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Supplementary Information for

Tumor cell-intrinsic PD-1 receptor is a tumor suppressor and mediates resistance to PD-1 blockade therapy

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Supplementary Information Text

Materials and Methods

Antibodies and reagents

The following antibodies were used for immunoblot: anti-PD-1 (ORIGEN), anti-PD-L1 (Cell Signaling Technology, CST),anti-phospho-Akt (anti-p-AKT) (CST), anti-Akt (CST), anti-phospho-p42/44 (anti-p-ERK) (CST), anti-p42/44 (CST), anti-phospho-S6 (CST), anti-S6 (CST), anti-GAPDH (ProteinTech), HRP-conjugated goat anti-rabbit (Beyotime Technology) and HRP-conjugated goat anti-mouse (Beyotime Technology).The following antibodies were used for flow cytometry: anti-PD-1 (ORIGEN), anti-PD-L1 (Abcam), Purified Mouse IgG2a, κ Isotype Ctrl Antibody (Biolegend), APC Goat anti-mouse IgG (minimal x-reactivity) Antibody (Biolegend), Rabbit IgG monoclonal [EPR25A]-Isotype Control (Abcam), Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Life technology), PE-conjugated anti-human PD-1 (MIH4, BD Pharmingen), FITC-conjugated anti-human CD31 (WM59, BD Pharmingen) PE-CY7-conjugated CD45 (HI30, BD Pharmingen). The following antibodies were used for IHC staining: anti-p-AKT (CST), anti-p-ERK (CST), anti-PD-1 (CST), anti-PD-L1 (CST), Mouse Anti-Human FC HRP antibodies (Cell Sciences). For *in vitro* and *in vivo* PD-1 blocking experiments, the following antibodies were used: Nivolumab (Selleck), Pembrolizumab (Selleck), Atezolizumab (Selleck) and Human IgG dry powder (Solarbio). Recombinant human B7-H1 Fc (PD-L1 Ig) (R&D Systems) and Natural Human IgG protein (Abcam) were used for treatment of tumor cells *in vitro*. CellTrace™ CFSE Cell Proliferation Kit (Invitrogen) was used for Cell proliferation assay with anti-PD-1 and anti-PD-L1 blocking antibodies *in vitro.*

RT-PCR and qRT-PCR

RNA was isolated using RNAiso Plus (TaKaRa) following the manufacturer's protocol. Standard cDNA was generated using PrimeScript® Reverse Transcriptase Master Mix (TaKaRa) according to the manufacturer's instructions. qRT-PCR was performed on QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher) using SYBR Premix Ex Taq (TaKaRa). The housekeeping gene, GAPDH，was amplified as control, the relative expression of PD-1/PD-L1 transcripts were calculated using the comparative cycle threshold (CT) ($2^{-\Delta\Delta Ct}$) method.

Immunoblot Analysis

Total cell lysates were extracted using cell lysis buffer supplemented with protease inhibitors and phosphatase inhibitors. Protein concentrations were determined using Enhanced BCA Protein Assay Kit (Beyotime Technology) according to the manufacturer's protocol. Equal amounts of total protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were incubated with the primary antibody overnight at 4°C after being blocked with 5% non-fat milk in TBS-T at room temperature for 1 h, followed by incubation with HRP-conjugated secondary antibodies (Cell Signaling Technology) at room temperature for another 1 h. Protein expression was detected with the ChemiScope 6000 Touch Imaging system (Clinx, SHH, China).

IHC

IHC analysis of p-AKT and p-ERK expression in tumor tissue sections was performed as described below. Briefly, 3-5 μm thick tumor tissue sections were deparaffinized in xylene and subsequently rehydrated with 100%, 95% and 75% ethanol and deionized H_2O, respectively. The sections were then placed on refractory plastic sections, placed in a citric acid buffer of pH 8.0, capped,

boiled for 10 minutes at high temperature until boiling, and then cooled down to room temperature. Subsequently, the sections were incubated with 1:200 diluted rabbit anti-p-AKT and rabbit anti-p-ERK antibody for 1 hour at room temperature, and then incubated with a 1:100 diluted biotin-conjugated goat anti-rabbit IgG for 30 minutes at room temperature. Finally, the color is developed using a DAB developer. IHC analysis of binding of in vivo administered anti-human PD-1/PD-L1 antibody to xenografts was performed using a similar experimental method as above. The sections only need to be incubated with a 1:100 diluted biotin-conjugated mouse anti-human IgG for 1 hour at room temperature, then the color reaction can be carried out. Images were captured from NIS-Elements software (BR 2.30, Nikon) and analyzed by Image-J software.

Animals

All protocols involving animals were previously approved by the Ethics Committee for the Use of Experimental Animals of the Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences (Suzhou, Jiangsu, China). Male mice that aged at least 6 weeks were purchased from Shanghai Model Organisms Center and randomly assigned to experimental groups. Stable *PDCD1*-, *PDCD1LG1*-knockdown and their respective control cell lines were injected subcutaneously (1×10^6 cells/inoculum) into the flanks of recipient NSG mice, as described (1). Tumor formation/growth was assessed every 2- 4 days as a time course until the experimental endpoint, and tumor volume was calculated by the formula: $(\text{width})^2 \times \text{length}/2$. Mice were euthanized 30 days after cell inoculation or if the longest dimension of the tumors reached 2.0 cm before 30 days. For PD-1- and PD-L1-treated experiments, mice were allocated randomly to each treatment group, which were injected intraperitoneally (200 μg per injection) with Nibvolumab, Atezolizumab and Human IgG, respectively, every other day

starting 1 day before the volume of the tumors reached 150-200 mm^3. Tumor formation/growth was assayed as described above, until the experimental endpoint of 15 days.

Cell Lines

All cell lines were obtained from ATCC and verified by short tandem repeat assays for their identification. These Cell lines were cultured in RPMI-1640 medium (Gibco) or DMEM (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) and 1% (v/v) penicillin/streptomycin (Gibco) and incubated at 37°C in a humidified incubator containing 5% CO_2.

Expression Vectors, shRNAs and siRNAs

Short hairpin RNAs (shRNAs) for human PD-1 and human PD-L1 were prepared by cloning double-stranded oligonucleotides into the vector pSIH1-H1-Puro (2). Human *PDCD1* or *PDCD1LG1* was cloned with 3×FLAG at the 3'-terminus into the pLVX-IRES-Neo lentivirus expression vector by Shanghai Generay Biotech Co. Ltd. The siRNAs for *PDCD1* or *PDCD1LG1* knockdown were generated by GenePharma (Shanghai, China).

