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Dendritic Cell and Regulatory T Cell numbers in relation to Breast Cancer Progression and Metastasis

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Abstract

Background: Breast cancer ranks as the second cause of cancer-related deaths in Egypt. Numerous immunomodulatory pathways underscore the immune system's involvement in cancer prognosis. Both Dendritic cells [DCs] and T regulatory cells [Tregs] are critical components of the immune system, and alterations in their numbers may contribute to breast cancer development, potentially impairing the body's ability to produce a durable anti-tumor immune response.

The aim of the work: The study aimed to assess the absolute numbers of dendritic cells and T regulatory cells in the peripheral blood of breast cancer patients with metastasis.

Patients and Methods: A case-control that was conducted involving 30 female patients with metastatic breast cancer, and 20 healthy subjects. Quantification of dendritic cell subsets and Tregs cells was performed using the TruCount assay via flow cytometry.

Results: Total dendritic cells, conventional dendritic cells, and plasmacytoid dendritic cells were all significantly decreased in breast cancer patients than in controls [p= 0.001 for all]. Among breast cancer patients, conventional dendritic cells were considerably lower than plasmacytoid dendritic cells [p = 0.0001]. Absolute counts of $CD3^+$ T cells, $CD4^+$ T cells, and the percentage of $CD4^+$ T cells among $CD3^+$ T cells were significantly decreased in breast cancer patients compared to controls [p = 0.001, 0.001, 0.007 respectively]. $CD25^+$ T cell counts did not show significant differences between breast cancer patients and controls. However, the percentage of $CD25^+$ T cells among $CD4^+$ T cells was significantly increased in breast cancer patients [p = 0.0001], and a significant increase in $CD25^+$ T cells was found in progesterone receptor-negative cases.

Conclusion: The study revealed a decrease in both total dendritic cells and dendritic cell subsets in the peripheral blood of breast cancer patients, alongside an increase in the percentage of regulatory T cells. These findings emphasize the significance of comprehending the role of these cells in immunoregulation.

Keywords: Breast cancer; Dendritic cells; Plasmacytoid Dendritic cells; T regulatory; TruCount; Flow cytometry.

INTRODUCTION

Breast cancer [BC] is one of the most frequently reported cancers among women worldwide [1]. In Egypt, BC ranked first, with an incidence rate for females of all ages estimated at 32.4% of newly diagnosed cancer cases [2]. By 2050, the number of cases is expected to triple [3].

Despite early detection and significant advances in treatment modalities currently available, BC remains the second leading cause of cancer-related deaths in Egyptian women, with the vast majority occurring due to incurable metastasis. The underlying mechanisms involved in metastasis are still poorly understood [4]. Research studying BC persistence and the potential to develop metastasis has initially focused on tumor cells themselves, while recently, the impact of the immune response on tumor fate has been under intense investigation [5].

The immune system's function in cancer, especially breast cancer, appears to be dual. Efficient elimination of incipient tumors that depends on a complex series of interactions between immune cells has been described [6]. However, tumors still arise and progress, suggesting an inefficient immune response. Tumor persistence, despite the existence of an immune system, may be attributed both to gene mutations in tumor cells and to the presence of extrinsic immunomodulatory pathways that impede the capacity of the immune system`s ability to regulate tumor growth, facilitating progression and subsequent metastatic spread. The tumor`s fate depends on the balance between anti-cancer and cancer-promoting mechanisms [5].

Among immune cells, dendritic cells [DCs] and T regulatory cells [Tregs], which are critical components of the immune system, seem to play a decisive role in determining the behavior of the elicited immune response to the tumor [7, 8].

DCs are a diverse group of bone marrow-derived antigen-presenting cells distributed virtually in every tissue across the body. They exist as distinct subsets that differ in their ontogeny, surface molecule expression, stages of maturation, and biological functions [9].

At least two main populations are identified: conventional DCs [cDCs], which have a myeloid origin, and plasmacytoid DCs [pDCs], which have a lymphoid origin. cDCs are identified by their expressions of CD11C/ or CD141/BDCA-3. cDCs are shown to exhibit stimulatory effects and are considered critically important for inducing protective $CD8^+$T cell responses [10].

pDCs are identified by the expression of CD123/BDCA-3 and are shown to be potent inducers of type I interferon. In their resting state, they show less antigen-presenting ability relative to cDCs and seem to play an important role in sustaining self-tolerance [11].

The DC compartment is further diversified by the inclusion of monocyte-derived DCs [moDCs] that are derived from monocytes recruited to inflamed sites and promote CD4+T cell polarization [12].

DCs show remarkable plasticity in their immunoregulatory potential by covering dual functions, either actively initiating and shaping the immune response or exhibiting suppressive effects [8].

Treg cells are a functionally unique subset of T lymphocytes shown to control immune responses by exerting potent immuno-suppressive activity on a wide range of immune cells [7].

