BMC Cancer

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The immune signatures predict gastric/gastroesophageal junction cancer response to first-line anti-PD-1 blockade or chemotherapy

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Abstract

Background Anti-programmed cell death-1 (PD-1) immunotherapy and platinum-based chemotherapy are key components of first-line treatment for advanced Gastric or Gastroesophageal Junction (G/GEJ) cancer. However, the role of immune cells infiltrating the tumor microenvironment (TME) in predicting both therapy responses is still unclear.

Methods ORIENT-16 is a randomized, double-blind, placebo-controlled, phase 3 clinical trial, and enrolled 650 patients with unresectable locally advanced or metastatic G/GEJ cancer between January 3, 2019, and August 5, 2020. For patients enrolled from the First Affiliated Hospital of Zhejiang University School of Medicine, we analyzed progression-free survival(PFS) and overall survival (OS) based on PD-L1 expression and landmark analysis, and developed a multiplexed immunofluorescence (mIF) assay for CD4, CD8, PD-L1, CD68 and FoxP3 coupled with digital image analysis and machine learning to assess prognostic survival associations of immune cells.

Results A total of 54 eligible patients were enrolled in this study, 35 received sintilimab plus platinum-based chemotherapy and 19 received placebo plus platinum-based chemotherapy. For patients with PD-L1 combined positive score (CPS) < 10, survival disparities between anti-PD-1 immunotherapy and chemotherapy emerged 300 days post-treatment. High PD-L1 expression correlated with longer survival in anti-PD-1 therapy but less benefit in platinum-based chemotherapy. The mIF analysis also demonstrated significantly higher stromal PD-L1 density in immunotherapy responders, but tended to be lower in chemotherapy responders. Besides, high tumor stromal CD8 expression could be used as a positive biomarker in anti-PD-1 immunotherapy, and high tumor stromal CD4 expression was found associated with worse prognosis in platinum-based chemotherapy.

Conclusions Increased PD-L1 expression was associated with an increased effect on anti-PD-1 immunotherapy and reduced benefit from chemotherapy. The signature of TME immune cells has the potential to predict the response of anti-PD-1 immunotherapy and chemotherapy in G/GEJ cancer.

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Trial registration ClinicalTrials.gov Identifier: NCT03745170.

Keywords Gastric/Gastroesophageal junction (G/GEJ) cancer, Anti-PD-1 blockade, Platinum-based chemotherapy, Tumor immune microenvironment (TME), Predictive biomarkers

Introduction

Gastric and gastroesophageal junction (G/GEJ) cancer is one of the most common cancers worldwide, ranking fifth in incidence and fourth in mortality [1]. In China, new G/GEJ cancer cases account for approximately 40% of all new cases globally, with the vast majority presenting at stage III or IV and missing the opportunity for surgical resection [2, 3]. Even after curative surgery, most patients develop local recurrence or distant metastases, with a 5-year survival rate of less than 25% [4–6].

Immune checkpoint inhibitors (ICIs) targeting programmed cell death-1 (PD-1) and programmed death-ligand 1 (PD-L1) have significantly revolutionized the treatment landscape of advanced G/GEJ cancers [7, 8]. The ORIENT-16 trials [9] demonstrated superior efficacy and safety of first-line sintilimab plus chemotherapy compared to chemotherapy alone in Chinese patients. However, no significant differences were observed for patients with a combined positive score (CPS) less than 5. It seems that not all G/GEJ patients could benefit from inhibitors. The predictive value of PD-L1 in precision medicine remains to be explored. In addition, as the cornerstone treatment modality, fluoropyrimidine plus platinum-based chemotherapy is still an indispensable strategy for unresectable advanced or metastatic human epidermal growth factor receptor 2 (HER2)-negative G/GEJ adenocarcinoma, but its clinical outcome is far from satisfactory [10–15]. Therefore, there is an urgent need to study the potential factors that affect anti-PD-1/PD-L1 immunotherapy and platinum-based chemotherapy.

Alongside a growing understanding of the tumor immune microenvironment, researchers are increasingly focusing on components that may influence the treatment response, such as T cells, B cells, neutrophils, and macrophages [16–19]. In this context, studies have suggested that a high abundance of infiltrating CD8 + or CD3 + T cells within tumor tissues indicates a neoplastic "hot tumor" or an immune-inflamed state, suggesting a robust immune response [20]. PD-1 and PD-L1 blockade can also elicit a strong natural killer (NK) cell response [21], whereas β-catenin within the tumor can hinder dendritic cell recruitment, leading to PD-1/PD-L1 resistance [22]. The immune status of an individual can also be used to assess the effects of chemotherapy. Recent advances highlight the TME's multidimensional role in therapy resistance. Machine learning frameworks like iMLGAM [23], DAS [24], and PTMLS [25], driven by multiomics-defined features such as post-translational modified proteins and tumor-related genes, revealed that immune-hot microenvironments correlate with superior ICB response. These underscore the need for composite biomarker strategies. Therefore, owing to the inherent heterogeneity of tumors and the complex immune response to treatment, single biomarkers are insufficient for identifying the potential benefits in gastric cancer treatments.

Advancements in multiplex immunofluorescence (mIF) allow simultaneous visualization of multiple antigen markers within the same tissue section. This technique, combined with digital image analysis and machine learning, facilitates characterization of the density and spatial distribution of the tumor immune microenvironment. However, mIF remains underutilized in the context of advanced gastric cancer. Consequently, factors within the tumor microenvironment that dictate the response to first-line anti-PD-1 immunotherapy and chemotherapy remain unclear.

In the present study, patients in the ORIENT-16 study from the First Affiliated Hospital of Zhejiang University School of Medicine were included. We further analyzed the survival curves using the landmark statistical method and performed exploratory analyses of the efficacy of anti-PD-1 immunotherapy and platinum-based chemotherapy in PD-L1 CPS ≥ 10 and CPS < 10 subgroups. Using multiplexed immunofluorescence combined with digital image analysis and supervised machine learning, we quantified the immune cells in the tumor microenvironment and assessed the relationship between immune cell densities and treatment outcomes. These data will be used to investigate the immune and stromal features of good responders and poor responders, with the goal of identifying the prognostic significance of immune biomarkers of anti-PD-1 immunotherapy or platinum-based chemotherapy.

Methods

Study design and participants

The ORIENT-16 study, a randomized, double-blind, phase 3 clinical trial, was conducted at 62 hospitals in China and registered on November 14, 2018, at ClinicalTrials.gov (NCT03745170) (https://clinicaltrials.gov/ct2/show/NCT03745170?term=NCT03745170%26;draw=2%26;rank=1). Our study is a secondary analysis of data from the ORIENT-16 study. Patients from the First Affiliated Hospital of Zhejiang University School of Medicine were collected, which has the largest number of participants in the ORIENT-16 study. The trial was conducted according to Good Clinical Practice Guideline. The

protocol and all amendments were approved by an independent ethics committee and were consistent with the ORIENT-16 study published previously [9].

