Research and Reports in Urology

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ORIGINAL RESEARCH

CD169$^+$ Macrophages Residing in the Draining Lymph Nodes and Infiltrating the Tumor Play Opposite Roles in the Pathogenesis of Bladder Cancer

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Purpose: CD169$^+$ macrophages are considered to enhance anti-tumor immunity by capturing lymph-borne dead tumor cells. The number of CD169$^+$ macrophages in regional lymph nodes (RLNs) is positively correlated with prolonged cancer-free survival in various human cancers. However, a recent study argued against this dogma; that is, CD169$^+$ macrophages infiltrating into the tumor were associated with poor prognosis in patients with breast cancer. To explain this discrepancy, we quantified the number of CD169$^+$ macrophages located in the bladder tumor and RLNs of the same patients and examined their relationship with the 5-year survival rate.

Patients and Methods: Tumor and RLN specimens resected from 40 invasive bladder cancer patients (29 males and 11 females; median age, 70.7 years; range, 49–81 years) who underwent radical cystectomy were evaluated using immunostaining.

Results: The number of CD169$^+$ macrophages in RLNs was associated with a good cancer prognosis, while CD169$^+$ macrophages infiltrating the tumor strongly correlated with a higher incidence of lymphovascular invasion.

Conclusion: CD169$^+$ macrophages play opposing roles in the induction of anti-tumor immunity based on their location in RLNs or tumors.

Keywords: anti-tumor immunity, tumor-associated macrophage, CD169

Introduction

CD169 is a macrophage-restricted cell surface receptor that is conserved across mammals.[1] CD169$^+$ macrophages mainly reside in secondary lymphoid tissues, such as lymph nodes and the spleen,[1,2] and capture blood- or lymph-borne antigens that flow into organs and relay them to neighboring antigen-presenting cells.[3–5] The interaction between CD169$^+$ macrophages and dendritic cells is important for the subsequent activation of antigen-specific adaptive immune responses.

CD169$^+$ macrophages are associated with immune responses against both pathogens and tumor-associated antigens.[6] In a previous study, we reported that a high density of CD169$^+$ macrophages in regional lymph nodes (RLNs) is positively correlated with good cancer prognosis in bladder cancer patients, suggesting their roles in enhancing anti-tumor immunity.[7] However, Casseta's group demonstrated that CD169 is expressed on tumor-associated macrophage (TAM) and those macrophages are associated with shorter disease-specific survival.[8] Therefore, the precise role of CD169$^+$ macrophages in the pathogenesis of cancer remains to be determined in humans.

We hypothesized that CD169$^+$ macrophages residing in draining lymph nodes and tumors drive anti-tumor immunity in opposing directions. To verify this, we retrospectively investigated the numbers of lymph node-resident CD169$^+$ macrophages and tumor-infiltrating CD169$^+$ macrophages in the same bladder cancer patient. Our study demonstrated

Received: 12 September 2022
Accepted: 15 December 2022
Published: 11 January 2023

that although the number of lymph node-resident $CD169^+$ macrophages was associated with good cancer prognosis, the number of tumor-infiltrating $CD169^+$ macrophages was associated with lymphovascular invasion (LVI), which is the essential step in cancer metastasis. This is the first report to investigate the significance of $CD169^+$ macrophage localization in bladder cancer in the same patient. We showed that lymph node-residing $CD169^+$ macrophages contribute to establishing anti-tumor immunity, whereas those in the tumor may support the progression of cancer metastasis.

Materials and Methods

Study Design

This retrospective analysis was conducted in accordance with the Declaration of Helsinki, with the approval of the ethical committees of Omori Red Cross Hospital and Teikyo University Hospital. This study aimed to investigate the association between the abundance of $CD169^+$ macrophages and prognosis in patients with invasive bladder cancer. After obtaining approval from the institutional review boards (Ethics Committee Omori Red Cross Hospital and Teikyo University Ethical Review Board for Medical and Health Research Involving Human Subjects), patients who written informed consented to participate in this study and underwent radical cystectomy for bladder cancer at Omori Red Cross Hospital or Teikyo University Hospital between January 2006 and May 2018 were reviewed.

Patients

Tumor and RLN specimens resected from 40 invasive bladder cancer patients (29 males and 11 females; median age, 70.7 years; range, 49–81 years) who underwent radical cystectomy were evaluated. Patients without RLN dissection were excluded from this study. Bladder cancer death was the endpoint of survival analysis.

