The tumor-infiltrating effector regulatory T cell:CD8+ lymphocyte ratio in invasive breast cancer

Morihito Okada (morihito1217@gmail.com)
"Hiroshima Daigaku" https://orcid.org/0000-0001-7068-6608
Noriko Goda
"Hiroshima Daigaku"
Shinsuke Sasada
"Hiroshima Daigaku"
Hideo Shigematsu
Kure iryo center
Norio Masumoto
"Hiroshima Daigaku"
Noriyuki Shiroma
"Hiroshima Daigaku"
Koji Arihiro
Hiroshima daigaku
Takayuki Kadoya
"Hiroshima Daigaku"
Yujiro Kidani
Osaka daigaku
Naganari Ohkura
Osaka Daigaku
Hiroyoshi Nishikawa
Nagoya Daigaku
Shimon Sakaguchi
Osaka Daigaku

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Abstract

Background Tumor-infiltrating lymphocytes (TILs) in breast cancer comprise immunostimulating and immunosuppressive components. Although FOXP3+ TILs are prototypical immunosuppressive TILs, only effector regulatory T cells (eTreg), a subset of immunosuppressive FOXP3+ TILs, are undetectable on immunohistochemical staining. This study aimed to evaluate the immunosuppressive potential of eTregs and the role of prototypical immunostimulatory CD8+ TILs in invasive breast cancer. Methods Fresh TILs extracted from 84 invasive breast cancer patients were analyzed via flow cytometry. We evaluated eTregs (CD4+FOXP3highCD45RA−), other FOXP3+ Treg subsets (naïve and non-Tregs), and total CD8+CD4- TILs. Clinicopathological factors, including histopathological characteristics, were also assessed. Results The median eTreg proportion of the total CD4+TILs was 18.7% (interquartile range [IQR], 16.4–25.5%); CD8+TILs, 124% (IQR, 87.5–140%). The proportion of eTregs to total FOXP3+ TILs varied (median, 65.6%; range, 10.1–93.2%). In an immunosuppression assay, only eTregs displayed potent immunosuppression; however, other Treg subsets did not. Among 39 patients who received neoadjuvant chemotherapy, eTreg subsets and pathological compete response (pCR) did not differ significantly, while pCR rates were significantly higher among individuals with a high than those with a low CD8+/eTreg ratio (90.2% vs 33.3%; P<0.05). Among all patients, a high CD8+/eTreg ratio tended to be associated with better disease-free survival rather than a low CD8+/eTreg ratio (P=0.09). Conclusions The CD8+/eTreg ratio is simple, optimal indicator of cancer immunity, and a high CD8+/eTreg ratio enhances the prognosis and treatment response in invasive breast cancer patients. However, further studies are required to validate the present findings.

Background

Breast cancer is a predominant malignant tumor with increasing morbidity and mortality rates among women worldwide.[1] Numerous studies have reported that tumor-infiltrating lymphocytes (TILs) in breast cancer are strongly associated with the therapeutic response and patient prognosis.[2-5] Evaluation of TILs typically involves enumeration of TILs in hematoxylin-eosin-stained tissue specimens. Subsequently, the qualitative assessments were performed because TILs are functionally heterogeneous and comprise immune-progressive or -suppressive components. [6]

Regulatory T cells (Tregs), prototypical immune-suppressive TILs, express transcription factor forkhead box P3 (FOXP3) and regulate anti-cancer immunity.[7-9] An increase in Tregs in breast cancer serves as an adverse prognostic factor[10-13]. However, subsequent studies have reported that Tregs predicted favorable outcomes[14-17] or that their utility as a prognostic factor was unclear.[18] The role of Tregs in breast cancer TILs remains controversial.[18] However, $CD8^+$ TILs are prototypical immunostimulatory TILs, and $CD8^+$ TILs are an independent predictors of therapeutic responses[20,21] or survival.[22] Furthermore, numerous studies reported the evaluation of $CD8^+/FOXP3^+$ ratio of TILs as a sensitive marker of tumor immune responses in breast cancer rather than evaluation of $FOXP3^+$ or $CD8^+$ TILs alone.[23-27] These studies

indicate that a balance in the TIL component, which have conflicting function, influence breast cancer prognosis.

