</sup> and FoxP3<sup>neg</sup> CD4<sup>+</sup> T cells following *in vitro* stimulation with PMA/ionomycin. The failure to produce effector cytokines after mitogenic stimulation is an intrinsic property of Treg populations, and as has been shown recently by other investigators [38, 39], can be measured in an assay that evaluates total FoxP3<sup>+</sup> CD4<sup>+</sup> T cells using minimal numbers of cells rather than just the CD25-expressing subset of CD4<sup>+</sup> T cells isolated from a large number of cells. We found that for all donor groups tested, FoxP3<sup>neg</sup> CD4<sup>+</sup> T cells included a large subset of cells able to secrete IFN- $\gamma$  (no significant difference among donor groups), but FoxP3<sup>+</sup> CD4<sup>+</sup> T cells



**Fig. 1** FoxP3 expression on peripheral blood CD4<sup>+</sup> T cells. PBMC were analyzed by flow cytometry and gated on CD3<sup>+</sup>, CD4<sup>+</sup>, and CD14<sup>neg</sup> events. Boxes indicate percentages of CD4<sup>+</sup> population and drawn according to isotype control. **a** Healthy volunteer, **b** early stage patient having completed initial surgery and about to begin adjuvant chemotherapy, **c** early stage patient having completed adjuvant chemotherapy, **d** stage IV patient having not received chemotherapy for 30 days. Data shown are representative of 14 healthy volunteers, 11 pre-chemotherapy patients, 11 post-chemotherapy patients, and 23 stage IV patients

did not secrete IFN- $\gamma$  (Fig. 3). These observations are consistent with the expected anergic properties recognized as a general phenomenon of Tregs [9, 38, 39].

We then directly measured functional suppressive activity of Tregs by isolating viable CD25-expressing CD4<sup>+</sup> T cells from donors and coculturing these cells with CD3-stimulated responder T cells. Although this assay is limited because not all FoxP3<sup>+</sup> CD4<sup>+</sup> T cells express CD25 and a measurable fraction of non-regulatory (FoxP3<sup>neg</sup>) T cells do express CD25, FoxP3 expression is mostly confined to CD25<sup>high</sup> cells in humans [39]. (It was not possible to isolate FoxP3-expressing CD4<sup>+</sup> T cells for this assay because cellular permeabilization and fixation required to detect intracellular FoxP3 kills the cells.) We found that Tregs isolated from either normal donors or patients with metastatic disease suppressed



**Fig. 2** Expression of surface and intracellular markers on FoxP3<sup>+</sup> CD4<sup>+</sup> T cells from healthy volunteers and patients. Peripheral blood from healthy volunteers (HV;  $n = 3$ ), stages I–III patients having completed initial surgery and about to begin adjuvant chemotherapy (PreC;  $n = 2$ ), stages I–III patients having completed adjuvant chemotherapy during the past 3 months (postC;  $n = 2$ ), and stage IV patients having not received chemotherapy for at least 30 days (Met;  $n = 2$ ) was eval-

uated via flow cytometry for the expression of CD25, CTLA-4, CD45RO, CD27, CD45RA and CCR7. Cells were gated on FoxP3<sup>+</sup> CD4<sup>+</sup> T cells. Light gray histograms represent isotype controls; dark gray histograms represent experimental results. Mean fluorescence intensity after background subtraction is indicated in the upper right corner

CD3-mediated proliferation of normal CD8<sup>+</sup> T cells (Supplemental Fig. 1), consistent with the known function of Tregs. We did not have sufficient cells from enough patients to explore if patient Tregs suppressed more potently than healthy volunteer Tregs.

