Cancer Biomarkers 20 (2017) 395–409
DOI 10.3233/CBM-170026
IOS Press

Infiltration of $\gamma\delta$ T cells, IL-17$^+$ T cells and FoxP3$^+$ T cells in human breast cancer

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Abstract.
BACKGROUND: Tumor-infiltrating lymphocytes (TILs) have a strong prognostic value in various forms of cancers. These data often refer to use of the pan-T cell marker CD3, or the cytotoxic T lymphocyte marker CD8α. However, T cells are a heterogeneous group of cells with a wide array of effector mechanisms ranging from immunosuppression to cytotoxicity.
OBJECTIVE: In this study we have investigated the prognostic effects of some unconventional T cell subtypes in breast cancer; $\gamma\delta$ T cells, IL-17$^+$ T cells and FoxP3$^+$ T cells (T_{regs}) in relation to the conventional CD3 and CD8α T cell markers.
METHODS: This was done using immunohistochemistry on a human breast cancer tissue microarray consisting of 498 consecutive cases of primary breast cancer.
RESULTS: Infiltration of $\gamma\delta$ T cells and T cell infiltration in general (CD3), correlated with a good prognosis, while T_{reg} infiltration with a worse. Infiltration of $\gamma\delta$ T cells was associated with a significantly improved clinical outcome in all breast cancer subtypes except triple negative tumors. Only infiltration of either CD3$^+$ or CD8α^+ cells was independently associated with better prognosis for all breast cancer patients.
CONCLUSIONS: This study sheds further light on the prognostic impact of various T cell subtypes in breast cancer.

Keywords: Breast cancer, T lymphocytes, TILs, prognosis, unconventional T cells

1. Introduction

Breast cancer is a heterogeneous disease consisting of different subtypes with varying prognosis [1]. It is however not only the breast cancer subtype that determines the prognostic outcome, but also the tumor microenvironment cell composition [2]. The cells of the immune system are an important part of the tumor microenvironment, where presence of tumor infiltrating lymphocytes (TILs) usually is associated with a better prognosis, while infiltration of myeloid cells is associated with a worse prognosis [3]. T lymphocytes is an important TIL population [3]. In breast cancer, infiltration of T cells has been linked to different outcomes in different breast cancer subtypes. In HER2$^+$ and triple negative breast cancers (TNBCs; ER$^-$PR$^-$HER2$^-$), infiltration of T cells has been associated with an improved prognosis [4], a finding that was even more evident in patients receiving neoadjuvant chemotherapy [5–7]. It has been postulated that this effect may be due to tumor cell death and expression of neoantigens that initiate tumoricidal immune responses [8,9].

T cells can grossly be divided into cytotoxic T lymphocytes (CTLs; CD8$^+$), T helper cells (T_h [T_{h1}, T_{h2}, T_{h9}, T_{FH}, T_{h17} and T_{regs}]; CD4$^+$), $\gamma\delta$ T cells and NKT cells [10]. Regulatory T cells (T_{regs}) are defined as CD4$^+$ CD25$^+$FoxP3$^+$ T cells, and T_{h17} cells as CD4$^+$RORγt$^+$ T cells with high production of the pro-inflammatory cytokine; IL-17A [10,11]. T_{regs} are associated with immune suppression [12] and infiltration of T_{regs} in ER$^+$ breast cancers has been shown to correlate with a worse prognosis [4,13], while studies regarding the prognostic effects of T_{h17} infiltration

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ISSN 1574-0153/17/$35.00

Table 1
Correlations between CD3, T cell subtypes and breast cancer molecular subtypes

Breast cancer molecular subtypes		CD3	$\gamma\delta$ T cells	Treg	IL-17$^+$ T cells	CD8
Triple-negative	Correlation coefficient	0.181**	0.107*	0.216**	−0.125**	0.151**
	P-value	< 0.001	0.03	< 0.001	0.012	0.001
	N	407	412	401	404	493
Luminal A	Correlation coefficient	−0.131**	−0.116*	−0.234**	0.021	−0.05
	P-value	0.008	0.018	< 0.001	0.678	0.267
	N	406	411	400	403	492
Luminal B	Correlation coefficient	0.000	0.034	.052	−0.006	−0.033
	P-value	0.996	0.493	0.303	0.904	0.462
	N	406	411	400	403	492
HER2	Correlation coefficient	0.144**	0.137**	0.231**	−0.023	0.033
	P-value (2-tailed)	0.004	0.005	< 0.001	0.650	0.466
	N	406	411	400	403	492
ER status	Correlation coefficient	−0.234**	−0.182**	−0.300**	0.096	−0.119*
	P-value	< 0.001	< 0.001	< 0.001	0.054	0.008
	N	408	413	402	405	498

Spearman's rho. 2-tailed P-value.

in breast cancer are limited [14–16]. When it comes to CTLs, infiltration of CD8$^+$ lymphocytes does not seem to have a prognostic impact in ER$^+$ breast cancer, but has been shown to correlate with an improved breast cancer specific survival (BCSS) in ER-negative tumors [17]. $\gamma\delta$ T cells are unconventional T cells that express invariant, canonical TCRγ and TCRδ chains. They are either CD4$^-$CD8$^-$ or express CD8$\alpha\alpha^+$ homodimers, and recognize antigen in an MHC/HLA independent manner [18]. The fact that $\gamma\delta$ T cells often express CD8 puts previous studies concerning the prognostic value of CD8$^+$ CTLs in breast cancer in a different light. $\gamma\delta$ T cells are T cells with dual functions and can thus be both tumor promoting and suppressing [19]. In breast cancer, $\gamma\delta$ T cell infiltration was reported to be associated with the HER2 subtype and poor prognosis in a small patient cohort [20]. However, contrasting data have been shown in recent publications where elevated expression levels of genes associated with $\gamma\delta$ T cells had a positive impact on patient survival [21,22].

There are many reports concerning the prognostic and predictive impact of infiltrating T cells on breast cancer survival, but often only CD3, CD8 or FoxP3-positive T cells have been evaluated [23]. Furthermore, the T cell subpopulations $\gamma\delta$ T cells, T_{h17} cells and T_{regs} all have been reported to have dual and opposing effects in different tumor types, therefore making them important to study for each cancer type [24,25]. Also, the presence of IL-17$^+\gamma\delta$ T cells has lately been proposed thus complicating the T_{h17} nomenclature [26]. In this study, we therefore decided to evaluate the prognostic impact of infiltrating $\gamma\delta$ T cells, IL-17$^+$ T cells and FoxP3$^+$ T cells (T_{regs}), as compared to the conventional TIL markers CD8α^+ and CD3$^+$ T cells, in tumors from a retrospective, consecutive cohort of 498 patients with primary breast cancer [27]. Our findings highlight the prognostic effect of each T cell subpopulation in breast cancer and will be important for the future understanding and use of novel drugs like immune checkpoint inhibitors in this disease.