Eligible patients were 18 years or older with histologically confirmed unresectable locally advanced, recurrent, or metastatic G/GEJ adenocarcinoma. Patients who had received previous adjuvant or neoadjuvant chemotherapy or radiotherapy were also allowed at least 6 months after the last administration for disease recurrence. Additionally, patients needed to have at least one measurable or evaluable lesion, as defined in the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines (version 1.1). Patients with known human epidermal growth factor receptor 2 (HER2)-positive status; a history of active, known, or suspected autoimmune disease; or prior systemic treatment for advanced or metastatic G/GEJ adenocarcinoma were excluded from the trial. Written informed consent was obtained from all patients prior to enrollment.

All patients were randomly assigned to sintilimab plus chemotherapy group (sintilimab plus platinum-based chemotherapy) and placebo plus chemotherapy group (placebo plus platinum-based chemotherapy). Randomization was performed using an interactive web response system and stratified according to the Performance Status (PS) score of the Eastern Cooperative Oncology Group Performance Status (ECOG) (0 or 1), liver metastasis (yes or no), peritoneum metastasis (yes or no), and PD-L1 (Combined Positive Score (CPS) < 10 or ≥ 10). The patients, investigators, and study sponsor remained blinded to the treatment assignment throughout the study.

Outcomes

The primary endpoints of overall survival (OS) and the secondary endpoints of progression-free survival (PFS), objective response rate (ORR) and safety for the primary analysis populations of ORIENT-16—the total, PD-L1 CPS ≥ 5, and CPS < 5 populations—have been reported. The efficacy outcomes for the PD-L1 CPS < 10 and CPS ≥ 10 subgroups (OS and PFS) were evaluated. Additionally, the prespecified exploratory endpoint evaluated the association between the efficacy of anti-PD-1 immunotherapy or platinum-based chemotherapy and PD-L1 expression levels as well as other biomarkers within the immune microenvironment. The efficacy endpoints were assessed by independent reviewers.

Biomarker analysis

Formalin-fixed, paraffin-embedded samples from primary or metastatic lesions were evaluated during screening for PD-L1 expression. The evaluation was performed by a central laboratory (Covance, Shanghai, China) using PD-L1 IHC 22C3 pharmDx assay (Agilent Technologies, USA). A scoring system called the combined positive score (CPS) was used, which was obtained by dividing the number of PD-L1-positive tumor cells, lymphocytes, and macrophages by the total number of viable tumor cells, and then multiplying by 100.

Based on the abundant literature on the modulatory effects of the tumor microenvironment, the research team decided on a biomarker panel after further discussion. This panel included T cells (CD4 [26], CD8 [20]), regulatory T cells (FoxP3 [27]), macrophages (CD68 [28]), immune escape markers (PD-L1 [29]), and Pan-CK. The Akoya OPAL Polaris Seven-Color Automation multiplex immunohistochemistry (mIHC) panels were used for mIF staining following the manufacturer's instructions. The concentration and staining order of the antibodies used in this study were optimized in advance. Primary antibodies raised against CD4 (Cell Signaling Technology, #25229, 1:50), CD8 (Cell Signaling Technology, #85336, 1:100), CD68 (Cell Signaling Technology, #76437, 1:200), FoxP3 (Cell Signaling Technology, #98377, 1:50), and PD-L1 (Cell Signaling Technology, #13684, 1:100), were used. Nucleic acids were stained with DAPI. Tumor parenchyma and stroma were differentiated based on pan-CK staining (Cell Signaling Technology, #4545, 1:250).

The immunofluorescence slides were scanned using the Vectra 3.0 Automated Quantitative Pathology Imaging System (Akoya Biosciences) equipped with a 20 × objective and visualized using Phenochart software. Two experienced pathologists reviewed all samples and excluded inappropriate regions from the analysis. Typical fields of view were selected with minimal overlap between regions and positioned to capture as much tissue as possible. The multispectral images were analyzed using the inForm software package(Akoya Biosciences). The density of various immune cell subsets was measured as the number of positively stained cells per square millimeter (cells/mm^2).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 9 and the IBM SPSS Statistics software (version 26.0). The chi-square test or Independent Samples t-test was performed to compare the baseline characteristics between the two treatment arms. Censoring for overall survival and progression-free survival occurred at the last date of survival and last tumor imaging assessment, respectively. Kaplan-Meier (KM) plots were generated to visualize the corresponding PFS and OS, and the stratified log-rank test was used to compare the treatment arms (sintilimab + chemotherapy vs. placebo + chemotherapy) and CPS score groups (≥ 10 vs. <10). Two-sided 95% confidence intervals (CIs) for the hazard ratios (HRs)

were calculated. Subgroup analyses using the stratified Cox proportional hazards model assessed PFS and OS according to CPS scores and baseline characteristics (age, histological type, and metastases), ensuring a minimum sample size of 15 per subgroup. The independent sample t-test and Mann-Whitney U test were used to assess the association between immune cells in tumor microenvironment and treatment efficacy in the exploratory multiplexed immunofluorescence analysis.

Results

Patients characteristics

From January 3, 2019, to July 20, 2020, 63 patients were screened for eligibility in our center. Of these, 54 were randomly assigned to treatment: 35 in the sintilimab plus chemotherapy group and 19 in the placebo plus chemotherapy group (Fig. 1A). The baseline characteristics were generally well balanced between the two treatment groups (Table 1). Among 54 patients, 39 (72.2%) had gastric adenocarcinoma (sintilimab plus

