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ORIGINAL ARTICLE

Prognostic Impact of Circulating CD28 Negative Suppressor T Cells and Memory B Cells on Treatment Outcomes of Patients with Breast Cancer

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ABSTRACT

Background: Circulating lymphocytes can put up understanding of the interactions between tumor cells and lymphocytes infiltrating the tumor microenvironment. **Objective:** In this sense, the prognostic value of the inflammatory cell in breast cancer (BC) peripheral blood needs to be evaluated; this would suggest that estimation and evaluation of these circulating lymphocytes could provide a prognostic value in BC. **Methods:** The present study was a prospective case-control study, 30 Patients with operable breast cancer; any T, any N, M0, treated surgically with either modified radical mastectomy or breast conservative surgery, and 20 healthy controls were included. For detection of lymphocyte subsets in peripheral blood; Fluorochrome-labeled monoclonal primary antibodies were used, the cells were analyzed by FACSCalibur flow cytometry with Cell Quest software, then patients were treated with chemotherapy, 3DCRT, and hormonal treatment, and followed up to determine relapses and recurrence-free survival (RFS). **Results:** Significant differences of different cell types B, T, NK, NKT, and $CD28^-$ T between BC patients and controls, significant differences of $CD8^+CD28^-$cells among different biologic subtypes, negative correlations between $CD8^+CD28^-$cells and memory B cells, and RFS. Significant differences of naïve B cells among different grades of BC, and negative correlation between B cells and NKT cells. **Conclusion:** NK, NKT, lymphocytes, and $CD28^-$Tcells significantly differed between healthy controls and BC patients; also, different B cells were associated with good response to treatment while $CD28^-$cells were associated with shorter RFS.

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Keywords: NK Cells, NKT Cells, B-Cells, T-Cells, Breast Cancer

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INTRODUCTION

Breast cancer (BC) is the commonest cancer throughout the world and considered a leading cause of death. It represents about 22% of all female cancers (1). Due to available strategies for early detection and treatment, 90% of women diagnosed with BC survive at least five years after their diagnosis despite this high incidence rate (2). Its occurrence is not only under genetic control but also involves interplay between cancer cells and the local microenvironment (TME) and immune cells (3). In description of the local inflammatory response, the prognostic value of inflammatory cell infiltrate in breast cancer was debatable in many studies. Mohammed *et al.* summarized sixty-six previous studies regarding this issue; 72%of the published studies clarified an association between inflammatory cell infiltrate and the outcomes in breast cancer, while 53% of these studies recorded favorable survival with accumulation of the inflammatory cell infiltrate within TME, the remaining ones detected an association with worse survival (4). Cellular immunity assumes a critical role in eradicating residual tumor cells and keeping up homeostasis since an active immune system targets pathogenic mutant cells or cancer cells and can lead to cancer cell apoptosis. In this way, the adequate function of cellular immunity and supporting function of humoral immunity could interfere with the progression of cancer and treatment failure (5). The $CD8^+CD28^-$phenotype ($CD8^+CD28$ null cells), a particular $CD8^+$T cell subtype, are considered to suppress CD4+ T cell function, either through direct cell-to-cell contact (6) or through the release of cytokines (7,8). The elevated levels of $CD8^+CD28^-$phenotype cells were positively correlated with higher levels of immunosuppressive cytokines, interleukin (IL)-6 and IL-10, but negatively correlated with interferon-γ (9). Predictive factors associated with greater probability of achieving a complete pathologic response (pCR) using neoadjuvant chemotherapy regimens are tumor size, histological type (ductal-lobular carcinoma), molecular tumor subtype (basal–luminal), hormone receptor status (negative-positive estrogen and progesterone receptors (ER, PR), expression of human epidermal growth factor receptor 2 (HER2), Ki-67, and the Scarff-Bloom-Richardson grade (10-11). Circulating lymphocytes may give knowledge into the potential connections between infiltrated lymphocytes and cancer cells within the cancer microenvironment. In this sense, the prognostic value of peripheral blood inflammatory cells of breast cancer patients needs to be evaluated; this would suggest that routine assessment and quantification of lymphocytes could provide clinically meaningful prognostic information in BC.

MATERIALS AND METHODS

Study Type, Settings, and Duration. The present study was a prospective case-control study conducted in South Egypt Cancer Institute and Assiut University Hospital. The Institutional Ethical Review Board of Faculty of Medicine, Assiut University approved the study protocol. All participants signed in written informed consents before inclusion in the study.

Study Participants. Thirty patients with operable BC, any T, any N, and M0, treated surgically with either modified radical mastectomy (MRM), or breast conservative surgeries (BCS) and histologically confirmed to be breast cancer, and presented to any one of the previously mentioned sites above during the study period, were invited to

participate in the study. Patients who underwent MRM or BCS, and those received neoadjuvant chemotherapy or even radiotherapy were excluded; flow cytometry was done before radical surgery, then they were treated according to international standardized guidelines of management of breast cancer. Patients were followed up for a median time of 42 months (range of follow up 10-60 months). Twenty Age-matched healthy women with no previous history of malignant disease were approached to participate in the study as a control group.

