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Gamma-delta T Cells in Bladder Cancer Draining Lymph Nodes

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ABSTRACT

Background: Gamma-delta (γδ) T cells are a distinct subset of T cells with a receptor composed of γ and δ chains. Their ability to directly recognize stress-induced molecules and non-peptide antigens expressed by cancer cells, along with their capacity to produce cytokines and interact with other immune cells, makes them potentially significant contributors to immune-based treatments.

Objective: To investigate the presence and frequency of Tγδ cells in tumor-draining lymph nodes of patients with bladder cancer (BC), and to assess their association with prognostic parameters.

Methods: Forty-nine fresh tumor-draining lymph nodes from untreated patients with BC were minced to obtain single cells. The cells were surface-stained with anti-CD3, anti-TCRγδ, and anti-HLA-DR antibodies, then acquired on a four-color FACSCalibur flow cytometer, and analyzed by FlowJo software.

Results: On average, 2.07%±1.99% of $CD3^+$ lymphocytes in regional nodes of BC exhibited a γδ T phenotype. A considerable percentage of these cells (37.90%±24.42%) expressed HLA-DR. Statistical analysis revealed that while the frequency of γδ T cells showed no variation among patients with different prognoses, the $HLA\text{-}DR^+$ subset was higher in T4 patients than in T2 patients (p=0.031). These cells also tended to be increased in stage III compared to stage II (p=0.077).

Conclusion: The data collectively indicated an association of HLA-DR expressing γδ T cells with prognostic factors related to tumor progression (higher T-group and stage), suggesting their potential involvement in disease progression. However, future research, including longitudinal studies with larger cohorts, needs to validate these findings and elucidate the functional roles of γδ T cells in the immune response against BC.

Keywords: Bladder Cancer, Draining Lymph Node, Gamma-delta T Cells, HLA-DR

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INTRODUCTION

Bladder cancer (BC), the fourth most common cancer in males worldwide, poses a major health challenge due to its high morbidity and mortality rates. Despite advances in medical research, survival rates for BC have not shown significant improvements, particularly for patients diagnosed at advanced stages. This underscores the urgent need for innovative therapeutic approaches (1). Understanding the role of the immune system and its components is a crucial step in developing effective treatments and improving patient outcomes due to its pivotal role in shaping the tumor microenvironment and influencing treatment responses (1, 2).

Gamma-delta (γδ) T cells are a distinct subset of T cells characterized by their T-cell receptors (TCRs), which are made up of γ and δ chains instead of the typical α and β chains found in most T cells. Unlike conventional T cells, γδ T cells recognize a diverse range of antigens without relying on major histocompatibility complex (MHC) presentation and can directly exert cytotoxic effects on infected or transformed cells (3, 4). Their ability to directly recognize stress-induced molecules and non-peptide antigens expressed by cancer cells, combined with their capacity to produce cytokines and interact with other immune cells, makes them potentially significant contributors to immune-based cancer treatments (5-7). A recent transcriptome analysis revealed that γδ T cells are one of the strongest predictors of favorable outcomes in various cancers (8). Research on BC patients and mouse models has also demonstrated that injections of γδ T cells extended survival. Additionally, when these cells were deleted, mice no longer responded to BCG, indicating a potential anti-tumorigenic function (9-12). However, the complex activity of γδ T cells within the tumor microenvironment highlights their ability to have both tumor-suppressing and tumor-promoting effects. These effects are determined by the surrounding cytokine environment and their interactions with other cellular components within the tumor (13-15). Therefore, in the present study, we focused on determining the presence and frequency of γδ T cells in tumor-draining lymph nodes of patients with BC and their association with prognostic parameters.

MATERIALS AND METHODS

Patients

Forty-nine tumor-draining lymph nodes, dissected as part of the routine diagnostic procedure, were obtained from patients with BC who underwent surgery. These patients had no history of prior chemotherapy, radiotherapy or immunotherapy. The clinical and pathological characteristics of the patients were obtained from their medical records and are summarized in Table 1. Tumor stage was determined based on the American Joint Committee on Cancer Staging Manual, 8th edition. The study was explained to the participants, and informed consent was obtained prior to study. The ethical committee of Shiraz University of Medical Sciences was approved the study (IR.SUMS.MED.REC.1403.174).

