CD4+CD25+ Regulatory T-Cell Frequency in HER-2/neu (HER)-Positive and HER-Negative Advanced-Stage Breast Cancer Patients

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Abstract

Purpose: CD4+CD25[bright] regulatory T cells (Tregs) are increased in patients with several malignancies and correlate with disease stage and prognosis. Breast cancer patients represent a heterogeneous population with unpredictable disease progression even at advanced stages. Circulating Tregs in correlation with HER-2/neu (HER) status and treatment with chemotherapy, either alone or in combination with trastuzumab therapy, were monitored in advanced-stage breast cancer patients.

Experimental Design: Circulating Treg frequency and absolute counts of 46 HER+ and 28 HER-, stage III and IV, breast cancer patients before therapy and during trastuzumab therapy and/or chemotherapy have been compared with 24 healthy donors and correlated with plasma HER extracellular domain concentration and clinical outcome.

Results: Treg frequency in HER+ patients was significantly increased compared with both HER- patients and healthy donors. Trastuzumab therapy, with or without combined chemotherapy, resulted in a progressive decrease of circulating Tregs. Percentage change in Tregs statistically correlated with percentage change in plasma HER extracellular domain. Furthermore, decrease in Tregs correlated with either objective clinical response or stable disease, whereas increased Treg frequency during trastuzumab therapy coincided with disease progression. No statistically significant change in Treg frequency following chemotherapy was observed in HER- patients.

Conclusions: Treg cell frequency does not directly correlate with clinical stage in breast cancer, as stage III and IV HER+ and HER- patients exhibit significantly different Treg profiles. Trastuzumab therapy, either alone or combined with chemotherapy, results in decreased Treg frequency in HER+ advanced patients with an objective clinical response.

Cancer progression and metastasis has been correlated with the presence of immunosuppressive factors. Reports on the implication of suppressor/regulatory cells in cancer development and progression date back more than 3 decades (1, 2). The identification of a better-characterized regulatory subpopulation among CD4+ cells, constitutively expressing high levels of CD25, CTLA-4, GITR, and Foxp3, displaying anergy when stimulated by T-cell receptor cross-linking in vitro, and actively inhibiting CD4+CD25+ T cells, CD8+ T cells, dendritic cells, natural killer cells, and natural killer T and B cells in a cell to cell contact and dose-dependent manner (3), led to a recent intensification of investigation on the role of these cells on tumor development, growth, and escape from immunosurveillance.

The biology of CD4+CD25[bright]Foxp3+ regulatory T cells (Tregs) in murine tumor models seems to be rather straightforward. Increased frequency of Tregs at the tumorsite has been documented in several murine models, suggesting that the tumor itself actively promotes the increase of Tregs either by activating naturally occurring Tregs or by converting non-Tregs into Tregs (4). The systemic increase of Tregs in cancer-bearing mice is still controversial (5, 6).

In humans, increased numbers of Tregs, either at the tumor site, the tumor-draining lymph nodes, or in the circulation, have been reported in patients with several types of malignancy, including gastrointestinal malignancies (7–9), ovarian cancer (10–13), non–small cell lung carcinoma (10, 14), melanoma (15), renal cell carcinoma (15), glioma (16), Hodgkin’s lymphoma (17), and chronic lymphocytic leukemia (18). Increased numbers of Tregs have been correlated with greater disease burden and poorer overall survival (8, 13).
Therapeutic approaches in cancer treatment may affect Treg cell frequency and function. Interleukin-2 therapy has been found to induce expansion of Tregs in melanoma and renal carcinoma patients treated with high-dose interleukin-2 (15, 19). On the contrary, some chemotherapeutic agents, such as cyclophosphamide and fludarabine, have been observed to reduce the number and function of Tregs (3).

Breast cancer is the most common malignancy among women worldwide. The development of optimal consensus treatment guidelines for breast cancer requires comprehensive analysis of the results of randomized clinical trials and the interpretation of their clinical, biological, and personal relevance for individual patients (20).

