Breast Cancer (2019) 26:180–189
https://doi.org/10.1007/s12282-018-0910-4

ORIGINAL ARTICLE

Coexistence of regulatory B cells and regulatory T cells in tumor-infiltrating lymphocyte aggregates is a prognostic factor in patients with breast cancer

Emi Ishigami[1] · Masahiro Sakakibara[2] · Junta Sakakibara[1] · Takahito Masuda[1] · Hiroshi Fujimoto[1] · Shouko Hayama[1] · Takeshi Nagashima[1] · Takafumi Sangai[1] · Ayako Nakagawa[1] · Yukio Nakatani[3] · Masayuki Otsuka[1]

Received: 15 December 2017 / Accepted: 14 September 2018 / Published online: 22 September 2018

Abstract

Background Tumors can acquire tolerance to tumor immunity and develop enhanced proliferation. Regulatory B cells (Bregs), whose role in immune tolerance is similar to that of regulatory T cells (Tregs), appear to be involved in tumor immunity. Recently, Bregs were found to induce Tregs against tumor immunity. However, the platform for the coexistence of Bregs and Tregs in cancer patients and its clinical significance remain unclear; thus, they were evaluated in breast cancer patients.

Methods In 489 breast cancer patients, CD25- and IL10-positive Bregs and Foxp3-positive Tregs were immunohistochemically evaluated in tumor-infiltrating lymphocyte aggregates (TIL aggregates) that consisted of CD19-positive B-cell follicles and CD3-positive T-cell parafollicles. Then the correlations of the localization and existence of these cells with metastasis-free survival (MFS) were evaluated in breast cancer patients.

Results TIL aggregates were observed in marginal regions of tumors in breast cancer patients. In the TIL aggregates, the existence of Bregs was closely related to that of Tregs ($p < 0.0001$). On multivariate analysis, the coexistence of Bregs and Tregs in TIL aggregates was correlated with MFS in breast cancer patients ($p = 0.007$). Furthermore, MFS was significantly shorter for patients with the coexistence of Tregs and Bregs in TIL aggregates than in those with Tregs alone without Bregs ($p = 0.0475$).

Conclusions The present results suggest that Bregs are related to the induction of Tregs in TIL aggregates and the development of metastasis of breast cancer cells. Bregs are expected to be a new diagnostic and therapeutic target in breast cancer patients.

Keywords Regulatory B cell · Breast cancer · Tumor immunity · Immune tolerance · Regulatory T cell

Introduction

A relationship between tumor growth and immune cells has been reported [1–3]. The initial focus was on the cytotoxic effect of tumor-infiltrating lymphocytes (TIL) against tumor [4–7]. Since then, it has been found that tumors acquire tolerance, through mechanisms such as regulatory T cells (Tregs), to tumor immunity, which enhances their proliferation [8–13]. Recently, it was reported that regulatory B cells (Bregs) are related to immune tolerance [14, 15]. Similar to Tregs, Bregs were found to be related to autoimmunity, transplantation immunity, and tumor immunity [16–22]. There have been reports that Bregs are related to tumor immunity in several cancers [23–33]. Olkhanud et al. reported that tumor-evoked Bregs induced Tregs and were related to lung metastasis of breast cancer [38]. However,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12282-018-0910-4) contains supplementary material, which is available to authorized users.

✉ Masahiro Sakakibara
sakaki@iuhw.ac.jp

[1] Department of General Surgery, Chiba University Graduate School of Medicine, Chiba, Chiba, Japan

[2] Department of Breast Surgery, International University of Health and Welfare Hospital, Nasushiobara, Tochigi, Japan

[3] Diagnostic Pathology, Chiba University Graduate School of Medicine, Chiba, Chiba, Japan

the platform where Bregs are evoked by tumor and where Bregs induce Tregs has been unclear. Furthermore, Bregs were expected to become diagnostic and therapeutic targets for breast cancer patients. However, the clinical significance of Bregs in breast cancer patients remains unclear. Therefore, in this study, Bregs were identified in tissue samples of breast cancer patients by immunohistochemistry (IHC), the platform where Bregs interact closely with tumor cells and Tregs was identified, and the clinical significance of the existence of Bregs in this platform was evaluated in breast cancer patients.

Patients and methods

Patients

This study included 258 invasive ductal carcinoma (IDC) patients with axillary lymph node metastases and 231 ductal carcinoma in situ (DCIS) patients who underwent surgery in the Department of Surgery at the Chiba University Hospital between 2000 April and 2011 December. Patients who received preoperative hormonal therapy or neoadjuvant chemotherapy were excluded. DCIS was diagnosed by examination of surgical specimens and did not include cases with micro-invasion. In DCIS patients, no distant metastases or deaths due to breast cancer were observed. All IDC patients received doxorubicin and cyclophosphamide (AC) (60/600 mg/m^2 q3w) or fluorouracil, epirubicin, and cyclophosphamide (FEC) (500/100/500 mg/m^2 q3w) and/or weekly paclitaxel (80 mg/m^2) or docetaxel (75 mg/m^2) chemotherapy. When four or more axillary lymph node metastases were observed, the chest wall and the supraclavicular area were irradiated. Endocrine therapy was performed after surgery for all estrogen receptor (ER) and/or progesterone receptor (PR)-positive patients. For human epidermal growth factor receptor-2 (Her2)-positive patients, trastuzumab treatment was given as adjuvant therapy. During follow-up of IDC patients, 47 patients (18.2%) developed distant metastases due to breast cancer, and 23 (8.9%) patients died due to breast cancer. The mean survival time was 106 months, whereas the mean metastasis-free survival (MFS) was 99 months (range 7–195 months). Follow-up data were collected up to June 1, 2017, and the median length of follow-up for censored cases was 113 months (range 12–206 months). The study was reviewed and approved by the Institutional Ethic Committee. Informed consent was obtained from all patients who participated.