Immunostaining

Obturator lymph nodes were examined among the available resected RLNs. Lymph nodes without cancer metastases were used in this study. The deepest invaded portion of the bladder cancer was selected for evaluation. Tumor tissues or RLNs were routinely fixed in 10% neutral buffered formalin and were embedded in paraffin. Thin-slice sections were prepared from the paraffin blocks. After deparaffinization and rehydration, the sections were immersed in a protease solution (Nichirei, Tokyo, Japan) for 10 min at room temperature to retrieve the antigen. They were rinsed twice in phosphate-buffered saline (PBS) and immersed in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. After rinsing with PBS, the sections were incubated with anti-CD169 (clone HSn 7D2; Santa Cruz Biotechnology, Dallas, TX), anti-CD68 (clone PG-M1; Dako, Glostrup, Denmark) primary antibodies for 1 h at room temperature. An isotype-matched mouse IgG (Dako) was used as a negative control. After rinsing in PBS, the sections were treated with peroxidase-labeled secondary antibody (MAX PO; Nichirei) for 30 min and then exposed to diaminobenzidine (DAB) to visualize immunoreactions. The sections were counterstained with hematoxylin and observed under a light microscope (BX53F; Olympus, Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed using JMP 14 software (SAS Institute, Cary, NC). A paired *t*-test was used to compare macrophage numbers between tumors and RLNs. Bivariate comparisons of clinicopathological features between CD169 low cases and high cases were performed using the chi-squared test. The association between multiple prognostic factors and cancer-specific survival was assessed using univariate and multivariate Cox proportional hazard model analyses. Multivariate analysis included pathological T stage, lymph node metastasis, and the number of $CD169^+$ macrophages in the RLNs. Survival curves were calculated using the Kaplan–Meier method, and differences between survival curves were analyzed using the Log rank test. Differences were considered statistically significant at $P < 0.05$.

Results

A total of 40 tumors consisting of one T0, one Tis, two T1, eleven T2, nineteen T3, and six T4 bladder cancers were analyzed. Among these, 28 cases were pure urothelial carcinoma, 5 were urothelial carcinoma with squamous

differentiation, 4 had urothelial carcinoma with glandular differentiation, 1 had squamous cell carcinoma, and 1 had sarcomatoid urothelial carcinoma. In the T0 patient, the tumor obtained from transurethral resection before cystectomy was urothelial carcinoma. Pelvic lymph node metastasis was pathologically confirmed in 12 patients (30.0%). The follow-up period after radical cystectomy ranged from 2.0 to 144.7 months (median 35.3 months). During this period, 16 patients died of bladder cancer. The 5-year cancer-specific survival rate in the study group was 50.6%.

Immunohistochemistry was performed to investigate the expression of CD169 and CD68 in tumors (Figure 1A) and RLNs (Figure 1B) obtained from patients with bladder cancer who underwent radical cystectomy. The numbers of $CD68^+$ and $CD169^+$ macrophages in the tumor and RLNs are summarized in Figure 2.

In all cases, $CD68^+$ macrophages were more abundant in RLNs than in the tumors. Our previous study showed that $CD169^+$ macrophages were rarely observed in the tumors. In this study, we carefully examined the sections and observed

