Original Article

Iranian Journal of Immunology

December 2025; 22(4):

Expression and Prognostic Significance of PD-1/PD-Ls in Breast Cancer Draining Lymph Nodes

Zahra Mansourabadi[1,2#], Ali Moazzeni[3#], Majid Akrami[4], Maryam Fakhimi[2,5], Zahra Faghih[2*]

[1]Department of Immunology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran; [2]Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran; [3]Department of Hematology, Faculty of Allied Medicine, Bushehr University of Medical Sciences, Bushehr, Iran; [4]Breast Diseases Research Center, Department of Surgical Oncology, Shiraz University of Medical Sciences, Shiraz, Iran; [5]Department of Dermatology, Carver College of Medicine, University of Iowa, Iowa city, Iowa, USA
[#]These authors contributed equally: Zahra Mansourabadi and Ali Moazzeni

ABSTRACT

Background: Tumor-draining lymph nodes play a pivotal role in orchestrating immune cell trafficking and initiating antitumor responses. Among immunoregulatory molecules, programmed cell death protein 1 (PD-1) has emerged as a central mediator in tumor-induced immunosuppression.

Objective: To investigate the expression patterns of PD-1 and its ligands (PD-L1, PD-L2) in the tumor-draining lymph nodes of patients with breast cancer (BC).

Methods: Lymph node samples were freshly collected from BC patients undergoing surgery. Mononuclear cells were isolated and analyzed by flow cytometry for surface markers CD45, PD-1, PD-L1, and PD-L2. Data were analyzed using FlowJo v10.8.1.

Results: PD-1 was detected on 9.48±5.19% of $CD45^+$ cells, whereas PD-L1 and PD-L2 were expressed at lower levels (1.73±0.85% and 1.68±0.84%, respectively). Despite a significant reduction in the percentage of $CD45^+$ lymphocytes, the frequencies of $PD\text{-}1^+$ and $PD\text{-}L2^+$ subsets were significantly elevated in patients with poorly-differentiated and advanced-stage tumors ($P<0.05$). Additionally, the frequency of $PD\text{-}1^+$ lymphocytes was significantly higher in patients with the triple-negative tumors ($P=0.014$) and in those negative for estrogen and progesterone receptor ($P=0.001$).

Conclusion: Elevated expression of PD-1 and its ligands in BC-draining lymph nodes is associated with adverse clinical features, suggesting their role in immune evasion. These findings along with higher frequency of PD-1+ lymphocyte in triple-negative patients may inform subtype-specific therapeutic strategies and predict responsiveness to PD-1/PD-Ls blockade therapies. Future studies should include functional analyses with broader immunophenotyping to further elucidate these mechanisms.

Keywords: Breast cancer, Lymph node, Immune checkpoint, PD-1, PD-L1, PD-L2

*Corresponding author:
Zahra Faghih, PhD;
Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, P.O. Box: 71348-45550, Shiraz, Iran
Email: Faghihz@sums.ac.ir

Cite this article as:
Mansourabadi Z, Moazzeni A, Akrami M, Fakhimi M, Faghih Z. Expression and Prognostic Significance of PD-1/PD-Ls in Breast Cancer Draining Lymph Nodes. *Iran J Immunol.* 2025; 22(4): ,
doi: 10.22034/iji.2025.107984.3084.

Received: 2025-07-28
Revised: 2025-10-11
Accepted: 2025-10-12

INTRODUCTION

Programmed cell death protein-1 (PD-1), also known as CD279, is a key immune checkpoint receptor in the CD28/CTLA-4 family. It is primarily expressed on activated T cells and plays a central role in maintaining immune tolerance. Upon binding to its ligands, PD-L1 or PD-L2, PD-1 triggers inhibitory signaling pathways that suppress immune activation, a mechanism frequently exploited by tumors to evade immune surveillance (1, 2).

Recent advances in immuno-oncology have highlighted the PD-1 axis as a key mediator of tumor immune evasion, particularly in cancers resistant to conventional therapies (3). Consistent with its immunosuppressive role, PD-1 expression on tumor-infiltrating lymphocytes has been associated with impaired immune function and poor clinical outcomes. Similarly, the overexpression of its ligand, PD-L1, is frequently linked to poor prognosis (4, 5). Therefore, characterizing the expression pattern of PD-1 and its ligands in draining lymph nodes as primary sites of immune activation (6), can provide valuable insights into the tumor immune microenvironment and its clinical relevance. Notably, the spatial distribution and density of these immune checkpoint molecules within lymphatic tissues have shown significant prognostic and predictive value for therapeutic outcomes across multiple cancer types (7, 8).

Hence, in this study, we investigated the presence and the expression levels of PD-1 and its ligands, PD-L1 and PD-L2, in the axillary lymph nodes of patients with breast cancer (BC), the most prevalent cancer in women, and assessed their association with tumor prognosis.

PATIENTS AND METHODS

Patients

Fourty-seven fresh tumor-draining lymph nodes were collected from patients with BC undergoing therapeutic surgery and used as the source of lymphocytes. None of the patients had a history of prior chemotherapy, radiotherapy or immunotherapy. Clinical and pathological data were obtained from patients' medical records (Table 1). Tumor staging was determined according to the 8th edition of the American Joint Committee on Cancer guideline.

