Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Peripheral TNF-α and $CD8^+/CD28^+$ T Lymphocytes as Alternatives for PD-L1 Prediction in Breast Cancer Tumor Microenvironment: Stratified by Neoadjuvant Therapy

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Background: Programmed death-ligand 1 (PD-L1) is an immunotherapy target; however, its detection is based on biopsy tissues, and repeated biopsies present clinical challenges. This study aimed to explore peripheral blood-based alternatives to PD-L1 tissue detection in breast cancer (BC), particularly stratification by neoadjuvant therapy (NAT).

Methods: A total of 134 cases were recruited, the peripheral lymphocyte subtypes and cytokines were detected by flow cytometry and PD-L1 expression in tumor microenvironment (TME) was detected by immunohistochemistry and assessed by two qualified pathologists.

Results: The patients with positive PD-L1 expression had peripheral $CD8^+/CD28^+$ T lymphocytes 20% higher than those with negative expression ($p = 0.008$) with the area under the receiver operating characteristic curve (AUC) being 0.64 ($p = 0.002$). Among patients with negative NAT, positive PD-L1 expression was associated with peripheral $CD8^+/CD28^+$ T lymphocytes that increased by 54% ($p = 0.003$), and the AUC being 0.68 ($p = 0.003$). In patients receiving NAT, positive PD-L1 expression was associated with peripheral TNF-α ($p = 0.010$), which increased from 0.45pg/mL to 0.64pg/mL in the PD-L1 positive group, and the AUC was 0.79 ($p = 0.012$). Among patients without NAT experience, a 1% increase in peripheral $CD8^+/CD28^+$ T lymphocytes was associated with a 21% higher probability of positive PD-L1 expression (OR = 1.21, 95% CI: 1.06–1.37) and among patients with NAT, the OR of peripheral TNF-α (>0.5pg/mL) increased to 24.5 for positive TME PD-L1 expression ($p = 0.008$).

Conclusion: Peripheral $CD8^+/CD28^+$ T cell percentages and TNF-α levels served as non-invasive biomarkers for TME PD-L1 expression in BC patients with and without NAT, respectively. These biomarkers warranted further validation in clinical implementation to guide precision immunotherapy.

Keywords: programmed death-ligand 1, T lymphocyte subtype, cytokine, neoadjuvant therapy, breast cancer

Introduction

In 2022, nearly 20 million new cancer cases and 9.7 million cancer deaths were reported globally.[1] Breast cancer (BC) ranked second in incidence (11.6% of total cases) and fourth in mortality (6.9% of total deaths).[1] Notably, China accounted for 18.4% of global BC cases and 25.1% of BC-related deaths,[2] highlighting its particularly urgent challenge within the worldwide burden. Projections indicated this upward trend would persist over the next 10 years,[3] underscoring the critical need for improved therapeutic strategies. Neoadjuvant therapy (NAT) emerged as a cornerstone of

Received: 11 May 2025
Accepted: 16 July 2025
Published: 19 July 2025

contemporary BC management, enabling tumor downsizing, enhancing surgical resectability, and facilitating early evaluation of treatment response.[4–6] The advent of immune checkpoint inhibitors revolutionized neoadjuvant immunotherapy, particularly through PD-1/PD-L1 blockade, which demonstrated significant clinical benefits.[7]

PD-L1 was the most reliable biomarker of the tumor microenvironment (TME) immune status and reflected NAT efficacy,[8–10] even in BC patients.[11,12] Emerging investigations had revealed the dynamic influence of NAT on PD-L1 expression in triple-negative breast cancer (TNBC),[13] thereby highlighting the indispensable significance of dynamically monitoring PD-L1 expression to refine NAT strategies. However, conventional PD-L1 detection using tissue biopsy had inherent limitations. Tumor heterogeneity frequently led to inconsistent immunohistochemical outcomes,[14] and during NAT, repeated biopsies posed significant clinical challenges owing to their invasive characteristics and sampling bias.[15,16] Biopsy introduced anxiety to patients, with an overall complication rate of 5.2%.[17] Additionally, anesthetics used during biopsy procedures exerted complex effects on tumor cells and immune cells,[18–20] thereby interfering with TME detection. For instance, lidocaine could suppress the viability and migration of BC cells by acting on TRPM7 channels.[21] General anesthetics like propofol also disrupted immune system function and cytokine regulation during the peri-procedural period,[22,23] making it difficult to accurately capture the true state of the TME. Thus, the traditional biopsy-dependent detection approach, hampered by anesthesia related interference and invasiveness, struggled to consistently and comprehensively reflect TME biomarker information.

Given these challenges, peripheral blood biomarkers such as cytokines and immune cell subsets emerged as promising alternatives.[24] Recent evidence has highlighted the dynamic association between peripheral blood and the TME immune status.[25,26] However, the predictive utility of these markers in BC remained poorly characterized, particularly in the context of NAT. This study addressed this gap by investigating whether peripheral blood could serve as a surrogate biomarker for TME PD-L1 expression, with a specific focus on stratifying patients based on NAT exposure. This study aimed to establish non-invasive monitoring tools that could potentially reduce reliance on tissue biopsies, improve real-time treatment decision-making, and optimize immunotherapy outcomes in BC patients undergoing NAT.

