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ORIGINAL ARTICLE

GM-CSF-producing lymphocytes in tumor-draining lymph nodes of patients with bladder cancer

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ABSTRACT. Background: **Bladder cancer (BC) is the tenth common cancer worldwide. Despite progress in treatment and the use of chemotherapoeutic drugs, the survival rate of BC patients is still low. Manipulation of the immune system was recently introduced as an interesting alternative treatment for this immunogenic cancer with fewer side effects. Accordingly, in the present study, we assessed the frequency of GM-CSF-producing lymphocytes in tumor-draining lymph nodes (TDLNs) of BC patients and evaluated their relationship with clinicopathological factors and survival rate.**
Methods: **Fifty-four patients with BC who had received no treatment were recruited. Mononuclear cells were isolated from fresh homogenized lymph nodes by centrifugation over Ficoll-Hypaque, activated and subsequently analyzed by flow cytometry for the cell surface expression of CD4 and CD8 and the intracellular production of GM-CSF.**
Results: **Flow cytometric analysis revealed that 4.97 $\pm$ 2.7% of lymphocytes in TDLNs of patients with BC produced GM-CSF. The mean frequency of GM-CSF-producing cells was 5.5% among $CD4^+$ lymphocytes and 11.7% in the $CD8^+$ population. Elevated frequencies of GM-CSF-producing lymphocytes, as well as a higher production of GM-CSF by CD4+ lymphocytes was observed in the patients with tumor-free lymph nodes, as compared to those with at least one tumor-infiltrated lymph node ($p < 0.05$). On the other hand, the lower frequency of GM-CSF-producing $CD4^+$ lymphocytes (ThGM) was associated with improved overall, but not one-year, survival. No other significant relationship was observed between clinicopathological parameters and the frequency of GM-CSF-producing subsets.**
Conclusion: **Collectively, our findings suggest a protective role for GM-CSF in the early stages of BC; however, the unfavorable association of ThGM frequency with survival rate may imply a more complex role for this cytokine in BC.**

Key words: Bladder cancer, GM-CSF, $CD4^+$, $CD8^+$, ThGM, TcGM

INTRODUCTION

Bladder cancer (BC), the cause of nearly 200,000 deaths with more than 540,000 new cases diagnosed annually, is classified as the tenth common cancer in both sexes [1]. Although the recent treatments and chemotherapy drugs have failed to significantly improve the survival rate, BC responds well to immunotherapy. BC is a highly immunogenic tumor with a high mutation rate triggering the immune responses [2]. Intravesical administration of Bacillus Calmette-Guérin, the first nonspecialized immunotherapy that received the United States Food and Drug Administration (FDA) approval for BC, stimulates the immune system and decreases the recurrence rate [3]. In addition, following the introduction of the immune-checkpoint inhibitors the immunotherapy approaches have become more prominent and blocking agents for programmed cell death protein-1 (PD-1) and its ligand programmed death-ligand-1 (PD-L1) were approved for late stage of BC [4].

To date, several subsets have been introduced in the heterogeneous family of $CD4^+$ T cells, including both helper (Th1, Th2, Th9 Th17, Th22, T follicular helper (Tfh) and Th25) and regulatory (Treg) subsets with unique cytokines, transcription factors and effector functions [5]. Recently, a new distinct subset of helper T cells, which produce abundant amount of granulocyte-macrophage-colony-stimulating factor (GM-CSF), has been also introduced by Zhang group as potential GM-CSF-producing T helper (ThGM) cells [6]. It is considered as a stable subset with an approximate frequency of 2% among other helper subsets and is characterized by the surface expression of chemokine receptors such as CCR10, CCR4, CCR6 and CXCR3 [7]. Differentiation of naïve T cells toward ThGM is supposed to be mediated

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through signal transducer and activator of transcription 5 (STAT5) signaling and depends on IL-7 and IL-2 in the absence of other cytokines [6, 8]. ThGM is more prone to apoptosis and activation-induced cell death (AICD) that can be rescued by inhibition of Fas/FasL interaction. This subset is suggested to primarily play a role in the initial phase of the immune responses [6]. ThGM also produces a high amount of GM-CSF, a pluripotent cytokine with the potential of both humoral and cellular immunity induction. However, other cells including macrophages, endothelial cells, and alveolar epithelial cells also produce GM-CSF.