They account roughly for 5–10% of the overall peripheral blood $CD4^+$T cells in healthy humans. Under physiologic conditions, Tregs are considered critical for the maintenance of self-tolerance and immune homeostasis [14, 15].

Phenotypically, multiple markers have been used to characterize Tregs. Constitutive expression of CD25 [IL-2 R alpha chain] is used as a diagnostic marker [16]. Tregs express other molecules collectively suggested to be involved in their suppressive function or movement, including cytotoxic T lymphocyte-associated antigen-4 [CTLA-4], glucocorticoid-induced TNF receptor [GITR], L-selectin, lymphocyte activation gene-3 [LAG-3], Toll-like receptors 4, 5, 7, 8, and the transcription factor FOXP3. Tregs are also characterized by low or absent expression of CD127 [IL-7R], while recently, glycoprotein A repetition predominant [GARP] has been widely accepted as an additional marker for activated Tregs [17].

In the present study, it is hypothesized that concomitant alteration in the numbers of DCs and Tregs may be involved in BC pathogenesis and, together, can hinder the generation of a protective anti-tumor immune response in patients with BC, thus facilitating metastatic spread.

In this study, Peripheral blood samples from BC patients and healthy controls were analyzed using specific markers to identify DC subsets [CD11c and CD123 for cDCs and pDCs, respectively] Both dendritic cell subsets may express low quantities of lymphocyte, natural killer cell, and monocyte lineage markers. To overcome this issue, the researchers also used a lineage cocktail of CD3, CD14, CD16, CD19, CD20, and CD56 antibodies [18,19]. [CD3, CD4, and CD25] were used as specific markers to identify Tregs [21]. These markers enable precise characterization of DC and Treg populations, providing insights into their potential roles in BC progression and metastasis.

THE AIM OF THE WORK

The present study was conducted to evaluate the absolute numbers of DCs and Tregs in the peripheral blood of BC patients with metastasis. Findings were correlated with pathological subtypes of BC and compared to apparently healthy controls.

PATIENTS AND METHODS

Study design: This is a case-control comparative study.

Study participants:

The study included a total of 50 female subjects classified into two age-matched groups.

1. BC patients group included 30 patients with stage IV BC with metastasis [liver, lung, ovary, or other], who did not start any treatment. These patients were selected from those attending the Cobalt Center, Ain Shams University Hospital, and Cancer Institute, Cairo University, in the period from January to December 2022. Diagnosis of BC was performed according to the guidelines of the National Comprehensive Cancer Network [22].

Information about patient history, metastatic sites, and the molecular subtypes defined on the basis of the profile of expression or absence of human epidermal growth factor receptor [HER2], estrogen receptor [ER], progesterone receptor [PR], and the tumor stage [tumor-node-metastasis] were obtained from the patient's medical records.

2- Control group: included 20 apparently healthy volunteer female blood donors, with no known medical conditions or history of cancer.

Inclusion and Exclusion Criteria:

Patients eligible to enroll in the study were over 18 years old and had histologically proven BC with metastasis. Individuals excluded from participating included BC patients presenting as secondary to primary cancer, patients with brain metastasis, individuals on immunosuppressive medications, chemotherapy, or undergoing radiation therapy, patients with organ failure or concomitant infectious diseases, or pregnant women at the time of the study.

A written informed consent was obtained from all participants before enrollment in the study, according to the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Damietta Faculty of Medicine, Al-Azhar University, Damietta, Egypt [IRB00012367-22-09-004].

Sample collection and processing: From each subject, both BC patients and healthy controls, 2 ml of venous blood were collected by venipuncture into an EDTA-coated vacutainer tube. Samples were transferred to the flow cytometry laboratory to be processed within 2 hours of collection [23].

Determination of absolute counts of DCs and Treg cells: Quantification of DC subsets and Treg cells was done by flow cytometry analysis of whole blood using the TruCount™ assay [BD Pharmangen, catalog No:340334], an optimized, reliable flow cytometry-based cell counting method that can detect rare cell subsets within whole blood samples by reference to a known number of beads contained within the TruCount™ tube [24]. Cells under investigation were identified using appropriate fluorochrome-labeled monoclonal antibodies according to the manufacturer's instructions.

Staining: Direct immunofluorescence staining of cells of interest was done for each sample in 2 separate TruCount tubes using the stain/lyse-no-wash protocol as previously described [24]. Briefly, 50 ul of well-mixed anticoagulated whole blood were incubated with combinations of pre-titrated monoclonal antibodies. For DC subsets, APC-CD11c, PE-CD123, and FITC-lineage cocktail [CD3, CD14, CD16, CD19, CD20, and CD56] [BD, catalog No: 347637, 340545, and 340546 respectively] were used. For Treg cells, FITC-CD3, PE-CD4, and APC-CD25 [BD, catalog No: 345763, 345769, and 340907 respectively] were used. The tubes were incubated in the dark for 15 minutes at room temperature. Erythrocytes were lysed by adding 450 ul of FACS lysing solution [BD, catalog No: 349202]; tubes were re-incubated in the dark for another 10 minutes at room temperature.

