Immunomodulation of FOXP3$^+$ Regulatory T Cells by the Aromatase Inhibitor Letrozole in Breast Cancer Patients

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Abstract

Purpose: We have shown previously that tumor infiltration by FOXP3$^+$ regulatory T cells (Treg) is associated with increased relapse and shorter survival of patients with both *in situ* and invasive breast cancer. Because estrogen regulates Treg numbers in mice and promotes the proliferation of human Tregs, we hypothesized that blocking estrogen receptor-α signaling would abrogate Tregs and be associated with response to hormonal therapy and increased survival.

Experimental Design: FOXP3$^+$ Tregs were quantified in tumor samples collected at baseline by incisional biopsy and after 6 months at definitive surgery in 83 elderly breast cancer patients ($T_{2\text{-}4}$ $N_{0\text{-}1}$) enrolled in a randomized phase II trial based on 6 months of primary letrozole (2.5 mg/d) or 6 months of letrozole plus oral "metronomic" cyclophosphamide (50 mg/d).

Results: Treg number ranged from 0 to 380 (median, 30) before treatment and from 0 to 300 (median, 8) after treatment. There was a significant reduction in Tregs in letrozole and letrozole-cyclophosphamide patients ($P < 0.0001$ and $P < 0.002$, respectively) after treatment. Treg number at residual histology was inversely related with response ($P < 0.03$ and $P = 0.50$, respectively) and a greater Treg reduction was observed in responding patients ($P < 0.03$).

Conclusion: This study suggests that aromatase inhibitors may have an indirect antitumor mechanism of action through reducing Tregs in breast tumors and may be of use in estrogen receptor-α-negative tumors in combination with immunotherapy approaches.

Cancer is rarely suppressed by the host immunoresponse even in the presence of high numbers of tumor-specific T cells. During the neoplastic process, tumor cells acquire immunotolerance and thereby evade tumor immunity through several specific immune evasion strategies such as the secretion of tumor-derived immunosuppressive factors (e.g., interleukin-10 and transforming growth factor-β; ref. 1), tumor-derived inhibitory factors (e.g., prostaglandin E_2; ref. 2), loss of MHC class I expression, or the loss of expression of tumor-specific antigens. In most cases, tumor-reactive T cells fail to eliminate tumor cells because they are maintained in an unresponsive state or the tumor microenvironment is not conducive to their function.

The failure of an anticancer immunoresponse may also reflect the presence of a specific subpopulation of CD4$^+$CD25$^+$ regulatory T cells (Treg), whose function is to protect against autoimmunity. In healthy humans, this population accounts for 5% to 10% of peripheral CD4$^+$ T cells. Recent investigations have clarified the *in vivo* behavior and functions of these cells. Tregs down-regulate the activation and expansion of self-reactive lymphocytes and are crucial for the repression of autoimmune disorders and transplant rejection. Although the role of Tregs in cancer has not been fully elucidated, in some malignancies, these cells are likely to be responsible for maintaining the self-tolerance that may hinder the generation and activity of antitumor reactive T cells.

Experimental murine tumor models have shown that Tregs are potent inhibitors of an antitumor immunoresponse (reviewed by ref. 3). In carcinoma patients, an accumulation of Tregs in the peripheral blood, in the tumor-draining lymph nodes, and in the primary tumor itself (4), has been observed, supporting a role for Tregs in cancer-induced immunosuppression. More recently, we have shown that tumor infiltration by Tregs is associated with the reduced survival of breast carcinoma patients (5).

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Translational Relevance

We have shown recently that high numbers of Tregs in *in situ* and invasive breast carcinomas give independent prognostic information even beyond 5 years when conventional pathologic factors lose their power. Because Tregs play a pivotal role in immunosuppression and tumor emergence, we performed a phase II randomized controlled trial of letrozole ± the immunomodulatory agent cyclophosphamide in breast cancer patients to assess the clinical utility of the number of Tregs in predicting the response to therapy. This question addresses second of the top 15 breast cancer research priorities determined by registrants at the San Antonio Breast and St Gallen meetings in 2005. We show a significant reduction in the numbers of Tregs in the primary tumor after treatment with an aromatase inhibitor but not by the addition of cyclophosphamide, showing that the effect is largely due to the aromatase inhibitor. Importantly, this reduction in Tregs is inversely related to the response. These novel findings suggest that letrozole has a significant immunomodulatory role and that aromatase inhibitors could be used in the future in combination with other immunotherapeutic approaches not only in patients with ER-positive but also in ER-negative tumors.

