Peptide Blocking of PD-1/PD-L1 Interaction for Cancer Immunotherapy
Chunlin Li, Nengpan Zhang, Jundong Zhou, Chen Ding, Yaqing Jin, Xueyuan Cui, Kefeng Pu, and Yimin Zhu

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 IFN-γ 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) February 2018.

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 lower 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-L2 (cat. #10022-H08H), GM-CSF (cat. #10015-HNAY), IL4 (cat. #11846-HNAA), IL2 (cat. #11848-HNAY1), bFGF (cat. #10014-HNAA), and recombinant mouse PD-L1 (cat. #50010-M08H) were purchased from Sino Biological Inc. Anti-human PD-L1 (cat. #557924, clone MHIH), anti-human PD-1 (cat. #557946, clone MHI4), 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. #2121030) were obtained from Thermo Fisher Scientific.

**Human serum**

Human serum (cat. #ZX101-1) was bought from Beijing Zomai Biotechnology Co., Ltd. RPMI1640 medium (cat. #SN30809.01), IMDM (cat. #SH30228.01), penicillin/streptomycin solution (cat. #SV30082.01), and FBS (cat. #SV30087.02) were purchased from HyClone. Histopaque-1077 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. #13588-1-AP) was obtained from Proteintech. The human IFNγ ELISA Kit (cat. #70-EK1802) was bought from Multisciences. Vita-Orange cell culture reagent (cat. #B34302) was bought from Bioseed. The PD-L1 antibody, durvalumab, was a gift from Dingfu Biotarget.

**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.
Measuring the $K_d$ value of TPP-1 to PD-L1

TPP-1 (TPP-1: SGQYASYHCWCWRDPGRSGGSK) 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: SGQWRWDSYRWHPSCACGGSK) was designed and synthesized and served as a control (26).

The affinity constant ($K_d$) and kinetics ($k_a$ and $k_d$) of TPP-1 peptide binding to PD-L1 were determined by surface plasmon resonance (SPR, BiacoreT200). PD-L1 was immobilized on the surface of a XanTex sensor chip. The remaining reactive sites were sized at GL Biochem Ltd, and the N-termini of the peptides Measuring the by BiacoreT200 Evaluation Software, version 2.0.

On the second day, the plate was blocked with 1% BSA in PBST, incubated with 4 ml of FITC-labeled peptides or PD-L1 anti-human CD3 L PBS twice and then incubated for another 30 minutes. Samples were analyzed by flow cytometry.

The 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 µL (1 µg/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 peptides (20 µmol/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 harvested and incubated with 0.25% trypsin and then centrifuged at 10,000 g/mL. Each sample was injected at a flow rate of 25 µL/minute for about 15 minutes. $K_d$, $k_a$, and $k_d$ were calculated by BiacoreT200 Evaluation Software, version 2.0.

Examination of binding specificity of the soluble peptides

For the fluorescence microscopy, CHO-K1 and CHO-K1/PD-L1 were seeded in a 12-well plate (2 x 10^5 cells/well). At 70% to 80% confluency, the cells were washed with PBS twice, followed by incubating with 1% BSA for 10 minutes. FITC-labeled peptides were added at a final concentration of 10 µmol/L and incubated for 30 minutes at room temperature. Afterward, the cells were washed with PBS twice and analyzed with a flow cytometer.

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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 µL (1 µg/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 nmol/L biotinylated PD-1 or durvalumab with FITC-labeled TPP-1 (at the final concentration of 40, 20, 10, 5, or 2.5 µmol/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 biotinylated PD-1 or durvalumab signals, 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 x 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 µL anti-human CD3 (1 µg/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 µL PBS twice. Afterwards, 100 µL PD-L1 at the final concentration of 0.1 µg/mL 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 different 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 x 10^6 cells/well in 150 µL medium) were added, followed by the centrifugation at 1,000 rpm for 2 minutes. Cells were incubated in a humid incubator with 5% CO2 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, TNPx (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 µg/mL) for 2 hours. The frozen CD4+ T cells were thawed and cultivated overnight. About 1 x 10^5 cells were seeded in each well with a DC:T ratio of 1:10 in 200 µL IMDM plus 10% FBS. IFNγ in supernatants and cell proliferation was quantified at day 3.
In vivo studies