Isolation of Mononuclear Cells and Flow Cytometric Analysis

Fresh lymph node specimens were mechanically dissociated in complete culture medium [RPMI 1640+ 10% fetal bovine serum (both from Gibco, USA)] to generate a single-cell suspension. Mononuclear cells populations, were isolated by filtering and centrifugation over a Ficoll-Hypaque (Lymphodex Innotrain, Germany) density gradient. Cell viability was assessed using Trypan blue (Biosera, France) exclusion and samples with >95% viability were used. A total of 250,000 cells were resuspended in 50 µl of staining buffer (1× PBS containing 2% FBS) and stained with fluorochrome-conjugated anti-human antibodies: CD45 (FITC, clone: HI30), PD-L2 (PerCP-Cy5.5, clone: MIH18) (both from BD Biosciences, USA), PD-1 (PE, clone: EH12.2H7), and PD-L1 (APC, clone: 29E2A3) (BioLegend, USA). Cells were incubated in the dark at room temperature for 15 min, washed, and resuspended in staining buffer, and acquired on a BD FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo v10.1.8 (BD Life Sciences). As PD-1, PD-L1, and PD-L2 are expressed on both immune and stromal cells, initial gating was based on CD45 expression –a pan marker of leukocytes– to distinguish immune cell populations ($CD45^+$) from non-immune cells ($CD45^-$; Fig. 1B). The percentages of PD-1-, PD-L1-, and PD-L2-expressing cells were then determined in each subset (Fig. 1C and D). Expression levels of markers were quantified using geometric mean fluorescence intensity (gMFI), normalized against the gMFI of the negative control population.

Table 1. Clinical and pathological characteristics of patients with breast cancer

Characteristics	Value (%)*
Age	48.53±10.82
Tumor type	
IDC	37 (78.7)
ILC	3 (6.4)
IMCF	3 (6.4)
Others	4 (8.5)
T grouping	
T1	19 (42.2)
T2	26 (57.8)
Unreported	2
Lymph node status	
Free (N0)	8 (17.4)
N1	26 (56.5)
N2	9 (19.6)
N3	3 (6.5)
Unreported	1
Stage	
I	6 (13.6)
II	27 (61.4)
III	11 (25)
Unreported	3
Histological grade	
Well	6 (15)
Moderate	25(62.5)
Poor	9 (22.5)
Unreported	7
Lymphatic invasion	
Negative	39 (90.7)
Positive	4 (9.3)
Unreported	4
Vascular invasion	
Negative	8 (18.6)
Positive	35 (81.4)
Unreported	4
Perineural invasion	
Negative	6 (14.3)
Positive	36 (85.7)
Unreported	5
Necrosis	
Negative	11 (24.4)
Positive	34 (75.6)
Unreported	2
ER	
Negative	7 (16.3)
Positive	34 (73.9)
Unreported	4
PR	
Negative	10 (23.3)
Positive	33 (76.7)
Unreported	4
HER2	
Negative	22 (52.4)
Equivocal	7 (16.7)
Positive	13 (31)
Unreported	5
Molecular subtype	
Luminal	17(50.0)
HER2-enriched	13 (38.2)
Triple negative	4 (11.8)
Unreported	13

*Clinical variables with missing data are designated as “unreported”, and valid percentages are presented for each variable.

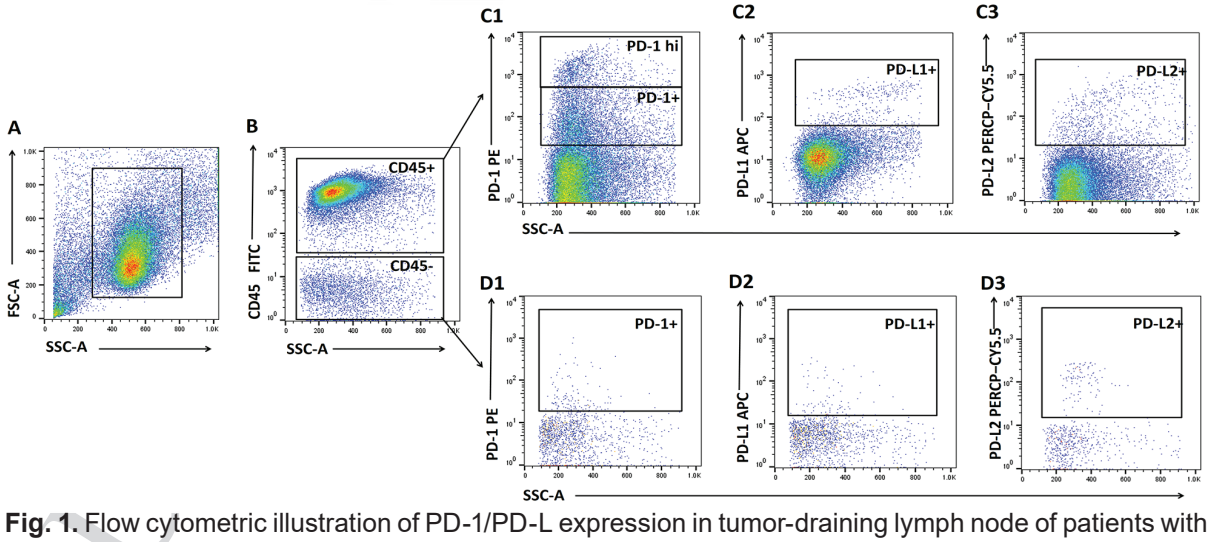

Fig. 1. Flow cytometric illustration of PD-1/PD-L expression in tumor-draining lymph node of patients with breast cancer. To assess the expression of PD-1/PD-Ls, mononuclear cells were first gated according to relative size and granularity (FSC/SSC) (A). cells were then sub-divided based on CD45 expression into $CD45^+$ and $CD45^-$ populations (B) Within each population, the frequency of PD-1 (C.1, D.1), PD-L1 (C.2, D.2), and PD-L2 (C.3, D.3) expressing cells was determined.