PD-1/PD-L1 Overexpression and Knockdown

Short hairpin RNAs (shRNAs) against human PD-1, human PD-L1 or scrambled shRNA-control were packaged into lentiviral particles by 293T-Lentix packaging cells co-transfected with the viral packaging plasmids pMD2.G and psPAX2, and viral supernatants were harvested 48-72 hours after transfection. 293T-Lentix cells co-transfected with human *PDCD1* or *PDCD1LG1* expression vector, pMD2.G and psPAX2 were utilized to package vectors into retroviral particles. Viral supernatants were harvested as above. NCI-H1299 or Calu-1 cell line were infected with filtered lentiviral and/or retroviral supernatant and selected in either 1.5-2 μg/ml puromycin (Puromycin

Dihydrochloride, Life Technologies) and/or 600-700 μg/ml neomycin (G418 sulfate, Life Technologies). RNAi was performed by the transfection of siRNA oligos using the jetPRIME® and jetPRIME® Buffer (PolyPlus-transfection), according to the manufacturer's instructions.Human *PDCD1* or PDDCD1LG1 knockdown or overexpression were confirmed by qRT-PCR and flow cytometry prior to all in vivo tumorigenicity studies described below.

Site-directed PD-1 Mutagenesis

To abrogate PD-1 signaling into the NCI-H1299 cells, tyrosine residues within the cytoplasmic PD-1 signaling motifs 'immunoreceptor tyrosine-based inhibitory motif' (ITIM) and/or 'immunoreceptor tyrosine-based switch motif' (ITSM) were mutated to phenylalanine using the GENEART Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer's protocol. The mutated human *PDCD1* variant was packaged into retroviral particles and used to infect the NCI-H1299 lung cancer cell line as described above. Wild-type or mutant *PDCD1* overexpression of all cell lines was confirmed by qRT-PCR and immunoblot.

Data Resources

The accession number for the transcriptome sequencing data reported in this paper is GEO Datasets: H3K4me1(ENCSR000AMU, ENCSR953XVZ, ENCSR617SRM, ENCSR356ANC), H3K4me2(ENCSR000AMV), H3K4me3 (ENCSR701FGA, ENCSR429VWL, ENCSR000DXY, ENCSR000DVC, EN CSR000DPO, ENCSR000DXZ, ENCSR000DWZ), H3K27ac (ENCSR453M UW, ENCSR948TOS, ENCSR738SXD, ENCSR000DPL, ENCSR550WUX, ENCSR540ADS) and H3K9ac (ENCSR813HFV, ENCSR000AMX).

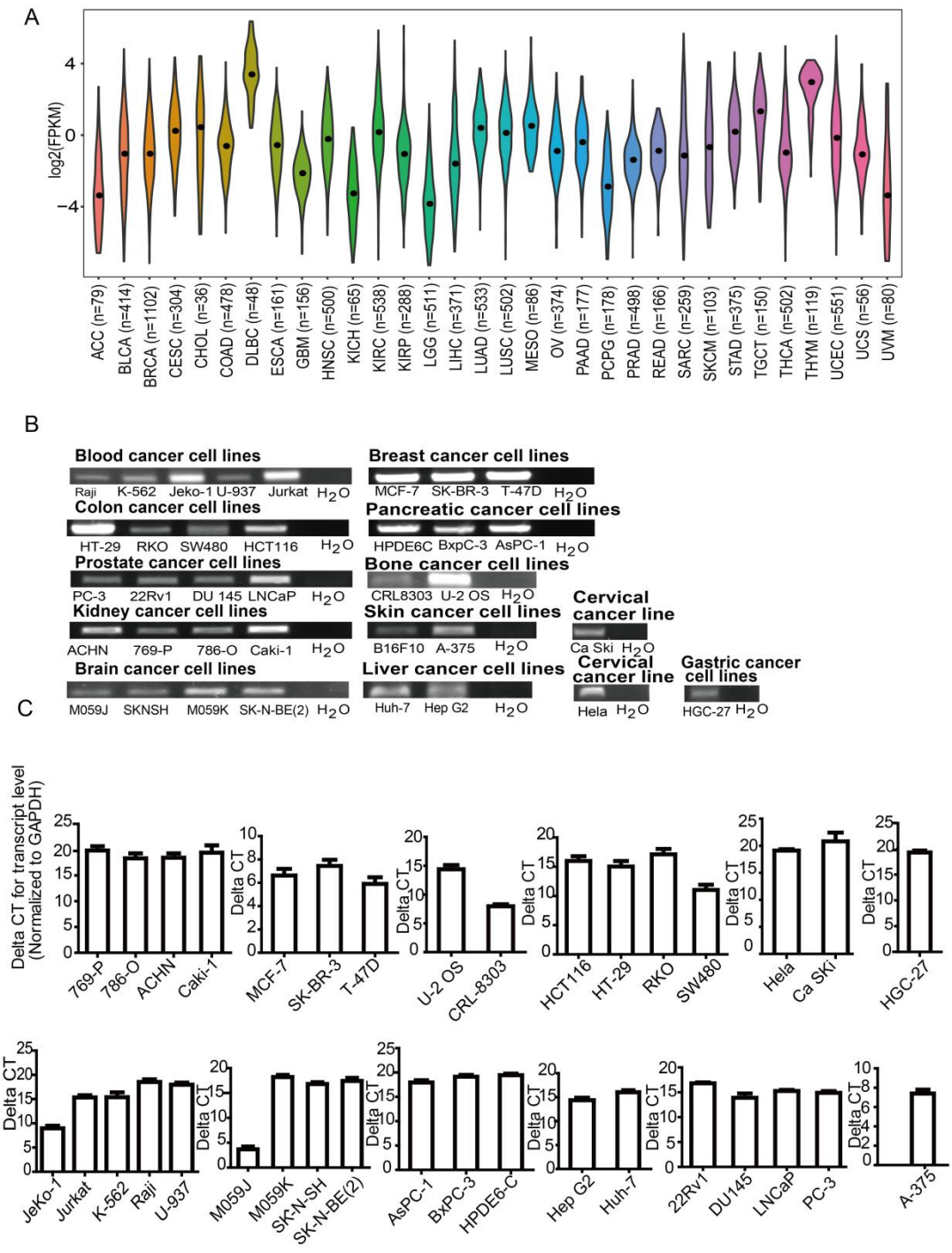

Fig. S1. *PDCD-1* is Transcribed in Various Tumor Cells.

(A) Violin plots showing the expression levels of *PDCD-1* in various kinds of clinical tumor tissues based on data from TCGA. Black dot indicates the median. (B-C) RT-PCR (B) and qRT-PCR (C) expression analysis of *PDCD-1* mRNA. Data are presented as the mean ± SD of three independent experiments.

