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Tumor-Infiltrating Immune Cells Are Associated With Prognosis of Gastric Cancer

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Abstract: Immune cells contribute to determining the prognosis of gastric cancer. However, their exact role is less clear.

We determined the prognostic significance of different immune cells in intratumoral tissue ($_T$), stromal tissue ($_S$), and adjacent normal tissue ($_N$) of 166 gastric cancer cases and their interactions, including $CD3^+$, $CD4^+$, $CD8^+$, $CD57^+$, $CD68^+$, $CD66b^+$, and $Foxp3^+$ cells, and established an effective prognostic nomogram based on the immune reactions.

We found high densities of $_TCD3^+$, $_TCD4^+$, $_TCD8^+$, $_SCD3^+$, $_SCD4^+$, $_SCD57^+$, $_SCD66b^+$, and $_NFoxp3^+$ cells, as well as high $_TCD8^+/_SCD8^+$ ratio, $_TCD68^+/_SCD68^+$ ratio, $_TCD3^+/_TFoxp3^+$ ratio, $_TCD4^+/_TFoxp3^+$ ratio, $_TCD8^+/_TFoxp3^+$ ratio, $_SCD3^+/_SFoxp3^+$ ratio, and $_SCD4^+/_SCD8^+$ ratio were associated with better survival, whereas high densities of $_TCD66b^+$, $_TFoxp3^+$, $_SFoxp3^+$ and $_NCD66b^+$ cells as well as high $_TCD57^+/_SCD57^+$ ratio, $_TCD66b^+/_SCD66b^+$ ratio, $_SCD8^+/_SFoxp3^+$ ratio, and $_TFoxp3^+/_NFoxp3^+$ ratio were associated with significantly worse outcome. Multivariate analysis indicated that tumor size, longitudinal tumor location, N stage, $_TCD68^+/_SCD68^+$ ratio, $_TCD8^+/_TFoxp3^+$ ratio, density of $_TFoxp3^+$ cells, and $_TCD66b^+/_SCD66b^+$ ratio were independent prognostic factors, which were all selected into the nomogram. The calibration curve for likelihood of survival demonstrated favorable consistency between predictive value of the nomogram and actual observation. The C-index (0.83, 95% CI: 0.78 to 0.87) of our nomogram for predicting prognosis was significantly higher than that of TNM staging system (0.70).

Collectively, high $_TCD68^+/_SCD68^+$ ratio and $_TCD8^+/_TFoxp3^+$ ratio were associated with improved overall survival, whereas high density of $_TFoxp3^+$ cells and $_TCD66b^+/_SCD66b^+$ ratio demonstrated poor overall survival, which are promising independent predictors for overall survival in gastric cancer.

(*Medicine* 94(39):e1631)

Abbreviations: CI = confidence interval, C-index = concordance index, DAB = diamiobenzidine, HD = high density, HR = hazard ratio, IHC = Immunohistochemistry, IL-10 = interleukin-10, LD = low density, MAC = macrophages cells, NKC = natural killer cells.

INTRODUCTION

Gastric cancer is one of the common malignancies with high incidence in the world, especially in East Asian countries.[1] Currently, the main treatment of gastric cancer consists of surgical resection plus standard D2 lymphadenectomy, adjuvant chemotherapy, and some molecular targeting therapy.[2–4] Although our cognitions on gastric cancer have been significantly developed in recent years, the prognosis was still undesirable yet. In addition, it is very common that gastric cancer patients with the same TNM stage have the different long-term survival. Therefore, in order to improve the long-term survival, it is important to better understand the mechanisms of disease progression and find new effective predictive prognostic factors as the targets of interventions. Although many predictive factors have been evaluated, such as clinicopathologic factors, biomarkers, genes, and microsatellite instability,[5–7] their prognostic accuracies are controversial and an ideal factors has not yet been found. Recently, it became clearer that there is a positive correlation between the presence of tumor-infiltrating inflammatory cells (TLCs) and survival of patients with malignancies.[8–14] The types, density, and location of immune cells are even more accurate in predicting prognosis than the currently used the TNM stage for colon cancer,[8] which suggests that evaluation of the TLCs might be more useful for further comprehension of tumor development, prediction of prognosis, and immunotherapy.

Editor: Natale Figura.
Received: June 21, 2015; revised: August 20, 2015; accepted: August 26, 2015.
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Funding: Our study was supported by National Natural Science Foundation of China (Grant number: 81301867, 81372344) and National High-Technology Research and Development Program (863 Program) of China (Grant number: 2015AA020306)

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Author contributions: Conceptions and design: Kai Liu, Kun Yang, Bin Wu, Hai-Ning Chen, Zong-Guang Zhou, Xian-Ming Mo, Jian-Kun Hu; Development of methodology: Kai Liu, Kun Yang, Bin Wu, Hai-Ning Chen, Li-Li Jiang, Fu-Xiang Ye, Jian-Kun Hu; Acquisition of data: Kai Liu, Kun Yang, Bin Wu, Hai-Ning Chen, Xiao-Long Chen, Li-Li Jiang, Du He, Zheng-Hao Lu, Lian Xue, Wei-Han Zhang, Xin-Zu Chen; Analysis and interpretation of data: Kai Liu, Kun Yang, Bin Wu, Hai-Ning Chen, Li-Li Jiang, Fu-Xiang Ye, Du He, Qiu Li, Jian-Kun Hu; Writing, review and/or revision of the manuscript: All authors: Administrative, technical, or material support: Qiu Li, Zong-Guang Zhou, Xian-Ming Mo, Jian-Kun Hu; Study supervision: Jian-Kun Hu

The authors have no conflicts of interest to disclose.

ISSN: 0025-7974
DOI: 10.1097/MD.0000000000001631

Recent studies have highlighted several types of TLCs, such as $CD3^+$ T cells, $CD8^+$ T cells, regulatory T cells (T_{regs}), natural killer cells (NKC), neutrophils or macrophages cells (MAC), are associated with disease outcomes for various human cancers.[8–15] For gastric cancer, it was reported that the combination of high numbers of intratumoral macrophage and T_{regs} was associated with improved survival.[15] However, others showed the T_{regs} played a role of immunosuppression and tumor progression in patients with gastric and esophageal cancers and led to a poorer prognosis.[16] Intratumoral high T_{regs} /$CD8^+$ T cells ratio was an independent predictor for the worse prognosis of gastric cancer.[17] However, $CD4^+$ and $CD8^+$ TLCs were not associated with overall survival.[17] It was also found that tumor-infiltrating neutrophils were significantly associated with higher survival rates in gastric cancer,[18] but the presence of intratumoral neutrophil was an independent factor of poor prognosis for patients with other cancers.[12]

Therefore, the above results provide strong evidence that immune cells contribute to determining the prognosis of gastric cancer. However, the exact role of immune cells in gastric cancer is less clear. On the other hand, whether immune cells play a protecting or promoting role only can be interpreted after understanding the definite functions of each cell phenotype in this process.[19] The aims of the present study were to determine the prognostic significance of different immune cells and their interactions in gastric cancer, including $CD3^+$ (Marker of T cells), $CD4^+$ (Marker of T helper cells), $CD8^+$ (Marker of cytotoxic T cells), $CD57^+$ (Marker of natural killer cells), $CD68^+$ (Marker of macrophage), $CD66b^+$ (Marker of neutrophil), and $Foxp3^+$ (Marker of T_{regs}) cells. This study also aimed to establish an effective prognostic nomogram based on the immune cells infiltration. To our limited knowledge, this is the first report demonstrating prognostic values of various kinds of immune cells and their combined effects between cells. In addition, this is also the first time that the tumor compartments were considered separately by intratumoral tissue ($_T$), stromal tissue ($_S$), and adjacent normal tissue ($_N$) simultaneously.

MATERIALS AND METHODS

Patients and Specimens

Formalin-fixed, paraffin-embedded specimens were obtained from 166 patients who under surgical resection for gastric adenocarcinoma in West China Hospital, Sichuan University between 2006 and 2009. Clinicopathological and follow-up data of these patients were collected from our prospective database of gastric cancer. Clinicopathological data including demographic parameters, tumor size (cm), Borrmann types, T, N, M, stage, and degree of tumor differentiation (well differentiated, moderate, poor, signet-ring cell, and mucinous type) were reviewed. Clinicopathological terminology was based on the Japanese classification of gastric carcinoma (3rd English version).[20] The West China Hospital research ethics committee approved retrospective analysis of anonymous data.

Follow-up

Overall survival was calculated from the time of surgery until death or the last observation for surviving patients. Follow-up assessments were performed every 3 to 6 months for the first 2 years, every 6 to 12 months for 3 to 5 years after surgery and then annually.[21] The postoperative follow-up was carried out by regular out-patient visits and telephone interviews. Follow-up information was updated to December, 2014. Reasons for those patients lost to follow-up were mainly due to cancellation of out-patient visit or change of telephone number and address. The overall follow-up rate was 90.36% (150/166). Sixteen patients were lost to follow-up.

