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Correlation between stromal Th and Tc lymphocytes and PD-L1 expression in early breast cancer tumors

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Abstract

Introduction. Prognostic and predictive value of PD-L1 as a biomarker in breast cancer remains controversial. While some studies suggest its association with negative prognostic parameters, others reported a highly significant association between PD-L1 expression and tumor-infiltrating lymphocytes, which are known to be an independent favorable prognostic factor. The aim of present study is to examine the relationship between immune response markers and PD-L1 expression in early breast cancer.

Material and methods. Immunohistochemical expression of PD-L1, along with density and composition of stromal lymphocytic infiltrate and peritumoral lymphoid aggregates was analyzed in 95 samples of invasive breast cancer.

Results. A strong positive correlation between PD-L1 expression and the density of stromal lymphocytic infiltrate and peritumoral lymphoid aggregates was identified and a cut-off value of 53% coverage of tumor stroma by lymphocytes, with which PD-L1 positivity can be predicted with excellent diagnostic accuracy, was determined for the first time using statistical methods. Additionally, PD-L1 positivity was observed significantly more often in tumors with higher absolute number of both CD4 and CD8 T-lymphocytes in the stromal infiltrate. No significant correlation with molecular subtype of breast cancer was found.

Conclusions. Our results indicate that the density of stromal lymphocytic infiltrate might be a better predictor of PD-L1 positivity in early breast cancer than the molecular subtype and that the key to the optimization of PD-L1 as a biomarker in breast cancer lies in its interpretation in the context of other immune response markers. *(Folia Histochemica et Cytobiologica 2023, Vol. 61, No. 4, 193–204*)

Keywords: PD-L1; early breast cancer; tumor-infiltrating lymphocytes; CD4 lymphocytes; CD8 lymphocyte; peritumoral lymphoid aggregates

Introduction

The importance of effective immune response in prevention of the development and progression of cancer has long been recognized [1] and huge efforts of the scientific community are still directed to the improvement of our understanding of the variety of mechanisms by which tumor cells manage to evade immune surveillance [2]. The immune system functions as a complex network of effector cells, molecules and biochemical processes whose main purpose is to defend the organism from foreign particles, while at the same time limiting autoreactivity and harmful effect of immune cells and the accompanying inflammatory process on the organism itself [3].

The immune system's response to the presence of tumor cells is reflected in the infiltration of the tumor by mononuclear immune cells, mainly different populations of T-lymphocytes, but also follicular dendritic cells, macrophages, B-lymphocytes and plasma cells, all together referred to as tumor-infiltrating lymphocytes (TILs) [4]. It has been believed that cytotoxic (CD8) T-lymphocytes, which eliminate tumor cells, after being activated by CD4 helper T-lymphocytes, either directly through the expression of Fas ligand on their surface or by releasing potent cytotoxins, are

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the key players in antitumor immune response [5]. On the other hand, CD4 lymphocytes have a much more complex role in mediation of the antitumor immune response because, depending on the predominant cytokines secreted in the tumor microenvironment, they can differentiate into different subpopulations of effector cells, displaying various properties [5].

An effective immune response relies directly on the fine balance between co-stimulatory and co-inhibitory signals mediated by members of the B7-1/B7-2-CD28/CTLA-4 protein superfamily [6–9]. One of the members of this superfamily is PD-1 molecule (programmed cell death receptor 1), a transmembrane protein that is expressed as a monomer on the surface of activated CD4 and CD8 T-lymphocytes, B-lymphocytes, NK-cells, monocytes and certain antigen-presenting cells [6, 10]. One of its ligands, PD-L1, is a transmembrane glycoprotein which in addition to its constitutive expression on the surface of inflammatory cells, can also be expressed on the surface of neurons, keratinocytes, syncytiotrophoblast cells, endocrine cells of the pancreatic islets, that is, in those tissues and organs where preservation of immune tolerance and prevention of the development of autoimmunity is of vital importance [11]. Signal transduction *via* PD-1/PD-L1 complex takes place in parallel with the establishment of contact between the T-cell receptor and the antigen presented as part of the major histocompatibility complex (MHC) molecule, with consequent effects on the cell cycle and metabolism of T-cells [2], which become anergic and enter the process of apoptosis [12].

So far, a large number of solid tumors that use this natural protection mechanism against the harmful effects of the immune responses and inflammation to avoid the immune surveillance of the organism, by expressing PD-L1 on the surface of tumor cells or inflammatory cells of the tumor microenvironment, have been identified [11, 13–15]. Therefore, the use of anti-PD-1/PD-L1 antibodies/inhibitors is one of the widely adopted therapeutic modalities in a large number of malignancies nowadays, primarily lung cancer [16–18].

Immune checkpoint inhibitor therapy in breast cancer, which is the most common malignancy and the leading cause of death from malignant diseases in female population worldwide [19], has so far found its place only in the treatment of triple-negative breast cancer (TNBC). A number of monoclonal antibodies to block the PD-1/PD-L1 axis have been approved by the United States Food and Drug Administration in the treatment of locally advanced, unresectable, and metastatic TNBC, as the most immunogenic subtype of breast cancer with potentially the greatest benefits from immunotherapy [20, 21].

On the other hand, the results of various studies suggest that a higher density of stromal tumor-infiltrating lymphocytes (sTILs), as well as the presence of peritumoral lymphoid aggregates [22], are indicators of a favorable response to chemotherapy and a generally better prognosis, not only in triple-negative (TNBC), but also in non-luminal HER-2 positive breast cancers [23, 24]. The latter two subtypes of breast cancer have been identified as tumors that show high expression of PD-L1 protein more often compared to luminal molecular subtypes [25].

However, a review of the literature reveals a large number of studies with conflicting results regarding the prognostic and predictive value of PD-L1 as a biomarker in breast cancer [26]. While some studies suggest the association between PD-L1 expression and negative prognostic parameters (such as tumor size, higher grade and proliferative activity, absence of steroid receptor expression) [25, 27–30] in some studies a highly significant association between PD-L1 expression and a high density of TILs (as an independent favorable prognostic factor) was shown [23, 24, 31, 32]. Finally, the predictive value of PD-L1 expression in the selection of candidates for immune checkpoint inhibitor therapy, even within the TNBC subtype, has been questioned by some recent studies [26, 33].

