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ORIGINAL PAPER

Importance of CD45RO+ tumor-infiltrating lymphocytes in post-operative survival of breast cancer patients

Simin Ahmadvand[1,2] · Zahra Faghih[2] · Mehdi Montazer[3] · Akbar Safaei[3] · Maral Mokhtari[3] · Peyman Jafari[4] · Abdol-Rasoul Talei[5] · Sedigheh Tahmasebi[5] · Abbas Ghaderi[1,2]

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Abstract

Purpose In recent years, the prognostic/predictive significance of tumor infiltrating lymphocytes (TILs) has become a topic of interest. Here, we aimed to evaluate the prognostic significance of CD3+, CD8+, CD45RO+ and Foxp3+ TILs in breast cancer, as well as the relation of these markers to other clinicopathological features of this disease.

Methods FFPE tumor samples from 94 females with invasive ductal carcinoma of the breast were retrospectively selected and immunohistochemically assessed for CD3, CD8, CD45RO and Foxp3 expression. Digital photos were acquired from the center (CT) and invasive margins (IM) of the tumors, after which positive cells were counted using ImageJ software.

Results We found that greater infiltrations of target lymphocyte subpopulations were associated with TNM stage III, lymph node metastasis, high histological grade, ER negativity and HER2 positivity. The ratios of CD8+ cytotoxic T cells to CD3+, CD45RO+ and Foxp3+ TILs were found to be relatively higher in tumors exhibiting the aforementioned characteristics. In univariate survival analyses, CD8+ TILs in the IM and total CD45RO+ TILs were found to be significantly associated with overall survival (OS). Infiltration of CD45RO+ TILs in the CT and lymph node status were variables that significantly correlated with disease-free survival (DFS). Multiple Cox regression analyses revealed independent significant prognostic effects of total CD45RO+ TILs and lymph node status (HR of 3.24 and 3.19, respectively) in predicting OS. Infiltration of CD45RO+ TILs in the CT (HR 3.12) and lymph node status (HR 3.15) also exhibited significant prognostic effects on DFS.

Conclusion From our data we conclude that CD45RO+ TILs serve as prognostic factors for predicting OS and DFS of breast cancer patients.

Keywords Breast cancer · Tumor-infiltrating lymphocytes · CD45RO+ memory T cell · CD8+ cytotoxic T cell · Prognostic marker

Simin Ahmadvand and Zahra Faghih contributed equally to this study.

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✉ Abbas Ghaderi
ghaderia@sums.ac.ir

[1] Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

[2] Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

[3] Department of Pathology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

[4] Department of Biostatistics, Shiraz University of Medical Sciences, Shiraz, Iran

[5] Breast Disease Research Center, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

1 Introduction

Breast cancer (BC) is the most common diagnosed cancer and second leading cause of cancer related deaths in women [1]. It is a heterogeneous disease comprised of several histological and molecular subtypes with different prognoses and clinical outcomes [2]. While BC histological subtypes are determined through morphological evaluation of hematoxylin and eosin (H&E)-stained tumor sections, its intrinsic molecular subtypes can be determined through expression patterns of surrogate markers that include hormone receptors (HR) such as the estrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor-2 (HER2), the proliferation marker Ki67, and histological grade [3]. BCs are stratified according to ER expression status as luminal (ER+) and non-luminal (ER-) tumors. Luminal tumors

are further subdivided into luminal A (HR+/HER2-/Ki67-) and luminal B (HR+/HER2-/Ki67+ and HR+/HER2+/Ki67-) subtypes. The HER2-enriched (HR-/HER2+) and basal-like (HR-/HER2−/basal marker+) subtypes belong to the non-luminal BC tumors [4, 5].

By uncovering key roles of the tumor microenvironment (TME) in controlling tumor behavior [6, 7], the prognostic significance of tumor infiltrating lymphocytes (TILs) as important components of the TME, and their possible predictive value have become topics of interest in cancer research. The significance of TILs has been demonstrated in the prognosis of a range of cancers, including head and neck [8], lung [9], renal [10], ovarian [11] and pancreatic cancers [12]. The most comprehensive studies on the prognostic value of TILs have been conducted on colorectal cancers (CRCs) [13–17]. Several studies have revealed a superiority of TILs over TNM-stage in predicting disease-free survival (DFS) and overall survival (OS) of CRC patients. In this context, studies by Galon et al. on lymphocytic infiltrates have led to the establishment of Immunoscore [14], an immune-based classification system for patients with CRC. According to this system, patients are stratified into 5 different groups, ranging from Immunoscore 0 (I0) to Immunoscore 4 (I4), based on the numeration of two lymphocytic populations (CD3+ and CD8+) in the center (CT) and invasive margin (IM) of the tumor samples. According to this classification, patients with high scores exhibit improved survival rates [18].

The prognostic significance of TILs in BC has been evaluated by many studies [19], but most of these studies have been focused on whole lymphocytic infiltrates and to a lesser extent on specific TIL subpopulations [20]. Some studies have reported positive relationships between the presence of TILs and prognosis [21, 22], whereas others have reported neutral [23, 24] or negative [25, 26] relationships. Here, we have taken into consideration the results of Galon et al. in CRC and investigated the prognostic importance of CD3+, CD8+ and CD45RO+ TILs, as well as Foxp3+ infiltrates, on invasive ductal carcinoma (IDC) of the breast. The relation of the expressions of these markers to clinical and pathological BC parameters was also studied.

2 Materials and methods

2.1 Patients and tissue samples

The study population was retrospectively selected from BC patients who underwent surgical resection of their primary tumor between 2009 and 2011 in the Shahid Faghihi Hospital of the Shiraz University of Medical Sciences, Shiraz, Iran. In total, 94 patients were enrolled with an IDC phenotype who did not receive pre-operative chemotherapy or radiotherapy, and for whom formalin-fixed and paraffin-embedded (FFPE) tissue blocks, as well as clinicopathologic and follow-up data were available. The patient data, including age, diagnosis date, survival status, date of last follow-up, date of recurrence and, for expired patients, date and cause of death, were obtained from their records. An expert pathologist reviewed the H&E-stained tumor sections to select slides with both CT and IM. Finally, corresponding FFPE tissue sections were prepared for immunohistochemical staining.

