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The comprehensive assessment of local immune status of ovarian cancer by the clustering of multiple immune factors

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Abstract

The aim of this study was to evaluate the local immune status of human ovarian cancers by the comprehensive analysis of tumor-infiltrating immune cells and immunosuppressive factors, and to elucidate the local immunity in clinical course. The numbers of CD1 α +, CD4+, CD8+, CD57+, forkhead box P3+ and programmed cell death-1+ cells were counted, and the intensity of immunosuppressive factors, such as programmed cell death-1 ligand (PD-L)1 , PD-L2, cyclooxygenase (COX)-1, COX-2 and transforming growth factor β 1, were evaluated in 70 ovarian cancer specimens stained by immunohistochemistry. Then hierarchical clustering of these parameters showed the four clusters into ovarian cancer cases. Cluster 1, which had significantly better prognosis than the others, was characterized by high infiltration of CD4+ and CD8+ cells. In conclusion the comprehensive analysis of local immune status led to subdivide ovarian cancers into groups with better or worse prognoses and may guide precise understanding of the local immunity.

Keywords

ovarian cancer; tumor immunity; tumor immune escape

1. Introduction

Ovarian cancer is the most lethal gynecologic cancer in the world with >200,000 patients diagnosed every year and over a half of them dying annually. These deaths are partly due to the fact that more than half of the patients with ovarian cancer are diagnosed at advanced tumor stages (stage III or IV). Although platinum or taxane-based chemotherapies are effective in the treatment of the majority of ovarian cancer cases, most of the patients suffer from recurrence and eventually develop chemo-resistance. Considering the high mortality rate of ovarian cancer due to the absence of curative treatment in the advanced stage or at recurrence, new therapeutic modalities other than chemotherapy and surgery are urgently needed [1; 2; 3].

Tumor immune therapy has long been considered as an alternative modality in the treatment of solid tumors including ovarian cancer. Nevertheless, there have been few reports on clinically successful immune therapies. The failure in immune therapies in such clinical trials is partly ascribed to the phenomenon designated as “tumor immune escape”. It is increasingly understood that the dynamic interaction between tumor cells and immune cells in the local microenvironment plays a pivotal role in cancer development and progression [4]. In the case of advanced cancers, tumor cells establish an immunosuppressive environment regionally and make it difficult to induce immune activation to eliminate cancer cells. In this situation, adoptive immunotherapy, such as a tumor vaccine, is not sufficient to eradicate tumors [5; 6].

The differences in the phenotypes or populations of tumor-infiltrating immune cells, such as CD4⁺ (helper) T cells, CD8⁺ (cytotoxic) T cells, CD57⁺ (NK) cells and CD11c⁺ (dendritic) cells, have been shown to be associated with different clinical outcomes of solid tumors including colorectal cancer [7], breast cancer [8], gastric cancer [9; 10], lung cancer [11; 12], hepatic cancer [13; 14], melanoma [15], kidney

cancer [16] and uterine cervical cancer [17]. In ovarian cancer, several recent studies have shown an association between tumor-infiltrating immune cells and clinical outcomes [18; 19]. We also reported that CD8⁺ T cell infiltration [20] and NK cell infiltration [21] are associated with a favorable prognosis in the ovarian cancer patient. On the other hand, regulatory T cells, most specific marker of which is FOXP3, in the tumor site play a suppressive role in the local tumor immunity, leading to tumor progression [22; 23].

With respect to the tumor, there are also a wide variety of mechanisms that enable tumor cells to evade an immune attack. These mechanisms include a loss of MHC [24], the upregulation of immunosuppressive factors, such as transforming growth factor β (TGF β) [25], IL-10, indoleamine 2,3-dioxygenase (IDO) [26] and cyclooxygenases (COX-1 and COX-2) [27] or upregulating negative regulatory signals, such as programmed cell death-1 (PD-1) ligands (PD-L1, PD-L2) and cytotoxic T lymphocyte antigen-4 (CTLA-4) [28; 29; 30; 31]. We reported that PD-L1 expression in ovarian cancer is inversely correlated with tumor-infiltrating CD8⁺ T cells and is associated with a poor prognosis of the patient [20]. The expression of the immune suppressive factors COX and UL-16 binding protein 2 is also inversely associated with CD8⁺ T cell infiltration and the prognosis of the patient with ovarian cancer [21; 32]. Thus, there are a variety of reports that suggest that a certain immunosuppressive factor influences the local tumor immunity. However, there are few comprehensive analyses that integrate various immune factors and evaluate the immune status as a whole.

Therefore, in this study, we attempted to explore the status of local immunity in ovarian cancers by integrating various immune parameters presented by the immunohistochemical analysis of clinical specimens. For this purpose, we employed bioinformatics analyses, such as hierarchical clustering, that allows the comprehensive

assessment of multiple factors and enables us to determine the relationships among them.

2. Materials and Methods

2.1. Patients and Samples

Formalin-fixed, paraffin-embedded specimens were obtained from 70 patients who underwent primary surgery for epithelial ovarian cancer at the Kyoto University Hospital. After surgery, all patients received platinum- and paclitaxel-based chemotherapy. The average age of the patients was 55 years old (range, 26- 78; standard deviation [SD], 11). At the end of the study, 29 (41%) patients had died from their disease, and 41 (59%) patients were alive. The mean follow-up period was 5 years (range, 0- 11; SD, 3.0). All 70 tissue specimens were collected under the approval of the Ethics Committee of the Kyoto University Hospital.

2.2 Immunohistochemistry

The primary antibodies and antigen retrieval methods are listed in Supplementary Table 1. Briefly, formalin-fixed, paraffin-embedded specimens were cut into 4 μ m-thick sections. The tissue sections were deparaffinized in xylene and dehydrated. For antigen retrieval for TGF β 1 and CD4 staining, the samples were boiled in Tris-EDTA buffer (pH 9.0) in a pressure cooker. For FOXP3 and PD-1 staining, the samples were boiled in citrate buffer (pH 6.0) in a pressure cooker. To block endogenous peroxidase activity, all of the sections were treated with 100% methanol containing H₂O₂. Nonspecific binding of IgG was blocked using normal rabbit serum (Nichirei, Tokyo, Japan). The sections were incubated with a mouse anti-TGF β 1 monoclonal antibody (Ab) (clone TB21), anti-CD4 monoclonal Ab (clone 1F6), anti-FOXP3 monoclonal Ab (clone 236A/E7) and PD-1 monoclonal Ab (clone NAT) overnight at 4°C. Then the sections were incubated with biotinylated rabbit anti-mouse secondary Abs (Nichirei), followed

by an incubation with a streptavidin-peroxidase complex solution. Signals were generated by incubation with 3, 3-diaminobenzidine. Finally, the sections were counterstained with hematoxylin and observed under a microscope.

2.3. Evaluation of the specimens

Immune cells in the intraepithelial space were counted using a microscopic field at $\times 200$ magnification (0.0625 mm^2). Five areas with the most abundant infiltration of immune cells were selected, and an average count was calculated. The result was interpreted as negative when fewer than five cells per 0.0625 mm^2 were observed and as positive when more than or equal to five cells were observed. The expression of TGF β 1 was evaluated according to the intensity of the staining and scored as follows: 0, negative; 1, very weak expression; 2, moderate expression; and 3, strongest expression. Cases with scores of 0 or 1 were defined as the low-expression group, and cases with scores of 2 or 3 were defined as the high-expression group. Two independent gynecological pathologists examined the immunohistochemical slides without any prior information regarding the clinical history of the patients.

