

# Regulation of PD-L1 expression in a high-grade invasive human oral squamous cell carcinoma microenvironment

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**Abstract.** Blockade of the programmed-death 1 receptor (PD-1)/programmed-death ligand (PD-L1) pathway efficiently reduces tumour growth and improves survival. Durable tumour regression with blockade of the PD-1/PD-L1 checkpoint has been demonstrated in recent clinical studies. Oral squamous cell carcinoma (OSCC) is highly immunosuppressive, and PD-L1 expression has been proposed as a potential mechanism responsible for this phenotype. Despite the fact that anti-PD-1 treatment can produce durable responses, such therapy appears to benefit only a subset of patients. Thus, it is important to understand the mechanisms underlying regulation of PD-L1 expression in the OSCC microenvironment. In this study, we showed that PD-L1 expression in high-grade invasive OSCC cell lines was lower than that in a low-grade invasive OSCC line and found a close correlation between PD-L1 expression and the epithelial-mesenchymal transition (EMT). PD-L1 expression was upregulated in macrophages and dendritic cells (DCs) in high-grade invasive human OSCC tissues or co-cultured with mesenchymal-phenotype OSCC cells *in vitro*. TLR4-inhibitory peptide successfully suppressed PD-L1 upregulation on macrophages and DCs co-cultured with mesenchymal-phenotype OSCC cells, suggesting that

some EMT-induced tumour antigen is critical for PD-L1 induction on tumour-associated macrophages and DCs. Further studies are necessary to explore the impact of EMT on the tumour immune microenvironment and to identify potential biomarkers for selecting patients who might preferentially benefit from PD-1/PD-L1 blockade or immunotherapies more broadly.

## Introduction

The programmed-death 1 receptor (PD-1, CD279)/programmed-death ligand 1 (PD-L1, B7-H1, CD274) pathway has emerged as a critical inhibitory pathway that regulates T cell responses and maintains immune suppression (1-4). Oral squamous cell carcinoma (OSCC) is a malignant tumour found most often in the head and neck region (5,6) that is highly immunosuppressive in general, and PD-L1 expression has been proposed as a potential mechanism facilitating this phenotype (7-9). PD-L1 is expressed in many tumours in a constitutive and interferon- $\gamma$  (IFN- $\gamma$ )-inducible manner. PD-L1 binds to an inhibitory receptor (PD-1), which is a member of the B7 family of receptors (4), and to the costimulatory molecule CD80 (B7-1) (10). PD-1 is expressed on activated T cells, and ligation of PD-1 by tumour-associated PD-L1 induces apoptosis or downregulation of effector cytotoxic T lymphocytes (CTL), resulting in an escape from T-cell mediated immune surveillance (11). Blockade of the PD-L1/PD-1 pathway efficiently reduces tumour growth and improves survival (7,12-15). Durable tumour regression with blockade of the PD-1/PD-L1 checkpoint has been demonstrated in recent clinical studies (1,2), leading to the recent registration of an anti-PD-1 antibody for advanced melanoma and non-small cell lung cancer. In addition, the clinical efficacy of an anti-PD-1 monoclonal antibody in OSCC has been observed in phase I studies (16). In general, the response to PD-1 blockade has been commonly correlated with PD-L1 expression in treated tumours (1).

Although anti-PD-1 treatment can produce durable responses, it appears to benefit only a subset of patients. Chen *et al* recently demonstrated a molecular link between the epithelial-mesenchymal transition (EMT) and intratumoural CD8<sup>+</sup> T cell suppression through PD-L1 regulation, in both animal models and human cell lines (17). EMT is a key

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**Abbreviations:** CAF, carcinoma-associated fibroblast; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; HGF, human gingival fibroblast; IFN- $\gamma$ , interferon- $\gamma$ ; NSCLC, non-small cell lung cancer; OSCC, oral squamous cell carcinoma; PD-1, programmed-death 1 receptor; PD-L1, programmed-death ligand; qPCR, quantitative real-time PCR; TLR4, toll-like receptor 4

**Key words:** oral cancer, PD-L1, epithelial-mesenchymal transition, tumour microenvironment

process that drives cancer metastasis and drug resistance, and has been associated with poor prognosis in multiple cancers, including OSCC (18-20). For instance, positive staining for the mesenchymal marker vimentin occurs in specimens from non-small cell lung cancer (NSCLC) patients who develop resistance to epidermal growth factor receptor (EGFR) inhibitors, suggesting that EMT has been triggered in such tumours (21-23). Previously, we found that loss of EGFR expression in OSCC was associated with EMT and might have functional implications in the resistance to cetuximab treatment (24). However, the impact of EMT on reprogramming the tumour immune microenvironment is largely unknown.

