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Cytokine profile of $CD4^+CD25^-FoxP3^+$ T cells in tumor-draining lymph nodes from patients with breast cancer

Andisheh Niakan[a,b,1], Zahra Faghih[a,1], Abdol-Rasoul Talei[c], Abbas Ghaderi[a,b,*]

[a] Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[b] Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[c] Breast Disease Research Center, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

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ABSTRACT

Background: A T cell subtype with the $CD4^+$**CD25**$^-FoxP3^+$ phenotype was recently described. We aimed to investigate the frequency of these cells and their ability to produce cytokines in tumor-draining lymph nodes from patients with breast cancer (BC).

Materials and methods: Mononuclear cells from lymph nodes of 20 patients with BC were activated and stained for appropriate markers. The cells were assayed with four-color flow cytometry.

Results: A very small fraction of $CD4^+$**CD25**$^-FoxP3^+$ cells produced cytokines at levels that were significantly lower than in the regulatory ($CD4^+$**CD25**$^+FoxP3^+$) and effector cell ($CD4^+$**CD25**$^+FoxP3^-$) subpopulations. The expression of IFNγ and IL-2 in the $CD4^+$**CD25**$^-FoxP3^+$ subset was significantly higher than in Treg cells, but lower than in the effector subset. Conversely, IL-22 expression in Treg cells was significantly higher than in the $CD4^+$**CD25**$^-FoxP3^+$ subpopulation. The expression of IL-10 in the $CD4^+$**CD25**$^-FoxP3^+$ subset was also significantly higher than in effector cells.

Conclusion: We suggest that $CD4^+$**CD25**$^-FoxP3^+$ cells in patients with BC are exhausted cells with an intermediate phenotype between effector and regulatory cells.

1. Introduction

Breast cancer (BC) is the most frequent type of cancer in women and an important public health problem worldwide (Ali et al., 2016; Erfani et al., 2006; Huang et al., 2015; Rad et al., 2015). The survival rates and prognosis for BC are dependent on many factors, one of which is the presence and extent of axillary lymph node involvement. In fact, the presence of metastases in the axillary lymph node drainage system reduces the 5-year survival rate (Ali et al., 2016; Gherghe et al., 2015). It is now well documented that the structure as well as the cellular composition of lymph nodes play a crucial role in tumor progression and dissemination (Faghih et al., 2014; Jafarinia et al., 2016; Mehdipour et al., 2019; Mehdipour et al., 2016).

Regulatory T cells (Treg), a suppressive subset of $CD4^+$ cells with a $CD25^+FoxP3^+CD127^{low/-}$ phenotype, are important regulators of immune responses. Disruption in the development and/or function of Treg cells is recognized as a primary cause of autoimmune and inflammatory diseases in humans and animals (Chaudhary and Elkord, 2016; Hoeppli et al., 2015; Plitas and Rudensky, 2016; Watanabe et al., 2010). Through various suppressive mechanisms, these cells have detrimental effects on the tumor microenvironment, allowing the tumor cells to escape eradication by the immune system (Facciabene et al., 2012). Tumor-derived $CD4^+$ Treg cells have been extensively studied in different types of cancer including BC, and in most cases, changes in their frequencies are associated with poor prognosis, invasive phenotype, increased relapse rate and diminished overall survival (Gupta et al., 2007; Liyanage et al., 2002; Ormandy et al., 2005; Shen et al., 2009; Whiteside, 2012; Woo et al., 2001).

Although the IL-2 receptor alpha chain (**CD25**) is commonly considered a crucial marker for both murine and human $CD4^+$regulatory T cells, recent studies have introduced a new subset of FoxP3-expressing $CD4^+$ cells which are negative for **CD25**. In the murine system, it has been reported that this T cell subset is able to recover CD25 expression after homeostatic expansion, and to reconstitute the peripheral reservoir of the differentiated Treg pool (Zelenay et al., 2005). In humans, this T cell subset has been studied mostly in patients with autoimmune diseases. An

* Corresponding author at: Shiraz Institute for Cancer Research, Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.
E-mail address: ghaderia@sums.ac.ir (A. Ghaderi).
[1] These authors contributed equally to the study.

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increase has been reported in the percentages of cells with a similar phenotype in patients with new-onset and active systemic lupus erythematosus (Bonelli et al., 2009; Zhang et al., 2008), multiple sclerosis (Fransson et al., 2010), rheumatoid arthritis (de Paz et al., 2012), and immune thrombocytopenia (Sollazzo et al., 2015) compared to healthy controls. Most of these studies suggest that this population may include dysfunctional regulatory T cells or activated/effector T cells. Several studies have further reported the presence of these cells in human tumors (Faghih et al., 2014; Jafarinia et al., 2016). In our previous study of patients with BC, we demonstrated that this subset was also present in draining lymph nodes, and that its frequency was elevated in node-positive (LN^+) patients with the invasive ductal carcinoma subtype of BC. This finding, along with the positive correlation observed between the $CD25^-$ subset and $CD25^+$ Treg cells as well as the number of involved lymph nodes (LNs) is evidence for the inhibitory role of these cells in breast tumor immunity (Faghih et al., 2014). A study of non-Hodgkin lymphoma also showed that the tumor microenvironment may be involved in the generation of intratumoral $CD4^+$**$CD25^-$**$FoxP3^+$ T cells. This is consistent with studies reporting that this subset is functionally similar to that of $CD4^+CD25^+FoxP3^+$ T cells, and is increased in a majority of malignancies (Yang et al., 2007). Functional studies of this subset are in progress; however, it is still unclear whether it comprises a unique subpopulation of Treg cells that do not express CD25, or whether it represents an effector/memory T cell subset which transiently expresses FoxP3. Therefore, in order to explore the function of $CD4^+$**$CD25^-$**$FoxP3^+$ T cells in cancer patients, the frequency of these cells and their ability to produce effector and inhibitory cytokines in tumor-draining lymph nodes (TDLNs) from patients with BC were investigated.