Immunohistochemical staining

Four-micrometer-thick, paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed by autoclaving (121 °C for 15 min) in citrate buffer at pH 6.0 for CD3 and IL-10 and at pH 9.0 for CD19, CD25, and Foxp3. Endogenous peroxidase activity was blocked by incubation for 15 min in 3% hydrogen peroxide solution. All slides were incubated with primary antibodies CD3 (LN-10, 1:200, Leica, Newcastle, UK), CD19 (LE-CD19, M729629, 1:100, Dako, Tokyo, Japan), CD25 (ab128955, anti-IL-2 receptor alpha antibody, 1:400, Abcam Cambridge, UK), IL-10 (E-10, 1:100, Santa Cruz Biotechnology, TX, USA), FoxP3 (ab22510, anti-FOXP3 antibody, 1:200, Abcam) at room temperature for 60 min. EnVision Kits (K4007, Dako, Glostrup, Denmark) were used for visualization. The sections were washed three times with phosphate-buffered saline following each procedure. All sections were counterstained with Mayer's hematoxylin. ER and PR were considered positive when $\geq 1\%$ of the tumor cells were immunohistochemically stained positive using standard methods and the anti-ER antibody, clone 1D5, or the anti-PR antibody, PgR636 (DAKO, Tokyo, Japan). Staining for Her2 was scored as one of four grades (score 0, 1, 2, 3), and tumors with scores of 3+ were considered to be Her2 positive (Her2, PN2A antibody, DAKO, Tokyo, Japan). Ki67 was defined as positive when $\geq 15\%$ of the tumor cells were immunohistochemically stained positive using standard methods and the anti-Ki67 antibody clone MIB-1, IgG isotype (DAKO, Tokyo, Japan). Immunohistochemical staining was evaluated by three investigators (E.I., M.S., Y.N.) who were blinded to clinical outcomes.

Assessment of Bregs and Tregs

In 489 breast cancer patients, TIL aggregates that consisted of CD19-positive B-cell follicles and CD3-positive T-cell parafollicles were detected. Some TIL aggregates were included in germinal center-like formations in B-cell follicles. Bregs were observed using IHC with CD25 or IL10 in B-cell follicles around the germinal center, in which all cells were CD19 positive. CD25-positive B cells in TIL aggregates were counted and scored as 0, none or less than 5 positive cells per 400× high-power field (HPF); 1, 5 and more, and less than 10 per HPF; 2, 10 and more, and less than 20 per 400× high-power field (HPF); and 3, 20 and more per HPF. Scores 1, 2, and 3 were considered CD25-positive Bregs. IL10-positive B cells in TIL aggregates were counted and scored as 0, none or less than 50 positive cells per 400× high-power field (HPF); 1, 50 and more, and less than 100 per HPF; and 2, 100 and more per HPF. Scores 1 and 2 were considered IL10-positive Bregs. Foxp3-positive T cells were counted and scored as 0, none or less than 5 per 400× high-power field (HPF); 1, 5 and more, and less than 20 per HPF; and 2, 20 and more per HPF. Scores 1 and 2 were considered Foxp3-positive Tregs.

Statistical analysis

Pearson's Chi-square tests were used for the analysis of correlations with the number of immune cells and patient characteristics in the two groups. MFS was analyzed by the log-rank test using Kaplan–Meier curves. Univariate and multivariate analyses using a Cox proportional hazards model were used for survival analysis. A p value < 0.05 was considered significant. These analyses were performed using statistical software.

Results

Lymphoid follicles including B cells and T cells were formed in marginal regions of tumor in patients with ductal carcinoma in situ and invasive ductal carcinoma of breast.

First, to identify where Bregs are produced, TIL were observed in the tumors of breast cancer patients. In DCIS patients, TIL were aggregated around DCIS lesions. Most were only one or two TIL aggregates around each DCIS

