CD8+ T Lymphocyte Subsets in Bladder Tumor Draining Lymph Nodes

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ABSTRACT

Background: Cytotoxic CD8+ T cells, as essential parts of the adaptive immune system, play pivotal roles in anti-tumor immune responses. It is well documented that cytokine expression profiles and activation status of these cells during anti-tumor immune responses affect the outcome of host-tumor interaction. Objective: To investigate the percentages of CD8+ lymphocytes and their subsets in tumor draining lymph nodes of patients with bladder cancer. **Methods:** Forty-five patients with bladder cancer, candidate for radical cystectomy, were recruited. Mononuclear cells were isolated from draining lymph nodes using Ficoll-Hypaque gradient centrifugation, and were activated by PMA/Ionomycin in the presence of Golgi inhibitors. The cells were then permeabilized and stained with appropriate flourochrome conjugated antibodies against CD3, CD8, IFN-γ, IL-17 and IL-4 molecules. Data were collected on a four-color flow cytometer and analyzed by CellQuestPro software. **Results:** Despite no difference in the frequency of IL-17 producing CD8+ (Tc17) lymphocytes, the mean expression of IL-17 in this subset was significantly elevated in high-grade patients (p=0.011). The percentage of double positive IFN-γ/IL-17 CD8+ lymphocytes was also significantly increased in node positive patients compared to node negative ones (p=0.046). Our results also demonstrated that the percentage of IFN-γ producing CD8+ (Tc1) lymphocytes was significantly increased in the patients with higher histological grade compared to those with lower ones (p=0.038). **Conclusion:** IFN-γ and IL-17 producing CD8+ T cells may increase in advanced stages of bladder cancer, but their correlation with tumor prognosis remains to be investigated.

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Keywords: Bladder Cancer, Lymph Node, CD8+ lymphocytes, Tc1, Tc2, Tc17

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INTRODUCTION

CD8+ cytotoxic T lymphocytes play a pivotal role in adaptive immune responses to malignancies (1). Analogous to CD4+ helper lymphocytes, CD8+ T cells are heterogeneous with respect to effector functions, transcription factors and cytokine profile; accordingly these cells are classified to different effector subsets (2). It is well documented that cytokine expression profiles and activation status of these cells during anti-tumor immune responses, could determine the outcome of host-tumor interaction. Tc1 lymphocytes are the activated functional CD8+ lymphocytes, which produce interferon (IFN)-γ and TNF-α and efficiently kill their targets by perforin or Fas mediated pathways. By contrast, Tc2 cells secret interleukin (IL)-4, IL-5, IL-13 and IL-10 and seem to be less cytotoxic than Tc1 cells (2). A subset of IL-17 producing cells, termed Tc17, has been recently introduced as a new subset of effector CD8+ T lymphocytes, which besides IL-17A, produces IL-17F, IL-21 and IL-22 cytokines. These cells; in common with Th17 cells, show the expression of RORγt, CCR6 and IL23R (2,3). Recent investigations demonstrated a role for Tc17 cells in the pathogenesis of several diseases including autoimmune disorders and cancers (4-7). A protective role against tumor development has also been reported for this subset (8).
In the present study, we aimed to evaluate the percentages of CD8+ lymphocytes and their subpopulations including Tc1, Tc2 and Tc17 in the Tumor Draining Lymph Nodes (TDLNs) of patients with Bladder Cancer(BLC). The comparison was also made between patients with different clinical and pathological characteristics.

MATERIALS AND METHODS

Subjects. During 2015-2016, forty-five untreated patients approved to suffer from bladder cancer, candidate for radical cystectomy, were recruited. None of the patients received any chemotherapy, radiotherapy and/or immunotherapy. All patients were admitted to the public hospitals affiliated to Shiraz University of Medical sciences (Shiraz, Iran). Lymph node samples were collected during surgery, as a part of their treatment planning, and were referred to cancer immunology laboratory for further investigation. The study was approved by the Ethics Committee at Shiraz University of Medical Sciences. Sample collection was made after obtaining informed consent. Clinical and pathological characteristics of the patients including age, sex as well as tumor type, tumor grade and stage, lymphovascular invasion, perineural invasion, fat invasion, perivesical fat invasion, and carcinoma in situ were obtained from patients' files. The stage of tumor was determined according to World Health Organization (WHO) classification of tumors, 2004 (9).
Preparation of Mononuclear Cells. Mononuclear cells were isolated from lymph nodes as previously described (10). Briefly, fresh fragments of lymph nodes were obtained and sliced into small pieces in culture medium (Biosera, UK) and filtered through a 40 μm cell strainer (BD Biosciences, US). Mononuclear cells were then separated using Ficoll-Hypaque density centrifugation (Biosera, UK). The number of viable cells was determined by using a hemocytometer and applying trypan blue vital staining procedure (Biosera, UK).

