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ORIGINAL ARTICLE

NK, NKT and Invariant-NKT Cells in Tumor Draining Lymph Nodes of Patients with Breast Cancer

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ABSTRACT

Background: NK (natural killer) and NKT (natural killer T) cells, as components of innate immune system, play a crucial role in tumor progression and dissemination. **Objective:** To investigate the percentages of NK cells, NKT cells, iNKT (invariant natural killer T) cells, total T lymphocytes as well as activated T lymphocytes, in tumor draining lymph nodes (TDLNs) of patients with breast cancer (BC) and their association with different clinic-pathological features of the patients. **Methods:** Axillary lymph nodes were obtained from 30 Iranian women with breast cancer. After routine pathological evaluations, mononuclear cells were separated from their lymph nodes and incubated with appropriate fluorochrome conjugated monoclonal antibodies specific for CD3, HLA-DR, CD16/56, and Vα24Jα18-TCR. Data were collected on a four-color flow cytometer and analyzed by CellQuest software. **Results:** The mean percentages of NK ($CD3^-CD16/56^+$), NKT ($CD3^+CD16/56^+$) and iNKT ($V\alpha24J\alpha18\text{-}TCR^+$) cells in TDLNs mononuclear cells of BC patients were 2.04%, 2.44% and 0.1%, respectively. A significant decrease in the percentages of NK and iNKT subsets in patients with grade I was observed compared to grade III (p=0.03 and p=0.01, respectively). Moreover, NK cells were increased in patients with grade III of BC compared to grade II (p= 0.003). **Conclusion:** The increase in the percentage of NK and iNKT cells in TDLNs of patients with higher grade of BC might suggest a suppressive phenotype for these cells in breast cancer, which merit more functional investigation.

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Keywords: Breast Cancer, Lymph Nodes, Invariant-NKT Cells, NK Cells, NKT Cells

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INTRODUCTION

Breast cancer (BC) is the most frequent malignancy in women all around the world (1), and the fifth leading cause of death in Iranian women (2). It is now well-documented that a developing tumor interacts with both adoptive and innate immune systems elements. Beside T cells as the vital and well documented adoptive immune cells in cancer therapy (3), natural killer (NK) and natural Killer T (NKT) cells have been usually considered as important components of innate immune system with the ability to control tumor progression and dissemination (4,5). These cells, which are introduced as $CD3^-$ $CD16/56^+$ and $CD3^+$ $CD16/56^+$ cells respectively, are capable of direct killing of the target cells, and secreting immune regulatory cytokines (6,7). Based on variation in their T cell receptors (TCRs) structure, two common types of NKT cells have also been reported. Type I or invariant NKT (iNKT) cells harbor Vα24-Jα18 TCRα chains in preferential combination with Vβ11 TCRβ in humans, whilst type II or variant NKT (vNKT) cells have more diversity in their TCR chains (8). Conducting immune surveillance and bridging between adoptive and innate immune systems have been considered to be the crucial roles of NK and iNKT cells in anti-tumor immune responses (9-11). While vNKT cells have been reported to down-regulate antitumor immunity, emerging evidence suggested iNKT cells promote anti-tumor immune responses. iNKT cells also share functional characteristics with conventional NK cells including direct cytotoxic activity against tumor cells and the production of cytokines such as IFN-γ (12,13). T lymphocytes, NK cells and NKT cells have been reported to be in the center of antitumor immune regulation paradigm with reciprocal effects (14,15). Regarding the central role of NK, NKT and T cells in antitumor immunity, the crosstalk between these antitumor immune elements, as well as the significance of tumor draining lymph nodes (TDLNs) in shaping the immune response, we aimed to investigated the percentages of NK, iNKT, total NKT, total T lymphocytes as well as activated T lymphocytes in TDLNs of the patients with BC. Correlation between the percentages of the aforementioned cell subsets with cancer progression factors was also investigated.

MATERIALS AND METHODS

Subjects. Thirty Iranian women with BC and no prior chemotherapy or radiotherapy were enrolled in this study. Lymph nodes were resected as a part of treatment procedure and used as the source of lymphocytes. Clinical and pathological characteristics of the enrolled patients were collected from their medical records (Table 1). The study was approved by the Ethics Committee of Shiraz University of Medical Sciences, and informed consent was obtained from all subjects prior to sampling.

Obtaining Mononuclear Cells from Lymph Nodes. Following surgery and after the clinical evaluation of the resected lymph nodes by a pathologist for routine lymph node assessment, lymph node fragments were minced into small pieces in the culture medium (RPMI 1640 containing 10% fetal bovine serum, Biosera, France). The cells were then filtered through a 40 μm cell strainer (BD Biosciences, USA) in order to obtain a homogenous cell suspension which was then employed to isolate mononuclear cells by Ficoll-Hypaque (Biosera, France) gradient centrifugation.