Isolation of Mononuclear Cells from Lymph Node

Fresh lymph nodes were mechanically minced in culture medium supplemented with 10% fetal bovine serum (FBS), and then filtered through a 40 μm cell strainer to obtain a single-cell suspension. Mononuclear cells were isolated using Ficoll-Hypaque gradient centrifugation. The Trypan blue exclusion test was used to check the cells' viability. Samples with a viability of more than 95% were then proceeded to further evaluation.

Flow Cytometry Analysis

To specifically phenotype γδ T cells, a density of 2.5×10^5 cells was suspended in 50 μl of staining buffer (1× PBS containing 2% FBS) and immediately surface-stained with

FITC-conjugated anti-CD3 (clone: UCHT1; BD Bioscience, USA), APC-conjugated anti-TCRγδ (clone: B1, BioLegend, USA), and PerCP-conjugated anti-HLA-DR (clone: L243). The cells were incubated for 15 minutes at room temperature in the dark to prevent photobleaching of the fluorophores. The cells were then washed thoroughly and resuspended in staining buffer. Sample acquisition was performed using a four-color FACSCalibur flow cytometer (BD Biosciences), capable of detecting four fluorochromes simultaneously. FlowJo software version 10.8.1 (BD Life Sciences) was used for data analysis. Lymphocytes were first isolated by gating based on their relative side scatter (SSC-H) and forward scatter (FSC-H) to exclude debris and unwanted cells (Fig. 1A). Next, $CD3^+$ cells were identified as the total T cell population (Fig. 1B). Further gating was performed within this population to isolate TCRγδ expressing cells, specifically identifying γδ T cells (Fig. 1C). Subsequently, HLA-DR-expressing subsets within γδ T population were determined (Fig. 1D).

Statistical Analysis

Data was analyzed using statistical package for the social sciences (SPSS) software version 26. Nonparametric tests, including Mann-Whitney U and Kruskal-Wallis tests, were applied for statistical analyses comparing patients with different clinicopathological characteristics. Data was presented as median, and mean±standard deviation (SD). *P*-values less than 0.05 were considered statistically significant. Graphs were created using the GraphPad Prism software package, version 8 (San Diego, CA, USA).

RESULTS

Table 1. Prognostic association of T cell subsets in patients with different cinicopathological characteristics of bladder cancer

Characteristics	N (valid %)	Median		
		$CD3^+$ lymphocytes (% of lymphocytes)	γδ T cells (% of $CD3^+$ T cells)	$HLA\text{-}DR^+$ γδ T (% of γδ T cells)
Gender				
Male	40 (81.6)	42.75	1.49	33.90
Female	9 (18.4)	9.00	9.00	6.00
P value		0.517	0.379	0.849
Tumor type				
Urothelial carcinoma (UC)	48 (98.00)	44.25	1.31	33.90
Non-UC	1 (2.00)	51.80	0.94	NA
P value		NA	NA	NA
Histological grade				
Low	3 (6.3)	41.50	0.62	38.00
High	45 (93.7)	44.50	1.33	32.70
P value		0.717	0.156	0.786
Unreported	1	46.40	2.49	NA
T-stage				
T1	4 (8.3)	52.20	1.19	26.15
T2	26 (54.2)	43.55	1.27	27.20
T3	8 (16.7)	50.85	1.31	22.88
T4	10 (20.8)	34.95	1.24	48.50
P value		0.19	1.000	0.154
Unreported	1	38.10	1.53	60.20
Lymph node involvement				
Positive	15 (30.6)	36.10	1.89	39.25
Negative	34 (69.4)	47.25	1.24	32.35