FOXP3 is a general Treg marker for immunohistochemical staining. However, $FOXP3^+$ cells are functionally heterogeneous and can be classified into the following three components via flow cytometry on the basis of FOXP3 and naïve T cell marker CD45RA expression levels: “naïve Tregs,” “effector Tregs (eTregs),” and “non-Tregs.” Only eTregs have an immunosuppressive function, while other cell subsets are non-suppressive and secrete inflammatory cytokines. [8,28] In colorectal cancer, variations in the tumor-infiltrating Treg component are caused by immunologically relevant genes, thus affecting patient prognosis.[29] Infiltration of only eTregs from total $FOXP3^+$ cells is undetectable via FOXP3 immunohistochemical staining. Variations in eTreg infiltration have not been investigated in breast cancer, and the role of eTregs in breast cancer is unclear. This study aimed to investigate the association of tumor-infiltrating eTreg and $CD8^+$TILs with clinical outcomes of invasive breast cancer patients.

Methods

Patients and treatments

In total, 84 early breast cancer patients who underwent complete resection between December 2015 and November 2016 were included. Patients with non-invasive breast cancer were excluded. A neoadjuvant chemotherapy (NAC) regimen comprised docetaxel (75 mg/m^2, every 3 weeks) for four cycles, followed by FEC (5-fluorouracil, 500 mg/m^2; epirubicin, 100 mg/m^2; cyclophosphamide, 500 mg/m^2, every 3 weeks) for four cycles. Patients with human epidermal growth factor receptor 2 (HER2)-positive breast cancer received trastuzumab (8 mg/m^2 as the first dose and 6 mg/m^2 thereafter) every 3 weeks together with docetaxel. Pathological complete response (pCR) was defined as the absence of invasive residual tumors in the primary lesion and axillary lymph nodes.[30] This study was approved by the Ethics Committee of Hiroshima University and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Breast cancer tissue collection and extraction of TILs

Fresh invasive breast cancer tissue specimens were harvested via core needle biopsy, vacuum-assisted biopsy (Mammotome elite, Cincinnati, OH, USA), or surgery. Biopsy specimens of patients receiving NAC were harvested before treatment. Tumor specimens were harvested as follows: 3 to 5 samples with 16-gauge biopsy needles, >6 samples with 13-gauge Mammotome needles, or an area of at least 10 mm × 10 mm × 2 mm shaved with a razor during surgery. Fresh TILs were extracted using a previously described protocol.[29] Fresh tissues were rapidly diced using tissue scissors and homogenized using a GentleMACS dissociator (Miltenyi Biotech, Bergisch Gladbach, Germany). TILs were harvested from the cell suspension.

Flow cytometry

Fresh TILs were washed with phosphate-buffered saline containing 2% fetal calf serum and APC-conjugated anti-CD4 mAb (BD Biosciences, Franklin Lakes, NJ, USA), V500-conjugated anti-CD8 mAb (BD Biosciences), FITC-conjugated anti-CD45RA mAb (BD Biosciences), and Fixable Viability Dye (eBioscience, San Diego, CA, USA). Intracellular FOXP3 was stained using an anti-FOXP3 mAb and FOXP3 Staining Buffer Set (eBioscience) in accordance with the manufacturer's instructions. Cells were analyzed using LSRFortessa (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA).

Determination of the TIL component

TILs were categorized in accordance with previously described protocols.[8,28] TILs were gated into $CD4^{-}CD8^{+}$ T cells and $CD4^{+}CD8^{-}$ T cells, and the $CD4^{+}CD8^{-}$ T cell fraction was further gated on the basis of FOXP3 and CD45RA expression as follows: naïve Tregs ($FOXP3^{low}CD45RA^{+}$), eTregs ($FOXP3^{high}CD45RA^{-}$), and non-Tregs ($FOXP3^{low}CD45RA^{-}$). The ratio of each TIL subpopulation to the total $CD4^{+}CD8^{-}$ TIL subset was determined. Moreover, lymphocytes were harvested from PBMCs and lymphocytes from normal breast tissue (LNBT).

Treg suppression assay

Naïve Tregs, eTregs, and non-Tregs were isolated from TILs, LNBTs, and PBMCs, using a FACS Aria II system (BD Biosciences). Responder $CD25^{-}CD4^{+}$ T cells (control) were purified from PBMCs, labeled with carboxyfluorescein succinimidyl ester (CFSE), and mixed with purified individual components at a ratio of 1:3 (responder T cell:individual components). Treg Suppression Inspector reagent (Miltenyi Biotec) was added in accordance with the manufacturer's instructions. Cells were cultured for 5 d, and the proliferation of CFSE-labeled cells was evaluated.