For each experiment, two quality control tubes were included, the first containing unstained cells to detect autofluorescence and the second an isotype control, mouse IgG1 PE and IgG2a APC [BD, Catalog No: 349043 and 340473 respectively], to detect nonspecific staining.

Data acquisition and analysis: Cells were directly analyzed on a previously adjusted FACSCalibur Flow Cytometer [BD] using Cell Quest software [BD] [23].

A minimum of 50,000 events were acquired. An acquisition gate was established based on forward and side-scatted light. The gate included both lymphocyte and monocyte populations and excluded debris. Cells in the gate were analyzed for the expression of marker[s] identifying cells of interest. All cells displaying fluorescence above the threshold delineated by the isotype control were considered positive for the marker. Fluorescent TruCount beads were identified by their small-sized fluorescence in all fluorescence channels, along with their respective cell type marker[s]. Once cells of interest were identified, statistics were obtained.

For DCs, cells stained negative or dim for the cocktail of lineage markers and positive for CD11c were identified as myeloid DCs, while cells stained negative or dim for the cocktail of lineage markers and positive for CD123 were identified as pDCs.

For Treg cells, gating for analysis of Treg cells was based first on the expression of CD3 and CD4. These cells were further analyzed for CD25 co-expression. The $CD4^+$ $CD25^{hi}$ were identified as a tail from the major population of $CD4^+$T cells [25].

Once the cell population of interest was identified, statistics were obtained, and the absolute number of cells per ml of blood sample was calculated by the formula:

Number of events in the region containing cells= [x number of beads per test/ Number of events in the absolute count bead region] x volume of sample.

Statistical Analysis: SPSS version 23 was used to handle and analyze the data. The Shapiro-Wilk test [or Kolmogorov-Smirnov test] was used to determine the normality of data distribution for each variable. Parametric quantitative data was represented as mean ± SD, whereas non-parametric data was represented as median and interquartile range [IQR]. Categorical data were presented as percentages. Depending on the normality findings, relevant tests were performed: for parametric data, Independent t-tests and paired t-tests were used; for non-parametric data, Mann-Whitney U and Kruskal-Wallis tests were used. The Pearson correlation coefficient was used to examine correlations in parametric data. A P-value of less than 0.05 was judged statistically significant.

RESULTS

This study examined the immunological profiles of female patients with breast cancer [BC] with metastases to healthy controls, focusing on dendritic cells [DCs] and CD3CD4CD25T cell subsets. Demographic profile of studied participants is shown in [Table 1].

Peripheral blood samples from all participants were analyzed by flow cytometry for absolute counts of total and two subsets of DCs [cDCs, pCs] and $CD3^+$ T, $CD4^+$T, $CD25^+$ T cells. The percentage of $CD4^+$ T cells was calculated from $CD3^+$ and the percentage of $CD4^+CD25^+$ T cells was calculated from $CD3^+$ $CD4^+$ T cells. Absolute counts of total DCs, cDCs subset, and pDCs subset were significantly decreases in BC patients compared to controls [p= 0.001, 0.001, 0.001, respectively] [Table 2]. In BC patients comparing the two subsets of

DCs, a significant reduction in cDCs as compared to pDCs was observed [p = 0.0001] [Table 3].

As regards pathological types of BC and sites of metastasis, non-significant differences in total DCs, cDCs and pDCs were detected between BC ductal and lobular types [Tables 4] or according to sites of metastasis [Table 5] or receptor expression [Table 6]. Absolute counts of $CD3^+$T cells, $CD4^+$T cells and the percentage of $CD4^+$T cells among $CD3^+$T cells were significantly decreased in BC patients compared to controls. $CD25^+$ T cell counts did not show significant differences between BC patients and controls. However, the percentage of $CD25^+$ T cells among $CD4^+$ T cells was significantly increased in BC patients [p= 0.001] [Table 7]. Similar to DCs, no significant differences in $CD3^+$T, $CD4^+$T, $CD25^+$T cells, and $CD25^+$T cells percentage among $CD4^+$T cells were observed in based on BC pathological type [Table 8] or sites of metastasis [Table 9].

As regards receptor expression, a significant increase in $CD25^+$ T cells was detected in PR negative cases compared to PR positive cases. Non-significant differences were detected between cases expressing the ER or HER2 receptor [Table 10]. There were no direct correlations found between counts of circulating total DCs, cDCs, pDCs, and the percentage of $CD25^+$T cells among BC patients [p > 0.05] [Table 11].

Overall, the study found substantial differences in DC and T cell subsets in BC patients with metastases versus healthy controls. These findings expand our understanding of the immunological dysregulation associated with metastatic breast cancer, which may have implications for future therapeutic options.

Figures [1 and 2] represented Flow cytometer data in cancer patients to detect DCs and Tregs, respectively.