2. Materials and methods

2.1. Breast cancer patients

The study cohort has been previously described [27–30] and included 498 patients that were diagnosed with invasive breast cancer between 1 January 1988 and 31 December 1992 at the Department of Pathology, Skåne University Hospital, Malmö. Patient characteristics are provided in Supplementary Table 1. Ethical approval for this study was obtained from the Ethics Committee at Lund University (Dnr 613/02). Informed consent was not required and patients were offered the option to opt out.

2.2. TMA, immunohistochemistry and staining assessment

Tissue microarrays (TMA) were constructed as previously described [27–30]. Analysis of ER, PR and HER2 status of the tumors in the TMA, was performed according to current Swedish guidelines. For antibodies and staining procedures see Supplementary Table 2.

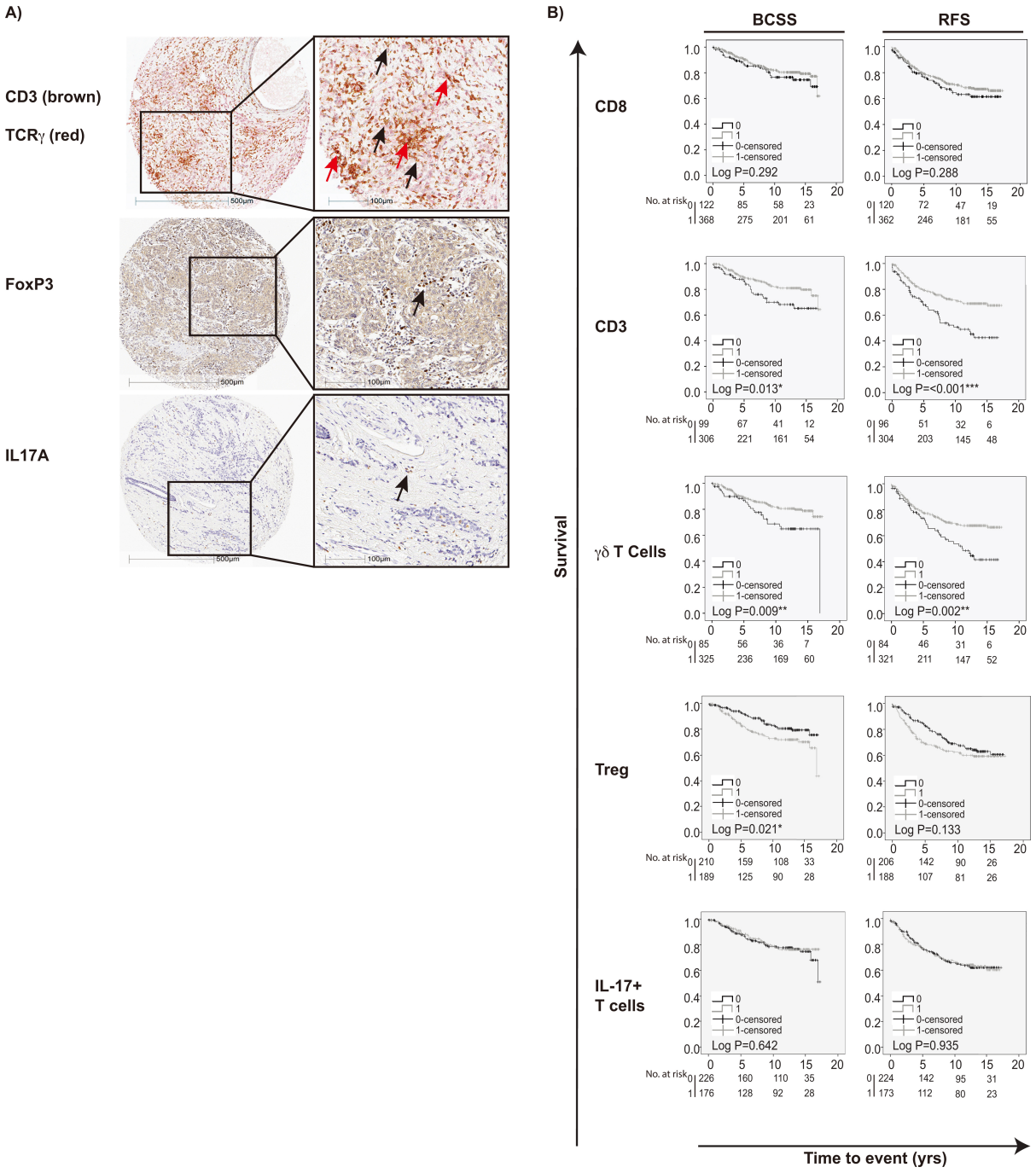

Fig. 1. IHC staining of T cell subpopulations in breast cancers and association to survival outcome. A) IHC stainings in breast cancer TMA showing CD3; brown staining, $\gamma\delta$ TCR; red membranous staining, FoxP3; brown staining and IL-17; brown staining. B) BCSS and RFS according to the infiltration of pan-T cell marker CD3, $\gamma\delta$T cells, T_{regs} and IL-17$^+$ T cells. Log-rank P value < 0.05 was considered significant.

Anti-TCRγ specificity was evaluated using sorted peripheral blood $\gamma\delta$ T cells as positive control (Supplementary Fig. 1). CD3 and TCRγ were manually annotated using a semiquantitative scoring system and denoted as 0 = none, 1 = low, 2 = moderate and 3 = high in each core. CD8 had been scored previously [31]. The total number of IL-17 and FoxP3 positive cells with lymphocytic morphology was an-

Table 2
Crosstab[a] for CD8α+, FOXP3+, IL-17+ and γδ TCR expression in breast cancer

	γδ T cells		
	0	1	*P*-value
Numbers (all breast cancers)			
CD8α+ T cells			
0	31	70	
1	52	257	0.003**,[a]
FOXP3+ T cells			
0	62	142	
1	20	169	< 0.001***,[a]
IL-17+ T cells			
0	47	173	
1	33	143	0.520[a]
Numbers (HER2+)			
CD8α+ T cells			
0	2	1	
1	0	20	< 0.001***,[a]

[a]Pearson's χ^2-test.

notated in each core using automated image analysis (Halo image analysis software, Indica Labs, Corrales, NM, USA). The total number of positive cells was then manually categorized as 0 = none, 1 = low, 2 = moderate and 3 = high. The core with the highest number of positive cells within each case was used in the subsequent statistical analyses.