Table 2. PD-1/PD-Ls expression in draining lymph nodes of patients with breast cancer

Cell subset	Min	Max	Median	Mean±SD
$CD45^+$ lymphocytes	86.50	99.80	98.20	97.08±3.16
$PD\text{-}1^+$	3.08	30.00	8.45	9.48±5.19
$PD\text{-}L1^+$	0.52	4.46	1.70	1.73±0.85
$PD\text{-}L2^+$	0.62	4.14	1.36	1.68±0.84
$CD45^-$ cells	0.17	13.50	1.82	2.91±3.15
$PD\text{-}1^+$	0.24	79.60	2.99	6.61±12.35
$PD\text{-}L1^+$	0.64	16.20	2.28	3.88±4.08
$PD\text{-}L2^+$	0.53	86.10	2.93	10.38±14.47

t-distributed Stochastic Embedding (t-SNE) and Phonograph Analyses

Advanced clustering and visualization were performed using the t-distributed stochastic neighbor embedding (t-SNE) algorithm and the Phenograph plugin (version 2.4). FCS files were concatenated and t-SNE was run with default parameters: perplexity=20, learning rate=200, 1000 iterations, and theta=0.5. Phenograph clustering (v.2.4) was applied to a down-sampled concatenated file containing 300,000 events with K=30. Forward scatter (FSC), side scatter (SSC), CD45, PD-1, PD-L1, and PD-L2 parameters were included as input parameters for the analyses.

Statistical Analysis

The statistical package for the social sciences (SPSS) software version 26 was used for data analysis. Comparisons between patient groups with different clinicopathological characteristics were conducted using the Mann-Whitney U test for two-group comparisons and the Kruskal-Wallis H test for multiple-group comparisons. Data are presented as median values and mean±standard deviation (SD). All statistical tests were two-tailed, with $P< 0.05$ considered statistically significant. Graphical representations were generated using GraphPad Prism software, version 8 (San Diego, CA, USA).

RESULTS

PD-1, PD-L1, and PD-L2 Expression on $CD45^+$ Immune Cells

As anticipated, the majority of lymph node cells were $CD45^+$, with a mean frequency of 97.08±3.16% (86.50-99.80) (Table 2). Phenotypic analysis showed that 9.48±5.19 (3.08-30.00) of $CD45^+$ lymphocytes expressed PD-1. Despite a significant decrease in the frequency of $CD45^+$ lymphocytes in patients with stage II compared to stage I (P=0.046), the PD-1-expressing $CD45^+$ cells were more prevalent in stage II than in stage I (P=0.031) and those with poorly-differentiated tumors (III>I; P=0.043) (Fig. 2A and B). PD-1 expression intensity (gMFI) was also higher in stage II (P=0.023) and III (P=0.015) compared to stages I, in larger tumors (T2>T1; P=0.035), and in lymph nodes-positive cases (LN+>LN−; P=0.035) (Fig. 2C). Patients with triple-negative breast cancer (TNBC) exhibited significantly higher proportions of $PD\text{-}1^+CD45^+$ cells compared to luminal subtypes (P=0.014; Fig. 2D). Similarly, estrogen/progesterone receptor (ER/PR)-negative tumors showed elevated $PD\text{-}1^+$ lymphocyte infiltration relative to receptor-positive cases (both P=0.001). PD-1 expression intensity (MFI) was also elevated in TNBC (P=0.008) and PR-negative tumors (P=0.029).

We also separately analyzed a distinct subset of lymphocytes with high PD-1 expression ($PD\text{-}1^{hi}CD45^+$). This population represented 1.39±1.17% of lymphocytes (range: 0.13–4.63%) within the tumor-draining lymph nodes of patients with BC. Similar to $PD\text{-}1^+$ subset, $PD\text{-}1^{hi}$ cells were significantly more frequent in patients with larger tumors (T2>T1; P=0.011), advanced disease stages (III/II>I; P=0.037), and poorly-differentiated tumors (III>I/II; P=0.011).

