

Evaluation of the Predictive Role of Tumor Immune Infiltrate in Patients with HER2-Positive Breast Cancer Treated with Neoadjuvant Anti-HER2 Therapy without Chemotherapy



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ABSTRACT

Purpose: Tumor-infiltrating lymphocytes (TIL) are associated with benefit to trastuzumab and chemotherapy in patients with early-stage HER2⁺ breast cancer. The predictive value of TILs, TIL subsets, and other immune cells in patients receiving chemotherapy-sparing lapatinib plus trastuzumab treatment is unclear.

Experimental Design: Hematoxylin and eosin-stained slides ($n = 59$) were used to score stromal (s-)TILs from pretreatment biopsies of patients enrolled in the neoadjuvant TBCRC006 trial of 12-week lapatinib plus trastuzumab therapy (plus endocrine therapy for ER⁺ tumors). A 60% threshold was used to define lymphocyte-predominant breast cancer (LPBC). Multiplexed immunofluorescence (m-IF) staining (CD4, CD8, CD20, CD68, and FoxP3) and multispectral imaging were performed to characterize immune infiltrates in single formalin-fixed paraffin-embedded slides ($n = 33$).

Results: The pathologic complete response (pCR) rate was numerically higher in patients with LPBC compared with

patients with non-LPBC (50% vs. 19%, $P = 0.057$). Unsupervised hierarchical clustering of the five immune markers identified two patient clusters with different responses to lapatinib plus trastuzumab treatment (pCR = 7% vs. 50%, for cluster 1 vs. 2 respectively; $P = 0.01$). In multivariable analysis, cluster 2, characterized by high CD4⁺, CD8⁺, CD20⁺ s-TILs, and high CD20⁺ intratumoral TILs, was independently associated with a higher pCR rate ($P = 0.03$). Analysis of single immune subpopulations revealed a significant association of pCR with higher baseline infiltration by s-CD4, intratumoral (i-) CD4, and i-CD20⁺ TILs.

Conclusions: LPBC was marginally associated with higher pCR rate than non-LPBC in patients with lapatinib plus trastuzumab treated HER2⁺ breast cancer. Quantitative assessment of the immune infiltrate by m-IF is feasible and may help correlate individual immune cell subpopulations and immune cell profiles with treatment response.

Introduction

We and others have shown that a chemotherapy-sparing neoadjuvant therapy regimen containing dual anti-HER2 therapy with lapatinib plus trastuzumab can achieve pathologic complete response (pCR) in as many as one-third of patients with HER2-positive (HER2⁺) breast cancer (1–3). Deescalation of treatment may decrease toxicity and cost in a substantial number of patients without compromising the clinical outcomes. Germane to the success of

deescalation approaches is the accurate selection of patients who are likely to benefit from such a strategy. Identifying major determinants of sensitivity and resistance to neoadjuvant lapatinib plus trastuzumab therapy can aid in distinguishing patients who can be optimally treated with this regimen from those who should also receive chemotherapy in addition to anti-HER2 agents (4).

The immune microenvironment of a tumor has recently emerged as a potential modulator of response to treatment and cancer

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Translational Relevance

While several clinical trials have suggested that combination of anti-HER2 agents without chemotherapy may be an effective treatment approach for a subset of patients with HER2⁺ breast cancer, there is still a lack of appropriate biomarkers to accurately select these patients. In addition, while the distribution of tumor-infiltrating lymphocytes has been proven to predict therapeutic response and long-term outcomes in HER2⁺ breast cancer, novel quantitative methods to more precisely assess their density and distribution are also lacking. Our study demonstrates that multiplexed imaging of pretreatment tumor specimens by multiplexed immunofluorescence is feasible and effective in quantifying different subtypes of lymphocytic infiltrates, and may allow for the identification of an immune infiltrate profile characterized by high CD4⁺, CD8⁺, and CD20⁺ cells, which is independently and significantly associated with pCR. Overall, our findings suggest that this technique bears the potential as a quantitative and qualitative assay to precisely delineate the immune infiltrates in tumor specimens and aid in predictive biomarker discovery.

progression (5, 6). Tumor-infiltrating lymphocytes (TIL) are mononuclear immune cells that infiltrate tumor tissue and have been described in most types of solid tumors, including breast cancer (5, 6). In the neoadjuvant setting, while high levels of TILs at baseline are associated with increased pCR rates and/or better event-free survival in patients with HER2⁺ breast cancer in some trials (7–9), these reports are not consistent in all studies (10–14). In some studies, TILs were significantly associated with pCR in univariate but not multivariate analysis (11, 12). These somewhat conflicting results about the predictive value of TILs in HER2⁺ breast cancer may be attributed either to the lack of an appropriate quantitative approach and/or a standard cutoff for TILs, or to the differences in the treatment regimens among the studies. Because most of these neoadjuvant clinical trials treated patients with chemotherapy in combination with different anti-HER2 agents, the predictive value of TILs for chemotherapy-sparing anti-HER2 therapy in HER2⁺ breast cancer patients is still unclear.

