

CD4+CD25^{high}, CD8+CD28⁻ cells and thyroid autoantibodies in breast cancer patients

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Abstract

Aim of the study: To investigate the percentage of CD4+CD25^{high} cells (including Treg cells) and CD8+CD28⁻ cells in breast cancer patients with and without high levels of autoimmune thyroid antibodies.

Material and methods: Thirty-five women with breast cancer (9 of them having high thyroid antibodies) and fourteen healthy subjects were enrolled in this study. Flow cytometry was used to count CD4+CD25^{high} cells and CD8+CD28⁻ suppressive cells (CD8 cell subtypes).

Results: In the patient group, the percentage of CD28⁻ cells in CD8+ lymphocytes were higher [67.50% (55.1180.33) vs. 51.56% (42.5766.38); $p = 0.021$] and the percentage of CD28+CD45RO⁻ cells (memory cells) in CD8+ lymphocytes were lower than in the control group. CD4+CD25^{high} cell percentage in CD4+ lymphocytes was elevated in the patient group [6.44% (4.528.74) vs. 2.97% (1.72-4.34); $p < 0.001$]. When the cytometric parameters were compared between patients (with high vs. normal thyroid antibodies), the distribution of CD8+ cell subgroups was also similar. CD4+CD25^{high} cells among CD4+ lymphocytes were decreased in patients with high levels of thyroid antibodies [5.19% (3.426.17) vs. 6.99% (4.829.95); $p = 0.043$].

Conclusions: CD4+CD25^{high} cells may play a role in autoimmunity of breast cancer patients, and may be a predictive marker. Advanced studies which evaluate the possible links between regulatory cells and autoimmunity should be established in cancer patients.

Key words: breast cancer, Treg cells, autoimmunity, thyroid antibody.

(Centr Eur J Immunol 2014; 39 (3): 338-344)

Introduction

The relation between cancer and autoimmunity has been known for decades. In cancer cases, some autoimmune diseases and/or autoimmune responses may also be seen. In fact, increased thyroid disorder prevalence and high levels of autoimmune thyroid antibodies have been reported in women with breast cancer [1-3]. On the other hand, the underlying mechanisms regarding the association between autoimmunity and cancer have not been uncovered so far.

CD4+CD25+ Treg cells and CD8+CD28⁻ cells maintain the balance between autoimmunity and self-tolerance. One of the factors that influence susceptibility to thyroid autoimmunity is regulatory cells. Thymectomy and radiation-induced suppressor T cell depletion causes autoimmune thyroiditis [4]. It has been demonstrated that

CD4+CD25+ Treg cells prevented experimental autoimmune thyroiditis [5, 6] while their depletion triggered it [7, 8]. CD8+CD28⁻ cells also suppress the autoimmune process [9, 10].

These regulatory cells have also a relationship with cancer. CD4+CD25+ Treg cells and CD8+CD28⁻ cells play a role in anti-tumor immune response. It has been shown that CD4+CD25+ Treg cells increased in peripheral blood of cancer patients and they augmented tumor-induced immunosuppression [11-16]. Elevated CD8+CD28⁻ cells have also been found in cancer patients (lung cancer, mesothelioma, etc.) [11, 17-19]. Also in breast cancer, circulating Treg cell levels have been elevated, particularly in the advanced stage and in HER2+ disease [20, 21]. Phenotyping of lymphocytes in breast cancer showed that CD8+ T cells were predominant in the tumor infiltrating lymphocytes of invasive breast cancer [22]. The presence

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of cells expressing the markers for Tregs (CD4+CD25+) and suppressor (CD8+CD28-) in the tumor microenvironment was also detected.

The underlying mechanism regarding the association between autoimmunity and cancer may be related to regulatory cells. Thus, in this study, we aimed to investigate the percentage of CD4+CD25^{high} cells and CD8+CD28- cells in breast cancer patients with and without high levels of autoimmune thyroid antibodies.

