

Immunosuppressive regulatory T cells are associated with aggressive breast cancer phenotypes: a potential therapeutic target

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FoxP3 is a marker for immunosuppressive CD25 + CD4 + regulatory T cells. These regulatory T cells are thought to play a role in inducing immune tolerance to antigens and may be selectively recruited by carcinomas. We investigated whether breast carcinomas had significant numbers of FoxP3-positive regulatory T cells by immunohistochemistry, and if their presence was associated with other prognostic factors, such as Nottingham grade, hormone receptor immunohistochemical profile, tumor size, or lymph node metastases. Ninety-seven needle core or excisional breast biopsies with invasive breast carcinoma diagnosed at the University of Washington were stained with antibodies to FoxP3, estrogen receptor, and Her2/neu. The numbers of FoxP3-positive cells present within the neoplastic epithelium, and immediately adjacent stroma were counted manually in three high-powered fields (HPFs; × 400) by two independent pathologists. The average scores were then correlated with the parameters of interest. A threshold of ≥ 15 FoxP3-positive cells/HPF was used to define a FoxP3-positive case in some analyses. Higher average numbers of FoxP3-positive cells present significantly correlated with higher Nottingham grade status ($P = 0.000229$). In addition, the presence of significant numbers (≥ 15/HPF) of FoxP3-positive cells in breast carcinoma was positively associated with higher Nottingham grade ($P = 0.00002585$). Higher average numbers of FoxP3-positive cells were also significantly associated with larger tumor size (> 2.0 cm; $P = 0.012824$) and trended toward an association with estrogen receptor negativity. Interestingly, 'triple-negative' (estrogen and progesterone receptor negative and Her2/neu negative) Nottingham grade III cases were also significantly associated with high numbers of FoxP3 cells. These results argue that regulatory T cells may play a role in inducing immune tolerance to higher grade, more aggressive breast carcinomas, and are a potential therapeutic target for these cancers.

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Regulatory T cells (T_{regs}) are a specialized subpopulation of T cells that act to suppress the activation of other immune cells and thereby maintain immune system homeostasis. Their importance is emphasized by studies showing that a deficiency of T_{regs} results in serious autoimmune disease affecting multiple organs.^{1,2} Furthermore, this subset of T cells has been implicated in a broad spectrum of other medical conditions, including specific autoimmune diseases such as multiple sclerosis, diabetes and inflammatory bowel disease, graft-

versus-host disease, allograft rejection,³ and protective immunity to viral infections.⁴

Functionally, T_{regs} are defined as a subset of T cells, which can suppress the proliferation of other T cell populations *in vivo* and *in vitro*. Phenotypically, T_{regs} are characterized predominantly as CD4 + CD25 + T cells, which also express FoxP3, a fork-head/winged-helix transcription factor involved in the development in T_{regs} . T_{regs} have also been shown to express cytotoxic T-lymphocyte antigen-4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR).^{1,5} However, there are smaller subsets of T_{regs} , which do not fulfill all these phenotypic criteria.⁶

In addition to their role in autoimmune and infectious diseases, T_{regs} have been shown to be important in the body's response to neoplasia. T cells targeted at tumor-associated antigens are detectable in blood and draining lymph nodes of

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individuals with a variety of neoplasms, even at late stages of disease.^{7–10} These tumor-specific T cells can be used to establish functional tumor-specific T-cell lines, which kill autologous tumor cells *in vitro* and *in vivo*.¹¹ However, the spontaneous clearance of established tumors by endogenous immune mechanisms is rare. Although there is increasing evidence that tumors induce some degree of immune tolerance, the underlying mechanisms are not completely understood. However, numerous studies suggest that T_{regs} may play an important role in suppressing this tumor-associated antigen-specific immunity.^{12–20} Higher numbers of T_{regs} have been found in the peripheral blood and neoplastic tissues of patients with a variety of tumors, including breast carcinoma,²¹ ovarian carcinoma,¹³ gastric carcinoma,²² melanoma,²³ and others. Many of these studies have found an association between high numbers of T_{regs} and a poor clinical patient outcome. The prognostic impact of downregulating the immune response is emphasized by recent studies, which suggest that other immune modulating molecules, such as B7-H1 and PD-1, present on tumor infiltrating lymphocytes correlate with a poor prognosis in breast carcinoma.²⁴ Given the proposed immune modulating effects of T_{regs} in a patient's tumor response, we attempted to correlate the number of T_{regs} present within breast carcinoma tissue sections with other defined prognostic indicators of clinical outcome.