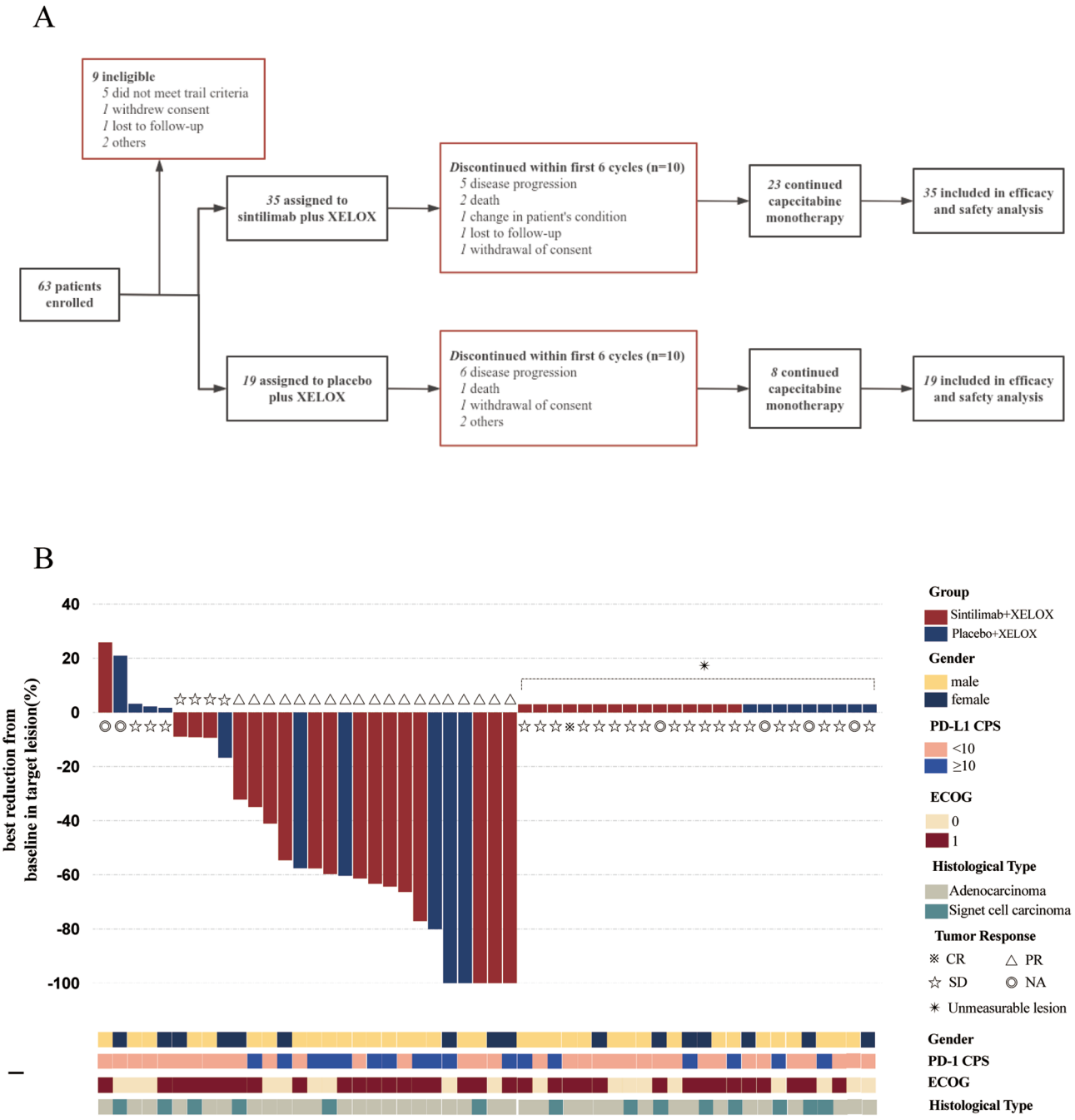


Fig. 1 Trial profile (**A**) and treatment response (**B**)

Table 1 Baseline characteristics of study patients

	Sintilimab + chemotherapy[a] (*N* = 35)	Placebo + chemotherapy (*N* = 19)	*P*-value
Age (years)			
Median	62	63	0.69
Range	41–74	44–72	
Sex, *n* (%)			
Female	9(25.7)	7(36.8)	0.39
Male	26(74.3)	12(63.2)	
ECOG performance status, *n* (%)			
0	10(28.6)	8(42.1)	0.31
1	25(71.4)	11(57.9)	
Primary tumor location, *n* (%)			
Gastric	35(100.0)	18(94.7)	0.75
Gastric-esophageal junction	0(0.0)	1(5.3)	
Histological type, *n* (%)			
Adenocarcinoma	25(71.4)	14(73.7)	0.86
Signet cell carcinoma	10(28.6)	5(26.3)	
Lauren's classification, *n* (%)			
Intestinal	6(16.7)	0(0.0)	0.2
Diffuse	5(14.3)	3(15.8)	
Mixed	2(5.6)	3(15.8)	
Unknown or unclassifiable	22(62.9)	13(68.4)	
Previous gastrectomy, *n* (%)			
No	28(80.0)	16(84.2)	0.79
Yes	7(20.0)	3(15.8)	
Perioperative chemotherapy, *n* (%)			
No	31(88.6)	17(89.5)	1
Yes	4(11.4)	2(10.5)	
Extent of disease, *n* (%)			
Metastatic	35(100.0)	16(84.2)	0.07
Locally advanced	0(0.0)	3(15.8)	
Site of metastases, *n* (%)			
Liver	8(22.9)	7(36.8)	NA
Peritoneum	14(40.0)	8(42.1)	
Lung	2(5.7)	2(10.5)	
Bone	3(8.6)	1(5.3)	
Lymph node	22(62.9)	11(57.9)	
Other	8(22.9)	6(31.6)	
Tumor cell PD-L1 expression			
CPS < 10	23(65.7)	14(73.7)	0.55
CPS ≥ 10	12(34.3)	5(26.3)	

ECOG Eastern Cooperative Oncology Group, *PD-L1* Programmed Death-Ligand 1, *CPS* the Combined Proportional Score

[a]Chemotherapy: XELOX(Capecitabine plus oxaliplatin)

chemotherapy, 25/35 [71.4%]; placebo plus chemotherapy, 14/19 [73.7%]). Thirty-seven (68.5%) patients had a PD-L1 CPS ≥ 10 (sintilimab plus chemotherapy, 12/35 [34.3%]; placebo plus chemotherapy, 5/19 [26.3%]). Distant lymph nodes (33/54 [61.1%]) and peritoneal metastases (22/54 [40.7%]) were the most common presentations in patients with advanced disease. Of the patients in the immunotherapy group, 28.6% ($n = 10/35$) discontinued treatment within the first six cycles, compared to 52.6% ($n = 10/19$) in the chemotherapy group. Disease progression was the main reason for discontinuation (sintilimab plus chemotherapy, 5/35 [14.3%]; placebo plus chemotherapy, 6/19 [31.6%]). 65.7% ($n = 23/35$) of the patients in the sintilimab plus chemotherapy group, compared to 42.1% ($n = 8/19$) in the placebo plus chemotherapy group subsequent treatment. Additionally, 14.3% ($n = 5/35$) and 15.8% ($n = 3/19$) of the respective groups underwent subsequent gastrectomy. The changes of best reduction from baseline in target leisions, as well as tumor response and some clinical data are presented in a waterfall plot (Fig. 1B).

Survival differences between anti-PD-1 therapy and chemotherapy: subgroup analyses and landmark analyses

The efficacy and safety analyses in the present study were in accordance with ORIENT-16 observations, and each is indicated with a note in the supplementary materials. A CPS threshold of 10 was used as the randomization stratification factor in our study. Patients with a CPS of 10 or more appeared to benefit significantly more from sintilimab immunotherapy, with an estimated hazard ratio (HR) of 0.14 (95% CI 0.02–1.01; $P<0.0001$) for PFS and 0.19 (95% CI 0.03–1.09; $P=0.0009$) for OS (Fig. 2A and B). Conversely, no significant benefits were observed in patients with CPS less than 10 (mPFS, HR 0.66 [0.30–1.48]; $P=0.27$; OS, HR 0.66 [0.30–1.48]; $p=0.25$) (Fig. 2C and D). Landmark analysis of the CPS < 10 population revealed temporal heterogeneity in treatment response: while early survival curves were comparable ($P=0.632$), a statistically significant divergence emerged beyond 300 days post-treatment ($P=0.008$; Fig. 3). Complete subgroup analyses are presented in Supplementary Figs. 2–3.

