CD8+ T cells located in tertiary lymphoid structures are associated with improved prognosis in patients with gastric cancer

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Received August 21, 2019; Accepted February 11, 2020

DOI: 10.3892/ol.2020.11828

Abstract. The presence of tumor infiltrating lymphocytes (TILs) and tertiary lymphoid structures (TLSs) in tumor tissues are of great prognostic significance in several types of human cancer. The present study investigated the density of TILs and TLSs in gastric cancer (GC) tissues and their association with pathological parameters. Moreover, the clinical significance of follicular CD8+ cytotoxic T cells present within the germinal centers of the tumor-associated TLSs was investigated. Immunohistochemistry and H&E staining were used to examine the infiltration and distribution patterns of TILs, TLSs and germinal center (gc) CD8+ TILs in tumor tissues obtained from 63 patients with GC. The number of TILs, TLSs, combination of TILs and TLSs (TILs-TLSs) and gcCD8+ TILs were used to define tumoral immune parameters, and the prognostic value of these parameters was assessed. The analysis revealed that patients with GC with increased levels of TILs, TLSs, or gcCD8+ TILs exhibited improved overall survival. In addition, gcCD8+ TILs levels were significantly associated with patient age, histological grade and pTN stage. Increased levels of TILs-TLSs were positively associated with nerve invasion, tumor thrombus, nodal metastasis and histological grade. Multivariate Cox regression analysis revealed that TILs-TLSs and gcCD8+ TILs were independent prognostic factors. The data obtained in the present study demonstrated that high levels of tumoral immune parameters are important independent prognostic predictors for human GC. The results also suggested a possible role of gcCD8+ TILs in tumor immune surveillance.

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Abbreviations: TILs, tumor infiltrating lymphocytes; TLSs, tertiary lymphoid structures; GC, gastric cancer; Tfc, follicular CD8+ cytotoxic T cells; gcCD8+ TILs, germinal center CD8+ TILs; SLO, secondary lymphoid organs; TME, tumor microenvironment; FDC, follicular dendritic cell; FRC, fibroblastic reticular cell; CT, center of the tumor; IM, invasive margin; pTN, pathological tumor and lymph node; AUC, area under the receiver operating charatcteristic curve

Key words: tumor infiltrating lymphocytes, tertiary lymphoid structures, follicular CD8+ T cells, gastric cancer

Introduction

Previous studies have, demonstrate that immune cell infiltration in human gastrointestinal cancers is associated with cancer progression and is a favorable prognostic predictor (1-5). Both cellular composition and organization of tumor infiltrating lymphocytes (TILs) are crucial for inhibiting cancer progression and are implicated in the success of cancer immunotherapy (1,2,6). Tertiary lymphoid structure (TLSs) are an important source of TILs, characterized by ectopic aggregated lymphocytes with high endothelial venules and have a similar function to secondary lymphoid organs (SLOs). The lymphocytes in TLSs have easy access to tumor antigens as TLSs are not encapsulated and embedded within the tumor microenvironment (5). The numbers of both TILs and TLSs within solid tumor tissues can be used for the assessment of tumor immune surveillance and are important prognostic factors for cancer (7-11).

Although TLSs are thought to be associated with anti-tumor immune responses (12-15), the functional role of their cellular components remains unclear. TLSs are divided into B- and T-zones, which consist of follicular dendritic cells (FDCs) and fibroblastic reticular cells (FRCs), respectively (16). TLSs can be further defined as primary and secondary TLSs, based on the absence or presence of a germinal center in B-cell follicles. Following stimulation by tumor antigens, B cells differentiate and form the germinal center, which is the site

of B cell proliferation, class switching and somatic hyper mutation (17-19). $CD4^+$ $CXCR5^+$ T follicular helper (Tfh) cells are a subtype of $CD4^+$ helper T cells, principally located in germinal centers, and play critical roles in recruiting, activating and regulating the germinal center (20-22). Previous studies suggested that Tfh cells mediate follicular and germinal center formation in TLSs (23,24). In recent studies, $CXCR5^+$ $CD8^+$ T cells were found within the lymphoid follicle, primarily within the germinal center, where they controlled viral replication during chronic HIV and lymphocytic choriomeningitis virus (LMCV) infection (25,26). The $CD20^+$ B cells and $CD8^+$ T cells co-localize in the tumor nest (20) and TLSs (21). This distribution pattern of follicular $CD8^+$ T cells might be involved in antitumor immune responses.

The present study investigated follicular $CD8^+$ T cells in the tumor tissues of patients with gastric cancer (GC). In addition, germinal center $CD8^+$ ($gcCD8^+$) TILs were quantified. The relationship between tumoral immune parameters such as TILs, TLSs, TILs-TLSs and $gcCD8^+$ TILs and clinical pathological parameters was determined.

