# Peripheral cytokine levels as predictive biomarkers of benefit from immune checkpoint inhibitors in cancer therapy

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**Research**

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# Abstract

**Background** Currently, only a small subset of cancer patients can benefit from anti-PD-1/PD-L1 monotherapy, indicating that further predictive biomarkers are needed.

**Methods** In the retrospective study, plasma samples were collected before anti-PD-L1/PD-L1 treatment in two subset of patients. A total of 59 immunological factors, including cytokines, chemokines, and soluble immune checkpoints, were measured by using a multiplex immunoassay kit. Moreover, multiplex immunohistochemistry (mIHC) was performed in a subgroup of patients.

**Results** In the discovery cohort, multiplex immunoassay profiling data revealed that both soluble PD-L1 and C-C motif chemokine 5 (CCL5/RANTES) showed rising trends across the three subgroups PD, SD and CR/PR. Further investigation demonstrated the predictive and prognostic value of the pre-treatment levels of PD-L1, CCL5/RANTES, and their combinatorial signature the “2-cytokine signature”. As expected, the signature-high patients displayed a remarkably increased disease control rate (DCR) and prolonged survival versus that of the lower subgroup. More importantly, the relevance between the three signatures and the efficiency of immunotherapy was confirmed in the pan-cancer validation cohort. Notably, the significant association between the “2-cytokine signature” and longer survival was validated. Further quantitative analyses of the tumor microenvironment composition suggested a link between the “2-cytokine signature” and NK cell infiltration.

**Conclusions** A combined peripheral signature comprising CCL5/RANTES and soluble PD-L1 appears to be an effective biomarker to predict benefit from anti-PD-1/PD-L1 monotherapy. Our study underscores that peripheral immunological features may play an essential role in guiding patient selection and are worthy of future prospective investigations.


# Background

In the past few years, remarkable achievements have been made in the field of cancer therapy due to the use of immune checkpoint inhibitors (ICIs) targeting the programmed cell death 1/programmed cell death ligand 1 (PD-1/PD-L1) axis. However, only a small proportion of participants benefit from anti-PD-1/PD-L1 monotherapy, ranging from 10–40% [1, 2]. Hence, there is an urgent need for effective biomarker-based patient selection [3].

The identification of peripheral biomarkers for response to immunotherapy possesses distinct advantages due to limited accessibility, multiple lesions, and heterogeneity of the tissue samples [4]. Recently, several immune-related analytes have been measured in liquid biopsies, such as circulating tumor cells (CTCs) [5], the circulating T-cell subpopulation [6], blood tumor mutational burden (TMB) of circulating tumor DNA (ctDNA) [7], and systematic immunological factors, including cytokines, chemokines and soluble immune checkpoint molecules [8, 9]. Importantly, as a broad category of small proteins, cytokines function as crucial regulators of the immune system [10]. However, the predictive values of cytokines have only been explored in limited cancer types, including lung cancer and melanoma

[11–13]. There are currently no validated cytokine biomarkers that can effectively identify potential responders to anti-PD-1/PD-L1 immunotherapy regardless of cancer type.

In this study, we measured the baseline expression levels of 59 systematic immunological factors, including cytokines, chemokines and soluble immune checkpoint molecules. The correlation between clinical benefit and the distribution pattern of peripheral factors was extensively explored in the discovery cohort, which included 21 esophageal squamous cell cancer (ESCC) patients treated with anti-PD-1 antibody. Subsequently, the candidate peripheral biomarkers were tested in a pan-cancer validation cohort. Moreover, we analyzed tumor infiltrating lymphocytes (TILs) using multiplex immunohistochemistry (mIHC) in a subset of tissue samples from the combined cohort, aiming to determine the association between peripheral and local immune systems.

## Methods

# Patient and study design

The two patient cohorts involved in this study were available from the Department of GI Oncology, The Fifth Medical Center, General Hospital of PLA. All patients who failed or did not tolerate standard treatments (In the discovery cohort, ESCC patients who failed or did not tolerate first-line chemotherapy; in the validated cohort, all patients who failed or did not tolerate at least first-line standard treatment) were treated with anti-PD-1/PD-L1 monotherapy. Informed consent was obtained for all investigations.

# Sample Collection And Multiplexed Bead Immunoassays

Plasma samples and tissue samples were collected at baseline before anti-PD-L1/PD-L1 treatment. EDTA anti-coagulated whole blood samples were obtained by venipuncture (10 mL, BD Vacutainer blood collection tube; BD Biosciences, Brea, CA, 111 USA) and centrifuged (1,000 × g, 15 min) to isolate the plasma. A total of 59 immunological factors were simultaneously measured in plasma samples using the 45-ProcartaPlex Human Cytokine/Chemokine/Growth Factor Panel (Affymetrix, Inc., Santa Clara, CA, USA) and the 14-ProcartaPlex Human Immuno-Oncology Checkpoint Panel (Affymetrix, Inc.). A signature score was calculated by averaging the included cytokines after a $\log_2$ transformation of the concentration value.

# Multiplex Immunohistochemistry (mihc) Analysis

Formalin-fixed paraffin-embedded (FFPE) tumor tissue sections were obtained from a subset of patients from the entire cohort (13 in the discovery cohort and 26 in the validation cohort). The multiplex IHC platform, which can detect the expression of multiple markers in a single section [14], was utilized to measure the expression of PD-1/PD-L1 and immune cell infiltration. The immune markers included CD8 + T-cells, CD68 + macrophages (M1), CD68 + CD163 + macrophages (M2) and CD57 + natural killer (NK)

cells. In all, antibodies against CD8 (ZA-0508, clone SP16; Zsbio; 1:100 diluted), PD-1 (ZM0381, UMAB199, Zsbio; 1:100 diluted), CD68 (ZM-0060, clone KP1; Zsbio; 1:400 diluted), CD163 (ZM0428, 10D6, Zsbio; 1:200 diluted), and PD-L1 (CST13684, E1L3N, CST, 1:100 diluted) were employed. Briefly, the FFPE slides were deparaffinized, rehydrated, and washed before epitope retrieval. Endogenous peroxidase was blocked using Antibody Diluent/Block buffer (72424205; PerkinElmer, Massachusetts, USA). For antibody incubation, one antigen required one round of labeling, including consecutive primary and secondary antibody incubation. Tyramide signal amplification (TSA) visualization was performed with the Opal seven-color IHC Kit (NEL797B001KT; PerkinElmer, Massachusetts, USA). Slides were scanned using PerkinElmer Vectra (Vectra 3.0.5; PerkinElmer, Massachusetts, USA). The percentage of positively stained cells in all nucleated cells was counted.

