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Poor clinical outcomes and immunoevasive contexture in CXCL13+CD8+ T cells enriched gastric cancer patients

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ABSTRACT

As an adverse survival prognosticator, chemokine (C-X-C motif) ligand 13 (CXCL13) has been studied in several types of malignancies. The secretion and physiological roles of CXCL13 in follicular helper T cells (T_{FH}) cells have been well described, while the clinical significance of $CD8^+$ tumor-infiltrating lymphocytes (TILs)-associated CXCL13 remains unknown. This study aims to investigate the clinical significance of $CXCL13^+CD8^+$ T cells in survival and chemotherapeutic responsiveness prediction in gastric cancer. In this study, 440 patients enrolled from Zhongshan Hospital with tumor microarray (TMA) specimens were randomly divided into testing set (n = 220) and validation set (n = 220) for analysis. $CXCL13^+CD8^+$ T cells were detected by multicolor immunohistochemistry. Fresh tumor tissue samples from another 60 gastric cancer patients were collected to detect $CXCL13^+CD8^+$ T cells functional status by flow cytometry (FCM). We found that high intratumoral $CXCL13^+CD8^+$ T cells infiltration predicted poor overall survival and inferior chemotherapeutic responsiveness in gastric cancer. $CXCL13^+CD8^+$T cells were associated with immunoevasive contexture with increased regulatory T (T_{reg}) cells and dysfunctional cytotoxic T lymphocytes (CTLs). Moreover, the combinational analysis of $CXCL13^+CD8^+$ T cells and $CD8^+$ T cells infiltration stratified patients into distinct risk groups with different clinical outcomes and chemotherapeutic responsiveness. Conclusively, intratumoral $CXCL13^+CD8^+$ T cells infiltration could be an independent prognostic and predictive marker for gastric cancer patients. $CXCL13^+CD8^+$ T cells represented an exhausted $CD8^+$ T cell subset, and might be a potential immunotherapeutic target in gastric cancer.

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Introduction

Gastric cancer ranks the fifth most frequently diagnosed malignancy and the third leading cause of cancer-associated mortality in the world.[1,2] Radical gastrectomy is the most effective treatment for gastric cancer patients.[3] Additionally, for patients with TNM stage II and III tumors, postoperative fluorouracil-based adjuvant chemotherapy (ACT) is a common first-line adjuvant therapy.[4] However, gastric cancer is a heterogeneous disease with distinct molecular phenotypes and immune contexture, which attenuates the effect of chemotherapeutic agents and makes it difficult to predict patient clinical outcomes and therapeutic responsiveness to ACT.[5,6]

In recent years, immune checkpoint inhibitors (ICIs), which aim at reactivating antitumor immune responses, have proven highly effective in a series of solid malignancies.[7] Unfortunately, the total response rate of gastric cancer patients to immunotherapy is less than 15%.[8,9] Multiple factors may affect immunotherapeutic responsiveness, including interferon signaling,[10] programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) interaction,[11] and the degree of cytotoxic T lymphocyte (CTL) infiltration.[12]

$CD8^+$ cytotoxic T cells in response to tumor antigens undergo substantial functional and phenotypic alternations, including reduced secretion of effector chemokines and elevated expression of inhibitory receptors.[13] Inhibitory receptors such as PD-1 constitute critical immune checkpoints in T cell activation, and their expression represents a major mechanism by which T cell proliferation and function are regulated and limited. Blockade of PD-1 and other inhibitory receptors reinvigorates $CD8^+$ T cell-mediated immunity and has revolutionized our approach to the treatment of cancers.[14] However, despite the exact clinical benefits of ICIs in many patients, the underlying molecular mechanisms that lead to T cell reactivation during immune checkpoints inhibition are not fully understood, and it remains to be elucidated why some patients respond yet others do not. Consequently, identification of immunotherapeutic responsiveness biomarkers and immune evasion regulators is of urgent need, and further classification of gastric cancer based on T cell functional phenotype might add more information to patient prognosis and decision-making for treatment strategies.

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Chemokine (C-X-C motif) ligand 13 (CXCL13) is a chemokine predominantly involved in the recruitment of B cells to lymph nodes and in the formation of germinal centers.[15] As a tumor-associated chemokine, CXCL13 plays an important part in tumor proliferation and migration.[16] Meanwhile, CXCL13 has been demonstrated as an adverse survival prognosticator in various cancer types, including oral squamous cell carcinoma,[17] non-small cell lung cancer,[18] gastric cancer,[19] hepatocellular carcinoma,[20] prostate cancer,[21] advanced colorectal cancer,[22] lymphoma,[23] and neuroblastic tumor.[24] CXCL13 is principally produced by stromal cells resident in B cell follicles, in murine secondary lymphoid organs.[25,26] However, recent studies have reported that CXCL13 can also be secreted by T cells, especially $CD4^+$ follicular helper T (T_{FH}) cells[27–29] and $CD8^+$ tumor-infiltrating lymphocytes (TILs).[30] The secretion and physiological roles of CXCL13 in T_{FH} cells have been well described,[27,31] while $CD8^+$ TILs-associated CXCL13 still requires further investigation.[32] Interestingly, although intratumoral CXCL13 expression was an adverse survival prognosticator, $CXCL13^+$ T_{FH} cell density paradoxically indicated better survival,[27] indicating the function and the potential effect of CXCL13 on tumor immune microenvironment might be more complicated than we thought. In our previous study, we have demonstrated that CXCL13 could predict poor survival outcomes and therapeutic responsiveness in gastric cancer.[19] However, the source of CXCL13 and the underlying mechanism for CXCL13-associated adverse clinical outcomes remain unknown in gastric cancer, and require further investigation.

In the current study, we inspected the clinical significance and functional features of $CXCL13^+CD8^+$ T cells in gastric cancer. $CXCL13^+CD8^+$ T cells represented a dysfunctional $CD8^+$ T cell subset, and were associated with immunoevasive tumor microenvironment, indicating that $CXCL13^+CD8^+$ T cells might be a potential immunotherapy target in gastric cancer.

