

# IJMCM



International Journal of Molecular and Cellular Medicine p-ISSN: 2251-9637 e-ISSN: 2251-9645

# Diversity of Memory CD8<sup>+</sup> T Cells in Tumor-Draining Lymph Nodes from Patients with Bladder Cancer

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Article type:	ABSTRACT
Original	The role of memory T cells in orchestrating memory responses to previously known tumor antigens is well
Article	documented. The aim of this study was to assess the frequency of different memory T cell subsets in tumor-
	draining lymph nodes of patients with bladder cancer (BC) and their prognostic significance. Mononuclear
	cells were isolated from 50 tumor-draining lymph nodes of untreated patients with BC and stained with
	antibodies against the markers CD8, CD95, CD45RO and CCR7. Data were collected using the FACSCalibur
	flow cytometer and analyzed using FlowJo software. Among the $CD8^{\scriptscriptstyle +}$ cytotoxic lymphocytes, the frequency
	$of\ different\ subsets\ was\ determined\ including\ total\ memory\ cells\ (CD8^+CD45RO^+CD95^+),\ T\ central\ memory\ cells\ (CD8^+CD45RO^+CD95^+),\ T\ centr$
	$(TCM:CD8^+CCR7^+CD45RO^+CD95^+), T\ effector\ memory\ (TEM:CD8^+CCR7^-CD45RO^+CD95^+), T\ stem\ cell\ and the constraints of $
	$memory\ (TSCM:\ CD8^+CCR7^+CD45RO^-CD95^+)\ and\ na\"{i}ve\ T\ cells\ (CD8^+CCR7^+CD45RO^-CD95^-).\ The$
	analysis revealed that on average $49.32\pm20.15$ (between $1.62\%$ and $87.20\%$ ) percent of CD8+ lymphocytes in
	draining lymph nodes of BC had a memory phenotype. TCM cells showed the highest frequency
	$(34.71\pm17.04)$ , while TSCM cells $(7.51\pm8.53)$ demonstrated the lowest. The total frequency of memory cells
	tended to be higher in patients with tumor invasion to muscle layer ( $P=0.052$ ) and stage III ( $P=0.042$ ) than in
	patients without invasion and stage I. The TCM subset was more frequent in patients with necrotic tumors
	than in patients without necrosis (P=0.048). TSCM significantly increased in patients with N2 compared to
	N0 (P=0.042). Conversely, the ratio of TSCM cells to total memory cells was higher in lower tumor stages
	(P=0.059), tumors without muscle invasion (P=0.026) and low T grouping (P=0.043). Overall the data
Received:	indicated an increase in the frequency of memory T cells and their TSCM and TCM cells with tumor
2024.04.14	progression. In contrast, the ratio of TSCM to total memory cells was higher in less advanced tumors. These
Revised:	results suggest that the immune system is frequently exposed to tumor antigens and strives to create a memory
2024.06.16	T cell reservoir, but this is suppressed by inhibitory factors provided by the tumor. These findings emphasize
Accepted:	the importance of understanding the dynamic interplay between memory T cell subsets and BC progression.
2024.06.23	<b>Keywords:</b> Bladder cancer, cytotoxic T cells, memory T cells, TSCM, TCM, TEM

**Cite this article**: Ariafar A, *et al.* Diversity of Memory CD8<sup>+</sup> T Cells in Tumor-Draining Lymph Nodes from Patients with Bladder Cancer. *International Journal of Molecular and Cellular Medicine*. 2024; 13(2):147-159. **DOI:** 10.22088/JMCM.BUMS.13.2.147

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Publisher: Babol University of Medical Sciences

