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Natural Killer Cell Subsets in Tumor Draining Lymph Nodes of Patients with Bladder Cancer and Their Clinical Implications

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ABSTRACT

Background: Natural killer (NK) cells are crucial innate components in anti-tumor immunity. However, the clinical impacts and their phenotypes in bladder cancer (BC) remain unclear.

Objective: To assess the clinical significance of NK cell subsets in tumor-draining lymph nodes of patients with BC.

Methods: In a cross-sectional study, pelvic lymph nodes were obtained from 49 untreated patients with BC. Mononuclear cells were isolated and immunophenotyped using CD3, CD56, CD16, CD27, and CD11b markers. NK cells were then classified based on their expression patterns of CD56/CD16 (conventional) and CD27/CD11b (new).

Results: On average, NK cells constituted 2.99±1.44% of the total lymphocytes in the draining lymph node of patients with BC. The $CD56^{dim}$ and regulatory NK subsets ($CD27^{+}CD11b^{+/-}$) were the predominant old and new NK, respectively. The NK cells significantly increased in patients with at least one involved node (LN^{+}) compared with those with free nodes (LN^{-}; p=0.022). Conversely, $CD56^{dim}CD16^{-}$ subset significantly decreased in higher stages (p=0.032) and in tumors with muscle invasion (p=0.038). Significant variations were also observed in different T-stages (p<0.05). Regarding new classification, the frequency of $CD11b^{+}$ regulatory NK cells was significantly lower in node-positive patients (p=0.025).

Conclusion: These findings emphasize the dynamic nature of NK cell subsets in bladder cancer and their potential relevance in disease progression and management, suggesting potential implications for therapeutic strategies targeting these specific subsets.

Keywords: Bladder Cancer, Draining Lymph Node, Natural Killer (NK) Cells, NK Subsets

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INTRODUCTION

Bladder cancer (BC) is the 10th most prevalent cancer worldwide, with approximately 550,000 new cases and 200,000 deaths annually. It mainly affects older individuals, and its incidence rises with age (1). BC is roughly three times more common in males than females (1, 2). The primary risk factors for BC development are exposure to environmental chemical carcinogens and, to a lesser extent, infection with human papillomavirus (HPV), chronic cystitis, radiation exposure, and treatment with the chemotherapy drug cyclophosphamide. Inactivation of tumor suppressor genes, such as *P53* and *Retinoblastoma* (*RB*), has also been implicated in bladder carcinogenesis through dysregulation of cell cycle control and apoptosis at the genetic level (3).

Management of BC mainly relies on tumor staging, which is determined by the presence of distant metastases, regional lymph node status, depth of muscle invasion, and the level of cell differentiation. In cases with muscle-invasive tumors, the treatment of choice is radical cystectomy with urinary diversion (4). However, primary tumors without muscle invasion, which account for approximately 70% of newly diagnosed cases, are generally managed with transurethral resection of bladder tumor (TURBT) as the first step. However, due to the high rate of recurrence and the transformation of 20% to 40% of tumors into muscle-invasive forms, cystectomy is the only definitive treatment in cases with no response to intravesical therapy (5). These findings highlight the importance of focusing on increasing the effectiveness of intravesical therapy using advanced immunotherapy and chemotherapy regimens. Introducing advanced, effective treatments requires understanding the immune response mechanisms to neoplasms and identifying the microenvironment in which cancer cells have grown and persist.

Natural killer (NK) cells are primarily recognized as a vital component of the innate immune system in the context of anticancer immune responses. Their ability to recognize cancerous cells independently of MHC recognition and to execute rapid, potent attacks by releasing cytotoxic granules underscores the crucial role of NK cells in combating circulating tumor cells and micrometastases (6, 7). Beyond their direct cytotoxic effect, however, they are now widely acknowledged for their immunoregulatory functions and the significant role they play in modulating adaptive immune responses (8). The MHC-independent killing of tumor cells by NK cells, in addition to their presence among tumor-infiltrating lymphocytes in the BC microenvironment, makes targeting NK cells a potential and promising therapeutic approach for advanced anti-tumoral management through monoclonal antibodies or engineered NK cells (9-13).