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

Characteristics	Value (Valid percent)
Age (years)	48.20 ± 9.41
Lymph node status	
Free from tumor cells (N0)	22 (73.3%)
Involved by tumor cells	8 (26.7%)
N1 (1-3)	3 (10%)
N2 (4-9)	1 (3.3%)
N3 (>9)	4 (13.2%)
Stage	
I	8 (26.7%)
II	17 (56.7%)
III	5 (16.7%)
IV	0 (0%)
Tumor type	
Infiltrative ductal carcinoma (IDC)	22 (73.3%)
Medullary carcinomas (MC)	6 (20%)
Invasive lobular carcinoma (ILC)	2 (6.6%)
Tumor size	
T1 (≤2 cm)	11 (36.7%)
T2 (2-5 cm)	18 (60%)
T3 (>5 cm)	1 (3.3%)
Histological grade	
Well differentiated (I)	5 (23.8%)
Moderately differentiated (II)	11 (52.4%)
Poorly differentiated (III)	5 (23.8%)
Unreported	9
Estrogen receptor (ER)	
Positive	16 (66.7%)
Negative	8 (33.3%)
Unreported	6
Progesterone receptor (PR)	
Positive	14 (58.3%)
Negative	10 (41.7%)
Unreported	6

Flow Cytometric Analysis. Anti-CD3 FITC/CD16^{+}CD56 PE (clone: SK7), Anti-HLA-DRPerCP (clone: L243) and Anti-Vα24Jα18 TCR Alexa Fluor®647 (clone: 6B11), all obtained from BD Biosciences, USA, were used to phenotype NK, iNKT, total NKT, and total and activated T lymphocytes. Cognate isotype control antibodies (all purchased from BD Biosciences, USA) were further used to check the background staining. The cell suspensions containing 5×10^5 cells were incubated with the foregoing antibodies as the test tube. The same amount of cells in isotype control tube was stained by applying isotype control antibodies. Unstained cells, treated in the same manner as stained cells, were used for a subsequent setup of the instrument. The cells were then subjected to flow cytometer analysis by use of four-color BD FACS Calibur flow cytometer (BD Biosciences, USA). The raw data were then collected and exposed

to dot plot analysis by applying CellQuest Pro software package (BD Biosciences, USA).

Statistical Analysis. Statistical analysis was performed using SPSS software package version 13 (SPSS GmbH Software, Germany). Mann-Whitney U and Kruskal Wallis tests were applied to the statistical analysis between two or more groups of patients with different clinico-pathological characteristics, respectively. Spearman's correlation test was employed to determine the correlation among various cell subsets. p-values less than 0.05 were considered as statistically significant.

RESULTS

The Clinical and Pathological Characteristics of the Patients.

Thirty untreated BC patients were enrolled in the present study. As summarized in Table 1, the mean age of patients was 48.2 ± 9.4 years. According to the patients' pathological reports, infiltrative ductal carcinoma was the most frequent tumor type (22/30, 73.3%). Eight patients (26.7%) had at least one involved lymph node (considered as LN^+ patients), and 56.7% of the patients (17/30) were diagnosed to be in stage II of the disease.

NK, NKT and T lymphocyte Frequencies in the TDLNs of Patients with BC.

Table 2 shows the frequency of various investigated lymphocyte subsets in draining lymph nodes of the patients with BC. As shown, the lymphocyte population in TDLNs was observed to encompass 51.1% $CD3^+$T cells (total T cell), among which 6.3% had an inactivated status (HLA-DR^+CD3^+).

Table 2. The mean percentage of total T, activated T, NKT and NK cells in tumor draining lymph nodes of patients with breast cancer.

Cell Subsets	CD Markers	Min	Max	Mean ± SD
Total T cell	$CD3^+CD16^-CD56^-$Lymphocytes	32.97	77.27	51.14 ± 11.68
Activated T	$CD3^+CD16^-CD56^-$HLA-DR^+ T cells	3.20	12.19	6.32 ± 2.08
NK cells	$CD3^-CD16^+CD56^+$Lymphocytes	0.65	4.77	2.04 ± 0.83
NKT cells	$CD3^+CD16^+CD56^+$Lymphocytes	0.60	3.80	2.44 ± 0.84
iNKT cells	$CD3^+CD16^+CD56^+Va24^+$Lymphocytes	0.00	0.58	0.10 ± 0.1

Two percent of the lymphocytes in the TDLNs of BC patients were observed to have an NK phenotype ($CD3^-CD16/56^+$). 2.4% of the cells simultaneously expressing CD3 and CD16/56 were considered as NKT cells in the present study. iNKT (Vα24Jα18-TCR^+) cells were shown to be 0.1% of lymphocytes in TDLNs (Figure 1).