Locally advanced breast cancer (LABC) and metastatic breast cancer (MBC) refer to a diverse and heterogeneous group of patients. Certain clinical characteristics have been used to predict which patients are likely to have a favorable or unfavorable course (21). Molecular prognostic and predictive factors have also been extensively evaluated to help individualize therapy of breast cancer more so than for any other solid tumors (22). For example, the value of HER-2/new (HER) amplification and/or overexpression is clearly recognized for selection of patients who are likely or not to benefit from trastuzumab. HER gene amplification and protein overexpression occur in ~25% of breast cancer patients, resulting in a clinically aggressive tumor type that is associated with shortened disease-free and overall survival (23). The monoclonal antibody trastuzumab, which is directed against an epitope on the external domain of the HER protein, has clinical activity in women with HER-overexpressing MBC and LABC when used as a single agent and, of particular interest, when used in combination with chemotherapy (24, 25).

However, even in breast cancer, molecular markers of prognosis and prediction are less than ideal, resulting in insufficient application of therapy that is either not needed or needed but ineffective. In the last years, major research effort is focused on identifying novel valuable markers for tailored treatment decision making. The role of immunosuppression in cancer evolution and treatment outcome is currently under thorough evaluation. Treg monitoring and manipulation in cancer patients has emerged as a new promising option (26). However, until now, there have been conflicting data in the literature about Tregs in breast cancer patients. In an early report (7), patients with stage I to III invasive ductal adenocarcinoma were found to have increased prevalence of circulating Tregs. Recently, node-negative breast cancer patients were found to have a greater percentage of circulating Tregs compared with normal subjects, with HLA-A2 patients having more Tregs than HLA-A2 patients (27). In contrast, Okita et al. (14) did not find increased percentage of CD4+CD25+ cells in the peripheral blood of breast cancer patients at different stages of the disease.

To further elucidate the perspectives of circulating Treg frequency in breast cancer patients, we studied two distinct patient subgroups based on HER expression (i.e., with HER+ or HER- tumors) at advanced stages of the disease before and during chemotherapy and/or antibody-based immunotherapy.

### Materials and Methods

**Patients.** Patients with MBC and/or LABC treated with chemotherapy and/or trastuzumab between June 2005 and June 2006 were eligible for participation. Peripheral blood from 24 healthy volunteers and 74 breast cancer patients (28 HER+ and 46 HER-) was collected. Chemotherapeutic drugs in both groups included taxanes (paclitaxel and docetaxel), carboplatin, gemcitabine, vinorelbine, and capcitabine used either as single agents or in combination. Conventional treatment schedules (weekly or triweekly administration of trastuzumab and/or chemotherapy) were applied in both HER+ and HER- groups of patients. Eligible patients underwent pretreatment imaging within 4 weeks of starting therapy. The trial was approved by the institutional review board, and all patients were required to give written informed consent.

**Study design.** Patients with a histologic diagnosis of invasive breast carcinoma and measurable locally advanced or metastatic disease were eligible. HER overexpression was confirmed either by immunohistochemical staining of 3+ or positivity by the fluorescence in situ hybridization technique. In patients with 2+ HER overexpression by immunohistochemical staining, confirmatory fluorescence in situ hybridization testing was required. Peripheral blood samples from cancer patients were collected before treatment initiation and before every other cycle for all patients, although blood samples were also collected from healthy volunteers. Clinical outcome was determined by computed tomography scans of the chest, abdomen, and pelvis and by magnetic resonance imaging of the brain according to Response Evaluation Criteria in Solid Tumors criteria.

**Quantification of absolute cell numbers.** Absolute counts of Tregs were done using TrueCount tubes (Becton Dickinson) and lysis-no-wash methods as described by the manufacturer. In brief, 50 µL of whole blood (with anticoagulant) were stained for 15 min at room temperature with pretitrated combinations of peridinin chlorophyll protein–conjugated anti-CD45, FITC-labeled anti-CD4, phycoerythrin-labeled anti-CD25 (Becton Dickinson); RBCs were lysed; and samples were analyzed on a FACSCalibur (Becton Dickinson) flow cytometer using CellQuest software (Becton Dickinson).

