# Functional $CD3^+CD8^+PD1^-$ T Cell Accumulation and PD-L1 Expression Increases During Tumor Invasion in DCIS of the Breast

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## Abstract

**We explored quantitative T-cell distribution among samples of 49 patients with ductal carcinoma in situ vs. those with minimal infiltration lesions to find the functional alterations. $CD3^+CD8^+$ programmed death 1-negative T cell and programmed death ligand 1-positive expression increased as disease progressed.**

**Background:** The changes in T cell subsets and programmed death ligand 1 (PD-L1) expression during the transition from ductal carcinoma in situ (DCIS) to early invasive breast cancer had not been well studied. **Patients and Methods:** A total of 85 DCIS patients were classified into 49 DCIS (clinical stage: Tis, noninvasive) and 36 with a minimally infiltrating lesion (MIL; $<$ 5 mm; clinical stage: T1a). We explored the quantitative alterations of T-cell markers and PD-L1 in these groups using the Opal multi-immunohistochemistry technique. **Results:** We observed increased infiltration of CD3-positive ($CD3^+$)$CD8^+$ programmed death 1 (PD1)-negative T cells and higher PD-L1 expression in DCIS with MIL. Elevated PD1 expression correlated with PD-L1 expression in MIL and DCIS. **Conclusion:** We conclude that during the transition from DCIS to an invasive lesion, the host cytolytic T cells begin interacting with the tumor and destroy the tumor tissue, leading to an adaptive upregulation of PD-L1 and tumor protection against immune destruction.

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**Keywords:** Breast cancer, Immune phenotype, Opal technique, PD1 immune microenvironment, T cells expression

## Introduction

Ductal carcinoma in situ (DCIS) of the breast, although generally associated with an excellent prognosis after local therapies, can be associated with invasive disease, recurrence, and rarely with metastases.[1] Prognosis for recurrence of DCIS is affected by factors such as tumor size, nuclear grade, margin status, and prognostic scores using clinical/pathological features such as The University of Southern California/Van Nuys prognostic index and multigene expression assays[2]; however, the host response to DCIS is not accounted for by these factors.

Emerging data support a role for the host immune response to invasive breast cancer, specifically infiltration of the tumor microenvironment by lymphocytes and myeloid cells in influencing prognosis and benefit from therapy.[3-6] Importantly, the relative composition of the immune cells and expression of programmed death ligand 1 (PD-L1) in invasive breast cancer varies depending on the subtype, with greater numbers of tumor infiltrating lymphocytes (TIL),[7] and $FoxP3^+$ regulatory T cells (Tregs)[8] in estrogen receptor (ER)-negative ($ER^-$) tumors, and higher expression of PD-L1 in $ER^-$ and triple-negative breast carcinomas.[9] In general, breast tumor PD-L1 expression is associated with increased TIL.[10,11] Although $CD8^+$ T-cell infiltration is typically associated with a better clinical outcome,[12] there are conflicting data on PD-L1 expression and the combination of high PD-L1 expression

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combined with high CD8$^+$ density has been associated with a worse clinical outcome in early stage breast cancer.[13] Among them, the expression of programmed death 1 (PD1) affects the prognosis most significantly. The published literature has shown the CD8+/PD-1+T cells exerted as cytotoxic effects in vitro,[14] whereas such biological functions were reversed in the presence of PD-L1 expressed in the tumor cells in vivo.[15] Therefore, we used the CD3$^+$CD8$^+$PD1$^-$ and CD3$^+$CD8$^+$PD1$^+$ T cell to observe the immune microenvironment.

Although there has been less study of DCIS, TIL and PD-L1 positivity have been reported to be greater in high-grade and ER$^-$ subtypes as observed in early invasive breast cancer.[16,17] However, there has been less study of the changes in composition of the T-cell infiltrate and PD-L1 expression during the transition from DCIS to invasive disease and whether any such changes might influence the development of invasive disease. One study[18] that compared invasive breast cancer and DCIS did observe fewer activated CD8$^+$ cytolytic T cells in invasive breast cancer, suggesting that immune evasion occurs during the DCIS to invasive cancer transition. We wished to study the immune composition of DCIS and DCIS undergoing early infiltration. To overcome previous limitations imposed by 1-color immunohistochemistry (IHC), we used a 7-color IHC method to characterize the T-cell infiltrate in DCIS and DCIS with minimal invasion (< 5 mm) which represents the Tis and T1a phases of clinical staging, respectively, and compared the areas of DCIS-only and areas with minimally infiltrating lesions (MIL) and correlated these findings with outcome.