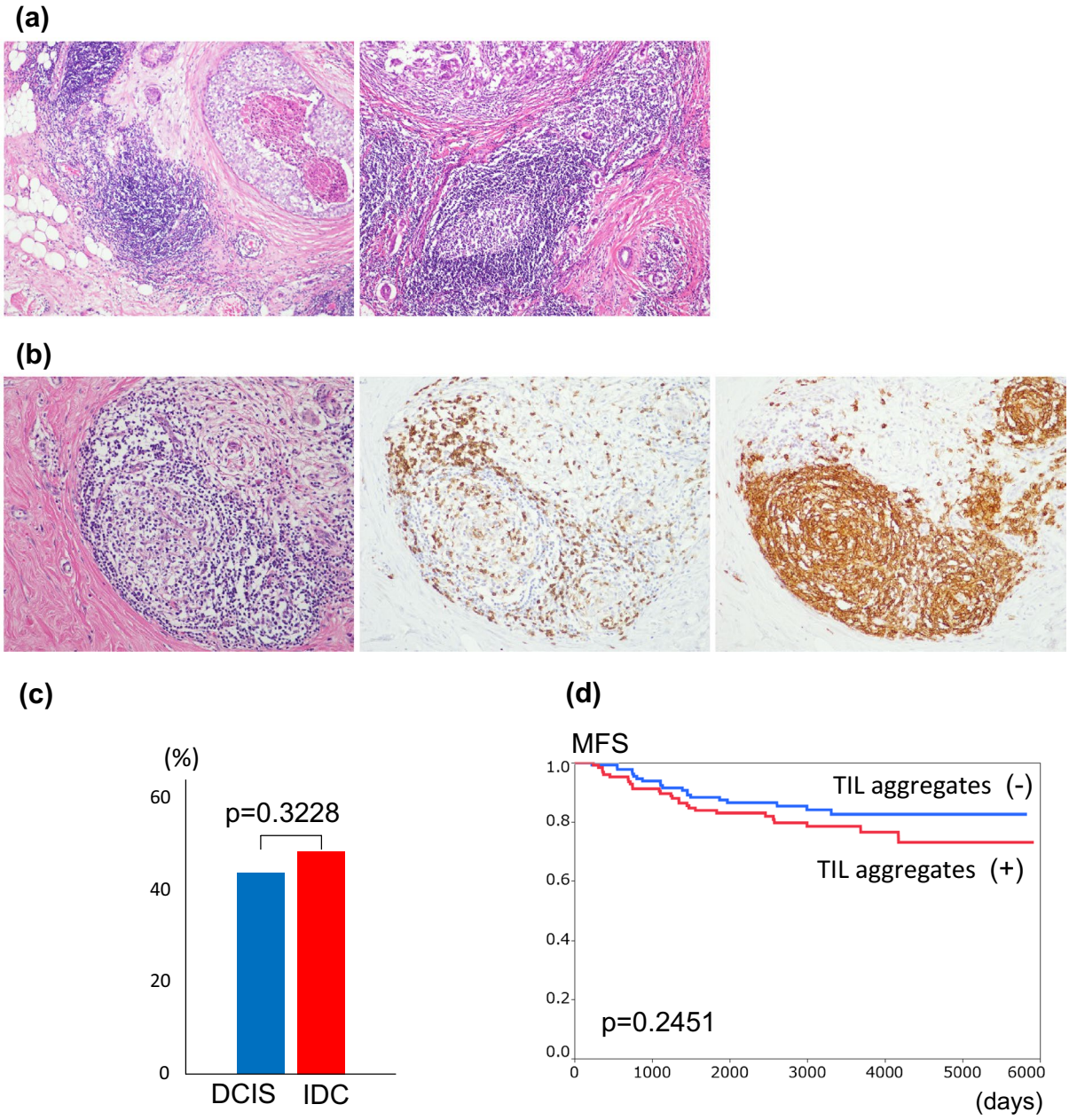

Fig. 1 TIL aggregates. TIL aggregates are observed in patients with ductal carcinoma in situ (DCIS) (**a**, left) and invasive ductal carcinoma (IDC) (**a**, right). TIL aggregates (**b**, left) consist of CD3-positive T cells (**b**, middle) and CD19-positive B cells (**b**, right). There is no significant difference in the rates of TIL aggregates between DCIS and IDC patients ($p = 0.3228$) (**c**). In IDC patients, there is no significant difference in metastasis-free survival (MFS) between patients with and without TIL aggregates ($p = 0.2451$) (**d**)

lesions (Fig. 1a). In IDC patients, most TIL were localized in marginal regions or superficial regions of tumors, and a few were seen in the tumor bed. In about half of the cases, TIL were aggregated like lymphoid follicle in marginal regions of the tumors (Fig. 1a). These aggregates were evaluated by IHC using CD19 and CD3 antibodies. Most of the TIL aggregates in the marginal regions of tumors were B cells (Fig. 1b). There were a small number of B cells without TIL aggregates, and most of the B cells were localized in these aggregates. Furthermore, close to these B-cell aggregates, there were T-cell aggregates resembling parafollicles (Fig. 1b). Although the number of TIL aggregates differed greatly between DCIS and IDC patients, there was no significant difference in the frequency of TIL aggregates between DCIS (43%) and IDC patients (48%) ($p=0.3228$) (Fig. 1c). The clinicopathological features of patients with and without TIL aggregates in IDC are shown in Table 1. In IDC patients, TIL aggregates were associated with a significantly higher rate of large tumor size, high histological grade, negativity for ER, negativity for PR, positivity for Her2, high expression of Ki67, and positivity for lymphatic invasion (ly) ($p=0.0045$, $p=0.0042$, $p=0.0015$, $p=0.0119$, $p<0.0001$, $p=0.0149$, respectively). However, there was no significant difference in metastasis-free survival (MFS) between patients with and without TIL aggregates ($p=0.2451$) (Fig. 1d). In DCIS patients, TIL aggregates were associated with a significantly higher rate of high nuclear grade and negativity for ER ($p<0.0001$, $p=0.0007$, respectively) (Supplemental Table).

CD25-positive regulatory B cells and IL10-positive regulatory B cells were found in TIL aggregates of breast cancer

Next, the presence of Bregs in TIL aggregates was evaluated. Of 231 DCIS patients with TIL aggregates, 49 patients (21.2%) showed CD25-positive B cells (which were also CD19 positive), and 58 patients showed IL10-positive B cells (25.1%) (Fig. 2a, b). In contrast, of 258 IDC patients with TIL aggregates, 129 (50%) showed CD25-positive B cells, and 119 showed IL10-positive B cells (46.1%) (Fig. 2c,

Table 1 Relationships between the existence of TIL aggregates and clinicopathological factors in invasive breast cancer patients

	($n=258$)	TIL aggregates positive ($n=125$)	TIL aggregates negative ($n=133$)	p value
Tumor size				0.0045*
T1	120 (46.5)	47 (37.6)	59 (44.4)	
T2, 3, 4	137 (53.1)	78 (62.4)	73 (54.9)	
Unknown	1 (0.4)		1 (0.8)	
Lymph node metastasis				0.3141
<4	193 (74.8)	90 (72.0)	103 (77.4)	
≥ 4	65 (25.2)	35 (28.0)	30 (22.6)	
Histological grade				0.0042*
1, 2	192 (74.4)	83 (66.4)	109 (82.0)	
3	66 (25.6)	42 (33.6)	24 (18.1)	
Estrogen receptor				0.0015*
Positive	205 (79.5)	89 (71.2)	116 (87.2)	
Negative	53 (20.5)	36 (28.8)	17 (12.8)	
Progesterone receptor				0.0005*
Positive	183 (70.9)	76 (60.8)	107 (80.5)	
Negative	75 (29.1)	49 (39.2)	26 (19.6)	
Her2				0.0119*
Positive	37 (14.3)	25 (20.0)	12 (9.0)	
Negative	221 (85.7)	100 (80.0)	121 (91.0)	
Ki67				<0.0001*
$\geq 15\%$	118 (45.7)	74 (59.2)	44 (33.1)	
$<15\%$	140 (54.3)	51 (40.8)	89 (66.9)	
ly				0.0149*
Positive	117 (45.3)	67 (53.6)	50 (37.6)	
Negative	136 (52.7)	57 (45.6)	79 (59.4)	
Unknown	5 (1.9)	1 (0.8)	4 (3.0)	

Her2 human epidermal growth factor receptor 2

*$p<0.05$

d). TIL aggregates of IDC patients contained significantly more CD25-positive B cells and IL10-positive B cells than those of DCIS patients ($p < 0.0001$, $p = 0.0025$, respectively) (Fig. 2e). In IDC patients with TIL aggregates, there was a significant correlation between the rates of CD25-positive B cells and of IL10-positive B cells ($p < 0.0001$) (Fig. 2f). Then, in this study, CD25-positive or IL10-positive B cells (which were also CD19-positive) were defined as Bregs. The clinicopathological features of IDC patients with and without Bregs are shown in Table 2. No factors were significantly different between these two groups. However, MFS was significantly shorter in patients with Bregs than in those without Bregs ($p = 0.0050$) (Fig. 2g).

Regulatory T cells were found in parafollicles in TIL aggregates of breast cancer.

Next, the presence of Tregs, which are known to be induced by Bregs, was evaluated. In IHC, most Foxp3-positive cells were present in superficial regions of tumors, and parafollicles in TIL aggregates were present in marginal regions of tumors (Fig. 3a, b). In this study, these Foxp3-positive cells were defined as Tregs. Of 258 IDC patients, 144 (55.8%) showed Tregs in superficial regions, and 121 (46.9%) showed Tregs in TIL aggregates. There was no significant correlation between the presence of Tregs in superficial regions and Tregs in TIL aggregates ($p = 0.1045$) (Fig. 3c). There was no significant difference in MFS between patients with and without Tregs in superficial regions ($p = 0.4370$) (Fig. 3d). In contrast, MFS was significantly shorter in patients with than in those without Tregs in TIL aggregates ($p = 0.0102$) (Fig. 3e). Furthermore, the rate of the presence of Tregs in TIL aggregates was significantly correlated to that of Bregs in TIL aggregates ($p < 0.0001$) (Fig. 3f).