Materials and methods

Patients and tissue samples. The present study retrospectively analyzed data of patients admitted to the Department of Pathology, Third Affiliated Hospital of Soochow University between 2006 to 2008. The patients were enrolled according to the following criteria: i) Pathologically-confirmed diagnosis of primary GC (adenocarcinoma); ii) did not receive pre-operative chemotherapy or radiotherapy; iii) presence of adequate paraffin-embedded fixed tissue blocks; iv) at least one slide contained the invasive margin of the tumor; and v) availability of complete medical records and follow-up information. A total of 63 patients with GC were included in this present study. Tumor clinical and pathological staging system was based on the Eighth Edition of the Union for International Cancer Control/American Joint Committee on Cancer. Three slides contain cancer tissue were available for each patient, and a total of 189 slides were reviewed in the present study. Patient survival data were available until the end of November 2011. Patient clinical data are presented in Table I. The present study was approved by the Ethics Committee of Soochow University, and complied with the Declaration of Helsinki. Informed consent to use the tissue sample for scientific research was obtained from all patients.

Pathomorphological evaluation of TILs and TLSs. Cancer tissue was fixed in 10% (v/v) formalin and embedded in paraffin until use. The H&E stained slides of the resected GC tissues were reviewed and scored independently by two pathologists who were blinded to the clinical data and prognosis of the patients. The pathologists were trained in the pathomorphological evaluation of TILs and TLSs, and any problematic cases were discussed with them during subsequent scoring. The TILs and TLSs in the center of the tumor (CT) and the invasive margin (IM) were examined (11,27,28). The TILs scoring system incorporated two aspects: i) The number of infiltrating lymphocytes; score 0, no infiltration; score 1, mild infiltration; score 2, moderate infiltration; score 3, extensive infiltration (Fig. 1); and ii) the percentage of the tumor area containing TILs in the CT or IM. The CT-TILs and IM-TILs location scores were defined as the number of infiltrating lymphocytes multiplied by the percentage, and then, the final score was computed by summation of the CT- and IM-TILs scores. TLSs were also evaluated in the CT and IM by measuring two factors:

Table I. Details of parameters in the study cohort.

	Total study cohort	
Parameters	n	%
Sex		
Male	49	77.8
Female	14	22.2
Age, years		
≤50	8	12.7
>50	55	87.3
Tumor size		
≤5 cm	39	61.9
>5 cm	24	38.1
Nerve invasion		
Yes	25	39.7
No	38	60.3
Tumor thrombus		
Yes	22	34.9
No	41	65.1
Nodal metastasis		
Yes	36	57.1
No	27	42.9
Histological grade		
I-II	30	47.6
III	33	52.4
pTN stage		
I	23	36.5
II	13	20.6
III	27	42.9
TILs		
High	44	69.8
Low	19	30.2
TLSs		
High	43	68.3
Low	20	31.7
TILs and TLSs		
High	32	50.8
Low	31	49.2
$gcCD8^+$ TILs[a]		
High	23	41.8
Low	32	58.2

[a]$gcCD8^+$ TILs were obtained in 55 patients. TILs, tumor infiltrating lymphocytes; TLSs, tertiary lymphoid structures.