Materials and methods

Patients and gastric tissue samples

The study was primarily based on two independent patient cohorts. Cohort 1 includes 496 patients from Zhongshan hospital, Fudan University. However, 56 patients were excluded due to data missing, dot loss or suffering from metastatic diseases. Consequently, we enrolled 440 patients in the current study. These patients received radical gastrectomy and standard D2 lymphadenectomy between August 2007 and December 2008. Four hundred and forty gastric tissue samples of Cohort 1 were formalin-fixed and paraffin-embedded (FFPE). Clinical data including sex, age, tumor size, tumor location, Lauren classification,[33] tumor grade, T classification, N classification, TNM stage, and application of fluorouracil-based ACT were retrospectively collected. Tumor stages were determined according to the 7th edition of the American Joint Committee on Cancer (AJCC) TNM classification. The 440 gastric cancer patients of Cohort 1 were randomly divided into two independent patient sets (Testing set, n = 220; Validation set, n = 220). After surgery, routine fluorouracil-based chemotherapy was administered to the patients with TNM stage II and III tumors. As the endpoint of this study, the overall survival (OS) was counted from the date of gastrectomy to the date of death or the last follow-up. All patients were observed until April 2014. Cohort 2 enrolled additional 60 gastric cancer patients from the Zhongshan hospital, Fudan University. The patients of Cohort 2 underwent radical gastrectomy and standard D2 lymphadenectomy between August 2018 and November 2018. Fresh tissue samples of these patients were collected during surgery and performed with flow cytometry (FCM). Corresponding 60 FFPE tissue blocks were also retrospectively acquired and constructed as an independent tissue microarray (TMA). Detailed patients characteristics were presented (Table 1). Written informed consent was obtained from each patient from Zhongshan hospital, Fudan University, and the study was approved by the institutional review board and ethics committee of Zhongshan hospital, Fudan University.

Pathological analysis on gastric cancer subtypes

The Lauren classification, first addressed by P LAUREN in 1965, was an attempt to classify gastric cancer into two histological main types: diffuse-type and intestinal-type carcinoma.[33] In this study, the Lauren classification was reviewed by the two pathologists (Dr. Lingli Chen and Dr. Peipei Zhang). In 2015, the Asian Cancer Research Group (ACRG) established clinically relevant molecular subtypes (ACRG classification) that encompassed the heterogeneity and provided useful clinical information for gastric cancer. The ACRG used gene expression data to describe four molecular subtypes linked to distinct patterns of molecular alterations, disease progression, and prognosis in gastric cancer, which included microsatellite instability (MSI) subtype, microsatellite stable/epithelial-to-mesenchymal transition (MSS/EMT) subtype, microsatellite stable/tumor protein 53 (TP53)-active (MSS/$TP53^+$) subtype and microsatellite stable/TP53-inactive (MSS/$TP53^-$) subtype.[34] In this study, we used the ACRG Gastric cohort GSE62254 downloaded from NCBI Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). The ACRG classification of gastric cancer was defined in GSE62254. In GSE62254, the $CXCL13^+CD8^+$ T cell signature comprised CD2, CD3E, CD5, CD7, CD8A, CD8B, CD27, CD28, HIF-1α, TGF-β1, TGF-β2, TGF-β3, CTGF, SMAD2, SMAD3, CXCL13 and CXCR5.[35,36] Signature score was defined as the mean of normalized expression of related genes [$\log_2$ (FPKM +1)]. Gene signature was listed in Supplementary Table S1.

Immunohistochemistry

The TMAs of cohort 1 and cohort 2 were constructed as previously described.[37] Double-staining immunohistochemistry was performed and each entire set of TMAs was stained at the same time to ensure an objective comparison between different samples. The slides were baked at 60°C for 6 hours, deparaffinized in xylene for three times, 15 minutes each time and rehydrated in graded alcohol. Then, they were heated in sodium citrate buffer (0.01 M sodium citrate buffer, pH = 6) for

Table 1. Association between $CXCL13^+CD8^+$ T cells infiltration and clinicopathological features in gastric cancer.

	Cohort 1 (n = 440)[a]						Cohort 2 (n = 60)[b]		
	Testing set (n = 220)			Validation set (n = 220)			Flow cytometry set (n = 60)		
	$CXCL13^+CD8^+$ T			$CXCL13^+CD8^+$ T			$CXCL13^+CD8^+$ T		
Features	Low	High	P	Low	High	P	Low	High	P
All patients	115	105		103	117		30	30	
Age			0.24			0.80			0.43
<65 year	69	71		66	73		11	14	
≥65 year	46	34		37	44		19	16	
Sex			0.31			0.64			0.095
Female	39	29		27	34		8	3	
Male	76	76		76	83		22	27	
Location			0.29			0.57			0.66
Distal	61	66		69	71		13	16	
Middle	19	16		11	17		4	2	
Proximal	35	23		23	29		13	12	
Tumor size			**0.049**			0.12			0.79
<4 cm	56	65		61	57		12	11	
≥4 cm	59	40		42	60		18	19	
Grade			0.37			0.96			0.081
G1	3	6		2	3		4	3	
G2	22	24		20	24		7	14	
G3	90	75		81	90		19	11	
G4	0	0		0	0		0	2	
Lauren classification			0.32			0.50			0.069
Intestinal type	67	68		67	71		10	17	
Diffuse type	48	37		36	46		20	13	
T classification			0.40			0.089			0.24
T1	20	26		18	14		3	3	
T2	17	13		20	13		2	7	
T3	21	23		14	27		9	10	
T4	57	43		51	63		16	10	
N classification			**0.026**			0.72			0.64
N0	31	49		41	45		8	11	
N1	16	11		11	12		8	6	
N2	22	15		25	23		4	6	
N3	46	30		26	37		10	7	
TNM stage			**0.010**			0.51			0.17
I	24	29		28	24		3	8	
II	21	33		23	28		12	7	
III	70	43		52	65		15	15	
Adjuvant chemotherapy[c]			**0.004**			0.81			NA
No	38	55		43	47		-	-	
Yes	77	50		60	70		-	-	

Abbreviations: TNM = Tumor-Node-Metastasis; NA = Not available; $P < 0.05$ marked in bold font shows statistical significance.