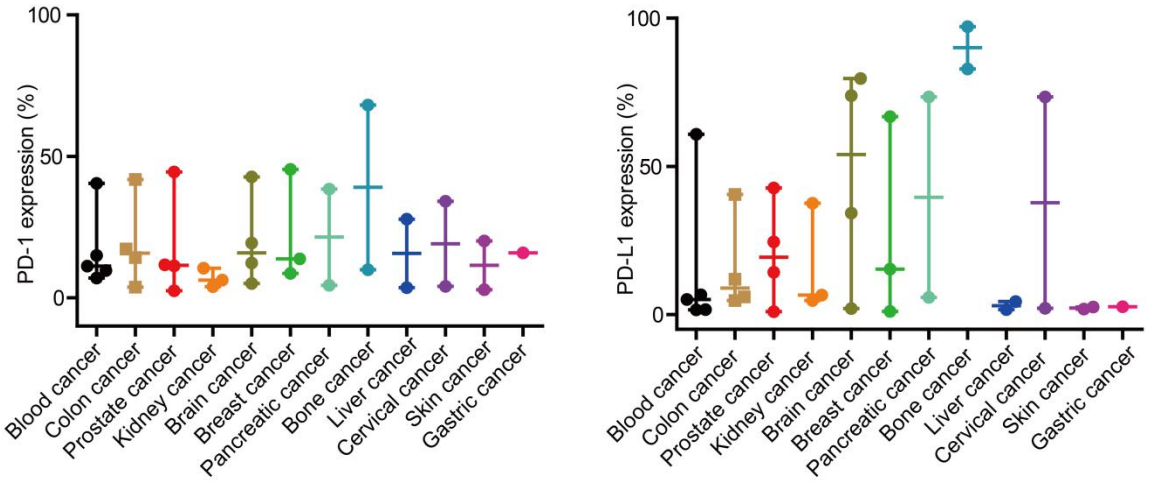

Fig. S2. PD-1 is Expressed in Human Tumor Cell Lines.

Percentages (median ± range) of PD-1 (left) and PD-L1 (right) surface protein expression in cell lines of our analysis from Table S1.

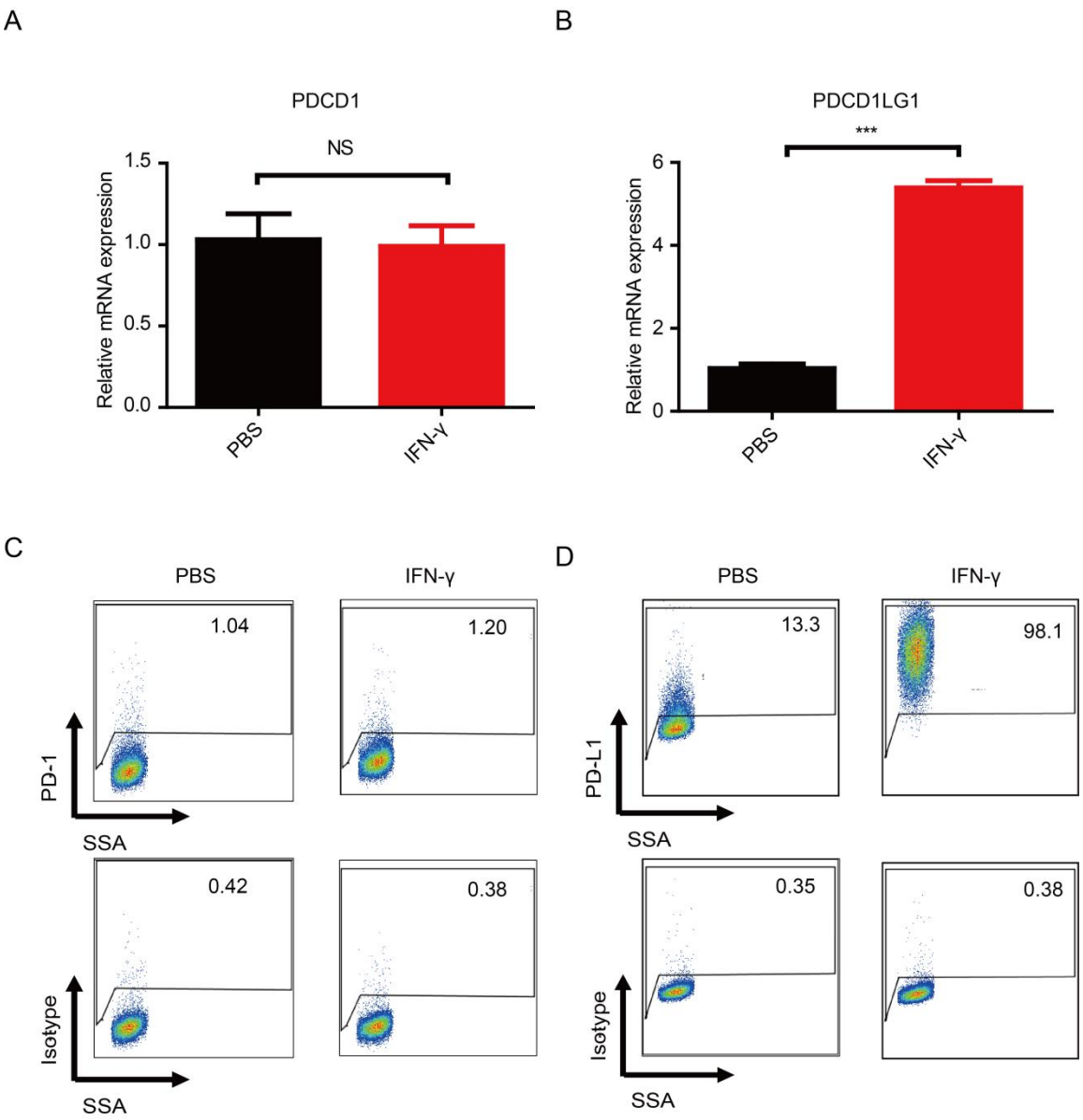

Fig. S3. IFN-γ Induces PD-L1 but not PD-1 Expression.

(A-B) qRT-PCR expression analysis for *PDCD1* (A) and *PDCD1LG1* (B) mRNA. (C-D) Representative flow cytometric plots of PD-1 (C) and PD-L1 (D) surface protein expression in NCI-H1299 cells after treatment with IFN-γ (100 ng/ml) for 48 hours. ***p<0.001. NS, no significance.