Characteristics	N (valid %)	Median		
		CD3$^+$ lymphocytes (% of lymphocytes)	γδ T cells (% of CD3$^+$ T cells)	HLA-DR$^+$ γδ T (% of γδ T cells)
P value		0.045	0.229	0.636
N-stage				
N0	34 (69.4)	47.25	1.24	32.35
N1	4 (8.2)	36.95	1.77	14.28
N2	10 (20.4)	36.40	1.94	48.25
N3	1 (2.0)	36.10	1.10	NA
P value		0.136	0.337	0.111
TNM-stage				
I	4 (8.3)	52.20	1.19	26.15
II	18 (37.5)	45.70	1.24	24.50
III	26 (54.2)	37.95	1.34	43.10
P value		0.105	0.774	0.166
Unreported	1	38.10	1.53	60.20
Muscle invasion				
Positive	44 (91.7)	44.25	1.28	32.70
Negative	4 (8.3)	52.20	1.19	26.15
P value		0.318	1.000	0.531
Unreported	1	38.10	1.53	60.20
Perivesical fat invasion				
Positive	16 (33.3)	44.25	1.31	43.10
Negative	32 (66.7)	45.65	1.27	29.15
P value		0.299	0.844	0.219
Unreported	1	38.10	1.53	60.20
Organ-confined tumor (OCT)				
OCT	27 (55.1)	48.80	1.26	28.50
non-OCT	22 (44.9)	36.90	1.62	43.10
P value		0.021	0.41	0.098
Lymphovascular invasion				
Positive	17 (36.2)	36.10	1.27	39.25
Negative	30 (63.8)	47.60	1.37	30.90
P value		0.06	0.595	0.201
Unreported	2	56.95	1.37	39.00
Perineural invasion				
Positive	25 (51.0)	38.70	1.34	37.35
Negative	20 (40.8)	48.60	1.23	32.35
P value		0.205	0.749	0.494
Unreported	4	38.05	1.37	39.00
Tumor necrosis				
Positive	18 (54.5)	44.85	1.18	25.90
Negative	15 (45.5)	46.40	1.54	37.90
P value		0.901	0.229	0.152
Unreported	16	39.80	1.34	38.00
Tumor shape				
Papillary	29 (87.9)	44.50	1.27	28.50
Flat (sessile)	4 (12.1)	54.20	1.37	51.65
P value		0.184	0.894	0.262
Unreported	16	44.00	1.89	32.70

γδ T cells: Gamma-delta T cells; NA: not-applicable

* Since there was only one patient in the N3 group, this group was excluded from the analysis

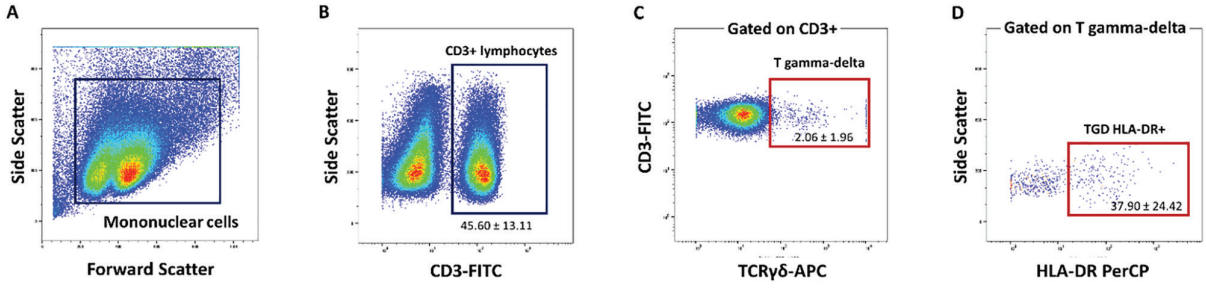

Fig. 1. Phenotype determination of gamma-delta (γδ) T cells in tumor-draining lymph nodes from patients with bladder cancer. Lymphocytes were selected based on their relative side scatter (SSC-H) and forward scatter (FSC-H) to exclude debris and unwanted cells (A). Next, CD3 positive cells were identified as total T cell population within the mononuclear/lymphocyte gate (B). Further gating was performed within this population to specifically isolate γδ T cells (C). HLA-DR expressing subsets were then determined among γδ T cells (D).