Materials and Methods

Ethical Approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Institutional Review Board of Beijing Shijitan Hospital, Capital Medical University (sjtkyll-1x-2018(56)), and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all the participants.

Study Design

This was a cross-sectional study. Circulating phenotypic lymphocytes and cytokines were detected in the preoperative blood, and PD-L1 expression was evaluated in the biopsy or surgical tissues. NAT treatment data were also collected from patients' medical records.

Patients

A total of 134 patients with BC were recruited at the Beijing Shijitan Hospital between November 2018 and November 2020. All enrolled patients had the Eastern Cooperative Oncology Group (ECOG) ≤ 2. The patient with skin or chest wall invasion were excluded, as were those diagnosed with inflammatory BC, autoimmune diseases, heart, brain, kidney, or other vital organ insufficiencies. For peripheral blood sampling, preoperative samples were collected from non-NAT patients, while samples from NAT-treated patients were obtained post-NAT completion. PD-L1 detection was performed using surgical specimens from NAT-undergoing patients and biopsy samples from non-NAT patients without surgical indications. Blood collection was executed using EDTA-K2 anticoagulant tubes. Venipuncture was performed at the antecubital vein. Post-collection, the tubes were inverted and gently mixed, then stored on ice or temporarily at 4°C, with clear specimen identification and complete records strictly ensured. For biopsy specimens, ultrasound-guided puncture was conducted, following local anesthesia, 1–2 strips of tissue were harvested, fixed in

formalin, and submitted for examination with proper labeling. For surgical specimens, once excised from the body, the harvested tissue blocks were fixed in formalin. Subsequently, after procedures including dehydration, they were embedded in paraffin to form tissue blocks, conforming to standard pathological processing protocols.

Immunohistochemistry (IHC) Staining and Scoring

The positive threshold for estrogen receptor (ER) and progesterone receptor (PR) detection by IHC was set at 1% tumor cell staining. Positive expression of HER-2 was defined as 3+ in IHC tests or positive in situ hybridization (ISH) test; negative expression was defined as -, 1+ in IHC test, or negative ISH test. The Ki-67 index was determined by IHC using 4μm-thick formalin fixed paraffin-embedded sections. The hotspot area was determined under a low-power field, and an index of ≥14% was defined as high expression. Molecular subtypes were defined as Luminal A (HER-2 negative, ER positive, Ki-67 low expression), Luminal B (HER-2 negative, ER positive, Ki-67 high expression), TNBC (HER-2 negative, ER negative, Ki-67 arbitrary), HER-2 overexpression/HR negative (HER-2 positive, ER negative, Ki-67 arbitrary) and HER-2 overexpression/HR positive (HER-2 positive, ER positive, Ki-67 arbitrary).

Monoclonal antibodies against PD-L1 (rabbit anti-human, #SP142) were purchased from Roche Shanghai Co. Ltd. Secondary antibodies were purchased from Beijing Zhongshan Jinqiao Biotechnology Co. Ltd. The EnVision two-step method was used to detect the expression of PD-L1 on the immune cells in the TME. Two pathologists interpreted the IHC staining of immune cells in the entire section. Tumor-infiltrating immune cells with brown staining accounting for more than 1% of the tumor area were determined to be positive for PD-L1 in the TME.[27,28] Expression percentage was defined as the percentage of positive PD-L1 immune cells in the tumor area.

Flow Cytometry Tests and Scoring

Six-milliliter venous blood was collected in EDTA-K2 anticoagulation tubes (Becton, Dickinson and Company), and a three-color flow cytometric analysis was performed to determine cell phenotypes using a Cytomics FC500 flow cytometer (Beckman Coulter). The monoclonal antibodies CD3-FITC (A07746, Beckman Coulter), CD4-PE (A07751, Beckman Coulter), CD8-PC5 (A07758, Beckman Coulter), CD127-PC7 (A64618, Beckman Coulter), CD28-PE (IM2071u, Beckman Coulter), and CD25-PC5 (IM2646, Beckman Coulter) were added to the flow tube. The lymphocyte subtypes were defined as the percentage of total circulating T lymphocytes (CD3+ cells). Five thousand lymphocytes were gated to calculate the percentage of positive T-lymphocyte subtypes using CXP analysis software (Beckman Coulter).[29,30]

Cytokine Detection

Peripheral cytokines, including interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17A, IL-12P70, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IFN-α, were detected using a multicytokine detection kit (Cellgene Biotech Co., LTD, China). The detection was performed according to the manufacturer's instructions. Twenty microliter capture microsphere solution, 25μL plasma sample or diluted standard sample, and 25μL fluorescence detection solution were added into a tube for 2.5-hour incubation. PBS (1 mL) was then added and centrifugated at 200 g for 5 min. The supernatant was discarded, and the precipitate was resuspended in 100 mL PBS. The detection process was conducted using a BD LSRFortessa flow cytometer (BD Biosciences, USA), and the results were analyzed using BD FACSDiva™ software (BD Biosciences, USA) software.[31] Cytokine levels were determined using a logistic curve-fitting equation, generated using a serially diluted standard sample.

