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Original Contribution

The predictive and prognostic value of Foxp3+/CD25+ regulatory T cells and PD-L1 expression in triple negative breast cancer

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ABSTRACT

Recent significant developments in cancer immunotherapy have led to important breakthroughs and paradigm shifts in the treatment of malignancy. Although breast cancer traditionally has been considered less immunogenic, triple-negative breast cancer (TNBC) is the most immunogenic subtype with more stromal tumor-infiltrating lymphocytes (TILs) and higher programmed death-ligand 1 (PD-L1) expression. The goal of this study is to evaluate regulatory T cells (Tregs) and PD-L1 expression in TNBC, as well as their associations with clinicopathologic features and the outcomes. Tissue microarrays (TMA) of biopsy and resection specimens of 43 TNBC patients who underwent breast biopsy, neoadjuvant chemotherapy, and mastectomy were prepared. The number of Foxp3+ Tregs, Foxp3+/CD25+ Tregs, and expression of PD-L1 in tumor cells (PD-L1 TCs) and TILs (PD-L1 TILs) were assessed by immunohistochemistry. PD-L1 expression combined positive score (PD-L1 CPS) was calculated according to the manufacturer's guidelines. PD-L1 expression was detected in 72% of the cases, and it expressed in a higher percentage and higher intensity in TILs than TCs in TNBC ($p = 0.006$ and 0.0005, respectively). PD-L1 TCs, PD-L1 TILs, and PD-L1 CPS were all positively associated with pathologic complete response (pCR) ($p = 0.04$, 0.03, and 0.02, respectively). PD-L1 TILs and PD-L1 CPS also correlated with TILs and tumor infiltrating lymphocyte volume (TILV). Foxp3+ Tregs and Foxp3+/CD25+ Tregs had strong positive correlation ($r = 0.89$), and they were positively associated with TILs, TILV, and PD-L1 expression. Foxp3+/CD25+ Tregs, PD-L1 TCs, and PD-L1 CPS were positively correlated with better overall survival ($p = 0.04$, 0.04 and 0.01, respectively).

1. Introduction

Triple negative breast cancer (TNBC), defined by the lack of both ER/PR expression and HER2 amplification/overexpression, is a highly heterogeneous disease. It reportedly constitutes approximately 15%–25% of all breast cancer [1,2]. Clinically, TNBC is characterized by aggressive clinical behavior, poor outcome and lack of targeted therapy [1]. Consequently, neoadjuvant chemotherapy (NACT) followed by mastectomy is considered as the standard-of-care treatment for earlier staged and locally advanced TNBC with the aim of achieving a tumor down-stage and improving the chance of breast conservation [2-4]. Recent developments in cancer immunotherapy have led to important breakthroughs and paradigm shifts in the treatment of malignancy [5-7]. Even though breast cancer traditionally has been considered less immunogenic, TNBC is the most immunogenic subtype due to genomic instability and higher rates of mutation, with more stromal tumor infiltrating-lymphocytes (TILs) and higher programmed death-ligand 1 (PD-L1) expression [5-7].

TILs, the adaptive host immune response, have emerged as a predictive and prognostic biomarker in TNBC. Compelling evidence including ours has demonstrated that the presence of high TILs and tumor infiltrating lymphocyte volume (TILV) is associated with high pathologic complete response (pCR) rate after NACT and improved overall survival in TNBC [8-12]. Among the different subtypes of TILs, considerable attention has been paid to regulatory T cells (Tregs) [13-16]. Tregs are a heterogeneous population of T cells that composed of discrete subsets with different phenotypes and functions. They are characterized by high expression of interleukin-2 receptor α chain (CD25) and Forkhead box protein 3 (Foxp3), and function by modulating the immune system, maintaining tolerance to self-antigens, and preventing autoimmune disease [14-16]. CD25 is a classic surface marker used for identification of Tregs before the discovery of Foxp3. Studies in mice models have shown that anti-CD25 antibody partially depletes Tregs in the peripheral lymphoid organs or blood, and inhibits tumor growth to

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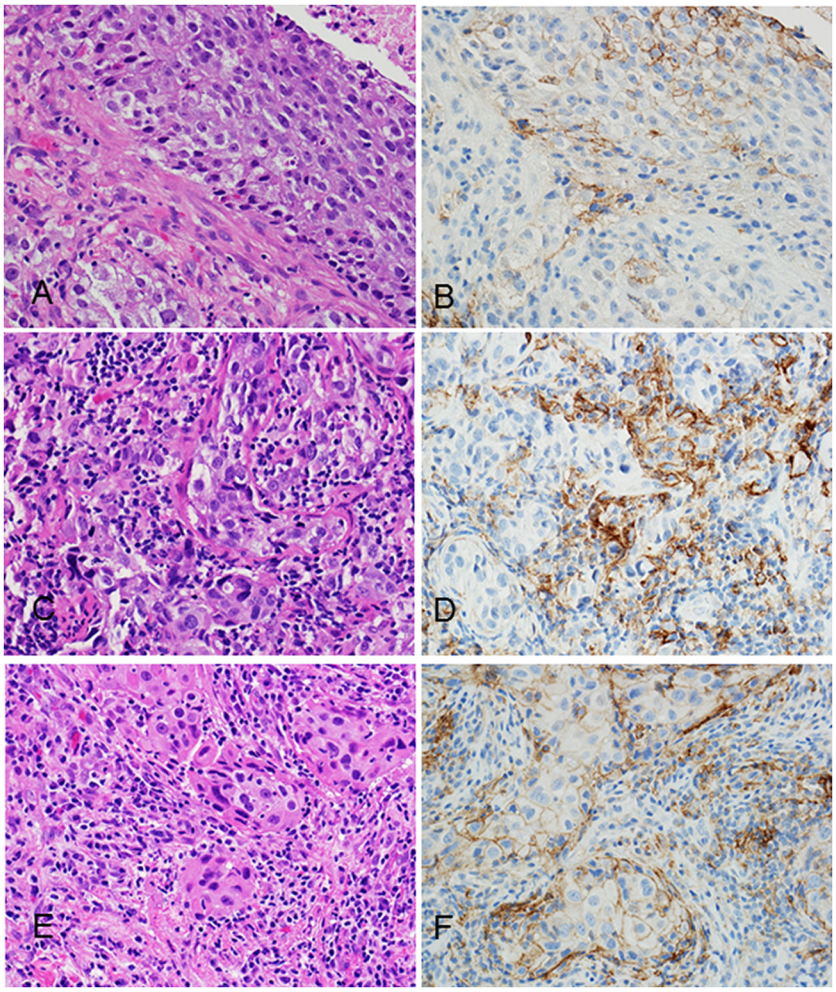