GM-CSF is recognized as an immune-modulatory cytokine with an essential role in the survival and activation of myeloid cells [9]. It enhances antigen-induced immunity by promoting dendritic cell differentiation, M1 macrophage polarization, antigen presentation, phagocytosis and the recruitments of monocytes and other myeloid populations from bone marrow to the circulation [10]. It has been also shown that GM-CSF promotes cytokine production from the helper and cytotoxic T cells as its absence leads to impairment in cytokine production by Th1 and Th2 [6]. Besides, GM-CSF regulates the differentiation of naïve T cells toward Th2 cells independent of IL-4 [11, 12]. But recent researches show the contradictory roles of GM-CSF in the context of tumors. While some studies reported an antitumor role for this cytokine, a number of investigations showed its association with poor prognosis [13]. Antitumor activity of GM-CSF has been commonly attributed to its role in the recruitment and activation of dendritic cells to present tumor-associated antigens to $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes. It activates other immune cells including granulocytes, macrophages and NK cells. However, migration and proliferation of cancer cells were also reported to be stimulated in the presence of GM-CSF in different cancer types [13]. To the best of our knowledge, there is no study investigating this cytokine or corresponding subsets in the draining lymph nodes of BC. Therefore, in the present study, we assessed the frequency as well as clinical relevance of $CD4^+$ helper and $CD8^+$ cytotoxic cells producing GM-CSF (ThGM and TcGM) in tumor-draining lymph node (TDLNs) from patients with BC.

METHODS

Cases

In the present study, 54 patients with BC referred to the hospitals affiliated to Shiraz University of Medical Sciences between 2014 and 2017 for surgical operation were recruited. The patients received no treatment, that is, chemotherapy, radiotherapy and immunotherapy. Their cancer was confirmed based on pathology reports. All participants signed informed consents and Shiraz University Ethics Committee approved the study (IR.SUMS.MED.REC.1398.72).

Isolation of mononuclear cells

A fresh part of lymph nodes dissected from the pelvic region during surgery was transferred to the laboratory in complete culture medium [RPMI 1640 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Gibco, USA)]. The samples were washed with 1x phosphate-buffered saline (PBS), mechanically minced into small pieces with a surgical scalpel blade and filtered through a 40 μm cell strainer (SPL Life Sciences, South Korea) to obtain a single-cell suspension. The mononuclear cells were then separated using Ficoll-Hypaque (Lymphodex: Innotrain, Germany) gradient centrifugation, washed two times with 1x PBS, counted and then prepared in optimal concentrations for further analysis.

Cell activation, staining and flow cytometry analysis

To determine the GM-CSF production by lymphocytes, cells were first activated with 50 ng/ml Phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml Ionomycin in complete culture media for 5 hours at 37 °C. To prevent cytokine secretion, Brefeldin A and Monensin (0.7 μl/ml, both from BD Biosciences, USA) were added to the activation media. Activated cells were then washed with staining buffer (1x PBS containing 3% FBS and 0.1% sodium azide), fixed by paraformaldehyde 1% (Sigma-Aldrich, Germany) and permeabilized with Perm/Wash buffer (BioLegend, USA). The cells were stained with PerCP-Cy5.5 anti-CD4 (clone: RPA-T4, BioLegend), PerCP-conjugated anti-CD8 (clone: SK1, BioLegend) and PE anti-GM-CSF (clone: BVD2-21C11, BD Biosciences). The stained cells were washed and fixed to be analyzed by a four-color FACSCalibur flow cytometer (BD Biosciences). The data were analyzed by FlowJo software (version 7.6.5, Ashland, OR, USA). Based on their forward and side scatters, lymphocytes were selected and then $CD4^+$ and $CD8^+$ were gated in the lymphocyte population. The percentages of GM-CSF-producing cells were determined in total lymphocytes, $CD4^+$ and $CD8^+$ T cells. Geometric mean fluorescence intensity (gMFI) of GM-CSF was assessed in each subset and normalized using the gMFI of the negative population and considered as a criterion for the expression level of this cytokine.