2. Materials and methods

2.1. Patients

Lymph node samples were obtained from 20 patients with BC who had undergone surgical tumor resection. None of the patients had a history of chemotherapy or radiotherapy prior to surgery. Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1395.S699). A part of the dissected lymph node was used in the present work, and the remaining part underwent routine pathological examination. Tumor involvement in the LNs was histologically determined by an expert pathologist. The main clinical and pathological characteristics of the patients are summarized in Table 1.

2.2. Isolation of mononuclear cells

To obtain a homogenous cell suspension, LNs were mechanically minced into small pieces in complete culture medium [RPMI 1640 (Biosera, France)] containing 10% FBS and 1% penicillin/streptomycin (both from Gibco, Life Technologies, USA), and were filtered through a 40-μm cell strainer (BD Biosciences, USA). Mononuclear cells were then isolated by centrifugation over a Ficoll-Hypaque gradient (InnotrainDiagnostik GmbH, Germany). The cells were then stained with trypan blue dye (Biosera, France) to evaluate viability.

2.3. Flow cytometric analysis

2.3.1. Antibodies

The following anti-human antibodies were used: FITC anti-CD25 (M-A251), PE anti-FoxP3 (259D/C7), Alexa Fluor®647 anti-CD127 (HIL-7R-M21), and PE anti-IL-17 (N49-653) from BD Bioscience; Alexa Fluor®488 anti-CD25 (M-A251), Alexa Fluor®647 anti-FoxP3 (206D), PerCP-Cy5.5 anti-CD4 (RPA-T4), PE anti-IFNγ (4S.B3), PE anti-IL-2 (MQ1-17H12), and PE anti-IL-10 (JE53-19F1) from Biolegend, USA; PE anti-IL-22 (142,928) from R&D system, USA; and their respective isotype controls (all from Biolegend).

Table 1
Clinical and pathological characteristics of the patients with breast cancer.

Characteristics	Value
Age (years)	46.9 ± 12.71(27–72)
Lymph node status	
Free (N0)	6 (30%)
Involved	14 (70%)
N1 (1–3)	7 (35%)
N2 (4–9)	5 (25%)
N3 (> 9)	2 (10%)
Stage	
I	5 (25%)
II	8 (40%)
III	7 (35%)
IV	0 (0%)
Tumor type	
Infiltrating/invasive ductal carcinoma (IDC)	20 (100%)
Tumor size (cm)	
T1 (≤2)	14 (70%)
T2 (2–5)	6 (30%)
T3 (> 5)	0 (0%)
Histological grade	
Well differentiated (I)	2 (10%)
Moderately differentiated (II)	13 (65%)
Poorly differentiated (III)	5 (25%)
Estrogen receptor (ER)	
Positive	14 (70%)
Negative	6 (30%)
Progesterone receptor (PR)	
Positive	14 (70%)
Negative	6 (30%)
Her2 expression	
Positive	3 (15%)
Negative	17 (85%)

2.3.2. Cell staining

To determine the frequency of different $FoxP3^+$ cell subsets, unstimulated mononuclear cells were primarily surface-stained for CD4, CD25, and CD127 molecules with appropriate fluorochrome-conjugated antibodies. The cells were then fixed with Fixation working solution (eBioscience, USA), permeabilized with the FoxP3 buffer set (eBioscience), and incubated with anti-FoxP3 antibody for intracellular staining. Then the cells were washed, resuspended in staining buffer, and assayed with flow cytometry.

To quantify cytokine expression in different subsets of $CD4^+$ lymphocytes, mononuclear cells were first stimulated in complete culture medium containing 50 ng/ml phorbol myristate acetate and 1 μg/ml ionomycin (both from Sigma-Aldrich, Germany) in the presence of brefeldin A, monensin (both from BD bioscience) and DNase (2U) (Fermentas, USA) for 5 h at 37 °C. After activation, the cells were washed and surface-stained for CD4 and CD25. Then the cells were fixed and permeabilized with the FoxP3 buffer set (eBioscience), and were stained for intracellular expression of FoxP3, IFNγ, IL-2, IL-10, IL-17 and IL-22. Then they were washed and assayed with flow cytometry. The data were collected with a four-color FACSCalibur flow cytometer and analyzed with the CellQuest Pro software package (both from BD bioscience). A minimum of 200,000 events were acquired.

The frequencies of **$CD25^+$** and **$CD25^-$** $FoxP3^+CD127^{Low/-}$ T cells were determined in $CD4^+$ populations. The percentage of cytokine-producing subsets was determined separately in the **$CD25^+$** and **$CD25^-$** $CD4^+$ lymphocyte gates. The geometric mean florescence intensity (MFI) of each cytokine was considered as an additional criterion for expression level per cell. All MFI values were normalized to the MFI of the negative population in each sample.