# Statistical analysis

Quantitative variables are presented as the mean ± standard deviation (SD). To determine differences between two subgroups of non-normally distributed quantitative variables, the Mann–Whitney U-test was performed. The Kruskal–Wallis test was used to test for differences between more than two populations when the variables are nonparametric. The Jonckheere–Terpstra test was used when the treatment effects were ordered, e.g., CR/PR, SD and PD. The Youden index, a summary measure of the ROC curve [15], was used to identify an “optimal” cutoff value on the signature score. For categorical variables, the chi-square test was used. The Kaplan–Meier estimator and log-rank test were performed to analyze overall survival (OS) and progression-free survival (PFS) analyses. Statistical analyses were conducted with SPSS 23.0 software.

## Results

# Patient characteristics

A total of 82 patients were included in the study, which comprised 21 ESCC patients in the discovery cohort and 61 pan-cancer patients in the validation cohort, including colorectal cancer (CRC), gastric cancer (GC), hepatocellular carcinoma (HCC), neuroendocrine carcinoma (NEC), and other cancer types. The clinical and treatment characteristics of all participants are shown in Table S1. All patients had regionally advanced or metastatic diseases. Patients with clinical benefit (PR/SD, also designated as disease control) and no benefit (PD) were examined by using imaging examinations according to the Response Evaluation Criteria in Solid Tumors (RECIST) v1.1.

# Identification Of Potential Predictive Biomarkers In Escc

To identify potential systemic biomarkers associated with the immune response, we profiled 59 peripheral immunological factors in a subset of plasma samples from ESCC patients (discovery cohort) by using multiplexed bead immunoassays (Fig. 1A). As Table S2 and Figure S1 reveals, the baseline levels of

several cytokines/chemokines/soluble checkpoint molecules were differentially expressed among the PR, SD and PD subgroups ($P < 0.05$ for a statistical test). Only three immunological factors showed statistical significance by all three tests (Kruskal-Wallis H test for distribution, Jonckheere-Terpstra test for ordered difference, and median test for median differences), indicating the distinct pattern of PD-L1, C-C motif chemokine 5 (CCL5/RANTES), and interleukin 10 (IL-10). Interestingly, in contrast to the decreasing trend of IL10, both PD-L1 and CCL5/RANTES showed a rising trend of therapeutic response across the three groups as PD, SD and PR (Figure S1 and Fig. 1B), indicating a “pro-inflammatory” role of the two factors.

# Association between peripheral immune signatures and clinical benefit to ICI treatment in ESCC

Previously, combinatory cytokine signatures have been reported to predict immune-related toxicities [16] and clinical outcomes in cancers [17, 18]. For instance, the expression of a cluster of upregulated cytokines was integrated into a toxicity score, which was associated with the development of immune-related toxicity [16]. Therefore, we tested the predictive and prognostic value of PD-L1, CCL5/RANTES, and their combinatorial signature (mean of log2 transferred value of PD-L1 and CCL5/RANTES) in the discovery cohort. As expected, the increased baseline levels of RANTES, PD-L1 and the “2-cytokine signature”correlated with a higher disease control rate (DCR: PR + SD), with AUC values of 0.818 (95% CI 0.616–1, $P = 0.014$), 0.750 (95% CI 0.521–0.979, $P = 0.053$), and 0.755 (95% CI 0.530–0.979, $P = 0.049$), respectively (Fig. 1C). Accordingly, when using the optimal cutoff value derived from ROC analyses (the Youden index), the peripheral signature-high patients demonstrated a significantly higher DCR than the signature-low subgroup (CCL5/RANTES: 88.9% vs. 16.7%, $P < 0.01$; PD-L1: 80.0% vs. 18.2%, $P < 0.01$; 2-cytokine signature: 80.0% vs. 18.2%, $P < 0.01$; Fig. 1D). Moreover, higher levels of the three signatures were associated with better PFS (CCL5/RANTES: HR, 0.222, log-rank test, $P < 0.001$; PD-L1: HR, 0.283, log-rank test, $P < 0.01$; 2-cytokine signature: HR, 0.283, log-rank test, $P < 0.01$; Fig. 2A). Besides, the associations between signature levels and OS were identical (Fig. 2B) (log-rank test, $P < 0.05$ for all comparisons).

# Validation of the peripheral immune signatures in a pan-cancer cohort

To confirm the predictive and prognostic value of the candidate biomarkers in the peripheral blood samples, we further profiled the expression levels of 59 immunological factors in an independent validation cohort (Table S3 and Figure S2-S3). Not surprisingly, plasma samples in the clinic benefit subgroup displayed higher levels of CCL5/RANTES, PD-L1 and the 2-cytokine signature than those from the no benefit subgroup ($P < 0.05$) (Fig. 3A). The AUC curves for the three immune signatures were 0.707 (95% CI 0.575–0.839, $P = 0.008$), 0.710 (95% CI 0.578–0.845, $P = 0.005$) and 0.722 (95% CI 0.587–0.857, $P = 0.003$) (Fig. 3B). By using the same stratification cutoff value as in the discovery cohort, higher levels of CCL5/RANTES, PD-L1, and 2-cytokine signature were associated with a higher DCR ($P < 0.05$ for all

comparisons, Fig. 3C). As expected, improved PFS was observed in patients with higher baseline levels of two signatures (CCL5/RANTES: HR, 0.505, log-rank test, $P < 0.05$; 2-cytokine signature: HR, 0.424, log-rank test, $P < 0.01$; Fig. 4A). However, only the stratification power of the 2-cytokine signature attained statistical significance for OS (HR, 0.431, log-rank test, $P < 0.05$; Fig. 4B).