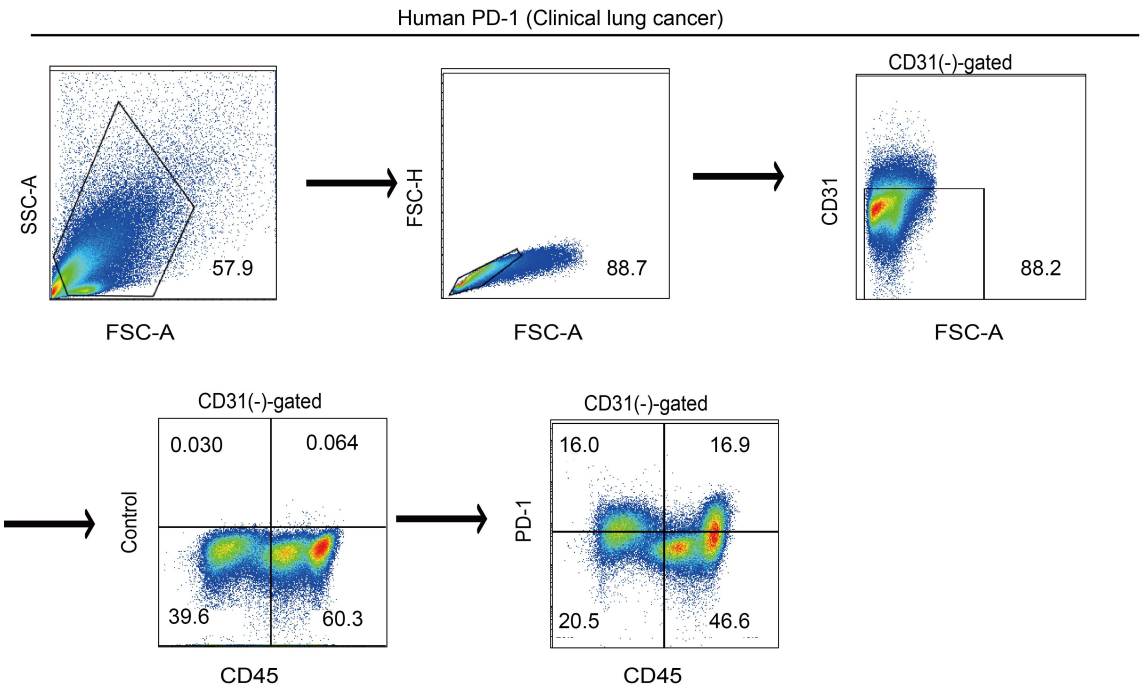

Fig. S4. The Procedure of Flow Cytometric Analysis.

Representative flow cytometric analysis of PD-1 surface protein expression by lung cancer cells negative for the CD31 endothelial cell marker and the CD45 lymphocyte common antigen in single-cell suspensions derived from a clinical tumor biospecimen.

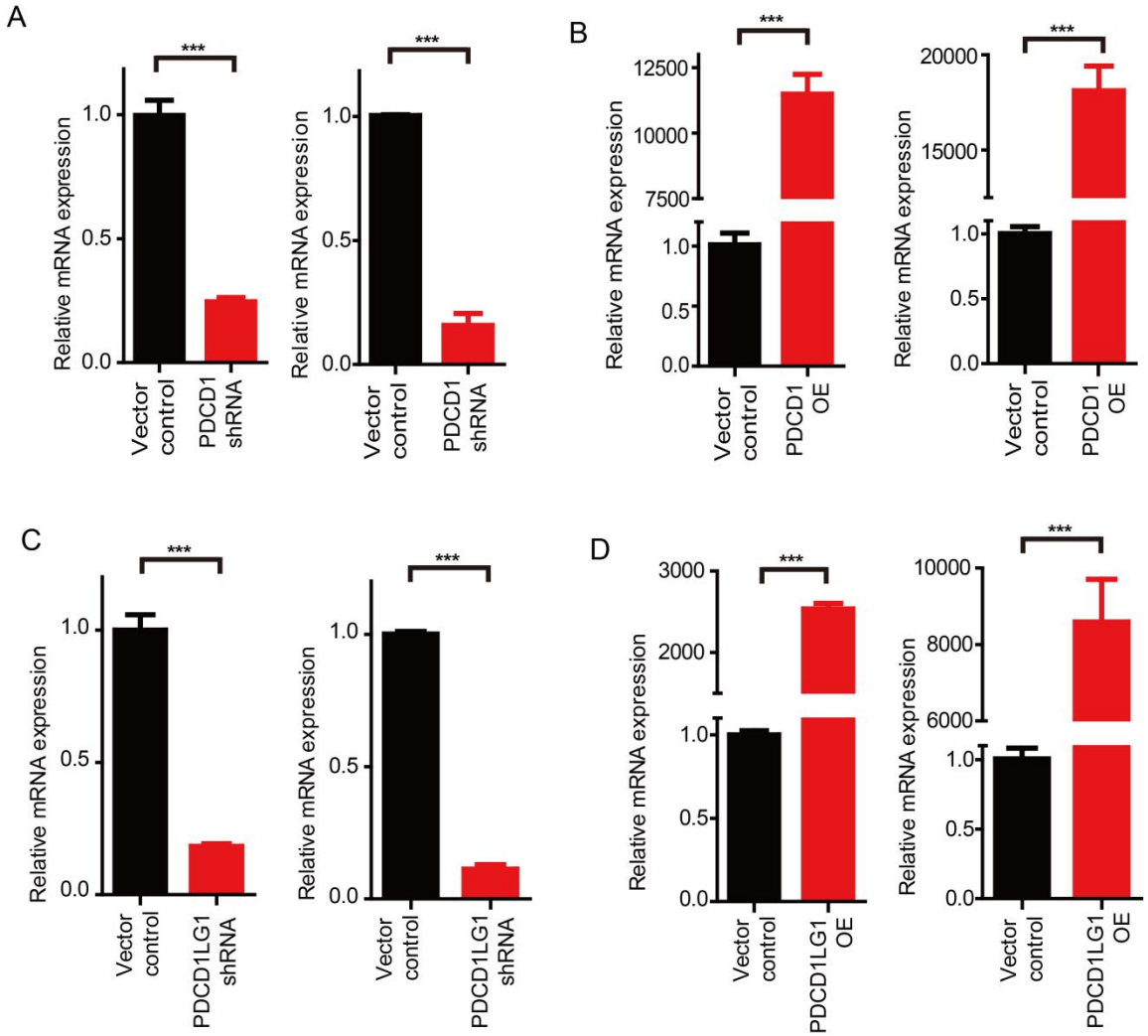

Fig. S5. Relative *PDCD1* and *PDCD1LG1* mRNA Expression.

Quantification of qRT-PCR from Cells (left: Calu-1 (A and C) and right: NCI-H1299 (B and D)) Transfected with the Indicated Plasmids. ***p<0.001.