[a]Cohort 1 originally included 496 patients from Zhongshan Hospital, Fudan University, while 56 patients were excluded due to data missing, dot loss or suffering metastatic diseases. The remaining 440 patients in Cohort 1 were randomly divided into Testing set (n= 220) and Validation set (n= 220).

[b]Cohort 2 included 60 patients from Zhongshan Hospital, Fudan University, of which the fresh samples were collected and performed with flow cytometry to detect CTL function.

[C]Patients with adjuvant chemotherapy received at least one cycle of fluorouracil-based chemotherapy.

antigen retrieval and blocked with 3% H_2O_2 in methanol at 37° C for 30 minutes. Next, 10% normal goat serum was incubated at 37°C to eliminate nonspecific reactions and then applied with anti-CD8A antibody (Abcam, ab199016, diluted at 1:500, Supplementary Table S2) for 2 hours at 37°C. Subsequently, anti-CXCL13 antibody (ThermoFisher, PA5-28827, diluted at 1:1000, Supplementary Table S2) was applied to incubate the slides overnight at 4°C. Next day, TMA slides were washed, incubated with HRP/Mouse and AP/Rabbit secondary antibody with 3,30-diaminobenzidine (DAB) and Vector Blue to visualize the reaction products, respectively, and mounted with aqueous mounting media. In our study, two pathologists (Dr. Peipei Zhang and Dr. Lingli Chen) who were blinded to the clinicopathological data scored all samples separately. The IHC staining sections were scanned at high magnification (×200) and captured by NIS-Elements F3.2 to identify the three independent microscopic fields with the densest infiltration of immune cells to ensure representativeness and homogeneity. Identical settings were used for each photograph. The mean count of their evaluation was adopted. Variations in the enumeration, exceeding 5 cells, were reevaluated separately by both pathologists to acquire consensus.

Flow cytometry

Fresh gastric cancer tissues were collected as soon as the tumors were resected during surgery. Single cells were isolated with the use of collagenase IV and then stained with appropriate monoclonal antibodies (mAbs) for 30 min at 4°C. If necessary, staining of intracellular molecules was performed with Fixation/Permeabilization Solution Kit (BD Biosciences) according to the manufacturer's instructions. Stained cells were washed and resuspended in phosphate-buffered saline/0.1% bovine serum albumin coupled with azide. Subsequently, flow cytometry was performed with FACSCelesta flow cytometer (BD Biosciences) and analyzed by FlowJo software (Tree Star).

Antibodies utilized in the current study were listed (Supplementary Table S2).

Statistical analysis

In Cohort 1, we chose the median value 4 cells/HPF (at ×200 magnification) as the cutoff value. The tumors infiltrated with $CXCL13^+CD8^+$ T cells <4 cells/HPF were defined as $CXCL13^+CD8^+$ T low subgroup, while the tumors infiltrated with $CXCL13^+CD8^+$ T cells ≥4 cells/HPF were defined as $CXCL13^+CD8^+$ T high subgroup. As to Cohort 2, the cutoff value was also the median value. Continuous variables were analyzed with the use of Student's *t* test or Mann–Whitney U-test. The Kruskal-Wallis test followed by Dunn's multiple comparisons test were performed to compare $CXCL13^+CD8^+$ T cell signature within distinct ACRG molecular subtypes. The relationship between $CXCL13^+CD8^+$ T cell density and patient characteristics was evaluated by chi-squared test or Fisher's exact test. Survival outcomes were analyzed through Kaplan–Meier curves, log-rank test, and multivariate analysis based on Cox proportional hazards method. All statistical analyses were 2-sided and *P*-value of < 0.05 was considered as statistically significant. Illustration and statistical analyses were conducted by GraphPad Prism (Version 8.00), Medcalc (Version 12.7.0), IBM SPSS Statistics (Version 25.0) or R software (Version 3.6.1).

Results

Intratumoral $CXCL13^+CD8^+$T cells predict poor prognosis in gastric cancer

In this study, we enrolled two independent gastric cancer patient cohorts (Table 1). Immunohistochemistry (IHC) was performed (Figure 1. a and Supplementary Figure S1. A). Compared with peritumor tissues, intratumor tissues showed significantly higher infiltration of $CXCL13^+CD8^+$ T cells ($P<$ .001; Supplementary Figure S1. B). Consequently, we were predominantly focused on intratumoral $CXCL13^+CD8^+$ T cells in our following study.

Gastric cancer is a heterogeneous disease. In 2015, the Asian Cancer Research Group (ACRG) established clinically relevant molecular subtypes that encompassed this heterogeneity and provided useful clinical information. The ACRG gastric cancer molecular subtypes included MSI subtype, MSS/EMT subtype, $MSS/TP53^+$ subtype and $MSS/TP53^-$ subtype.[34] In this study, we inspected the infiltration of $CXCL13^+CD8^+$ T cells in 4 ACRG gastric cancer subtypes, respectively. However, no certain type displayed significant accumulation of $CXCL13^+CD8^+$ T cells (Figure 1. b). Therefore, we assumed that $CXCL13^+CD8^+$ T cells might indicate a novel subtype of gastric cancer. The association between $CXCL13^+CD8^+$ T cells infiltration and clinicopathological parameters was also inspected (Table 1). Altogether, these data underline that there exists an abnormally increased infiltration of $CXCL13^+CD8^+$T cells in tumor microenvironment, which might indicate a novel subtype of gastric cancer.

To further investigate the clinical significance of intratumoral $CXCL13^+CD8^+$ T cells in gastric cancer, we inspected the association between $CXCL13^+CD8^+$ T cell infiltration and survival status. Notably, we found that the patients who had dismal survival outcomes tended to have significantly more $CXCL13^+CD8^+$ T cells infiltrating into the tumor site (Figure 1. c). Furthermore, we applied Kaplan–Meier curves and log-rank test to compare the overall survival (OS) between $CXCL13^+CD8^+$ T high and $CXCL13^+CD8^+$ T low subgroups. In both Testing set and Validation set, higher infiltration of $CXCL13^+CD8^+$ T cells predicted significantly poorer OS (Figure 1. d). Multivariate Cox regression analysis indicated that $CXCL13^+CD8^+$ T cells could serve as a potential independent prognostic factor for survival outcomes in both Testing set and Validation set (Figure 1. e). Collectively, these results indicate that the infiltration of $CXCL13^+CD8^+$ T cells could be an independent adverse prognosticator in gastric cancer.