# Correlation Between Peripheral Immune Signatures And Tumor Infiltrating Lymphocytes

The above data indicated that CCL5/RANTES and PD-L1 are peripherally correlated with the clinical benefit and prognosis of patients with various cancer types receiving ICI treatment. Cytokines are a broad category of small proteins that allow components of the immune system to communicate with one another to induce a coordinated response [10]. We therefore asked whether different peripheral expression patterns were associated with local immune features in the tumor microenvironment (TME).

In the combined cohort, we obtained only 39 FFPE tissue samples for the mIHC assay (discovery cohort: 13 samples; validation cohort: 26 samples). To determine the correlation between the peripheral and local immune systems, we measured the expression of PD-1/PD-L1 and immune cell infiltration, which includes CD8 + T-cells, CD68 + macrophages (M1), CD68 + CD163 + macrophages (M2) and CD57 + NK cells(Fig. 5A). Intriguingly, in the 2-cytokine signature-high group, we observed enrichment of CD57+, CD57+/PD-L1+, and CD68+/CD163+/PD-L1 + subpopulations in the stromal region and total area when compared to the signature-low group (Fig. 5B). However, no difference was found in CD8 + T-cells between the high- and low- 2-cytokine signature subgroups (data not shown). In addition, we observed no significant difference in infiltrating immune cells and PD-1/PD-L1 expression between the high-RANTES versus low-RANTES or high-PD-L1 versus low-PD-L1 subgroups (data not shown).

In view of the correlation identified between peripheral and local immune features, we next asked whether the infiltrating immune cells correlated with clinical benefit to immunotherapy. As expected, abundant infiltration of CD8 + T-cells and CD57 + NK cells were local immunological correlates of the DCR (Fig. 6). Specifically, higher percentages of CD8 + T-cells and CD57 + NK cells were observed in the DCR subgroup (Fig. 6A and B). More importantly, the enrichment of these immune cells can stratify patients with longer OS (Fig. 6C). Although improvement of PFS was observed in high-TILs subgroups when compared with low-TILs, statistical significance was not attained (Figure S4). Collectively, our data suggest that higher levels of the predictive peripheral 2-cytokine signature are associated with abundant NK cell infiltration in the TME, which may, in turn, contribute to the better prognosis of patients receiving ICI treatment.

# Discussion

Cytokines can be secreted by cells of the innate and/or adaptive immune system in response to microbes and tumor antigens and are thus intricately involved in all immune response signaling [19]. Indeed, correlations have been found between peripheral cytokine molecules and clinical response to ICI

immunotherapy in some cancer types, especially in lung cancer and melanoma [11–13]. However, no validated pan-cancer peripheral immunological biomarkers have been reported. Here, we performed systematic immunological factor profiling on 59 factors and identified that CCL5/RANTES, PD-L1, and the combined signature are potential predictors for clinical benefit in cancer patients receiving anti-PD-1/PD-L1 monotherapy regardless of cancer type.

In recent years, studies on ICI immunotherapy in esophageal cancer (EC) have received considerable attention. Kudo and colleagues have reported a single-arm, multicenter phase 2 study that assessed the safety and activity of nivolumab (anti-PD-1) monotherapy in EC patients. Nivolumab treatment revealed an objective response rate (ORR) and DCR of 17% and 42% in this cohort, respectively [20]. Most recently, the global phase III ATTRACTION-3 study proved that nivolumab was associated with a significant improvement in OS when compared with chemotherapy in unresectable advanced or recurrent EC [21]. In addition, in the global phase III KEYNOTE-181 trial, pembrolizumab did not improve the OS or PFS in the intent-to-treat (ITT) population when compared with chemotherapy as second-line therapy. Intriguingly, a significant benefit was observed in patients with a PD-L1 CPS $\geq$ 10 [22], indicating a role for PD-L1 expression as a prognostic biomarker in EC. Notably, here, we showed that the higher baseline levels of CCL5/RANTES and PD-L1 in the plasma were associated with a stronger DCR (DCR > 80% in higher signature subgroups, Fig. 1) and better prognosis (Fig. 2) when compared with the low-signature subgroups. Our data raise the possibility that peripheral immune factors may function as potential predictors for clinical benefit in EC patients, and future studies with larger sample sizes would be helpful to verify their clinical utility.

Furthermore, the predictive values of the three immunological correlates, CCL5/RANTES, PD-L1 and the 2-cytokine signature, were further validated in an independent pan-cancer cohort (Fig. 3). CCL5/RANTES belongs to the family of C-C chemokines, whose members also include CCL3 (MIP-1a) and CCL4 (MIP-1b) [23]. CCL5/RANTES is expressed by T lymphocytes, macrophages and certain types of tumor cells, which may play an active role in amplifying the antitumor response by recruiting a variety of lymphocytes into inflammatory sites, including T-cells and macrophages [23, 24]. On the other hand, CCL5/RANTES has also been associated with cancer progression and metastasis, and elevated levels of CCL5/RANTES in tissues or plasma are indicative of unfavorable outcomes in patients with several cancer types [25–27]. Here, we present novel evidence showing that plasma CCL5/RANTES might be a favorable prognostic biomarker for multiple cancers, including EC, CRC, and HCC with ICI treatment (Table S1).

Previously, the relevance between soluble immune checkpoint molecules and the efficiency of immunotherapy in melanoma has also been reported [12]. However, evidence of soluble PD-L1 (sPD-L1) and therapeutic effectiveness is still obscure in other cancers. Here, we show that higher pretreatment PD-L1 levels in plasma may indicate an improved clinical benefit rate in both ESCC and other cancer types with anti-PD-1/PD-L1 monotherapy. In fact, sPD-L1 can be produced by tumor cells and activated mDCs [28], and moderate baseline levels of sPD-L1 may indicate existing antitumor immune responses [29]. In addition, other studies have documented that the plasma level of soluble PD-L1 is related to the severity of cancer with traditional therapeutic strategies [30]. Collectively, these observations indicate that cancer

patients with higher pretreatment sPD-L1 levels might be an indication of the effectiveness of ICI immunotherapy.