## Patients and Methods

### *Ethical Approval*

This study was in accordance with the ethical standards of Beijing Shijitan Hospital, and approved by the Beijing Century Altar Ethics Committee. Informed consent was obtained from all individual participants included in the study.

### *Human Tissues and Histologic Evaluation*

After the institutional review board approved the protocol and waived the informed consent requirement (because this was a retrospective study), 85 archived, surgically resected, formalin-fixed, paraffin-embedded (FFPE) tissue samples (collected between 2012 and 2017) were obtained from the Beijing Shijitan Hospital, Capital Medical University. Forty-nine cases of DCIS, enriched for large lesions, clinical stage Tis without infiltrating lesions, were age- and size-matched with 36 cases of DCIS with MIL defined as an invasive component of no more than 5 mm, which represents early infiltration (clinical stage T1a). Diagnoses of DCIS and MIL were confirmed from hematoxylin and eosin-stained slides by a pathologist (F.Y.), and full slides were also evaluated on the basis of the guidelines from the International Immuno-Oncology Biomarkers Working Group ($\kappa > 0.75$). Of the total number of cases, 35 pure DCIS and 19 MIL also contained peritumoral tissue available for analysis.

Patient characteristics and clinical parameters including age, surgery type, and clinical status were obtained via medical record review. Pathologic characteristics including tumor stage, grade, size, composition, and hormone receptor status (ER and progesterone receptor assessed using standard IHC) were obtained from pathology reports. HER2 and Ki-67 were determined using standard IHC on freshly cut sections as a part of this study. HER2 positivity was confirmed using fluorescence in situ hybridization analysis for HER2 2+ tumors according to IHC.

### *Immunofluorescence Staining and Antibody*

Histology and multicolor immunofluorescence analyses (multiplex quantitative tissue imaging analysis) were performed from 5-μm sections of FFPE tissues. Slides were deparaffinized in xylene and rehydrated in a series of graded alcohols. In a serial fashion, each antigen was labeled with distinct fluorophores. Nuclei were subsequently visualized with 4′,6-diamidino-2-phenylindole (1:2000), and a cover slip was applied to the slides using Prolong Gold Antifade Mountant (P36934; ThermoFisher, Shanghai, China). Multiplex antibody panels applied in this study were: panel 1: anti-CD3 (MAB9626, 1:100, Opal 690; Abnova, Taipei, Taiwan]), PD1 (43248, 1:200, Opal 570; CST, Danvers, MA), PD-L1 (13684, 1:200, Opal 620; CST), anti-CD8 (22510 1:50, Opal 520; Abcam, Cambridge, MA); and panel 2: FoxP3 (13653T, 1:100, Opal 520; CST), CD4 (10400-R113, 1:100, Opal 690; Sino, Beijing, China), PD1 (43248, 1:200, Opal 570; CST; Figure 1).

### *Digital Image Acquisition and Analysis*

For the analysis, fluorescently labeled slides were scanned using the Vectra 2.0 (PerkinElmer, Waltham, MA) and TissueFAXS Fluo slide scanning system (TissueGnostics, Vienna Austria) based on a Zeiss Axio Imager Z2 upright epifluorescence microscope. Images were captured using a Zeiss 20 Plan-Apochromat air objective (0.8 numerical aperture). Analysis was performed using inForm 2.1 (PerkinElmer) and TissueQuest 5.0 (TissueGnostics) analysis software that identifies cells on the basis of segmentation of DAPI-stained nuclei on the basis of thresholds set for intensity to identify all DAPI nuclei. Measures of cell counts, mean intensity, percentage of positive cells of the total number of cells (% positive cells/all nucleated cells), and positive cell density were determined using the inForm 2.1 (PerkinElmer) and TissueQuest 5.0 (TissueGnostics) analysis platform.