Flowcytometric Analysis. Isolated mononuclear cells were divided into two separate tubes. The first tube was surface-stained by APC-conjugated anti-CD3 and PerCP-conjugated anti-CD8 antibodies (both from BD Biosciences, USA) in order to evaluate the percentage of total CD3+CD8+ cytotoxic T cells. To assessthe cytokine expression pattern, mononuclear cells, were activated in the second tube for 5 hours in culture medium containing 50 ng/ml PhorbolMyristate Acetate (PMA) and 1 µg/ml Ionomycin (Both from Sigma-Aldrich, Germany) in the presence of 0.7 µl BrefeldinA and Monensin (Both from BD Biosciences) as cytokine secretion blockers. Activated cells were then washed and fixed using 1% paraformaldehyde, and permeabilized by usingBD Perm/Wash™buffer (BD Biosciences, USA) according to the manufacturer's instruction. The permeabilized cells were then intracellularly stained with PerCP-conjugated anti-CD8, FITC–conjugated anti-IFN-γ, PE-conjugated anti-IL-4, and AlexaFluor® 647–conjugated anti-IL-17a antibodies (all from BD Biosciences, USA). Corresponding isotype control antibodies were also used to stain the cells in a separate tube in order to exclude background staining.Acquisition of the stained samples were finally performed on a four-color FACSCaliburflowcytometer (BD Biosciences, USA),and the collected raw data were analyzed by using CellQuestPro software package (BD Biosciences, USA).

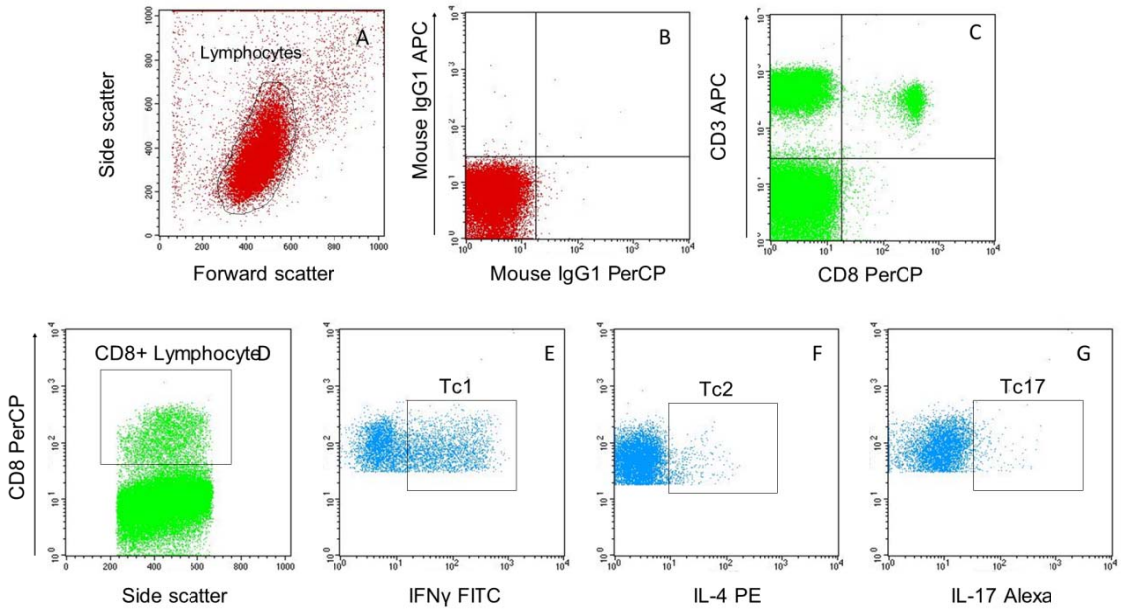

Figure 1. Flowcytometric plots of CD8+ cells effector subsets. Total frequency of CD8+ T cells (C) was determined in lymphocyte gate (A). The percentage of different CD8+ effector subpopulations, IFN-γ, IL-4, and IL-17 CD8+ expressing cells, demonstrating Tc1 (E), Tc2 (F), and Tc17 (G) cells, respectively, were assessed in CD8+ lymphocytes population (D). Tc1: T cytotoxic type 1, Tc2: T cytotoxic type 2, Tc17: T cytotoxic type 17