2.2 Immunohistochemistry

Four serial sections of 3 μm thickness were prepared from each FFPE tissue block and mounted on positively charged glass slides. Sections were heated at 61 °C for 15 min, deparaffinized in xylene for 30 min, and rehydrated in decreasing graded ethanol solutions (100% and 96%). Antigen retrieval was performed by boiling the sections in high pH retrieval solution (tris-EDTA, pH 9) in a pressure cooker for about 16 min. We applied 10% H_2O_2 and 10% goat serum to block endogenous peroxidase and non-specific hydrophobic interactions, respectively. Primary antibodies directed against CD3 [Dako, IS50330–2, ready to use (RTU)], CD8 [Dako, IS62330–2, Clone C8/144B, RTU], CD45RO [Dako, M074201–2, Clone UCHL1, 1/200 dilution] and Foxp3 [Abcam, ab20034, Clone 236A/E7, 1/100 dilution] were added to the sections and incubated for 1 h at room temperature. Visualization was performed using a diaminobenzidine (DAB)/peroxidase-based Dako REAL™ EnVision™ Detection System (K500711–2, Dako) according to the manufacturer's instructions. Finally, tissues were counterstained with hematoxylin, dehydrated in an increasing graded ethanol series, cleaned in xylene, and mounted with mounting medium. Proper controls including positive (immunostaining of human tonsil tissue for the same markers), negative (no primary antibody) and isotype-matched controls were used to validate the IHC procedure and to obtain reliable results. The IHC scores for ER, PR and HER2 were obtained from pathology reports.

2.3 Digital microphotography and positive cell quantification

An expert pathologist blinded to the patients' clinical characteristics and outcomes took the digital photos. A CD3-stained slide of each patient was selected as a reference and scanned with an optical microscope equipped with a digital camera (Olympus DP72) to determine predominant tumor areas that had the highest CD3+ cell

infiltration in both CT and IM. Next, photos of 3 other markers were taken from the same regions as of the CD3 marker (Online Resource 1). All images were taken at a magnification of 200×, which corresponds to a 0.1 μm pixel size resolution. Necrotic areas and regions with any other artifacts were excluded. Finally, IHC quantification was performed through analysis of the images by a semi-automatic image analysis workstation, ImageJ, and the results were reported as the number of positive-stained cells per area unit (square millimeter). Since each image represented about 0.15 mm^2 of the true area of the tumor section, the number of positive cells was adjusted to the area unit (mm^2) of the tumor surface.

2.4 Statistical analysis

Statistical analyses were conducted by SPSS, version 21. The non-parametric Mann-Whitney U test was used to determine differences in TIL infiltration between patient groups. Receiver operating characteristic (ROC) curves were used to determine the optimal cut-off points for the subpopulation of TILs, referring to survival and disease relapse. Survival times were defined as OS, i.e., interval between diagnosis and cancer-related death or last contact, and DFS, i.e., interval between the operation and any type of relapse or last follow-up. Univariate Cox regression analysis was used to investigate variables that exhibited significant associations with OS and DFS. All variables with p values < 0.1 were included in the multiple Cox regression model. Kaplan-Meier curves were used to visualize cumulative survival rates based on categorical variables that remained in the last step of the multiple Cox regression analysis. P values < 0.05 were considered significant.

3 Results

3.1 Clinicopathological characteristics of the breast cancer patients

The study population consisted of 94 females with an IDC phenotype who underwent radical mastectomy or quadrantectomy as their first-line treatment. The patients' mean age at diagnosis was 49.52 years (25 to 81 years). The mean follow-up period was 62.4 months (8.2 to 90.1 months). During this time, 32 (34%) patients experienced recurrence and 23 (24.5%) suffered from cancer-related deaths. In accordance with the 7th edition of the AJCC TNM classification system [27], most (60.6%) patients were in stage II and none had distant metastases (stage IV) at the time of surgery. Based on HR and HER2 expression, 51 (54.3%) tumor samples were of the luminal (ER+/PR±/HER2-) subtype and 43 (45.7%) samples were of the non-luminal subtype, including HER2-enriched (ER-/PR-/HER2+) and triple negative (ER-/PR-/HER2-) tumors. Detailed clinicopathological characteristics of the patients are listed in Table 1.

3.2 Extent of CD3+, CD8+, CD45RO+ and Foxp3+ infiltrates in the centers and invasive margins of breast tumors

Tissue samples were immunohistochemically stained for the pan T cell marker CD3, the cytotoxic T cell co-receptor CD8, the memory T cell marker CD45RO and the regulatory T lymphocyte transcription factor, Foxp3 (Fig. 1). The extent of lymphocytic infiltration in CT and IM was quantified using ImageJ software. Overall, TILs were more abundant in the IM than in the CT of the tumors. In both CT and IM, CD45RO+ cells were the more numerous subpopulation, whereas Foxp3+ regulatory T cells were the least frequent subset in the BC tissues. The mean as well as minimum and maximum numbers of the investigated subsets are listed in Table 2.

3.3 CD3+, CD8+, CD45RO+ and Foxp3+ TIL distribution among patient groups

First, we categorized continuous variables and those that divide the data into more than two groups into a series of binary variables. The distribution of target cells between patient groups was studied using the Mann-Whitney U test. Of the patients included, 50 (53.2%) were 50 years or younger, and 44 (46.8%) were older than 50 years. A comparison of the infiltrated immune cells between both age groups revealed a significantly larger whole T cell population (CD3+ cells) in the IM of patients younger than 50 years compared to that in the older patients ($p = 0.009$).

As listed in Tables 3 and 4, TIL subpopulations in both CT ($p < 0.01$) and IM ($p < 0.001$) of poorly differentiated tumors (grade III) were remarkably more numerous than those in well and moderately differentiated tumors (grades I/II). Additionally, we found that patients with at least one metastatic lymph node, as well as those with TNM-stage III, showed significantly higher numbers of T cells (CD3+) as well as CD45RO+ infiltrates in both CT and IM of their tumors. However, the CD8/CD3 ratio in the CT ($p = 0.04$) and the CD8/Foxp3 ratio in the IM ($p = 0.02$) of the low grade (I, II) tumors were significantly higher than their ratios in the CT and IM of high-grade (III) tumors, respectively. In case of TNM-stage, the CD8/CD3 ratio in both CT ($p = 0.007$) and IM ($p = 0.004$) of early stage (I, II) patients was higher than that in the CT and IM of stage III patients. In patients with no lymph node metastasis,