Although NK cells mediate anti-neoplastic immune responses, they seem to be immunosuppressed in patients with cancer. NK cell abnormalities in such microenvironments can promote tumor cell survival. In addition, substantial evidence indicates that NK cells are a heterogeneous population consisting of various functional subsets. According to conventional classification, two distinct subsets of NK cells have been documented: regulatory $CD56^{bright}CD16^{dim}$, and cytotoxic $CD56^{dim}CD16^{bright}$ NK cells (14, 15). In the context of BC, it has been shown that while $CD56^{dim}$ NK cells were observed to be dominant, they are dysfunctional and more prevalent in higher-stage tumors. On the other hand, $CD56^{bright}$ NK cells are more cytotoxic and have prognostic relevance, making them a promising target in BC treatment (11, 12). In addition, NK cells have recently been categorized into tolerant ($CD27^{-}CD11b^{-}$), cytotoxic ($CD11b^{+}$ $CD27^{-}$), and regulatory ($CD27^{+}$ $CD11b^{+/-}$) cell subsets based on differences in their phenotypes and functions. The tolerant subset, primarily composed of $CD56^{bright}$ NK cells, is characterized by dominant inhibitory signals. In contrast, the cytotoxic subset (mainly $CD56^{dim}$) and the

$NK^{regulatory}$ subset (mostly $CD56^{bright}$) possess dominant activating signals (15, 16). To the best of our knowledge, no study could be found on draining lymph nodes in BC investigating new classification. Therefore, in the present study, we sought to phenotype NK cells in the draining lymph nodes of patients with BC, based on both conventional (CD56/CD16) and new (CD27/CD11b) classification systems.

MATERIALS AND METHODS

Study Design and Patient Selection

This cross-sectional study enrolled 49 BC patients who were newly diagnosed for BC based on their histopathological records and underwent radical cystectomy for pelvic lymph node dissection. The patients with a history of chemotherapy, radiotherapy, and immunotherapy were excluded. They were recruited from hospitals affiliated with Shiraz University of Medical Sciences, Shiraz, Iran. The Institutional Review Board reviewed and approved the study (IR.SUMS.MED.REC.1402.199).

Isolation of Mononuclear Cells from Lymph Nodes

Pelvic lymph nodes (one node per patient) were obtained during radical cystectomy as part of routine pathological evaluation. To obtain a single-cell suspension, lymph nodes were mechanically minced into small pieces in a complete culture medium containing 10% fetal bovine serum (FBS, Gibco, USA) and filtered through a 40-µm cell strainer (SPL Life Sciences, Korea). The mononuclear cells were then separated using Ficoll-Hypaque (Biosera, France) gradient centrifugation, washed, and dissolved in a staining buffer (phosphate-buffered saline containing 2% FBS) for the following cell staining procedure. The Trypan blue dye exclusion test was used to determine the number of viable cells. Samples with over 95% viability were used for further analysis.

Flow Cytometry Analysis

Fluorochrome-conjugated anti-human antibodies, PerCP-CD3 (UCHT1), FITC-CD16 (B73.1), PE-CD56 (5.1H11), PerCP.Cy5.5-CD27 (M-T271) (all from Biolegend, USA), and PE-CD56/CD16 and APC-CD11b (ICRF44) (BD Biosciences, USA) were used for immunophenotyping NK cells, their functional subsets, and NKT cells. For this purpose, 2.5×10^5 cells in 50 µl staining buffer were distributed in each flow cytometry tube. The cell suspension was incubated with the adjusted concentrations of antibodies in the test tube. Unstained cells were used as the controls. The cells were then subjected to flow cytometry analysis using a four-color FACSCalibur flow cytometer (BD Biosciences). At least 100,000 events were acquired per sample. The FlowJo™ v10.8 software (BD Life Sciences) was subsequently used to analyze the data.

Gating Strategy

Representative flow plots illustrating the gating strategy are shown in Fig. 1. To determine the frequency of total NK cells and their functional subsets, first lymphocytes were gated based on their relative size (FSC-H) and granularity (SSC-H) (a). Total NK cells were then identified as $CD3^-CD16^+/CD56^+$ lymphocytes (b). NKT cells were phenotyped as $CD3^+CD56^+/CD16^+$ cells among lymphocytes (b). Based on the old classification system, $CD56^+$, $CD16^+$, and $CD16^+CD56^+$ subsets as well as $CD56^{bright}$, $CD56^{dim}$, and their CD16 expressing subsets were further determined within the CD16+/$CD56^+$ lymphocytes (c, d). For the new classification, due to the restriction in the number of fluorochromes and in the absence of CD3, NK subsets were delineated based on the expression of CD27 and CD11b markers within the total NK/NKT cell population (e) as follows: cytotoxic ($CD27^-CD11b^+$), tolerant ($CD27^-CD11b^-$), and regulatory ($CD27^+CD11b^{+/-}$) subsets (f). The frequency of each NK subset was reported in NK/NKT population.

