Peptide Blocking of PD-1/PD-L1 Interaction for Cancer Immunotherapy
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Abstract
Immunotherapy has become a promising alternative therapeutic approach for cancer patients. Interruption of immune checkpoints, such as CTLA-4 and PD-1, has been verified to be a successful means for cancer therapy in clinical trials. mAb targeting PD-L1 has been approved to treat urothelial carcinoma, non-small cell lung cancer, or Merkel cell carcinoma by the FDA. However, the high cost of the antibody can limit its application. In our study, targeting PD-L1 peptide (TPP-1), which specifically binds to PD-L1 with high affinity, was identified through bacterial surface display methods. Using a T-cell activation assay and mixed lymphocyte reaction, TPP-1 was verified to interfere with the interaction of PD-1/PD-L1. To examine the inhibitory effect of TPP-1 on tumor growth in vivo, a xenograft mouse model using H460 cells was established. The growth rate of tumor masses in TPP-1 or PD-L1 antibody–treated mice was 56% or 71% lower than that in control peptide–treated mice, respectively, indicating that TPP-1 inhibits, or at least retards, tumor growth. IHC of the tumors showed that IFNγ and granzyme B expression increased in the TPP-1 or PD-L1 antibody–treated groups, indicating that TPP-1 attenuates the inhibitory effect of PD-L1 on T cells and that T cells may get reactivated. On the basis of our data, TPP-1 peptide could work as an alternative to antibodies for tumor immunotherapy. Cancer Immunol Res; 6(2): 178–88. ©2017 AACR.

Introduction
To become fully activated, T cells need to receive antigen-independent costimulatory signals through the corresponding receptors. However, coinhibitory receptors are also expressed on T cells to regulate their activation, tolerance, and immunopathology, thereby balancing the immune system (1). Cytotoxic T lymphocyte–associated protein 4 (CTLA-4, CD152; ref. 2) is a critical inhibitory receptor expressed on cytotoxic T lymphocyte cells and acts as an important brake in the regulation of cell-cycle progression. Ipilimumab, an mAb targeting CTLA-4, was the first immunotherapeutic antibody drug approved by the FDA in 2011 (3) for the treatment of advanced melanoma. Although cancer immunotherapy with anti-CTLA-4 has achieved modest success in clinical practice in the past several years, data collected from both animal experiments and clinical trials indicate a potential risk of autoimmunity or other severe adverse events related to anti-CTLA-4 administration (3, 4). Programmed cell death 1 (PD-1, CD279) and programmed cell death ligand 1 (PD-L1, B7-H1, CD274) are recognized as promising targets for cancer immunotherapy (2, 4).

Preliminary clinical findings of the mAbs targeting PD-1/PD-L1 show equivalent antitumor activity but moderate side effects compared with targeting CTLA-4 (5, 6). B7-1 (CD80) and B7-2 (CD86), the ligands of CTLA-4, are expressed on antigen-presenting cells (APC). Blockade of CTLA-4 primarily activates T cells in secondary lymphoid organs (4, 7) and, therefore, has a systemic impact that is not confined to the tumor microenvironment (TME) only, which may explain why mAbs targeting the CTLA-4 pathway can induce severe autoimmune disease or other adverse events.

PD-L1 is expressed on many types of cancers (8–10), and its high expression in tumor cells or presence in TME can be indicative of tumor-infiltrating lymphocytes (11). Blockade of PD-1/PD-L1 can strengthen the function of effector T cells and increase their production of cytokines through reactivation (1, 12). It mainly has local and regional influence on the immune system, although some systemic side effects can occur. Compared with CTLA-4 antibody, which can have severe systemic side effects, the blockade of PD-1/PD-L1 pathway could be viewed as relatively safer than that of CTLA-4 (4). Therefore, the application of drugs targeting PD-1/PD-L1 immune checkpoint could be a more promising approach for the cancer immunotherapy. Three PD-L1 mAbs, atezolizumab, durvalumab (MEDI4736), and avelumab (13–15), have been approved by the FDA. Although the antibodies have made great progress in cancer treatment, their application in patients is still limited due to high production costs and immunogenicity.

Specific binding peptides have the advantage of much lower production cost and amenability to chemical synthesis. Peptide therapeutics, such as the GLP-1 peptide (approved by the FDA for the treatment of type II diabetes), have gained wide application in the clinic (16). Peptides targeting cancer cells, cytokines, and...
membrane receptors have been investigated for the diagnosis and treatments of cancers (17–19). It has also been demonstrated that shielding the critical region of peptides by glycosylation or PEG conjugation is effective at reducing immunogenicity (20–22). Peptides with low molecular weights have the ability of good tissue penetration compared with antibodies. Because of their low molecular weight, peptides could be delivered into the core of tumor masses (23). In this report, bacterial surface display methods (24) were used to screen a PD-L1–targeting peptide with high affinity and specificity. In vitro and in vivo experiments showed that one of PD-L1–binding peptides could interfere with PD-1/PD-L1 biological function and, therefore, could be applied as a potential drug for tumor immunotherapy.