2.4. Hierarchical clustering and statistics

Hierarchical clustering analysis of our immunohistochemical data was performed using the software Cluster 3.0 that was originally designed for manipulating cDNA microarray data [33]. Following the instructions of the software, the eleven parameters (six tumor-infiltrating immune cells and five immune suppressive factors) were normalized, and a complete-linkage hierarchical clustering was conducted. The dendrogram and heat map were graphically viewed using Java TreeView [33]. Cluster and Treeview software are freely available programs that can be accessed at

<http://jtreeview.sourceforge.net/>.

2.5. Statistical analysis

Fisher's exact test and the χ^2 test were used to analyze the associations between each cluster and various clinic-pathological factors. Spearman's correlation coefficient test was employed to analyze the associations among 11 immunological factors. Univariate analysis for overall survival was performed and evaluated with the log rank test, and Kaplan–Meier curves were generated. A multivariate Cox proportional-hazard model was used to evaluate the independency of Cluster 1 as a prognostic factor. Two-sided P values of <0.05 were considered to be significant.

3. Results

3.1. Expression of immune-suppressive factors in ovarian cancer specimens and patient prognosis

Immunohistochemical expression of TGF β 1, PD-L1, PD-L2, COX-1 and COX-2 were evaluated in 70 ovarian cancer tissues (Figure 1). High expression (score 2 or 3) of TGF β 1 was observed in 22 cases (31.4%) and low expression (scored 0 or 1) was observed in 48 cases (68.6%). There was no correlation between the expression of these factors and clinicopathological characteristics such as age, histological type, FIGO stage, TNM classification, and residual tumor state (Supplementary Table 2) [20; 32].

The log rank test showed that the 5-year survival rate of patients with high expression of TGF β 1, COX-1 or COX-2 was not significantly different from the patients with low expression (Supplementary Figure 1). Only the high expression of PD-L1 was an independent worse prognostic factor, whereas PD-L2 expression was not related to patient prognosis [20].

3.2. Tumor-infiltrating immune cell count and prognosis

The number of tumor-infiltrating CD1 α ⁺ (dendritic cells), CD4⁺ (helper T cells), CD8⁺ (killer T cells), CD57⁺ (NK cells), FOXP3⁺ (regulatory T cells) and PD-1⁺ immune cells was evaluated using the same 70 ovarian cancer specimens (Figure 1). The average numbers of these cells were shown in Supplementary Table 3, respectively. There was positive correlation between tumor-infiltrating FOXP3⁺ cells and several clinicopathological factors such as age, histology, tumor status and residual tumor, while there was no correlation between the number of CD4⁺, CD8⁺, CD57⁺ or PD-1⁺ cells and clinicopathological characteristics (Supplementary Table 4 and 5).

A significant correlation was found between parameters below; CD4+ cell infiltration vs. CD8+ cell infiltration, COX-1 and COX-2 expression, CD4+ cell vs. PD-1+ cell infiltration, CD8+ cell vs. PD-1+ cell infiltration, CD57+ cell vs. PD-1+ cell infiltration), FOXP3 cell infiltration vs. PD-L2 expression and FOXP3 vs. CD4+ cell infiltration (Supplementary Table 6), although a negative correlation between COX-1 vs. COX-2 expression, CD8+ cell infiltration vs. PD-L1 expression, CD8+ cell infiltration vs. COX-1 expression and CD8+ cell infiltration vs. COX-2 expression [20; 32].

The log rank test showed that the overall survival rate of patients with high levels of CD1 α +, CD4+, CD57+, FOXP3+ or PD-1+ immune cells was not significantly different from patients with low infiltration (Supplementary Figure 1), whereas a high infiltration of CD8+ cells was the only beneficial prognostic factor ($p < 0.001$) [20]. Combination of any two factors such as CD8+ and PD-L1 low did not serve as a superior prognostic factor compared with single factor. Besides we found a higher ratio of CD8/FOXP3 in Cluster 1 than that in Cluster 2-4, although there was no statistic significance (mean \pm SD, Cluster 1, 3.4 ± 2.4 vs. Cluster 2-4, 1.9 ± 1.9).

3.3 The correlation among eleven immunological factors

The correlation among eleven immunological factors (the expression of PD-L1, PD-L2, COX-1, COX-2 and TGF β 1 and the number of tumor-infiltrating immune cells expressing CD1 α +, CD4+, CD8+, CD57+, FOXP3+ and PD-1+) was examined (Supplementary Table 6). The expression of PD-L1 or COX expression was negatively correlated with the number of CD8+ cells in the tumor site, respectively [20; 32]. In this study, we found that the number of CD4+ cells was positively correlated with the number of CD8+ cells (correlation coefficient ($R=0.240$; $p=0.045$) and FOXP3+ cells ($R=0.410$; $p < 0.001$). In addition, the number of PD-1+ cells showed a positive

correlation with the number of CD4⁺ cells (R=0.302; p=0.011), CD8⁺ cells (R=0.366; p=0.002) and CD57⁺ cells (R=0.365; p=0.002). The number of FOXP3⁺ cells was negatively correlated with PD-L2 expression (R=-0.262; p=0.028).

3.4. Evaluation of the local immune status by hierarchical clustering of immune factors in ovarian cancer

Hierarchical clustering analysis of the expression levels of five immune suppressive factors and the cell counts of the six tumor-infiltrating immune cells were used to divide the 70 ovarian cancers into 2 major clusters and subdivided one of the major clusters into three clusters, which were designated as Cluster 1 and Clusters 2, 3 and 4, respectively (Figure 2). When Cluster 1 was compared to the other clusters (Clusters 2-4), it was characterized as having significantly higher immune cell infiltration, such as CD4⁺ cells (p=0.004), CD8⁺ cells (p<0.0001) and PD-1⁺ cells (p=0.0037), and as having lower expression of immunosuppressive factors such as TGFβ1, PD-L1, PD-L2, COX-1 and COX-2 (Figures 3A-3C, 3F and 4).

The characteristics of the other three clusters were relatively common in terms of low immune cell infiltration and partially high expression of immune suppressive factors with the following patterns: Cluster 2, high COX-1 expression (p<0.0001) and high CD57⁺ cell (NK cell) infiltration (p=0.0042); Cluster 3, high PD-L2 (p=0.0002), low FOXP3⁺ cells (p=0.0288) and low PD-1⁺ cell infiltration (p=0.011); and Cluster 4, low CD4⁺, low CD8⁺, low CD1α⁺, low CD57⁺, low PD-1, high PD-L1, high TGFβ1, and high COX-2 (Figures 3 and 4).

3.5. Univariate analysis and correlation between four clusters and clinicopathological factors

The Kaplan–Meier curve and log rank test showed that the overall survival rate of patients in Cluster 1 was significantly better than those in the other clusters (5-year survival rate in Cluster 1 vs. Clusters 2-4, 84.6% vs. 55.2%; $p=0.041$) (Figure 5 and Table 1). The progression-free survival rate of patients in Cluster 1 was not significantly, but was relatively, better than other clusters (5-year survival rate of Cluster 1 vs. Clusters 2-4, 78.6% vs. 44.4%; $p=0.061$).

There was no statistical correlation between the four clusters and the clinicopathological factors such as primary tumor status, lymph node metastasis, distant metastasis, residual tumor status, the age of the patient, histology, and adjuvant chemotherapy (Table 2).