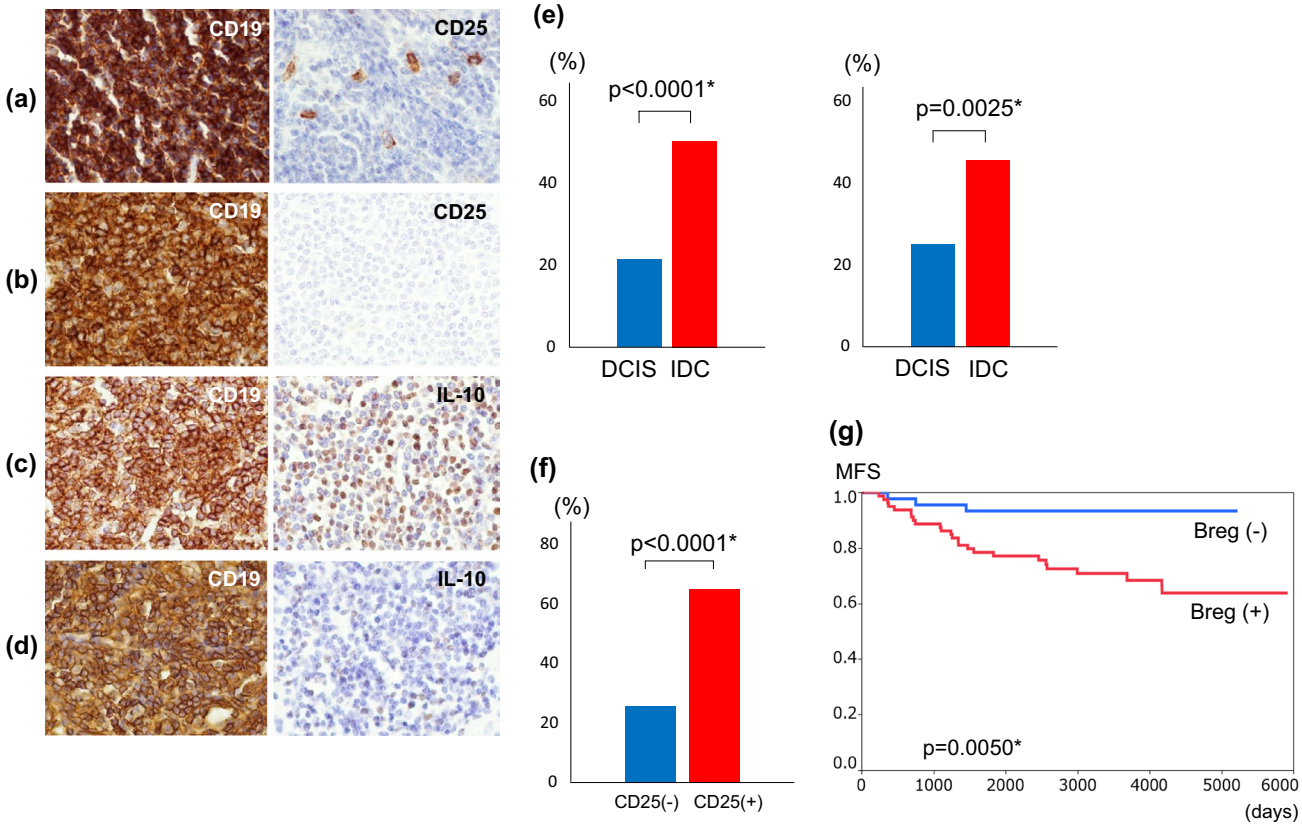

Fig. 2 Regulatory B cells in TIL aggregates. Using serial sections, immunohistochemistry for CD19, CD25, and IL10 was performed. CD25-positive cells and IL10-positive cells were evaluated in lesions in which all cells were CD19-positive B cells in TIL aggregates in patients with ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC), and patients with CD25-positive (**a**), CD25-negative (**b**), IL10-positive (**c**), and IL10-negative tumors were identified (**d**). Rates of patients with CD25-positive ($p < 0.0001$) (**e**, right) and IL10-positive ($p = 0.0025$) (**e**, left) cells are significantly higher in IDC patients than in DCIS patients. The rate of patients with CD25-positive cells is significantly correlated with that of IL10-positive cells ($p < 0.0001$) (**f**). Cells that are CD25 positive or IL10 positive in TIL aggregates are considered regulatory B cell (Breg) positive in this study. Metastasis-free survival (MFS) is significantly shorter for patients with Bregs in TIL aggregates than in those without Bregs ($p = 0.0050$) (**g**)

Table 2 Relationship between clinicopathological factors and the existence of Bregs in TIL aggregates in invasive breast cancer patients

	($n=125$)	Breg positive ($n=79$)	Breg negative ($n=46$)	p value
Tumor size				0.3793
T1	47 (62.4)	32 (40.5)	15 (32.6)	
T2, 3, 4	78 (37.6)	47 (59.5)	31 (67.4)	
Lymph node metastasis				0.1090
<4	90 (72.0)	53 (67.1)	37 (80.4)	
≥ 4	35 (28.0)	26 (32.9)	9 (19.6)	
Histological grade				0.1748
1, 2	83 (66.4)	49 (62.0)	34 (73.9)	
3	42 (33.6)	30 (38.0)	12 (26.1)	
Estrogen receptor				0.6093
Positive	89 (71.2)	55 (69.6)	34 (73.9)	
Negative	36 (28.8)	24 (30.4)	12 (26.1)	
Progesterone receptor				0.9903
Positive	76 (60.8)	48 (60.8)	28 (60.9)	
Negative	49 (39.2)	31 (39.2)	18 (39.1)	
Her2				0.1942
Positive	25 (20.0)	13 (16.5)	12 (26.1)	
Negative	100 (80.0)	66 (83.5)	34 (73.9)	
Ki67				0.7720
$\geq 15\%$	74 (59.2)	46 (58.2)	28 (60.9)	
$<15\%$	51 (40.8)	33 (41.8)	18 (39.1)	
ly				0.4236
Positive	67 (53.6)	40 (50.6)	27 (58.7)	
Negative	57 (45.6)	38 (48.1)	19 (41.3)	
Unknown	1 (0.8)	1 (1.3)		