Five- to 6-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 × 10⁶), along with 5 × 10⁵ 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 × b², 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 Prísm 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 × 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_5CWCWRX_5 was constructed into pBAD33-eCPX (Supplementary Fig. S2A). The library size was about 5 × 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, IFNγ, 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 fluorescent 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

durvalumab (solid lines of Fig. 3D and F). The

could effectively bind to PD-L1, even in the presence of PD-1 or

based analysis. The ELISA results showed that the TPP-1 peptide

investigated either using

fl

durvalumab was

peptides on the binding of PD-L1 to PD-1 or durvalumab was

membrane.

these results con

fl

PD-L1 mAbs. Similar results were seen for CHO-K1 cells using

TPP-1 had low binding (Supplementary Fig. S5B). TPP-1 pep-

for MDA-MB-435 cells (expression of PD-L1 is relatively low),

MB-231 cells (Fig. 3C; Supplementary Fig. S5B). In contrast,

sion. TPP-1 had higher binding to CHO-K1/PD-L1 and MDA-

PD-L1, and MDA-MB-231 cell lines had higher PD-L1 expres-

231, and MDA-MB-435 was measured by

copy. The expression of PD-L1 in CHO-K1/PD-L1, MDA-MB-

environment, the binding ability of TPP-1 to CHO-K1/PD-L1

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 $9.467 \times 10^{-6}$ mol/L ($3.022 \times 10^{-4}$

Ms$^{-1}$ for $k_+ \text{ and } 3.192 \text{ s}^{-1}$ for $k_-)$.

hPD-L2 and mPD-L1 had the most similar amino acid

sequences to PD-L1 as described in previous reports (33, 34), as

be 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-

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 (Supple-

mentary Fig. S7B). When an antagonist was added, the release of

IFN$\gamma$ and proliferation was seen, with durvalumab blocking

PD-L1 at 68 $\mu$mol/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 compar-

ison, 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 DGs 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 (Supplemen-

tary 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.

A

B

CD3

PD-L1

(Durvalumab (68 $\mu$mol/L)

TPP-1

(4 $\mu$mol/L)

SPP-1

(20 $\mu$mol/L)

Control

Durvalumab (68 $\mu$mol/L)

TPP-1 (4 $\mu$mol/L)

TPP-1 (20 $\mu$mol/L)

SPP-1 (20 $\mu$mol/L)

IFN$\gamma$ (pg/mL)

IFN$\gamma$ (pg/mL)
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 $\mu$m. 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.

Prediction of possible binding sites of TPP-1 to PD-L1

The secondary structure of TPP-1 was composed of a random coil and beta-sheet predicted by LOMETS (Supplementary Fig. S11A). The binding model of TPP-1 to PD-L1 (PDB ID of PD-L1: 3BIS) was predicted by ZDOCK (Supplementary Fig. S11B). The binding position of PD-1 (light sea green) to PD-L1 (space fill model in dark khaki) is shown in Supplementary Fig. S11C. These results, combined with the ligand inhibition assay, indicate that the binding site of TPP-1 may be close to the PD-1- and PD-L1–binding pocket.

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 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 bioluminescence 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 by IHC. IFNγ and GZMB are biomarkers of T-cell activation and are believed to be produced by CD4+ Th type I 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 inhibitory 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 enxatide 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.
Taken together, we have shown that TPP-1 peptide can specifically interact with PD-L1 and block PD-1/PD-L1 interaction. It has the potential as a substitution for antibodies in cancer immunotherapy. Peptides with high affinity and specificity may be effective candidates for targeting therapy once the proper delivery systems have been established.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C. Li, Y. Zhu
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


Writing, review, and/or revision of the manuscript: C. Li, Y. Zhu, K. Pu, Y. Zhu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Li, N. Zhang, J. Zhou, Y. Zhu
Study supervision: C. Li, Y. Zhu

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Chunlin Li, Nengpan Zhang, Jundong Zhou, et al.


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