Material and methods

Patients and study design

Thirty-five women with breast cancer as a patient group and fourteen age-matched healthy women as a control group were enrolled in this study (control group). Age values (median) of the patient and control groups were 42 (range 29-78) and 40 (29-55), respectively. None of the patients had received radiotherapy, hormonotherapy, or chemotherapy in

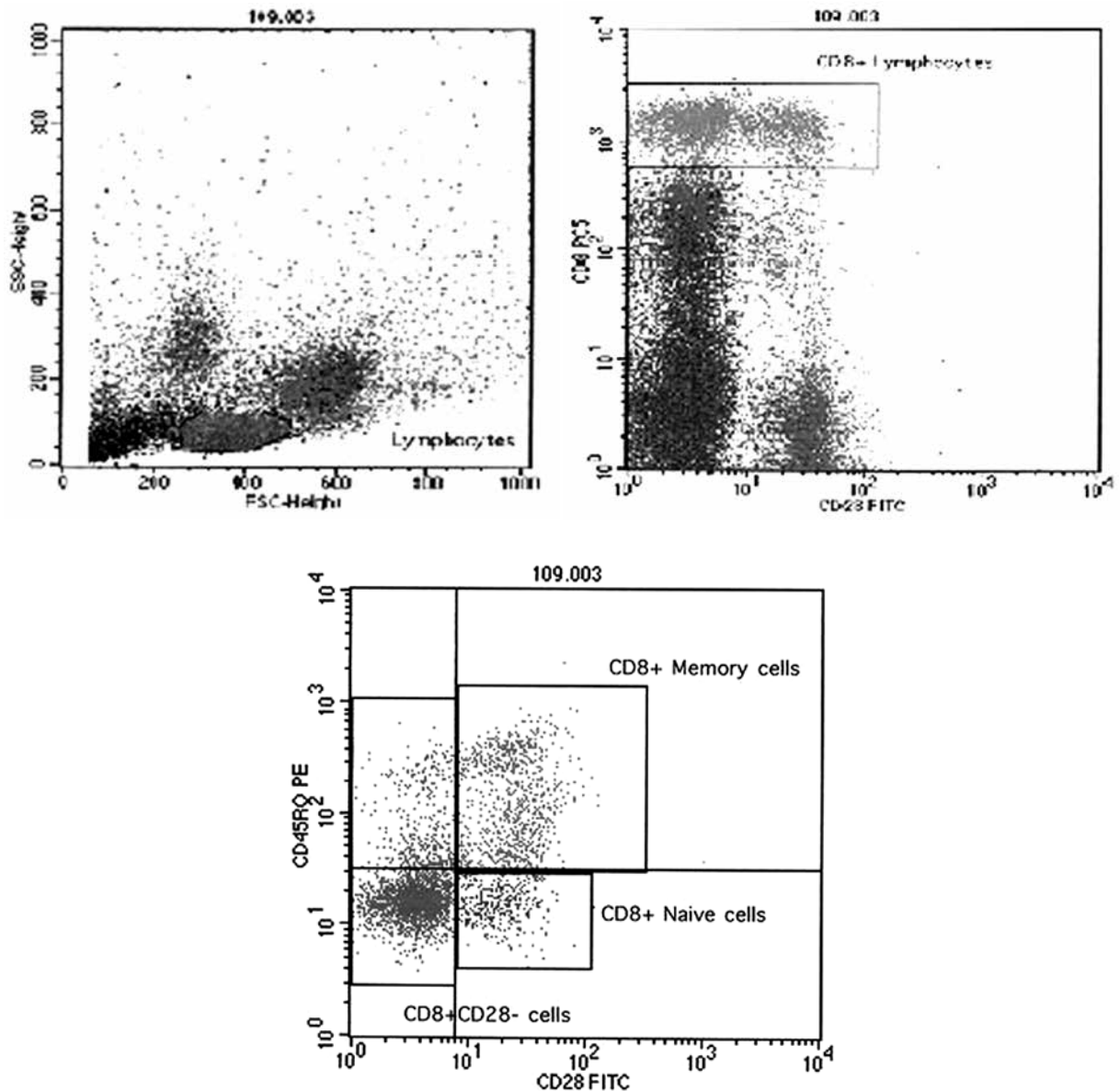


Fig. 1. CD8+ lymphocyte subtypes in FACS. The lymphocytes (in FS/SS histogram) and CD8^{bright} cells (in lymphocytes) were gated. The percentage of CD8+ lymphocyte subtypes (%) was calculated in CD8+ lymphocyte gated CD28/CD45RO histogram