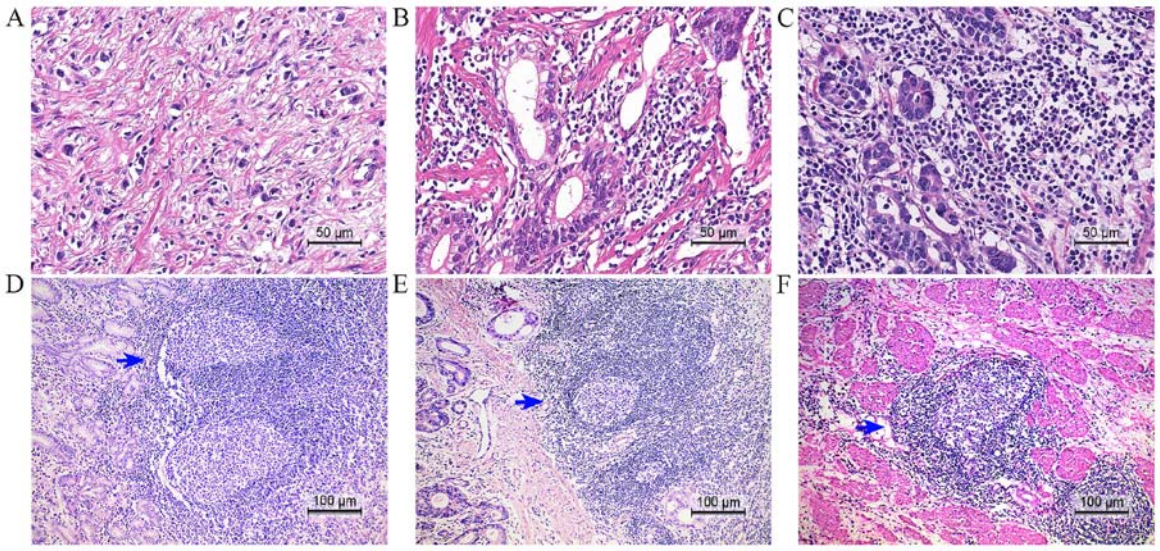

Figure 1. Pathomorphological images of inflammatory cell reactions in gastric cancer. (A) Low and (B and C) high levels of tumor infiltrating lymphocytes. Magnification, x200; scale bar, 50 μm. Tertiary lymphoid structures (marked with blue arrows) in different gastric anatomical layers: (D) In the mucosa, (E) in the submucosa and (F) in the muscularis propria. Magnification, x100; scale bar, 100 μm.

The number of TLSs (aggregates of lymphocytes with and without germinal centers were counted) and the percentage, similar to the TILs, in the CT or IM (29,30). The CT-TLSs and IM-TLSs location scores were defined as the numbers multiplied by the percentage, and then, the final score of TLSs was computed by summation of the CT- and IM-TLS scores.

Immunohistochemistry and evaluation of the gcCD8$^+$ T cells. Formalin-fixed and paraffin-embedded (FFPE) tissues were cut into 4 μm thick consecutive sections deparaffinized in xylene and rehydrated in graded ethanol solutions. Monoclonal mouse antibody against human CD8 (MAB-0021, ready to use, Maixin Biotechnology Limited Corporation) was used to stain the T lymphocytes. The CD8 antigen was retrieved by boiling the slides in citrate buffer (10 mmol/l; pH 6.0) under high pressure for 2 min. Then, the sections were immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, rinsed three times in PBS, and then incubated with primary antibodies at 4˚C overnight. The negative control was performed with PBS. The sections were incubated with horseradish peroxidase-labeled goat anti-mouse secondary antibody (ready to use, Maixin Biotechnology Limited Corporation). Diaminobenzene was used as the chromogen and hematoxylin as the nuclear counterstain. The scoring system for gcCD8$^+$ TILs was assessed in every slide. The number of germinal centers and gcCD8$^+$ TILs in each germinal center were counted. The average values were subsequently obtained.

Statistical analysis. Statistical analyses were performed using SPSS (version 24.0; IBM Corp.) and GraphPad Prism software (version 6; GraphPad Software, Inc.). Data were analyzed using the χ^2 test, Kaplan-Meier method and Cox regression analysis as appropriate. All tests were two-sided and P<0.05 was considered to indicate a statistically significant difference.

Results

Relationship between tumoral immune parameters and clinical pathological parameters. The TILs, TLSs, and gcCD8$^+$ TILs were defined as tumoral immune parameters in the present study (Tables I and II). In order to make the scoring system more facilitative to statistical analysis, these variables were converted into binary variables. TILs were sub-grouped by the TIL final score: TIL^{hi} 44 cases, score >0.45 [area under the receiver operating characteristic curve (AUC)=0.35; sensitivity, 82.1%; specificity, 50.0%], and TIL^{low} 19 cases, score ≤0.45 (Table I; Fig. 1A-C). TLSs were sub-grouped by the TLS final score: TLS^{hi} 43 cases, score >0.42 (AUC=0.39; sensitivity, 79.5%; specificity, 50.0%), and TLS^{low} 20 cases, score ≤0.42 (Table I; Fig. 1D-F). Considering the combination of TILs and TLSs, the samples were divided into two groups. The TIL-TLS^{hi} group (32 cases) consisted of samples that scored as both TIL^{hi} and TLS^{hi}. The TIL-TLS^{low} group (31 cases) consisted of samples that scored as TIL^{hi}-TLS^{low}, TIL^{low}-TLS^{hi} and TIL^{low}-TLS^{low} (Table I). Based on the density of gcCD8$^+$ TILs, 55 samples (specimens from eight cases were excluded due to absence of germinal centers) were divided into two groups: gcCD8^{hi} TIL, 23 cases, score >6.93 (AUC=0.19; sensitivity, 59.5%; specificity, 94.4%; Fig. 2A-F), and gcCD8^{low} TIL, 32 cases, score ≤6.93 (Table I; Fig. 2G-L).