Table 1 Clinicopathological characteristics of breast cancer patients

Characteristics		*n* (%)	Mean (min–max)
Age (years)	Total	94 (100)	42.59 (25–81)
	≤ 50	50 (53.2)	
	> 50	44 (46.8)	
Survival	Alive	69 (73.4)	
	Dead (cancer caused)	23 (24.5)	
	Dead (other causes)	2 (2.1)	
Recurrence	Positive	32 (34)	
	Negative	62 (66)	
Recurrence type	Local	5 (16.1)	
	Distant metastasis	22 (71)	
	Local and distant	4 (12.9)	
	Unknown	1	
Operation types	Mastectomy	50 (53.2)	
	Quadrantectomy	44 (46.8)	
Survival times (days)	Overall survival (OS)		1872.31 (248–2705)
	Disease-free survival (DFS)		1652.99 (129–2681)
Tumor size (cm)	Total		2.60 (0.5–5.0)
	≤ 2.6	51 (54.3)	
	> 2.6	43 (45.7)	
T-stage	T1	27 (28.7)	
	T2	63 (67)	
	T3	0 (0)	
	T4	4 (4.3)	
N-stage	N0	45 (50.5)	
	N1	27 (28.6)	
	N2	10 (11)	
	N3	9 (9.9)	
	Unknown	3	
TNM-stage	I	14 (14.9)	
	II	57 (60.6)	
	III	23 (24.5)	
Histological grade	I	18 (19.4)	
	II	46 (49.5)	
	III	29 (31.2)	
	Unknown	1	
LVI	Positive	40 (43.5)	
	Negative	52 (56.5)	
	Unknown	2	
ER[a]	Positive	51 (54.3)	
	Negative	43 (45.7)	
PR[a]	Positive	41 (43.6)	
	Negative	53 (56.4)	
HER2[a]	Positive	33 (35.1	
	Negative	61 (64.9)	
BC subtype	Luminal (ER+/HER2-)	51 (54.3)	
	Non-luminal (HER2-enriched and TNBC)	43 (45.7)	

LVI lymphovascular invasion, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2, *BC subtype* breast cancer subtype, *TNBC* triple negative breast cancer

[a] The IHC results of these markers were obtained from pathology reports

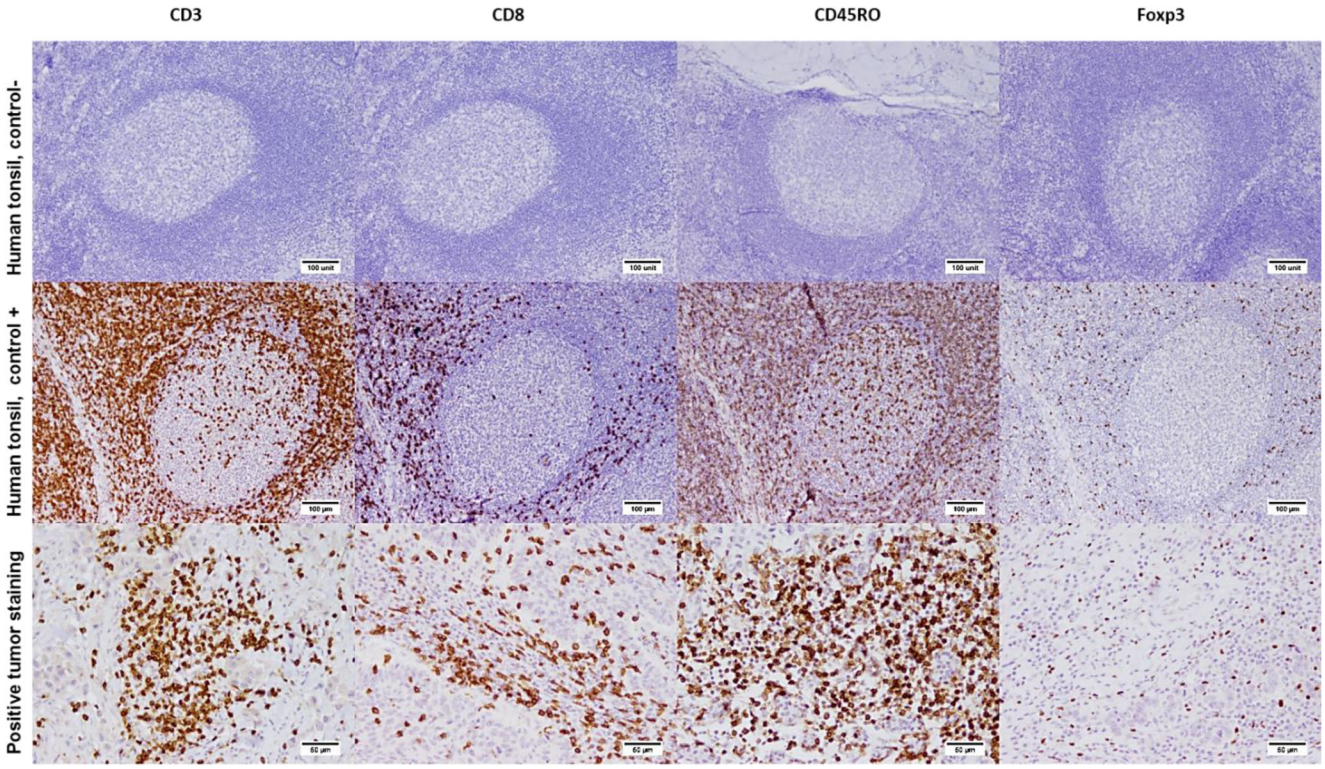

Fig. 1 CD3, CD8, CD45RO and Foxp3 staining patterns and isotype controls. Images in the first row illustrate the staining patterns of isotype (CD3 and CD8) and negative (CD45RO and Foxp3) human tonsil controls (no positive staining). In the second and the third rows representative images of positive staining in human tonsil control and breast tumor tissues, respectively, are shown. Brown spots represent positively stained cells

the CD8/CD3 ratio in the IM ($p = 0.04$) as well as the CD8/Foxp3 ratio in the CT ($p = 0.02$) were significantly higher than those in patients with at least one tumor-involved lymph node (Online Resource 2).

3.4 CD3+, CD8+, CD45RO+ and Foxp3+ TIL distribution between luminal and non-luminal breast cancers

Based on ER, PR and HER2 expression, we classified patients into two groups, i.e., luminal ($n = 51$, 54.3%) and non-luminal ($n = 43$, 45.7%). By doing so, we observed significantly more infiltrations of all subpopulations of immune cells in the IM ($p \leq 0.001$ for all) as well as infiltrations of CD3+ ($p = 0.013$) and Foxp3+ ($p = 0.002$) TILs in the CT of the non-luminal tumors (Tables 3 and 4).