Statistical analysis

Data were statistically analyzed by using SPSS (version 20, SPSS Inc., USA). Nonparametric Mann–Whitney U and Kruskal–Wallis H tests were used to compare the frequency of cells between two and more groups, respectively. After obtaining significant results through Kruskal–Wallis H, post hoc Dunn's multiple comparison test was applied to find the difference between the two groups in multiple comparisons. Based on the ROC curve, cutoffs were defined for quantitative data to classify them into high and low groups. The correlations between the prevalence of cell subsets with each other and with the patients' age were assessed by Spearman rank correlation. Survival analysis was done by univariate Kaplan–Meier and multivariate Cox proportional regression. *P* values less

than 0.05 (two tailed) were considered statistically significant. Graphs were depicted using GraphPad Prism 6 software (San Diego, CA, USA).

RESULTS

Clinical and pathological characteristics of the patients

In the present study, 54 patients (46 males and 8 females) with the mean age of 64.17 ± 11.85 years old were enrolled. According to the TNM stage most patients (n = 21, 39.6%) were reported to be in stage II. Other features of tumors such as histological grade, lymph node involvement, T and N stages, tumor necrosis, carcinoma in situ, invasion of tumor to the adjacent muscle, perivesical fat, perineural, lymphovascular, and lamina propria were also collected and included in the analysis (summarized in *table 1*).

Table 1
Clinical and pathological characteristics of patients with bladder cancer

Characteristics	Value (%)*
Age (years)	64.17 ± 11.85
Gender	
Male	46 (85.2)
Female	8 (14.8)
Histological grade	
Low	8 (15.7)
High	43 (84.3)
Unreported	3
Stage	
1	5 (9.6)
2	22 (42.3)
3	9 (17.3)
4	16 (30.8)
Unreported	2
Lymph node status	
Free	38 (71.7)
Involved	15 (28.3)
Unreported	1
T stage	
T1	5 (9.6)
T2	31 (59.6)
T3	7 (13.5)
T4	9 (17.3)
Unreported	2
Lymphovascular invasion	
Positive	19 (38)
Negative	31 (62)
Unreported	4
Perineural invasion	
Positive	27 (56.3)
Negative	21 (43.8)
Unreported	6
Muscular invasion	
Positive	47 (90.4)
Negative	5 (9.8)
Unreported	2
Perivesical fat invasion	
Positive	16 (30.8)
Negative	35 (69.2)
Unreported	3
Tumor necrosis	
Positive	21 (52.5)
Negative	18 (47.5)
Unreported	15
Urothelial CIS	
Positive	15 (40.5)
Negative	22 (59.5)
Unreported	17

* The frequencies were reported as valid percentages omitting missing data.

Frequency of GM-CSF-producing lymphocytes in draining lymph node of patients with bladder cancer

The frequency of GM-CSF-producing cells and the expression intensity of GM-CSF in draining node of BC were determined and reported in total lymphocytes and their $CD4^+$ and $CD8^+$ subsets (*figure 1*). According to flow cytometry analyses, 4.97 ± 2.7% of lymphocytes in TDLNs of patients with BC expressed GM-CSF. The mean frequency of GM-CSF-producing cells was 5.5% among $CD4^+$ lymphocytes and 11.7% in $CD8^+$ population. The frequencies of GM-CSF-producing cells and the gMFIs of this cytokine in different cell subsets are summarized in *table 2*.