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded tissue specimens were consecutively sliced into 4 μm-thick sections. Primary polyclonal antibodies including anti-CD3 clone SP7 (dilution 1:250; Thermo Scientific, Fremont, CA), anti-CD4 clone 113 (dilution 1:200; Sino Biological, BDA, Beijing, PR China), anti-CD8 clone SP16 (dilution 1:150; Thermo Scientific, Fremont, CA), anti-CD57 clone NK1 (dilution 1:100; Thermo Scientific, Fremont, CA), anti-CD66b clone 80H3 (dilution 1:100; LifeSpan Biosciences, Seattle, WA), anti-CD68 clone KP1 (dilution 1:800; eBioscience, San Diego, CA), and anti-Foxp3 clone 236A/E7 (dilution 1:100; eBioscience, San Diego, CA) were used. A 2-step protocol (Novolink Polymer Detection System, Novocastra, Newcastle, UK) was performed on the paraffin sections for the immunohistochemistry. According to the manufacturer's instructions, paraffin sections were deparaffinaged in xylene and received gradient elution in ethanol. Then the slides were incubated in 0.3% H_2O_2 to block the endogenous peroxidase activity. Antigen retrieval was carried out by immersing the slides in the hot water of 95 centigrade degree for ~45 min. Incubation with primary antibodies was performed followed by washing with phosphate-buffered saline and then incubation with secondary antibodies using GTVision™ III Detection System/Mo&Rb (Gene Tech, Shanghai, PR China). The sections were pigmentized in 3, 3-diamiobenzidine (DAB) solution (dilution 1:50; Gene Tech, Shanghai, PR China) for ~5 s under the monitoring of microscopic observation. Then all the sections were counterstained with haematoxylin. Negative control sections without primary antibodies were all performed in every series.

Evaluation of Immunohistochemical Variables

The number of immune cells was determined separately in the following compartment: (*I*) within the intratumoral tissue; (*II*) within the tumor stromal tissue at the invasive border; and (*III*) within the peripheral normal tissue (normal tissue with distance >1 microscope field at ×200 magnification from invasive border). Each section was evaluated for immune cells by microscopic examination (×400; BX51; Olympus, Tokyo, Japan). Five noncontiguous microscopic areas that represent the densest immune cells were randomly selected for each compartment on each sample in order to ensure representativeness and homogeneity. The numbers of immune cells in the 5 fields were accumulated and then averaged to calculate the mean number for 1 computerized 400× microscopic field (0.1590 mm^2/field). The photographs were captured with a light microscope (BX51; Olympus, Tokyo, Japan) that connected with a personal computer and displayed on a high-resolution color 14-inch monitor. The evaluation of cells was performed by 2 independent pathologists that were blinded to clinicopathologic data. Variations in the enumeration, within a range of 5%, were re-evaluated and a consensus decision was made.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 (SPSS®, Chicago, IL). Variables of normality were tested, and if conforming to the normal distribution, data were

expressed as mean ± standard deviation. Two independent *t* tests for quantitative data and chi-square test or Fisher's exact test for categorical data were performed; data were expressed as medians with a range taking the Spearman test into consideration. For all immunohistochemical variables, the median was used as the cutoff point for division of subgroups.[11–13] Survival curves were derived from Kaplan–-Meier estimates, and the curves were compared by log-rank tests. Significant factors were identified by univariate analysis, and further examined by multivariate analysis. The multivariate regression was performed using the Cox proportion hazards model. A nomogram was formulated according to the results of multivariate analysis with R project (http://www.r-project.org/). The nomogram's predictive accuracy was measured via a concordance index (C-index) (the larger the C-index, the more accurate the prediction) and assessed by comparing prediction by nomogram and actual observed survival. A calibration curve showed as the plot of predicted probabilities from the nomogram versus the actual probabilities was generated. Comparisons between the nomogram and TNM staging systems[20] were performed in R and were evaluated by the C-index. Two-sided P value <0.05 was considered as statistical significance.

RESULTS

Patient Characteristics

The characteristics of patients are presented in Table 1. The mean age was 55.30 ± 11.87 years (range, 19–79 years), and 75.3% of patients were men. Only 22 patients (13.3%) had early gastric cancer, and 142 patients (85.5%) had poor differentiation. Eighty eight (53.0%) patients had postoperative chemotherapy. The median number of harvested lymph nodes was 25.5 (11–69) in this study. Seventy-nine (47.6%) patients had died at the end of follow-up. The median duration of follow-up for patients was 65.88 months. The median survival for all patients was 79.57 months (95% confidence interval [CI]: 49.06–110.07 months). The 5-year survival for the study population was 52.0%.

Immune Cells in Gastric Cancer and Correlations of Different Immune Cells

$CD3^+$, $CD4^+$, $CD8^+$, $CD57^+$, $CD68^+$, and $CD66b^+$ cells showed cell membrane staining, whereas $Foxp3^+$ cells exhibited distinct nuclear staining. The distribution and density of positive cells varied substantially among samples. Representative images are shown in Figure 1. The densities of intratumoral $CD8^+$ T cells ($_TCD8^+$) and $_TFoxp3^+$ cells were highest compared to stromal tissue and adjacent normal tissue, whereas the densities of $CD3^+$ T cells, $CD57^+$ cells, and $CD66b^+$ cells were the highest in stromal tissue. The density of $CD68^+$ cells was the highest in adjacent normal tissue. The density of $CD4^+$ cells was significantly higher in intratumoral tissue than that of stromal tissue, but higher without significance compared to adjacent normal tissue. The average counts of immune cells are shown in Table 2. The densities of $CD3^+$, $CD4^+$, and $CD8^+$ T cells were strongly associated with each other in intratumoral tissues. The densities of $CD8^+$ and $CD57^+$ cells, as well as $CD4^+$ and $CD68^+$ cells, were also significantly correlated in intratumoral tissues. The density of $Foxp3^+$ cells was negatively correlated with $CD3^+$, $CD8^+$, $CD57^+$, $CD4^+$, and $CD68^+$ cells in intratumoral tissues with significant difference. Other correlations between the immune cells are listed in Table 3.

Association of TLCs with Clinicopathologic Factors

Associations between the densities of TLCs and clinicopathologic factors are listed in Table 1. The densities of $_TCD3^+$, $_TCD4^+$, and $_TCD8^+$ cells were associated with M1 significantly. Tumors with more lymph nodes metastasis were found to have lower densities of $_TCD4^+$ and $_TCD57^+$ cells. The density of $_TCD8^+$ cells was significantly lower in tumors showing more advanced stages and therefore more palliative resections. Significant association was observed between the density of $_TCD68^+$ cells and gender. However, the density of $_TCD66b^+$ cells was not associated with either of these features. As expected, $_TFoxp3^+$ cell density was higher in tumors reported as M1 or more advanced stages. In terms of lymphadenectomy and chemotherapy, no differences were observed between patients with lower densities of TLCs and those with higher densities of TLCs.

Univariate Analysis

Table 4 shows the results of univariate survival analysis for the clinicopathologic features and for immunohistopathologic variables. Clinical factors statistically associated with overall survival were age, longitudinal tumor location, tumor size, Borrmann type, T, N, distant metastasis (M), stage, resection type, and lymphadenectomy. Densities of $_TCD3^+$, $_TCD4^+$, $_TCD8^+$, $_TCD66b^+$, $_TFoxp3^+$, $_SCD3^+$, $_SCD4^+$, $_SCD57^+$, $_SCD66b^+$, $_SFoxp3^+$, $_NCD66b^+$, and $_NFoxp3^+$ cells were associated with overall survival. Neither $_TCD68^+$ cells, $_SCD68^+$ cells, nor $_NCD68^+$ cells were associated with survival. High densities of $_TCD3^+$, $_TCD4^+$, $_TCD8^+$, $_SCD3^+$, $_SCD4^+$, $_SCD57^+$, $_SCD66b^+$, and $_NFoxp3^+$ cells were associated with better survival, whereas high densities of $_TCD66b^+$, $_TFoxp3^+$, $_SFoxp3^+$, and $_NCD66b^+$ cells were associated with significantly worse outcome. Patients with low density of $_TFoxp3^+$ cells had longer overall survival (median, 74.4 months) than did those with high density (median, 34.98 months). Due to the existence of synergistic or antagonistic effects between different kinds of immune cells and between the different locations of immune cells, the combined influences were also evaluated. Our results suggest the subgroup of patients with high $_TCD57^+/_SCD57^+$ ratio, $_TCD66b^+/_SCD66b^+$ ratio, $_SCD8^+/_SFoxp3^+$ ratio, and $_TFoxp3^+/_NFoxp3^+$ ratio demonstrated worse survival. The survival for patients with high $_TCD8^+/_SCD8^+$ ratio, $_TCD68^+/_SCD68^+$ ratio, $_TCD3^+/_TFoxp3^+$ ratio, $_TCD4^+/_TFoxp3^+$ ratio, $_TCD8^+/_TFoxp3^+$ ratio, $_SCD3^+/_SFoxp3^+$ ratio, and $_SCD4^+/_SCD8^+$ ratio were significantly improved.