**Materials and Methods**

**Reagents and cell culture**

Recombinant human PD-L1 (cat. #10084-H08H), PD-2 (cat. #10292-H08H), GM-CSF (cat. #10015-HNAY), IL2 (cat. #11846-HNAAE), IL2 (cat. #11848-HNAY1), bFGF (cat. #10014-HNAAE), and recombinant mouse PD-L1 (cat. #50010-M08H1) were purchased from Sino Biological Inc. Anti-human PD-L1 (cat. #557924, clone MHIH), anti-human PD-1 (cat. #557946, clone MHIH), and anti-human CD4 (cat. #561841, clone RPA-T4) were purchased from BD Biosciences. Dynabeads MyOne Streptavidin C1 magnetic beads (cat. #65002), Biotin-XX Microscale Protein Labeling and Detection Kit (cat. #B30756), streptavidin PE (SAPE) conjugates (cat. #S-866), DynaMag-Spin Magnet (cat. #12320D), Dynabeads human T-activator CD3/CD28 (cat. #11131D), and Ham’s F-12K medium (cat. #21270030) were obtained from Thermo Fisher Scientific. Human serum (cat. #ZX101-1) was bought from Sigma Aldrich. BSA V (cat. #A8020) was obtained from Solarbio. Purified functional-grade anti-human CD3 (cat. #16-0039-81) was obtained from eBioscience. Anti-IFNγ (cat. #BA0952) was obtained from BOSTER. Anti-granzyme B (GZMB; cat. #557924-1-AP) was obtained from Proteintech. The human IFNγ ELISA Kit (cat. #70-EK1802) was bought from Multisciences. Vita-Orange cell line MIH4, and anti-human CD4 (cat. #561841, clone RPA-T4) were kindly provided by Professor Guangli Suo (Suzhou Institute of Nano-tech and Nano-Bionics, CAS, Suzhou, China). MDA-MB-231 and MDA-MB-435 were cultured in DMEM medium. CHO-K1 over-expressing PD-L1 (CHO-K1/PD-L1) and its parent cell line, CHO-K1, and the plasmid of plvx-puro/luciferase were kindly provided by Icartab Biomed of Suzhou and were maintained in F-12K medium. The media were all supplemented with 10% FBS and 1% penicillin/streptomycin, and all cultured at 37°C, 5% CO2. All cells lines were tested to be free from mycoplasma contamination by a mycoplasma genus-specific PCR.

**Screening of binding peptides to PD-L1 from a random display library**

A random bacterial surface display library was kindly provided by Professor Patrick S. Daugherty (University of California, Santa Barbara, Santa Barbara, CA). Peptides with 15 randomized amino acid sequences were displayed at the N-terminus of enhanced circularly permuted OmpX (eCPX), an outer membrane protein of *Escherichia coli*. These bacteria express the peptide and eCPX simultaneously under the control of the araBAD promoter (24), and the library size consisted of 5 × 108 clones. PD-L1 was biotinylated and binding (the quantity of biotin) was detected according to the protocol for the Biotin-XX Microscale Protein Labeling Kit with FluorReporter Biotin Quantitation Assay Kit.

Isolation of peptide–bacteria complexes was performed as previously reported with some modification (26). Briefly, the library size was reduced by magnetic-activated cell sorting (MACS) with 40 nmol/L biotinylated PD-L1. After MACS, the new library was grown further, and the library size was estimated. MACS was repeated until the library size was smaller than 1 × 107, and then FACS was performed using a FACS Aria II (Becton Dickinson). The concentration range of PD-L1 that we used for screening was from 40 to 10 nmol/L. The incubation duration and temperature for the peptide library with biotinylated PD-L1 was 45 minutes at 4°C. Following PD-L1 incubation, SAPE (final concentration, 5 nmol/L) was added and incubated for 30 minutes. The bacteria with higher fluorescence intensity were sorted by flow cytometry and cultured in LB medium overnight for the next screening round. To improve specificity, 1% (v/v) human serum was added in the last three to five rounds. Once the binding rate and mean fluorescence intensity stopped increasing after two consecutive rounds, the bacterial clones were collected and sent to GENEWIN Inc. for DNA sequencing.