Intratumoral $CXCL13^+CD8^+$T cells indicate inferior responsiveness to fluorouracil-based adjuvant chemotherapy in gastric cancer

Subsequently, to discover whether $CXCL13^+CD8^+$ T cells could contribute to the chemoresistance to fluorouracil, we investigated the association between $CXCL13^+CD8^+$ T cell infiltration and responsiveness to adjuvant chemotherapy, based on the overall survival outcomes of the patients with TNM stage II/III tumors. Notably, the $CXCL13^+CD8^+$ T cell high subgroup conferred significantly inferior responsiveness to fluorouracil-based ACT, regarding OS (P= .003 for interaction, Figure 2. a). The same result was also observed when we further stratified patients according to TNM stage. In TNM stage III tumors, $CXCL13^+CD8^+$ T cell high subgroup also had inferior responsiveness to fluorouracil-based ACT (P= .028 for interaction, Figure 2. c). In TNM stage II tumors, although no significant results was observed according to interaction test, there was a trend toward better OS in $CXCL13^+CD8^+$ T cell low subgroup (Figure 2. b). And we assumed that the negative results of interaction test in TNM stage II tumors might result from the relatively small cohort of stage II patients (n = 105). Conclusively, these results suggest that intratumoral $CXCL13^+CD8^+$ T cells could potentially indicate inferior therapeutic responsiveness to fluorouracil-based ACT in gastric cancer.

$CXCL13^+CD8^+$T cells potentially indicate a dysfunctional T cell phenotype in gastric cancer

Since we have highlighted the clinical significance of $CXCL13^+CD8^+$ T cells, and found $CXCL13^+CD8^+$ T cells could predict poor OS and inferior chemotherapeutic responsiveness in gastric cancer (Figures 1 and 2), we wondered whether this subgroup of $CD8^+$ T cells were associated with immunoevasive tumor microenvironment. To validate this hypothesis, we conducted IHC staining on several significant tumor-infiltrating immune cells, including $CD4^+$ T cells, forkhead box P3 positive ($FoxP3^+$) regulatory T (T_{reg}) cells, $CD8^+$ T cells, $CD66b^+$ neutrophils, $CD56^+$ Natural Killer (NK) cells, $CD68^+$ macrophages, $CD163^+$ M2-polarized macrophages and $CD11c^+$ dentritic cells (DC, Figure 3. a and Supplementary Figure S2). Notably, only