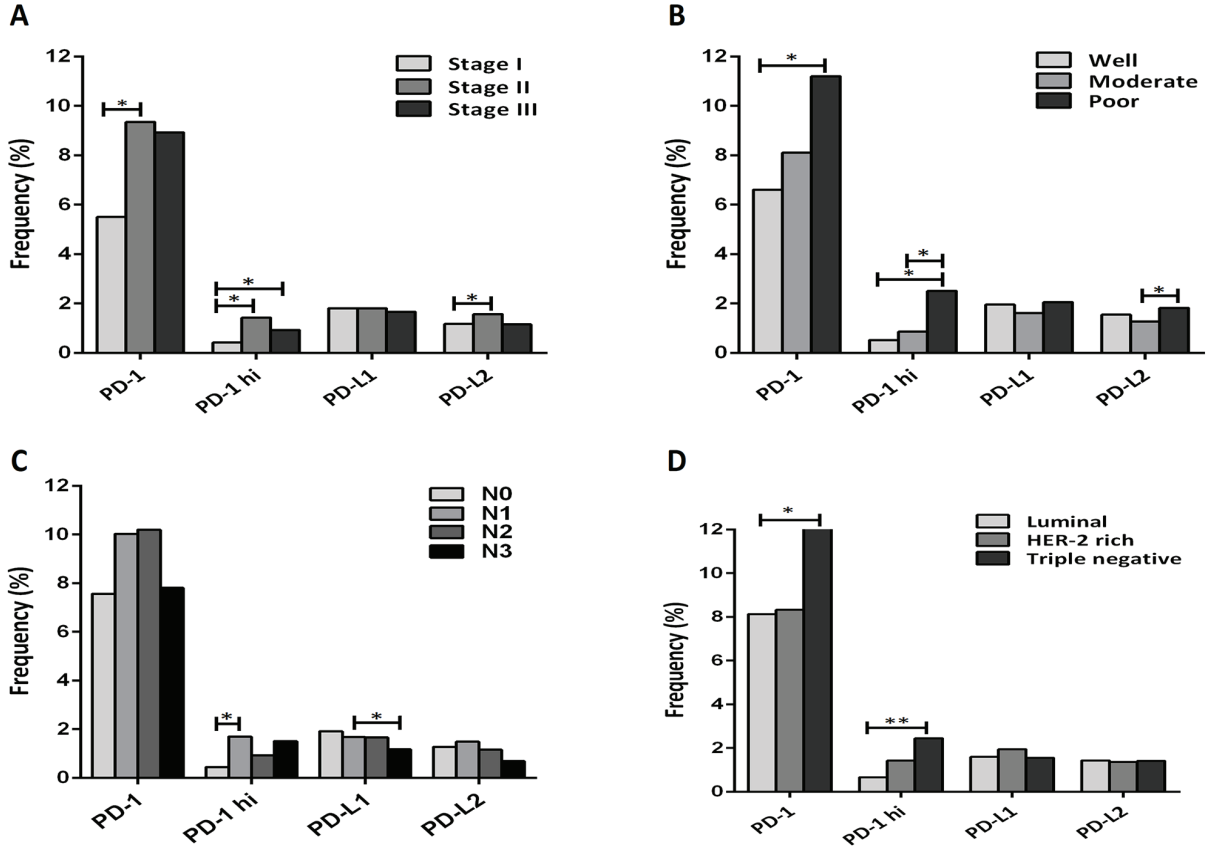

Fig. 2. Statistical analyses of PD-1/PD-L expression in breast cancer-draining nodes across clinicopathological subgroups. Frequencies of PD-1^{+}, PD-1^{hi}, PD-L1^{+} and PD-L2^{+} cells are shown across tumor stages (A), grades (B), lymph node status (C) and molecular subtypes (D). Data are present as median. * P<0.05. ** P<0.01.

Their frequency was also elevated in LN+ patients compared with LN- cases (P=0.011). Consistent with this, CD45^{+}PD-1^{hi} cells were significantly higher in N1-patients (with 1-3 tumor-infiltrated nodes) compared with node-negative individuals (P=0.012). The mean expression of PD-1 within this subset was higher in stage III than in stage II (P=0.029). Additionally, CD45^{+}PD-1^{hi} cells were found at significantly higher frequencies in patients with invasive ductal carcinoma with medullary features (IDCMF; P=0.024), in those with TBNC (P=0.008), and ER/PR-negative tumors (both P<0.001) compared with patients with invasive ductal carcinoma (IDC), other molecular subtypes and ER/PR-positive tumors, respectively.

In contrast to PD-1, the expression of PD-L1 and PD-L2 displayed differential expression on CD45^{+} lymphocytes and was relatively low at 1.73±0.85% and 1.68±0.84, respectively (Table 2). Despite this low frequency, statistical analyses demonstrated that the mean expression intensity of PD-L1 (gMFI) was significantly higher in CD45^{+} cells of patients with stage II compared with those in stage III (P=0.005, Fig. 2B). Furthermore, N1 patients exhibited significantly higher PD-L1 expression in CD45^{+} cells than N2 (with 4-9 tumor-infiltrated nodes) and N3 (with 10 or more tumor-infiltrated nodes) patients (P=0.026 and P=0.008, respectively; Fig. 2C). For PD-L2, both patients with stage II (P=0.046) and those with poorly-differentiated tumors (P=0.037) demonstrated higher frequencies of CD45^{+}PD-L2^{+} cells compared with stage I and moderately differentiated cases (Fig. 2A and B). Additionally, ER-negative patients showed a trend toward higher PD-L2 expression (gMFI) in CD45+ lymphocytes relative to ER-positive individuals (P=0.056).