**Construction of the focused library**

The focused library with the format X5CWCWRX5 on the N-terminus of eCPX was constructed by using degenerate code NVS oligonucleotides (27, 28) as the forward primer. Construction of the focused bacterial display peptide library was performed as described previously (29). In short, the procedure involved preparing the vector and the insert DNA segment, performing test ligations, producing electro-competent *E.coli* cells (MC1061), transforming the ligation, and counting the library size. Ten clones from LB agar plates were randomly selected and were sequenced to evaluate the quality. Focused library screening procedures were performed as already described in the methods. Starting concentration of PD-L1 was 10 nmol/L, and 3% (v/v) human serum was added in the following screening.

**Binding property analysis of peptides displayed on bacterial surfaces**

The binding properties of clones to PD-L1 were compared under four conditions: (i) at the concentration of 2 nmol/L, (ii) at different concentrations ranging from 0.2 to 4.8 nmol/L, (iii) undergoing rigid washing or normal washing procedures; and (iv) with the addition of BSA, IL2, IFNγ, or bFGF. The bacterial clones undergoing “rigid washing” were suspended in PBS (pH 7.4) and incubated at 4°C for 5 minutes, whereas the clones undergoing “normal washing” were suspended only in PBS. The binding ability of clones under the above conditions was measured by flow cytometry.
Measuring the $K_D$ value of TPP-1 to PD-L1

TPP-1 (TPP-1: SGQYASHWCW/DPGRSGSK) was synthesized at GL Biochem Ltd, and the N-termini of the peptides were labeled with FITC. A scrambled TPP-1 peptide named SPP-1 (SPP-1: SGQCWRDWSYRWHPSCACGGSK) was designed and synthesized and served as a control (26).

SPP-1 (SPP-1: SGQGYRWDSYRWHPSCACGGSK) was designed and synthesized and served as a control (26). The affinity constant ($K_D$) and kinetics ($k_{on}$ and $k_{off}$) of TPP-1 peptide binding to PD-L1 were determined by surface plasmon resonance (SPR, Biacore200). PD-L1 was immobilized on the surface of a XanTex sensor chip. The remaining reactive sites were blocked with 1% ethanolamine at the flow rate of 10 $\mu$L/minute for 7 minutes. TPP-1 was dissolved and diluted with HEPES buffer (HBS, pH 7.4). Peptide concentrations were 0.625, 1.25, 2.5, 5.0, and 10.0 $\mu$g/mL. Each sample was injected at a flow rate of 25 $\mu$L/minute for about 15 minutes. $K_D$, $k_{on}$, and $k_{off}$ were calculated by Biacore200 Evaluation Software, version 2.0.

Examination of binding specificity of the soluble peptides

The binding specificity of TPP-1 to PD-L1 was examined with both a fluorescence-based ELISA and cell-based analyses. For fluorescence-based ELISA analysis, fluorescence plates (Costar) were coated with 100 $\mu$L (1 $\mu$g/mL) of human PD-L1 (hPD-L1), human PD-L2 (hPD-L2), mouse PD-L1 (mPD-L1), or BSA in PBS (pH 7.4) overnight at 4°C. Plates were then blocked with 1% BSA in PBST (PBS with 0.05% Tween 20) for 1 hour at room temperature. After washing twice with PBST, the pellets (20 $\mu$mol/L) were added into each well and incubated for another 1.5 hours at room temperature. Afterward, the plates were washed three times with PBST. The relative fluorescence intensity of each well was determined by Cytation 3 (BioTek) using 488 nm excitation and 515 nm emission wavelengths.

Cell-based analysis was performed on both attached and detached cells by flow cytometry and fluorescence microscopy, respectively. CHO-K1, CHO-K1/PD-L1, MDA-MB-231, and MDA-MB-435 cells were coated with 0.25% trypsin and diluted to a final concentration of 1 $\times$ 10^6 cells/mL. Afterward, cells were incubated with 4 $\mu$mol/L FITC-labeled peptides or PD-L1 antibody (BD Biosciences) for 40 minutes on an inversion shaker at 4°C, and then each tube was washed with PBS twice and analyzed with a flow cytometer.

For the fluorescence microscopy, CHO-K1 and CHO-K1/PD-L1 were seeded in a 12-well plate (2 $\times$ 10^5 cells/well). At 70% to 80% confluency, cells were washed with PBS twice, followed by blocking with 1% BSA for 10 minutes. FITC-labeled peptides were added at a final concentration of 10 $\mu$mol/L and incubated for 30 minutes at room temperature. Afterward, cells were washed with PBS twice and fixed with 4% paraformaldehyde. Cell nuclei were stained with Hoechst 33,342 (2 $\mu$g/mL, Sigma Aldrich) for 10 minutes and then washed with PBS twice. Cells were visualized under a fluorescent microscope (Nikon Ti).