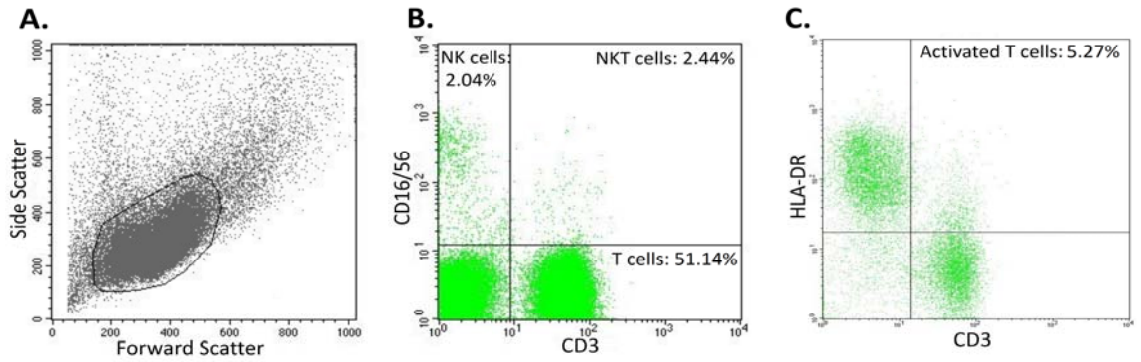

Figure 1. Flow cytometric analysis of mononuclear cells obtained from tumor draining lymph nodes of patients with breast cancer. A. Lymphocytes were gated on the forward and side scatter plot. The lymphocytes gate were then applied to the B and C dot plots to analyze NK ($CD3^-CD16/CD56^+$), NKT ($CD3^+CD16^+CD56^+$) and activated T cells ($CD3^+CD16^-CD56^-HLA\text{-}DR^+$). iNKT cells dot plot has not been shown.

Statistical analysis showed that the frequency of NK cells increased significantly in patients with higher grades (grade III; n=5) compared to those with lower ones, meaning grade II (n=11; p=0.003) and grade I (n=5; p=0.032) (Figure 2). Furthermore, a positive correlation was further detected between the NK cells frequency and tumor grade (R=0.530, p=0.013). The frequency of NKT cells in patients with grade III was higher, though not significantly, compared to those with grade I (p=0.05). The percentage of iNKT (Vα24Jα18-TCR) cells showed a significant increase in patients with grade III in comparison to those with grade I (p=0.016) (Figure 2).

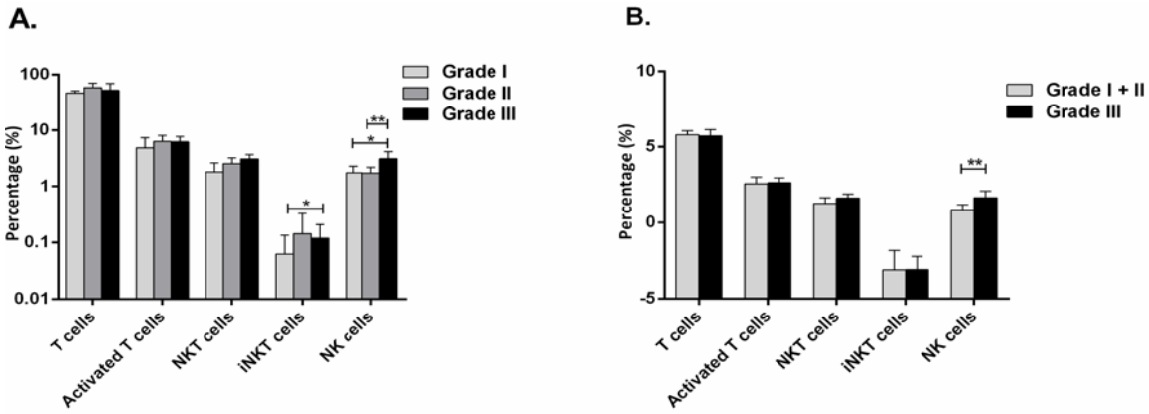

Figure 2. The frequency of total T, activated T, NKT, iNKT and NK cells in draining lymph nodes of breast cancer patients with different grade.

Regarding other clinico-pathological characteristics, no significant differences were observed regarding the frequencies of the investigated lymphocyte subsets between patients with different stages, lymph node involvements, tumor sizes, tumor types, as well as hormone (estrogen and progesterone) and growth factor (Her2) receptor

expressions. A correlation study was also performed to investigate any correlation between the frequencies of different cell subsets. However, no significant correlation was observed between the percentages of other investigated lymphocytes in the TDLNs of BC patients.