Fig. 1. Immunohistochemical stains of PD-L1.
A–B, High PD-L1 expression in TCs only; C–D, High PD-L1 expression in TILs only; E–F, High PD-L1 expression in both TCs and TILs.

improve survival in several types of cancer [17,18]. Foxp3 functions as a master regulator in the development and control of Tregs and it is now considered as the most specific and reliable marker of Tregs [14-16]. Due to the ability to inhibit anti-tumor immunity, high level of tumor-infiltrating Foxp3+ Tregs is expected to be associated with poor prognosis [19-23]. However, recent studies have challenged this idea and shown that Foxp3 + Tregs could improve survival in some tumors, including breast cancer [23-27]. Others fail to show any correlation of Foxp3+ Tregs with survival [28]. To date, the prognostic significance of Foxp3+ Tregs in breast cancer still remains controversial and further research is required to fully understand its significance [24-28].

In addition, TNBC is also more likely to have increased PD-L1 expression in the tumor microenvironment [29-31]. PD-L1 expression ranges from 5–85% in TNBC, while 0–46% in the other breast cancer subtypes [29]. Programmed cell death protein 1 (PD-1) is an immune checkpoint inhibitor which is expressed on the surface of immune effector cells. It is activated mainly by PD-L1, which is expressed in both tumor cells and infiltrating immune cells. Blockage of PD-L1/PD-1 axis in the tumor microenvironment has been shown to promote tumor regression, and has emerged as a promising therapeutic candidate for targeted therapy to enhance antitumor immunity [32]. Early data from clinical trials on anti-PD1/PD-L1 monoclonal antibodies have shown promising results in advanced breast cancer, especially in TNBC with an overall response rate varied from 5 to 30% in heavily pretreated patients [33]. In the neoadjuvant setting, preliminary results from very limited studies have suggested that high pCR rates is associated with combined blockade of the PD-1/PD-L1 pathway and chemotherapy [6,34]. Multiple trials have been initiated to evaluate the combination of checkpoint inhibitors with other anticancer agents in the treatment of breast cancer, especially in TNBC [6,34]. Despite the promising results, there have been conflicting results as to whether PD-L1 expression has favorable or adverse prognostic significance in breast cancer. Some studies found a positive correlation between PD-L1 expression and a favorable prognosis [35-39]. Others found a negative or no correlation between PD-L1 expression and prognosis [40-44], while Mori et al. found correlation to prognosis only when PD-L1 expression was seen in combination with low TILs score [42].

Although this is an exciting time for immunotherapy, we are still far from understanding the complexity of the tumor-immune microenvironment. In this pilot study, we aimed to investigate the predictive and prognostic value of PD-L1 expression and Tregs in a cohort of TNBC, and their association with clinicopathological features and the outcomes.

2. Materials and methods

2.1. Study patients and pathologic sample selection

The study was approved by Institutional Review Board at University of Texas McGovern Medical School (IRB# HSC-MS-14-1022). We retrospectively identified 58 TNBC patients who underwent breast biopsy, standard neoadjuvant therapy, and mastectomy in our institution

Table 1
Correlation of PD-L1 expression with clinicopathologic features.

	PD-L1 H[a] TCs	PD-L1 L[a] TCs	*P*	PD-L1 H[a] TILs	PD-L1 L[a] TILs	*P*	CPS H[a]	CPS L[a]	*P*
Age (years)									
< 50	7	16	0.5	17	6	0.1	12	15	0.4
≥50	8	12		10	10		5	11	
Tumor size (cm)									
< 2 cm	4	3	0.2	4	3	0.8	3	4	0.8
2–5 cm	6	9		6	9		6	10	
≥5 cm	6	15		9	12		8	12	
Lymph node status									
Positive	8	11	0.4	3	18	0.7	10	11	0.3
Negative	7	17		4	18		7	15	
TILs									
Low (< 60%)	10	19	0.8	11	18	0.03	7	22	0.003
High (≥60%)	5	9		10	4		10	4	
Stroma ratio									
Poor (< 50%)	12	22	0.9	15	19	0.5	13	19	0.4
Rich (≥50%)	3	6		3	6		3	8	
TILV									
Low (< 1600)	11	21	0.1	10	22	0.005	9	23	0.009
High (≥1600)	4	7		9	2		8	3	
Response to NACT									
pCR	9	11	0.04	14	6	0.03	12	8	0.02
RD	4	19		7	16		6	17	

TILs, tumor-infiltrating lymphocytes; TILV, tumor-infiltrating lymphocytes volume; NACT, neoadjuvant chemotherapy; pCR, pathologic complete response; RD, residual disease; PD-L1, programmed death ligand 1; CPS, combined positive score; TCs, tumor cells.

[a] Mean of PD-L1 expression in TCs (20) and TILs (60), and CPS (20) was used as the cut-off for H (high) and L (low).

during 2007–2017. Paraffin tissue blocks of the biopsy and resection specimens from 43 patients were available for further studies.