Therefore, numerous authors share the opinion that it is necessary to optimize the use of PD-L1 as a biomarker in breast cancer [22, 26, 34] and that the analysis of its expression should be placed in the context of the density and composition of sTILs [22, 26, 34, 35]. Despite the complexity of anti-tumor immune responses, the presence of stromal TILs is considered to be a robust prognostic and predictive biomarker in breast cancer [36] and guidelines for its routine scoring have been provided by the International TILs Working group [37]. According to these, sTILs should be scored as a continuous variable (the mean percentage of tumor stroma covered by mononuclear cells), although clinically significant cut-off value of sTILs density still remains unknown [37].

In addition, as the focus of researchers has so far been almost exclusively placed on locally advanced and metastatic TNBC, data on PD-L1 expression and characteristics of intratumoral inflammatory infiltrate in other molecular subtypes, especially in early breast cancer, are still insufficient in the literature [25].

Therefore, the aim of this study is to examine the relationship between the density and composition of stromal TILs and the characteristics of peritumoral lymphoid aggregates and PD-L1 expression in dif-

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ferent molecular subtypes of early breast cancer and determine the threshold value percentage of sTILs at which PD-L1 positivity can be predicted in early breast cancer.

Material and methods

Patients. The study included all female patients who were diagnosed with stage IA-IIIA invasive breast cancer (according to the eighth edition of the TNM classification [38]) on surgically obtained material at the Center for Pathology of the Clinical Center of Montenegro, Montenegro, between 2016 and 2020 and which did not previously receive neoadjuvant therapy. The study was conducted according to the ethical principles governing medical research and human subjects as laid down in the Helsinki Declaration and approved by the Ethical Committee of the Medical Faculty of the University of Montenegro (approval number 1492/2, dated 23.09.2022).

Based on the existing pathomorphological reports with data on the degree of immunohistochemical (IHC) expression of steroid hormone receptors (ER — estrogen and PR — progesterone receptors), human epidermal growth factor receptor 2 (HER-2) status and Ki67 proliferation index value, according to St. Gallen consensus [39], the samples were classified into five groups with the following molecular subtypes: Luminal A, Luminal B HER-2 positive, Luminal B HER-2 negative, non-luminal HER-2 positive and TNBC.

ER and PR were considered positive if the Allred score was ≥ 3, while the presence of HER-2 overexpression was assessed according to current ASCO/CAP (American Society of Clinical Oncology/College of American Pathologists) guidelines [40] on immunohistochemically stained sections and sections stained by DDISH (Dual-color dual-hapten *in situ* hybridization) method in the case of an equivocal result of the previously conducted IHC analysis.

Finally, 95 samples (19 from each of the five molecular subtypes) which contained enough material in the available archived paraffin blocks for the planned further immunohistochemical analysis were selected, whereby we standardized the sample in relation to age, so that the average age of the subjects was very similar in each of the five investigated groups. The mean age of study participants was 59.68 ± 11.71 (mean ± SD) years.

Analysis of HE stained histological sections from original surgical specimens. By examining archived microscopic slides, stained with the standard hematoxylin-eosin (HE) method, the histological type and grade of the tumor was confirmed. The density of sTILs was scored as a continuous variable according to the International TIL Working group recommendations, that is: all mononuclear immune cells were taken into account and polymorphonuclear granulocytes were excluded based on morphology [37].

In addition, the density of sTILs was also recorded semiquantitatively, using the methodology suggested by Cimino-Mathews *et al*., as mild (< 5% of the tumor stroma), moderate (focal infiltrate in 5–50% of the tumor stroma) and diffuse (diffuse infiltrate in ≥ 50% of the tumor stroma) [22] (Fig. 1).

Finally, presence of peritumoral lymphoid aggregates (PLA) was recorded and scored using the same methodology [22] as absent, focal (rare isolated clusters of lymphocytes), moderate (multiple lymphoid aggregates) and highly developed (multiple lymphoid aggregates with well-developed germinal centers) (Fig. 2).

Tissue microarray (TMA) preparation, immunohistochemical staining and analysis. In selected samples, two TMAs measuring 3 mm were formed from the archived paraffin blocks for each patient using the Quick Ray Manual Tissue Microarrayer set (Untima Co., Ltd., Gyeonggi-do, South Korea). The obtained preparations were first stained with the standard HE technique, in order to confirm the adequacy of the sample and facilitate orientation during the interpretation of immunohistochemically stained sections.

For the purpose of antigen retrieval, TMAs were treated in 10 mM citrate buffer, pH 6.0, in microwave oven for 10 min, and then washed out with deionized water. Endogenous peroxidase was blocked using a 3% hydrogen peroxide solution at room temperature for 10 min. The sections were than incubated with