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#### Introduction

Tumor-draining lymph nodes are emerging as the primary site of orchestration of immune responses within the complex landscape of the tumor microenvironment (1). Memory T cells, the crucial components of the adaptive immune system, have been shown to populate the lymph nodes and contribute to memory immune responses to previously encountered tumor antigens (2). These cells have been reported to mediate a protective response by promoting tumor-immune balance through the secretion of cytokines and direct tumor cell killing (3, 4). This superior antitumor efficacy is likely related to their lower activation threshold compared to naïve T cells, their enhanced ability to migrate to lymph nodes, and their prolonged persistence and mediation of primary immunosurveillance of peripheral tissues. This diverse population comprises various subsets that differ in specific markers and functions, including T stem cell memory (TSCM), T central memory (TCM) and T effector memory (TEM). TSCM cells, the less differentiated subset with robust proliferation potential, have the ability to self-renew and differentiate into other memory subsets. TCM cells are predominantly found in secondary lymphoid organs and promote long-term immune memory. The third subset, TEM cells, are fast responding cells that rapidly perform effector functions such as cytokine release and cytotoxic activities without further differentiation (5).

CD8<sup>+</sup> cytotoxic T cells and their memory counterparts in primary tumor lesions or circulating blood are generally considered favorable prognostic indicators. Their higher density is often associated with favorable clinical outcomes in a variety of cancers (6). Accordingly, the aim of the present study was to investigate the prevalence of different subsets of CD8<sup>+</sup> memory T cells in the draining lymph nodes of patients with bladder cancer (BC), one of the most common cancers, especially in men. The investigation of these cells in the tumor-draining lymph nodes could provide valuable insights into the complex immune landscape of BC and suggest potential approaches for therapeutic interventions.

#### Materials and methods

#### **Patients**

Fifty patients with BC who had undergone surgical tumor resection were included in the present study. None of the patients had received chemotherapy or radiotherapy in the past. Clinical and pathologic information was obtained from the patients' medical records (summarized in Table 1). Informed consent was obtained from all patients before sampling. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.MED.REC.1402.368).

#### Mononuclear cell isolation

To obtain mononuclear single cells, fresh lymph nodes were mechanically minced in RPMI 1640 (Biosera, France) with 10% fetal bovine serum (FBS, Gibco, USA), and 1% penicillin/streptomycin (Biosera) and filtered through a 40- $\mu$ m cell strainer (BD Biosciences, USA). Mononuclear cells were then isolated from the single cell suspension by Ficoll-Hypaque (Biosera) gradient centrifugation. The mononuclear ring was harvested, washed and dissolved in 1x PBS. A cell density of  $250\times10^3$  was then distributed into flow cytometry tubes (BD Biosciences) for further analysis.

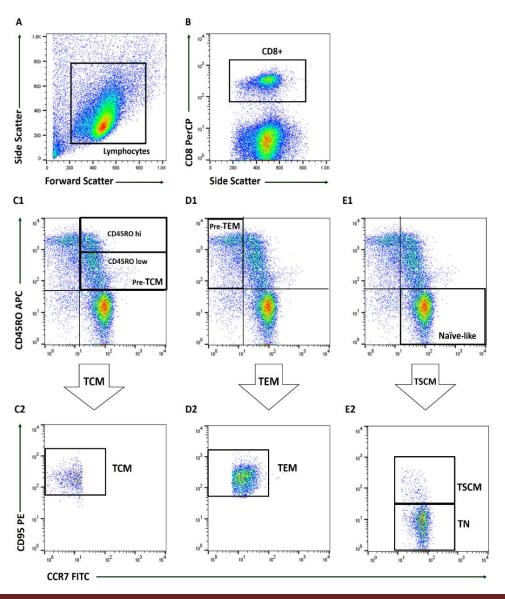
#### Flow cytometry analysis

Cells were surface-stained with fluorochrome-conjugated anti-human antibodies, including FITC anti-