### *Statistical Analysis*

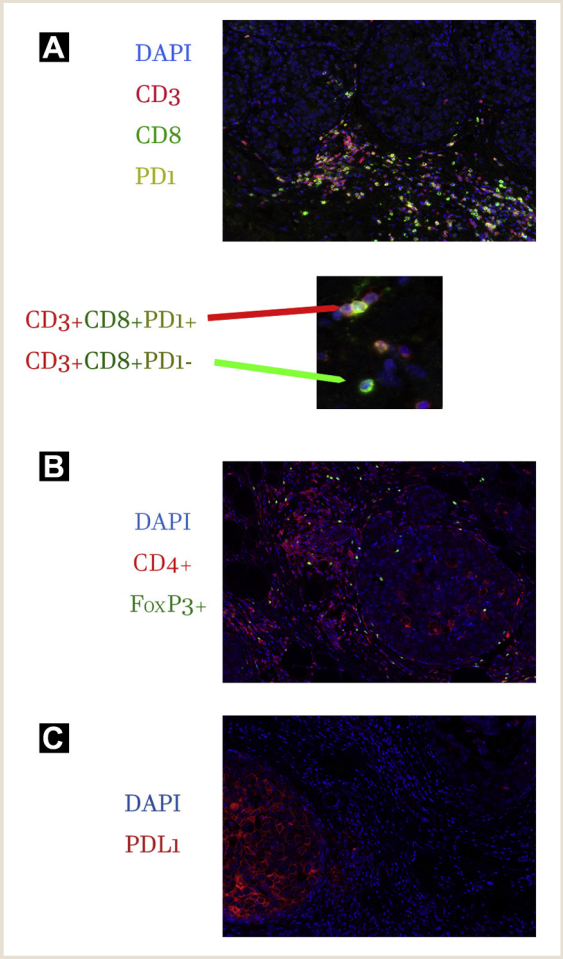
The fluorescence intensity of each pixel point reflects the expression level of the marker to which the fluorescence value corresponds. We calculated the amount of each marker expressed by each cell in a representative field. The number of cells positive for a particular marker and the proportion of the total cell number was also calculated. All data are represented as mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism software version 7.0 (San Diego, CA). *P* values were calculated using an unpaired 2-tailed Student *t* test.

## Results

### *Patient Characteristics*

The clinical and pathological characteristics of 85 cases are shown in Table 1. The mean age was 53 years (range, 30-91 years). Most cases were ER$^+$ and HER2$^-$. Twelve patients had breast-conserving surgery followed by radiotherapy. Seventy-three patients underwent total mastectomy without radiotherapy. After 5 years of follow-up, 1 patient had local recurrence and 1 patient had distant metastasis.

**Figure 1** Using the Multi-Target Immunofluorescence Staining to Mark Different CDs. (A) CD3, CD8, and Programmed Death 1 (PD1) Are Shown to Reflect the Immune Effect of $CD8^+$ T Cells. (B) CD4 and FoxP3 Reflect the Immune Effect of $CD4^+$ T Cells. (C) Programmed Death Ligand 1 (PD-L1) Reflects the Inhibited Immune Effect




Abbreviations: DAPI = 4′,6-diamidino-2-phenylindole; FoxP3 = forkhead box P3.

**Table 1** Clinical and Pathological Characteristics of the Study Cohort (n = 85)

| Variable | Patients, n (%) |
|---|---|
| **Age, y** | |
| ≤50 | 32 (38) |
| >50 | 53 (62) |
| **ER** | |
| Positive | 67 (79) |
| Negative | 18 (21) |
| **Progesterone Receptor** | |
| Positive | 66 (78) |
| Negative | 19 (22) |
| **HER2** | |
| Positive | 25 (30) |
| Negative | 60 (70) |
| **DCIS Nuclear Grade** | |
| Low | 21 (25) |
| Intermediate | 16 (19) |
| High | 48 (56) |
| **Molecular Subtype** | |
| Luminal A | 38 (45) |
| Luminal B | 30 (35) |
| **HER2-Enriched** | 14 (16) |
| Basal-like | 3 (4) |
| Pure DCIS | 49 (58) |
| DCIS with invasive lesion | 36 (42) |
| **Size of Invasive Lesion** | |
| ≤1 mm | 18 (21) |
| >1 mm and ≤5 mm | 18 (21) |
| **Operation** | |
| Lumpectomy | 12 (14) |
| Mastectomy | 73 (86) |
| **Radiotherapy** | |
| Yes | 12 (14) |
| No | 73 (86) |
| **Endocrine therapy** | |
| Yes | 59 (69) |
| No | 26 (31) |
| **$ER^+$ Endocrine Therapy** | |
| Yes | 57 (85) |
| No | 10 (15) |
| **Chemotherapy** | |
| Yes | 13 (15) |
| No | 72 (85) |
| **Chemotherapy** | |
| $HER2^+$ | 11 (85) |
| $HER2^-$ | 2 (15) |

Abbreviations: DCIS = ductal carcinoma in situ; ER = estrogen receptor.