Another interesting finding in our study is that increased peripheral levels of the combined 2-cytokine signature, CCL5/RANTES and PD-L1, are indicative of better clinical benefit in both cohorts (Fig. 1–4). More intriguingly, the high 2-cytokine signature subgroup demonstrated enriched immune cell infiltration in the TME, including CD57 + NK cells, CD57+/PD-L1 NK cells (PD-L1 + NK) and CD68+/CD163+/PD-L1 M2 macrophages (Fig. 5). Further evidence also shows that higher CD57 + NK cells may be correlated with higher DCR in the combined cohort (Fig. 6). NK cells are a group of innate cytolytic effector cells that participate in immune surveillance, and NK infiltration in tumors has been associated with an improved prognosis for cancer patients [31–33].

Specifically, the antitumor efficacy of anti-PD-L1 immunotherapy may be associated with the activation of PD-L1 + NK cells in leukemia [31]. In HCC, a 14-gene immune signature comprising CCL5/RANTES, CXCL10 and CCL2, whose expression correlates with markers of T helper 1 (Th1), CD8 + T and NK cells, is known to predict favorable prognosis in patients with liver cancer, suggesting a chemokine-NK axis [33]. In the present investigation, we discovered a peripheral predictive biomarker as the 2-cytokine signature and then illustrated its correlation with NK cell infiltration and therapeutic efficacy to anti-PD-1/PD-L1 therapy. Thus, the enrichment of NK cells in the TME may offer a potential explanation as to why signature-high patients may benefit from ICI immunotherapy.

# Conclusions

Our study identified a novel subset of peripheral predictive biomarkers, including CCL5/RANTES, PD-L1 and their combined 2-cytokine signature. We showed that increased pretreatment levels of these peripheral immunological factors were associated with improved DCR in patients with varied cancer types receiving ICI immunotherapy. Further quantitative analyses of the TME composition revealed a link between the 2-cytokine signature and NK cell infiltration. It would be worthwhile to perform mechanistic studies exploring the underlying mechanism by which CCL5/RANTES and PD-L1 function integratively during anti-PD-1/PD-L1 immunotherapy.

# List Of Abbreviations

PD-1: Programmed cell death protein-1; PD-L1: Programmed cell death ligand-1; ESCC: Esophageal squamous cell cancer; TILs: Tumor infiltrating lymphocytes; mICH: Multiplex immunohistochemistry; CCL5: C-C motif chemokine 5; RNATES: Regulated upon activation normal T cell expressed and secreted factor; IL-10: Interleukin 10; DCR: Disease control rate; OS: Overall survival; PFS: Progression-free survival; ICI: Immune checkpoint inhibitors; AUC: Area under curve; ROC: Receiver operating characteristic; HR: Hazard ratio; ORR: Overall response rate; TME: Tumor microenvironment.

# Declarations

**Ethics approval and consent to participate:** This study was approved by the Internal Review and the Ethics Boards of the Fifth Medical Center, General Hospital of the PLA and were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient.

**Consent for publication:** Not applicable.

**Availability of data and materials:** All data and materials are available upon reasonable request from the readers.

**Competing interests:** HZ has received research funding from The National Key Sci-Tech Special Project of China. The remaining authors declare no competing financial interests.

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**Authors' contributions:** HZ and JX designed the study. SJ acquired the data. HC, KY, BM, YH and GZ analyzed the data. HC and KY drafted the manuscript. All authors read and approved the final manuscript.

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# Supplementary Figure Legends

**Figure S1. Cytokine levels (concentration in log10) in esophageal squamous cell cancer (ESCC) patients receiving anti-PD-1/PD-L1 treatment.** Patients were grouped by CR/PR, SD and PD. Statistically significant comparisons among the three subgroups (Table S1) revealed 14 candidate cytokines.

**Figure S2. Heatmap cluster analysis of the data from the 59 cytokine factors in the validation cohort.**

**Figure S3. Cytokine levels (concentration in log10) in ESCC patients receiving anti-PD-1/PD-L1 treatment. Patients were grouped by CR/PR, SD and PD.** Statistically significant comparisons among the three subgroups (Table S3) revealed 10 candidate cytokines.

Figure S4. Kaplan–Meier curves comparing PFS in infiltrating immune cell-high versus immune cell-low subgroups.