Molecular and Histological Classification of BC Were Evaluated as Routine Hospital Tests. A spectrum of four main subtypes of BC with distinct biological features, subsequently, different response patterns to treatment modalities and clinical outcomes were known, traditional classification system of biological types was used in this study, using grade, ER, PR, Her2neu, Ki-67, subset cluster analysis uncovered two main subtypes of BC, ER-positive and ER-negative BCs, The ER-positive group are characterized by luminal cells expressing different genes including cytokeratin 8 and 18 and estrogen receptor genes, so-called luminal subtype. They were further sub-classified into luminal A (represented 50-60% of all BCs, had low grade, low degree of nuclear pleomorphism, low mitotic activity, high level of ER, low level of proliferation-related genes and positive cytokeratin 8,18(CK8,18)), and luminal B represented 15-20% of BC with increased expression of proliferation-related genes such as v-MYB (avian myeloblastosis viral oncogene homolog-like), GTP cyclohydrolase (GCH), nanosecond electrical pulse 1(NSEP1), and cyclin E1. The cutoff parameter to define luminal A or B is Ki-67 index with 14% or less characterizes luminal A, and more than 14% characterizes luminal B, ER-negative BCs are classified into HER2 positive, basal-like, and normal like. HER2 positive BC represents 15-20%, highly proliferative, 75% of them with high grade, 40% have mutated P53 gene, and about 50% express ER, subsequently are highly aggressive. Basal-like, 8-37% of all breast cancers, are associated with high grade, expresses high levels of basal myoepithelial markers such as CK5, CK14, CK17, laminin, without expression of ER, PR, and HER2 hence termed triple-negative, in addition, they overexpress P-cadherin, epidermal growth factor receptor (EGFR), fascin, mutated P53, inactive retinoblastoma (Rb) genes, BRCA1,2 and others. Normal breast-like type representing 5-10%, has an intermediate prognosis between luminal and basal-like cancers as they lack ER, PR, HER2, also are negative CK5, and EGFR, however, they were grouped with triple negative.

Sample Taking. 2 ml of venous blood was taken from all participants by clean venupuncture and collected into a sterile tube containing sodium heparin anticoagulant.

Antibodies. Fluorochrome-labeled monoclonal primary antibodies were used Fluoroisothiocyanate (FITC)-conjugated CD28 (CD28.2), phycoerythrin (PE)-conjugated CD8 (RPA-T8), peridinium-chlorophyll-protein (Per-CP)-conjugated CD4 (L200), FITC-conjugated-CD27 (M-T271), PE-conjugated-CD16 (3G8), PE-conjugated-CD56 (NCAM16.2), Per-CP-conjugated CD19 (4G7) and allophycocyanin (APC) conjugated CD3 (SP34-2). All were purchased from Becton Dickinson (BD) Biosciences, San Jose, CA, USA.

Flow cytometry. For detection of lymphocyte subsets in peripheral blood, 50 µl of blood sample was stained with 5 µl of monoclonal antibodies. After incubation for 20 minutes at 4°C in the dark, RBCs lysis was done and washed with phosphate buffer saline (PBS). The cells were resuspended in PBS then acquired and analyzed by FACSCalibur flow cytometry with Cell Quest software (BD Biosciences, USA). Mouse anti-human IgG was used as an isotype-matched negative control for each sample. Staining protocol was used

to analyze the expression of lymphocytes population by forward and side scatter histogram. Then, the percentages of $CD3^{+}CD16^{+}CD56^{+}$ (NKT) cells, $CD3^{-}CD16^{+}CD56^{+}$ (NK), $CD19^{+}$ (B-lymphocytes), $CD19^{+}$ $CD27^{-}$ (naïve B cells) $CD19^{+}$ $CD27^{+}$ (total memory B cells), $CD8^{+}$ $CD28^{-}$T cells ($CD8^{+}$ CD28 null), and $CD4^{+}$ $CD28^{-}$T cells ($CD4^{+}$ CD28 null) as shown in figure 1.