2.3. Statistical analysis

Spearman's Rho test was applied using non-dichotomized CD3, TCRγ, FoxP3 and IL-17 scoring for associations between T cell populations and breast cancer subtypes. For all other analyses dichotomized variables were constructed; CD3 was dichotomized into low (0, 1) or high (2, 3), TCRγ into absence (0) or presence (1, 2, 3), FoxP3 into low (0, 1) or high (2, 3) and IL-17 into low (0, 1) or high (2, 3). Pearson χ^2-test was used for crosstabs. Kaplan-Meier analysis was used to evaluate the impact of different T cell populations on breast cancer specific survival (BCSS) and recurrence free survival (RFS). Log rank test was applied to analyze any significant differences in Kaplan-Meier survival plots. Cox regression proportional hazards analysis was used to obtain hazard ratios (HR) for BCSS and RFS according to CD8α, CD3, TCRγ, FoxP3 and IL-17 density in both uni- and multivariable analysis, adjusted for dichotomized clinicopathological parameters (age, lymph node metastasis, tumor size, Nottingham histological grade [NHG], ER status, HER2 status, triple negative, luminal A and luminal B), and Ki67. All P values were two-tailed. P value $\leqslant 0.05$ was considered significant. All calculations and statistical analyses were performed with IBM SPSS Statistics version 23.0 (SPSS Inc).

3. Results

3.1. Infiltration of alternative T cell subpopulations and associations to molecular subtypes of primary breast cancer

Representative staining patterns of the analyzed T cell-subpopulations and correlations between different T cell populations and breast cancer subtypes are shown in Fig. 1a and Supplementary Fig. 1.

As shown in Table 1, infiltration of CD8α positive cells correlated positively with TNBC and inversely with ER-positive breast cancers. Infiltration of both CD3 and γδ T cells was associated with TNBC and HER2+ breast cancers, but inversely associated with both the luminal A subtype as well as with ER-positive breast cancers. T_{reg} infiltration was associated with the TNBC and HER2+ breast cancers, but inversely associated with both the luminal A subtype and ER-positive breast cancers. IL-17+ T cell infiltration was inversely associated with the TNBC subtype. It is known that γδ T cells can express CD8αα homodimers [18], but also IL-17A and the transcription factor FoxP3 [19]. As shown in Table 2, there was a significant correlation between CD8α and γδ TCR ($p = 0.003$), as well as for FoxP3 and γδ TCR ($p \leqslant 0.001$), but not with IL-17 expression and γδ TCR (Table 2).

3.2. Prognostic significance of alternative T cell subpopulations in the entire cohort

We next investigated the prognostic impact of individual T cell subsets (CD3, CD8α+, γδ T cells, T_{regs} and IL-17+ T cells) on BCSS and RFS in the entire cohort. Kaplan-Meier analysis revealed that tumor infiltration of any kind of T cells (CD3) correlated with a significantly improved BCSS and RFS (Fig. 1B). There was no significant association between CD8α+ lymphocyte infiltration and BCSS or RFS. γδ T cell infiltration was associated with a significantly prolonged BCSS and RFS. In contrast, T_{regs} were associated with poor BCSS, while IL-17+ T cells did not have any prognostic impact (Fig. 1B).

Univariable Cox regression analysis showed that presence of T cells (CD3), had an overall significant positive impact on BCSS (HR = 0.56; 95% CI = 0.35–0.89; $p = 0.015$) and RFS (HR = 0.50; 95% CI = 0.35–0.72; $p \leqslant 0.001$). This was also true for γδ T

Table 3
Univariable Cox regression analysis for BCSS and RFS

	BCSS			RFS		
	HR (CI 95%)	P-value	N	HR (CI 95%)	P-value	N
Univariable Cox regression analysis with no stratification						
CD8						
0	1.00		122	1.00		120
1	0.78 (0.49–1.24)	0.29	368	0.82 (0.56–1.19)	0.29	362
CD3						
0	1.00		99	1.00		96
1	0.56 (0.35–0.89)	0.015*	306	0.50 (0.35–0.72)	< 0.001***	304
γδ T cells						
0	1.00		85	1.00		84
1	0.53 (0.33–0.86)	0.01**	325	0.56 (0.38–0.81)	0.002**	321
Treg						
0	1.00		210	1.00		206
1	1.67 (1.08–2.60)	0.022*	189	1.30 (0.92–1.83)	0.134	188
IL-17$^+$ T cells						
0	1.00		226	1.00		224
1	0.90 (0.58–1.40)	0.643	176	1.02 (0.72–1.44)	0.935	173
Univariable Cox regression analysis stratified for treated patients						
CD8						
0	1.00		38	1.00		37
1	0.47 (0.25–0.88)	0.019*	120	0.58 (0.34–0.99)	0.044*	120
CD3						
0	1.00		39	1.00		39
1	0.72 (0.37–1.39)	0.324	106	0.59 (0.35–0.99)	0.046*	105
γδ T cells						
0	1.00		34	1.00		34
1	0.53 (0.28–1.01)	0.054	115	0.59 (0.35–1.02)	0.058	114
Treg						
0	1.00		74	1.00		73
1	1.22 (0.66–2.27)	0.528	68	1.20 (0.73–1.98)	0.469	68
IL-17$^+$ T cells						
0	1.00		89	1.00		88
1	0.98 (0.52–1.85)	0.952	56	0.96 (0.57–1.62)	0.886	56
Univariable Cox regression analysis stratified for untreated patients						
CD8						
0	1.00		56	1.00		55
1	0.84 (0.35–2.01)	0.69	161	0.71 (0.39–1.31)	0.28	160
CD3						
0	1.00		38	1.00		37
1	0.35 (0.14–0.87)	0.023*	130	0.36 (0.20–0.68)	0.002**	130
γδ T cells						
0	1.00		32	1.00		31
1	0.50 (0.20–1.30)	0.157	137	0.46 (0.24–0.89)	0.021*	137
Treg						
0	1.00		84	1.00		83
1	2.59 (1.00–6.67)	0.050*	81	1.23 (0.67–2.24)	0.502	81
IL-17$^+$ T cells						
0	1.00		86	1.00		86
1	1.02 (0.43–2.37)	0.988	80	1.23 (0.67–2.26)	0.501	79

Abbreviations: BCSS. breast cancer specific survival; RFS. recurrence free survival; HR. hazard ration.

cells (BCSS [HR = 0.53; 95% CI = 0.33–0.86; p = 0.01] and RFS [HR = 0.56; 95% CI = 0.38–0.81; p = 0.002]), but not for CD8α. In contrast, tumor infiltration of T_{regs} was associated with a decreased BCSS (HR = 1.67; 95% CI = 1.08–2.60; p = 0.022). IL-17$^+$ T cell infiltration showed no significant impact on BCSS or RFS (Table 3).