Table1. Clinicopathologic features of patients with bladder cancer.						
Characteristics	N (%)					
Age	63.78±12.0					
Tumor type						
Urothelial carcinoma (UC)	48 (98)					
Non-UC	1 (2)					
Unreported	1					
T grouping						
T1 1	4 (8.2)					
T2	27 (55.1)					
T3	8 (16.3)					
T4	10 (20.4)					
Unreported	1					
Lymph node involvement						
Negative	35 (70.0)					
Positive	15 (30.0)					
Lymph node status	·					
Free (N0)	35 (70.0)					
N1	4 (8.0)					
N2	10 (20.0)					
N3	1 (2.0)					
Stage	,					
I	4 (8.2)					
II	19 (38.8)					
III	26 (53.1)					
Unreported	1					
Histological grade						
Low	3 (6.1)					
High	46 (93.9)					
Unreported	1					
Lymphovascular invasion						
Negative	31 (64.6)					
Positive	17 (35.4)					
Unreported	2					
Muscle invasion						
Negative	4 (8.2)					
Positive	45 (91.8)					
Unreported	1					
Perineural invasion						
Negative	21 (45.7)					
Positive	25 (54.3)					
Unreported	4					
Necrosis						
Negative	21 (60.0)					
Positive	14 (40.0)					
Unreported	15					
Carcinoma in situ						
Negative	17 (53.1)					
Positive	15 (46.9)					
Unreported	18					

CCR7 (3D12), PE anti-CD95 (Dx2), APC anti-CD45RO (UCHL1) and PerCP anti-CD8 (Sk1) (all from BioLegend, USA) for 20 minutes at room temperature (RT) in the dark. Then, they were washed twice with 1×PBS and detected using a four-color FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software version 10.8.1 (BD Life Sciences).

As illustrated in Figure 1, dead cells were first excluded due to their low forward and side scatter (A). Cytotoxic cells were then selected based on their distinguishable expression of CD8 within the lymphocyte gate (B). Subsequently, different memory and naïve subsets were defined based on the expression of CCR7,



**Fig. 1**. Schematic representation of the phenotype of CD8<sup>+</sup> memory T cell subsets in tumor-draining lymph nodes of patients with bladder cancer. Mononuclear cells were separated based on their relative size and granularity (FSC/SSC) (A). Cytotoxic cells were then determined by the CD8 expression (B), and the frequency of TCM (C1, 2), TEM (D1, 2), TSCM and naïve cells (E1, 2) was determined by the expression of CD45RO, CCR7 and CD95. TCM: T central memory, TEM: T effector memory, TSCM: T stem cell memory, TN: T naïve

CD45RO and CD95. The cells expressing CD95, CCR7 and CD45RO were classified as TCM (C1, C2); the population exhibiting a CCR7<sup>-</sup>CD45RO<sup>+</sup>CD95<sup>+</sup> phenotype was identified as TEM (D1, D2); and a subset of cells with the naïve phenotype – CCR7<sup>+</sup>CD45RO<sup>-</sup>— but positive for CD95, was considered as TSCM (E1, E2). The CCR7<sup>+</sup>CD45RO<sup>-</sup> cells that did not express CD95 were categorized as naïve T cells (E1, E2). Based on the variable expression of CD45RO on TCM cells, two subgroups with the phenotype CD45RO<sup>hi</sup> TCM and CD45RO<sup>low</sup> TCM were also included in the analyses (C1). The geometric mean fluorescence intensity (gMFI) of CD95 was considered as a criterion for the expression level at the single cell level.

#### Unsupervised analysis: t-distributed stochastic embedding (t-SNE) and Phenograph

The t-distributed stochastic neighbor embedding (t-SNE) was implemented to reveal unbiased inherent relationships between data points in a low-dimensional space. For this purpose, the FCS files of all patients were concatenated into a single file after the exclusion of unwanted cells and then the t-SNE and Phenograph (v.2.4) plugins were applied in FlowJo software. The parameters were used in both analyses with the default values. The FSC, SSC, CD8, CCR7, CD45RO, and CD95 parameters were used to delineate the t-SNE and Phenograph clustering.

#### Statistical analysis

The Statistical Package for Social Sciences version 26 (SPSS GmbH Software, Germany) was used for all statistical analyses, and P values of less than 0.05 were considered significant. Mann–Whitney U and Kruskal-Wallis H nonparametric tests were used to identify statistically significant differences in subgroup frequencies among patients. The graphs were created using the GraphPad Prism 9 software package (San Diego CA, USA).