3.6. Multivariate analysis

Multivariate analysis showed that Cluster 1 was an independent favorable prognostic factor for overall survival (RR, 4.93) (Table 1). Other factors contributing to overall poor survival were tumor status (RR, 5.36), lymph node metastasis (RR, 2.78), and residual tumor status (RR, 5.86) (Table 1).

4. Discussion

Recent studies have shown that local tumor immunity is closely associated with clinical course of cancer patient, and several immunological factors, including CD8 T cell count shown in our previous study, serve as prognostic indicator. However, these analyses were mainly done using single factor or combination of several factors, and there are few papers which tried to clarify the immunological background by analyzing multiple immune factors simultaneously. The application of hierarchical clustering allowed us to manage the complex data sets of immunohistochemical staining with multiple antibodies [34; 35] and to identify new groups of patients with similar local immunological patterns that may be caused by similar consequences. Ovarian cancers were divided into two groups, Cluster 1 and Clusters 2-4, by hierarchical clustering analysis according to the local immunological state. The patients in Cluster 1 had a significantly better prognosis than those in other clusters ($p=0.041$, Figure 5B). In this group, immune cells, including CD4⁺ cells, CD8⁺ cells and PD-1⁺ cells, were highly infiltrated into tumor sites compared to the other clusters ($p=0.004$, $p<0.001$ and $p=0.0037$, respectively), while the expression of TGF β 1, PD-L1, PD-L2, COX-1 and COX-2 were significantly low. In this group, PD-1⁺ cells may represent T cells in the late active phase [36], though its significance is to be clarified. Thus, Cluster 1 was characterized by high immune cell infiltration and low expression of all immunosuppressive factors studied (Figures 3 and 4), suggesting that host-tumor immunity in the tumor microenvironment is still maintained in this group, which may lead to the significantly better prognosis. Besides a ratio of CD8/FOXP3 ratio in Cluster 1 was higher than that in Cluster 2-4, although there was no statistic significance, which is a similar tendency to the previously published report [19].

Clusters 2-4 were characterized by a low level of immune cell infiltration and high expression of immunosuppressive factors and had significantly worse prognoses than Cluster 1. Cluster 2 was characterized by a significantly high expression of COX-1, whereas Cluster 3 and 4 had significantly high expressions of COX-2 ($p=0.0053$ and $p=0.0048$, respectively). The immunoregulatory function of COX-2-induced prostaglandin E2 (PGE₂) is known to be important in inducing immune tolerance in the tumor microenvironment [37]. Secreted from tumor cells, PGE₂ alters the Th1/Th2 balance, suppresses lymphocyte proliferation, and regulates the function of antigen presenting cells [37; 38]. There is a report that expression of COX-2 is an independent prognostic factor in human ovarian carcinoma [39]. Hence, high expression of COX-2 in Cluster 3 and Cluster 4 may contribute to poorer prognosis associated with low CD8+ cell infiltration (Figures 3-5 and Supplementary Table 6). Similarly, COX-1 expression was inversely correlated with CD8+ cell infiltration in Cluster 2, which may partly explain the poor prognosis of Cluster 2.

Cluster 3 was characterized by high PD-L2 expression and low PD-1+ cell infiltration and had a worse prognosis. We previously reported that the patient with high expression of PD-L2 had a tendency for poor prognosis, although the difference was not statistically significant. In this respect, high expression of PD-L2 may partly explain the poor prognosis of this group, possibly by negatively influencing the infiltration of CD8+, CD4+ and PD-1+ cells. Cluster 4 was characterized by high expression of PD-L1, TGFβ1 and COX2 and low CD8+ cell infiltration. Previous studies on PD-L1 expression in malignant tumors, such as in kidney, bladder, breast, gastric, pancreatic and ovarian cancer, have shown that PD-L1 has a negative impact on the survival of the patient [29]. In addition, PD-L1 expression was inversely correlated with intraepithelial infiltrating CD8+ T cells, suggesting that PD-L1 inhibits the intratumoral infiltration of

CD8⁺ T cells. TGF β signaling has been implicated in tumor progression, metastasis and immunosuppression in the advanced tumor phase [25]. These results suggest that PD-1 ligand and/or COX expression are associated with an unfavorable clinical outcome of the patient by influencing the local immune environment.

Recently, three phases of cancer immunoediting, namely, “elimination”, “equilibrium” and “escape” [4; 6; 40], have been proposed. In the first “elimination” phase, innate and adaptive immune cells recognize and eliminate tumor cells by immunosurveillance, protecting the host against cancer. In the second “equilibrium” phase, ongoing tumor growth and immune surveillance enter into a dynamic balance with one another, yielding in a protracted period. In the last “escape” phase, the tumor avoids immune-mediated destruction and develops into a clinically apparent neoplasm [4]. This hypothesis is mainly applied to the process of cancer development in which immunosurveillance is gradually impaired. However, in clinical situations, cancer patients sometimes experience an asymptomatic period coexisting with known cancer lesions, or even a spontaneous regression, without any medical interventions, suggesting that the balance between tumor growth and host immunity significantly influences the clinical course of cancer patients. Nevertheless, there have been few studies that intended to comprehensively analyze the local immune status of each case, which would thereby establish the means to predict the clinical outcome. In this study, Cluster 1 may represent a phenotype of the “equilibrium” phase, where immune cells infiltrate into the tumor site to eliminate the tumor. Clusters 2, 3 and 4 may be in “escape” phase in which the local immune environment has already fallen into an immunosuppressive status. For an effective immune therapy, an understanding of the immune status in each case is particularly important. This study provides a model to analyze the complicated immune reaction in a local tumor site.

This study may also provide a future direction for order-made immunotherapy in each ovarian cancer patient. Currently, therapeutic modalities to target specific immunosuppressive factors are being developed. Blocking antibodies against PD-1 (MDX-1106) have been developed and are in Phase I clinical trials for advanced refractory malignancies [41]. Phase II and III clinical trials using selective COX2 inhibitors, celecoxib and rofecoxib, in combination with a chemotherapeutic have shown a clinical benefit [42]. Clinical trials focusing on the inhibition of the TGF β signaling pathway by a monoclonal antibody or a small molecule inhibitor of the TGF β receptor I kinase are being performed. To select the most efficient single or combined immune targeting therapies, precise assessments with multiple immune parameters in each case is essential.

In conclusion, hierarchical clustering of tumor-infiltrating immune cells and immunosuppressive factors was used to identify a subgroup of ovarian cancer patients with a better prognosis. This study also suggested that immunosuppressive factors might influence the pattern of tumor-infiltrating immune cells. The approach to comprehensively analyze multiple immune factors shown here may lead to a precise understanding of the local immune status and provide a tool for the application of immune therapies to treat ovarian cancer patients.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1

Univariate and multivariate analysis demonstrating the independent risk factors, including Cluster 1, on overall survival of patients with ovarian cancer ($n = 70$).

	n	Univariate hazard ratio*	Overall survival		
			p	Multivariate hazard ratio*	p
Cluster			0.041		0.035
Cluster 1	38	1		1	
Clusters 2-4	32	3.98 (1.04- 5.90)		4.93 (1.11- 21.76)	
Tumor status			<0.001		0.013
pT1 + pT2	31	1		1	
pT3	39	7.90 (2.73-22.83)		5.36 (1.42- 20.20)	
LN metastasis			0.003		0.041
pN0	56	1		1	
pN1	14	3.24 (1.50- 7.00)		2.78 (1.04- 7.38)	
Distant metastasis			0.047		0.122
pM0	57	1		1	
pM1	13	2.28 (1.01- 5.16)		2.44 (0.79- 7.58)	
Residual tumor			<0.001		0.001
Optimal	49	1		1	
Suboptimal	21	4.54 (2.17- 9.50)		5.86 (1.98- 17.34)	
Histology			0.477		0.103
Serous type	33	1.33 (0.59- 3.02)		3.27 (1.20- 8.93)	
Non-serous type	37	1		1	
Chemotherapy			0.122		0.936
Paclitaxel	31	1		1	
No paclitaxel	39	1.79 (0.86- 3.77)		1.03 (0.48- 2.23)	
Age			0.486		0.562
< 55	32	1		1	
≥55	38	1.31(0.62- 2.77)		1.268 (0.57- 2.83)	

(*) The numbers in parenthesis represent the 95% confidence interval (C.I.)