To predict the efficacy and optimize anti-PD-1 therapy, alone or in combination, it is important to understand the mechanisms controlling PD-L1 expression. In this study, we focused on the regulation of PD-L1 expression in OSCC, and the mechanism of regulation of PD-L1 expression in the tumour microenvironment.

## Materials and methods

**Cell culture.** Three human OSCC cell lines established from tumour biopsies with different grades of invasive abilities were used, including OSC-20 cells (low-grade invasive cells), OSC-19 cells (low-grade invasive cells), and TSU cells (high-grade invasive cells). The OSC-20 cell line was derived from a 58-year-old female with tongue cancer metastatic to the cervical lymph nodes (25). OSC-19 was derived from a 61-year-old male with tongue cancer metastatic to the cervical lymph nodes (26). The TSU cell line was established from a patient with gingival squamous cell carcinoma who had developed marked leukocytosis (27). In addition, normal human gingival fibroblasts (HGFs; ATCC no. CRL-2014) obtained from the American Type Culture Collection (Manassas, VA, USA) served as a control. Macrophages and dendritic cells (DCs) were generated from human peripheral blood mononuclear cells (PBMCs), as described previously (28,29). PBMCs were obtained by venepuncture into an 8-ml Vacutainer CPT Cell-Preparation Tube (BD Vacutainer Systems, Franklin Lakes, NJ, USA). Briefly, monocyte-derived macrophages were generated by incubating monocytes ( $1 \times 10^6$ /ml) in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 50 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ, USA) at 37°C in a CO<sub>2</sub> incubator for 7 days. Induced macrophages were examined using an anti-macrophage antibody (MAC387; Abcam, Tokyo, Japan). Monocyte-derived DCs were generated by incubating monocytes at  $1 \times 10^6$  cells/ml in G4 medium (G4 Dendritic Cell Generation kit; Humanzyme, IL, USA) at 37°C in a CO<sub>2</sub> (5%) incubator for 7 days. Induced DCs were examined using an anti-DC antibody (CD83; Abcam). Carcinoma-associated fibroblasts (CAFs) were generated by the addition of recombinant TGF- $\beta$ 1 to confluent growth-arrested cell monolayers, as previously described (30,31). Recombinant TGF- $\beta$ 1 (R&D Systems Europe Ltd., Abingdon, UK; 50 ng/ml) was prepared in serum-free culture medium. CAFs were examined using anti- $\alpha$ SMA (R&D Systems Europe Ltd.) antibodies. Culture inserts (1- $\mu$ m pore size) were purchased from Corning Japan (Tokyo, Japan). MyD88 homodimerization-inhibitor peptide and toll-like receptor 4 (TLR4)-inhibitory peptide were

purchased from Imgenex (San Diego, CA, USA) and Novus Biologicals (Littleton, CO, USA), respectively, and used in the cell-blocking study.

**Tissue samples.** Experiments using human tissues were approved by the ethics committee of the Kanazawa University Graduate School of Medical Science (IRB no. 2014-004, 352-2), and written informed consent was obtained from patients providing tissue specimens. The subjects included 24 patients with primary OSCC who underwent surgical resection at the Department of Oral and Maxillofacial Surgery at Kanazawa University Hospital between 2000 and 2008. The patients with carcinoma were aged from 43 to 89 years ( $64.4 \pm 11.2$  years, mean  $\pm$  SD). The grade of tumour differentiation was determined according to the criteria proposed by the World Health Organization (32). The mode of tumour invasion was assessed according to the classification by Yamamoto *et al* (33).

**RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR).** The mRNA expression levels of PD-L1, PD-L2, E-cadherin, N-cadherin, Vimentin, and Snail1 were analysed using a Rotor-Gene Q 2plex System (Qiagen, Hilden, Germany) with FAM/ZEN/IBFQ probes (Integrated DNA Technologies, Inc., Coralville, IA, USA; DNA sequences not opened). Total RNA was extracted using the RNeasy Protect Mini kit (Qiagen), and cDNA was obtained using the PrimeScript first-strand cDNA Synthesis kit (Takara, Tokyo, Japan). All reactions were performed according to the manufacturer's instructions. We amplified 18S rRNA as an internal standard using HEX/ZEN/IBFQ probes (Integrated DNA Technologies, Inc.; DNA sequences not opened). Relative expression levels were calculated using the  $\Delta\Delta$ Ct method for qPCR (34), which presents the data as fold-differences in expression level relative to a calibrator sample; in this case, the mean expression of 3 experimental measurements of 18S rRNA in control cells or vehicle-treated cells.