### *Frequency of Peritumoral T-Cell Subsets Varies According to Molecular Subtypes of DCIS*

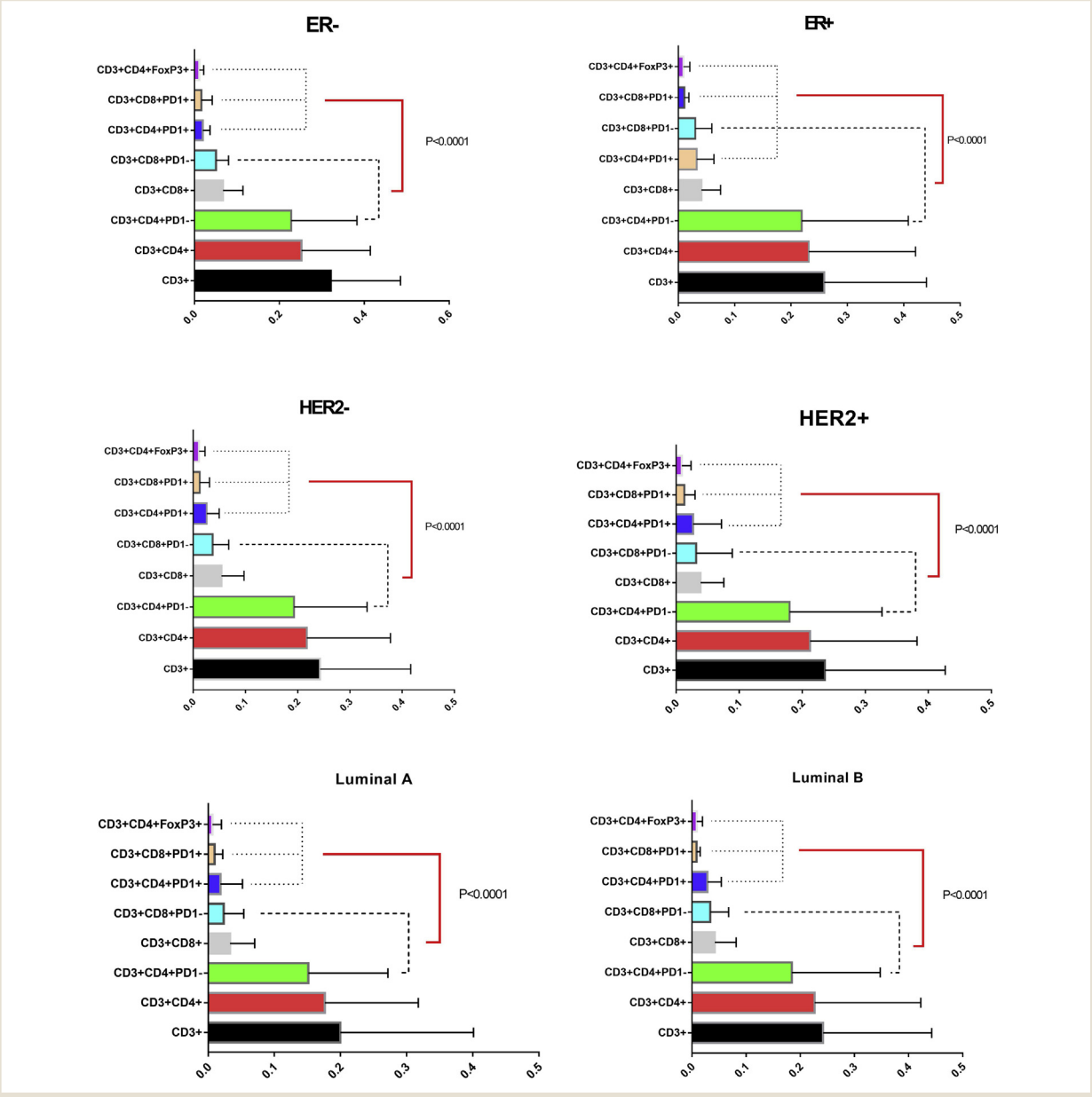
We used multiple-target immunofluorescence staining to analyze the composition of different lymphocyte subsets in the peritumoral tissues of all 85 DCIS specimens and then compared their frequency in the different molecular subtypes of DCIS. T helper cells ($CD3^+CD4^+PD1^-$) were the most frequent T-cell subtype, followed in order by $CD8^+$ T cytotoxic cells ($CD3^+CD8^+PD1^-$), Treg ($CD3^+CD4^+FoxP3^+$), and T exhaust cells ($CD3^+CD8^+PD1^+$), independent of molecular subtype, or hormone receptor status (Figure 2).