2.2. Tissue microarray (TMA) construction

The H&E slides of all initial core biopsies of TNBC cases and follow-up surgical specimens with residual tumor were reviewed by a breast pathologist and representative tumor areas were selected and marked directly on the H&E slides. The cylindrical tissue sample was cored (diameter 3 mm) from the corresponding tumor area in the paraffin block and extruded directly into the recipient block. Multiple 4-μm sections were cut with a microtome and transferred to glass slides for further immunohistochemistry analysis.

2.3. Foxp3 and Foxp3/CD25 immunohistochemistry staining and analysis

The Foxp3/CD25 double staining was performed as previous described [45]. Briefly, antibody cocktail was made by combining rabbit anti-human CD25 (1:100, Abcam, ab128955, Cambridge, MA) and mouse anti-human Foxp3 (1:200, Abcam, ab20034, Cambridge, MA). All primary antibodies were diluted in GeneTex antibody diluent (GeneTex, GTX28208, Irvine, CA). For Foxp3/CD25 double staining, the secondary antibodies were anti-mouse-HRP and anti-rabbit-AP (Biocare Medical, MRCT525H, Pacheco, CA). After xylene x 3 times deparaffin treatment and sequential alcohol rehydration, sections of TMA were treated with TE buffer (PH = 9.0) at 100 °C for 45 min using a steamer for antigen retrieval. After cooling in room temperature for 30 min, endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. Then nonspecific binding was blocked with non-immune horse serum (VECTOR LABS, S-2000, Burlingame, CA) for 20 min. The primary antibody of anti-Foxp3/CD25 cocktail was incubated for 1 h at room temperature in a moisture chamber. Five times washing were performed with 1× PBS washing buffer between the primary and secondary antibodies. The double secondary antibodies for Foxp3/CD25 were incubated at room temperature for 30 min. Signals were visualized after adding 3, 3′-diaminobenzidine tetrahydrochloride (DAB) and Vulcan Fast Red Chromogens. Tonsil tissue was used as the positive control, and TMA section with mouse serum was served as the negative control. Three representative images were taken at 200× magnification by a breast pathologist (SZ). The number of Foxp3+ and Foxp3+/CD25+ TILs were counted using ImageJ software (National Institute of Health, Maryland, MD).

2.4. PD-L1 immunohistochemistry staining and expression scoring

PD-L1 immunohistochemistry staining using the PD-L1 IHC 22C3 PharmDx qualitative assay (Agilent technologies, California) strictly followed the manufacturer's protocol on the 4-μm thick unstained TMA sections using EnVision FLEX visualization system on Autostainer Link 48. Tonsil and placenta tissue were used as positive controls, as these organs have been shown to constitutively express PD-L1.

PD-L1 TCs and PD-L1 TILs were manually analyzed by a breast pathologist (SZ). PD-L1 expression was assessed as the percentage of positively stained cells and the staining intensity. Membranous PD-L1 stain in TCs or cytoplasmic and/or membranous PD-L1 stain in TILs is considered as positive. The intensity of expression was scored as 0 (no expression), 1 (weak expression), 2 (moderate expression), or 3 (strong expression). The final PD-L1 expression score (for TCs or TILs) was calculated as percentage of PD-L1 positive cells x intensity of PD-L1 expression. The PD-L1 expression Combined Positive Score (CPS) was calculated according to the manufacturer's guidelines. It was calculated as the number of PD-L1-stained cells (tumor cells, lymphocytes, and macrophages), divided by the total number of viable tumor cells, and multiplied by 100. CPS ≥ 1 is considered as positive for PD-L1 expression.

2.5. Statistical analysis

The continuous variables obtained were analyzed using the Student *t*-test, whereas the categorical data were evaluated using chi-squared test, as appropriate. The correlation of two factors was performed using Pearson correlation coefficient calculator. Overall survival (calculated from the date of diagnosis to the date of death from any cause or to the follow-up cutoff) was plotted on Kaplan-Meier curves. Patients who survived to the study cutoff and those lost to follow-up were considered censored data in the analysis. Differences between the survival curves were determined using the log-rank test. A p value of less than 0.05 was considered statistically significant. The Kaplan-Meier survival curve was performed using SPSS software (IBM, New York, NY).

The cutoffs for high (H) or low (L) were defined according to the mean of each variable as follows: 20 for PD-L1 TCs, 63 for PD-L1 TILs, 20 for CPS, 34 for Foxp3, and 7 for Foxp3/CD25.

3. Results

3.1. Clinicopathologic features of study patients

Briefly, all cases were histologically confirmed as invasive carcinoma of no special type (ductal, not otherwise specified) with an ER/PR/HER2 triple-negative phenotype. The mean and median age at diagnosis was 46 and 46 (range, 24–64). Clinical stages at diagnosis were stage I (4, 9%), stage II (24, 56%), and stage III (15, 35%). All patients received standard NACT (4 cycles of doxorubicin + cyclophosphamide followed by 12 cycles of paclitaxel weekly) before mastectomy. Among them, 20 patients (47%) achieved pCR following NACT whereas the rest of patients (53%) still had residual disease. The follow-up period ranged from 8 to 135 months.