When adjusted for clinicopathological parameters

Table 4
Multivariable Cox regression analysis for BCSS and RFS with no stratification

	BCSS			RFS		
	HR (CI 95%)	P-value	N	HR (CI 95%)	P-value	N
Age (yrs)						
< 50	1.00		52	1.00		52
$\geqslant 50$	0.44 (0.22–0.87)	0.019*	263	0.65 (0.38–1.13)	0.124	261
Lymph node status						
Negative	1.00		182	1.00		181
Positive	6.53 (3.43–12.41)	< 0.001***	133	2.58 (1.69–3.95)	< 0.001***	132
Ki67 grade						
0–10	1.00		120	1.00		120
11–$\geqslant 25$	2.27 (1.08–4.76)	0.031*	195	1.46 (0.89–2.39)	0.131	193
Size (mm)						
< 20	1.00		186	1.00		186
$\geqslant 20$	2.13 (1.22–3.73)	0.008**	129	1.55 (1.02–2.36)	0.039*	127
NHG						
I and II	1.00		208	1.00		208
III	2.59 (1.34–5.02)	0.005**	107	2.46 (1.51–4.00)	< 0.001***	105
HER2						
Negative	1.00		299	1.00		297
Positive	0.79 (0.21–2.96)	0.722	16	1.26 (0.44–3.66)	0.666	16
Triple negative						
Negative	1.00		291	1.00		289
Positive	6.06 (1.32–27.70)	0.02*	24	13.36 (2.78–64.31)	0.001***	24
ER status						
Negative	1.00		45	1.00		44
Positive	3.20 (0.70–14.73)	0.136	270	6.40 (1.30–31.48)	0.022*	269
Luminal A						
Negative	1.00		152	1.00		151
Positive	0.34 (0.18–0.64)	0.001***	163	0.91 (0.57–1.46)	0.69	162
Luminal B						
Negative	1.00		286	1.00		284
Positive	0.63 (0.26–1.55)	0.315	29	1.09 (0.54–2.20)	0.814	29
CD8						
0	1.00		81	1.00		80
1	0.44 (0.24–0.82)	0.009**	234	0.57 (0.36–0.92)	0.022*	233
CD3						
0	1.00		74	1.00		72
1	0.49 (0.25–0.98)	0.043*	241	0.42 (0.25–0.71)	0.001***	241
γδ T cells						
0	1.00		60	1.00		59
1	0.68 (0.35–1.32)	0.252	255	0.92 (0.55–1.54)	0.748	254
Treg						
0	1.00		156	1.00		154
1	1.62 (0.88–2.98)	0.121	159	1.38 (0.88–2.16)	0.159	159
IL-17+ T cells						
0	1.00		172	1.00		171
1	1.31 (0.74–2.32)	0.352	143	1.41 (0.92–2.16)	0.115	142

Abbreviations: BCSS. breast cancer specific survival; RFS. recurrence free survival; HR. hazard ration; NHG. Nottingham histologic grade.

in multivariable Cox regression analysis, CD8α^+ T cells were independently associated with an improved BCSS (HR = 0.44 95% CI = 0.24–0.82; $p = 0.009$) and RFS (HR = 0.57; 95% CI = 0.36–0.92; $p = 0.022$), while CD3$^+$ lymphocytes were independently associated with an improved BCSS (HR = 0.49 95% CI = 0.25–0.98; $P = 0.043$) and RFS (HR = 0.42; 95% CI = 0.25–0.71; $p = 0.001$; Table 4).