The TILs were significantly associated with the tumor size, nerve invasion, lymph nodal metastasis, histological grade and pTN stage (P=0.007, 0.001, 0.004, 0.026 and 0.018, respectively; Table II). The data showed that the TLSs were not significantly associated with clinical pathological parameters. The combination of TILs-TLSs was significantly associated with nerve invasion, tumor thrombus, lymph nodal metastasis and histological grade (P=0.003, 0.006, 0.029 and 0.016, respectively; Table II). The data also showed the gcCD8$^+$ TILs was significantly associated with patient age, histological grade and pTN stage (P=0.028, 0.010 and 0.048, respectively; Table II). The higher levels of gcCD8$^+$ TILs were

Table II. Association between clinicopathological parameters and tumoral immune parameters.

	gcCD8$^+$ TILs				TILs				TLSs				TILs-TLSs			
Parameters	Low	High	χ^2	P-value	Low	High	χ^2	P-value	Low	High	χ^2	P-value	Low	High	χ^2	P-value
Sex																
Male	23	19	0.854	0.355	17	32	2.153	0.142	15	34	0.131	0.718	26	23	1.311	0.252
Female	9	4			2	12			5	9			5	9		
Age (years)																
≤50	6	0	4.841	**0.028**	4	4	1.713	0.191	3	5	0.140	0.708	5	3	0.648	0.421
>50	26	23			15	40			17	38			26	29		
Tumor size																
≤5 cm	18	17	1.804	0.179	7	32	7.246	**0.007**	12	27	0.045	0.832	16	23	2.741	0.098
>5 cm	14	6			12	12			8	16			15	9		
Nerve invasion																
Yes	12	8	0.043	0.836	14	11	13.140	**<0.001**	10	15	1.303	0.254	18	7	8.616	**0.003**
No	20	15			5	33			10	28			13	25		
Tumor thrombus																
Yes	13	4	3.383	0.066	10	12	3.755	0.053	10	12	2.932	0.087	16	6	7.483	**0.006**
No	19	19			9	32			10	31			15	26		
Nodal metastasis																
Yes	20	9	2.932	0.087	16	20	8.139	**0.004**	12	24	0.098	0.755	22	14	4.763	**0.029**
No	12	14			3	24			8	19			9	18		
Histological grade																
I-II	11	16	6.631	**0.010**	5	25	4.950	**0.026**	8	22	0.682	0.409	10	20	5.773	**0.016**
III	21	7			14	19			12	21			21	12		
pTN stage																
I	9	13	6.053	**0.048**	2	21	8.072	**0.018**	6	17	0.642	0.725	7	16	5.399	0.067
II	6	5			5	8			5	8			7	6		
III	17	5			12	15			9	18			17	10		

Values in bold indicate P<0.05. TILs, tumor infiltrating lymphocytes; TLSs, tertiary lymphoid structures.