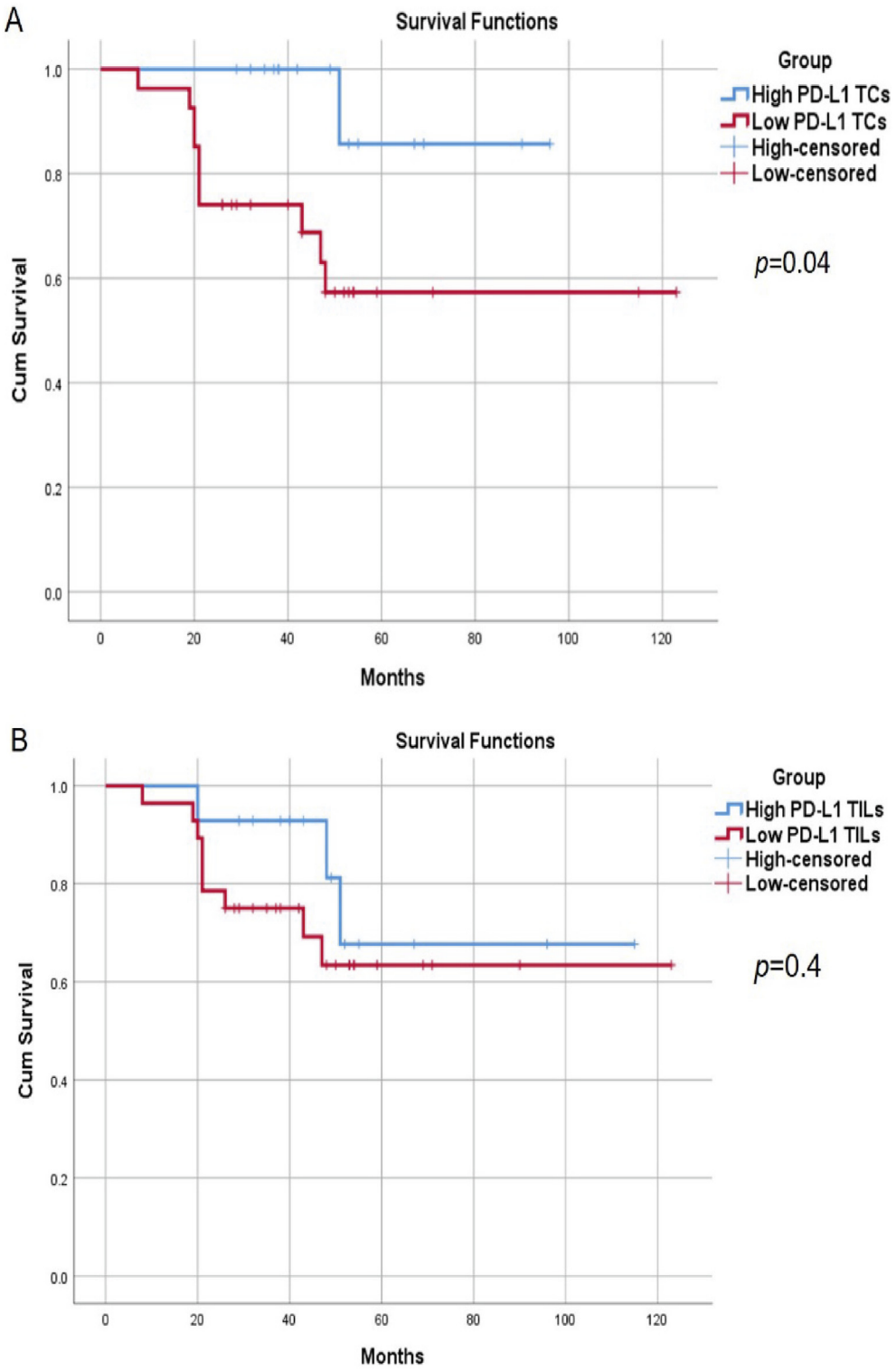

Fig. 2. Correlation of PD-L1 TCs (A), PD-L1 TILs (B), PD-L1 CPS (C), Foxp3+ Tregs (D), and Foxp3+/CD25+ Tregs (E) with overall survival. Mean of PD-L1 expression in TC (20) and TILs (60), CPS (20), Foxp3+ Tregs (34), and Foxp3+/CD25+ Tregs (7) were used as the cut-off for H (high) and L (low).

3.2. The association of PD-L1 expression with clinicopathologic features and overall survival

Among the cases available for PD-L1 assessment, PD-L1 expression (CPS ≥1) was identified in 31 cases (72%) including 4 cases (9%) expressed by TCs (PD-L1 TCs) only, 16 cases (37%) by TILs (PD-L1 TILs) only, and 11 cases (26%) by both TCs and TILs. Strong PD-L1 expression (score 3) was observed in 20 cases, including 3 cases in TCs only (Fig. 1A–B), 15 cases in TILs only (Fig. 1C–D), and 2 cases in both TCs and TILs (Fig. 1E–F). PD-L1 expression in > 50% of the TCs was present in 4 cases, in > 50% of TILs was presented in 7 cases, and in > 50% of both TCs and TILs was observed in 2 cases. Overall, PD-L1 expression tends to be expressed in a higher percentage and higher intensity in TILs than TCs in TNBC (p = 0.006 and 0.0005, respectively).

We then evaluated the association of PD-L1 expression with clinicopathologic features (Table 1) and overall survival (Fig. 2A–C). The