Survival analyses of both anti-PD-1 therapy and chemotherapy: predictive role of PD-L1 expression

We performed a comprehensive evaluation of treatment efficacy stratified by PD-L1 expression levels in patients receiving either anti-PD-1 immunotherapy or platinum-based chemotherapy (Fig. 4A-F). For patients receiving sintilimab, the mPFS was 12.34 months in patients with CPS of 10 or more and 6.47 months in patients with CPS less than 10 ($P=0.13$) (Fig. 4C). Similarly, the mOS also improved in the CPS ≥ 10 group by 9.88 months (23.18 months vs. 13.30 months; HR 0.51 [95% CI: 0.23–1.13]; $P=0.12$) (Fig. 4D). Although these differences did not reach statistical significance, the data suggest a potential

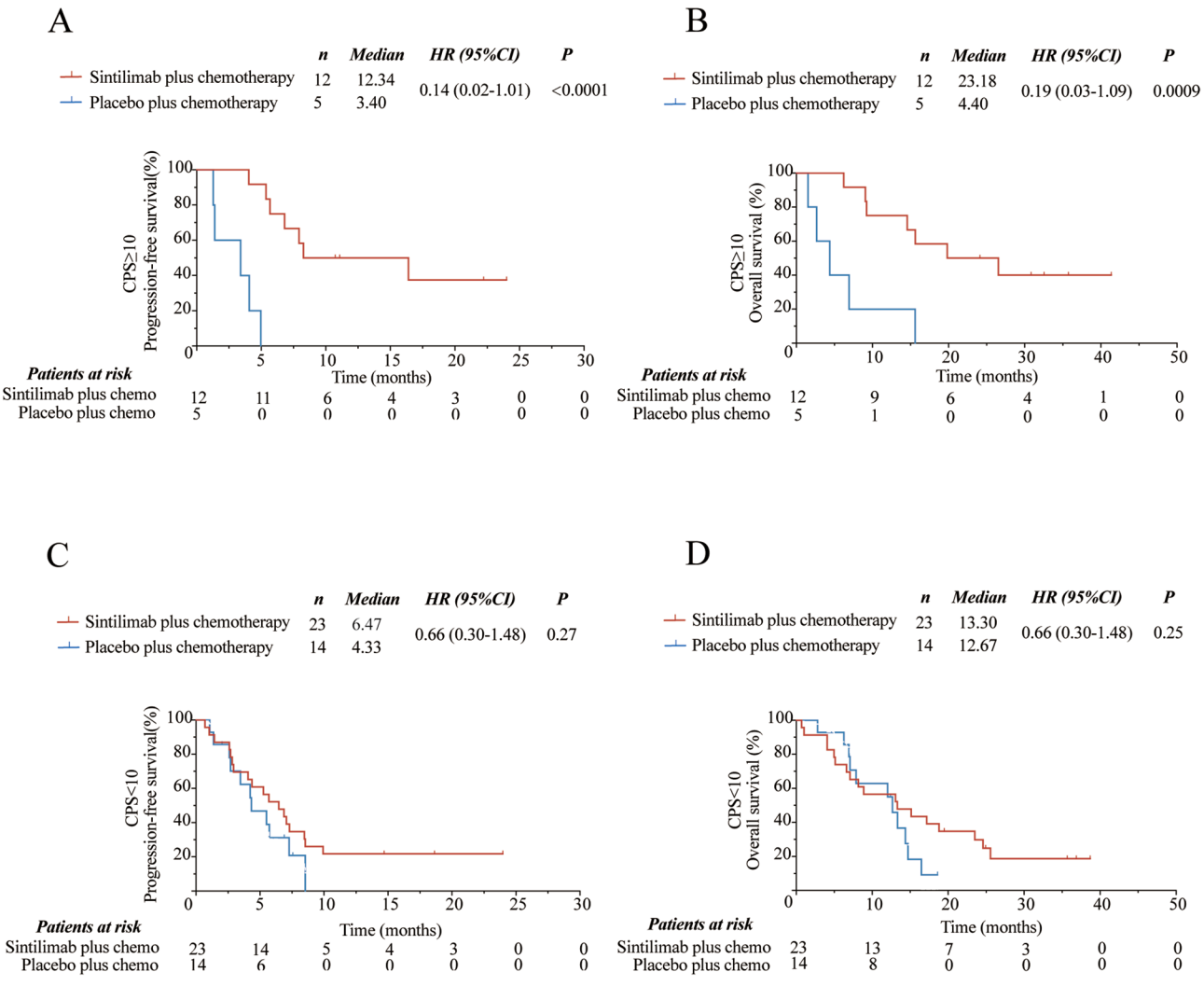


Fig. 2 Kaplan-Meier plot of progression-free survival and overall survival. Progression-free survival in patients with PD-L1 CPS ≥ 10 (**A**) and PD-L1 CPS < 10 (**C**); overall survival in patients with PD-L1 CPS ≥ 10 (**B**) and PD-L1 CPS < 10 (**D**); ✳Small lesions (longest diameter < 10 mm or pathological lymph node short diameter ≥ 10 mm to < 15 mm) were taken as unmeasurable lesions. HR, hazard ratio