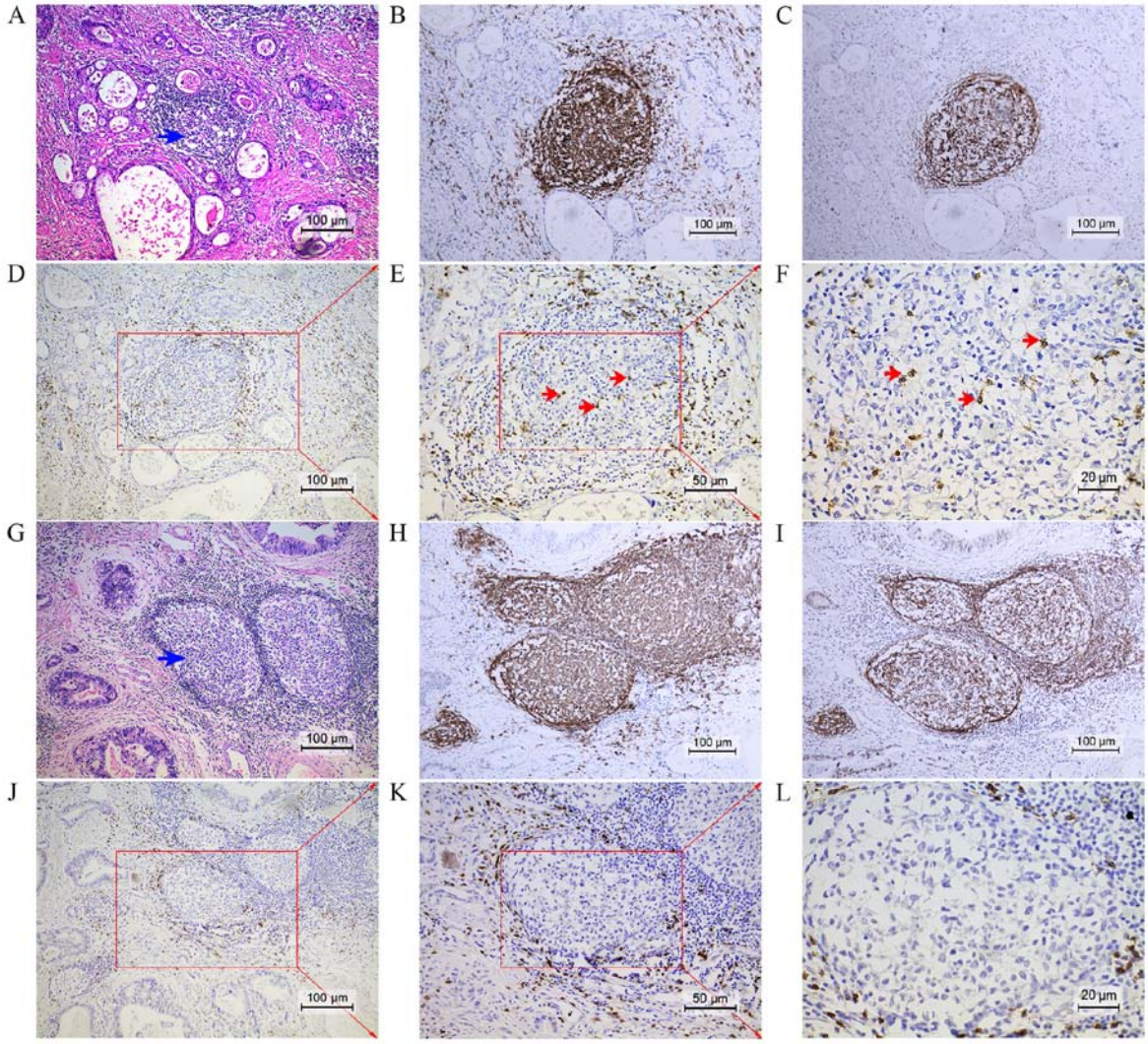

Figure 2. Two cases of (A-F) gcCD8^{hi} TILs and (G-L) gcCD8^{low} TILs were presented. (A) Case 1 had the TLS with a follicle and germinal center (marked with blue arrow) in HE slides. (B) CD20 staining revealed B lymphocyte aggregation and the germinal center. (C) CD21 staining revealed the follicular dendritic cell network in the germinal center. (D) Enlarged images of the red square area revealed follicular CD8^{+} TILs around the germinal center. (E and F) gcCD8^{+} TILs were detected in the germinal center (red arrows). (G) TLS marked with a blue arrow were also observed in Case 2. (H-J) The same expression pattern of CD20, CD21 and CD8 was observed. (K-L) Conversely, there were no gcCD8^{+} TILs that infiltrated into the germinal center. (A-D and G-J) Magnification, x100; scale bar, 100 μm. (E and K) Magnification, x200; scale bar, 50 μm. (F and L) Magnification, x400; scale bar, 20 μm. TLS, tertiary lymphoid structure; CD, cluster of differentiation; HE, hematoxylin and eosin; TILs, tumor infiltrating lymphocytes.

associated with lower histological grade and lower pTN stage. These data suggested that the gcCD8^{+} TILs might be involved in antitumor immunity in GC.

Association between tumoral immune parameters and patient prognosis. Kaplan-Meier survival analysis showed that the patients with TIL^{hi}, TLS^{hi}, TIL-TLS^{hi} or gcCD8^{hi} TIL had favorable prognosis than those with lower levels (P=0.0008, 0.0108, 0.0005 and 0.0003, respectively; Fig. 3A-D). The prognostic value of tumoral immune parameters in patients with advanced GC (pTN III stage) was investigated. The patients in the TIL-TLS^{hi} and gcCD8^{hi} TIL groups had an improved overall survival (OS) compared with patients in the TIL-TLS^{low} and gcCD8^{low} TIL groups (P=0.012 and 0.002; Table III; Fig. 3G and H).

In the univariate Cox regression analysis (analysis^{a}), smaller tumor size, lower histological grade, absence of tumor thrombus or lymph nodal metastasis, lower pTN stage and higher levels of tumoral immune parameters were favorable prognostic factors for OS (Table IV). Considering the possible interference among TILs, TLSs and TILs-TLSs, we did a cohort of multivariate Cox regression analysis. Multivariate Cox regression analysis^{b} (based on the clinicopathological feature, TILs, TLSs and gcCD8^{+} TILs) revealed that the gcCD8^{+} TILs were the only independent prognostic factor for OS (HR=0.087; 95% CI: 0.011-0.692; P=0.021; Table IV). Multivariate Cox regression analysis^{c} (based on the clinicopathological feature, TILs-TLSs and gcCD8^{+} TILs) demonstrated that TILs-TLSs and gcCD8^{+}TILs were independent prognostic factors (HR=0.247, 95% CI: 0.069-0.882, P=0.031; HR=0.067, 95% CI: 0.008-0.561, P=0.013, respectively). Moreover, the multivariate Cox regression analysis^{d} was designed to determine the association between tumoral immune parameters (TILs, TLSs and gcCD8^{+} TILs). The result showed TILs and gcCD8^{+} TILs could be used as independent prognostic factors (HR=0.322, 95% CI: 0.124-0.835, P=0.020; HR=0.059, 95% CI: 0.008-0.451, P=0.006, respectively). The tumoral immune