Variables

Tumor size was categorized according to diameter as ≤2 cm (T_1), ≤5 cm (T_2), and >5 cm (T_3). Tumor staging was performed according to the tumor node metastasis (TNM) classification system in accordance with the guidelines of the 8th edition of the American Joint Committee on Cancer. Clinical TNM (cTNM) stage was classified as I ($T_1N_0M_0$), II ($T_{0\sim1}N_1M_0$, $T_2N_{0\sim1}M_0$, $T_3N_0M_0$), and III ($T_{0\sim2}N_2M_0$, $T_3N_{1\sim2}M_0$, $T_4N_{0\sim3}M_0$, and $T_{0\sim4}N_3M_0$). Histological grade was defined as scores estimated according to glandular duct formation, nuclear pleomorphism, and mitotic ability. The score ranges for grades I, II, and III are 3–5, 6–7, and 8–9, respectively.

Statistical Analysis

All statistical analyses were performed using the R software (version 4.4.2). Pearson's chi-square and Fisher's exact tests were used to compare the relationships between clinical characteristics and PD-L1 expression. Differences between peripheral blood lymphocyte subtypes, cytokines, and TME PD-L1 expression were assessed using the Wilcoxon rank-sum test. The association between peripheral lymphocyte subtypes, cytokines, and positive TME PD-L1 expression rate was estimated using Spearman correlation tests. The receiver operating characteristic (ROC) curve was plotted between peripheral lymphocyte subtypes, cytokines, and TME PD-L1 expression, and the validity was estimated using the area under the curve (AUC). A Logistic Regression Model was used to control for confounding factors. TNF-α was set as a categorical variable in the multivariate logistic analysis, with a cutoff value in the ROC analysis. All analyses were two-sided, and $p < 0.05$.

Results

Association Between TME PD-L1 Expression and Clinicopathological Characteristics

The PD-L1 expression level was not associated with age, BMI, tumor size, lymph node metastases, TNM stage, pathological diagnosis, axillary lymph node metastases, sentinel lymph node metastases, histological grade, ER expression, PR expression, HER-2 expression and molecular subtype in NAT and non-NAT BC patients ($p > 0.05$, Table 1). However, non-NAT BC patients with positive PD-L1 expression in the TME had a higher Ki-67 index ($p = 0.030$, Table 1). Significant differences existed between patients with and without NAT. The NAT group had a lower median age ($p = 0.037$, Table 1), larger tumor size ($p < 0.001$, Table 1), higher proportion of lymph node metastases ($p < 0.001$, Table 1), distinct TNM stage distribution ($p < 0.001$, Table 1), more frequent axillary lymph node metastases ($p = 0.032$, Table 1), differing histological grade composition ($p = 0.042$, Table 1), and higher Ki-67 index ($p = 0.021$, Table 1).

Table 1 Association of PD-L1 Expression with Clinical-Pathological Characteristics Stratified by Different NAT Statuses

	PD-L1 Expression Rate in Patients with Negative NAT (n=104)			PD-L1 Expression Rate in Patients with NAT (n=30)			NAT (n=134)		
	≤1%	>1%	*p*	≤1%	>1%	*p*	No	Yes	*p*
Age, median (IQR)[a]	60.0(18.0)	57.0(20.0)	0.901	44.0(16.0)	53.0(20.0)	0.161	58.0(19.0)	45.5(20.0)	0.037
BMI, median (IQR) [a]	25.2(4.7)	25.4(4.5)	0.872	24.7(3.2)	27.2(7.0)	0.786	25.4(4.2)	25.0(6.3)	0.649
Tumor size(cm), median (IQR) [a]	1.8(1.2)	1.9(0.9)	0.549	3.1(1.8)	3.1(1.8)	0.252	1.9(1.0)	3.1(1.6)	<0.001
Lymph node metastases, n (%)[b]			0.594			0.689			<0.001
No	30(76.9)	44(72.1)		3(27.3)	7(41.2)		74(74.0)	10(35.7)	
Yes	9(23.1)	17(27.9)		8(72.7)	10(58.8)		26(26.0)	18(64.3)	
TNM stage, n (%)[b]			0.380			0.072			<0.001
I	23(59.0)	35(56.5)		3(27.3)	0(0.0)		58(57.4)	3(10.3)	
II	10(25.6)	22(35.5)		4(36.4)	10(55.6)		32(31.7)	14(48.3)	
III	6(15.4)	5(8.1)		4(36.4)	8(44.4)		11(10.9)	12(41.4)	
Pathological diagnosis, n (%)[b]			0.405			0.524			0.052
Invasive ductal carcinoma	28(71.8)	49(79.0)		10(100.0)	16(88.9)		77(76.2)	26(92.9)	
Other cancers	11(28.2)	13(21.0)		0(0.0)	2(11.1)		24(23.8)	2(7.1)	
Axillary lymph node metastases, n (%)[b]			0.893			0.648			0.032
No	33(84.6)	51(83.6)		4(50.0)	8(66.7)		84(84.0)	12(60.0)	
Yes	6(15.4)	10(16.4)		4(50.0)	4(33.3)		16(16.0)	8(40.0)	
Sentinel lymph node metastases, n(%)[b]			0.461			0.370			0.101
No	33(84.6)	48(78.7)		9(90.0)	17(100.0)		81(81.0)	26(96.3)	
Yes	6(15.4)	13(21.3)		1(10.0)	0(0.0)		19(19.0)	1(3.7)	

(*Continued*)

Table 1 (Continued).