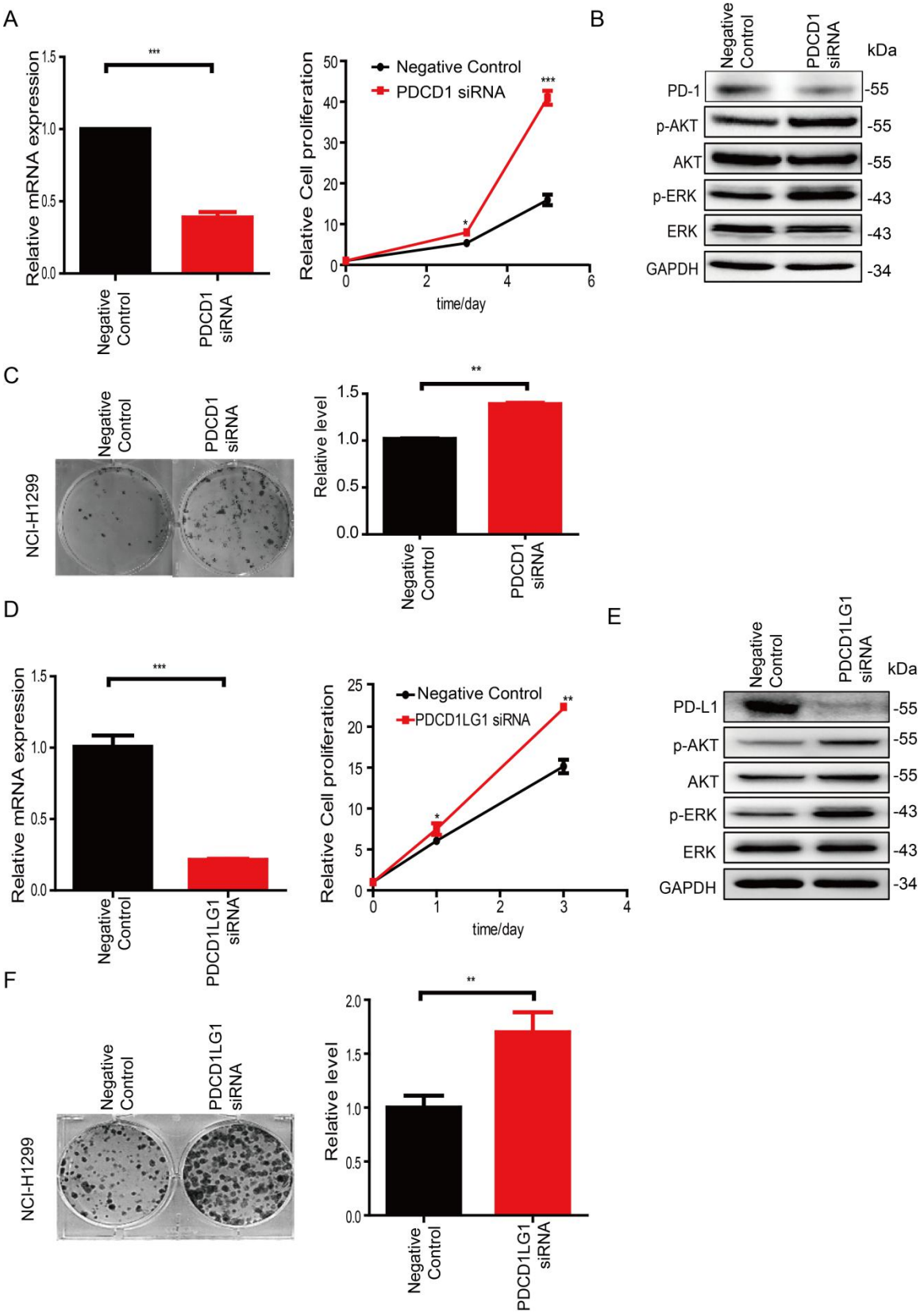

Fig. S6. Tumor Cell-Intrinsic PD-1 and PD-L1 Inhibit Tumor Cell Growth and Activation of Signaling.

(A and D) qRT-PCR assay (left) for the relative expression of *PDCD1* and *PDCD1LG1*. CTG assay assessing cell proliferation (right) in NCI-H1299 cells transfected with the indicated siRNAs. (B and E) Immunoblot analysis of the indicated proteins in NCI-H1299 cells transfected with the indicated siRNAs. (C and F) Representative colony formation assay (left) and quantification data (right) in NCI-H1299 cells transfected with the indicated siRNAs. Data are presented as the mean ± SEM from three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

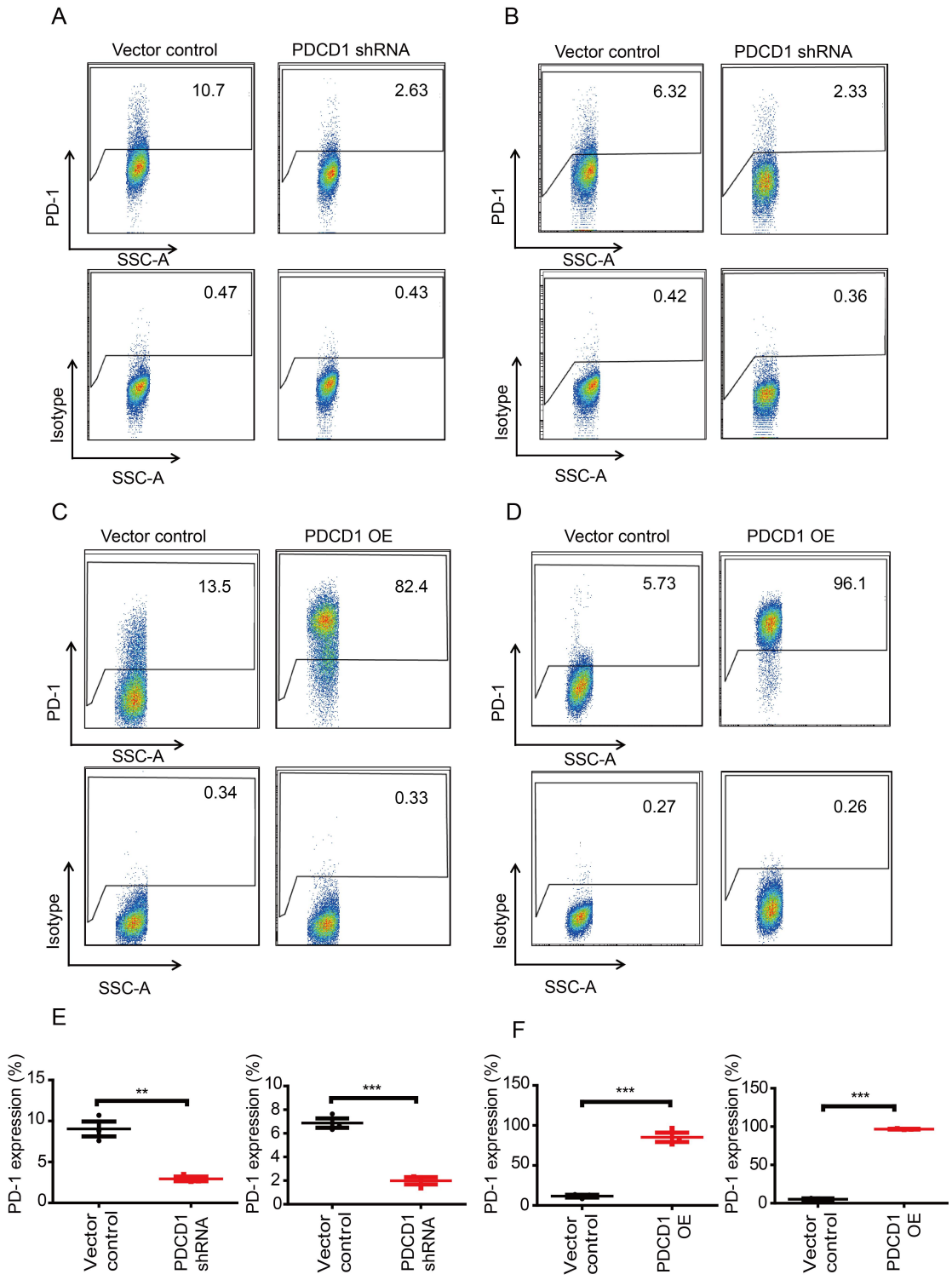

Fig. S7. Expression of Surface PD-1 in Calu-1 and NCI-H1299.