# Figures

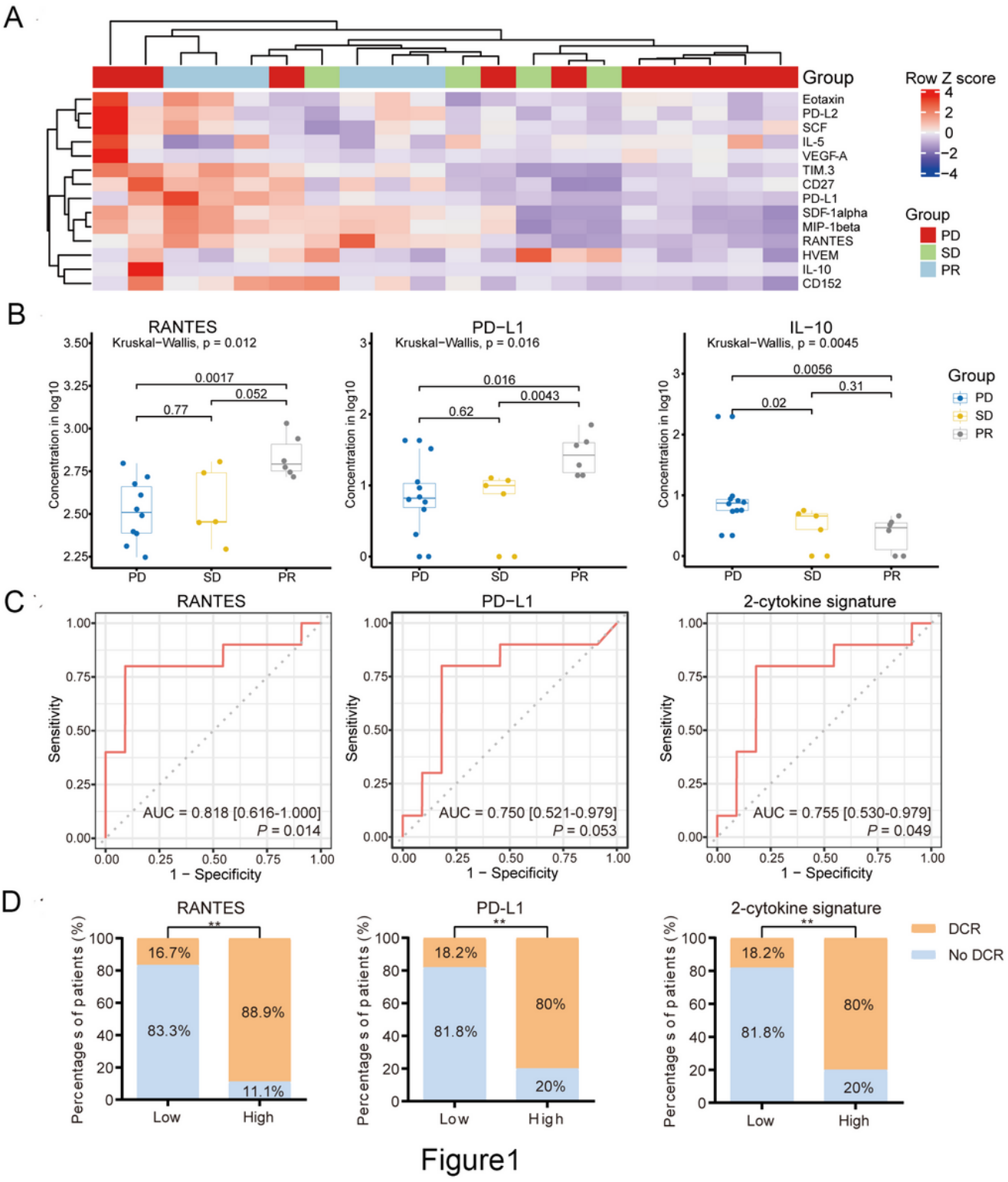



Figure1

Figure 1

Pretreatment RANTES and PD-L1 levels in plasma correlate with an increased DCR in discovery cohort. A. Heatmap cluster analysis of the 59 cytokine datasets in the discovery cohort (esophageal squamous cell cancer, n=21). B. Plasma cytokine levels (concentration in log10) of RANTES, PD-L1 and IL-10 in the discovery cohort.

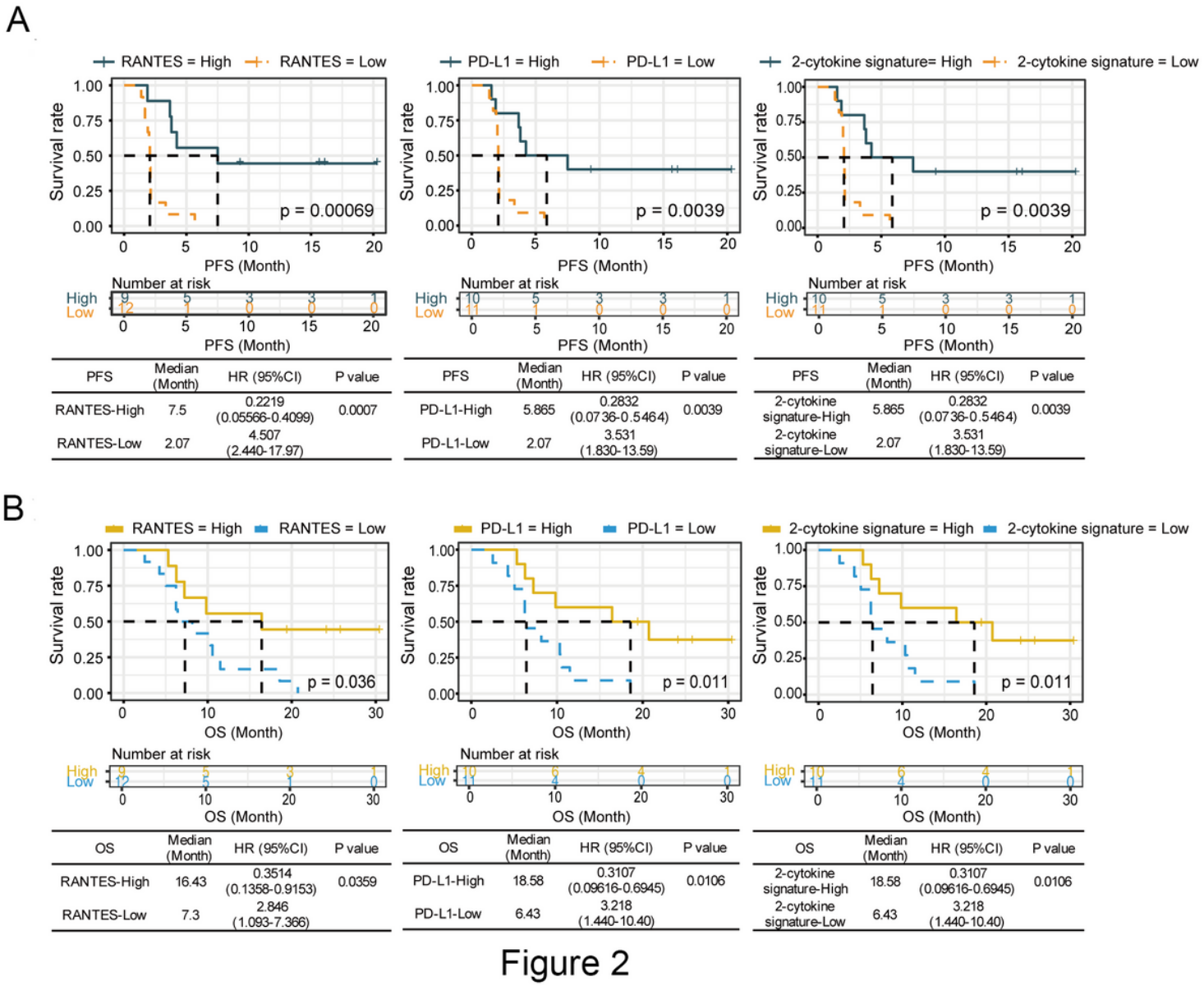



Figure 2

## Figure 3

The survival curves in the discovery cohort. A. Overall survival. B. Progression-free survival. The cutoff point between high and low levels was determined using the Youden index of RANTES, PD-L1 and the 2-cytokine signature. Significant differences were found in all of the comparisons between the high and low levels.