**Phenotypic characterization of Tregs.** Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll-Hypaque centrifugation using standard procedures. Four-color flow cytometry analysis was done on peripheral blood mononuclear cells using the following antibodies: FITC-labeled anti-CD4, CD45RO, CD45RA, CD62L, and CD27; phycoerythrin-labeled anti-CD25 (Becton Dickinson); allophycocyanin-labeled anti-GITR (R&D Systems); and CD4-allophycocyanin (Becton Dickinson). Intracellular Foxp3 was determined with allophycocyanin-conjugated anti-Foxp3 (eBioscience) according to the manufacturer’s instructions.

**Peripheral blood Treg isolation and functional characterization.** CD4+ cells were isolated from peripheral blood mononuclear cells by negative selection using a combination of anti-glycoporphin-A, anti-CD14, anti-CD56, anti-CD19, and anti-CD6 monoclonal antibodies and anti-mouse Ig-coated microbeads and sequentially passed through an LS and an LD column (Milteny Biotec). CD4+CD25bright and CD4+CD25- cell subpopulations were separated by cell sorting from the purified CD4+ cell fraction stained with anti-CD4-FITC and anti-CD25-phycoerythrin with a Coulter Epics Alr cell sorter (Beckman Coulter). The purity of the isolated populations was always >98%

CD4+CD25bright Tregs were expanded, for 7 days, with CD3/CD28 beads (T-cell expander, Dynal Biotech) at a 1:1 cell/bead ratio in αMEM with 10% FCS, l-glutamine, gentamicin (all from Life Technologies Ltd.), and 100 IU/mL human interleukin-2 (Proleukin, Chiron B.V.) and tested for suppressive activity. In brief, 5 × 10⁴ autologous CD4+CD25- cells were mixed with 5 × 10⁴ irradiated (3,000 rad) alloimmune mature dendritic cells, produced as described previously (28), with or without 5 × 10⁴ expanded CD4+CD25bright cells, in U-bottomed 96-well plates for 5 days, and [3H]thymidine (30-40 Ci/mmol; Amersham Pharmacia Biotech) incorporation (1 µCi/well, added for the last 16 h of culture) was measured.

**Plasma HER extracellular domain and cytokine determination.** Plasma samples were collected before the initiation of therapy and before each cycle of treatment. Commercially available ELISA kits were used for measurements of interleukin-10 (Diaclone), transforming growth factor-β (R&D Systems), interleukin-8 (R&D Systems), and interferon-γ (R&D Systems).
growth factor-β (TGF-β; R&D Systems), and HER extracellular domain (HER-ECD; Immuno 1, Bayer Diagnostics).

Statistical analysis. GraphPad Prism version 4 (GraphPad Software, Inc.) was used for the statistical analysis of data. The two-tailed Mann-Whitney U test, Wilcoxon matched pairs test, and Spearman correlation test were used for statistical evaluation, and P values ≤0.05 were considered statistically significant.

Results

Patients’ characteristics and clinical results. Seventy-four patients and 24 healthy volunteers consented to participate in the study. The summary of patients’ characteristics is presented in Table 1. Twenty (27%) patients had LABC and 54 (73%) patients had MBC. Forty-six (62%) patients had HER+ tumors. The group of HER+ patients slightly differed from the HER- patient group in age (53.33 ± 11.57 versus 58.52 ± 11.41; P = 0.0424). Objective clinical responses were documented in 12 patients with LABC and in 15 patients with MBC. There were 3 complete responses and 24 partial responses, whereas stable disease was documented in 21 MBC and 9 LABC patients. Twenty of 46 HER+ patients had objective clinical response (3 complete responses and 17 partial responses), whereas only 7 of 28 HER- patients had partial clinical responses. Progressive disease was documented in eight HER+ and five HER- patients.

Tregs are increased in patients with HER+ tumors. We determined the absolute numbers and the percentages of circulating Tregs in the peripheral blood of breast cancer patients with advanced disease (LABC or MBC).