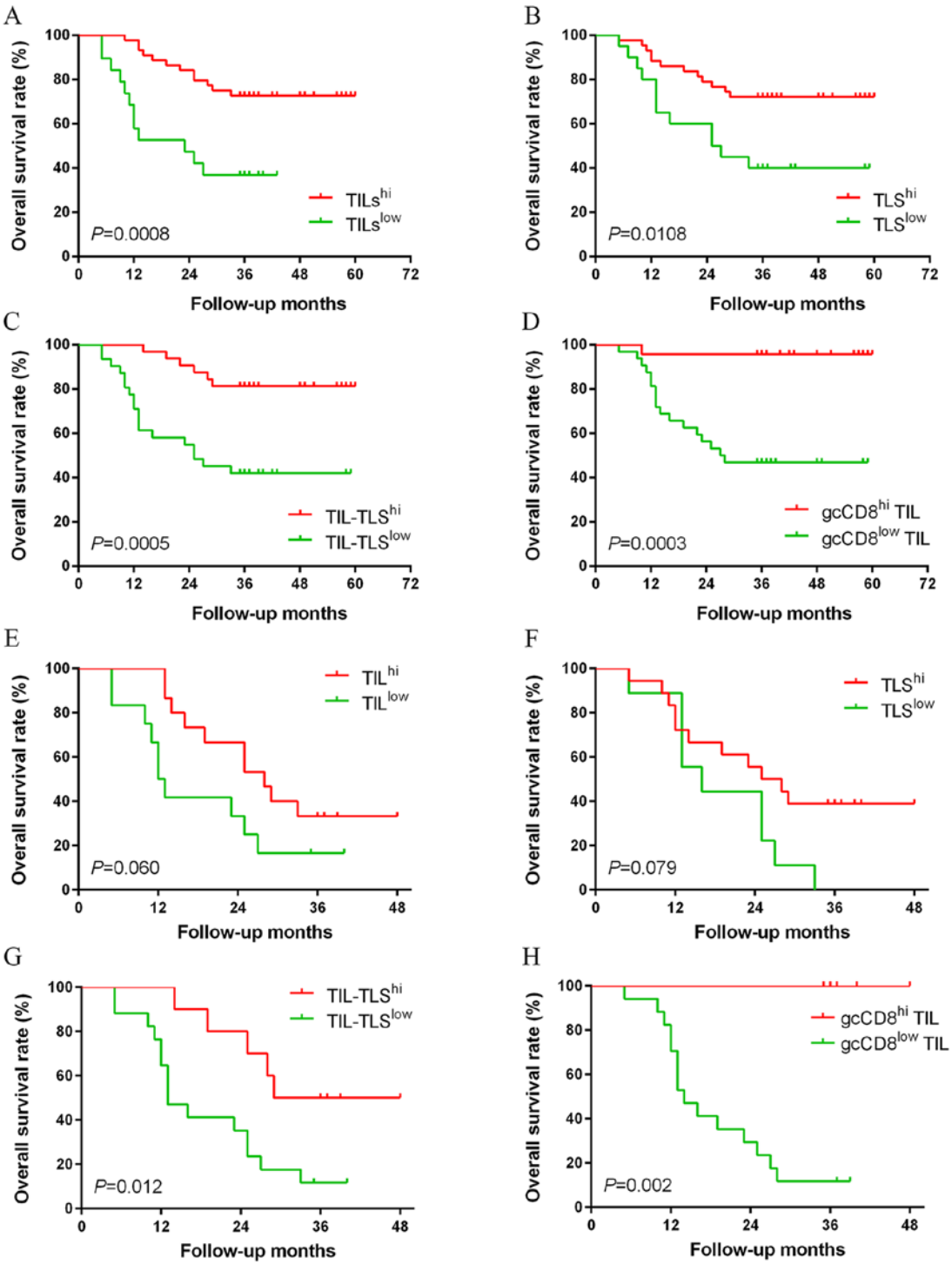

Figure 3. Kaplan-Meier survival curves for tumoral immune parameters. Kaplan-Meier analysis of overall survival for 63 patients stratified by (A) TILs, (B) TLSs and (C) TILs-TLSs in gastric cancer, and for 55 patients stratified by (D) gcCD8+ TILs. In pTN III stage, there was no statistically significant difference between the (E) IL or (F) TLS levels and OS. However, patients with high levels of (G) TILs-TLSs and (H) gcCD8 TILs had an improved OS. TILs, tumor infiltrating lymphocytes; TLSs, tertiary lymphoid structures; OS, overall survival.

parameters exhibited an HR <1 in the Cox regression analysis, suggesting that may protect against tumor progression.