#### **Results**

#### Clinicopathologic features of the patients

Lymph nodes were obtained from 50 untreated patients with BC (mean age= $63.78\pm12.0$  years) who had undergone radical cystectomy. As summarized in Table 1, 30% of patients had at least one tumor-infiltrated node (15/50) and 91.8% had tumor invasion into the muscle layer (45/49). In addition, most patients were in stage III (26/50, 53.1%) and had a high histologic grade (46/50, 93.9%).

## CD8<sup>+</sup> memory T cell subsets in tumor-draining lymph nodes and their association with clinicopathologic features

As shown in Table 2, CD8<sup>+</sup> lymphocytes accounted for  $7.02 \pm 2.80\%$  (range 2.67-15.50) of the total mononuclear cells in the tumor-draining lymph nodes of patients with BC. Of these,  $49.32 \pm 20.15$  had a memory phenotype and expressed CD45RO<sup>+</sup>CD95<sup>+</sup>. Further analysis revealed that TCM cells were the most common memory subset ( $34.71\pm17.04$ ), while TSCM cells had the lowest prevalence among the memory subsets with a frequency of  $7.51 \pm 8.53$ . The average frequencies of the different memory T cell subsets among CD8<sup>+</sup> lymphocytes are reported in Table 2.

In the next step, the association of the different memory and naïve subsets with clinicopathologic features was investigated. As illustrated in Figure 2, statistical analyses indicated that the total number of CD8<sup>+</sup> memory T cells was significantly more frequent in patients with stage III (unadjusted P=0.042)

and positive muscle invasion (P=0.052) than in stage I and non-invaded patients. These cells also showed a significant increase in patients older than 60 years compared to those younger than 60 (P=0.001).

As for the TSCM subset, although its frequency was significantly higher in T1 patients with a tumor invading the lamina propria and T4, where the extravesical tumor directly invades external organs, than in patients with T3 (tumor invades perivesical soft tissue; P=0.024 and P=0.043, respectively). The percentage of TSCM was significantly higher in patients with N2 (with more than one positive lymph node in the pelvis) than in patients with free lymph nodes (N0; P=0.042).

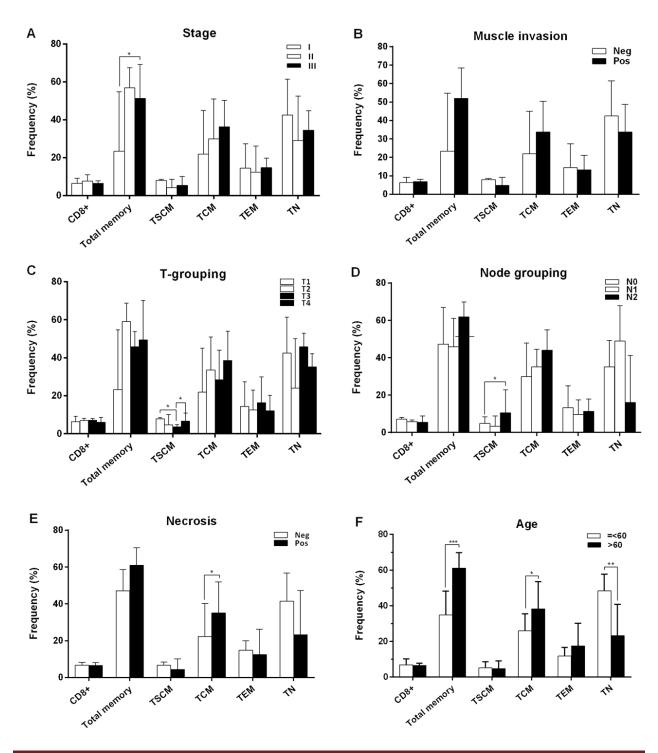
Patients with tumor necrosis revealed a higher frequency of TCM (P=0.048) and its high CD45RO-expressing subset (P=0.03) compared to patients without necrosis. On the other hand, the mean expression of CD45RO in TCM cells was significantly higher in patients with T3 compared to those with T4 (P=0.033). The TCM cells also demonstrated a significantly higher frequency in patients older than 60 years compared to those younger than 60 (P=0.027), while the naïve cells were more frequent in patients younger than 60 years (P=0.008).