Multivariate Analysis

Clinicopathologic features and immunohistopathologic variables showing significances by univariate analysis were adopted as covariates when multivariate Cox proportional hazards analysis was performed. Details of the results are presented in Table 5. The analysis revealed that tumor size, longitudinal tumor location, and N stage were factors to show independent prognostic significances. High $_TCD68^+/_SCD68^+$ ratio and $_TCD8^+/_TFoxp3^+$ ratio were associated with improved overall survival, whereas high density of $_TFoxp3^+$ cells and $_TCD66b^+/_SCD66b^+$ ratio demonstrated a significant association with poor survival (Figure 2).

Prognostic Nomogram for Overall Survival and its Predictive Accuracy

Figure 3A revealed the prognostic nomogram integrating all significant independent factors identified in multivariate

TABLE 1. Characteristics of Patients and Association of Intratumoral Infiltrating Cells With Clinicopathologic Factors

		CD3$^+$			CD4$^+$			CD8$^+$			CD57$^+$			CD66b$^+$			CD68$^+$			Foxp3$^+$		
Clinicopathologic Factors	**N**	**LD**	**HD**	P^*	**LD**	**HD**	P^*	**LD**	**HD**	P^*	**LD**	**HD**	P^*	**LD**	**HD**	P^*	**LD**	**HD**	P^*	**LD**	**HD**	P^*
Gender				0.37			0.86			0.21			0.11			0.37			0.05			0.86
Female	41	60	65		62	63		66	59		67	58		60	65		68	57		62	63	
Male	125	23	18		21	20		17	24		16	25		23	18		17	26		21	20	
Age (years)				0.87			0.15			0.08			0.08			0.63			0.87			0.43
<60	101	50	51		46	55		45	56		45	56		52	49		50	51		53	48	
≥60	65	33	32		37	28		38	27		38	27		31	34		33	32		28	35	
Longitudinal location				0.20			0.52			0.08			0.48			0.56			0.17			0.18
Upper third	44	19	25		22	22		29	15		26	14		23	21		26	18		16	28	
Middle third	26	15	11		16	10		12	14		12	18		10	16		11	15		13	13	
Lower third	93	49	44		43	50		40	53		43	50		49	44		46	47		52	41	
Whole stomach	3	0	3		2	1		2	1		2	1		1	2		0	3		2	1	
Circumferential location				0.49			0.87			0.16			0.10			0.74			0.22			0.47
Less curvature	91	41	50		45	46		45	46		44	47		46	45		49	42		47	44	
Great curvature	22	11	11		12	10		9	13		13	9		10	12		13	9		10	12	
Anterior wall	10	3	7		4	6		7	3		7	3		7	3		3	7		4	6	
Posterior wall	26	16	10		12	14		10	16		8	18		12	14		13	13		16	10	
Full circle	17	12	5		10	7		12	5		11	6		8	9		5	12		6	11	
Gross type				0.45			0.41			0.22			0.30			0.64			0.39			0.29
Borr-0	14	5	9		6	8		7	7		7	7		9	5		7	7		8	6	
Borr-1	6	2	4		5	1		3	3		1	5		2	4		4	2		3	3	
Borr-2	88	43	45		43	45		39	49		41	47		41	47		38	50		45	43	
Borr-3	47	28	19		22	25		25	22		28	19		25	22		28	19		25	22	
Borr-4	11	5	6		7	4		9	2		6	5		6	5		6	5		2	9	
Differentiation				0.28			0.66			0.97			0.84			0.52			0.76			0.14
Well	5	1	4		2	3		2	3		3	2		3	2		2	3		4	1	
Moderate	19	8	11		10	9		10	9		11	8		9	10		8	11		11	8	
Poor	55	27	28		31	24		26	29		25	30		23	32		31	24		3	25	
Signet-ring cell	73	42	31		35	3		38	35		36	37		39	34		36	37		29	44	
Mucinous	14	5	9		5	9		7	7		8	6		9	5		6	8		9	5	
Vessels invasion				0.08			0.56			0.33			0.56			0.85			0.08			0.08
No	133	62	71		65	68		64	69		68	65		66	67		62	71		62	71	
Yes	33	21	12		18	15		19	14		15	18		17	16		21	12		21	12	
Tumor size (cm)				0.91			0.26			0.62			0.34			0.28			0.26			0.14
≤2	26	13	13		10	16		13	13		10	16		13	13		14	12		18	8	
~5.0	79	41	38		37	42		37	42		38	41		45	34		35	44		35	44	
~8.0	44	20	24		25	19		22	22		24	20		17	27		27	17		23	21	
>8.0	17	9	8		11	6		11	6		11	6		8	9		7	10		7	10	
Depth of infiltration (T)				0.13			0.46			0.07			0.74			0.48			0.30			0.42
T1	22	7	15		10	12		11	11		10	12		14	8		10	12		15	7	
T2	17	8	9		6	11		4	13		7	10		10	7		9	8		7	10	
T3	3	1	2		2	1		1	2		1	2		2	1		0	3		1	2	
T4a	114	59	55		58	56		59	55		61	53		53	61		57	57		55	59	
T4b	10	8	2		7	3		8	3		4	6		4	6		7	3		5	5	
Nodal status (N)				0.70			0.000			0.06			0.000			0.70			0.42			0.23
N0	48	22	26		20	28		19	29		19	29		26	22		19	29		29	19	
N1	32	16	16		9	23		14	18		18	14		17	15		15	17		14	18	
N2	21	9	12		9	12		11	10		6	15		8	13		12	9		12	9	
N3a	46	27	19		32	14		24	22		24	22		24	22		26	20		22	24	
N3b	19	9	10		13	6		15	4		16	3		8	11		11	8		6	13	
Distal metastasis (M)				0.04			0.01			0.000			0.10			0.82			0.10			0.01
M0	145	68	77		66	78		64	80		69	75		72	72		68	76		78	68	
M1	22	16	6		17	5		19	3		14	0		11	11		15	7		5	17	
Stage				0.13			0.31			0.02			0.33			0.33			0.13			0.02
Ia	15	5	10		8	7		7	8		7	8		9	6		7	8		10	5	
Ib	8	2	6		2	6		3	5		2	6		7	1		4	4		3	5	
IIa	8	2	6		3	5		3	5		4	4		2	6		5	3		3	5	
IIb	33	19	14		12	21		11	22		12	21		17	16		10	23		24	9	
IIIa	12	6	6		6	6		6	6		5	7		5	7		5	7		7	5	

		CD3$^+$			CD4$^+$			CD8$^+$			CD57$^+$			CD66b$^+$			CD68$^+$			Foxp3$^+$		
Clinicopathologic Factors	**N**	**LD**	**HD**	***P****	**LD**	**HD**	***P****	**LD**	**HD**	***P****	**LD**	**HD**	***P****	**LD**	**HD**	***P****	**LD**	**HD**	***P****	**LD**	**HD**	***P****
IIIb	25	13	12		8	17		11	14		14	11		13	12		11	14		10	15	
IIIc	43	20	23		27	16		23	20		25	18		19	24		26	17		21	22	
IV	22	16	6		17	5		19	3		14	8		11	11		11	7		5	17	
Lymphadenectomy				0.63			0.34			0.06			0.30			0.29			0.27			0.25
D1	34	19	15		14	20		22	12		21	13		13	21		19	15		16	18	
D1+	35	16	19		16	19		12	23		15	20		20	15		13	22		22	13	
D2	96	48	48		53	43		49	47		47	49		50	46		50	46		44	52	
D2+	1	0	1		0	1		0	1		0	1		0	1		1	0		1	0	
Curative degree				0.17			0.65			0.02			0.65			0.17			0.65			0.65
R0	161	79	82		80	81		78	83		80	81		82	79		80	81		80	81	
R1/R2	5	4	1		3	2		5	0		3	2		1	4		3	2		3	2	
Chemotherapy				0.76			0.06			0.53			0.12			0.53			0.06			0.53
No	78	38	40		33	45		41	37		44	34		41	37		33	47		37	41	
Yes	88	45	43		50	38		42	46		39	49		42	46		50	38		46	42	

Clinicopathological terminology was based on the Japanese classification of gastric carcinoma (3rd English version). HD = high density, LD = low density.

* Comparisons were performed with the chi-square test for categorical variables.

analysis. The C-index of our nomogram for predicting the prognosis was 0.83 with the 95% CI from 0.78 to 0.87. The calibration curve for likelihood of survival at 3 or 5 years demonstrated favorable consistency between the predictive value of the nomogram and actual observation (Figure 3B and C). Compared with the TNM staging system (0.70, 95% CI: 0.65–0.76), the C-index of our nomogram was statistically higher ($P < 0.001$), which validate the nomogram as a useful model to predict the long-term survival of gastric cancer patients.