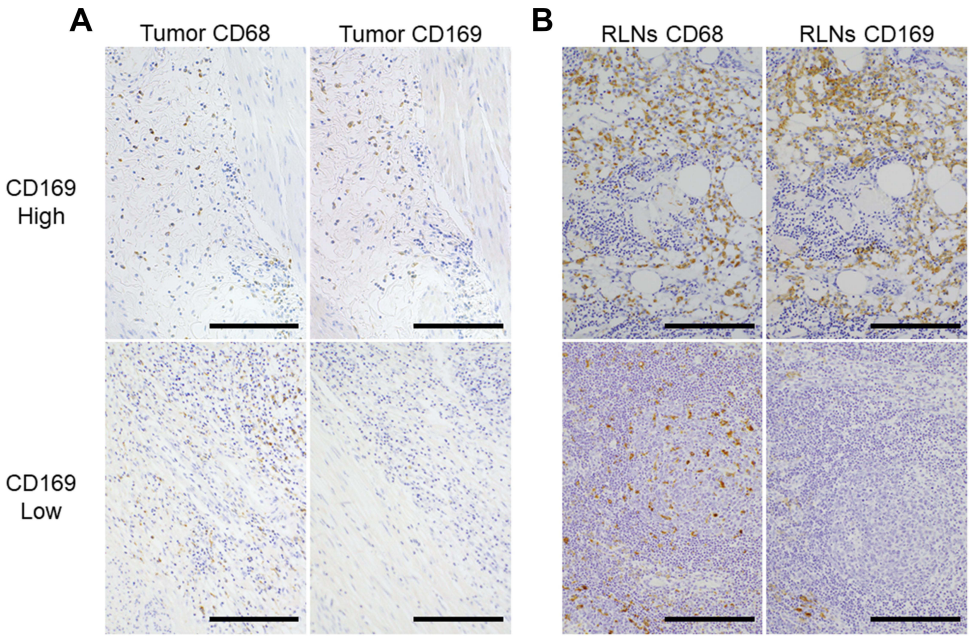

Figure 1 Immunohistochemistry (IHC) for CD68 and CD169 in the bladder tumor and RLNs. Scale bar = 200 μm. Tumors (**A**) and RLNs (**B**) were stained for CD68 (left columns) and CD169 (right columns). In both (**A**) and (**B**), the pair of upper panels is the CD169 High case, and the pair of lower panels is the CD169 Low case.

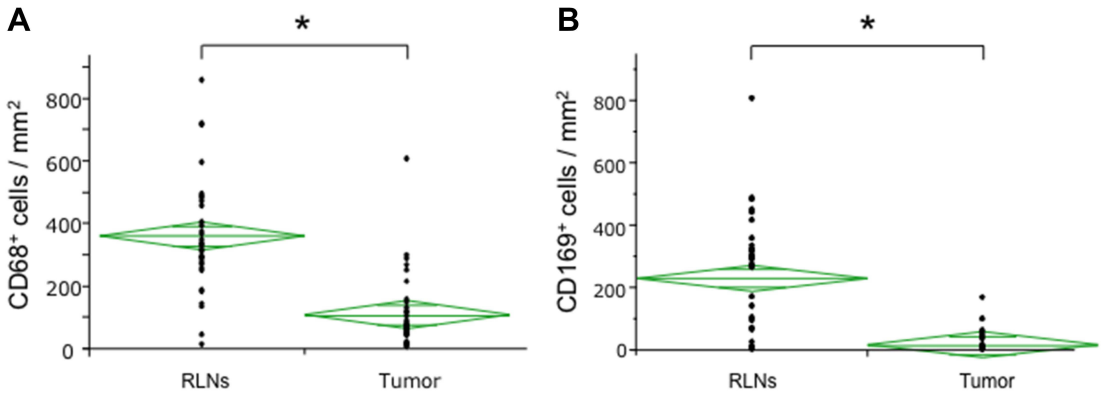

Figure 2 (**A**) Numbers of $CD68^+$ macrophages in RLNs and tumor. $CD68^+$ macrophages were more abundant in RLNs than in tumors significantly (*=$p < 0.0001$). (**B**) Numbers of $CD169^+$ macrophages in RLNs and tumor. $CD169^+$ macrophages were also more abundant in RLNs than in tumors significantly (*=$p < 0.0001$). Although occasional infiltration of $CD169^+$ macrophages in tumor was observed in some cases, the number of CD169+ macrophages in tumor was much smaller than that in the RLNs.

occasional $CD169^+$ macrophage infiltrations in some cases, although the number of $CD169^+$ macrophages in the tumor was much smaller than that in the RLNs.

Next, we analyzed the correlation between clinicopathological features and the expression of CD169 in tumor tissue and RLNs. We classified patients into two groups: CD68/169-low and -high cases, based on the median for macrophages in the RLNs and the 3rd quartile for macrophages in the tumor. The borderlines of CD68-low and -high cases in RLNs, CD169-low and -high cases in RLNs, CD68-low and -high cases in tumor, and CD169-low and -high cases in tumor, were set at 343 cells/mm^2, 282 cells/mm^2, 126 cells/mm^2, and 12 cells/mm^2, respectively. The number of $CD169^+$ macrophages in the tumor was not associated with age, gender, number of total macrophages in the tumor, pathological T stage, presence of lymph node metastasis, number of $CD169^+$ macrophages in RLNs, or history of neoadjuvant chemotherapy. Interestingly, the number of $CD169^+$ macrophages in the tumor was significantly positively correlated with the frequency of LVI ($P = 0.024$, Table 1). The number of $CD169^+$ macrophages in RLNs was not correlated with age, gender, number of total macrophages in RLNs, pathological T stage, the lymph node metastasis, LVI, or history of neoadjuvant chemotherapy (Table 2).