Discussion

The present study demostrated that patients with GC with higher levels of TILs, or TLSs, or gcCD8+ TILs had an improved OS. Multivariate Cox regression analysis revealed that TILs-TLSs and gcCD8+ TILs were independent prognostic factors. In addition, gcCD8+ TILs were significantly associated with patient age, histological grade and pTN stage. Higher levels of TILs-TLSs was positively associated with the nerve invasion, tumor thrombus, lymph nodal metastasis and histological grade. The data obtained in the present study suggested that high levels of tumoral immune parameters were associated with improved prognosis in patients with GC.

Table III. Kaplan-Meier analysis of tumoral immune parameters.

A, Tumoral immune parameters in all cases

Parameters	Cases, n	Status of death	Mean OS, months	Log-Rank χ^2	P-value
TILs					
High	44	12	49.25	11.198	**0.001**
Low	19	12	24.21		
TLSs					
High	43	12	48.14	6.501	**0.011**
Low	20	12	33.40		
TILs-TLSs					
High	32	6	53.03	12.148	**<0.001**
Low	31	18	33.42		
gcCD8$^+$ TILs					
High	23	1	57.83	13.322	**<0.001**
Low	32	17	36.16		

B, Tumoral immune parameters in pTN III stage cases

Parameters	Cases, n	Status of death	Mean OS, months	Log-Rank χ^2	P-value
TILs					
High	12	10	30.33	3.527	0.06
Low	15	10	18.58		
TLSs					
High	18	11	19.11	3.089	0.079
Low	9	9	18.89		
TILs-TLSs					
High	10	5	35.50	6.310	**0.012**
Low	17	15	19.00		
gcCD8$^+$ TILs					
High	5	0	-	9.334	**0.002**
Low	17	15	18.65		

Values in bold indicate P<0.05. TILs, tumor infiltrating lymphocytes; TLSs, tertiary lymphoid structures.

Cancer progression is a multi-step process that involves genetic, epigenetic, as well as histopathological change (3,31-33). Infiltrating immune cells play an important role in preventing or promoting cancer progression (34). Previous studies have demonstrated that high levels of TILs may inhibit the progression of breast and colorectal cancer (8,31,35). The present study revealed that a high level of TILs was associated with improved prognosis in patient with GC. Univariate and multivariate Cox regression analyses revealed that TILs could be used as an independent favorable prognostic factor.

Previous studies demonstrated that TLSs are required for the development of the T- and B-cell mediated immune response against human cancer and various other diseases (25,26,31,36,37). TLSs have been previously described in several types of cancer, such as lung cancer and breast cancer, and are associated with patient prognosis (10,17,29). TLSs are organization by heterotopic lymphoid tissues, and exhibit similar organization and functionality to SLOs. The basic components of TLSs include the T-zone, comprising of T cells and FRCs, and the B-zone, comprising of B cells and FDCs. TLSs are involved in establishing a systemic memory response to protect patients against circulating metastatic cancer cells, therefor inhibiting tumor recurrence for several years (8,31). The present study revealed that the high levels of TLSs were associated with improved outcome in patients with GC, and could therefor be used as an independent prognostic factor. The combination of TILs and TLSs is superior to TILs or TLSs alone for predicting survival. In advanced cancer, the high levels of TILs-TLSs were associated with improved OS. Based on the results obtained in the present study, TLSs are an important niche in which tumor antigen specific T and B cells are generated.

Despite the association of gcCD8$^+$ T cells with improved prognosis, the role of gcCD8$^+$ T cells in antitumor immune

Table IV. Univariate and multivariate Cox regression analyses of clinicopathological parameters and tumoral immune parameters.

A, Univariate analysis

Variables	Unfavorable/favorable	HR	95% CI	P-value
Sex	Male/Female	0.863	0.343-2.176	0.755
Age, years	≤50/>50	0.747	0.255-2.187	0.594
Tumor size, cm	≤5/>5	3.643	1.587-8.363	**0.002**
Histological grade	I-II/III	4.624	1.722-12.421	**0.002**
Neural Invasion	No/Yes	2.168	0.969-4.847	0.060
Tumor thrombus	No/Yes	6.619	2.716-16.134	**0.001**
Lymphatic metastasis	No/Yes	11.528	2.702-49.184	**0.001**
pTN stage	I-II/III	18.732	2.525-138.976	**0.004**
TILs	High/Low	0.279	0.125-0.623	**0.002**
TLSs	High/Low	0.370	0.166-0.825	**0.015**
gcCD8+ TILs	High/Low	0.062	0.008-0.470	**0.007**
TILs-TLSs	High/Low	0.376	0.222-0.636	**0.000**

B, Multivariate Cox regression analysis

Variables	Unfavorable/favorable	HR	95% CI	P-value
Tumor size, cm	≤5/>5	0.896	0.198-4.046	0.886
Histological grade	I-II/III	1.009	0.277-3.671	0.990
Tumor thrombus	No/Yes	2.227	0.482-10.748	0.299
Lymphatic metastasis	No/Yes	1.251	0.135-11.560	0.844
pTN stage	I-II/III	5.594	0.336-93.237	0.230
TILs	High/Low	0.503	0.169-1.492	0.215
TLSs	High/Low	0.609	0.203-1.831	0.377
gcCD8+ TILs	High/Low	0.087	0.011-0.692	**0.021**