To explore the difference between the lymphocytes of DCIS and the lymphocytes of MIL, we evaluated the proportion of the various T cell subsets in the peritumoral/stromal areas (representative IHC analysis is shown in Figure 3A). As shown in Figure 3B, significantly more $CD3^+$ T cells were observed in DCIS than in MIL. Further, there was a trend for a greater infiltration with $CD3^+$ T cells in DCIS than the DCIS areas in MIL specimens. To study the specific

types of infiltrating T cells, we focused on CD3$^+$CD8$^+$PD1$^-$ cells, which represent T cells with a cytolytic role, and CD3$^+$CD8$^+$PD1$^+$ and CD3$^+$CD4$^+$FoxP3$^+$, which represented T cells with immunosuppressive functions. Compared with DCIS, MIL contained more CD3$^+$CD8$^+$PD1$^-$ cytotoxic T cells, whereas there were no significant differences of suppressive immune cells (CD3$^+$CD8$^+$PD1$^+$ and CD4$^+$FoxP3$^+$ T cells) between them. Moreover, the CD3$^+$CD8$^+$PD1$^-$ cytotoxic T cells were mainly expressed in carcinoma in situ parts in MIL compared with carcinoma in situ. These data suggest that an invasive component to DCIS is associated with an effector T-cell response.

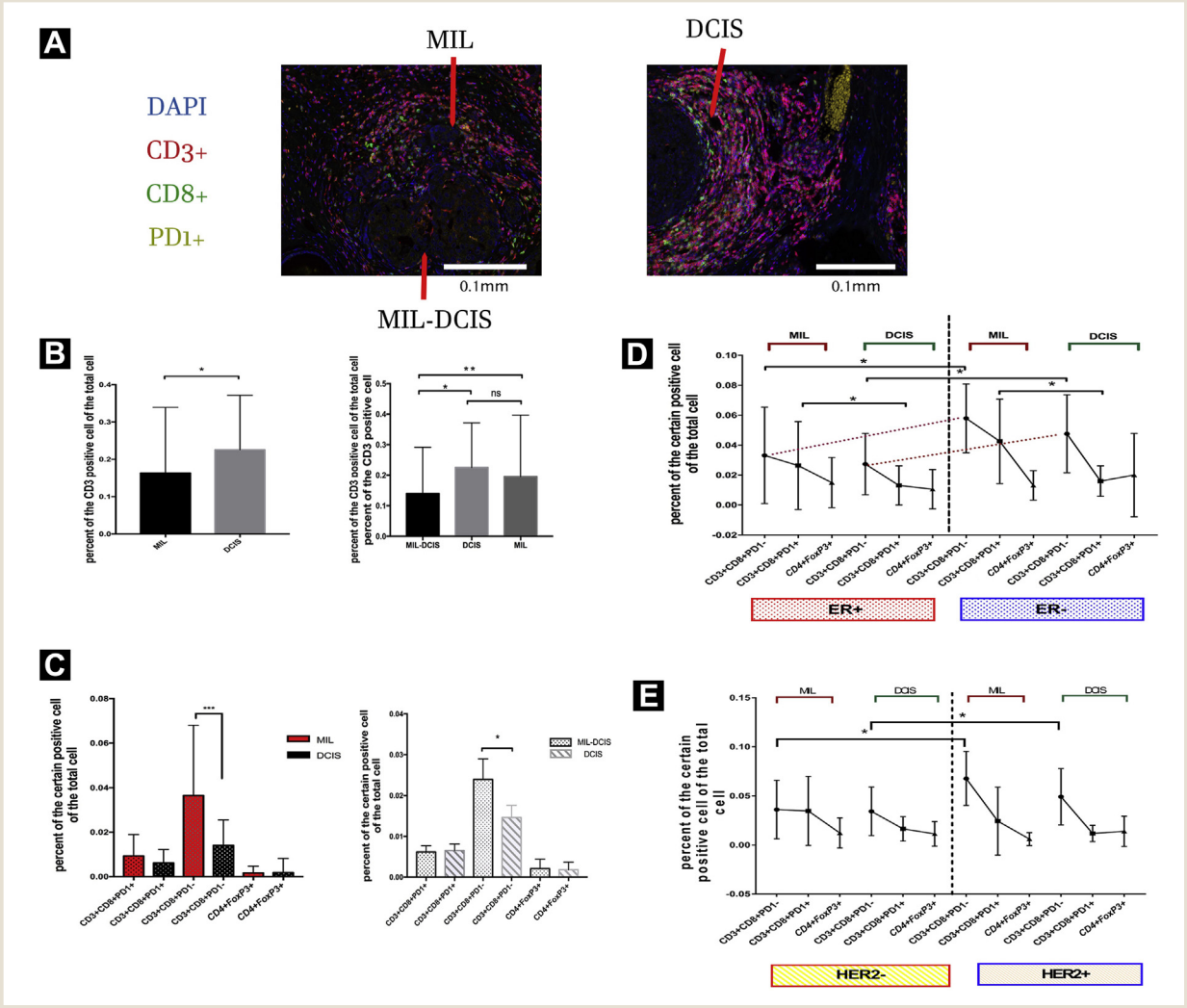
We evaluated how the various T cell subset proportions differ between the molecular subtypes of DCIS and MIL (Figure 3D). Although CD3$^+$CD8$^+$PD1$^-$ T cells were significantly higher in ER$^-$ breast cancer, compared with ER$^+$ breast cancer, there was no obvious difference between ER$^+$ MIL and ER$^+$ DCIS, nor between ER$^-$ MIL and ER$^-$ DCIS. In contrast, the CD3$^+$CD8$^+$PD1$^+$ T cells were greater in patients with MIL compared with DCIS in the ER$^+$ and ER$^-$ groups. We also assessed the relative proportion of the different T-cell subsets in HER2$^+$ compared with HER2$^-$ tumors (Figure 3E). In DCIS and MIL, the CD3$^+$CD8$^+$PD1$^-$ T cells were increased in HER2$^+$

