

# Relationship between expression of PD-L1 and PD-L2 on esophageal squamous cell carcinoma and the antitumor effects of CD8<sup>+</sup> T cells

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**Abstract.** The programmed death-1 (PD-1)/programmed death-ligands (PD-Ls) signal pathway has been implicated as a potential immune escape mechanism in several human cancers. However, the studies of PD-1/PD-Ls pathway in esophageal squamous cell carcinoma (ESCC) are not yet sufficient. The current study investigated the expression of PD-L1, PD-L2 and PD-1 in ESCC tissues. The correlations between the expression of these proteins and clinical histopathological parameters were analyzed. Then the stable transfected Ec109 cell lines overexpressing PD-L1/PD-L2 were established by plasmid transfection successfully. Ec109 and CD8<sup>+</sup> T cells were co-cultured to analyze the effects of PD-1/PD-Ls signal pathway on the function of CD8<sup>+</sup> T cells including proliferation, apoptosis and interferon- $\gamma$  production. We found that PD-L1-positive patients had significantly poorer prognosis than the negative patients, while their prognosis was not related to PD-L2 expression. The count of PD-1<sup>+</sup> TILs (tumor-infiltrating lymphocytes) was negatively correlated with both PD-L1 and PD-L2 expression. In functional studies, we found that PD-1/PD-Ls signal pathway was able to downregulate the function of CD8<sup>+</sup> T lymphocyte and its function could be restored by blocking the signal pathway. This indicates that PD-1/PD-Ls may prevent effective antitumor immunity, which provides important evidence to delineate the cellular immune deficiency mechanism in ESCC. Therefore, PD-1/PD-Ls are predicted to become novel targets for ESCC immunotherapy.

## Introduction

Esophageal cancer is the sixth leading cause of cancer-related death worldwide and one of the most difficult malignant tumors to treat and cure (1). Squamous cell carcinoma is responsible for 95% of all esophageal cancers worldwide (2,3). Although advances have been made in the therapy with multimodal treatment strategies, including neoadjuvant chemotherapy or radiochemotherapy, esophageal cancer still remains one of the most deadly human malignancies. In addition, neoadjuvant chemotherapy and radiochemotherapy also bring many complications (4-6). Therefore, it is urgently necessary to study the pathogenesis of esophageal cancer and explore new treatment approaches.

The ability to evade immune surveillance is a well accepted feature of malignant tumors. Recently, manipulation of costimulatory signaling has been implicated as a potential immune escape mechanism in human cancer (7-9). Costimulatory signaling plays a key role in the initiation and termination of immune responses by regulation of T-cell activation (10).

Programmed death-1 (PD-1) is a costimulatory molecule that provides an inhibitory signal in T-cell activation. PD-1 belongs to the CD28 family (11,12). PD-1 is expressed on T cells, B cells and myeloid cells. Programmed death-ligands (PD-Ls) are ligands for PD-1, including PD-L1 and PD-L2, which are cell-surface glycoprotein belonging to the B7 family (13-16). Previous studies have shown that PD-1/PD-Ls interaction inhibits the function of T cell (14,16).

Recently, aberrant PD-L1 and PD-L2 expression by cancer cells has been reported in many human malignancies (17-19). A series of clinical trials concerning the systemic administration of therapeutic antibodies for blocking PD-1 or PD-L1 have shown a promising clinical effect in various tumors (20,21). However, further studies on the PD-1/PD-Ls pathway in esophageal squamous cell carcinoma (ESCC) are required. There is no previous study on the expression of PD-L1, PD-L2 and PD-1 simultaneously in ESCC tissues. The association between their expression and ESCC

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prognosis is still controversial, and the mechanism of the PD-1/PD-Ls pathway in ESCC is not clear. Our research is likely to provide important evidence to delineate the cellular immune deficiency mechanism in ESCC and a potential strategy for immunotherapy against ESCC.

## Materials and methods

**Tissue samples.** We examined 106 patients with esophageal cancer who underwent surgery at Department of Surgery, the Affiliated Cancer Hospital of Zhengzhou University, between January 2008 and December 2009. The patients had undergone primary surgical resection with curative intent without preoperative chemotherapy or radiotherapy. Seventy-six patients were male and 30 were female. The median age of the patients was 59 years, with a range of 38 to 80 years. Tumor stage was defined according to the pathological tumor node metastasis (pTNM system) classification proposed by the International Union against Cancer (UICC/AJCC, 7th edition) [stage I (n=17), stage II (n=61), stage III (n=23) and stage IV (n=5)]. The median follow-up time for all patients was 55 months. Postoperative pathohistologic analysis indicated that all tumors in this study were ESCC. We also obtained 30 cases of paracancerous tissue (>5 cm away from the cancer margin) as control.

**Immunohistochemistry (IHC).** The IHC streptavidin-peroxidase staining method was performed on 5  $\mu$ m-thick formalin-fixed and paraffin-embedded tissue sections. The sections were deparaffinized in xylene, rehydrated in gradient ethanol solutions. The antigen retrieval was conducted in 0.01 mol/l citrate (pH 6.0). Slides were incubated overnight with rabbit anti-human PD-L1 polyclonal antibody (1:40; ab58810; Abcam, Cambridge, MA, USA), rabbit anti-human PD-L2 polyclonal antibody (1:60; AB21968a; Sangon, Shanghai, China), mouse anti-human PD-1 monoclonal antibody (1:50; ab52587; Abcam) and phosphate-buffered saline as blank control. Incubation of the biotinylated secondary antibody with horseradish peroxidase and 3,3'-diaminobenzidine chromogen (all from ZSGB-Bio, Beijing, China) was performed sequentially. Next, the slides were counterstained with hematoxylin and then covered with neutral balsam.

