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PD-L1 (B7-H1) expression and the immune tumor microenvironment in primary and metastatic breast carcinomas***

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Summary

Programmed death ligand 1 (PD-L1) expression by tumor-infiltrating lymphocytes (TILs) and tumor cells in breast cancer has been reported, but the relationships between PD-L1 expression by TIL, carcinoma cells, and other immunologic features of the breast tumor microenvironment remain unclear. We therefore evaluated the interrelationships between tumor cell surface and TIL PD-L1 expression, lymphocyte subpopulations, and patterns of immune cell infiltration in cohorts of treatment-naïve, primary breast cancers (PBCs) (n = 45) and matched PBC and metastatic breast cancers (MBC) (n = 26). Seventy-eight percent of untreated PBCs contained PD-L1⁺ TILs, but only 21% had PD-L1⁺ carcinoma cells. Carcinoma PD-L1 expression localized to the tumor invasive front and was associated with high tumor grade ($P = .04$). Eighty-nine percent of PD-L1⁺ carcinomas contained brisk TIL infiltrates, compared to only 24% of PD-L1⁻ carcinomas; this included CD3⁺ ($P = .02$), CD4⁺ ($P = .04$), CD8⁺ ($P = .002$), and FoxP3⁺ T cells ($P = .02$). PD-L1⁺ PBCs were more likely to contain PD-L1⁺ TIL than PD-L1⁻ PBCs ($P = .04$). Peripheral lymphoid aggregates were present in 100% of PD-L1⁺ compared to 41% of PD-L1⁻ PBC ($P < .001$). No patient with PD-L1⁺ PBC developed distant recurrence, compared to 15% of patients with PD-L1⁻ PBC. For the matched PBC and MBC cohort, 2 patients (8%) had PD-L1⁺ tumors, with 1 case concordant and 1 case discordant for carcinoma PD-L1 expression in the PBC and MBC. Our data support PD-L1 expression by tumor cells as a biomarker of active breast tumor immunity and programmed death 1 blockade as a therapeutic strategy for breast cancer.

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Supplementary data

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Keywords

PD-L1; Tumor infiltrating lymphocytes; Primary breast cancer; Metastatic breast cancer; Tumor microenvironment

1. Introduction

In large randomized trials of modern chemotherapy, stromal tumor-infiltrating lymphocytes (TILs) [1] or both intratumoral and stromal TILs [2,3] were independent prognostic factors for survival in triple-negative primary breast cancer (PBC). Brisk TIL infiltrates also predicted benefit from adjuvant trastuzumab-based chemotherapy in human epidermal growth factor receptor 2–positive (HER-2⁺) PBC [2]. These studies evaluated TIL by hematoxylin and eosin (H&E) staining but are limited by their failure to more precisely characterize TIL phenotype. Assessing TIL phenotype by immunohistochemistry (IHC) provides additional critical information about the relative numbers and spatial relationships of distinct immune cell subsets in the breast tumor microenvironment (TME). Higher numbers of both CD8⁺ T lymphocytes and CD20⁺ B lymphocytes are independent predictors of patient survival [4,5] and response to neoadjuvant therapy [6]. Conversely, higher numbers of FoxP3⁺ regulatory T cells (Treg) are associated with shorter relapse-free survival [7].

PBC can be classified into 5 intrinsic molecular subtypes based on gene expression profiles [8]. These subtypes include the luminal A (LumA), luminal B (LumB), HER-2–enriched (HER-2⁺), basal-like (BLC), and claudin-low subtypes. These subtypes can also be defined by IHC, where LumA cancers are ER⁺PR⁺HER-2⁻Ki-67^{low}, LumB cancers are ER⁺PR⁺HER-2⁻Ki-67^{high} or ER⁺PR⁺HER-2⁺, HER-2–enriched cancers are ER⁻PR⁻HER-2⁺, and BLC are typically ER⁻PR⁻HER-2⁻ (triple-negative breast cancers [TNBCs]) with cytokeratin 5/6 or epidermal growth factor receptor expression [9]. TNBCs are thought to be more immunogenic than other PBC, as high expression of B-cell metagenes relative to interleukin 8 metagenes in 32% of TNBC was associated with better prognosis [10].

The programmed death (PD) 1 pathway is a major immune response checkpoint and target for cancer immuno-therapy [11,12]. PD-1 is a member of the B7-CD28 family of coregulatory molecules expressed by activated lymphocytes [13]. The major ligand for PD-1, PD-L1 (B7-H1), can be expressed by tumor cells themselves, as well as by immune cells [14] including infiltrating T cells, B cells, macrophages, and dendritic cells, and the presence of tumoral PD-L1⁺ TIL correlates with clinical response to PD-1 pathway blockade with anti-PD-1 [15] or anti-PD-L1 targeted immunotherapy [16]. PD-L1 expression can be induced on tumor cells by inflammatory cytokines, providing a mechanism of adaptive immune resistance [17]. Some data suggest the PD-1 pathway may be an active immune checkpoint in breast cancer [12]. Tissue microarray analysis (TMA) analysis of 660 PBCs showed that infiltration by PD-1⁺ TIL was associated with worse survival in LumB and BLC [18]. TMA analysis of 650 PBCs showed PD-L1⁺ carcinoma cells in 23.4% of breast tumors, with worse survival in LumB, HER-2⁺, and BLC [19]. PD-L1 expression strongly

correlated with the presence of PD-1⁺ TIL. Another study found 19% of TNBCs expressed PD-L1, where carcinoma PD-L1 expression was associated with CD8⁺ T-cell infiltration relative to PD-L1⁻ TNBCs [20].

Here, we characterize PD-L1 expression by TIL and carcinoma cells in 45 PBCs across the 3 clinically relevant subtypes and in a TMA cohort of matched PBC and metastatic breast carcinoma (MBC). We examine distinct subtypes of TIL, their architecture, and their association with PD-L1⁺ carcinoma cells and TIL. We also explore the association of immunologic features of the breast TME with clinicopathological characteristics of the tumors and clinical outcome.