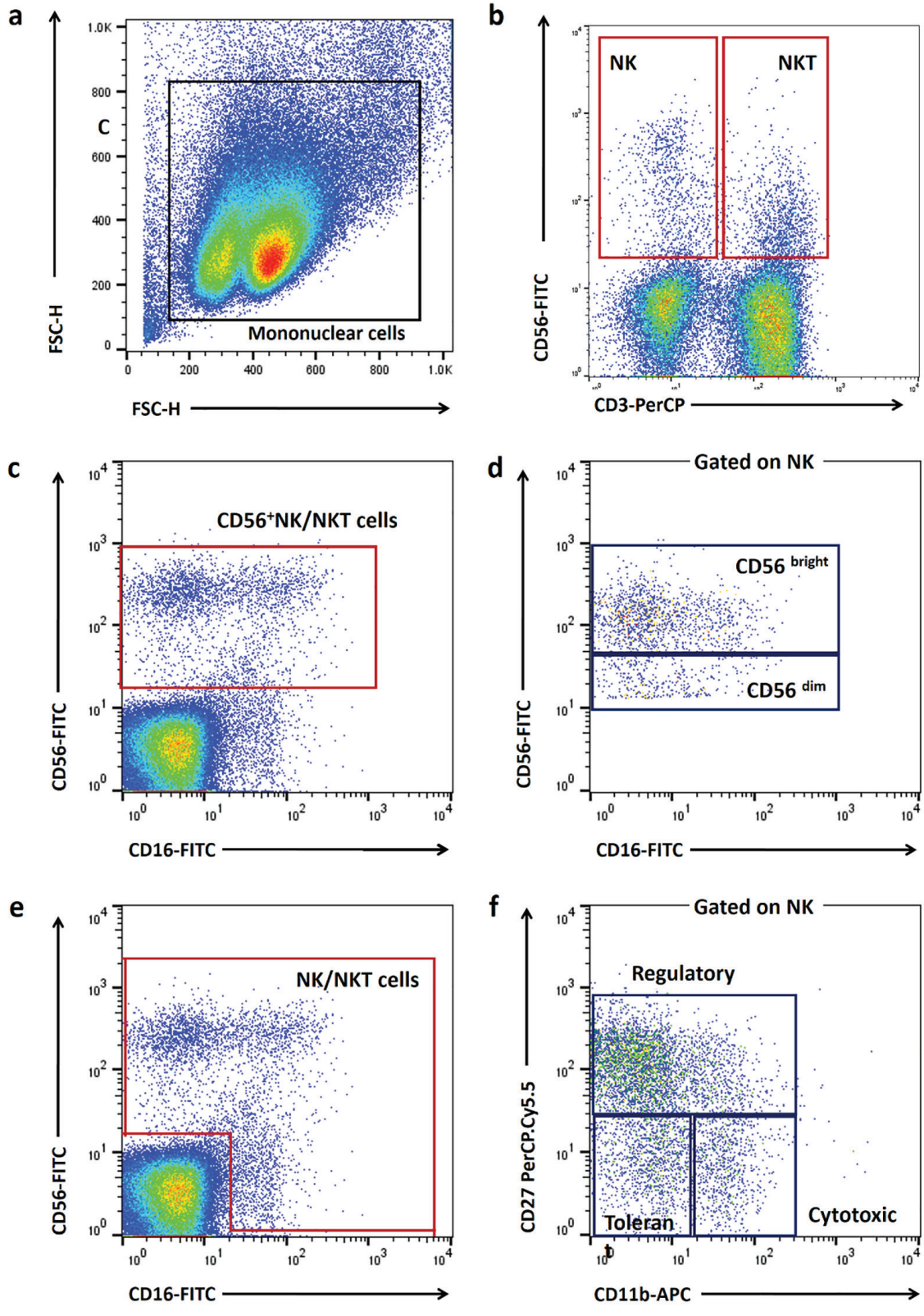

Fig. 1. Representing plot of NK cell subsets phenotype. To phenotype NK cells and their subsets, first lymphocytes were gated based on their relative size (FSC-H) and granularity (SSC-H) (a). Total NK cells were then identified as $CD3^{-}CD16^{+}/CD56^{+}$ lymphocytes and NKT cells were phenotyped as $CD3^{+}CD56^{+}/CD16^{+}$ cells (b). $CD56^{bright}$ and $CD56^{dim}$ NK cells along with their CD16 expressing subsets were determined in $CD56^{+}$ NK cells (c, d). New subsets were delineated among total NK/NKT cells (e) based on the expression of CD27 and CD11b markers as follows: cytotoxic ($CD27^{-}CD11b^{+}$), tolerant ($CD27^{-}CD11b^{-}$), and regulatory ($CD27^{+}CD11b^{+/-}$) subsets (f).

Statistical Analysis

Statistical analyses were conducted using SPSS version 20 software (SPSS GmbH Software, Germany). Nonparametric Mann-Whitney U or Kruskal-Wallis tests were used to compare the frequencies of total NK cells and their subsets among the patients with different clinicopathological groups. $P<0.05$ was considered significant. GraphPad Prism version 9 for windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com) was used to depict the figures.

Table 1. Clinicopathological characteristics of the patients with bladder cancer

Characteristics		N (valid percentage)
Gender	Male	42 (85.7%)
	Female	7 (14.3%)
Tumor Type	Urothelial Carcinoma	49 (100%)
Histological Grade	Low	3 (6.3%)
	High	45 (93.8%)
	Unreported	1
T-stage	T1	4 (8.3%)
	T2	26 (54.2%)
	T3	8 (16.7%)
	T4	10 (20.8%)
	Unreported	1
Lymph Node Involvement	Positive	13 (26.5%)
	Negative	36 (73.5%)
N-stage	N0	36 (73.5%)
	N1	4 (8.2%)
	N2	8 (16.3%)
	N3	1 (2%)
TNM-stage	I	4 (8.2%)
	II	19 (38.8%)
	III	25 (50.0%)
	IV	0
	Unreported	1
OCT	OCT	28 (58.3%)
	non-OCT	20 (41.7%)
	Unreported	1