TILs are usually detected using standard hematoxylin and eosin (H&E)-stained tissue sections under light microscopy and classified as either stromal-TILs (s-TILs) or intratumoral-TILs (i-TILs; refs. 5, 15), following the International TILs Working Group guidelines (15). S-TILs are the lymphocytes present in tumor stroma, whereas i-TILs are those in direct contact with the tumor epithelial cells (15). In breast cancer, s-TILs have been demonstrated to be the predominant population and are considered a more suitable predictive and prognostic marker compared with i-TILs (15). Although the evaluation of TILs by standard H&E staining is simple, inexpensive, and to some extent reproducible, limitations of this approach include its semiquantitative nature, and its inability to discriminate immune cell subtypes and their spatial organization within the tumoral architecture. The limitations of manual TIL assessment may be overcome by IHC/immunofluorescence (IF) staining and digital image analysis, which can provide a more accurate measurement and phenotyping of immune cells in tissue sections.

In this study, we measured s-TILs in baseline tumor samples from patients on the TBCRC 006 trial, treated with neoadjuvant lapatinib plus trastuzumab with endocrine therapy in ER-positive cases for 12 weeks, by the standard H&E-based method, and investigated their

role in predicting response to neoadjuvant anti-HER2 treatment without chemotherapy. We also employed an advanced multispectral imaging-based technology in a subset of tumor samples, to characterize stromal and intratumoral immune cell subpopulations in a quantitative manner and to define their association with treatment outcome.

Materials and Methods

Patients and specimens

The phase II neoadjuvant clinical trial TBCRC 006 (1) is a multicenter single-arm study conducted through the Translational Breast Cancer Research Consortium (Fig. 1). Institutional review board and scientific committee approval were obtained at the lead site (Baylor College of Medicine, Houston, TX) and other participating sites. Written informed consent was obtained from all patients. This study was performed in compliance with the Declaration of Helsinki and all applicable US Federal and international regulations and ethical standards. Sixty-six patients with stage II/III HER2⁺ invasive breast cancer were enrolled, and 64 were eligible and evaluable for response. HER2 positivity was assessed by IHC or FISH, according to the 2007 American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines (16). Patients were treated for 12 weeks with lapatinib (1,000 mg orally every day) and trastuzumab (4 mg/kg loading dose followed by 2 mg/kg per week). Patients also received the aromatase inhibitor letrozole 2.5 mg orally once per day (combined with a luteinizing hormone–releasing hormone agonist of choice in premenopausal women) if their tumor was estrogen receptor (ER) and/or progesterone receptor (PR) positive by IHC (according to ASCO/CAP 2010 guidelines; ref. 17).

Tumor biopsies were collected before treatment (baseline) and placed in formalin for subsequent paraffin embedding (formalin-fixed paraffin embedded tissue, FFPE) or flash frozen on dry ice. Median tumor size was 6 cm (range, 1.5–30 cm). The overall pathologic complete response (pCR) rate was 27% (36% in ER-negative tumors and 21% in ER-positive tumors).

Pathologic assessment

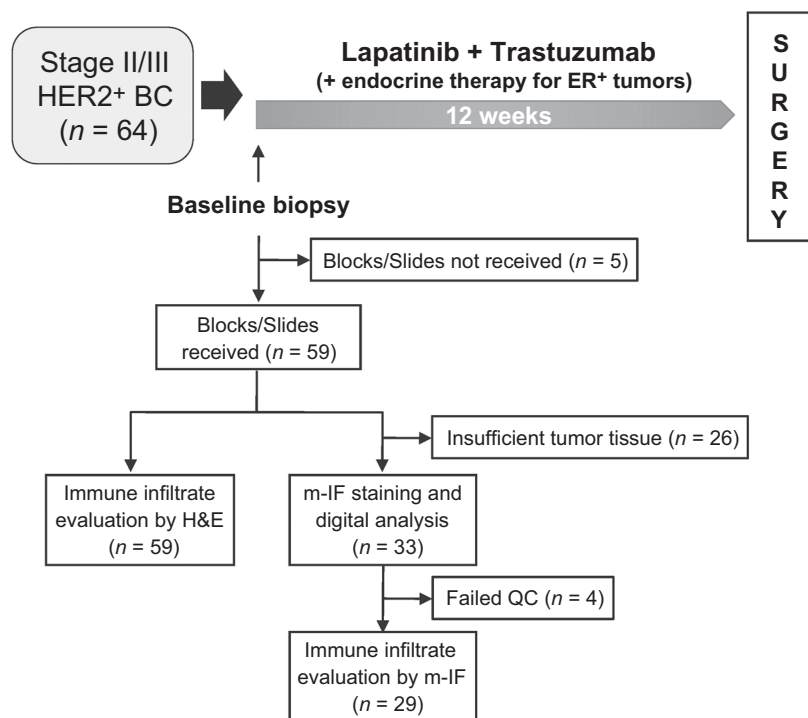
ER, PR, Ki67, and PTEN evaluation was performed by IHC; HER2 evaluation was by IHC and FISH assay; and *PIK3CA* mutational analysis was performed on baseline samples as described previously (1, 18, 19).