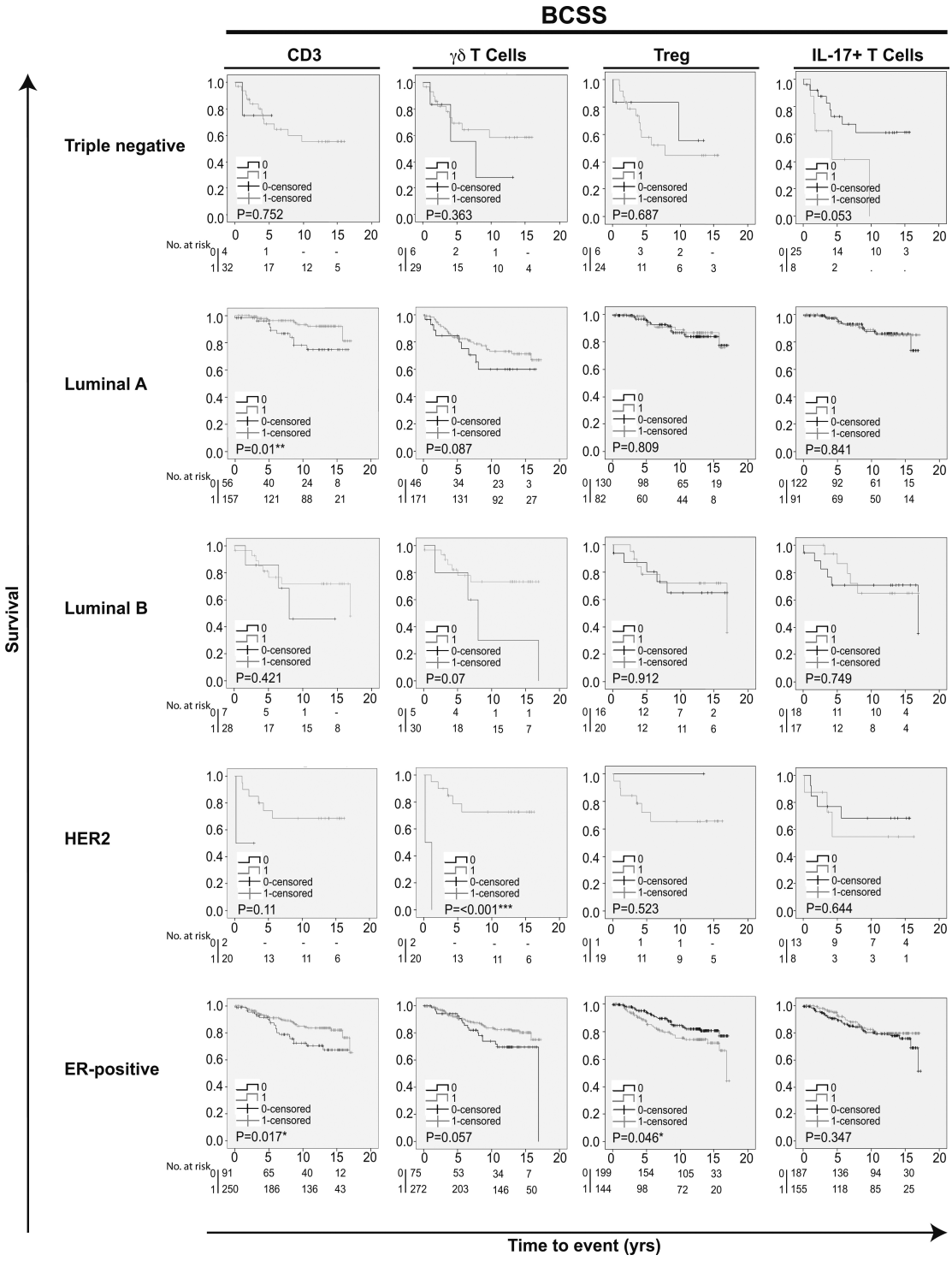

Fig. 2. Kaplan-Meier estimates of breast cancer specific survival according to different infiltrating T cell subpopulations in breast cancer. Impact of pan-T cell CD3, $\gamma\delta$ T cells, T_{regs} and IL-17^+ T cells on BCSS in different breast cancer subtypes. Log-rank P value < 0.05 was considered significant.

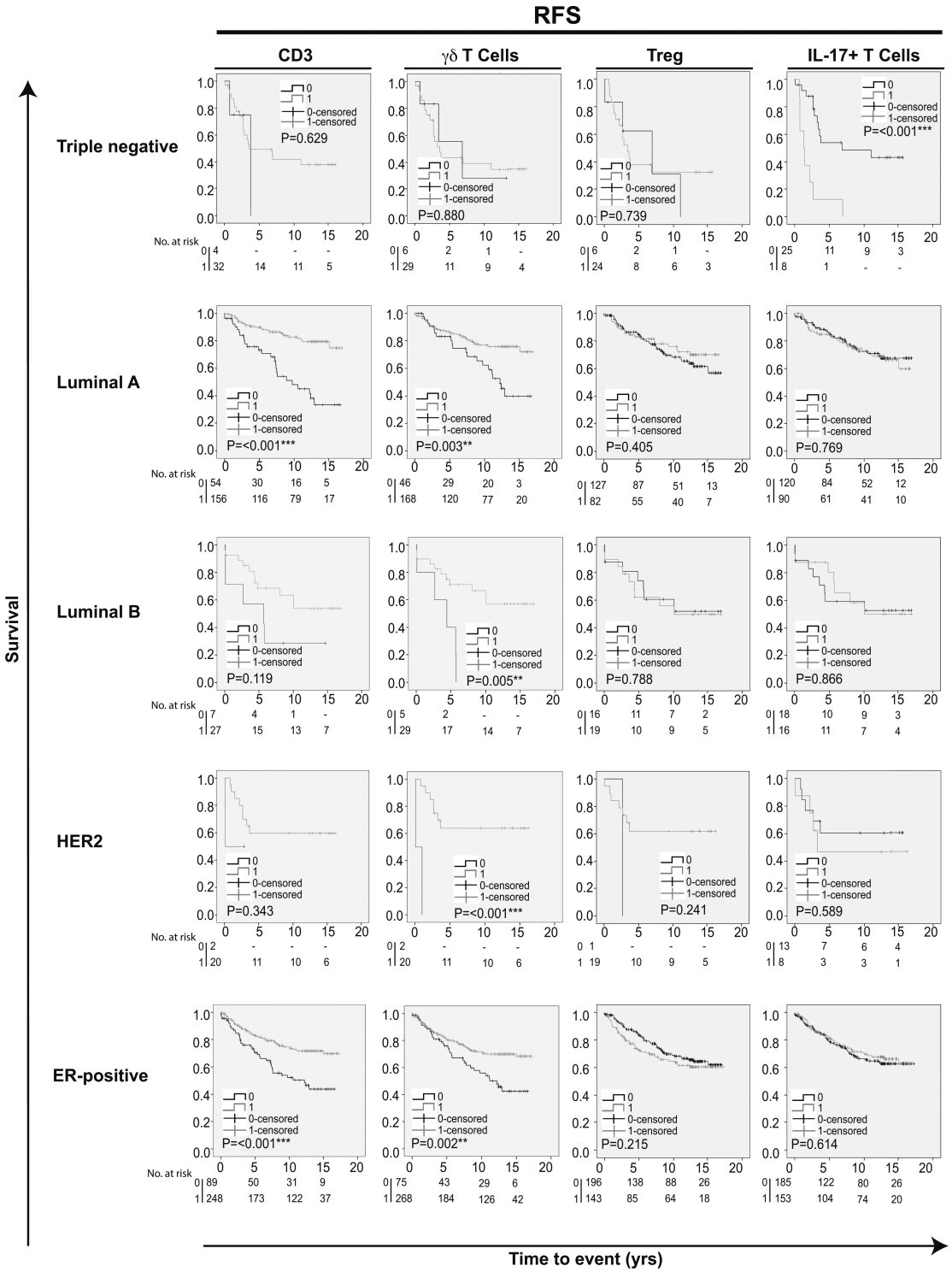

Fig. 3. Kaplan-Meier estimates of recurrence free survival according to different infiltrating T cell subpopulations in breast cancer. Impact of pan-T cell CD3, $\gamma\delta$ T cells, T_{regs} and IL-17$^+$ T cells on RFS in different breast cancer subtypes. Log-rank P value < 0.05 was considered significant.