Materials and methods

Patient Samples

Ninety-seven formalin-fixed, paraffin-embedded needle core or excisional breast biopsy tissue specimens with invasive breast carcinoma were obtained from the archives of the Department Of Pathology at the University Of Washington. Histologic grading was carried out using the Nottingham-combined histologic grade (Elston–Ellis modification of Scarff–Bloom–Richardson grading system).²⁵ Using this classification system, 19 cases met criteria for Nottingham grade I, 26 cases were characterized as Nottingham grade II, and 52 cases were characterized as Nottingham grade III.

Immunohistochemical Staining

All tissues were deparaffinized followed by the blockade of endogeneous peroxidases and antigen retrieval using antigen unmasking solution (Vector; USA). All tissues were immunohistochemically stained for FoxP3 (e-Bioscience Inc., San Diego, CA, USA) clone 236A/E7 using a dilution of 1:200 following an 18-min pretreatment with EDTA; estrogen receptor (ER; Immunotech/Beckman Coulter, Fullerton, CA, USA) clone 1D5 using a dilution of 1:1000, following a 15-min pretreatment

in citrate buffer, pH=6.0; progesterone receptor (PR; BioGenex, San Ramon, CA, USA) clone PR88 using a dilution of 1:100 following an 18-min pretreatment in citrate buffer pH=6.0; and Her-2/neu (Dako, Carpinteria, CA, USA) using a dilution of 1:800, following a 15-min pretreatment in citrate buffer, pH=6.0. The slides were then counterstained in hematoxylin, dehydrated, and mounted. Positive and negative controls were performed to ensure that the staining procedure was successful. Previously characterized breast tissue was used as the positive control for ER, PR, and Her-2/neu. Reactive tonsil was used as the positive control for FoxP3. Negative control staining was performed on the tissue of interest using normal mouse or normal rabbit sera (MP Biomedicals LLC, Solon, OH, USA).

The numbers of FoxP3-positive cells present within neoplastic epithelium and immediately adjacent stroma (within the same high-powered field; HPF) were counted manually in three HPFs ($\times 400$) by two independent pathologists. The average scores were then correlated with parameters of interest, such as hormone receptor status and Nottingham grade.

Our study utilized FoxP3 antibody clone 236A/E7 (ab20034), which has been shown to have the best performance characteristics and the most suitable for reliable immunohistochemical analysis on paraffin-embedded sections.^{26,27}

Statistical Analysis

We analyzed whether there was a statistically significant correlation between the number of T_{regs} and the Nottingham grade, tumor size, lymph node status, ER status, and Her2/neu positivity in our set of invasive breast carcinomas. The average number of T_{regs} present was compared using a two-tailed student's *t*-test assuming unequal variance (Microsoft® Office Excel, Microsoft® Corporation, Redmond, WA, USA). We also attempted to corroborate proposed thresholds used in the literature for determining significant numbers of T_{regs} within the histologic sections when compared with other established prognostic indicators. Similar to Bates *et al*²¹, we selected a threshold of ≥ 15 FoxP3-positive cells/HPF to define a FoxP3-positive case. Using this threshold, contingency tables were created and analyzed using a Cochran–Armitage trend test.

Results

Within the invasive breast carcinoma samples, there was a significant correlation between the average number of FoxP3-positive cells and the Nottingham histologic grade ($P = 0.00023$). Although the average number of FoxP3-positive cells increased with increasing histologic grade (see Table 1, Figure 1), there was no clear cutoff for the number of FoxP3-

Table 1 Correlation of average number of FoxP3-positive cells with pathologic features

	<i>Number of cases</i>	<i>Average number FoxP3-positive cells/HPF</i>	<i>P-value</i>
Nottingham grade I–II	45	15	0.0002
Nottingham grade III	52	28	
ER positive	50	19	0.0677
ER negative	47	25	
Her2/neu overexpressed	37	21	0.5596
Her2/neu not overexpressed	60	23	
Size ≥ 2.0 cm	36	28	0.0128
Size < 2.0 cm	44	18	
Lymph node positive	29	25	0.2218
Lymph node negative	52	20	
ER, PR, and Her2/neu negative ('triple negative')	33	26	0.0701
Nontriple negative'	64	20	
ER, PR, and Her2/neu negative (triple negative) and Nottingham grade III	26	30	0.0093
Nontriple negative, non-Nottingham grade III	71	19	

ER = estrogen receptor; HPF = high-powered field; PR = progesterone receptor.

