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ORIGINAL ARTICLE

Possible involvement of regulatory T cells in tumor onset and progression in primary breast cancer

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Abstract
Background The *FOXP3* mRNA expression and the other regulatory T cell-related molecules were investigated and compared with clinicopathological parameters in human primary breast cancer.
Method This study included 136 breast cancer patients operated in our department from 2003 to 2006. Total RNA was extracted from frozen normal breast and breast cancer tissues, and the expression of *FOXP3*, *IL-10*, *TGFβ1* and *CCL22* mRNA was evaluated using quantitative real-time RT-PCR.
Result *FOXP3*, *IL-10, TGFβ1* and *CCL22* mRNA expressions were significantly higher in cancer tissue than in normal tissue, not only at pT1, 2, and 3 stages but also at the DCIS stage. There were positive correlations between *FOXP3* and *IL-10*, *FOXP3* and *TGFβ1,* as well as *FOXP3* and *CCL22* mRNA expressions, respectively. *FOXP3* and *IL-10* mRNA expressions were significantly upregulated in PgR-negative or HER2-positive tumors.
Conclusion These results suggest that regulatory T cells are involved in tumor onset and progression in human primary breast cancer, possibly contributing to poor prognosis of patients with breast cancer.

Keywords FOXP3 · CCL22 · IL-10 · TGFβ1 · Regulatory T cells

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Introduction

Regulatory T (Treg) cells have been highlighted in tumor immunology [1, 2]. Although Treg cells protect hosts in vivo against the development of autoimmunity, they also suppress the reaction of lymphocytes to tumor antigens in some malignant neoplasms [3]. This suppression helps the neoplasms to escape from the host immune system [4]. Therefore, to explore the existence of Treg cells in cancer patients and the involvement of Treg cells in tumor onset and progression is an important issue to be clarified. It has been reported that Treg cells are increased in peripheral blood in patients with non-small cell lung cancer [5, 6] and gastrointestinal malignancies [7], in sentinel lymph nodes in breast cancer [8], and in tumor nodules in head and neck [9], prostate [10], and breast [11, 12] cancers. Although these reports have suggested Treg cell involvement in tumor progression, it still requires more clinical samples to define Treg cell significance in tumor onset and progression of breast cancer.

Treg cells are originally characterized by the co-expression of CD4 and CD25 molecules [13, 14]. Treg cell activity is known to be closely associated with the expression of forkhead/winged helix transcription factor, Foxp3. Although Foxp3 is not induced upon activation of $CD25^-$ T cells, naive $CD4^+CD25^-$T cells transform into Treg cells

when *Foxp3* is introduced via retrovirus or enforced transgene expression [15, 16]. Recently, $CD4^+CD25^-$T cells were induced to express *FOXP3* and achieve regulatory function by stimulation through the T-cell receptor (TCR) in humans [17] and transforming growth factor-β (TGFβ) in mice [18]. From these findings, FOXP3 can be considered to be a critical regulator of Treg cell development and function in mice and humans. Although the precise mechanisms of immunosuppression by Treg cells remain to be determined, Treg cells can inhibit immune cell functions either directly through cell-to-cell contact or indirectly through the secretion of anti-inflammatory mediators such as IL-10 and TGFβ1. The antigens recognized by Treg cells appear to be self, tissue-specific antigens [19]. More recently, the molecular mechanisms of Treg cell migration have been precisely demonstrated: Treg cells express chemokine receptor CCR4 and show demonstrable chemotactic responses to the CCR4 ligands CCL22 and CCL17 [20].

Based on the above findings, we studied Treg cell involvement in the onset and progression of 136 human breast cancer samples by using RT-PCR for *FOXP3*, *IL-10*, *TGFβ1* and *CCL22* mRNA expressions. We analyzed the relationship between these molecular expressions and known clinicopathological parameters of prognosis such as the size of the primary tumor, histologic tumor grade, lymph node status, estrogen (ER) and progesterone receptor (PgR) expression, HER-2/neu expression, and lymphatic and vascular invasion.

Materials and methods

Patients enrolled

Adult female patients with primary breast cancer who underwent breast surgery at the Hiroshima University Hospital between 2003 and 2006 were enrolled in this study. The study was approved by the institutional review board of Hiroshima University Hospital, and written informed consent was obtained from all enrolled patients. Normal breast tissues were taken from a site distant from the tumorous lesion ($n = 11$).