### Relative frequencies of memory and naïve CD8<sup>+</sup> T cells and their associations with clinicopathologic features

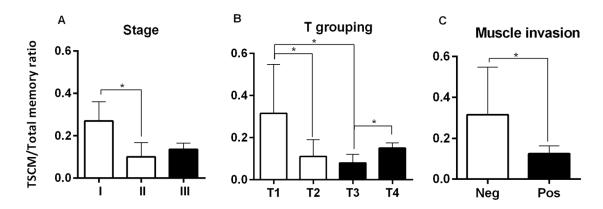
Next, the ratio of TSCM cells and naïve subset to different memory CD8<sup>+</sup> T cells was calculated and their associations with clinicopathologic features were analyzed (Figure 3). The analyses of the current study showed that the ratio of TSCM cells to total memory cells significantly increased in patients with T1 (P=0.043, P=0.012), T4 (P=0.014), stage I (P=0.019) and without muscle invasion (P=0.026) compared to those with T2, T3, stage II and muscle-invasive tumor. In addition, the ratio of TSCM cells to TCM cells was significantly higher in patients with T1 (P=0.024) and T4 (P=0.025) than in patients with T3 tumors. Furthermore, the ratio of naïve cells to TEM, TCM and total memory cells was significantly higher in patients under 60 years of age (P=0.001, P=0.015, and P=0.007, respectively).

Table 2. Frequency of memory CD8+ T cells in draining lymph nodes of patients with bladder cancer.								
Cell subset	Marker	Min	Max	Media	Mean (%) ±			
		(%)	(%)	n (%)	SD			
Cytotoxic lymphocytes	CD8 <sup>+</sup>	2.67	15.50	6.79	$7.02\pm2.80$			
CD8+CD45RO+	CD8 <sup>+</sup> CD45RO <sup>+</sup>	16.00	86.00	53.90	$53.20 \pm 18.02$			
CD8+CD95+	CD8 <sup>+</sup> CD95 <sup>+</sup>	19.00	96.00	56.80	61.01±21.43			
Cytotoxic memory cells	CD8+CD45RO+CD95+	1.62	87.20	49.40	49.32±20.15			
TCM	CD8 <sup>+</sup> CCR7 <sup>+</sup> CD45RO <sup>+</sup> CD95 <sup>+</sup>	9.53	74.90	33.60	34.71±17.04			
CD45ROhi TCM	$CD8^{+}CCR7^{+}CD45RO^{hi}CD95^{+}$	1.02	51.40	15.80	17.50±11.49			
CD45ROlow TCM	CD8+CCR7+CD45ROlowCD95+	3.80	67.20	15.30	17.58±12.75			
TEM	CD8 <sup>+</sup> CCR7 <sup>-</sup> CD45RO <sup>+</sup> CD95 <sup>+</sup>	0.03	58.50	12.50	16.94±12.47			
TSCM	CD8 <sup>+</sup> CCR7 <sup>+</sup> CD45RO <sup>-</sup> CD95 <sup>+</sup>	1.07	54.70	4.94	7.51±8.53			
TN	CD8+CCR7+CD45RO-CD95	2.34	72.60	33.30	33.45±19.83			

TCM: T central memory, TEM: T effector memory, TSCM: T stem cell memory, TN: T naïve



**Fig. 2**. Statistical analyses of CD8<sup>+</sup> memory T cell subsets in bladder cancer draining lymph nodes from patients with different clinicopathologic features. The frequencies of CD8+, memory, TSCM, TCM, TEM, and naïve T cells at different stages (A), muscle invasion status (B), T groupings (C), node groupings (D), necrosis status (E) and age (F); Data are expressed as median ± IQR. \*Differences are significant at P<0.05. \*\* Differences are significant at P<0.01. TCM: T central memory, TEM: T effector memory, TSCM: T stem cell memory, TN: T naïve