3.5 Prognostic significance of CD3+, CD8+, CD45RO+ and Foxp3+ TILs

We used the Mann-Whitney U test to initially evaluate the abundance of immune stained cells between patients who suffered from cancer-related deaths ($n = 23$, 24.5%) and those that were still alive at their last follow-up ($n = 69$, 73.4%), as well as patients with ($n = 32$, 34%) and without ($n = 62$, 66%) recurrence. We found that the TIL distributions in the primary tumors of survivors and non-survivors showed no significant differences, whereas there were statistically more CD45RO+

Table 2 Mean frequency of immune cells in the center and invasive margin of breast tumors

	CT				IM			
	CD3	CD8	CD45RO	Foxp3	CD3	CD8	CD45RO	Foxp3
Mean (cells/mm^2)	1562.13	707.72	1884.90	214.91	3266.76	1232.28	3529.70	321.72
SEM	123.05	61.33	164.09	21.51	234.27	88.66	250.75	28.76
Min–Max	49.33–4958	49–3897	74–7622	0–1332	247–11,149	173–6586	419–11,618	0–1505

CT center of tumor, *IM* invasive margin, *SEM* standard error of mean

Table 3 Comparison of the frequency of immune cells in tumor centers of breast cancer patients with different clinicopathological features

Variable		*n* (%)	Mean (± SEM)			
			Center of tumor			
			CD3	CD8	CD45RO	Foxp3
Age (years)	≤ 50	50 (53.2)	1678.81 (168.18)	756.77 (72.08)	1988.13 (237.81)	216.57 (24.56)
	> 50	44 (46.8)	1429.54 (180.46)	651.98 (102.55)	1767.59 (224.88)	213.03 (36.83)
P value			0.22	0.09	0.64	0.45
Survival	Alive	69 (73.4)	1554.00 (138.00)	730.35 (74.45)	2010.51 (98.70)	213.42 (21.26)
	Dead	23 (24.5)	1556.14 (290.73)	628.46 (111.70)	1546.49 (301.39)	216.63 (61.54)
P value			0.56	0.34	0.14	0.27
Recurrence	No	62 (66)	1610.89 (142.58)	709.76 (63.16)	2080.35 (201.59)	226.37 (22.71)
	Yes	32 (34)	1467.66 (235.58)	703.77 (133.95)	1506.20 (274.47)	192.70 (45.68)
P value			0.22	0.33	0.02	0.06
H. grade	I, II	64 (68)	1293.45 (146.11)	601.64 (63.06)	1575.19 (174.89)	174.97 (25.94)
	III	29 (30.9)	2148.55 (197.49)	927.13 (133.87)	2590.00(334.96)	301.10 (35.27)
P value			<0.001	0.01	0.003	<0.001
TNM stage	I, II	71 (75.5)	1338.25 (123.59)	666.00 (57.55)	1677.68 (182.69)	211.92 (25.71)
	III	23 (24.5)	2253.24 (287.81)	836.52 (177.38)	4773.54 (612.34)	381.80 (71.11)
P value			0.002	0.58	0.003	0.49
Tumor size	≤ 2.5	51 (54.3)	1436.47 (158.41)	680.03 (92.52)	1751.81 (200.18)	208.94 (31.74)
	> 2.5	43 (45.7)	1711.17 (192.06)	740.57 (77.94)	2042.74 (269.56)	222.00(28.54)
P value			0.26	0.30	0.61	0.58
Node status	LN+	45 (47.9)	1860.96 (194.02)	787.14 (103.99)	2122.42 (221.05)	229.67 (27.20)
	LN-	46 (48.9)	1272.47 (145.91)	622.03 (66.84)	1693.95 (250.80)	184.46 (24.15)
P value			0.03	0.35	0.03	0.22
LVI	Positive	40 (42.6)	1474.45 (191.77)	689.43 (79.23)	1798.81 (225.97)	228.16 (38.40)
	Negative	52 (55.3)	1621.35 (167.52)	710.12 (92.85)	1944.87 (240.93)	199.23 (25.08)
P value			0.44	0.86	0.89	0.58
BC subtype	Non-luminal	43 (45.7)	1805.25 (172.33)	776.14 (99.61)	2175.82(265.90)	254.12 (26.02)
	Luminal	51 (54.3)	1357.15 (170.41)	650.04 (75.65)	1639.60 (199.00)	181.85 (32.54)
P value			0.013	0.23	0.10	0.002
HER2 status	Positive	33 (35.1)	1761.79 (234.64)	737.76 (129.59)	2116.10 (297.32)	230.22 (30.39)
	Negative	61 (64.9)	1454.12 (140.46)	691.48 (64.24)	1759.82 (195.10)	206.63 (28.90)
P value			0.29	0.94	0.46	0.19
ER status	Positive	51 (54.3)	1357.15 (170.41)	650.04 (75.65)	1639.60 (199.00)	181.85 (32.54)
	Negative	43 (45.7)	1805.25 (172.33)	776.14 (99.61)	2175.82 (265.90)	254.12 (26.02)
P value			0.01	0.23	0.1	0.002
PR	Positive	41 (43.6)	1268.22 (179.44)	599.82 (81.05)	1635.82 (237.56)	157.02 (24.96)
	Negative	53 (56.4)	1789.49 (163.06)	791.19 (87.84)	2077.58 (224.03)	259.69 (31.76)
P value			0.01	0.08	0.08	<0.001

CT center of tumor, *IM* invasive margin, *SEM* standard error of mean, *LVI* lymphovascular invasion, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2, *BC subtype* breast cancer subtype, *TNBC* triple negative breast cancer

TILs in the CT of the non-recurrent patients compared to the CT of recurrent patients ($p = 0.02$).