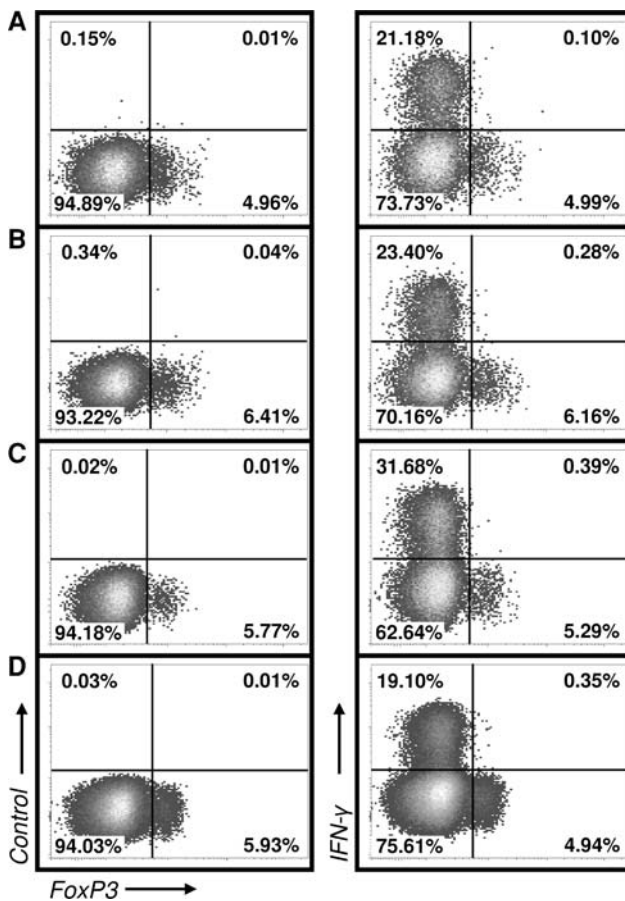
#### Differences in the percentage, but not absolute counts of FoxP3 cells

Five parameters were then compared among the four donor groups: (1) ALC, (2) the percentage of CD4<sup>+</sup> T cells among all lymphocytes, (3) the absolute count of CD4<sup>+</sup> T cells (cells/ $\mu$ l), (4) the percentage of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells among all CD4<sup>+</sup> T cells and (5) the absolute counts of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells (Table 1; Fig. 4). For ALC, there was a highly significant difference among donor groups ( $P = 0.0003$ , Fig. 4a). There was no significant difference between healthy volunteers and patients pre-chemotherapy, but ALC was significantly lower both for patients post-chemotherapy and those with metastatic disease ( $P < 0.05$ ). For the percentage of CD4<sup>+</sup> T cells, there was no significant difference among groups ( $P = 0.55$ , Fig. 4b), but because of changes in the ALC, the average absolute count of CD4<sup>+</sup> T cells was significantly different among donor groups ( $P = 0.0019$ , Fig. 4c). In patients with metastatic disease, the average count was significantly lower than in healthy or patients pre-chemotherapy ( $P < 0.05$ ). In addition, there was a trend toward lower CD4<sup>+</sup> T cell counts in patients post-

chemotherapy. Indeed, CD4<sup>+</sup> counts were  $<200$  cells/ $\mu$ l, which defines AIDS in HIV-infected persons, in 27% of patients post-chemotherapy and 22% of patients with metastatic disease compared with 0% of healthy individuals or patients pre-chemotherapy. Overall, these results suggest that after chemotherapy and/or in the context of metastatic tumor burden, breast cancer patients can be relatively lymphopenic and moderately to severely compromised with regard to CD4<sup>+</sup> T cell numbers.