Her2 human epidermal growth factor receptor 2

*$p<0.05$

Coexistence of regulatory B cells and regulatory T cells in TIL aggregates was an independent prognostic factor in breast cancer patients

The rate of the presence of Tregs in TIL aggregates was correlated to that of Bregs in ILFs. To study the correlation between Bregs and Tregs in TIL aggregates in greater depth, the correlations of the 4-level presence (score 0–3) of CD25-positive Bregs and of the 3-level presence (score 0–2) of IL10-positive Bregs with the 3-level presence (score 0–2) of Tregs in TIL aggregates were evaluated. The presence of both CD25-positive Bregs and of IL10-positive Bregs was increased in proportion to the presence of Tregs in TIL aggregates (Fig. 4a). These results suggested that the presence of Tregs was closely correlated to the presence of Bregs in TIL aggregates. Finally, the clinical significance of the coexistence of Bregs and Tregs in TIL aggregates was evaluated in IDC patients. Fifty-one patients had Bregs and Tregs in TIL aggregates. On univariate analysis of clinicopathological factors (tumor size, histological grade, nodal status, ER expression, PgR expression, Her2 expression, Ki67 expression, and ly status), including the coexistence of Bregs and Tregs in TIL aggregates, large tumor size (T2–4), histological grades 3 and 4 and more lymph node metastases, ER negative, ly positive, and the coexistence of Bregs and Tregs in TIL aggregates were significantly correlated to MFS ($p<0.0001$, $p<0.0001$, $p=0.0040$, $p=0.0045$, $p=0.0029$, $p<0.0001$, $p=0.0149$, respectively) (Table 3). On multivariate analysis of these 6 factors with correlations to MFS on univariate analysis, large tumor size (T2–4), histological grade 3, and coexistence of Bregs and Tregs in TIL aggregates were significantly correlated to MFS ($p=0.009$, $p=0.042$, $p=0.007$, respectively) (Table 3). Furthermore, 60 patients had Tregs in TIL aggregates, and 51 patients had the coexistence of Bregs and Tregs in TIL aggregates. Thus, 9 patients had no Bregs, only Tregs, in TIL aggregates. To confirm the clinical significance of the coexistence of Bregs and Tregs in TIL aggregates, MFS was compared between patients with the coexistence of Bregs and Tregs in TIL aggregates and those with Tregs alone in TIL aggregates. MFS was significantly shorter in the coexistence group than in the Treg-alone group ($p=0.0475$) (Fig. 4a). These data indicated the clinical significance of the coexistence of Bregs and Tregs in TIL aggregates in IDC patients.

Discussion

To the best of our knowledge, this is the first report to show the platform where Bregs were evoked by tumor, where Bregs induced Tregs, and the clinical significance of the coexistence of Bregs and Tregs in TIL aggregates in patients with breast cancer.

This study focused on TIL aggregates in breast cancer and found CD25-positive or IL10-positive Bregs. TIL aggregates were formed in about half of IDC patients, especially in patients with high-grade malignancies. However, there was no difference in MFS between patients with and without TIL aggregates. In addition, TIL aggregates were observed in DCIS patients. These data suggest that all TIL aggregates are not related to invasion and metastasis of breast cancer cells and may be reasonably considered to be the host's response to tumor proliferation. In contrast, in about half of IDC patients with TIL aggregates in marginal regions of the tumors, Bregs were observed in TIL aggregates. MFS was shorter in patients with Bregs than in those without Bregs in TIL aggregates. Additionally, The positive rate of Bregs in DCIS patients was significantly lower than that in IDC

