

# The Role of Peripheral CD4<sup>+</sup>PD-1<sup>+</sup> and CD8<sup>+</sup>PD-1<sup>+</sup> T Lymphocytes in Triple Negative Breast Cancer Patients

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**Chen *et al.*: Role of CD4<sup>+</sup>PD-1<sup>+</sup> and CD8<sup>+</sup>PD-1<sup>+</sup> T Lymphocytes in Triple Negative Breast Cancer**

**Programmed cell death protein-1 and programmed death ligand-1 pathway activation play a vital role that can be modulated by tumor cells to blunt host immune surveillance. Periphery programmed cell death protein-1 expression level on cluster of differentiation 4<sup>+</sup> and cluster of differentiation 8<sup>+</sup> T lymphocytes of 185 female patients first diagnosed with triple negative breast cancer in Tianjin Medical University Cancer Institute and Hospital were analyzed by multicolor flow cytometry prior to any anti-tumor treatment. Patients who received chemotherapy alone for 2-8 cycles and received sequential samples were assessed with response evaluation criteria in solid tumors 1.1. Peripherally, the rate of cluster of differentiation 4<sup>+</sup> programmed cell death protein-1<sup>+</sup> and cluster of differentiation 8<sup>+</sup> programmed cell death protein-1<sup>+</sup> lymphocytes in general group were significantly higher than in the control group (p<0.0001). Post treatment programmed cell death protein-1 expression level on cluster of differentiation 4<sup>+</sup> T lymphocytes and cluster of differentiation 8<sup>+</sup> T lymphocytes was significantly lower than the baseline level in better response to neoadjuvant chemotherapy group (all p<0.05). In the contrary, programmed cell death protein-1 expression on both cluster of differentiation 4<sup>+</sup> T lymphocytes and cluster of differentiation 8<sup>+</sup> T lymphocytes showed no difference between pretreatment and post treatment in triple negative breast cancer patients with poor response to neoadjuvant treatment (all p>0.05). Peripheral cluster of differentiation 4<sup>+</sup> programmed cell death protein-1<sup>+</sup> and cluster of differentiation 8<sup>+</sup> programmed cell death protein-1<sup>+</sup> T lymphocytes of patients with triple negative breast cancer are associated with clinical response to neoadjuvant treatment.**

**Key words:** Triple negative breast cancer, T lymphocytes, programmed cell death protein-1, programmed death ligand-1

Triple Negative Breast Cancer (TNBC) which is a specific subtype of breast cancer with poor prognosis negatively express Estrogen Receptor (ER), Progesterone Receptor (PR) or Human Epidermal Growth Factor Receptor 2 (HER2) that constitutes 12 %-18 % of breast cancer patient<sup>[1]</sup>. Neoadjuvant Chemotherapy (NAC) is the standard treatment for early stage TNBC. However TNBC patients still have poor progression-free survival and overall survival due to chemotherapy resistance<sup>[2]</sup>. The interaction of Programmed Cell Death Protein-1 (PD-1)/Programmed Death Ligand-1 (PD-L1) results in activating the tumor immune escape, enhance tumor invasion and progression<sup>[3]</sup>. Anti-PD-1 agents and anti-PD-L1 agents activated the antitumor immune response which are associated with a better response rate and improved survival rate in different studies<sup>[4]</sup>.

The anti-tumor effects of immune system is composed of T and B lymphocytes and Natural Killer (NK) cells<sup>[5,6]</sup>. Several studies reveal that higher level of Tumor-Infiltrating Lymphocytes (TILs) in the host microenvironment is closely related with better long-term prognosis and better response to anti-tumor treatment in different types of carcinomas<sup>[7,8]</sup>. While Cluster of Differentiation (CD) 4-positive (+) T lymphocytes act as T helper cells (Th cells) and CD8-positive (+) T lymphocytes shows cytotoxic function<sup>[9,10]</sup>. TNBC is the most immunogenic breast cancer subtype, compared with other subtypes. The rate of PD-1 and PD-L1 expression was absolutely higher in TNBC subtype, while T cells with PD-1 expressed character by “exhausted T cells”. In fact, the expression level of immune checkpoints like PD-1/PD-L1 on T lymphocytes in peripheral blood patients is also described here.