Table [1]: Demographic profile of studied participants

Variable		BC patients [n=30]	Control [n=20]	P value
Age [years]	Min. – Max.	20 – 60	20 – 60	0.223
	Mean ± SD	45.7 ± 9.8	41.9 ± 11.9	
Pathologic characteristics	Ductal No [%]	24 [80%]	-	-
	Lobular No [%]	6 [20%]		
Expression/absence of receptors	ER No [%]	17 [56.7%]		-
	PR No [%]	19 [63.3%]		
	HER – 2 No [%]	13 [43.3%]	-	
	TN No [%]	3 [10%]		
Sites of metastasis among patients	Bone No [%]	9 [30%]	-	
	Liver No [%]	8 [26.7%]		
	Lung No [%]	8 [26.7%]		-
	Skin No [%]	3 [10%]	-	
	Pleura No [%]	2 [6.6%]		

ER: Estrogen receptor, PR: Progesterone receptor, HER-2: Human epidermal growth factor-2, TN: triple negative

Table [2]: Absolute counts of total DCs, cDCs and pDCs as compared to controls

	BC Patients[N=30]	Controls [N=20]	*P* value
Total DCs [Mean + SD [x 10^6]	7.3 [6.7 ± 3.8]	22 [23.84 ± 6.4]	0.001*
cDCs [Mean + SD [x 10^6]	2.3 [2.16 ± 1.3]	7.3 [7.57 ± 1.69]	0.001*
pDCs [Mean + SD [x 10^6]	4.6 [4.45 ± 2.6]	14.3 [15.27 ± 4.35]	0.001*

By Kruskal Wallis test * Statistically significant

Table [3]: Comparison of cDCs and pDCs in BC patients

	Mean ± SD [x 10^6]	*P* value
cDCs	2.16 ± 1.3	0.0001*
pDCs	4.45 ± 2.6	

* Statistically significant

Table [4]: Comparing total DCs, cDCs, pDCs according to pathological type of the tumor.

	Ductal [N=24]	Lobular [N= 6]	*P* value
Total DCs [Mean + SD [x 10^6]	6.1 [6.27 ± 3.6]	7.9 [8.00 ± 4.31]	0.34
cDCs [Mean + SD [x 10^6]	1.7 [1.9 ± 1.31]	2.7 [2.7 ± 1.4]	0.27
pDCs [Mean + SD [x 10^6]	4.1 [4.27 ± 2.5]	5.3 [5.27 ± 2.6]	0.32

By Mann Whitney U test

Table [5]: Comparing total DCs, cDCs, and pDCs according to sites of metastasis

	Bone [N= 9]	Liver [N= 8]	Lung [N=8]	Pleura + skin [N=5]	*P* value
Total DCS [Mean + SD [x 10^6]	7.4 [7.2 ± 2.13]	6.3 [7.5 ± 4.6]	5.3 [5.3 ± 4.13]	8.14 [6.3 ± 4.6]	0.72
cDCs [Mean + SD [x 10^6]	2.2 [2.2 ± 1.03]	2.05 [2.5 ± 1.43]	1.8 [1.7 ± 1.43]	2.7 [2.1 ± 1.6]	0.74
pDCs [Mean + SD [x 10^6]	4.6 [5.0 ± 2.03]	4.4 [4.0 ± 3.03]	3.4 [3.4 ± 2.53]	5.7 [4.2 ± 3.0]	0.73

By Kruskal Wallis test

Table [6]: Comparing total DCs, cDCs, pDCs according to receptor expression

Receptor type			
ER	**Positive [N=17]**	**Negative [N=13]**	*P* value
Total DCs [Mean ± SD [x10^6]	5.90 ± 4.63	7.4 ± 2.36	0.30
cDCs [Mean ± SD [x10^6]	1.90 ± 1.5	2.30 ± 1.6	0.48
pDCs [Mean ± SD [x10^6]	3.80 ± 2.96	5.20 ± 1.90	0.16
PR	**Positive [N=19]**	**Negative [N=11]**	
Total DCs [Mean ± SD [x10^6]	6.2 ±3.50	7.30 ±4.26	0.28
cDCs [Mean ± SD [x10^6]	1.9 ±1.21	2.50 ±1.40	0.14
pDCs [Mean ± SD [x10^6]	4.2 ±2.56	4.70 ±2.70	0.39
HER-2	**Positive [N=13]**	**Negative [N=17]**	
Total DCs [Mean ± SD [x10^6]	6.80 ± 3.70	6.50 ± 3.96	0.71
cDCs [Mean ± SD [x10^6]	2.00 ±1.03	2.10 ±1.36	0.82
pDCs [Mean ± SD [x10^6]	4.6 ±2.60	4.30 ±2.60	0.33

By Kruskal-Wallis test; Positive= expression of the receptor, Negative= no expression of the receptors, TNBC cases were not analyzed due to a low number [less than 5].