2. Materials and methods

2.1. Case selection of PBC

This study was reviewed and approved by the Institutional Review Board of the Johns Hopkins Medical Institutions. Surgically resected PBCs were randomly selected from the pathology archives collected over a 10-year period (2001-2011). Selection criteria included a diagnosis of infiltrating ductal carcinoma (IDC) or infiltrating mammary carcinoma (IMC); known estrogen receptor (ER), progesterone receptor (PR), and HER-2 IHC results; no prior neoadjuvant chemotherapy; and sufficient archival tissue in formalin-fixed, paraffin-embedded blocks to perform our planned IHC analyses. The patients were treatment naive at the time of tissue collection (ie, received no neoadjuvant therapy) and were all offered standard adjuvant therapy as per the clinical standard of care. Hormone receptor and HER-2 expression were used to classify cases into the following phenotypes: luminal (LUM) (ER⁺/PR⁺/HER-2⁻), HER-2⁺ (ER⁻/PR⁻/HER-2⁺), and triple-negative BLC (ER⁻/PR⁻/HER-2⁻ and cytokeratin 5/6 positive and/or epidermal growth factor receptor positive), using a previously described and validated IHC surrogate for molecular breast cancer subtype [9]. All cases classifying as LUM in this cohort were ER⁺ and PR⁺ (ie, none were ER⁺/PR⁻). The final 45 cases were selected to be evenly divided across these 3 breast cancer subtypes.

2.2. TMA construction of matched PBC and MBC

We evaluated 3 previously described TMAs containing cores from 16 patients with PBC and matched, surgically resected MBC at first relapse [21,22]. We subsequently constructed 2 additional TMAs from archived formalin-fixed, paraffin-embedded blocks of 10 additional patients with matched PBC and MBC, giving a total cohort of 26 matched PBC and MBC for this study. Each TMA consisted of 99 spots measuring 1.4 mm in diameter, with 5 to 10 cores per tumor to minimize sampling error. ER, PR, and HER-2 expressions were used to classify cases into the following phenotypes: LUM (ER⁺/PR⁺/HER-2⁻ or ER⁺/PR⁻/HER-2⁻), HER-2⁺ (ER⁻/PR⁻/HER-2⁺), and triple-negative carcinoma (TNBC) (ER⁻/PR⁻/HER-2⁻) [9].

2.3. Histologic quantification of TIL and lymphoid aggregates

Microscopy was performed with an Olympus BX43F compound light microscope, and still images were acquired with an Olympus DP73 digital camera. TIL on H&E-stained whole

sections of 45 PBCs were qualitatively scored by a histologic intensity score; TIL included both lymphocytes and macrophages. TIL location was recorded as peritumoral (P), intratumoral (I), or both peritumoral and intratumoral (PI). TIL intensity was scored as none (0), mild (1; rare TIL; $\leq 5\%$ of tumor area), moderate (2; focal infiltrate; 5%-50% of tumor area), and diffuse/severe (3; diffuse infiltrate; $>50\%$ of tumor area) [17,23]. In addition, the presence or absence of peritumoral lymphoid aggregates was recorded as absent (0), focal (1; rare, isolated aggregates of lymphoid cells), present (2; multiple lymphoid aggregates), and well developed (3; present with well-developed germinal centers). The qualitative TIL scores were correlated to digital TIL quantification on the same cases.

2.4. Immunohistochemistry for TIL subsets

Whole tumor sections of 45 PBCs and the TMAs were stained for CD20 (monoclonal, clone MS/L26, catalog no. 760-2531; Ventana Medical Systems, Tucson, AZ), CD3 (mouse monoclonal, clone PS1, catalog no. ORG-8982; Leica Microsystems, Bannockburn, IL), CD4 (rabbit monoclonal, clone Sp35, catalog no. 790-4423; Ventana Medical Systems), CD8 (mouse monoclonal, clone C8/C8144B, catalog no. 760-4250; Cell Marque, Rocklin, CA), and FoxP3 (mouse monoclonal, clone 236A/E7, catalog no. 14-4777-80, dilution 1:50; eBioscience; San Diego, CA) as previously described [22]. The immunostains were performed individually (ie, no dual labeling) on serial unstained sections. TILs in the previously constructed TMAs (the first 16 patients with matched PBC/MBC) were previously reported [22].

TIL subsets in the 45 treatment-naive PBCs were quantitated as total TIL/unit area (square millimeter) by digital image analysis on scanned whole slides of CD3, CD4, CD8, CD20, and FoxP3 stained slides detailed in Supplementary Methods [24]. TIL subsets within lymphoid aggregates was determined by manual inspection of stained whole slide sections.

TILs present in TMAs were quantified by manual expression scoring blinded to the clinicopathological patient characteristics [22]. Briefly, on the TMAs, B and T lymphocytes were considered tumor infiltrating if they were located within 1 high-power field (HPF; $\times 40$) of carcinoma cells. CD20, CD3, CD4, and CD20 positivities were defined as membranous lymphocyte staining, and FoxP3 positivity was defined as any nuclear lymphocyte staining. The total number of TIL/HPF was counted in each TMA core and averaged across the case to give the mean number of TIL/HPF.

2.5. Expression and quantification of PD-L1 staining on carcinomas and TILs

IHC for PD-L1 (B7-H1) was performed on whole sections of 45 PBCs and on TMAs of 26 matched PBC/MBC using the murine anti-human PD-L1 antibody, clone 5H1 ($2 \mu\text{g}/\text{mL}$) paired with a isotype-matched mouse IgG1 control using established methods [15,17]. The percentage carcinoma cells exhibiting clear membrane PD-L1 expression were scored in 5% increments from 0% to 100%, with less than 5% labeling considered negative. The average PD-L1 labeling was taken across all cells present on the whole slide or TMA cores for a tumor. PD-L1 staining was separately recorded in adjacent ductal carcinoma in situ (DCIS) if present. The percentage PD-L1 expression by TILs was also recorded in 5% increments and scored as none (0), focal (1+; $\leq 5\%$), moderate (2+; 5%-50%), or severe (3+;

51%-100%). A board-certified pathologist (A. C. M.) scored all cases; those with a positive or borderline PD-L1 score were independently reviewed by a second board-certified pathologist (J. M. T.). There were no differences in scoring between pathologists. Both pathologists were blinded to tumor clinicopathological characteristics and patient outcomes at the time of scoring.

2.6. Statistical analysis

Associations between PD-L1 expression on tumor cells, immune cell infiltrates, and other tumor features were evaluated using the Fisher exact test. For continuous variables, the *P* values are derived from 2-sided *t* tests, with *P* values of less than .05 considered significant. Statistical analyses were performed using SAS software (version 9.2; SAS Institute, Cary, NC).