Table 2**Correlations between the four clusters and clinicopathological characteristics in ovarian cancer (*n* = 70)**

	n	Cluster 1	Cluster 2	Cluster 3	Cluster 4	p
Age						0.922
<55	32	6	8	10	10	
≥55	38	8	5	11	12	
Stage						0.972
I	27	6	3	9	9	
II	4	1	0	1	2	
III	26	5	5	8	8	
IV	13	2	5	3	3	
Histology						0.672
Serous	33	7	9	8	9	
Clear cell	22	5	2	9	6	
Endometrioid	11	1	1	4	5	
Mucinous	2	0	0	0	2	
Others	2	1	1	0	0	
Tumor status						0.889
pT1+pT2	31	6	4	10	11	
pT3	39	8	9	11	11	
LN metastasis						0.566
Positive	14	2	5	4	3	
Negative	56	12	8	17	19	
Distant metastasis						0.499
Positive	13	2	5	3	3	
Negative	57	12	8	18	19	
Residual tumor						0.773
Optimal	49	10	7	16	16	
Suboptimal	21	4	6	5	6	
Chemotherapy						0.965
No paclitaxel	39	9	7	12	11	
Paclitaxel	31	5	6	9	11	

Figure 1

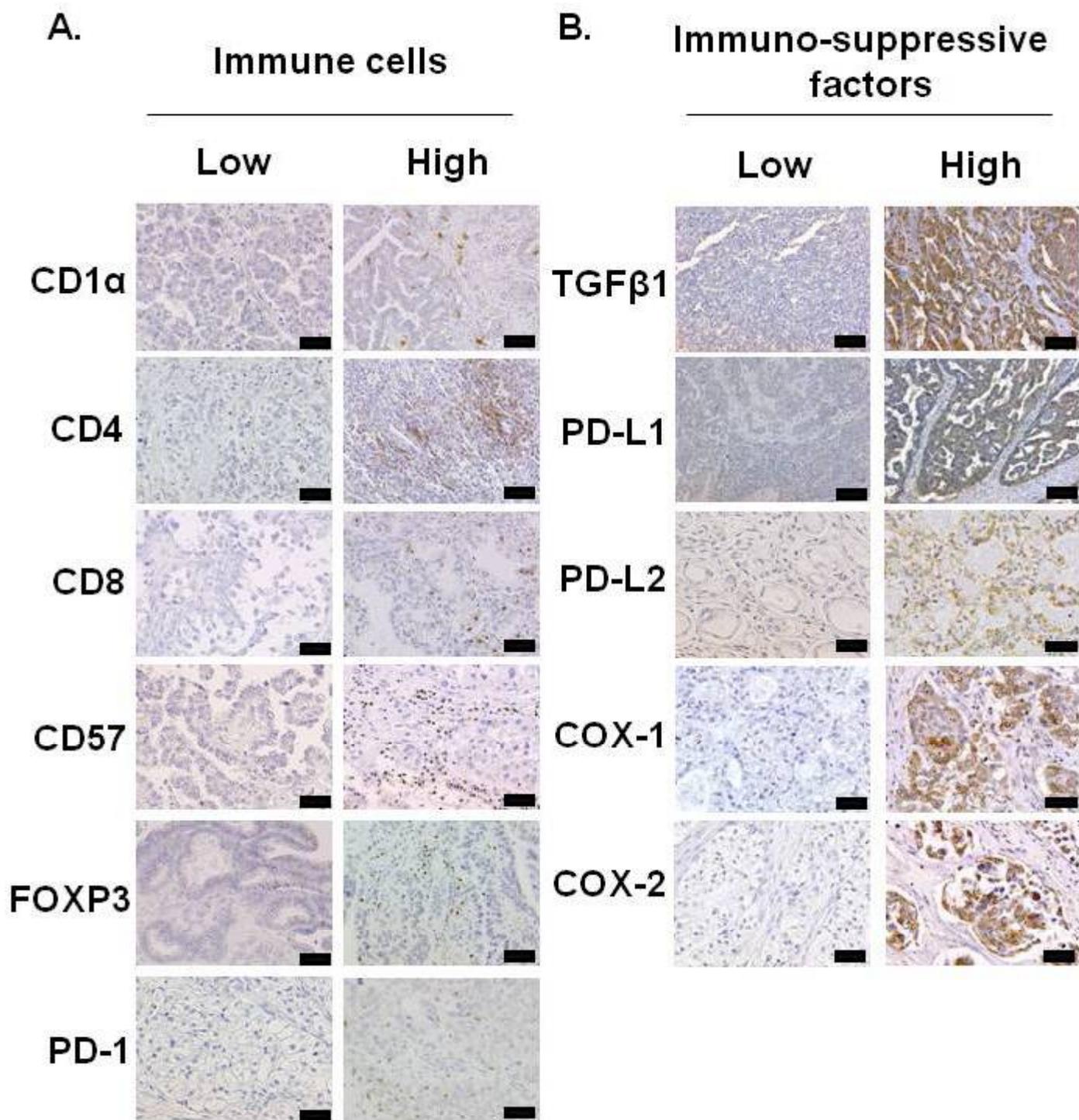


Figure 2

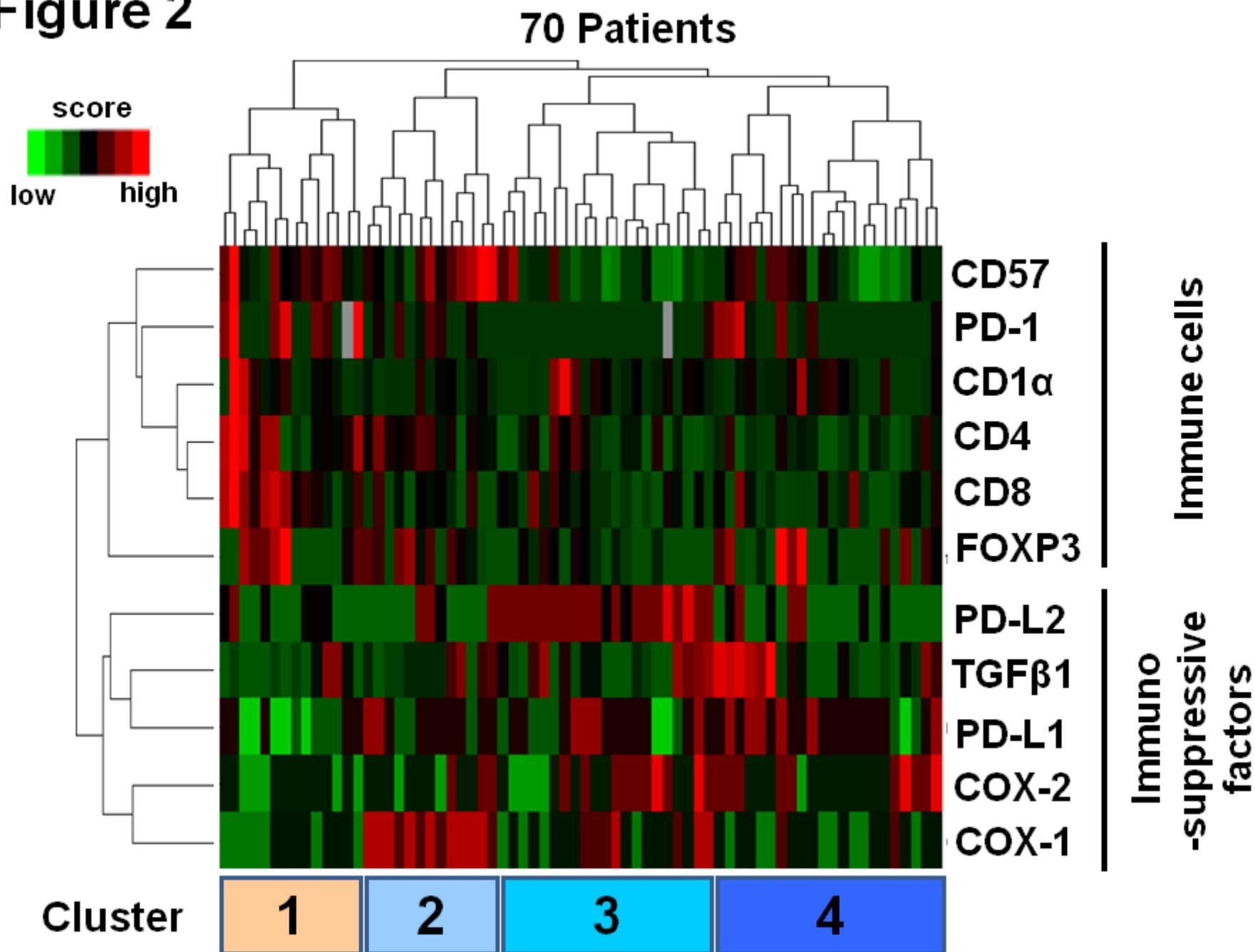


Figure 3

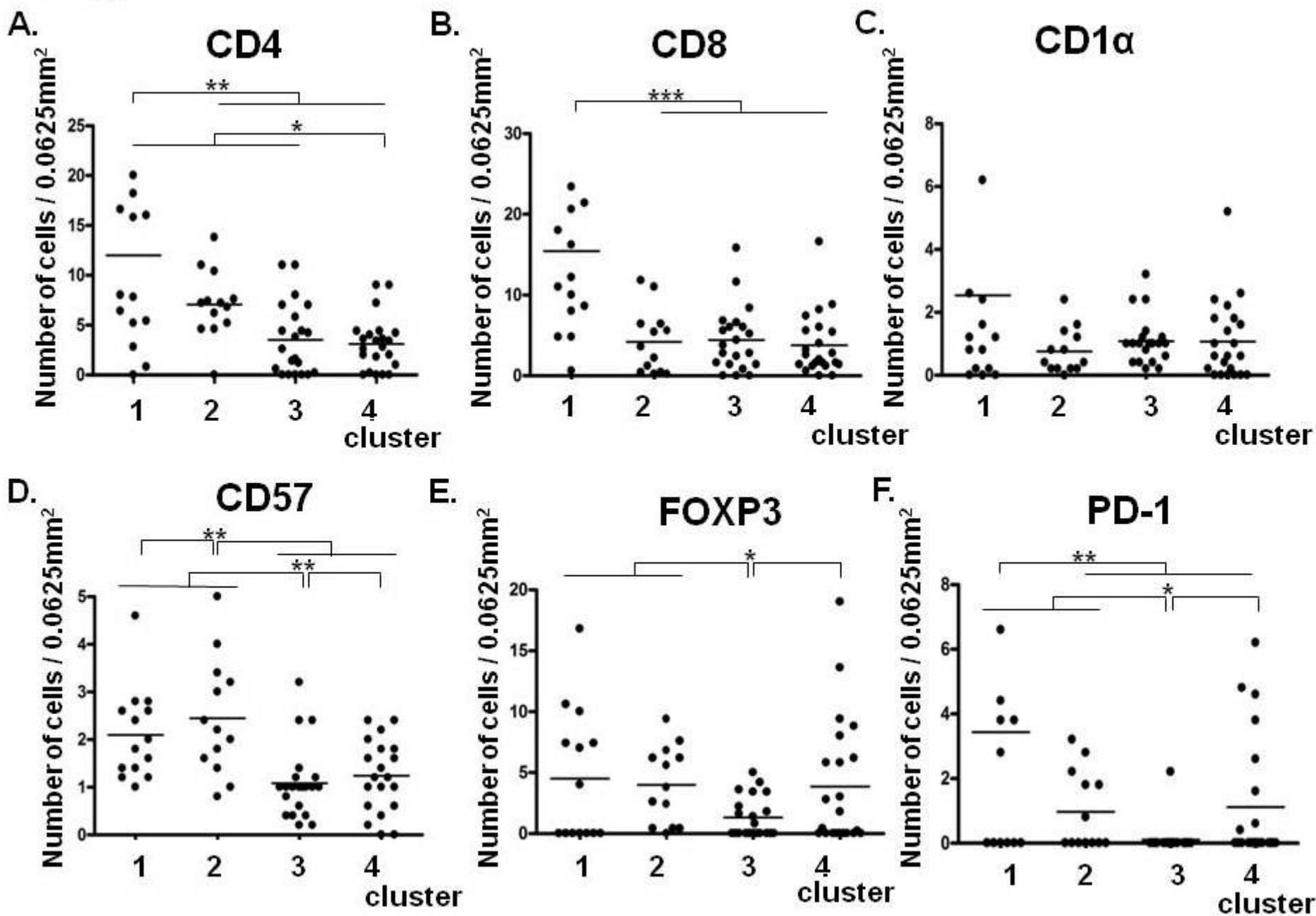


Figure 4

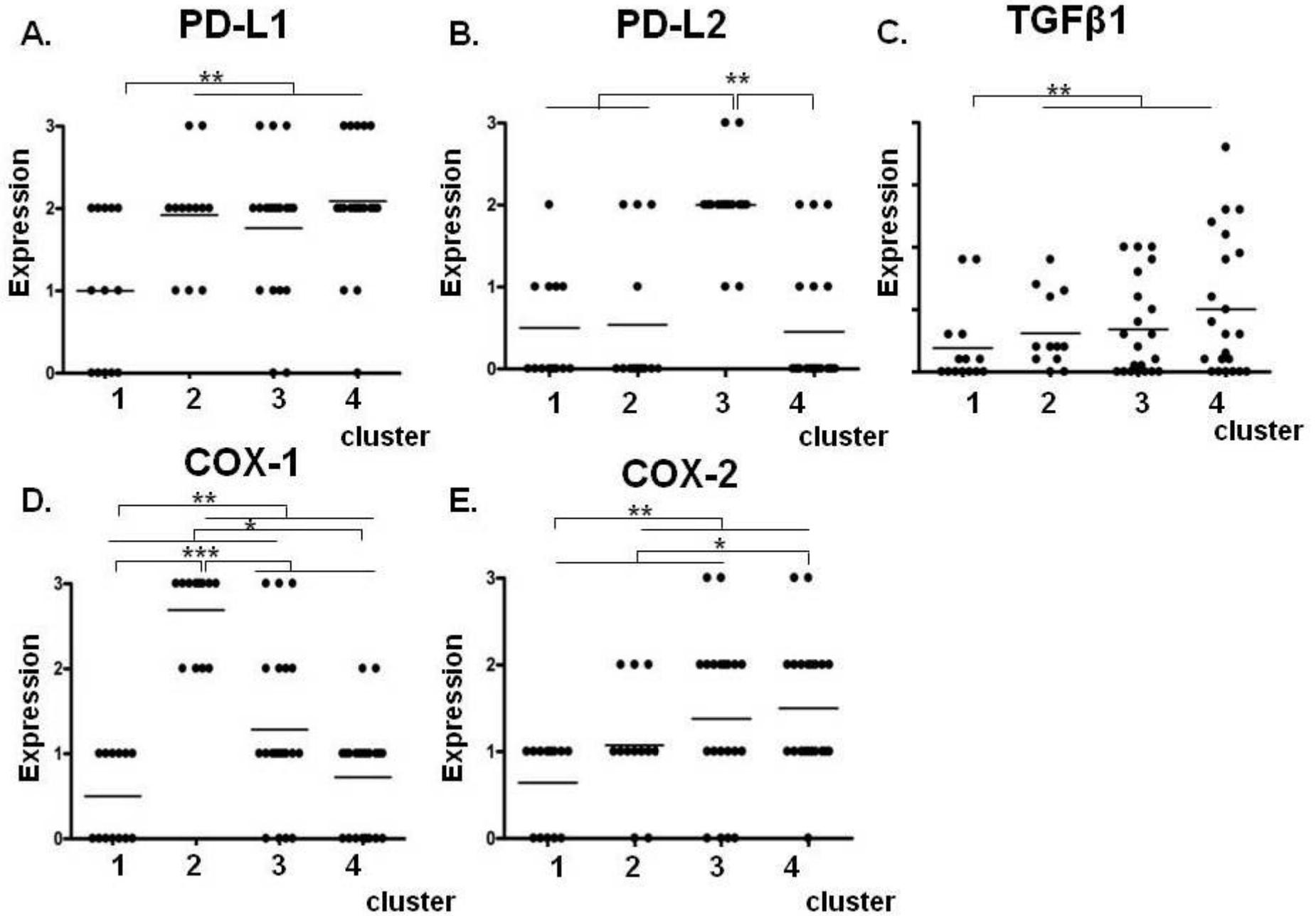
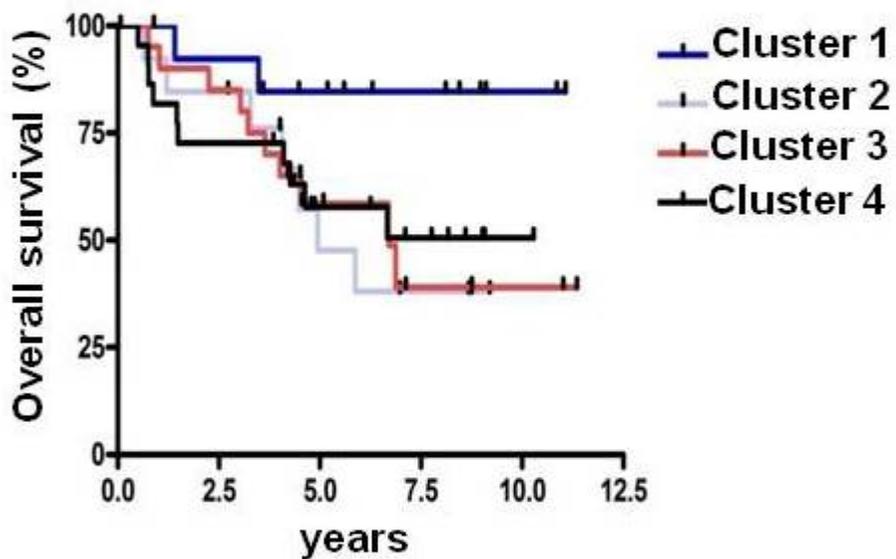
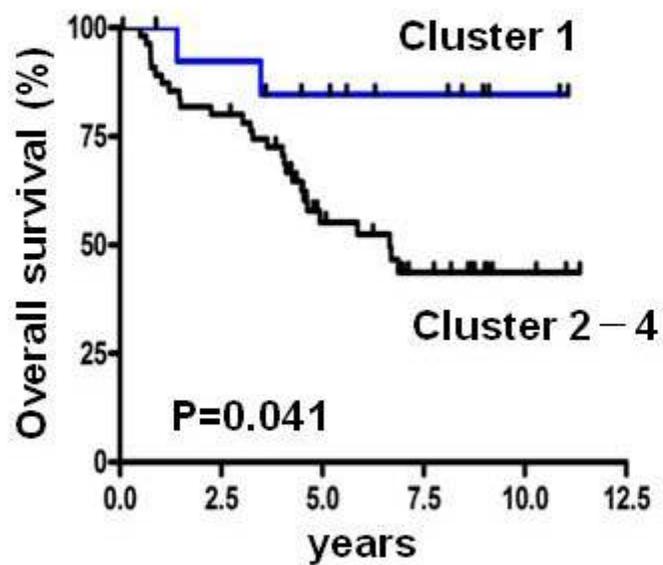


Figure 5

A.



B.