Table [7]: Absolute counts of CD3$^+$ T, CD4$^+$T, and CD25$^+$T cells, CD4$^+$T cells percentage among CD3$^+$T cells and CD25$^+$T cells percentage among CD4$^+$T cells

	BC Patients [N=30]	Controls [N=20]	*P* value
CD 3$^+$ T cells [x10^6] [Mean ± SD]	0.9 [1.2 ± 0.8]	5.5 [2.49 ± 0.9]	0.001*
CD4$^+$ T cells [x10^6] [Mean ± SD]	0.45 [0.6 ± 0.5]	1.5 [1.52 ± 0.5]	0.001*
CD4$^+$ T cells [%] [Mean ± SD]	48 [51.09 ±17.1]	60.77 [61.47±7.03]	0. 007 *
CD25$^+$ T cells [x10^6] [Mean + SD]	0.14 [0.14 ± 0.1]	0.19 [0.19 ± 0.05]	0.451
CD25$^±$ T cells [%] [Mean ± SD]	30.9 [37.88 ± 35.38]	13 [13.08 ±2.7]	0.001*

By Kruskal Wallis test * Statistically significant

Table [8]: Comparing absolute counts of CD3$^+$ T, CD4$^+$T, and CD25$^+$T cells and CD25$^+$T cells percentage among CD4$^+$T cells according to BC pathological type

	Ductal [N=24]	Lobular [N=6]	*P* value
CD 3$^+$ T cells [x10^6] [Mean ± SD]	1.02 [1.45± 0.86]	0.63 [0.64 ±0.15]	0.02
CD4$^+$ T cells [x10^6] [Mean ± SD]	0.47 [0.71±0.61]	0.44 [0.39 ± 0.14]	0.23
CD4$^+$ T cells [%] [Mean ± SD]	47 [49.1±17.37]	64 [59.1±14.56]	0.23
CD25$^+$ T cells [x10^6] [Mean ± SD]	0.14 [0.18±0.10]	0.11 [0.25±0.32]	0.49
CD25$^+$ T cells [%] [Mean ± SD]	30.9 [31.0±11.6]	34 [65.36±74.2]	0.29

By Mann Whitney U test

Table [9]: Comparing absolute counts of CD3$^+$ T, CD4$^+$T, and CD25$^+$T cells and CD25$^+$T cells percentage among CD4$^+$T cells according to sites of metastasis

	Bone [N=9]	Liver [N=8]	Lung [N=8]	Pleura & skin [N=5]	*P* value
CD 3$^+$ T cells [x10^6] [Mean±SD]	0.9 [1.25±0.85]	1.04 [1.46±0.9]	0.7 [1.31±0.99]	0.9[1.06±0.6]	0.859
CD4$^+$ T cells [x10^6] [Mean±SD]	0.49 [0.5±0.49]	0.52 [1.46±0.9]	0.44[0.67±0.66]	0.5[0.5±0.18]	0.21
CD4$^+$ T cells [%] [Mean±SD]	48.9 [44.8±20.5]	64.6 [58.66±14.4]	42.5[50.86±16.7]	47.1[50.7±14.9]	0.60
CD25$^+$ T cells [x10^6] [Mean±SD]	0.18 [0.148+0.09]	0.17 [0.3+0.58]	0.11[0.15+0.10]	0.11[0.13±0.04]	0.15
CD25$^+$ T cells [%] [Mean±SD]	44.8 [42.0 ± 10.6]	24.7 [28.01±12.3]	25.8[28.0 ± 12.0]	25.9[28.6 ± 11.5]	0.088

By Kruskal Wallis test

Table [10]: Comparing absolute counts of CD3$^+$ T, CD4$^+$T, and CD25$^+$T cells and CD25$^+$T cells percentage among CD4$^+$T cells according to receptor expression

Receptor type			
ER	**Positive [N=17]**	**Negative [N=13]**	***P* value**
CD 3$^+$ T cells [x10^6] [Mean ± SD]	1.11±0.66	1.5±1.0	0.48
CD4$^+$ T cells [x10^6] [Mean ± SD]	0.54±0.36	0.79±0.75	0.91
CD4$^+$ T cells [%] [Mean ± SD]	49.48±13.23	53.2+21.6	0.24
CD25$^+$ T cells [x10^6] [Mean ± SD]	0.14±0.07	0.263±0.22	0.22
CD25$^+$ T cells [%] [Mean ± SD]	29.27 ± 10.17	49.1±51.4	0.23
PR	**Positive [N=19]**	**Negative [N=11]**	
CD 3$^+$ T cells [x10^6] [Mean ±SD]	1.3±0.8	1.2±0.87	0.59
CD4$^+$ T cells [x10^6] [Mean ± SD]	0.63±0.6	0.67±0.53	0.18
CD4$^+$ T cells [%] [Mean ± SD]	46.2±17.1	59.56±14.0	0 .08
CD25$^+$ T cells [x10^6] [Mean ± SD]	0.17±0.10	0.23±0.24	0 .81
CD25$^+$ T cells [%] [Mean ± SD]	34.0 ± 11.5	44.0 ± 57.14	0.043*
HER-2	**Positive [N=13]**	**Negative [N=17]**	
CD 3$^+$ T cells [x10^6] [Mean ± SD]	1.11±0.25	0.86±0.4	0.16
CD4$^+$ T cells [x10^6] [Mean ± SD]	0.6±0.07	0.48±0.25	0.10
CD4$^+$ T cells [%] [Mean ± SD]	36.89±18.28	31.06±19.6	0.9
CD25$^+$ T cells [x10^6] [Mean ± SD]	0.16±0.05	0.13±0.09	0.22
CD25$^+$ T cells [%] [Mean ± SD]	31.12±13.97	28.8±20.26	0.054