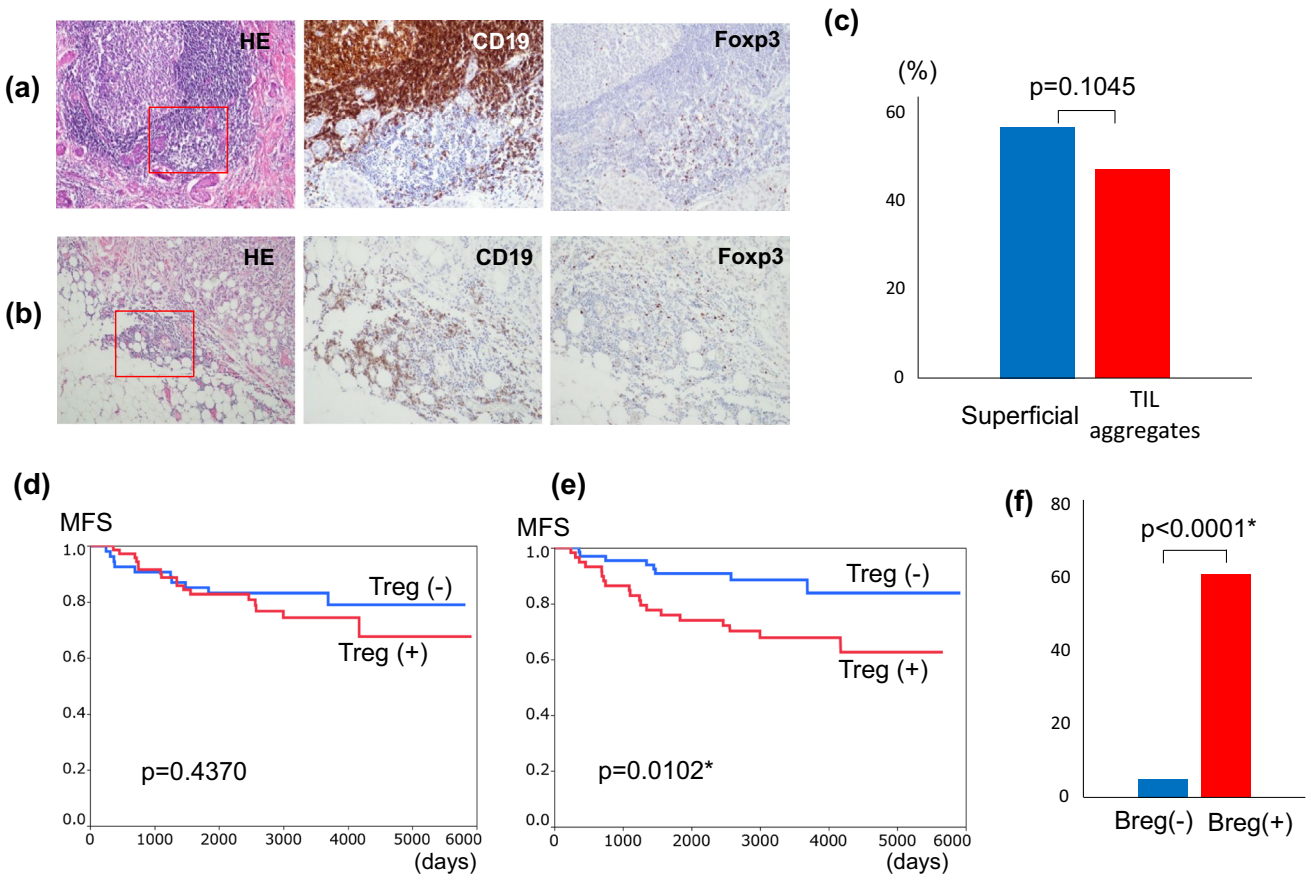

Fig. 3 Regulatory T cells in TIL aggregates. Foxp3-positive Tregs are localized in parafollicles in TIL aggregates (**a**) and superficial regions of tumor (**b**). There is no difference in the positive rate of patients with Tregs in TIL aggregates and Tregs in superficial regions ($p=0.1045$) (**c**). Metastasis-free survival (MFS) of patients with Tregs in superficial regions is not different from that without ($p=0.4370$) (**d**). In contrast, MFS of patients with Tregs in TIL aggregates is significantly shorter than that in patients without ($p=0.0102$) (**e**). The rate of patients with Tregs in TIL aggregates is significantly correlated to that with Bregs in TIL aggregates ($p<0.0001$) (**f**)

patients. These data indicate that Bregs in TIL aggregates are related to invasion and metastasis of breast cancer cells. TIL aggregates in marginal regions show lymphoid follicle-like structure and these TILs are considered as a platform to produce Bregs. Furthermore, Tregs may be induced by Bregs in these TIL platforms.

Recently, there are many articles about the immune reaction surrounding DCIS [34–37]. These reports showed that DCIS which was microinvasive, of high grade and high malignancy (ER negative, Her2 positive or high proliferation) was with higher immune reaction (CD4 T cells, CD 8 T cells and B cells) than DCIS which was not microinvasive, of low grade and low malignancy. These immune reactions were considered as a barometer for systemic therapies in DCIS patients. In this study, we evaluated TIL aggregates and Bregs in patients with pure DCIS. Compared to IDC patients, the absolute number of TIL aggregates in tumor was low in DCIS patients. However, some DCIS cases of high grade and ER negative showed TIL aggregates around DCIS lesion. Similar to IDC patients, most TIL aggregates may be only host's response to tumor proliferation. However, about 20% patients showed Bregs in TIL aggregates. Because the absolute number of TIL aggregates as platform that Bregs induced Tregs is low, Bregs of DCIS patients may not cause metastasis directly. However, TIL aggregates are increased in high-grade and high-malignancy DCIS. Furthermore, MFS of IDC patients with Breags in TIL aggregates was significantly shorter than that without Bregs. Then, DCIS patients with Bregs in TIL aggregates, especially with high grade and high malignancy, should be given consideration to systemic therapy.

In this study, we considered CD25-positive B cells or IL10-positive B cells as Bregs. There was significant correlation between the positive rates of CD25 and IL10 in CD19-positive B cells (data not shown). Patients with CD25-positive B cells were in strict correspondence with patients with IL10-positive B cells. Naturally, there were patients with single-positive CD25 or IL10. There was no