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The purpose of this study was to investigate the character of PD-1 and PD-L1 expression level on T lymphocyte subsets in peripheral blood of patients with TNBC who received NAC to elucidate clinical significance.

## MATERIALS AND METHODS

### Subjects:

A total of 185 patients diagnosed with TNBC, who primarily received NAC from 2020-2021 and without any previous treatment in Tianjin Hospital of ITCWM Nankai Hospital were obtained in this study. All patients received endoscopic tests and confirmed with TNBC histologically were included in the study. The exclusion criteria were as follows. Age < 18 y or age > 75 y; patients with Eastern Cooperative Oncology Group (ECOG) performance status scale > 2; patients who received anti-tumor treatment previously; patients who has undergone blood transfusion; patients who were diagnosed with current or past history of other carcinomas; patients with autoimmune diseases (e.g. rheumatoid arthritis, systemic lupus erythematosus, hyperthyroidism or chronic liver disease); patients with infectious diseases (e.g. tuberculosis, bacterial, fungal or viral infections and hepatitis B or hepatitis C). The studies involving human participants were reviewed and approved by the ethics committee of Tianjin Medical University Cancer Institute and Hospital.

### Tumor response to NAC:

All the patients received Magnetic Resonance Imaging (MRI) scan and ultrasonography to evaluate tumor response to NAC before and after every two cycles of NAC. Tumor response to NAC was analyzed by two clinical doctors independently by Response Evaluation Criteria in Solid Tumors (RECIST) guidelines (version 1.1). The end point of our study is pathological response to neoadjuvant chemotherapy in patients with TNBC. According to histopathological criteria for the assessment of therapeutic response in breast cancer (2007 version), the tumor response was classified in accordance with the RECIST v1.1. Complete Response (CR), Partial Response (PR), Stable Disease (SD), Progressive Disease (PD) were gathered and assessed based on modified European Group for Blood and Marrow Transplantation (EBMT) criteria.

### Blood sample collection:

5 ml of heparinized peripheral blood samples of each patient with TNBC and healthy people were collected at the appointed time before treatment. Plasma samples

of each person were obtained *via* separation through standard density gradient centrifugation within 30 min.

### Flow cytometry:

Flow cytometry was used to analyze the phenotype of T lymphocytes which was performed using a Becton-Dickinson FACSCalibur™ flow cytometer. To identify the PD-1 expression of CD4<sup>+</sup> and CD8<sup>+</sup> subsets, cells were phenotypically characterized *via* incubation with relevant Fluorescein Isothiocyanate (FITC)-Phycoerythrin (PE) and monoclonal Antibodies (mAbs). Subsets were performed using the following markers, mouse anti-human CD3-FITC, mouse anti-human CD4-FITC, mouse anti-human CD8-FITC, PE mouse anti-human CD279 (PD-1), PE mouse anti-human CD274 (PD-L1).

### Statistical analysis:

CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes were then analyzed for the percentage of PD-1 expression *via* comparison with the control. Data were obtained and analyzed using the Prism software. Two or more intergroup variable comparisons were performed using Statistical Package for the Social Sciences (SPSS) software (version 16.0) by student's t-test and one-way Analysis of Variance (ANOVA). A 2-tailed p-value less than 0.05 was considered as statistically significant.

## RESULTS AND DISCUSSION

185 patients were involved in our study and all the patients received modified radical mastectomy or breast-conserving surgery. 185 patients lies within the range of 26-71 y, while 149 patients (80 %) were under 60 y old. 18 patients received 2 cycles of NAC, 135 patients received 4-6 cycles and 32 patients completed more than 6 cycles. 182 patients received taxane and anthracycline-based regimen sequentially or in combination and 28 patients received vinorelbine plus platinum based regimen when first line of treatment failed. Of 185 patients, 145 (78 %) neutropenia or neuropathy patients were of grade 1 or 2, while 64 thrombocytopenia patients were of grade 1 or 2. The rate of chemotherapy-induced grade 3 or 4, nausea and vomiting patients were 19 (10 %) of 182 patients. 5 patients required regimen dose reduction of 20 %, due to circumstances including grade 3 or 4, neutropenia and thrombocytopenia, no discontinuation of treatment due to adverse effects were observed (Table 1).