By Kruskal Wallis test * Statistically significant; Positive= expression of the receptor, Negative= no expression of the receptors, TNBC cases were not analyzed due to a low number [less than 5].

Table [11]: Correlation between total DCs, cDCs, pDCs and $CD25^{+}$T cells percentage

	$CD25^{+}$ T cells [%]	
	r	p
Total DCs	0.085	0.657
cDCs	0.086	0.053
pDCs	0.090	0.637

r: Pearson correlation coefficient

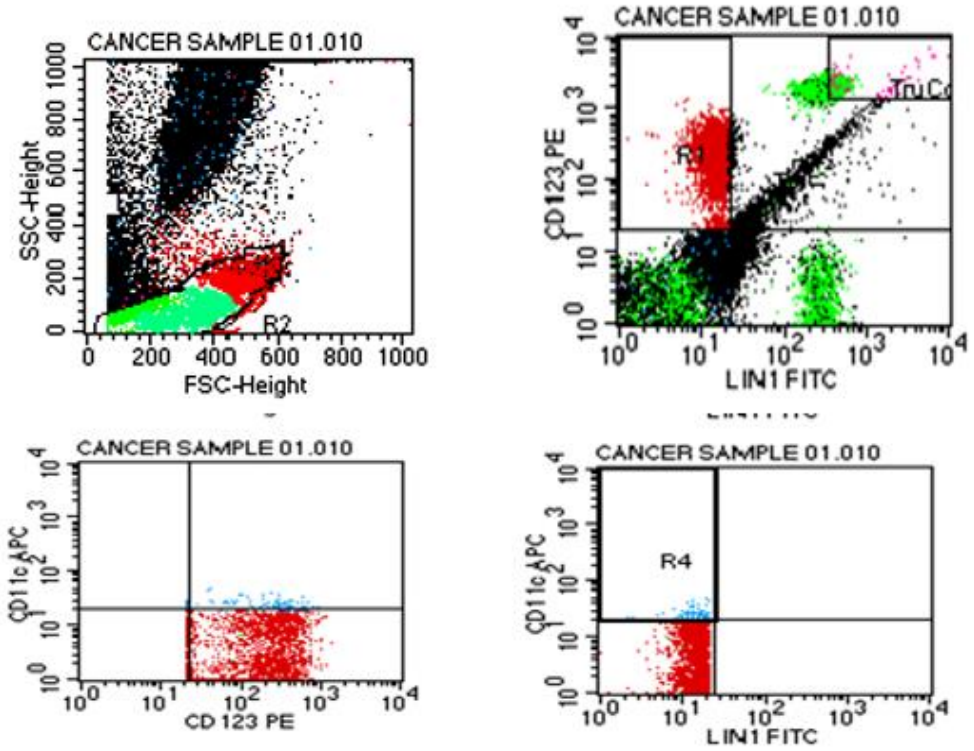

Figure [1]: Flow cytometer data in cancer patients to detect DCs

The image is from our Flow cytometer analysis of DCs subsets isolated from whole blood in breast cancer patient samples.

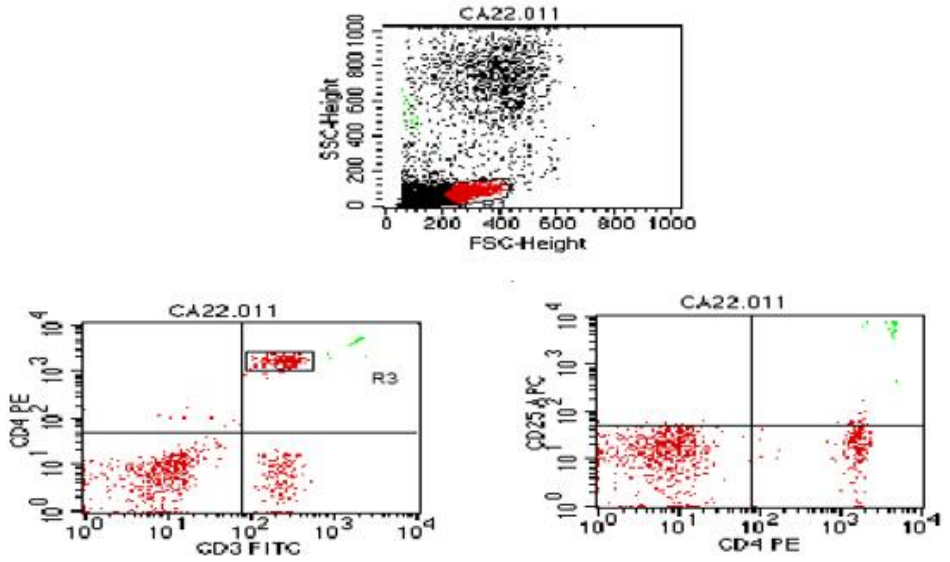

Figure [2] : Flow cytometer data in cancer patients to detect Tregs.