RT-PCR

Total RNA was extracted from stored tumor tissues with an RNeasy Mini Kit (QIAGEN, Hilden, Germany). DNase treatment was performed with the RNase-Free DNase Set (QIAGEN) to avoid amplification of contaminating genomic DNA. The quality and quantity of the obtained total RNA were determined by absorbance at 260 and 280 nm and were adjusted to a concentration of 0.10 μg/μL. In addition, the integrity of RNA in each preparation was tested by RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The prepared total RNA served as the template for first-strand cyclic DNA (cDNA) synthesis using Ready-To-Go™ You-Prime-First-Strand Beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) with 0.5 μg of Oligo (dT)15 primer (Novagen, Darmstadt, Germany) according to the manufacturer's instructions. Each cDNA product was diluted tenfold with RNase-free water to avoid inhibition of the PCR reaction by reagents used in RNA extraction or reverse transcription. For relative quantification by PCR, each cDNA product was analyzed at a final Mg^{2+} concentration of 3 mM in a LightCycler with software (version 3.5; Roche Molecular, Mannheim, Germany) by using the FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany). For each primer pair, a standard curve was developed. The PCR condition was 95°C for 10 min for the initial denaturation, followed by 35 cycles of 1 second at 95°C, 10 s at 60°C, and 10 s at 72°C, followed by a melting program (at 60–95°C) to check the proper melting temperature of the product. Annealing temperatures and elongation times were optimized for primer generation and exclusion of artifacts. Primers for FoxP3, IL-10, TGFβ1, CCL22 and GAPDH were from Search-LC GmbH (Heidelberg, Germany).

All clinical samples were amplified by PCR, and the quality of amplification was checked with Light Cycler R software (version 3.5; Idaho Technology Inc., Salt Lake City, UT, USA) by calculating the ratio of the crossing point (threshold cycle number for the beginning of the exponential phase) and the logarithm concentration of the amplified copy number for each standard sample [21].

Statistical analysis

The Mann–Whitney *U* test and Kruskal–Wallis test were used when testing categorical or continuous categorical data against categories. Spearman's rank correlation and simple regression analysis were used to identify associations in the expression of each parameter with StatView software (version 5 for Windows; Abacus Concepts, Inc., London, UK). *P* values <0.05 were considered significant.

Results

Patient and tumor characteristics

Background data of patients with regard to age, histologic type of tumor, pathologic (p)TNM classification, ER, PgR, HER2, and lymphatic and vascular invasion status are summarized in Table 1. The enrolled patients were 58.1 ± 13.0 years old (mean ± standard deviation; range

Table 1 Patients and tumor charateristics

	Total (n = 136)	%
Age (year)		
Mean ± SD	58.1 ± 13.0	
Range	33–88	
pT category		
pTis (DCIS)	12	9
pT1	84	62
pT2 pT3	40	29
pN category		
pN0	94	69
pN1 pN2 pN3	37	27
pNX	5	4
Tumor histologic type		
Noninvasive ductal carcinoma	12	9
Invasive ductal carcinoma	107	79
Special types	17	13
Tumor grade (SBR)		
Grade 1	31	23
Grade 2	51	38
Grade 3	53	39
Unknown	2	1
Lymphatic invasion score		
0	87	64
1.2.3	49	36
Vascular invasion score		
0	136	100
1.2.3	0	0
Hormone receptor status		
ER-positive	120	88
ER-negative	16	12
PgR-positive	102	75
PgR-negative	34	25
HER2 status		
Negative	106	78
Positive	28	21
Unknown	2	1

SD standard deviation, *ER* estrogen receptor, *PgR* progesterone receptor, *SBR* Scarff–Bloom–Richardson

33–88 years). Of the 136 patients enrolled, 12 had pTis status, 84 had pT1 status, and 40 patients pT2 or pT3 status. Ninety-four patients had pN0 status, and 37 had pN1, N2, and N3 status. Seventy-nine percent of the tumors were determined to be invasive ductal carcinomas according to standard histopathology (hemtoxylin and eosin staining). Greater than 70% of the tumors expressed estrogen (88%) or progesterone (75%) receptors. Twenty-one percent of the patients had positive HER2 status and 78% of the patients negative HER2 status according to HercepTest-immunostaining and fluorescence in situ hybridization (FISH). One-third of the patients had Scarff–Bloom–Richardson grades 1, 2, or 3. Approximately two-thirds of the patients showed a lymphatic invasion score of 0, and none of the patients had vascular invasion.