3. Results

3.1. Treatment-naive PBC

3.1.1. Clinicopathological features of 45 PBCs—The clinicopathological features of 45 patients with newly diagnosed PBC are detailed in Table 1. Briefly, the 45 cases were equally distributed between LUM, HER-2⁺, and BLC. The mean patient age was 54 years, with 60% white and 36% black patients. All cases were Elston grade II (29%) or III (71%), with BLC and HER-2⁺ PBCs having higher grade than LUM cancers. Most patients had stage 2 disease (60%) and negative sentinel lymph nodes (54%); the median tumor size was 2.3 cm. Four BLC patients (27%) carried *BRCA* mutations (3 *BRCA1*, 1 *BRCA2*). LUM patients had fewer nodal metastases, with lower-grade tumors that were more often multifocal. Five patients died due to MBC within 5 years of initial diagnosis; 3 had BLC and 1 each had LUM or HER-2⁺ cancer. Five additional patients died due to unknown or other causes. There was no difference in survival probability between the tumor subtypes (BLC, HER-2, or LUM) in this series, although survival analysis is limited by the small sample size when divided into subgroups (n = 15 each).

3.1.2. TIL distribution and PD-L1 expression by TIL in PBCs—We first evaluated breast TILs by histopathologic intensity scoring on a scale from 0 to 3. All 45 tumors contained TILs, with slightly more than half containing TILs with PD-L1 expression scores of 2 or 3 (moderate-diffuse) (Tables 2 and 3). Most TILs were peritumoral rather than intratumoral (Fig. 1). We used whole section image analysis to confirm our histologic scoring and to also quantify TIL subsets within PBCs (Table 2). There were about twice as many peritumoral than intratumoral TIL. Tumors containing TIL with higher levels of PD-L1 expression had more CD3⁺ (*P* = .01) and CD4⁺ (*P* = .04) T cells than PBCs containing TIL with lower levels of PD-L1 expression (Table 3). Tumors containing PD-L1⁺ TIL were more likely to have PD-L1 expression by the carcinoma cells (*P* = .04). More PD-L1⁺ TIL were seen in HER-2⁺ carcinomas (*P* = .01; Table 3), as described further below, but there was no association of TIL PD-L1 expression with other standard clinicopathological parameters and no association of TIL PD-L1 expression with overall survival (Supplementary Fig. 1A).

Notably, we found lymphoid aggregates in 53% of treatment-naive PBCs (Fig. 1C-F). Lymphoid aggregates were localized at the tumor edge and contained a mix of CD3⁺ T cells and CD20⁺ B cells, with both T cells and B cells at the periphery and within the lymphoid aggregate center. Lymphoid aggregates were seen in 63% tumors with PD-L1⁺ TIL compared to 13% tumors with PD-L1⁻ TIL ($P = .017$).

3.1.3. Cell surface PD-L1 expression by carcinoma cells in PBCs—We found that 21% of PBCs expressed PD-L1 on the surface of the carcinoma cells (Fig. 1G and Table 4). PD-L1 expression localized to the tumor invasive front of carcinoma cell nests and was associated with high tumor grade independent of subtype ($P = .04$). Diffuse/severe TIL infiltration was present in 89% of PD-L1⁺ tumors compared to 24% of PD-L1⁻ tumors ($P = .002$). PD-L1 expression levels ranged from 5% to 20%, with 5 PBCs displaying 5% and 2 each displaying 10% or 20%. There was 100% concordance between PD-L1 expression by the PBC and any associated DCIS ($P = .008$). Three PD-L1⁺ PBCs had associated DCIS on the same slide, all of which was PD-L1⁺. Seven PD-L1⁻ PBCs had associated DCIS, all of which was PD-L1⁻. Nonspecific PD-L1 staining was noted in the central necrosis of some DCIS foci. Of the 4 *BRCA*-mutated tumors, 2 had PD-L1⁺ carcinoma cells. There was no association of carcinoma cell PD-L1 expression with any standard pathologic parameter except grade. No patient with PD-L1⁺ disease developed distant recurrence, compared to 15% of patients with PD-L1⁻ disease. Kaplan-Meier analysis showed a trend toward higher probability of overall survival in patients with PD-L1⁺ carcinomas, but this was not statistically significant ($P = .27$) (Supplementary Fig. 1B).

PD-L1⁺ PBCs contained more CD3⁺, CD4⁺, CD8⁺, and FoxP3⁺ T cells than PD-L1⁻ PBCs, with the greatest difference in CD8⁺ T cells ($P = .002$); there was no difference in the CD8/*FoxP3* ratio ($P = .99$) (Table 4). PD-L1⁺ PBCs were more likely to contain PD-L1⁺ TILs ($P = .04$). Most PBCs showed either similar levels of PD-L1 expression by tumor cells and TIL or higher PD-L1 expression by TIL than tumor cells. Higher PD-L1 expression by tumor cells relative to TIL was seen in 22% of the PD-L1⁺ tumors, whereas 78% of cases displayed equal PD-L1 expression in TIL and carcinoma cells. Lymphoid aggregates were seen in 100% of PD-L1⁺ tumors, compared to 41% of PD-L1⁻ tumors ($P < .001$). Furthermore, 78% of the PD-L1⁺ tumors displayed diffuse, well-formed lymphoid aggregates with central germinal centers, compared to just 9% of the PD-L1⁻ PBCs ($P < .001$).

3.1.4. PD-L1 expression by TIL and breast carcinoma cells in breast cancer subtypes—We also examined immune parameters by subtype (Tables 2-4). BLCs had more diffuse TIL infiltrates (60%) compared to LUM (14%; $P = .0209$) and HER-2 (36%; P , nonsignificant) (Table 2). However, HER-2⁺ carcinomas contained more PD-L1⁺ TILs (86% TIL scores 2-3) compared to either BLC carcinomas (33%) or LUM carcinomas (43%) ($P = .01$) (Table 4). BLCs had greater numbers of CD8⁺ T cells ($P = .034$) and FoxP3⁺ T cells ($P = .034$) than LUM breast cancers, but no other statistical differences emerged in this small series (Table 2). Of the PD-L1⁺ carcinomas, 56% were HER-2⁺, 33% were BLC, and 11% were LUM. Within the subtypes, 36% of HER-2⁺ PBC, 20% of BLC, and 7% of LUM PBCs had PD-L1⁺ carcinoma cells. The percentage of PD-L1 expression by

tumor cells ranged from 5% to 20%, with 5 cases displaying 5% and 2 each displaying 10% and 20%.