**Evaluation of IHC.** IHC results for all examined costimulatory molecules were evaluated by scanning each slide under low-power magnification (x40) to identify regions containing positive immunoreactivity. Immunostaining was further evaluated at high-power magnification (x200). Tumor samples were examined by two observers in a blinded manner. Expression of PD-L1, PD-L2 and PD-1 was evaluated as staining on the cell membrane and cytoplasm. PD-L1 and PD-L2 staining-positive cases were determined by staining intensity and the positive cell percentage according to the methods previously published (22,23). The staining intensity grading: 0 point (no staining), 1 point (faint yellow), 2 points (clay-bank), 3 points (sepia). The percentage of the tumor cell population staining was scored as follows: 1 point (<10%), 2 points (10-50%), 3 points (>50%). The positive cases were determined according to the two items multiplied by products:

positive (>3 points), negative ( $\leq$ 3 points). The mean count of PD-1<sup>+</sup> TILs (tumor-infiltrating lymphocytes) of 106 cases was used as threshold and the cases were divided into high PD-1<sup>+</sup> TILs group and low PD-1<sup>+</sup> TIL group according to the threshold.

**Cells and cultures.** The Ec109 cells were cultured in RPMI-1640 (Biological Industries, Kibbutz Beit. Haemek, Israel) supplemented with 10% fetal bovine serum (FBS), (Biological Industries), 2 mmol/l glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C in 5% CO<sub>2</sub>. The lymphocytes were provided by the Biological Treatment Center of the Second Affiliated Hospital, Zhengzhou University. Cells were plated into culture flasks with the medium at the concentration of 2x10<sup>6</sup>/ml and placed at 37°C in 5% CO<sub>2</sub>. The lymphocytes were sampled and counted every 2-3 days maintained at 1-4x10<sup>6</sup>/ml by supplementing culture medium or subculture. The cells were harvested between day 10 and day 17. Lymphocyte culture medium was similar with cancer cell medium above, except added with 1,000 U/ml interleukin (IL)-2 (KEXIN, Beijing, China).

**Magnetic activated cell sorting (MACS).** The CD8<sup>+</sup> T cells were generated using miniMACS system. The lymphocytes were labeled with anti-CD8 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The isolation was carried out according to the manufacturer's instructions. The purity of CD8<sup>+</sup> T cells was measured by flow cytometry (FCM). Then CD8<sup>+</sup> T cells were cultured in lymphocyte culture medium.

**Transfection.** The PD-L1 and PD-L2 cDNA were digested with *KpnI/XhoI* and constructed into pcDNA3.1 expression vector by Sangon Biotech (Shanghai, China). Ec109 cells were cultured in a 6-well plate (1x10<sup>6</sup>/well). When the cells were 70% confluent, they were used for transfection with pcDNA3.1/PD-L1, pcDNA3.1/PD-L2 or pcDNA3.1, respectively. The complex of DNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was prepared according to the manufacturer's instructions and was added to the culture wells. The culture plate was shaken gently so that the complex of DNA-Lipofectamine 2000 distributed well. Ec109 cells were cultured for another 4 h, and then culture medium was replaced by fresh medium. The transfected Ec109 cells (Ec109/PD-L1, Ec109/PD-L2 and Ec109/mock) were cultured for 48 h. Then G418 (400  $\mu$ g/ml) was used to select the stable transfection clones.

**FCM.** FCM was performed by standard method. The data were acquired by using a FACSCanto cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and analyzed by CellQuest Pro software. Monoclonal antibody used to measure the purity of CD8<sup>+</sup> T cells before and after MACS included mouse anti-human-CD8-PE, -CD3-PerCP (both from Miltenyi Biotec). The following monoclonal antibodies were used to measure PD-1 expression of CD8<sup>+</sup> T cells and PD-L1, PD-L2 expression of Ec109 cells before and after transfection: mouse anti-human -PD-L1-APC (Biolegend, San Diego, CA, USA), -PD-L2-Fitc and -PD-1-PE (both from Miltenyi Biotec). IgG isotype controls were used in FCM.

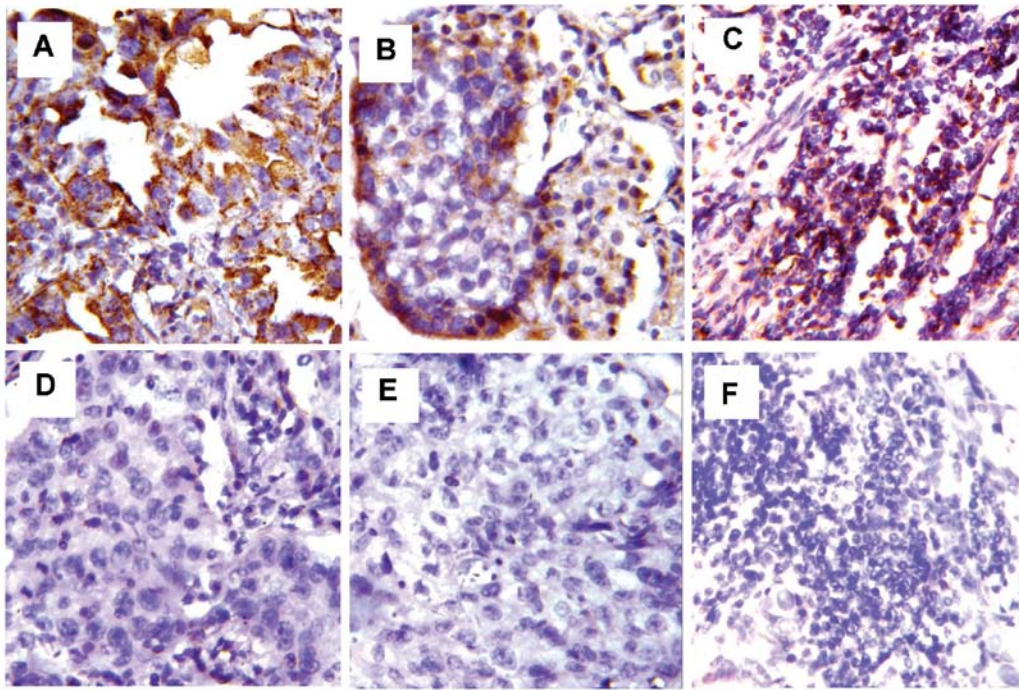


Figure 1. Immunohistochemical staining for human ESCC tissues. (A) PD-L1 positive, (B) PD-L2 positive, (C) PD-1 positive, (D) PD-L1 negative, (E) PD-L2 negative and (F) PD-1 negative. Original magnification, x200.