Next, we analyzed the association between clinicopathological features and cancer-specific survival using a Cox proportional hazard model in 40 patients with bladder cancer. In univariate analysis, the pathological T stage was negatively, and the number of $CD169^+$ macrophages in RLNs was positively correlated with favorable cancer-specific survival ($P = 0.037$ and 0.015, respectively, Table 3). In a subsequent multivariate analysis including pathological T stage, lymph node metastasis, and the number of $CD169^+$ macrophages in RLNs, the number of CD169+ macrophages in RLNs was identified as the strongest independent favorable prognostic factor ($P = 0.013$, Table 3).

We also evaluated the prognostic value of $CD169^+$ macrophages in bladder cancer patients. The RLNs CD169-high cases showed a longer 5-year cancer-specific survival rate (80.0%) compared to the RLNs CD169-low cases (30.1%; $P = 0.0073$, Figure 3A). These findings further suggested the importance of RLNs-resident $CD169^+$ macrophages in

Table 1 Associations Between Clinicopathological Features and $CD169^+$ Macrophages in Tumor

Characteristics		n	CD169+ MACs in Tumor		*P*-value
			Low (n=30)	High (n=10)	
Age	< 70	19	16	3	0.195
	≥ 70	21	14	7	
Gender	Male	29	22	7	0.839
	Female	11	8	3	
$CD68^+$ total MACs in tumor	Low	30	22	8	0.668
	High	10	8	2	
Pathological T stage	T0, 1, 2	15	12	3	0.567
	T3, 4	25	18	7	
LN metastasis	No	28	23	5	0.121
	Yes	12	7	5	
$CD169^+$ MACs in RLNs	Low	19	13	6	0.360
	High	21	17	4	
Lymphovascular invasion	Negative	20	18	2	<u>0.024</u>
	Positive	20	12	8	
Neoadjuvant chemotherapy	Not done	33	24	9	0.449
	Done	7	6	1	

Abbreviations: MAC, macrophage; LN, lymph node; RLN, regional lymph node. Underlined numbers represent statistically significant results.

Table 2 Associations Between Clinicopathological Features and the $CD169^+$ Macrophages in RLNs

Characteristics		n	$CD169^+$ MACs in RLNs		*P*-value
			Low (n=19)	High (n=21)	
Age	< 70 ≥ 70	19 21	9 10	10 11	0.987
Gender	Male Female	29 11	15 4	14 11	0.382
Total macrophages in RLNs	Low High	20 20	12 7	8 13	0.112
Pathological T stage	T0, T1, T2 T3, T4	15 25	8 11	7 14	0.567
LN metastasis	Negative Positive	28 12	13 6	15 6	0.836
Lymphovasucular invasion	Negative Positive	20 20	10 9	10 11	0.751
Neoadjuvant chemotherapy	Not Done Done	33 7	17 2	26 5	0.262

Abbreviations: RLN, regional lymph node; MAC, macrophage; LN, lymph node.

Table 3 Associations Between Clinicopathological Features and the Bladder Cancer-Specific Survival

Clinicopathological Feature		n	Univariate Analysis		Multivariate Analysis		
			HR	*P*-value	Hazard Ratio	95% CI	*P*-value
Age (years)	< 70 ≥ 70	19 21	1.81	0.251	ND	ND	ND
Pathological T stage	T0, 1, 2 T3, 4	15 25	3.11	<u>0.037</u>	2.73	0.81–9.19	0.103
LN metastasis	Negative Positive	28 12	2.51	0.070	1.61	0.56–4.64	0.375
$CD68^+$ MACs in tumor	Low High	30 10	1.22	0.728	ND	ND	ND
$CD169^+$ MACs in tumor	Low High	30 10	1.84	0.281	ND	ND	ND
$CD68^+$ MACs in RLNs	Low High	20 20	0.52	0.206	ND	ND	ND
$CD169^+$ MACs in RLNs	Low High	19 21	0.21	<u>0.015</u>	0.20	0.05–0.72	<u>0.013</u>
$CD169^+$ / $CD68^+$ MACs in RLNs	< 0.6 ≥ 0.6	18 22	1.68	0.262	ND	ND	ND

Abbreviations: HR, hazard ratio; CI, confidence interval; ND, not done. Underlined numbers indicate statistically significant results.

improving cancer prognosis. On the contrary, the tumor CD169-high cases showed a shorter 5-year cancer-specific survival rate (40.0%) compared the tumor CD169-low cases (50.3%), although the association was not statistically significant ($P = 0.2573$, Figure 3B).