Muscle Invasion	Positive	44 (91.7%)
	Negative	4 (8.3%)
	Unreported	1
PVFI	Positive	16 (33.3%)
	Negative	32 (66.7%)
	Un-reported	1
LVI	Positive	15 (31.9%)
	Negative	32 (68.1%)
	Unreported	2
PNI	Positive	25 (54.3%)
	Negative	21 (45.7%)
	Unreported	4
Tumor Necrosis	Positive	21 (60%)
	Negative	14 (40%)
	Unreported	14
Age (years)	Mean±SD	64.24±11.57

TNM: Tumour, Node and Metastasis; OCT: Organ Confined Tumor; PVFI: Perivesical Fat Invasion; LVI: Lymphovascular Invasion; PNI: Perineural Invasion

RESULTS

Demographics and Histopathological Features of Patients

A total of 49 patients with BC were included in this study. The mean age was 64.24±11.57 years (range, 39–83 years). The majority of cases were male (n=42, 85.7%). All the patients had a final diagnosis of urothelial carcinoma. In terms of TNM stage, the patients were mostly in stage III (n=25, 51.0%), followed by stage II (n=19, 38.8%). Nodal involvement was observed in 26.5% of cases (n=13). The detailed histopathological features of the study population are shown in Table 1.

NK Cells Distribution in Tumor Draining Lymph Nodes of Patients with Bladder Cancer

The frequencies of different NK subsets are shown in Table 2. Phenotype analysis revealed that, on average, 2.99±1.44 percent of lymphocytes in the draining lymph nodes of patients with BC exhibited the NK cell phenotype (expressing CD16, CD56, or both while being negative for CD3). Among the lymphocytes, 1.05±0.83 percent simultaneously expressed CD3, a pan-marker of T cells, representing NKT cells. According to the conventional and new classifications, all subsets could be found in BC draining nodes. However, the $CD56^{dim}$ and regulatory NK subset ($CD27^{+}CD11b^{+/-}$) were the most prevalent subsets with mean frequencies of 57.54±25.49% and 53.56%±14.32, respectively (Table 2).