Table 2. Frequency of gamma-delta T cells in tumor draining lymph nodes of patients with bladder cancer

Cell subset	Min	Max	Median	Mean±SD
$CD3^+$ lymphocytes (% of lymphocytes)	23.20	75.80	44.50	45.60±13.11
γδ T cells: $CD3^+$ $TCR\gamma\delta^+$ (% of $CD3^+$ T cells)	0.12	8.77	1.28	2.06±1.96
$HLA\text{-}DR^+$ γδ T (% of γδ T cells)	2.66	99.10	33.90	37.90±24.42

γδ T cells: Gamma-delta T cells

Patient Demographics and Clinical Characteristics

After pathological confirmation, a total of 49 untreated patients with BC with a mean age of 64.65±11.59 years were recruited for this study. According to the pathological reports, most patients were in stage III (26/48, 54.2%) and 15 out of the 49 patients had at least one involved lymph node (30.6%). The primary clinicopathological characteristics of the patients are summarized in Table 1.

γδ T Cells in Tumor Draining Lymph Nodes of Patients with Bladder Cancer

We first determined the frequency of T cells, γδ T cells (n=49) and the expression of HLA-DR on the surface of γδ T cells (n=28) in tumor draining lymph nodes of patients with BC. The descriptive statistics for these markers, including minimum, maximum, mean, and median frequencies are presented in Table 2. Our phenotyping results showed that on average, 2.06%±1.96% of $CD3^+$ lymphocytes in the regional draining nodes of BC had a γδ T phenotype, as demonstrated by the expression of TCRγδ. A considerable percentage of these cells (37.90%±24.42%) also expressed HLA-DR, which is an activation marker for T cells.

We then examined the distribution of these cells among patients with different clinical and pathological characteristics (Table 1). Statistical analysis revealed that while the frequency of γδ T cells did not vary among patients with different prognoses, their subsets did vary across different T-stages. In this regard, $HLA\text{-}DR^+$ subsets were higher in T4 patients than in T2 patients (p=0.031). $HLA\text{-}DR^+$ γδ T cells also tended to be higher in stage III than in stage II (p=0.077). In addition, the percentage of total $CD3^+$ T lymphocytes increased significantly in patients with organ-confined tumors (p=0.021) and LN^- (p=0.045). It also tended to be higher in patients with stage II (p=0.066) and those without lymphovascular invasion (p=0.060) compared to patients with non-organ confined tumors, LN^+ patients, those in stage III, and those positive for lymphovascular invasion, respectively.

No significant associations were observed

with other clinicopathological characteristics including sex, age, shape of tumor, histological grade, invasion to the muscle and perivesical fat layers, tumor necrosis, perineural invasion, and carcinoma in situ.

DISCUSSION

In the present study, we investigated the frequency of γδ T cells and their HLA-DR expressing subset in the tumor draining lymph nodes of patients diagnosed with BC. Our findings revealed that nearly 2% of T lymphocytes exhibited a γδ T cell phenotype. A considerable percentage of these cells (more than 35%) expressed the HLA-DR activation marker. This subset showed an association with prognostic factors related to tumor progression including higher T-groups and tumor stages.

γδ T cells are unconventional, innate-like T cells that make up a small portion of T cells (about 2%) and are predominantly found among intraepithelial lymphocytes. Their rapid, non-MHC-restricted responses to various stimuli make them particularly interesting in tumor studies including BC. However, in our study, no association was observed between the frequency of γδ T cells and BC prognosis. Nevertheless, most studies have reported that these cells are associated with favorable clinical outcomes. It has been also shown that the tumor reactivity of γδ T cells could be boosted by BCG and zoledronate treatments, potentially leading to improved survival (9, 10). Other studies have consistently shown that this T subset enhances the cytotoxic effects of chemotherapy (i.e. carboplatin and mTOR inhibitors), leading to increased therapeutic efficacy and reduced adverse responses (11, 12). This favorable role could be attributed to the fact that these cells can rapidly respond to tumor cells by recognizing non-classical antigens independently of MHC presentation. They exhibit direct cytotoxicity by releasing substances like perforin and granzyme, interacting with death receptors such as Fas and TRAIL on tumor cells, and releasing pro-inflammatory cytokines, including interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) (6, 14, 16). These findings suggest that γδ T cells may play a protective role in BC and could serve as a new prognostic biomarker for patients with BC undergoing specific immune or chemotherapy treatments.