Ligand inhibition assay

Examination of the interference of TPP-1 peptides on the binding of PD-L1 to PD-1 or durvalumab was performed with a fluorescence-based ELISA and cell-based analyses. For fluorescence-based ELISA analysis, each well of a 96-well plate was precoated with 100 $\mu$L (1 $\mu$g/mL) PD-L1 in PBS overnight at 4°C. On the second day, the plate was blocked with 1% BSA in PBST, and then 100 $\mu$mol/L biontinated PD-1 or durvalumab with FITC-labeled TPP-1 (at the final concentration of 40, 20, 10, 5, or 2.5 $\mu$mol/L) was added into each well and incubated at room temperature for 1.5 hours. After being washed twice with PBST, the relative FITC fluorescence intensity was determined by Cytation 3 (488 nm excitation and 515 nm emission). For the detection of the biontinated PD-1 or durvalumab signals, SAPE (at the final concentration of 5 nmol/L) was added into wells and incubated for 30 minutes at room temperature. After being washed twice with PBST, the relative PE fluorescence intensity was determined by Cytation 3 (488 nm excitation and 575 nm emission). Cell-based analysis was performed with a flow cytometer. CHO-K1/PD-L1 cells were incubated with TPP-1 peptide (at the final concentration of 40, 20, 10, or 0 $\mu$mol/L) plus 100 nmol/L biontinated PD-1 or durvalumab for 60 minutes on an inversion shaker at 4°C. SAPE (5 nmol/L) was added after being washed twice with PBS and then incubated for another 30 minutes. Samples were analyzed by flow cytometry.

T-cell activation assay

Human peripheral blood mononuclear cells (PBMC) were isolated from the blood of a healthy donor by the histopaque-1077 as per the manufacturer's instructions. CD4^+ T cells were isolated from PBMCs using FACS, and the sorting efficiency was detected by a CD4-APC antibody. To generate CD4^+ T-cell blasts, cells were adjusted to 5 $\times$ 10^6 cells/mL in IMDM with 10% FBS and IL2 (20 ng/mL), followed by the addition of human T-activator CD3/CD28 beads ( bead-to-cell ratio was 1:1). Fresh medium was added every other day, and cells were harvested and frozen at day 7 for later use.

For each test well in the 96-well plate, 100 $\mu$L anti-human CD3 (1 $\mu$g/mL) was added into each well and incubated at 4°C overnight. On the second day, the antibody solution was aspirated out, and wells were washed with 200 $\mu$L PBS twice. Afterwards, 100 $\mu$L PD-L1 at the final concentration of 0.1 $\mu$mol/L was added into the wells, and plates were held in the 37°C incubator for 3 hours, followed by washing with PBS twice. Next, durvalumab and peptides (TPP-1 or SPP-1) at various concentrations were added into the corresponding wells, and the plates were incubated at 37°C for 1 hour followed by two washes with PBS. Finally, CD4^+ T cells (at the concentration of 5 $\times$ 10^5 cells/well in 150 $\mu$L medium) were added, followed by the centrifugation at 1,000 rpm for 2 minutes. Cells were incubated in a humid incubator with 5% CO_2 at 37°C for 3 days. Culture supernatants were collected, and the quantitation of IFNγ was detected using the human IFNγ ELISA Kit. T-cell proliferation rate was evaluated by the ViTa-Orange cell viability reagent (has greater linear relationship between cell number and OD450 value, according to the reagent instructions).

Mixed lymphocyte reaction

Fresh PBMCs were cultured in IMDM at 37°C for 2 hours and washed twice with PBS after removing the supernatant. The remaining cells were cultured in IMDM plus 10% FBS, GM-CSF (25 ng/mL), and IL4 (50 ng/mL). Fresh medium was added every 3 days. On day 6, TNFα (50 ng/mL) was added to induce dendritic cells (DC) for 24 hours. PD-L1 expression was detected by flow cytometry. Mature DCs were frozen for later use.

Before mixed lymphocyte reactions (MLR), DCs were treated with mitomycin C (MMC, 100 $\mu$g/mL) for 2 hours. The frozen CD4^+ T cells were thawed and cultivated overnight. About 1 $\times$ 10^5 cells were seeded in each well with a DC:T ratio of 1:10 in 200 $\mu$L IMDM plus 10% FBS. IFNγ in supernatants and cell proliferation was quantified at day 3.
In vivo studies

Five to six-week-old female Balb/c nude mice (SLAC Laboratory Animal) were used in this experiment to evaluate antitumor activity in vivo, and mice were housed in environmentally controlled, specific pathogen-free conditions. All animal studies were approved by a local Ethics Committee for Animal Experiments.