FOXP3 and *IL-10* mRNA expressions

We used quantitative RT-PCR to analyze *FOXP3* and *IL-10* mRNA expressions in breast cancer tissue and compared expression levels against each clinicopathological factor (Table 2). There was no correlation between *FOXP3* mRNA expression and pT category (data not shown). However, *FOXP3* mRNA expression in cancer tissue was significantly upregulated when compared with normal breast tissue expression ($P < 0.01$), even at the ductal carcinoma in situ (DCIS) stage ($P < 0.01$) (Fig. 1a). Similar results were obtained in *IL-10* mRNA expression (Fig. 1b). The upregulation of *FOXP*3 and *IL-10* mRNA expressions persisted at the pT1 and pT2, 3 stages ($P < 0.01$) (Fig. 1a, b), showing the expression levels of 7.4 ± 10.9, 170.5 ± 207.9, 205.1 ± 184.1, 165.7 ± 200.9 and 170.3 ± 231 copies in *FOXP3* mRNA, and 61.73 ± 118.4,

Table 2 Relationship between *FOXP3, IL-10, TGF* and clinicopathological factors (only correlative parameters and their values)

	n (%)	Mean ± SD	P value
FOXP3			
Tumor grade (SBR)			
Grade 1	31 (22%)	108.8 ± 129.6	
Grade 2	51 (38%)	152.2 ± 162.3	
Grade 3	53 (39%)	222.9 ± 267.6	0.0981
Hormone receptor status			
PgR-positive	102 (75%)	144.2 ± 180.8	
PgR-negative	34 (25%)	249.5 ± 260.9	0.0075
HER2 status			
Negative	106 (79%)	150.4 ± 187.3	
Positive	28 (21%)	255.0 ± 234.6	0.007
IL-10			
pN category			
pN0	94 (69%)	97.2 ± 146.5	
pN1 pN2 pN3	37 (27%)	122.9 ± 130.2	0.067
Hormone receptor status			
PgR-positive	102 (75%)	91.1 ± 121.9	
PgR-negative	34 (25%)	138.4 ± 183.2	0.0332
HER2 status			
Negative	106 (79%)	93.3 ± 133.3	
Positive	28 (21%)	143.9 ± 162.6	0.0445

SD standard deviation, *SBR* Scarff–Bloom–Richardson, *PgR* progesterone receptor