3.2. Matched PBC and MBC

3.2.1. Clinicopathological features of 26 matched PBC and MBC—The clinicopathological features are detailed in Supplementary Table 1. Briefly, most patients (88%) with matched PBC and MBC had IDC or mammary carcinoma not otherwise specified; the remainder (12%) had invasive lobular carcinoma. The distribution of tumor phenotype was 61% LUM, 31% TNBC, and 8% HER-2⁺. There was complete concordance between ER, PR, and HER-2 expression between the matched PBC and MBC. Most tumors at presentation were Elston grade III (65%) with a median tumor size of 2.4 cm and positive lymph nodes (58%). The mean interval time to first relapse was 3.8 years; 3 patients (12%) presented with MBC at diagnosis. Only patients with solitary metastases were studied; these sites included brain (42%), lung/pleura (31%), gastrointestinal tract/ovary (15%), liver (8%), and perivertebral (4%). Sites of metastasis for HER-2⁺ and TNBCs were limited to the brain and lung/pleura. Fourteen (54%) patients had died of metastatic disease at last follow-up.

3.2.2. PD-L1 expression and immunologic features by lymphocyte subset in matched PBC and MBC—Approximately half (54%) of PBC contained PD-L1⁺ TIL. Patients with PD-L1⁺ TIL in the PBC were more likely to have diffuse immune cell infiltrates and PD-L1⁺ TIL in the matched MBC ($P = .047$). PD-L1 expression by tumor cells was not evaluable in 2 cases due to processing loss (1 LUM and 1 HER-2⁺). Only 2 patients (8%) had PD-L1⁺ carcinoma cells. In 1 HER-2⁺ patient, both the PBC and lung MBC were PD-L1⁺. In 1 TNBC patient, the PBC was PD-L1⁻, but the MBC to the left lung was PD-L1⁺ (Fig. 2). This latter patient with the PD-L1 status conversion from the PBC to the MBC received standard adjuvant therapy in the 5.5-year interval between the primary and the development of the metastasis, which consisted of right axillary nodal and chest wall irradiation followed by 6 cycles of cyclophosphamide, doxorubicin, and 5-fluorouracil. Both of the PD-L1⁺ lung metastases displayed moderate to diffuse TIL infiltration, compared to only 36% of metastases where the PBC was PD-L1⁻. In addition, both of these metastases displayed moderate PD-L1⁺ TIL, compared to only 14% of metastases where the PBC was PD-L1⁻. In looking at all lung metastases ($n = 7$) relative to other site metastases ($n = 17$), lung metastases had more moderate-diffuse immune cells and more PD-L1⁺ TIL infiltrates (Supplementary Table 2).

Lymphocyte analysis was performed and reported on the first 16 cases in a previous study [21,22]. The same manual analysis was performed on the additional 10 cases added to this series, and the data are reanalyzed here. MBCs have fewer TILs compared to the matched PBCs as measured by average CD3, CD4, CD8, CD20, and FoxP3 cells per HPF. In contrast to the previous observation that only CD8 cells were decreased in luminal metastases, here we find that all T-lymphocyte subsets are decreased, if minimally, in the luminal metastases. As we previously reported, TNBC metastases have fewer CD3⁺ T cells than the luminal metastases, and brain metastases have fewer CD3⁺ T cells than the lung, spine, and gastrointestinal/ovary metastases.

4. Discussion

These analyses of PD-L1 expression and other immuno-logic features of the breast TME support the following 7 findings. First, treatment-naive PBCs contained both PD-L1⁺ TILs (78%) and PD-L1⁺ carcinoma cells (21%) localized to the invasive fronts of tumor cell nests. Second, tumor cell surface PD-L1 expression was associated with high tumor grade, independent of tumor subtype. Third, 10 cases of concurrent DCIS and invasive PBC were 100% concordant for PD-L1 expression. Fourth, PD-L1⁺ carcinomas were more likely to contain PD-L1⁺, CD3⁺, CD4⁺, CD8⁺, and FoxP3⁺ T cells, but not CD20⁺ B cells. Fifth, all (100%) PD-L1⁺ PBCs were associated with peripheral lymphoid aggregates, where less than half (41%) of PD-L1⁻ PBCs had lymphoid aggregates. Sixth, although follow-up remains short, no patient with PD-L1⁺ disease recurred, whereas 15% of patients with PD-L1⁻ disease developed a distant recurrence. Finally, our TMA series of matched PBC and MBC revealed that only 2 (8%) of 26 of patients' tumors expressed PD-L1 in either the primary tumor or the metastasis, implying that PD-L1 expression may impact disease progression. This is consistent with our data on clinical recurrence rate. Taken together, our findings support breast tumor cell surface PD-L1 expression as an informative marker of active breast tumor immunity. Our findings also support PD-1 pathway blockade as a treatment for PD-L1 expressing breast cancers.

Importantly, digital image analysis confirmed our visual histologic impression that most TILs are peritumoral and that TILs increase in severity across the histologic TIL intensity scores of 0 to 3. Our findings validate the use of histologic TIL intensity scores in estimating the degree of immune infiltration in PBCs. To our knowledge, this is one of the first studies to correlate the histologic TIL intensity score with discrete TIL counts via digital image analysis in breast cancer. Similar scoring systems have been used in other tumor types, such as melanoma, non-small cell lung carcinoma, renal cell carcinoma, and colorectal carcinoma [17,23], setting the stage for reduction to practice and widespread clinical use of the histologic TIL intensity score.

Several groups have examined TIL and the expression of PD-1 and PD-L1 in breast cancers [12]. Of the PBCs, 19% to 23% express PD-L1 on the tumor cell surface when analyzed on a TMA platform [19]. Brisk PD-1⁺ TIL infiltrates are associated with more aggressive phenotype and worse clinical outcomes in PBCs [18,25,26]. Consistent with our data, PD-L1⁺ TILs have also been associated with high-risk features of PBCs, including grade [20,26]. Most recently, both PD-1⁺ TILs and PD-L1⁺ carcinoma cells were more commonly found in TNBC (70% and 59%, respectively) than in other breast cancer subtypes (25%-60% and 20%-33%, respectively) [27]. This study reported breast carcinoma PD-L1 expression in 45% of PBCs and concurrent PD-1 and PD-L1 expression in 29% of PBCs. Another study reported PD-L1 messenger RNA expression in 55% to 60% of PBCs by TMA; this was associated with higher levels of TILs and better clinical outcomes [29]. These reported rates of PD-L1 expression are higher than what we observed and may relate to differences in the PD-L1 assay and extent of tumor examined. Many of these studies are limited by lack of assessment of whole tumor sections and tumor architecture, TIL subtype by IHC, and lack of distinction between PBCs and MBCs. Notably, several genetic lesions, including *TP53* and *PIK3CA* mutations, have been associated with an active PD-1 pathway

in breast cancers [20,27]. Our study included 4 patients with *BRCA* mutations; half of these patients had PBCs that express PD-L1. The relationships between PD-L1 expression, tumor immunity, and mutational load remain an active area of research.

