

IL-17 and IL-4 Producing CD8+ T Cells in Tumor Draining Lymph Nodes of Breast Cancer Patients: Positive Association with Tumor Progression

Zahra Faghih^{1,2}, Somayeh Rezaeifard¹, Akbar Safaei³, Abbas Ghaderi^{1,2}, Nasrollah Erfani^{1*}

¹Cancer Immunology Group, Shiraz Institute for Cancer Research, School of Medicine, ²Department of Immunology, School of Medicine, ³Department of Pathology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Background: CD8+ cytotoxic T lymphocytes have been recently divided based on their cytokine expression profile. **Objective:** To evaluate the percentages of CD8+ lymphocytes and their effector subsets including Tc1, Tc2 and Tc17 in the tumor draining lymph nodes (TDLNs) of patients with breast cancer. **Methods:** Single cell suspensions were obtained from TDLNs of 42 patients with breast cancer. Staining of the cell surface markers and intracellular cytokines was performed using appropriate fluorochrome-conjugated antibodies. The data was acquired on a four-color flow cytometer and was analyzed by CellQuestPro software package. The percentages of different CD8+ cell subtypes (Tc1, Tc2 and Tc17) were quantified in CD8+ T lymphocytes. The comparison was made between LN+ versus LN- patients, as well as patients in different clinico-pathological status. **Results:** The percentage of Tc1, Tc2 and Tc17 subsets were not significantly different between LN+ and LN- patients. Despite no difference in the percentages of Tc1 cells in LN+ patients with infiltrative ductal carcinoma (IDC), the mean expression of IFN- γ by Tc1 cells decreased significantly in comparison to LN- patients. On the other hand, the percentages of Tc2 and Tc17 effector subsets were increased in advanced stages ($p=0.018$ and $p=0.009$, respectively). **Conclusion:** As the first study to investigate various effector subtypes of CD8+ lymphocytes in TDLNs of patients with breast cancer, our data collectively suggests a positive association between IL-17- and IL-4-producing CD8+ T cell percentages (Tc2 and Tc17) in TDLNs with breast cancer progression. Although the number of Tc1 cells seems not to be affected by cancer progression, down-regulation of IFN- γ by these cells seems to be associated with tumor metastasis to TDLNs. These findings may have implications in cancer immunotherapy based on CD8+ effector subsets.

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Keywords: Breast Cancer, Tc1, Tc2, Tc17, Lymph Node

*Corresponding author: Dr. Nasrollah Erfani, Cancer Immunology Group, Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, Tel: (+) 98 711 2303687; Fax: (+) 98 711 2304952, e-mail: erfani@sums.ac.ir

INTRODUCTION

Cytotoxic CD8⁺ T cells are the essential part of the specific immune system with crucial roles in controlling malignancies (1). Anti-tumor activities of CD8⁺ T cells probably occur through their ability to kill tumor cells as well as cytokine secretion, particularly IFN- γ . Adoptive transfer of these cells has been reported to delay tumor growth (2) while their removal promotes tumor progression (3).

Similar to CD4⁺ T cells, CD8⁺ T lymphocyte may be differentiated into different effector subtypes characterized by their cytokines expression profiles, after encounter with antigens. Type 1 CD8⁺ T cells (Tc1) produce IFN- γ and TNF- α and destroy their target cells through perforin or Fas mediated pathways, while type 2 preferentially tend to produce IL-4, IL-5 and IL-10, and primarily use perforin route as their killing strategy. Both subtypes have been found in patients with various disorders, but their role in anti-tumor immune responses is still unclear (4-6).