In view of these data, many groups are investigating methods of reducing Treg numbers or blocking their activity within the tumor microenvironment, with the aim of enabling an effective antitumor immunoresponse in an otherwise nonresponding host by activating tumor-specific and nonspecific effector cells (6). It has been shown in both mice and humans that estrogen is able to promote immunotolerance by expanding the Treg compartment (7, 8). Thus, we hypothesized that aromatase inhibitors may derive a proportion of their therapeutic benefit via inhibiting the estrogenic effects on Treg numbers and activity in addition to depriving the tumor cells of their hormonal proliferative stimulus. Furthermore, hypoxia (9) and vascular endothelial growth factor (VEGF; ref. 10) may also inhibit T-cell development and may contribute to tumor-induced immunosuppression. As VEGF blockade has been reported to reduce intratumoral Tregs (11), we also hypothesized that abrogation of this angiogenic factor may also target Treg-mediated immunomodulation in breast cancer patients.

To ask the question whether Treg numbers are modulated by estrogen and/or metronomic cyclophosphamide, we used a primary systemic therapy breast cancer model where tumor biopsy specimens are obtained at both diagnosis (baseline sample) and definitive surgery to provide information on the interaction between biological markers and treatment. This approach allows the efficacy of aromatase inhibitors on Treg number and patient response together with tumoral VEGF expression to be explored. We have recently conducted a randomized phase II trial aimed to investigate the activity of the addition of low-dose metronomic oral cyclophosphamide to letrozole as an antiangiogenic therapy (12). Because low doses of oral cyclophosphamide also have a known immunomodulatory role including actions on Tregs, in this study, Treg numbers were evaluated in tumor specimens taken at baseline and after patients were treated with letrozole or letrozole-cyclophosphamide to enable testing for synergistic effects with letrozole. The primary aim of the study was to test for changes in Treg numbers in patients from the baseline number according to treatment allocation. Secondary aims were (*a*) to correlate Treg numbers with baseline conventional prognostic and predictive parameters, (*b*) to explore the potential of Treg numbers as a biomarker predicting tumor response, and (*c*) to explore the association between changes in Treg numbers and tumor response.

Patients and Methods

Patients. Patients included in this study were 114 elderly women (ages >70 years) or unfit for chemotherapy with T_{2-4} N_{0-1} and estrogen receptor (ER)-positive breast cancer, prospectively enrolled between November 2000 and January 2004 in a single-center, randomized, phase II trial of letrozole arm and letrozole plus metronomic cyclophosphamide arm (12). Eligibility criteria included an Eastern Cooperative Oncology Group performance status of ≤2, adequate bone marrow reserve (WBC count, >3.5 × 10^9/L; platelets, >100 × 10^9/L; hemoglobin, >10 g/dL), hepatic function (aspartate aminotransferase/alanine aminotransferase bilirubin and alkaline phosphatase levels <1.25 times the upper limit of normal value), and renal function (serum creatinine <1.25 the upper limit of normal value). Patients with nonmalignant systemic disease that precluded them from receiving study therapy and patients with second primary malignancies were not eligible. The study was approved by the local ethical committee (file no. RaLCTrVs1Ott_2000). Written informed consent was obtained from all patients before randomization.

Fifty-seven patients in the letrozole arm received 2.5 mg/d (1 tablet) letrozole (Femara; Novartis), whereas 57 patients on letrozole-cyclophosphamide arm received 2.5 mg/d letrozole and 50 mg/d (1 tablet) cyclophosphamide (Endoxan; Baxter; ref. 13). These drugs were given continuously for 6 months until definitive surgery.

Treatment evaluation. On first presentation, an incision biopsy was done on each patient and a small tissue sample (0.5-0.8 cm) was removed. Each month, the size of the primary tumor and the size of the axillary lymph nodes, when appreciable, were measured with a caliper by the same clinician. Response was assessed according to the WHO criteria by the clinical measurement of the changes in the product of the two largest diameters recorded in two successive evaluations. Pathologic complete response was defined as the absence of neoplastic cells in the breast and in the axillary lymph nodes after histologic examination. Surgery was planned after full clinical reassessment. Quadrantectomy or modified radical mastectomy was done when indicated in association with full axillary node dissection. All patients subjected to quadrantectomy underwent irradiation of the residual breast (60 Gy delivered over 6 weeks).