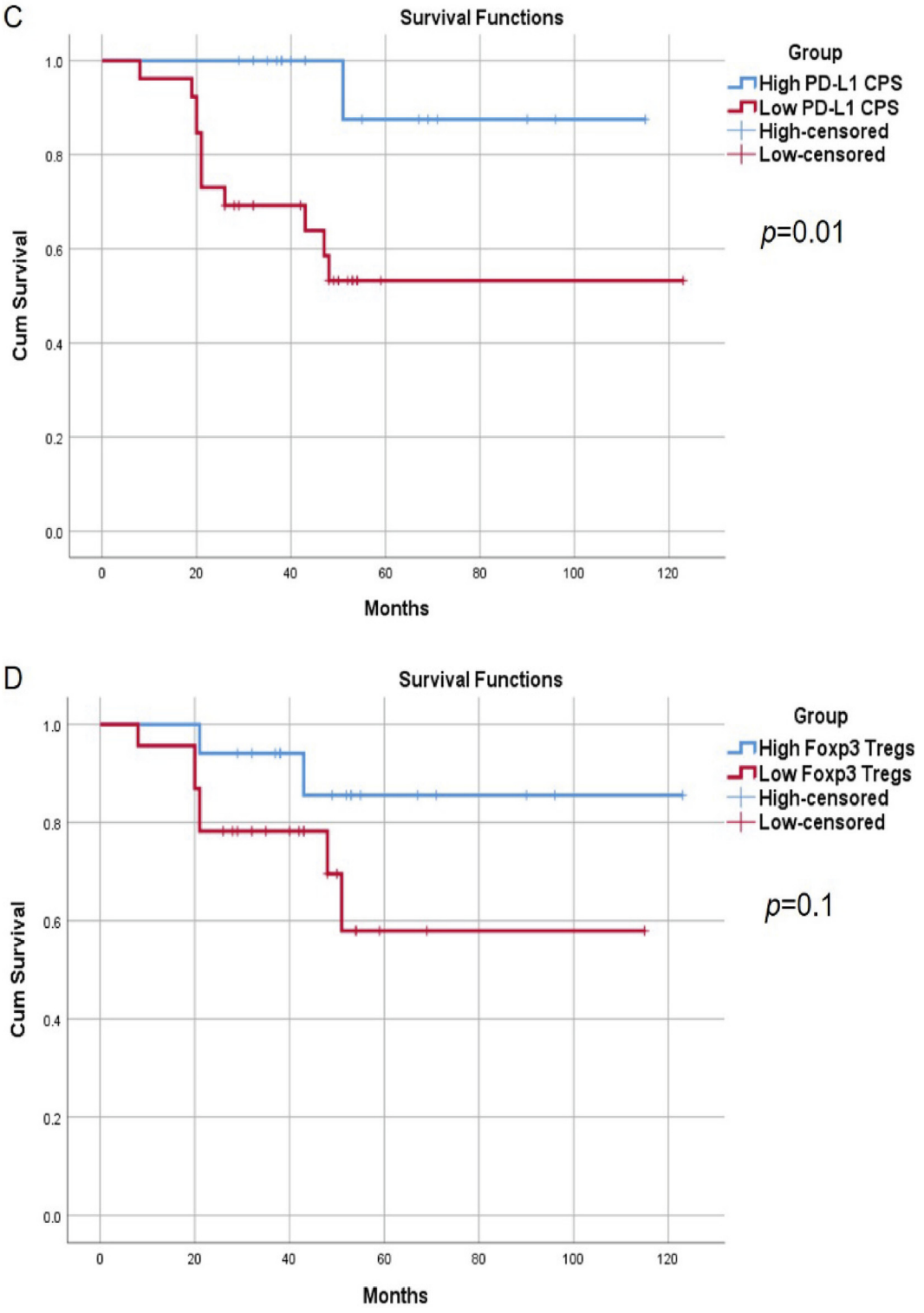

Fig. 2. (*continued*)

clinicopathologic features included patient's age, tumor size, pre-NACT lymph node (LN) status, TILs, stroma ratio, TILV, and response to NACT. Our results showed that PD-L1 TILs was positively associated with stromal TILs ($p = 0.03$), TILV ($p = 0.005$), and pCR rate ($p = 0.03$), while PD-L1 TCs was only positively associated with pCR rate ($p = 0.04$). Similar to PD-L1 TILs, CPS score was also positively correlated with stromal TILs ($p = 0.003$), TILV ($p = 0.009$), and pCR rate ($p = 0.02$). PD-L1 TILs, PD-L1 TCs, or PD-L1 CPS was not correlated with patient's age, tumor size, pre-NACT LN status, and stroma ratio. Lastly, PD-L1 TCs (Fig. 2A) and PD-L1 CPS (Fig. 2C) were significantly associated with better overall survival ($p = 0.04$ and $p = 0.01$, respectively), while PD-L1 expression in TILs (Fig. 2B) was not.