Supplementary Figure 1

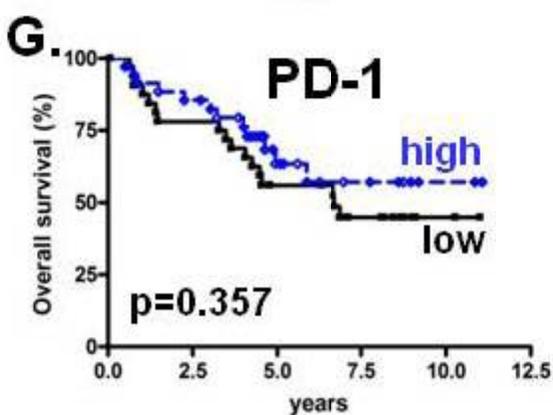
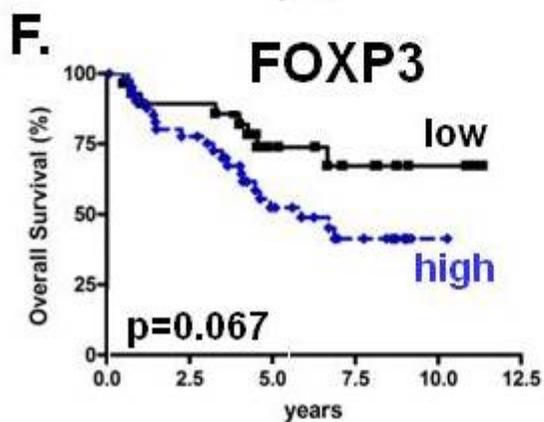
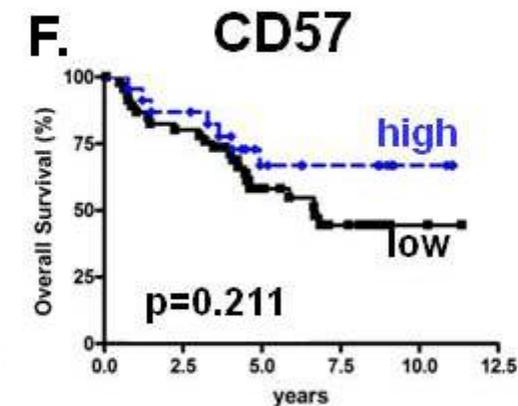
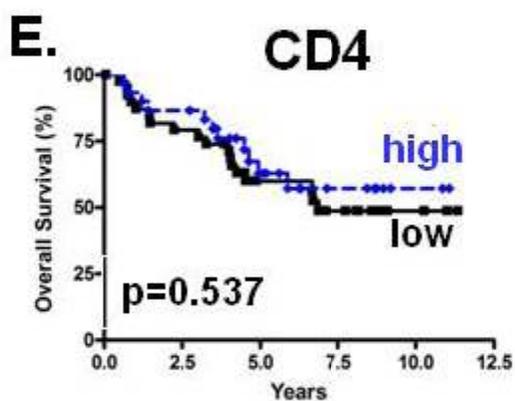
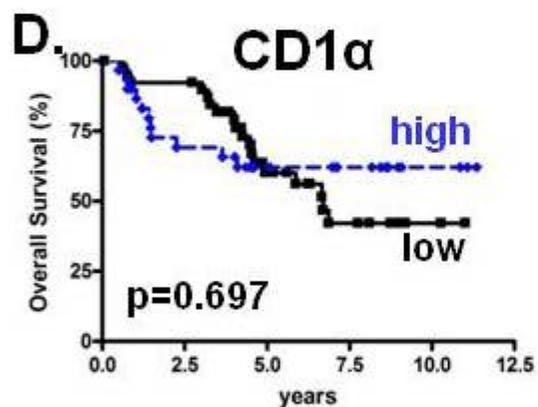
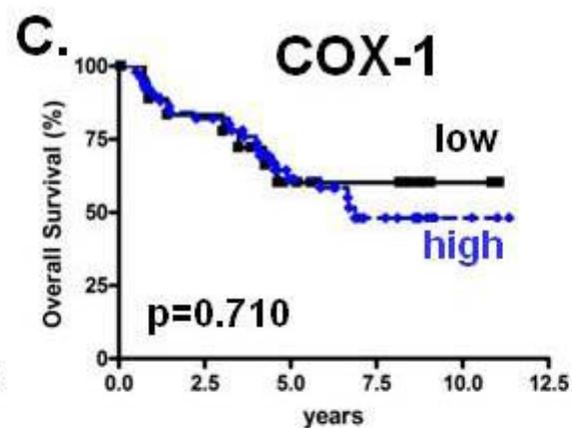
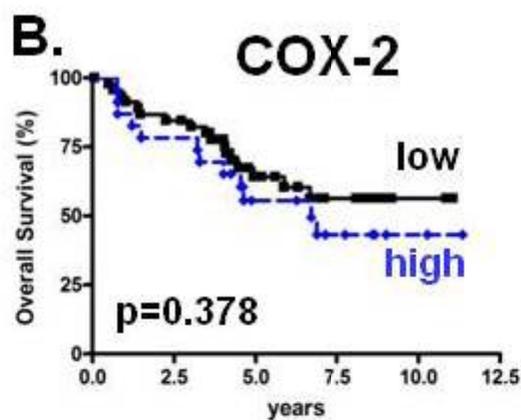
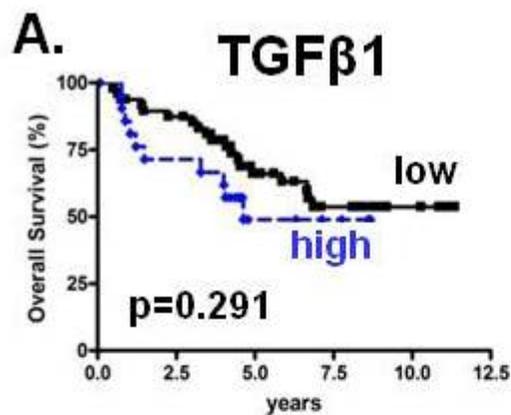


Figure legends

Figure 1

Immunohistochemical staining of human ovarian cancer tissue.

(A) Representative staining patterns of ovarian cancers with low expression or with high expression of immunosuppressive factors, such as TGF β 1, PD-L1, PD-L2, COX-1, and COX-2, are shown. (B) Representative staining patterns with low or high infiltrating immune cells, such as CD1 α +, CD4+, CD8+, CD57+, FOXP3+ or PD-1+ cells, in the tumor site are shown. Original magnification; (A and B) x200. White bar, 200 μ m.

Figure 2

Graphic representation of the immune status of 70 ovarian cancer tissues.

Patterns of immune status were classified into four clusters by hierarchical clustering based on six phenotype of immune cells, such as CD1 α +, CD4+, CD8+, CD57+, FOXP3+ or PD-1+ cells, in the tumor site and five immunosuppressive factors, such as TGF β 1, PD-L1, PD-L2, COX-1 and COX-2. Separated clusters are indicated by dendrograms. The color bar indicates that red is the high score (expression or infiltration), while green is the low score.

Figure 3

The patterns of immune cell infiltration into tumor sites in each cluster.

The dot plots represent the number of immune cells in the four clusters; (A) CD4; (B) CD8; (C) CD1 α ; (D) CD57; (E) FOXP3; and (F) PD-1 (*p<0.05, **p<0.01, ***p<0.0001).

Figure 4

The patterns of immunosuppressive factor expression in each cluster.

The dot plots represent the expression levels of immunosuppressive factors in the four clusters; (A) PD-L1; (B) PD-L2; (C) TGF β 1; (D) COX-1; and (E) COX-2 (*p<0.05, **p<0.01, ***p<0.0001).

Figure 5

Overall survival analyses of patients with ovarian cancer according to the four clusters.

(A) Kaplan–Meier curves according to Cluster 1 and the other clusters.

(B) Kaplan–Meier curves according to Cluster 1 and the combination of other clusters.

Supplementary Figure 1

Overall survival analyses of patients with ovarian cancer according to each immunological factor.

Kaplan–Meier curves according to the expression of the immunosuppressive factors (A) TGF β 1, (B) COX-1, and (C) COX-2 and to the number of (D) CD1 α + cells, (E) CD4+ cells, (F) CD57+ cells, (G) FOXP3+ cells and (H) PD-1+ cells infiltrating into tumor site

Supplementary Table 1

Primary antibodies used for immunohistochemistry.

Antigens	Clone	Host	Supplier	Antigen retrieval*	Reference
Immunosuppressive factor					
PD-L1	27A2	mouse	MBL	MW (CB)	30
PD-L2	Poly	goat	R&D systems	MW (EDTA)	30
COX-1	160110	mouse	Cayman Chemical	MW (CB)	44
COX-2	160112	mouse	Cayman Chemical	MW (CB)	44
TGF β 1	TB21	mouse	Abcam	PC (EDTA)	
Immune cell					
CD1 α	HI149	mouse	Becton Dickinson	MW (CB)	44
CD4	1F6	mouse	Nichirei	PC (EDTA)	
CD8	C8/144B	mouse	Nichirei	MW (CB)	30
CD57	HNK-1	mouse	Becton Dickinson	None	31
PD-1	NAT	mouse	Abcam	PC (CB)	
FOXP3	236A/E7	mouse	eBioscience	PC (CB)	

*Antigen retrieval: MW, Microwave; PC, Pressure cooker; CB, Citrate Buffer (pH 6.0);

EDTA, Tris-EDTA buffer (pH 9.0).