Ectopic lymphoid-like structures [12], also termed *lymphoid aggregates* [23] and *tertiary lymphoid structures* [28,30], have emerged as a biomarker of an ongoing antitumor immune response, identifying an inflamed TME that might be more responsive to cancer immunotherapy [31]. These aggregates can range from clusters of T and B cells to highly organized, functional germinal centers. They may be observed in treatment-naïve tumors, including melanoma, colorectal cancer, non-small cell carcinoma, and breast cancer and are frequently associated with improved survival [31,32]. They are thought to depend on active antigenic stimulation and reflect an ongoing adaptive immune response [33]. Similar ectopic lymphoid structures may be induced by cancer therapies. In breast cancer, tertiary lymphoid nodules and T-bet⁺ TIL were associated with response to trastuzumab-based neoadjuvant therapy and improved survival [34]. In pancreatic cancer, vaccination with a cell-based granulocyte-macrophage colony-stimulating factor-secreting vaccine induced intratumoral lymphoid nodules not found in immunotherapy-naïve tumors [31]. These lymphoid aggregates seem to represent foci of immunoregulation [12] and may be dominated by immunosuppressive pathways that favor tumor progression or by proinflammatory pathways that promote tumor regression [31,34]. Ultimately, they may mark tumors that can be manipulated with immunotherapy to promote inflammation and tumor rejection.

There has been little investigation of the possible role of the PD-1 pathway in breast tumor progression. Here, we examined DCIS, invasive PBCs, and matched PBCs and MBCs at first relapse. We show for the first time 100% concordance for PD-L1 expression between DCIS and associated invasive PBCs present in the same tumor; DCIS that express PD-L1 are associated with PD-L1⁺ invasive cancers, and DCIS that do not express PD-L1 are associated with PD-L1⁻ invasive cancers. We also used a TMA platform to analyze PD-L1 expression in a series of 26 matched PBCs and MBCs at first relapse. Only 8% of tumor cells expressed PD-L1 in either the PBC or the MBC, and only 4% of PBCs were PD-L1⁺. Several factors may account for this discrepancy between our data from the series of PBCs (21% PD-L1⁺) and the cohort of matched PBCs and MBCs (8% PD-L1⁺). First, most tumors in the matched cohort were LUM, which have lower rates of PD-L1 expression relative to HER-2⁺ PBCs and BLC/TNBC. Second, the matched PBC/MBC cohort was selected based on the presence of metastasis. Furthermore, carcinoma cell PD-L1 expression may be associated with lower relapse rates [29]. The very low rates of PD-L1⁺ tumors in this matched cohort could thus reflect selection bias. Third, it is possible that the lower PD-L1 positivity seen in the metastatic cohort was due to the TMA methodology used because PD-L1 positivity is most pronounced at the periphery of the tumors and the TMA cores are taken from various locations within the parent tumor (ie, there is no specific selection to the edge of the tumor or areas of TIL infiltration). The lower rate of PD-L1⁺ tumors may thus be due to sampling error. However, the latter seems less likely as multiple cores per tumor (5-10) including the tumor leading edges were included in the TMAs with the goal of minimizing such sampling error.

We did find 1 case where the PBC and MBC were concordant for PD-L1 expression and another where the PBC and MBC were discordant for PD-L1 expression—here the metastasis was PD-L1⁺. This discordance may reflect the dynamic nature of PD-L1 expression. A previous study of melanoma and renal cell carcinomas reported heterogeneity of PD-L1 status between different sites of metastases in the same patient [35]. In addition, PD-L1 may be constitutively up-regulated by tumor cells as the result of oncogene signaling or induced by inflammatory cytokines secreted in the context of an immune response in the TME as a mechanism of immune evasion [17]. Regardless, our observations support real-time analysis of the TME if using PD-L1 expression to identify patients most likely to respond to PD-1-directed therapies, that is, retesting PD-L1 status in the MBC even in patients with PD-L1⁻ PBC. Taken together, our data support the possibility of targeting the PD-1 pathway as adjuvant therapy in breast carcinoma and also suggest that combinatorial therapy (such as chemotherapy or target-based therapy such as trastuzumab) may be necessary in the metastatic setting promote an inflamed phenotype more responsive to PD-1 blockade and other immunotherapy.