More recently, a new subset of CD8⁺ effector cells has been introduced which secretes IL-17 with little or no IFN- γ and IL-4 (7). These cells, known as Tc17, are phenotypically and functionally different from other cytotoxic T cells. These cells express low levels of granzyme B, perforin and FasL, and do not show in-vitro cytotoxic activities (8). The involvement of Tc17 cells in the pathogenesis of some autoimmune diseases, such as SLE (9) and psoriasis (10) has been already reported. Other investigations suggest a role for Tc17 cells in tumor immunology as well (11,12). Although the role of CD8⁺ lymphocytes in tumor immunology is well established, there are few studies dealing with the subsets of CD8⁺ lymphocytes in cancer. The aim of the present study was to evaluate the percentages of CD8⁺ lymphocytes and their effector subsets including Tc1, Tc2 and Tc17 in the tumor draining lymph nodes (TDLNs) of patients with breast cancer. The comparison was made between node positive (LN+) versus node negative (LN-) patients, as well as between patients in different pathological conditions.

MATERIALS AND METHODS

Patients. This study was carried out on 42 untreated Iranian women with breast cancer (BC) who had undergone surgical resection. Mapping of sentinel lymph nodes (SLNs) was performed by blue dye or radioactive tracer techniques, and the involvement of regional LNs by tumor cells was histologically determined. Patients who had at least one resected LN involved by tumor cells were referred as LN+ patients. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences and all samples were obtained after giving informed consent. The main characteristics of the patients are summarized in Table 1.

Preparation of Lymph Node Mononuclear Cells. For obtaining the single-cell suspension, fresh fragments of lymph nodes were minced into small pieces in culture medium (Biosera, UK) and filtered by a 70 μ m cell strainer (BD Biosciences, USA). Mononuclear cells were then isolated using Ficoll-Hypaque (Biosera, UK) gradient centrifugation.

Flow Cytometric Analysis. Having a four-color flow cytometer but more than 4 markers to be assessed, mononuclear cells were divided into two tubes. To have the parentages of total T and CTL cells, the cells in the first tube were directly stained by

APC-conjugated anti-CD3 and PerCP-conjugated anti-CD8 antibodies. Intracellular cytokine staining needs the cells to be activated, permeabilized and then stained by the fluorochrome conjugated antibodies. Accordingly, the cells in the second tube were activated in the culture medium containing 50 ng/ml PMA and 1 μ g/ml Ionomycin (Both from Sigma-Aldrich, Germany) in the presence of 0.7 μ l Brefeldin A and Monensin (Both from BD Biosciences) for 5 hours at 37°C. The cells were then washed, fixed with 1% paraformaldehyde and permeabilized using BD Perm/Wash™ buffer (BD Biosciences). After that, the permeabilized cells were incubated with PerCP-conjugated anti-CD8, FITC-conjugated anti-IFN- γ , PE-conjugated anti-IL-4, and Alexa Fluor® 647-conjugated anti-IL-17a antibodies. As our anti-CD8 antibody (SK1) was able to recognize denatured epitopes, CD8 staining in the second tube was simultaneously done with other antibodies after fixation/permeabilization of the cells. The data was collected on a four-color FACSCalibur flow cytometer (BD Biosciences) and analyzed by CellQuestPro software package (BD Biosciences). The data for all samples were acquired with the same instrumental setting and the care was taken to make sure that the acquired data follow the same scatter and florescent light pattern.

Statistical Analysis. All the statistical analyses were performed using SPSS 13 software package (SPSS GmbH Software, Germany) and p values less than 0.05 were considered statistically significant. GraphPad Prism5 software package (Inc; San Diego CA, USA, 2003) was used for drawing the statistical graphs.

RESULTS

After assessment of total CD3+ T cells and CD3+CD8+ lymphocytes, the percentage of different CD8+ cell subtypes (Tc1, Tc2 and Tc17) was quantified in the CD8+ lymphocytes gate (Figure 1).