Supplementary Table 2**Correlations between the expression of TGF β 1 and clinicopathological characteristics****in ovarian cancer (*n* = 70)**

	n	TGF β 1		p
		Low	High	
Age				
<55	32	19	13	0.207
\geq 55	38	29	9	
Stage				0.883
I	27	21	6	
II	4	2	2	
III	26	19	7	
IV	13	8	5	
Histology				0.571
Serous	33	23	10	
Clear cell	22	17	5	
Endometrioid	11	6	5	
Mucinous	2	1	1	
Others	2	1	1	
Tumor status				0.849
pT1+pT2	31	22	9	
pT3	39	26	13	
LN metastasis				0.741
Positive	14	9	5	
Negative	56	39	17	
Distant metastasis				0.593
Positive	13	8	5	
Negative	57	40	17	
Residual tumor				0.773
Optimal	49	35	14	
Suboptimal	21	13	8	
Chemotherapy				0.732
No paclitaxel	39	27	12	
Paclitaxel	31	21	10	

Supplementary Table 3

The average numbers of tumor-infiltrating CD1 α +cells, CD4+cells, CD8+cells, CD57+cells, FOXP3+cells or PD-1+ immune cells was counted using the same 70 ovarian cancer specimens.

	Average number	Standard Deviation	range
CD1a	1.4	6.7	0-18.4
CD4	5.8	6.7	0-44.8
CD8	6.6	8.3	0-56.8
CD57	1.6	1.0	0-5.0
FOXP3	3.3	4.3	0-19
PD-1	1.2	2.5	0-12.6

Supplementary Table 4

Correlations between tumor-infiltrating CD1 α + cells or CD57+ cells and clinicopathological characteristics in ovarian cancer (n = 70)

	n	CD1 α + cells		p	CD4+ cells		p	CD57+ cells		p
		Low	High		Low	High		Low	High	
Age				0.072			0.533			0.448
<55	32	22	10		17	15		20	12	
\geq 55	38	18	20		23	15		27	11	
Stage				0.640			0.447			0.438
I	27	15	12		19	8		21	6	
II	4	2	2		2	2		1	3	
III	26	13	13		14	12		16	10	
IV	13	10	3		5	8		9	4	
Histology				0.700			0.057			0.973
Serous	33	20	13		13	20		20	13	
Clear cell	22	13	9		18	4		15	7	
Endometrioid	11	4	7		7	4		8	3	
Mucinous	2	0	2		2	0		2	0	
Others	2	1	1		0	2		2	0	
Tumor status				0.917			0.176			0.725
pT1+pT2	31	17	14		21	10		22	9	
pT3	39	23	16		19	20		25	14	
LN metastasis				0.131			0.365			0.065
Positive	14	5	9		6	8		6	8	
Negative	56	35	21		34	22		41	15	
Distant metastasis				0.198			0.231			0.881
Positive	13	10	3		5	8		9	4	
Negative	57	30	27		35	22		38	19	
Residual tumor				0.792			0.065			0.824
Optimal	49	27	22		32	17		33	16	
Suboptimal	21	13	8		8	13		14	7	
Chemotherapy				0.066			0.071			0.388
No paclitaxel	39	18	21		26	13		24	15	
Paclitaxel	31	22	9		14	17		23	8	

Supplementary Table 5

Correlations between tumor-infiltrating FOXP3+ cells or PD-1+ cells and clinicopathological characteristics in ovarian cancer (*n* = 70)

	n	FOXP3+ cells		p	PD-1+ cells		p
		Low	High		Low	High	
Age		28	42	0.040	48	22	0.976
<55	32	17	15		22	10	
≥55	38	11	27		26	12	
Stage				0.055			0.305
I	27	16	11		22	5	
II	4	3	1		2	2	
III	26	6	20		16	10	
IV	13	3	10		8	5	
Histology				0.0043			0.999
Serous	33	6	27		24	9	
Clear cell	22	16	6		16	6	
Endometrioid	11	5	6		8	3	
Mucinous	2	1	1		2	0	
Others	7	2	0		2	0	
Tumor status				0.0027			0.155
pT1+pT2	31	19	12		24	7	
pT3	39	9	30		24	15	
LN metastasis				0.502			0.949
Positive	14	4	10		10	4	
Negative	56	24	32		38	18	
Distant metastasis				0.286			0.784
Positive	13	3	10		8	5	
Negative	57	25	32		40	17	
Residual tumor				0.0069			0.955
Optimal	49	25	24		33	16	
Suboptimal	21	3	18		15	6	
Chemotherapy				0.238			0.515
No paclitaxel	39	18	21		28	11	
Paclitaxel	31	10	21		20	11	

Supplementary Table 6

Correlations among 11 immunological factors analyzed by Spearman's test.

Factor	PD-L1	PD-L2	COX-1	COX-2	TGFβ1	CD1a	CD4	CD8	CD57	PD-1	FOXP3
PD-L1	1	-	-	-	-	-	-	-	-	-	-
PD-L2	0.032	1	-	-	-	-	-	-	-	-	-
COX-1	0.793	-	1	-	-	-	-	-	-	-	-
COX-2	0.165	-0.013	0.261	1	-	-	-	-	-	-	-
COX-2	0.174	0.915	0.029	-	1	-	-	-	-	-	-
TGFβ1	0.192	0.097	-0.050	-0.098	1	-	-	-	-	-	-
TGFβ1	0.111	0.424	0.679	0.418	-	1	-	-	-	-	-
CD1α	-0.050	-0.066	-0.011	-0.023	-0.215	0.073	1	-	-	-	-
CD1α	0.678	0.590	0.930	0.848	0.114	0.123	-	1	-	-	-
CD4	0.072	0.151	0.038	-0.170	0.114	0.123	0.240	0.255	1	-	-
CD4	0.553	0.212	0.752	0.159	0.348	0.312	0.045	0.033	-	1	-
CD8	0.037	-0.140	0.038	-0.170	0.114	0.123	0.240	0.255	0.366	0.365	1
CD8	0.762	0.248	0.752	0.159	0.348	0.312	0.045	0.033	0.002	0.002	-
CD8	-0.258	-0.055	-0.270	-0.245	0.055	0.127	0.240	0.255	0.002	0.002	0.211
CD8	0.031	0.650	0.024	0.041	0.652	0.295	0.045	0.033	0.002	0.002	-
CD57	-0.064	-0.078	0.034	-0.167	-0.063	0.112	0.232	0.255	0.033	0.365	1
CD57	0.600	0.521	0.779	0.168	0.607	0.355	0.054	0.033	0.002	0.002	-
PD-1	0.102	-0.111	0.007	-0.052	0.045	0.081	0.302	0.366	0.365	0.002	1
PD-1	0.400	0.362	0.955	0.666	0.711	0.504	0.011	0.002	0.002	0.002	-
FOXP3	-0.016	-0.262	0.106	-0.109	0.072	0.058	0.410	0.062	0.073	0.211	1
FOXP3	0.898	0.028	0.385	0.370	0.556	0.636	<0.001	0.613	0.548	0.079	-

Coefficient R-values and p-values are listed in the upper and lower sections of the columns

The data in the brown columns shows significant correlation, respectively.