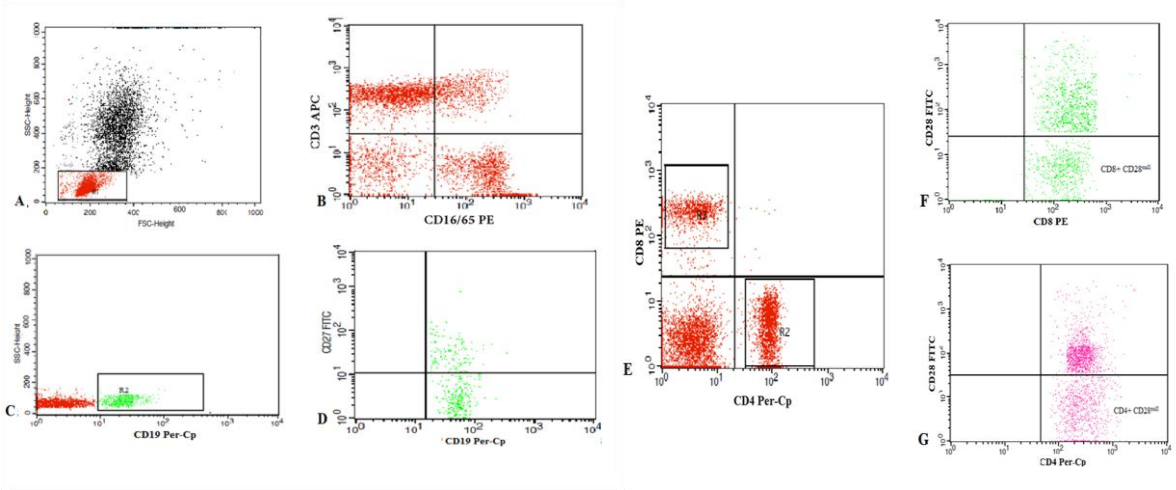

Figure 1. Representative flow cytometric analysis data showing (A) lymphocytes were gated according to forward and side scatter (R1) (B) the identification of NK cells $CD3^{-}CD16^{+}CD56^{+}$ and NKT cells $CD3^{+}CD16^{+}CD56^{+}$ (C) B lymphocyte Cells $CD19^{+}$ were gated for further analysis of expression of CD27 (D) Identification of $CD19^{+}CD27^{+}$memory B lymphocytes and naïve B lymphocytes $CD19^{+}CD27^{-}$ (E) $CD4^{+}$ and $CD8^{+}$T lymphocytes were selected for analysis of their expression of CD28, (F)and (G) showed the expression of CD28 on $CD8^{+}$ and $CD4^{+}$ T lymphocytes respectively.

Follow-up. Patients were followed up during treatment to evaluate their response to chemotherapy (mainly FEC regimen consisted of 5-FU, epirubicin, and cyclophosphamide, and EC+T epirubicin, and cyclophosphamide followed by taxotere ±herceptin), also after 3 dimensional conformal radiotherapy (3DCRT), and during hormonal treatments, they were followed up by clinical examination, multislice computed tomography chest and pelvi-abdomen (not in all cases), chest X-ray, abdominal ultrasound, Echo, bone scan, complete blood count, blood chemistries, and tumor marker CA15-3 according to standardized guidelines.

Statistical Analyses. We hypothesized that B-cell subsets, $CD4^{+}CD28^{-}$ cells and $CD8^{+}CD28^{-}$ cells in the peripheral blood of breast cancer patients and healthy controls differ. G power program version 3.1 was used to calculate our sample size of the study, and the minimum required sample size was 30 patients to detect an effect size of 0.5 of the difference between means of two independent groups with p-value <0.05 and a power of 86% as shown in figure 2.

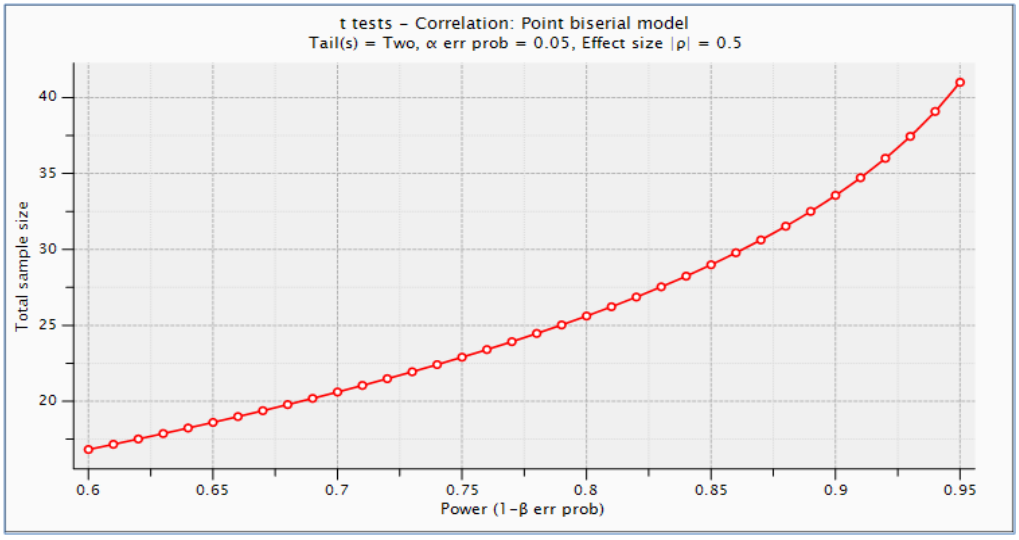

Figure 2. X-Y plot for the range of values of sample size correlated with study power.

Statistical package for social sciences (SPSS) version 20 was used for data analysis. All quantitative data were expressed as mean ± standard deviation (SD). Differences in a mean between the different groups of subjects were calculated using the independent t-test, one-way ANOVA, and LSD. Pearson correlation to detect the relation between different quantitative variables, Kaplan-Meier test to graph the survival curves of different biologic subtypes, and log-rank test to find a difference in survival curves, P-value <0.05 was considered significant. Recurrence-free survival (RFS) was defined as the time interval between diagnosis of BC and date of recurrence whether local or distant.