Histopathologic grade and immunohistochemistry. Tumor grade was evaluated using the Nottingham grading system. Immunohistochemical evaluation was done on paraffin-embedded tumor samples obtained at diagnosis and at definitive surgery. Bcl-2, HER-2, ER, progesterone receptor, and Ki-67 staining were done at the Pathology Unit of the Azienda Ospedaliera Istituti Ospitalieri of Cremona is described fully elsewhere (14). VEGF was detected with mouse monoclonal VG1 (dilution 1:2 for 60 min). The production of anti-FOXP3 antibody clone 236A/E7 as a marker of Tregs and its validation on transfected cell lines with positive and negative controls have been published previously (5). Sections were dewaxed in citroclear (HD Supplies) and antigen retrieval was done by microwave pressure-cooking for 3 min at full pressure in 50 mmol/L Tris and 2 mmol/L EDTA (pH 9). Before staining the sections, endogenous peroxidase was blocked, the slides were incubated for 30 min with the primary antibody (neat supernatant for 30 min) and washed with PBS, and the immunodetection was done with using the DAKO EnVision system according to the manufacturer's protocol (DAKOCytomation).

Immunohistochemistry for FOXP3 was done on 5 μm sections of tissue microarrays containing two of 1 mm tumor cores taken from selected morphologically representative tumor regions of each paraffin-embedded breast tumor (n = 121) or on whole sections (n = 42) from both the initial excisional biopsy and the tumor remaining at definitive surgery. Quality control was assessed on each block by H&E staining. Slides were counterstained with hematoxylin and mounted. The number of FOXP3$^+$ Tregs was counted and the highest value used in the analysis.

The clinician who evaluated all clinical responses (A.B.) was blinded to treatment allocation. The pathologists, either in Cremona or in Oxford, were blinded to treatment assignment, patient outcome, and whether the samples they examined were obtained from incisional biopsy or definitive surgery.

Statistical analysis. In a previous study of Tregs in invasive breast cancer, we showed that, using the median Treg number (≥15) to stratify patients into high and low Treg counts, patients with high Treg numbers in their primary tumor showed a significantly worse outcome in a multivariate analysis (15). Therefore, we analyzed Tregs as stratified by this previously defined cut-point when exploring for associations between Treg number and clinicopathologic and molecular variables (Table 1). Nonparametric statistical methods (Mann Whitney U test for unpaired data, Wilcoxon's matched-pairs signed-rank test for paired data, and Spearman ρ for simple correlation analysis) were used for comparing continuous variables. Association of categorized variables was done by χ^2 or χ^2 for trend when indicated. Multiple group comparison for Treg numbers at baseline was done by ANOVA. To take into account a possible confounding effect of baseline Tregs, analysis of covariance was done instead of ANOVA for multiple group comparisons when considering the reduction in Tregs and Tregs in residual tumor. As Treg number after chemotherapy was not normally distributed, a square root transformation was used in the analysis of covariance.

Table 1. Patients' characteristics

	Letrozole, *n* (%)	Letrozole + cyclophosphamide, *n* (%)
Age (y), median (range)	75 (64-89)	74 (67-87)
Progesterone receptor status		
Positive	24 (58.5)	25 (61.0)
Negative	17 (41.5)	16 (39.0)
Not evaluable	1	—
Tumor-node-metastasis		
T_2	30 (71.4)	32 (78.0)
T_{3-4}	12 (28.6)	9 (22.0)
N_0	23 (54.8)	26 (63.4)
N_1	19 (45.2)	15 (36.6)
Primary histology		
Ductal carcinoma	30 (71.4)	47 (82.5)
Lobular carcinoma	12 (28.6)	10 (17.5)
Grading		
2	13 (30.9)	8 (19.5)
3	29 (69.1)	33 (80.5)
Not evaluable	—	1
pER-α positivity	31 (73.8)	28 (87.5)
Not evaluable		9
ER-β positivity	23 (85.2)	16 (84.2)
Not evaluable	15	22
p53 positivity	22 (52.4)	16 (39.0)
Bcl-2 positivity	37 (88.1)	38 (92.7)
c-erbB2 positivity	4 (9.5)	2 (4.9)
Ki-67, mean (95% CI)*	15.8 (12.5-19.1)	17.3 (13.0-21.6)
Treg, mean (95% CI)	62.3 (37.5-87.0)	43.7 (24.9-62.5)
Distribution of Treg		
<15	11 (26.2)	16 (39.0)
>15	31 (73.8)	25 (61.0)

*95% Confidence interval.