We also examined the HLA-DR expression on γδ T cells and found that a significant portion of these cells expressed HLA-DR (more than one third). However, the expression levels varied widely, ranging from 2.66% to 99.10%. In addition to genetic diversity that may influence HLA-DR expression levels, the activation status of the cells, as well as the environmental and pathological factors may contribute to the variation within γδ T cells population. The human leukocyte antigen–DR isotype (HLA-DR), a common marker on professional antigen-presenting cells, is considered an activation marker for T cells but its exact role in γδ T cell function is not clear. However, this marker might play a role in γδ T cells as professional antigen-presenting cells (17). Our analysis revealed intriguing associations between the HLA-DR-expressing γδ T cell subset and various clinicopathological characteristics of BC. Specifically, these cells were significantly elevated in patients in the higher T group (T4>T2) and tumor stages (III>II), suggesting their potential involvement in disease progression. To the best of our knowledge, there is no report on HLA-DR expression on γδ T cells in BC draining nodes, but these T cells have been associated with immune reconstitution after allogeneic stem cell transplantation, and have been found to be higher in patients with autoimmune rheumatic diseases such as systemic sclerosis (18). HLA-DR expression on cytotoxic T cells could differentiate patients with axillary lymph node metastasis from those without infiltration. These cells exhibited increased expression of effector molecules such as

IFN-γ and Granzyme B, indicating their immune competent profile. This information can assist in predicting patients' response to neoadjuvant chemotherapy (19, 20). On the other hand, there is evidence that γδ T cells could promote tumor growth through the release of pro-inflammatory cytokines like IL-17, enhancing angiogenesis, and inhibiting antitumor responses (15). Our findings corroborate these studies that underscore the dual roles of γδ T cells in cancer, where they can exhibit both tumor-suppressing and tumor-promoting effects.

We also observed an increase in the total $CD3^+$ T lymphocytes in patients with organ-confined tumors and favorable prognostic indicators including negative lymph node involvement, lower tumor stage, and absence of lymphovascular invasion. This confirms that T cells are critical determinants of the immune landscape and therapeutic outcomes in cancer. These findings further emphasize the complex interplay between immune surveillance and tumor progression in BC, highlighting the importance of conducting subset-specific studies as previously noted (21-23).

Our sample size was relatively modest, which may have affected the statistical power to detect subtle associations with certain clinicopathological parameters. The cross-sectional nature of the study also limited our ability to determine the temporal dynamics of immune cell changes during disease progression. Future research directions could include conducting longitudinal studies with larger cohorts to validate these findings and elucidate the functional roles of γδ T cells in the immune response against BC.

CONCLUSION

Taken together, our data indicated that although the frequency of γδ T cells in draining lymph nodes showed no association with BC prognosis, an association of HLA-DR expressing γδ T cells with poor prognostic factors related to tumor progression, including higher T-group and tumor stage suggests their potential involvement in disease progression. These findings, along with the association of total T population with favorable prognostic factors further emphasize the complex interplay between the immune system and tumor cells. This highlights the importance of conducting subset-specific studies. Investigating the mechanisms underlying γδ T cell activation and their interactions with other immune and tumor cells in the regional lymph node may provide novel insights into immune evasion mechanisms and potential targets for immunotherapy.

AUTHORS' CONTRIBUTION

ZF designed the study, analyzed the data, and wrote and edited the manuscript. AA and HA provided the samples and secured the grants. ZM performed the experiments and analyzed the data. All authors reviewed and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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