RESULTS

Clinicopathologic features of patients.

Thirty BC patients and twenty healthy controls were enrolled in this study: the age of all study participants ranged from 27 to 70 years. Mean age for both patients and controls was (51.03 ± 10.33 and 50.14 ± 9.04 years) respectively (Table 1). The study participants' status (age, molecular classification, pathology, lymph node status, TNM stage, response, performance status by Eastern Cooperative Oncology Group-performance scale (ECOG)) were presented in Table 1.

Differential expressions of lymphocytes subsets between patients and controls.

We evaluated NK cells ($CD3^{-}CD16^{+}CD56^{+}$) and NKT cells ($CD3^{+}CD16^{+}CD56^{+}$) percentages in BC patient samples and found to be significantly higher than control samples as shown in table 2 and figure 1.

Table 1. Clinicopathologic Features of 30 breast cancer patients.

Feature	N= 30
Age	51.03 ± 10.33
Molecular classification	
Her2	8
Luminal A	10
Luminal B	6
Triple negative	6
Pathologic type	
IDC	25
ILC	2
Mixed IDC&ILC	1
Mucinous carcinoma	2
T-stage	
1	6
2	17
3	5
4	2
N-stage	
0	6
1	10
2	7
3	4
Nx	3
TNM staging	
1A	3 (10%)
2A	7 (23.3%)
2B	4 (13.3%)
3A	9 (30%)
3B	2 (6.7%)
3C	5 (16.7%)
Grade	
I	7
II	18
III	5
ECOG-PS	
0	3
1	12
2	14
3	1
Site	
Right	13 (43.3%)
left	17 (56.7%)
Response to treatment	
CR	16
PR	9
SD	3
PD	2
Local relapse	5 (16.67%)
Distant relapse	12 (40%)
-Single site	6 (20%)
-Multiple sites	6 (20%)

Her2; human epidermal growth factor receptor 2, ECOG-PS; Eastern Cooperative Oncology Group-performance status, IDC; infiltrating ductal carcinoma, ILC; infiltrating lobular carcinoma, CR;complete response, PR;partial response, SD;stable disease, PD; progressive disease Nx; lymph node status could not be assessed.

Further investigation of $CD19^+$ B lymphocytes, $CD19^+CD27^+$memory B lymphocytes, and naïve B lymphocytes ($CD19^+CD27^-$) showed that both total B and naïve B lymphocytes were significantly higher in patients than control samples. However, total memory B cells were significantly lower in patients than control samples as shown in table 2 and figure 1.

Table 2. Percentage of lymphocyte subsets in breast cancer patients and controls.

	Patients (n=30)	Control samples (n=20)	p-value
B lymphocytes	15.24±4.83	11.67±1.9	0.001*
Total memory	22.05±7.01	34.16±5.16	0.0001*
Naïve B lymphocytes	76.28±5.7	64.91±6.96	0.0001*
NK cells	10.20±2.44	8.38±1.34	0.002*
NKT cells	8.33±2.10	6.83±1.77	0.01*
CD4	44.12±8.43	56.40±5.43	0.0001*
CD8	17.91±4.59	15.01±4.20	0.02*
CD4/CD8 ratio	1.31±0,44	1.13±0.27	0.0001*
CD4+CD28- T cells	12.99 ± 4.83	7.57 ± 1.66	0.0001*
CD8+CD28- T cells	35.44 ±14.00	16.15 ± 6.10	0.0001*

Data analyzed with independent sample t-test, * Statistical significant difference

Also, the percentages of $CD4^+CD28^-$ and $CD8^+CD28^-$cells were significantly higher in BC samples in comparison to the control group (table 2 and figure 1). One way ANOVA test was conducted to evaluate the association between $CD8^+$ $CD28^-$cells and BC biologic subtypes; we found there was a statistically significant difference in the mean of cell percentages between the different biologic types (p=0.045),furthermore, Post-Hoc with LSD was run, and significant difference was mainly confirmed between triple negative and luminal B (p=0.008) and between Her2neu and the later (p=.046), However, there were no differences between triple negative and Her2 neu with Luminal A (figure 3).

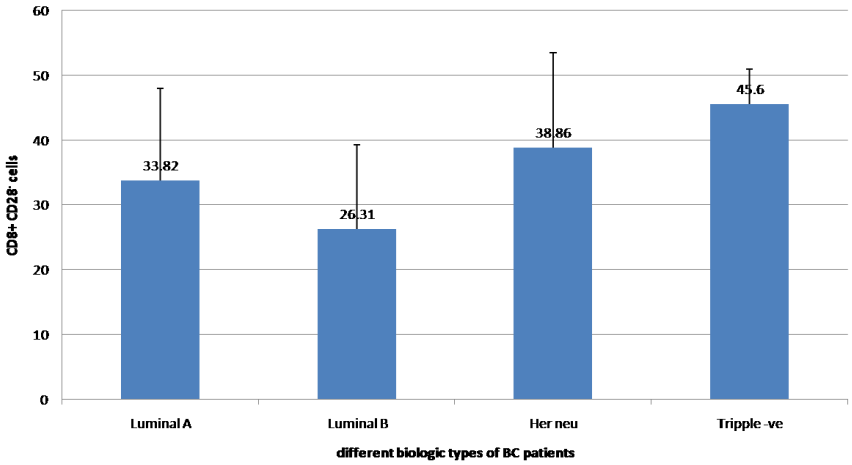

Figure 3. Difference of the mean percentage of CD8+CD28-cells between breast cancer biologic subtypes, one way ANOVA test, p=0.045.