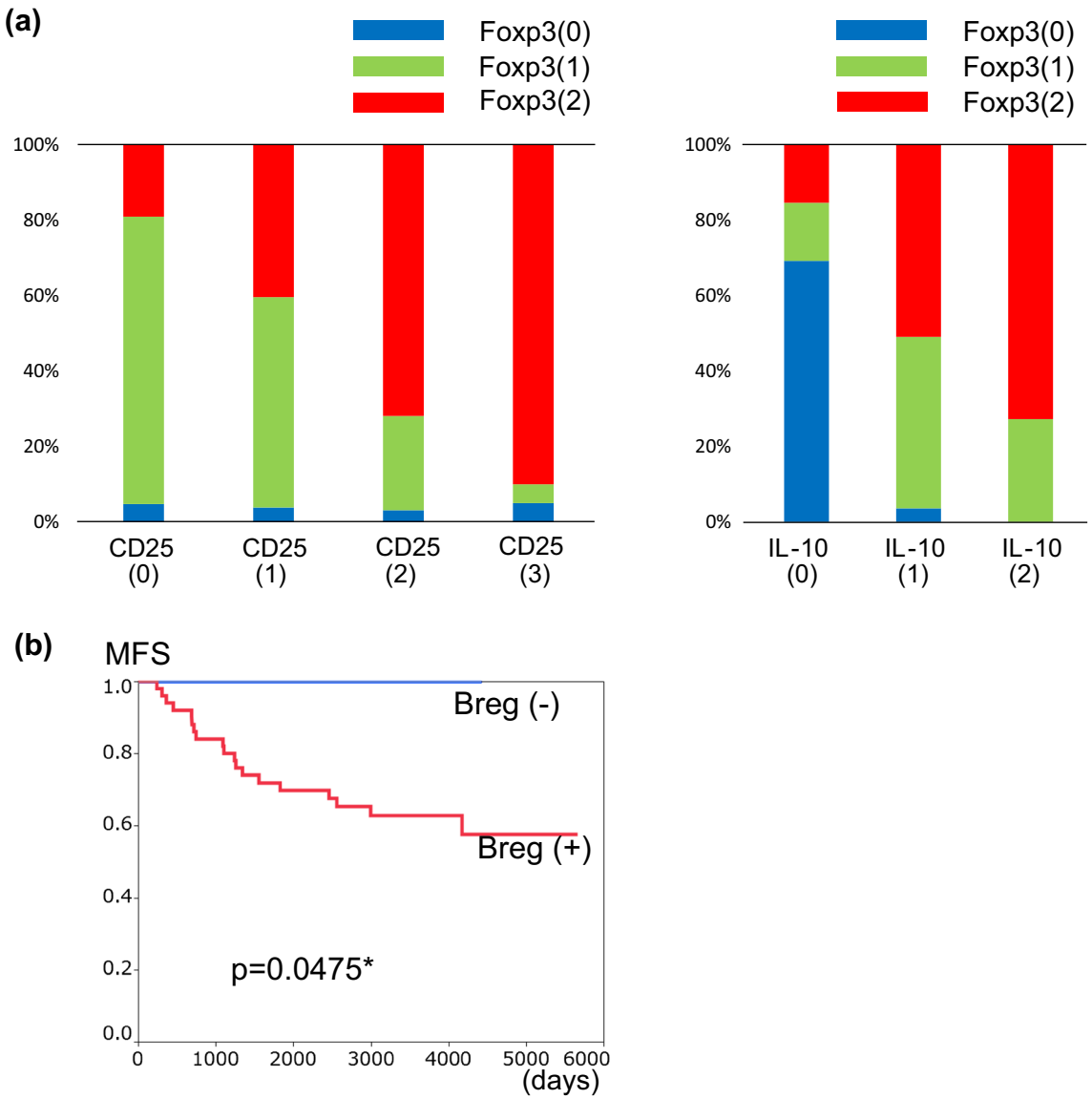

Fig. 4 Coexistence of regulatory B cells and regulatory T cells in TIL aggregates. To evaluate the correlation of regulatory B cells (Bregs) and regulatory T cells (Tregs) in TIL aggregates, the correlations of the numbers of CD25-positive Bregs (4 levels: score 0–3) and IL10-positive Bregs (3 level: score 0–2) to Foxp3-positive Tregs (3 levels: score 0–2) were examined. The numbers of CD25-positive Bregs and IL10-positive Bregs are closely related to the number of Foxp3-positive Tregs (**a**). Metastasis-free survival (MFS) is significantly shorter for patients with the coexistence of Bregs and Tregs in TIL aggregates than in those with Tregs alone ($p = 0.0475$) (**b**)

clinical difference among patients with CD25 and IL10 double positive, CD25 single positive and IL10 single positive (data not shown). Then, we regarded CD25-positive B cells and IL10-positive B cells as Bregs collectively.

Recently, Olkhanud et al. reported that tumor-evoked CD19-positive and CD25-positive Bregs induced Tregs, and they were related to lung metastasis of breast cancer cells in vivo [38]. In the present study, there was a close relationship between CD25-positive B cells and Tregs in TIL aggregates in breast cancer patients. Furthermore, there was also a close relationship between IL10-positive B cells and Tregs in TIL aggregates. Thus, CD25-positive or IL10-positive B cells were considered Bregs in the present study, and the coexistence of Bregs and Tregs in TIL aggregates was shown to be an independent prognostic factor in breast cancer patients. Furthermore, a comparison of MFS between patients with coexistence of Bregs and Tregs in TIL aggregates and patients with Tregs alone confirmed the clinical significance of the coexistence of Bregs and Tregs in TIL aggregates.

There have been two previous reports about the relationship between the localization of Tregs in tumors and

Table 3 Metastasis-free survival analysis of the 258 invasive breast cancer patients using a Cox proportional hazards model

	Univariate			Multivariate		
	HR	95% CI	*p* value	HR	95 %CI	*p* value
T2–4/T1	3.22	1.69–6.65	< 0.001*	2.44	1.24–5.16	0.009*
Histological grade (3/1, 2)	2.86	1.60–5.07	< 0.001*	1.99	1.02–3.84	0.042*
Node metastasis (≥ 4/< 4)	2.41	1.33–4.28	0.004*	1.47	0.76–2.75	0.245
Estrogen receptor (−/+)	1.96	1.02–3.60	0.045*	1.01	0.47–2.06	0.978
Progesterone receptor (−/+)	1.08	0.56–1.98	0.810			
Her2 (+/−)	1.46	0.63–2.96	0.351			
Ki67 (≥ 15%/< 15%)	1.71	0.97–3.10	0.066			
ly	1.91	1.07–3.50	0.029*	1.32	0.69–2.56	0.397
CD25 or IL10 Bregs and Tregs	2.98	1.63–5.30	< 0.001*	2.44	1.29–4.53	0.007*

HR hazard ratio, *CI* confidence interval, *Her2* human epidermal growth factor receptor 2

*$p < 0.05$

prognosis in breast cancer patients [39, 40]. Menetrier-Caux et al. showed that Tregs in TIL aggregates in marginal regions of tumors, not Tregs in the tumor bed, were correlated with a worse prognosis in breast cancer patients [39]. Kim et al. showed similarly that Tregs in peripheral regions, not Tregs in the tumor bed, were correlated with a worse prognosis in breast cancer patients [40]. In the present paper, it was clearly demonstrated that Tregs in parafollicles in TIL aggregate with Bregs, not Tregs in superficial regions of tumors.

Previous study demonstrated that Bregs induce Tregs. In this study, the number of Tregs was closely correlated with that of Bregs in TIL aggregates in marginal regions of tumors. Furthermore, MFS was significantly shorter in the coexistence of Tregs and Bregs in TIL aggregate than in the Tregs alone. These data suggested the clinical importance of Bregs in breast cancer progression.

In this study, Bregs were evaluated in serial sections of specimens. In patients with TIL aggregates, some showed germinal center formation in B-cell follicles in TIL aggregates. There was no difference in MFS between patients with and without germinal center formation in TIL aggregates (data not shown). Most Bregs in TIL aggregates were not in the germinal centers, but in B-cell follicles around the germinal centers. CD3-positive T cells were in germinal centers of TIL aggregates and not in B-cell follicles around germinal centers. All lymphoid cells in B-cell follicles around germinal centers were considered CD19-positive B cells. Thus, IHC was performed with CD25 and IL10, and these positive cells were considered CD19-positive and CD25-positive Bregs and CD19-positive and IL10-positive Bregs.

In conclusion, the present results suggest that TIL aggregates are formed by proliferation of breast cancer cells as a host response, but if Bregs are induced in TIL aggregates, Bregs may induce Tregs in TIL aggregates against tumor immunity and encourage metastasis of breast cancer cells. Bregs are expected to become a new diagnostic and therapeutic target in breast cancer patients.

Acknowledgements The authors would like to thank the patients, nurses, and physicians at Chiba University Hospital. This work was greatly assisted by the excellent technical help and advice of Hiroko Kawamura.

Compliance with ethical standards

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

References

1. Disis ML. Immune regulation of cancer. J Clin Oncol. 2010;28:4531–8.
2. Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Investig. 2007;117:1137–46.
3. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3:991–8.
4. West NR, Kost SE, Martin SD, Milne K, Deleeuw RJ, Nelson BH, et al. Tumour-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. Br J Cancer. 2013;108:155–62.
5. Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol. 2010;28:105–13.
6. Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol. 2013;31:860–7.
7. Savas P, Salgado R, Denkert C, Sotiriou C, Darcy PK, Smyth MJ, et al. Clinical relevance of host immunity in breast cancer: from TILs to the clinic. Nat Rev Clin Oncol. 2016;13:228–41.

