Association of CD4+ CD25+ FOXP3+ regulatory T-cells and human papilloma virus infection in Egyptian Women with breast cancer

Amany Tawfeik
Molecular Immunology Unit for Infectious Diseases, Department of Microbiology, Faculty of Medicine for Girls, Al-Azhar University

Ahmed Mora
Department of Chemistry, Faculty of Science, Al-Azhar University

Ahmed Osman
Biochemistry Department, Faculty of Science, Ain Shams University. Biotechnology Program, Basic and Applied Sciences Institute, Egypt-Japan University of Science and Technology

Nabila Elsheikh
Molecular Immunology Unit for Infectious Diseases, Department of Microbiology, Faculty of Medicine for Girls, Al-Azhar University

Mohamed Elrefaei (elrefaei.mohamed@mayo.edu)
Mayo Clinic

Research article

Keywords: T regulatory cells, CD8+ Tc cells, Her-2, Fox3P

Posted Date: January 8th, 2020

DOI: https://doi.org/10.21203/rs.2.17376/v2

Abstract

Several subsets of regulatory CD4+ T cells (CD4+ Tregs) have been described in peripheral blood and tumor microenvironment and blood of breast cancer (BC) patients and may play a key role in the progression of BC. High-risk human papilloma virus (HPV) have a causal role in a significant proportion of cervical, and head, and neck tumors and may play an important role in evoking neoplasia in BC. In this study we assessed the prevalence of CD4+Tregs (CD4+CD25+ FOXP3+ cells) and CD3+ CD8+ T cells by flow cytometry in peripheral blood from a total of 55 Egyptian women, including 20 treatment-naïve BC, 15 with breast benign lesions (BBL) and 20 healthy volunteers (HV). High-risk HPV genotype type 16, 18, and 31 was investigated in breast tissue from all BC and BBL patients using Real-Time PCR. HPV was detected in 4 BC, but in none of BBL patients. The frequency of CD4+ Tregs was significantly higher in BC compared to BBL and HV, ($p < 0.001$). In addition, we observed a significantly higher frequency of CD3+ CD8+ T cells in peripheral blood of patients with late stage III compared to early stage I and II BC ($p = 0.011$). However, there was no significant association between the ratio of CD8+ T cell to CD4+ Tregs frequencies and the expression of Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2). In conclusion, CD4+ Tregs may contribute to progression BC in Egyptian women with HPV infection. The potential role CD4+ Tregs as a prognostic or predictive parameter should be analyzed in a larger longitudinal study with sufficient follow-up time.

Background

Breast cancer (BC) is the second-leading cause of death from cancer in Egyptian women. The overall incidence of cancer is 157.0 per 100,000 Egyptian females with the highest incidence being BC (32%). A 3-fold increase in incidence of cancer is predicted by 2050 [1]. While the etiology of BC is still unknown, numerous risk factors have been identified. Increased public awareness and regular screening can play a vital role in the prevention, early detection and treatment of BC [2]. High-risk human papilloma virus (HPV) has a causal role in a significant proportion of cervical, head, and neck tumors and may play an important role in evoking neoplasia in breast cancer. [3].

The expression of hormone receptors such as estrogen receptor (ER) and progesterone receptor (PR) in BC predicts the response to growth factors, patient prognosis, and response to targeted therapy [4]. Patients positive for these receptors tend to have improved prognosis and better response to hormonal therapy [5]. In contrasts, BC patients expressing Human Epidermal Growth Factor Receptor 2 (HER2+) have increased disease recurrence and poor prognosis [6]. Triple negative breast cancer is a unique subtype constituting about 20% of breast cancer cases is characterized by the lack of ER, PR, and HER2 expression and poor clinical outcome [7, 8].

Several subsets of regulatory CD4+ T cells (CD4+ Tregs) have been described in peripheral blood and tumor microenvironment from breast cancer patients with invasive high-grade breast carcinomas, and may play a key role in the progression of breast cancer expression [9-13]. CD4+ Tregs express high levels of FOX P3, CD25, tumor necrosis factor receptor-related protein (GITR), CTLA-4, and CD103 [14-16]. In

addition, decreased expression of CD127 on human CD4+ Tregs is inversely correlated with FoxP3 expression [17, 18]. Naturally" occurring CD4+ Tregs are derived in the thymus, while "Adaptive" CD4+ Tregs are believed to come from mature T cells in the periphery under specific conditions of persistent antigenic stimulation [19]. CD4+Tregs exert their non-antigen specific inhibitory effects on T cell proliferation and cytokine production through a cell-cell contact-dependent mechanism [20, 21]. These cells have also been shown to inhibit the cytotoxic function and decrease the expression of major histocompatibility class I molecules on target cells. CD4+ Tregs were shown to secrete immunosuppressive cytokines interleukin-10 and TGF-β that suppress the induction of antitumor immunity [22, 23].