DISCUSSION

Immune cells in the tumor microenvironment have been reported to impact on cancer development, progression, and cancer-related immune reactions, which has emerged as the hotspot of cancer research.[22] In the present study, we performed an immunohistochemical evaluation of immune cells in gastric cancer. To our limited knowledge, this is the first report demonstrating prognostic values of various kinds of immune cells and their combined effects between different cells. In addition, this is also the first time that different immune cells

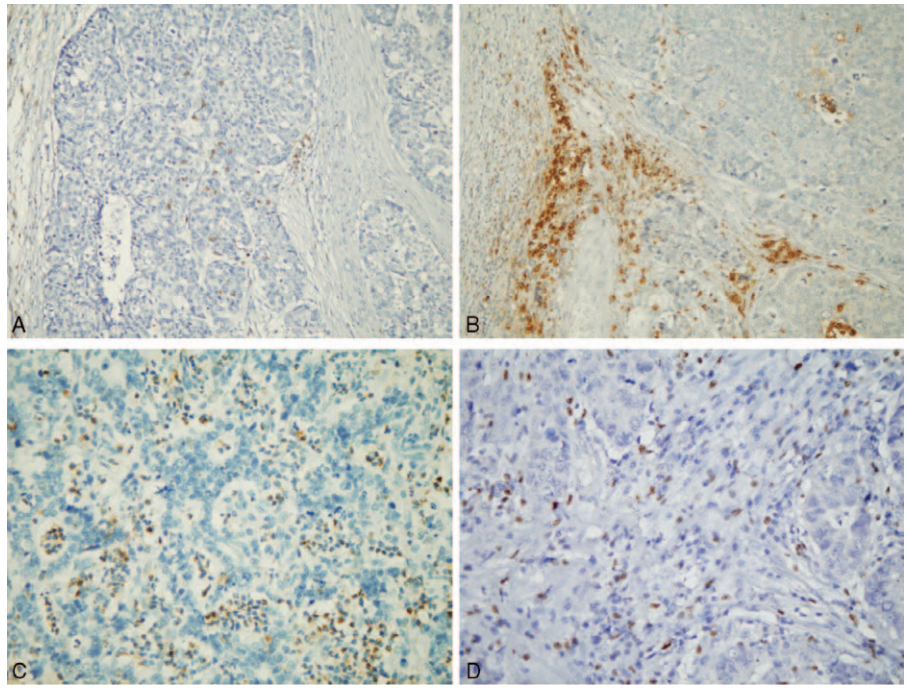

FIGURE 1. Representative pictures of CD8, CD68, CD66b, and Foxp3 immunostainings. (A) CD8$^+$ (x200); (B) CD68$^+$ (x200); (C) CD66b$^+$ (x400); and (D) Foxp3$^+$ (x400).

TABLE 2. Descriptive Statistics of Immunohistochemical Variables

Variables	Mean	Median	Variance	Minimum	Maximum	Skewness
Density of $_{T}CD3^{+}$ cells	129.0998	121.4550	5986.332	2.53	432.56	0.697
Density of $_{T}CD4^{+}$ cells	116.8565	108.3900	4568.909	1.79	294.58	0.303
Density of $_{T}CD8^{+}$ cells	132.7859	133.5100	6733.207	1.14	389.58	0.353
Density of $_{T}CD57^{+}$ cells	84.0251	77.5150	2957.216	0.00	280.33	0.753
Density of $_{T}CD68^{+}$ cells	178.5727	165.6500	12122.743	12.57	467.30	0.633
Density of $_{T}CD66b^{+}$ cells	91.8708	88.7350	2291.675	12.52	230.64	0.876
Density of $_{T}Foxp3^{+}$ cells	20.8371	16.5200	331.170	0.84	98.76	1.487
Density of $_{S}CD3^{+}$ cells	149.1126	127.1300	11956.879	10.91	492.63	0.873
Density of $_{S}CD4^{+}$ cells	97.2548	89.4850	4619.855	2.73	290.40	0.557
Density of $_{S}CD8^{+}$ cells	99.7528	85.3000	7848.429	2.34	490.35	1.784
Density of $_{S}CD57^{+}$ cells	128.7413	109.4400	6763.731	0.00	387.34	0.569
Density of $_{S}CD68^{+}$ cells	169.0667	153.3250	12898.634	12.78	476.90	0.760
Density of $_{S}CD66b^{+}$ cells	113.5694	98.7900	3160.095	10.34	290.34	0.657
Density of $_{S}Foxp3^{+}$ cells	12.1183	10.4400	83.689	0.00	54.43	1.788
Density of $_{N}CD3^{+}$ cells	115.9961	100.8000	5312.879	2.15	325.76	0.899
Density of $_{N}CD4^{+}$ cells	109.2323	90.3450	6552.099	0.50	398.23	0.936
Density of $_{N}CD8^{+}$ cells	106.3078	89.7400	7476.740	0.00	409.17	1.207
Density of $_{N}CD57^{+}$ cells	85.9158	77.4950	4537.068	0.00	398.45	1.455
Density of $_{N}CD68^{+}$ cells	263.7969	267.4150	16504.161	23.11	579.27	0.148
Density of $_{N}CD66b^{+}$ cells	61.3124	48.6050	1713.086	3.45	235.86	1.281
Density of $_{N}Foxp3^{+}$ cells	14.1402	10.0850	169.664	0.00	76.43	2.153
$_{T}CD3^{+}/\ _{S}CD3^{+}$ ratio	1.3946	0.8350	3.464	0.11	17.00	4.806
$_{T}CD4^{+}/\ _{S}CD4^{+}$ ratio	2.3692	1.0700	18.256	0.03	43.64	6.387
$_{T}CD8^{+}\ _{S}CD8^{+}$ ratio	2.1993	1.3250	6.424	0.12	19.49	3.066
$_{T}CD57^{+}/\ _{S}CD57^{+}$ ratio	1.0042	0.7000	1.822	0.11	10.93	5.130
$_{T}CD68^{+}/\ _{S}CD68^{+}$ ratio	1.8301	1.0000	6.554	0.06	19.46	3.557
$_{T}CD66b^{+}/\ _{S}CD66b^{+}$ ratio	1.0773	0.7100	2.604	0.15	19.51	9.345
$_{T}Foxp3^{+}/\ _{S}Foxp3^{+}$ ratio	2.3309	1.4900	11.588	0.10	30.78	4.919
$_{T}CD4^{+}/\ _{T}CD8^{+}$ ratio	2.4238	0.9300	77.135	0.01	102.11	9.650
$_{T}CD3^{+}/\ _{T}Foxp3^{+}$ ratio	17.0958	7.3100	627.323	0.11	139.01	2.634
$_{T}CD4^{+}/\ _{T}Foxp3^{+}$ ratio	15.8539	7.2350	640.243	0.04	180.18	3.863
$_{T}CD8^{+}/\ _{T}Foxp3^{+}$ ratio	17.5204	8.6750	697.533	0.02	181.67	3.494
$_{S}CD4^{+}/\ _{S}CD8^{+}$ ratio	2.3080	1.1550	17.745	0.01	33.56	5.117
$_{S}CD3^{+}/\ _{S}Foxp3^{+}$ ratio	23.4630	11.5150	1420.679	0.28	339.74	4.711
$_{S}CD4^{+}/\ _{S}Foxp3^{+}$ ratio	1.7204	0.8250	20.587	0.02	53.27	9.400
$_{S}CD8^{+}/\ _{S}Foxp3^{+}$ ratio	1.9560	0.9050	14.001	0.02	29.89	5.074
$_{T}CD3^{+}/\ _{N}CD3^{+}$ ratio	1.4314	1.1550	1.273	0.01	8.51	2.751
$_{T}CD4^{+}/\ _{N}CD4^{+}$ ratio	1.7896	1.1100	5.188	0.11	17.98	4.282
$_{T}CD8^{+}\ _{N}CD8^{+}$ ratio	1.9833	1.3300	4.245	0.02	11.77	2.902
$_{T}CD57^{+}/\ _{N}CD57^{+}$ ratio	1.8018	1.0900	6.911	0.07	21.25	4.477
$_{T}CD68^{+}/\ _{N}CD68^{+}$ ratio	0.7451	0.7000	0.289	0.06	4.99	4.620
$_{T}CD66b^{+}/\ _{N}CD66b^{+}$ ratio	2.2357	1.6700	7.878	0.37	27.15	6.214
$_{T}Foxp3^{+}/\ _{N}Foxp3^{+}$ ratio	2.5063	1.6100	17.469	0.06	42.50	6.178
$_{N}CD4^{+}/\ _{N}CD8^{+}$ ratio	3.4853	1.0500	298.822	0.09	216.56	11.603
$_{N}CD3^{+}/\ _{N}Foxp3^{+}$ ratio	27.7552	9.3700	18021.303	0.18	1710.72	12.110
$_{N}CD4^{+}/\ _{N}Foxp3^{+}$ ratio	18.4843	8.7700	994.247	0.03	201.13	3.310
$_{N}CD8^{+}/\ _{N}Foxp3^{+}$ ratio	22.0432	6.6200	4306.716	0.02	684.32	7.960

N = adjacent normal tissue, S = stromal tissue, T = intratumoral tissue.

were examined in intratumoral area, stromal area, and adjacent normal tissues simultaneously.