2.4. Statistical analysis

The results were analyzed with SPSS (version 16, SPSS Inc., USA). The nonparametric Mann–Whitney U test and Kruskal–Wallis H test

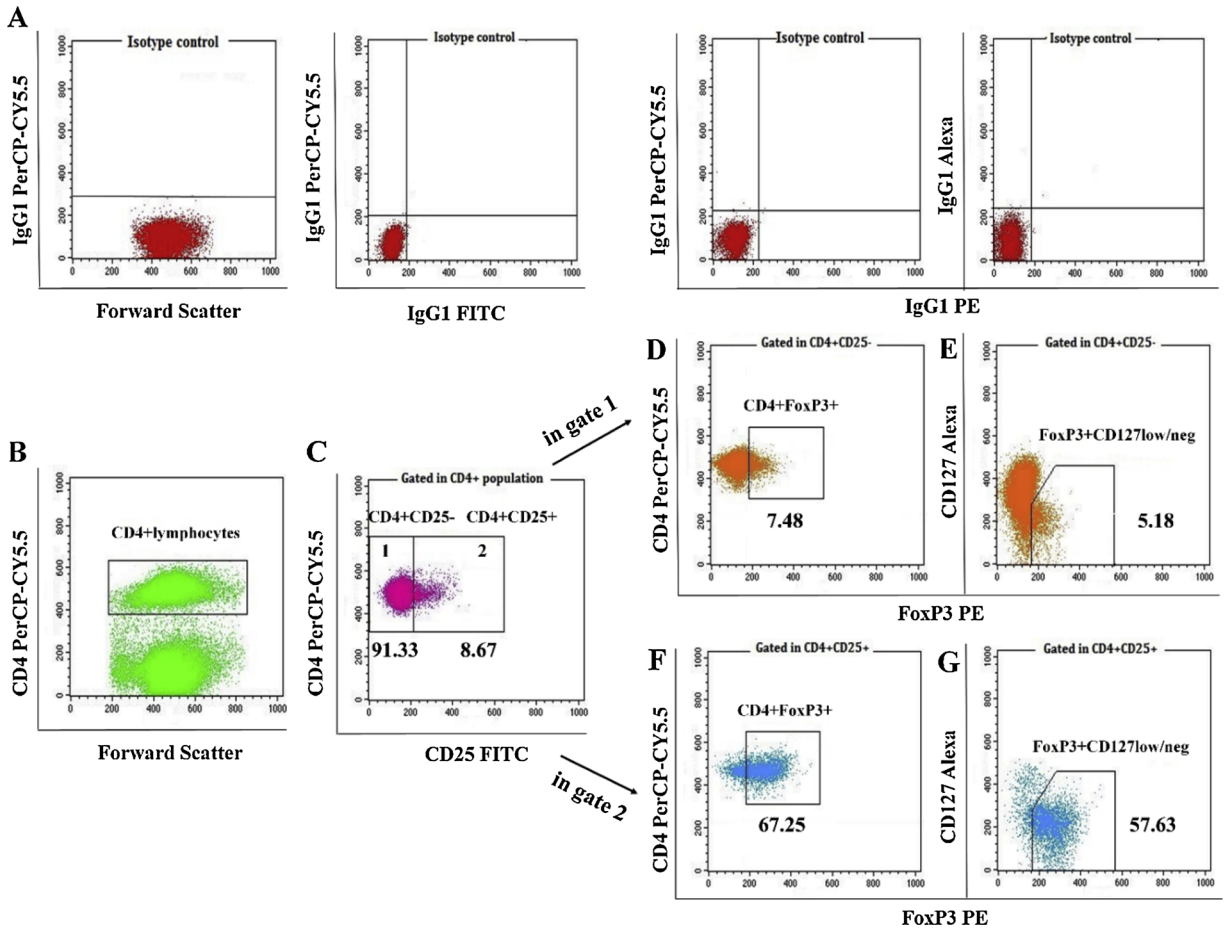

Fig. 1. Flow cytometric analysis of $CD4^+$ T cell subpopulations in TDLNs from patients with BC.
After antibody concentrations were adjusted and confirmed with isotype controls (**A**), CD4-expressing cells were primarily gated in the lymphocyte population (**B**). Then $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were gated (**C**) and the percentages of $CD4^+FoxP3^+$ and $FoxP3^+CD127^{low/-}$ cells were determined in gate **1** ($CD4^+CD25^-$) (**D-E**) and gate **2** ($CD4^+CD25^+$) (**F-G**).
TDLN: Tumor-draining lymph node; BC: Breast cancer.

Table 2
Frequency of different $CD4^+$ subpopulations in TDLNs from patients with BC.

Subpopulations	Min	Max	Median	Mean ± SEM
$CD4^+FoxP3^+$	5.14	22.26	11.74	12.87 ± 1.11
$CD4^+CD25^+FoxP3^+CD127^{LOW/-}$	22.96	75.6	56.09	57.63 ± 3.00
$CD4^+CD25^-FoxP3^+CD127^{LOW/-}$	1.75	11.41	4.35	5.18 ± 0.61
$CD4^+CD25^+FoxP3^+$	32.20	83.93	70.42	67.25 ± 2.97
$CD4^+CD25^-FoxP3^+$	2.47	15.30	6.61	7.48 ± 0.72
$CD4^+CD25^+FoxP3^-$	16.07	67.80	29.58	32.74 ± 2.97

TDLN: Tumor-draining lymph node; BC: Breast cancer.

were used for between-group comparisons. The correlations between the prevalence of each subset and the patients' age, tumor size and the number of involved LNs was determined with Spearman's rank correlation. P values less than 0.05 (two-tailed) were considered statistically significant. All graphs were prepared with GraphPad Prism 6 software (GraphPad Software, Inc., USA).