**Real-time quantitative PCR (qRT-PCR).** Ec109 cells before and after transfection in logarithmic growth phase were collected. The total RNA was extracted using the RNA extraction kit spin column method (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. Finally, 50  $\mu$ l RNA was collected and RNA purity (D260/D280) was 1.8-2.0, tested using an ultraviolet spectrophotometer (SMA4000; Merinton, Beijing, China). Subsequently, reverse transcription was conducted according to the reverse transcription kit recommendations (Thermo Fisher Scientific, Inc., Waltham, MA, USA). In total, 20  $\mu$ l cDNA was obtained and stored at  $-20^{\circ}\text{C}$  until use. The following primers synthesized by Sangon Biotech Co., Ltd., Shanghai, China were used for cDNA amplification system: PD-L1: forward, 5'-GCATGGAGAGG AAGACCTGA-3' and reverse, 5'-TTGTAGTCGGCACCA CCATA-3'; PD-L2: forward, 5'-CAGCAATGTGACCCTGG AAT-3' and reverse 5'-GGACTTGAGGTATGTGGAACG-3';  $\beta$ -actin as control. qRT-PCR was performed using the SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. Samples were denatured for 15 min at  $95^{\circ}\text{C}$ , followed by 40 cycles including denaturation at  $95^{\circ}\text{C}$  for 15 sec, annealing at  $52^{\circ}\text{C}$  for 30 sec and extension at  $72^{\circ}\text{C}$  for 34 sec, then by continuous fluorescence measurement during heating from  $60^{\circ}\text{C}$  to  $90^{\circ}\text{C}$  ( $0.1^{\circ}\text{C}/\text{s}$ ). The data was normalized to the  $\beta$ -actin expression of Ec109 cells and analyzed using the ABI 7500 Fast system (Applied Biosystems, Foster City, CA, USA).  $\Delta\text{CT} = \text{CT}(\text{target gene}) - \text{CT}(\beta\text{-actin})$ .

**Co-culture.** To delineate the role of PD-Ls in tumor-T-cell interactions in ESCC, co-culture experiments were carried out by simulating the tumor microenvironment. The following monoclonal antibodies were used to block PD-L1, PD-L2 and PD-1: mouse anti-human-PD-L1, mouse anti-human-PD-L2

(Biolegend), rabbit anti-human-PD-1 (Miltenyi Biotec). The experiments were divided into 6 groups for research on the PD-L1 signal: group (A)  $\text{CD8}^{+}$  T cells + Ec109/PD-L1 cells + IgG antibody; group (B)  $\text{CD8}^{+}$  T cells + Ec109/PD-L1 cells + PD-L1 antibody; group (C)  $\text{CD8}^{+}$  T cells + Ec109/PD-L1 cells + PD-1 antibody; group (D)  $\text{CD8}^{+}$  T cells + Ec109/mock cells + IgG antibody; group (E)  $\text{CD8}^{+}$  T cells + Ec109/mock cells + PD-L1 antibody; group (F)  $\text{CD8}^{+}$  T cells + Ec109/mock cells + PD-1 antibody. Another 6 groups for research on the PD-L2 signal: group (A)  $\text{CD8}^{+}$  T cells + Ec109/PD-L2 cells + IgG antibody; group (B)  $\text{CD8}^{+}$  T cells + Ec109/PD-L2 cells + PD-L2 antibody; group (C)  $\text{CD8}^{+}$  T cells + Ec109/PD-L2 cells + PD-1 antibody; group (D)  $\text{CD8}^{+}$  T cells + Ec109/mock cells + IgG antibody; group (E)  $\text{CD8}^{+}$  T cells + Ec109/mock cells + PD-L2 antibody; group (F)  $\text{CD8}^{+}$  T cells + Ec109/mock cells + PD-1 antibody. Each group was repeated at least five times.

**CCK-8 cell proliferation assay.** According to the above groups,  $\text{CD8}^{+}$  T cells ( $5 \times 10^4/\text{well}$ ) were co-cultured in 96-well plates with mitomycin ( $15 \mu\text{g}/\text{ml}$ ) treated Ec109 cells ( $1 \times 10^4/\text{well}$ ) at a ratio of 5:1. Cell co-cultures were maintained in complete media with recombinant human IL-2 ( $1,000 \text{ U}/\text{ml}$ ) and the antibodies ( $10 \mu\text{g}/\text{ml}$ ). The proliferation of  $\text{CD8}^{+}$  T cells was estimated by CCK-8 (Beyotime, Jiangsu, China). After co-cultured for 48 h, 20  $\mu$ l CCK-8 was added to each well. The absorbance of each well was measured with a microplate reader at 450 nm.

**Apoptosis.** According to the above groups,  $\text{CD8}^{+}$  T cells ( $5 \times 10^4/\text{well}$ ) were co-cultured in 96-well plates with Ec109 cells ( $1 \times 10^4/\text{well}$ ) at a ratio of 5:1. Cell co-cultures were maintained in complete media with recombinant human

Table I. The correlation between PD-L1, PD-L2 expression and clinicopathological characteristics of ESCC patients.

Characteristics	Total (n)	PD-L1		P-value	PD-L2		P-value
		Positive	Negative		Positive	Negative	
Gender							
Male	76	42	34	0.624	44	32	0.908
Female	30	15	15		17	13	
Age (years)							
≥60	58	33	25	0.478	35	23	0.834
<60	48	24	24		28	20	
Tumor location							
Proximal third	6	4	2	0.460	4	2	0.414
Middle third	83	42	41		45	38	
Distal third	17	11	6		12	5	
Grading							
G1	22	10	12	0.513	11	11	0.152
G2	47	28	19		24	23	
G3	37	19	18		26	11	
Pathologic status							
Stage I	17	10	7	0.744	9	8	0.364
Stage II	61	30	31		32	29	
Stage III	23	14	9		16	7	
Stage IV	5	3	2		4	1	

IL-2 (1,000 U/ml) and the antibodies (10 µg/ml). The apoptosis of CD8<sup>+</sup> T cells was estimated by Annexin V-FITC/PI (Miltenyi Biotec) following its manufacturer's instructions after co-cultured for 48 h.