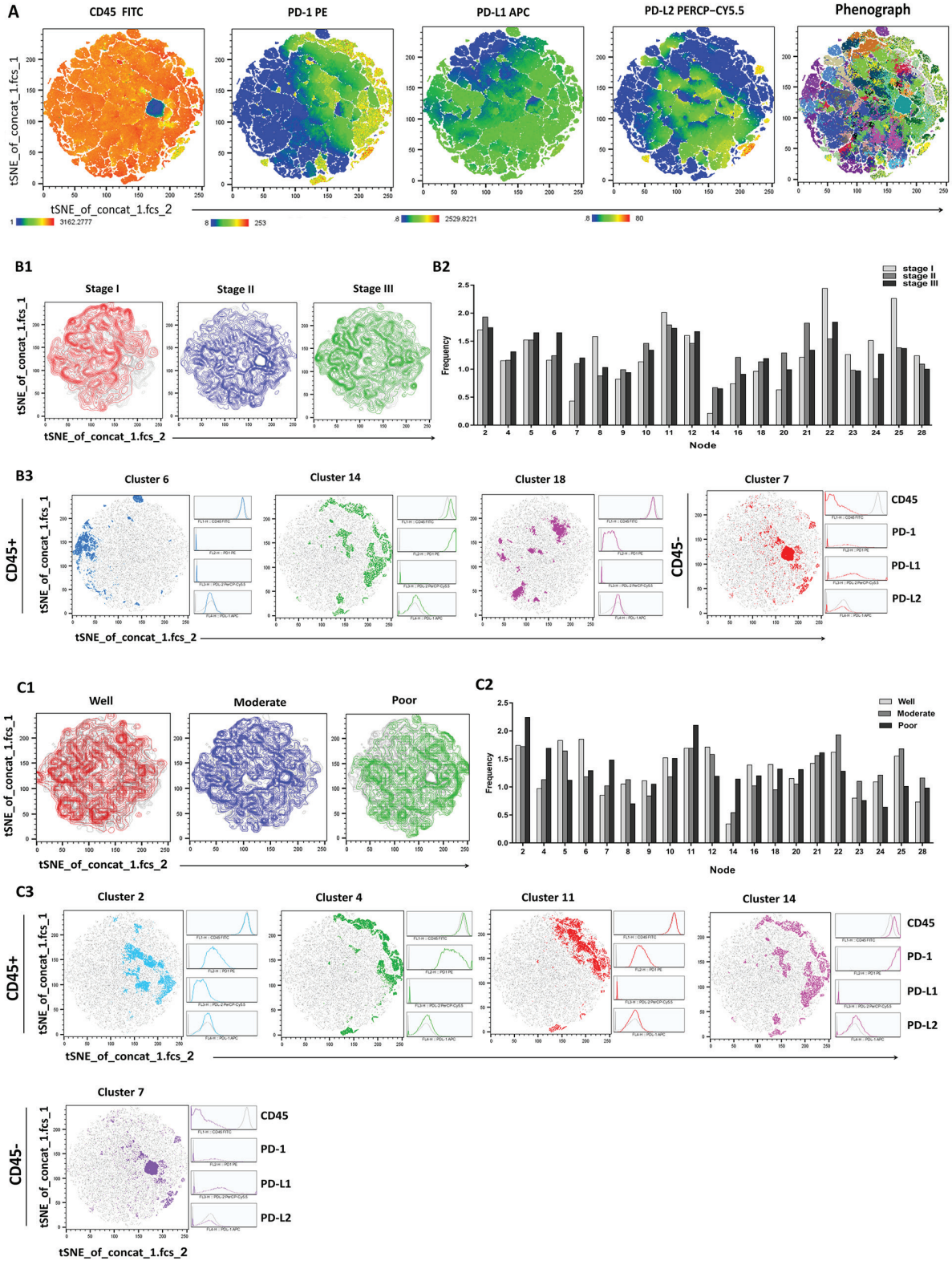

Fig. 3. Representative plots illustrating unbiased clustering of PD-1, PD-L1, and PD-L2 expression profiles in tumor-draining lymph nodes from breast cancer patients. (A) The tSNE maps demonstrating CD45, PD-1, PD-L1 and PD-L2 expression in breast tumor-draining lymph nodes. B1 and C1: Frequent clusters identified by Phenograph overlaid on the tSNE maps. B2 and C2: Bar charts showing the distribution of highly prevalent nodes clusters across tumor stages and grades. B3 and C3: Contour density plots illustrating cell populations among patients with stage I, II and III, and with well-, moderately- and poorly-differentiated tumors.

Expression of PD-1 and Its Ligands (PD-L1/ PD-L2) on CD45⁻ Non-immune Cells

CD45- cells comprised for 2.91±3.15% (range: 0.17-13.50) of total lymph node cells. Within this population, 6.61±12.35% expressed PD-1. Compared with CD45+ lymphocytes, ligand expression was higher in CD45⁻ cells with PD-L1 and PD-L2 detected in 3.88±4.08% and 10.38±14.47% of cells, respectively (Table 2). Statistical analysis showed that the frequency of CD45- cells was significantly higher in patients with stage II compared to those in stage I of the disease (P=0.041). PD-1 expression intensity (gMFI) within CD45-PD-1+ cells was higher in patients whose tumors lacked necrosis compared to those with necrotic tumors (P=0.012). This subset was also more frequent in N2 versus N1 patients (P=0.040). In terms of ligands, PD-L1+CD45⁻ cells were more prevalent in stage III patients than in stage II patients (P=0.026). Additionally, PD-L2 expression intensity (MFI) in CD45- cells was significantly higher in draining nodes of patients with a TBNC compared with those with the HER-2-enriched subtype (P=0.039).

High-dimensional Analysis Reveals Heterogeneous Subsets of Immune Cells with Distinct CD45, PD-1, PD-L1, and PD-L2 Expression Profiles

To expand on our findings, we utilized t-SNE and Phenograph analyses as unsupervised machine learning algorithms to evaluate the relationships among cells in high- and low-dimensional spaces, which may go unnoticed when using traditional sequential two-dimensional dot plots. The results were presented in Fig. 3A. Using the defined parameters, Phenograph analysis identified 30 distinct clusters. Patients were then stratified patients by key clinicopathological characteristics, including tumor grade and stage, and the distribution patterns of the 20 most abundant clusters was compared across subgroups. Three clusters (6, 14, 18) increased with tumor progression from stage I to stage III (Fig. 3B). These populations shared similar expression levels of CD45, PD-L1, and PD-L2, but differed in PD-1 expression. Four additional clusters (2, 4, 11, and 14) showed stepwise increases with higher tumor grades and exhibited comparable CD45, PD-L1, and partial PD-L2 expression, but lacked PD-1 expression (Fig. 3C). Phenograph analysis also identified a distinct CD45- population (cluster 7), that expanded with increasing tumor grade and stage. This subset demonstrated detectable expression of PD-1, PD-L1, and PD-L2.

DISCUSSION

In this study, we investigated the clinical relevance of PD-1 and its ligands, PD-L1 and PD-L2, within both CD45-positive and CD45-negative populations in BC-draining lymph nodes. Our findings showed that, with tumor progression, the overall frequency of CD45+ immune cells significantly declined, whereas PD-1 and PD-Ls expression within this population markedly increased.

CD45+ cells constituted the predominant population in regional lymph nodes of patients with BC, comprising more than 95% of all cells (86.5-99.8%). Our findings showed a decline in the frequency of immune cells with tumor progression, reflected by reduced CD45+ cell proportions in higher disease stages (stage II<stage I). This decrease may indicate a broad suppression of immune activity, independent of functional status. Although data specific to draining lymph nodes are lacking, immunohistochemical studies of various tumor types have similarly reported that higher percentages of CD45+ cells and/or tumor-infiltrating lymphocytes are associated with better clinical outcomes and improved survival, confirming that the general evaluation of immune cells could be a reliable prognostic marker (9).

We next analyzed the expression of PD-1 and its ligands, PD-L1 and PD-L2, within both CD45+ and CD45- populations. PD-1 expressing cells accounted for nearly 10% of CD45+ cells in the axillary lymph nodes. PD-1

is widely recognized as a negative regulator of immune activity, particularly in T cells, where ligand engagement suppresses proliferation and contributes to tolerance or tumor immune evasion (10). Reports on the expression of PD-1 in BC, especially in tumor-draining lymph nodes remain limited and inconsistent, with clinical associations varying by tumor subtype and disease stage (11-16).