Phenotype Determination. The percentage of CD3+CD8+ lymphocytes subsets was evaluated in lymphocyte gate. As illustrated in Figure 1, IFN-γ, IL-4, and IL-17 CD8+ expressing cells were considered as Tc1, Tc2, and Tc17 cells, respectively. The percentages of different CD8+ effector subpopulations were reported in CD8+ lymphocyte gate. Geometric Mean Fluorescent Intensity (MFI) of the IFN-γ, IL-4, and IL-17 normalized by the MFI of negative cells was considered as a criterion for cytokine expression level per cell.

Statistical Analysis. Data assembly and analysis were done by using SPSS 16 software package (SPSS GmbH Software, Germany). The frequency of the cells and their cognate cytokines was recorded as mean ± SEM. Alterations in the percentages of lymphocyte subpopulations among patients with different clinical and pathological characteristics were assessed using non-parametric Mann–Whitney U and Kruskal–Wallis tests. Non-parametric Spearman correlation rank test was used to determine any correlation between the percentages of various investigated subsets. P-values less than 0.05 were considered statistically significant. For drawing the statistical graphs, GraphPad Prism5 software package was used (Inc.; San Diego CA, USA, 2003).

RESULTS

Clinical and pathological characteristics of the patients. Forty-five patients with bladder cancer with the mean age of 65.4 ± 11.74 years were recruited. According to pathological records, most patients had Transitional Cell Carcinoma (TCC, 93.33%) as their tumor type. Thirty-eight patients had tumors with high histological grade (84.44%) and 16 patients (35.56%) were in stage II. The main clinico-pathological characteristics of the patients are summarized in Table 1.

CD8+ lymphocyte Subsets in TDLNs of BLC Patients with Involved and Free Lymph Nodes as well as Different Cancer Stages. Table 2 illustrates the mean frequency of CD8+ T lymphocytes and their effector subsets in bladder cancer patients. As indicated, total frequency of CD8+ lymphocytes in draining lymph nodes of BLC patients was 7.68 ± 0.57. Comparing LN+ patients (with at least one involved lymph node) and LN- patients, revealed no significant difference in the frequency of CD8+ lymphocytes and their functional subsets – Tc1, Tc2 and Tc17 cells. However, the percentage of IFN-γ/IL-17 double positive CD8+ lymphocytes was significantly higher in LN+ patients (p=0.046, Figure 2). We then divided patients into N0, N1, N2, and N3 groups, based on TNM staging system, the numbers of involved regional lymph node as well as the site of involved lymph node (s) (Table 1). Accordingly, the MFI of IL-17 expression by IFN-γ/IL-17 double positive CD8+ lymphocytes was observed to be significantly higher in N2 patients (patients with more than one involved regional lymph node in their pelvis) than N0 group (p=0.004). The percentage of CD8+ lymphocytes subpopulations, including Tc1, Tc2 and Tc17, as well as the mean expression of their cognate cytokines was not significantly different between patients with other clinico-pathological features including cancer stage, though a trend to decrease was observed in CD8+ T cells as tumor progressed from stage I to stage IV (p=0.05).

Table 1. The clinical and pathological characteristics of patients with bladder cancer.

Characteristics	Value (%)
Age (years)	65.4±11.74
Gender	
Male	37 (82.20)
Female	8 (17.80)
Tumor type	
TCC (Transitional Cell Carcinoma)	42 (93.33)
SCC (Squamous Cell Carcinoma)	3 (6.67)
Histological grade	
Low	7 (15.56)
High	38(84.44)
Stage	
1	5 (11.11)
2	16 (35.56)
3	11 (24.44)
4	13 (28.89)
Lymph node status*	
Free (N0)	33 (73.34)
Involved	12 (26.66)
N1	2 (4.44)
N2	9 (20.00)
N3	1 (2.22)
Lymphovascular invasion	
Positive	15 (33.30)
Negative	30 (66.70)
Perineural invasion	
Positive	21 (46.70)
Negative	24 (53.30)
Muscular invasion	
Positive	39 (86.70)
Negative	6 (13.30)
Perivesical fat invasion	
Positive	11(24.45)
Negative	34(75.55)
Urothelial CIS	
Positive	13 (28.90)
Negative	30 (66.70)
Missing	2 (4.40)