Association of GM-CSF-producing lymphocytes with prognostic factors

In the next step, we analyzed the association of GM-CSF-producing $CD4^+$ and $CD8^+$ lymphocytes with BC prognostic factors. Our statistical analyses revealed that the frequency of GM-CSF-producing lymphocytes tended to be higher in patients with free lymph nodes (LN^-) compared to those with at least one involved node (LN^+: 5.0 ± 4.7 vs. 3.7 ± 1.2, P = 0.051). In addition, the GM-CSF expression intensity (gMFI) in $CD4^+$ lymphocytes was significantly higher in LN^-patients compared to LN^+ ones (LN^+; 9.3 ± 5.2 vs. 8.1 ± 3.2, P = 0.047). The gMFI of GM-CSF in $CD4^+$ cells was also associated with node grouping, as the gMFI of GM-CSF was significantly higher in patients with free nodes (N_0) compared to those with at least two affected nodes (N_2, P = 0.006). A trend toward a higher gMFI was also observed in the $CD8^+$ subset of LN^-patients compared to LN^+ ones (P = 0.059). Moreover, the intensity of GM-CSF in $CD8^+$ T cells was significantly higher in patients with stage III than stage IV (P = 0.004). Other prognostic factors including histological grade, T stage, tumor necrosis, carcinoma in situ, invasion of the tumor to adjacent muscle, perivesical fat, perineural, lymphovascular, and lamina propria showed no significant associations with the frequency of GM-CSF-producing cells.

Correlation between different T cells subpopulations

The nonparametric Spearman test was used to assess any correlation between the frequency of GM-CSF-producing $CD4^+$ and $CD8^+$ lymphocytes and the age of patients. The results indicated that TcGM cells were directly correlated with ThGM cells (R = 0.75, P < 0.001). The expression intensity of GM-CSF in $CD8^+$ lymphocytes was also in direct correlation with the gMFI of GM-CSF in $CD4^+$ cells (R = 0.72, P < 0.001, *figure 2*). No significant correlation was