The relationship between the severity of BC and the frequency of CD4+ Tregs is not well defined. It has been reported that the number of CD4+ Tregs was significantly higher in patients with invasive high-grade BC, hormone-receptor-negative BC, and may correlate with both shorter relapse-free and overall survival time [11, 24, 25]. Increased frequency of CD4+ Tregs in BC patients may play a key role in the prognosis of BC [26, 27]. These findings suggest that quantification of CD4+ Tregs may be valuable for assessing BC progression and as an important therapeutic target. In contrast, the frequency of CD4+ Tregs did not directly correlate with the clinical stage of breast cancer in other studies [14, 28]. The aim of this study was to assess the frequency of CD4+ Tregs and CD8+ T cells in Egyptian women with BC in relation to high-risk HPV infection compared with healthy volunteers.

Methods

Population and source of samples

All women over the age of 18 visiting the oncology clinic at Al-Azhar university hospital were invited to volunteer in the study. All new cases of histologically confirmed BC were enrolled. Women that were diagnosed with benign breast lesion, as well as women residing in the same areas and admitted to the hospital, for a wide spectrum of acute, non-neoplastic conditions unrelated to known or likely risk factors for breast cancer were invited to volunteer as controls in the study. Women with gynecological, hormonal or neoplastic diseases were excluded from the study. Peripheral blood and frozen breast tissue samples were collected according to the ethical regulations at Al-Azhar university hospital. Demographic and clinical information was obtained from medical records. The study received approval from the Institutional Review Board at Al-Azhar University. Specimens were collected from 20 treatment-naïve women with primary BC, 15 benign breast lesions (BBL), and 20 healthy volunteers (HV) after providing informed written consent. To avoid a potential influence of major surgical procedures, as well as of neoadjuvant treatment, blood was drawn after core biopsy but before definitive surgery.

Detection and genotyping of high risk HPV

HPV DNA detection and typing were carried out using the “Advanced HPV high-risk Detection and Typing PCR kits (genesig, England), following manufacture protocol. Three high risk HPV types; 16, 18, and 31 were investigated. Each Kit is an in vitro Real -Time amplification test for qualitative detection of HPV specific genotype. It is based on two major processes: isolation of DNA from specimens and Real Time amplification of the E1 – E2 region of HPV. The β-globine gene was used as an internal control. The PCR samples were analyzed by the “One step Plus^{TM} Real Time PCR System with Notebook Computer from Applied Biosystem.

Clinicopathological risk factors were assessed, including age, lymph node metastasis, tumor size, histologic grade, tumor stage, hormonal receptor status including: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Tumor stages were classified according to the American Joint Committee on Cancer (AJCC)-TNM (tumor, node, metastases) classification.

Immunophenotyping of CD4+ Tregs and CD8+ T cells

Blood samples from patients with primary BC, BBL, and HV were procured from treatment-naïve patients prior to any surgery to avoid a potential influence of major surgical procedures and neoadjuvant treatment. Flow Cytometry testing was performed in a single laboratory on fresh blood samples the same day in was collected as previously described [29]. Two ml peripheral blood sample was collected in EDTA tubes. 100 ul of whole blood was incubated for 15 minutes at room temperature with antibodies against CD3, CD4, CD8, CD25, and intracellular transcription factor FOXP3, using immobilization buffer (BD, Biosciences, San Jose, USA). Red cell lysis was performed with FACS Lysing Solution (BD Biosciences). Isotype controls, IgG1a FITC / IgG2 PE and IgG APC were used for detection of non-specific binding background. Compensation settings were established before sample acquisition using color calibrate beads. A minimum of 30,000 CD3+ T cells per sample was acquired using a 4 color FACSCalibur (Beckton Dickenson (BD), USA) and analysis was performed by Cell-Quest Pro software (BD, USA). Gating was performed using the fluorescence-minus-one (FMO) control for each marker. Results were expressed as percent of CD25+ FOXP3+ CD4+ Tregs from the CD4+ lymphocyte gate or CD3+ CD8+ T cells from the lymphocyte gate after subtraction of the isotype control background values (<0.1%).

Statistical analysis

Statistical analysis was done using IBM SPSS® Statistics version 23 (IBM® Corp., Armonk, NY, USA). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). All

tests were two-tailed. When more than two groups of data sets were compared, one-way analysis of variance (ANOVA) was performed as described. A p-value < 0.05 was considered significant.

Results

Patient demographics and disease characteristics

The mean age of patients with BC, BBL, and HV was 50.6 +11.7, 55.0 +13.7, 64.3 +9.9 respectively (table 1). There was no significant difference in age between patients with BC compared to BBL (P = 0.188). HPV type 16 and 18 was detected in breast tissue from 4/20 BC patients compared to 0/15 of BBL patients and none of the healthy volunteers. Metaplastic carcinoma is an aggressive type of BC characterized by low hormone receptor expression and poor prognosis [30]. Metaplastic carcinoma and invasive duct carcinoma were found in 3/4 (75%) and 1/4 HPV infected patients respectively compared to 0/15 and 13/15 HPV negative BBL patients respectively. These results suggest that HPV infection may be associated with more aggressive BC in Egyptian women. Ten of 20 patients with BC (50%) were diagnosed with early stage disease (stage I and II) while 6/20 (30%) were diagnosed with advanced disease (stage III).

BC tumors are regarded to be hormonal receptor (HR) positive if at least 1% stained positive for ER or PR assays as previously described [31]. The number of BC patients expressing ER, PR, or HER2 was 13/20 (65%), 10/20 (50%), and 4/20 (20%) respectively (Table 2). While 3/20 (15%) BC patients lacked expression of all 3 hormonal receptors (triple negative).