*N0: Cancer has not spread to the regional lymph nodes.
N1: Cancer has spread to a single regional lymph node in the pelvis.
N2: Cancer has spread to more than one regional lymph node in the pelvis.
N3: Cancer has spread to the common iliac lymph nodes, which are located behind the major arteries in the pelvis, above the bladder.

Table 2. The mean percentage of T cells, CD8+ T lymphocytes, their effector subsets and the Mean fluorescent intensity (MFI) of their cognate cytokines in TDLNs of the patients with bladder cancer.

Cell Subset	Markers	Min	Max	Mean ± SEM
CD8+ T cells	CD3+CD8+ lymphocytes	3.26	17.29	7.68 ± 0.57
Tc1 cells	CD8+IFN-γ+ lymphocytes	2.27	59.27	24.03 ± 2.1
Tc2 cells	CD8+IL-4+ lymphocytes	1.32	31.15	10.82 ± 1.17
Tc17 cells	CD8+IL-17+ lymphocytes	1.23	39.8	8.36 ± 1.07
Tc1/Tc2 ratio		0.74	24.55	3.96 ± 0.82
Tc1/Tc17 ratio		0.80	23.44	4.70 ± 0.78
Tc2/Tc17 ratio		0.15	16.58	2.00 ± 0.43
	Cytokine expression (MFI)			
IFN-γ	IFN-γ in CD8+ lymphocytes	1.66	34.66	9.84 ± 0.99
IL-4	IL-4 in CD8+ lymphocytes	0.65	7.35	4.57 ± 0.21
IL-17	IL-17 in CD8+ lymphocytes	3.11	10.55	5.38 ± 0.23

***** TDLNs: Tumor Draining Lymph Nodes

CD8+ lymphocytes Subsets in Patients with Different Histological Grades. The percentage of IFN-γ producing CD8+ lymphocytes (Tc1 subset) was observed to be significantly higher in the patients with high histological grade in comparison to those with lower histological grade (p=0.038, Figure 3).

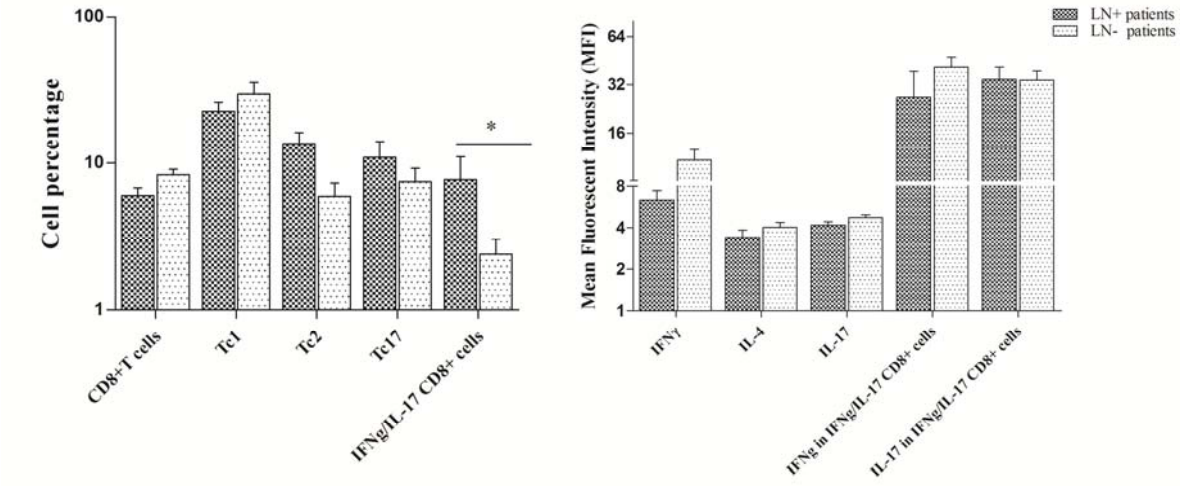

Figure 2. The percentage of different CD8+ lymphocyte subpopulations and the mean expression of their cognate cytokine in TDLNs of bladder cancer patients with different node status.