Pathological assessment and evaluation of stromal TILs

Histological characteristics including nuclear grade, estrogen receptor (ER) and HER2 status, Ki-67 labeling index, and stromal TILs, were assessed by two pathologists indipendently. ER and HER2 status was assessed in accordance with the American Society of Clinical Oncology/College of American Pathologists Guidelines[33,33]. Breast cancer subtypes were defined as ER(+), HER2(+), and triple-negative (TN; ER(-) and HER2(-)). The Ki-67 labeling index was scored as high (≥20%) and low (<20%). Stromal TILs were assessed via hematoxylin-eosin (HE)-stained slides of maximum tumor lesions, using the method of the International TILs Working Group 2014[34]. Lymphocyte predominant breast cancer (LPBC) was defined as stromal TILs ≥60%.

Statistical analyses

Basic statistics for TIL subpopulations were expressed as the median and the interquartile range (IQR). The Wilcoxon rank sum test was performed for multiple pairwise comparisons. Kaplan-Meier curve analysis was performed for disease-free survival with the rog-rank test. Receiver operating characteristic curves were constructed to determine the cutoffs of parameters predicting pCR. Statistical significance

was set at $P < 0.05$. All statistical analyses were performed using JMP Pro14 SAS software (SAS Institute Inc., Cary, NC, USA).

Results

Clinical and demographic data

Table 1 summarizes the clinicopathological characteristics of 84 patients with invasive breast cancer. The T status of 90.3% of patients was T1 and T2, and 61.9% of patients did not experience nodal metastasis. We intended to harvest core needle biopsy specimens before NAC for $HER2^+$ and TN subtypes. Among 39 patients receiving NAC, 20 achieved pCR. Furthermore, 29.8% cases were of LPBC on H&E staining.

Detection of eTregs and other functionally heterogenous $FOXP3^+$ TILs

From fresh TILs, $CD4^+CD8^-$ T cells were categorized as eTregs, naïve Tregs, and non-Tregs via flow cytometric analysis for CD45RA and FOXP3 (Figure 1a, left). Treg proportions varied in each case (Figure 1a, center-sample 1 and right sample2). In the suppression assay, though eTregs demonstrated strong immunosuppressive activity, naïve Tregs and non-Tregs displayed no immunosuppressive effects (Figure 1b). The proportion of eTreg among total $FOXP3^+$ TILs varied markedly (median, 65.6%; range, 10.1–93.2%) (Figure 1c). The median number of total TILs was 6.9×10^5 (IQR [interquartile range], $1.2–82 \times 10^5$) cells, which was sufficient for the analyses. Among $CD4^+$ TILs, the median percentage of total $FOXP3^+$ cells was 30.7% (IQR, 24.9–40.6), with naïve Tregs (2.0% IQR, 0.9–3.3), eTregs (18.7% IQR, 16.4–25.5), and non-Tregs (10.0% IQR, 4.9–11.55) (Figure 1d). Blood cells (PBMCs: $FOXP3^+$ [8.5% (4.3–9.8)]), naïve Tregs (2.1% [1.9–2.6]), eTregs (4.0% [3.8–4.2]), and non-Tregs (2.2% [1.8-2.9]) and normal breast tissue (LNBT: $FOXP3^+$ [median, 9.9%; IQR, 7.2–11.9]), naïve Tregs (1.2% [1.0–1.3]), eTregs (4.0% [3.9–4.3]), and non-Tregs (5.0% [3.8–6.9]) were also compared (Figure 1d). eTreg cells were increased in TIL populations unlike among PBMCs and LNBTs (Figure 1d).

Association between eTregs and $CD8^+$TILs

The median percentage of $CD8^+$ TILs to $CD4^+$TILs was 124% (IQR, 87.5–140). Table 2 shows the association of clinicopathological features with $FOXP3^+$, eTregs, and $CD8^+$TILs. $CD8^+$TILs were associated with HER2 amplification and LPBC. Treg components were similar among different breast cancer subtypes. $FOXP3^+$ expression and the number of eTregs were not significantly associated with clinicopathological factors.