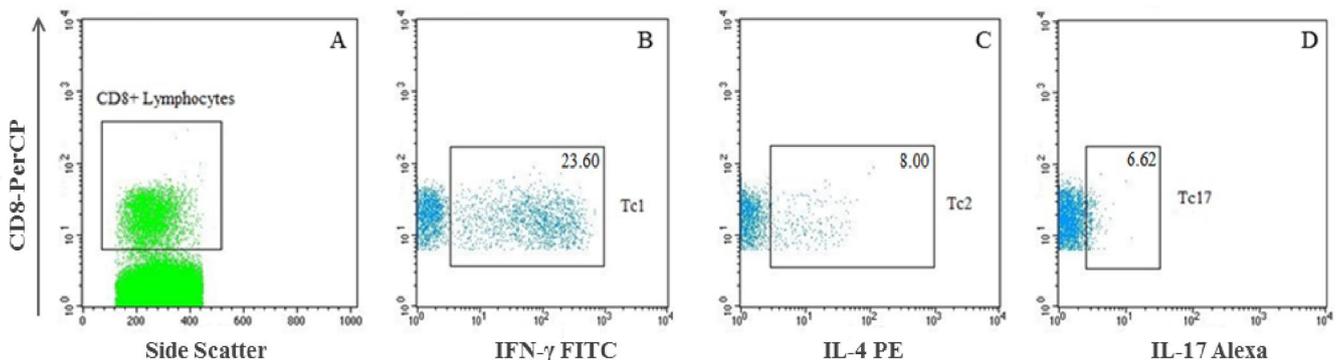


Figure 1. Flow cytometric plots of various effector subsets of CD8+ cells, the percentages of different CD8+ effector subtypes were determined in CD8+ lymphocytes population (A) including IFN- γ , IL4, and IL17 CD8+ producing cells representing Tc1 (B), Tc2 (C), and Tc17 (D) cells, respectively. Tc1: T cytotoxic type 1, Tc2: T cytotoxic type 2, Tc17: T cytotoxic type 17.

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

Characteristics	Value
Age (years)	49.57 ± 10.03
Lymph node status	
Free (N0)	29 (69%)
Involved	13 (31%)
N1 (1-3)	5 (11.9%)
N2 and N3 (>4)	8 (19.1%)
Stage	
I	10 (23.8%)
II	24 (57.1%)
III	8 (19.1%)
IV	0 (0%)
Tumor type	
IDC	33 (78.6%)
MC	6 (14.3%)
Others (ILC, SC)	3 (7.2%)
Tumor size (cm)	
T1 (≤2)	17 (40.5%)
T2 (2-5)	23 (54.8%)
T3 (>5)	2 (4.8%)
Histological grade	
Well differentiated (I)	8 (25%)
Moderately differentiated (II)	17 (53%)
Poorly differentiated (III)	7 (22%)
Missing	10
Estrogen receptor (ER)	
Positive	24 (67%)
Negative	12 (33%)
Missing	6
Progesterone receptor (PR)	
Positive	20 (56%)
Negative	16 (44%)
Missing	6
HER2 expression	
Positive	21 (58%)
Negative	15 (42%)
Missing	6
Lymph node type	
SLN	28 (66.6%)
Non-SLN	14 (33.4%)

* All the percentages in this table are valid percentages, not considering unknown status. IDC: infiltrative ductal carcinoma, MC: medullary carcinomas, ILC: invasive lobular carcinoma, SC: secretory carcinoma, SLN: sentinel lymph node

Geometric mean fluorescent intensity (MFI) was considered as the criterion of mean cytokine expression level (IFN- γ , IL-4, and IL-17) per cell. The mean percentages of CD8+ T cells and their effector subsets in breast cancer patients are presented in Table 2. Besides doing the analysis among the whole breast cancer patients (including all tumor types), all the analyses were also repeated separately in patients with infiltrating ductal carcinoma (IDC) tumor type as presented in Figures 2 to 4.

Table 2. The mean percentage of CD8+ T lymphocytes, their effector subsets and MFI of their cognate cytokines in TDLNs of breast cancer patients.