The image is from our Flow cytometer analysis of Treg subsets isolated from whole blood in breast cancer patient samples.

DISCUSSION

Solid tumors, including BC, have the ability to create a highly suppressive microenvironment that impedes immune clearance and manifests as detectable local and systemic changes in the relative proportions and phenotypes of regulatory cell populations, including DCs [26] and Tregs [27].

These immune cells play important roles in regulating immune responses, and dysregulation can lead to tumor escape and metastasis. The present study aimed to investigate the levels of circulating DCs [total, cDCs, pDCs], as well as $CD4^+CD25^+$ regulatory T [Tregs] cells in patients with metastatic BC. The study's rationale is to better understand how the balance of pro-inflammatory [DCs] and anti-inflammatory [Treg cells] immune cell populations changes in metastatic BC patients. This disparity has the potential to establish an immune-suppressive situation favorable for tumor growth.

Flow cytometry analysis was used to investigate these immune cells and obtain insights into their inter-relationship within the pathogenesis of BC malignancy. Blood samples were used as it permits the application of flow cytometry for better characterization of cells compared to what could be achieved with immuno-histochemistry performed for tissue section samples [28].

Results of the present study indicate significantly reduced numbers of total DCs and their two subsets [cDCs and pDCs] in the peripheral blood of BC patients compared to normal controls. This aligns with Pinzon-Charry *et al* who reported similar reductions in DCs numbers in stage IV BC patients [29]. Previous studies have also found similar reductions in DCs counts in patients with primary and metastatic malignancies, indicating a consistent pattern of DCs modifications across cancer types and illness stages [30].

Some mechanisms were hypothesized to explain this observed deficiency. These include reduced development and maturation or increased death of DCs precursors in the bone marrow [31-33] as a systemic response to tumor-derived factors [34].

Limited local production of factors important for DCs differentiation and expansion [35], tumor-induced DC apoptosis [36], and Treg-mediated cytolysis were also suggested [37].

In the context of BC progression, low DCs count in peripheral blood has important impacts as DCs are required to initiate and regulate immune responses against tumor cells. This may reflect their inadequate function that may participate in the formation of an immunosuppressive environment, allowing tumor cells to avoid immune monitoring and enhance metastasis [38].

In this study, BC patients showed a substantial drop in cDCs compared to pDCs [$p < 0.0001$]. pDCs, previously proven to be poor activators of naïve T cells [39] were less significantly reduced than cDCs, which are excellent against malignancies [40].

Several studies in cancer contexts, including BC, revealed a DC compartmentalization pattern in which pDCs were able to enter the tumor bed, perhaps playing a detrimental function. In contrast, cDCs were selectively excluded [41]. This distribution may impede the formation of a protective immune response and correlate negatively with positive clinical outcomes [42].

No differences in total DCs or DCs subtypes were observed when comparing different BC pathological types or as regarded ER, PR, and HER-2 expression states. This is in contrast to Paek *et al*., where decreased numbers of DCs have been detected in HER-2-negative compared to HER-2-positive cases [30].

There observed decrease in total $CD3^+$ T cells and $CD4^+$ T cells, and a reduced percentage of $CD4^+$ T cells among the overall $CD3^+$T cell population in the peripheral blood of BC patients as compared to normal controls. Previous studies reported similar results in patients with primary and metastatic cancers. This reduction suggests that the immune cell composition in the peripheral blood of BC patients differs from that of healthy controls. This shift in the proportion of T cell subsets could have serious consequences for the immunological response to malignancy [43].

In the context of cancer, including BC, multiple preclinical and clinical studies have described a big role for increased frequencies of $CD4^+CD25^+$Tregs in peripheral blood [21] and in tumor tissues in inducing a potent suppressive microenvironment that limits the efficiency of anti-tumor immune responses with a significant functional impact on tumor survival, growth, progression, and metastasis [44].

Many studies have demonstrated that Tregs accumulation in patients with malignant tumors, including BC, is not restricted to the tumor site but is also observed in peripheral blood [45].

This is consistent with results reported in numerous previous studies showing significantly higher frequencies of absolute counts and ratios of circulating Treg cells in patients with advanced BC [21, 46] and several other types of solid malignancies compared to healthy controls being significantly higher in stages III and IV than in stages I and II [47-50].