8. Facciabene A, Motz GT, Coukos G. T-regulatory cells: key players in tumor immune escape and angiogenesis. Cancer Res. 2012;72:2162–71.
9. Whiteside TL, Schuler P, Schilling B. Induced and natural regulatory T cells in human cancer. Expert Opin Biol Ther. 2012;12:1383–97.
10. Nishikawa H, Sakaguchi S. Regulatory T cells in tumor immunity. Int J Cancer. 2010;127:759–67.
11. Jacobs JF, Nierkens S, Figdor CG, de Vries IJ, Adema GJ. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? Lancet Oncol. 2012;13:e32–42.
12. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004;10:942–9.
13. Nakamura R, Sakakibara M, Nagashima T, Sangai T, Arai M, Fujimori T, et al. Accumulation of regulatory T cells in sentinel lymph nodes is a prognostic predictor in patients with node-negative breast cancer. Eur J Cancer. 2009;45:2123–31.
14. Mauri C, Bosma A. Immune regulatory function of B cells. Annu Rev Immunol. 2012;30:221–41.
15. Rosser EC, Mauri C. Regulatory. B cells: origin, phenotype, and function. Immunity. 2015;42:607–12.
16. DiLillo DJ, Matsushita T, Tedder TF. B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. Ann N Y Acad Sci. 2010;1183:38–57.
17. Mauri C. Regulation of immunity and autoimmunity by B cells. Curr Opin Immunol. 2010;22:761–7.
18. Wolf SD, Dittel BN, Hardardottir F, Janeway CA. Jr. Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. J Exp Med. 1996;184:2271–8.
19. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. Nat Immunol. 2002;3:944–50.
20. Matsumoto M, Baba A, Yokota T, Nishikawa H, Ohkawa Y, Kayama H, et al. Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation. Immunity. 2014;41:1040–51.
21. Shen P, Roch T, Lampropoulou V, O'Connor RA, Stervbo U, Hilgenberg E, et al. IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. Nature. 2014;507:366–70.
22. Wang RX, Yu CR, Dambuza IM, Mahdi RM, Dolinska MB, Sergeev YV, et al. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. Nat Med. 2014;20:633–41.
23. Horikawa M, Minard-Colin V, Matsushita T, Tedder TF. Regulatory B cell production of IL-10 inhibits lymphoma depletion during CD20 immunotherapy in mice. J Clin Investig. 2011;121:4268–80.
24. Li Y, An J, Huang S, He J, Zhang J. Esophageal cancer-derived microvesicles induce regulatory B cells. Cell Biochem Funct. 2015;33:308–13.
25. Zhou J, Min Z, Zhang D, Wang W, Marincola F, Wang X. Enhanced frequency and potential mechanism of B regulatory cells in patients with lung cancer. J Transl Med. 2014;12:304.
26. Wei X, Jin Y, Tian Y, Zhang H, Wu J, Lu W, et al. Regulatory B cells contribute to the impaired antitumor immunity in ovarian cancer patients. Tumour Biol. 2016;37:6581–8.
27. Qian L, Bian GR, Zhou Y, Wang Y, Hu J, Liu X, et al. Clinical significance of regulatory B cells in the peripheral blood of patients with oesophageal cancer. Cent Eur J Immunol. 2015;40: 263–5.
28. DiLillo DJ, Weinberg JB, Yoshizaki A, Horikawa M, Bryant JM, Iwata Y, et al. Chronic lymphocytic leukemia and regulatory B cells share IL-10 competence and immunosuppressive function. Leukemia. 2013;27:170–82.
29. Shao Y, Lo CM, Ling CC, Liu XB, Ng KT, Chu AC, et al. Regulatory B cells accelerate hepatocellular carcinoma progression via CD40/CD154 signaling pathway. Cancer Lett. 2014;355:264–72.
30. Wang WW, Yuan XL, Chen H, Xie GH, Ma YH, Zheng YX, et al. $CD19^{+}CD24^{hi}CD38^{hi}$Bregs involved in downregulate helper T cells and upregulate regulatory T cells in gastric cancer. Oncotarget. 2015;6:33486–99.
31. Shimabukuro-Vornhagen A, Schlosser HA, Gryschok L, Malcher J, Wennhold K, Garcia-Marquez M, et al. Characterization of tumor-associated B-cell subsets in patients with colorectal cancer. Oncotarget. 2014;5:4651–64.
32. Zhou X, Su YX, Lao XM, Liang YJ, Liao GQ. CD19(+)IL-10(+) regulatory B cells affect survival of tongue squamous cell carcinoma patients and induce resting CD4(+) T cells to CD4(+) Foxp3(+) regulatory T cells. Oral Oncol. 2016;53:27–35.
33. Schioppa T, Moore R, Thompson RG, Rosser EC, Kulbe H, Nedospasov S, et al. B regulatory cells and the tumor-promoting actions of TNF-alpha during squamous carcinogenesis. Proc Natl Acad Sci USA. 2011;108:10662–7.
34. Beguinot M, Dauplat MM, Kwiatkowski F, Lebouedec G, Tixier L, Pomel C, et al. Analysis of tumour-infiltrating lymphocytes reveals two new biologically different subgroups of breast ductal carcinoma in situ. BMC Cancer. 2018;18:129.
35. Campbell MJ, Baehner F, O'Meara T, Ojukwu E, Han B, Mukhtar R, et al. Characterizing the immune microenvironment in high-risk ductal carcinoma in situ of the breast. Breast Cancer Res Treat. 2017;161:17–28.
36. Thompson E, Taube JM, Elwood H, Sharma R, Meeker A, Warzecha HN, et al. The immune microenvironment of breast ductal carcinoma in situ. Mod Pathol. 2016;29:249–58.
37. Abba MC, Gong T, Lu Y, Lee J, Zhong Y, Lacunza E, et al. A molecular portrait of high-grade ductal carcinoma in situ. Cancer Res. 2015;75:3980–90.
38. Olkhanud PB, Damdinsuren B, Bodogai M, Gress RE, Sen R, Wejksza K, et al. Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4(+) T cells to T-regulatory cells. Cancer Res. 2011;71:3505–15.
39. Menetrier-Caux C, Gobert M, Caux C. Differences in tumor regulatory T-cell localization and activation status impact patient outcome. Cancer Res. 2009;69:7895–8.
40. Kim S, Lee A, Lim W, Park S, Cho MS, Koo H, et al. Zonal difference and prognostic significance of foxp3 regulatory T cell infiltration in breast cancer. J Breast Cancer. 2014;17:8–17.