Association between eTregs and $CD8^+$TILs and response to neoadjuvant chemotherapy

The therapeutic responses of 39 patients receiving NAC were evaluated. TN and HER2 subtypes achieved a higher pCR rate than the luminal subtype (TN:72%, HER2(+):55%, and ER(+) 38% $P<0.05$). No significant

difference was observed between eTregs and pCR (eTreg high 50.3% vs $CD8^+$ low 55.5%; P> 0.05) with respect to the TIL component. Patients displaying a high $CD8^+$/eTreg ratio achieved significantly higher pCR than those displaying a low $CD8^+$/eTreg ratio (91.7% vs 33.3% P=0.001), while no significant difference was observed between the $CD8^+$/$FOXP3^+$ subset and pCR rate ($CD8^+$/$FOXP3^+$ high 58.3% vs $CD8^+$/$FOXP3^+$ low 52.5%; P> 0.05) (Figure 2). To stratify high and low group of each parameter, receiver operating characteristic curves were constructed to determine the cutoff values of parameters predicting pCR (eTreg: cutoff value:9.1% of $CD4^+$cells; high: n=20, low: n=19, $CD8^+$: cutoff value:113% of $CD4^+$cells high: n=18, low=21, $CD8^+$/eTreg: cutoff value:13.3, high n= 13 , low n=26 . $CD8^+$/$FOXP3^+$: cutoff value:4.6, high n=12, low n=27).

Association of eTregs and $CD8^+$TILs with the survival of breast cancer patients

On survival analysis of all patients, eTreg-high and -low groups did not differ significantly (Figure 3a); however, the high $CD8^+$/eTreg group displayed an almost significantly greater disease-free survival than the low group (90.2% vs. 69.6% [CI], 0.53 to 1.92; P=0.0916) (Figure 3d), which served as a better prognostic marker than $CD8^+$ cells ($CD8^+$ high group 83% vs. $CD8^+$ low group 72% [CI], 0.65 to 2.49; P=0.316) (Figure 3b). The $CD8^+$/$FOXP3^+$ ratio did not differ significantly between the high and low groups (Figure 3c). The cut-off value of high and low groups of each parameter were same as analysis of pCR (eTreg: high: n=43, low:n=39, $CD8^+$: high: n=40, low=44, $CD8^+$/eTreg: high n= 21 , low n=63 . $CD8^+$/$FOXP3^+$: high n=33, low n=51).

Discussion

This study focused on immunologically functional heterogenous TILs and evaluated the balance between the immunostimulatory and immunosuppressive potential of TILs for breast cancer patients. To our knowledge, this study is the first to assess eTregs among breast cancer TILs and verify the association between the functional balance of TILs and clinical outcomes of breast cancer. The present results indicate the functional heterogeneity of $FOXP3^+$ regulatory T cells in fresh TIL samples. Tregs are a subset of $CD4^+$ T cells and essential effector cells to maintain immune homeostasis.[7,8,35] Although Tregs have been considered homogeneously immunosuppressive TILs in breast cancer, the present results show that only eTregs played an immunosuppressive role, and eTregs were more abundant in breast cancer tissues than among PBMCs and LNBTs, concurrent with previous reports on other cancers, such as colorectal,[29] and gastric cancers.[36] Heterogeneity among infiltrating Tregs indicates that T cells are activated and acquire functions in the tumor microenvironment. The present results indicate that low proportions of eTregs among the total $FOXP3^+$ TILs among breast cancer patients can result in an overestimation of their immunosuppressive function upon immunohistochemical staining for FOXP3. This implies the possibility of conflicting findings among studies on immunohistochemical evaluation of FOXP3 for breast cancer prognosis. LPBC is considered a robust biomarker for pathological responses to NAC, regardless of molecular subtypes[2,4] and survival in TN and HER2(+) subtypes.[3] This study indicates

that the $CD8^+$/eTreg ratio is a promising predictor of pCR and progression-free survival compared to the $CD8^+$/$FOXP3^+$ ratio, with a reasonable theoretical basis. This study indicates the tendency of a better prognosis based on the $CD8^+$/$FOXP3^+$ ratio, resulting from a low number of patients and short follow-up durations potentially attributable to the lack of statistical power. Anti-CD15 antibodies can specifically identify eTregs.[38] Dual immunohistochemical staining for FOXP3 and CD15s may be a promising method to detect eTreg infiltration with a large sample size.