With respect to the association between TLCs and clinicopathologic factors, we found low-density $_{T}CD4^{+}$ and $_{T}CD8^{+}$ cells correlated positively with M1, lymph nodes metastasis, and more advanced stages. $_{T}Foxp3^{+}$ cell density was higher in tumors reported as M1 or more advanced stages. These results are in agreement with the hypothesis that $CD4^{+}$ and $CD8^{+}$ cells regulate the immune system positively, whereas $Foxp3^{+}$ T_{regs} negatively. It is reported the $CD4^{+}/CD25^{+}$ T_{regs} populations in peripheral blood and tumor tissues of patients with gastrointestinal malignancies were significantly higher compared with healthy volunteers, which might means T_{regs} could aggress to peripheral blood with the progression of cancer.[16,22] Therefore, Shen et al considered that tumor-related factors may induce the recruitment of $CD4^{+}$ TICs and Foxp3+ T_{regs}.[17]

TABLE 3. Correlation Between Different Immune Cells

		$_{T}$CD3	$_{T}$CD4	$_{T}$CD8	$_{T}$CD57	$_{T}$CD68	$_{T}$CD66b	$_{T}$Foxp3	$_{S}$CD3	$_{S}$CD4	$_{S}$CD8	$_{S}$CD57	$_{S}$CD68	$_{S}$CD66b	$_{S}$Foxp3	$_{N}$CD3	$_{N}$CD4	$_{N}$CD8	$_{N}$CD57	$_{N}$CD68	$_{N}$CD66b
$_{N}$Foxp3	*r*	−0.075	−0.123	−0.129	−0.117	−0.110	−0.022	0.355	0.015	−0.015	−0.064	−0.069	0.102	−0.022	0.245	−0.070	−0.041	−0.078	−0.019	−0.047	0.092
	P	0.334	0.114	0.096	0.132	0.158	0.775	0.000	0.850	0.850	0.414	0.376	0.190	0.776	0.001	0.370	0.602	0.320	0.804	0.545	0.239
$_{N}$CD66b	*r*	−0.109	−0.117	−0.201	−0.004	0.007	0.487	0.191	−0.036	−0.104	0.091	−0.253	0.032	0.114	0.009	−0.059	−0.117	−0.119	−0.103	−0.035	
	P	0.163	0.132	0.010	0.962	0.931	0.000	0.014	0.648	0.183	0.242	0.001	0.685	0.143	0.908	0.446	0.133	0.127	0.188	0.657	
$_{N}$CD68	*r*	0.145	0.092	0.039	0.043	0.671	−0.141	−0.109	0.038	−0.007	0.111	0.112	0.365	−0.004	−0.019	0.005	0.172	0.160	0.131		
	P	0.062	0.239	0.621	0.580	0.000	0.071	0.164	0.626	0.932	0.153	0.150	0.000	0.960	0.809	0.945	0.026	0.039	0.092		
$_{N}$CD57	*r*	0.109	0.068	0.009	0.249	0.062	−0.054	−0.188	0.055	0.046	−0.049	0.434	−0.067	0.104	−0.121	0.044	0.086	0.044			
	P	0.164	0.385	0.905	0.001	0.427	0.486	0.015	0.481	0.556	0.528	0.000	0.391	0.182	0.119	0.574	0.272	0.576			
$_{N}$CD8	*r*	0.193	0.226	0.419	0.128	0.181	−0.045	−0.266	0.185	0.282	0.444	0.295	0.017	0.010	−0.090	0.125	0.325				
	P	0.013	0.003	0.000	0.101	0.020	0.566	0.001	0.017	0.000	0.000	0.000	0.825	0.901	0.249	0.109	0.000				
$_{N}$CD4	*r*	0.245	0.520	0.211	0.202	0.207	−0.054	−0.201	0.281	0.457	0.181	0.190	−0.039	0.068	−0.008	0.177					
	P	0.001	0.000	0.006	0.009	0.007	0.488	0.010	0.000	0.000	0.020	0.014	0.619	0.386	0.920	0.022					
$_{N}$CD3	*r*	0.400	0.215	0.205	0.020	−0.015	0.046	−0.081	0.388	0.131	0.009	0.130	0.064	−0.029	−0.052						
	P	0.000	0.006	0.008	0.802	0.849	0.557	0.302	0.000	0.093	0.912	0.096	0.416	0.714	0.505						
$_{S}$Foxp3	*r*	−0.151	−0.065	−0.077	−0.047	−0.132	0.007	0.462	−0.178	−0.048	0.036	−0.174	0.091	−0.204							
	P	0.052	0.409	0.324	0.544	0.090	0.925	0.000	0.022	0.536	0.642	0.025	0.244	0.008							
$_{S}$CD66b	*r*	0.017	0.089	−0.161	0.097	−0.027	0.227	−0.059	0.115	−0.016	−0.122	0.226	−0.140								
	P	0.833	0.254	0.039	0.214	0.726	0.003	0.450	0.141	0.833	0.117	0.003	0.073								
$_{S}$CD68	*r*	−0.068	−0.044	−0.110	−0.193	0.336	−0.085	0.128	−0.105	0.064	0.136	−0.203									
	P	0.387	0.574	0.158	0.013	0.000	0.274	0.101	0.180	0.410	0.080	0.009									
$_{S}$CD57	*r*	0.371	0.196	0.147	0.322	0.004	−0.098	−0.239	0.274	0.159	−0.099										
	P	0.000	0.011	0.058	0.000	0.959	0.207	0.002	0.000	0.041	0.207										
$_{S}$CD8	*r*	0.069	0.126	0.229	0.064	0.227	−0.072	−0.088	0.066	0.068											
	P	0.379	0.106	0.003	0.412	0.003	0.355	0.258	0.395	0.387											
$_{S}$CD4	*r*	0.211	0.463	0.235	0.076	0.075	0.106	−0.296	0.165												
	P	0.006	0.000	0.002	0.333	0.335	0.175	0.000	0.033												
$_{S}$CD3	*r*	0.539	0.177	0.218	0.094	0.028	0.054	−0.187													
	P	0.000	0.023	0.005	0.230	0.720	0.489	0.016													
$_{T}$Foxp3	*r*	−0.176	−0.270	−0.206	−0.234	−0.214	0.087														
	P	0.024	0.000	0.008	0.002	0.006	0.266														
$_{T}$CD66b	*r*	0.027	0.028	−0.179	−0.150	−0.030															
	P	0.731	0.724	0.021	0.054	0.704															
$_{T}$CD68	*r*	0.152	0.194	0.068	0.083																
	P	0.050	0.012	0.383	0.289																
$_{T}$CD57	*r*	0.102	0.105	0.218																	
	P	0.192	0.180	0.005																	
$_{T}$CD8	*r*	0.286	0.243																		
	P	0.000	0.002																		
$_{T}$CD4	*r*	0.204																			
	P	0.008																			

N = adjacent normal tissue, = correlation coefficient, S = stromal tissue; T = intratumoral tissue.

TABLE 4. Univariate Analyses of Factors Associated With Survival

Variables	HR	95% CI (lower)	95% CI (upper)	Log-Rank Test *P*
Gender				0.129
Male	1			
Female	0.653	0.377	1.132	0.129
Age (years)				0.019
<60	1			
≥60	1.7	1.091	2.648	0.019
Longitudinal location				0.001
Upper third	1			
Middle third	0.896	0.480	1.670	0.729
Lower third	0.370	0.222	0.615	0.000
Whole stomach	1.067	0.255	4.470	0.929
Circumferential location				0.135
Less curvature	1			
Great curvature	1.188	0.624	2.263	0.600
Anterior wall	0.863	0.309	2.411	0.778
Posterior wall	1.085	0.581	2.025	0.798
Full circle	2.592	1.254	5.360	0.010
Gross type				0.002
Borr-0	1			
Borr-1	4.964	0.829	29.736	0.079
Borr-2	4.407	1.064	18.260	0.041
Borr-3	5.586	1.322	23.610	0.019
Borr-4	14.707	3.203	67.530	0.001
Differentiation				0.229
Well	1			
Moderate	2.240	0.275	18.250	0.451
Poor	3.845	0.523	28.284	0.186
Signet-ring cell	3.553	0.487	25.917	0.211
Mucinous	1.549	0.173	13.867	0.696
Vessels invasion				0.205
No	1			
Yes	1.416	0.827	2.424	0.205
Tumor size (cm)				0.017
≤2	1			
∼5.0	1.850	0.859	3.982	0.116
∼8.0	2.580	1.153	5.774	0.021
>8.0	3.826	1.558	9.397	0.003
Depth of infiltration (T)*				0.001
T1	1			
T2	1.826	0.515	6.473	0.351
T3/T4a†	4.048	1.471	11.140	0.007
T4b	9.781	2.838	33.713	0.000
Nodal status (N)*				0.000
N0	1			
N1	1.502	0.706	3.197	0.291
N2	2.749	1.269	5.953	0.010
N3a	3.078	1.604	5.903	0.001
N3b	7.384	3.479	15.673	0.000
Distal metastasis (M)*				0.000
M0	1			
M1	4.494	2.546	7.933	0.000
TNM Stage*				0.000
Ia	1			
Ib	4.369	0.730	26.158	0.106
IIa	1.166	0.106	12.870	0.900
IIb	3.029	0.678	13.536	0.147