Standard assessment of TILs in H&E sections

Visual assessment of TILs was performed using full-face H&E slides by light microscopy according to the 2014 Guidelines from the International TILs Working Group (15). Briefly, all mononuclear cells, including lymphocytes and plasma cells and excluding granulocytes and other polymorphonuclear leukocytes, were identified as TILs. TILs within the borders of the invasive tumor stroma (s-TIL) were quantified as a percentage of occupied stromal areas (% s-TILs). TILs around ductal carcinoma *in situ*, in necrotic areas, outside tumor border, and in normal breast tissue were not quantified. A threshold of 60% was used to distinguish LPBC from non-LPBC. Of the 64 patients enrolled on the TBCRC 006 trial, slides from baseline core biopsies were available for 59 (92.2%) patients.

Multispectral fluorescent IHC

Multiplexed IF (m-IF) staining and multispectral image analysis (20) were also performed. Briefly, 4- μ m thick FFPE slides were deparaffinized in a Leica autostainer using the following protocol:

**Figure 1.**

Evaluable tumor specimens for baseline immune infiltrate analysis in the TBCRC 006 trial. Patients enrolled in the TBCRC 006 trial were treated for 12 weeks with neoadjuvant lapatinib plus trastuzumab (with endocrine therapy in ER-positive cases) followed by surgery. A total of 59 baseline tumor samples (pretreatment) were analyzed for immune infiltration: all the samples by H&E staining and 33 also by multiplexed immunofluorescence. QC, quality control.

xylene 10 minutes, 100% ethanol 10 minutes, 95% ethanol 5 minutes, 70% ethanol 5 minutes, ddH₂O briefly, 10% buffered formalin 10–20 minutes, ddH₂O briefly. Antigen retrieval was performed using citrate buffer pH 6.0 and microwave treatment (45 seconds 100% power, 15 minutes 20% power). Slides were blocked with Antibody Diluent (Biogenex Technologies) for 10 minutes. Primary antibodies were diluted in Antibody Diluent and incubated for 30 minutes at room temperature. Primary antibodies (Supplementary Table S1) were subsequently removed by vacuum, and slides were washed in TBST. Supplementary Table S1 reports the list of primary antibodies used in this study. Anti-rabbit or anti-mouse IgG, HRP-linked antibodies (Life Technologies) were added drop-wise to slides. Slides were incubated for 10 minutes at room temperature and then washed in TBST. Tyramide (TSA)-conjugated fluorophores (Opal, PerkinElmer, Inc.) were added to slides at 1:50 dilution and incubated for 10 minutes at room temperature. TSA was vacuumed off and slides were washed in TBST. This process was repeated recursively for 6 antibodies. 4',6-diamidino-2-phenylindole (Life Technologies) was diluted 1:500 in TBST and added to slides. Slides were incubated for 10 minutes at room temperature. Slides were then rinsed with ddH₂O, cover slipped with VECTASHIELD Hard Mount (Vector Laboratories), and stored at 4°C in a covered slide box. Slides were scanned using a Vectra automated multispectral microscope and images were analyzed using the inForm analysis software (PerkinElmer). For the majority of the biopsies, the entire tumor and tumor microenvironment were included in the analyzed fields (5 fields 2 × 2, using 20× objective). For biopsies of larger size, 5 fields (2 × 2, using 20× objective) were selected for a significant sampling of the tumor and tumor microenvironment. A tissue segmentation algorithm was applied to define stromal and tumoral areas (20). Then, single or multiple IF markers were used to localize, phenotype, and quantitate the density (number of positive cells per mm²) of specific cell types in both the stromal and tumoral areas (20).

Hierarchical clustering of immune cell markers

Distance metric equal to (1 – correlation) for standardized log₂ values (counts/mm²) from each case were used to conduct an unsupervised hierarchical clustering of the five m-IF immune markers. A centroid-linkage hierarchical clustering was performed using the dChip software (<https://sites.google.com/site/dchipsoft/home>).