3.3. The association of Tregs with clinicopathologic features and overall survival

Next, we quantified two subsets of Tregs, Foxp3+ Tregs and

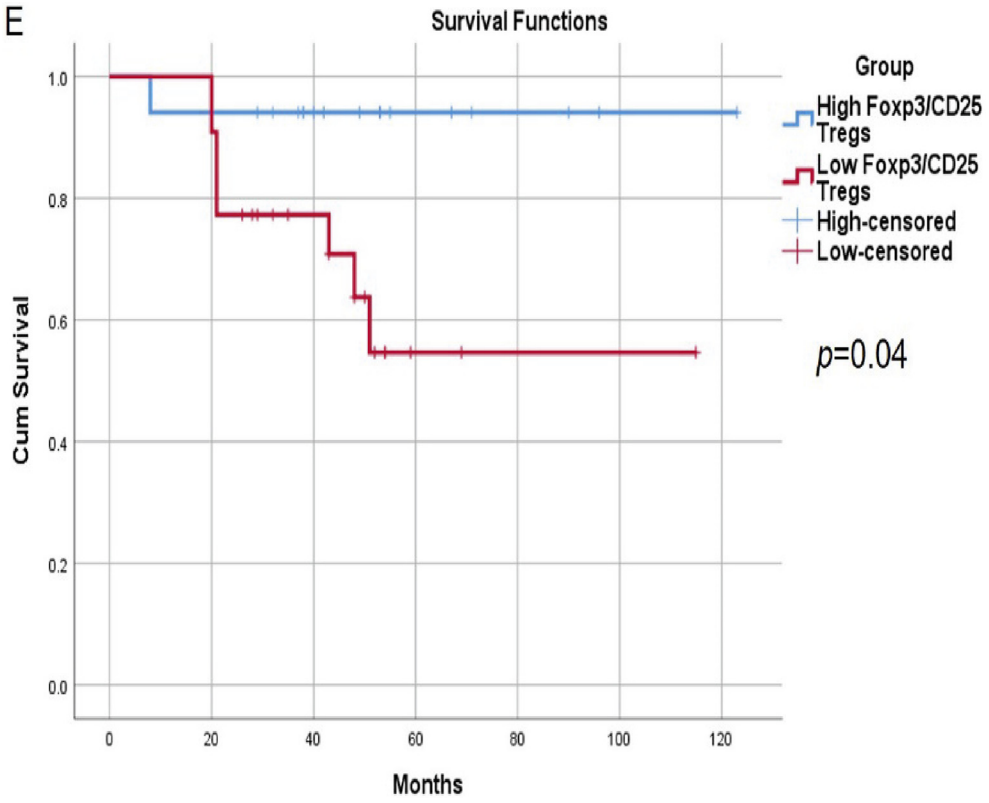

Fig. 2. (*continued*)

Foxp3+/CD25+ Tregs, in the tumor sections and evaluated their correlation with clinicopathologic features (Table 2) and overall survival (Fig. 2D–E). Our result showed Foxp3+ and Foxp3+/CD25+ Tregs were positively associated with TILs ($p = 0.04$ and 0.013, respectively), TILV ($p = 0.026$ and 0.005, respectively) and PD-L1 expression (Table 2). Most importantly, although Foxp3+ Tregs and Foxp3+/CD25+ Tregs had a strong positive correlation ($r = 0.89$), they were not identical. Some cases showed good correlation between Foxp3+ and Foxp3+/CD25+ cells (Fig. 3A and B), but other cases showed poor correlation between them (Fig. 3C and D). While Foxp3+ Tregs did not show any correlation with overall survival (Fig. 2D), Foxp3+/CD25+ Tregs were positively associated with patient's overall survival ($p = 0.04$) (Fig. 2E). None of them was associated with patient's age, tumor size, Pre-NACT LN status, and stroma ratio. In addition, they did not correlate with pCR rate.

3.4. Assessment of the effect of NACT on PD-L1 expression and Tregs

Lastly, we compared the PD-L1 expression and Tregs longitudinally between biopsy and resection specimens of the residual disease group. Although PD-L1 TCs and PD-L1 TILs were not significantly different before and after NACT, PD-L1 CPS was significant decreased after the NACT ($p = 0.01$). In addition, Foxp3+ Tregs and Foxp3+/CD25+ Tregs were also significantly reduced after the NACT ($p = 0.005$ and 0.03, respectively).

4. Discussion

TILs are a heterogeneous population and various TILs subtypes have different roles in tumor progression, either suppressing tumor growth by destroying cancer cells, or promoting tumor progression by selecting those tumor cells that could survive in an immunocompetent host. Increasing evidence has demonstrated the prognostic and predictive roles of TILs in breast cancer regardless of their cellular subtypes [8-12]. Tregs are a specialized subpopulation of TILs that suppress the activation of other immune cells, thereby maintaining systemic immune homeostasis. In previous studies, Tregs were mostly evaluated by using Foxp3 immunohistochemistry alone [19-28]. However, Foxp3+ T lymphocytes are heterogeneous cells, and it is evident that Foxp3 alone is not sufficient to identify heterogeneous Tregs [46-48]. For example, in a recent study of a cohort of colorectal cancer patients, no association of Tregs and outcome was found when Tregs were identified by Foxp3 expression alone. However, when the transcription factor, B lymphocyte-induced maturation protein-1 (Blimp-1), was used alongside Foxp3 to identify a subpopulation of Tregs, there was a positive association with patient outcome [47]. In another cohort study of patients with B-cell lymphoma, Tregs identified by expression of Foxp3 alone were associated with good patient outcome, but cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)+/Foxp3+ double-positive Tregs were associated with poor patient outcome [48]. These results emphasized the importance of using more than one marker to identify Tregs subsets and to evaluate their functions in human cancer tissue [45-48].

In addition, the impact of Tregs in TNBC has been reported in limited studies, and most studies were focused on the prognostic value with conflicting results. Lee et al. has assessed the quantity of Foxp3 Tregs in TNBC and investigated the correlation of Foxp3 Tregs with prognostic factors. They found Foxp3+ Tregs were associated with progression-free survival and overall survival. In addition, Foxp3+ Tregs had stronger prognostic significance than Foxp3- Tregs in TNBC [25]. Miyashita et al. studied the significance of CD8+/Foxp3+ Tregs in the residual tumor of TNBC patients not achieving pCR after NACT and demonstrated that a high CD8/Foxp3 ratio in residual tumors had significantly better recurrence-free survival and breast cancer-specific survival [22]. West et al. showed high Foxp3+ TILs levels were strongly associated with prolonged recurrence-free survival, particularly among basal-like tumors, for which Foxp3 status was an independent prognostic factor [26]. However, a very recent study by Althobiti et al. did not show any significant association between

Table 2
Correlation of Foxp3+ Tregs and Foxp3+/CD25+ Tregs with clinicopathologic features.