	PD-L1 Expression Rate in Patients with Negative NAT (n=104)			PD-L1 Expression Rate in Patients with NAT (n=30)			NAT (n=134)		
	≤1%	>1%	*p*	≤1%	>1%	*p*	No	Yes	*p*
Histological grade, n (%)[b]			0.363			0.163			0.042
I	5(16.1)	9(17.0)					14(16.7)	0(0.0)	
II	24(77.4)	35(66.0)		8(80.0)	14(100.0)		59(70.2)	22(91.7)	
III	2(6.5)	9(17.0)		2(20.0)	0(0.0)		11(13.1)	2(8.3)	
Estrogen receptor expression, n (%)[b]			0.298			0.671			0.247
No	5(12.8)	13(21.0)		2(18.2)	6(33.3)		18(17.8)	8(27.6)	
Yes	34(87.2)	49(79.0)		9(81.8)	12(66.7)		83(82.2)	21(72.4)	
Progesterone receptor expression, n (%)[b]			0.209			0.694			0.298
No	7(17.9)	18(29.0)		3(27.3)	7(38.9)		25(24.8)	10(34.5)	
Yes	32(82.1)	44(71.0)		8(72.7)	11(61.1)		76(75.2)	19(65.5)	
HER-2 expression, n (%)[b]			0.122			0.234			0.142
No	33(86.8)	45(73.8)		9(81.8)	10(55.6)		78(78.8)	19(65.5)	
Yes	5(13.2)	16(26.2)		2(18.2)	8(44.4)		21(21.2)	10(34.5)	
Ki-67 index (%), median (IQR) [a]	20.0(20.0)	30.0(53.0)	0.030	60.0(70.0)	50.0(50.0)	0.443	30.0(30.0)	55.0(70.0)	0.021
Molecular subtype, n (%)[b]			0.687			0.177			0.266
Luminal A	11(28.2)	13(21.3)		3(27.3)	0(0.0)		24(24.0)	3(11.1)	
Luminal B	19(48.7)	26(42.6)		4(36.4)	6(37.5)		45(45.0)	10(37.0)	
TNBC	3(7.7)	5(8.2)		2(18.2)	2(12.5)		8(8.0)	4(14.8)	
HER-2+/HR-	2(5.1)	7(11.5)		0(0.0)	3(18.8)		9(9.0)	3(11.1)	
HER-2+/HR+	4(10.3)	10(16.4)		2(18.2)	5(31.3)		14(14.0)	7(25.9)	

Notes: [a]Wilcoxon rank-sum test; [b]Pearson's chi-square or Fisher's exact tests.
Abbreviation: NAT, neoadjuvant therapy.

Association Between Peripheral Blood Lymphocyte Subtypes and Cytokines and TME PD-L1 Expression

Among all BC patients, those with positive TME PD-L1 expression exhibited a 20% higher relative proportion of peripheral $CD8^+/CD28^+$ T lymphocytes compared to those with negative PD-L1 expression (10.70% vs 8.90%, $p = 0.008$, Table 2). In patients with positive PD-L1 expression and not receiving NAT, peripheral $CD8^+/CD28^+$ T lymphocytes displayed a 54% elevation compared to PD-L1 negative expression (10.70% vs 6.95%, $p = 0.003$, Table 2). For patients who received NAT,

Table 2 Relationship Between Peripheral Lymphocyte Subtypes, Cytokines and PD-L1 Expression in Breast Cancer Patients