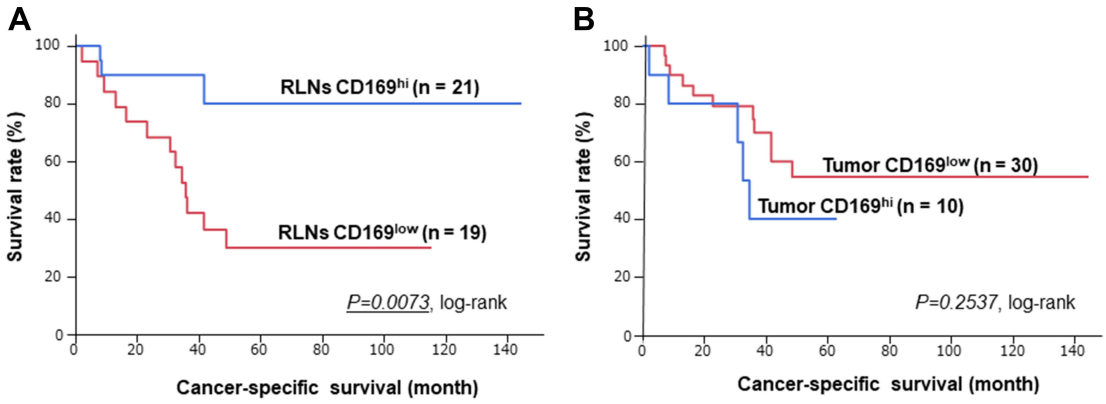

Figure 3 Kaplan-Meier cancer-specific survival curves for patients with bladder cancer. (**A**) Patients were divided into two groups according to the number of $CD169^+$ macrophages in RLNs. (**B**) Patients were divided into two groups according to the number of $CD169^+$ macrophages in the tumor.

Discussion

$CD169^+$ macrophages residing in tumor RLNs have been considered to enhance anti-tumor immunity and contribute to favorable prognosis.[2,7,9–11] However, in this study, we showed that $CD169^+$ macrophages infiltrating tumors are not correlated with longer cancer-specific survival; instead, they promote LVI in bladder cancer. A large number of intratumor $CD169^+$ macrophages tended to positively correlate with poor prognosis, although the association was not statistically significant.

LVI is the essential step in lymph node metastasis and systemic dissemination of cancer cells.[12] It is known as an independent and important poor prognostic factor.[13–15] From this line of evidence, it was reasonable that intratumor $CD169^+$ macrophages positively correlated with short cancer survival in our study. CD169, also known as Siglec1 or sialoadhesin, was originally defined as a tissue-resident macrophage-restricted surface protein that binds sized particles.[1–5] Recently, however, it was found that TAMs also express CD169 in human breast cancer,[8] human bladder cancer,[16] and mouse breast cancer.[17] The $CD169^+$ TAMs in human breast cancer recruit blood-borne monocytes by producing CCL8 and promote tumor metastasis by inducing MMP9 production from tumor cells.[8] This report indicates that CD169 expression in macrophages is not necessarily associated with favorable cancer prognosis; rather, CD169 expression in TAMs is associated with poor cancer prognosis. Our study highlights the bidirectional role of $CD169^+$ macrophages in anti-tumor immunity. We showed that although the number of RLN $CD169^+$ macrophages was linked to a high cancer survival rate, the number of tumor-infiltrating $CD169^+$ macrophages was correlated with higher LVI incidence, which is a crucial step in the invasion-metastasis cascade.

Conclusion

Overall, by analyzing the prognostic significance of $CD169^+$ macrophages in RLNs and tumors in the same patient with bladder cancer, we demonstrated that the two populations of $CD169^+$ macrophages play opposing roles in regulating anti-tumor immunity. Since $CD169^+$ macrophages in RLNs and tumors can elicit different immune responses, it is recommended to distinguish their localization when examining the prognostic correlation with $CD169^+$ macrophages.

Funding

This work has conducted with financial support by a Urological Research Fund of Teikyo University Hospital Mizonokuchi, Tokyo, Japan (Type A).

Disclosure

The authors report no conflicts of interest in this work.

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