Human Tregs were defined as lymphocytes expressing CD25 higher than the CD4-CD25+ population and CD4 slightly lower than CD4+CD25intermediate cells (29). This cell population was further phenotypically and functionally characterized (Fig. 1). CD4+CD25bright cells were found to be mostly Foxp3+, CD45RO+, CD45RA-, CD62L+, CD27+, and GITR+ and to suppress alloresponses of CD4+CD25+ autologous T cells.

When the percentage of Tregs in total CD4+ T cells was determined in all, HER+ and HER-, 74 breast cancer patients and compared with the healthy control group, a slight, although statistically significant, increase was observed (7.94 ± 2.56 versus 6.77 ± 1.29; P = 0.0407; Fig. 2A). When HER+ and HER- patients were evaluated separately, striking differences were observed. The frequency of Tregs among total CD4+ cells in HER- patients (6.15 ± 1.36) did not differ from that of healthy donors (P = 0.0554). On the contrary, HER+ patients exhibited significantly increased frequencies of circulating Tregs (8.92 ± 2.54; P = 0.0002).

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Fig. 1. CD4+CD25bright T cells represent Tregs. A, dot plot of total peripheral blood mononuclear cells. Gray, CD4+ T cells. Tregs are gated based on relatively low/intermediate expression of CD4 within the CD4+ T-cell compartment and high CD25 expression. B, standard contour plots showing the phenotypic characterization of Tregs from a representative HER+ patient. PE, phycoerythrin; APC, allophycocyanin. C, sorted and in vitro – expanded CD4+CD25bright Tregs suppress alloresponses of autologous CD4+CD25 T cells (data from a representative normal donor). DC, dendritic cells.
As expected, HER+ \((n = 29)\) patients had higher plasma HER- ECD concentrations than HER- \((n = 23)\) patients (31.32 ± 53.19 versus 3.68 ± 3.34; \(P = 0.0001\); Fig. 2B). Plasma concentrations of TGF-\(\beta\), known to promote Tregs (30) and found to be increased in breast cancer patient sera (27), did not differ between HER- and HER+ patients (4,983 ± 2,981 versus 4,359 ± 2,999; \(P = 0.2805\); Fig. 2C). Interleukin-10 was not detectable in breast cancer patients’ plasma, either positive or negative for HER expression (data not shown).

The absolute number of circulating Tregs did not correlate with disease status in breast cancer patients but rather was related to the number of circulating CD4+ T cells: patients with decreased numbers of total CD4+ cells (and total lymphocyte counts; data not shown) had relatively lower numbers of circulating Tregs than patients with higher CD4+ cell counts or healthy donors (Fig. 3A and B).

The percentage of Tregs in HER+ and HER- patients strongly correlated with plasma HER-ECD concentration \((n = 52; P = 0.0001)\); a not statistically significant correlation with plasma TGF-\(\beta\) amount was observed \((P = 0.0793; \text{Fig. 3C and D})\).

Treg frequency is decreased after trastuzumab antibody-based immunotherapy. HER+ breast cancer patients with MBC or LABC are routinely treated with trastuzumab, either as monotherapy or combined with chemotherapy. In this group of patients, the frequency of Tregs was monitored before and after each cycle of trastuzumab therapy. Samples were collected the day before the next cycle of treatment. The percentage of circulating Tregs among the CD4+ population significantly decreased from the first cycle of trastuzumab administration (data not shown), reaching normal levels after the third cycle (from 8.92 ± 2.54 to 6.98 ± 1.87 after the third cycle; \(P = 0.0018\), Wilcoxon matched pairs test, compared with samples before treatment; \(P = 0.7029\), Mann-Whitney \(U\) test, compared with healthy volunteers). No difference in the frequency of Tregs was observed in HER+ patients treated with similar chemotherapeutic regimens as HER+ patients. As shown in Fig. 4A, when HER+ patients were evaluated according to their clinical response after two or three cycles of therapy with trastuzumab (with or without chemotherapy), Treg frequency was found to be decreased in 19 of 20 patients responding with partial response or complete response \((P = 0.0001,\) Wilcoxon matched pairs test), whereas only 10 of 20 patients with stable disease exhibited reduced percentage of Tregs \((P = 0.0030)\). In contrast, in HER- patients, either with partial response or stable disease, no statistically significant differences were observed in Treg percentage \((P = 0.6875\) and 0.6554, respectively), implying that the decrease observed in Tregs after trastuzumab therapy might be attributed to this particular antibody treatment and not to chemotherapy alone. Furthermore, after sequential measurements of Treg frequency in normal volunteers, at time intervals expanding from 3 to 11 months, no statistically significant differences could be detected \((P = 0.3652)\).