	PD-L1 Expression Rate in all Patients (n=134)			PD-L1 Expression Rate in Patients with Negative NAT (n=104)			PD-L1 Expression Rate in Patients with NAT (n=30)		
	≤1%	>1%	*p*	≤1%	>1%	*p*	≤1%	>1%	*p*
$CD3^+$ lymphocytes (%), median (IQR) [a]	72.90(14.20)	73.10(10.03)	0.358	72.60(13.28)	72.80(10.35)	0.317	75.50(18.40)	74.50(8.00)	0.760
$CD4^+$ lymphocytes (%), median (IQR) [a]	42.50(10.35)	44.10(9.60)	0.440	42.40(10.88)	43.20(10.90)	0.647	44.60(6.90)	45.80(6.30)	0.580
$CD8^+$ lymphocytes (%), median (IQR) [a]	26.70(15.80)	23.90(9.45)	0.184	26.55(15.35)	23.90(9.35)	0.284	29.40(24.10)	23.30(10.30)	0.450
CD8/CD4 ratio, median (IQR) [a]	0.62(0.50)	0.56(0.33)	0.240	0.61(0.50)	0.58(0.34)	0.490	0.62(0.60)	0.55(0.28)	0.250
$CD4^+/CD25^+/CD127^-$ lymphocytes (%), median (IQR) [a]	1.60(1.65)	1.40(1.70)	0.588	1.30(1.78)	1.40(1.70)	0.139	2.10(2.10)	1.40(2.60)	0.090
$CD8^+/CD28^+$ lymphocytes (%), median (IQR) [a]	8.90(6.75)	10.70(6.60)	0.008	6.95(6.30)	10.70(6.40)	0.003	11.70(7.90)	10.70(5.30)	0.980
$CD8^+/CD28^-$ lymphocytes (%), median (IQR) [a]	46.40(27.05)	46.00(23.95)	0.944	40.90(30.20)	43.20(22.95)	0.550	54.70(18.80)	49.40(35.20)	0.380
IL-2, median (IQR) [a]	0.59(0.32)	0.68(0.32)	0.352	0.59(0.38)	0.66(0.38)	0.273	0.73(0.28)	0.68(0.23)	0.940
IL-4, median (IQR) [a]	1.36(1.04)	1.27(0.69)	0.340	1.36(1.09)	1.27(0.79)	0.387	1.54(0.69)	1.10(0.52)	0.580
IL-6, median (IQR) [a]	2.77(2.00)	2.45(2.00)	>0.999	2.77(2.00)	2.37(2.00)	0.421	2.07(2.00)	2.69(3.00)	0.190
IL-10, median (IQR) [a]	1.28(0.58)	1.33(0.80)	0.329	1.33(0.86)	1.33(0.95)	0.173	1.11(0.22)	1.17(0.68)	0.760

(*Continued*)

https://doi.org/10.2147/BCTT.S532688

Table 2 (Continued).

	PD-L1 Expression Rate in all Patients (n=134)			PD-L1 Expression Rate in Patients with Negative NAT (n=104)			PD-L1 Expression Rate in Patients with NAT (n=30)		
	≤1%	>1%	*p*	≤1%	>1%	*p*	≤1%	>1%	*p*
TNF-α, median (IQR) [a]	0.56(0.40)	0.60(0.30)	0.429	0.60(0.40)	0.56(0.30)	0.707	0.45(0.30)	0.64(0.30)	0.010
IFN-γ, median (IQR) [a]	0.85(0.50)	0.85(0.36)	0.914	0.85(0.50)	0.85(2.66)	0.961	0.89(0.69)	0.85(0.45)	0.680
IL-17A, median (IQR) [a]	2.17(1.93)	1.90(1.69)	0.679	2.26(1.78)	1.90(1.80)	0.689	1.90(2.19)	2.06(1.62)	0.980

Note: [a]Wilcoxon rank-sum test.
Abbreviation: NAT, neoadjuvant therapy.

peripheral TNF-α levels increased from 0.45pg/mL in the PD-L1 negative group to 0.64pg/mL in the positive group (p = 0.010, Table 2). Nevertheless, the proportion of $CD4^+/CD25^+/CD127^-$ lymphocytes in the PD-L1 positive expression group showed a 3% reduction compared to the PD-L1 negative expression group (p = 0.090, Table 2).

Correlation Between Peripheral Blood Molecular Markers and TME PD-L1 Expression Percentage

In all patients, the median PD-L1 expression percentage was 2.5% and positively correlated with $CD4^+$ lymphocytes (r = 0.184, p = 0.037, Table 3) and $CD8^+/CD28^+$ lymphocytes (r = 0.283, p = 0.001, Table 3). In BC patients without NAT experience, TME PD-L1 expression percentage was positively correlated with $CD8^+/CD28^+$ lymphocyte levels (r = 0.319, p = 0.001, Table 3). In BC patients with NAT experience, TME PD-L1 expression percentage was positively correlated with TNF-α levels (r = 0.438, p = 0.025, Table 3).

In all patients, the AUC of peripheral $CD8^+/CD28^+$T lymphocytes for predicting TME PD-L1 expression status was 0.64 (95% CI 0.54–0.74, p = 0.008, Figure 1A), with a sensitivity of 0.43 and a specificity of 0.82 at the optimal cut-off point. Notably, among patients without NAT experience, the AUC of peripheral $CD8^+/CD28^+$ T lymphocytes increased to 0.68 (95% CI 0.57–0.79, p = 0.003, Figure 1B) for TME PD-L1 expression status prediction, accompanied by a sensitivity of 0.82 and a specificity of 0.53 at the optimal cut-off point. Among patients with NAT experience, the

Table 3 Correlation Between Peripheral Blood Molecular Markers and PD-L1 Expression in Tumor Microenvironment in Breast Cancer Patients