Table 1. Patients with high thyroid antibodies

	TSH (uIU/ml)	ft4 (ng/dl)	ft3 (pg/ml)	Anti-TPO (IU/ml)	Anti-TG (IU/ml)
Range	(0.27-4.2)	(0.93-1.7)	(2.0-4.4)	(0-34)	(0-112)
Patient 1	2.12	0.99	2.55	292	57
Patient 2	22.60	0.95	2.46	476	1431
Patient 3	1.52	1.10	2.33	110	47
Patient 4	0.20	1.50	2.00	388	8
Patient 5	1.71	1.36	2.68	153	470
Patient 6	103.00	0.36	0.90	72	1722
Patient 7	21.28	0.61	2.36	124	22
Patient 8	3.43	1.02	2.95	488	3570
Patient 9	2.73	1.25	2.72	501	43

Anti-TPO – thyroid peroxidase antibody; Anti-TG – Anti-thyroglobulin antibody

Table 2. CD4+CD25^{high} T cells and CD8 cell subtypes in healthy women and breast cancer patients

% [median (25-75 percentile)]	Control	Breast cancer patients	p*
CD4+ cells in lymphocytes	37.51 (29.51-43.39)	37.37 (25.81-42.64)	0.723
CD4+CD25 ^{high} T cells in CD4+ lymphocytes	2.97 (1.72-4.34)	6.44 (4.52-8.74)	< 0.001
CD8+ cells in lymphocytes	26.58 (22.18-30.50)	20.94 (16.44-29.36)	0.063
Memory (CD28+CD45RO+) cells in CD8+ lymphocytes	38.04 (24.56-51.25)	26.94 (15.99-36.80)	0.054
Naive (CD28+CD45RO-) cells in CD8+ lymphocytes	25.92 (5.61-51.12)	6.44 (3.35-10.31)	0.004
CD28- cells in CD8+ lymphocytes	51.56 (42.57-66.38)	67.50 (55.11-80.33)	0.021

*Mann-Whitney U test

Table 3. CD4+CD25 high T cells and CD8 cell subtypes in both breast cancer patients with high and with normal thyroid antibodies

% [median (25-75 percentile)]	Normal	High	p*
Patients with thyroid antibodies...			
CD4+ cells in lymphocytes	33.50 (21.69-43.17)	40.13 (33.45-41.99)	0.450
CD4+CD25 ^{high} cells in CD4+ lymphocytes	6.99 (4.82-9.95)	5.19 (3.42-6.17)	0.043
CD8+ cells in lymphocytes	20.64 (16.27-29.71)	22.63 (18.51-26.80)	0.753
Memory (CD28+CD45RO+) cells in CD8+ lymphocytes	26.54 (15.47-37.19)	26.94 (14.41-43.42)	0.910
Naive (CD28+CD45RO-) cells in CD8+ lymphocytes	7.03 (3.04-10.50)	5.69 (3.33-22.67)	0.940
CD28- cells in CD8+ lymphocytes	70.14 (56.25-80.50)	60.35 (43.85-75.56)	0.345

*Mann Whitney U test

the last three months. The patients had no bone marrow metastasis. Some patients had been operated before the study.

Exclusion criteria were as follows: Male breast cancer patients and patients having concomitant chronic infectious disease including human immunodeficiency virus and tuberculosis, bone marrow metastasis, previous autoimmune disorder, thyroidectomy, corticosteroid or immunosuppressive therapy were excluded. The study has been approved by the local Ethical Committee.

Demographic and clinical data were assessed. Peripheral blood was collected for flow cytometry, complete blood count, serum free thyroxine (ft4), free triiodothyronine (ft3), thyroid stimulating hormone, anti-thyroglobulin (anti-TG) antibody, and anti-thyroid peroxidase antibody tests (anti-TPO). Those patients who had serum anti-TG antibody or anti-TPO antibody values at least twice the upper normal levels (115 IU/ml and 34 IU/ml values, respectively) were grouped as “patients with high

thyroid antibody levels” and others were grouped as “patients with normal thyroid antibody levels”.

Flow cytometry

Peripheral blood samples were obtained and studied immediately. Flow cytometry was used to count CD4+CD25^{high} T cells, and CD8+CD28⁻ suppressive cells (CD8 cell subtypes). Flow cytometry was performed on a Becton Dickinson FACSCalibur. Data were obtained and analyzed using CellQuest software.

Monoclonal antibodies: Anti-human monoclonal antibodies conjugated with fluorochromes and appropriate isotype controls were used: Fluorescein isothiocyanate (FITC) conjugated anti-CD28 (BD Pharmingen Catalog No: 555728), anti-CD4 (Caltag Lab Catalog No: MHCD0401), phycoerythrin-cyanine 5 (PC5) conjugated anti-CD8 (eBioscience Catalog No: 15-0088), anti-CD25 (BD Pharmingen Catalog No: 555433), and PE conjugated anti-CD45RO (BD Pharmingen Catalog No: 555493).