Prevalence of CD4+ Tregs

We chacaterized CD4+ Tregs by the expression of CD4, CD25, and FOXP3 [14]. Representative plots of the gating and frequency of frequency of CD4+ Tregs are shown in Fig. 1. We observed a significantly higher frequencey of CD4+ Treg in patients with BC compared to BBL and HV (p<0.001; table 1). In contrast, there was no significant difference in the total percentage of CD8+ and CD4+ T cells between the three groups (Table 1).

While invasive duct carcinoma (IDC) was the most prevalent BC subtype, there was no significant difference in frequency of CD4+ Tregs between patients with IDC and other pathological subtypes (P = 0.687; Fig. 2A). In addition, we observed no significant difference in frequency of CD4+ Tregs between BC patients with and without high-risk HPV infection (P = 0.750; Fig. 2B), lymph node metastases (P = 0.195; Fig. 2C), poorly differentiated grade III tumor (P = 0.211; Fig. 2D), late stage III tumor (P = 0.391; Fig. 2E), and triple negative tumor (P = 0.091; Fig. 2F).

Tumor-infiltrating CD8+ T lymphocytes in BC patients were reported to have antitumor activity resulting in a favorable effect on patients' survival [32]. Decreased number of tumor infiltrating CD8+ T cells in BC was significantly associated with lymph node metastasis and a higher disease stage. In this study we

observed a significantly higher frequencey of CD8+ T cells in peripheral blood of patients with late stage III compared to early stage I and II BC (p = 0.011; Fig. 3A). In contrast, there was no significant difference in the frequency of CD4+ T cells (Fig. 3B; P = 0.792) or CD4+ Tregs (Fig. 3C; P = 0.972) between early compared to late stage BC patients. In addition, there was no significant association between the ratio of CD8+ T cell to CD4+ Tregs frequencies and the expression of ER, PR, or HER2 (data not shown). The prognostic role of CD8+ T cell frequency as a prognostic marker in BC is not well defined and should be assessed in a large prospective study.

Discussion

The adaptive immune system plays an important role in BC development and prognosis. Cytotoxic CD8+ T lymphocytes and CD4+ T helper cells may promote tumor eradication. In contrast, CD4+ Tregs have an immunosuppressive effect that may promote tumor growth and progression of BC. In addition, heterogeneous expression of hormone receptors and growth factors in BC affects patient-specific adaptive immune responses [33]. In this study we assessed the prevalence and prognostic value of CD4+ T regs and CD8+ T cells in Egyptian females with breast cancer. We observed a significantly higher frequency of CD4+ Tregs in patients with BC compared to BBL and HV. These results suggest that the composition of T cell subsets in peripheral blood of Egyptian patients with BC may favor an immunoregulatory phenotype that is distinct from BBL and HV. Our finding is consistent with previously reported analysis of freshly isolated lymphocytes from normal and malignant breast tissue samples [34].

We assessed the histologic grade and disease stage as variables to gain further insight into the relationship between the frequency of CD4+ Tregs and tumor biology. In addition, we analyzed the frequency of CD4+ Tregs among clinically defined surrogates of BC molecular subtypes including ER+, Her2+, and triple negative BC in order to explore whether any particular clinical disease correlates were associated with increased number of CD4+ Tregs. Increased CD4+ Tregs frequency was associated with poor prognostic characteristics such as higher histological grade, lymph node metastasis [14, 35, 36].

We observed no significant increase in frequency of CD4+ Tregs in BC patients with poor prognostic characteristics such as higher histological grade, lymph node metastasis, and presence of high-risk HPV which may be due to the relatively small number of patients in the study. Increased number of CD4+ T regs was reported in the peripheral blood of patients with Her-2/neu-positive early breast cancer [9]. However, concurrent HPV infection and increased CD4+ Treg frequency did not seem to influence BC prognosis [37]. We postulate that circulating CD4+ Tregs could be used as a prognostic or predictive parameter in Egyptian women with BC and should be assessed in a larger longitudinal study with sufficient follow-up time.

It has long been hypothesized that hormone dependent oncogenic viruses, such as HPV may have causal roles in some cancers [38]. Expression of and high risk HPV DNA in BC [39] and HPV proteins in cervical cancer [40] was recently reported. In this study, we demonstrated the presence of high-risk HPV in the tissues of 20% of breast cancer specimens. These results suggest a possible causal role for high-risk

HPV in Egyptian women with BC and potential prevention of some breast cancers by HPV vaccination [3].