Table 2. Contingency tables of Tregs according to clinical and immunohistochemical prognostic variables

	Treg ≤15 (*n* = 27), *n* (%)	Treg >15 (*n* = 56), *n* (%)	*P*
T stage			
T_{1-2}	19 (22.9)	43 (51.8)	0.53
T_{3-4}	8 (9.6)	13 (15.7)	
Nodes			
N_0	17 20.5)	32 (38.5)	0.61
N_1	10 (12.0)	24 (28.9)	
Histology			
Ductal	20 (24.1)	43 (51.8)	0.79
Lobular	7 (8.4)	13 (15.7)	
Grade			
1	8 (9.6)	21 (25.3)	0.48
2	19 (22.9)	35 (42.2)	
HER-2			
Negative	25 (30.1)	52 (62.2)	0.11
Positive	2 (2.4)	4 (4.8)	
Bcl-2			
Negative	2 (2.4)	6 (7.2)	0.63
Positive	25 (30.1)	50 (60.2)	
Progesterone receptor			
Negative	16 (19.5)	17 (20.7)	<0.02
Positive	11 (13.4)	38 (46.3)	
Ki-67, mean (95% CI)	14.7 (11.2-18.3)	17.4 (13.8-21.0)	0.49
VEGF			
High	12 (15.6)	36 (46.7)	0.13
Low	12 (15.6)	17/53 (22)	

Results

Patient demographics and disease characteristics in the two treatment arms are shown in Table 1. Of the 114 patients enrolled, 83 were available for FOXP3 staining at baseline, 42 that were randomized in the letrozole arm and 41 in the letrozole-cyclophosphamide arm. Thirty-one patients did not have Treg numbers assessed due to insufficient material. FOXP3 staining was available after treatment in 73 patients, 34 randomized in letrozole arm and 39 in letrozole-cyclophosphamide arm. For the CONSORT diagram, see Supplementary Fig. S3.

Relationship between baseline Treg number and prognostic and predictive parameters. FOXP3$^+$ Treg cell number ranged from 0 to 380 (median, 30). Treg numbers were only significantly positively associated with progesterone receptor expression ($P < 0.02$), but there was no correlation with T status, N status, tumor grade, Bcl-2, HER-2, Ki-67 index, hypoxia-inducible factor-1α, or VEGF expression (all $P > 0.05$; Table 2).

Treatment-induced changes in Treg numbers. Treatment-induced changes in Treg numbers were assessed in 71 matched cases. At baseline, no significant difference in Treg number between treatment arms was observed (Table 1; $P = 0.22$). At the end of treatment, Treg numbers decreased in 55 (77.5%) patients [27 of 33 (81.8%) in letrozole arm and 28 of 38 (73.7%) in letrozole-cyclophosphamide arm], there was no

change in 2 (2.8%) patients, and there was an increase in 14 (19.7%) patients. There was a significant reduction in Treg number for all patients ($P < 0.00001$) and within each arm, letrozole ($P = 0.0001$) and letrozole-cyclophosphamide ($P < 0.002$), after treatment (Fig. 1A-C). Changes in Treg numbers before and after treatment were slightly greater in letrozole-cyclophosphamide patients than letrozole patients without attaining the statistical significance ($P = 0.09$); Treg numbers at the end of treatment did not differ between treatment arms ($P = 0.50$).

Treg number and treatment response prediction. Sixty-eight of the 83 (81.9%) cases in which Tregs were assessed at baseline attained a clinical response (complete + partial), 40 cases (48.2%) showed a clinical complete response, and 28 (33.7%) had a partial response. At post-treatment residual histology, 3 (3.6%) patients had a pathologic complete response. As published previously, there was a significant difference in treatment responses in favor of the association of the two drugs (87.4% versus 71.9%, respectively; ref. 12). Although Treg number at baseline showed an inverse relationship with clinical response that failed to attain statistical significance ($P = 0.5$; Fig. 2A), Treg number at residual histology did show a significant ($P < 0.03$) inverse relationship with response (Fig. 2B). There was also a significant trend toward a greater reduction in Treg numbers in responding patients compared with nonresponding patients in all patients ($P < 0.03$) and in the letrozole arm ($P < 0.03$) and with a similar but nonsignificant trend in the letrozole-cyclophosphamide arm ($P < 0.09$; Fig. 2C). There was no difference in Treg numbers between patients attaining a complete response and those attaining a partial response, in all, letrozole, or letrozole-cyclophosphamide patients ($P = 0.44$, 0.97, and 0.30, respectively).