Cell preparation and surface staining: Human peripheral blood mononuclear cells were isolated using Histopaque

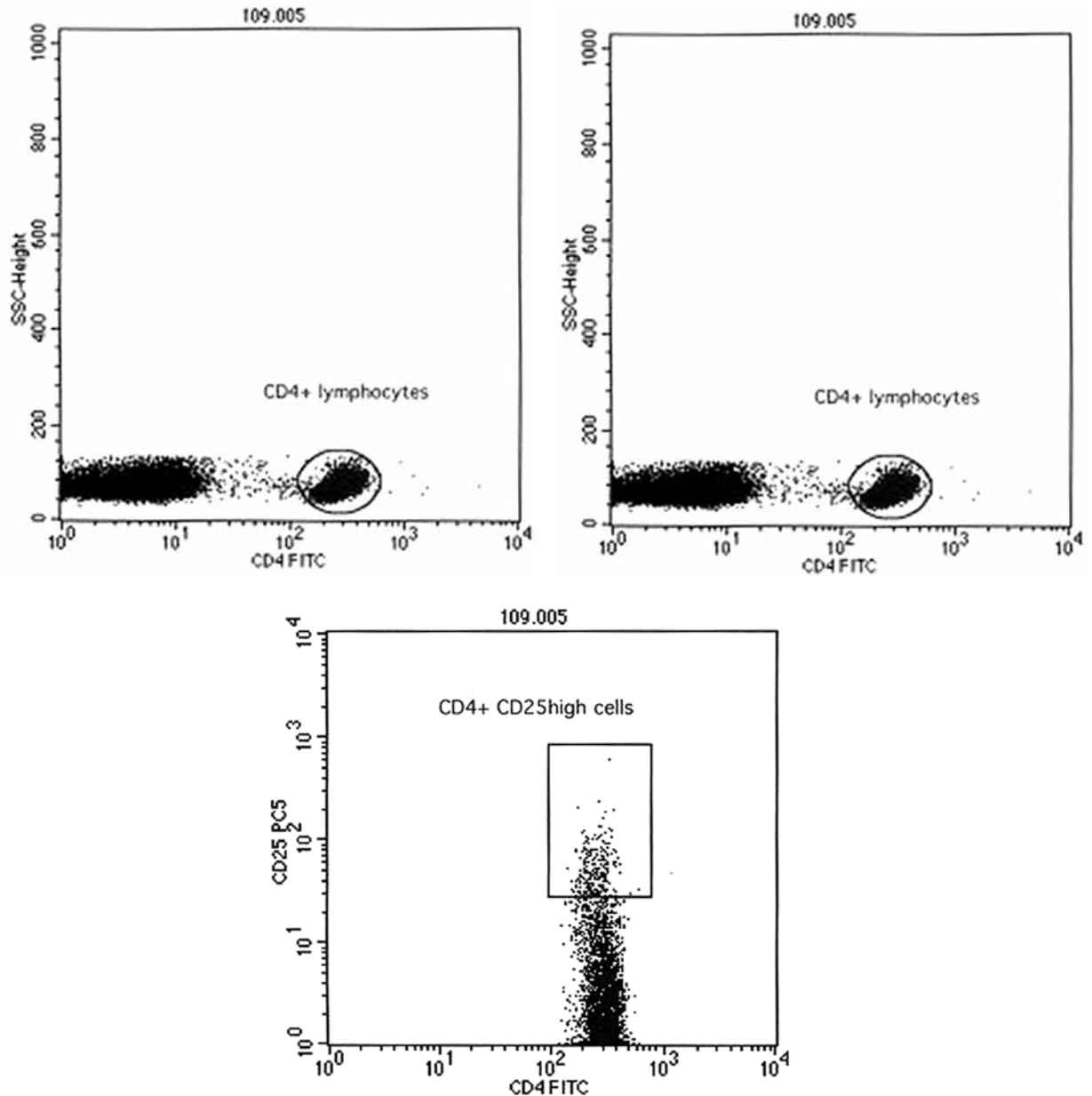


Fig. 2. CD4+CD25+ cells in FACS. The lymphocytes (in FS/SS histogram) and CD4+ cells (in lymphocytes) were gated. The percentage of CD4+CD25^{high} cells (%) was calculated in CD4+ lymphocyte gated CD4/CD25 histogram

(Sigma Catalog No: 1077) gradient centrifugation. 100- μ l aliquots were transferred to polypropylene test tubes (12 \times 75 mm; BD Bioscience Catalog No: 352052) and 20 μ l of conjugated monoclonal antibodies or isotype controls was added to each tube. Flow cytometric analysis was performed by BD FACS Calibur after the appropriate staining protocol.