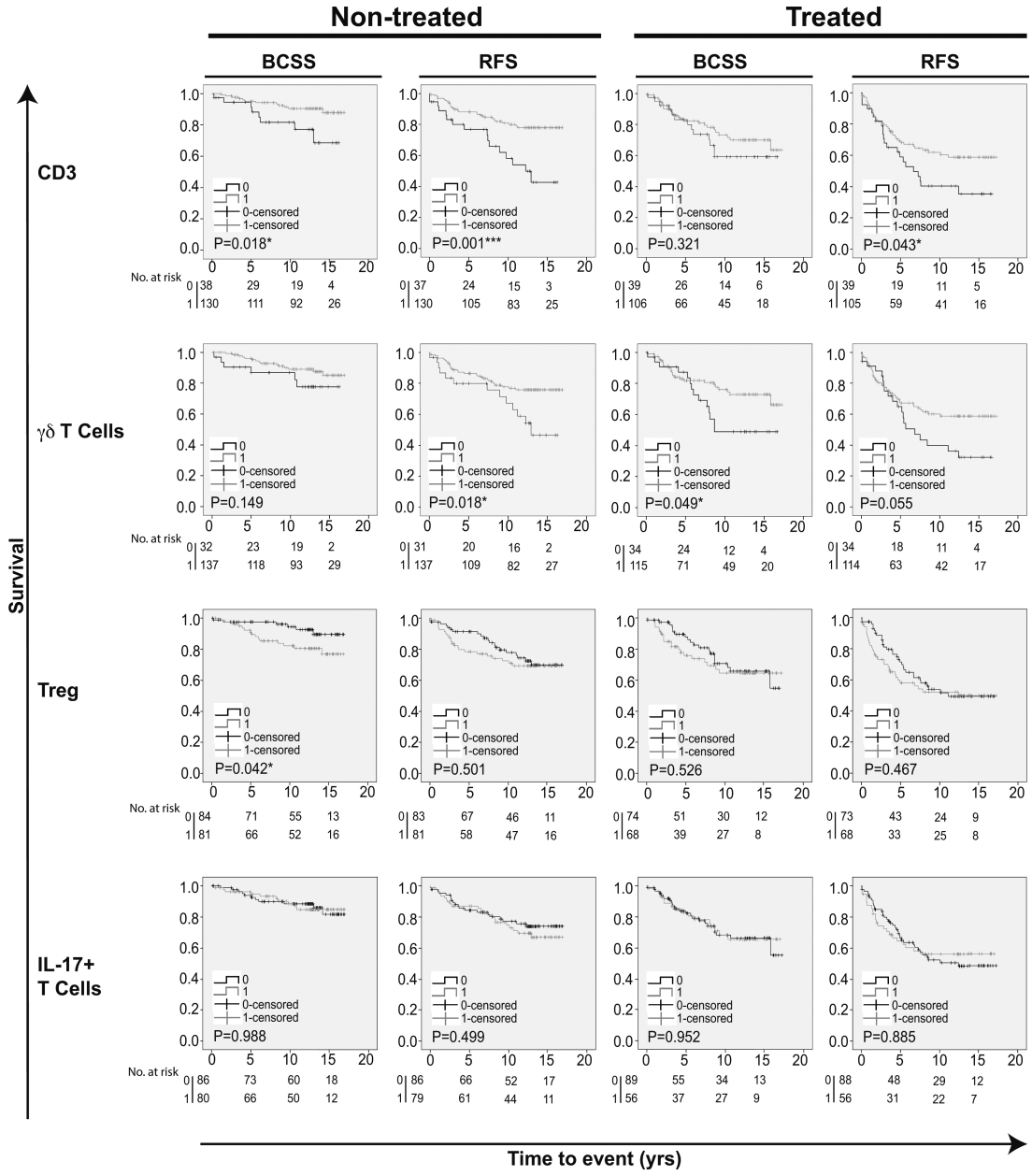

Fig. 4. Kaplan-Meier estimates on survival according to different infiltrating T-cell populations in patients receiving and not receiving adjuvant endocrine therapy. Impact of pan-T cell CD3, $\gamma\delta$ T cells, T_{regs} and IL-17$^+$ T cells on both BCSS and RFS in breast cancer patients receiving and not receiving adjuvant endocrine therapy. The study cohort was conceived before clinical use of ER-testing, hence both groups includes both ER-positive and ER-negative patients. Log-rank P value < 0.05 was considered significant.

3.3. Prognostic value of alternative T cell subpopulations according to breast cancer subtype

Kaplan-Meier analyses were also performed in strata according to different subtypes of breast cancer. This revealed that tumor infiltration of T cells overall (CD3) was associated with an improved prognosis specifically in Luminal A and ER-positive breast cancers (BCSS and RFS; Figs 2 and 3). CD8α^+ and $\gamma\delta$ T cell lymphocyte infiltration was associated with a prolonged BCSS only in the HER2$^+$ breast cancers (Fig. 2), and interestingly, 20 out of 23 HER2$^+$ cases scored positive for both CD8α^+ and $\gamma\delta$ T cell infiltration ($p \leqslant$

0.001; Table 2). Importantly, $\gamma\delta$ T cell infiltration was associated with an improved RFS in Luminal A, Luminal B, HER2 and ER-positive breast cancers (Fig. 3). Tumor infiltration of T_{regs} was associated with a poor BCSS only in ER-positive breast cancers (Fig. 2). In contrast, infiltration of IL-17$^+$ T cells was associated with a poor RFS in TNBCs (Fig. 3).

3.4. Prognostic impact of T cell infiltration in relation to endocrine therapy

Next, we evaluated whether tumor infiltration of different T cell populations had any impact on prognosis in relation to endocrine therapy. The study cohort was conceived before clinical use of ER-testing, hence both groups include both ER-positive and ER-negative patients [27]. Kaplan-Meier analysis in strata according to treatment revealed that tumor infiltration of CD8α^+ T cells only had a positive impact on BCSS and RFS in the treated group (Fig. 2B). CD3, however, was significantly associated with a prolonged RFS in the treated group, and with a prolonged BCSS and RFS in the untreated group (Fig. 4). Infiltration of $\gamma\delta$ T cells was significantly associated with an improved BCSS in patients receiving adjuvant treatment, and with a significantly improved RFS, but not BCSS, in the untreated group (Fig. 4). Infiltration of T_{regs} in tumors of untreated patients was significantly associated with a poor outcome on BCSS, while IL-17$^+$ T cells showed no impact on BCSS or RFS in either group (Fig. 4).

Univariable Cox regression analysis of the endocrine therapy treated group revealed that CD8α^+ T cell infiltration was associated with an improved BCSS (HR = 0.47; 95% CI = 0.25–0.88; $p = 0.019$) and RFS (HR = 0.58; 95% CI = 0.34–0.99; $p = 0.044$). In the endocrine therapy untreated group, CD3 was associated with improved BCSS (HR = 0.35; 95% CI = 0.14–0.87; $p = 0.023$) and RFS (HR = 0.36; 95% CI = 0.20–0.68; $p = 0.002$), while in the endocrine therapy treated patient group, CD3 was associated only with a prolonged RFS (HR = 0.59; 95% CI = 0.35–0.99; $p = 0.046$; Table 3). Presence of tumor infiltrating $\gamma\delta$ T cells was significantly associated with an improved RFS in the untreated group only (HR = 0.46; 95% CI = 0.24–0.89; $p = 0.021$) while T_{regs} were significantly associated with an impaired BCSS (HR = 2.59; 95% CI = 1.00–6.67; $p = 0.050$; Table 3).