	Foxp3+ H[a] Tregs	Foxp3+ L[a] Tregs	*P*	Foxp3+/CD25+ H[a] Tregs	Foxp3+/CD25+ L[a] Tregs	*P*
Age (years)						
< 50	9	13	0.7	8	14	0.5
≥50	9	10		9	10	
Tumor size (cm)						
< 2	4	4	0.85	4	4	0.5
2–5	9	12		9	12	
≥5	5	7		4	8	
Lymph node status						
Positive	9	11	0.9	7	13	0.4
Negative	9	12		10	11	
TILs						
Low (< 60%)	7	19	0.004	7	19	0.013
High (≥60%)	11	4		10	5	
Stroma						
Poor (< 50%)	14	20	0.4	13	21	0.35
Rich (≥50%)	4	3		4	3	
TILV						
Low (< 1600)	9	19	0.026	8	21	0.005
High (≥1600)	9	4		9	3	
Response to NACT						
pCR	10	11	0.6	9	12	0.7
RD	8	12		8	12	
PD-L1 expression						
PD-L1 H[a] TCs	9	4	0.03	9	4	0.02
PD-L1 L[a] TCs	9	18		8	19	
PD-L1 H[a] TILs	12	7	0.03	12	8	0.025
PD-L1 L[a] TILs	6	15		5	15	
CPS H[a]	11	6	0.03	12	6	0.005
CPS L[a]	7	16		5	17	

TILs, tumor-infiltrating lymphocytes; TILV, tumor-infiltrating lymphocytes volume; NACT, neoadjuvant chemotherapy; pCR, pathologic complete response; RD, residual disease; PD-L1, programmed death ligand 1; CPS, combined positive score; Tregs, regulatory T cells; Foxp3, Forkhead box protein 3.

[a] Mean of PD-L1 expression in TC (20) and TILs (60), CPS (20), Foxp3+ Tregs (34), and Foxp3+/CD25+ Tregs (7) were used as the cut-off for H (high) and L (low).

Foxp3+ T cells and patient outcome in TNBC [49].

In our study, we quantified Foxp3+ and Foxp3+/CD25+ subsets of Tregs, and evaluated the predictive and prognostic values of these two populations in TNBC. As we hypothesized, although there was a strong correlation between Foxp3+ Tregs and Foxp3+/CD25+ Tregs, they were not identical. Foxp3+/CD25+ Tregs were associated with better overall survival, but Foxp3+ Tregs were not. Foxp3+ Tregs and Foxp3+/CD25+ Tregs were positively associated with TILs, TILV, and PD-L1 expression, but none of them correlated with pCR rate in the setting of neoadjuvant chemotherapy. Our results supported that Foxp3 alone was not sufficient to evaluate Tregs due to the heterogeneity of regulatory T cells. The addition of more detailed markers, used in conjunction with one another, will allow for a deeper understanding of the role of Tregs in cancer microenvironment and whether they can be used as biomarkers to improve targeted therapies.

PD-L1 expression has been studied extensively in breast cancer. These included 3 largest studies with a total of 6527 patients. In general, PD-L1 expression in all breast cancer subtypes varied between 0% and 83%, with the majority studies showing expression below 50% [29]. Eight studies investigated expression in each specific subtype and found that PD-L1 expression ranged from 2.3 to 37% in luminal A subtype, 9–46% in luminal B subtype, 0–33% in HER2-positive subtype, and 5–80% in basal-like/triple-negative BC subtype [29]. In addition, there have been conflicting results in the literature in regard to whether PD-L1 expression was a favorable or adverse prognostic factor. Mittendorf et al. reported membranous PD-L1 expression in 19% of their TNBC patient cohort (n = 105) with no outcome data reported [30]. Their study also found that PD-L1 expression was significantly higher in TNBC than non-TNBC and a greater number of intratumoral CD8+ T cells were associated with PDL1-positive disease than PDL1-negative disease [30]. Beekers et al. investigated the expression of PD-L1 expression in different cellular comportment in a cohort of 191 TNBC. They found that PD-L1 was expressed in the tumor cell membrane (64%), cytoplasm (80%) and stromal (93%) cellular compartments. They also reported PD-L1 expression, in particular the cytoplasmic PD-L1 expression in the TCs and TILs, was associated with better survival [39]. Cerbelli et al. reported PD-L1 expression in 35% of tumor cells and 81% of TILs in a cohort of 54 TNBC patients. They also found a significant association of PD-L1 expression with TILs. In addition, PD-L1 expression in tumor cells but not TILs was found to correlate with better response to NACT [50]. Lastly, a recent study by Tomioka et al. showed TNBCs patients with combined Low-TILs and High-PD-L1 status has unfavorable prognosis [42,51].

The conflicting results were very likely due to different PD-L1 antibodies, different cutoff values, and the heterogeneity of breast cancer. Using a FDA-approved PD-L1 monoclonal antibody 22C3, our study showed 72% of TNBC cases with PD-L1 expression, including 35% of cases with PD-L1 expression in TCs and 63% of cases with PD-L1 expression in TILs. Our result also showed a positive correlation of PD-L1 expression, in particular PD-L1 TILs and CPS, with TILs, TILV, and pCR rate following NACT. Most importantly, our study was the first to use CPS scoring system to evaluate PD-L1 expression in tumor cells and immune cells in tumor microenvironment. We demonstrated that PD-L1 TCs and PD-L1 CPS were positively associated with better overall survival and raised the importance of evaluating PD-L1 expression and calculating CPS in TNBC. Our results in general were in keeping with most recent studies in TNBC, although other studies of mixed cohorts have shown associations of PD-L1 with a poor outcome. To our