**Analysis of cytokine secretion.** According to the above groups, CD8<sup>+</sup> T cells (5×10<sup>4</sup>/well) were co-cultured in 96-well plates with Ec109 cells (1×10<sup>4</sup>/well) at a ratio of 5:1. Cell co-cultures were maintained in complete media with recombinant human IL-2 (1,000 U/ml) and the antibodies (10 µg/ml). The interferon (IFN)-γ was estimated by human IFN-γ pre-coating ELISA kit (Dakewe, Beijing, China) following its manufacturer's instructions after co-cultured for 48 h.

**Statistical analysis.** SPSS 17.0 software was used for statistical analysis. The significance of the difference between PD-Ls expression and several clinical and pathologic variables was assessed by the Chi-square test. The Kaplan-Meier method was used to estimate the probability of survival. Quantitative values were expressed as mean ± standard deviation or median and range. The t-test and one-way analysis of variance were used to analyze the differences between groups. All statistical tests were conducted as two-sided, and P<0.05 was considered to indicate a statistically significant difference.

## Results

**The expression of PD-L1, PD-L2 in ESCC and the correlation with clinicopathological parameters.** Expression of PD-L1, PD-L2 and PD-1 was evaluated as staining on the

cell membrane and cytoplasm (Fig. 1). The expression of PD-L1 and PD-L2 was detected in tumor cells. The positive expression of PD-L1 and PD-L2 in ESCC tissues was 46.2 and 42.5%, respectively. But no immunoreactivity was found in surrounding normal esophageal tissues. We examined the relationship between PD-L1 and PD-L2 expression and various clinical pathological parameters. However, there was no significant relationship between either PD-L1 or PD-L2 expression with gender, age, tumor location, tumor grade or pathologic stage (Table I).

**The correlation between PD-1<sup>+</sup> TILs with the expression of PD-L1 and PD-L2.** PD-1 was predominantly expressed in the tumor stromal lymphocytes. The count of PD-1<sup>+</sup> TILs in the 106 ESCC cases (the range of the count of PD-1<sup>+</sup> TILs in the 106 ESCC cases were 0-16; mean, 6.1) was significantly increased in contrast to that in the normal tissues (0-7; mean, 2.59) (P=0.008). The mean value of 6.1 was used as the threshold and, accordingly, the 106 tumor cases were divided into PD-1<sup>+</sup> TIL high-density group (60 cases) and low-density group (46 cases). The expression of PD-L1 and PD-L2 was found to inversely correlate with PD-1<sup>+</sup> TILs (P<0.05) (Table II).

**The correlation between PD-L1, PD-L2 expression and prognosis.** As shown in Fig. 2, the overall survival of PD-L1 positive patients was significantly worse than that of negative patients (P=0.027). However, the overall survival of patients positive for PD-L2 tended to be worse than that of negative patients but the difference was not statistically

Table II. The correlation between PD-1<sup>+</sup> TILs with the expression of PD-L1 and PD-L2.

PD-1 <sup>+</sup> TILs	Total (n)	PD-L1		P-value	PD-L2		P-value
		Positive	Negative		Negative	Positive	
Low-density	46	18	28	0.011	20	26	0.017
High-density	60	39	21		41	19	
Total (n)	106	57	49		61	45	