**Western blot analysis.** The cultured cells were lysed with Pierce RIPA buffer (Thermo Scientific, Waltham, MA, USA). Lysates mixed with sample buffer were electrophoretically separated and transferred onto membranes. Membranes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan), followed by incubations with an anti-PD-L1, E-cadherin, N-cadherin, Vimentin, or Snail1 antibody (Abcam) and an anti-human  $\beta$ -actin antibody (Cell Signaling Technology, Tokyo, Japan). After washing with Tris-buffered saline (TBS) with 0.05% Tween, membranes were incubated with a horseradish peroxidase-conjugated anti-mouse IgG. After washing with TBS-0.05% Tween, membranes were incubated with the ECL Prime Western Blotting Detection reagent (GE Healthcare, Little Chalfont, UK). Signals were detected and analysed using C-DiGit (M&S TechnoSystems, Tokyo, Japan).

**EMT induction.** OSC-20 cells were seeded at 70% confluence and cultured for 48 and 72 h in Dulbecco's modified Eagle's medium (Sigma-Aldrich Japan, Tokyo, Japan) with 0.5% FBS (Hyclone, Logan, UT, USA) to induce EMT. Recombinant human TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN, USA) was added to a final concentration of 5 ng/ml.

**Immunohistochemistry.** Each specimen was fixed in 10% buffered formalin and then embedded in paraffin to prepare serial sections. Immunohistochemical staining was performed using antibodies against PD-L1, Snail, Vimentin, CD83, macrophages (MAC387),  $\alpha$ SMA, and fibroblast activation protein (FAP) (Abcam). Primary antibodies were detected using EnVision Single reagents (Dako Japan, Tokyo, Japan), Alexa Fluor 488-labeled goat anti-mouse IgG (Cell Signaling Technology), and Alexa Fluor 546-labeled goat anti-rabbit IgG (Thermo Fisher Scientific, Rockford, IL, USA). Staining specificity was confirmed using the Universal Negative Control for IS-Series Rabbit Primary Antibodies reagent (IS600; Dako Japan) as a negative control, instead of the matching primary antibody. After mounting with ProLong Gold Antifade reagent (Thermo Fisher Scientific, Tokyo, Japan), the tissues were subjected to fluorescence microscopy. Three regions were image-captured under a light microscope at x100 magnification, and the images were evaluated based on number of stained tumour cells, following the method described by Kimura *et al* (24). PD-L1 expression was evaluated based on the extent of immunolabelling in tumour cell membranes and was classified on a four-point scale: 0 (no labelling, or labelling in <10% of tumour cells); 1 (weak labelling, homogeneous or patchy, in >10% of tumour cells); 2 (moderate labelling, homogeneous or patchy, in >10% of tumour cells); 3 (intense labelling, homogeneous or patchy, in >10% of tumour cells). These scores were subsequently grouped into 2 categories: low expression (0 or 1) and high expression (2 or 3).

**Statistical analysis.** Statistical analyses were conducted using JMP 12.0 software (SAS Institute, Inc., Cary, NC, USA). The qPCR data are presented as the mean  $\pm$  standard error of the mean (SEM). Differences between groups were tested for statistical significance using the 2-tailed Mann-Whitney U test. Differences were considered significant at  $P < 0.05$ .

## Results

**PD-L1 and PD-L2 expression in 3 different grades of invasive OSCC cell lines.** PD-L1 and PD-L2 mRNA expression on 3 human OSCC lines with different grades of invasiveness (OSC-19, OSC-20, and TSU) were analysed. The qPCR data are presented as fold-differences relative to the mean values from 3 experiments with HGF cells (Fig. 1A). Among the 3 OSCC cell lines, the PD-L1 mRNA expression level ranged from 5.73 to 20.48-fold higher than in HGF cells (Fig. 1A), while PD-L2 mRNA expression ranged from 0.38 to 1.48-fold higher than in HGF cells (Fig. 1A). In particular, the expression of PD-L1 mRNA in low-grade invasive cells (OSC-20 cells) was highest among the 3 OSCC cell lines. Upregulation of PD-L1 mRNA in high-grade invasive cells (TSU cells) was not as remarkable compared to that in the other 2 low-grade invasive cells (OSC-20 and OSC-19 cells). PD-L2 expression on the 3 different invasive human OSCC lines was much lower, and differences were negligible compared to that of PD-L1. We further analysed PD-L1 expression at the protein level. Both low-grade invasive cells (OSC-20 and OSC-19) had a PD-L1 protein expression level higher than that of TSU cells. HGF cells exhibited negligible PD-L1 protein expression (Fig. 1B).