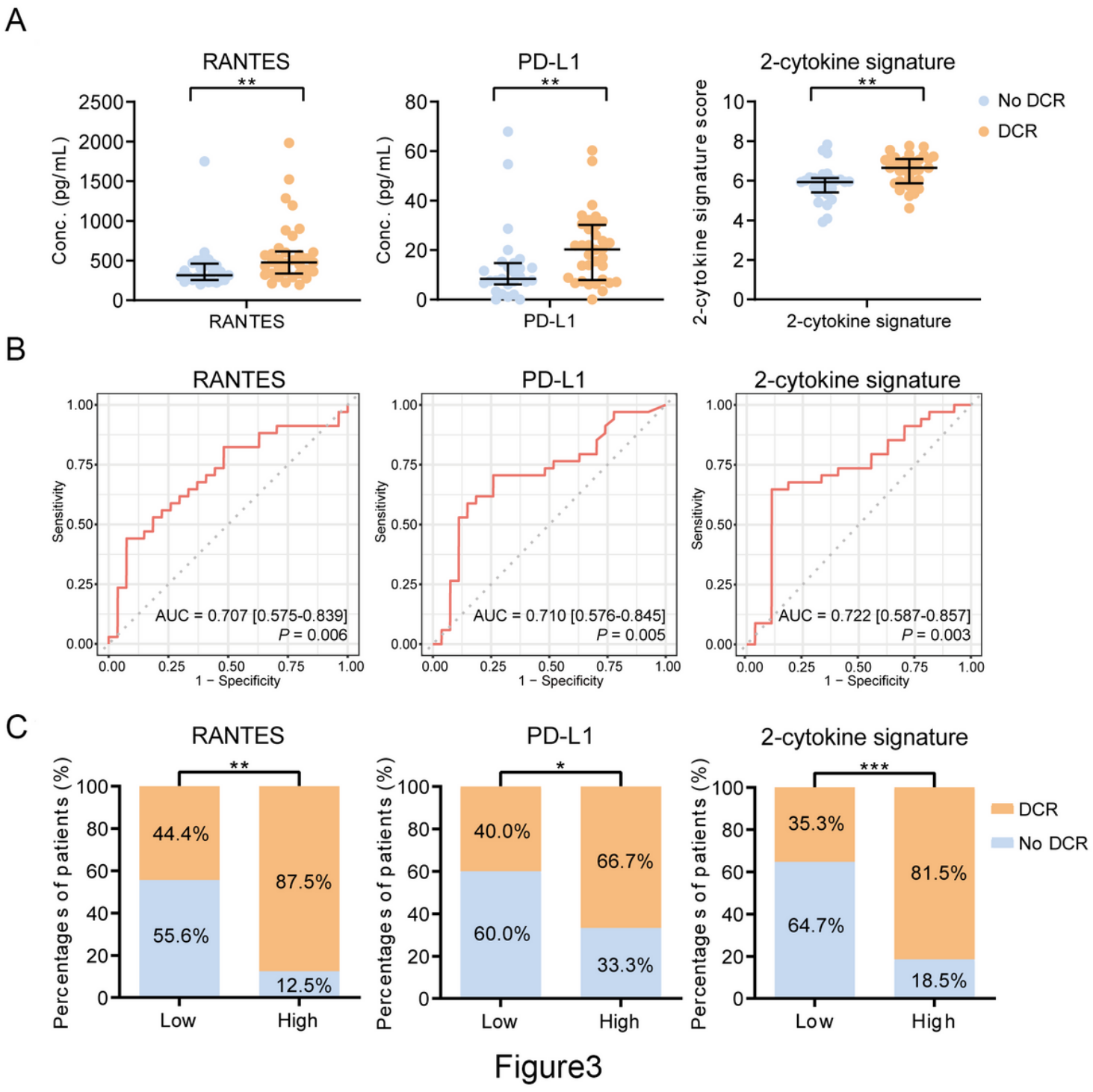
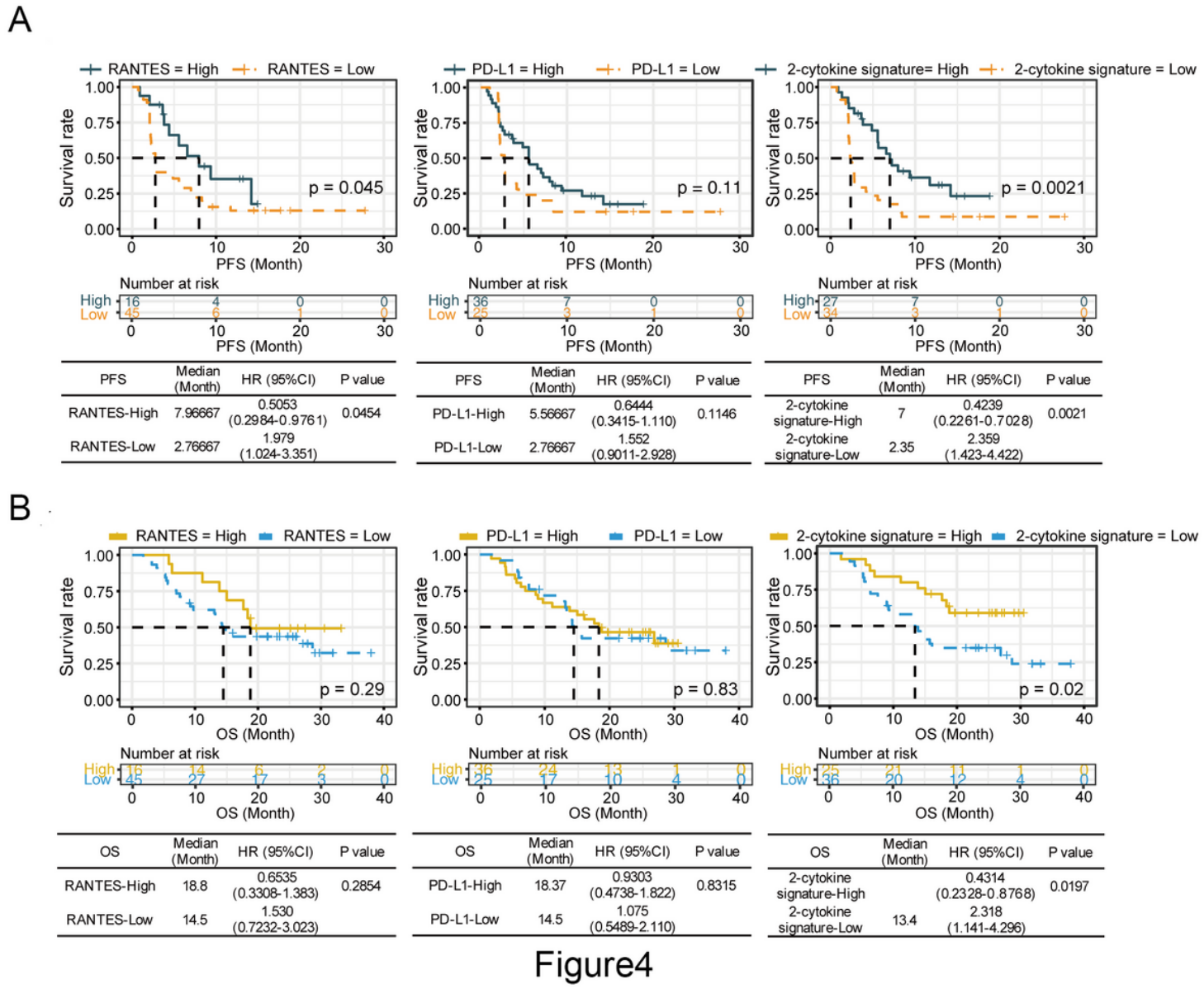