Discussion

Tregs are responsible for inhibition of autologous T-cell proliferation (15) in the periphery, and their presence in the tumor microenvironment is associated with suppression of antitumor immunity as shown by an increase in the efficacy of cancer vaccination in mice depleted of Treg cells (6). Elevated numbers of Tregs have been reported in several primary human cancers, including lung, pancreas, breast, and ovarian tumors (5, 16, 17), as well as in human melanoma lymph node metastases. It has been shown that tumor infiltration by Tregs is significantly correlated with worse prognosis in terms of overall survival in ovarian cancer (17) and we have reported similar association in breast cancer patients (5). Indeed, in the latter, we showed that elevated Treg numbers also gave prognostic information beyond 5 years, a time when conventional prognostic factors used at diagnosis lose their power.

FOXP3 is a member of the *forkhead box* family of transcription factors that was initially thought to be a master regulatory gene for lineage commitment and or development of $CD4^+CD25^+$ Tregs (18). The absence of functional FOXP3 favors autoimmune disease development (19) and $Foxp3^{-/-}$ mice have been shown to lack the $CD4^+CD25^+$ Treg population, which leads to hyperactivation of $CD4^+$ T cells. However, only the human $CD4^+CD25^{hi}$ population homogeneously express FOXP3, a minority of $FOXP3^+$ cells lack CD25 expression, and a small number are $CD8^+$. Thus, $FOXP3^+$ Tregs represent a more distinct T-cell population than described in studies relying on a $CD4^+CD25^+$ or $CD4^+CD25^{hi}$ profile alone. Although recent data have now indicated that some elements of the Treg signature are independent of this transcription factor (20), FOXP3 remains the best single marker of Tregs.