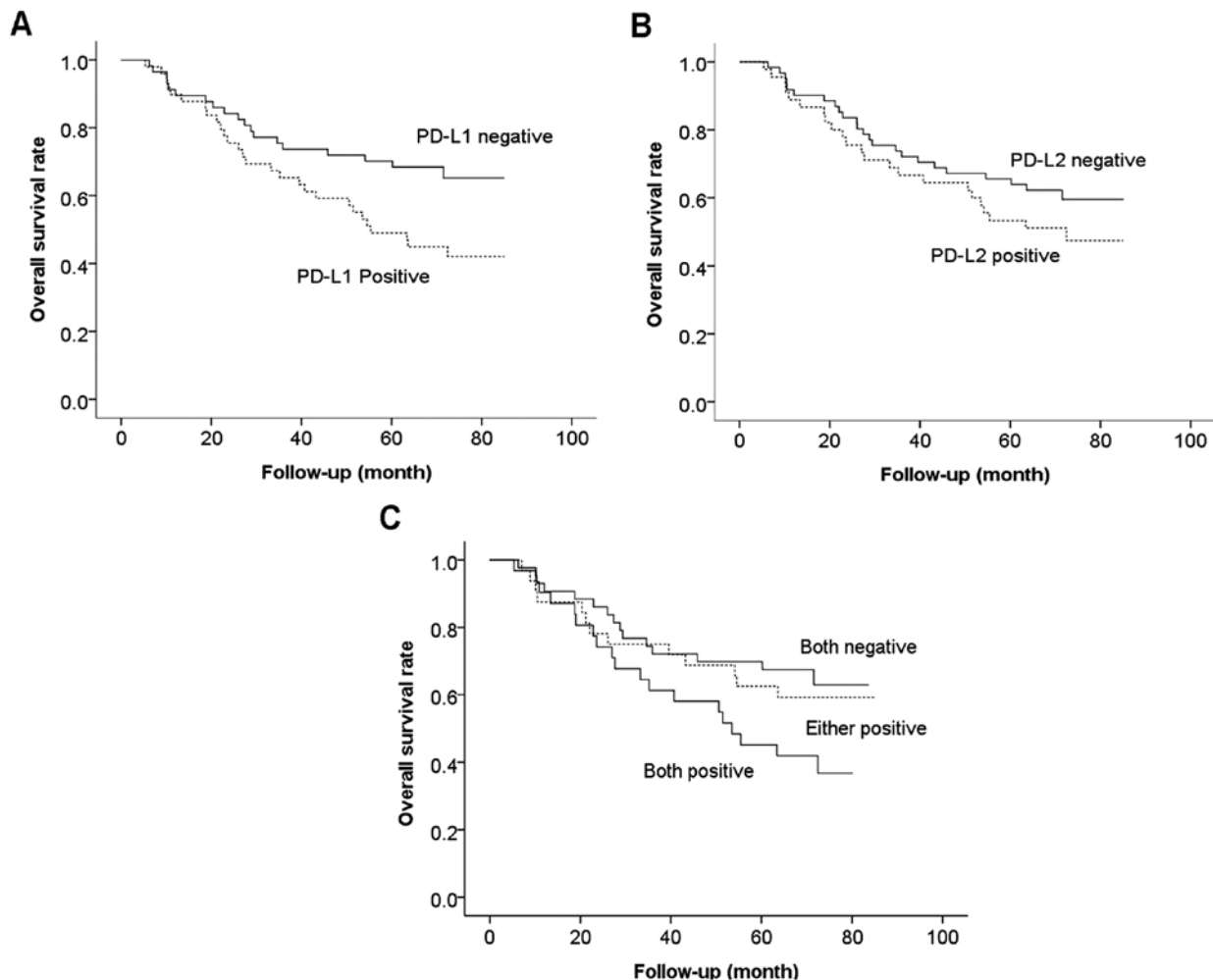


Figure 2. The correlation between PD-L1 and PD-L2 expression and prognosis. (A) PD-L1 positive patients had a poorer prognosis than the negative patients ( $P=0.027$ ). (B) The overall survival of patients positive for PD-L2 had a tendency to be worse than that of negative patients but the difference was not statistically significant ( $P=0.243$ ). (C) Overall survival of patients with tumors positive for both PD-L1 and PD-L2 was significantly worse than that with tumors negative for both ( $P<0.001$ ).

significant ( $P=0.243$ ). Furthermore, 31 patients had tumors positive for both PD-L1 and PD-L2, 32 patients had tumors positive for either PD-L1 or PD-L2 and 43 patients had tumors negative for both PD-L1 and PD-L2. Overall survival of patients with tumors positive for both PD-L1 and PD-L2 was significantly worse than that with tumors negative for both ( $P<0.001$ ). In addition, overall survival of patients positive for either PD-L1 or PD-L2 had a tendency to be better than that with both positive and worse than that with both negative, although the differences were not statistically significant ( $P=0.094$ ).

*The expression of PD-L1, PD-L2 and PD-1 on Ec109 and CD8<sup>+</sup> T cells.* As shown in Fig. 3, the expression of PD-L1 on Ec109 cell line was not high ( $24.5\pm4.2\%$ ), and Ec109 cell line did not express PD-L2 and PD-1. We subsequently examined the expression of PD-L1 and PD-L2 on Ec109 cells before and after transfection. Ec109/PD-L1 cells and Ec109/PD-L2 cells were selected with high levels of PD-L1 (97.3) and PD-L2 (93.6%) for further study. Transcriptions of PD-L1 gene and PD-L2 gene were identified by qRT-PCR indicated that the stable transfected Ec109 cell line was successfully established (Fig. 4). The purity of CD8<sup>+</sup> T cells before and

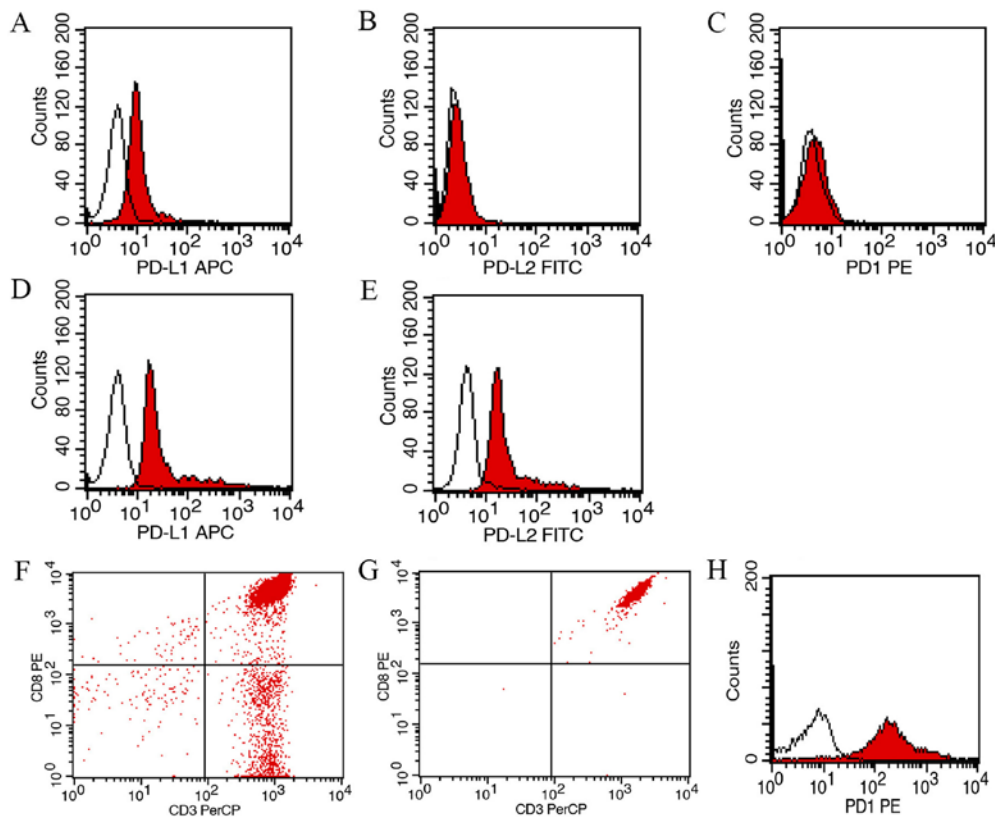


Figure 3. The expression rate of PD-L1 on Ec109 cell line was  $24.5 \pm 4.2\%$  (A). Ec109 cell line did not express PD-L2 and PD-1 (B and C). After transfection, Ec109/PD-L1 cells and Ec109/PD-L2 cells were selected with high levels of PD-L1 (97.3%, D) and PD-L2 (93.6%, E). The purity of CD8<sup>+</sup> T cells before and after separation by MACS was 75.2% (F) and 99.7%, respectively (G). In addition, the expression of PD-1 on CD8<sup>+</sup> T cells separated by MACS was 91.2% (H).