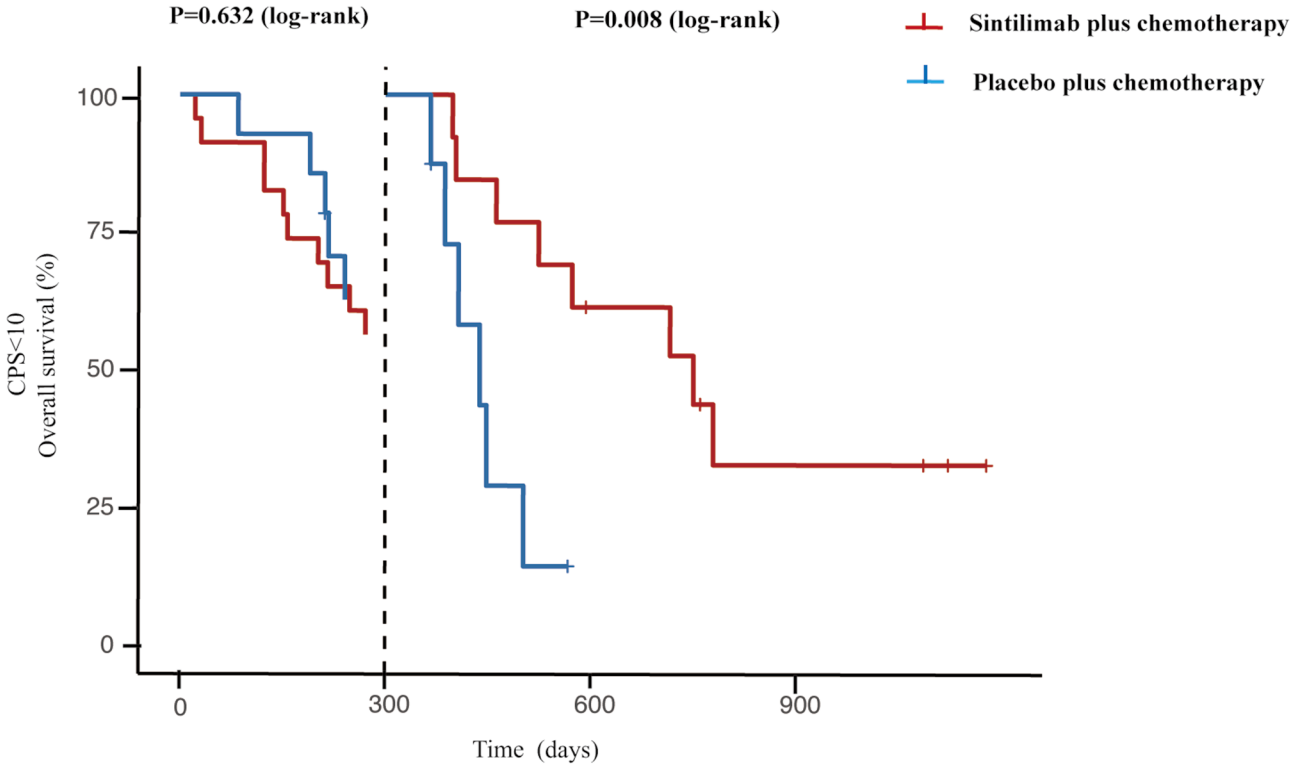


Fig. 3 Survival curves using the LANDMARK method of overall survival

clinical benefit of immunotherapy in patients with higher PD-L1 expression.

Conversely, in chemotherapy-alone group, patients with CPS ≥ 10 exhibited a concerning trend toward poorer outcomes. Their mPFS was shorter than the CPS < 10 subgroup (3.40 vs. 4.33 months; $P = 0.07$) (Fig. 4E), with a more pronounced reduction in mOS (4.40 vs. 12.67 months; $P = 0.11$) (Fig. 4F). This exploratory analysis suggests that patients with high PD-L1 expression may be insensitive to platinum-based chemotherapy and highlights the need for PD-L1-guided treatment selection in G/GEJ cancer management.

Relationships with TME immune cell subtypes and both anti-PD-1 therapy and chemotherapy: multiplex Immunofluorescence analyses

To further explore whether the tumor immune microenvironment could predict the treatment response, biopsy samples of all patients collected before treatment were analyzed separately by multiplex immunofluorescence. Patients with no tumor progression within half a year were defined as well-responders, and those with tumor progression were defined as poor-responders. Among 54 initially enrolled patients, 33 baseline samples met quality control criteria and were included in the final analysis (sintilimab plus chemotherapy group, $n = 18$; placebo plus chemotherapy group, $n = 15$).

In patients receiving anti-PD-1 immunotherapy (Fig. 5A), patients in the well-responder group showed higher densities of PD-L1 expression than those in the poor-responder group ($P = 0.02$). Interestingly, a strong positive association between CD8 + T cell infiltration and treatment response were revealed in our mIF analysis, with well-responders demonstrating higher CD8 + T cell densities ($P = 0.04$), whereas there were no major differences in CD4 + T cells, CD68 + macrophages, or Foxp3 + regulatory T cells (Tregs) between the well-responder and poor-responder groups, both in the context of tumor and stromal cells (Fig. 5B).

Notably, as shown in Fig. 5C, in patients receiving platinum-based chemotherapy alone, the number of CD4 + T cells was significantly higher in patients with poor chemotherapeutic efficacy ($P = 0.03$). Similarly, the expression levels of PD-L1 in the poor-responder group were also higher than those in the well-responder group ($P = 0.07$), consistent with our results of clinical analysis, although not reaching statistical significance limited by the small sample size (Fig. 5D). However, no differential expression patterns were detected for CD8 + T cells, CD68 + macrophages, or FoxP3 + Tregs in chemotherapy-treated patients.