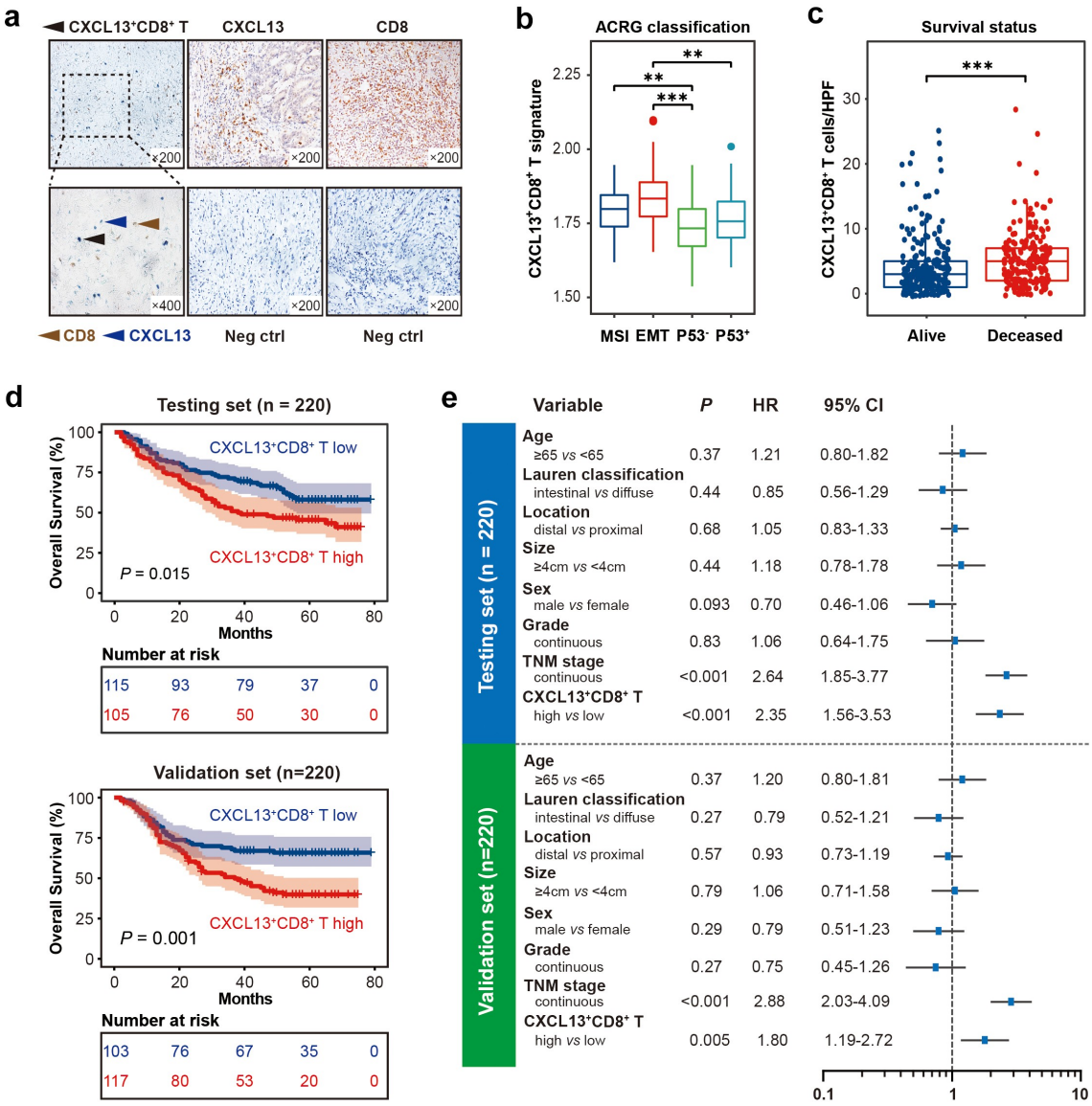

Figure 1. Intratumoral CXCL13$^+$CD8$^+$T cells predict poor prognosis in gastric cancer. (a) Immunohistochemical double-staining of CD8 (brown) and CXCL13 (blue) in gastric cancer tissues. Black arrowhead showed CXCL13$^+$CD8$^+$ T cells (left panel). Immunohistochemistry (IHC) staining for CXCL13 (median panel), CD8 (right panel) and corresponding negative control were also shown. Neg ctrl refers to negative control. (b) Association between CXCL13$^+$CD8$^+$ T cell signature and different ACRG classifications. Kruskal-Wallis test followed by Dunn's multiple comparisons test, *P< .05, **P< .01, ***P < .001, ns refers to not significant. (c) Association between the number of CXCL13$^+$CD8$^+$ T cells and patient survival outcomes. Mann-Whitney U test, *P< .05, **P< .01, ***P < .001, ns refers to not significant. (d-e) Kaplan-Meier curves (d) for overall survival (OS) according to the number of intratumor CXCL13$^+$CD8$^+$ T cells and multivariate analysis (e) based on clinicopathological characteristics in Testing set (n = 220) and Validation set (n = 220), respectively. The OS was compared between CXCL13$^+$CD8$^+$ T high and CXCL13$^+$CD8$^+$ T low subgroups. Log-rank test was performed for Kaplan-Meier curves. HR refers to hazard ratio, CI refers to confidence interval.

CD8$^+$ T cells showed elevated infiltration in CXCL13$^+$CD8$^+$ T high subgroup (Figure 3. a). We assumed if CXCL13$^+$CD8$^+$ T cells could potentially represent a subset of CD8$^+$ T cells featured by a certain functional phenotype. Interestingly, we observed that a high ratio of CXCL13$^+$CD8$^+$ T cells/CD8$^+$ T cells indicated decreased expression of effector molecules interferon-γ (IFN-γ), granzyme B (GzmB) and perforin, yet elevated expression of immune checkpoints, including PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) within CD8$^+$ T cells (Figure 3. b and Supplementary Figure S3). Conclusively, these data suggest that CXCL13$^+$CD8$^+$ T cells potentially indicate a dysfunctional T cell phenotype in gastric cancer.

Stratification of patients based on CXCL13$^+$CD8$^+$T cells and CD8$^+$T cells infiltration predicts prognosis and chemotherapeutic responsiveness in gastric cancer

Since CXCL13$^+$CD8$^+$ T cells potentially indicated a dysfunctional T cell phenotype in gastric cancer, we combined CXCL13$^+$CD8$^+$ T cells and CD8$^+$ T cells infiltration and assumed if we could provide a new stratification system to predict patient survival and therapeutic responsiveness more precisely. The cutoff value for CD8$^+$ T high/low subgroup was also the median value. Four patients were excluded due to the dot loss after IHC staining for CD8. Interestingly, we found that CD8$^+$ T cells could only stratify patient survival outcome in CXCL13$^+$CD8$^+$ T high subgroup, and CXCL13$^+$CD8$^+$ T low subgroup experienced better OS than CXCL13$^+$CD8$^+$ T high/