This study has several limitations. First, the number of cases was small and the follow-up period was short. Our findings do not elucidate the mechanism underlying an increase in the eTreg population in the tumor microenvironment. Second, we did not evaluate other TIL components. Recently, an exhaustive and promising evaluation of breast cancer TILs via single-cell RNA sequencing has been reported.[39] The genomic background or mechanisms underlying these variations in TIL components warrant further elucidation in future studies. In addition, eTregs express immune checkpoint components, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death-1 (PD-1), such that regulation of tumor-infiltrating Tregs serve as potential targets for cancer immunotherapy.[40-43] Further studies are required to determine the association between our present findings and those of studies evaluating the response to immune checkpoint inhibitors in breast cancer patients.

Conclusions

That the $CD8^+$/eTreg ratio is a simple and optimal marker for cancer immunity, and an increase in this ratio indicates a better prognosis and treatment response in invasive breast cancer patients; however, further large-scale studies are required to validate the present findings.

Abbreviations

TIL: tumor-infiltrating lymphocytes; eTregs: effector regulatory T cells; FOXP3: forkhead box P3; NAC: neoadjuvant chemotherapy; HER2: human epidermal growth factor receptor 2; pCR: Pathological complete response; LNBT: lymphocytes from normal breast tissue; CFSE: carboxyfluorescein succinimidyl ester; IQR: interquartile range.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Hiroshima University and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Concent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable reqest.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

NG, SS, HS designed the study. NG, SS, HS, NM, NS, KA collected data. NG, SS, HS, NM, TK analysed the data. NG, SS, HS, NM, TK organized the data. NG, YK, HN, SS, MO reviewed the papers and revised manuscript. All the authors have read and approved the final manuscript. All authors contributed to data analysis, drafting of paper and manuscript revisions and agree to be accountable for aspects of the work.

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Tables

Table 1. Patient characteristics

	Number (%)
Age (year), median (range)	57 (33–83)
T status	
T1	22 (26.1)
T2	54 (64.2)
T3	4 (4.7)
T4	4 (4.7)
Nodal metastasis	
Negative	52 (61.9)
Positive	32 (38.1)
Nuclear grade	
1	4 (4.8)
2	27 (32.1)
3	53 (63.1)
LVI positive	31 (36.9)
ER positive	50 (59.5)
HER2 positive	28 (33.3)
Triple Negative	20 (23.8)
Ki-67 labeling index	
< 20%	20 (23.8)
≥20%	64 (76.2)
Stromal TILs	
Non-LPBC	59 (70.2)
LPBC	25 (29.8)
Neoadjuvant chemotherapy	39 (46.4)
Non-pCR	19 (48.7)
pCR	20 (51.3)

ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; LPBC, lymphocyte predominant breast cancer; LVI, lymphovascular invasion; pCR, pathological complete response; TILs, tumor-infiltrating lymphocytes.

Table 2. Association of clinicopathological features with $FOXP3^+$, eTregs, and $CD8^+$TILs

	n	Total FOXP3+		eTreg		CD8+	
		Median (IQR)	*P*	Median (IQR)	*P*	Median (IQR)	*P*
Age							
< 50	28	31.7 (24.9–38.8)	0.913	21.2 (15.6–26.5)	0.605	113.0 (93.3–141.0)	0.849
≥ 50	56	30.7 (24.2–40.9)		20.1 (16.5–24.9)		116.0 (86.0–140.5)	
T status							
T1	22	34.8 (25.5–40.3)	0.401	22.4 (18.0–26.0)	0.344	113.5 (76.5–153.5)	0.665
T2–4	62	30.0 (24.4–40.3)		19.9 (16.1–25.5)		115.5 (89.5–139.5)	
Nodal metastasis							
Negative	52	30.7 (23.8–38.8)	0.289	20.0 (15.7–24.9)	0.384	117.0 (86.0–150.3)	0.522
Positive	32	32.3 (26.0–42.7)		21.4 (16.6–28.6)		108.5 (88.0–133.0)	
Nuclear grade							
1–2	31	33.8 (24.5–42.6)	0.347	20.0 (13.0–26.8)	0.792	113.0 (86.0–128.0)	0.319
3	53	29.3 (24.5–38.7)		20.3 (16.5–25.4)		117.0 (87.0–154.0)	
LVI							
Negative	57	30.7 (24.7–39.4)	0.427	20.0 (14.8–23.0)	0.193	117.0 (87.0–147.5)	0.515
Positive	27	33.8 (23.7–42.0)		22.0 (16.5–29.0)		104.0 (86.0–132.0)	
ER							
Negative	34	28.7 (25.7–38.2)	0.417	20.3 (16.1–25.8)	0.971	114.0 (90.5–153.5)	0.444
Positive	50	32.6 (23.8–41.9)		20.1 (16.2–25.5)		115.0 (85.8–138.3)	
HER2							
Negative	56	32.7 (25.1–40.9)	0.553	20.2 (14.2–25.4)	0.439	113.0 (83.8–131.0)	0.031
Positive	28	28.7 (23.2–38.7)		20.2 (17.1–26.5)		130.5 (93.3–192.8)	
Ki-67 proliferation index							
<20%	20	33.3 (26.0–40.6)	0.834	20.2 (13.0–26.1)	0.896	108.5 (87.5–131.8)	0.386
≥20%	64	30.7 (24.2–40.1)		20.2 (16.5–25.5)		117.0 (86.5–150.3)	
Stromal TILs							
Non-LPBC	25	36.7 (25.5–42.4)	0.136	20.8 (18.8–26.4)	0.178	155.0 (92.0–213.5)	0.002
LPBC	59	30.6 (24.1–38.3)		19.9 (13.0–25.5)		110.0 (86.0–128.0)	