	All Patients (n=134)		Patients with Negative NAT (n=104)		Patients with NAT (n=30)	
	Correlation Coefficient	*P*	Correlation Coefficient	*P*	Correlation Coefficient	*P*
$CD3^+$ lymphocytes [a]	0.035	0.691	0.039	0.700	−0.032	0.867
$CD4^+$ lymphocytes [a]	0.184	0.037	0.179	0.076	0.093	0.625
CD8+ lymphocytes [a]	−0.069	0.437	−0.072	0.479	−0.064	0.735
CD8/CD4 ratio [a]	−0.161	0.069	−0.158	0.119	−0.156	0.410
$CD4^+/CD25^+/CD127^-$ lymphocytes [a]	0.108	0.223	0.168	0.097	−0.136	0.473
$CD8^+/CD28^+$ lymphocytes [a]	0.283	0.001	0.319	0.001	0.130	0.495
$CD8^+/CD28^-$ lymphocytes [a]	0.126	0.155	0.184	0.069	−0.020	0.916
IL-2 [a]	0.039	0.680	0.065	0.542	−0.092	0.656
IL-4 [a]	−0.162	0.082	−0.148	0.163	−0.243	0.232
IL-6 [a]	0.010	0.918	−0.095	0.369	0.307	0.127
IL-10 [a]	−0.076	0.417	−0.092	0.351	0.001	0.997
TNF-α[a]	0.034	0.717	−0.082	0.439	0.438	0.025
IFN-γ[a]	−0.099	0.288	−0.104	0.327	−0.100	0.627
IL-17A [a]	−0.067	0.476	−0.061	0.566	−0.058	0.778

Note: [a]Spearman correlation tests.

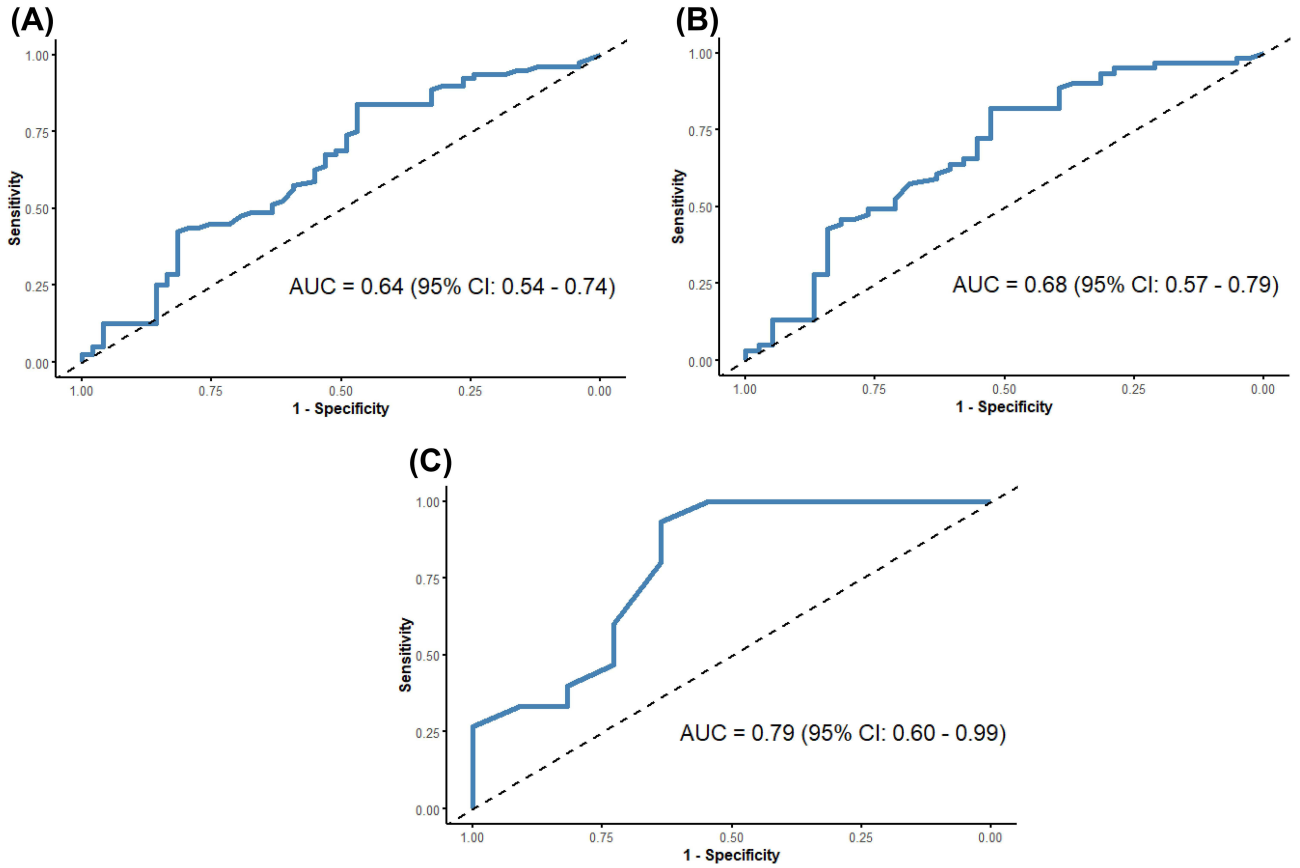

Figure 1 ROC analysis between peripheral biomarkers and TME PD-L1 expression status. (**A**) $CD8^+/CD28^+$ T lymphocytes in all breast cancer patients, (**B**) $CD8^+/CD28^+$ T lymphocytes in breast cancer patients with negative neoadjuvant therapy, (**C**) TNF-α in breast cancer patients with neoadjuvant therapy.