Predictive value of the CD4<sup>+</sup>PD-1<sup>+</sup> and CD8<sup>+</sup>PD-1<sup>+</sup> T lymphocytes count in peripheral blood was explained here. The peripheral percentage of PD-1<sup>+</sup>CD4<sup>+</sup> T

lymphocytes was 24.9 %±10.9 % at baseline in general TNBC group was significantly higher compared with control group (18.4 %±7.6 %). Similarly, PD-1<sup>+</sup>CD8<sup>+</sup> T lymphocytes level in peripheral blood at baseline was 23.0 %±11.3 % which were higher compared with control group (14.0 %±4.8 %). PD-1 expression level on CD4<sup>+</sup> lymphocytes (at baseline vs. post-surgery: 24.9 %±10.9 % vs. 17.5 %±8.9 %) and CD8<sup>+</sup> lymphocytes (at baseline vs. post-surgery: 23.0 %±11.3 % vs. 16.6 %±9.1 %) decreased significantly after receiving surgery (fig. 1A and fig. 1B).

Predictive value of the CD4<sup>+</sup>PD-1<sup>+</sup> and CD8<sup>+</sup>PD-1<sup>+</sup> T lymphocytes count for early tumor response was explained here. 26 patients were confirmed with pathologic CR after surgery. 81 patients achieved

clinically PR with no pathologic CR after surgery. 69 patients experienced SD and 9 patients had PD. In the subgroup analysis, assessed in response to Neoadjuvant Therapy (NAT), the percentage of PD-1<sup>+</sup>CD4<sup>+</sup> and PD-1<sup>+</sup>CD8<sup>+</sup> T lymphocytes decreased after NAC in CR group and PR group (both p value<0.001). The percentage of CD4<sup>+</sup>PD-1<sup>+</sup> and CD8<sup>+</sup>PD-1<sup>+</sup> T lymphocytes show no significant difference between baseline and post-NAT in SD and PD subgroups (p>0.05) as shown in Table 2.

Several immune checkpoint proteins expressed in most solid tumors, including breast cancer, lung cancer and renal cell cancer may be associated in inhibiting self-immune responses<sup>[11,12]</sup>.

**TABLE 1: CLINICOPATHOLOGICAL CHARACTERISTICS OF PATIENTS WITH TNBC**

Category	Number (N)	Percentage (%)
Age		
<60 y	149	80.54
≥60 y	36	19.46
Pre-NAT tumor stage		
cT1	21	11.35
cT2	130	70.27
cT3	31	17.30
cT4	2	0.01
Unknown	1	0.01
Pre-NAT nodal status		
Node positive	108	58.38
Node negative	76	41.08
Unknown	1	0.01
Tumor grading		
G1	2	0.01
G2	148	80.00
G3	35	18.91
Post-NAT tumor stage		
ypT0-T1	72	38.92
≥ypT2	91	49.19
Unknown	22	11.89
Post-NAT nodal status		
ypN0-N1	121	65.41
≥N2	63	34.05
Unknown	1	0.01
Ki-67 index		
<14 %	76	41.08
≥14 %	109	58.92

Note: T0: No evidence of cancer; T1: Tumor is smaller than 20 mm; T2: Tumor is larger than 20 mm; T3: Tumor is larger than 50 mm; T4: Tumor has grown into the chest wall; skin and inflammatory breast cancer; G1: Tumor cells are well-differentiated; G2: Moderately differentiated; G3: Poorly differentiated; N0: No regional lymph node metastasis; N1: Cancer has spread to 1 to 3 regional lymph nodes and N2: Cancer has spread to 4 or more regional lymph nodes and yp: Neoadjuvant pathologic stage groups