The FITC-conjugated TPP-1 peptides (4 mg/kg) were injected subcutaneously, and the relative FITC fluorescence intensity was measured by the IVIS Lumina II system (PerkinElmer) at time points of 0, 6, 12, 24, 48, and 72 hours. The half-life of the peptide in subcutaneous tissue was calculated to determine and assess the peptide dosing interval.

The H460 cells were transfected with the plvx-puro/luciferase lentiviral vector, followed by selection with puromycin (1 mg/mL), and clones with stable expression were established through monoclonal cultivation. Human PBMCs were enriched and activated as described above. On the fifth day, PBMCs were added into the plates of luciferase-expressing H460 cells (H460-luc), which were treated with mitomycin C, followed by coculturing another 3 days. H460-luc cells (2 × 10^6), along with 5 × 10^5 PBMCs in 0.1 mL PBS, were injected subcutaneously into the flank of the mice. TPP-1 (2 or 4 mg/kg), SPP-1 (4 mg/kg), and durvalumab (0.1 mg/kg) were injected subcutaneously of each group every other day eight times. The group injected with only tumor cells, followed by treating with TPP-1 (4 mg/kg), was appointed as control. The tumor volumes were measured with calipers in two dimensions (tumor volumes = 1/2 × a × b^2, a is the length and b is the width). The tumor mass was evaluated by measuring the bioluminescence with the IVIS Lumina II system (PerkinElmer) every week for a total of five times. All the mice were killed on day 40, and the tumor tissues were collected. The expression human IFNγ and GZMB in tumor tissues of each group were determined by IHC with corresponding antibodies. Digital pictures were taken by a Zeiss Scope A1, and the percentage of immuno-positive stained area was assessed using a digital image analyzing software (ImageJ, http://rsb.info.nih.gov/ij/; ref. 30).

Structure prediction and model construction of TPP-1 to PD-L1

The three-dimensional model of TPP-1 was predicted through the local meta-threading server LOMETS (31), and the binding model complex of TPP-1 to PD-L1 was described by ZDOCK (32). The Vector NTI-3D Molecular Viewer software (Thermo Fisher Scientific) was used to visualize the complex of TPP-1 and PD-L1.
Statistical analysis
All the data were expressed as means ± SEM. Histograms and line charts were generated by GraphPad Prism 6.0. t tests were used to determine the P values. A P value < 0.05 was considered to be statistically significant.

Results
Identification of the PD-L1–binding peptides from a random library
A random bacterial surface display library was used to screen and identify the PD-L1–binding peptides. MACS and FACS were used to enrich the binding peptide for PD-L1 (screening procedures shown in Fig. 1A). After one cycle of MACS, the library size was about $5 \times 10^6$ peptides, and FACS was then used for screening. After eight cycles of FACS, the percentage of bacteria in the sorting gate increased from 2.1% (40 nmol/L PD-L1) to 54.1% (10 nmol/L PD-L1 with 1% human serum; Fig. 2A). Forty bacterial clones were randomly selected for sequencing, and nine different peptide sequences were obtained. The sequences were aligned using Vector NTI software (Fig. 2B), and the consensus sequence “CWCWR” was detected using WebLogo 3.3 (Fig. 2C). The soluble peptides of the CWCWR sequence were synthesized, and the binding specificity was tested by flow cytometry with MDA-MB-231 and MDA-MB-435 (Supplementary Fig. S1), which had high and low PD-L1 expression, respectively. The results indicated that the specificity of the consensus sequence needed to be improved.

Identification of peptides with high affinity and specificity
To improve the affinity and specificity of the PD-L1–binding peptides, a focused library with the format $X_5$CWCWR$X_5$ was constructed into pBAD33-eCPX (Supplementary Fig. S2A). The library size was about $5 \times 10^7$ (count of clones on the LB agar plates). The sequencing results (Supplementary Fig. S2B) indicated that the quality of this library met requirements and could be used for the following screening. After one cycle of MACS and 13 cycles of FACS, the mean fluorescence intensity of PE-A increased from 31 (10 nmol/L PD-L1) to 1,287 (2 nmol/L PD-L1 with 3% human serum; Fig. 2D). A total of 20 clones were selected, and seven different peptide sequences were obtained (Fig. 2E). No obvious consensus sequence was identified.

Binding properties of peptides displayed on the bacterial surface
The binding properties of peptides displayed on the surface of bacteria were preliminarily analyzed by flow cytometry. With 2 nmol/L PD-L1, clone 1 and clone 3 showed significantly higher binding ability than clone 2 and random library clones (Supplementary Fig. S3A). With the increasing of PD-L1 concentration from 0.2 to 4.8 nmol/L, the binding properties of clone 1 and
clone 3 showed the same trend (Supplementary Fig. S3B). Binding abilities of clone 1 and clone 3 both declined significantly when undergoing the rigid washing procedure after incubation with PD-L1 (Supplementary Fig. S3C). The decline extent of both clone 1 and clone 3 was similar. The additions of BSA, IL4, IFNg, or bFGF into corresponding incubation systems did not influence the binding ability (Supplementary Fig. S3D). All these results show that clone 1 and clone 3 were better than clone 2 in binding affinity and specificity, with no significant differences between themselves.