A positive correlation was observed in multiple studies between the number of tumor infiltrating cytotoxic CD8+ T cells and clinical outcome in BC that may improve patient survival [13, 26, 36, 41]. However, CD8+ T cell infiltrates were also shown to be associated with higher histological grade and basal phenotype, and inversely associated with ER and PR expression. In addition, CD4+ Tregs and CD8+ T cells may play a role in the dissemination of circulating breast cancer cells [42]. These results suggest that cytotoxic CD8+ T cells are heavily involved in antitumor immune responses. In our study, we performed multivariate least square regression analysis after controlling for age to determine the relationship between clinical disease stage and the frequency of CD4+ Tregs and CD8+ T cells. We observed no significant difference in the frequency of peripheral blood CD4+ Tregs between early and late stages of BC. In contrast, a significantly higher frequency of CD8+ T cells in the peripheral blood correlated with late stage BC suggesting that the total CD8+ T cell counts are not constant during the course of BC and may serve as an important biomarker of disease. In addition, the ratio of CD4+ Tregs and CD8+ T cells was different between early and late stage of BC suggesting that different mechanisms control CD4+ Tregs and CD8+ T cells homeostasis. Our results are consistent with previously reported analysis of freshly isolated lymphocytes from a small set of normal and malignant breast tissue samples [43]. The ratio of CD4+ Tregs and CD8+ T cells as a prognostic marker of disease should be evaluated more clearly in a larger prospective study.

In vitro data often do not adequately address the complexity of in vivo immune processes. However, our study provides important information on the role of various risk factors including HPV in the pathogenesis of breast cancer in Egypt. The cross-sectional design does not permit definitive analysis of the predictive value of CD4+ Tregs and CD8+ T cell frequencies in BC. Nevertheless, we believe that our study provided an important evaluation of CD4+ Tregs and CD8+ T cell frequencies in Egyptian women with BC. Our results support the design of subsequent longitudinal studies to directly examine the relationship between the frequency of CD4+ Tregs and CD8+ T cells in the peripheral blood and the response to breast cancer treatment.

Declarations

Ethics approval: The study received approval from the Institutional Review Board at Al-Azhar University.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: This work was funded by the Science and Technology Development Fund (STDF), Egypt. Grant # 22914.

Authors contributions:

AT, AM, and AO acquired, analyzed, and interpreted the patient data. AT, NE, and ME were major contributors in writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements: Not applicable.

References

1. Ibrahim AS, Khaled HM, Mikhail NN, Baraka H, Kamel H: **Cancer incidence in egypt: results of the national population-based cancer registry program**. *J Cancer Epidemiol* 2014, **2014**:437971.
2. El Saghir NS: **Responding to the challenges of breast cancer in egypt and other arab countries**. *J Egypt Natl Canc Inst* 2008, **20**(4):309-312.
3. Heng B, Glenn WK, Ye Y, Tran B, Delprado W, Lutze-Mann L, Whitaker NJ, Lawson JS: **Human papilloma virus is associated with breast cancer**. *Br J Cancer* 2009, **101**(8):1345-1350.
4. Early Breast Cancer Trialists' Collaborative G: **Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials**. *Lancet* 2005, **365**(9472):1687-1717.
5. Arpino G, Weiss H, Lee AV, Schiff R, De Placido S, Osborne CK, Elledge RM: **Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance**. *J Natl Cancer Inst* 2005, **97**(17):1254-1261.
6. De Laurentiis M, Arpino G, Massarelli E, Ruggiero A, Carlomagno C, Ciardiello F, Tortora G, D'Agostino D, Caputo F, Cancello G *et al*: **A meta-analysis on the interaction between HER-2 expression and response to endocrine treatment in advanced breast cancer**. *Clin Cancer Res* 2005, **11**(13):4741-4748.
7. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V: **Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry**. *Cancer* 2007, **109**(9):1721-1728.
8. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA: **Triple-negative breast cancer: clinical features and patterns of recurrence**. *Clin Cancer Res* 2007, **13**(15 Pt 1):4429-4434.
9. Decker T, Fischer G, Bucke W, Bucke P, Stotz F, Gruneberger A, Gropp-Meier M, Wiedemann G, Pfeiffer C, Peschel C *et al*: **Increased number of regulatory T cells (T-regs) in the peripheral blood of patients with Her-2/neu-positive early breast cancer**. *J Cancer Res Clin Oncol* 2012, **138**(11):1945-1950.
10. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, Banham AH: **Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse**. *J*

Clin Oncol 2006, **24**(34):5373-5380.

11. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS *et al*: **Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma**. *J Immunol* 2002, **169**(5):2756-2761.
12. Wang Y, Sun J, Zheng R, Shao Q, Gao W, Song B, Chen X, Qu X: **Regulatory T cells are an important prognostic factor in breast cancer: a systematic review and meta-analysis**. *Neoplasma* 2016, **63**(5):789-798.
13. Bailur JK, Gueckel B, Derhovanessian E, Pawelec G: **Presence of circulating Her2-reactive CD8 + T-cells is associated with lower frequencies of myeloid-derived suppressor cells and regulatory T cells, and better survival in older breast cancer patients**. *Breast Cancer Res* 2015, **17**:34.
14. Khalife E, Khodadadi A, Talaeizadeh A, Rahimian L, Nemati M, Jafarzadeh A: **Overexpression of Regulatory T Cell-Related Markers (FOXP3, CTLA-4 and GITR) by Peripheral Blood Mononuclear Cells from Patients with Breast Cancer**. *Asian Pac J Cancer Prev* 2018, **19**(11):3019-3025.
15. Wang J, Yang J: **Identification of CD4(+)CD25(+)CD127(-) regulatory T cells and CD14(+)HLA(-)DR(-)/low myeloid-derived suppressor cells and their roles in the prognosis of breast cancer**. *Biomed Rep* 2016, **5**(2):208-212.
16. Fontenot JD, Gavin MA, Rudensky AY: **Foxp3 programs the development and function of CD4+CD25+ regulatory T cells**. *Nat Immunol* 2003, **4**(4):330-336.
17. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B *et al*: **CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells**. *J Exp Med* 2006, **203**(7):1701-1711.
18. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R *et al*: **Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells**. *J Exp Med* 2006, **203**(7):1693-1700.
19. Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR, Vignali DA: **The development and function of regulatory T cells**. *Cell Mol Life Sci* 2009, **66**(16):2603-2622.
20. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA: **CD4+CD25high regulatory cells in human peripheral blood**. *J Immunol* 2001, **167**(3):1245-1253.
21. Scotto L, Naiyer AJ, Galluzzo S, Rossi P, Manavalan JS, Kim-Schulze S, Fang J, Favera RD, Cortesini R, Suciu-Foca N: **Overlap between molecular markers expressed by naturally occurring CD4+CD25+ regulatory T cells and antigen specific CD4+CD25+ and CD8+CD28- T suppressor cells**. *Hum Immunol* 2004, **65**(11):1297-1306.
22. Strauss L, Bergmann C, Szczepanski M, Gooding W, Johnson JT, Whiteside TL: **A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment**. *Clin Cancer Res* 2007, **13**(15 Pt 1):4345-4354.
23. Levings MK, Sangregorio R, Sartirana C, Moschin AL, Battaglia M, Orban PC, Roncarolo MG: **Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin**

10, and are distinct from type 1 T regulatory cells. *J Exp Med* 2002, **196**(10):1335-1346.

24. Liu S, Foulkes WD, Leung S, Gao D, Lau S, Kos Z, Nielsen TO: **Prognostic significance of FOXP3+ tumor-infiltrating lymphocytes in breast cancer depends on estrogen receptor and human epidermal growth factor receptor-2 expression status and concurrent cytotoxic T-cell infiltration**. *Breast Cancer Res* 2014, **16**(5):432.
25. Banin-Hirata BK, de Oliveira CEC, Losi-Guembarovski R, Ozawa PMM, Vitiello GAF, de Almeida FC, Derossi DR, Andre ND, Watanabe MAE: **The prognostic value of regulatory T cells infiltration in HER2-enriched breast cancer microenvironment**. *Int Rev Immunol* 2018, **37**(3):144-150.
26. Matsumoto H, Thike AA, Li H, Yeong J, Koo SL, Dent RA, Tan PH, Iqbal J: **Increased CD4 and CD8-positive T cell infiltrate signifies good prognosis in a subset of triple-negative breast cancer**. *Breast Cancer Res Treat* 2016, **156**(2):237-247.
27. Xu L, Zhou Y, Xiao DM, Qin M, Luo JM, Tang XY: **[The change of CD4+ CD25high CCR6+ regulatory T cells in breast cancer patients]**. *Sichuan Da Xue Xue Bao Yi Xue Ban* 2010, **41**(3):415-419.
28. Perez SA, Karamouzis MV, Skarlos DV, Ardavanis A, Sotiriadou NN, Iliopoulou EG, Salagianni ML, Orphanos G, Baxevanis CN, Rigatos G *et al*: **CD4+CD25+ regulatory T-cell frequency in HER-2/neu (HER)-positive and HER-negative advanced-stage breast cancer patients**. *Clin Cancer Res* 2007, **13**(9):2714-2721.
29. Elrefaei M, Burke CM, Baker CA, Jones NG, Bousheri S, Bangsberg DR, Cao H: **HIV-specific TGF-beta-positive CD4+ T cells do not express regulatory surface markers and are regulated by CTLA-4**. *AIDS Res Hum Retroviruses* 2010, **26**(3):329-337.
30. Zhang Y, Lv F, Yang Y, Qian X, Lang R, Fan Y, Liu F, Li Y, Li S, Shen B *et al*: **Clinicopathological Features and Prognosis of Metaplastic Breast Carcinoma: Experience of a Major Chinese Cancer Center**. *PLoS One* 2015, **10**(6):e0131409.
31. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, Fitzgibbons PL, Francis G, Goldstein NS, Hayes M *et al*: **American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer**. *Arch Pathol Lab Med* 2010, **134**(6):907-922.
32. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, Ellis IO, Green AR: **Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer**. *J Clin Oncol* 2011, **29**(15):1949-1955.
33. Varn FS, Mullins DW, Arias-Pulido H, Fiering S, Cheng C: **Adaptive immunity programmes in breast cancer**. *Immunology* 2017, **150**(1):25-34.
34. Ruffell B, Au A, Rugo HS, Esserman LJ, Hwang ES, Coussens LM: **Leukocyte composition of human breast cancer**. *Proc Natl Acad Sci U S A* 2012, **109**(8):2796-2801.
35. Zhou Y, Shao N, Aierken N, Xie C, Ye R, Qian X, Hu Z, Zhang J, Lin Y: **Prognostic value of tumor-infiltrating Foxp3+ regulatory T cells in patients with breast cancer: a meta-analysis**. *J Cancer* 2017, **8**(19):4098-4105.