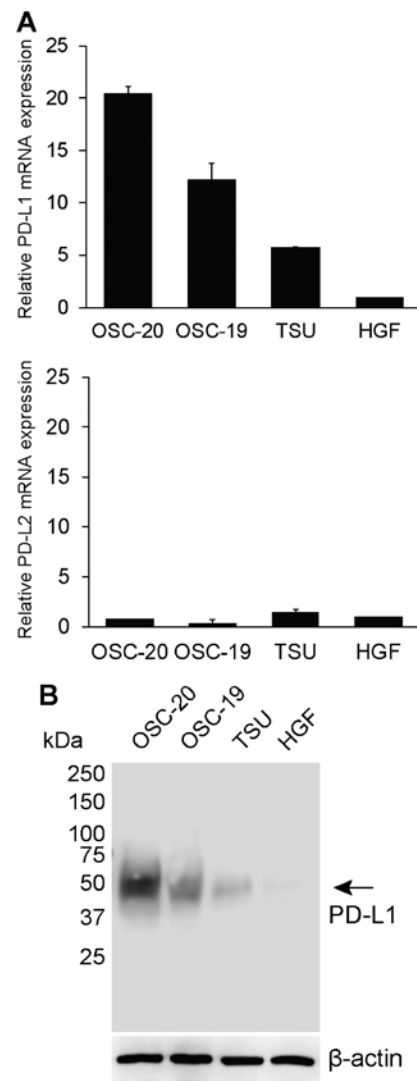


Figure 1. PD-L1 and PD-L2 expression on 3 OSCC cell lines with different grades of invasiveness. (A) Relative mRNA-expression levels of PD-L1 and PD-L2 in the OSC-20, OSC-19, TSU, and HGF cell lines. Expression levels are displayed as fold-differences relative to control cells (HGF cells). (B) Expression of the PD-L1 protein in OSC-20, OSC-19, TSU, and HGF cells was assessed by immunoblot analysis. The arrow indicates PD-L1.  $\beta$ -actin was detected as a loading control.

**The relationship between EMT and PD-L1 expression in human OSCC cell lines.** To evaluate whether PD-L1 expression is associated with EMT features, we analysed the mRNA- and protein-expression levels of E-cadherin, N-cadherin, vimentin, and Snail1 in the human OSC-19, OSC-20, and TSU lines with different degrees of invasiveness. The mRNA expression levels of these EMT-associated genes in OSC-19 and TSU cells are presented as fold-differences relative to those in OSC-20 cells. TSU cells displayed a mesenchymal phenotype manifested by the loss of E-cadherin and acquisition of N-cadherin, vimentin, and Snail compared to the corresponding expression levels in OSC-20 and OSC-19 cells (Fig. 2A and B). OSC-20 cells (epithelial phenotype) were exposed to TGF- $\beta$ 1 to determine whether such exposure could change the mRNA- and protein-expression levels of EMT-associated genes and PD-L1 (Fig. 2C and D). The mRNA-expression levels are presented as fold-differences relative to that of vehicle control-treated