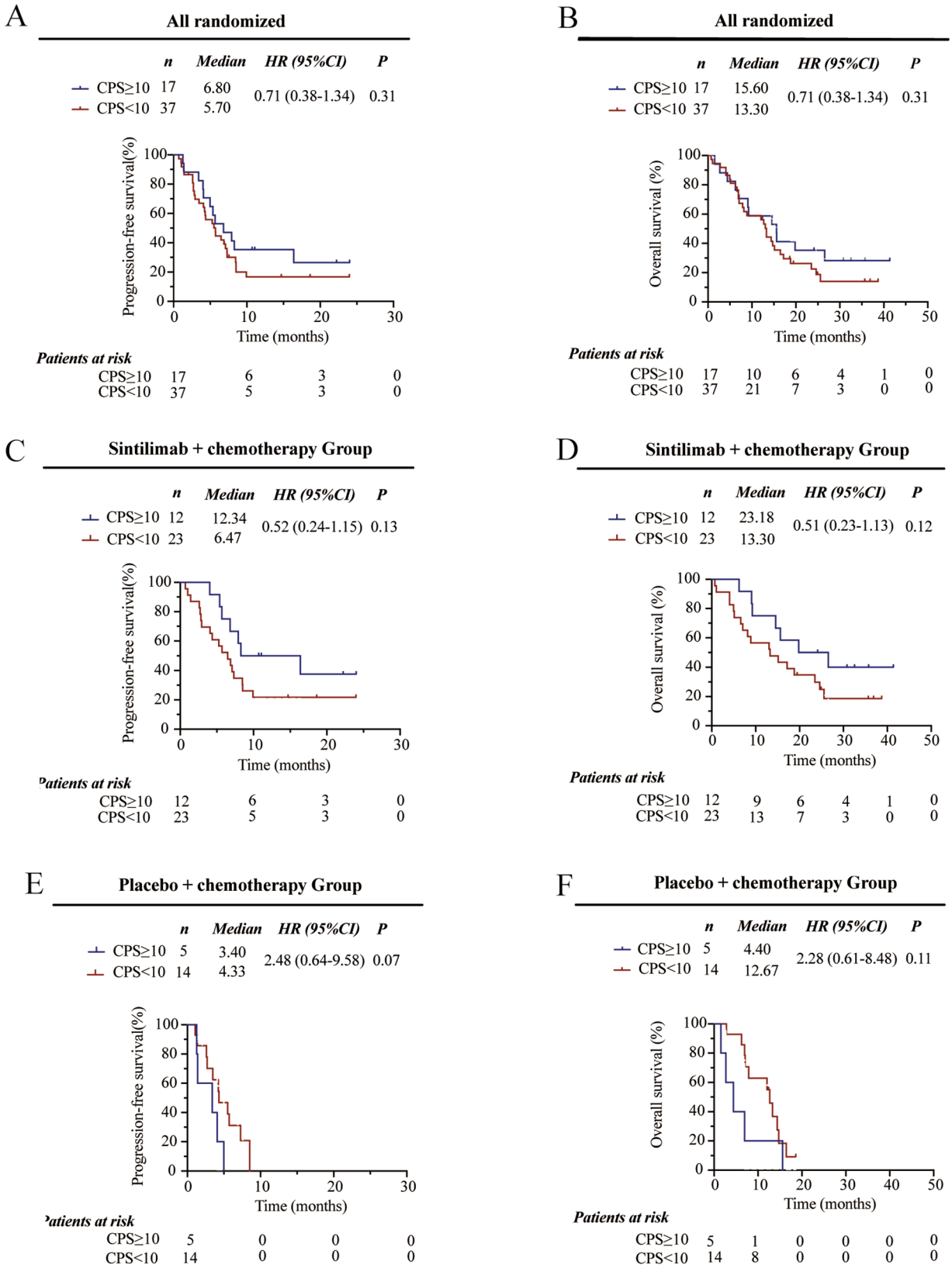


Fig. 4 Progression-free survival and overall survival subgroup analysis according to PD-L1 CPS. Progression-free survival in all randomized patients (**A**) and in patients receiving immunotherapy plus chemotherapy (**C**) and placebo plus chemotherapy (**E**). Overall survival in all randomized patients (**B**) and in patients receiving immunotherapy plus chemotherapy (**D**) and placebo plus chemotherapy (**F**). CI, confidence interval; CPS, combined positive score; HR, hazard ratio

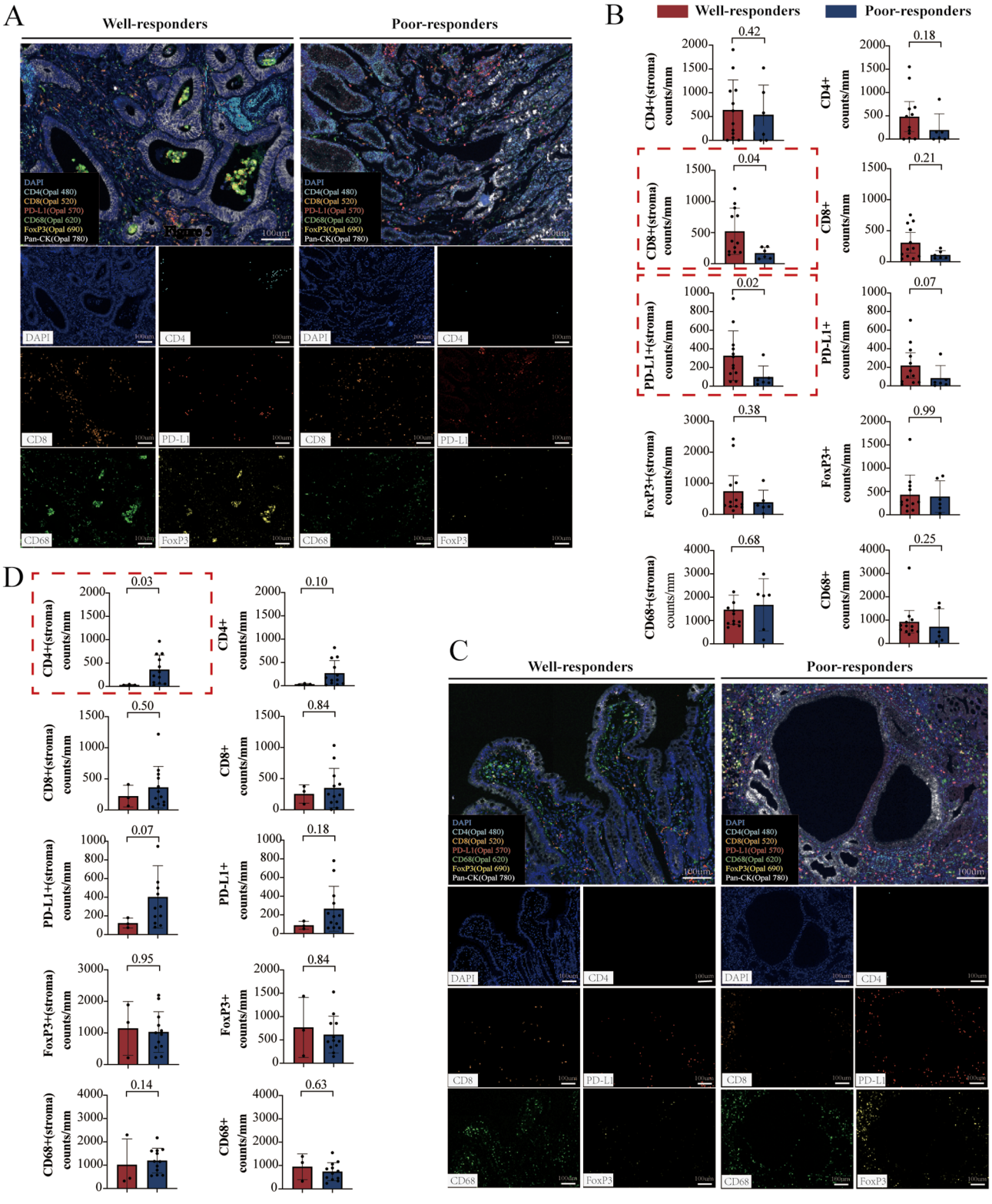


Fig. 5 The merged and single-stained images for four representative multiplex immunofluorescence (mIF) of tumor microenvironment (TME). Typical mIF images of a patient receiving sintilimab plus chemotherapy (**A**) and placebo plus chemotherapy (**D**). Immune cell infiltration levels in the tumor tissue before sintilimab plus chemotherapy (**B**) and placebo plus chemotherapy (**C**) between patients in the well-responder group and those in the poor-responder group assessed by multiplex immunofluorescence (mIF). Scale bar:100 μm

Discussion

In recent years, immunotherapy for advanced G/GEJ cancer has attracted widespread attention. Prospective clinical trials, such as CheckMate 649 [7], ORIENT-16 [9], and RATIONALE 305 [30] have demonstrated an effective clinical response in patients with advanced HER2-negative gastric cancer. Our results, consistent with the results of the ORIENT-16 study, further demonstrate the efficacy and safety of adding anti-PD-1 sintilimab to platinum-based chemotherapy, which resulted in a statistically significant and clinically meaningful improvement in both progression-free survival and overall survival compared to chemotherapy alone, not only in the overall population but also in patients with strong PD-L1 expression.