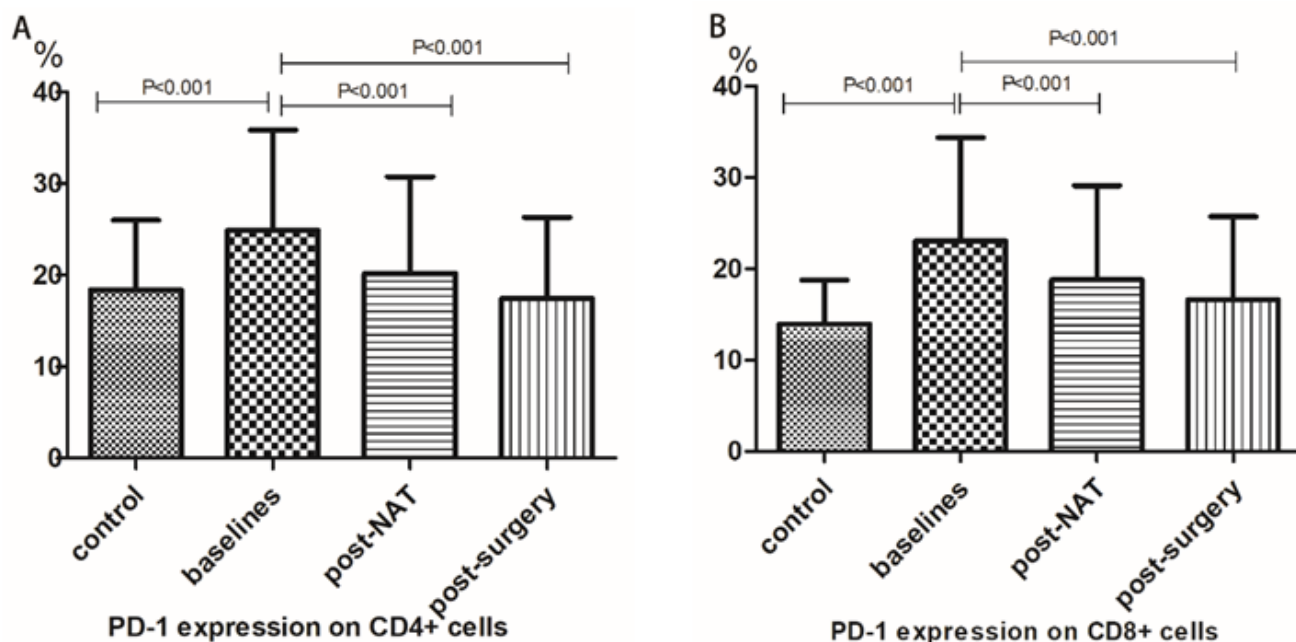


Fig. 1: The difference between PD-1 expression level of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in patients with TNBC and control groups at three phases (baseline, post-NAT and post-surgery), (A) The PD-1 expression on CD4<sup>+</sup> T lymphocytes in patients with TNBC and control group and (B) The PD-1 expression on CD8<sup>+</sup> T lymphocytes in patients with TNBC and control group

TABLE 2: THE PERCENTAGE OF PD-1<sup>+</sup>CD4<sup>+</sup> AND PD-1<sup>+</sup>CD8<sup>+</sup> T LYMPHOCYTES ASSOCIATED WITH CLINICAL RESPONSE TO NAC

Group	N	PD-1 <sup>+</sup> CD4 <sup>+</sup>	p	PD-1 <sup>+</sup> CD8 <sup>+</sup>	p
CR group	26				
Baseline		26.3±11.3		23.7±11.2	
Post-NAT		14.8±9.0	<0.001	12.9±7.5	<0.001
Post-surgery		13.0±6.8	0.07	12.1±6.9	<0.001
PR group	81				
Baseline		24.2±11.3		22.4±11.5	
Post-NAT		18.0±9.4	<0.001	16.9±9.5	<0.001
Post-surgery		15.6±8.0	<0.001	15.1±8.1	<0.001
SD group	69				
Baseline		25.7±10.9		24.2±11.3	
Post-NAT		24.7±10.9	0.19	23.1±10.9	0.12
Post-surgery		21.1±9.5	<0.001	20.1±9.9	<0.001
PD group	9				
Baseline		20.8±7.8		18.6±10.2	
Post-NAT		21.1±9.0	0.80	20.4±7.7	0.22
Post-surgery		19.3±6.1	0.42	17.2±7.5	0.56

In TNBC, PD-1 and PD-L1 expression has been reported frequently than other subtypes of breast cancer, while PD-1 inhibitor like pembrolizumab already has begun to show anti-tumor activity in this phase I trial<sup>[13]</sup>. In this famous phase III, randomized IMpassion130 trial, TNBC patients treated with atezolizumab combined with nab-paclitaxel had better progression-free survival, compared with placebo plus nab-paclitaxel in PD-L1 positive subgroup<sup>[2]</sup>. A new era with breast cancer

immunotherapy was opened using anti-PD-1 and anti-PD-L1 immunotherapies to treat TNBC patients.