It has been recently documented that a decrease >20% of the pretreatment value of serum/plasma HER-ECD correlated with clinical response to trastuzumab therapy (31). In this study, the percentage change in plasma HER-ECD concentration strongly correlated with the percentage change in Treg frequency \((P = 0.0006)\) after treatment with trastuzumab (Fig. 4C). No correlation of the percentage change in Tregs with the percentage change in TGF-\(\beta\) after trastuzumab therapy was observed \((P = 0.9534; \text{Fig. 4D})\).

Disease recurrence during trastuzumab therapy correlates with increase in Treg frequency. In the group of patients initially responding to trastuzumab by decreased Treg frequency after one to three cycles of antibody therapy and partial response or stable disease, disease progression either coincided or was preceded by an increase in Treg frequency. In Fig. 5, the follow-up measurements of Tregs from six patients with recurrence are...
presented. In four of these patients, the increase in Tregs was observed at least 1 month before the clinical substantiation of disease progression. In the two remaining patients with progressive disease, increased Treg frequency was determined after progressive disease confirmation, although in these particular patients, among the first patients enrolled in this study, blood sampling was requested after clinical evaluation. Two additional HER+ patients developed progressive disease, but they were not found to respond to trastuzumab therapy by either Treg frequency or plasma HER-ECD decrease (data not shown).

Discussion

The present study is the first, to the best of our knowledge, to evaluate circulating Tregs in correlation with HER status in breast cancer patients. We found that MBC patients with HER+ tumors exhibited an overall significantly increased frequency of circulating Tregs within the CD4+ T-cell compartment compared with healthy volunteers. On the contrary, the percentage of circulating Tregs did not differ between HER- patients and healthy donors. Furthermore, the influence of anti-HER antibody-based immunotherapy with trastuzumab and/or chemotherapy on Treg frequency was also examined. Trastuzumab therapy, but not chemotherapy alone, led to an overall reduction, to normal levels, in the frequency of Tregs. Remarkably, a good clinical response to trastuzumab therapy was associated with a significant reduction in Treg frequency, whereas disease recurrence correlated with a significant increase in the percentage of circulating Tregs.

Increased Treg frequency has been associated with advanced stages and poor prognosis in cancer patients (32), although differences among patients with various malignancies or even the same type of cancer have not yet been explained (3). Our data show that, within a defined group of breast cancer patients with progressed disease and similar clinical outcome, there is at least one factor that separates these patients into two different subgroups according to circulating Treg frequency—the overexpression of HER, a self-antigen expressed by normal epithelial cells and several types of cancer cells, which is released by cells into the systemic circulation (33). The specificity of Tregs in cancer patients remains unclear (32), although some evidence supports that Tregs associated with tumors are tumor antigen-specific Tregs (34). Whether systemically circulating tumor-associated self-antigens at increased concentrations, such as HER, carcinoembryonic antigen, and prostate-specific antigen, induce the expansion of circulating antigen-specific Tregs in cancer patients as described for endogenous systemic antigens (35, 36) remains to be elucidated.