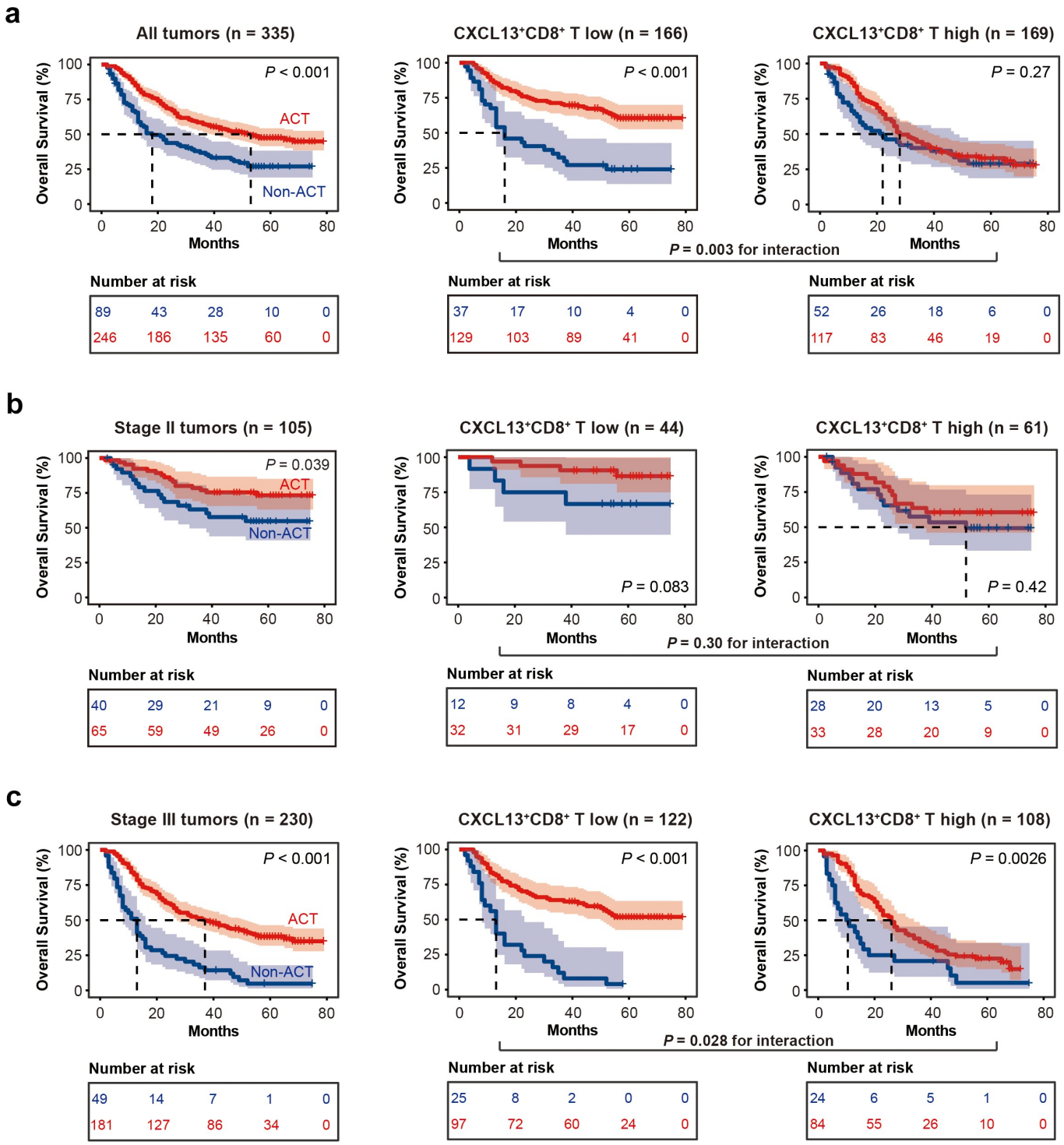

Figure 2. Intratumoral CXCL13$^+$CD8$^+$T cells indicate inferior responsiveness to fluorouracil-based adjuvant chemotherapy in gastric cancer. (a) Kaplan-Meier curves for TNM stage II/III patients with or without adjuvant chemotherapy (ACT) treatment in CXCL13$^+$CD8$^+$ T high/low subgroups. (b) Kaplan-Meier curves for TNM stage II patients with or without ACT treatment in CXCL13$^+$CD8$^+$ T high/low subgroups. (c) Kaplan-Meier curves for TNM stage III patients with or without ACT treatment in CXCL13$^+$CD8$^+$ T high/low subgroups. Log-rank test was performed for Kaplan-Meier curves.

CD8$^+$ T high subgroup or CXCL13$^+$CD8$^+$ T high/CD8$^+$ T low subgroup (Figure 4. a), we trichotomized the patients into three risk subgroups, defined as low-risk group (CXCL13$^+$CD8$^+$ T low), intermediate risk group (CXCL13$^+$CD8$^+$ T high/CD8$^+$ T high), and high-risk group (CXCL13$^+$CD8$^+$ T high/CD8$^+$ T low). Consistent with our hypothesis, low-risk group showed superior responsiveness to fluorouracil-based ACT (P = .007 for interaction, Figure 4. b-e). Consequently, these results indicate that the combination of CXCL13$^+$CD8$^+$ T cells and CD8$^+$ T cells could be applied to stratify patients into various risk subgroups, thus predicting distinct prognosis and responsiveness to fluorouracil-based chemotherapy.