**Fig. 3.** Statistical analyses of TSCM/total memory CD8<sup>+</sup> T cell ratio in bladder cancer drainage lymph nodes from patients with different clinicopathologic features. The TSCM/memory CD8<sup>+</sup> T cell ratio at different stages (A), T groupings (B) and muscle invasion status (C). Data are expressed as median ± IQR. \*Differences are significant at and \*\* P<0.01 and \*\*\* P<0.001. TSCM: T stem cell memory

#### Correlations between the frequencies of different CD8+ lymphocyte subsets

The correlations between the different memory and naïve subsets and between the subsets and patients' age were evaluated using the non-parametric Spearman correlation test. The results showed that the percentage of CD8+CD45RO+ cells had a strong negative correlation with naïve cells (P<0.001, R=-0.885) and age (P=0.001, R=0.559). TSCM cells were positively correlated with TCM cells (P<0.001, R=0.551) and TCM cells with low CD45RO expression (P<0.001, P=0.626). On the other hand, TSCM cells showed a negative correlation with TEM (P=0.005, P=0.005, P=0.005), naïve T cells (P=0.025, P=0.328) and mean expression of CD45RO on TCM cells (P<0.001, P=0.505). TCM cells indicated a strong negative correlation with naïve T cells (P<0.001, P=0.505), but a positive strong relationship with age (P=0.007, P=0.39). Naïve T cells also suggested a negative significant correlation with both the total number of memory cells (P<0.001, P=0.001, P=0.001,

#### An unbiased analysis revealed various subsets of immune cells with different expression of memoryrelated markers

The t-SNE and Phenograph are methods for unbiased visualization of data by constructing a probability distribution over pairs of objects. The present study used these sophisticated analysis techniques to gain deeper insights into the population of memory T cells in draining lymph nodes in BC. The data of the current study indicated that based on the defined parameters, 46 unique nodes could be detected by phenograph analysis. The distribution of the most common nodes (20 nodes) was then compared in patients with various clinicopathologic features, including T grouping, node grouping, stage, muscle invasion, and lymph node involvement. As illustrated in Figure 4, nodes 6, 10, 26, and 29 have an increased frequency with good prognosis, including T1 group, stage I, and tumor-free muscle. These populations showed partially similar expression of CD8 and CCR7 but differed in the expression of CD95 and CD45RO.

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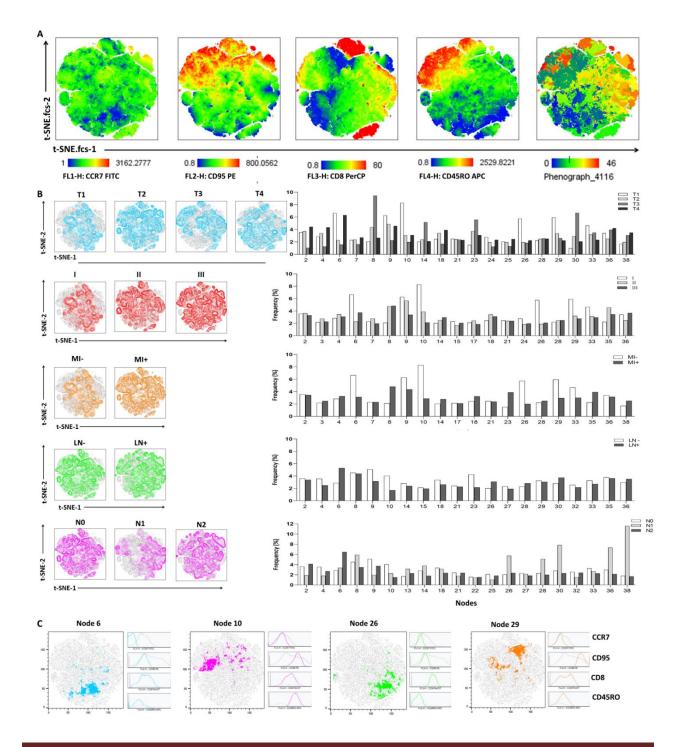