In contrast to the percentage of total CD4<sup>+</sup> T cells, which did not differ among groups, the percentage of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells among all CD4<sup>+</sup> T cells was highly significantly different among donor groups ( $P < 0.0001$ , Fig. 4d). It was higher in patients post-chemotherapy ( $P < 0.05$ ) and in patients with metastatic disease ( $P < 0.001$ ) compared with either healthy volunteers or patients pre-chemotherapy. The absolute number of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells, however, was not significantly different among donor groups ( $P = 0.84$ , Fig. 4e). This steady state of FoxP3<sup>+</sup> CD4<sup>+</sup> T cell numbers appeared to be accomplished by an inverse correlation of percent FoxP3<sup>+</sup> CD4<sup>+</sup> and ALC; that is, an elevated percentage of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells significantly correlated with a lower ALC ( $r = -0.405$ ,  $P = 0.002$ ). Thus, in contrast to CD4<sup>+</sup> T cell counts which are relatively lower in patients after chemotherapy or patients with metastatic disease, counts of FoxP3<sup>+</sup> CD4<sup>+</sup> are similar among donor groups despite the important differences in treatment history and tumor burden.





**Fig. 3** Functional analysis of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells. Cells were stimulated with PMA/ionomycin in the presence of monensin and stained for surface markers followed by intracellular staining for FoxP3 and IFN- $\gamma$ . PBMC gated were CD3<sup>+</sup>, CD4<sup>+</sup>, and CD14<sup>neg</sup> events. *Quadrants* show percentages of the CD4<sup>+</sup> gate and are drawn according to isotype controls. **a** Healthy volunteer, **b** early stage patient having completed initial surgery and about to begin adjuvant chemotherapy, **c** early stage patient having completed adjuvant chemotherapy, **d** stage IV patient having not received chemotherapy for 30 days. Representative plots of three subjects from each donor group

CD25<sup>+</sup> FoxP3<sup>+</sup> CD4<sup>+</sup> T cell subset shows similar pattern as total FoxP3<sup>+</sup> CD4<sup>+</sup> T cells

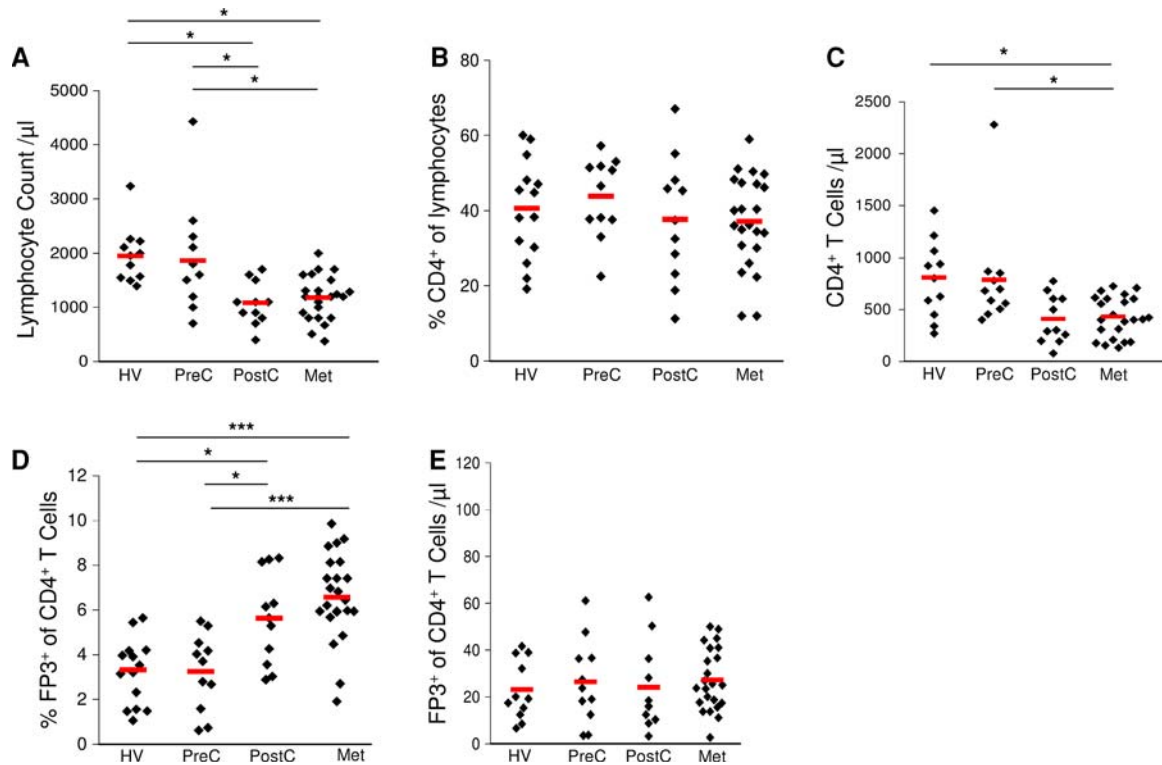
We also evaluated whether the patterns we observed for FoxP3<sup>+</sup> CD4<sup>+</sup> T cell homeostasis among the four donor groups also applied to the CD25<sup>+</sup> subset of Treg. In humans, not all FoxP3<sup>+</sup> CD4<sup>+</sup> T cells express CD25, and it remains unclear whether these cells differ functionally from CD25<sup>low</sup> CD4<sup>+</sup> FoxP3<sup>+</sup> T cells in vivo in humans. We found, using multi-parameter flow cytometry, that CD25<sup>bright</sup> FoxP3<sup>+</sup> CD4<sup>+</sup> T cells were readily detected in donors from each group (Fig. 5a) and that this subpopulation was equally anergic as total FoxP3<sup>+</sup> CD4<sup>+</sup> T cells with regard to mitogen-induced IFN- $\gamma$  secretion (data not shown). The percentage of CD25<sup>bright</sup> FoxP3<sup>+</sup> CD4<sup>+</sup> T cells was highly significantly different among donor groups