Higher rate of TILs has been demonstrated to be associated with better response to anti-tumor therapy and better survival outcomes in breast cancer. Recently, some studies demonstrated that the tumor immune environment have been correlated with the effectiveness and prognosis of both immunotherapy and traditional anti-tumor therapy<sup>[14]</sup>. Although tumor



biopsy samples are directly related with the tumor microenvironment, samples are difficult to obtain each two cycles of NAC. Peripheral blood biomarkers were detected in more investigates, which may be applied in the clinic. Absolute lymphocyte count may not predict the clinical response and effect in patients who received NAC accurately in various researches<sup>[15,16]</sup>.

Periphery proliferating Kiel 67<sup>+</sup> (Ki67<sup>+</sup>) CD8<sup>+</sup> effector-like T cells may be another biomarker associated with improved clinical outcomes of patients with Non-Small Cell Lung Cancer (NSCLC) who received anti-PD-1 immunotherapy<sup>[17]</sup>. In patients with prostate cancer, Kwek *et al.* demonstrated that improved overall survival was correlated with PD-1 on CD4 effector T cells (Teff) expression level at baseline<sup>[18]</sup>. Zgodzinski *et al.* revealed that PD-1 and its legend PD-L1 were expressed on T lymphocytes on tumor cells, peripheral blood and lymph nodes in gastric cancer<sup>[19]</sup>. In advanced breast cancer, 15 patients enrolled in one single arm phase II clinical trial received albumin bound paclitaxel intravenous and imiquimod cream topically to investigate the modulation of TILs<sup>[20]</sup>.

The level of T lymphocytes in peripheral blood is intrinsically dynamic in response to NAT. The aim of this study was to detect the frequencies of PD-1<sup>+</sup> expression on CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocyte in TNBC patients who received NAC. Several studies prove that up-regulation of PD-1 expressed in T lymphocytes were activated by PD-1/PD-L1 pathway which may inhibit T cell proliferation during the interaction of T cells, which may lead to immune functional defect in TNBC patients<sup>[21]</sup>.

In our study, the first objective is to assess the difference between pretreatment PD-1 expression level and post-surgery PD-1 expression level. The second objective is to assess the association of PD-1 expression level with Polymerase Chain Reaction (PCR) rate. In result, this trial also revealed that peripheral baseline PD-1 expression level on CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes was highly significant than post-NAT in CR subgroup. To the contrary, there is no difference between baseline level and post-NAT levels of peripheral PD-1 expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells in non-CR group.

According to our results, the PD-1 expression level of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes of patients with TNBC are significantly higher than healthy control group. The periphery PD-1 expression level on CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes can also be detected in CR group who have better response to NAT.

In our research, CD4<sup>+</sup>PD-1<sup>+</sup> and CD8<sup>+</sup>PD-1<sup>+</sup> T

lymphocytes as immunological biomarkers are identified as predictors in response to NAC in TNBC. PD-1 abnormal expression in peripheral T lymphocytes may predict T lymphocytes inhibition which will lead to impaired immune reaction in TNBC patients. More investigations are needed to find the relationship between peripheral blood components and clinical outcomes like progression-free survival and overall survival.

#### **Ethical approval:**

The study protocol was reviewed and approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital.

#### **Author's contributions:**

Conception and design was done by Yu Chen and Li Zhang; administrative support was given by Yu Chen; provision of study materials or patients was given by Yu Chen; collection and assembly of data was done by Yu Chen and Li Zhang; data analysis and interpretation was done by Yu Chen and Li Zhang; manuscript writing was done by all the authors and final approval of the manuscript was given by all the authors.

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#### **Conflict of interests:**

The authors declared no conflict of interest.

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