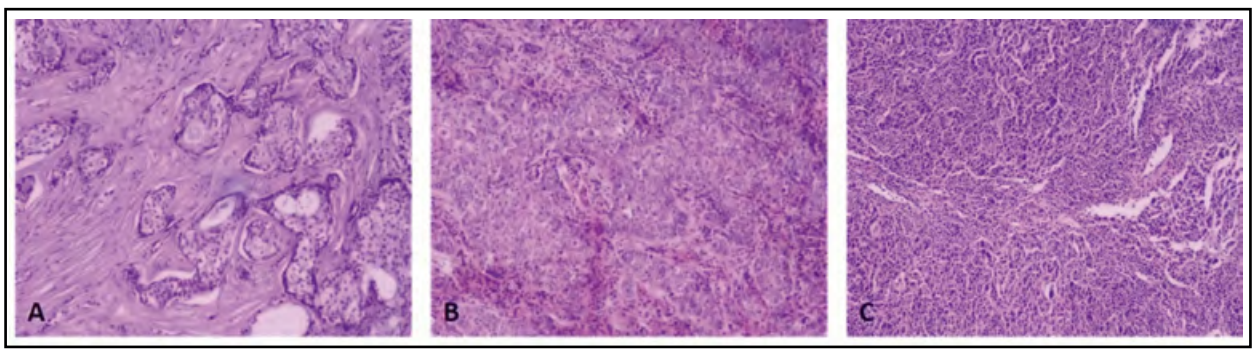

Figure 1. Semiquantitative stromal tumor-infiltrating lymphocytes (sTILs) density scoring was applied to the early breast cancer tumors: **A.** Mild stromal infiltrate (a small number of mononuclear cells is seen in an area that comprises < 5% of the tumor stroma). **B.** Moderate (focal) stromal infiltrate (clusters of mononuclear cells are covering around 15% of the tumor stroma). **C**. Diffuse stromal infiltrate is completely covering the tumor stroma. Hematoxylin-eosin (HE) staining, magnification: 100×. The sTILs scoring was determined as described in Methods.

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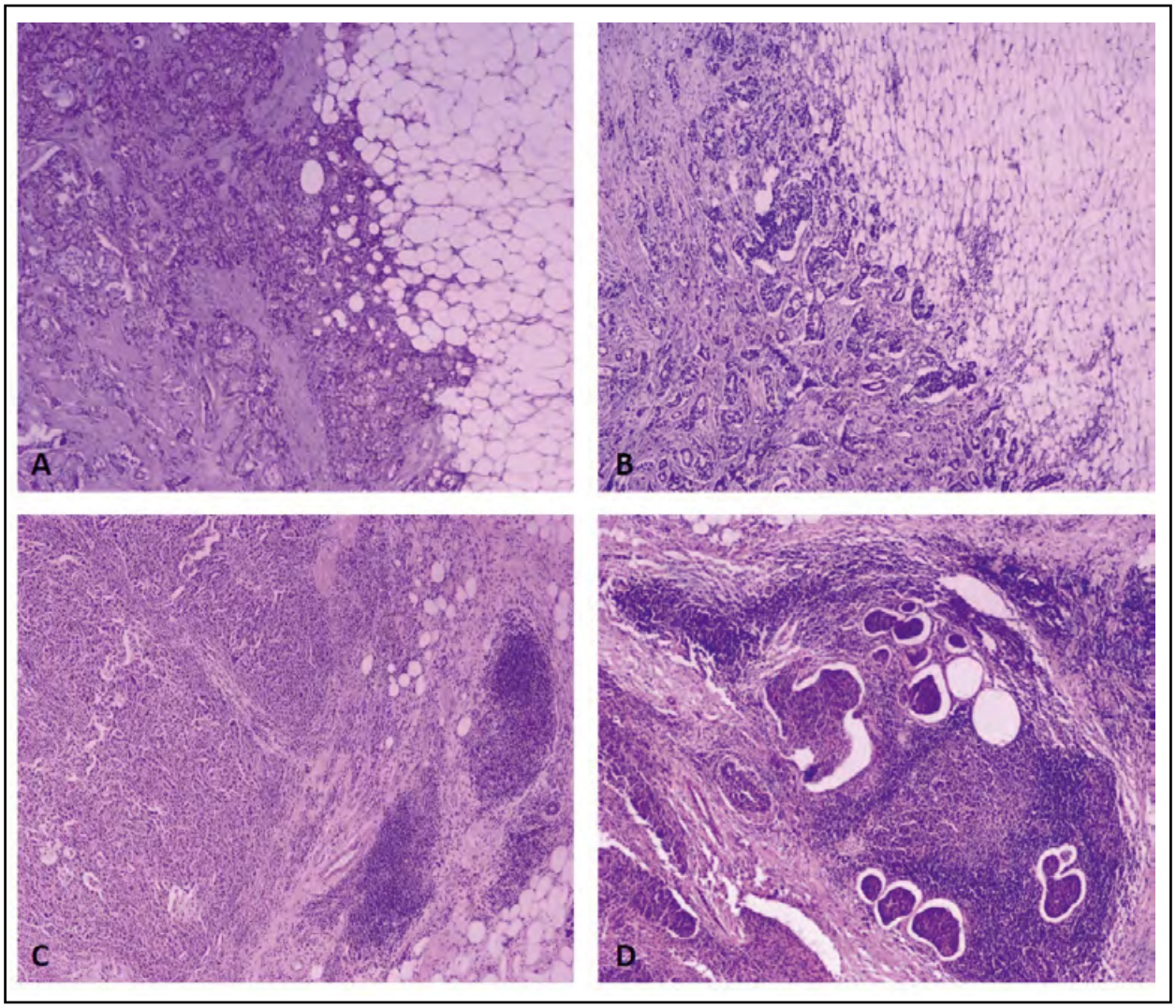

Figure 2. Peritumoral lymphoid aggregates (PLAs) scoring/classification in early breast cancer tumors: **A.** PLAs absent. **B.** Focal PLAs presence (rare isolated clusters of lymphocytes are seen at the invasive front of the tumor). **C.** Moderate PLAs distribution (multiple lymphoid aggregates are present at the tumor border). **D.** Highly developed PLAs (multiple lymphoid aggregates with well-developed germinal centers at the invasive front of the tumor). HE staining, magnification: 40×.

the primary antibody in a moist chamber, at room temperature, for 1 h. The streptavidin-biotin-peroxidase technique was used for antigen visualization according to the standard LSAB+ procedure (DAKO, Carpinteria, CA, USA and Glostrup, Denmark). After each incubation, sections were washed out with Tris-buffered saline solution (TBS, 0.05 M, pH 7.6). The following primary antibodies were used: CD4 (Monoclonal Mouse Anti-Human CD4, Clone 4B12 FLEX Ready to use DAKO, 1:100) and CD8 (Monoclonal Mouse Anti-Human CD8, T-cell, Clone C8/144B FLEX Ready to use DAKO, 1:100).