The present study has also demonstrated increased ratios of $CD3^+CD4^+CD25^+$ Tregs among $CD4^+$ T cells in the peripheral blood of patients with BC patients, but there were no significant differences in the absolute count of $CD3^+CD4^+CD25^+$ cells between BC patients and the control group.

Analyzing the percentage of $CD25^+$ Tregs in the $CD4^+$ T cell population can reveal important information about the relative number of $CD25^+$ Tregs in this subgroup of cells. This allows for an assessment of $CD25^+$ Tregs regulatory capacity in comparison to the entire $CD4^+$ T cell population, which is especially important in the setting of immune regulation in BC. This could help find new therapeutic targets for immune regulation in cancer therapy techniques. No correlation was found in the present study between increased ratios of $CD4^+CD25^+$ Tregs and ER and HER-2 expression states. In contrast, Decker *et al*., reported increased numbers of Tregs in the peripheral blood of patients with HER-2/neo positive early BC [51]. Estrogen's impact on BC biology is mostly related to tumor growth and proliferation [52]. Thus, the absence of a significant difference in Tregs between ER-positive and ER-negative patients is to be expected, as estrogen may not have a direct impact on Tregs levels.

A significant association was found between $CD4^+CD25^+$ Tregs and PR expression. This finding supports prior research that suggests progesterone receptors play a crucial role in modulating immune responses [53].

According to research, progesterone can modulate immune cell recruitment and function, such as Tregs [54, 55]. As a result, the observed difference in Tregs between PR-positive and PR-negative BC patients may be due to the effect of progesterone signaling on immune cell dynamics in the tumor microenvironment [55].

BC patients suffer from metastatic spread into a broad spectrum of anatomic locations. The most common sites of metastasis reported in the present study are the liver, lung, and bone. Similar results have been described in previous studies [56].

Tumor-induced tolerogenic DCs were shown to foster the expansion of Tregs [57, 58]. Reciprocally, in a positive feedback loop, tumor-induced Tregs cells talk back to DCs [59].

DC interacting with Tregs would alter cytokine profile with augmented production of anti-inflammatory cytokines and strained production of inflammatory cytokines [60].

The dynamic bi-directional interactions between DCs and Tregs put them at the center of tumor-induced suppressive networks that functionally compromise the efficacy of antitumor immune responses, contribute to tumor survival, allow its growth, and ultimately facilitate and support metastatic dissemination [61].

This study initially hypothesized that there could be a correlation between DC subsets and Tregs in BC patients, but the results showed a lack of association between circulating total DCs, cDCs, pDCs, and $CD25^{+}$Tregs percentage. The absence of a significant correlation observed in this study suggests that other factors or mechanisms may be driving the regulation of these immune cell populations independently of each other. Further investigation is required to explain the complex interplay between DCs and Tregs in BC and to understand how their interactions contribute to tumor immune evasion and disease progression. Finally, insights into the unique attributes of DCs and Treg cells and the combined roles they play locally and systemically in BC pathogenesis, progression, and metastasis are important. Targeting DC and Treg may correct immune deviation, and restore an effective anti-tumor immune response [62].

Strengths and limitations: The study recruited a homogeneous population of advanced cases of BC [stage IV with metastasis], but the small sample size was a crucial limitation that was responsible for the absence of some statistical relations [only 3 cases of TNBC were included]. Samples from peripheral blood samples offer a systemic perspective, while local tumor microenvironments provide insights into the immune landscape. Differences in results may arise due to differences in immunological context and composition between tumor microenvironment and peripheral blood.

Conclusion: According to the findings of this study, the number of total DCs and both conventional and plasmacytoid DC subsets decreased in the peripheral blood of patients with breast cancer, while the percentage of Tregs increased. Understanding the biology of these cells, which appear to be essential control components of immunoregulation, will be critical for creating effectors targeted at therapeutically regulating immune responses.

Recommendations: The research should be expanded to include larger cohorts of patients with different stages of the disease in addition to longitudinal studies comparing immune cell numbers and interactions locally and systemically in BC patients who develop or never develop metastasis. More research is needed to better understand the complicated interplay between DCs and Tregs in breast cancer, as well as how these interactions contribute to tumor immune evasion and disease progression.

List of abbreviation:

Breast cancer [BC]; Dendritic cells [DCs]; Conventional Dendritic cells [cDCs]; Plasmacytoid Dendritic cells [pDCs]; T regulatory cells [Tregs]; Cytotoxic T lymphocyte-associated antigen-4 [CTLA-4]; Glucocorticoid-induced TNF receptor [GITR]; L-selectin, lymphocyte activation gene-3 [LAG-3]; Glycoprotein A repetition predominant [GARP]; Human epidermal growth factor receptor [HER2]; Estrogen receptor [ER], Progesterone receptor [PR].

Declarations:

- **Ethics approval and consent to participate:** A written informed consent was obtained from all participants before enrollment in the study, according to the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Damietta Faculty of Medicine, Al-Azhar University, Damietta, Egypt [IRB00012367-22-09-004].

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

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

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