Figures

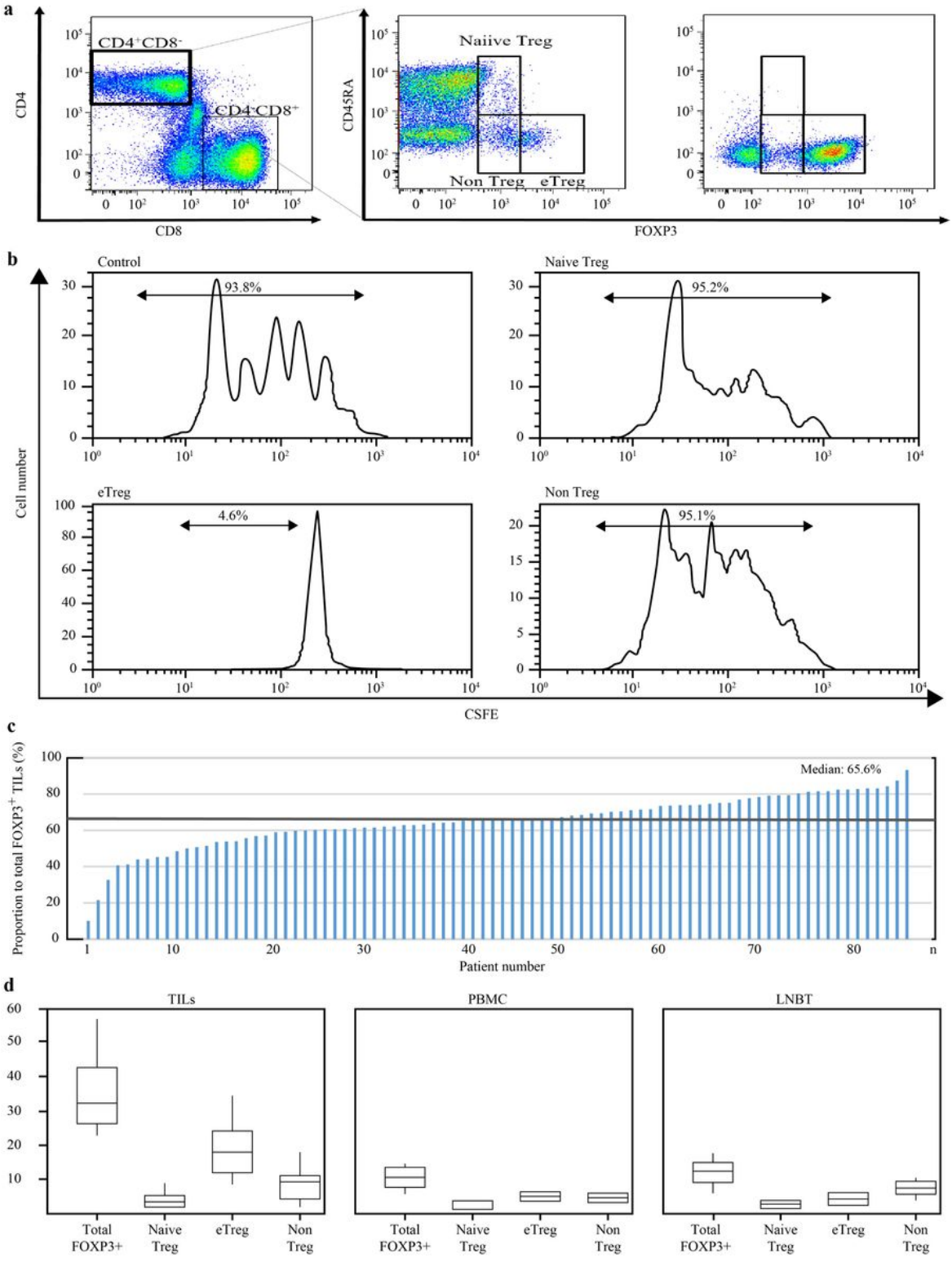

Figure 1

a: Detection of the TIL subpopulation via flow cytometry in invasive breast cancer tissue. CD4−CD8+ T cells and CD4+CD8− T cells among TILs were separated via flow cytometry (left), and the CD4+CD8− cells were further classified into naïve Tregs, eTregs, and non-Tregs via FOXP3 and CD45RA staining. The variation in the Treg subpopulation in each case (center-sample 1 and right sample 2). b: Suppression assay of breast cancer TILs. eTreg cells showing potent immunosuppressive activity, whereas naïve Treg