**Figure 2** Different T Lymphocyte Subsets in Different Types of Breast Cancer




Abbreviations: ER = estrogen receptor; FoxP3 = forkhead box P3.

**Figure 3** The Different Immune Status of Minimally Infiltrating Lesion (MIL) and Pure Ductal Carcinoma in Situ (DCIS). (A) Immunofluorescent Staining of Tumor Sections for the Indicated Markers in DCIS and MIL, Respectively. (B) Different Ratio of Positive CD3 T Cells in the MIL, MIL-DCIS, and Pure DCIS. (C) Compared With the Carcinoma in Situ, There Were Obviously More $CD3^+CD8^+$ Programmed Death 1-negative ($PD1^-$) Killer T Cells in the MIL, Whereas There Were No Significant Differences Between Them in the Suppressive Immune Cells $CD3^+CD8^+PD1^+$ and $CD3^+CD4^+FoxP3^+$ Cells. (D) Different Types of Immune Cell Populations Around the Estrogen Receptor-Positive ($ER^+$) Breast Cancer and $ER^-$ Breast Cancer. (E) Different Types of Immune Cell Population Around the $HER2^+$ Breast Cancer and $HER2^-$ Breast Cancer. *$P$ < .05; **$P$ < .001; ***$P$ < .0001




Abbreviation: DAPI = 4′,6-diamidino-2-phenylindole; FoxP3 = forkhead box P3.