Moreover, only significant accumulation of CD28-CD4+cells in Her2neu subtype when compared to luminal A in Post-Hoc analysis (p=0.046). Conversely, significant negative

correlation was found between $CD8^+$ $CD28^-$ and total memory B cells (r= -0.2 p=.021). Additionally, Significant negative correlation between $CD8^+CD28^-$ cells and recurrence-free survival (r= -o.556, p<0.001). As expected; a significantly positive correlation was observed between total memory B and RFS (r=0.553, p<0.002), the median RFS of different biological subtypes were demonstrated in figure 4.

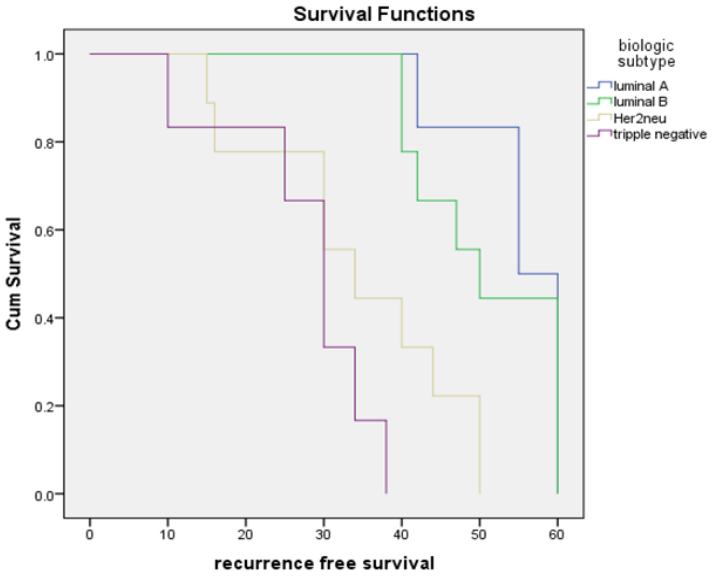

Figure 4. It shows the median RFS for different biologic subtypes; for luminal A was 55 ± 4.41 (95% CI=46.358-63.642), for luminal B, it was 50 ± 4.47 (95% CI= 41.235-58.765), for Her2neu, it was 34±5.963 (95% CI=22.313-45.687), and for triple negative BC, it was 30±2.887 (95% CI=24.342-35.658), log rank=20.295, p<0.0001.

A statistically significant difference in naïve B cell mean percentages between different grades of the tumor was observed (p=0.01); there were significantly different cell mean percentages between grade 1 and grade 3 (p=0.01), also between grade 2 and grade 3 (p=0.02), but no significant difference was observed between grades 1 and 2 (figure 5).

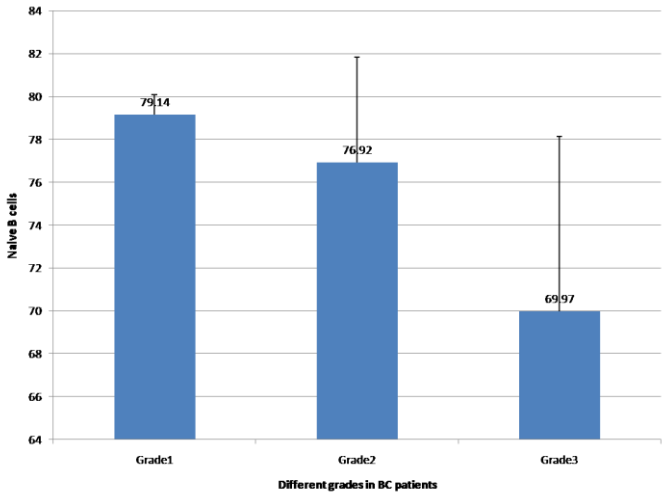

Figure 5. Differences in the mean naïve B cell percentages among different grades of BC, one way ANOVA test, p=0.01.

Upon analyzing the differences in immune cells mean percentages in different TNM stages; we didn't detect any significant difference of total memory B cells (p=0.671),

naïve B cells (p=0.667), NK cells (p=0.9), NKT cells (p=0.581), CD28-CD4 cells (p=0.8), CD28-CD8 cells (p=0.58), even after running Post-hoc test with LSD, no significant differences between the previously mentioned cells and TNM stage with exception of B lymphocytes where there was significant accumulation of these cells in stage 1A compared with stage 3A (p=0.027), and between the former and stage 3C (p=0.038), (figure 6).