Cell subset	Markers	Minimum	Maximum	Mean \pm SD
CD8+ CTL	CD3+CD8+ lymphocytes	2.68	16.02	8.07 \pm 3.71
Tc1 cells	CD8+IFN- γ + lymphocytes	6.49	66.3	23.60 \pm 12.84
Tc2 cells	CD8+ IL-4+ lymphocytes	2.58	30.78	8.00 \pm 5.49
Tc17 cells	CD8+ IL-17+ lymphocytes	0.98	29.09	6.62 \pm 5.15
Cytokine expression (based on MFI)				
IFN- γ	IFN- γ MFI in CD8+ lymphocytes	7.02	115.22	45.62 \pm 26.04
IL-4	IL-4 MFI in CD8+ lymphocytes	4.85	14.42	9.42 \pm 2.29
IL-17	IL-17 MFI in CD8+ lymphocytes	3.27	15.71	5.53 \pm 2.70

* The percentage of different CD8+ T subsets as well as MFI of all cytokines was determined in CD8+ lymphocyte gate. TDLN: tumor draining lymph node, Tc: T cytotoxic, MFI: geometric mean fluorescent intensity

The Clinical and Pathological Characteristics of Patients. As illustrated in Table 1, 28 SLNs and 14 non-sentinel axillary LNs (non-SLNs) were obtained from 42 untreated women with BC. The mean age of patients was 49.71 ± 10.11 yrs. According to their pathological reports, 13 out of 42 patients (31%) had at least one affected LN (LN+ patients). The most frequent tumor type was IDC (33/42, 78.6%). More than half of the patients were diagnosed as stage II, and none was in stage IV.

Comparison of CD8+ T cell Subpopulations in TDLNs of LN+ and LN- Patients. As demonstrated in Table 3, the percentages of Tc2 and Tc17 cells were increased in the TDLNs of LN+ patients, while the frequency of Tc1 cells were decreased in these patients. Concordant to Tc1 reduction, a trend toward lower MFI of IFN- γ expression in CD8+ lymphocytes was observed in LN+ patients in comparison to LN- patients, although the difference was not significant (48.32 ± 23.48 vs. 38.20 ± 32.70 , $p=0.06$). The geometric MFI of IFN- γ expression by CD8+ cells was also decreased in patients with more than 9 positive nodes (N3, 20.28 ± 5.93) in comparison to patients with free nodes (N0, 48.32 ± 23.48 , $p=0.027$). On the other hand, the frequency of Tc2 (19.61 ± 9.69) and Tc17 cells (17.14 ± 1.03) were increased in N3 patients in comparison to the N0 group (7.12 ± 3.15 , $p=0.002$ and 5.68 ± 2.92 , $p=0.003$; respectively).

Table 3. The mean percentage of CD8+ T lymphocytes, their effector subsets and MFI of their cognate cytokines in TDLNs of LN+ and LN- patients and patients in different stages.

	LN- patients	LN+ patients	Stage I	Stage II	Stage III
Cell subset	Mean \pm SD				
T cells	55.47 \pm 10.46	58.30 \pm 12.24	59.53 \pm 14.21	55.25 \pm 9.53	56.13 \pm 11.70
CTLs	7.49 \pm 3.60	9.33 \pm 3.76	7.26 \pm 5.09	8.25 \pm 3.40	8.47 \pm 3.14
Tc1 cells	24.22 \pm 13.82	21.81 \pm 10.04	20.09 \pm 9.79	25.06 \pm 14.77	24.87 \pm 10.24
Tc2 cells	7.12 \pm 3.15	10.53 \pm 9.38	7.82 \pm 3.76	6.38 \pm 2.82	15.74 \pm 11.07
Tc17 cells	5.68 \pm 2.92	9.35 \pm 8.70	5.91 \pm 2.85	5.37 \pm 2.93	13.86 \pm 10.70
Cytokine expression in CD8+ lymphocytes (based on MFI)					
IFN- γ	48.32 \pm 23.48	38.2 \pm 32.70	38.03 \pm 15.56	54.01 \pm 29.59	27.06 \pm 14.40
IL-4	9.51 \pm 2.08	9.17 \pm 2.95	9.97 \pm 2.26	9.43 \pm 2.14	8.13 \pm 3.09
IL-17	4.96 \pm 1.11	7.17 \pm 4.84	5.26 \pm 1.09	5.34 \pm 2.78	6.00 \pm 4.77

As depicted in Figure 2, repeating the analysis separately in breast cancer patients with IDC tumor type indicated that the mean expression of IFN- γ is significantly decreased in LN+ patients in comparison to LN- ones (36.89 \pm 13.26 and 46.48 \pm 5.27, respectively, $p=0.04$; Figure 2B), and also in N3 patients in comparison to N0 patients (20.28 \pm 5.93 and 46.48 \pm 21.72, $p=0.019$, respectively, Figure 2D). Notably, the nodes of all six patients with medullary carcinoma (MC) were free of tumors.