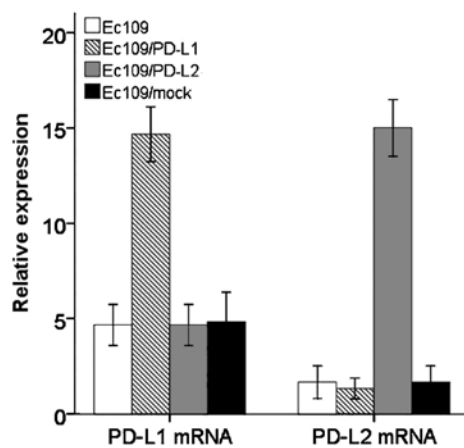


Figure 4. The levels of PD-L1 and PD-L2 mRNA in Ec109 cells before and after transfection were detected by qRT-PCR. The expression of both PD-L1 in Ec109/PD-L1 cells and PD-L2 in Ec109/PD-L2 cells were significantly elevated compared with normal Ec109 cells and Ec109/mock cells ( $P < 0.001$ ).

after separation by MACS was 75.2 and 99.7%, respectively. In addition, the expression of PD-1 in CD8<sup>+</sup> T cells separated by MACS was 91.2% (Fig. 3).

**Functional significance of PD-L1 and PD-L2 for purified allogeneic CD8<sup>+</sup> T cells.** After co-cultured, the Ec109/PD-L1 cells caused a decrease in CD8<sup>+</sup> T cell proliferation, IFN- $\gamma$  production and increased apoptosis compared with the control group. However, blockade of PD-L1 or PD-1 with antibodies

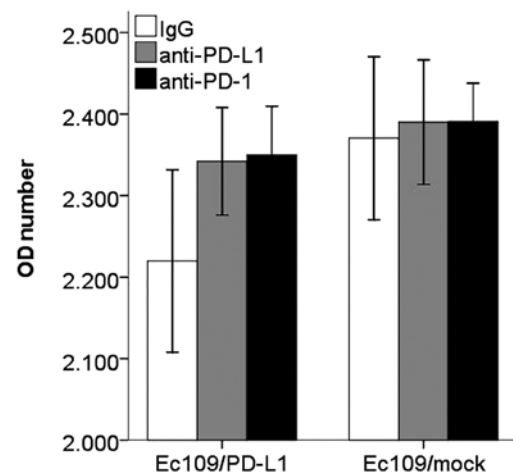


Figure 5. The effect of PD-L1 on the proliferation of CD8<sup>+</sup> T cells. The Ec109/PD-L1 IgG group caused decreased proliferation of CD8<sup>+</sup> T cells, compared with the control Ec109/mock IgG group ( $P = 0.001$ ). However, blockade of PD-L1 or PD-1 with antibodies resulted in enhanced proliferation of CD8<sup>+</sup> T cells ( $P = 0.006$  and  $P = 0.004$ , respectively).

resulted in enhanced CD8<sup>+</sup> T cells proliferation, IFN- $\gamma$  production and decreased apoptosis (Figs. 5-8).

There was significant inhibitory effect of PD-L2 on the CD8<sup>+</sup> T cell IFN- $\gamma$  secretion, and this inhibitory effect could be restored with PD-L2 or PD-1 blocking antibody (Fig. 9). However, CD8<sup>+</sup> T cells proliferation and apoptosis in PD-L2 signal test were not altered significantly (Figs. 10 and 11).



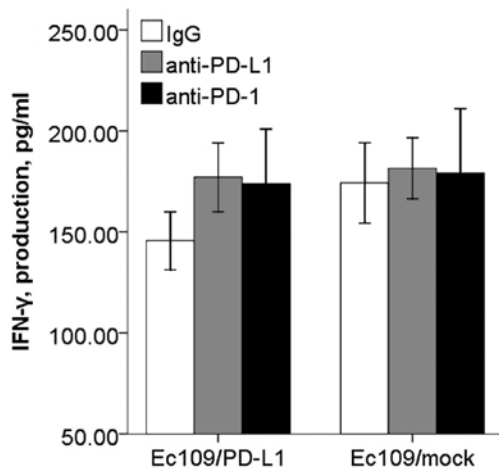


Figure 6. The effect of PD-L2 on the proliferation of CD8<sup>+</sup> T cells. There was no significant difference between each group (Ec109/PD-L2 + IgG group vs. Ec109/mock + IgG group,  $P=0.951$ ; Ec109/PD-L2 + IgG group vs. Ec109/PD-L2 + PD-L2 antibody group,  $P=0.916$ ; Ec109/PD-L2 + IgG group vs. Ec109/PD-L2 + PD-1 antibody group,  $P=0.892$ ).

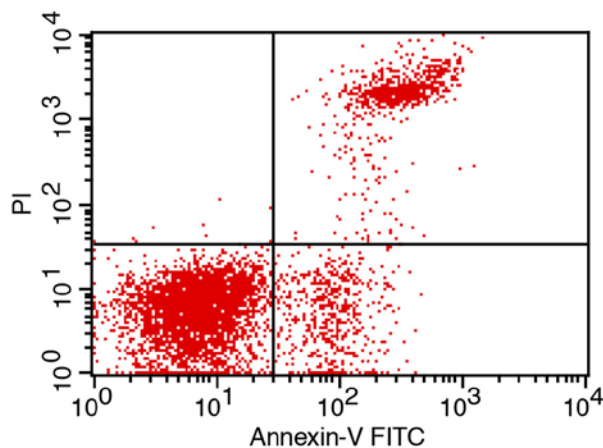


Figure 7. Flow cytometry of CD8<sup>+</sup> T cell apoptosis. According to the size and different structures of cells, CD8<sup>+</sup> T cells and Ec109 cells can be divided into two groups by FCM. The apoptosis percentage of CD8<sup>+</sup> T cells was estimated by Annexin V-FITC.