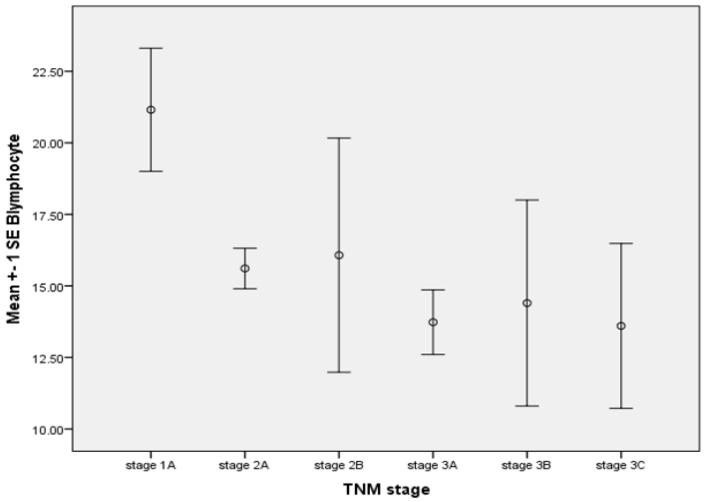

Figure 6. Difference of mean B lymphocyte percentages according to different TNM stage encountered in our patients, one way ANOVA test, Post-Hoc with LSD.

Furthermore, a statistically significant difference in naïve B cell mean percentages in different clinical responses was observed (p=0.01). Post-hoc analysis with LSD was run to determine significance among subgroups; there was statistically significant higher naïve B cell in CR than PD (p=0.002) also higher in PR than PD (p=0.009) finally higher in SD than PD (p=0.007) (figure 7). On the other hand, we found a significant negative correlation between NKT cells and B lymphocytes (r=-0.477, p=0.008). Also, significant negative correlation between NKT cells and CD4 lymphocytes (r=-0.364, p=0.04) consistent with these results, a significant mild positive correlation between B and CD4 lymphocytes was observed. Significant progressive accumulation of $CD8^+$ $CD28^-$ cells with unfavorable biologic subtypes (p=0.04) namely, Her2neu and triple negative subtypes had significantly lower mean percentages than luminal B (p=0.046, 0.008 respectively), and more advancing N stage (p=0.04), namely N1 to N3, implicating that elevated levels of $CD8^+CD28^-$cells were associated with poorer prognostic types and high risk of progression especially among patients received 1^{st} line chemotherapy treatment (figure 8).

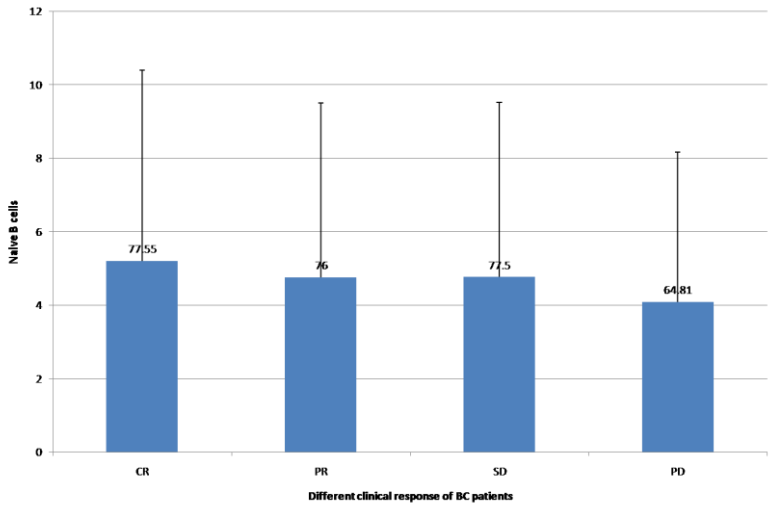

Figure 7. It shows significant differences in the mean naïve B cell percentages among different clinical responses of BC patients, one way ANOVA test, p=0.01.

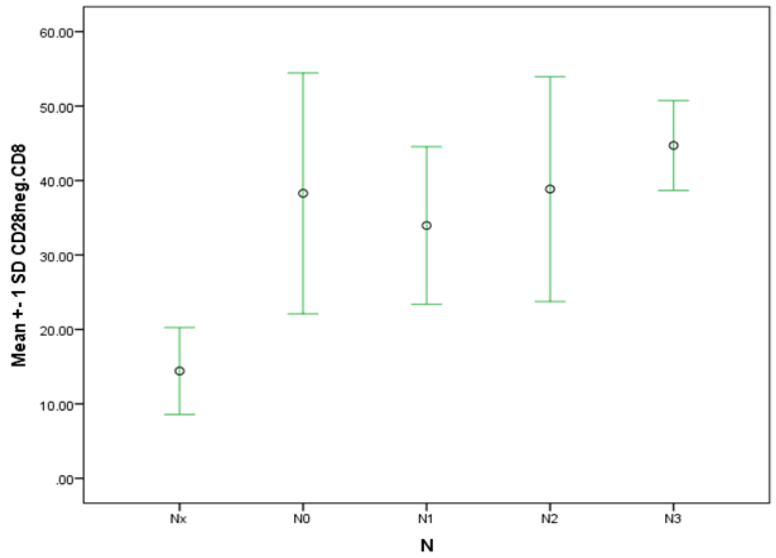

Figure 8. It shows difference of mean CD8+CD28-cell percentages among different N stage, one way ANOVA test, p=0.04.