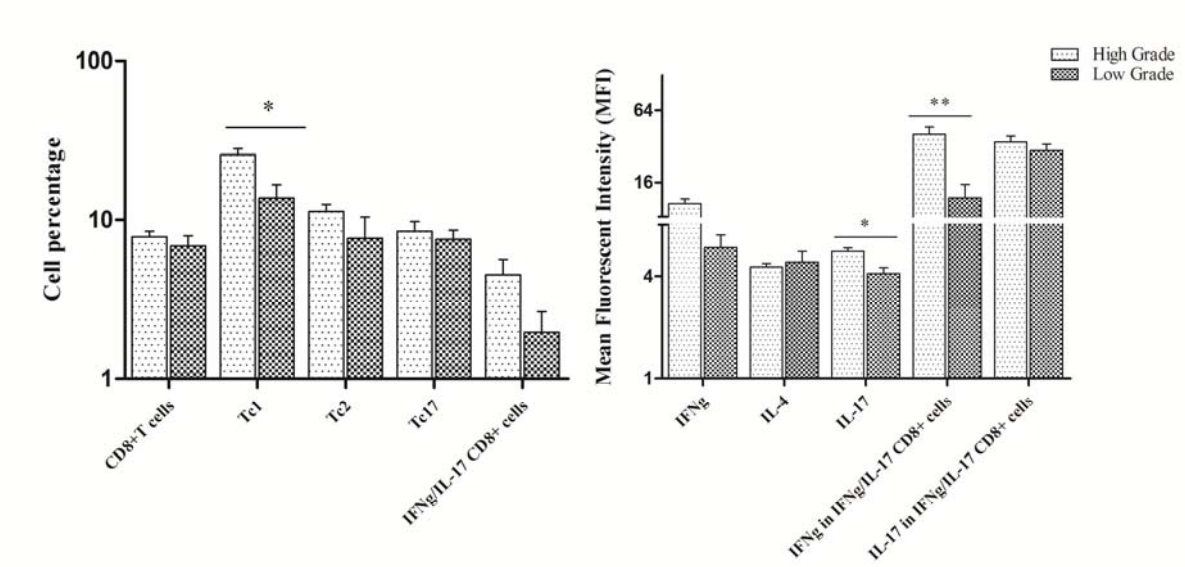

Figure 3. The percentage of different CD8+ lymphocyte subpopulations and the mean expression of theircognate cytokine in Tumor Draining Lymph Nodes of bladder cancer patients in different histological grades.

Besides, the ratio of Tc1/Tc17 (p=0.02), the MFI of IFN-γ expression in IFN-γ/IL-17 double positive CD8+ lymphocytes (p=0.008), as well as the MFI of IL-17 expression in Tc17 cells (p=0.011), were also significantly increased in the patients with higher histological grade.

Association of Cytotoxic T Cell Subsets with Other Clinico-Pathological Characteristics. In addition to LN involvement, tumor stage and tumor grade, association study was also performed between the percentage of CD8+ cell subsets and other clinical and pathological characteristics of the patients including age, sex as well as tumor type, tumor grade and stage, lymphovascular invasion, perineural invasion, fat invasion, perivesical fat invasion, carcinoma in situ. Results demonstrated that in the patients positive for perineural invasion, the percentage of CD8+ cytotoxic T cells was significantly lower than negative ones (p=0.018). Moreover, the percentage of Tc17, IFN-γ/IL-17 double positive CD8+ lymphocytes, as well as the MFI of IL-17 expression in these lymphocytes were observed to be significantly increased in the patients with fat invasion (p=0.049, p=0.024, p=0.037, respectively). In addition, Tc1/Tc2 ratio was significantly higher in the patients with invasion to lamina propria (p=0.031). On the other hand, Tc1/Tc2 and Tc1/Tc17 ratios were significantly decreased in the patients positive for lymphovascular invasion (p=0.007, p=0.019, respectively). No further association was observed between CD8+ subsets and other clinicopathological characteristics.