C, Multivariate Cox regression analysis

Variables	Unfavorable/favorable	HR	95% CI	P-value
Tumor size, cm	≤5/>5	1.208	0.273-5.355	0.803
Histological grade	I-II/III	0.806	0.208-3.116	0.754
Tumor thrombus	No/Yes	1.742	0.381-7.970	0.474
Lymphatic metastasis	No/Yes	0.980	0.103-9.296	0.986
pTN stage	I-II/III	7.159	0.419-122.410	0.174
gcCD8+ TILs	High/Low	0.067	0.008-0.561	**0.013**
TILs-TLSs	High/Low	0.247	0.069-0.882	**0.031**

D, Multivariate Cox regression analysis

Variables	Unfavorable/favorable	HR	95% CI	P-value
TILs	High/Low	0.322	0.124-0.835	**0.020**
TLSs	High/Low	0.396	0.148-1.056	0.064
gcCD8+ TILs	High/Low	0.059	0.008-0.451	**0.006**

Values in bold indicate P<0.05. TILs, tumor infiltrating lymphocytes; TLSs, tertiary lymphoid structures; HR, hazard ratio.

responses and germinal center B cell responses remains unknow. Previous studies investigating chronic infection with LMCV and HIV revealed that there are two subsets of CD8+ effector T cells, namely CXCR5+ PD-1+ CD8+ T and

$CXCR5^-$ $PD\text{-}1^+$ $CD8^+$ T cells (26,38). The $CXCR5^+$ subset exhibits stem cell-like properties and is localized in the B cell follicle and germinal center during chronic infection. The $CXCR5^+$ subset undergo self-renewal and can differentiate into $CXCR5^-$ $CD8^+$ T cells. The *de novo* converted $CXCR5^-$ subset exhibits increased cytotoxicity and removes virus infected cells. Hornquist *et al* (39) studied the role of $CD8^+$ T cells in the regulation of gut mucosal immune responses and showed that $CD8^+$ T cells inhibited local B cell responses. By contrast, B cells and plasma cells have been shown to impede T cell-mediated antitumor immune responses (40-42). Based on the aforementioned findings, $gcCD8^+$ T cells are likely to promote cell-mediate antitumor immune responses and inhibit humoral immunity. A diagrammatic representation based on two hypotheses is presented in Fig. S1. Based on the results obtained from studies investigating chronic HIV or LMCV infection (43,44), it is hypothesized that $PD1^+$ $CD8^+$ TILs may be further divided into $CXCR5^+$ and $CXCR5^-$ subsets, which are regulated by the inhibitor of DNA binding 2 (ID2)/transcription factor E2-α axis. The $CXCR5^+$ subset located in the B cell follicle and the germinal center. The follicular $CXCR5^+$ $CD8^+$ T cells subset can undergo proliferation and differentiate into the $CXCR5^-$ subset following ID2 upregulation. 2. According to the studies by Hornquist *et al* (39) and Li *et al* (44), germinal center $CXCR5^+$ $CD8^+$ T cells can exert a suppressive effect on germinal B cell responses and inhibit the generation of plasma cells. Some mechanisms in these hypotheses were demonstrated. Elimination of immunosuppressive B cells expressing IgA, IL-10 and PD-L1 allows $CD8^+$ cytotoxic T cells eradication of oxaliplatin-resistance prostate cancer (40). The expression of CXCR5 on $CD8^+$ T cells was necessary for T cells infiltrating into B-cells follicular (43). The present study revealed that the phenotype of TILs, TLSs and $gcCD8^+$ TILs exhibited significant heterogeneity in patients with GC. TILs, TLSs and $gcCD8^+$ TILs may be had the potential function associated with GC immunotherapy. Further investigation is required to validate the hypotheses proposed in the present study.

Acknowledgements

Not applicable.

Funding

This work was supported by grants from the National Natural Science Foundation of China (No. 31701111), Key R&D Project of Science and Technology Department of Jiangsu Province (BE2015633). Changzhou Health and Family Planning Commission Youth Talent Science and Technology Project (QN201709). This work was also supported in part by Roswell Park Cancer Institute/University of Pittsburgh Cancer Institute Ovarian Cancer Specialized Programs of Research Excellence Grants (P50CA159981).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QL, DZ and WH designed the study and wrote the manuscript. DZ, TC, ZY, XG and LC performed all of the experiments. XZ and BX performed the statistical analysis. BL and JJ conceived and designed the study, guided the experiments and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Soochow University, and complied with the Declaration of Helsinki. Informed consent to use the tissue sample for scientific research was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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