In summary, we demonstrate PD-L1 expression by breast carcinoma cells in up to 21% of PBCs, with greater expression in HER-2⁺ and BLC than in luminal cancers. PD-L1 expression is associated with tumor grade, brisk PD-L1⁺ T cell (but not B cell) infiltrates, and tumor-associated lymphoid aggregates. These features may reflect an inflamed TME in PBCs, suggesting that PD-L1⁺ breast cancers may be more responsive to immunotherapies. We also found that MBCs were less likely to be PD-L1⁺, although selection bias may limit the relevance of this observation. Further investigation of the role of PD-L1 expression in reflecting an inflamed or suppressive TME and in predicting response to PD-1 immunotherapies in breast tumor progression is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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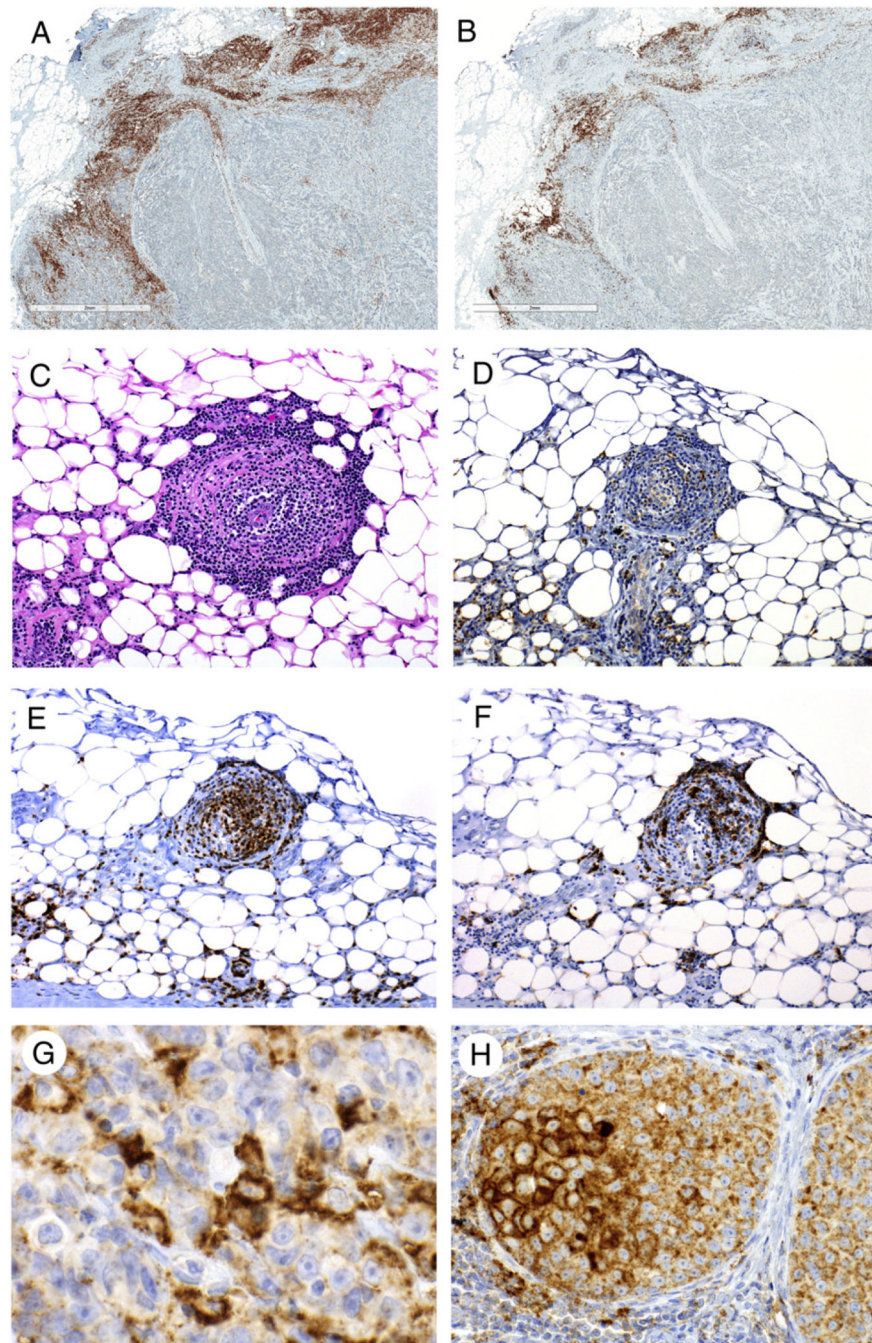


Fig. 1. Immunologic features of the primary breast carcinoma (PBC) tumor microenvironment. Tumor-infiltrating CD3⁺ T lymphocytes (A) and CD20⁺ B lymphocytes (B) are preferentially located at the peritumoral interface with the surrounding stroma. Lymphoid aggregates were seen in 53% of PBCs, including 100% of PD-L1⁺ carcinomas (C). The cellular composition of lymphoid aggregates in the PBCs shows scattered PD-L1⁺ lymphocytes (D), CD3⁺ T lymphocytes (E), and CD20⁺ B lymphocytes (F). PD-L1 expression by carcinoma cells was seen in 21% of treatment-naive PBCs (G). All PD-L1⁺

invasive PBCs with associated ductal carcinoma in situ (DCIS) showed PD-L1 expression by carcinoma cell within the DCIS (H).

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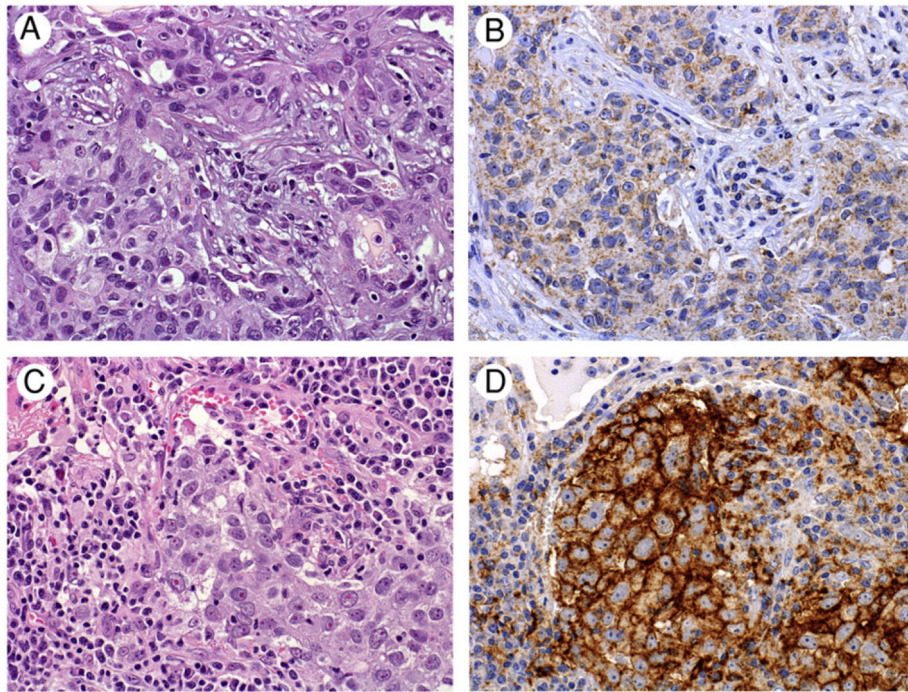


Fig. 2. Carcinoma PD-L1 expression in a patient with metastatic breast carcinoma. This patient's triple-negative primary breast carcinoma (A, H&E) was PD-L1⁻ (B), but the lung metastasis (C, H&E) was PD-L1⁺ (D).