(A-B) Representative flow cytometry scatter plots showing expression of cell surface PD-1 in Calu-1 (A) and NCI-H1299 (B) cells 72 hours after transfection with the indicated plasmids. (C-D) Representative flow cytometry scatter plots showing expression of cell surface PD-1 in Calu-1 (C) and NCI-H1299 (D) cells 72 hours after transfection with the indicated plasmids. (E-F) Quantitation of

Calu-1 (left) or NCI-H1299 (gight) cells expressing surface PD-1 with *PDCD1* knockdown (E) or overexpression (F). Data are presented as the mean ± SD from three independent experiments. **p<0.01, ***p<0.001. These results from Fig. 2 A and E experiments.

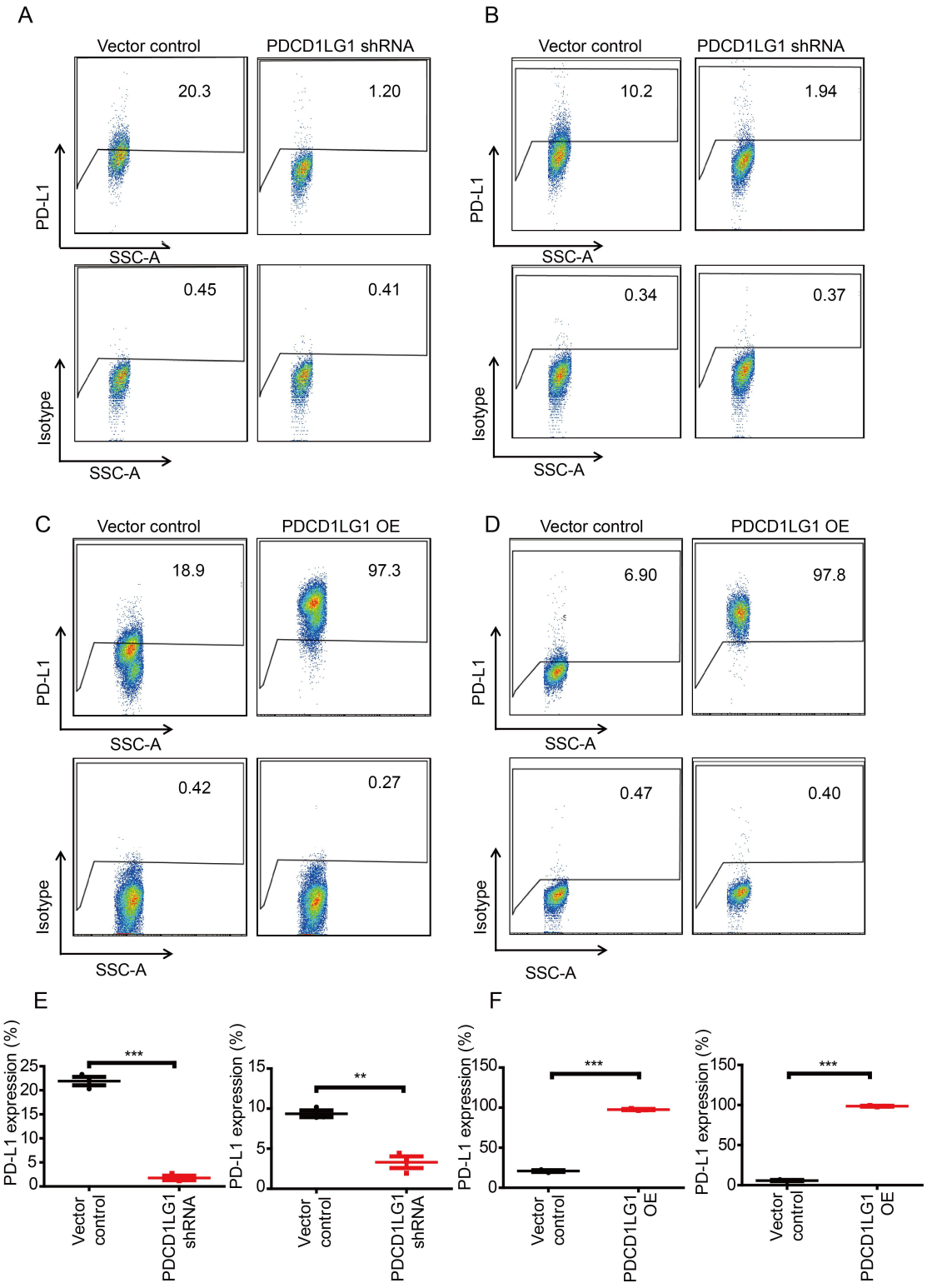

Fig. S8. Expression of Surface PD-L1 in Calu-1 and NCI-H1299.

(A-B) Representative flow cytometry scatter plots showing expression of cell surface PD-L1 in Calu-1 (A) and NCI-H1299 (B) cells 72 hours after transfection with the indicated plasmids. (C-D) Representative flow cytometry

scatter plots showing expression of cell surface PD-L1 in Calu-1 (C) and NCI-H1299 (D) cells 72 hours after transfection with the indicated plasmids. (E-F) Quantitation of Calu-1 (left) or NCI-H1299 (gight) cells expressing surface PD-L1 with *PDCD1LG1* knockdown (E) or overexpression (F). Data are presented as the mean ± SD from three independent experiments. **p<0.01, ***p<0.001**.** These results from Fig.3 A and E experiments.

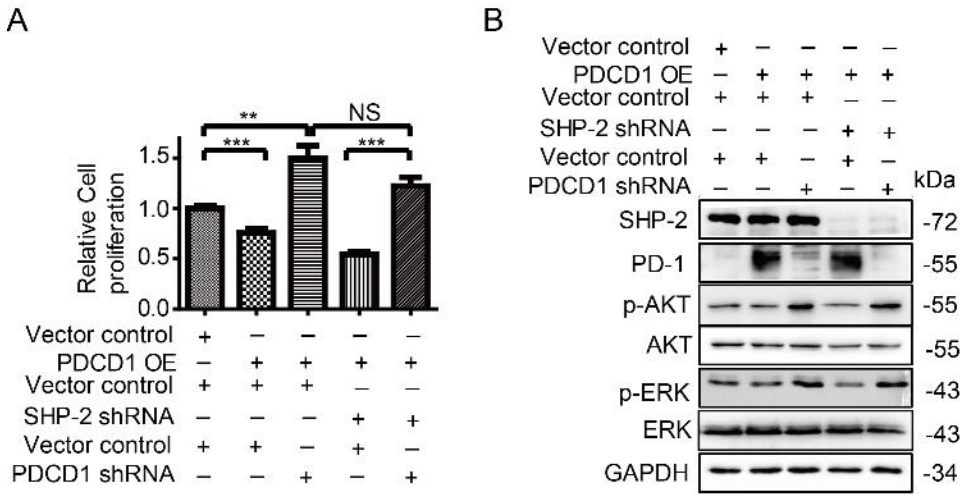

Fig. S9. SHP2 is Dispensable for Function of Tumor Cell-Intrinsic PD-1.