Our findings demonstrated that PD-1 expression among $CD45^+$ cells was associated with poor clinical features, including larger tumor size, poor differentiation, higher stage, and regional nodes involvement. Consistent with this, Yuan et al. reported a positive association between PD-1 expression and high histological grade in metastatic axillary lymph nodes (14). Similarly, in bladder cancer-draining pelvic lymph nodes, mean expression of PD-1 increased among $CD45^{high}$ cells (likely lymphocytes) with tumor progression (17). Although additional data from lymph nodes are scarce, studies on primary breast tumors have shown that infiltration of PD-1^+ lymphocytes is more pronounced in aggressive tumors and correlates with poorer clinical outcomes (15, 16).

We next evaluated the expression of PD-1 ligands, PD-L1 and PD-L2, on the $CD45^+$ cells within draining lymph nodes. PD-L1, which is broadly expressed in inflamed tissues, and PD-L2, typically restricted to antigen-presenting cells, are generally believed to negatively regulate immune responses to tumors following engagement with their receptor, PD-1 (18, 19). Although the surface expression of PD-L1 and PD-L2 on $CD45^+$ lymphocytes was relatively low (<1%), we observed a noteworthy association between PD-L1 expression and intermediate stages of tumor progression (N1>N2, II>III). This pattern may reflect an active antitumor immune response, consistent with reports that identify PD-L1 as a favorable prognostic indicator in certain BC subtypes (20, 21).

While PD-L1 expression in the breast tumor microenvironment has been widely studied and often associated with poor prognostic markers (19-26), data on its expression in draining lymph nodes remain limited (14, 27). Immunohistochemical studies of metastatic lymph nodes have shown that immune cells frequently expressed PD-L1, consistent with patterns observed in tumor tissue (14, 28). However, other reports describe higher PD-L1 levels in axillary LNs than in matched primary tumors (8, 14, 27). For instance, Li et al. found that PD-$L1^+$ immune infiltrates in metastatic lymph nodes were significantly associated with increased distant metastasis and reduced disease free survival (8). Yuan et al similarly reported elevated PD-L1 expression in metastatic lymph nodes from patients with poor prognostic features, including high Ki-67, advanced TNM stage, and multiple involved lymph nodes, and older age (older than 50 years), though they did not distinguish between tumor and immune cell expression (14). Comparable findings have been documented in pelvic lymph nodes of patients with bladder cancer (17). By contrast, Alves et al. found no association between PD-L1 expression in metastatic nodes of BC and clinicopathological characteristics of the primary tumor (27).

For PD-L2, the second known ligand of PD-1, we observed higher frequencies of PD-L2-expressing $CD45^+$ cells in more advanced tumors (stage II > I; grade III > II) and ER-negative subtypes. To our knowledge, this is the first study to evaluate PD-L2 expression in BC-draining lymph node. Prior investigations have focused primarily on PD-L2 in breast tumor tissues (20, 29-31). Consistent with our findings, PD-L2 expression in BC has been linked to ER negativity, lymph node involvement, distant recurrence, HER2 overexpression, and younger age at diagnosis (20). Notably, a subset of ER+ tumors also exhibited elevated PD-L2 levels associated with poor clinical outcomes, supporting a potential immunosuppressive role for PD-L2 in BC (29). Similar patterns have been reported in bladder cancer (17). Given PD-L2's higher binding affinity for PD-1 compared to PD-L1 (32), its upregulation in BC underscores its possible functional significance in tumor immune

evasion. However, the functional implications of PD-1/PD-Ls signaling remain complex, as recent studies have shown that PD-1 can also be upregulated following immune cell activation, particularly in T cells. Given that a substantial proportion of immune cells in lymph nodes are T cells (33), both the timing and magnitude of PD-1 expression likely influence whether T cells remain activated or become exhausted, thereby shaping responses to PD-1–targeted therapies, especially in patients with advanced disease. Additionally, environmental stimuli such as IL-4 and IFN-γ, have been shown to induce PD-1/PD-L upregulation, with PD-L2 being particularly responsive (34). The increased presence of IL-4-producing cells (Th2 phenotype) reported in draining nodes of BC patients (33, 35-37) may therefore contribute to the elevated frequency of PD-1$^+$ cells observed in more advanced tumors.

In examining subtype-specific heterogeneity, we found that the frequency of PD-1$^+$CD45$^+$ cells was markedly more frequent in patients with TNBC. Previous studies have also reported subtype-dependent differences in PD-1/PD-L expression, although the findings remain inconsistent. Several reports support our results, indicating increased PD-1 or PD-L1 expression in triple negative patients (24, 28, 31, 38-41). However, other studies have described the highest PD-L1 levels in luminal A tumors and the lowest in triple negative subtypes (11), or its association with both HER2-enriched and triple negative basal subtypes (20). These discrepancies likely reflect subtype-specific tumor microenvironments, potentially shaped by intrinsic hormone-related pathways that differentially regulate immune checkpoint expression (42, 43).

We also examined PD-1 and PD-Ls expression in CD45$^-$ cells within breast cancer-draining lymph nodes. This CD45$^-$ compartment likely includes non-immune cells stromal elements and, in LN$^+$ cases, disseminated tumor cells. CD45$^-$ cells represented roughly 3% of total lymph node cellularity, and more than 6% expressed PD-1. Compared to CD45$^+$ lymphocytes, the expression of PD-Ls, particularly PD-L2, was higher in this population (4-10%). These findings, together with the significant increase in the frequency of CD45$^-$ cells during tumor progression (stage II>I), suggest either a gradual replacement of immune cells with tumor cells or a reduction in CD45$^+$ immune cells due to tumor-mediated immunosuppression. Our data further indicate an active PD-1/PD-Ls signaling axis within the CD45$^-$compartment. Although no studies have examined this population in BC–draining nodes, an association between CD45$^-$ cells and reduced survival has been reported in the bladder cancer-draining lymph node (17), collectively supporting an inhibitory role for these cells in advanced BC.