AUC of peripheral TNF-α for predicting TME PD-L1 expression status was 0.79 (95% CI 0.60–0.99, p = 0.012, Figure 1C), with a sensitivity of 0.93 and a specificity of 0.64 at the optimal cut-off point.

In multivariate analysis, for all patients, a 1% rise in the peripheral percentage of $CD8^+/CD28^+$ T lymphocytes was associated with a 14% higher probability of positive TME PD-L1 expression (OR = 1.14, 95% CI = 1.03–1.26, p = 0.015, Table 4). Among patients without NAT, the probability of positive TME PD-L1 expression was increased by 21% (OR = 1.21, 95% CI = 1.06–1.37, p = 0.006, Table 4). Among the patients with NAT, with peripheral TNF-α levels higher than

Table 4 Multivariate Analysis Between Factors and TME Expression of PD-L1

	All Patients† (n=134)						Patients with Negative NAT‡ (n=104)					
	Univariate Analysis			Multivariate Analysis			Univariate Analysis			Multivariate Analysis		
	OR	95% CI	p	OR	95% CI	p	OR	95% CI	p	OR	95% CI	p
CD8+/CD28+T lymphocytes	1.10	1.02–1.18	0.015	1.14	1.03–1.26	0.015	1.12	1.03–1.22	0.011	1.21	1.06–1.37	0.006
Age	1.01	0.98–1.04	0.438	1.03	0.98–1.07	0.296	1.00	0.97–1.04	0.885	1.00	0.94–1.06	0.996
TNM stage												
I	1.00	–	0.382	1.00	–	0.403	1.00	–	0.389	1.00	–	0.323
II	1.70	0.76–3.81	0.199	1.55	0.50–4.82	0.446	1.45	0.58–3.61	0.429	1.81	0.41–8.01	0.437
III	0.97	0.37–2.54	0.944	0.57	0.14–2.34	0.438	0.55	0.15–2.01	0.363	0.30	0.04–2.24	0.240

(*Continued*)

https://doi.org/10.2147/BCTT.S532688

Table 4 (Continued).

	All Patients† (n=134)						Patients with Negative NAT‡ (n=104)					
	Univariate Analysis			Multivariate Analysis			Univariate Analysis			Multivariate Analysis		
	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI	p	OR	95% CI	*p*
Tumor grade												
I	1.00	–	0.821	1.00	–	0.296	1.00	–	0.389	1.00	–	0.272
II	0.85	0.26–2.77	0.788	0.28	0.05–1.42	0.123	0.81	0.24–2.72	0.733	0.32	0.06–1.88	0.208
III	1.25	0.25–6.24	0.785	0.24	0.03–2.16	0.201	2.50	0.38–16.42	0.340	1.06	0.07–15.90	0.967

Note: †further adjusting age, TNM stage, histological grade, Ki−67 index and HER-2 expression. ‡Further adjusting age, TNM stage, histological grade, Ki−67 index and Marital status.

the median level (0.5pg/mL), the OR associated with the probability of positive TME PD-L1 expression was increased to 24.5-fold (OR = 24.50, 95% CI 2.28–262.53, $p = 0.008$), with further adjustment for age, TNM stage, histological grade, and $CD4^+/CD25^+/CD127^-$ lymphocytes ($p = 0.045$).

Discussion

Peripheral biomarkers for predicting TME PD-L1 expression were feasible. PD-1/PD-L1 expression in peripheral T lymphocytes of patients with BC had been reported to be consistent with PD-L1 expression on the TME.[29] This study provided novel evidence that TNF-α and $CD8^+/CD28^+$ T lymphocytes were associated with PD-L1 expression in BC patients, particularly in the context of NAT. These findings underscored the potential of noninvasive liquid biopsy approaches for PD-L1 assessment, addressing limitations of traditional tissue biopsies.

PD-L1 expression was known to be heterogeneous and dynamically influenced by treatment modalities.[16,32,33] If the initial biopsy was negative, multiple additional biopsies may be required.[34] Moreover, diagnostic biopsies did not fully reflect PD-L1 expression in TNBC patients.[35] Dynamic monitoring of PD-L1 expression through peripheral alternatives during treatment overcame repeated biopsy drawbacks. In recent years, liquid biopsy technology has become an effective screening tool for the early diagnosis of BC. Jafari et al[36] found liquid biopsy analytes enhanced BC diagnosis accuracy and efficiency. Peripheral biomarkers could also monitor disease progression during therapy and potentially revealed the mechanisms of mutation and resistance.[37]

Consistently, serum TNF-α concentration had been reported to be an indicator of the response to NAT in locally advanced BC.[38] Studies performed by Bulska-Będkowska W et al[39] elucidated that increased baseline serum TNF-α (sTNF-R1) level was associated with pathological response to NAT in BC patients. Jabeen et al[40] also found that serum TNF-α concentration was lower in BC patients who achieved a pathologic complete response to NAT. Mechanistically, TNF-α was found to drive PD-L1 upregulation via the JNK/c-Jun/TNF-α signaling axis,[41] reflecting the dynamic microenvironmental interactions triggered by NAT. This suggested that TNF-α might serve as a dynamic marker for PD-L1-mediated immune resistance during NAT.