( $P < 0.0001$ , Table 1; Fig. 5b). The percentage was higher in patients with metastatic breast cancer when compared with healthy volunteers ( $P < 0.001$ ) or patients either pre-chemotherapy ( $P < 0.001$ ) or post-chemotherapy ( $P = 0.003$ ); however, similar to that for total FoxP3<sup>+</sup> CD4<sup>+</sup> T cells, there was no significant difference in the absolute count of CD25<sup>bright</sup> FoxP3<sup>+</sup> CD4<sup>+</sup> T cells among donor groups ( $P = 0.21$ , Fig. 5c).

## Discussion

It is now generally accepted that Tregs frustrate productive tumor immune surveillance and represent a major obstacle for the successful development of cancer immunotherapy. In patients with breast cancer, increased Treg infiltration of primary tumors independently predicts a worse clinical course [29, 30]. Ex vivo, Tregs isolated from patients with breast cancer inhibit T cell proliferation [8, 9, 40]. In mice genetically engineered to develop invasive breast cancer, therapeutic elimination of Tregs markedly improves the efficacy and curative potential of tumor vaccination [18], further underscoring the importance of Treg biology in the natural history and therapy of breast and other cancers. In this study, we have determined the relative and absolute numbers of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells in the peripheral blood of patients with early or late stage breast cancer compared with healthy volunteers to understand the dynamic changes in the Treg compartment as a function of stage and treatment. Although the percentage of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells among all CD4<sup>+</sup> T cells was significantly higher in patients with metastatic breast cancer and those with stages I–III breast cancer who had completed chemotherapy, the absolute counts of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells in peripheral blood were stable in all donor groups evaluated, despite the variations in total absolute CD4 counts. Similar results were obtained for the CD25<sup>+</sup> subset of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells. Overall, these findings demonstrate that the circulating Treg pool as measured by absolute counts is no higher in patients than in healthy individuals, and that a homeostatic mechanism appears to govern a stable absolute count of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells regardless of disease stage or treatment status.