Figure3

## Figure 5

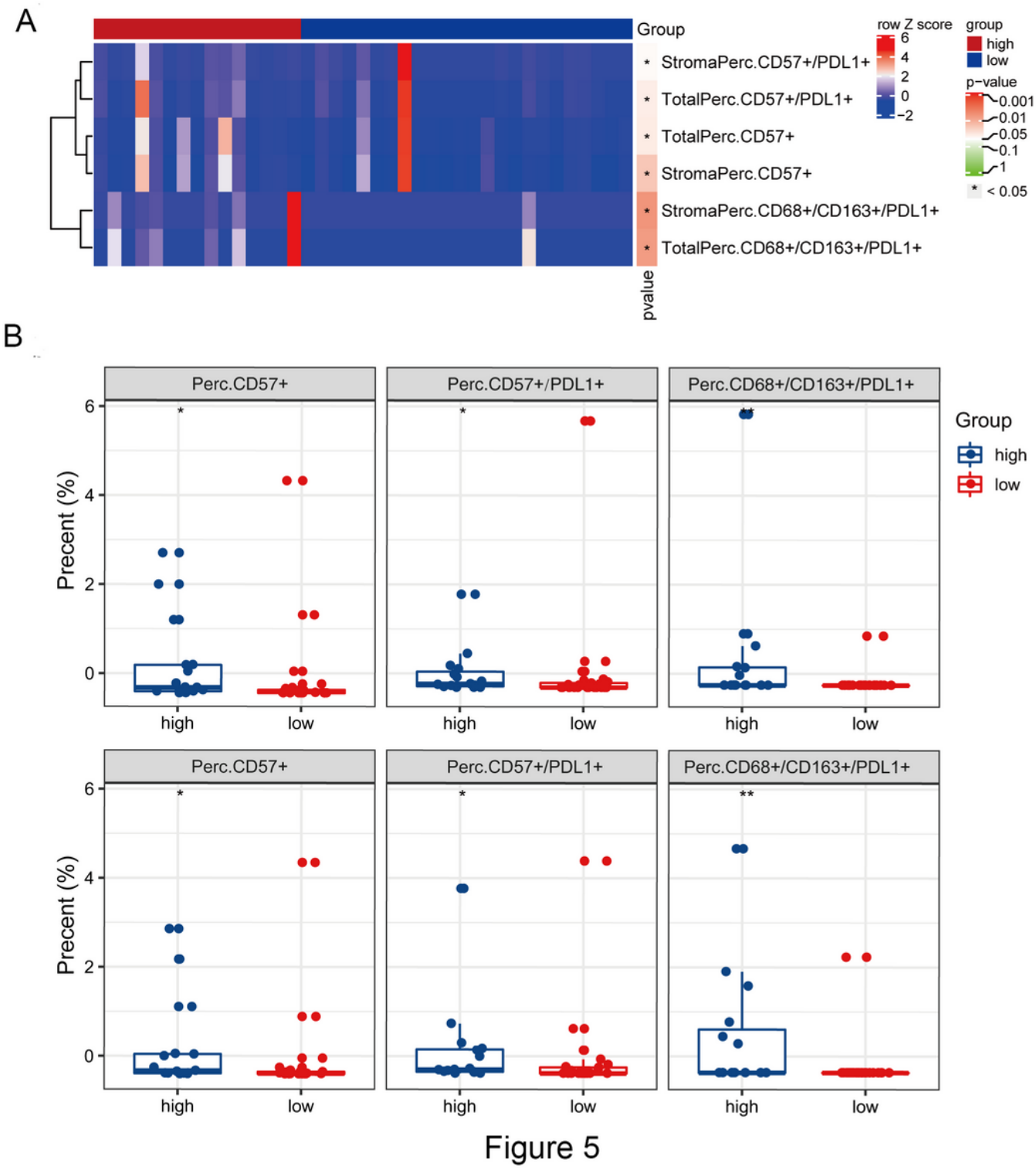
Baseline plasma cytokine levels are associated with disease control. A. Baseline plasma concentrations of RANTES, PD-L1 and the 2-cytokine signature (calculated by the average concentration of RANTES and PD-L1 in log10) in the validation cohort. Statistical comparisons across median cytokine levels in DCR (disease control rate) versus no DCR by Mann-Whitney U-test. B. ROC curves for the correlation of RANTES, PD-L1 and the 2-cytokine signature with DCR in the validation cohort. C. Comparison of the frequency of DCR patients with different cytokines or 2-cytokine signature levels in the validation cohort.