3. Results

3.1. Clinical and pathological characteristics of the patients

After confirmation by pathological analysis, 20 untreated women with BC aged 27 to 72 years (46.9 ± 12.71) were enrolled in the study. Twenty axillary LNs were obtained from these patients. As shown in Table 1, the tumor type was reported to be infiltrating ductal carcinoma in all patients. According to TNM staging based on the American Joint Committee on Cancer Classification and Stage Group (AJCC, seventh edition) (Edge and Compton, 2010), most patients were in stage II (40%) and III (35%). No patients were in stage IV at the time of surgery. According to histopathological staining, 10 of the LNs were affected by tumor cells (node-positive) and 10 were tumor-free (node-negative). However, 14 patients (70%) had at least one involved LN and were considered LN^+ patients, while in 6 patients (30%) all lymph nodes were tumor-free (LN^- patients).

3.2. Phenotype determination of $CD4^+$ subpopulations in TDLNs from patients with BC

To determine the frequency of various subsets, the $CD4^+$ lymphocyte population was first gated in the mononuclear cell population. The frequencies of $CD4^+CD25^+$, $CD4^+CD25^-$, $CD25^+FoxP3^+$, $CD25^-FoxP3^+$, $CD25^+FoxP3^+CD127^{low/-}$ and $CD25^-FoxP3^+CD127^{low/-}$ cells were then determined among $CD4^+$ cells. The gating strategies are shown in Fig. 1, and all frequencies are summarized in Table 2.

3.3. Frequency of different $FoxP3^+$ subsets in TDLNs from patients with BC

As shown in Table 2, 12.87 ± 1.11% of $CD4^+$ lymphocytes expressed intracellular FoxP3 transcription factor. The frequencies of $CD4^+$ **$CD25^+$**$FoxP3^+CD127^{low/-}$ cells and $CD4^+$**$CD25^-$**$FoxP3^+CD127^{low/-}$ cells

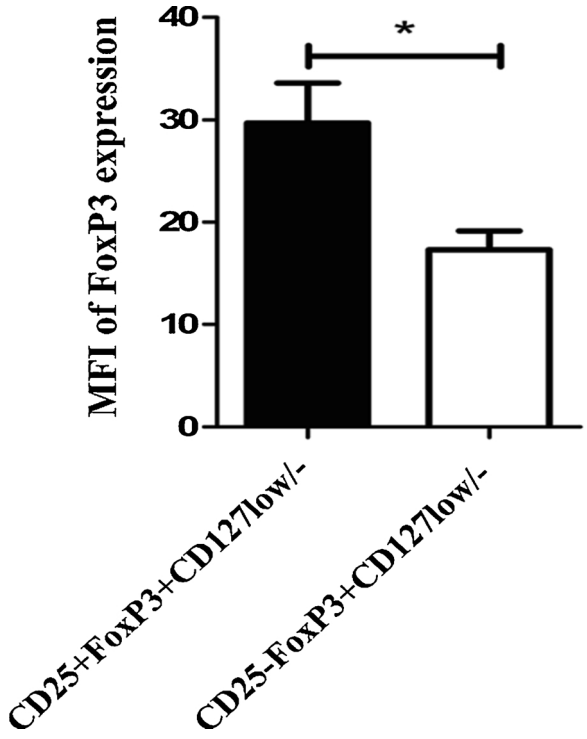

Fig. 2. Comparison of mean FoxP3 expression between $CD25^+$ and $CD25^-$ subsets in TDLNs from patients with BC.
Mean florescence intensity of FoxP3 was compared between **CD25**$^+$ and **CD25**$^-$ $CD4^+FoxP3^+CD127^{low/-}$ cells. Data are shown as the mean ± SEM. *Significant difference at the < 0.05 level (2-tailed).
SEM: Standard error of the mean; TDLN: Tumor-draining lymph node; BC: Breast cancer.

among $CD4^+$ lymphocytes were 57.63 ± 3.00 and 5.18 ± 0.61, respectively. As a criterion for regulatory function, the MFI of FoxP3, the specific transcription factor for Treg cells, was first compared between **CD25**$^+$ and **CD25**$^-$ $CD4^+FoxP3^+CD127^{low/-}$ cells. The results showed that the MFI of FoxP3 in $CD4^+$**CD25**$^+FoxP3^+CD127^{low/-}$ cells was significantly higher than in the **CD25**$^-$ subset (P = 0.02) (Fig. 2).

3.4. Cytokine production by $CD4^+CD25^-FoxP3^+$ and $CD4^+CD25^+FoxP3^+$ populations in TDLNs from patients with BC

Due to the limitation of color-staining, we excluded CD127 from the staining panel in this step. Accordingly, to investigate functionality, the frequencies of different cytokine-producing cells (IFNγ, IL-2, IL-22, IL-17 and IL-10) in $CD4^+$**CD25**$^-FoxP3^+$ populations were determined and compared to $CD4^+$**CD25**$^+FoxP3^+$ (regulatory) and $CD4^+$**CD25**$^+FoxP3^-$ (effector) cell populations (Fig. 3). A very small fraction of $CD4^+$**CD25**$^-FoxP3^+$ cells produced cytokines. Our analysis showed that the frequency of these cytokine-producing cells was significantly lower than in the regulatory subset (P < 0.0001 for IFNγ, IL-2, IL-10 and IL-22; P = 0.002 for IL-17, Fig. 4A). However, mean of IFNγ and IL-2 expression in the $CD4^+$**CD25**$^-FoxP3^+$ subset was significantly higher than in the regulatory cell subset (P = 0.026 and P = 0.005, respectively). The MFI of IL-22 in the regulatory cell subset was significantly higher than in $CD4^+$**CD25**$^-FoxP3^+$ cells (P < 0.0001, Fig. 4B).