TPP-1 binds specifically to PD-L1 with high affinity

The peptides of clone 1 and clone 3 were synthesized, and solubility tests indicated that clone 3 was easier to dissolve in the

Figure 3.
The physicochemical properties of TPP-1 peptide. A, The binding affinity of TPP-1 peptide to PD-L1 examined by an SPR method. B, The binding specificity of TPP-1 to hPD-L1, hPD-L2, mPD-L1, and BSA analyzed by fluorescence-based ELISA (n = 5). C, The binding ability of TPP-1 to CHO-K1 (dotted) and CHO-K1/ PD-L1 (solid) determined by flow cytometry (n = 3). D, The changes in fluorescence signals of TPP-1-FITC (solid lines) and PD-1-PE (dotted lines) with the increased concentration of peptide analyzed by fluorescence-based ELISA (n = 3). E, PD-1-binding capacity to CHO-K1/PD-L1 cells in the presence of different concentrations of TPP-1 peptides was analyzed by flow cytometry (n = 3). F, Changes in fluorescent signals of TPP-1-FITC (solid lines) and durvalumab-PE (dotted lines) with increasing concentrations of peptide analyzed by fluorescence-based ELISA (n = 3). G, Durvalumab-binding capacity to CHO-K1/PD-L1 cells in the presence of different concentrations of TPP-1 peptides was analyzed by flow cytometry (n = 3). Data, mean ± SEM; †, P < 0.05; ‡, P < 0.01, unpaired t test.
signals of PD-1

The $K_D$, $k_a$, and $k_d$ of the binding between TPP-1 and PD-L1 were determined by the SPR method (Fig. 3A). According to our preliminary data, the single mode was used to determine the affinity and kinetics. Five different concentrations of TPP-1 were tested, and the $K_D$ value was $9.467 \times 10^{-8}$ mol/L ($3.022 \times 10^{-4}$ Ms$^{-1}$ for $k_a$ and $3.192$ s$^{-1}$ for $k_d$).

hPD-L2 and mPD-L1 had the most similar amino acid sequences to PD-L1 as described in previous reports (33, 34), as well as similar spatial structure. The ELISA results (Fig. 3B) showed that TPP-1 had no cross-reactivity to mPD-L1 and hPD-L2, indicating its specificity to PD-L1.

To evaluate the binding specificity in a more physiologic environment, the binding ability of TPP-1 to CHO-K1/PD-L1 cells was examined by flow cytometry and fluorescence microscopy. The expression of PD-L1 in CHO-K1/PD-L1, MDA-MB-231, and MDA-MB-435 was measured by flow cytometry (Supplementary Fig. S3A). Compared with MDA-MB-435, CHO-K1/PD-L1, and MDA-MB-231 cell lines had higher PD-L1 expression. TPP-1 had higher binding to CHO-K1/PD-L1 and MDA-MB-231 cells (Fig. 3C; Supplementary Fig. S5B). In contrast, for MDA-MB-435 cells (expression of PD-L1 is relatively low), TPP-1 had low binding (Supplementary Fig. S5B). TPP-1 peptide at higher concentrations had comparable staining with PD-L1 mAbs. Similar results were seen for CHO-K1 cells using fluorescence microscopy (Supplementary Fig. S6). Together, these results confirm that TPP-1 can not only bind specifically to free PD-L1 but also to cells that expressed PD-L1 on their cell membrane.

**TPP-1 can block the interaction of PD-1 and PD-L1**

In the ligand inhibition assay, the interference of TPP-1 peptides on the binding of PD-L1 to PD-1 or durvalumab was investigated either using fluorescence-based ELISAs or cell-based analysis. The ELISA results showed that the TPP-1 peptide could effectively bind to PD-L1, even in the presence of PD-1 or durvalumab (solid lines of Fig. 3D and F). The fluorescence signals of PD-1–PD-L1 were decreased with the increased concentration of TPP-1 peptide. However, the fluorescent signals of durvalumab–PE did not change significantly (dotted lines of Fig. 3D and F). From these results, we assumed that TPP-1 at the tested concentrations did not block durvalumab (100 n mol/L) but could interfere the interaction between PD-1 (100 n mol/L) and PD-L1. However, our results did not make clear whether the binding site of TPP-1 to PD-L1 was overlapped with that of durvalumab. It has been shown that the binding site of durvalumab to PD-L1 is highly overlapped with that of PD-1 (35). Considering the relative lower affinity of TPP-1 to PD-L1, we hypothesized that for TPP-1 to block PD-L1/PD-L1 interaction, the binding site of TPP-1 to PD-L1 would need to be overlapped with that of PD-1. Our hypothesis was validated by flow cytometry (Fig. 3E and G). As the concentration of TPP-1 was increased, the PD-1–binding capacity toward CHO-K1/PD-L1 was decreased. Durvalumab did not show the same trend.