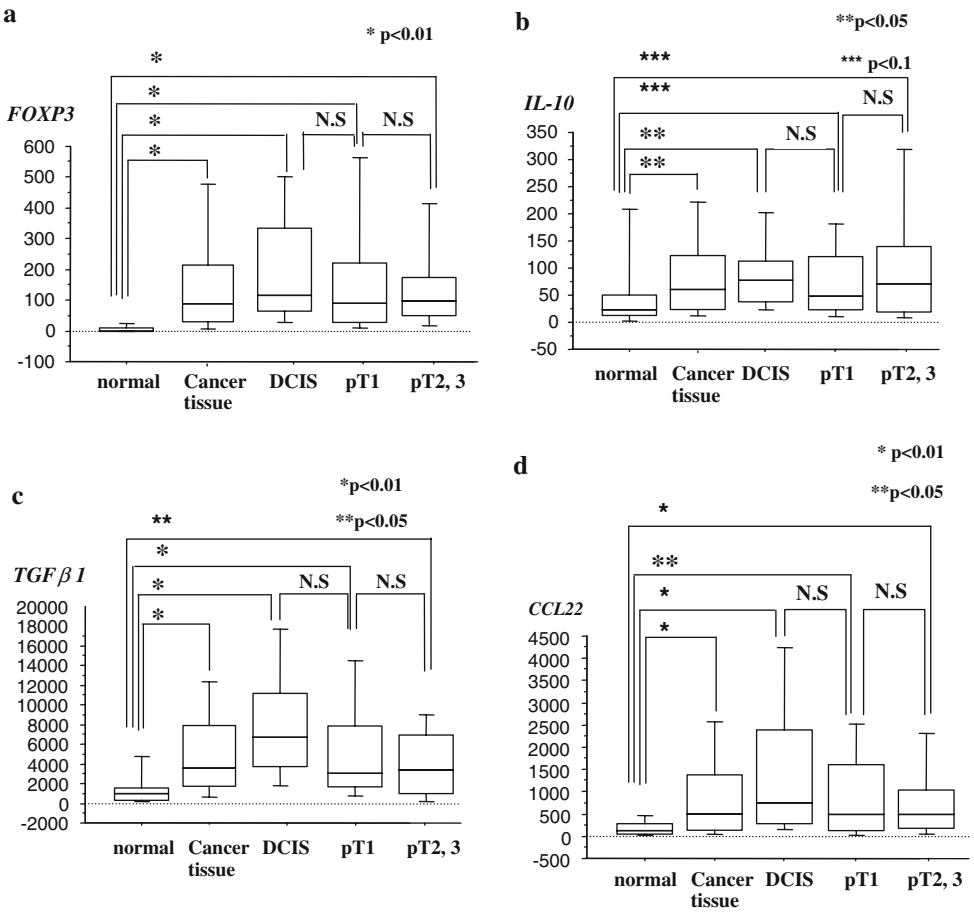

Fig. 1 *FOXP3*, *IL-10*, *TGFβ1,* and *CCL22* mRNA expression in normal breast and cancer tissues. Clinical samples were subjected to quantitative RT-PCR analysis specific for *FOXP3*, *IL-10, TGFβ1*, and *CCL22*. Copy numbers of expressed genes are indicated

102.7 ± 140.1, 93.7 ± 87.8, 92.9 ± 121.4 and 126.4 ± 184.5 copies in *IL-10* mRNA, for normal breast, total cancer tissue, DCIS, pT1, and pT2, 3 stages, respectively (Fig. 1a, b). *FOXP3* and *IL-10* mRNA expressions showed no correlations with pN category, tumor histologic type, or lymphatic invasion score (data not shown). However, *FOXP3* mRNA expression was positively correlated with tumor grade, and also significantly higher in PgR-negative cancers or HER2-positive cancers (Table 2). Similarly, *IL-10* mRNA expression was positively correlated with PgR-negative cancers or HER2-positive cancers (Table 2).

TGFβ1 and CCL22 mRNA expressions

We also used quantitative RT-PCR to analyze *TGFβ1* and *CCL22* mRNA expressions in breast cancer tissue and compared expression levels against each clinicopathological factor. *TGFβ1* and *CCL22* mRNA expressions showed no correlation with pT category (data not shown). However, *TGFβ1* and *CCL22* mRNA expressions in cancer tissue were significantly upregulated when compared with normal breast tissue ($P < 0.01$), even at the DCIS stage ($P < 0.01$) (Fig. 1c, d). *TGFβ1* and *CCL22* mRNA expressions were not correlated with pN category, a tumor histologic type, tumor grade, lymphatic invasion score, hormone receptor status, or HER2 status, (data not shown).

Correlation between *FOXP3* mRNA expression and expressions of *IL-10*, *TGFβ1,* and *CCL22* mRNA

The relationships between *FOXP3* mRNA expression and expressions of *IL-10*, *TGFβ1*, and *CCL22* mRNA were analyzed (Fig. 2a–c). Positive correlations were observed between expressions of *FOXP3* and *IL-10*, *FOXP3* and *TGFβ1, and FOXP3* and *CCL22* mRNA (Spearman's $\rho = 0.640$, $P < 0.0001$; $\rho = 0.307$, $P = 0.0004$; $\rho = 0.557$, $P < 0.0001$, respectively) (Fig. 2a–c).

Discussion

It has been reported that low level *Foxp3* mRNA expression is detectable in breast epithelium and breast cancer cell lines, where *Foxp3* functions as a breast cancer suppressor gene [22], indicating attention to understand the origin of *FOXP3*-expressing cells; those are breast epithelium, breast cancer cells, or Treg cells. However, they demonstrated that