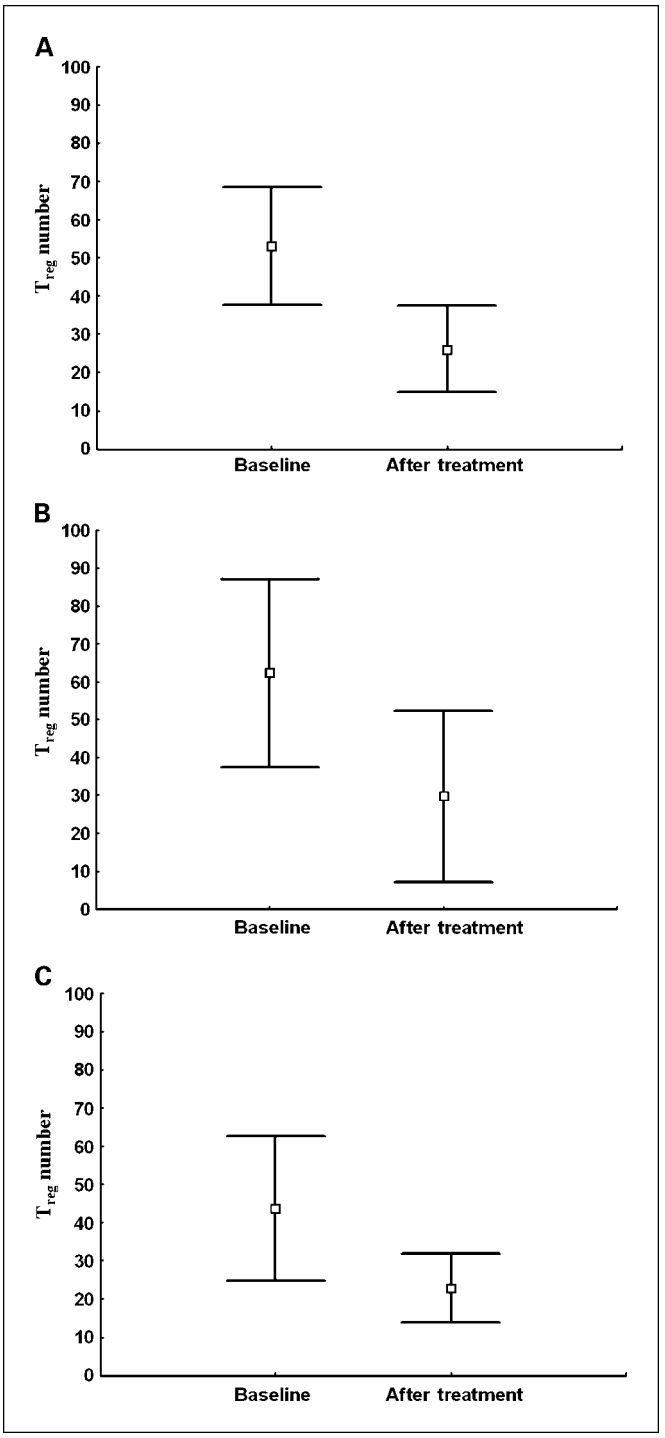

Fig. 1. Treg evaluation before and after treatment in all matched paired patients (*A*) and patients randomized to receive letrozole (*B*) or letrozole-cyclophosphamide (*C*). Mean and 95% CI.

In this study, we used our FOXP3 antibody as a marker to monitor the effect of the drugs on Treg numbers in breast tumor biopsies and we have identified for the first time the

immunomodulating potential of letrozole. Estrogen is capable of augmenting FOXP3 expression and converting $CD4^+CD25^-$ T cells into $CD4^+CD25^+FOXP3^+$ Tregs *in vitro* and *in vivo* (8, 21). The elimination of Tregs using a variety of strategies has been shown to enhance antitumor immunity in some but not all experimental systems (22). The significant reduction in Treg numbers induced by letrozole, the inverse association of absolute numbers, and the reduction in Treg numbers in patients exhibiting a clinical response post-letrozole-based treatment suggest that tumoral Treg numbers can be modulated by aromatase inhibition; thus, estrogen inhibition is another mechanism to restore an antitumor response.