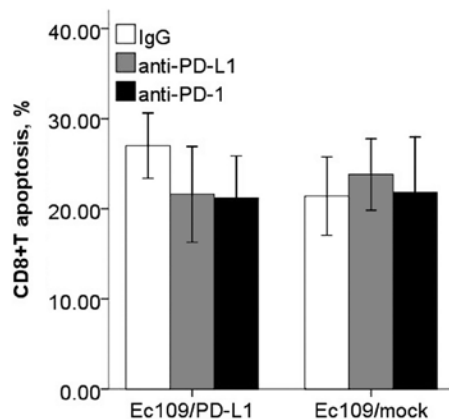


Figure 8. The effect of PD-L1 on the apoptosis of CD8<sup>+</sup> T cells. The Ec109/PD-L1 IgG group caused increased apoptosis of CD8<sup>+</sup> T cells, compared with the control Ec109/mock IgG group ( $P=0.030$ ). However, blockade of PD-L1 or PD-1 with antibodies resulted in reduced apoptosis of CD8<sup>+</sup> T cells ( $P=0.036$  and  $P=0.025$ , respectively).

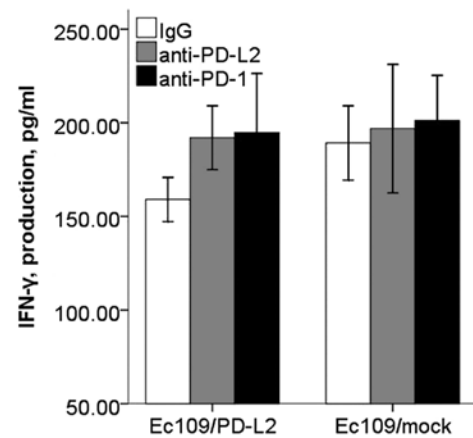


Figure 9. The effect of PD-L2 on apoptosis of CD8<sup>+</sup> T cells. There was no significant difference between each group (Ec109/PD-L2 + IgG group vs. Ec109/mock + IgG group,  $P=0.605$ ; Ec109/PD-L2 + IgG group vs. Ec109/PD-L2 + PD-L2 antibody group,  $P=0.756$ ; Ec109/PD-L2 + IgG group vs. Ec109/PD-L2 + PD-1 antibody group,  $P=0.354$ ).

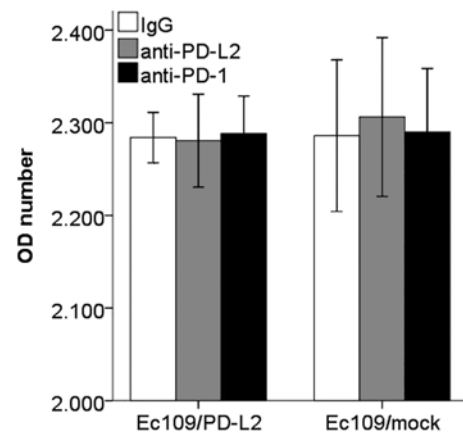


Figure 10. The effect of PD-L1 on the IFN- $\gamma$  secretion of CD8<sup>+</sup> T cells. The Ec109/PD-L1 IgG group caused decreased IFN- $\gamma$  secretion of CD8<sup>+</sup> T cells, compared with the control Ec109/mock IgG group ( $P=0.017$ ). However, blockade of PD-L1 or PD-1 with antibodies resulted in enhanced IFN- $\gamma$  secretion of CD8<sup>+</sup> T cells ( $P=0.010$  and  $P=0.018$ , respectively).

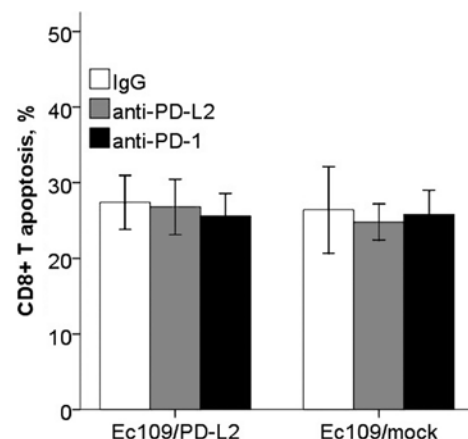


Figure 11. The effect of PD-L2 on the IFN- $\gamma$  secretion of CD8<sup>+</sup> T cells. The Ec109/PD-L2 IgG group caused decreased IFN- $\gamma$  secretion of CD8<sup>+</sup> T cells, compared with the control Ec109/mock IgG group ( $P=0.023$ ). However, blockade of PD-L2 or PD-1 with antibodies resulted in enhanced IFN- $\gamma$  secretion of CD8<sup>+</sup> T cells ( $P=0.014$  and  $P=0.008$ , respectively).

## Discussion

Despite the presence of large numbers of TILs in cancer tissues, the immune system often fails to prevent tumor development and progression (24-27). Recent studies have suggested a novel mechanism that tumor may evade host immune response through the expression of PD-L1 and PD-L2. PD-L1 and PD-L2 have been thought to be involved in the negative regulation of cellular and humoral immune responses by engaging PD-1 receptor on activated T and B cells (28,29).

However, there is no previous study on the expression of PD-L1, PD-L2 and PD-1 simultaneously in ESCC tissues. Our results show PD-L1, PD-L2 and PD-1 were aberrantly over expressed in ESCC tissues. PD-L1 and PD-L2 proteins both located on cytoplasm and cell membrane of tumor cells. We found that 46.2% of ESCC tissues evaluated in this study were positive for PD-L1 and 42.5% ESCC tissues were positive for PD-L2. Furthermore, PD-L1-positive patients had significantly poorer prognosis than the negative patients. Though PD-L2 expression was correlated with an impaired survival, this difference was not statistically significant. However, there was no significant relationship between either PD-L1 or PD-L2 expression with the age, gender, lesion location, differentiated degree and pathologic stage.

PD-L1 expression has been detected in most human cancers, such as gastric, pancreatic, kidney, breast, ovarian and bladder urothelial cancers. In renal cell carcinoma, tumor PD-L1 expression has been shown to correlate with rapid cancer progression, cancer death and overall mortality (22,30). In urothelial cell carcinoma, tumor-associated PD-L1 expression was found to be significantly associated with a high frequency of postoperative recurrence, poorer survival rate, and advanced tumor stage (31). In pancreatic cancer, patients with cancer-cell associated PD-L1-positive expression had a significantly poorer prognosis than patients with PD-L1-negative tumors (32,33). In gastric carcinoma, patients with PD-L1-positive tumors also had a significantly decreased probability of survival compared with patients with PD-L1-negative tumors (34).