IHC staining for PD-L1 was performed using PD-L1 (Monoclonal Rabbit Anti-Human PD-L1, Clone SP142, Roche/Ventana Medical Systems, Tucson, AZ, USA) antibody on the Ventana Benchmark GX (Ventana Medical Systems, Tucson, AZ, USA) automated platform, according to the manufacturer's instructions.

Palatine tonsil was used as an external positive control for immunohistochemical staining.

Zeiss Axiolab 5 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with the field diameter of 0,65 mm, was used for morphological analysis, where one high power field (HPF, 400×), corresponded to field area of 0.332 mm^2.

For both CD4 and CD8 markers, strong membrane positivity was interpreted as positive result. Quantification of CD4 and CD8 positive sTILs was performed manually by counting positive cells on 9 HPFs what corresponded to 3 mm^2 *per* TMA (that is 18 HPFs, corresponding to 6 mm^2 *per* patient) and the obtained values were expressed as the average number of cells/mm^2/patient. The values were used to calculate the CD4/CD8 ratio.

While assessing PD-L1 expression in immune cells, all the cells showing partial/complete membrane/cytoplasmic staining

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of any intensity were taken into account and the immune cell score (ICS) algorithm was used for scoring [35]. The final result was the mean ICS value, obtained by evaluating both TMAs of each individual patient's tumor. According to the currently valid protocol for evaluating PD-L1 expression in TNBC, an ICS ≥ 1% was considered a positive finding [35].

Statistical analysis. The data were analyzed using IBM SPSS Statistics version 23.0 software (IBM SPSS for Windows, Armonk, NY, USA). Statistical hypotheses were tested using non-parametric statistical tests (Fisher's exact test and Mann-Whitney U test), and the correlation between parameters was described with the help of Phi correlation coefficient. Statistical "ROC" curves and the formula for calculating Youden's index (J = sensitivity + specificity – 1) were used to display the cut-off value of sTILs percentage that predicts PD-L1 positivity. For all the statistical analyses, the level of significance was 0.05.

Results

Qualitative study

All of the three analyzed markers (CD4, CD8 and PD-L1) demonstrated their expected immunohistochemical staining patterns.

As seen in Fig. 3, strong CD4 membranous staining was seen in T-lymphocytes of the interfollicular area of the control tonsillar tissue, along with moderate positivity of germinal center macrophages and absent reaction in squamous epithelium. In breast cancer strong CD4 immunoreactivity (Ir) was seen in stromal T-lymphocytes, with a few positive intratumoral T-lymphocytes located between cancer cells, which were omitted from further analysis.

The CD8 immunohistochemical staining of the tonsillar tissue showed strong membranous reaction, primarily in T-lymphocytes of the interfollicular area, with a few positive intraepithelial T-lymphocytes and absent reaction in squamous epithelium of the tonsil. In breast cancer, strong positivity in stromal T-lymphocytes, with a few positive intratumoral T-lymphocytes (which were not considered in further analysis) was noted (Fig. 4).

Staining pattern and interpretation of PD-L1 immunoreactivity are shown in Fig. 5. In control tissue (tonsil), moderate to strong cytoplasmic/membranous staining was noted in lymphocytes and macrophages in germinal centers, while superficial squamous epithelium remained negative. In PD-L1 positive breast tumors, immunoreactivity was usually seen in clusters of immune cells within the tumor stroma (ICS ≥ 1%), whereas PD-L1 negative tumors showed either no IHC reaction, or positive reaction that was noted only in a few individual cells within the tumor stroma (ICS < 1%).

Quantitative analysis

Out of 95 early breast cancer samples included in the study, positive PD-L1 expression was found in 20 cases (21.5%). Statistical analysis found no significant association between PD-L1 expression and tumor size (U = 717.00; P = 0.76), pT (Fisher exact test = 1.92; P = 0.37), or the pN stage of the disease (Fisher exact test = 0.52; P = 0.93), as well as the molecular subtype of the tumor (Fisher's exact test = 8.25; P = 0.07).
On the other hand, we found a significant connection between PD-L1 expression and the histological grade of the tumor (Fisher exact test = 12.81; P = 0.003),

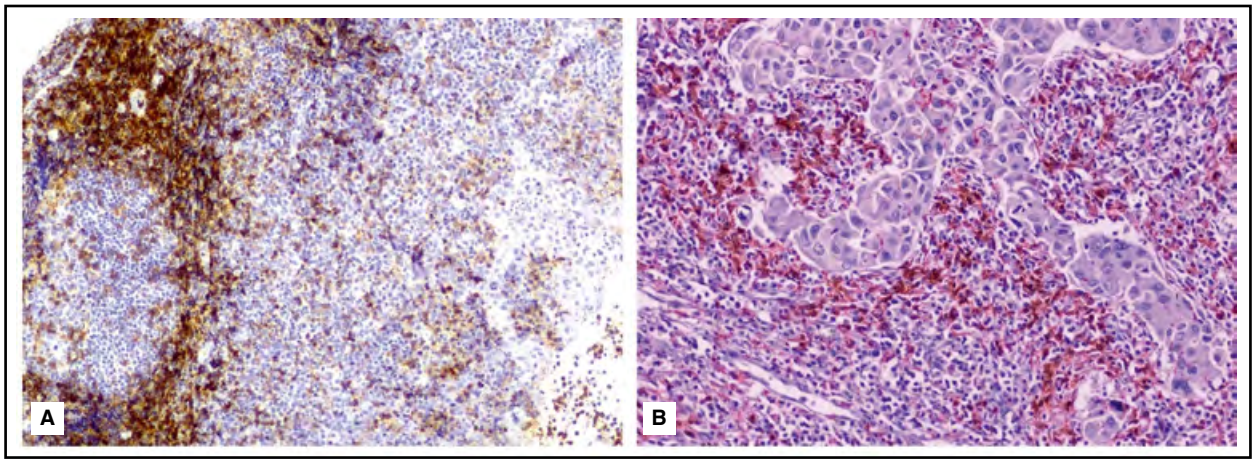

Figure 3. Immunohistochemical staining of CD4+ lymphocytes in early breast cancer tumors. **A.** Control tissue (palatine tonsil) — strong membranous staining is seen in T-lymphocytes of the interfollicular area, along with moderate positivity in the germinal center macrophages and absent reaction in squamous epithelium. IHC, magnification: 40×; **B.** Tumor-infiltrating lymphocytes in breast cancer tissue — strong immunoreactivity is present in stromal T-lymphocytes, with a few positive intratumoral T-lymphocytes located between cancer cells. Immunohistochemistry (IHC), magnification: 100×. Tissue microarrays of 95 breast cancer patients were processed for IHC as described in Methods.