(A) The CTG assay assessing the cell proliferation of NCI-H1299 cells transfected with the indicated plasmids or shRNAs. (B) Immunoblot analysis of the indicated proteins in cells transfected with the indicated plasmids or shRNAs. Data are presented as the mean ± SEM from three independent experiments. **p<0.01,***p<0.001. NS, no significance.

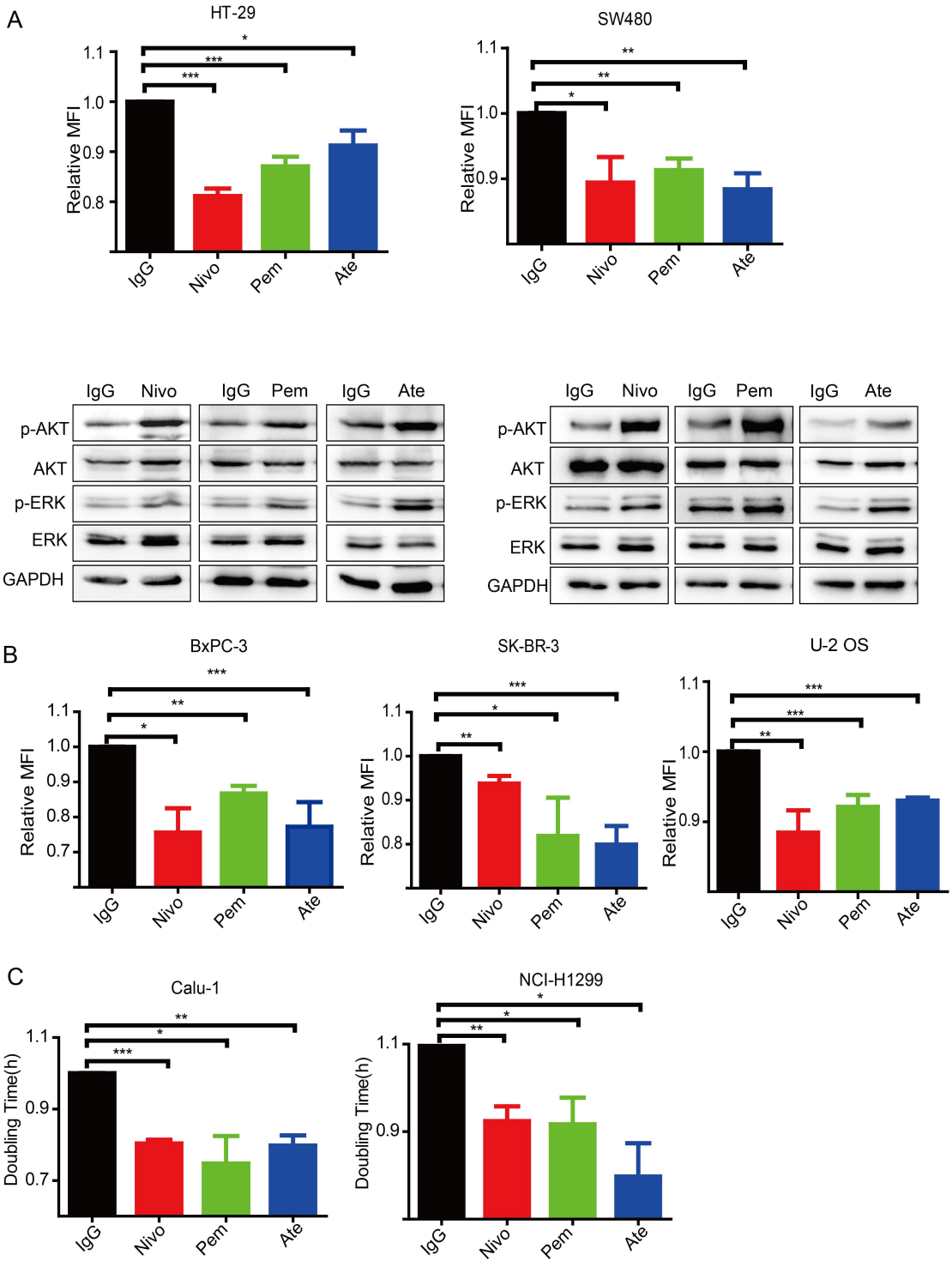

Fig. S10. The Function of Clinical Available Antibodies Targeting PD-1/PD-L1.

(A) CFSE assay assessing the relative proliferation of HT-29 and SW480 cells treated with the indicated antibodies (100 μg/ml) for 48 hours as MFI (top). Immunoblot analysis of cells after treated with the indicated antibody (100 μg/ml) for 6 hours (bottom). (B) CFSE assay assessing the relative

proliferation of BxPC-3, SK-BR-3 and U-2 OS cells treated with the indicated antibodies (100 µg/ml) for 48 hours as MFI. (C) xCELLilence RTCA assay assessing the relative proliferation of Calu-1 and NCI-H1299 cells treated with the indicated antibodies (100 µg/ml) for 48 hours. Data are presented as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