**TPP-1 reverses PD-L1–mediated inhibition of T-cell activation**

T-cell activation assays were performed to determine whether TPP-1 could block PD-L1 in culture and, thereby, activate the CD4$^+$ T cells. IFN$\gamma$ production and cell proliferation were evaluated. In the presence of CD3 antibody, T cells released more IFN$\gamma$ and proliferated significantly, which was reversed by the addition of PD-L1 at an ED$_{50}$ of about 5 to 10 $\mu$g/mL (Supplementary Fig. S7A), and TPP-1 peptide alone or TPP-1 plus PD-L1 did not activate T cells without anti-CD3 (Supplementary Fig. S7B). When an antagonist was added, the release of IFN$\gamma$ and proliferation was seen, with durvalumab blocking PD-L1 at 68 nmol/L. Similarly, at a TPP-1 concentration of 20 $\mu$mol/L, both IFN$\gamma$ release (Fig. 4A) and T-cell proliferation (Supplementary Fig. S7C) increased significantly by comparison, confirming that PD-L1 can block the function and T-cell–activating ability of TPP-1.

MLRs were conducted to confirm the activity of TPP-1 in a more physiologic environment, as shown in the schematic illustration (Fig. 1B). Matured DCs had a 16-fold higher PD-L1 expression compared with the control (Supplementary Fig. S8A). CD4$^+$ T cells were activated by CD3/CD28 antibody and IL2. At a TPP-1 concentration of 20 $\mu$mol/L, IFN$\gamma$ release was significantly higher than control and SPP-1 (Fig. 4B), and all the groups showed similar outcomes for proliferation (Supplementary Fig. S8B).

**Figure 4.**

TPP-1 reactivated T-cell function verified by T-cell activation assays (A; $n = 3$) and MLR assay (B; $n = 5$). The extent of T-cell activation was positively correlated to the amount of IFN$\gamma$ production. Data, mean ± SEM; *, $P < 0.05$, unpaired t test.
Figure 5. TPP-1 inhibited the tumor growth in a tumor xenograft model via reactivating T-cell function. A, Representative bioluminescence images of tumor-bearing mice for each group (n = 5) on day 21. B, Total bioluminescent signal (p/s) changes for each group at day 7, 14, 21, 28, and 35. C, Tumor volume (mm$^3$) changes measured on day 7, 12, 17, 22, 27, and 28. D, Representative images of IFN$\gamma$ and GZMB immunostaining on tumor tissues for each group. Scale bars, 200 μm. Magnification, ×40. E, Quantification of IFN$\gamma$ and GZMB expression (n = 5); data, mean ± SEM; *, P < 0.05; **, P < 0.01, one unpaired t test per row.
TPP-1 inhibits human tumor growth via a T-cell–dependent mechanism

The in vivo activities of TPP-1 were evaluated in a xenograft mouse model. The H460-luc cell line had high expression of luciferase and PD-L1 (Supplementary Fig. S9). A mixture of cells, which contained previously activated PBMCs and H460-luc cells at a ratio of 1:4, were subcutaneously injected into mice, and peptides were injected every other day based on the half-life of the peptide in subcutaneous tissue (Supplementary Fig. S10). The representative bioluminescence images of tumor-bearing mice for each group are shown in Fig. 5A. The total radiance (p/s) (Fig. 5B) and tumor volumes (Fig. 5C) of each group were recorded. These results confirm that durvalumab and TPP-1 can inhibit tumor growth (compared with SPP-1 and control) in this xenograft mouse model. The growth rate in TPP-1- or durvalumab-treated mice was 56% or 71% lower than that in control peptide–treated mice, respectively. When administered in the absence of T cells (control group), TPP-1 had no effect on the growth of the H460-luc tumors (Fig. 5).

To further verify that the inhibitory effects of TPP-1 on tumor growth were mediated by the reactivation of T cells, the expression of IFNγ and GZMB in tumor tissues was examined. When T cells are activated, cytokines, such as IFNγ and GZMB, will be secreted to the TME to initiate killing of tumor cells. The results from IHC (Fig. 5D and E) showed that the expression of IFNγ and GZMB increased significantly in the durvalumab and TPP-1 groups compared with those in control and SPP-1 groups, which indicated that our peptide inhibited tumor growth by reactivating T cells through blocking the interaction between PD-1 and PD-L1.