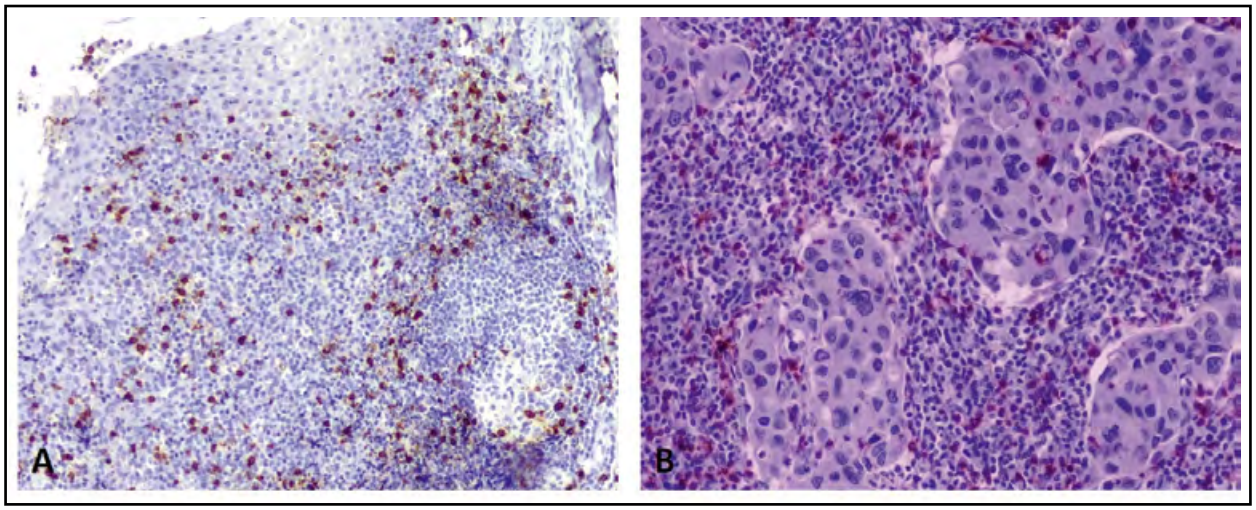

Figure 4. Immunohistochemical staining of CD8+ lymphocytes in early breast cancer tumors. **A.** Control tissue (palatine tonsil) — strong membranous reaction is seen primarily in T-lymphocytes of the interfollicular area, with a few positive intraepithelial T-lymphocytes and absent reaction in squamous epithelium. Immunohistochemistry (IHC), magnification: 40×. **B.** Breast cancer tumor-infiltrating lymphocytes: strong immunoreactivity is seen in stromal T-lymphocytes, with a few positive intratumoral T-lymphocytes. IHC, magnification: 100×. Tissue microarrays of 95 breast cancer patients were processed for IHC as described in Methods.

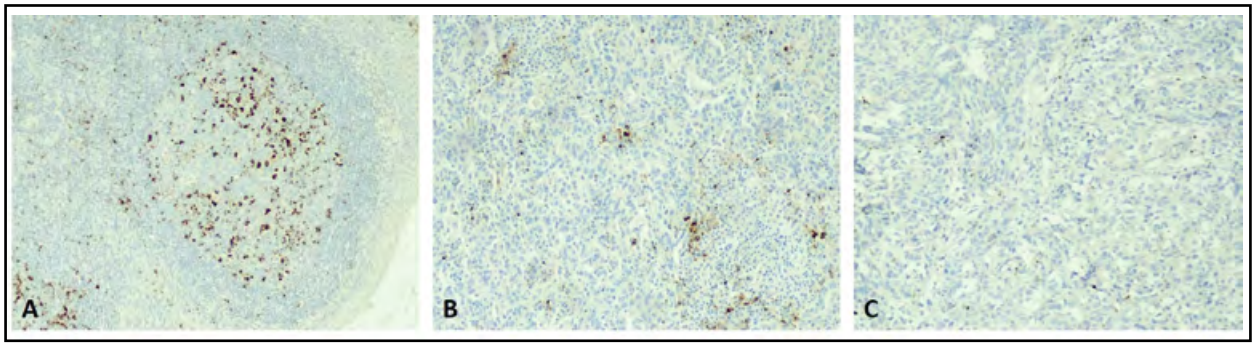

Figure 5. Staining pattern and interpretation of PD-L1 immunohistochemical stainings. A. Control tissue (palatine tonsil) - moderate to strong cytoplasmic/membranous staining is seen in lymphocytes and macrophages in germinal centers, while superficial squamous epithelium remains negative. IHC, Magnification: 40×. B. Breast cancer tumor with positive PD-L1 expression. Immunoreactivity is seen in clusters of immune cells within the tumor stroma (ICS ≥ 1%). IHC, magnification: 100×; C) Breast cancer tumor with negative PD-L1 expression - positive reaction is noted only in a few individual cells within the tumor stroma (ICS < 1%). IHC, magnification: 100×. Tissue microarrays of 95 breast cancer patients were processed for immunohistochemistry and ICS was determined as described in Methods. Abbreviations: ICS — immune cell score; PD-L1 — Programmed cell death receptor ligand 1.

with a moderate positive correlation between these two parameters (Phi = 0.38; P = 0.003). The Z test showed that positive PD-L1 expression occurs statistically significantly more often in patients diagnosed with grade G3 breast cancer, compared to patients diagnosed with grade G1 and G2.