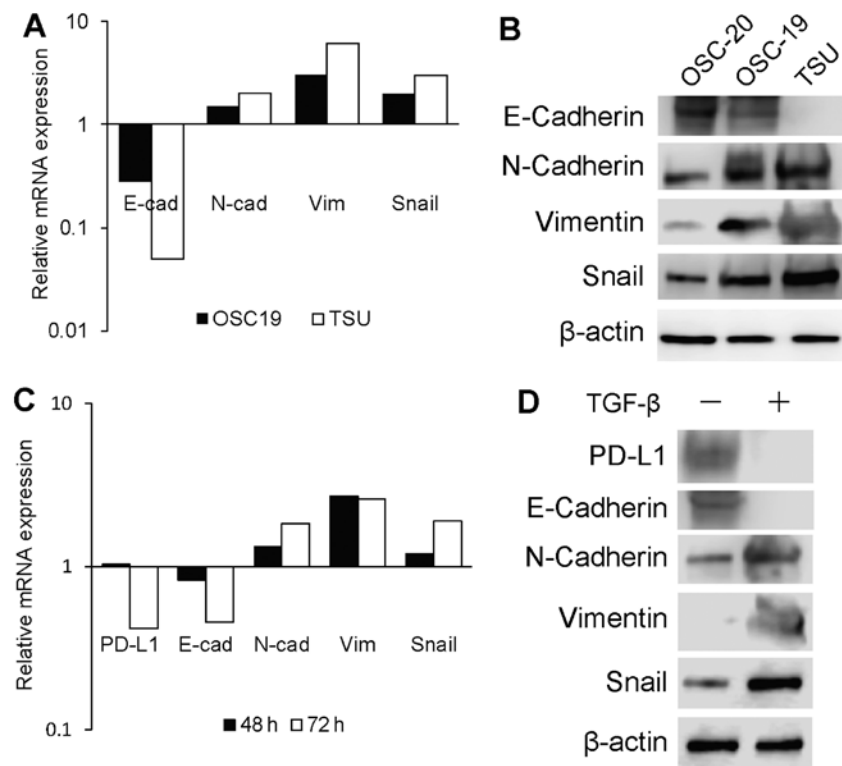


Figure 2. Relationship between EMT and PD-L1 expression on human OSCC cell lines. (A) Relative mRNA expression levels of the EMT-associated genes E-cadherin, N-cadherin, vimentin, and Snail, in OSC-20, OSC-19, and TSU cell lines. Expression differences in OSC-19 and TSU cells are displayed as fold-changes relative to the expression levels in OSC-20 cells. (B) Expression of the EMT-associated protein in OSC-20, OSC-19, and TSU cells was assessed by immunoblot analysis.  $\beta$ -actin was detected as a loading control. (C) Relative mRNA expression levels of PD-L1 and the EMT-associated genes E-cadherin, N-cadherin, vimentin, and Snail in TGF- $\beta$ -treated OSC-20 cells. OSC-20 cells were treated with human TGF- $\beta$  or a vehicle control for 48 and 72 h. Expression differences are displayed as fold-changes relative to the control vehicle at 48 and 72 h. (D) Expression of the EMT-associated protein in TGF- $\beta$ -treated OSC-20, OSC-19, and TSU cells was assessed by immunoblot analysis.  $\beta$ -actin was detected as a loading control.

cells. Serial examination of EMT markers (loss of E-cadherin and upregulation of N-cadherin, vimentin, and Snail) at 48 and 72 h showed that TGF- $\beta$ 1 treatment resulted in EMT in OSC-20 cells (Fig. 2C). We further confirmed E-cadherin downregulation and N-cadherin, vimentin, and Snail upregulation at the protein level at 72 h after TGF- $\beta$ 1 treatment (Fig. 2D). Strikingly, the total PD-L1 mRNA and protein levels were reduced in OSC-20 cells after EMT induction by TGF- $\beta$ 1 treatment.

**Immunohistochemical analysis of human OSCC tissues.** To confirm downregulation of PD-L1 on high-grade invasive cells compared to low-grade invasive cells, we further studied immunohistochemical analysis using 3-, 4C-, and 4D-grade invasive OSCC tissues. Previously, we demonstrated that OSC-20 and OSC-19 cells implanted into nude mice successfully reproduced the same types of diffuse invasive tumours (grades 3 and 4C in terms of the mode of invasion, respectively) (35), and repeated inoculation with TSU cells successfully reproduced the formation of grade 4D OSCC tumours in nude mice (36). With the low-grade invasive OSCC lines (grades 3 and 4C, mode of invasion), immunohistochemical analysis demonstrated that PD-L1 was detected in cancer cells, but not in stromal cells (Fig. 3A). However, PD-L1 signals were detected in stromal cells of high-grade invasive OSCC (grade 4D, mode of invasion), whereas PD-L1 was not detected in the cancer cells (Fig. 3A). Table I displays the correlations between

PD-L1 expression and the clinicopathological factors. PD-L1 expression in high-grade invasive OSCCs (4D, mode of invasion) was significantly downregulated compared to low-grade invasive OSCCs (3 and 4C, mode of invasion) ( $P=0.0392$ ). In contrast, PD-L1 expression did not correlate with tumour size ( $P=0.873$ ), lymph node metastasis ( $P=0.542$ ), local recurrence ( $P=0.083$ ), or histological differentiation ( $P=0.145$ ). Immunohistochemical analysis demonstrated that signals of EMT-related protein (Vimentin and Snail) were detected in cancer cells in human OSCC tissues classified as grade 4D in terms of the mode of invasion (Fig. 3B). To analyse which cells expressed PD-L1 in the cancer stroma, we investigated cells surrounding the cancer cells by studying several biomarkers. Immunofluorescence analysis demonstrated that most CD83 (DC marker)-positive cells expressed PD-L1 and a fraction of MAC387 (macrophage marker)-positive cells expressed the PD-L1 protein in high-grade invasive OSCC tissues (grade 4D, mode of invasion; Fig. 3C). However,  $\alpha$ SMA or FAP (CAF marker)-positive cells did not co-localize with PD-L1 expressing cells (Fig. 3C).

**PD-L1 expression in macrophages or DCs co-cultured with high-grade, invasive OSCC cells.** The above immunohistochemical analysis demonstrated that macrophages and DCs expressed PD-L1 in stromal cells in high-grade invasive OSCC tissues. Thus, we performed further *in vitro* studies to confirm PD-L1 expression in macrophages and DCs *in vitro*. We found