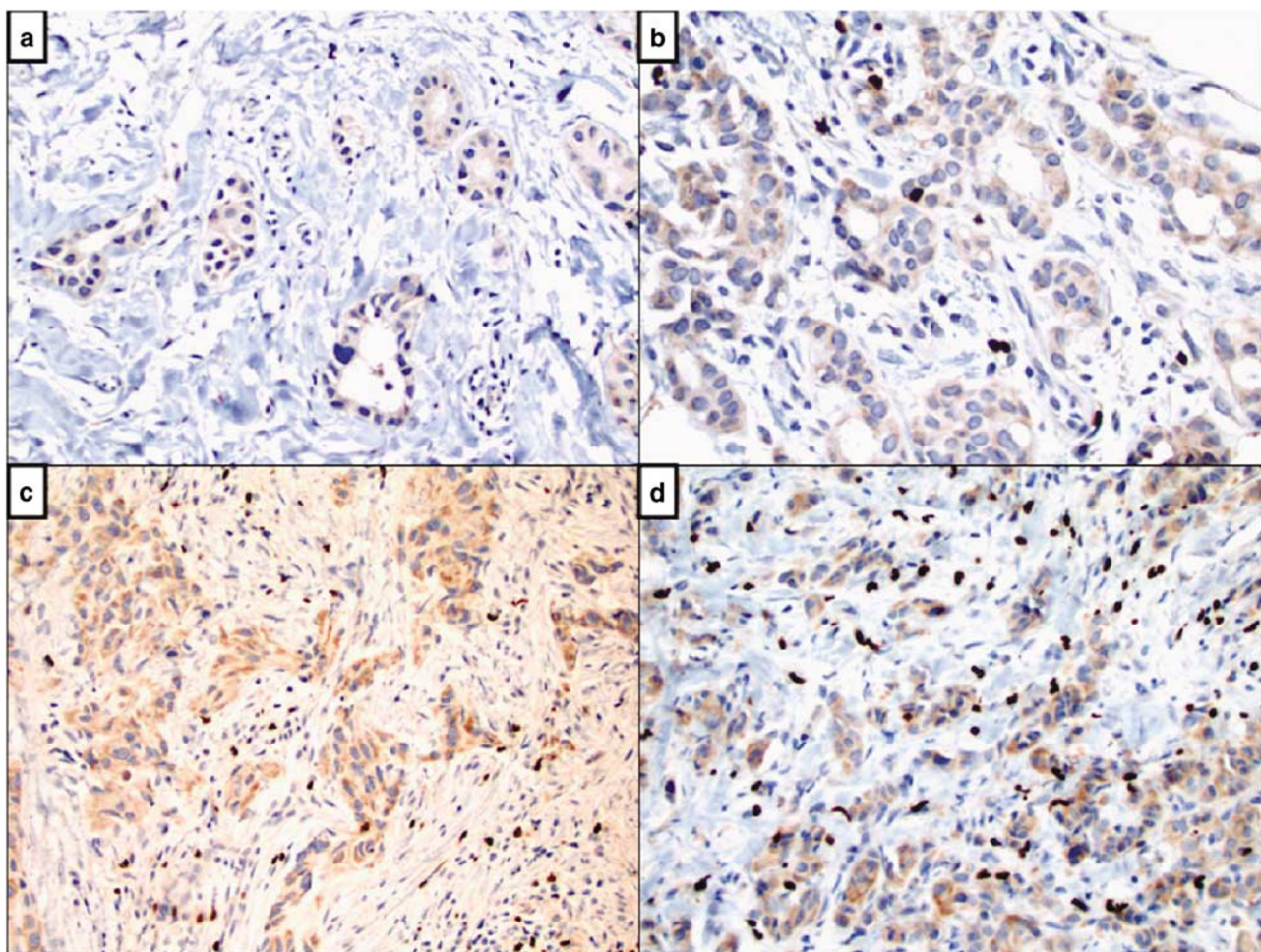


Figure 1 Examples of FoxP3-positive infiltrates in breast cancers of different Nottingham grades. (a) Absence of FoxP3-positive cells in a Nottingham grade I breast cancer ($\times 200$). (b) Few (< 15 /HPF) FoxP3-positive cells in a Nottingham grade I breast cancer ($\times 400$). (c) Moderate numbers of FoxP3-positive cells (≥ 15 /HPF) in a Nottingham grade II cancer ($\times 200$). (d) High numbers of FoxP3-positive cells (> 15 /HPF) in a Nottingham grade III cancer ($\times 200$).