For survival analyses, we converted the TIL subpopulations from continuous to categorical variables based on their corresponding cut-off values according to ROC analysis of survival and recurrence status as binary classifiers. Examples of high and low infiltrations of each subpopulation are depicted in the Online Resource 3. Cut-off points were also determined for the total number of each subpopulation of TILs (defined as the combined frequency of each TIL

Table 4 Comparison of the frequency of immune cells in tumor invasive margins of breast cancer patients with different clinicopathological features

Variable		*n* (%)	Mean (± SEM)			
			Invasive margin			
			CD3	CD8	CD45RO	Foxp3
Age (years)	≤ 50	50 (53.2)	3825.80 (350.53)	1377.88 (141.10)	3983.67 (364.66)	340.89 (37.67)
	> 50	44 (46.8)	2631.48 (277.18)	1066.83 (96.65)	3013.82 (326.65)	299.92 (44.33)
P value			0.009	0.07	0.06	0.28
Survival	Alive	69 (73.4)	3436.89 (287.47)	1284.10 (107.73)	3458.34 (279.85)	330.32 (34.65)
	Dead	23 (24.5)	2732.64 (404.89)	1085.33 (163.13)	3527.33 (578.74)	275.62 (52.37)
P value			0.21	0.13	0.75	0.37
Recurrence	No	62 (66)	3285.44 (294.05)	1271.9 (116.31)	3313.69 (284.98)	314.70(32.65)
	Yes	32 (34)	3230.56 (392.33)	1155.48 (131.92)	3948.21 (485.72)	335.31 (56.74)
P value			0.84	0.41	0.4	0.97
H. grade	I, II	64 (68)	2803.14 (282.55)	1084.18 (115.15)	2936.88 (283.47)	234.33 (29.34)
	III	29 (30.9)	4098.07 (338.65)	1530.18 (114.07)	4662.85 (415.46)	497.59 (51.27)
P value			<0.001	<0.001	<0.001	<0.001
TNM stage	I, II	71 (75.5)	2978.41 (261.49)	1165.59 (80.96)	3126.76 (250.89)	302.25 (30.31)
	III	23 (24.5)	4156.87 (478.44)	1438.17 (262.42)	4773.54 (612.34)	381.80 (71.11)
P value			0.009	0.44	0.01	0.28
Tumor size	≤ 2.5	51 (54.3)	3392.88 (336.17)	1266.22 (140.59)	3506.05 (353.92)	340.98 (43.62)
	> 2.5	43 (45.7)	3117.18 (324.21)	1192.03 (100.34)	3557.74 (357.02)	298.87 (36.00)
P value			0.70	0.95	0.74	0.76
Node status	LN+	45 (47.9)	3870.47 (360.62)	1346.25 (148.20)	4228.41 (395.77)	363.97 (44.09)
	LN-	46 (48.9)	2746.58 (294.56)	1122.87 (101.16)	2921.93 (299.49)	282.06 (37.84)
P value			0.02	0.33	0.01	0.09
LVI	Positive	40 (42.6)	3316.43 (385.78)	1311.03 (168.10)	3695.68 (417.90)	328.07 (38.63)
	Negative	52 (55.3)	3087.60 (282.58)	1150.32 (93.64)	3280.19 (304.28)	306.44 (41.86)
P value			0.85	0.54	0.59	0.33
BC subtype	Non-luminal	43 (45.7)	4219.15 (380.60)	1522.45 (152.30)	4526.62 (383.96)	427.36 (47.20)
	Luminal	51 (54.3)	2463.76 (239.51)	987.63 (88.90)	2689.15 (283.30)	232.64 (30.20)
P value			<0.001	<0.001	<0.001	<0.001
HER2 status	Positive	33 (35.1)	3889.11 (416.25)	1477.76 (193.08)	3898.83 (454.91)	375.98(53.25)
	Negative	61 (64.9)	2930.08 (275.16)	1099.49 (84.74)	3330.00 (297.63)	292.36 (33.43)
P value			0.02	0.06	0.28	0.07
ER status	Positive	51 (54.3)	2463.76 (239.51)	987.63 (88.90)	2689.15 (283.30)	232.64 (30.20)
	Negative	43 (45.7)	4219.15 (380.60)	1522.45 (152.30)	4526.62 (383.96)	427.36 (47.20)
P value			<0.001	<0.001	<0.001	<0.001
PR	Positive	41 (43.6)	2370.41 (263.80)	916.28 (90.45)	2605.04 (319.76)	188.91 (23.86)
	Negative	53 (56.4)	3960.16 (333.88)	1476.74 (132.02)	4244.99 (340.65)	424.45 (42.67)
P value			<0.001	<0.001	<0.001	0.005

CT center of tumor, *IM* invasive margin, *SEM* standard error of mean, *LVI* lymphovascular invasion, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2, *BC subtype* breast cancer subtype, *TNBC* triple negative breast cancer

subpopulation in CT and IM). The optimal cut-off points, their corresponding sensitivities and specificities, and event-related values are listed in Tables 5 and 6.

Univariate Cox regression analysis was performed to determine whether individual variables had a significant relationship to survival times. We found that OS was significantly influenced only by the frequency of two subpopulations of immune cells: CD8+ cells in the tumor IM with a hazard ratio (HR) of 2.348 (95% CI 1.029–5.359, $p = 0.043$) and the total population of CD45RO+ cells (HR: 2.640; 95% CI: 1.164–5.984, $p = 0.020$) (Table 7). Our data revealed no significant relationship between the clinicopathological variables and

Table 5 Cut-off points of target cells based on patient survival

Region	Variable	Cut-off point, cells/mm^2	Sensitivity%	Specificity%	AUC	Death-related values
CT	CD3	1295	60.9	52.2	0.540	Smaller
	CD8	629	65.2	52.2	0.566	Smaller
	CD45RO	1319.65	60.9	52.2	0.602	Smaller
	Foxp3	160.33	50.7	49.3	0.576	Smaller
IM	CD3	2429.67	60.9	58.0	0.587	Smaller
	CD8	925	65.2	63.8	0.605	Smaller
	CD45RO	2886	60.9	50.7	0.521	Smaller
	Foxp3	259	60.9	49.3	0.562	Smaller
Total	CD3	1714	60.9	65.2	0.584	Smaller
	CD8	4847	65.2	50.7	0.594	Smaller
	CD45RO	3108	52.2	76.8	0.576	Smaller
	Foxp3	542.5	73.9	42.0	0.576	Smaller

CT center of tumor, *IM* invasive margin, *AUC* area under curve

OS. However, although insignificant, node negative patients showed an improved OS compared to node positive patients, with a HR of 2.365 (95% CI: 0.940–5.947, $p = 0.067$). Univariate Cox proportional hazards model for DFS revealed that both node negativity (HR: 2.352; 95% CI: 1.091–5.068, $p = 0.029$) and higher numbers of CD45RO+ cells in the CT (HR: 2.249; 95% CI: 1.064–4.754, $p = 0.034$) significantly reduced the risk of post-operative disease relapse. Neither CD3+ T cells nor Foxp3+ regulatory T cells showed any significant effect on both OS and DFS.