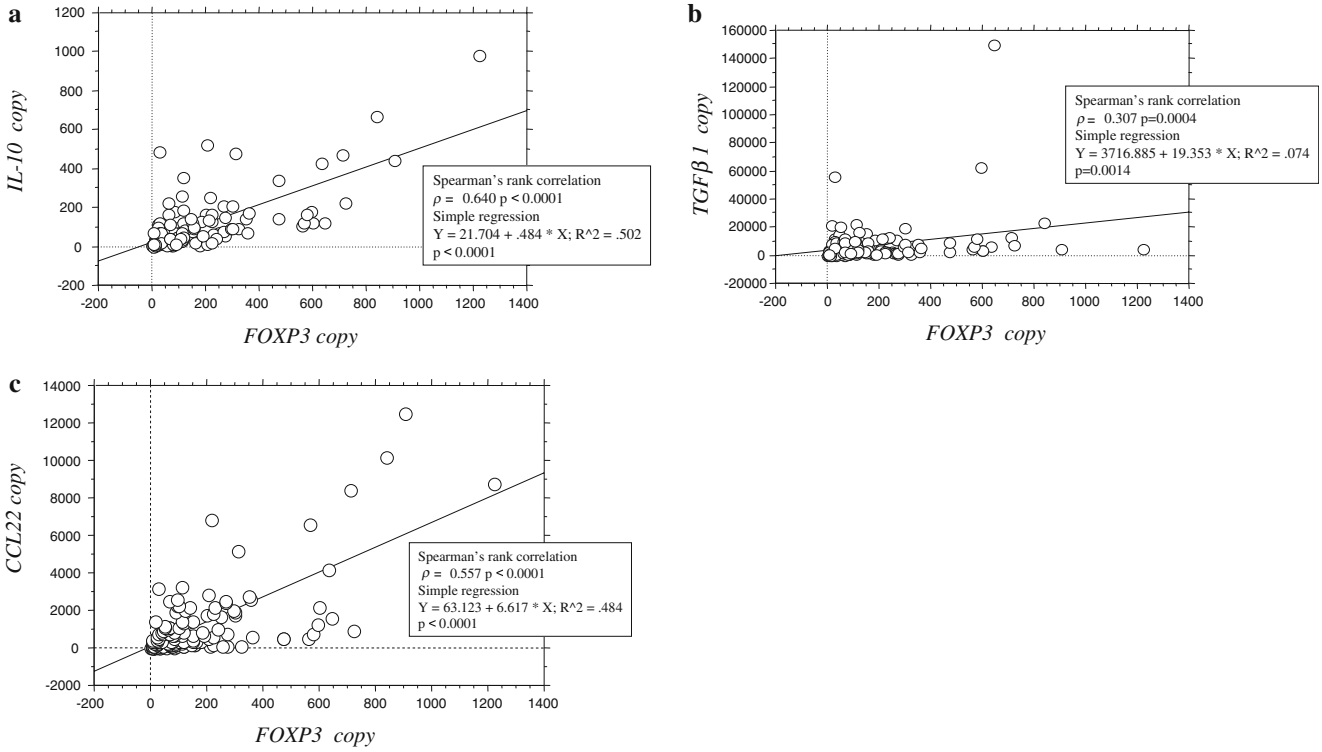

Fig. 2 Regression analysis between *FOXP3* mRNA expression and *IL-10*, *TGFβ1*, *CCL22* mRNA expressions. Copy numbers for *FOXP3* mRNA were plotted in comparison with copy numbers for *IL-10* (**a**), *TGFβ1* (**b**), and *CCL22* (**c**) mRNA for each patient, and regression analysis was performed to assess the correlations between these values

deletion, functionally significant somatic mutations, and down-regulation of the *FOXP3* gene were commonly found in human breast cancer samples. In our study, it was observed that *FOXP3* expression was higher in tumor tissue than in normal breast tissue. Together, it is strongly suggested that the *FOXP3* expression in breast cancer tissue, we observed here, indicates the tumor-infiltrating Treg cell origin.

We hypothesized that Treg cell accumulation in tumor tissue would be augmented in parallel with tumor progression. In this study, higher expression of *FOXP3* mRNA in tumor tissue than in normal breast tissue was observed even at the DCIS stage and persisted at the T1 and T2, 3 stages, indicating that Treg cell accumulation in tumor tissue is an early event in tumor development and progression. Leong et al. [23] have also shown the existence of Treg cells in breast cancer tissue and that there is no significant correlation between tumor stage and CD4+CD25+ T cells, although they have not mentioned about stage 0 (DCIS). Interestingly, expression of *CCL22*, which has been shown to be a ligand of CCR4 in Treg cells [20], was also found to be higher in tumor tissue at the DCIS stage than in normal breast tissue in our study. Miller et al. [24] have indicated that Treg cells are an important cellular component of early-stage prostate tumors, and that supernatants from cultured prostate tissue samples and prostate cancer ascites fluid induce migration of Treg cells and contain the chemokine CCL22 as detected by ELISA. It is therefore likely that the homing receptors for Treg cells are induced and expressed in tumor tissues at earlier phases of tumor development, and the subsequent Treg cell accumulation helps tumors to escape from the host immune system, resulting in tumor progression.