Statistical analysis

Spearman correlations, Fisher exact test, and Wilcoxon rank sum test were used to examine the associations between immune cells assessed by H&E or by m-IF and other tumor baseline biomarkers. The predictive value of s-TILs by H&E was tested by considering s-TILs as a continuous variable, and then according to the LPBC versus non-LPBC categorical definition. Association of immune cell variables with pCR was determined by univariate and multivariable logistic regression analysis. ORs with a 95% confidence interval (95% CI) were estimated. In the multivariable model, the likelihood ratio test was used to evaluate the contribution of s-TILs, immune profiles by m-IF, and the clinicopathologic factors (age: ≤50 years, >50 years; categorical tumor size: ≤5 cm, >5 cm; positive nodes: yes, no). All statistical tests were two-sided and considered significant when $P \leq 0.05$.

Results

Visual assessment of baseline s-TILs by H&E

Pretreatment s-TILs evaluation using H&E slides was conducted on 59 available samples out of the 64 patients who were evaluable for pCR (Fig. 1). In our cohort, 12 patients (20%) exhibited an LPBC profile and 37 (80%) presented with a non-LPBC profile (Supplementary Fig. S1). Patient demographic and clinical characteristics across baseline LPBC- and non-LPBC groups are reported in (Supplementary Table S2). The two groups did not significantly differ in age, race, ethnicity, menopausal status, performance status, tumor size, axillary lymph node

Table 1. Association between pCR and s-TILs.

	pCR n (%)	Non-pCR n (%)	OR (95% CI)	Fisher exact P
Non-LPBC (n = 47)	9 (19.1)	38 (80.9)	1	0.0575
LPBC (n = 12)	6 (50)	6 (50)	4.2 (1.1-16.2)	

involvement, expression of hormonal receptors (ER and PR), and deregulation of PI3K/PTEN pathway (Supplementary Table S2). We further investigated the correlations between s-TILs as continuous variable and levels of ER, PR, HER2 (HER2 H-score, and HER2/CEP17 ratio) and Ki67 (Supplementary Table S3). Our analysis showed a positive association of s-TILs with Ki67 (Spearman correlation = 0.304, $P = 0.0241$), but not with other tumor biomarkers (Supplementary Table S3).

We next evaluated the association of s-TILs with pCR. Levels of s-TILs considered either as a categorical or a continuous parameter were not significantly associated with pCR (high vs. low, $P = 0.057$; 10% increase OR = 1.22; 95% CI, 0.97–1.53, $P = 0.094$). The pCR rate, however, was numerically higher in patients with LPBC compared with patients with non-LPBC (50% vs. 19%) (Table 1). Similar results were found when we investigated the association of s-TILs with pCR in ER-positive or ER-negative subgroups (Supplementary Table S4).

Quantitative assessments of baseline immune cells by m-IF staining and digital analysis

Next, we characterized and quantified various subtypes of stromal and intratumoral immune infiltrates [cytotoxic T cells (CD8⁺), helper T cells (CD4⁺/FoxP3⁻), regulatory T cells (Treg; FoxP3⁺), B cells (CD20⁺), and macrophages (CD68⁺)] using m-IF labeling and digital image analysis of 33 available tumor biopsies at baseline (Figs. 1 and 2A). In 29 of 33 m-IF-stained samples, we successfully measured the density of s-TILs and i-TILs using the multispectral imaging data. One sample did not show the presence of tumor cells while three samples failed the m-IF staining quality control check. Importantly, baseline clinicopathologic characteristics did not significantly differ between groups of patients with ($n = 29$) or without ($n = 30$) m-IF data (Supplementary Table S5). Mean baseline values of s- and i-TIL density for the whole group are shown in Supplementary Table S6. The density of total s-TILs (total number of cells positive/mm²) determined by m-IF significantly and positively correlated with the percentage of s-TILs assessed on H&E-stained tissue sections from the same breast cancer specimens ($r = 0.55$, $P = 0.002$; Fig. 2B). Furthermore, the densities of individual immune subtypes within stromal and intratumoral compartments were highly correlated (Supplementary Table S7). Infiltration of CD4⁺, CD8⁺, and CD20⁺ TILs showed strong positive correlations among one another in stromal as well as intratumoral areas (Supplementary Table S7). Finally, we also observed a significant correlation between levels of s-TILs assessed by H&E and s-CD20⁺, i-CD20⁺, s-CD8⁺, i-CD8⁺, and i-CD4⁺ cells (Supplementary Table S7).

Exploratory analysis of TILs as a predictor of response to chemotherapy-sparing neoadjuvant lapatinib plus trastuzumab therapy in HER2⁺ breast cancer