Also, a statistically significantly higher value of the Ki67 proliferative index was determined in patients with positive PD-L1 expression (48.50 ± 21.527; mean ± SD), compared to those without the expression of this marker (33.07 ± 23.980; mean ± SD), (U = 437.00; P = 0.006).

The analysis of parameters related to the antitumor immune response, a strong positive correlation between PD-L1 expression and type of sTIL was found (Phi = 0,88; P < 0,001), and the Z test showed that positive PD-L1 expression was statistically significantly more frequent in tumors with diffuse, compared to moderate and mild types of stromal TILs, regardless of the molecular subtype.

Furthermore, it was determined that the absolute number of both CD4– (U = 61.50; P < 0.001) and CD8– (U = 90.50; P < 0.001) lymphocytes *per* mm^2 in the tumor stroma was statistically significantly higher in tumors with positive PD-L1 expression (Fig. 6), although no significant predominance of a certain lymphocyte subpopulation was found, that is CD4/CD8 ratio did not differ from the tumors in which PD-L1 expression was absent or present (Fisher exact test = 0.851; P = 0.42).

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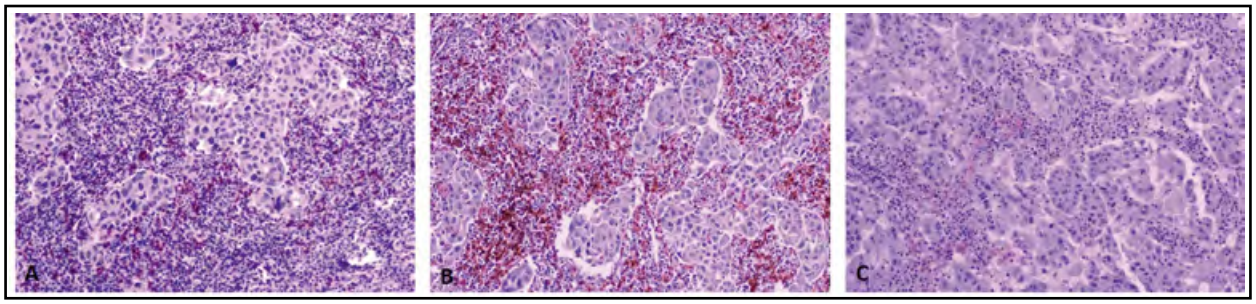

Figure 6. Diffuse stromal mononuclear immune cell infiltrates, with high absolute numbers of (**A**) CD8 lymphocytes, and (**B**) CD4 lymphocytes, that were detected in (**C**) PD-L1 positive breast cancer tumor. IHC, magnification: 100×.

In addition to the type of sTIL, statistically significant difference was found between tumors with positive and negative PD-L1 expression in relation to the characteristics of peritumoral lymphoid aggregates (Fisher's exact test = 12.87; P = 0.003). The correlation coefficient revealed a moderate positive correlation between PD-L1 expression and the type of peritumoral lymphoid aggregate (Phi = 0.37; P = 0.003), and the Z test showed that moderate peritumoral lymphoid aggregates were noted statistically significantly more often compared to focal and absent lymphoid aggregates in tumors with positive PD-L1 expression. No difference was found between the presence of highly-developed lymphoid aggregates in relation to other types of peritumoral lymphoid aggregates in patients with positive PD-L1 expression in breast cancer tumors. However, these observations were not related to tumor molecular subtype.

The results of the association between PD-L1 expression and clinico-pathological features and anti-tumor immune response markers are summarized in Table 1 for discrete variables and Table 2 for continuous variables.

In further analysis, Mann-Whitney test showed a statistically significant difference between the mean value of sTIL density, expressed as the percentage of

Table 1. PD-L1 expression in relation to clinico-pathological characteristics (histological grade, molecular subtype, pT and pN stage of the disease) and anti-tumor immune response markers (density of sTILs and peritumoral lymphoid aggregates and CD4/CD8 ratio) in 95 examined samples of breast cancer (discrete variables)

		PD-L1		Total	P
		Negative	Positive		
Histological grade	G1 (well differentiated) G2 (moderately differentiated) G3 (poorly differentiated)	4 (4.2%) 49 (51.5%) 22 (23.2%)	0 (0%) 5 (5.3%) 15 (15.8%)	4 (4.2%) 54 (56.8%) 37 (39.0%)	0.003
Molecular subtype	Non-luminal HER2+ TNBC Luminal B HER2+ Luminal B HER2- Luminal A	14 (14.7%) 11 (11.6%) 16 (16.8%) 16 (16.8%) 18 (18.9%)	5 (5.3%) 8 (8.4%) 3 (3.2%) 3 (3.2%) 1 (1.1%)	19 (20.0%) 19 (20.0%) 19 (20.0%) 19 (20.0%) 19 (20.0%)	0.07
pT stadium	T1 T2 T3	35 (36.8%) 37 (38.9%) 3 (3.2%)	7 (7.4%) 11 (11.6%) 2 (2.1%)	42 (44.2%) 48 (50.5%) 5 (5.3%)	0.37
pN stadium	N0 N1 N2	52 (54.7%) 11 (11.6%) 12 (12.6%)	13 (13.7%) 4 (4.2%) 3 (3.2%)	65 (68.4%) 15 (15.8%) 15 (15.8%)	0.93
Density of sTILs	Mild Moderate Diffuse	22 (23.2%) 50 (52.6%) 3 (3.1%)	0 (0%) 1 (1.1%) 19 (20.0%)	22 (23.2%) 51 (53.7%) 22 (23.1%)	< 0.001
Peritumoral lymphoid aggregates	Absent Focal Moderate Well developed	7 (7.4%) 27 (28.4%) 37 (38.9%) 4 (4.2%)	0 (0%) 1 (1.1%) 15 (15.8%) 4 (4.2%)	7 (7.4%) 28 (29.5%) 52 (54.7%) 8 (8.4%)	0.003
CD4/CD8 ratio	CD4/CD8 < 1 CD4/CD8 > 1	22 (23.2%) 53 (55.8%)	8 (8.4%) 12 (12.6%)	30 (31.6%) 65 (68.4%)	0.42

Abbreviations: PD-L1 — Programmed death receptor ligand 1; sTils — stromal tumor-infiltrating lymphocytes.