The frequencies of cytokine-producing cells were also compared between the $CD4^+$**CD25**$^-FoxP3^+$ subset and effector cells. Statistical analysis revealed that the percentages of IFNγ-, IL-2-, IL-10-, IL-17- and IL-22-producing cells in the $CD4^+$**CD25**$^-FoxP3^+$ subpopulation were also significantly lower than in effector cells (P < 0.0001 for IFNγ, IL-2, IL-10 and IL-22; P = 0.002 for IL-17). In addition, mean IL-2 expression was greater in effector cells than in the $CD4^+$**CD25**$^-FoxP3^+$ subset (P = 0.006), while the MFI of IL-10 in the $CD4^+$**CD25**$^-FoxP3^+$ subset was significantly higher (P = 0.028) (Fig. 4A, 4B).

3.5. Frequency of different $CD4^+$ cytokine-producing subsets in TDLNs from patients with BC and different clinical and pathological characteristics

In the next step, we compared the frequency of different FoxP3-expressing cells among patients with different clinical and pathological characteristics. Comparison of the percentages of $CD4^+FoxP3^+CD127^{low/-}$ (**CD25**$^+$ and **CD25**$^-$) cells in patients with different node status disclosed no differences between positive and negative nodes (Fig. 5A); however, the percentage of $CD4^+$**CD25**$^+FoxP3^+CD127^{low/-}$ cells was significantly higher in LN^+ patients than in patients with tumor-free nodes (P = 0.028, Fig. 5B). No other significant differences were observed between the percentages of these two subsets among patients with different clinical and pathological characteristics, including stage (Fig. 5C) and invasive status. No significant differences were found in the percentages of cytokine-producing $CD4^+$ lymphocytes, including the $CD4^+$**CD25**$^-FoxP3^+$ subset, among patients with different stages, node status, tumor size, invasive status or hormone receptor status.

3.6. Correlations among different $CD4^+$ lymphocyte subsets in TDLNs from patients with BC

The correlations between different $CD4^+$ lymphocyte subpopulations were determined with the nonparametric Spearman correlation test. Our analysis indicated a strong positive correlation between the percentages of **CD25**$^+$ and **CD25**$^-$ subsets of $CD4^+FoxP3^+CD127^{low/-}$ cells (R = 0.571, P = 0.008). A significant direct correlation was also seen between tumor size in LNs and the frequency of these two subsets (R = 0.556, P = 0.011 for **CD25**$^-$ and R = 0.55, P = 0.012 for **CD25**$^+$) as well as the frequency of the $CD4^+$**CD25**$^-IFN\gamma^+FoxP3^+$ subset (R = 0.494, P = 0.027). Moreover, there was a direct correlation between the $CD4^+$**CD25**$^-FoxP3^+CD127^{low/-}$ subset and IFNγ production by $CD4^+$ lymphocytes (R = 0.576, P = 0.008) (Fig. 6).

4. Discussion

Our previous study reported that a subset of $CD4^+$ lymphocytes expressing FoxP3 was present in TDLNs from patients with BC, and these cells were more frequent in LN^+ patients with the invasive ductal carcinoma subtype of BC. This finding, together with the positive correlations observed for the $CD25^-$ subset with $CD25^+$ Treg cells and the number of involved LNs, prompted us to further investigate this subset in breast tumor immunity (Faghih et al., 2014). To determine the functional activity of this subset, we determined the cytokine profile of $CD4^+$**CD25**$^-FoxP3^+$ cells in TDLNs from women with BC in comparison to regulatory ($CD4^+$**CD25**$^+FoxP3^+$) and effector ($CD4^+$**CD25**$^+FoxP3^-$) cells.

Frequency analysis demonstrated that approximately 4–5% of $CD4^+$ lymphocytes in TDLNs from patients with BC had the $CD4^+$**CD25**$^-FoxP3^+CD127^{low/-}$ phenotype; this proportion falls within the range of conventional Treg ($CD4^+CD25^+FoxP3^+CD127^{low/-}$) frequencies. Because FoxP3 is a lineage-specific transcription factor for Treg cells (Tanaka and Sakaguchi, 2017), we primarily compared mean expression of the FoxP3 molecule between the $CD25^-$ subset and conventional $CD25^+$ Tregs. Our results indicated that mean FoxP3 expression was significantly lower in the $CD25^-$ subset compared to $CD25^+$ cells. Similar results were also reported regarding colon cancer (Jafarinia et al., 2016). FoxP3 expression has been shown to be directly associated with the suppressive activities of Tregs (Rudensky, 2011), implying that the $CD4^+$**CD25**$^-FoxP3^+CD127^{low/-}$ subset has a weaker inhibitory function compared to conventional Tregs.

In the next step, to elucidate the functionality of $CD4^+$**CD25**$^-$ $FoxP3^+$ cells, we determined their cytokine profile and compared them