Variables	HR	95% CI (lower)	95% CI (upper)	Log-Rank Test *P*
IIIa	6.952	1.474	32.790	0.014
IIIb	3.847	0.843	17.566	0.082
IIIc	8.918	2.113	37.646	0.003
IV	21.300	4.859	93.362	0.000
Resection type				0.000
Distal gastrectomy	1			
Total gastrectomy	2.524	1.500	4.246	0.000
Proximal gastrectomy	3.447	1.917	6.199	0.000
Lymphadenectomy				0.000
D1	1			
D1+	0.330	0.174	0.627	0.001
D2/ $D2^+$	0.338	0.204	0.559	0.000
Curative degree				0.672
R0	1			
R1/R2	1.284	0.404	4.080	0.672
Chemotherapy				0.459
No				
Yes	0.846	0.544	1.316	0.459
$_TCD3^+$ cells	0.492	0.311	0.778	0.002
$_TCD4^+$ cells	0.557	0.357	0.870	0.010
$_TCD8^+$ cells	0.571	0.365	0.892	0.014
$_TCD57^+$ cells	0.673	0.432	1.049	0.080
$_TCD68^+$ cells	0.658	0.421	1.028	0.066
$_TCD66b^+$ cells	1.676	1.071	2.624	0.024
$_TFoxp3^+$ cells	2.345	1.481	3.713	0.000
$_SCD3^+$ cells	0.582	0.371	0.912	0.018
$_SCD4^+$ cells	0.615	0.394	0.960	0.032
$_SCD8^+$ cells	1.546	0.988	2.419	0.057
$_SCD57^+$ cells	0.589	0.377	0.919	0.020
$_SCD68^+$ cells	1.564	0.997	2.451	0.051
$_SCD66b^+$ cells	0.608	0.387	0.955	0.031
$_SFoxp3^+$ cells	2.069	1.309	3.269	0.002
$_NCD3^+$ cells	1.036	0.667	1.611	0.874
$_NCD4^+$ cells	0.704	0.452	1.095	0.120
$_NCD8^+$ cells	0.655	0.420	1.022	0.062
$_NCD57^+$ cells	0.728	0.467	1.135	0.162
$_NCD68^+$ cells	0.826	0.531	1.286	0.398
$_NCD66b^+$ cells	1.824	1.165	2.856	0.009
$_NFoxp3^+$ cells	0.573	0.364	0.901	0.016
$_TCD3^+/\ _SCD3^+$ ratio	0.799	0.514	1.244	0.321
$_TCD4^+/\ _SCD4^+$ ratio	1.010	0.650	1.571	0.963
$_TCD8^+\ _SCD8^+$ ratio	0.355	0.223	0.567	0.000
$_TCD57^+/\ _SCD57^+$ ratio	1.963	1.241	3.106	0.004
$_TCD68^+/\ _SCD68^+$ ratio	0.292	0.150	0.568	0.000
$_TCD66b^+/\ _SCD66b^+$ ratio	3.679	2.236	6.054	0.000
$_TFoxp3^+/\ _SFoxp3^+$ ratio	1.338	0.860	2.083	0.197
$_TCD4^+/\ _TCD8^+$ ratio	1.424	0.910	2.229	0.122
$_TCD3^+/\ _TFoxp3^+$ ratio	0.225	0.137	0.371	0.000
$_TCD4^+/\ _TFoxp3^+$ ratio	0.304	0.190	0.486	0.000
$_TCD8^+/\ _TFoxp3^+$ ratio	0.203	0.123	0.335	0.000
$_SCD4^+/\ _SCD8^+$ ratio	0.432	0.273	0.683	0.000
$_SCD3^+/\ _SFoxp3^+$ ratio	0.372	0.234	0.592	0.000
$_SCD4^+/\ _SFoxp3^+$ ratio	0.802	0.516	1.247	0.328
$_SCD8^+/\ _SFoxp3^+$ ratio	2.411	1.528	3.804	0.000
$_TCD3^+/\ _NCD3^+$ ratio	0.667	0.426	1.043	0.076
$_TCD4^+/\ _NCD4^+$ ratio	1.004	0.646	1.561	0.985
$_TCD8^+\ _NCD8^+$ ratio	1.224	0.786	1.906	0.371
$_TCD57^+/\ _NCD57^+$ ratio	1.052	0.676	1.635	0.823
$_TCD68^+/\ _NCD68^+$ ratio	0.813	0.522	1.264	0.357

Variables	HR	95% CI (lower)	95% CI (upper)	Log-Rank Test *P*
$_{T}CD66b^{+}/\ _{N}CD66b^{+}$ ratio	0.777	0.499	1.210	0.264
$_{T}Foxp3^{+}/\ _{N}Foxp3^{+}$ ratio	2.486	1.565	3.951	0.000
$_{N}CD4^{+}/\ _{N}CD8^{+}$ ratio	1.028	0.661	1.599	0.902
$_{N}CD3^{+}/\ _{N}Foxp3^{+}$ ratio	1.195	0.768	1.860	0.430
$_{N}CD4^{+}/\ _{N}Foxp3^{+}$ ratio	0.772	0.496	1.201	0.251
$_{N}CD8^{+}/\ _{N}Foxp3^{+}$ ratio	0.763	0.491	1.187	0.231

CI = confidence interval; HR = hazard ration; N = adjacent normal tissue; S = stromal tissue; T = intratumoral tissue.
* TNM stage and histologic grade are based on the Japanese classification of gastric carcinoma: 3rd English edition.
† T3 of invasive depth was incorporate into T4a due to only 3 patients in the T3 subgroup and $D2^{+}$ of lymphadenectomy was incorporate into D2 due to only 1 patient in the $D2^{+}$ subgroup.

Univariate survival analysis in the present study confirmed high densities of $_{T}CD3^{+}$, $_{T}CD4^{+}$, and $_{T}CD8^{+}$ as well as $_{S}CD3^{+}$ and $_{S}CD4^{+}$ cells resulted in improved survival in gastric cancer. In contrast, neither $_{S}CD8^{+}$, $_{N}CD3^{+}$, $_{N}CD4^{+}$, nor $_{N}CD8^{+}$ cells were associated with survival. Furthermore, $_{T}CD8^{+}/_{S}CD8^{+}$ ratio and $_{S}CD4^{+}/_{S}CD8^{+}$ ratio could be expected to have antitumor reactivity. $_{T}CD3^{+}/_{S}CD3^{+}$ ratio, $_{T}CD4^{+}/_{S}CD4^{+}$ ratio, $_{T}CD4^{+}/_{T}CD8^{+}$ ratio, $_{N}CD4^{+}/_{N}CD8^{+}$ ratio, $_{T}CD3^{+}/_{N}CD3^{+}$ ratio, $_{T}CD4^{+}/_{N}CD4^{+}$ ratio, and $_{T}CD8^{+}/_{N}CD8^{+}$ ratio had no prognostic role in gastric cancer. However, none of the factors mentioned above were found to be associated with overall survival in multivariate survival analysis. Some research has reported that the density of $_{T}CD3^{+}$ TICs decreased during tumor progression,[23] and survival outcomes were improved in patients with a higher density of $_{T}CD3^{+}$ cells.[24] Patients in the high-density groups for $_{T}CD3^{+}$ and $_{T}CD8^{+}$ cells had a significantly longer survival time.[25] However, other studies also reported $CD8^{+}$ T cells producing interleukin-17 could promote tumor progression.[26] We think there are likely several aspects that are responsible for our results and the discrepant research. First, $CD4^{+}$ lymphocytes include a group of heterogeneous T lymphocytes, which can secret diverse cytokines.[27] The presence of specific T-cells could be modulated by other components. Second, the activation status, rather than just the existence of $CD8^{+}$ cells has great prognostic significance.[28,29] It has been reported the activity and the number of cancer peptide-specific T cells need to be enhanced by vaccination with the appropriate cancer antigenic peptides.[30] Third, our