To investigate the immune subpopulation profiles and their relationship with response to dual anti-HER2 therapy, we performed an unsupervised hierarchical clustering using the five immune cell markers employed to characterize immune infiltrates. Clustering analysis

generated two groups of tumors arbitrarily defined as cluster 1 ($n = 15$, 51.7%) and cluster 2 ($n = 14$, 48.3%) (Fig. 2C). Cluster 2 group represented tumors with higher median levels of CD4⁺, CD20⁺, and CD8⁺ s-TILs and higher CD20⁺ i-TILs compared with cluster 1 (Supplementary Table S8). No significant differences were found regarding clinicopathologic characteristics between the 2 cluster groups (Supplementary Table S9). A significant predictive value for pCR was demonstrated for the cluster 2 profile in a univariate analysis (OR: 14; 95% CI, 1.43–137.2, $P = 0.0235$; Table 2). Importantly, independent predictive value of cluster 2 was also confirmed in a logistic regression multivariate analysis including age, tumor size, and axillary lymph node involvement (Table 2). In evaluating individual immune subsets, we found that patients who achieved pCR had higher levels of s-CD4⁺ ($P = 0.006$), i-CD4⁺ ($P = 0.01$), and i-CD20⁺ ($P = 0.04$) cells compared with patients who did not (Fig. 3A). Conversely, s- or i- infiltration of CD8⁺, CD68⁺, FoxP3⁺, and s-CD20⁺ cells (Fig. 3A) as well as three different TIL ratios (FoxP3⁺/CD4⁺, FoxP3⁺/CD8⁺, and CD8⁺/CD4⁺) did not correlate with treatment outcome (data not shown).

Within the subset of pretreatment tumor samples analyzed by m-IF, the median level of s-TILs by H&E was higher in patients who achieved a pCR after neoadjuvant LT compared with those who did not (Fig. 3B). Furthermore, a significant association between s-TILs either as a continuous ($P = 0.0371$) or categorical ($P = 0.0188$) variable and pCR was observed in univariate analysis (Supplementary Table S10). In multivariable analyses, only the LPBC profile was found to be independently associated with pCR (Supplementary Table S10). Finally, using a likelihood ratio test, we investigated the added predictive information of cluster profiles to standard s-TILs and clinical prognostic factors. Our exploratory analysis revealed that cluster profiles added significant predictive information beyond that provided by conventional clinicopathologic variables and s-TILs as a continuous variable ($P = 0.018$). In contrast, the cluster profile did not add additional information to the model with s-TILs as categorical variable ($P = 0.088$).

Discussion

In the last few years, the tumor immune microenvironment has become an area of intense research in breast cancer (5, 6, 22). The tumor–host immune interaction may be particularly significant in determining the therapeutic effect of the mAbs trastuzumab and/or pertuzumab, because their therapeutic efficacy depends, in part, on innate and adaptive immune-mediated mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (22). This tumor–host immune interaction may be even more important in the context of lapatinib plus trastuzumab therapy, which was used in our study. In fact, it has been suggested that the synergistic effect of lapatinib and trastuzumab may stem, at least partly, from an increased trastuzumab-mediated ADCC due to a lapatinib-induced accumulation of HER2 at the cell surface, as shown in preclinical models (22). Previous reports have shown that in patients with HER2⁺ breast cancer, high levels of TILs and the activation of immune pathways assessed by immune-related genes and gene signatures have been associated with more favorable prognosis in the adjuvant and metastatic settings as well as with increased rates of pCR upon neoadjuvant treatment (5, 6, 8, 9, 21–24). Most published data, however, stem from trials that studied regimens of anti-HER2 therapy in combination with chemotherapy. Although some of these trials included a chemotherapy alone control arm, the predictive value of tumor immune infiltrates inferred from such trials