However, the efficacy of anti-PD-1 treatment in tumors with low PD-L1 expression remains unclear. The results of patients with a CPS less than 5 have been shown to have a non-significant survival benefit in the ORIENT-16 study. The level of PD-L1 expression was reclassified and two PD-L1 subgroups (CPS < 10 and CPS ≥ 10) were identified in our study, finding that for patients with CPS less than 10, no significant difference was observed between the anti-PD-1 immunotherapy plus chemotherapy group and placebo plus chemotherapy group ($P = 0.761$). This observation aligns with Zhao et al.'s findings [31], which demonstrated no significant differences in OS and PFS between ICI-chemotherapy combinations and chemotherapy alone in the CheckMate-649 PD-L1 CPS 1–4 and KEYNOTE-062 PD-L1 CPS 1–9 subgroups ($P \geq 0.05$). Interestingly, our landmark analysis identified a delayed survival advantage emerging approximately 300 days post-treatment in low PD-L1 expressors (CPS < 10) receiving ICI-chemotherapy combination therapy. This long-term survival discrepancy is likely attributable to the tailing effect of anti-PD-L1 treatment, wherein sustained immune activation through tumor-immune interactions and memory T cell formation drives prolonged clinical benefit, as opposed to the direct cytotoxic mechanism of chemotherapy. Supporting this interpretation, Gauci et al. [32] conducted a long-term analysis of solid tumor patients responding to anti-PD-1/PD-L1 therapy and found that 48/76 patients developed an objective response at 3 months regardless of PD-L1 expression. Similarly, the tailing effect, which depends on the immune status of an individual and tumor heterogeneity, was also embodied in long-term follow-up data of some clinical trials such as CheckMate153 of NSCLC [33] and RATIONALE-305 [34] of advanced GC, especially striking in patients with high PD-L1 expression. This conclusion has also been validated in patients with CPS < 10 in our study and a time point of 300 days was proposed. Moreover, consolidation chemotherapy was initiated after six cycles of combination therapy in both groups and continued for up to a maximum of 2 years. Therefore, we proposed an alternative hypothesis that applications of immunotherapy might increase the sensitivity of the tumor to subsequent chemotherapy. Exposure to immune checkpoint inhibitors may elicit the antitumor activity of chemotherapy, which can lead to a high response rate [35]. A retrospective CLARITY study [36] also supported that for NSCLC patients who progressed to previous ICIs administered as first- or second-line therapy, salvage chemotherapy could yield a survival-favorable response. While the precise mechanisms require further elucidation, these findings have important clinical implications: For CPS < 10 patients with limited life expectancy, early combination therapy may offer restricted benefit; For most patients, immunotherapy should be initiated as early as possible in the treatment course and could be continued as maintenance therapy after 4–6 cycles of complete routine first-line therapy, and disease control (CR/PR/SD) was reached in order to increase the survival benefit.

In addition, we found that only a subset of patients could benefit from anti-PD-1 immunotherapy or platinum-based chemotherapy due to the high heterogeneity of gastric cancer (GC). For patients receiving anti-PD-1 immunotherapy, elevated PD-L1 expression was correlated with prolonged PFS and OS, consistent with landmark trials including KEYNOTE-061 [37] and KEYNOTE-062 [38]. This notion was further supported by PD-L1 staining of mIF analysis. While PD-L1 expression demonstrates significant prognostic value in advanced G/GEJ cancer, our data suggest it should not be considered as a standalone predictive biomarker for immunotherapy response.

The potential immune microenvironment [39] on the prognostic impact of ICIs on gastric cancer, such as interferon (IFN) [40], neutrophil-to-lymphocyte ratio (NLR) [41], and specific genes like ARID1A [42], POLE or POLD1 [43], and TP53 [44], have attracted significant attention in recent year. Interestingly, our mIF analysis revealed significantly greater CD8 + T cell infiltration in well responders versus poor responders, consistent with their established role as the key effector of antitumor immunity. However, the role of CD8 + T cells in the immunotherapy response remains unclear, which may be due to the functional heterogeneity of CD8 + T cell subtypes on one hand. Tc1 cells, which possess typical CD8 + T cell-related cytotoxic signatures, have been shown to promote the production of perforin, granzymes B and interferon(IFN)-γ [45] in order to kill tumor cells. Tc2 [46] and Tc22 [47] cells expressing granzyme B are highly cytolytic and demonstrate robust cytotoxic abilities. Tissue-resident memory T (TRM) cells, identified by CD103 (ITGAE) expression, correlate with improved survival [48]. Conversely, Tc9 and Tc17 subtypes [49]

have been shown to produce relatively little IFN-γ and granzyme B, and are thought to promote disease progression [50–52]. In our mIF analysis, the prognosis of patients with high CD8 + T expression was significantly better than that of patients with low CD8 + T expression, suggesting that CD8 Tc subsets with strong cytotoxic functions such as Tc1s, Tc2s, or Tc22s account for the majority of the TME in our gastric cancer samples. However, no trials included in this analysis provided CD8 + T cell subset analysis, and the underlying predictive mechanism remains unclear and requires further investigation.

On the other hand, some researchers have uncovered that a high degree of CD8 + T cell infiltration could increase the expression of immune checkpoint receptors such as PD-1 and CTLA-4 and sensitize tumors to ICI, which may be another reason for the good immunotherapy response of CD8 + T cells. Chiaravalli et al. [53] established a positive correlation between CD8 + tumor-infiltrating lymphocytes (TILs), PD-L1 expression, and favorable clinical outcomes, supporting that CD8 + T cell-targeted strategies could potentiate the efficacy of anti-PD-1 immunotherapy. The immune checkpoint receptor CTLA-4 is expressed on CD8 + effector T cells, and CTLA-4 blockade enhances the response of CD8 + T cells in the TME [54]. Currently, the combination of anti-PD-1 and anti-CTLA-4 mAb (such as nivolumab plus ipilimumab) has been approved for the treatment of melanoma [55], non-small cell lung cancer [56], hepatocellular carcinoma [57], mismatch repair deficient colorectal cancer [58], while lack of sufficient evidence on advanced gastric cancer. The COMPASSION-04 clinical trial [59] presented at 2024 American Association for Cancer Research (AACR) reported encouraging efficacy and safety profiles for cadonilimab, a PD-1/CTLA-4 bispecific antibody, in first-line G/GEJ adenocarcinoma regardless of PD-L1 status. Furthermore, several clinical trials of anti-CTLA immunotherapy for gastric cancer are still underway. Taken together, our results demonstrate that CD8 + T cells play a pivotal role in predicting ICI treatment responses. A combination of PD-1 and CTLA-4 blockade could have synergistic effects by potentiating the antitumor activity and provide more therapeutic options for patients with low PD-L1 expression, and, hopefully, to be a new first-line treatment option for gastric or GEJ adenocarcinoma.