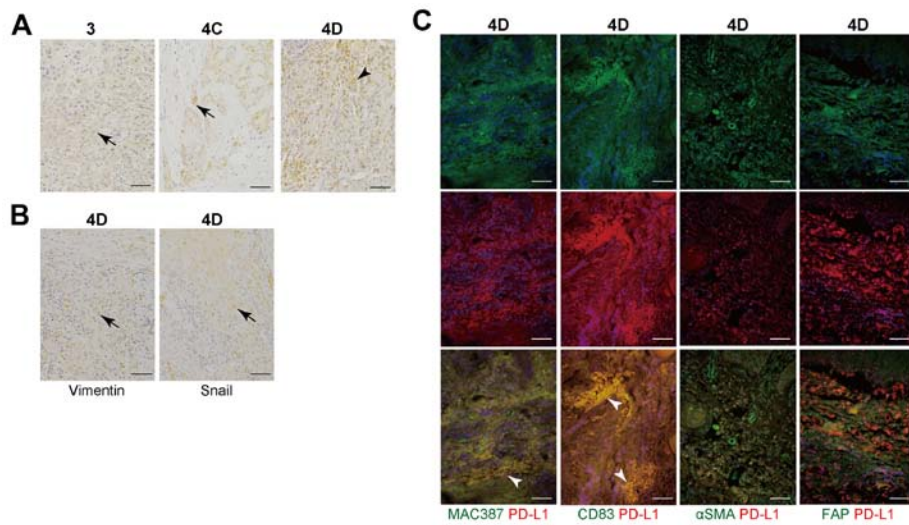


Figure 3. Immunohistochemical analysis of human OSCC tissues. (A) Localization of PD-L1 in OSCC tissues (mode of invasion: grade 3, 4C or 4D). Positive staining of cancer cells (arrow) or stroma cells (arrowhead). (B) Localization of vimentin or snail in OSCC tissues (mode of invasion: grade 4D). Positive staining of cancer cells (arrow). (C) Fluorescent images of MAC387 (green), CD83 (green),  $\alpha$ SMA (green), FAP (green), PD-L1 (red), and DAPI (blue) in OSCC tissues (mode of invasion: grade 4D). Double-positive staining of cells (arrowhead). Scale bar, 100  $\mu$ m.