and non-Treg cells did not display immunosuppressive effects. Arrows and “%” indicates the proliferation of co-cultured responder T cell labeled with carboxyfluorescein succinimidyl ester. c: the proportion of eTreg to total FOXP3+ TILs. d: Proportions of FOXP3+ cells and Treg subpopulations in breast cancer, PBMCs, and LNBT. LNBT, lymphocytes from normal breast tissue; PBMCs, peripheral blood mononuclear cells; TIL, tumor-infiltrating lymphocytes.

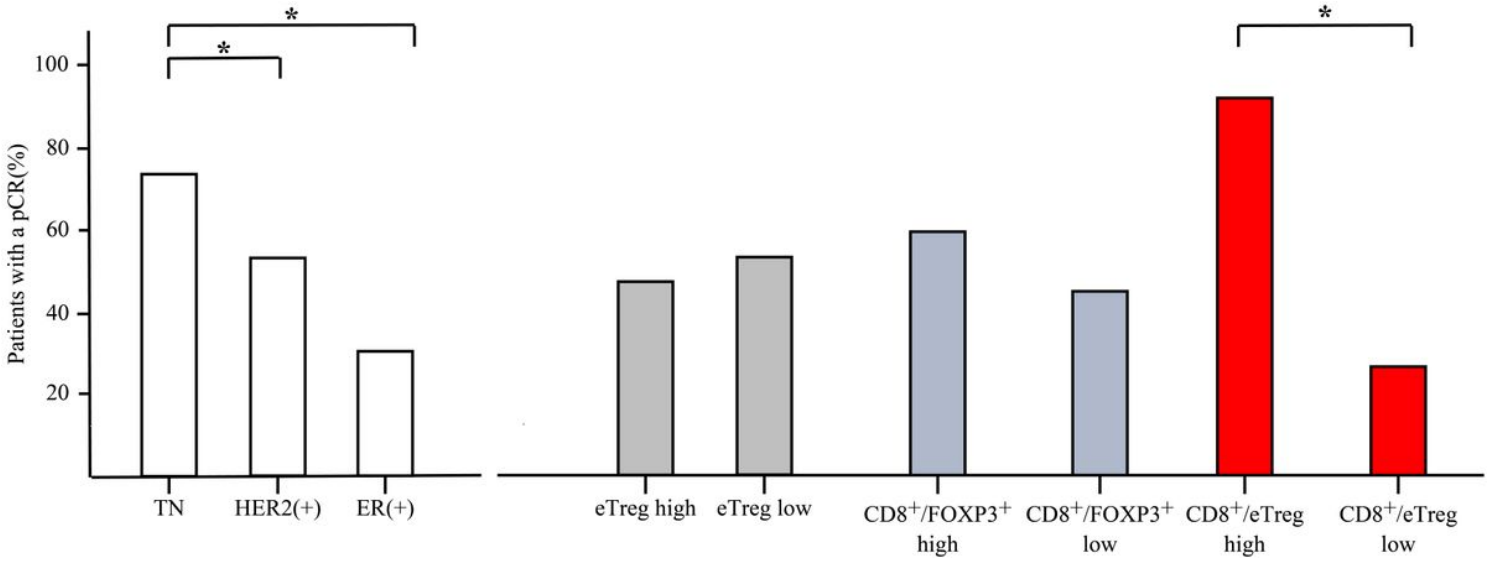

Figure 2

pCR rate of each breast cancer subtypes and variation of TIL subpopulations. pCR, pathological complete response.