All variables in the univariate analysis that had a p value < 0.1 were entered into a multiple Cox regression model with backward elimination of non-significant variables at the 5% level. Accordingly, total CD45RO+ TILs (HR: 3.248; 95% CI: 1.312–8.041, $p = 0.011$) showed a strong prognostic effect on the OS of BC patients. We found that lymph node status was another independent prognostic factor for OS with a HR of 3.192 (95% CI: 1.230–8. 281, $p = 0.017$) (Table 8). With regard to DFS, CD45RO+ TILs in the CT with a HR of 3.128 (95% CI: 1.390–7.036, $p = 0.006$), as well as nodal status with a HR of 3.152 (95% CI: 1.428–6.955, $p = 0.004$) were found to serve as two independent prognostic variables. Kaplan-Meier curves for the cumulative risk of death and recurrence of BC patients based on these prognostic markers are depicted in Fig. 2.

3.6 Prognostic significance of CD45RO+ TILs in LN+ patients

Variables that remain in the final step of multiple Cox analysis are those that can independently predict patient outcomes.

Table 6 Cut-off points of target cells based on disease recurrence

Region	Variable	Cut-off point, cells/mm^2	Sensitivity%	Specificity%	AUC	Relapse-related values
CT	CD3	1295	65.6	58.1	0.577	Smaller
	CD8	629	65.6	50.0	0.561	Smaller
	CD45RO	1319.67	68.8	58.1	0.640	Smaller
	Foxp3	160.33	62.5	53.2	0.615	Smaller
IM	CD3	2898.33	53.1	46.8	0.512	Smaller
	CD8	1023.67	62.5	58.1	0.551	Smaller
	CD45RO	2528.33	62.5	46.8	0.552	Greater
	Foxp3	234.33	56.3	50.0	0.502	Greater
Total	CD3	4292.33	53.1	51.6	0.529	Smaller
	CD8	1714	62.5	51.6	0.565	Smaller
	CD45RO	4600	56.5	56.3	0.530	Smaller
	Foxp3	542.5	68.8	43.5	0.546	Smaller

CT center of tumor, *IM* invasive margin, *AUC* area under curve

Table 7 Univariate Cox regression analysis of disease free survival (DFS) and overall survival (OS) of patients with invasive ductal carcinoma of the breast

Variable		OS					DFS				
		β	S.E	HR	95% CI for HR	*P* value	B	S.E	HR	95% CI for HR	*P* value
Age (Y)	≤ 50			1 (Ref)					1 (Ref)		
	> 50	0.088	0.418	1.09	0.481–2.477	0.833	0.213	0.354	1.237	6.18–2.477	0.547
Grade	I&II			1 (Ref)					1 (Ref)		
	III	−0.105	0.475	0.900	0.355–2.284	0.825	−0.037	0.396	0.964	0.444–2.095	0.964
TNM	I&II			1 (Ref)					1 (Ref)		
	III	0.571	0.455	1.770	0.726–4.313	0.209	0.586	0.383	1.796	0.848–3.806	0.126
T (cm)	≤ 2.5			1 (Ref)					1 (Ref)		
	> 2.5	0.092	0.418	1.096	0.483–2.486	0.827	0.054	0.354	1.056	0.527–2.114	0.879
Node	LN-			1 (Ref)					1 (Ref)		
	LN+	0.861	0.471	2.365	0.940–5.947	0.067	0.855	0.392	2.352	1.091–5.068	0.029
LVI	Positive			1 (Ref)					1 (Ref)		
	Negative	0.772	0.427	2.164	0.936–5.003	0.071	0.557	0.361	1.746	0.860–3.545	0.123
BC subtype	Luminal			1 (Ref)					1 (Ref)		
	HER2&TN	−0.351	0.439	0.704	0.298–1.664	0.424	0.127	0.355	1.13	0.566–2.276	0.721
ER	Negative			1 (Ref)					1 (Ref)		
	Positive	.351	.439	1.421	0.601–3.358	0.424	−0.127	0.355	0.881	0.439–1.766	0.721
PR	Positive			1 (Ref)					1 (Ref)		
	Negative	0.209	0.422	1.232	0.539–2.816	0.621	0.416	0.366	1.517	0.741–3.105	0.255
HER2	Positive			1 (Ref)					1 (Ref)		
	Negative	0.195	0.453	1.215	0.500–2.955	0.667	−0.022	0.372	0.979	0.472–2.030	0.953
$CD3^+$ CT	High			1 (Ref)					1 (Ref)		
	Low	0.351	0.427	1.420	0.614–3.281	0.412	0.658	0.372	1.931	0.930–4.006	0.077
$CD3^+$ IM	High			1 (Ref)							
	Low	0.541	0.428	1.718	0.743–3.973	0.206	−0.150	0.355	0.861	0.429–1.726	0.673
$CD8^+$ CT	High			1 (Ref)					1 (Ref)		
	Low	0.484	0.438	1.623	0.688–3.830	0.269	0.533	0.373	1.704	0.820–3.540	0.153
$CD8^+$ IM	High			1 (Ref)					1 (Ref)		
	Low	0.854	0.421	2.348	1.029–5.359	0.043	0.603	0.366	1.828	0.892–3.744	0.099
CD45RO + CT	High			1 (Ref)					1 (Ref)		
	Low	0.350	0.427	1.419	0.614–3.280	0.413	0.811	0.382	2.249	1.064–4.754	0.034
CD45RO + IM	High			1 (Ref)					1 (Ref)		
	Low	0.357	0.427	1.429	0.618–3.303	0.404	−0.369	0.365	0.692	0.338–12.415	0.313
Foxp3+ CT	High			1 (Ref)					1 (Ref)		
	Low	0.317	0.427	1.372	0.594–3.172	0.459	0.399	0.731	1.490	0.356–6.237	0.585
Foxp3+ IM	High			1 (Ref)					1 (Ref)		
	Low	0.203	0.421	1.225	0.536–2.798	0.630	−0.334	0.357	0.716	0.356–1.441	0.350
$CD3^+$ T	High			1 (Ref)					1 (Ref)		
	Low	0.753	0.428	2.123	0.918–4.910	0.078	0.037	0.355	1.038	0.518–2.080	0.917
$CD8^+$ T	High								1 (Ref)		
	Low	0.472	0.438	1.603	0.679–3.780	0.281	0.417	0.366	1.518	0.741–3.110	0.254
CD45RO+ T	High			1 (Ref)					1 (Ref)		
	Low	0.971	0.418	2.640	1.164–5.984	0.020	0.355	0.357	1.426	0.708–2.871	0.320
Foxp3+ T	High			1 (Ref)					1 (Ref)		
	Low	0.526	0.475	1.692	0.667–4.295	0.268	0.349	0.382	1.417	0.671–2.994	0.361