Analysis: In anti-CD28/anti-CD45RO/anti-CD8 triple staining test tube, CD8^{bright} lymphocytes were gated in CD28/CD8 dotplot. Then CD8+CD28⁻ cells, CD8+CD28+C-CD45RO+ (Memory), and CD8+CD28+CD45RO⁻ (naive) cells were counted in CD8^{bright} lymphocyte gated CD28/CD45RO dotplot (Fig. 1). Anti-CD4/anti-CD25 double staining was used for CD4+CD25^{high} T cells count. CD4+ lymphocytes were gated in FS/CD4 dotplot. CD4+CD25^{high} lymphocytes were counted in CD4/CD25 histogram (Fig. 2).

Statistics

In statistical analysis, Mann-Whitney *U* test was used to compare immunological and hematological parameters between breast cancer patients and healthy controls, and between patients with high vs. normal levels of thyroid antibodies. Results are presented as medians and percentiles.

Results

Nine patients had high levels of at least one of anti-TPO and anti-TG antibodies (Table 1). Three of them had met-

astatic disease. Twenty-six patients had normal levels of thyroid antibodies and they were euthyroid. Four patients with normal antibodies had metastatic disease. White blood cells (WBC), lymphocytes, granulocytes, red blood cells, platelet counts; CD4+ and CD8+ cells in lymphocytes were not different between the groups (patient vs. control).

Flow cytometry indicated significant differences between patient and control groups with respect to the distribution of CD8+ T cell subgroups. In the patient group, the percentage of CD28⁻ cells in CD8+ lymphocytes was higher [67.50% (55.1180.33) vs. 51.56% (42.5766.38); $p = 0.021$] and the percentage of CD28+CD45RO⁻ cells (naive cells) in CD8+ lymphocytes was lower than in the control group [6.44% (3.3510.31) vs. 25.92% (5.6151.12); $p = 0.004$] (Table 2). CD4+CD25^{high} cells percentage in CD4+ lymphocytes were elevated in the patient group [6.44% (4.528.74) vs. 2.97% (1.724.34); $p < 0.001$] (Table 2).

When the cytometric parameters were compared between patients (with high vs. normal thyroid antibodies), CD4+ and CD8+ cells in lymphocytes were not different. The distribution of CD8+ cell subgroups was also similar. CD4+CD25^{high} cells among CD4+ lymphocytes were decreased in patients with high levels of thyroid antibodies [5.19% (3.426.17) vs. 6.99% (4.829.95); $p = 0.043$] (Table 3, Fig. 3). We did not find any significant correlation between CD4+CD25^{high} cells or CD8+CD28⁻ cells and high thyroid autoantibodies level.

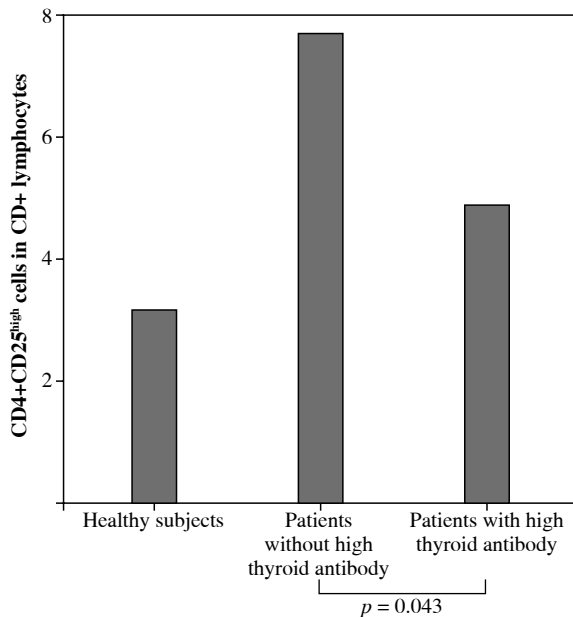


Fig. 3. CD4+CD25^{high} cell percentages (%) in breast cancer patients with and without high thyroid autoantibodies and healthy women. Treg cells among CD4+ lymphocytes were decreased in patients with high levels of thyroid antibodies compared in patients without