CONCLUSION

This study provides one of the first characterizations of differential expression of PD-1/PD-Ls, particularly PD-L2, in breast cancer-draining lymph nodes. Increased expression of PD-1 and its ligands in advanced disease, larger tumors, lymph node-positive cases, and poorly-differentiated tumors, together with the known immunoregulatory functions of this pathway, suggest that PD-1/PD-L pathway may contribute to immune suppression in BC. Further functional studies with larger cohorts are needed to clarify the specific roles of these molecules in BC immunity, which may ultimately support the development of more effective therapeutic strategies and improve prediction of responses to PD-1/PD-L–targeted treatments.

ETHICAL APPROVAL

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1396.S787). Informed consent was obtained from all participants prior to sample collection.

FUNDING

This work was financially supported by Shiraz University of Medical Sciences (Grant number: 12835) and Shiraz Institute for Cancer Research (Grant number: ICR-100-504).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Sharpe AH, Pauken KE. The diverse functions of the PD1 inhibitory pathway. Nature Reviews Immunology. 2018;18(3):153-67.
2. Peng S, Bao Y. A narrative review of immune checkpoint mechanisms and current immune checkpoint therapy. Ann Blood. 2022;7:33.
3. Salmaninejad A, Valilou SF, Shabgah AG, Aslani S, Alimardani M, Pasdar A, et al. PD-1/PD-L1 pathway: Basic biology and role in cancer immunotherapy. Journal of cellular physiology. 2019;234(10):16824-37.
4. Han Y, Liu D, Li L. PD-1/PD-L1 pathway: current researches in cancer. American journal of cancer research. 2020;10(3):727.
5. Yang H, Zhou X, Sun L, Mao Y. Correlation between PD-L2 expression and clinical outcome in solid cancer patients: a meta-analysis. Frontiers in oncology. 2019;9:47.
6. Koukourakis MI, Giatromanolaki A. Tumor draining lymph nodes, immune response, and radiotherapy: Towards a revisal of therapeutic principles. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer. 2022;1877(3):188704.
7. Taube JM, Klein A, Brahmer JR, Xu H, Pan X, Kim JH, et al. Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. Clin Cancer Res. 2014 Oct 1;20(19):5064-74. PubMed PMID: 24714771. Pubmed Central PMCID: PMC4185001. Epub 20140408. eng.
8. Li M, Li A, Zhou S, Xu Y, Xiao Y, Bi R, et al. Heterogeneity of PD-L1 expression in primary tumors and paired lymph node metastases of triple negative breast cancer. BMC cancer. 2018 Jan 2;18(1):4. PubMed PMID: 29291717. Pubmed Central PMCID: PMC5748959. Epub 2018/01/03. eng.
9. Paijens ST, Vledder A, de Bruyn M, Nijman HW. Tumor-infiltrating lymphocytes in the immunotherapy era. Cellular & molecular immunology. 2021;18(4):842-59.
10. Liu J, Chen Z, Li Y, Zhao W, Wu J, Zhang Z. PD-1/PD-L1 checkpoint inhibitors in tumor immunotherapy. Frontiers in pharmacology. 2021;12:731798.
11. Tsang JY, Au W-L, Lo K-Y, Ni Y-B, Hlaing T, Hu J, et al. PD-L1 expression and tumor infiltrating PD-1+ lymphocytes associated with outcome in HER2+ breast cancer patients. Breast cancer research and treatment. 2017;162:19-30.
12. Ye Q, Wang C, Xian J, Zhang M, Cao Y, Cao Y. Expression of programmed cell death protein 1 (PD-1) and indoleamine 2, 3-dioxygenase (IDO) in the tumor microenvironment and in tumor-draining lymph nodes of breast cancer. Human pathology. 2018;75:81-90.
13. Nayek S, Sau S, Majumdar K, Basu A, Bandyopadhyay A. Histomorphology of immunomodulation and PD1 expression in breast cancer tumor draining lymph nodes: Determinants of Breast Cancer Screening. Archives of Breast Cancer. 2022:213-20.
14. Yuan C, Liu Z, Yu Q, Wang X, Bian M, Yu Z, et al. Expression of PD-1/PD-L1 in primary breast tumours and metastatic axillary lymph nodes and its correlation with clinicopathological parameters. Scientific reports. 2019;9(1):14356.
15. Muenst S, Soysal S, Gao F, Obermann E, Oertli D, Gillanders W. The presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer. Breast cancer research and treatment. 2013;139:667-76.
16. Matikas A, Zerdes I, Lövrot J, Sifakis E, Richard F, Sotiriou C, et al. PD-1 protein and gene expression as prognostic factors in early breast cancer. ESMO open. 2020;5(6):e001032.
17. Ariafar A, Ghaedi M, Rezaeifard S, Shahriari S, Zeighami S, Ghaderi A, et al. Clinical relevance and prognostic significance of PD-1/PD-Ls in non-metastatic bladder cancer: a role for PD-L2. Molecular Immunology. 2020;124:35-41.
18. Wang Y, Du J, Gao Z, Sun H, Mei M, Wang Y, et al. Evolving landscape of PD-L2: bring new light to checkpoint immunotherapy. British Journal of Cancer. 2023;128(7):1196-207.
19. Vranic S, Cyprian FS, Gatalica Z, Palazzo J, editors. PD-L1 status in breast cancer: Current view and perspectives. Seminars in cancer biology; 2021: Elsevier.
20. Baptista MZ, Sarian LO, Derchain SF, Pinto GA, Vassallo J. Prognostic significance of PD-L1 and PD-L2 in breast cancer. Human pathology. 2016;47(1):78-84.
21. Stovgaard ES, Dyhl-Polk A, Roslind A, Balslev E, Nielsen D. PD-L1 expression in breast