We next assessed the associations between the frequency of various NK subsets and tumor characteristics. Our results demonstrated that the frequency of NK cells was significantly higher in draining lymph nodes of the patients with tumor-infiltrated nodes (LN^{+}) compared with the LN^{-} patients (p=0.022, Fig. 2a). In addition, the percentages of total NK/NKT cells and their $CD56^{dim}CD16^{-}$ subset were significantly lower in draining nodes of the patients with muscle invasion (p=0.020 and p=0.038, respectively; Fig. 2b). Additionally, the total frequency of NK/NKT cells was significantly lower in the draining nodes of the patients with TNM stages II (p=0.016) and III (p=0.043) compared with stage I.

Table 2. Frequency of different subtypes of natural killer cells in tumor-draining lymph nodes from the patients with bladder cancer

	NK Cell Subsets	Phenotype	Min	Max	Mean±SD (%)	Median
	Total NK/NKT	$CD16+/CD56^{+}$	1.70	8.45	4.04±1.59	3.81
	NKT	$CD16+/CD56^{+}CD3^{+}$	0.13	4.42	1.05±0.83	0.85
	NK	$CD16+/CD56^{+}CD3^{-}$	1.01	7.06	2.99±1.44	2.59
Conventional	$CD56^{+}$	$CD56^{bright}$	1.39	81.20	41.28±26.30	48.40
		$CD56^{bright}CD16^{+}$	0.26	81.10	11.79±13.27	10.18
		$CD56^{bright}CD16^{-}$	0.97	69.50	30.78±20.09	34.80
		$CD56^{dim}$	19.00	98.70	57.54±25.49	49.90
		$CD56^{dim}CD16^{+}$	11.50	89.90	38.53±23.42	31.20
		$CD56^{dim}CD16^{-}$	2.32	50.40	20.30±10.83	18.20
	Double positive	$CD56^{+}CD16^{+}$	3.98	51.50	20.36±9.59	19.00
	Tolerant/Regulatory	$CD56^{+}CD16^{-}$	0.80	90.10	42.25±17.44	38.30
	Cytotoxic	$CD56^{-}CD16^{+}$	5.92	93.60	35.77±19.17	35.00
New	Cytotoxic	$CD56^{+}/CD16^{+}CD27^{-}CD11b^{+}$	5.18	47.70	24.26±11.55	25.40
	Tolerant	$CD56^{+}/CD16^{+}CD27^{-}CD11b^{-}$	1.16	42.00	18.66±9.17	16.50
	Regulatory	$CD56^{+}/CD16^{+}CD27^{+}CD11b^{+/-}$	17.8	92.2	53.56±14.32	53.20
	$CD11b^{+}$ Regulatory	$CD56^{+}/CD16^{+}CD27^{+}CD11b^{+}$	7.13	52.90	22.77±10.47	21.10
	$CD11b^{-}$ Regulatory	$CD56^{+}/CD16^{+}CD27^{+}CD11b^{-}$	5.31	54.00	25.23±12.30	25.30