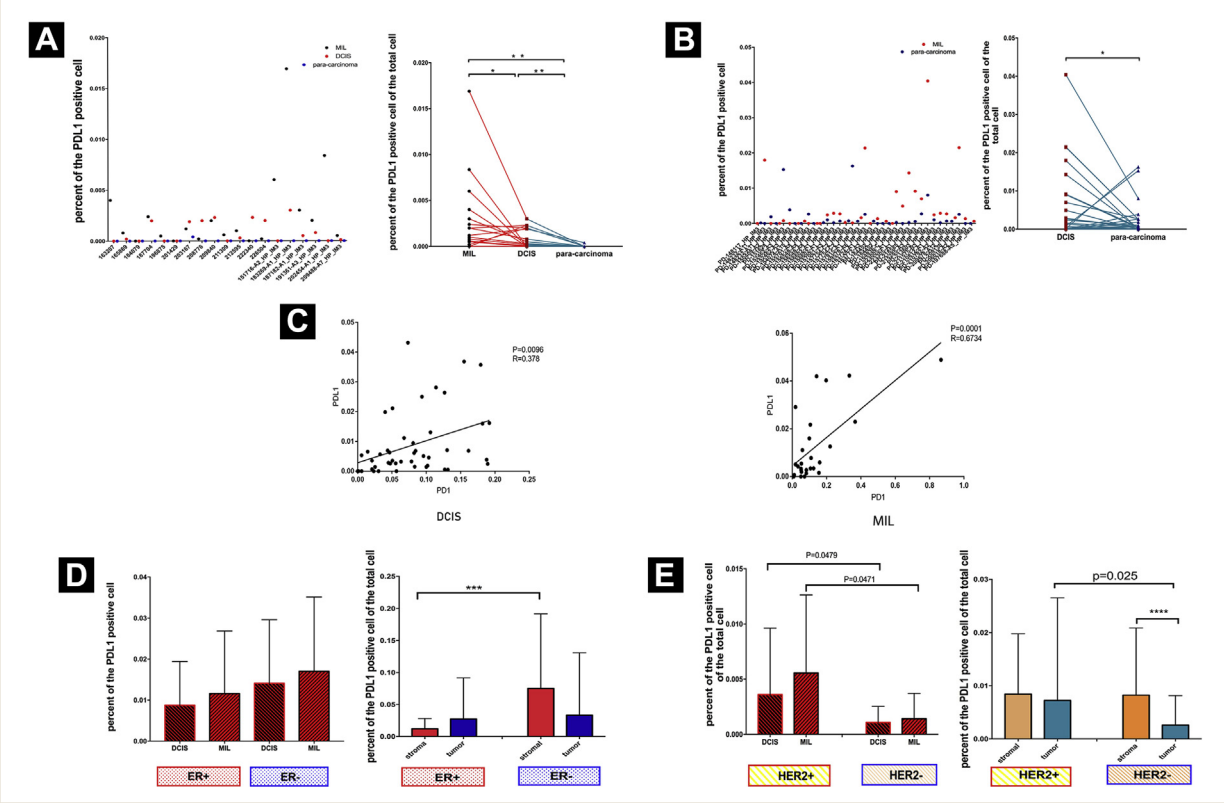
patients compared with $HER2^-$ patients whereas the other T-cell subsets did not differ between these 2 groups. These data suggest that hormone receptor signaling and HER2 overexpression affect the $CD3^+CD8^+PD1^-$ T cells.

### *Elevated PD-L1 Expression Predicts Tumor Infiltration*

To study the effects of the PD1/PD-L1 interaction on the immune microenvironment, we quantitated the PD-L1 expression in the stroma and tumor cells using a fluorescence-based assay. PD-L1 expression of stromal cells was significantly higher in $ER^-$ breast cancer than that in $ER^+$ breast cancer (Figure 4D). The overall PD-L1 expression was higher in $HER2^+$ breast cancer (DCIS and MIL) than that of $HER2^-$ breast cancer; however, among $HER2^+$ cancers, stromal cells and tumor cells had similar PD-L1 expression, whereas in $HER2^-$ cancers, PD-L1 expression of stromal cells was higher than that of tumor cells (Figure 4E).

We hypothesized that pure DCIS would have different PD-L1 expression than DCIS with an invasive component. To compare the expressions of PD-L1 in DCIS and in the DCIS portion of MIL, we chose specimens from 19 MIL patients and 35 DCIS patients who had peritumoral tissue available. In MIL, PD-L1 expression was generally higher than in the carcinoma in situ,