In spite of major advances in screening, surgery, radiation, endocrine therapy, and chemotherapy for patients with early-stage breast cancer, there has been only modest progress in improving survival for women with advanced-stage disease. The median survival for patients with metastases remains 18 to 24 months. New treatments for MBC have almost certainly extended survival, but the overall improvement has been modest and to a certain extent due to advances in imaging and the lead-time bias associated with the earlier diagnosis of metastases (37). Trastuzumab, a humanized monoclonal antibody directed against the ECD of the transmembrane glycoprotein HER, provides clinicians with a valuable tool in the treatment of women with HER+ LABC and MBC (38). However, certain aspects relating to trastuzumab administration should be continued in the stable or responding
patient. It is also unclear whether continuing trastuzumab administration following disease progression in patients receiving trastuzumab and chemotherapy improves response to subsequent chemotherapy treatment. Another important aspect of trastuzumab use is the obvious need for early recognition of patients not responding or even developing resistance (39, 40).

Serum concentrations of HER-ECD have been extensively evaluated as a candidate predictive marker during trastuzumab treatment in breast cancer patients (41). Although serum HER-ECD levels represent a promising surrogate marker, various technical variables limit its wide application. The most important issues that have to be clarified are the optimal serum HER-ECD concentration threshold for differentiation between positive and negative HER status and the relatively low sensitivity and specificity of the method currently applied (42). Recently, a pooled analysis of seven clinical trials of first-line trastuzumab therapy (with or without chemotherapy) with serial measurements of serum HER-ECD indicated that patients with <20% decrease in serum HER-ECD levels have lower clinical benefit (31).

In this study, we detected a strong positive correlation between the percentage change in Treg frequency and the percentage change in plasma HER-ECD during trastuzumab therapy. Although the specificity of these circulating Tregs has not been investigated, the finding that changes in circulating Treg frequency paralleled with changes in plasma HER-ECD implies that at least a fraction of these cells recognize and respond to systemically circulating HER protein. The mechanism(s) underlying the reduction in the percentages of circulating Tregs in patients undergoing trastuzumab-based immunotherapy is at present unclear. Because patients responding to trastuzumab therapy by exhibiting a decrease in plasma HER-ECD also show a decrease in Treg frequency, it would be tempting to speculate that HER might be eliminated from the circulation by antigen-trastuzumab complex formation and uptake by phagocytes through FcγR binding. The diminished amount of circulating antigen would thus possibly result in decreased expansion of antigen-specific Tregs (35, 36). HER-trastuzumab complex formation could also lead to activation, maturation, and enhanced antigen cross-presentation by antigen-presenting cells (43, 44) and, along with decreased Treg frequency, could account for an enhanced antitumor response, possibly contributing to the clinical status amelioration of patients. This hypothesis is currently under investigation.

In this study, therefore, we present novel data about circulating CD4+CD25+ Tregs in breast cancer patients, either expressing HER or not, which may improve our understanding of the mechanisms underlying the systemic generation of Tregs in cancer. Our findings strongly support the concept that malignant disease severity does not necessarily correlate with

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**Fig. 4.** Treg frequency is decreased after trastuzumab immunotherapy. Treg frequencies were monitored before and after treatment (following the second or third cycle of trastuzumab therapy and/or chemotherapy) in HER+ and HER- patients and classified according to their clinical response (PR, partial response; CR, complete response; SD, stable disease). A, sequential measurements of Treg frequencies in normal donors at two randomly selected time points were used as a control. Correlation of the percentage change of Treg frequency with the percentage change in plasma HER (B) and TGF-β (C) after trastuzumab therapy in HER+ patients. Data were analyzed with the Wilcoxon matched pairs test.
increased frequency of circulating Tregs but rather that this depends on particular characteristics of the tumor, such as systemic release of tumor-associated self-antigens. Moreover, expanding on the information obtained from the current study could help to clarify which patients would benefit from a systemic elimination of Tregs by agents, such as denileukin diftitox (Ontak), thus preventing the unnecessary exposure of patients to the side effects of such treatments. Furthermore, our data suggest that antibody-based immunotherapy, apart from eliminating cancer cells, which is the primary goal, might also indirectly contribute to the elimination of tumor-specific Tregs, thereby improving immune responses against tumors when combined with active immunotherapy. Finally, the measurement of circulating Tregs could serve as a valuable surrogate marker for assessing the response to trastuzumab therapy.

Acknowledgments

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