Although, there was no significant impact of different types of immune cells on the pattern of distant relapse nor local relapse, but upon subgrouping, we found a statistically significant difference in the mean percentage of NK cells between those patients without distant relapse and multiple sites of relapse with mean difference of 2.261 ± 1.093 p=0.048, but not with others, also, significant accumulation of CD4+CD28- cells in those with single site of relapse than no relapse with mean difference of 5.073 ± 2.150, p=0.026, (figure 9, 10)

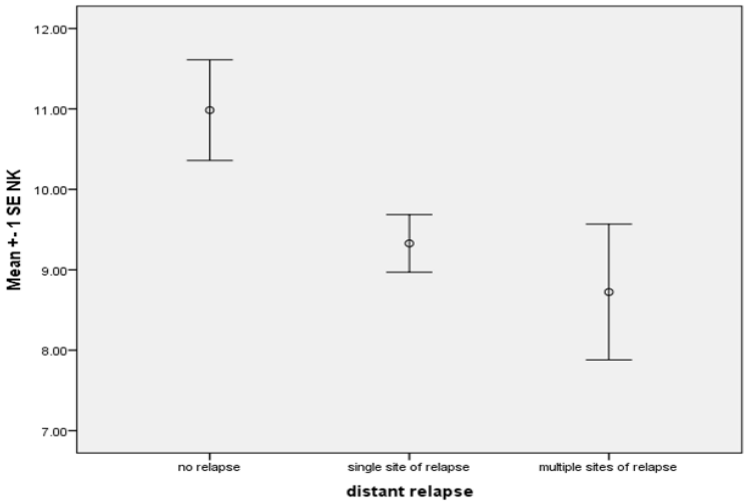

Figure 9. It shows significant accumulation of NK cells in patients without relapse compared with multiple sites of relapse, one way ANOVA test, p=0.048.

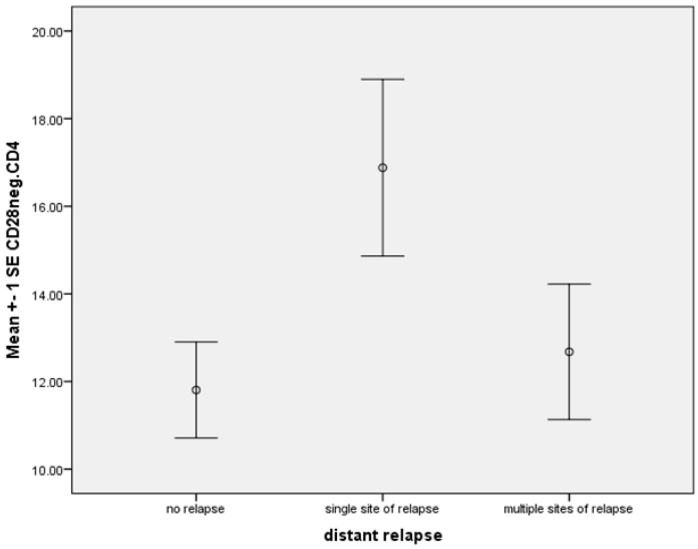

Figure 10. It shows significant accumulation of CD28-CD4+ cells in those patients without relapse compared with those with single site of relapse, one way ANOVA test, p=0.026.

DISCUSSION

Immunity in breast cancer is thought to be reached by components of innate immune system including the natural killer cells (12) and natural killer T cells, in addition to helper (13) and cytotoxic T cells (14), together with humoral immunity through immune response creating B cells (15). In this study, we found significant differences in B-cell percentages between BC patients and control samples. The higher percentage of total B cells and CD4+ T cells in BC patients indicated that B cells participated in anti-tumor immune responses and the possibility of a shift toward a TH2 type response characterized by the increased percentages of B cells and $CD4^+$ T cells (16). Our results coincided with