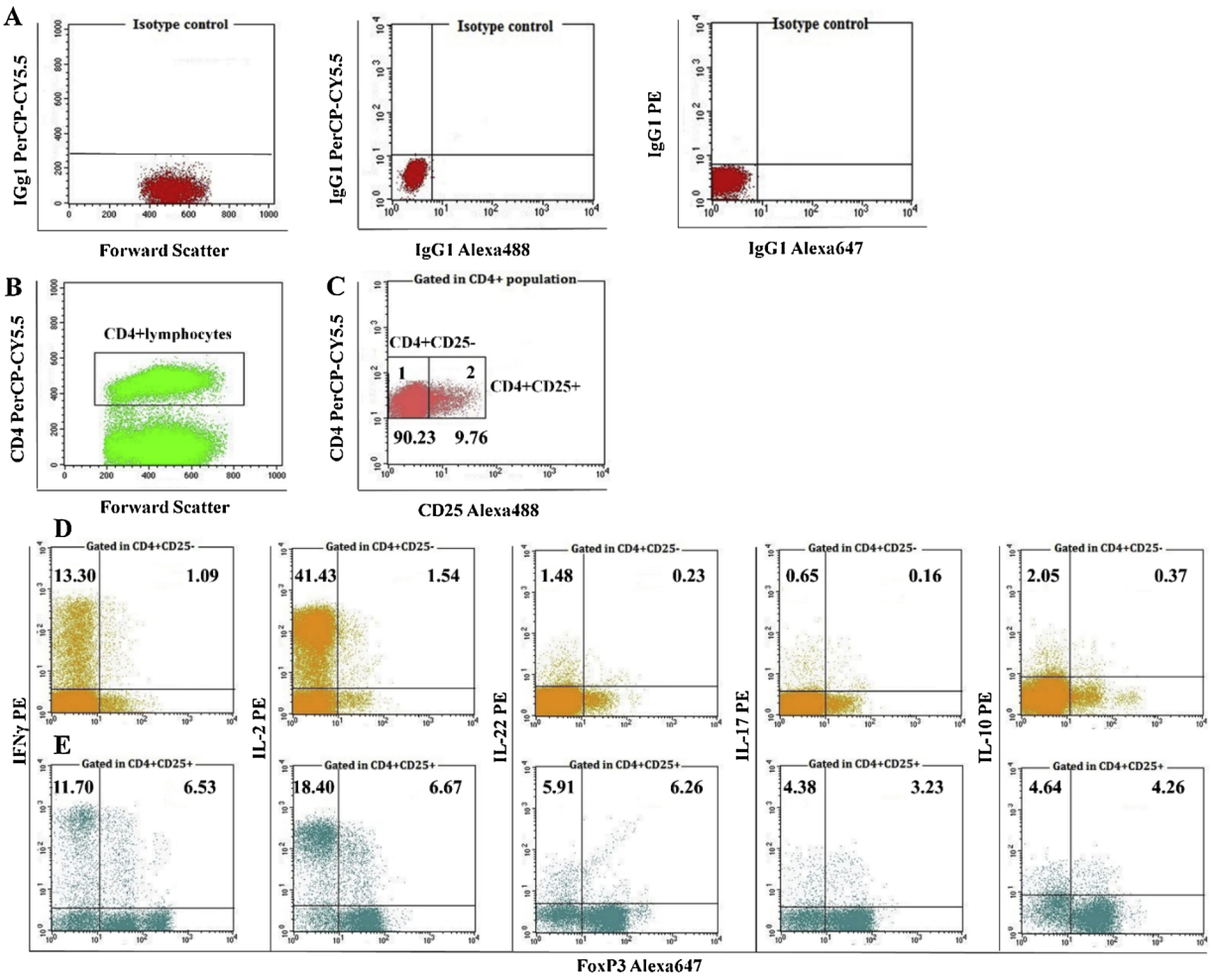

Fig. 3. Flow cytometric analysis of cytokine production by different T cell subpopulations in TDLNs from patients with BC.
After antibody concentrations were adjusted and confirmed with isotype controls (**A**), $CD4^+$ T cells were gated in the mononuclear cell population, and the frequencies of $CD4^+CD25^-$ (gate **1**) and $CD4^+CD25^+$ cells (gate **2**) were then determined in the $CD4^+$ cell gate (**B, C**). The percentages of IFNγ-, IL-2-, IL-22-, IL-17- and IL-10-producing cells were determined in gates **1** (**D**) and **2** (**E**).
TDLN: Tumor-draining lymph node; BC: Breast cancer.

to $CD25^+$ regulatory and effector subsets. Due to the limited options for color-staining, we were obliged to exclude CD127 from our cytokine analysis. Nevertheless, we observed that in TDLNs from patients with BC, a very small fraction of the **$CD25^-$** subset produced inflammatory and inhibitory cytokines, namely IFNγ, IL-2, IL-17, IL-22 and IL-10. Because CD25 is the alpha chain in the high-affinity IL-2 receptor, **$CD25^-$** cells may not respond to IL-2, and hence may be unable to expand and produce effector cytokines. Although this subset contained smaller subpopulations producing IFNγ, IL-2, IL-10, IL-17 and IL-22 compared to both regulatory and effector subsets, mean expression of effector cytokines such as IFNγ and IL-2 in this subset was significantly higher than in regulatory T cells. As FoxP3 is known to strongly inhibit the production of IFNγ and IL-2 by T cells, it can be concluded that the lower expression of FoxP3 in the **$CD25^-$** subset may result in less suppression of the synthesis of these effector cytokines. Greater effector capacity has also been proposed for $CD4^+$**$CD25^-$**$FoxP3^+$ cells in colorectal cancer, given that there are more IL-2- and IFNγ-producing cells in this subset than among regulatory T cells (Jafarinia et al., 2016). On the other hand, it has been reported that after activation, effector T cells can transiently express FoxP3, albeit at lower levels compared to conventional Tregs; moreover, this temporary expression by effector T cells is insufficient to suppress the expression of known targets of FoxP3 repressor activity, including CD127, IL-2 and IFNγ (Allan et al., 2007; H.-x. Yang et al., 2009). In addition, Yang and colleagues observed that $CD4^+CD25^-FoxP3^-$ cells upregulated CD25 and FoxP3 expression after long-term activation; however, they gradually downregulated these two molecules, such that these cells became $CD25^-$ while retaining a low level of FoxP3 expression (Z.-Z. Yang et al., 2007). Therefore, it can be concluded that at least a proportion of $CD4^+$**$CD25^-$**$FoxP3^+$ cells may retain an effector phenotype after losing their surface CD25 expression due to long-term activation, while maintaining FoxP3 expression at low levels.