Correlations AmongDifferent CD8+ Lymphocyte Subsets. Non-parametric Spearman correlation rank test was used to determine whether there is any correlation between the percentage of CD8+ T cells or their effector subpopulations in TDLNs of patients with bladder cancer. Analysis showed a positive strong correlation between the percentage of Tc2 and Tc17 effector subsets (R=0.519, p=0.001). Furthermore, the percentage of IFN-γ/IL-17 double positive CD8+ lymphocytes was strongly correlated with Tc2 and Tc17 cells (R=0.579, p=0.001 and R=0.865, p=0.001, respectively). Whilst, the percentage of CD8+ T lymphocytes had a negative correlation with Tc1 and Tc17 cells (R=-0.393, p=0.015 and R=-0.399, p=0.013, respectively). There was also a direct correlation between the mean expression of IFN-γ and IL-4 in CD8+ lymphocytes (R=0.376, p=0.015).

DISCUSSION

CD8+ cytotoxic T cells are considered as one of the main crucial players in antitumor immune responses (1). Infiltration of these cells to tumor site has already been reported in different types of tumors; and in most cases, was correlated with good prognosis (11, 12). In the present study, we determined the frequency of CD8+ T lymphocytes and their effector subpopulations in draining lymph nodes of bladder cancer patients, as the main site of eliciting anti-tumor immune response. We also investigated their association with the major clinico-pathological characteristics of the disease.

Our analysis revealed that 3 to 17 percent of lymphocyte population in TDLNs of patients with BLC was CD8+ T cells. Despite no difference in the frequency of total CD8+ T lymphocytes, as well as Tc2 and Tc17 subsets, the percentage of IFN-γ producing CD8+ (Tc1) lymphocytes was significantly elevated in the patients with higher histological grade compared to patients with lower histological grade. Additionally, the mean expression of IL-17 in Tc17 subset (based on the MFI of IL-17 expression) was also observed to be significantly higher in these patients. The percentage of double positive IFN-γ/IL-17 CD8+ lymphocytes was significantly increased in node positive patients comparing to node negative ones. High levels of infiltrating CD8+ cells within the tumor tissue or in peripheral blood has already been indicated to be correlated with better disease-free and overall survival of advanced BLC patients comparing to the patients with fewer frequency of CD8+ lymphocytes but in the similar stage (13-15). Consistently, our results indicated that as tumor progressed from stage I to stage IV, total frequency of CD8+ T cells tended to decrease.

Besides Tc1 and Tc2 CD8+ lymphocytes, IL-17 secreting CD8+ T (Tc17) lymphocytes are considered as a distinct subset, which produce low level granzyme B and perforin and exhibits no strong cytotoxicity (2,16). Some recent studies showed the presence of IL-17 producing CD8+ T cells in tumor microenvironment and draining lymph nodes of various types of cancer (4,17,18). Despite no difference in the frequency of Tc17 lymphocytes, in the present study we found that the mean expression of IL-17 in Tc17 cells to be significantly elevated in high-grade BLC patients. Recent investigation on TDLNs of patients with breast cancer by our group also indicated an increase in the percentages of Tc17 cells along with Tc2 lymphocytes in LN+ patients; the finding which observed to be associated with tumor progression (4). Consistently, a positive correlation between Tc2 and Tc17 percentages in TDLNs of patients with BLC was also observed in the present study. Zhang *et al.* have already reported higher proportion of

Tc17 cells in both peripheral blood and tumor tissues of the patients with Uterine Cervical Cancer (UCC). This increase was also indicated to be associated with pelvic LN metastases and increased microvessel density in UCC patients. A positive correlation between Tc17 cells and Foxp3 expressing T cells infiltrated to UCC tissues was also reported in this study (19). Correlation of enriched IL-17 producing cells, especially CD8+ ones with microvessel density as well as poor survival was also observed in hepatocellular carcinoma (20). Although in some cancers such as thyroid carcinoma, lower proportion of Tc17 cells was detected in the peripheral blood of patients, and it was negatively correlated with the tumor size (21), these observations collectively suggest the increase in the numbers and/or activity of Tc17 cells along with tumor progression. Increase in the numbers and/or activity of Tc17 cells may in turn promote tumor progression through fostering angiogenesis or supporting inhibitory milieu in tumor microenvironment. Tc17 cells are indicated to have the ability to inhibit, indirectly, anti-tumor immune responses in stomach cancer by recruiting the myeloid derived suppressor (MDSC) cells, which in turn inhibit IFN-γ production and replication of CD8+ cytotoxic cells (22,23).