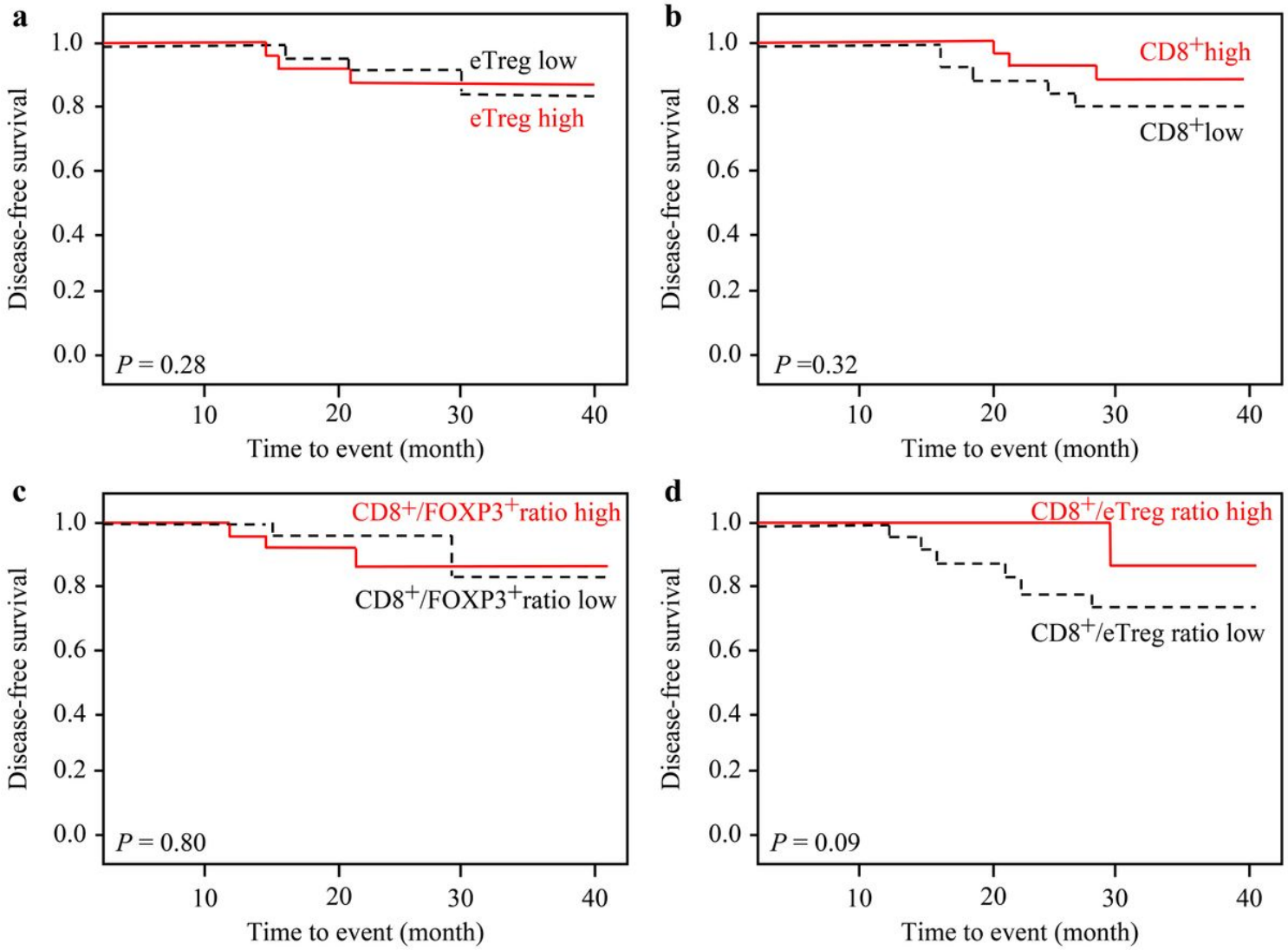

Figure 3

Disease-free survival a:eTreg high/low group, b: CD8+ high/low group, c CD8+/FOXP3+ high/low group, e CD8+/eTreg high/low group.