TABLE 5. Multivariate Analyses of Factors Associated With Survival Outcomes

Variables	HR	95% CI (lower)	95% CI (upper)	*P* value
Longitudinal location				0.035
Upper third	1			
Middle third	0.992	0.317	3.105	0.989
Lower third	0.441	0.113	1.712	0.237
Whole stomach	10.367	0.826	130.150	0.070
Tumor size (cm)				0.023
≤2	1			
~5.0	1.854	0.179	19.181	0.605
~8.0	3.319	0.614	17.932	0.163
>8.0	8.729	1.289	59.119	0.026
Nodal status (N)*				0.034
N0	1			
N1	2.715	0.059	125.648	0.610
N2	15.062	0.605	374.890	0.098
N3a	18.507	2.650	129.261	0.003
N3b	24.595	1.680	360.041	0.019
Resection type				0.049
Distal gastrectomy	1			
Total gastrectomy	1.059	0.232	4.829	0.941
Proximal gastrectomy	6.447	0.836	49.707	0.074
$_{T}Foxp3^{+}$ cells	5.580	1.350	23.070	0.018
$_{T}CD68^{+}/\ _{S}CD68^{+}$ ratio	0.158	0.045	0.550	0.004
$_{T}CD66b^{+}/\ _{S}CD66b^{+}$ ratio	3.639	1.107	11.962	0.033
$_{T}CD8^{+}/\ _{T}Foxp3^{+}$ ratio	0.109	0.015	0.790	0.028

CI = confidence interval, HR = hazard ration, N, adjacent normal tissue, S, stromal tissue, T, intratumoral tissue.
* TNM stage and histologic grade are based on the Japanese classification of gastric carcinoma: 3rd English edition.

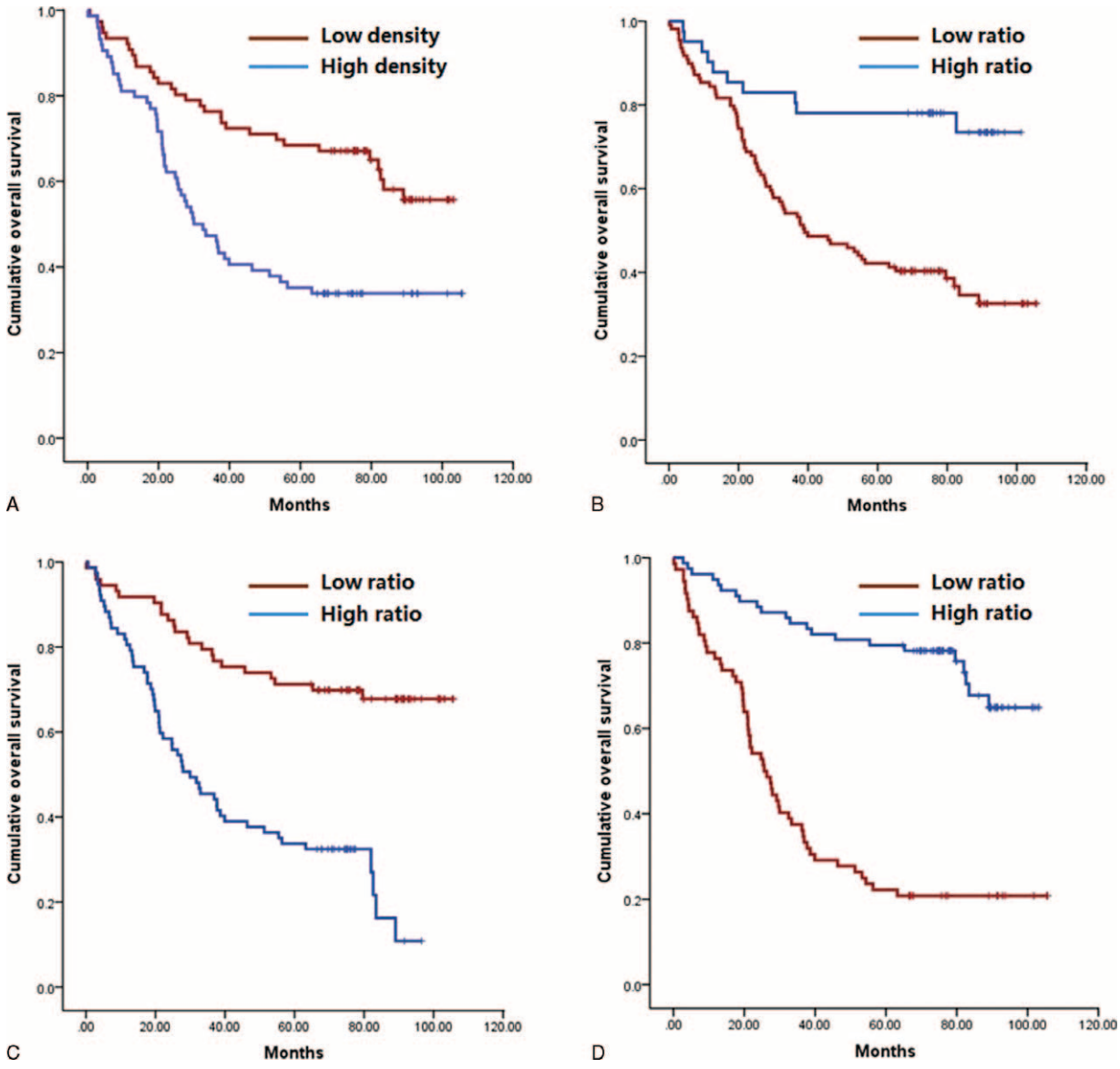

FIGURE 2. Kaplan–Meier analysis of overall survival. (A) High density of $_{T}Foxp3^{+}$ cells versus low density of $_{T}Foxp3^{+}$ cells; (B) high $_{T}CD68^{+}/_{S}CD68^{+}$ ratio versus low $_{T}CD68^{+}/_{S}CD68^{+}$ ratio; (C) high $_{T}CD66b^{+}/_{S}CD66b^{+}$ ratio versus low $_{T}CD66b^{+}/_{S}CD66b^{+}$ ratio; and (D) high $_{T}CD8^{+}/_{T}Foxp3^{+}$ ratio versus low $_{T}CD8^{+}/_{T}Foxp3^{+}$ ratio.

results showed the highest densities of various immune cells were distributed in different locations. Salama et al also reported that lymphocyte densities in both normal and tumor tissues had stronger prognostic significance in colorectal cancer.[31] So the immune cells in different locations of the tumor microenvironment also could influence each other.

CD57 is a marker of natural killer (NK) cells. Our results of univariate analysis indicated the high density of $_{S}CD57^{+}$ cells was associated better overall survival, whereas $_{T}CD57^{+}/_{S}CD57^{+}$ ratio predicted the poor prognosis. Neither density of $_{S}CD57^{+}$ cells nor $_{T}CD57^{+}/_{S}CD57^{+}$ ratio was correlated to the overall survival in multivariate analysis. NK cells could attack tumor cells directly, representing an antitumor immunity.[32] It has been reported that the recruitment of NK cells could exhibit strong antitumor activity and generate a better prognosis in gastric adenocarcinoma.[33] However, an increased proportion of $CD57^{+}$ cells in the circulation indicates a poor prognosis in advanced gastric cancer.[34] One reasonable explanation for this is that the activity of NK cells is regulated by different cytokines and many cell subsets, even cancer itself. It has been reported that gastric cancer cells may decrease NK cytotoxicity through releasing the negative regulated cytokine, IL-10.[35]

Neutrophils comprising CD66b have been identified as a poor prognostic factor in many kinds of cancers, including gastric cancer.[12,36–38] Our study is consistent with these studies, because we observed that densities of $_{T}CD66b^{+}$, $_{N}CD66b^{+}$, cells and $_{T}CD66b/_{S}CD66b^{+}$ ratio were associated with poor outcome in univariate analysis although the density of $_{S}CD66b^{+}$ cells was showed to result in improved survival. Also, the