Table 1

Clinicopathological characteristics of primary surgical breast cancer specimens

Characteristic	Total patients	Luminal phenotype	HER-2 ⁺ phenotype	Basal-like phenotype
Total sample size, n (%)	45 (100%)	15 (33%)	15 (33%)	15 (33%)
Age (y)				
Median age (range)	56 (31-88)	59 (41-73)	58 (31-88)	48 (34-79)
<50	19 (42%)	5 (33%)	6 (40%)	8 (53%)
>50	26 (58%)	10 (67%)	9 (60%)	7 (47%)
Patient race				
Black	16 (36%)	3 (20%)	4 (27%)	9 (60%)
White	27 (60%)	12 (80%)	9 (60%)	6 (30%)
Other	2 (4%)	0	2 (14%)	0
Histology ^a : IDC or IMC	45 (100%)	15 (100%)	15 (100%)	15 (100%)
Histologic grade ^b				
Grade 1	0	0	0	0
Grade 2	13 (29%)	10 (67%)	2 (14%)	1 (7%)
Grade 3	32 (71%)	5 (33%)	13 (86%)	14 (93%)
Tumor size (cm)				
Median size (range)	2.3 (0.7-12)	2.4 (1.1-5.8)	2.1 (0.7-6)	2.5 (2-12)
<2	10 (22%)	3 (20%)	6 (40%)	1 (7%)
2-5	30 (67%)	11 (73%)	8 (53%)	11 (73%)
>5	5 (11%)	1 (7%)	1 (7%)	3 (20%)
Multifocal disease	8 (18%)	4 (27%)	3 (20%)	1 (7%)
Associated DCIS	37 (82%)	14 (93%)	14 (93%)	9 (60%)
Stage				
1	11 (24%)	4 (27%)	5 (33%)	2 (14%)
2	27 (60%)	8 (53%)	8 (53%)	11 (73%)
3	7 (16%)	3 (20%)	2 (14%)	2 (14%)
4	0	0	0	0

Abbreviations: DCIS, ductal carcinoma in situ; HER-2, human epidermal growth factor receptor 2; IDC, infiltrating ductal carcinoma; IMC, infiltrating mammary carcinoma; TNBC, triple-negative breast cancer.

^aThe IMCs were all the luminal subtype.

^bOf the grade 2 tumors, 10 were luminal, 2 were HER-2⁺, and 1 was TNBC.

Table 2

Immune parameters of primary surgical breast cancer specimens

	All cases evaluable	Tumor phenotype		
		Luminal	HER-2+	Basal-like
Total breast tumors (n = 43)	43 (100%)	14 (33%)	14 (33%)	15 (35%)
PD-L1 expression in tumor				
Negative	34 (79%)	13 (93%)	9 (64%)	12 (80%)
Positive	9 (21%)	1 (7%)	5 (36%)	3 (20%)
Associated DCIS				
PD-L1 negative	10 (23%)	5	4	1
PD-L1 positive	7 (70%)	4 (80%)	2 (50%)	1 (100%)
PD-L1 positive	3 (30%)	1 (20%)	2 (50%)	0
TIL intensity score ^a				
0	0	0	0	0
Focal: 1	7 (16%)	5 (38%)	1 (7%)	1 (7%)
Moderate: 2	20 (47%)	7 (50%)	8 (57%)	5 (33%)
Diffuse: 3	16 (37%)	2 (14%)	5 (36%)	9 (60%)
PD-L1+ TIL ^b				
0	8 (19%)	2 (14%)	1 (7%)	5 (33%)
Focal: 1	12 (28%)	6 (43%)	1 (7%)	5 (33%)
Moderate: 2	20 (47%)	5 (36%)	11 (79%)	4 (27%)
Diffuse: 3	3 (7%)	1 (7%)	1 (7%)	1 (7%)
2 or 3	23 (53%)	6 (43%)	12 (86%)	5 (33%)
Lymphoid aggregate score				
0	20 (47%)	6 (43%)	7 (50%)	7 (50%)
Focal: 1	10 (23%)	6 (43%)	3 (21%)	1 (7%)
Moderate: 2	3 (7%)	0	0	3 (20%)
Germinal centers: 3	10 (23%)	2 (14%)	4 (29%)	4 (27%)
TIL count/mm ² , median (range)				
CD3	1128 (71-8465)	751 (178-3944)	1037 (264-4833)	1430 (71-8465)
CD4	1546 (67-12639)	888 (215-3350)	1991 (405-5658)	1572 (67-13639)
CD8	926 (2-3842)	625 (2-2198)	879 (175-3842)	1302 (84-3770)
FoxP3	397 (64-1822)	248 (75-1027)	268 (87-1220)	604 (64-1822)
CD20	283 (6-5776)	98 (6-1959)	199 (28-2346)	644 (45-5776)
CD8/FoxP3 ratio	2.2 (0.8-7.9)	2.5 (0.8-7.9)	2.2 (0.9-5.6)	2.1 (1.0-5.2)
CD4/FoxP3 ratio	4.64 (0.86-11.01)	5.2 (2.0-7.2)	4.8 (0-10.4)	2.6 (0.9-11.0)

Abbreviations: DCIS, ductal carcinoma in situ; HER-2, human epidermal growth factor receptor 2; TIL, tumor-infiltrating lymphocyte.

^aImmune infiltrates included TILs and accompanying histiocytes and were scored as “none” (0), “focal” (1), “moderate” (2), or “diffuse” (3); see “Materials and methods” section.

^bTumor cells or infiltrating immune cells were considered PD-L1⁺ if greater than or equal to 5% of cells had membranous PD-L1 labeling. The proportion of TILs expressing PD-L1 was graded as “none” (0), “focal” (1); “moderate” (2), or “severe” (3).

Table 3

Relationship of TIL PD-L1 expression to clinical and immune parameters in primary breast carcinomas

	TIL PD-L1 expression ^a			P ^b
	PD-L1 ^{neg} (0,1)	PD-L1 ^{mod} (2)	PD-L1 ^{diffuse} (3)	
Total evaluable tumors (n = 43)	20 (47%)	20 (47%)	3 (7%)	
Tumor phenotype				.01*
Luminal	8 (40%)	5 (25%)	1 (33%)	
HER-2 ⁺	2 (10%)	11 (55%)	1 (33%)	
Basal-like	10 (50%)	4 (20%)	1 (33%)	
Median age (y, range)	57 (34-79)	51 (33-72)	68 (57-88)	.57*
Patient race				.43
Black	9 (45%)	6 (30%)	0	
White	11 (55%)	13 (65%)	3 (100%)	
Other	0	1 (5%)	0	
Tumor grade				.07
1	0	0	0	
2	9 (45%)	3 (15%)	0	
3	11 (55%)	17 (85%)	3 (100%)	
Median tumor size (cm, range)	2.5 (1.2-12)	2.3 (1.1-6)	3.3 (2-4.5)	.10*
Multifocal disease	3 (15%)	5 (25%)	0	.84
Stage				.91
1	4 (20%)	5 (25%)	0	
2	13 (65%)	11 (55%)	3 (100%)	
3	3 (15%)	4 (20%)	0	
Associated DCIS	n = 5	n=5		.17
PD-L1 negative	5 (100%)	2 (40%)	0	
PD-L1 positive	0	3 (60%)	0	
TIL intensity score ^c				.49
0	0	0	0	
Focal: 1	5 (25%)	2 (10%)	0	
Moderate: 2	10 (50%)	9 (45%)	1 (33%)	
Diffuse: 3	5 (25%)	9 (45%)	2 (67%)	
Carcinoma cell PD-L1 status ^d				.04
Carcinoma PD-L1 ⁺	1 (5%)	7 (35%)	1 (33%)	
Carcinoma PD-L1 ⁻	19 (95%)	13 (65%)	2 (67%)	
Lymphoid aggregate score				.08
0	13 (65%)	7 (35%)	0	
Focal: 1	2 (10%)	7 (35%)	1 (33%)	
Moderate: 2	1 (5%)	1 (5%)	1 (33%)	
Germinal centers: 3	4 (20%)	5 (25%)	1 (33%)	
Quantitative TIL count/mm ²				