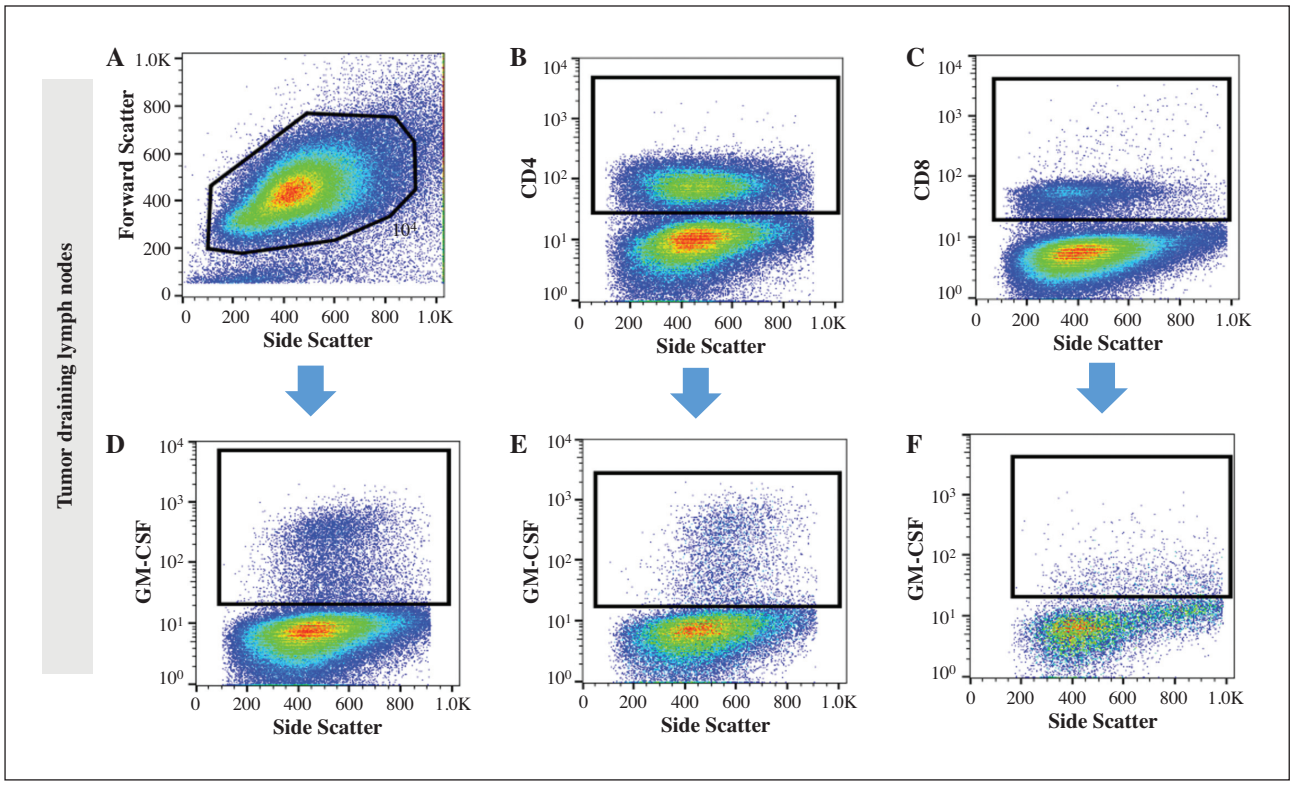

Figure 1
Gating strategy to determine the frequency of GM-CSF-producing $CD4^+$ and $CD8^+$ cells in tumor-draining nodes from patients with bladder cancer. First, lymphocytes were selected based on their size and granularity (A). Then, $CD4^+$ (B) and $CD8^+$ (C) were primarily gated on the lymphocyte population. Finally, the percentages of GM-CSF-producing cells were determined in total lymphocytes (D), $CD4^+$ cells (E) and $CD8^+$ cells (F).

Table 2
Frequency of GM-CSF-producing lymphocytes in draining lymph nodes of patients with bladder cancer

Subset	Mean ± SD	Median (IQR)
GM-CSF$^+$ lymphocytes	4.97± 2.7	4.2 (3.1-6.5)
CD4$^+$ lymphocytes	30.3± 8.2	29.9 (24.1-35)
ThGM	5.5± 4.3	3.6 (2.4-7.1)
CD8$^+$ lymphocytes	5.3± 2.2	5.2 (3.7-6.5)
TcGM	11.7± 7	9.8 (6.3-16.2)
GM-CSF in TcGM	11.8± 24.7	8.2 (6.5-9.6)
GM-CSF in ThGM	11.9± 16.2	9.2 (7.3-11.3)

*The gMFI of positive cells were normalized with the gMFI of negative cells. gMFI: geometric mean fluorescence intensity; IQR: interquartile range; SD: standard deviation; TcGM: $CD8^+$cytotoxic cell producing GM-CSF; ThGM: $CD4^+$ helper cell producing GM-CSF.

observed between the frequencies of GM-CSF-producing cell subsets and the age of patients.

Survival analysis

Patients enrolled in the study were followed up from 41 to 2156 days. Univariate log rank regression test was performed to find the correlation of survival with different prognostic factors as well as the frequencies of different investigated subsets. Next, the variables with $P < 0.2$ were entered to the multiple Cox regression model with backward method. Based on this analysis, lower frequency of ThGM was associated with improved overall survival ($P = 0.047$, 95% CI = 1.01–4.78, hazard ratio = 2.19), whereas such association was not observed in 1-year survival (*figure 3*). No association was observed between other factors and survival rates.

DISCUSSION

To the best of our knowledge, this is the first study that determined the frequency of GM-CSF-producing cells in TDLNs of patients with BC. We observed that 5.5% of $CD4^+$ and 11.7% of $CD8^+$ lymphocytes produced GM-CSF. Investigation of the association of GM-CSF-producing lymphocytes and its subsets with BC prognostic factors revealed that the frequency of