Discussion

The interference of PD-1/PD-L1 interaction has yielded impressive progress in the immunotherapy of cancers. In this report, PD-L1–targeting peptides were identified with bacterial surface display methods through random library and focused library screening, sequentially. The binding property of a candidate peptide named TPP-1 was investigated. The results showed that TPP-1 could bind specifically to PD-L1 with high affinity and block PD-1/PD-L1 interaction. TPP-1 lead to the reactivation of the T cells in vitro and in vivo assays, which indicates that TPP-1 could work as inhibitory candidate for PD-L1 to treat cancers.

The $K_D$ value is an important parameter to evaluate the binding affinity of two molecules. The $K_D$ value of PD-L1 with its natural binding ligand, PD-1, is about 500 nmol/L (34). In our study, the $K_D$ value of PD-L1 with TPP-1 peptide is about 95 nmol/L (around five times less than that with PD-1), meaning that the affinity between TPP-1 and PD-L1 is higher than that between PD-1 and PD-L1. The binding site of TPP-1 to PD-L1 is close to the interactive site of PD-1 and PD-L1 (36) predicted by ZDOCK software, which may indicate that TPP-1 could interfere with the binding ability of PD-1 to PD-L1 and potentially act as blockade molecule.

A xenograft mouse model was used to evaluate the ability of TPP-1 to reactivate T cell–mediated tumor cell killing. The bioluminescence signal and tumor volumes were assessed to evaluate the antitumor activity of TPP-1 mediated by the reactivation of T cells. Around 4 weeks after inoculation of previously activated T cells with H460-luc cells, the bioluminescence signals and tumor volumes in all five groups increased significantly compared with those in the first 3 weeks. However, there existed significant differences among the groups. The volume of tumors and the bioluminescent signals of tumor masses in durvalumab and TPP-1 groups did not increase as fast as those in control and SPP-1 groups. This may indicate that T cells were reactivated and had cytotoxic effects on cancer cells. To test our speculation, the expression of IFNγ and GZMB was examined. IFNγ and GZMB are biomarkers of T-cell activation and are believed to be produced by CD4$^+$ Th type 1 cells or CD8$^+$ cytotoxic lymphocytes (37). Expression of these two cytokines in our study could represent the activation extent of T cells in vivo. The expression of IFNγ and GZMB increased in durvalumab and TPP-1 (4 mg/kg) in comparison with control and SPP-1 groups, which could explain why tumor growth was inhibited by the activation of T cells. However, we also noticed that after 4 weeks, tumors seemed to recover from inflammatory effects, and the speed of tumor growth rose (Fig. 5B and C). A reason this occurred might be due to H460 cell lines having high expression of PD-L1, which could be stimulated by inflammatory factors (especially IFNγ) in the TME (38), which would help tumor cells escape from host immune recognition (39, 40). Monotherapy targeting only one immune checkpoint, such as CTLA-4, PD-1/PD-L1, was not effective at this stage, which is consistent with other researchers’ conclusions (12). The combinatorial therapy of our peptides with drugs targeting checkpoints or other treatments (41, 42), such as traditional chemotherapy or radiotherapy, may be a better choice.

Peptides are emerging as an attractive area of therapeutic agents because as potential drugs, they have many advantages, such as low molecular weight, low price, facile synthesis, and so on. In our study, we also showed that our TPP-1 peptide at higher concentrations had comparable effects on inhibiting tumor growth as durvalumab. Although some peptide drugs have been approved for clinical use, like goserelin for breast cancer and prostate cancer (43, 44) and exenatide for type II diabetes (45), challenges still exist in the development and wide application of peptide drugs. Peptides used orally are easy to be digested in the stomach, but after they pass the stomach, hydrophilic peptides are difficult to be absorbed by the intestine. If peptide drugs are directly injected intravenously, fast renal clearance, as well as enzymatic degradation in plasma, may occur. However, many attempts have achieved great progress. Researchers have conjugated Fc domains to peptides to avoid enzymatic digestion and designed nanocarriers for peptide delivery to prolong their circulation time (46, 47). With the appearance and development of new techniques, we believed that challenges for the development of peptide drugs can be overcome, and peptides as drugs would be widely used.
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Li, Y. Zhu

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Development of methodology: C. Li, Y. Zhu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Li, C. Ding, X. Cui
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Li, Y. Zhu

References


Effect of PD-L1-Targeting Peptide on Cancer Immunoresponse

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Peptide Blocking of PD-1/PD-L1 Interaction for Cancer Immunotherapy

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