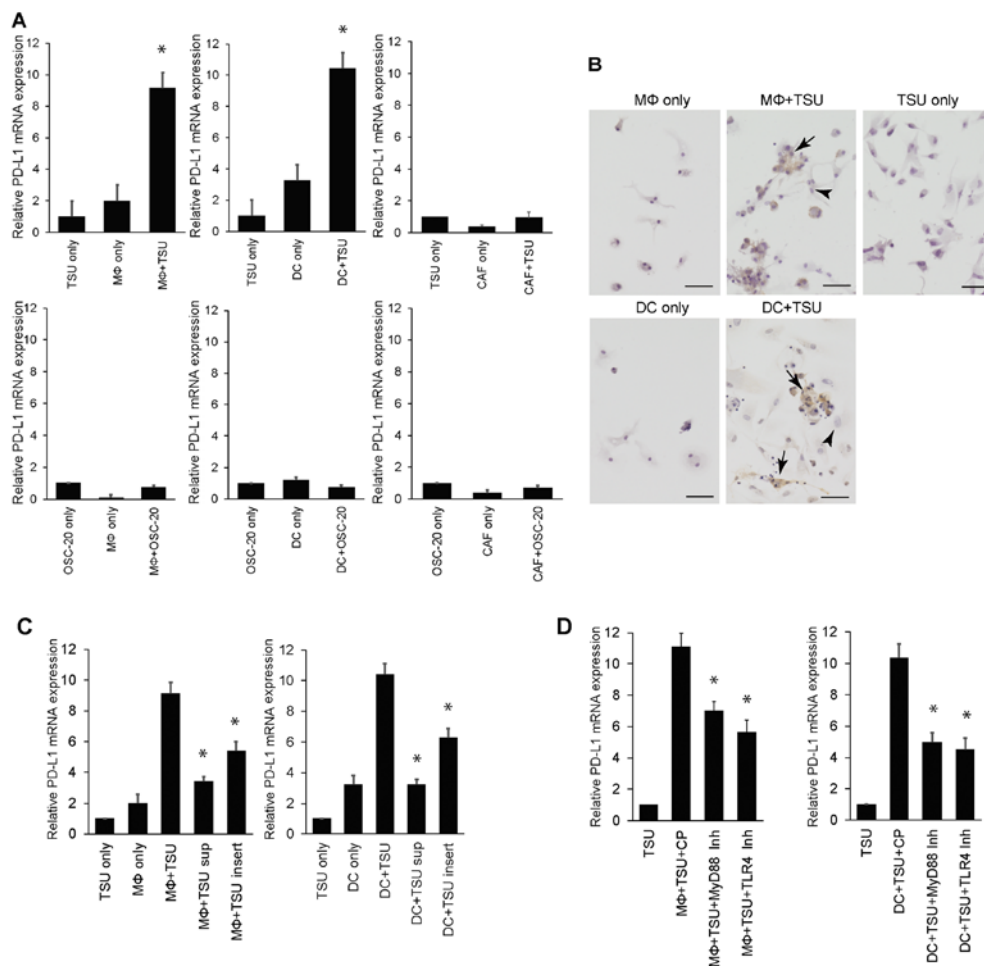


Figure 4. PD-L1 expression on macrophages or DCs co-cultured with high-grade invasive OSCC cells. (A) Expression of PD-L1 mRNA in macrophages or DCs or CAFs co-cultured with TSU or OSC-20 cells. Expression levels are displayed as fold-differences relative to the corresponding mRNA levels in control TSU or OSC-20 cells. The data shown represent the mean  $\pm$  SEM. \* $P < 0.05$ . (B) Immunohistochemical analysis of macrophages or DCs co-cultured with TSU cells, or macrophages, DCs, and TSU cells cultured separately. Positive immunostaining of macrophages or DCs (arrow) and a lack of staining of TSU cells (arrowhead). Scale bar, 50  $\mu$ m. (C) Expression of PD-L1 mRNA in macrophages or DCs after adding the supernatant from a TSU cell culture or TSU cells in a culture insert. Expression levels are displayed as fold-differences relative to the corresponding mRNA levels in control TSU cells. The data shown represent the mean  $\pm$  SEM. \* $P < 0.05$ . (D) PD-L1 upregulation on macrophages or DCs co-cultured with TSU cells was suppressed by a MyD88 homodimerization-inhibitor peptide or TLR4-inhibitory peptide, but not by a control peptide (CP). Expression levels are displayed as fold-differences relative to the corresponding levels observed in control TSU cells. The data shown represent the mean  $\pm$  SEM. \* $P < 0.05$ .



Table I. Correlation between PD-L1 expression and the OSCC clinicopathological factors of the patients.

Factor	PD-L1 expression		P-value
	High (n=13)	Low (n=11)	
T category			
T1	4	3	0.873
T2	7	5	
T3	0	1	
T4	2	2	
N category			
Negative	12	7	0.542
Positive	1	4	
Local recurrence			
Negative	10	5	0.083
Positive	3	6	
Differentiation			
Well	9	7	0.145
Moderately	3	1	
Poorly	1	3	
Mode of invasion			
Grade 3	6	2	0.0392 <sup>a</sup>
Grade 4C	5	3	
Grade 4D	2	6	

<sup>a</sup>P<0.05.

that PD-L1 expression increased significantly (~10-fold) in macrophages or DCs co-cultured with TSU cells, relative to that observed in TSU cells, macrophages or DCs cultured separately. Differences in PD-L1 expression were negligible between TSU cells co-cultured with CAFs and TSU cells or CAFs cultured separately (Fig. 4A). In addition, PD-L1 expression in macrophages or DCs co-cultured with OSC-20 cells, as well as in separate cultures of OSC-20 cells and macrophages or DCs, did not change (Fig. 4A). Differences in PD-L1 expression were negligible between OSC-20 cells co-cultured with CAFs and OSC-20 cells or CAFs cultured separately (Fig. 4A).

Next, macrophages or DCs co-cultured with TSU cells were immunostained using an anti-PD-L1 antibody to determine which cell type expressed PD-L1. PD-L1 signals were detected in macrophages or DCs, but not TSU cells (Fig. 4B). In particular, macrophages or DCs attached to TSU cells that expressed PD-L1. PD-L1 was not detected in the separate cultures of TSU cells, macrophages, or DCs (Fig. 4B). PD-L1 upregulation was not observed after adding the supernatant from a TSU cell culture to macrophages or DCs (Fig. 4C). PD-L1 upregulation was inhibited after adding TSU cells in culture inserts to macrophages or DCs (Fig. 4C). Further, we used a blocking peptide to study the mechanism of toll-like receptors in PD-L1 induction in macrophages or DCs co-cultured with TSU cells. PD-L1 upregulation in macrophages or DCs co-cultured with TSU cells was significantly

suppressed by treatment with the MyD88 homodimerization-inhibitor peptide or TLR4-inhibitory peptide (Fig. 4D).

## Discussion

PD-L1 is a ligand of the inhibitory receptor PD-1, and the PD-L1/PD-1 pathway is involved in the induction and maintenance of peripheral immunotolerance in cancer tissues (11,37). Previous data demonstrated that constitutive expression of PD-L1 occurs in various human cancers, including squamous cell carcinomas of the lung, esophagus, and head and neck; other types of carcinomas of the colon, ovaries, bladder, and breast; melanoma; and glioma (7,12,13,37-45). Tumour-associated PD-L1 is closely correlated with a poor prognosis and/or higher malignancy grade. In general, PD-1 blockade has been commonly correlated with PD-L1 expression in treated tumours (1). Here, we demonstrated that PD-L1 expression in 3 OSCC cell lines was significantly upregulated compared to that in normal cells; thus, PD-L1 may play a greater role in immunotolerance in the cancer microenvironment than PD-L2 does. However, it was surprising that PD-L1 expression in high-grade invasive OSCC cell lines was lower than that in a low-grade invasive OSCC line because these data contradicted previous findings with other tumour types (7,12,13,37-45). While the molecular mechanisms that account for this difference remain to be determined, we suggest that they may be confined to cancer subtypes with a unique molecular profile. Clearly, further investigation will be required to test this hypothesis.