In endocrine therapy treated patients, CD8α^+T cells remained an independent prognostic factor for a prolonged BCSS and RFS in multivariable Cox regression analysis (HR = 0.21; 95% CI = 0.09–0.52; $p = 0.001$ and HR = 0.41; 95% CI = 0.20–0.84; $p = 0.014$, respectively; Table 5). Infiltration of $\gamma\delta$ T cells was independently associated with an improved BCSS (HR = 0.32; 95% CI = 0.12–0.82; $p = 0.018$; Table 5) only in endocrine therapy treated patients. In endocrine therapy untreated patients, however, CD3 was an independent factor of a prolonged BCSS (HR = 0.03; 95% CI = 0.00–0.24; $p = 0.001$) and RFS (HR = 0.21; 95% CI = 0.07–0.64; $p = 0.006$), whereas presence tumor infiltrating T_{regs} was revealed to be an independent factor of an impaired BCSS (HR = 17.31; 95% CI = 2.45–122.16; $p = 0.004$; Table 6).

4. Discussion

In this study, we investigated the prognostic value of infiltrating $\gamma\delta$ T cells, T_{regs} and IL-17$^+$ T cells, as compared to the common TIL markers CD3 and CD8α, in invasive breast cancer. The analyses were also performed in relation to adjuvant endocrine therapy.

We show that infiltration of T cells (CD3) had a positive effect on prognostic outcome in breast cancer, which supports previous studies evaluating TILs as a prognostic parameter. However, since CD3 is a pan-T cell marker, further investigation was needed to identify specific T cell subtypes that may play a role in breast cancer progression and as potential responders to immune-therapies.

The only T cell subpopulation with results similar to that of CD3, turned out to be $\gamma\delta$ T cells that were associated with an improved survival in general. When stratified according to breast cancer subtype, infiltration of $\gamma\delta$ T cells was the only T cell population that correlated with a positive outcome in all subtypes (luminal A, luminal B, HER2-positive and ER-positive breast cancers), except TNBCs. It is likely that the TNBC environment not only recruits more $\gamma\delta$ T cells, but also has a negative effect on $\gamma\delta$ T cell activity. This is a controversy that would merit further investigation at the functional level in the future.

In multivariable Cox regression analyses however, both CD3 and CD8α^+ T cell infiltration remained independent factors of a prolonged BCSS as well as RFS. Interestingly, in contrast to general T cell infiltration (CD3), both infiltrating CD8α^+ and $\gamma\delta$ T cells were revealed to be independent prognostic factors in patients receiving endocrine treatment. The fact that several immune cells can express CD8α, including $\gamma\delta$ T cells, makes it difficult to evaluate whether it is conventional

Table 5
Multivariable Cox regression analysis for BCSS and RFS stratified for treated patients

	BCSS			RFS		
	HR (CI 95%)	P-value	N	HR (CI 95%)	P-value	N
Age (yrs)						
< 50	1.00		6	1.00		6
$\geqslant 50$	0.33 (0.07–1.64)	0.176	105	0.19 (0.06–0.58)	0.004**	105
Lymph node status						
Negative	1.00		26	1.00		26
Positive	27.39 (4.93–152.07)	< 0.001***	85	7.22 (2.39–21.82)	< 0.001***	85
Ki67 grade						
0–10	1.00		40	1.00		40
11–$\geqslant 25$	2.36 (0.71–7.85)	0.162	71	1.29 (0.58–2.89)	0.536	71
Size (mm)						
< 20	1.00		47	1.00		47
$\geqslant 20$	2.64 (1.07–6.51)	0.035*	64	2.32 (1.18–4.55)	0.014*	64
NHG						
I and II	1.00		62	1.00		62
III	1.76 (0.56–5.52)	0.333	49	1.82 (0.82–4.03)	0.142	49
HER2						
Negative	1.00		106	1.00		106
Positive	0.99 (0.13–7.91)	0.995	5	0.69 (0.09–5.07)	0.715	5
Triple negative						
Negative	1.00		102	1.00		102
Positive	12.21 (0.82–182.39)	0.07	9	15.78 (1.11–224.73)	0.042*	9
ER status						
Negative	1.00		15	1.00		15
Positive	3.78 (0.21–67.21)	0.365	96	6.39 (0.37–108.91)	0.2	96
Luminal A						
Negative	1.00		64	1.00		64
Positive	0.24 (0.09–0.66)	0.006**	47	0.66 (0.32–1.37)	0.265	47
Luminal B						
Negative	1.00		101	1.00		101
Positive	0.34 (0.07–1.70)	0.189	10	1.09 (0.37–3.22)	0.882	10
CD8						
0	1.00		27	1.00		27
1	0.21 (0.09–0.52)	0.001***	84	0.41 (0.20–0.84)	0.014*	84
CD3						
0	1.00		30	1.00		30
1	0.73 (0.26–2.06)	0.55	81	0.66 (0.32–1.36)	0.262	81
$\gamma\delta$ T cells						
0	1.00		21	1.00		21
1	0.32 (0.12–0.82)	0.018*	90	0.68 (0.31–1.50)	0.342	90
Treg						
0	1.00		55	1.00		55
1	1.09 (0.39–3.06)	0.865	56	1.71 (0.83–3.55)	0.148	56
IL-17$^+$ T cells						
0	1.00		64	1.00		64
1	1.57 (0.66–3.71)	0.309	47	1.53 (0.79–2.96)	0.21	47

Abbreviations: BCSS. breast cancer specific survival; RFS. recurrence free survival; HR. hazard ration; NHG. Nottingham histologic grade.

CTLs that has the major effect on prognosis in our investigation [32–35]. Some studies have proposed that $\gamma\delta$ T cells can acquire both pro- or anti-tumor properties depending on the tumor microenvironment [25]. Furthermore, the regulation of $\gamma\delta$ T cells by inhibitory co-receptors is slightly different as compared to conventional $\alpha\beta$ T cells [36,37]. Indeed, the majority of tumors that scored positive for $\gamma\delta$ T cell infiltration in our cohort also scored positive for CD8α^+ T cells and these may therefore be $\gamma\delta$ T cells, rather than conventional TCR $\alpha\beta^+$ CTLs. This was especially clear in the HER2 group, however, due to the small size of this

Table 6
Multivariable Cox regression analysis for BCSS and RFS stratified for untreated patients