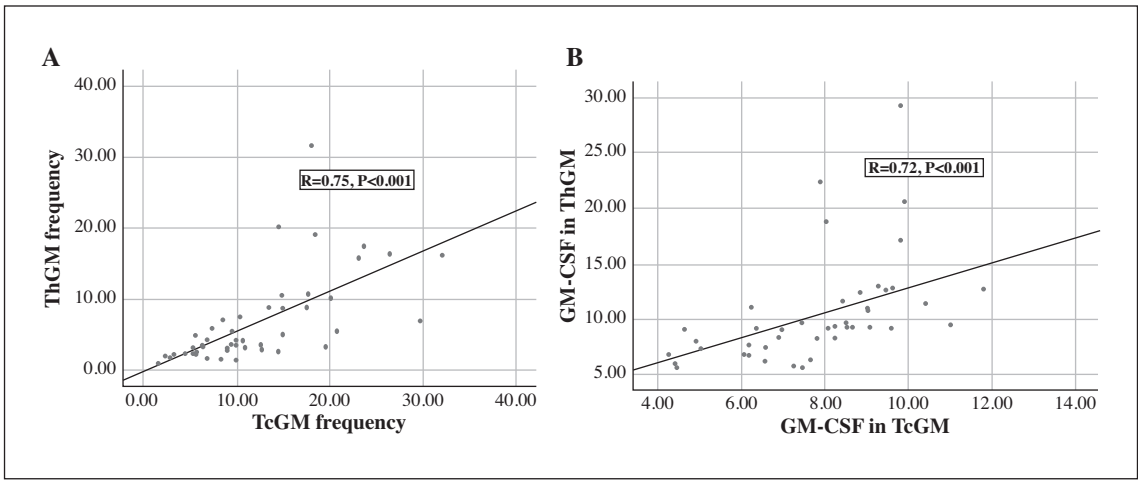

Figure 2
Correlation of frequency (A) and intensity level of GM-CSF (B) between $CD4^+$ and $CD8^+$ lymphocytes producing GM-CSF in patients with bladder cancer. ThGM: $CD4^+$ helper cell producing GM-CSF, TcGM: $CD8^+$ cytotoxic cell producing GM-CSF, MFI: mean fluorescence intensity.

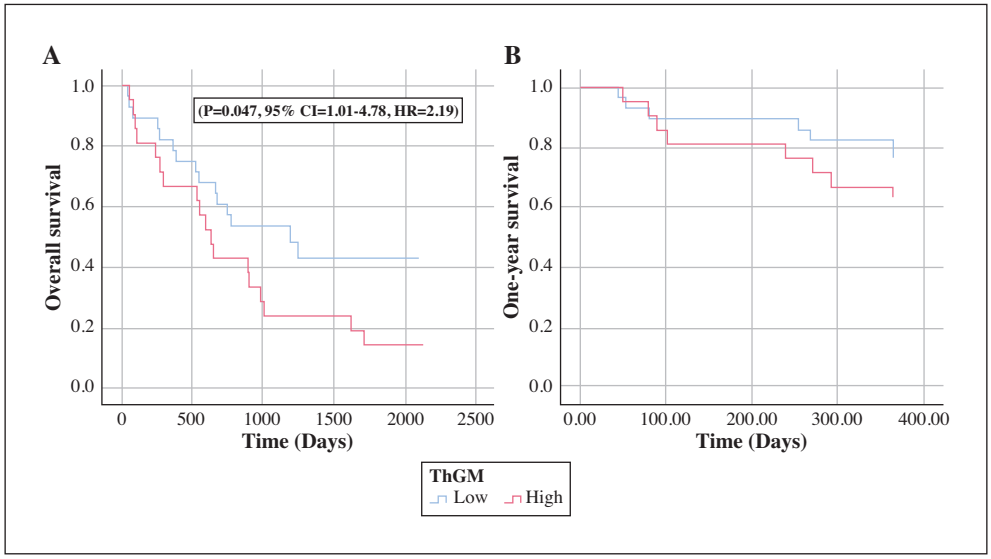

Figure 3
Overall (A) and one-year survival (B) of patients with low and high frequencies of ThGM lymphocytes.