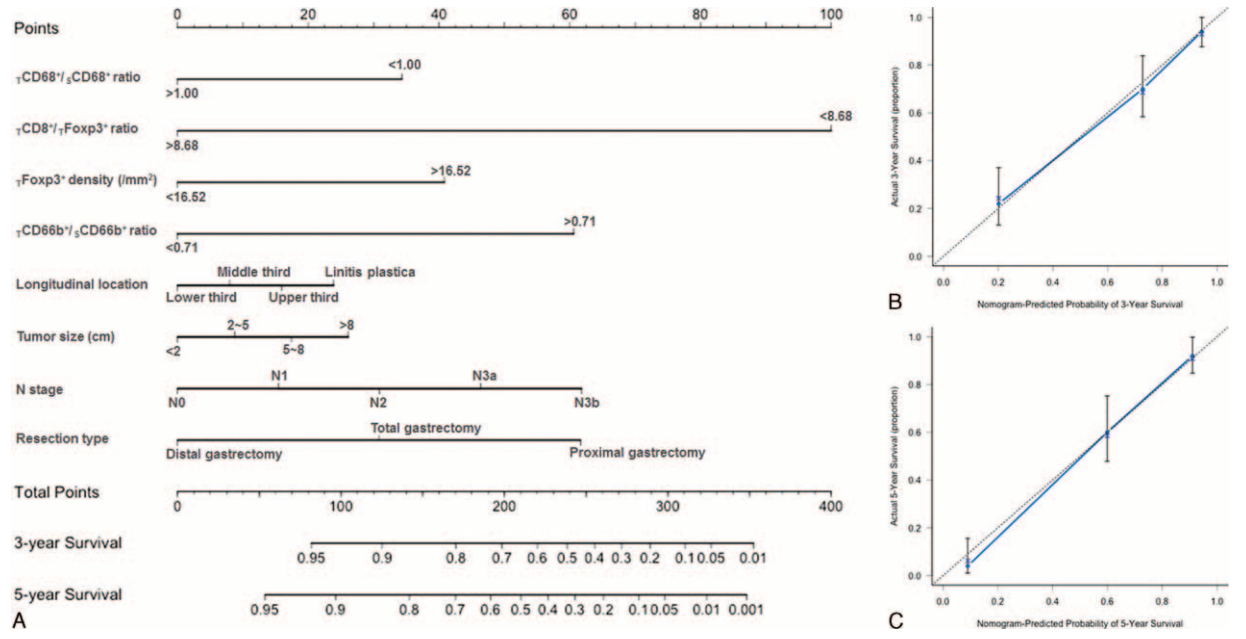

FIGURE 3. Gastric cancer survival nomogram (A). The calibration curve for predicting patient survival at 3 years (B) and 5 years (C).

$_{T}CD66b/_{S}CD66b^{+}$ ratio was identified to be an unfavorable factor in multivariate analysis. Neutrophils are considered to have a protumorigenic role by promoting neoangiogenesis and reducing antitumor immune response.[27] Nevertheless, Caruso et al reported that female patients, compared to male patients with higher density of intratumoral neutrophil have about a 39% reduction in their risk of mortality.[18]

Macrophage, which was recognized as CD68 positive, demonstrated poor survival outcomes in gastric cancer patients.[39] It is suggested that tumor-infiltrating macrophages may cause increased CD44 expression through suppressing miR-328, resulting in tumor progression.[40] And tumor-infiltrating macrophages could express thymidine phosphorylase, which is associated with tumor angiogenesis and poor survival in intestinal type gastric cancer.[41] In the present study, both the univariate and multivariate analyses showed only $_{T}CD68^{+}/_{S}CD68^{+}$ ratio could favor overall survival. This may be explained by 3 possibilities. First, there is an inverse relationship between tumor-infiltrating macrophage cells and other subtypes. Wang et al reported the combination of high numbers of intratumoral $CD68^{+}$ macrophage and $Foxp3^{+}$ T_{regs} was associated with improved survival. Our results also showed the densities of $CD4^{+}$ cells and $CD68^{+}$ cells were significantly correlated in tumor tissues, and density of $Foxp3^{+}$ T_{regs} was negatively correlated with $CD68^{+}$ cells. Second, macrophages in different locations of the tumor microenvironment may have opposite functions and influences between each other, as illustrated by our study. Third, $CD68^{+}$ macrophages display polarized versatile infiltration profiles comprising $CD11c^{+}$ proinflammatory macrophages and $CD206^{+}$ immunosuppressive macrophages in gastric cancer.[42]

T_{regs} are generally considered to be immunosuppressive and block of effective antitumor immunity, therefore are associated to poor outcome in several kinds of tumors.[10,11,13,17,31] Various surface antigens, such as CD4, CD25, Foxp3, CTLA-4, and so on, are expressed on T_{regs}, among which Foxp3 is considered as the most specific marker for T_{regs} and it is possible to define T_{regs} more strictly as $CD4^{+}/CD25^{+}$ regulatory T cells.[11,17,31,43] This is the reason why in the present study, we used Fxop3 + to identify T_{regs}. In univariate analysis, we demonstrated that densities of $_{T}Foxp3^{+}$ and $_{T}Foxp3^{+}$ cells, $_{S}CD8^{+}/_{S}Foxp3^{+}$ ratio and $_{T}Foxp3^{+}/_{N}Foxp3^{+}$ ratio were associated with worse survival and showed stronger prognostic significance. However, density of $_{N}Foxp3^{+}$ cells was associated with better prognosis, which opposed to previous results.[31,44] A high $_{T}CD3^{+}/_{T}Foxp3^{+}$ ratio, $_{T}CD4^{+}/_{T}Foxp3^{+}$ ratio, $_{T}CD8^{+}/_{T}Foxp3^{+}$ ratio and $_{S}CD3^{+}/_{S}Foxp3^{+}$ ratio were associated with improved survival in gastric cancer, and we found that the $_{T}Foxp3^{+}/_{S}Foxp3^{+}$ ratio, $_{S}CD4^{+}/_{S}Foxp3^{+}$ ratio, $_{N}CD3^{+}/_{N}Foxp3^{+}$ ratio, $_{N}CD4^{+}/_{N}Foxp3^{+}$ ratio and $_{N}CD8^{+}/_{N}Foxp3^{+}$ ratio were not prognostic for survival. In multivariate analysis, only density of $_{T}Foxp3^{+}$ cells as negative prognostic factor and $_{T}CD8^{+}/_{T}Foxp3^{+}$ ratio as the positive factor were identified for survival. These results are in keeping with many reports,[16,17,22,45] as well as ovarian cancer, colorectal cancer, breast cancer, and hepatocellular cancer.[10,11,13,31] It has been proven in tumor models that the ratio of T_{regs} to effector T cells, instead of just the presence or absence of T_{regs}, played a more important role in determination of tumor development.[46] It has been reported that T_{regs} can inhibit the function of effector T cells by direct touch or secretion of immune-suppressive cytokines.[47,48] Our results have also shown that density of $_{T}Foxp3^{+}$ cells has a strong negative correlation with that of $_{T}CD8^{+}$ cells. This can partly explain the reason why the prognostic significance of $_{T}CD8^{+}$ cells was apparent in univariate analysis but not remarkable in multivariate analysis. Therefore, a combination of attenuation of T_{regs} and concomitant stimulation of tumor-specific effector T cells may be an effective immunotherapy strategy to improve the prognosis for patients with gastric cancer.[11,17]

Except for the immune parameters, the multivariate analysis revealed that some clinicopathological factors such as tumor size, longitudinal tumor location, and N stage, had independent prognostic significances. It has been reported that

the T stage was a significant prognostic factor for gastric cancer. However, the T stage has not been identified as a significant prognostic factor in the present study, which may be attributed to 2 reasons. First, type II error probably existed in our results because the sample size may be relative small. Second, many variables were included into the multivariate analysis in our Cox model just like TICs, N stage, M stage, radical degree, and so on. Thus, there might be some interactions among these included factors. And the prognostic effect of T stage may be neutralized by other factors.

Our nomogram showed good performance in predicting survival, which was supported by the C-index and the calibration curve. Our nomogram also demonstrated more accuracy than the conventional TNM system for predicting prognosis in gastric cancer. These results could provide a possibility for doctors to predict the prognosis of gastric cancer accurately in clinic through evaluating the resected specimens with these identified novel independent predictors. However, we should notice that the prognostic accuracy of the suggested nomogram has been conducted in the same population where the nomogram was calculated as internal validation in the present study. Although internal validation could prevent against overinterpretation of current data, they cannot ensure external applicability. Whether our nomogram can be universally applied is still to be determined. Therefore, the nomogram needs to be validated externally in the future and it is a question that requires careful clinical judgment. On another hand, the immune reactions, which were proven to be associated with the overall survival in our multivariate analyses, were included in the nomogram comprising more prognostic variables than the traditional staging system. Thus, it can be inferred that part of the prognostic value of TNM system might derive from major underlying differences of quality and density of infiltrating immune cells.[8] However, the exact mechanisms of how immune cells influence the overall survival and interact with each other are far from completely understood. Therefore, further studies are needed to focus on the relationship between the tumor microenvironment and immune cells.

In conclusion, high ${}_{T}CD68^{+}/{}_{S}CD68^{+}$ ratio and ${}_{T}CD8^{+}/{}_{T}Foxp3^{+}$ ratio were associated with improved overall survival, whereas high density of ${}_{T}Foxp3^{+}$ cells and ${}_{T}CD66b^{+}/{}_{S}CD66b^{+}$ ratio demonstrated poor overall survival, which are promising independent predictors for overall survival in gastric cancer.

ACKNOWLEDGMENTS

Authors thank Ying Jie Xiu and Jian Ping Liu from Department of Pathology in West China Hospital for the assistance of pathological evaluation. This work was internal supported by Volunteer Team of Gastric Cancer Surgery (VOLTGA), West China Hospital, Sichuan University, PR China. The authors thank Dr. Trevor Hamilton (Department of Surgical Oncology, Princess Margaret Cancer Centre, University of Toronto, Canada), a native English-speaker, for his kind language modification of the manuscript.

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