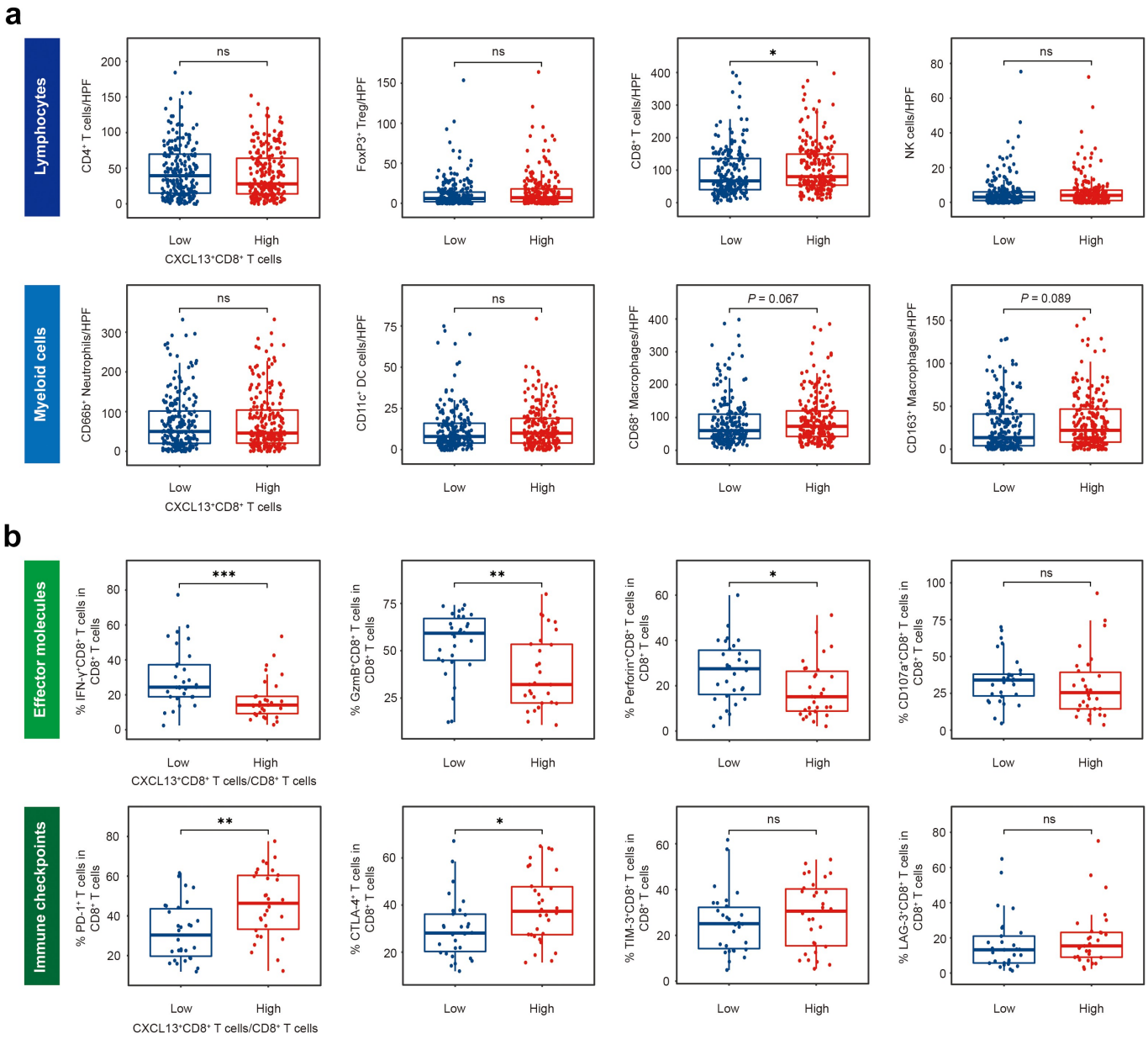

Figure 3. Intratumoral CXCL13$^+$CD8$^+$T cells are associated with a dysfunctional T-cell phenotype in gastric cancer. (a) Association between the intratumoral infiltration of CXCL13$^+$CD8$^+$ T cells and significant immune cells, including CD4$^+$ T cells, FoxP3$^+$ T_{reg} cells, CD8$^+$ T cells, CD66b$^+$ neutrophil cells, CD56$^+$ NK cells, CD68$^+$ macrophages, CD163$^+$ macrophages and CD11c$^+$ DC cells. Unpaired *t* test (CD4, CD8, CD66b, CD56, CD68, CD163, CD11c), Mann-Whitney U test (FoxP3), *P< .05, **P< .01, ***P < .001, ns refers to not significant. (b) Flow cytometry to detect the expression of effector/activated molecules (IFN-γ, granzyme B, perforin, CD107a) and immune checkpoints (PD-1, CTLA-4, TIM-3, LAG-3) on CD8$^+$ T cells in CXCL13$^+$CD8$^+$ T cells/CD8$^+$ T cells high or low subgroups. Mann-Whitney U test, *P< .05, **P< .01, ***P < .001, ns refers to not significant.

Conclusively, our risk classification model could be a reliable prognostic and predictive factor in gastric cancer.

Discussion

It is generally believed that CD8$^+$ T cells act as a positive prognosticator in most cancer types, as they could attack cancer cells directly.[38] However, emerging studies have reported that several certain subtypes of intratumoral CD8$^+$ T cells are associated with poor clinical outcomes,[39] which attributes to T cell dysfunction.[32] Previous studies, which investigate T cells in human melanoma and hepatocellular carcinoma, have identified *CXCL13* as a significantly upregulated gene in dysfunctional CD8$^+$ T cells.[28,29,40] Until now, few studies have investigated the mechanism of CXCL13 up-regulation in malignancies. Some studies indicated that CXCL13 expression might be regulated by phosphatidylinositol 3-kinase (PI3K)-AKT pathway[41] or Wnt/β-Catenin signal pathway.[42] The up-regulated CXCL13 could interact with its specific receptor C-X-C motif chemokine receptor 5 (CXCR5), and form the CXCL13-CXCR5 axis, which plays an important role in tumor proliferation and migration.[16] Although previous studies reported that the expression of CXCL13 receptor CXCR5 was significantly up-regulated in gastric cancer,[43] and CXCR5 could be expressed on CD8$^+$ T cell,[44] CD4$^+$ T_{FH} cells[45] and myeloid-derived suppressor cells (MDSCs),[46] the clinical