Table 2. PD-L1 expression in relation to the density of sTILs expressed as a continuous variable, area density of CD4 and CD8 lymphocytes and Ki67

	PD-L1	Mean value ($\bar{x} \pm SD$)	P
sTILs (%)	Negative Positive	15.31 ± 14.32 68.75 ± 16.85*	< 0.001
CD4 (*per* mm²)	Negative Positive	31.65 ± 30.18 175.25 ± 87.62*	< 0.001
CD8 (*per* mm²)	Negative Positive	28.16 ± 33.79 150.60 ± 78.19*	< 0.001
Ki67 (%)	Negative Positive	33.07 ± 23.980 48.50 ± 21.527*	0.006

Abbreviations as in the legend to Table 1.

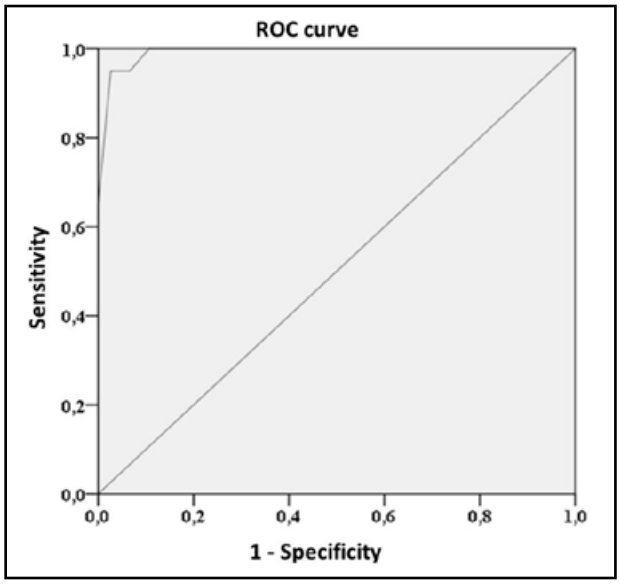

Figure 7. ROC curve for predicting PD-L1 positivity based on the density of stromal tumor-infiltrating lymphocytes. The curve was constructed by plotting the true positive rate (the proportion of observations that were correctly predicted to be positive) against the false positive rate (the proportion of observations that were incorrectly predicted to be positive out of all negative observations). The area under the ROC curve for assessing the positivity of PD-L1 expression based on the density of stromal tumor-infiltrating lymphocytes speaks in favor of excellent diagnostic accuracy (AUC = 0.992; P < 0.001). Abbreviations: AUC — area under the curve; ROC — receiver operating characteristic.

tumor stroma covered by lymphocytes, between breast cancers with positive and negative PD-L1 expression (U = 12,50; P < 0.001). The area under the ROC curve for assessing the positivity of PD-L1 expression based on the density of sTILs speaks in favor of excellent diagnostic accuracy (AUC = 0,992; P < 0.001), and based on the highest value of Youden's index (Youden's index = 0.923), a cut-off value of 53% was established (Fig. 7).

Discussion

To date, a large number of studies with conflicting results in terms of clinical, prognostic and predictive significance of the presence of PD-L1 expression in breast cancer has been published [25, 26, 28, 31–34, 41, 42]. On the other hand, we are witnessing that the development and clinical application of targeted therapy in the form of PD-1/PD-L1 inhibitors is progressing at a much faster pace compared to basic mechanistic studies and the understanding of all aspects of PD-1/PD-L1-mediated signaling [43]. Therefore, the need to take a few steps back in order to optimize the routine use of PD-L1 as a biomarker in breast cancer has increasingly been appreciated.

However, one must take into account the problematic comparability of the results published so far, which is primarily due to the application of different methodologies for the evaluation of PD-L1 expression. While some authors used molecular techniques for these purposes, most used immunohistochemistry, while applying a wide range of different antibodies and different scoring systems (including the assessment of PD-L1 expression in different cell populations), as well as very different threshold values at which the expression is of this marker was considered positive [34].

In our study, apart from the strict criteria for the inclusion of patients and tumor samples (treatment naive, surgically obtained specimens of early breast cancer, age standardized groups), PD-L1 expression was determined using Ventana PD-L1 (SP142) immunohistochemical assay and interpreted according to the official accompanying guide (designed and approved as companion diagnostic test to evaluate the clinical benefit from the use of atezolizumab in locally advanced and metastatic TNBC) [44], all of which we consider to be advantages in terms of obtaining relevant results.

Analyzing PD-L1 expression in immune cells of the tumor microenvironment of different molecular subtypes of early breast cancer in our study, we determined positive PD-L1 expression in 21.5% of cases, which is comparable to the results published by Muenst *et al.* who reported PD-L1 expression in 23% of 650 breast cancer samples [28] and Sabatier *et al.* who found PD-L1 upregulation in 20% of 5,454 samples of breast cancer [29].

Higher frequency of PD-L1 positivity was reported by Ghebeh *et al*. [27] who found PD-L1 expression in 50% of 44 analyzed breast cancers samples, while Mittendorf *et al*. reported a lower percentage of PD-L1 positive tumors (19% positive cases in their sample of 105 TNBC cases, compared to 42.1% positive TNBC tumors in our study). Although these

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authors also used immunohistochemistry, the variations in frequency of positive PD-L1 expression may be attributed to the differences in the experimental design, as they used a different primary antibody and scoring system, along with a very different cut-off values for PD-L1 positivity.

The highest percentage of PD-L1 positive tumors was reported by mRNA expression studies. For example, Schalper *et al.* reported PD-L1 expression in 58% of breast cancer specimens [45]. However, higher frequency of PD-L1 expression in these studies might be explained by the fact that mRNA expression may not always correlate with actual protein expression [28].