In recent years, several studies have reported increased percentages of $CD4^+$**$CD25^-$**$FoxP3^+$ cells in the peripheral blood of patients with autoimmune diseases, noting that the increases correlated directly with disease activity. These findings further suggest that these cells may be a subset of activated/effector T cells (Bonelli et al., 2009; de Paz et al., 2012; Fransson et al., 2010; Zhang et al., 2008).

However, our comparison of cytokine profiles in the $CD4^+$**$CD25^-$**$FoxP3^+$ subset versus effector T cells (which were negative for FoxP3 but expressed CD25) revealed that in addition to lower percentages of cytokine-producing cells, the levels of IFNγ and IL-2 expression in the $CD4^+$**$CD25^-$**$FoxP3^+$ population were lower than cytokine levels in effector T cells. In contrast, mean IL-10 expression in the $CD4^+$**$CD25^-$**$FoxP3^+$ subset was significantly higher than in effector cells. These findings corroborate the notion that although $CD4^+$**$CD25^-$**$FoxP3^+$ cells may have stronger effector functions than the regulatory cell population, they might also have a stronger

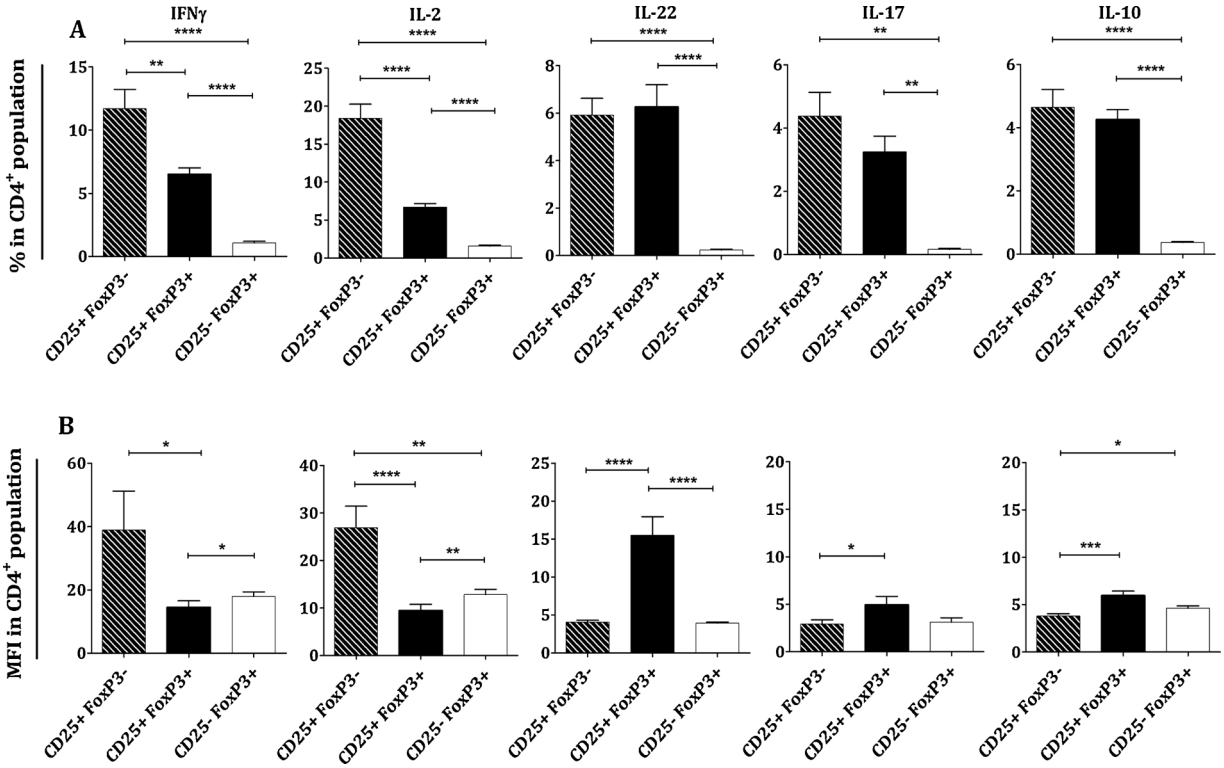

Fig. 4. Percentages of cytokine-producing cells in different FoxP3-expressing subpopulations in TDLNs from patients with BC.
The percentages of cytokine-producing $CD4^+CD25^+FoxP3^-$ (effector) and $CD4^+CD25^+FoxP3^+$ (regulatory) cells as well as $CD4^+CD25^-FoxP3^+$ cells (**A**) and the mean fluorescence intensity of their corresponding cytokines in these subpopulations (**B**) were determined and reported for the corresponding population. Data are shown as the mean ± SEM.
* Significant difference at the < 0.05 level, ** Significant difference at the < 0.01 level.
*** Significant difference at the < 0.001 level, **** Significant difference at the < 0.0001 level. SEM: Standard error of the mean; TDLN: Tumor-draining lymph node; BC: Breast cancer.