Fig. 4. Representative plots of unbiased cluster definition of CD8, CCR7, CD45RO and CD95 expression in bladder cancer draining lymph nodes. A: t-SNE and Phenograph maps demonstrating the expression of CD8, CCR7, CD45RO and CD95 in draining lymph nodes of patients with bladder cancer, B: Contour density plots of the frequent clusters identified by Phenograph overlaid with the t-SNE map (left) and bar charts of the distribution of high-prevalence nodes in different T groupings, stages, node groupings, muscle invasion and lymph node involvement status, and C: Dot-blot representation of the nodes with the highest expression associated with good prognosis, including T1 grouping, stage I and tumor-free muscle

#### **Discussion**

In the present study, the frequency of different CD8<sup>+</sup> memory T cells in the drainage nodes patients with BC, which are the main site for triggering anti-tumor immune responses, was investigated. Overall, the data of the current study collectively suggested an increased frequency of less differentiated memory subsets, TSCM and TCM cells, with tumor progression reflected in by more involved nodes, T4 and tumor necrosis.

CD8+ T cells, known as cytotoxic T cells, are one of the most important subsets of T cells in the antitumor immune response. Infiltration of these cells into the TME has been observed in various tumor types and generally correlates with a good prognosis. CD8+ T cells accounted for 6-7% of the total lymphocytes in the draining nodes of patients with BC (7). About half of them indicated memory phenotype, reaching more than 80% in some cases. Prognostic analysis revealed that the total frequency of CD8+T cells increased with tumor progression and invasion into the muscle layer and at higher stages (III>I). This increase could be due to frequent exposure of T cells to tumor antigens leading to their activation and differentiation into a memory phenotype. Accordingly, our previous study on BC tumor tissue also indicated a positive correlation between lymphocytes with a memory phenotype (CD45RO+lymphocytes) and muscle layer invasion, higher grade and higher T-grouping (8). CD45RO+ cells were also more common in advanced breast tumors (9) and renal cell carcinomas (10), which had higher TNM stages and grades. Similarly, memory T cells correlated with reduced 10-year overall survival in a subset of high-risk head and neck squamous cell carcinoma patients, including those with poorly differentiated tumors and tumorinfiltrated regional nodes (11). The lower proportion of memory CD8+ T cells was associated with lymph node metastases in gastric cancer (12). On the other hand, there are some reports, including a meta-analysis on different types of solid tumors, indicating an inverse association between high density of intratumoral CD45RO+ T cells and TNM stage, tumor size and lymph node metastasis, providing a favorable clinical outcome (13-15). In addition, no significant association was found between memory cell frequency and clinicopathologic features in esophageal squamous cell carcinoma (16) and oral squamous cell carcinoma (17). These controversies reflect the need for further studies to thoroughly evaluate the prognostic relevance of memory T cells in different cancer types, taking into account their diverse phenotype and function in the TME, lymph nodes and blood circulation. Memory T cells are typically classified into different subsets based on their phenotypic characteristics, which play different roles in immunity. The TCM subset, which generally resides in the lymphoid organs, was the most frequent CD8+ memory T-cell subset in the tumordraining lymph nodes of patients with BC. This subset had a higher frequency in patients with tumor necrosis. Whereas, the mean expression of CD45RO, a marker for memory T cells, was higher in TCM cells from patients with low T-grouping (T3>T4). However, as far as we know, there is no data on draining nodules in BC. Consistently, our previous study on draining nodules from patients with breast cancer showed that the TCM subset was significantly higher in tumor-involved nodules than in tumor-free nodes. In addition, the expression of CD95 on the surface of these cells as well as the CD45ROhi and CD45ROlow subsets was higher in patients with poor prognosis (stage II>I and N1>N0) (18). Consistent with our results, Zhang et al. suggested a positive association of CD8+ TCM in the periphery with clinical stage and node involvement of patients with gastric cancer. However, the percentage of this subset decreased significantly in peripheral blood compared to healthy controls (12).