Similar to the results published by other authors [25, 29], we found a statistically significant association between PD-L1 expression and poor prognostic factors, such as histological grade and tumor mitotic index, but not tumor size and the stage of disease, which could be explained by the fact that present study was performed in early breast cancer cases.

Also, we observed a statistically significant association between PD-L1 expression and the density of sTILs, which is in accordance with previously published results [22, 25, 31]. To our best knowledge, we could present for the first time, the cut-off value of the percentage of sTIL (53%) at which PD-L1 positivity can be predicted with excellent accuracy in early breast cancer tumors.

Although current literature lacks sufficient data on the dynamic changes in the distribution and quantity of different subpopulations of T cells during breast cancer progression, it has been suggested that CD4 and CD8 T-lymphocytes might have opposing roles in breast cancer progression and outcome [46]. While most authors agree that CD8 lymphocytes represent a key cell population that controls the effectiveness of the antitumor immune responses in general [5, 45, 47], the role of CD4 lymphocytes, and their subtypes, in the antitumor response and tumor progression is much more complex and dynamic [5]. For example, Huang *et al.* showed that in early lesions of breast cancer, Th1 cells represented the dominant subpopulation of CD4 lymphocytes in the stromal infiltrate and that the increase in the number of CD4 cells, with the predominance of FoxP3 regulatory and Th17 subpopulations of T-lymphocytes, was associated with the progression of the disease [46]. The same authors demonstrated a positive correlation between the number of CD4 T-lymphocytes in the stromal infiltrate and the advanced stage of the disease.

Similar to the results of previously conducted research, our study showed higher number of CD8 T-lymphocytes in the stromal infiltrate of PD-L1 positive compared to PD-L1 negative tumors [29, 48], although in our study the same observation was made for CD4 lymphocytes. However, we did not find a statistically significant predominance of one subpopulation of T-lymphocytes over the other (CD4 *vs.* CD8 cells) in PD-L1 positive *vs.* PD-L1 negative tumors. This could be a consequence of selecting patients with early breast cancer as a studied cohort, since it was demonstrated that with disease progression CD4 T-lymphocytes infiltrate the tumor more rapidly compared to CD8 T-lymphocytes, which makes them a predominant lymphocyte population in late breast cancer [46].

Considering the dynamic changes in the composition of the intratumoral lymphocytic infiltrate (predominantly within the population of CD4 T-lymphocytes) during the development of malignant breast tumors, in future research it would be interesting to analyze the expression of certain subpopulations of CD4 lymphocytes, especially FoxP3-positive regulatory T-lymphocytes, whose presence is considered the main obstacle in achieving effective antitumor immunity and good response to immunotherapy [46].

In addition to sTILs, we also analyzed the association between PD-L1 expression and presence and characteristics of peritumoral lymphoid aggregates and found that PD-L1 positivity occurs significantly more often in tumors with a higher density of peritumoral infiltrates. This has been previously reported by Cimino-Mathews *et al.*, who identified presence of peritumoral lymphoid aggregates in 59% of PD-L1 positive breast cancer cases [22].

Peritumoral lymphoid aggregates, also termed tertiary lymphoid structures (TLS), have emerged as another biomarker of active antitumor immune response [22]. Apart from T-lymphocytes, these structures are known to contain a significant number of B-lymphocytes [22], whose role in this process has been intensively investigated recently. Generally, presence of TLS at the border between the tumor and the surrounding tissue was found to be associated with a good prognosis and favorable response to immune checkpoint inhibitor therapy, even in tumors with a low mutational burden [49]. When it comes to studies on breast cancer, the presence of TLS was found to be associated with a favorable prognosis [22, 50–52]. Although the available literature lacks studies that analyzed the presence of B-lymphocytes in peritumoral lymphoid aggregates in the context of PD-L1 expression, we believe that this should become subject of future studies.

Contrary to the results published so far, in which the presence of PD-L1 expression has been dominantly associated with TNBC [25, 26, 31, 53, 54] in our study this association did not reach statistical significance

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(P = 0.07), although the highest percentage of PD-L1 positive tumors belonged to this molecular subtype of breast cancer. The discrepancy between our result and the results of these studies is probably due to the relatively small size of our sample. However, since it has been demonstrated that tumor immunogenicity in breast cancer decreases with progression of the disease [55], this difference might also be due to the fact that present study was performed in early stages of breast cancer, where increased sTILs may occur more often across other (non-TNBC) molecular subtypes [56].

In conclusion, our results indicate that the density of sTILs might be a better predictor of PD-L1 positivity in early breast cancer than the molecular subtype itself. Thus, when assessing a patient's suitability for PD-L1 inhibitor therapy and consequently setting the indication for the evaluation of PD-L1 status in early breast cancer, we believe that all patients whose tumors show a density of sTIL above 53% should be taken into account, regardless of HER2 expression and hormone receptor status.

The higher density of sTILs and peritumoral lymphoid aggregates, as well as the absolute number of CD4 and CD8 lymphocytes in the stromal mononuclear cells' infiltrates in PD-L1 positive tumors suggest that the interpretation of PD-L1 expression should be done exclusively in the context of the density and composition of the intra- and peritumoral lymphoid infiltrates. In order to optimize the use of PD-L1 as a biomarker in breast cancer, studies on larger samples are necessary to improve our understanding of the dominant subpopulations of T and B lymphocytes in PD-L1 positive cancers and define their associations with the course of the disease and patient survival.

Article information and declarations

Data availability statement

The data that support the findings of this study are not openly available due to sensitivity and patient privacy protection, but are available from the corresponding author upon reasonable request. Data are located in controlled access data storage at the Clinical center of Montenegro.

Ethics statement

The study was conducted according to the ethical principles governing medical research and human subjects as laid down in the Helsinki Declaration and approved by the Ethical Committee of the Medical faculty of the University of Montenegro.

Author contributions

All authors contributed to the study design and write up of the manuscript. JV collected the sample, performed the microscopic evaluation and statistical analysis of obtained data.

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Conflict of interest

All authors declare that they have no conflicts of interest.

Supplementary material

All the materials, including tables and figures, will be provided within the manuscript, as a single document.

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