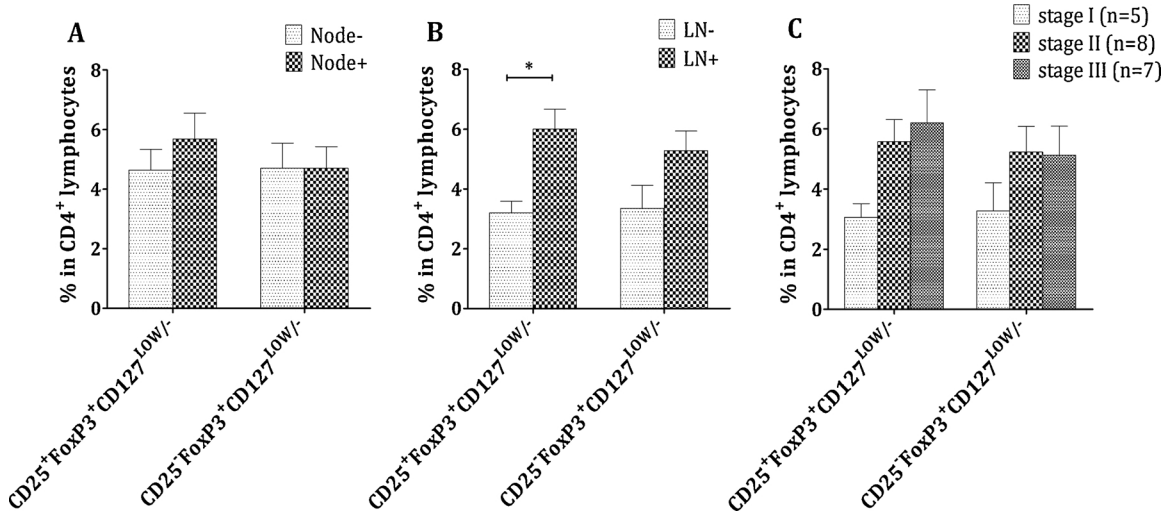

Fig. 5. Percentages of $CD25^+$ and $CD25^-$ $FoxP3^+$ subsets in patients with different clinicopathological characteristics.
The percentages of $CD4^+FoxP3^+CD127^{low/-}$ (**$CD25^+$** and **$CD25^-$**) cells were compared between positive and negative nodes (**A**), LN^+ and LN^- patients (**B**), and TDLNs from patients with BC in different stages (**C**). Data are shown as the mean ± SEM.
* Significant difference at the < 0.05 level (2-tailed).
SEM: Standard error of the mean; TDLN: Tumor-draining lymph node; BC: Breast cancer.

suppressive function than effector subsets due to their IL-10 production. The suppressive function and immunoregulatory effects of this T cell subset have consistently been documented in non-Hodgkin lymphoma, where they were able to repress the proliferation of autologous infiltrating $CD8^+$ T cells (Han et al., 2011; Yang et al., 2007). Yang and colleagues. further showed that in non-Hodgkin lymphoma, the phenotype of intratumoral $CD4^+$**$CD25^-$**$FoxP3^+$ T cells was very similar to regulatory T cells, yet different from effector T cells with regulatory activities (Yang et al., 2007). Bonelli et al. proposed that these cells are dysfunctional Tregs whose suppressive capacity is not cytokine-

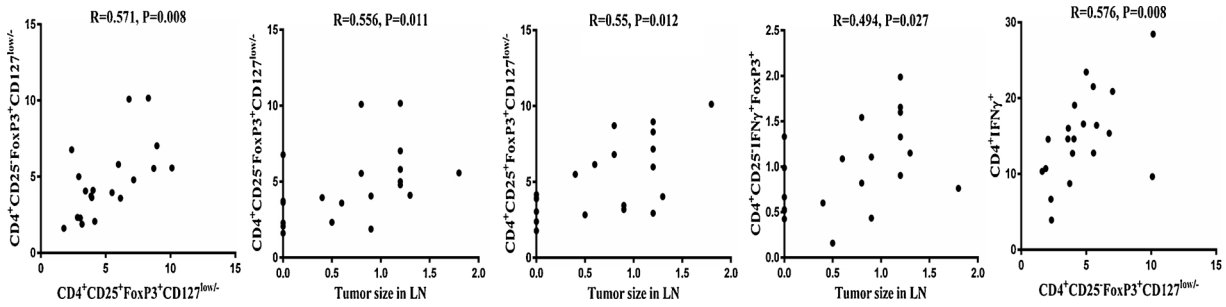

Fig. 6. Correlations among different $CD4^+$ lymphocyte subsets in TDLNs from patients with BC.
Correlations between different $CD4^+$ lymphocyte subpopulations were determined with the nonparametric Spearman correlation test.
TDLN: Tumor-draining lymph node; BC: Breast cancer.

mediated, since they were not able to suppress IFNγ production by responder T cells and had less suppressive potential compared to $CD4^+CD25^+CD127^-$ cells. These authors also showed that neither $CD4^+CD25^+CD127^-$ nor $CD4^+CD25^-CD127^-$ populations in peripheral blood mononuclear cells of patients with systemic lupus erythematosus were able to produce IFNγ, IL-10 or TGF-β after stimulation with anti-CD3 (Bonelli et al., 2009). Similar results were observed in the present study, in which a very small fraction of the $CD4^+CD25^-CD127^-$ population produced cytokines.

5. Conclusion

Taken together, our results indicate that $CD4^+\mathbf{CD25}^-FoxP3^+$ cells in TDLNs from patients with BC are probably an exhausted population, with phenotypes different from both $CD4^+\mathbf{CD25}^+Foxp3^+$ conventional Treg cells and $CD4^+\mathbf{CD25}^+Foxp3^-$ effector cells. These cells are probably a heterogeneous population of effector and regulatory T cells and/or an intermediate population between these two subsets, since they expressed intermediate levels of effector and regulatory cytokines. Further studies are recommended with larger sample sizes to analyze other effector and regulatory markers and transcription factors, in order to determine the exact phenotype and function of $CD4^+\mathbf{CD25}^-FoxP3^+$ cells.

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Ethical approval

All procedures involving human participants were in accordance with the ethical standards of the Ethics Committee of Shiraz University of Medical Sciences and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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