Previously, we demonstrated that high-grade invasive OSCC cells have a mesenchymal phenotype (24). In this study, we confirmed the EMT-associated genes were upregulated in high-grade invasive OSCC tumour cells (TSU). Interestingly, PD-L1 expression in TSU OSCC cells (with a mesenchymal phenotype) was downregulated compared to that in OSC-19 and OSC-20 cells (with an epithelial phenotype). We also demonstrated that TGF- $\beta$ 1 caused OSC-20 cells (with an epithelial phenotype) to undergo an EMT-associated gene switch, resulting in lost PD-L1 expression. These results indicated that a close correlation existed between PD-L1 expression and EMT, and that the net effect of TGF- $\beta$ 1 signalling was PD-L1 downregulation with a concomitant EMT-associated gene switch in tumour cells. However, further studies are needed to explain why OSCC maintains high-grade invasive ability even after PD-L1 downregulation in cancer cells. Our above data were developed in cell line models, which do not reflect contributions of the tumour microenvironment and immune responses, which play important roles in therapeutic responses. Further studies of the precise molecular mechanisms underlying the association between EMT and the regulation of PD-L1 expression in a human tumour microenvironment should be conducted.

By analysing the immunomarkers PD-L1, vimentin, and snail1, we confirmed the loss of PD-L1 expression and the mesenchymal phenotype of cancer cells in high-grade invasive human OSCC tissues (grade 4D). Interestingly, strong PD-L1 signals were detected in stromal cells of high-grade invasive human OSCC tissues (grade 4D), rather than in cancer cells. In low-grade invasive human OSCC tissues (grade 3 and 4C), PD-L1 expression was seen only in cancer cells, but not in

stromal cells. These data suggested that invasiveness and the EMT signature were inversely correlated with PD-L1 expression in cancer cells and directly correlated with PD-L1 expression in stromal cells. Potentially, some interaction occurred between EMT-induced OSCC cells and stromal cells. A previous report provided evidence that PD-L1 is not only expressed by tumour cells, but also by tumour-associated macrophages, DCs, and CAFs (46,47). In this study, we detected a strong presence of PD-L1 in macrophages or DCs in cancer stroma tissues via immunofluorescence analysis. While the expression levels of PD-L1 in tumour cells, tumour-infiltrating immune cells have recently been shown to correlate with clinical responses to anti-PD-1 therapy (48-50). Only a subset of patients with PD-L1-expressing tumours had clinical responses and other patients (without PD-L1 staining) demonstrated clinical benefits, indicating that additional factors in the tumour microenvironment (such as stroma cell PD-L1 expression) may exist that define the subgroup of patients who derive benefit.

Here, we showed that macrophages or DCs co-localized with PD-L1 protein expression in cancer stromal tissues by immunofluorescence staining. We performed further *in vitro* analysis of the effects of direct interactions between cancer cells and macrophages or DCs on PD-L1 expression. We showed here that PD-L1 expression was upregulated in macrophages or DCs co-cultured with TSU cells, but upregulation was not observed in CAFs co-cultured with TSU cells. Immunostaining of co-cultured macrophages or DCs and TSU cells revealed that PD-L1 upregulation was observed only in macrophages or DCs, but not in co-cultured TSU cells. Although the association between PD-L1 expression and macrophages or DCs reported here is suggestive of an adaptive resistance mechanism, it is still necessary to explain why PD-L1 was predominantly expressed by macrophages or DCs, rather than cancer cells. If cancer cell-derived IFN- $\gamma$  is indeed a major mediator of PD-L1 expression in oral cancer, as described in other settings (51), then oral cancer cells may be less IFN- $\gamma$ -responsive than macrophages or DCs. Another possibility is that IFN- $\gamma$  is expressed at lower levels in TSU cells, as we showed here that the supernatant of cultured TSU cells or TSU cells in culture insert did not induce PD-L1 expression in macrophages or DCs. Alternatively, perhaps while cleaning up tumour debris, macrophages and DCs begin to present high levels of tumour antigens, making them prime targets for T cell recognition. In fact, in this study, a TLR4-inhibitory peptide successfully suppressed PD-L1 upregulation in macrophages or DCs co-cultured with TSU cells. In this scenario, PD-L1 expression may provide a means for protecting macrophages or DCs against cell death, and tumour antigen could be induced by EMT, because PD-L1 upregulation was not observed in macrophages or DCs co-cultured with OSC-20 cells with an epithelial phenotype (Fig. 4A).

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