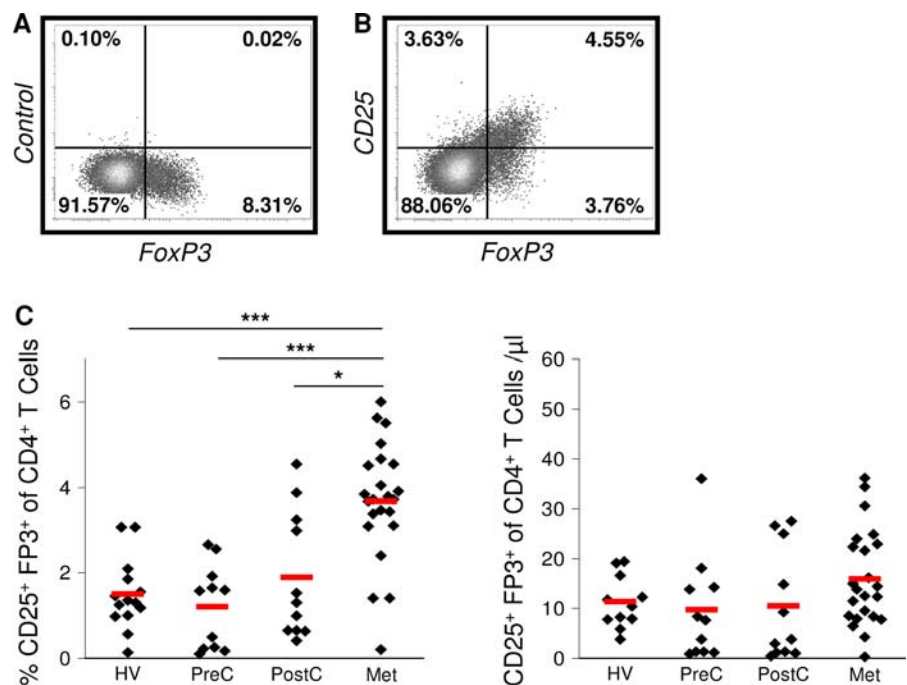
Several prior studies have examined circulating Tregs in cancer patients by exclusively employing percentages and not absolute counts of Tregs in blood, using CD25 rather than FoxP3 to identify Tregs, or performing comparisons to normal donors of unstated ages. The latter is particularly important because Treg prevalence appears to increase with age in both mice and humans [41–43], which might confound analyses if adult cancer patients who are generally older are compared with samples from younger blood donors. In this study, we calculated both percentages and absolute counts of Tregs, using FoxP3 as the primary Treg



**Fig. 4** FoxP3<sup>+</sup> CD4<sup>+</sup> T cells in healthy volunteers and breast cancer patients. Peripheral blood was evaluated from healthy volunteers (HV; 14), stages I–III patients having completed initial surgery and about to begin adjuvant chemotherapy (PreC; *n* = 11), stages I–III patients having completed adjuvant chemotherapy during the past 3 months (postC; *n* = 11), and stage IV patients having not received chemother-

apy for at least 30 days (Met; *n* = 23). **a** lymphocyte count per  $\mu$ l of blood, **b** percent CD4<sup>+</sup> T cells of total lymphocytes, **c** number of CD4<sup>+</sup> T cells per  $\mu$ l of blood, **d** percentage of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells among total CD4<sup>+</sup> T cells, **e** number of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells per  $\mu$ l of blood. Each symbol represents one donor, and bars represent means. \**P* < 0.05 for the comparison shown; \*\*\**P* < 0.001

**Fig. 5** FoxP3 and CD25 expression on peripheral blood CD4<sup>+</sup> T cells. **a** PBMC from a patient with stage IV disease were analyzed by flow cytometry and gated on CD3<sup>+</sup>, CD4<sup>+</sup>, and CD14<sup>neg</sup> events. Quadrants show percentages of the CD4<sup>+</sup> gate, **b** percentage of CD25<sup>+</sup> FoxP3<sup>+</sup> CD4<sup>+</sup> T cells among total CD4<sup>+</sup> T cells for the four donor groups, **c** number of CD25<sup>+</sup> FoxP3<sup>+</sup> CD4<sup>+</sup> T cells per  $\mu$ l of blood. For (b) and (c), each symbol represents one donor, and bars represent means. \**P* < 0.05 for the comparison shown; \*\*\**P* < 0.001