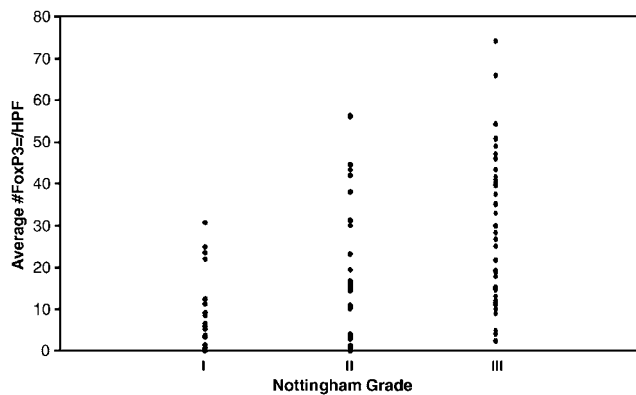


Figure 2 Scatter Plot of the number of FoxP3-positive cells present in each histologic Nottingham grade.

positive cells present within each Nottingham histologic grade (see Figure 2). Using the ≥ 15 FoxP3-positive cells/HPF threshold, 76.9% of Nottingham grade III cases were FoxP3-positive compared to 21.0% of Nottingham grade I cases. A statistically significant association was found between higher Nottingham grade cases and the presence of ≥ 15 FoxP3-positive cells/HPF ($P = 0.000026$).

Examples of immunohistochemical stains for FoxP3 are represented in Figure 1 and the immunohistochemical stain results are listed in Table 1. We found that ER-negative cases had a higher average number of FoxP3-positive cells present (25 FoxP3-positive cells/HPF for ER-negative cases vs 19 FoxP3-positive cells/HPF for ER-positive cases), but the association did not reach statistical significance ($P = 0.068$). Her2/neu status did not correlate with FoxP3 status ($P = 0.56$; see Table 1). Carcinomas that were negative for ER, PR, and Her2/neu (so called 'triple negative' carcinomas) also showed an association with the average number of FoxP3-positive cells present, but it was not statistically significant ($P = 0.07$; see Table 1). However, Nottingham grade III carcinomas that were also triple negative contained an average of 30 FoxP3-positive cells/HPF, whereas nontriple negative cases of lower histologic grade contained an average of 19 FoxP3-positive cells/HPF. This association was statistically significant ($P = 0.0093$).

We also correlated the average number of FoxP3-positive cells present with tumor size and lymph node metastasis. Information regarding lymph node status and the size of the invasive carcinoma within the resection specimen was available for 80 of our 97 cases. Although no statistically significant association was found between the average number of FoxP3-positive cells present within the carcinoma and the presence of lymph node metastasis ($P = 0.222$; see Table 1), we did find a significant association between larger tumor size (> 2.0 cm) and increasing numbers of FoxP3-positive cells within and surrounding the tumor ($P = 0.013$). Carcinomas measuring ≥ 2.0 cm

contained an average of 28 FoxP3-positive cells/HPF, whereas carcinomas less than 2.0 cm contained an average of 18 FoxP3-positive cells/HPF.

Discussion

A variety of prognostic and pathologic factors have been used to predict the survival in breast carcinoma patients, including tumor size and grade, nodal status, tumor necrosis, and protein markers such as ER, progesterone receptor, and Her2/neu. Although the current prognostic factors predict relapse in the first 5 years after therapy, it is unclear whether these parameters are useful in predicting long-term survival or late relapse.^{28–30} It has been hypothesized that immunologic factors, specifically T_{regs} , play a significant role in tumor development and progression due to their ability to induce immune tolerance to a cancer. Given their possible role in tumor progression, T_{regs} are increasingly being looked at as both prognostic factors and therapeutic targets. Recent studies have also shown that successful chemotherapeutic treatments for breast carcinoma, which resulted in a complete pathologic response also resulted in the disappearance of FoxP3-positive T cells and an increased number of CD8+ cytotoxic T cells.³¹