Comparison of CD8+ T cell Subpopulations in Different Stages. Similar to the variations observed in LN+ patients, with the increase of tumor stage from I to III, the percentages of Tc2 and Tc17 cells also tend to increase; whereas the frequency of Tc1 lymphocytes is decreased (Table 3). As shown in Figure 3, these variations were only significant in patients with IDC tumor type. Detailed analysis of this group of the patients revealed that the percentage of Tc2 (19.61 \pm 9.69 vs. 7.82 \pm 3.76, $p=0.018$) and Tc17 (17.14 \pm 10.35 vs. 5.91 \pm 2.85, $p=0.009$) were significantly higher in patients with stage III in comparison to stage I. These differences were higher between stages II and III ($p=0.004$ for both Tc2 and Tc17). On the other hand, the level of IFN- γ expression observed to be decreased in patients with stage III (5.93 \pm 3.42) in comparison to ones in stage II (53.77 \pm 30.50, $p=0.018$, Figure 3B). It should be noted that all of the patients with MC tumor type were in stage II and the comparison was not possible in this group of the patients.

Association between the Frequency of Cytotoxic T Cell Effector Subsets and Other Clinical and Pathological Characteristics of the Patients. The percentage of each cell type was compared between the two types of investigated LNs (sentinel vs. non-sentinel), and between patients with different clinical and pathological characteristics, including tumor size and type, histological grade, pre-tumoral invasion, and hormone (ER and PR) and growth factor (Her2) receptors expression. Same analysis was also separately performed in the IDC subgroup.