36. Meng S, Li L, Zhou M, Jiang W, Niu H, Yang K: **Distribution and prognostic value of tumorinfiltrating T cells in breast cancer**. *Mol Med Rep* 2018, **18**(5):4247-4258.
37. Lukesova E, Boucek J, Rotnaglova E, Salakova M, Koslabova E, Grega M, Eckschlager T, Rihova B, Prochazka B, Klozar J *et al*: **High level of Tregs is a positive prognostic marker in patients with HPV-positive oral and oropharyngeal squamous cell carcinomas**. *Biomed Res Int* 2014, **2014**:303929.
38. Lawson JS, Glenn WK, Heng B, Ye Y, Tran B, Lutze-Mann L, Whitaker NJ: **Koilocytes indicate a role for human papilloma virus in breast cancer**. *Br J Cancer* 2009, **101**(8):1351-1356.
39. Khodabandehlou N, Mostafaei S, Etemadi A, Ghasemi A, Payandeh M, Hadifar S, Norooznezhad AH, Kazemnejad A, Moghoofei M: **Human papilloma virus and breast cancer: the role of inflammation and viral expressed proteins**. *BMC Cancer* 2019, **19**(1):61.
40. Miao H, Cao B, Ge W, Zhao W: **Expression of p16 and p27 protein in cervical exfoliated cells and its relationship with high risk human papilloma virus in cervical lesions**. *J Biol Regul Homeost Agents* 2019, **33**(1):197-203.
41. Kuznetsova M, Lopatnikova J, Shevchenko J, Silkov A, Maksyutov A, Sennikov S: **Cytotoxic Activity and Memory T Cell Subset Distribution of in vitro-Stimulated CD8(+) T Cells Specific for HER2/neu Epitopes**. *Front Immunol* 2019, **10**:1017.
42. Xue D, Xia T, Wang J, Chong M, Wang S, Zhang C: **Role of regulatory T cells and CD8(+) T lymphocytes in the dissemination of circulating tumor cells in primary invasive breast cancer**. *Oncol Lett* 2018, **16**(3):3045-3053.
43. Rech AJ, Mick R, Kaplan DE, Chang KM, Domchek SM, Vonderheide RH: **Homeostasis of peripheral FoxP3(+) CD4 (+) regulatory T cells in patients with early and late stage breast cancer**. *Cancer Immunol Immunother* 2010, **59**(4):599-607.

Tables

ble,1. Patient demographics and disease characteristics

	BC (n=20)	BBL (n= 15)	HV (n=20)	*P*
Age (y)	50.6±11.7	55.0±13.7	64.3 ± 9.9	.188
HPV				
Positive	4	0	0	ND
Negative	16	15	20	
Lymphocyte subsets				
CD4+ Tregs	4.39 ±1.31	1.73 ± .74	1.73 ± .63	**0.001**
CD8+	22.51±5.51	23.93±6.30	23.95±8.08	.802
CD4+	43.16± 8.05	43.16 ±7.39	40.17±7.68	.359
Histology type, n (%)				
Carcinoma in situ	1 (5.3%)			
Invasive duct carcinoma	14 (73.7%)			
Invasive mammary carcinoma	1 (5.3%)			
Metaplastic carcinoma	3 (15.8%)			
Benign mammary lesion		4 (33.3%)		
Fibroadenoma		3 (25.0%)		
Fibrocystic changes		1 (8.3%)		
Benign ductal epithelial cells		1 (8.3%)		
Benign mastitis		1 (8.3%)		
Benign unspecified		2 (16.7%)		
Unknown	1	3		
Tumor size				
< 2 cm	2 (10.5%)	1 (11.1%)		
2-5 cm	12 (63.2%)	3 (33.3%)		0.313
>5 cm	5 (26.3%)	5 (55.6%)		
Unknown	1	3		

ND: not done because the group is < 5.; BBL= breast benign lesions; HV= healthy volunteers; p<0.05 is significant

Table 2. Frequency of CD4+ Tregs and CD8+ T cells in BC patients according to age, HPV, and tumor characteristics