NK Cell: Natural Killer Cell; NKT Cell: Natural Killer T Cell

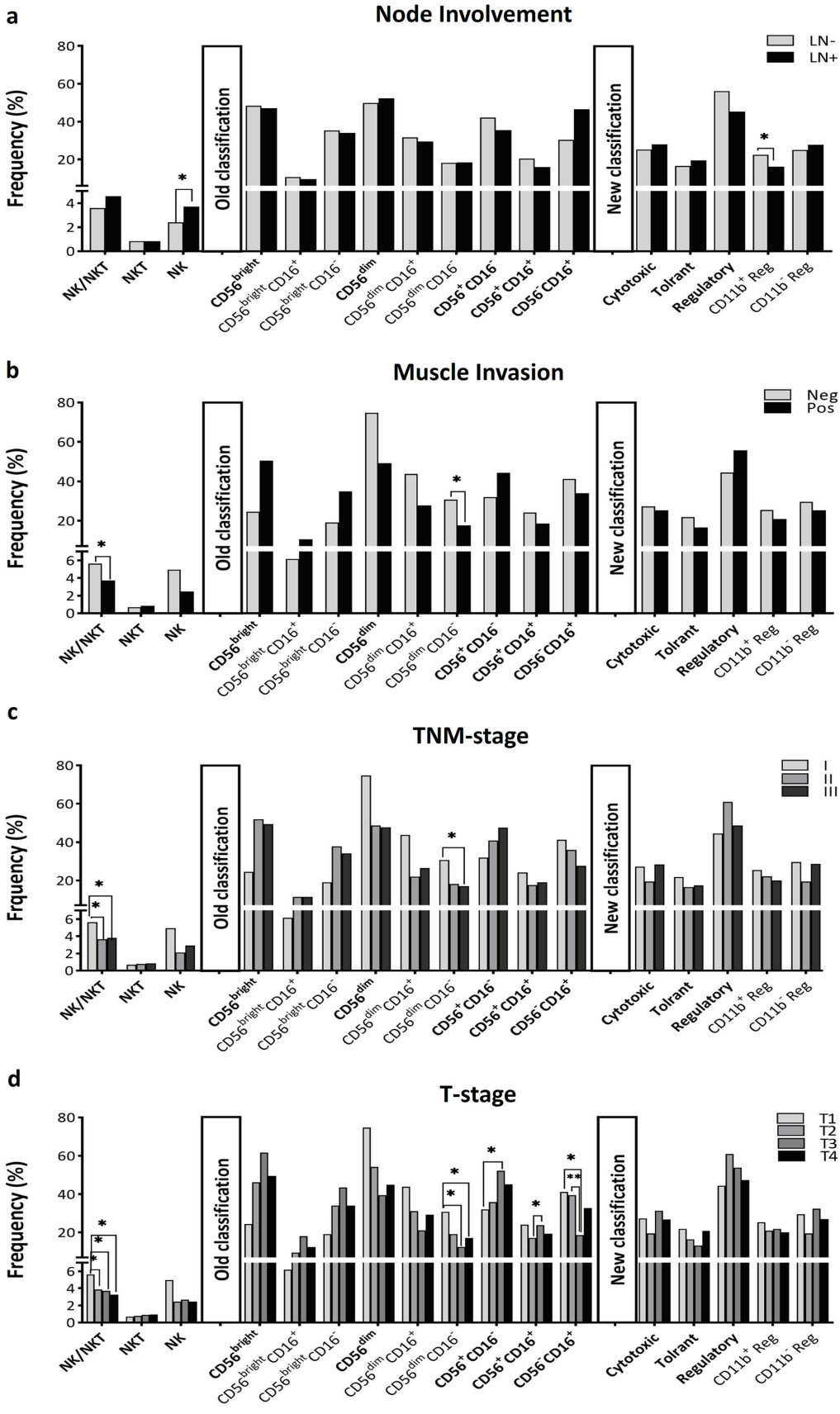

Fig. 2. Association of NK subsets and clinicopathological characteristics of bladder cancer (n=49). NK subsets showed significant variations among the patients with different statuses of regional draining node involvement (a), muscle invasion (b), stages (c), and T-stage (d). The data are presented as median. *Significant at the 0.05 level (2-tailed), **Significant at the 0.01 level (2-tailed).

Furthermore, the percentage of $CD56^{dim}CD16^{-}$ cells was higher in stage I than in stage II (p=0.032), as illustrated in Fig. 2c. Significant variations were also observed in the frequencies of different NK subsets in the patients in different T-stages (p<0.05); however, none of the p-values survived Bonferroni corrections. In general, total NK/NKT cells, $CD56^{dim}CD16^{-}$, and $CD56^{-}CD16^{+}$ subsets were more frequent in lower T-stages. On the other hand, $CD56^{+}CD16^{-}$ and double positive $CD56^{+}CD16^{+}$ were higher in T3, as detailed in Fig. 2d. The patients with tumor necrosis also had decreased frequencies of total NK cells (p=0.022), NKT (p=0.044), $CD56^{dim}$ (p=0.031), and $CD56^{dim}CD16^{-}$ (p=0.020) but increased prevalence of $CD56^{bright}CD16^{-}$ (p=0.031) (data not shown). In addition, perivesical fat invasion showed positive associations with the higher frequencies of $CD56^{+}CD16^{+}$ (p=0.043) and $CD56^{+}CD16^{-}$ (p=0.022) populations while demonstrating a negative association with $CD56^{-}CD16^{+}$ (p=0.007) (data not shown). Double-positive NK cells also significantly increased in the patients with perineural invasion (p=0.041) (data not shown).