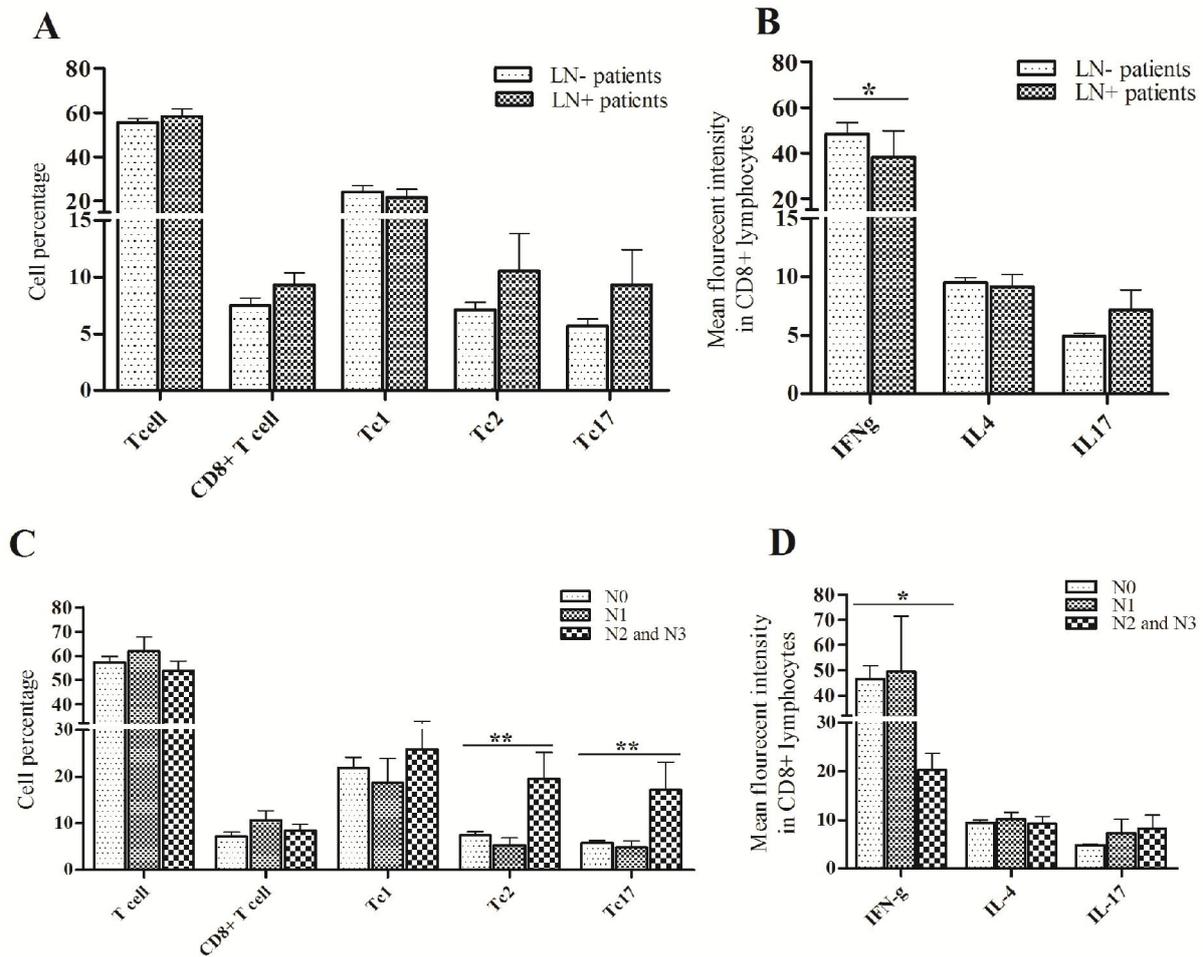


Figure 2. The percentages of different CD8+ lymphocyte subsets and the MFI of their cytokine expression in TDLNs of IDC patients with different node status.

Data are shown as mean \pm SEM. TDLN: tumor draining lymph node, Tc: T cytotoxic, LN+: lymph node positive, LN-: lymph node negative, IDC: infiltrative ductal carcinoma, MFI: mean fluorescent intensity.

*Differences are significant at $p < 0.05$ (2-tailed), ** Differences are significant at $p < 0.01$ (2-tailed)

The results indicated a negative correlation between the frequency of T lymphocytes (CD3+ cells) and the size of tumor ($R=-0.037$, $p=0.02$). IL-4 expression in CD8+ lymphocytes was increased in TDLNs of patients with moderate differentiation (tumor grade 2) compared with ones with poor differentiation (tumor grade 3) ($p=0.019$). In addition, IL-17 expression per Tc17 cells (based on MFI) was significantly increased in ER positive patients ($p=0.04$). No significant association was observed between CD8+ subpopulation and their effector subsets with other clinical parameters. Similar results were obtained in IDC patients.

Correlations between the Percentages of Different CD8+ Lymphocyte Subsets. We then sought to determine whether there is any correlation between the prevalence of different CD8+ effector subtypes in TDLNs of patients with breast cancer. Notably, using non-parametric correlation, it was demonstrated that there was a strong positive correlation between the percentage of Tc2 and Tc17 effector subsets ($R=0.723$, $p<0.001$). There was similar correlation in IDC patients, whereas no relationship was observed between different cell subsets in patients with MC.