cancer: expression in subtypes and prognostic significance: a systematic review. Breast cancer research and treatment. 2019;174:571-84.
22. Kitano A, Ono M, Yoshida M, Noguchi E, Shimomura A, Shimoi T, et al. Tumour-infiltrating lymphocytes are correlated with higher expression levels of PD-1 and PD-L1 in early breast cancer. ESMO open. 2017;2(2):e000150.
23. Ghebeh H, Mohammed S, Al-Omair A, Qattant A, Lehe C, Al-Qudaihi G, et al. The B7-H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. Neoplasia. 2006;8(3):190-8.
24. Muenst S, Schaerli A, Gao F, Däster S, Trella E, Droeser R, et al. Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. Breast cancer research and treatment. 2014;146:15-24.
25. Loi S, Michiels S, Adams S, Loibl S, Budczies J, Denkert C, et al. The journey of tumor-infiltrating lymphocytes as a biomarker in breast cancer: clinical utility in an era of checkpoint inhibition. Annals of Oncology. 2021;32(10):1236-44.
26. Buisseret L, Garaud S, de Wind A, Van den Eynden G, Boisson A, Solinas C, et al. Tumor-infiltrating lymphocyte composition, organization and PD-1/PD-L1 expression are linked in breast cancer. Oncoimmunology. 2017;6(1):e1257452.
27. Alves AM, Paredes J, Schmitt F. Expression of PD-L1 in primary breast carcinoma and lymph node metastases. Surgical and Experimental Pathology. 2019;2(1):1-6.
28. Tawfik O, Kimler BF, Karnik T, Shehata P. Clinicopathological correlation of PD-L1 expression in primary and metastatic breast cancer and infiltrating immune cells. Human pathology. 2018;80:170-8.
29. Chervoneva I, Peck AR, Sun Y, Yi M, Udhane SS, Langenheim JF, et al. High PD-L2 predicts early recurrence of ER-positive breast cancer. JCO Precision Oncology. 2023;7:e2100498.
30. Yearley JH, Gibson C, Yu N, Moon C, Murphy E, Juco J, et al. PD-L2 expression in human tumors: relevance to anti-PD-1 therapy in cancer. Clinical cancer research. 2017;23(12):3158-67.
31. Asano Y, Kashiwagi S, Goto W, Takada K, Takahashi K, Morisaki T, et al. Prediction of treatment responses to neoadjuvant chemotherapy in triple-negative breast cancer by analysis of immune checkpoint protein expression. Journal of Translational Medicine. 2018;16(1):1-12.
32. Youngnak P, Kozono Y, Kozono H, Iwai H, Otsuki N, Jin H, et al. Differential binding properties of B7-H1 and B7-DC to programmed death-1. Biochemical and biophysical research communications. 2003;307(3):672-7.
33. Faghih Z, Erfani N, Haghshenas MR, Safaei A, Talei A-R, Ghaderi A. Immune profiles of CD4+ lymphocyte subsets in breast cancer tumor draining lymph nodes. Immunology letters. 2014;158(1-2):57-65.
34. Rozali EN, Hato SV, Robinson BW, Lake RA, Lesterhuis WJ. Programmed death ligand 2 in cancer-induced immune suppression. Clinical and Developmental Immunology. 2012;2012.
35. Faghih Z, Rezaeifard S, Safaei A, Ghaderi A, Erfani N. IL-17 and IL-4 producing CD8+ T cells in tumor draining lymph nodes of breast cancer patients: positive association with tumor progression. Iranian Journal of Immunology. 2013;10(4):193-204.
36. Faghih Z, Sadat Shobeiri S, Ariafar A, Sarkarian M, Zeighami S, Nazari N, et al. CD8+ T lymphocyte subsets in bladder tumor draining lymph nodes. Iranian Journal of Immunology. 2016;13(4):237-48.
37. Zareinejad M, Mehdipour F, Roshan-Zamir M, Faghih Z, Ghaderi A. Dual Functions of T Lymphocytes in Breast Carcinoma: From Immune Protection to Orchestrating Tumor Progression and Metastasis. Cancers. 2023;15(19):4771.
38. Tatara T, Mukohara T, Shimono Y, Yamasaki T, Imamura Y, Funakoshi Y, et al. Expression of programmed death-1 in sentinel lymph nodes of breast cancer. Journal of Surgical Oncology. 2018;117(6):1131-6.
39. Soliman H, Khalil F, Antonia S. PD-L1 expression is increased in a subset of basal type breast cancer cells. PloS one. 2014;9(2):e88557.
40. Mittendorf EA, Philips AV, Meric-Bernstam F, Qiao N, Wu Y, Harrington S, et al. PD-L1 expression in triple-negative breast cancer. Cancer immunology research. 2014;2(4):361-70.
41. Zhang M, Sun H, Zhao S, Wang Y, Pu H, Zhang Q. Expression of PD-L1 and prognosis in breast cancer: a meta-analysis. Oncotarget. 2017;8(19):31347.
42. Segovia-Mendoza M, Morales-Montor J. Immune Tumor Microenvironment in Breast Cancer and the Participation of Estrogen and Its Receptors in Cancer Physiopathology. Front Immunol. 2019;10:348. PubMed PMID: 30881360. Pubmed Central PMCID: PMC6407672. Epub 20190301. eng.
43. Shuai C, Yang X, Pan H, Han W. Estrogen Receptor Downregulates Expression of PD-1/PD-L1 and Infiltration of CD8(+) T Cells by Inhibiting IL-17 Signaling Transduction in Breast Cancer. Front Oncol. 2020;10:582863. PubMed PMID: 33102239. Pubmed Central PMCID: PMC7545792. Epub 20200925. eng.