marker, and compared the results to a group of healthy volunteers that was not statistically different in age to three patient groups. Moreover, functional anergy for FoxP3<sup>+</sup> CD4<sup>+</sup> T cells, but not FoxP3<sup>neg</sup> CD4<sup>+</sup> T cells, was demonstrated for cells from each type of donor in this study. Measuring Tregs in peripheral blood, as we have done here, may not optimally reflect the physiology of Tregs at the tissue level, especially within the tumor microenvironment. Immunohistology analysis comparing tumor tissue to peripheral blood may help reveal whether changes in the percentage of Tregs in PBMC has an influence on the composition of tumor infiltrate. However, in this study, we did not evaluate intratumoral Tregs either because tumor tissue was not available for this purpose or there was no tumor to evaluate (e.g., healthy volunteers or patients in remission after surgery and/or adjuvant chemotherapy).

Although a higher percentage of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells among total CD4<sup>+</sup> T cells in peripheral blood of patient with metastatic breast cancer has been previously observed [8, 9], our observation that breast cancer patients after adjuvant chemotherapy (but not before) also exhibit a relatively higher percentage of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells has not been previously reported. This relative increase in FoxP3<sup>+</sup> CD4<sup>+</sup> T cells was not related to tumor burden because all patients were in complete remission. Some of the agents administered to our patients in the adjuvant setting, cyclophosphamide in particular, are considered Treg toxic, even selectively so [18]. However, it does not appear likely from our data that Tregs are depleted systemically by standard adjuvant chemotherapy either in relative or absolute numbers. This notion carries important clinical implications: for example, vaccines and other immunotherapies delivered to patients on completion of adjuvant therapy may need to include strategies aimed expressly at overcoming the immunosuppressive effects of Tregs even though patients achieve minimal residual disease. Overall, we observed reductions in total lymphocytes and in conventional (non-Treg) CD4<sup>+</sup> T cells in patients compared with healthy volunteers, and this might be due to the effects of chemotherapy or tumor burden. Reductions in such cells may also explain the apparent relative increase in Tregs, as measured as a percentage of total CD4<sup>+</sup> T cells. However, when the absolute count of Tregs was measured, we found a relatively constant number within the four donor populations.

Finally, our data suggest that homeostatic mechanisms governing the peripheral blood count of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells fundamentally differ from those governing the total CD4<sup>+</sup> T cell count; such differential regulation would explain, for example, why the average absolute CD4 count in patients with metastatic disease or those post-chemotherapy was lower than that for healthy volunteers, yet the absolute count of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells was not. Regulatory and non-regulatory T cells might be regulated differentially

by cytokines such as IL-2 or exhibit differential susceptibility to effects of chemotherapy or macroscopic tumor burden. Although our data do not allow us to distinguish among these possibilities, the manifestation of this physiology is a circulating concentration of Tregs that is no higher in early or late stage breast cancer patients than in healthy individuals. In future studies, it would be interesting to perform a comprehensive serum cytokine analysis to explore this hypothesis; however, such an analysis may be revealing only at the microenvironmental level. An alternative approach to explore the role of cytokines in Treg modulation would be to deliver in vivo monoclonal antibody that blocks cytokines or their receptors. Along these lines, we have initiated a clinical trial to deliver anti-CD25 monoclonal antibody to patients (in order to block IL-2) and subsequently measure effects on Tregs in vivo. In preliminary data recently reported [44], we have found that treatment with anti-CD25 monoclonal antibody results in a protracted loss of Tregs from peripheral blood of patients with stage IV breast cancer. Studies to investigate the mechanism of this effect (IL-2 deprivation vs. antibody-mediated cytotoxicity vs. other) are ongoing.

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