DISCUSSION

T lymphocytes and natural killer (NK) and natural killer T (NKT) cells have been shown to be the main players of adoptive and innate immune responses against cancer, respectively. Tumor draining lymph nodes (TDLNs) have been well documented as the main lymphoid organs in anticancer immunity and the place for crosstalk among different armaments of the immune system. The present study investigated the frequency of various immune cell subsets including T lymphocytes, NK, NKT cells, and iNKT lymphocytes in the TDLNs of patients with BC and their association with the clinical and pathological characteristics of the disease. Based on our results, the total T cells comprised 51.1% of LN lymphocytes, among which 6.32% were observed to have activated phenotype. In addition, 2%, 2.4% and 0.1% of lymphocytes in the TDLNs of BC patients were observed to be with NK, NKT and iNKT phenotypes, respectively. Although no significant differences were observed in the frequencies of T and activated T cells among patients with different clinico-pathological features, the frequency of NK cells was significantly increased in patients with poor differentiated tumor (grade III). Furthermore, a significant positive correlation was detected between the frequency of NK cells in TDLNs and tumor grade. Such increase suggests the association between TDLN NK cells and the poor prognosis of patients with BC. Natural killer (NK) cells are historically considered as the first line of host defense against tumor cells; moreover, their reciprocal interactions with T lymphocytes and dendritic cells (DC) in the paracortex of lymph node were shown to be crucial for a mutual activation of all three cell types (16). The majority of previous studies have confirmed the important role of NK cells in tumor surveillance because the infiltration of tumors with NK cells has been shown to represent a positive prognostic marker in different carcinomas (17-19). Several studies have further reported a positive correlation between the frequency of intra-tumoral NK cells and patients' survival (20). Nevertheless, recently emerged evidence shows that NK cells can have cytotoxic, regulatory and tolerant subsets (21). Sungur *et al.* observed that the distinct suppressor subset of NK cells in tumor microenvironment was able to suppress antitumor immune responses in patients with gastrointestinal stromal tumors (22). Furthermore, $CD11b^{-}CD27^{-}$ NK cells, introduced as tolerant NK cells, had a negative impact on the outcome of patients with lung carcinoma (23). The frequency of non-cytotoxic and immature NK cells subsets increased in the peripheral blood and tumor tissues of patients with advance BC (24). The higher frequency of NK cells in the TDLNs of the BC patients with worse prognostic conditions in the current study implies that these accumulated NK cells may mostly be suppressor subsets. To be confirmed, this hypothesis requires an accurate assessment of NK cell subsets and functional studies in the TDLNs and tumor tissue of the BC patients. The present study also focused on the frequency of total NKT lymphocytes and iNKT subset in the TDLNs of BC patients. The frequency of the total NKT cells was higher, though not significantly, in patients with grade III. Similar to NK cells, NKT cells have been considered as a heterogeneous innate immune cell

population, with immune regulatory functions in different diseases such as cancer (25). These cells are usually categorized into two different subsets (12). While vNKT cells have been reported to down-regulate cancer immune surveillance, iNKT cells promote anti-tumor immune responses (13). Although the current study did not investigate vNKT subset among total NKT population, the significantly higher frequency of iNKT cells in patients with higher tumor grade might be relevant to the impaired cytotoxic function of this cell subset. The defective function of iNKT cells have been shown in several studies on human and animal tumors such as advance prostate cancer. α-Galcer and IL-12 were effective in the retrieval of impaired iNKT cells' activity (26). However, activated iNKT cells were anergic with imperfect effector functions (27). On the other hand, iNKT cells were divided into functionally distinct subsets including NKT1, NKT2, or NKT17, which secrete cytokines corresponding to conventional helper T cells counterparts (28). Therefore, our forthcoming studies should be focused on investigating the dominant subset of iNKT cells population in the TDLNs of BC patients because type 2 cytokines secreted by NKT2 cells might suppress anti-tumor immune responses (27,29), resulting in worse prognostic features.

In conclusion, our data showed that NK cells and iNKT cells in TDLNs are associated with poor prognosis in patients with breast cancer. Accordingly, the phenotype of these cells is suggested to be a suppressive phenotype, and/or the cells with impaired function, a hypothesis which merits more investigations. The results of the present study are approximately consistent with our previous studies showing an inhibitory milieu in draining nodes of BC patients with advanced tumor (30-32). However, our sample size was almost too small, there were no data regarding the percentages of the investigated subsets in the blood circulation of the patients, and there was a lack of a functional study, all considered as limitations of the current study.

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