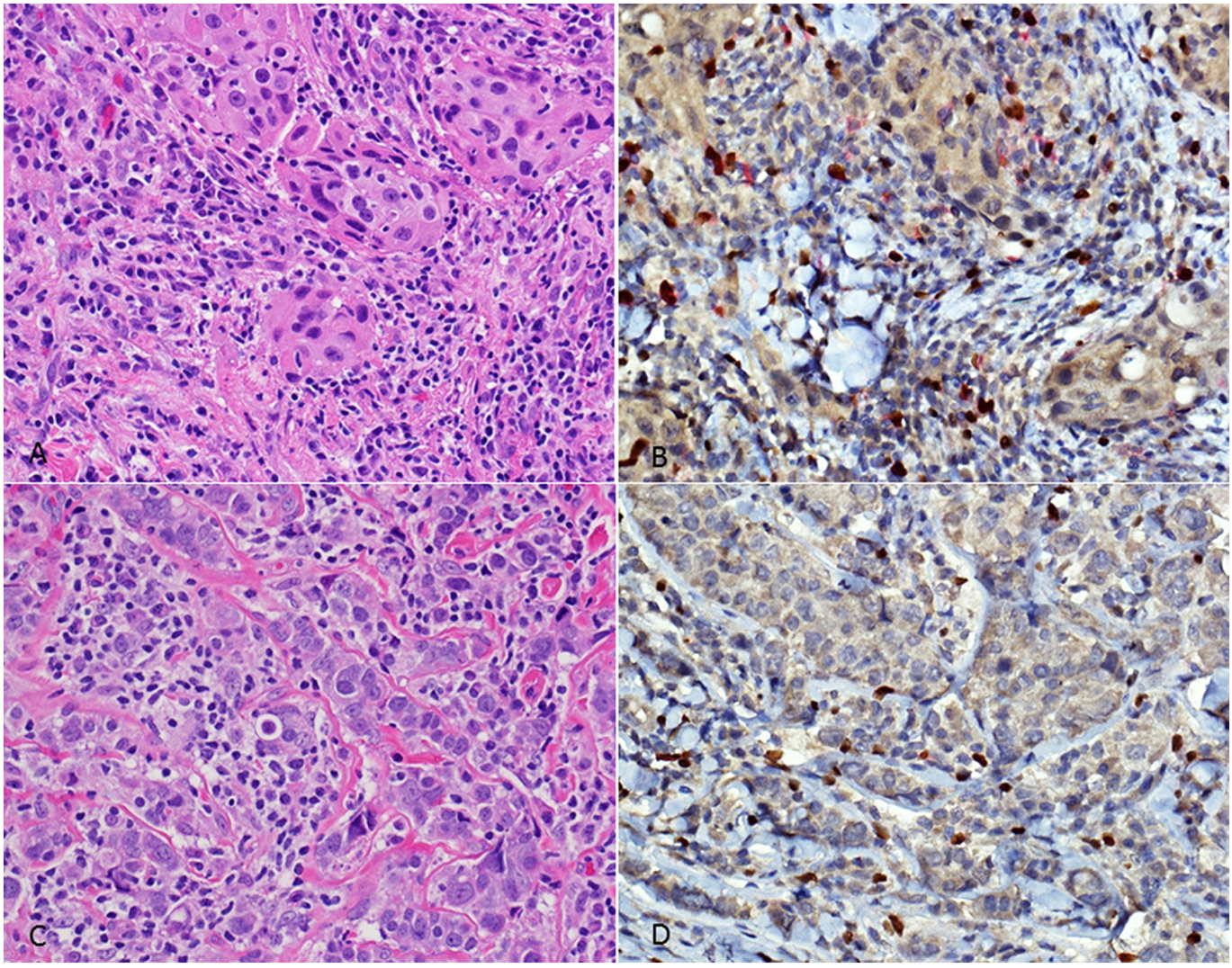

Fig. 3. Foxp3+ and Foxp3+/CD25+ cells. A&B: This triple negative breast cancer (A; H&E, 400×) had very good correlation between the Foxp3+ and Foxp3+/CD25+ cells, and most Foxp3+ cells were also positive for CD25 (B; double immunohistochemical staining for Foxp3/CD25). C&D: This triple negative breast cancer (C; H&E, 400×) showed very poor correlation between the Foxp3+ and Foxp3+/CD25+ cells, and most Foxp3+ cells were actually negative for CD25(D; double immunohistochemical staining for Foxp3/CD25).

knowledge, this is the first study thoroughly evaluating PD-L1 expression not only through examining its expression (density and percentage) in TCs and TILs, but also through evaluating of CPS in a cohort of TNBC patients. Our data suggested PD-L1 expression score correlated with better response to neoadjuvant chemotherapy and better overall survival, and has both predictive and prognostic significance in TNBC.

Lastly, our result showed Tregs positively correlated with PD-L1 expression, which has been reported in previous studies [51,52]. Tregs and the PD-L1/PD-1 pathway play pivotal role in maintaining peripheral immune tolerance, and evidence suggested they may act through the same pathway. PD-L1 was found to modulate signaling molecules that are critical for the conversion of naive T cells to Tregs. It upregulates Foxp3 expression and enhances Tregs suppressive function. The finding of correlation between high Tregs and PD-L1 expression raise the possibility that PD-L1 signals might play an important role in immune modulation through regulating Tregs in the complex tumor-immune microenvironment of breast cancer, although the exact underlying mechanism is still not fully elucidated. Our study is the first to show the correlation of PD-L1 expression and Foxp3+/CD25+ Tregs in TNBC and suggested that PD-L1 expression combined with Foxp3+/CD25+ Tregs could be used together to stratify TNBC patients who may respond to NACT and have better overall survival.

In summary, we evaluated the predictive and prognostic value of PD-L1 expression and Tregs in TNBC. Our result demonstrated that Foxp3/CD25 double staining is a better marker than Foxp3 alone to evaluate Tregs. Foxp3+/CD25+ Tregs and PD-L1 CPS were positively associated with TILs, TILV, and pCR rate. Neoadjuvant chemotherapy (NACT) significantly reduced Foxp3+ Tregs, Foxp3+/CD25+ Tregs, and PD-L1 CPS in the resection specimens of residual disease (RD) group. In addition, Foxp3+/CD25+ Tregs, PD-L1 TCs, and PD-L1 CPS correlated with better overall survival. Therefore, PD-L1 expression and Foxp3+/CD25+ Tregs have both therapeutic and prognostic value in TNBC. The relative limited numbers of TNBC cases is a potential limitation of our study and further studies are aimed to validate the results in big cohorts of TNBC patients.

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Conflict of interest disclosure

We have no conflict of interest to declare.

Disclosure

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