Our further analysis showed that *FOXP3* mRNA expression was upregulated in PgR-negative and Her2-positive breast cancer tissues and significantly correlated with tumor grade. It has been reported that PgR status significantly improves outcome prediction over ER status alone for adjuvant endocrine therapy of breast cancer patients [25]. *HER-2/neu* gene amplification and protein overexpression are associated with worsened prognoses in patients with breast cancer [26], even in DCIS tumors [27], suggesting the involvement of *FOXP3* expression as a prognostic factor in breast cancer. Bates et al. [11] have investigated the expression of FOXP3 protein in breast cancer by immunohistochemistry and demonstrated that high Treg cell numbers are present in high-grade tumors, in patients with lymph node involvement, and in ER-negative tumors, and HER2-positive tumors, consistent with our observations, and that breast cancer patients who express FOXP3 have poorer prognoses. FOXP3-expressing Treg cells can avoid the anti-tumor activity of immune effector cells in breast cancer tissue, resulting in poor prognosis of breast cancer patients.

It has been shown in some studies that Treg cells may mediate immunosuppression through the secretion of IL-10 [28], and in other studies that TGFβ or direct cell-to-cell contact may be important for suppression [29]. Gupta et al. [12] have demonstrated that intratumoral *FOXP3* mRNA and protein levels were correlated with the levels of TGFβ1, vascular endothelial growth factor and intratumoral microvessel density. In our study, *FOXP3* expression was positively correlated with *IL-10* and *TGFβ1* expression in breast cancer tissue, and *IL-10* was much more highly correlated with *FOXP3* expression than *TGFβ1*. This finding requires careful interpretation. IL-10 has been shown to be secreted by not only Treg cells but also T helper-type (Th)-2 cells [30]. In addition, it has been demonstrated that tumor cells themselves can produce and secrete IL-10 protein [31], indicating that IL-10 expression in breast cancer tissue does not always require Treg cells. TGFβ1 is also produced from not only Treg cells, but many cell types including tumor cells, and it is implicated in several aspects of breast cancer onset and progression [32]. However, it has been reported that Treg cells may stimulate Th-2 responses. Suto et al. [33] reported that Treg cells induce Th-2 cell proliferation in patients with allergic inflammation of the airways. In previous studies of sentinel lymph node immunity in breast cancer, we observed that upregulation of Treg and Th-2 cell responses developed in parallel after metastasis occurred in SNs [8]. Moreover, IL-10-producing Th-2 cells can regulate Th1 effector cell function, resulting in cancer-related imbalance of immune effector cells [31, 32, 34]. Collectively, although the relationship between Treg cells and IL-10 or TGFβ1 expression should be carefully interpreted, our results may indicate that TGFβ1 and especially IL-10 are involved in Treg-associated immunosuppression in breast cancer tissues. To our knowledge, this is the first report showing a significant correlation between FOXP3 and IL-10 or TGFβ1 expression by regression analysis in breast cancer tissue in situ. IL-10 or TGFβ1 expression in breast cancer tissue in situ should be further assessed in relation to Treg cells, Th2 cells, and tumor cells because of the complexity of the tumor microenvironment. This issue is an interesting question that is being currently addressed in our institute.

Chemotherapeutic agents that attenuate Treg cell function may allow successful chemotherapy for breast cancer, since Treg cells may play a large role in progression of breast cancer. There are several types of combination chemotherapy regimens for breast cancer treatment, and representative protocols often contain an alkylating agent cyclophosphamide [35]. Recent evidence has indicated that cyclophosphamide reduces the number of Treg cells [36, 37]. The chemotherapeutic activity of cyclophosphamide may be dependent, in part, on the downregulation of Treg cells in vivo. Bates et al. [11] have also pointed out the requirement of targeting Treg cells in breast cancer treatment for preventing late-relapses. More recently, not only chemotherapeutic agents but also molecular targeting agents including anti-CD25 [38], anti-GITR antibodies [39], and IL-2-immunotoxin conjugates [40] have been developed to effectively attenuate Treg cell function. Our results suggest that the success of these clinical investigations for Treg cell attenuation may enhance and improve the efficacy of breast cancer treatment in the future.

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