Peripheral $CD8^+/CD28^+$ T lymphocyte levels had been reported as alternative markers of lymph node metastasis in early BC patients.[31] Notably, in patients without NAT, Geng et al[42] demonstrated that elevated peripheral $CD8^+/CD28^+$ T cell counts correlated with superior responses to PD-1/PD-L1 inhibitors. This indicated that $CD8^+/CD28^+$ T cells function as biomarkers for lymph node metastasis and immune competence in non-NAT patients, highlighting their utility in assessing tumor-immune interactions.

In immunotherapy, peripheral biomarkers were increasingly used for dynamic monitoring of TME changes. In melanoma, circulating soluble PD-L1 and lymphocyte subsets emerged as predictors for response to PD-1/PD-L1 blockade.[43] In gastric cancer, in-depth understanding of the PD-1/PD-L1 axis promoted exploration of combination therapies targeting other immune checkpoints and TME characteristics-tailored treatment strategies.[44] For solid tumors, next-generation chimeric antigen receptor T cell (CAR-T) therapies were overcoming TME barriers by enhancing mitochondrial function or reprogramming lactate metabolism.[45] Moreover, disrupting small extracellular vesicle-

mediated communication between tumor cells and tumor-associated macrophages enabled reprogramming of immunosuppressive macrophages, providing a novel approach to enhance anti-tumor immunity.[46] Collectively, these cross-tumor findings suggested that mechanisms of immune biomarkers and liquid biopsy approaches might offer novel monitoring strategies for immunotherapy.

Breakthroughs in liquid biopsy technology had provided new tools for minimally invasive diagnosis and dynamic monitoring. Circulating tumor DNA (ctDNA) was used for real-time tumor tracking,[47] molecular barcoding technology enabled precise detection of low-frequency variants,[48] and methylation biomarkers facilitated early diagnosis of BC.[49] These innovations made it possible to quantify signaling pathways and gene expression mechanisms via liquid biopsy platforms. For example, pathway-effect mutations inferred from circulating RNA or methylation signatures could be validated by longitudinally tracking molecular features in patient samples, enabling real-time monitoring of treatment response and detection of minimal residual disease. When integrated with mechanistic research on TNF-α and $CD8^+$/$CD28^+$ T cells in BC, future studies might leverage liquid biopsy technology to integrate multi-dimensional biomarkers, promoting a paradigm shift in immunotherapy monitoring from "static assessment" to "dynamic tracking", and thus constructing more accurate predictive models for immunotherapy efficacy.

This study had several limitations. First, the small sample size might have affected the reliability and generalizability of the findings. Second, the expression of PD-L1 was heterogeneous and dynamic, and this study only conducted a cross-sectional survey, which might lead to uncertainty regarding its relationship with peripheral biomarkers. Third, some patients who did not receive NAT were ineligible for surgery and thus provided biopsy samples as a replacement for surgical specimens. However, this study suggested that peripheral biomarkers could predict TME PD-L1 expression with respect to the NAT regimen. In future studies, more extensive exploration and analysis of the expression of relevant markers and treatment outcomes of NAT should be conducted by increasing the sample size, thus further validating the results of this study.

Conclusions

Peripheral TNF-α cytokine expression was associated with TME PD-L1 expression in BC patients receiving NAT. Peripheral $CD8^+$/$CD28^+$T lymphocytes were associated with TME PD-L1 expression in BC patients without NAT. Therefore, peripheral biomarkers might serve as an alternative to PD-L1 biopsy. Further studies were warranted for the practical application of alternative peripheral markers in predicting TME PD-L1 expression.

Data Sharing Statement

The datasets generated and analyzed during the current study are not publicly available because of privacy and ethical issues but are available from the corresponding author upon reasonable request.

Ethics Approval and Informed Consent

All procedures performed in studies involving human participants were in accordance with the ethical standards of Institutional Review Board of Beijing Shijitan Hospital, Capital Medical University (sjtkyll-1x-2018(56)) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Acknowledgments

This paper has been uploaded to ResearchSquare as a preprint: https://www.researchsquare.com/article/rs-3927200/v1

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This research was funded by the Beijing Municipal Committee of Science and Technology (grant number Z19110006619041), the High Level Public Health Technical Talents Construction Project from the Beijing Municipal Health Commission (2022-2-025), and the Beijing Hospital's Authority (XMLX202114). The supporting organizations had no role in the study design, data collection, analysis, or interpretation.

Disclosure

The authors declare that they have no competing interests in this work.

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