the study of Tsuda *et al.* (17) and Mohammed *et al*. (4). The significant increase in naïve and decrease in memory B cells BC patients than control samples could be a time sequence, wherein naive cells responded first, followed by the production of memory B cells. Conversely, Tsuda *et al.* found that naïve and memory B cell subsets were higher in BC patients, but this increase was not statistically significant. Also, they found that memory B cells significantly expanded when patients classified to High-Band Low-B groups, High-B was defined as values above the highest B-cell proportion observed in healthy donors (17). This difference could be explained by the accumulating evidences suggesting that B cells within the BC patients were highly variable as a result to some factors inducing B cell subtype switching, and other chemotactic factors that regulated B cell tumor infiltration (15). B cells may act as a marker to predict breast cancer survival and the treatment response because high B cell numbers correlated with improved prognosis (18). This supported our finding of the presence of a correlation between total memory B cells and recurrence-free survival, the higher percentage of naïve B cell found in patients with complete clinical response and tumor grade 1, however, Eiró and Rody study results were contradictory (19,20). NK and NKT cell subsets collaborate to constitute protective antitumor immunity (21). Our investigation documented a higher and significant increase in the percentage of NK in the blood of women with BC. This was consistent with Murta *et al.* (22) as well as Muraro *et al.* who demonstrated an increase in the percentage of NK cells, but only in women with HER2/neu negative tumors (23). In our study, 22 of the 30 patients studied had HER2/neu negative cancers; a previous data indicated that both adaptive and innate immune mechanisms appeared to contribute to this anticancer response (24). On the contrary, Verma *et al.* reported no difference in the percentage of NK cells (25). This may be attributed to advanced tumor state in his study that may inhibit aspects of innate immunity, migration of these cells to tumor site, or the lymphatic drainage around the tumor (26,27). However, the significant decrease in NK cells in patients with multiple sites of relapse than those without relapse suggested that tumor burden depresses immune reactions and limits their potential protective effects (28), and this finding was also supported by Konjevic *et al.* who demonstrated a significantly decreased NK cell activity in patients with breast cancer with progression of disease (29). In a mouse model of breast cancer metastases, inhibition of iNKT cells by tumor down-regulation of CD1d molecules facilitated metastatic breast cancer progression (30). In our study, a higher frequency of NKT cells in BC patients compared with healthy donors was found. These observations supported our finding of a significant increase in NK cells as NKT cells can produce IFN-γ that activates NK cells (31). This was in contrast with Horlock *et al.* who documented no significant differences in NKT cell numbers between both populations (32). CD28 stimulates T-cell activation and survival; in addition its expression on naïve T cells occurs early in the newborns. $CD28^-$T cells undergo progressive age related changes in the peripheral blood to decline in the elderly (33). Expansion of $CD8^+CD28^-$lymphocyte in patients with different malignancies was recorded (34), and this expansion of $CD8^+CD28^-$lymphocytes in cancer patients was found to be associated with advanced stages and worse survival (35,36). The previous findings were concomitant with our results which revealed a significantly higher percentage of $CD8^+CD28^-$ lymphocyte subpopulations in BC patients than in control samples, in addition, this higher mean was found in patients with triple-negative type and advanced node stage indicating the association of cells with poor prognostic types, on the same track, they significantly increased with single site of relapse than no relapse. Taken together, our results indicated that $CD8^+CD28^-$ had an intimate association with poor

patient's response. This also supported by Song et al. who demonstrated a significant increase in the percentage of $CD8^+CD28^-$T cells in metastatic BC patients, subsequently associated with shortening progression-free survival (9). Similarly, Song *et al.* demonstrated that higher proportion of $CD8^+CD28^-$T cells in the peripheral blood was associated with shorter progression-free and overall survival, in addition, among patients who received first-line chemotherapy; it predicted disease progression and recurrence. Furthermore, among patients receiving more lines of chemotherapy, it predicted death (37). Also, a significant negative correlation was observed between $CD8^+$ $CD28^-$T cells and total memory B these observations supported by previous studies demonstrated that $CD8^+CD28^-$T cells secreted suppressive cytokines such as IL-10, and transforming growth factor (TGF)-b (38). Also, they interacted directly with antigen-presenting cells via cell-to-cell contact, up-regulated the inhibitory receptors immunoglobulin-like transcript 3 (ILT3) and ILT4 and decreased co-stimulatory molecules (39). Our results provide, for the first time, higher percentage of $CD4^+CD28^-$T cells in BC patients in comparison to control samples with significant accumulation of cells in those with a single site of relapse than no relapse. Regarding these points, many studies had reported the expansion of $CD4^+CD28^-$T cells in patients with various immune disorders, including autoimmune diseases (40), chronic inflammatory disorders (41,42), and immunodeficiency diseases (43). Patients suffering from these disorders commonly showed an age related decrease in the proliferation of CD4+$CD28^-$T cells, to indicate premature aging of the immune system in these patients (44), in addition, this premature aging is detected in younger individuals complaining from cancers. The sample size has a limited power for assessing the relations between null cells and different biologic breast types. The small sample size is the crucial study limitation; in addition, heterogeneity of these studied patients including heterogeneity of biologic subtypes, staging, and treatments received also resulted in absence of several relations worthy to be studied. There is a great need to develop approaches for primary therapy of breast cancer that maintain immune functions since antibody dependent cellular cytotoxiciy-promoting therapeutic monoclonal antibodies, such as trastuzumab, pertuzumab, and anticancer vaccination, are being increasingly developed especially in the adjuvant setting. A paucity of studies examining the impact of $CD28^-$Tcells in breast cancer patients with regard mainly to treatment outcomes, our study was considered an early one to detect the negative association between $CD28^-$T cells and survival and need to be supported by further studies. In conclusion, NK, NKT, different B cells, and $CD28^-$Tcells were significantly different between healthy controls and BC patients. Also, different B cells were associated with good response to treatment while $CD28^-$cells were associated with shorter RFS.

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