The cutoff point between high and low levels was determined using the Youden index of each cytokine and 2-cytokine signature. Fisher's exact test was used to compare the variables. AUC, area under the curve. *P <0.05; **P <0.01.




## Figure 7

The survival curves in the validation cohort. A. Overall survival. B. Progression-free survival. The cutoff point between high and low levels was determined using the Youden index of each cytokine or 2-cytokine signature. Significant differences were found in all of the comparisons between the high and low levels.




## Figure 9

Comparison of tumor microenvironment composition among high- and low-2-cytokine signatures. A. Heatmap cluster analysis in the combined cohort. Slides were stained with 6 markers (CD163, CD68, PD-L1, CD8, PD1, CD57) by multi-IHC. B. Comparison of differences in TILs in the tumor microenvironment between the high- and low-2-cytokine signature subgroups. *P <0.05; **P <0.01.

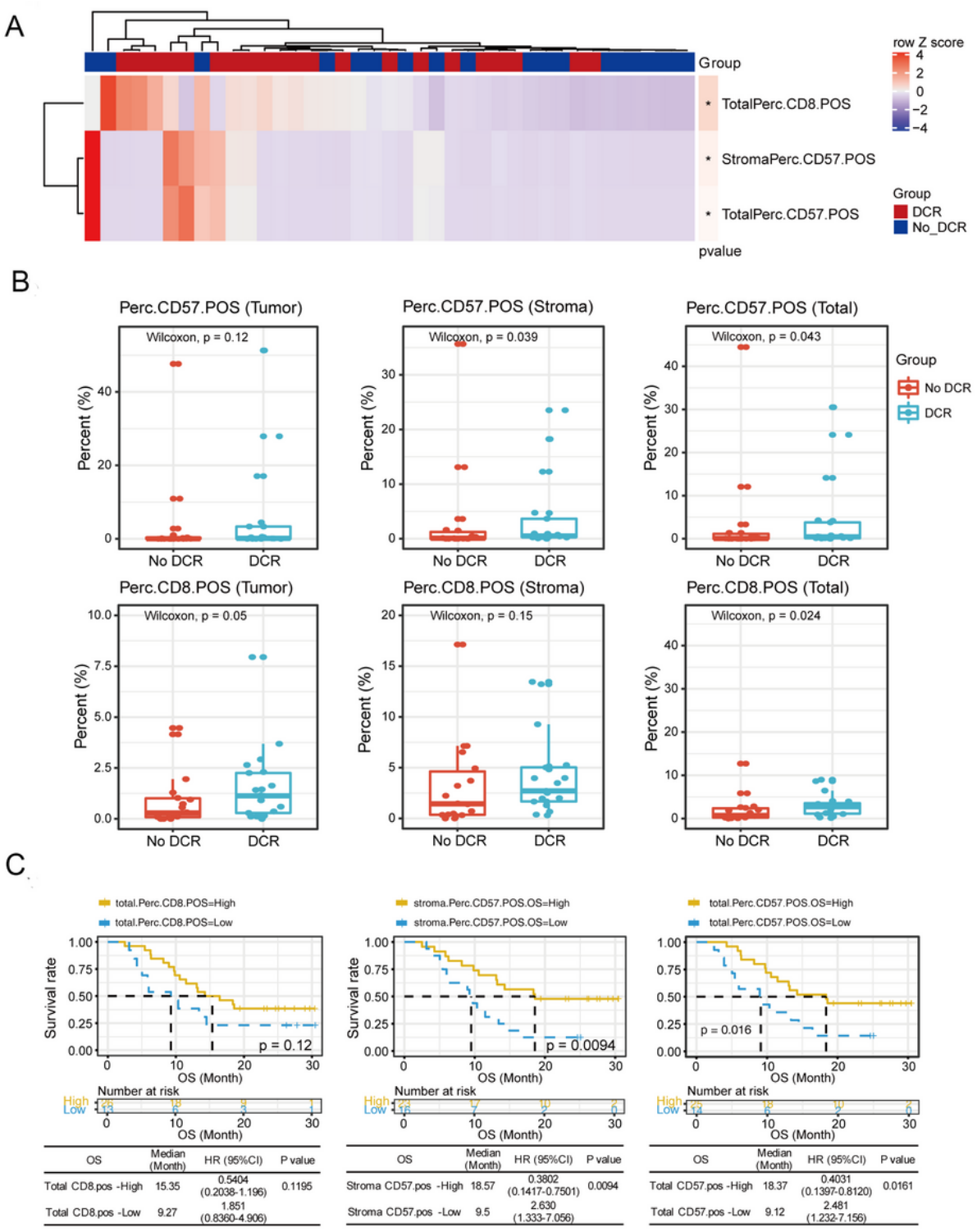



Figure 6

## Figure 11

Comparison of tumor microenvironment composition among DCR and no DCR patients. A. Heatmap cluster analysis of CD8 and CD57 expression in the combined cohort. Slides were stained with 6 markers (CD163, CD68, PD-L1, CD8, PD1, CD57) by multi-IHC. B. Comparison of differences in TILs in the tumor microenvironment between DCR and no DCR subgroups. C. Kaplan–Meier curves comparing OS time in infiltrating immune cell-high versus immune cell-low subgroups.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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- FigureS3.jpg
- Supplementarytables.pdf
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