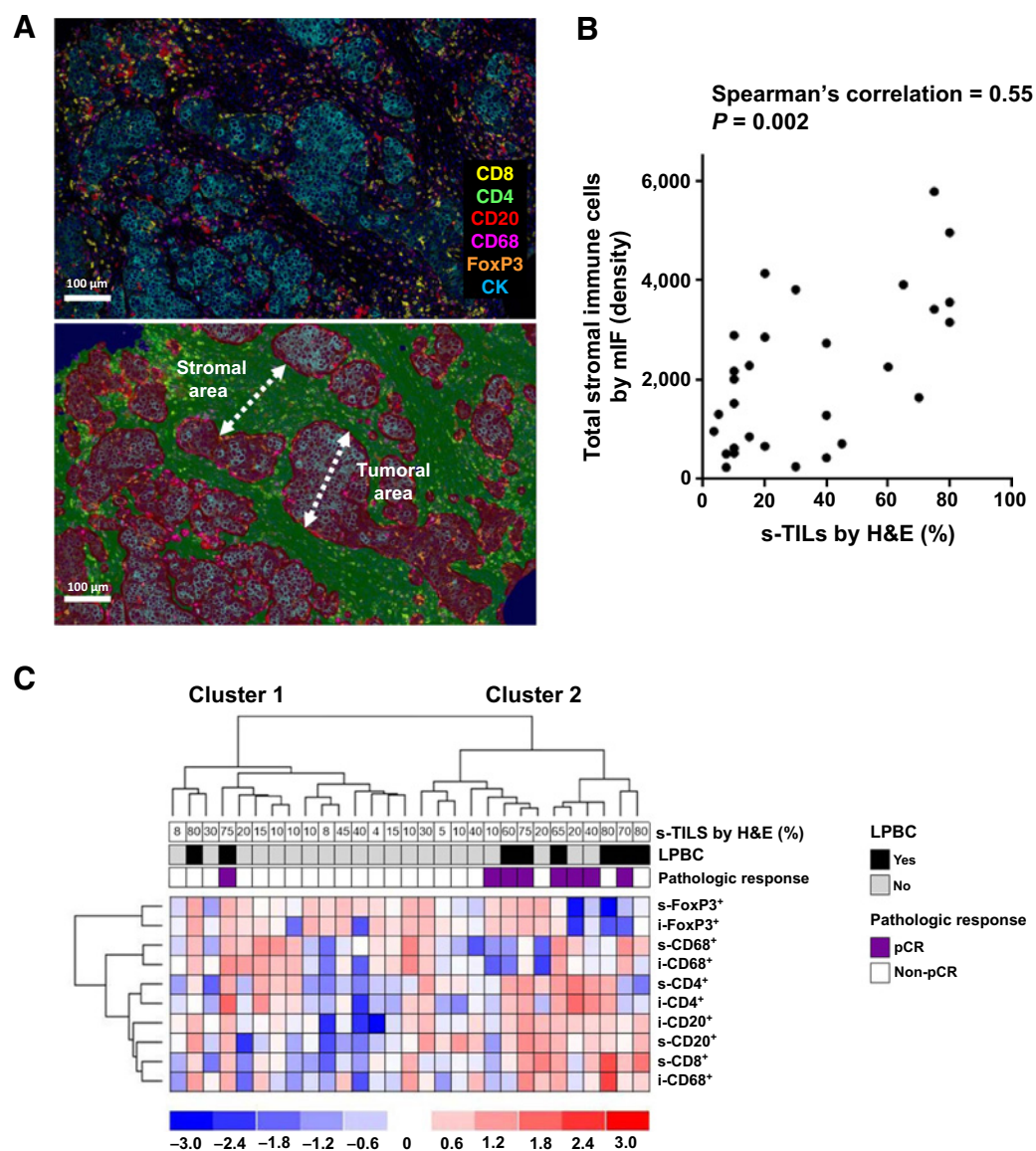


Figure 2.

Multiplexed immunofluorescence (m-IF) of tumor infiltrates of TBCRC 006 baseline specimens. **A**, Top, a composite image obtained using the mIF labeling and multispectral imaging. The m-IF stains included anti-CD8 (yellow), anti-CD4 (green), anti-CD20 (red), anti-CD68 (magenta), and anti-FoxP3 (orange), and anti-CK (cyan). Bottom, the stromal and tumoral areas defined using the inForm tissue segmentation algorithm. **B**, Spearman correlation between total stromal TILs by mIF and s-TILs assessed by H&E. **C**, Unsupervised hierarchical clustering analysis of pretreatment TIL subpopulations.

may not be reflective of the predictive role of immune infiltrates in the context of anti-HER2 treatment alone without the confounding effect of chemotherapy.

Recently, the predictive role of TILs has been evaluated in the PAMELA trial, where the chemo-free lapatinib plus trastuzumab (with endocrine therapy for ER-positive cases) regimen given for 18 weeks resulted in an overall pCR rate of 30% (3). In this study, high TILs at baseline significantly correlated with pCR in the univariate analysis, but the association between TILs and pCR did not maintain statistical significance in multivariate analysis corrected for intrinsic subtype, hormone receptor status, histologic grade, and nodal status. Interestingly, TILs measured at day 15 of treatment and a combined score

taking into account TILs and tumor cellularity at day 15 of treatment were independently associated with pCR (12).

In our study, s-TILs were evaluated using full-face H&E slides, in accordance with the current recommended methodology. In line with the previous studies (13, 14), high levels of s-TILs were observed in 20% of patients and were not associated with deregulation of the PI3K pathway (due to *PIK3CA* mutations or loss/low *PTEN* protein levels). In contrast to previous reports, however, we did not find a significant association between increased s-TILs and ER-negative status (11, 13, 14), although we did notice a positive correlation of s-TILs with the Ki67 index, similar to the CherLob study (11). We further observed a numerically higher, but not statistically significant,

Table 2. Univariate and multivariate analysis of correlation between immune infiltrate and pCR.

		OR (95% CI)	P
Univariate model			
Cluster	1	1	0.0235
	2	14 (1.43-137.3)	
Multivariate model			
Cluster	1	1	0.0355
	2	19.073 (1.221-298.035)	
Age	>50	1	0.4163
	≤50	2.561 (0.265-24.73)	
Tumor size	>5 cm	1	0.3727
	≤5 cm	3.439 (0.228-51.986)	
Nodes	Y	1	0.7281
	N	1.523 (0.142-16.349)	

pCR rate in patients with LPBC than in non-LPBC (50% vs. 19%). Notably, in our cohort, no patients with levels of s-TILs lower than 10% achieved a pCR. Similar results have also been reported in the NeoALTTO and the NeoSphere trials, where patients with very low levels of TILs (s-TILs ≤ 5%) showed the lowest pCR rates (13, 14).