GM-CSF-producing lymphocytes was relatively higher in patients with free nodes. Besides, the expression of GM-CSF in $CD4^+$ lymphocytes was remarkably higher in patients with less progressed tumors. In this regard, we observed more GM-CSF levels in $CD4^+$ and $CD8^+$ subsets in patients with free tumor nodes (LN^-vs. LN^+), those with the lower number of involved nodes (N_0 vs. N_2) and lower stage of the disease (III vs. IV).

GM-CSF is a hematopoietic growth factor with various immunomodulatory effects [14]; however, its prognostic significance in cancer is less investigated. The literature review revealed no study addressing GM-CSF-producing lymphocytes in draining lymph nodes of BC; however, there are rare studies on tumor tissue and urine [15, 16]. Nevertheless, the higher production of GM-CSF in patients with less progressed tumor, those without lymph node involvement or fewer numbers of involved nodes and lower stages suggested a protective role for this cytokine in BC. Consistently, in a recent study, Morizawa et al. reported that GM-CSF expression by BC tumor cells was associated with a decreased risk of recurrence and cancer-specific mortality [15]. Similarly, favorable outcomes and prognosis were observed in the lung, esophageal and colorectal cancers in patients with higher levels of GM-CSF in tissue or serum [17, 18]. It has been also shown that immunotherapy with GM-CSF alone or with chemotherapy could be effective in BC animal models or improve patients' outcomes [19-21]. In a recent study, Hori et al. showed that the administration of GM-CSF in a murine model of BC, contrary to other CSFs, inhibited lymphangiogenesis in the tumor milieu. They also showed that GM-CSF decreased the recruitment of tumor-associated macrophages (M2 type) [22]. Similar results were

obtained in renal cell carcinoma and melanoma [9, 20]. These observations emphasize the role of GM-CSF in the recruitment, differentiation, and enhancement of the antitumor activity of immune cells [23, 24]. Besides, direct tumor inhibition was also proposed for GM-CSF through immune-independent mechanisms [25, 26].

In our study, a significant inverse association was found between the frequency of ThGM and overall survival, while this association was not observed when 1-year survival rate was considered. Regarding the protective role of GM-CSF in preventing lymph node involvement, it could be assumed that GM-CSF may play a protective role in the initial phase of cancer, but chronic secretion of this cytokine promoted inflammation and tumor growth and subsequently led to lower survival. Consistently, Chen et al. mentioned time course as an important factor that influences the role of GM-CSF in colon cancer [27]. GM-CSF level was reported to be higher in the urine of patients with metastatic high-grade bladder tumors compared to nonmetastatic patients and controls [15, 16]. Morizawa et al. also showed that urine GM-CSF level before treatment was correlated with its expression in the tumor and cancer-specific mortality; accordingly, they considered urine GM-CSF an indication of poor prognosis [15]. GM-CSF was also associated with a poor prognosis in head and neck cancer with a positive correlation with the increased recurrence rate [28]. It implies a complicated role for GM-CSF in the context of different tumors or stages, as different doses or its combinations with other cytokines differentially induce and recruit populations with mature or immature phenotypes. In this regard, it has been shown that a high dose of GM-CSF differentiates cells toward suppressive populations, that is, $FOXP3^+$ regulatory (Treg) and myeloid-derived suppressor cells, which in turn impair the antigen-specific T cell responses particularly $CD8^+$ T cells [29-33] or GM-CSF alone or with IL-4 that can enrich different populations of mature and immature antigen-presenting cells [3].

We also found a strong positive correlation between GM-CSF-producing $CD4^+$ and $CD8^+$ cells and GM-CSF expression level in both subsets. This relationship may show a positive loop between TcGM and ThGM in which each subset increases the production of GM-CSF in the other and they enhance the infiltration of each other or other immune cells to inflammatory sites such as tumor milieu [23, 34].

Collectively, our findings suggested a protective role for GM-CSF in the early stages of BC, supported by the observation of higher GM-CSF production in the draining lymph nodes of patients with less progressed tumors. On the other hand, the negative association of the ThGM frequency with the survival rate may imply a more complex role for this cytokine in BC. However, our results should be confirmed by a larger sample size and functional investigations.

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Conflicts of interest. The authors declare no conflicts of interest.

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