	Item	n (%)	CD4+ T regs	*p*	CD8+ T cells	*P*
1.	Age			.121		.779
	< 50	7(35%)	3.77±1.08		21.95±6.42	
	> 50	8(40%)	4.76±1.62		20.40±4.13	
	Unknown	5(25%)				
2.	HPV					
	+ ve	4 (20%)	4.23±1.10		22.57±8.88	
	- ve	16(80%)	4.43±1.39		22.49±4.77	
3.	Grade:			ND		ND
	G1&G2	16(80%)	4.17±1.35		22.08±6.05	
	G3	3(15%)	5.04±.71		23.74±2.33	
	Unknown	1(5%)				
4.	Pathology			.687		.500
	IDC	14(70%)	4.37±1.40		22.60±4.91	
	Others	5(25%)	4.14±1.10		21.63±7.91	
	Unknown	1(5%)				
5.	LN metastasis			.195		.105
	+ve	8(40%)	4.12±1.57		19.74±3.98	
	-ve	8(40%)	4.77±.76		24.87±7.06	
	Unknown	4(20%)				
6.	Stage:			.792		**.011**
	Early (I&II)	10(50%)	4.52±1.41		19.22±4.33	
	Advanced (III)	6(30%)	4.32±.97		27.44±5.35	
	Unknown	4(20%)				
7.	ER:			ND		ND
	+ve	13 (65%)	4.46±1.40		22.85±6.17	
	-ve	4 (20%)	3.68±1.24		20.57±5.54	
	Unknown	3 (15%)				
8.	PR:			.161		.193
	+ve	10 (50%)	4.74±1.28		24.42±5.76	
	-ve	7 (35%)	3.61±1.29		19.30±5.14	
	Unknown	3 (15%)				
9.	Her 2:			ND		ND
	+ve	4 (20%)	4.04±1.24		22.85±4.94	
	-ve	13 (65%)	5.06±1.69		20.56±9.23	
	Unknown	3 (15%)				
10.	Triple negative:			ND		ND
	Yes	3 (15%)	3.17±.87		23.09±2.78	
	No	14 (70%)	4.51±1.36		22.14±6.49	
	Unknown	3 (15%)				

Figures

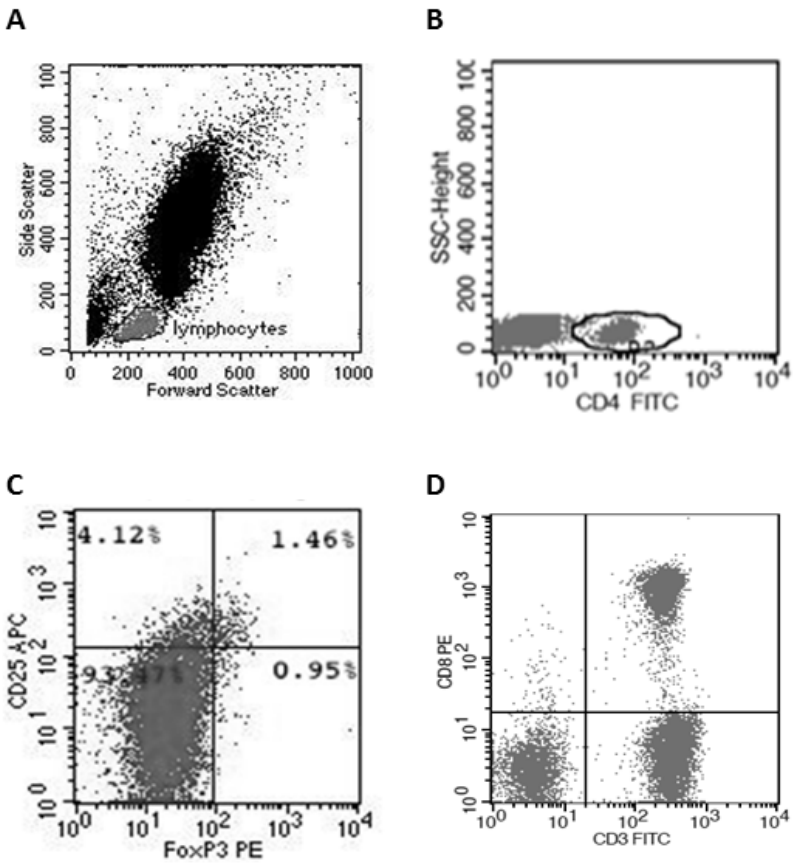

Figure 1

Flow cytometric analysis. (A) Using typical forward and side scatter characteristics, a gate was first set on lymphocytes. (B) From the lymphocyte gate, CD4+ T cells were determined. (C) From the CD4+ gate, the CD25+ FoxP3+ CD4+ Tregs are shown. (D) From the lymphocyte gate, CD3+ CD8+ T cells are shown.