	TIL PD-L1 expression ^a			<i>P</i> ^b
	PD-L1 ^{neg} (0,1)	PD-L1 ^{mod} (2)	PD-L1 ^{diffuse} (3)	
Total CD3, median (range)	732 (71-2294)	1716 (435-8465)	1871 (1653-2792)	.01*
Total CD4, median (range)	711 (67-6369)	2378 (405-13639)	2526 (2422-2718)	.04*
Total CD8, median (range)	806 (2-3169)	1232 (175-3842)	1053 (643-1386)	.32*
Total FoxP3, median (range)	261 (64-1115)	472 (87-1822)	422 (289-425)	.25*
Total CD20, median (range)	152 (6-1706)	333 (28-5776)	530 (58-596)	.08*
CD8/FoxP3 ratio, median (range)	2 (0.8-7.9)	2.2 (0.9-5.3)	2.5 (2.2-3.3)	.67*
CD4/FoxP3 ratio, median (range)	2.5 (0.86-11)	4.8 (2.4-9.1)	6.4 (5.98-8.4)	.14*

Abbreviations: DCIS, ductal carcinoma in situ; HER-2, human epidermal growth factor receptor 2; TIL, tumor-infiltrating lymphocytes and macrophages.

^aThe proportion of TILs expressing PD-L1 was graded as “none” (0), “focal” (1); “moderate” (2), or “severe” (3).

^bThe *P* values of categorical variables are derived from Fisher exact test, and the *P* values of continuous variables are derived from 2-sided *t* tests. The *P* values with asterisk (*) indicate that *P* values are derived from comparison between the group PDL1 = 0/1 and group PDL1 = 2/3.

^cImmune infiltrates included TILs and accompanying histiocytes and were scored as “none” (0), “focal” (1), “moderate” (2), or “diffuse” (3); see “Materials and methods” section.

^dTumor cells were considered PD-L1⁺ if greater than or equal to 5% of cells had membranous PD-L1 labeling.

Table 4

Relationship of tumor cell PD-L1 expression to clinical and immune parameters in primary breast carcinomas

	Tumor cell PD-L1 expression ^a		<i>P</i> ^b
	PD-L1 ⁻	PD-L1 ⁺	
Total evaluable tumors (n = 43)	34 (79%)	9 (21%)	
Tumor phenotype			.19
Luminal	13 (38%)	1 (11%)	
HER-2 ⁺	9 (26%)	5 (56%)	
Basal-like	12 (35%)	3 (33%)	
Median age (y, range)	57 (33-79)	48 (36-88)	.82
Patient race			.18
Black	13 (38%)	2 (22%)	
White	21 (62%)	6 (67%)	
Other	0	1 (11%)	
Tumor grade			.04
1	0	0	
2	12 (35%)	0	
3	22 (65%)	9 (100%)	
Median tumor size (cm, range)	2.5 (1.1-12)	2.1 (1.4-4.5)	.08
Multifocal disease	7 (21%)	1 (11%)	1.00
Stage			1.00
1	7 (21%)	2 (22%)	
2	21 (62%)	6 (67%)	
3	6 (18%)	1 (11%)	
Associated DCIS	n = 7	n=3	.008
PD-L1 negative	7 (100%)	0	
PD-L1 positive	0	3 (100%)	
TIL intensity score ^c	0	0	.002
0	7 (21%)	0	
Focal: 1	19 (56%)	1 (11%)	
Moderate: 2			
Diffuse: 3	8 (24%)	8 (89%)	
PD-L1 ⁺ TIL ^d			.04
None or focal: 0-1	19 (56%)	1 (11%)	
Moderate: 2	13 (38%)	7 (78%)	
Diffuse: 3	2 (6%)	1 (11%)	
Lymphoid aggregate score			<.001
0	20 (59%)	0	
Focal: 1	8 (24%)	2 (22%)	
Moderate: 2	3 (9%)	0	
Germinal centers: 3	3 (9%)	7 (78%)	

	Tumor cell PD-L1 expression ^a		<i>P</i> ^b
	PD-L1 ⁻	PD-L1 ⁺	
Quantitative TIL count/mm ²			
Total CD3: median (range)	769 (71-3944)	2460 (1506-8465)	.02
Total CD4: median (range)	1053 (67-6369)	2907 (2240-13 639)	.04
Total CD8: median (range)	777 (2-3169)	1462 (643-3842)	.002
Total FoxP3: median (range)	318 (64-1115)	965 (247-1822)	.02
Total CD20: median (range)	207 (6-1959)	846 (58-5776)	.16
CD8/FoxP3 ratio: median (range)	2.2 (0.8-7.9)	2.2 (0.9-5.3)	.99
CD4/FoxP3 ratio: median (range)	4.2 (0.86-11.0)	6.5 (2.4-9.08)	.16

Abbreviations: DCIS, ductal carcinoma in situ; HER-2, human epidermal growth factor receptor 2; TIL, tumor-infiltrating lymphocytes and macrophages.

^aTumor cells were considered PD-L1⁺ if greater than or equal to 5% of cells had membranous PD-L1 labeling.

^bThe *P* values of categorical variables are derived from Fisher exact test, and the *P* values of continuous variables are derived from 2-sided *t* tests.

^cImmune infiltrates included TILs and accompanying histiocytes and were scored as “none” (0), “focal” (1), “moderate” (2), or “diffuse” (3); see “Materials and methods” section.

^dThe proportion of TILs expressing PD-L1 was graded as “none” (0), “focal” (1); “moderate” (2), or “severe” (3).