We observed associations between elevated numbers of FoxP3-positive cells and the more aggressive tumor phenotypes of advanced Nottingham grade (grade III) and larger tumor size (> 2.0 cm). One of the most aggressive breast cancer phenotypes, the so-called 'triple negative' (ER, PR, and Her2/neu) Nottingham grade III cancer, was also significantly associated with higher average numbers of FoxP3-positive cells. These findings suggest that there may be more immune tolerance induction to these more aggressive tumor types. In addition, it may offer a novel therapeutic target for these cases, which are not candidates for hormonal therapy or trastuzumab treatment.

Although there is no clearly defined cutoff for what constitutes a clinically significant threshold for total numbers of T_{regs} in breast cancers, some investigators have suggested ≥ 15 FoxP3-positive cells/HPF as a threshold.²¹ Our data corroborated the association between ≥ 15 FoxP3-positive cells/HPF as well as increased mean numbers of FoxP3 positive with advanced Nottingham grade. We also noted a trend toward the increased number of FoxP3-positive cells and negative ER status; however, it did not reach statistical significance. Two recently published studies have found similar association with higher numbers of FoxP3-positive cells in breast cancer tissue with both higher Nottingham grade and ER-negative status.^{21,24} We did not observe an association between the number of FoxP3-positive T_{regs} present and expression of Her2/neu by immunohistochemistry. Other authors have found an association with Her2/neu over-

expression and ≥ 15 FoxP3-positive cells/HPF.²¹ This difference could be due to the lower percentage of Her2/neu-positive cases in the previous study compared to our study (11 vs 38%, respectively). Further study may clarify this difference in results. Curiously, the role of the FoxP3 gene in Her2/neu-positive breast carcinomas may extend beyond its role in T_{regs} . A recent study suggests that the FoxP3 gene may play a role as an X-linked breast cancer suppressor gene and an important regulator of the Her2/neu oncogene.³²

Interestingly, a recent study examined the location of FoxP3-positive cells in gastric cancer cases and described the distribution patterns as diffuse, peritumor, or follicular. The authors showed a difference in patient survival based on these localization patterns.³³ Although the highest mean numbers of FoxP3-positive cells were found in stage IV disease in this study, the peritumor pattern of staining was significantly associated with better survival and early stage disease at diagnosis. This may be due to the localization of T_{reg} migration factors at the tumor–stroma interface in earlier stage disease.³³ Although our study did not address specific localization patterns of the FoxP3-positive cells, the majority of cases did have FoxP3-positive cells within the invasive carcinoma and the immediately adjacent stroma. Further investigation into the distribution patterns of FoxP3-positive T cells are necessary to determine whether there is a similar association with stage and survival in breast cancer patients.

T_{regs} may play a role in inducing immune tolerance to higher grade, hormone receptor negative breast carcinomas. T_{regs} in the tumor microenvironment are thought to function as mediators of immune evasion mechanisms. Therefore, reducing T_{reg} function in cancer patients could be therapeutic, analogous to the benefits seen in mouse models.^{34–37} This concept has been translated into clinical practice with the use of immunotherapeutic interventions for cancer (so called ‘tumor vaccines’), such as denileukin diftitox (Ontak, a recombinant fusion protein consisting of IL-2 and diphtheria toxin). A recent report supported this idea by demonstrating that denileukin diftitox-reduced T_{reg} numbers, and overall suppression mediated by the CD4 + CD25 + cell population in a mouse model of breast cancer, with improved immunity and tumor regression.³⁸ The therapeutic effects of Ontak have also been investigated in a variety of neoplasms, including cutaneous T-cell leukemia/lymphoma,³⁹ ovarian, breast, and pancreatic carcinomas.⁴⁰ Similarly, antibodies against T_{regs} have been used in the management of malignant melanomas with promising results.⁴¹

In conclusion, our study supports other reports that T_{regs} are associated with more aggressive breast cancer phenotypes. The association of high numbers of T_{regs} with more aggressive cancers, in conjunction with early immunotherapeutic data, suggests that T_{regs} may

represent an important potential therapeutic target in the treatment of aggressive breast carcinomas.

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Disclosure/conflict of interest

The authors have no conflicts of interest to disclose.

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