The evaluation of TILs using an H&E-based method is semiquantitative and provides limited information about the complexity of the tumor immune microenvironment, which may be crucial for a better understanding of the biology and clinical activity of the immune infiltrates. Several studies have investigated the predictive and/or prognostic value of specific immune cell markers, checkpoints, and gene expression signatures in breast cancer using different methodologic approaches (5, 6, 14, 15, 25-27). A correlative analysis from the

Cancer and Leukemia Group B (CALGB) 40601 has identified an immune-cell infiltrate signature (IgG signature) as an independent predictor of pCR (27). Similarly, in the GeparSixto trial, s-TILs as well as 12 immune-activating and immune-suppressive markers were found to be predictive of pCR in patients with triple-negative and HER2⁺ breast cancer (26). Therefore, we attempted to further investigate the composition of the immune infiltrates by adapting an m-IF technology to quantify and appropriately classify the various immune cell subtypes present in the tumor (20). While our analysis has been conducted only in a subset of the patients, we have clearly demonstrated that this approach is feasible using FFPE tumor material. More importantly, the significant correlation observed between the classical visual assessment (15) of TILs and the automated digital image-based quantification of immune cells further validates this new analytic approach.

In this study, we used an m-IF panel of 5 phenotypic biomarkers which allowed us to simultaneously identify cytotoxic (CD8⁺) and helper (CD4⁺/FoxP3⁻) T cells, Treg cells (CD4⁺/FoxP3⁺), B cells (CD20), and macrophages (CD68). Our profiling of the immune context resulted in identification of a group of patients (cluster 2) characterized by high CD4, CD8, and CD20 infiltration, which was independently and significantly associated with pCR. Interestingly, evaluation of the individual immune subsets showed that high levels of CD4⁺ T-cells and CD20⁺ B cells rather than CD8⁺ T cells were more frequent in patients who achieved a pCR. Our results are in line with previous data on the predictive/prognostic values of TIL subpopulations in breast cancer. Several IHC studies reported increased CD8⁺ lymphocyte infiltration as a positive predictive and prognostic marker in breast cancer (28-30). Similarly, high levels of CD4⁺ T cells, which play a key role in recruiting and modulating cytotoxic T cells in antitumor immunity (31), have been associated with better survival (32, 33) and higher pCR rate (34).

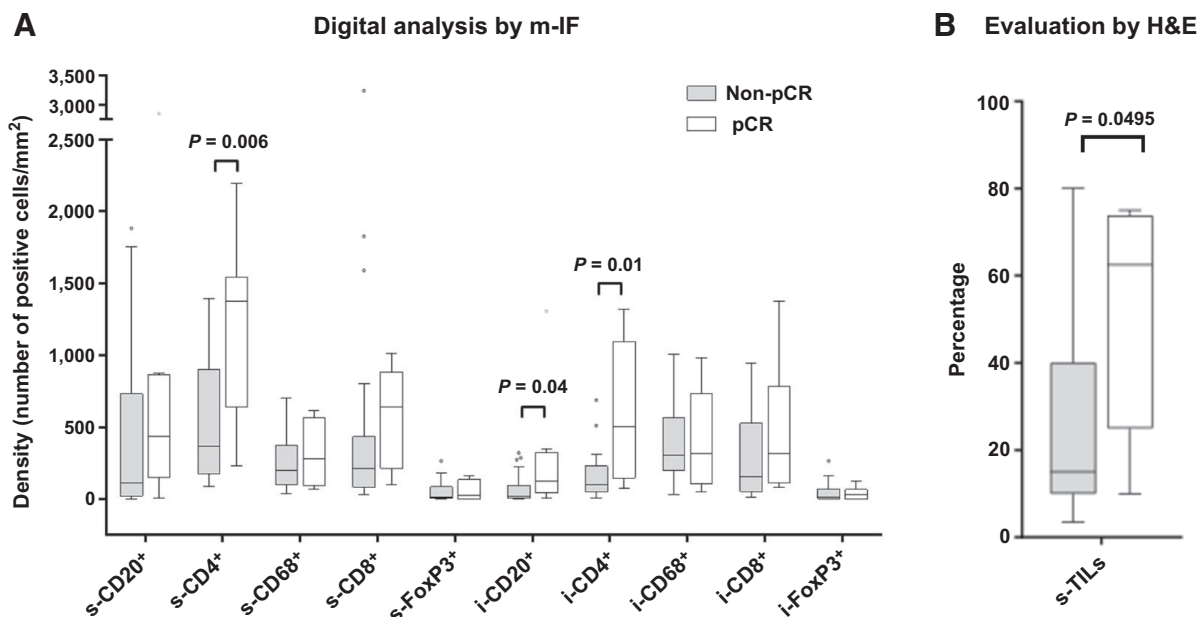


Figure 3. Association between immune cell density and response to neoadjuvant trastuzumab plus lapatinib therapy. Box plots of density of individual immune cell subpopulations in the stromal (s-) and intratumoral (i-) areas (A), and box plots of percentage of s-TILs assessed by H&E (B) in patients achieving a pCR (white) versus residual disease (non-pCR, gray). P values were not adjusted for multiple comparisons.