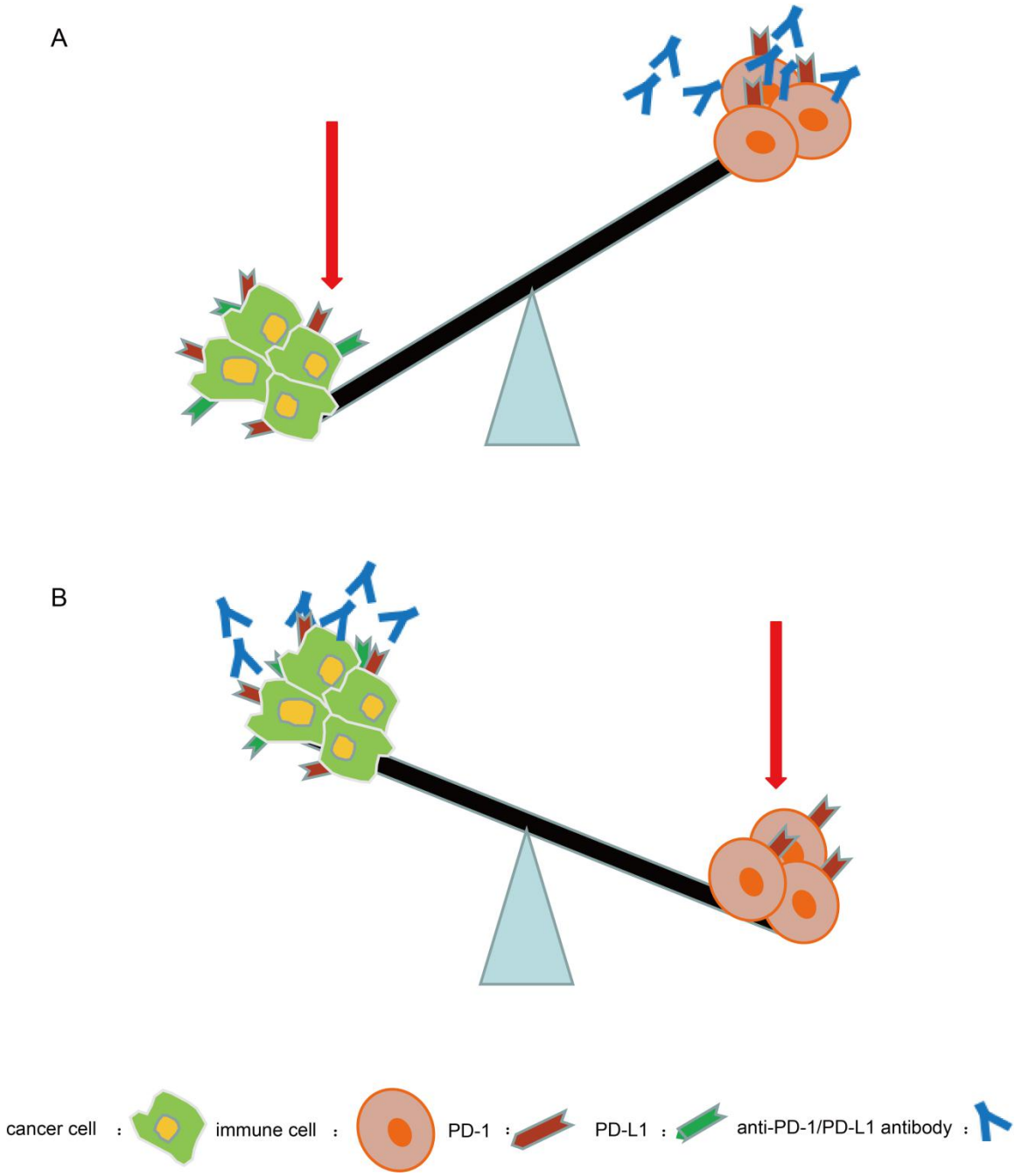

Fig. S11. A Proposed Model for PD-1/PD-L1-targeted Antibodies Balance Activation between T Cells and Tumor Cells.

(A) Antibodies targeting PD-1/PDL1 efficiently activate T cells, thus tumor cells are destructive. (B) HPD occurs after PD-1/PD-L1-targetd antibodies activate tumor cells and overwhelm activation of T cells.

Table S1. PD-1 and PD-L1 Surface Protein Expression.

Percentages of PD-1 and PD-L1 surface protein expression in various human cancer cell lines detected by flow cytometry. + represents positive population and - represents negative population.

	PD1+	PDL1+	PD1+PDL1+	PD1-PDL1-
Blood cancer cell lines				
Raji	6.27	0.95	0.75	92
K-562	11.7	3.35	3.29	81.6
JeKo-1	39.1	0.28	1.36	59.3
U-937	0.68	51.8	9	38.6
Jurkat	9.98	3.91	1.21	84.9
Colon cancer cell lines				
HT-29	1.66	3.57	2.14	92.6
RKO	4.59	27.8	12.7	54.9
SW480	37.9	0.85	3.91	57.3
HCT116	6.20	3.89	8.07	81.8
Prostate cancer cell lines				
PC-3	19.2	17.4	25.3	38.1
22Rv1	4.61	17.8	6.71	70.9
DU 145	11.2	0.50	0.52	87.8
LNCaP	1.69	13.5	0.79	84
Kidney cancer cell lines				
ACHN	1.77	2.03	4.52	91.7
769-P	1.52	2.31	2.43	93.7
786-O	1.66	28.8	8.8	60.7
Caki-1	1.83	58.2	11.3	28.7
Brain cancer cell lines				
M059J	1.95	33.1	40.8	24.2
SK-N-SH	6.22	21.0	13.2	59.5
M059K	1.4	68.8	10.9	18.9
SK-N-BE(2)	4.35	1.38	0.69	93.3
Breast cancer cell lines				
MCF-7	13.0	0.26	0.78	86.0
SK-BR-3	0.034	21.4	45.4	33.2
T-47D	1.78	8.49	6.89	82.8
Pancreatic cancer cell lines				
BxPC-3	1.13	2.47	3.35	93
AsPC-1	1.135	36.1	37.3	25.3
Bone cancer cell lines				

CRL8303	0.037	29.1	68.1	2.79
U-2 OS	0.68	73.6	9.29	16.4
Liver cancer cell lines				
Huh-7	27.1	1.02	0.70	71.2
Hep G2	1.06	1.79	2.58	94.6
Cervical cancer cell lines				
Hela	3.37	1.40	0.69	94.5
Ca Ski	1.22	40.5	32.9	25.3
Skin cancer cell line				
A-375	2.25	1.93	0.64	95.2
B16-F10	19.5	1.26	0.58	78.7
Gastric cancer cell line				
HGC-27	14.7	1.49	1.20	82.6

Table S2. Primers Used for qRT-PCR, Site-directed PD-1 Mutagenesis and the cDNA Target Sequences of shRNAs or siRNAs.

Gene	Species	Forward (5'-3')	Reverse (5'-3')
PDCD1	Human	TCGTGCTAAACTGGTACCGC	CTGACCACGCTCATGTGGAA
PDCD1 LG1	Human	GGACAAGCAGTGACCATCAAG	CCCAGAATTACCAAGTGAGTCCT
GAPDH	Human	CAATGACCCCTTCATTGACC	TTGATTTTGGAGGGATCTCG
PD-1 ITIM Site	Human	CTGTGTTCTCTGTGGACTTCGGGGAGCTGGATTTCC	GGAAATCCAGCTCCCCGAAGTCCACAGAGAACACAG
PD-1 ITSM Site	Human	CCTGAGCAGACGGAGTTCGCCACCATTGTCTTTCC	GGAAAGACAATGGTGGCGAACTCCGTCTGCTCAGG
PDCD1 shRNA	Human	GCTTCGTGCTAAACTGGTACC	N/A
PDCD1 LG1 shRNA	Human	CTGGCACATCCTCCAAATGAAAGGACTC	N/A
SHP2 shRNA	Human	GGCTGAGTTGGTCCAGTATTACATGGAAC	N/A
PDCD1 siRNA	Human	CCCTGTGGTTCTATTATATTT	N/A
PDCD1 LG1 siRNA	Human	GGAGAATGATGGATGTGAATT	N/A

References

1. T.Schatton *et al.,* Identification of cells initiating human melanomas. *Nature* **451**, 345-349(2008).
2. S. jiang *et al.,* Cholesterol induces epithelial-to-mesenchymal transition of prostate cancer cells by suppressing degradation of EGFR through APMAP. *Cancer Res*.**79**,3063-3075(2019).