The research on PD-L2 in malignant tumors is still relatively rare. In a study of pancreatic cancer, no correlation was found between PD-L2 expression and survival (32). In ovarian cancer, although PD-L2 expression was correlated with an impaired survival, this did not reach statistical significance (35). Similarly, in hepatocellular carcinoma a minority had high PD-L2 expression, and again, although PD-L2 expression was correlated with an impaired disease-free survival, this difference was not statistically significant (36). Only one report suggested that the expression of PD-L2 was significantly correlated with poorer prognosis in ESCC (23). Thus, the majority of studies have found a significant correlation between impaired survival and PD-L1 expression, but much less so for PD-L2. Currently, the expression of PD-L2 research conclusion remains controversial in tumor tissues.

TILs are considered as a manifestation of the host immune response (37). Several clinical studies have suggested that TILs play a critical role and have prognostic significance in certain human tumors including esophageal cancer (38-40). PD-1 mainly expressed on TILs. In our research, we showed

for the first time that the count of PD-1<sup>+</sup> TILs was negatively correlated with both PD-L1 and PD-L2 expression in ESCC. The results of the current study indicated that the expression of PD-L1 and PD-L2 on ESCC inhibits PD-1<sup>+</sup> TILs activity or promote PD-1<sup>+</sup> TILs apoptosis, ultimately promoting immune evasion via the PD-1/PD-Ls pathway.

In order to further study the effects of PD-L1 and PD-L2 on immune cells, we measured the PD-L1, PD-L2 and PD-1 expression of Ec109 cells by FCM. The percentage of PD-L1 positive cells on Ec109 was 24.5±4.2%. The Ec109 cells did not express PD-L2 and PD-1. Then stable transfected Ec109 cell line was established and PD-L1/PD-L2 gene was expressed successfully. Transcription of PD-L1 gene and PD-L2 gene were identified by qRT-PCR. This has not been reported in ESCC, and can be used as a model applied to further studies on PD-L1 and PD-L2.

Furthermore, we have analyzed the PD-1/PD-Ls signal pathway on the function of CD8<sup>+</sup> T cells for the first time by co-culturing Ec109 and CD8<sup>+</sup> T cells. We chose the CD8<sup>+</sup> T lymphocytes in the function experiment, because CD8<sup>+</sup> T cells are generally thought to play a central role in antitumor immune response and the presence of CD8<sup>+</sup> T was reported as a prognostic factor in esophageal cancer (38,39). CD8<sup>+</sup> T cells can also produce IFN- $\gamma$ , which is an important activator of macrophages and inducer of class II major histocompatibility complex (MHC) molecule expression, and IFN- $\gamma$  has antiviral, immunoregulatory and antitumor properties (41).

The Ec109 cells and purified activated CD8<sup>+</sup> T cells were co-cultured for 48 h. PD-L1 significantly inhibited the CD8<sup>+</sup> T cells proliferation, IFN- $\gamma$  secretion and enhanced the apoptosis, which could be restored with the presence of PD-L1 and PD-1 blocking antibody. PD-L2 significantly inhibited the IFN- $\gamma$  secretion of CD8<sup>+</sup> T cells, and this could be restored with the presence of PD-L2 and PD-1 blocking antibody. But no significant result was obtained in the proliferation and apoptosis experiments. PD-1/PD-Ls interactions lead to phosphorylation of two tyrosines at the intracellular tail of PD-1. These tyrosines are part of an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). ITSM then recruits either of two structurally-related protein tyrosine phosphatases (42), which suppress activation of PI3K/Akt (43). Consequently, the survival factor Bcl-xL is downregulated and expression of transcription factors associated with effector cell function including GATA-3, T-bet and Eomes are lost (44). The net result of these PD-1-induced cascades is an impairment of proliferation, cytokine production, cytolytic function, and survival of the CD8<sup>+</sup> T cells (45).

However, the results of PD-L1 and PD-L2 are not identical. These data indicate that PD-L1 and PD-L2 may have different roles in tolerance induction, as Rozali *et al* (46) concluded that PD-L2 could play a role in the modulation of T-cell function, but the exact molecular pathway was yet to be elucidated. Of note, PD-1 may not be the only receptor for PD-L2. This can be inferred from helminth infection and allergic animal models, showing enhanced disease severity when PD-L2 blocking antibodies were used, but not when PD-1 blocking antibodies were used (47,48). Furthermore, PD-L2 mutants with abolished PD-1 binding capacity could



still exert functional effects on T cells from normal and PD-1-deficient mice (49). Thus, the role of PD-L2 still is not clear.

Activation of the immune system is recognized as an important treatment strategy against cancer (50). In fact, therapeutic antibodies for blocking PD-1 and PD-L1 have been developed and are undergoing human clinical testing (51,52). Although PD-1 and PD-L1 directed therapy is currently undergoing investigation in several types of malignancies, including both solid tumors and hematologic malignancies, PD-1 and PD-L1 therapy has been most studied in patients with metastatic melanoma. Antibodies targeting PD-1 in clinical development include nivolumab, pembrolizumab and pidilizumab. The first antibody to target PD-L1 in clinical trials was MDX-1105. Antibodies currently in clinical development that target PD-L1 include MPDL3280A, MEDI4736 and MSB0010718C. These clinical trials result in durable responses and relative safety in patients with a wide range of cancers (20,21). These therapeutic antibodies for blocking PD-1 and PD-L1 have broad application prospects.

However, immunotherapy in ESCC is still immature. Our finding revealed that PD-L1, PD-L2 and PD-1 were aberrantly expressed in ESCC and they might thwart effective antitumor immunity by interaction with tumor-T-cell, which provides an important clue to reveal the cellular immune deficiency mechanism in ESCC. Thus, more research in animal models, and in human are necessary to fully delineate the immune regulation functions of PD-Ls as well as molecule mediation mechanism involved in ESCC. How to selectively block these inhibitory molecules will be an attractive approach for ESCC immunotherapy.

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