While numerous biomarkers have been identified for predicting response to ICIs in GC, the relationship between TME immune profiles and chemotherapy sensitivity remains poorly understood. Generally, chemotherapeutic drugs can specifically induce a cellular immune response, which results in tumor cell death or stimulates immune effector molecules to relieve the suppressed immune response. Therefore, tumor immune status can be used to evaluate the effects of chemotherapy. Interestingly, we observed that those with a CPS of 10 or more were more insensitive to chemotherapy and had significantly shorter PFS than patients with a CPS of less than 10. To further validate the association between PD-L1 expression and chemotherapy efficacy, we also analyzed the microenvironmental components using mIF analysis and found that elevated PD-L1 expression correlates with chemoresistance. Lou et al. [60] used intracellular PD-L1 as an RNA-binding protein that enhances the efficacy of chemotherapy by inhibiting DNA damage response and repair. Another study by Frank Sinicrope [61] examined the potential role of tumor cell-intrinsic PD-L1 in regulating chemosensitivity in human CRC cells and found that the deletion of PD-L1 could suppress BH3-only BIM and BIK proteins, causing chemoresistance and survival reduction. In contrast to the ability of PD-L1 mentioned above, knockdown of PD-L1 has been shown to sensitize breast cancer [62], small-cell lung cancer [63], and lymphoma cells [64] to chemotherapy-induced apoptosis. This apparent contradiction may reflect the functional diversity of PD-L1 and tumor cell type specificity. In GC, Shin K et al. [65] evaluated the correlation among serum-derived exoPD-L1, plasma sPD-L1, immune-related markers, and circulating immune cells and established sPD-L1 as a prognostic marker, with low sPD-L1 levels predicting better outcomes in chemotherapy-treated GC patients. In the tumor microenvironment, low PD-L1 expression also predicted sensitivity in our study. However, the limited existing literature on the role of PD-L1 against traditional chemotherapy highlight the need for the mechanistic studies.

In addition, we found significantly higher expression of CD4 + T cells in the poor-responder group than in the well-responder group, suggesting that CD4 + T cell abundance may serve as a negative predictor of chemotherapy efficacy. Immunohistochemistry (IHC) of the T-cell marker CD4 + was performed on GC patients who received XELOX therapy to evaluate TILs at Fudan University [66]. As a result, from patients in the XELOX non-sensitive group (XNSG), we observed that the percentage of CD4 positive cells was significantly higher than that in the XELOX sensitive group (XSG) (54.2% and 2.9%, respectively) ($P < 0.05$). Similarly, the prognostic significance of CD4 + subsets was further supported by esophageal cancer (EC) studies demonstrating that Tregs - a specialized CD4 + population - negatively impact outcomes following chemoradiotherapy [67], which may be partially related to the expression of inhibitory receptors (such as IL-10) and depletion of T cells. Consequently, the density of CD4 + T cells may help stratify patients likely to benefit from platinum-based chemotherapy regimens. Despite the close association between CD4 + T cell density and chemotherapy response, the mechanism of

chemoresistance in CD4 + Ts is far from fully understood. What's more, the exploratory mIF analysis, while revealing significant associations between immune cell densities and treatment response, should be further validated in a large-scale sample due to the limited cohort size for subgroup stratification.

Limitations

Despite its strengths and implications for future research, our study has some limitations. First and most importantly, combined with results from the ORIENT-16 clinical trial, the subgroup analyses regarding PD-L1 expression should be stratified based on CPS 0–5, 5–10 or CPS ≥ 10 for more precision treatment. There were a limited number of data and also a limited number of patients to allow meaningful analyses of this subgroup. Additionally, although we observed some significant tendency toward treatment response in patients with different components of the tumor microenvironment, the trend is still require further evaluation in larger patient populations. Finally, we only performed a quantitative analysis of each biomarker separately and only a single molecular marker for each cell type was used to identify immune cells in tumor tissue. Further studies, including colocalization analyses and spatial transcriptomics, are essential, with the potential to provide additional biological insights into the TME in GC and present an opportunity to improve patient selection for novel diagnostics and therapeutics.

Conclusions

In conclusion, we identified the predictive value of PD-L1 in both anti-PD-1 immunotherapy and platinum-based chemotherapy by further analyzing the survival curves stratified by PD-L1 expression and an interesting phenomenon was discovered by landmark statistical method that clear differences between anti-PD-1 immunotherapy and chemotherapy would display around 300 days after first-day treatment for patients with CPS < 10. Multiplexed immunofluorescence exploration of the tumor microenvironment showed that immune cells such as CD4 and CD8 were closely correlated with therapy efficacy and would offer critical insights into the complex and heterogeneous immune landscape associated with immunotherapy and chemotherapy treatment response.

Abbreviations

AACR	American Association for Cancer Research
CIs	Confidence Intervals
CR	Complete Response
CPS	Combined Positive Score
EC	Esophageal Cancer
ECOG	the Eastern Cooperative Oncology Group
GC	Gastric Cancer
G/GEJ Cancer	Gastric and Gastroesophageal Junction Cancer
HER2	Human Epidermal Growth Factor Receptor 2
HRs	Hazard Ratios
ICIs	Immune Checkpoint Inhibitors
IFN	Interferon
mIF	multiplex Immunofluorescence
NK cell	Natural Killer cell
NLR	Neutrophil-to-Lymphocyte Ratio
NSCLC	Non-Small Cell Lung Cancer
ORR	Objective Response Rate
OS	Overall Survival
PD-1/PD-L1	Programmed Death-1/Programmed Death-ligand 1
PFS	Progression-free Survival
PR	Partial Response
PS score	Performance Status score
RECIST guidelines	Response Evaluation Criteria in Solid Tumors guidelines
SD	Stable Disease
TILs	Tumor-Infiltrating Lymphocytes
TME	Tumor Microenvironment
TRM cells	Tissue-resident Memory T cells
XNSG	XELOX non-sensitive group
XSG	XELOX sensitive group

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-025-14805-6.

Supplementary Material 1.

Supplementary Material 2.

Acknowledgements

The authors declare that no external funding or assistance was received for this study.

Authors' contributions

All authors contributed to the conception and design of this study. The ideas for this review have been provided by H.J. The clinical data was analyzed by H.W. and W.S., and the multiplex immunofluorescence was performed by H.W. and Q.L. Q.C. and Y.H. made the data curation, while Q.W., and D.H were responsible for data visualization. The first draft of the manuscript was written by H.W. and W.S., and H.J. and Y.D. critically revised the manuscript. All authors commented on the previous versions of the manuscript and approved the final manuscript.

Funding

This study is supported by grants from the Natural Science Foundation of Zhejiang Province (Grant No.: LMS25H160019) and Development Center for Medical Science and Technology National Health Commission of the People's Republic of China (Grant No.: WKZX2024CX102213).

Data availability

All data generated or analysed during this study are included in the supplementary data files.

Declarations

Ethics approval and consent to participate

Our study was in accordance with the Good Clinical Practice Guideline and in compliance with the Helsinki Declaration. The study protocol was approved by the institutional Ethics Committee of the First Affiliated Hospital of College of Medicine, Zhejiang University, and was prospectively registered at ClinicalTrials (NCT03745170). All patients provided written informed consent before enrollment.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 9 June 2025 / Accepted: 4 August 2025
Published online: 25 August 2025

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