Discussion

In this study to our best notice for the first time in the literature, the relationships of CD4+CD25^{high} cells and CD8+CD28⁻ cells in breast cancer patients with thyroid autoimmunity was investigated. Although we found increased CD4+CD25^{high} (these cells include Treg cells) cells and CD8+CD28⁻ cells in breast cancer patients, CD4+CD25^{high} cells were lower in patients with elevated thyroid antibodies when compared with those having normal thyroid antibodies, on the other hand CD8+CD28⁻ cells were not different between patients with and without thyroid autoantibodies.

This study has several limitations. We were not able to use CD4/CD25/FoxP3, CD4/CD25/CD127, or CD3/CD8/CD28/CD45RO staining because of technical barriers. Also CD4+CD25^{high} cells may contain activated non-regulatory T cells and some CD8+ cells may be NK cells. The numbers of patient and control groups were relatively small and study population was heterogeneous (lymph node status, surgery, receptor status etc.). This study could be considered as a preliminary study. Survival and some other clinical parameters have not been evaluated. Besides, the suppressive function of the aforementioned cells has not been shown in vitro. It is reported that CD4+ CD25+ Treg cells are lower in autoimmune disease [23]. We did

not compare the patients with high level thyroid antibody with non-cancer autoimmune patients because the study was performed only in medical oncology and biochemistry departments.

We have previously investigated CD4+CD25^{high} cells and CD8+CD28⁻ cells in advanced stage lung cancer patients [11]. The percentage of CD8+CD28⁻ cells, CD28⁻/CD28⁺ cell ratio in CD8⁺ lymphocytes and CD4+CD25^{high} cells were elevated in the patient group. Meloni *et al.* [18] also showed that these regulatory cells were increased in peripheral blood of patients with pleural mesothelioma and lung cancer. Tsukishiro *et al.* [24] investigated CD8 T cells and they found elevated CD8+CD28⁻ cells in circulation of patients with head and neck cancer. In breast cancer patients, peripheral CD4+ CD25+ Treg cells' increasing is controversial. Some authors found higher CD4+ CD25+ Treg cell percentages in breast cancer patients [21, 25], but others did not [26, 27]. In our presented study, CD4+ CD25^{high} cells and CD8+CD28⁻ cells were elevated in breast cancer patients.

Wei *et al.* [12] have reviewed the role of CD4+CD25+ Treg cells in the balance between anti-tumor immunity and autoimmunity. Treg cells were accepted as suppressing both anti-tumor immunity and autoimmunity. Several studies have shown that Treg cells played a role also in autoimmunity and self-tolerance [4-8, 28]. Previously, it has been demonstrated that depletion of CD4+CD25+ Treg cells resulted in inhibition or slowing of malignant tumors, but that augmented antitumor immune response – via Treg cell depletion – may be complicated by autoimmunity [12]. For example, Phan *et al.* [29] investigated Treg depletion (via CTLA-4 blockage) plus vaccination in 14 metastatic melanoma and they showed that autoimmune manifestations were present in 43% of the patients.

It is also known that Treg cells have a role in autoimmune thyroid disorders. For example, Klatka *et al.* [30] examined CD4+CD25+ cells in Graves' disease. CD4+CD25+ cells were reported to be decreased. We have also found decreased CD4+CD25^{high} cells in breast cancer patients with high autoimmune thyroid antibodies in the study.

Smyth *et al.* [31] investigated the prevalence of thyroid autoantibodies, and its association with disease prognosis in breast cancer patients. An elevated anti-TPO level was more frequent in their patients when compared with the control group. Survival analysis demonstrated that the patients with higher anti-TPO levels were associated with better disease-free and overall survival compared with those who had normal anti-TPO levels. In our study, we have shown the presence of a relationship between two prognostic factors (thyroid autoimmunity and Treg cells) in patients with breast cancer.

In conclusion, this preliminary study shows that the percentage of CD4+CD25^{high} cells is lower in breast cancer patients having thyroid autoantibodies when compared

to those not having them. It suggests that CD4+CD25^{high} (probable Treg) cells play a role in autoimmunity of breast cancer patients. This finding may be used as a predictive marker. Comprehensive clinical studies investigating regulatory cells and autoimmunity are needed in cancer patients.

The authors declare no conflict of interest.

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