LVI lymphovascular invasion, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2, *BC subtype* breast cancer subtype, *TNBC* triple negative breast cancer

Table 8 Multiple Cox regression analysis of disease free survival (DFS) and overall survival (OS) of patients with invasive ductal carcinoma of the breast

Variable		β	S.E	HR	95% CI for HR	*P* value
OS						
Node	LN-			1 (Ref)		
	LN+	1.161	0.486	3.192	1.230–8.281	0.017
CD45RO+ T	High			1 (Ref)		
	Low	1.178	0.462	3.248	1.312–8.041	0.011
DFS						
Node	LN-			1 (Ref)		
	LN+	1.148	0.404	3.152	1.428–6.955	0.004
CD45RO+ CT	High			1 (Ref)		
	Low	1.140	0.414	3.128	1.390–7.036	0.006

However, since lymph node involvement itself is one of the strongest prognostic factors in BC, we performed an additional analysis to assess the prognostic significance of CD45RO+ TILs in only LN+ patients. Of 45 patients with involved lymph nodes, 42.2% ($n = 19$) experienced recurrence and 28.9% ($n = 13$) died. Univariate Cox regression analysis for DFS revealed a prognostic significance of CD45RO+ TILs even in LN+ patients (HR: 2.52, 95% CI: 1.007–6.320, $p = 0.048$). The results for OS prediction did not reach significance, which may be due to its smaller number of events (HR: 2.10, 95% CI: 0.67–7.1, $p = 0.19$).

4 Discussion

The majority of studies aimed at investigating TILs in BC has focused on the prognostic impact of the total population of lymphocytic infiltrates. Fewer studies have focused on the prognostic significance of specific subpopulations. Thus, here we aimed to evaluate the prognostic significance of immune cells in invasive ductal carcinoma of the breast with a primary focus on T cells by immunohistochemical staining for CD3, CD8, CD45RO and Foxp3 in primary tumor samples. We found that TILs were more abundant in the IM than in the CT of the tumors. In both regions, the CD45RO+ subpopulation was the most prevalent population and its frequency was even higher than that of CD3+ T cell infiltrates. This observation indicates that in the TME other cells, most likely differentiated B lymphocytes and NK cells, may also express CD45RO as has been reported by others [28–30]. An analysis of TIL subpopulations and clinicopathological parameters revealed significantly more T cell infiltrates in the IM of primary tumors of patients younger than 50 years than in tumors of older patients. This remarkably lower abundance of CD3+ T cells in the tumors of older patients could simply be an indicator of immunosenescence, which may explain the adverse effect of ageing on immune system functions, especially cell-mediated immunity [31].

Histological grade is one of the clinicopathological parameters inversely related to prognosis. Low grade tumor cells are slow growing and morphologically resemble normal cells. However, high grade transformed cells are poorly differentiated (or undifferentiated), more aggressive, and tend to grow faster. In the current study we observed an association between higher numbers of TILs and a higher histological grade (grade III). Similar results were reported by Tian et al. [32] and Ladoire et al. [33]. It may be assumed that wide phenotypic differences between poorly differentiated transformed cells and normal cells can lead to increased stimulation of immune cells and their further recruitment into the tumor microenvironment.

We also classified the study population into luminal and non-luminal groups based on ER, PR and HER2 expression. We observed more TILs in the non-luminal HER2-enriched and triple negative (TNBC) tumors than in the luminal subtypes. Several studies, including those that have assessed TILs by H&E staining and those that have evaluated specific TIL subpopulations by IHC, also reported increased TIL infiltrations in non-luminal subtypes [34–37]. TNBC and HER2-enriched BCs have been reported to exhibit the highest rates of genetic mutations [38, 39]. The increased frequency of TILs in these two subtypes may be explained by the increased mutation rates in the tumors, which has probably led to the emergence of new antigens that have the capability to provoke immune responses and TIL recruitment. On the other hand, luminal subtypes, which have been reported to exhibit the lowest mutational loads, show the lowest lymphocytic infiltration among all subtypes of BC [38, 39].

Similar to several other studies [40, 41], we found that higher numbers of the TIL subpopulations were associated with other aggressive phenotypes of the disease, including lymph node metastasis, advanced TNM-stage, HER2 positivity and ER negativity. However, a high ratio of cytotoxic TILs (CD8+) to whole T cell infiltrates (CD3+) and regulatory TILs (Foxp3+) reached relative significance in CT and IM of cases positive for the aforementioned aggressive features. These