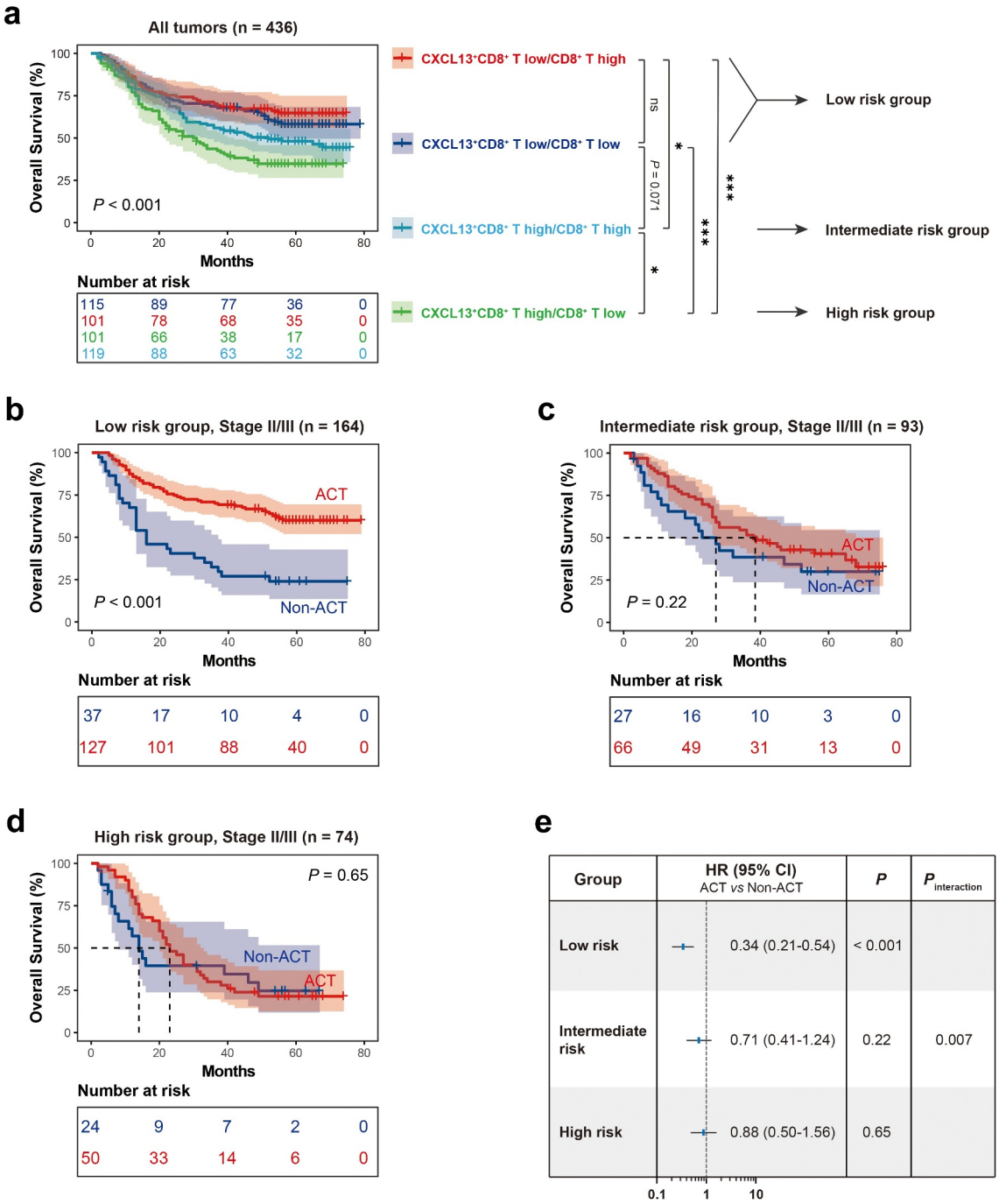

Figure 4. Stratification of patients based on CXCL13$^+$CD8$^+$T cells and CD8$^+$T cells infiltration predicts prognosis and chemotherapeutic responsiveness in gastric cancer. (a) CD8$^+$ T cells could only stratify patient survival outcome in CXCL13$^+$CD8$^+$ T high subgroup, while CXCL13$^+$CD8$^+$ T low subgroup experienced better OS than CXCL13$^+$CD8$^+$ T high/CD8$^+$ T high subgroup or CXCL13$^+$CD8$^+$ T high/CD8$^+$ T low subgroup. The patients were further trichotomized into three risk subgroups, defined as low risk group (CXCL13$^+$CD8$^+$ T low), intermediate risk group (CXCL13$^+$CD8$^+$ T high/CD8$^+$ T high), and high risk group (CXCL13$^+$CD8$^+$ T high/CD8$^+$ T low). (b-e) Low risk group showed superior responsiveness to fluorouracil-based ACT (P = .007 for interaction). HR refers to hazard ratio, CI refers to confidence interval, ACT refers to adjuvant chemotherapy.

significance and the potential impact of CXCR5 on gastric cancer was still obscure, and would be further investigated in our following studies.

Immune checkpoint blockade (ICB) which reactivates tumor-specific T cells through targeting the PD-1/PD-L1 axis has emerged as a promising treatment strategy for various malignancies.[47] However, very few patients (13%) responded to ICB in gastric cancer,[8,9] and efforts to improve ICB treatment efficacy were confounded by a lack of understanding of the underlying mechanisms of immune evasion.[48] Notably, prior studies have reported that not all PD-1$^+$ cells may respond to anti-PD-1 treatment equally,[49,50] and not all PD-1$^+$ T cells indicated exhausted or dysfunctional T cells, since PD-1 expression might begin to rise once activated.[51] A prior study has described PD-1^{hi} CD8$^+$ T cells as a highly distinct cellular pool. Exhausted PD-1^{hi} T cells were characterized by high levels of CXCL13 production, and CXCL13$^+$PD-1^{hi}CD8$^+$ T cells could mediate the

tolerance to immunotherapy.[30] Notably, three recent studies that investigated T cells in human malignancies have identified *CXCL13* as one of the significantly up-regulated genes in highly exhausted TILs,[28,29,40] and *CXCL13* mRNA expression was accompanied by constitutive protein secretion from $CD8^+$ T cells,[27,30] suggesting a possible involvement of these cells in the formation of immune evasion and resistance to ICB.

In this study, we described a subset of intratumoral $CD8^+$ T cells which expresses CXCL13 in gastric cancer, and analyzed the prognostic and predictive value of $CXCL13^+CD8^+$ T cells in gastric cancer. We found that higher infiltration of $CXCL13^+CD8^+$ T cells could indicate poor prognosis and inferior therapeutic responsiveness to fluorouracil-based adjuvant chemotherapy in gastric cancer. Furthermore, we found that $CXCL13^+CD8^+$ T cells represented a dysfunctional phenotype of $CD8^+$ T cells, with decreased IFN-γ, granzyme B, and perforin level yet elevated PD-1 and CTLA-4 expression. These results indicated that higher infiltration of $CXCL13^+CD8^+$ T cells might be correlated with an immunoevasive tumor microenvironment, which provided an explanation for the poor survival and inferior responsiveness to ACT in gastric cancer.

However, our study was retrospective and required a further validation to confirm our findings within the framework of larger and multi-centered clinical cases. Besides, the underlying mechanism that $CXCL13^+CD8^+$ T cells orchestrated immune evasion was poorly understood and needed further investigation and elucidation in the following studies. In conclusion, our study suggested that intratumoral $CXCL13^+CD8^+$ Tcells could be an independent prognosticator for poor OS and inferior responsiveness to ACT in gastric cancer. Moreover, $CXCL13^+CD8^+$ Tcells indicated adysfunctional phenotype of $CD8^+$ Tcells, and could identify an immunoevasive subtype gastric cancer, indicating that $CXCL13^+CD8^+$ Tcells might be apotential immunotherapy target in gastric cancer.

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Authors' contributions

K. Jin, Y. Cao, Y. Gu and H. Fang for acquisition of data, analysis and interpretation of data, statistical analysis and drafting of the manuscript; Y. Fei, J. Wang, X. Liu, K. Lv, X. He, C. Lin, H. Liu, H. Li and H. He for technical and material support; R. Li, H. Zhang and J. Xu for study concept and design, analysis and interpretation of data, drafting of the manuscript, obtained funding and study supervision. All authors read and approved the final manuscript.

Availability of data and material

All data generated that are relevant to the results presented in this article are included in this article. Other data that were not relevant for the results presented here are available from the corresponding author Dr. Xu upon reasonable request.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Consent for publication

All authors provide their consent for publication of the manuscript.

Ethics approval and consent to participate

The study was approved by the Clinical Research Ethics Committee of Zhongshan Hospital, Fudan University. Written informed consent was obtained from each patient included and this study was performed in accordance with the Declaration of Helsinki.

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