IL-17 producing cells, including Tc17 lymphocytes, have been recently reported to be able to convert into IFN-γ producing lymphocytes, e. g. Tc1-like cells, in inflammatory microenvironment (8,24). We found, in the present study, the percentage of double positive IFN-γ/IL-17 producing CD8+ lymphocytes to be significantly increased in node positive patients compared to node negative patients. Information regarding double positive IFN-γ/IL-17 producing CD8+ lymphocytes is scarce. Weather these cells are transient cells as the consequence of Th17 conversion to Tc1-like lymphocytes, or they are an independent subset with distinct effector function, remains to be elucidated. Some studies suggest that IFN-γ/IL-17 producing CD8+ lymphocytes lack cytotoxic function (3). Our findings might suggest a negative role for these cells in anti-tumor immune responses, through providing suitable inflammatory condition for tumor cells to infiltrate into lymph nodes. Consistently, mean expression of IL-17 cytokine in these double positive cells was observed to be significantly increased in the patients with higher numbers of involved nodes (N2 group). Furthermore, we also found that the percentage of these cells was strongly correlated with the frequency of Tc2 and Tc17 cells. Beside concordant mechanisms in their differentiation and elevation, this observation might suggest a promoting role for IL-17/IFN-γ producing cells in BLC tumor progression as previous studies also indicated a negative role for Tc2 and Tc17 cells in some other cancers (4,19,20,25). Despite this, results of several adoptive transfer studies indicated, that through switching to Tc1 phenotype, Tc17 cells may promote neutrophil recruitment and consequently suppress tumor progression (26,27). Furthermore, IL-17 producing cells which simultaneously express IFN-γ were found in elevated numbers in both human- and mouse inflammatory tissues and/or blood from patients with chronic inflammatory disorders (24, 28) suggesting an immune augmenting role for these lymphocytes. The origin and the exact role of double positive IFN-γ/IL-17 producing CD8+ lymphocytes in bladder cancer merits more investigations.

Our results also demonstrated that the percentage of IFN-γ producing CD8+ (Tc1) lymphocytes was significantly higher in patients with high histological grade comparing to those with low grade. Although the number of individuals with low grade tumor was low (only 7 patients), this finding seems to be contrary to other previous studies in melanoma; demonstrating the anti-tumorigenic activity of Tc1 cells in these cancers

(29). It is also contrary to our observation in salivary gland tumors in which we indicated that mean percentages of Tc1 cells and the ratios of Tc1/Tc2 lymphocytes were significantly lower in the patients with malignant tumors (30). The role of IFN-γ producing cells in anti-tumor immune responses is well-documented. IFN-γ could directly inhibit tumor cell growth, or indirectly influence the development, recruitment, and/or activation of immune cells (31). The pro-tumor effects of IFN-γ, from the other side, have also been reported in some clinical settings. It is suggested that IFN-γ induces proliferative and anti-apoptotic signals, and/or promote tumor escape from recognition and cytolysis by CTLs and NK cells (31). Under which conditions and through which mechanisms IFN-γ play its role as a tumorigen or anti-tumorigen, probably depends on the contexts of tumor especially tumor type and specificity, microenvironmental factors, as well as types of receptor and the intensity of signaling. Finally, comprehensive interpretation of IFN-γ-related antitumor immunity requires complete information regarding both suppressive and activating immune mechanisms and the balance between theses effector elements. Beside the dual role of IFN-γ, the exact role of Tc1 in anti-cancer scenario also remains to be elucidated.

To best of our knowledge, current study is the first to investigate, simultaneously, various effector subtypes of CD8+ lymphocytes in TDLNs of patients with BLC. As mentioned in the manuscript we compared LN+ patients (patients with at least one involved lymph node) with LN- patients (patients with no involved lymph node), and no data was available regarding involvement of investigated lymph nodes. Despite of this, our data collectively suggest a positive association between IL-17/IFN-γ producing CD8+ T cells in TDLNs and bladder cancer progression. Up-regulation of IL-17 by Tc17 cells seems to be associated with tumor metastasis to TDLNs. Results of this study provides new insights into bladder cancer TDLN organization in terms of CD8+ T cell subsets, and may have implications in cancer immunotherapy based on CD8+ effector subsets.

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