**Figure 4** Expression of Programmed Death Ligand 1 (PD-L1) in Minimally Infiltrating Lesion (MIL) and Pure Ductal Carcinoma in Situ (DCIS). (A) In the MIL, After the Tumor Infiltrated the Stroma, the PD-L1 Level Increased Correspondingly. (B) The Expression of PD-L1 Was Also Significantly Higher in the Carcinoma in Situ Than That in the Paracarcinoma Tissues. (C) With the Increase of PD-L1 Expression, Stroma PD1 Also Increased Accordingly, and the Correlation Between Them Was More Similar in the MIL. (D) Expression of PD-L1 in Different Sites in Estrogen Receptor-Positive (ER$^+$) and ER$^-$ Breast Cancer. (E) PD-L1 Expression Level Was Increased in Carcinoma of the Situ Part, the MIL Part, and the Tumor Tissue Part in HER2$^+$ Breast Cancer. *$P$ < .05; **$P$ < .001; ***$P$ < .0001




Abbreviation: ns = not significant.

which was higher than PD-L1 expression in the paracarcinoma tissues (Figure 4A and B). We also observed a correlation of PD-L1 and stromal PD1 in DCIS and MIL, but the correlation between them was closest in the MIL (Figure 4C).

## Discussion

Although the immune milieu of invasive breast cancer has been extensively studied, the local response to DCIS and changes in the immune milieu that accompany the development of microinvasive malignancy (early infiltration) have only been reported in a limited fashion, in part because of limitations imposed in standard IHC. More recently, the Opal technology has allowed multiple IHC parameters to be simultaneously analyzed in tissue specimens. Using this technology, we observed that in all breast cancer molecular subgroups, there was a greater frequency of cytolytic T cells, and a lower frequency of inhibitory T cells. Further, the number of CD3$^+$CD8$^+$PD1$^-$ cytotoxic T cells in the MIL was increased. It can be seen in Figure 3A that exhausted T cells expressing PD1 mainly accumulate in the infiltrated part of MIL, whereas in the lymphocytes around the DCIS part of the MIL, the number of CD3$^+$CD8$^+$PD1$^-$ cytotoxic T cells was higher than that of the carcinoma in situ. This suggests that when tumor invades the stroma, the tumor tissues can better interact with the stromal immune cells, resulting in the increase of cytotoxic T cells and also increase PD1$^+$ T cells. However, the tumor invasion might be the result of tissue destruction by immune cell infiltration and consistent with that reported in the literature.[19] We also studied the possible influences of ER and HER2 on the immune microenvironment. We observed, as have others,[20,21] that overall in DCIS, the immune response was more potent in ER$^-$ breast cancer than in ER$^+$ breast cancer, and the frequency of cytotoxic T cells was increased in HER2$^+$ breast cancer, but there was no difference between the MIL and carcinoma in situ. These data suggest that the increased CD3$^+$CD8$^+$PD1$^-$ T-cell expression in the MIL had little to do with whether there was ER expression and HER2 expression in the breast cancer, but rather, it was the early invasion that led to changes in the immune environment around them.

## Conclusion

We observed that PD-L1 expression was greatest in tumors with minimal infiltration, so we hypothesized that the main reason for the decrease of immune cells in invasive cancer was the associated increase in PD-L1 expression. In a comparison between $ER^+$ and $ER^-$ breast cancers, we found that the expression of PD-L1 in the stromal cells mainly accounts for the differences between the 2 groups. The reason for the higher PD-L1 expression in the stroma is suspected to be the increased interferon-γ secreted by the cytotoxic T cells,[22-24] which are increased in quantity in $ER^-$ breast cancer. In contrast, for $HER2^+$ DCIS, the expression of PD-L1 occurs mainly in tumor tissues. It has been reported that signal transducer and activator of transcription 3 in the HER2 pathway can directly increase the expression of PD-L1 in tumor tissue.[25-27] According to the results, our currently related results could lead us to perform more precise immunotherapy in the future.

### *Clinical Practice Points*

- The changes in T-cell subsets and PD-L1 expression during the transition from DCIS to early invasive breast cancer has not been well studied.
- We explored the quantitative alterations of T-cell markers and PD-L1 in 49 DCIS patients (clinical stage: Tis, noninvasive) and 36 patients with an MIL (< 5 mm; clinical stage: T1a) using the Opal multi-IHC technique.
- We observed increased infiltration of $CD3^+CD8^+PD1^-$ T cells and higher PD-L1 expression in DCIS with MIL.
- Elevated PD1 expression correlated with PD-L1 expression in MIL and DCIS.
- Our results could lead us to perform more precise immunotherapy in the future.

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## Disclosure

The authors have stated that they have no conflicts of interest.

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