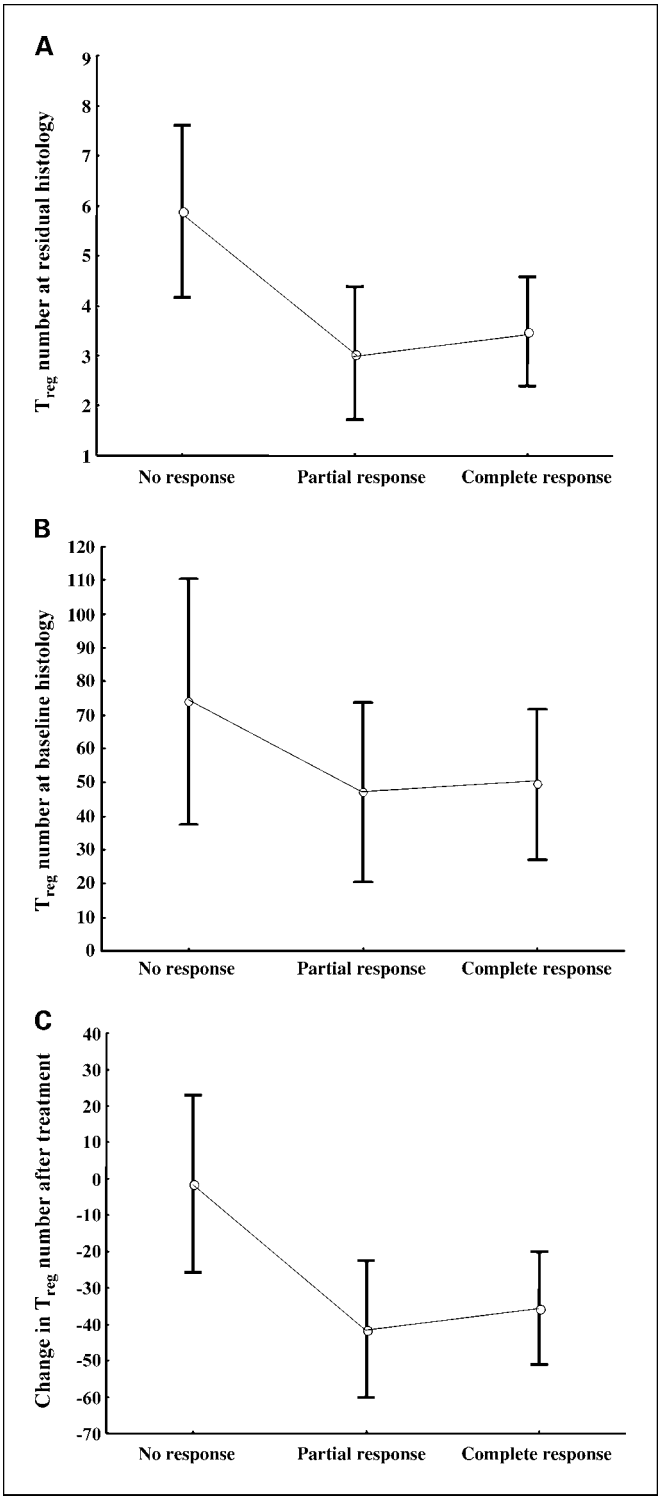

Fig. 2. *A,* Treg number at baseline histology stratified by response. Mean and 95% CI. *B,* Treg number at residual histology stratified by response. Mean and 95% CI of square root transformed values. *C,* Treg changes after treatment according to clinical disease response to treatment (all patients). Mean and 95% CI.

Nevertheless, cyclophosphamide has also been reported to have anti-$CD4^+CD25^+$ T-cell activity and at low dose enhances immunoresponses probably due to its ability to selectively kill Treg cells (23). However, in this study, we detected significantly lower Treg numbers in both letrozole and letrozole-cyclophosphamide patients at post-residual histology, compared with baseline Treg number, with no significant further Treg reduction on the addition of cyclophosphamide to letrozole. We also observed an association between Treg reduction in both arms with clinical response, although Treg reduction was slightly greater (but not significantly so) in the letrozole-cyclophosphamide arm, again suggesting that the modulation of Tregs was due to the effect of the aromatase inhibitor rather than cyclophosphamide. The reasons for this absence of synergy are unclear, but it is possible that the administered dose of cyclophosphamide is effective as an antiangiogenic agent (12), with the drug kinetics not enabling drug to gain access to the tumor itself to act as an immunomodulator. Nevertheless, because cyclophosphamide is also reported to inhibit the suppressive capacity of Tregs (24), although there was no significant difference in Treg numbers between the two arms, we cannot rule out the possibility that Treg functionality is further compromised in the presence of cyclophosphamide. It would thus be of great interest to investigate this possibility.

Prostaglandin E_2 is a potent stimulator of aromatase expression via cyclic AMP. In breast cancer, prostaglandin E_2 promotes cell proliferation (25), tumor progression (26), and inhibition of T-cell-mediated antitumor responses through several pathways (27). One such is stimulation of expression of FOXP3 in Treg cells by tumor-derived prostaglandin E_2, thereby increasing their protumor activity (27). Thus, letrozole by blocking the aromatase enzyme might also interfere with the prostaglandin E_2-aromatase pathway resulting in a reduction in FOXP3 expression/Treg stimulation. Nevertheless, even in patients showing a reduction in Treg number, Tregs were detectable, suggesting that these may still be mediating tumor tolerance, thereby enabling tumors to antagonize drug effect and tumor regression.

Although the antiestrogenic effect and restoration of antitumor immunity are potential mechanisms leading to this response, other pathways such as hypoxia-inducible factor-1α are also important in resistance/responsiveness (28). Thus, hypoxia is reported to alter the balance between T-cell subsets and has been shown to inhibit T-cell-mediated immunoresponses (reviewed in ref. 9). However, we observed no association between the expression of hypoxia-inducible factor-1α and that of a hypoxia-inducible factor-1 target gene, VEGF, with Treg numbers. One possible reason for this is that expression of these markers was determined in tumor cells rather than in $FOXP3^+$ Tregs themselves.

Another potential mechanism of the immunomodulation is through changes in expression of receptors and their cognate ligands that have been shown to be important in immunotrafficking and inflammatory processes. Thus, it is recognized

that estrogen enhances CXCL12 expression (29), a major chemokine involved in Treg recruitment; indeed, hypoxia upregulates its receptor CXCR4 (SDF-1; ref. 30).

In conclusion, this study shows for the first time in a human *in vivo* model a novel role for the aromatase/estrogen pathway in breast tumor immunity and the potential immunomodulating activity of an aromatase inhibitor. Our findings suggest that the development of treatment strategies adopting the combination of aromatase inhibitor-based treatment with vaccine-based strategies may synergize to overcome Treg reconstitution from $CD4^+CD25^-$ cells and increase the treatment response and overall survival of patients.

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