In the case of the new classification, we observed that the frequency of $CD11b^{+}$ regulatory NK cells was significantly lower in the patients with tumor-infiltrated lymph nodes (LN^{+} patients) than in those without tumor metastasis (LN^{-}, p=0.025, Fig. 2a). Further analysis revealed that the frequencies of this subset, as well as total regulatory NK cells, tended to be decreased in the patients with free nodes compared with those in the N2 group (two or more involved node) (non-adjusted p=0.094 and p=0.054, respectively). The $CD11b^{+}$ regulatory subset was also higher in the patients with organ-confined tumors (p=0.015) and those without necrosis in their tumor tissues (p=0.016) (data not shown). No further associations were found between the frequency of NK subsets and other clinicopathological and demographic features of the patients.

DISCUSSION

This study assessed the frequency and clinical implications of different NK cell subsets in tumor-draining lymph nodes of the patients with BC. Our analysis revealed that the $CD56^{+}CD16^{-}$, representing the tolerant/regulatory subset in conventional classification, and the $CD27^{+}CD11b^{+/-}$ regulatory NK cells in the new system were the predominant NK/NKT populations in the draining node of BC. We further demonstrated that the frequencies of total NK/NKT cells and their cytotoxic subsets significantly decreased with tumor progression (higher tumor stage, invasion into the muscle, and necrotic tumors). Intriguingly, there was an increase in the percentage of NK cells (with negative expression of CD3) in the patients with tumor-infiltrated nodes.

Nearly 4 percent (1.7-8%) of total lymphocytes in BC draining lymph nodes exhibited NK/NKT phenotypes as indicated by the expression of CD56, CD16, or both. The lower frequency of these cells showed negative associations with higher rates of muscle invasion, higher T- and TNM stages, and necrotic tumors. However, considering NK cells as CD3 negative population, a positive association was observed with involved nodes. To the best of our knowledge, there is no study on NK cells in the draining lymph nodes of BC; the existing literature is limited and mostly focuses on exploring the role of these cells in peripheral blood and tumor tissues. The results of these studies as well as studies on other solid tumors generally indicated a reduction in NK cell activity and its cytotoxicity with tumor progression (11, 12, 17, 18). However, Krpina et al. observed that patients who later experienced recurrence of non-muscle invasive BC within the first two years after surgery had significantly higher bladder wall stromal NK cells at the time of initial diagnosis than the non-recurrent cases. However, this difference was limited to the patients with early-stage (Ta) disease,

those with fewer tumors, and those with smaller tumors (<3 cm) (19). These findings collectively suggest that the infiltration of NK/NKT cells in draining lymph nodes or tissues can provide a potent immune response against BC in its early stages. It is crucial to note, however, that NK cells constitute a heterogeneous population with distinct functional subsets exhibiting both regulatory and cytotoxic effects (15, 16).

NK cells are conventionally classified into two main groups based on the expression levels of CD56 and CD16 surface markers. The first subset, $CD56^{bright}$ NK cells, is characterized by high expression of CD56 (also known as neural cell adhesion molecule, NCAM) and low or no expression of CD16 (FcγRIIIa) ($CD56^{bright}CD16^{dim/-}$). They show lower cytotoxicity but possess significant immunoregulatory and cytokine-producing capabilities. The second subset, $CD56^{dim}$ NK cells, express low levels of CD56 but high levels of CD16 ($CD56^{dim}CD16^{+}$). They are highly cytotoxic and responsible for directly killing target cells. Other subsets, including $CD56^{dim}CD16^{-}$ and $CD56^{-}CD16^{bright}$, constitute a small percentage of the NK cell population. $CD56^{dim}$ cells are typically found in peripheral blood and tissues, while the $CD56^{bright}$ subset is supposed to be more prevalent in secondary lymphoid tissues, including lymph nodes (14, 15, 20). However, in our study, the $CD56^{bright}$ and $CD56^{dim}$ phenotypes had relatively similar frequencies, constituting 41% and 57% of the total NK/NKT population in BC draining lymph nodes, respectively. Our results also indicated that a $CD16^{-}$ subset of $CD56^{dim}$ cytotoxic NK cells, was more prevalent in the patients without muscle invasion and decreased with tumor progression in higher stages. Regarding other subsets, the $CD56^{bright}$ regulatory NK cells seemed to be associated with an indicator of worse prognosis since they are more frequent in necrotic tumors. Similar associations have also been reported in breast cancer (21). However, further studies are needed to elucidate the functional implications of this observation on BC. Controversially, the Svatek group observed that intratumoral BC $CD56^{dim}$ NK cells were less cytotoxic and accumulated in higher-stage tumors. In comparison, NK subsets with high expression of CD56 ($CD56^{bright}$) exhibited a more activated phenotype and enhanced production of interferon-gamma (IFN-γ) with a favorable prognostic significance (11, 12). These controversial results, along with the observations that non-specific expression of CD56 in bladder tumors, irrespective of the type of expressing cells, were associated with poor survival (12), indicating that using one or two markers i.e. CD56 is not enough; additional reliable markers are needed for determining the functional NK subsets.