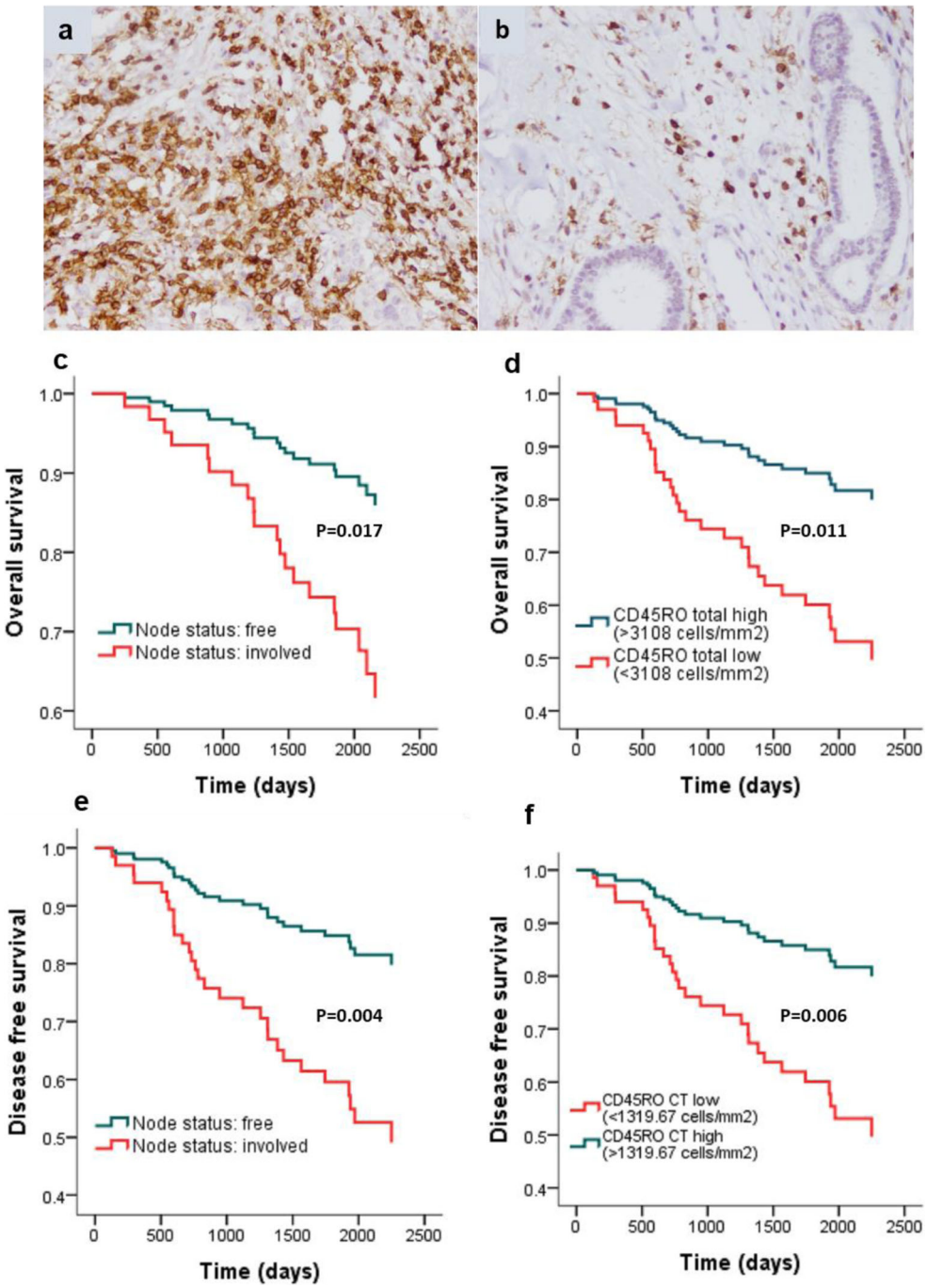

Fig. 2 Kaplan-Meier curves of overall and disease-free survival. Examples of high (**a**) and low (**b**) CD45RO+ TILs in tumour centers are shown (200×). Kaplan-Meier curves of OS and DFS according to lymph node metastasis (**c** and **e**) and CD45RO+ TIL status (**d** and **f**) indicate that patients with no lymph node metastases and those with high CD45RO+ TIL infiltrates exhibit a significantly improved survival. TILs: Tumor-infiltrating lymphocytes, OS: overall survival, DFS: disease-free survival

data underscore the importance of studying TIL subpopulation ratios, in addition to their absolute numbers.

Our survival analysis showed that certain immune cell subpopulations may play a prognostic role in both OS and DFS of BC patients. According to univariate Cox analysis for OS, among all clinicopathological and immunological parameters tested, the prevalence of CD8+ cytotoxic T cells in invasive tumor margins (IM; HR: 2.34) and total CD45RO+ TILs (HR: 2.64) showed significant prognostic relevance. We included all variables that had a p value < 0.1 in the multiple survival analyses. The results showed that total CD45RO+ TILs (HR: 3.24), as well as lymph node involvement status (HR: 3.19), were the two markers that could independently predict prognosis. The same approach was applied to determine significant prognostic variables in DFS. In this case, lymph node status (HR: 3.15) and CD45RO+ TILs in the CT (HR: 3.12) were the two remaining variables in the model.

Lymph node metastasis is one of the most important prognostic factors in BC patients. Patients with tumor-involved lymph nodes are at a high risk of distant metastasis and disease

relapse. It is now well-documented that the host genetic background and reactions of cellular and molecular components of the immune system within the tumor draining lymph nodes may dramatically affect tumor cell infiltration, colonization and metastasis [42–46]. Interestingly, hazard ratios obtained from our survival models for CD45RO+ cell infiltration into the primary tumor samples showed a prognostic power as strong as lymph node metastasis. We found that CD45RO+ infiltrates may be considered as prognostic factors, even in patients with tumor-involved lymph nodes. In addition, we found that the biomarkers that reached significance had the advantage of predicting OS and DFS regardless molecular subtypes.

Several studies have reported the prognostic importance of CD8+ and CD45RO+ T cell infiltrates in primary tumors of different origins [9, 10, 34, 47, 48]. In BC, Ali et al. evaluated the prognostic value of CD8+ TILs in a large number of BC patients. Based on the location of the infiltrates in tissues, they defined two TIL compartments that included intra-tumoral and stromal TILs with or without direct contact with tumor cells, respectively. Their results indicated that in ER negative non-luminal BC (HER2-enriched and TNBC) higher numbers of both intra-tumoral and stromal CD8+ TILs significantly reduced the relative risk of BC-related death, whereas in ER+ HER2+ luminal tumors similar results were only observed for intra-tumoral TILs [49]. Yajima et al. also reported a positive relation between higher amounts of CD45RO+ cells and a longer DFS, but not OS, in BC patients [50].

In contrast to skin and mucosal tissues, immune cell infiltration in normal breast tissue is uncommon. A remarkable population of inflammatory cells has been detected in surgically removed tumors [51]. Considering CD45RO as a marker of effector and memory T cells, we could deduce from our results that higher numbers of CD45RO+ TILs may indicate that the elicited immune responses against tumor cells was adequate enough to generate immunological memory which, in turn, may be used to eliminate residual tumor cells and micro-metastases, and to improve patient survival after surgical removal of the primary tumor. Finally, it should be noted that although we found that a higher number of Foxp3+ regulatory T cells, like other TIL subpopulations, was associated with more severe clinicopathologic BC features, they showed a neutral prognostic effect on post-operative patient survival.

Taken together, our survival analyses revealed a prognostic effect for CD45RO+ TILs, which was as strong as the prognostic impact of lymph node metastasis, regardless molecular BC subtype. We also found that a higher frequency of CD3+, CD8+, CD45RO+ and Foxp3+ TILs was associated with more aggressive features of the disease, including the non-luminal subtypes. Of note, new therapeutic approaches that potentially target these TIL subpopulations could be designed. We conducted the present study on patients with invasive ductal carcinoma of the breast. In the future, these studies should be repeated with other histological subtypes of BC with larger sample sizes to clarify the role of immune cells in each BC subtype.

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Compliance with ethical standards

Ethical approval For our retrospective study formal consent is not required.

This article does not contain any studies with animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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