CD4⁺ TILs play a central role in recruiting and modulating B-cell and cytotoxic T-cell antitumor immunity. Notably, it has been shown that an adequate CD4⁺ T-cell activity is required for the CD8⁺ cytotoxic T cells to fully function (31). Importantly, in the context of HER2⁺ disease, a recent study demonstrated that levels of IFN γ -secreting CD4⁺ T cells were higher in patients who achieved pCR than in those who did not (98% vs. 33%) after neoadjuvant chemotherapy plus trastuzumab treatment (35).

Although not as well established as for T cells, a role for B cells in antitumor response has been suggested (36, 37). The presence of high stromal tumor-infiltrating B lymphocytes (CD20⁺ cells) assessed by IHC (34, 38) or by multiplexed quantitative immunofluorescence (25) has been linked to a favorable response to neoadjuvant chemotherapy in patients with breast cancer. The expression of B-cell related genes and gene signatures has also been strongly associated with good prognosis in breast cancer (39, 40). In contrast, in two neoadjuvant trials of anti-HER2 treatment in combination with chemotherapy, B-cell signatures did not correlate with pCR (11, 27). In our quantitative m-IF analysis, higher levels of both stromal and intratumoral CD20⁺ B cells defined the Cluster 2 patients with higher pCR rates. Therefore, our data support a potential role for B cells in response to a dual anti-HER2 blockade in the absence of chemotherapy, which needs validation in future studies. Concordantly, patients who achieved pCR also showed numerically higher levels of stromal CD20⁺ cells compared with patients who did not, although this difference did not reach statistical significance.

Our work presents some limitations. In our study, the lack of a statistically significant association of s-TILs with pCR could be ascribed to the small sample size in the TBCRC 006 trial or to a lack of biological association between TILs and response to dual HER2-targeted therapy (plus endocrine therapy for ER-positive cases) in the absence of chemotherapy. Other limitations include the limited number of immune markers used for the characterization of the immune infiltrate and the lack of data to address its role in affecting long-term outcomes. Finally, despite intriguing results from the clustering analysis, we recognize that larger datasets are required to develop an m-IF-based predictor that could prospectively assign a molecular immunologic subtype to each HER2⁺ breast cancer case.

In conclusion, our study presents a strong rationale for conducting quantitative assessment of the immune cell infiltrates using m-IF to correlate the individual immune cell profile with response. We have identified an immune profile in patients with HER2⁺ breast cancer that is an independent predictor of pCR. Future and ongoing studies with larger patient populations combined with external validation of the predictive value of the immune subsets will further elucidate the repertoire of immune infiltrates that provide predictive and/or prognostic information to guide the treatment of patients with HER2⁺ breast cancer.

Disclosure of Potential Conflicts of Interest

C.C. Hoyt is an employee/paid consultant for and holds ownership interest (including patents) in Akoya Biosciences, Inc. K. Roman is an employee/paid

consultant for Akoya Biosciences. C. Wang is an employee/paid consultant for Akoya Biosciences. Y. Zheng is an employee/paid consultant for Akoya Biosciences. J.S. Reis-Filho is an employee/paid consultant for Paige AI, Volition RX, Goldman Sachs, Repare Therapeutics, Ventana Medical Systems, Roche Tissue Diagnostics, Genentech, InVivo, and Novartis. A. Prat is an employee/paid consultant for Roche and Oncolytics Biotech, and is an unpaid consultant/advisory board member for Roche, Novartis, NanoString Technologies, Pfizer, Amgen, Lilly, and Daiichi Sankyo. C.K. Osborne is an unpaid consultant/advisory board member for Tolmar, Pfizer, Genentech, and Eli Lilly, and reports receiving other remuneration from O'Melveny and Myers. M.F. Rimawi is an employee/paid consultant for Genentech, MacroGenics, Daiichi Sankyo, and Novartis, and reports receiving commercial research grants from Pfizer and GlaxoSmithKline. R. Schiff is an employee/paid consultant for MacroGenics and reports receiving commercial research grants from AstraZeneca, GlaxoSmithKline, Gilead Sciences, and PUMA Biotechnology. No potential conflicts of interest were disclosed by the other authors.

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