Recent studies have proposed another phenotypic classification system for NK cells based on the expression of the CD27, a member of the TNF-receptor family, and CD11b, representing different developmental stages of NK cells. Based on the differential expression of these markers, NK cells can be divided into four phenotypic subsets and three functional groups: regulatory ($CD27^{+}CD11b^{-/+}$), cytotoxic ($CD27^{-}CD11b^{+}$), and tolerant ($CD27^{-}CD11b^{-}$) NK cells (15, 16). Our findings showed that BC-draining lymph nodes contain all NK subsets. However, these subsets might not be present simultaneously in one patient. Regulatory NK cells (more than 50%), followed by the cytotoxic NK subset (around 25%), were the dominant subsets in BC lymph nodes. Importantly, due to restriction in the number of antibodies, we could not incorporate CD3 as a negative lineage marker in our analysis. Thus, a portion of these identified cells is probably NKT cells. In addition, prognostic analyses revealed that the infiltration of tumor cells into draining lymph nodes was associated with a considerably lower frequency of regulatory $CD11b^{+}$ NK subset compared with those without metastasis. Regarding the immunomodulatory capabilities of $CD11b^{+}$ subset, this decline may reflect a phenotypic shift during tumor progression

toward a state of tolerance. To the best of our knowledge, no study has been conducted on the new classification of NK subsets in BC. Nevertheless, a study by Jin et al. on tumor-infiltrating NK cells in non-small cell lung cancer identified a substantial accumulation of $CD11b^{-}CD27^{-}$ tolerant subset, also known as double negative (DN) NK cells, within tumor tissues (22). Tolerant and regulatory subsets were also more frequent than cytotoxic subset in breast tumor tissues (21). These NK cells appeared to be immature and inactive, and their positive associations with higher stages and tumor size suggested that tolerant NK cells might be dysfunctional or exhausted cells in the context of cancer, leading to tumor progression through impaired immunosurveillance (22). These findings, however, call for more future research on BC and other solid tumors.

CONCLUSIONS

Our analyses collectively indicated that the total frequency of NK/NKT cells, regardless of their phenotypes, was associated with a favorable prognosis in BC. However, NK cells showed a negative association. Increased NK cells with regulatory phenotype along with lower $CD56^{dim}$ cytotoxic cells in more advanced tumors, in addition to the fact that NK cell activity is firmly regulated by a balance in activating and inhibitory environmental signals, might imply a shift in NK cell phenotype from cytotoxicity to regulatory during tumor growth. This change could occur due to NK suppression in advanced tumors, as shown in BC and other cancers (17, 22-25). The small sample size and the cross-sectional nature of our analysis, along with the limitations of using only four fluorochromes in a single tube, restricted the comprehensive functional phenotyping of NK cells. Yet, our findings highlight the clinical relevance of NK cells and their subsets in the immune response against BC and the potential of NK cell-based immunotherapies in BC treatment.

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AUTHORS' CONTRIBUTION

AA was the principal investigator involved in study design, investigated and supervised the findings of this work. EK analyzed data and provided manuscript draft. AM provided clinical samples. ZF was the principal investigator involved in study design, supervision of lab experiments and data analysis. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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