TEM cells, another important memory subset, exhibit higher expression of receptors responsible for migration to inflamed tissue and have stronger immediate effector functions than TCM cells. The data revealed that 17% of CD8<sup>+</sup>T cells had an effector memory phenotype, but no correlation was observed with clinicopathologic features. In a study of breast cancer draining lymph nodes, a higher frequency of CD8<sup>+</sup> TEM was previously observed in N1 patients compared to node-free patients (18). Such an increase was also reported in the metastatic lymph nodes of patients with lung cancer (19). These cells were also more frequent in the peripheral blood of patients with gastric cancer than in healthy controls, although their prevalence decreased significantly after tumor resection (12).

The least differentiated memory T cells, TSCM cells, present a phenotype similar to naïve and stem cells and respond rapidly to antigens to form a pool of memory and effector cells, a reservoir of responding cells (20). Our statistical analysis revealed that TSCM cells might play a prognostic role in BC progression despite their low frequency among memory subsets, as their frequency increased in patients with N2-node clustering. However, their frequency varied depending on the invasion of the tumor into different layers of the bladder (T-grouping). It was higher in less advanced tumors (T1), decreased in the middle stage (T3) and increased again in those patients with highly invasive tumors (T4). These fluctuations could be due to the fact that they differentiate into other subsets to form effector subsets, but as the tumor progresses, the suppressive milieu of the tumor blocks their differentiation in the late stages. A reduction of naïve and TSCM CD8+T cells was also observed in the circulation of patients with advanced non-small cell lung cancer, probably due to their recruitment to the lymph node or tumor site (21). In addition, the presence of CD4+TSCM cells in the tumor stroma and their quantity correlated negatively with the TNM stage in patients with colorectal cancer. The absolute value and frequency of CD4+TSCM were able to clearly distinguish colorectal cancer, benign tumors, and healthy controls (22).

When examining the ratio of the different subsets, our data indicated a correlation between a higher ratio of CD8<sup>+</sup> TSCM to total memory T cells and good prognostic conditions, i.e. lower stage (I>II, III), no invasion into the muscle layer and lower T grouping (T1>T2, T3). However, to our knowledge, there is no data in the literature on the ratio of TSCM/memory T cells in tumors, the ratio of CD8<sup>+</sup> TCM/TEM has been proposed as a predictive biomarker for response to checkpoint inhibitors in patients with non-small cell lung cancer (23). It was also shown that a higher ratio of naïve CD4<sup>+</sup> cells and memory cells was associated with prolonged progression-free survival in patients with non-small cell lung cancer. Although this was not mentioned, this population of naïve cells could include in part TSCM cells (24). The density of cytotoxic effector/memory T cells in the tumor compartment was shown to be an independent prognostic biomarker for overall and disease-free survival in patients with triple-negative breast cancer (25).

To our knowledge, this is the first report on the study of CD8<sup>+</sup> memory T cell subsets in tumor-draining lymph nodes of patients with BC. The results indicated that the composition and frequency of CD8<sup>+</sup> memory T cell subsets in tumor-draining lymph nodes of bladder cancer had significant correlations with various clinicopathologic parameters. In the current study, a general increase in the overall frequency of memory T cells was observed in advanced tumor stages and patients with tumor-infected muscle. Furthermore, the

prevalence of TSCM and TCM cells was higher in patients with lymph node metastases and necrotic tumors, respectively. On the other hand, the higher ratio of TSCM to total memory cells was observed in lower tumor stages, tumors without muscle invasion, and low T-grouping. These data suggested that the immune system was frequently exposed to tumor antigens and made an effort to form a memory T cell reservoir, but this was suppressed by inhibitory factors provided by the tumor. The higher ratio of TSCM to total memory cells in lower tumor stages, tumors without muscle invasion and lower T grouping may reflect the immune system's response to tumor antigens in the absence of a large tumor burden. These findings emphasize the importance of understanding the dynamic interplay between memory T cell subsets and bladder cancer progression.

#### Acknowledgments

This work was financially supported by the Shiraz University of Medical Sciences (grant number: 28208) and the Shiraz Institute for Cancer Research (grant number: ICR-100-504).

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