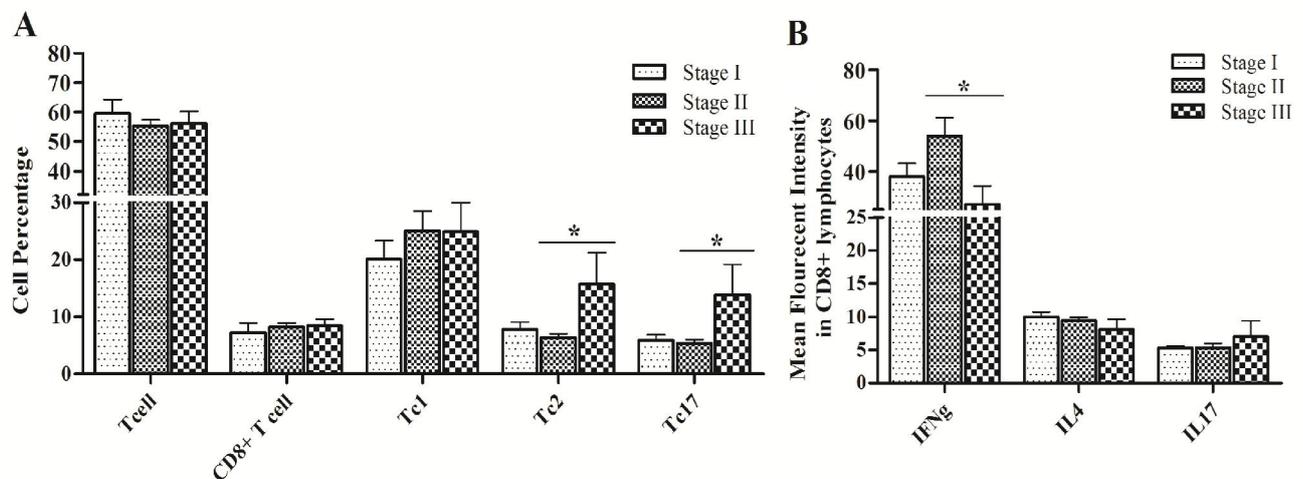


Figure 3. The percentages of different CD8+ lymphocyte subsets and the MFI of their cytokine expression in TDLNs of IDC patients in different stages. Data are shown as mean \pm SEM. TDLN: tumor draining lymph node, Tc: T cytotoxic, IDC: infiltrative ductal carcinoma, MFI: geometric mean fluorescent intensity.

*Difference is significant at $p<0.05$ level (2-tailed), ** Difference is significant at $p<0.01$ level (2-tailed).

DISCUSSION

The role of CD8+ cytotoxic T cells in anti-tumor immunity is well established. These cells are considered as one of the most important infiltrating cells into many types of tumor tissues including breast carcinoma. Although many studies demonstrated CD8+ T cells as the most abundant phenotype in breast tumor microenvironment (14-19), the predictive importance of these cells in BC is still controversial. Some reports indicated

that higher levels of infiltrating CD8⁺ T cells are associated with better survival of BC patients (20), whereas others inconsistently showed no association with prognosis or even association with poor prognosis (16,22). This discrepancy in the results may be explained by differences in methods (immunohistochemical versus flow cytometric analysis), as well as the clinico-pathological status of the investigated subjects. In fact, breast carcinoma demonstrates considerable heterogeneity at the molecular pathology level (23). Some recent studies have only shown an association of CD8 expression in the tumor tissue with a favorable outcome in basal like subgroup of BC patients (17,24). The discrepancies in results regarding the CD8⁺ T cells in tumors may also arise from heterogeneity in CD8⁺ lymphocyte population. In fact, CD8⁺ CTLs are divided into several subsets based on cytokine expression pattern, similar to their CD4⁺ Th counterparts. Each subset may accordingly render different effector functions and consequently may have different effects on the patients' outcome. None of the previously published investigations dealt with the subset determination. In the present study, we aimed to investigate not only CD8⁺ T lymphocyte population in the TDLNs (the main site of anti-tumor response), but also to determine the cell subsets and their association with patients' clinico-pathological characteristics, including LN involvement, tumor stage, etc.

Our analysis indicated an increase in the prevalence of Tc17 (IL-17+CD8⁺) and Tc2 (IL-4+CD8⁺) lymphocytes in TDLNs of BC patients in advanced stages. These findings, accompanied by the elevation of the mentioned subsets in patients with higher number of positive nodes (N3), may suggest a negative role for Tc2 and Tc17 cells in the progression of breast cancer. We also observed a strong positive correlation between the percentages of these two CD8⁺ effector subtypes in TDLNs, suggesting concordance mechanisms in their elevation.