	BCSS			RFS		
	HR (CI 95%)	P-value	N	HR (CI 95%)	P-value	N
Age (yrs)						
< 50	1.00		37	1.00		37
$\geqslant 50$	0.47 (0.12–1.89)	0.288	105	1.15 (0.47–2.81)	0.767	104
Lymph node status						
Negative	1.00		123	1.00		123
Positive	16.11 (3.95–65.65)	< 0.001***	19	2.95 (1.24–7.02)	0.014*	18
Ki67 grade						
0–10	21.00		62	1.00		62
11–$\geqslant 25$	6.20 (2.33–295.19)	0.008**	80	1.92 (0.82–4.47)	0.131	79
Size (mm)						
< 20	1.00		116	1.00		116
$\geqslant 20$	13.97 (3.99–65.23)	0.001***	26	2.52 (0.97–6.51)	0.057	25
NHG						
I and II	1.00		108	1.00		108
III	5.22 (1.20–22.81)	0.028*	34	3.82 (1.66–8.81)	0.002**	33
HER2						
Negative	1.00		133	1.00		132
Positive	0.44 (0.04–4.98)	0.511	9	2.55 (0.60–10.81)	0.203	9
Triple negative						
Negative	1.00		131	1.00		130
Positive	10.20 (0.76–137.56)	0.08	11	16.33 (1.58–169.16)	0.019*	11
ER status						
Negative	1.00		23	1.00		22
Positive	7.87 (0.66- 93.92)	0.103	119	8.02 (0.79–81.24)	0.078	119
Luminal A						
Negative	1.00		60	1.00		59
Positive	0.11 (0.02–0.57)	0.009**	82	1.78 (0.66–4.82)	0.256	82
Luminal B						
Negative	1.00		131	1.00		130
Positive	0.28 (0.03–2.51)	0.255	11	3.14 (0.73–13.50)	0.124	11
CD8						
0	1.00		40	1.00		39
1	0.36 (0.06–2.03)	0.244	102	0.67 (0.31–1.44)	0.303	102
CD3						
0	1.00		32	1.00		31
1	0.03 (0.00–0.24)	0.001***	110	0.21 (0.07–0.64)	0.006**	110
γδ T cells						
0	1.00		28	1.00		27
1	2.64 (0.33–20.96)	0.358	114	1.30 (0.43–3.92)	0.644	114
Treg						
0	1.00		68	1.00		67
1	17.31 (2.45–122.16)	0.004**	74	1.50 (0.68–3.31)	0.317	74
IL-17+ T cells						
0	1.00		74	1.00		74
1	2.12 (0.54–8.28)	0.279	68	1.65 (0.77–3.52)	0.195	67

Abbreviations: BCSS. breast cancer specific survival; RFS. recurrence free survival; HR. hazard ration; NHG. Nottingham histologic grade.

patient group no conclusions should be drawn. In line with our findings, it has already been shown that γδ T cell gene expression signatures were associated with a favorable prognosis across multiple malignancies, also in breast cancer [21,22].

In the herein studied cohort, infiltration of T_{regs} was associated with a worse prognosis, but not in multivariable Cox regression analyses. When stratified according to breast cancer subtypes it was revealed that infiltration of T_{regs} was associated with worse prognosis primarily in ER-positive breast cancers, indicating that T_{regs} could serve as a potential therapeutic

target in ER-positive breast cancers specifically. Also, a significant association between presence of FoxP3$^+$ and $\gamma\delta$ T cells was found. However, the fact that the prognostic impact of infiltrating $\gamma\delta$ T cells and T_{regs} was contradictory suggests that these are not regulatory FoxP3$^+\gamma\delta$ T cells [38].

We show that infiltration of IL-17$^+$ T cells in general has no prognostic impact. However, when stratified according to breast cancer subtype it was revealed that infiltration of IL-17$^+$ T cells was associated with a worse prognosis in TNBC, specifically. TNBCs often produce inflammatory mediators and it is likely that T_{h17} cells are either attracted to or even play a role in regulating the TNBC microenvironment [39]. Studies on the clinical relevance of T_{h17} infiltration in breast tumors has been performed previously, but on small patient cohorts and with opposing results [14–16,40]. Nonetheless, these studies point out the complex nature of T_{h17} cell behavior in a tumor context. Tumor infiltrating T_{h17} cells have been associated with both tumor promoting and inhibiting properties [39]. Indeed, reports investigating the plasticity of T_{h17} in breast cancer have shown that T_{h17} even might be immunosuppressive [16,41]. Lately, the presence of IL-17$^+$ $\gamma\delta$ T cells that promotes myeloid cell accumulation and polarization has been reported as an important mechanism in breast cancer progression and metastasis in mice [26] and supporting evidence was recently shown also for human colorectal cancers [42]. The strength of our study lies in the size of the breast cancer cohort, but also that IL-17$^+$ T cell and $\gamma\delta$ T cell infiltration was analyzed in parallel. We found no correlation between presence of IL-17$^+$ cells and $\gamma\delta$ T cells in this study.

5. Conclusions

In conclusion, our results demonstrate that in breast cancer, infiltration of T cells (CD3) and $\gamma\delta$ T cells are associated with good clinical outcome, while T_{regs} are associated with worse clinical outcome. Infiltration of $\gamma\delta$ T cells was associated with a significantly improved prognosis in all breast cancer subtypes except TNBC. However, when adjusted for clinicopathological parameters, only CD8α T cells and CD3 infiltration proved to be independently associated with clinical outcome. Furthermore, except for CD3, infiltration of $\gamma\delta$ T cells was the only marker that correlated with an improved clinical outcome in both endocrine treated and untreated patients. When adjusted for clinicopathological parameters, both CD8α T cells and $\gamma\delta$ T cell infiltration was independently associated with an improved prognosis for patients receiving adjuvant endocrine therapy, while CD3 and T_{reg} infiltration was associated with clinical outcome in untreated patients. Finally our findings indicate that in breast tumors, the infiltrating CD8α^+ T cells may indeed be $\gamma\delta$ T cells, which merit further investigation concerning regulation and checkpoint inhibitors. This study renders new light on previously unexplored aspects of tumor infiltrating lymphocytes, and highlights potential prognostic aspects of $\gamma\delta$ T cells, IL-17$^+$ T cells and T_{regs} in breast cancer, with particular reference to molecular subtypes and endocrine adjuvant treatment.

Acknowledgments

The authors wish to thank Ms Elise Nilsson and Mrs Kristina Ekström-Holka for professional help with IHC. This work was generously supported by grants from the Swedish Cancer Society, Gunnar Nilsson Cancer Foundation, MAS Cancer Foundation, Åke Wibergs Foundation and Percy Falks Foundation.

Conflict of interest

The authors declare no conflict of interest.

Supplementary data

The supplementary files are available to download from http://dx.doi.org/10.323/CBM-170026.

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