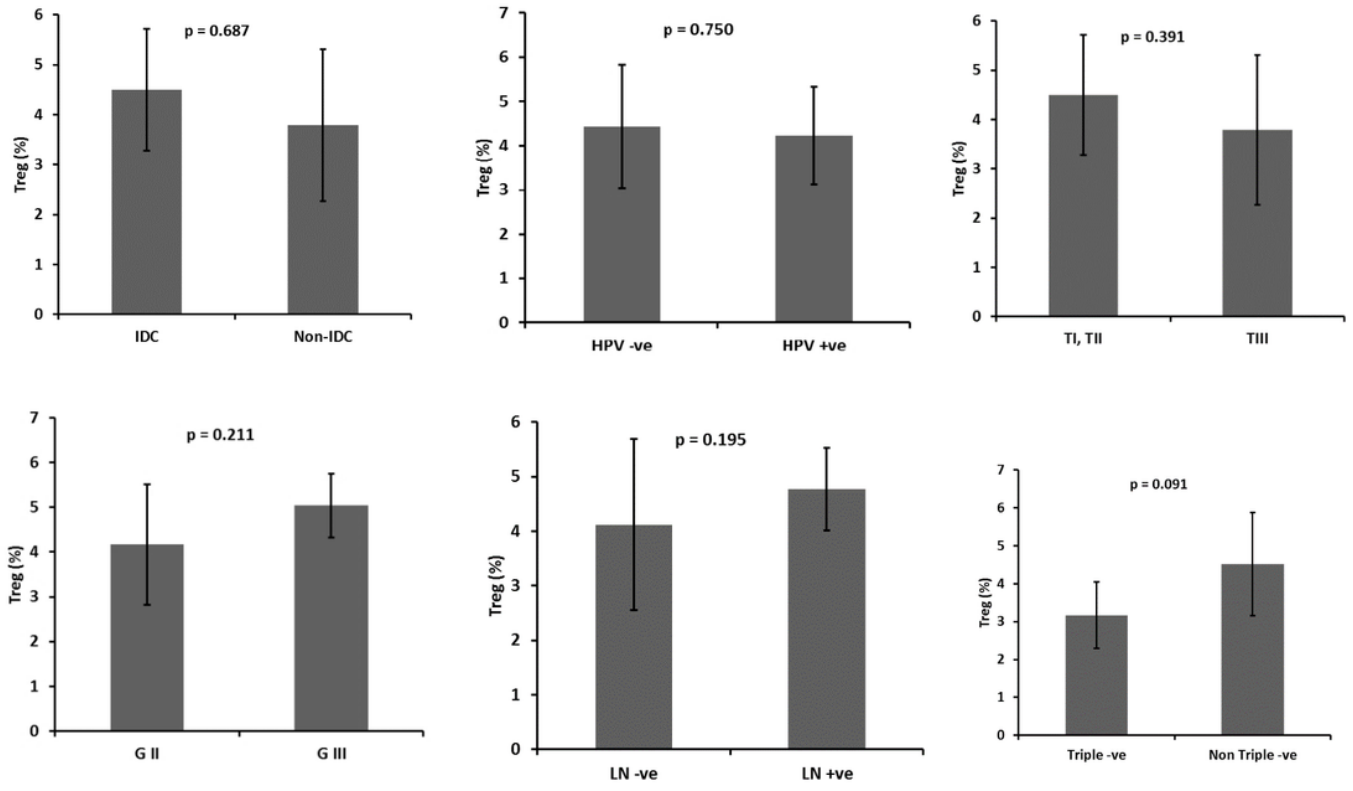

Figure 2

Relation between the frequency of CD25+ FOXP3+ CD4+ Tregs in (A) HPV +ve vs. HPV -ve, (B) IDC vs. non-IDC, (C) small tumor size (TI, TII) versus large tumors (TIII), (D) well differentiated (G2) versus poorly differentiated (G3), (E) node negative patients versus node positive patients, and (F) Triple -ve vs. non-triple -ve patients. Groups were compared using t tests (A, B, C, E, F) and ANOVA (D). Bars represent standard deviation.

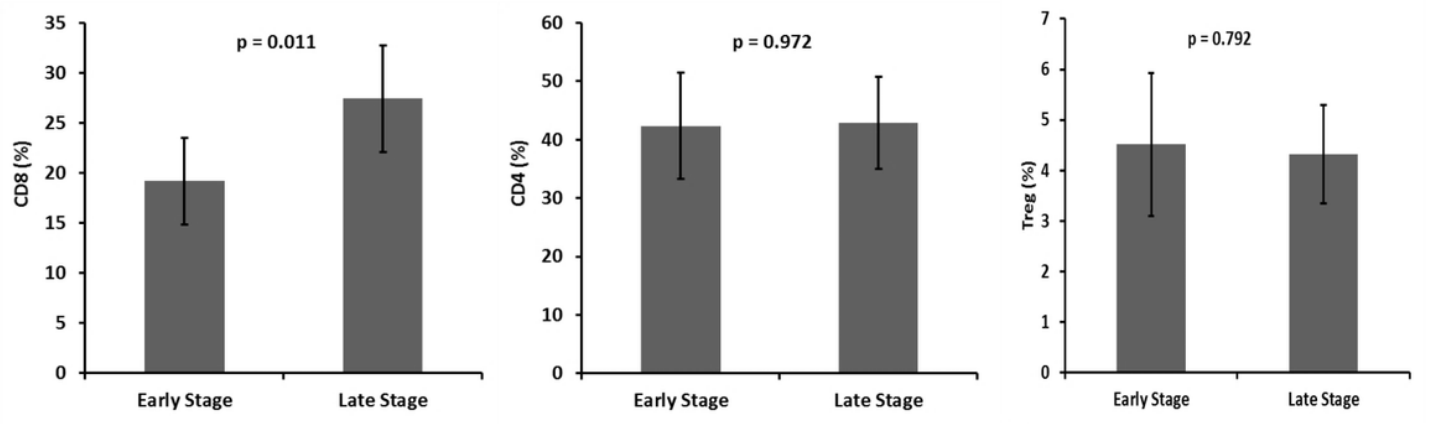

Figure 3

Frequency of (A) CD3+ CD8+ T cells, (B) CD4+ T cells, and (C) CD25+ FOXP3+ CD4+ Tregs in relation to early (I, II) versus late (III) stage of BC. Mann-Whitney U test was performed to access statistical significance. Bars represent standard deviation. A significant difference in the frequency of CD3+ CD8+ T cells was found between early and late tumor stages (P = 0.011).