Despite recent advances in understanding the differentiation of Th17 cells in human cancer, little is known about phenotype, function and the mechanisms underlying the regulation of Tc17 in human tumors. Some recent studies have shown that both CD4⁺ and CD8⁺ IL-17-producing T cells are present in the tumor microenvironment of various cancers (25-27). Consistently, we observed an increase in the percentages of Tc17 effector subsets in TDLNs of BC patients along with tumor progression. These findings are supported by the results of Nam *et al.* who reported an increase in IL-17 mRNA following the presence of the tumor cells in the regional LNs of mammary carcinoma. This increase was specifically observed in CD8⁺ cells but not in CD4⁺ lymphocytes (27).

Zheng *et al.* demonstrated that highly enriched IL-17-producing cells in human hepatocellular carcinoma are positively correlated with microvessel density as well as poor survival of the patients. The majority of these cells were reported to be IL-17-producing CD8⁺ lymphocytes (Tc17 cells) (28,29). The cells were also indicated to have reduced levels of granzyme B and perforin, which is consistent with the disability of Tc17 cells to exert a cytotoxic effect on target cells *in vitro* (28,29). It has also been shown that in hepatocellular carcinoma and stomach cancer, Tc17 cells indirectly inhibit anti-tumor immune responses through recruiting the myeloid derived suppressor (MDSC) cells. MDSC cells in turn inhibit IFN- γ production and prevent replication of CD8⁺ cytotoxic cells (30). There are, however, several adoptive transfer studies indicating that the cells with Tc17 phenotype are able to switch to Tc1 phenotype (11), and recruit neutrophils (31) and consequently participate in cancer abolition. Differences in the origin of Tc17 cells in these studies (intrinsic versus adoptive

transfer) in conjunction with differences in the type of tumor may explain contradictions observed in these studies.

We also found a reduction in IFN- γ expression by Tc1 effector subsets in LN+ patients in comparison to LN- ones (based on the MFI of IFN- γ intensities in this subset). This observation may suggest a decrease in the activity of Tc1 subset along with tumor progression. This finding seems to be consistent with other studies based on adoptive transfer technology, indicating the anti-tumorigenic activity of Tc1 cells in different types of cancer including breast carcinoma (12,32). It has been reported that the effector function of Tc1 cells in cancer is crucially dependent on IFN- γ production (33). These observations in concordance with our findings support the hypothesis that Tc1 cells exert an anti-tumor role in BC patients especially through IFN- γ production.

To our knowledge, there is only one study that investigated Tc1 and Tc2 subsets in LNs of BC patients (34). The results of this study indicated no significant difference in the frequency of Tc1 cells between SLNs and non-SLNs and also among patients in different clinico-pathological stages, as we have also observed in the current study. They concluded that the Tc1 to Tc2 ratio is stable in different clinical stages and during tumor progression. Contrary to their latter finding, we have observed an increase in the percentage of Tc2 effector subset in advanced stages, whereas Tc1 activity (based on IFN- γ production) was lowered accompanying tumor cell metastasis to LNs and also in advanced stages. Moreover, in their study in mice, Reome et al. showed that aside from early IL-4 production by tumor infiltrated Th2 lymphocytes; Tc2 effector subpopulation is also localized in the tumor site and produces elevated levels of both IL-4 and IL-10 along with tumor progression (35). These cytokine-releasing profile and kinetics seem to be in concordance with what we observed regarding Tc2 subtype in the current study. Despite of this, it should not be ignored that there are some adoptive transfer studies showing that Tc2 cells possess cytotoxic and inhibitory properties against tumor cells (36,37). Further studies are required to explain the inconsistency between different investigations.

To best of our knowledge this is the first study that simultaneously investigates various effector subtypes of CD8+ lymphocytes especially Tc17 in TDLNs of patients with BC. Taken together, our data suggest a positive association between Tc2 and Tc17 percentages in TDLNs and breast cancer progression. Down-regulation of IFN- γ by Tc1 cells seems to be associated with tumor metastasis to TDLNs. Results of the present study collectively provides new insights into the TDLN composition in terms of CD8+ T cell subsets, and supports the suggestion that similar to CD4+ subset, probably under certain conditions, CD8+ lymphocytes may promote tumor progression. These findings may have implications in cancer immunotherapy based on CD8+ effector subsets.

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