A CTLA-4 Antagonizing DNA Aptamer with Antitumor Effect

Bo-Tsang Huang,1 Wei-Yun Lai,1 Yi-Chung Chang,1 Jen-Wei Wang,1 Shauh-Der Yeh,1,2 Emily Pei-Ying Lin,1,3 and Pan-Chyr Yang1,4

INTRODUCTION

The concept of immune checkpoint blockade has revolutionized anti-cancer strategies in nearly all cancer fields.1 Monoclonal antibodies targeting the cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death protein 1 (PD)-1/programmed death-ligand 1 (PD-L1) axes are now part of routine practice.2 Recent aptamer-related research has further extended the field into functional aptamer therapeutics, especially in the field of immuno-oncology, an area full of new hope and uncertainty.10,21 Because the immune checkpoint landscape encompasses multiple positive and negative regulators, aptamers may have several advantages over mAbs in cancer immunotherapy per se. For example, it is possible to engineer aptamers with dual desirable functions, either being a dual antagonist or an agonist-antagonist integrative.10,22 In addition, the small size of the aptamer (6–30 kDa) facilitates its rapid renal clearance, with a half-life ranging from hours to 2 days.23,24 This allows for better managing in case of side effects, which are now recognized as an important issue in cancer immunotherapy.21

CTLA-4 is expressed on T cells. It is a homolog of CD28, which binds to the surface antigens B7-1 (CD80) and B7-2 (CD86).25 CTLA-4 expression is initiated upon T cell activation, which attenuates CD28 co-stimulation and inhibits signaling by competing for B7 binding.26 Blocking of CTLA-4 and B7 conjugation reshapes the host immune response and exerts a sustained anti-tumor effect in some cancer subpopulations.27–29 CTLA-4 and PD-1/PD-L1 axes blockade constitutes the backbone of the current cancer immunotherapy.3 As potential advantages of the aptamer are recognized, it is worth developing a CTLA-4-antagonizing aptamer. Although a

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A CTLA-4 Antagonizing DNA Aptamer with Antitumor Effect

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tetrameric CTLA-4 RNA aptamer had been reported, it does not function as an effective antagonist in a monomeric form.30 It indeed, however, can serve as a carrier for targeting delivery, such as the case of the aptamer-siSTAT3 chimera against T-regulatory cells.31 Based on this unmet need, we developed a novel CTLA-4-antagonizing DNA aptamer, aptCTLA-4, by the integration of two high-throughput platforms: SELEX (systematic evolution of ligands by exponential enrichment) and next-generation sequencing (NGS). We showed that aptCTLA-4 is biologically functional and binds to CTLA-4 with high affinity and promotes T cell activity in the tumor microenvironment.

RESULTS

Cell-Based SELEX and NGS for aptCTLA-4 Identification

The CTLA-4-targeting aptamers were selected from a single-stranded DNA (ssDNA) library composed of $10^{15}$ molecules using 12 round cell-based SELEX (Figure 1A). The ssDNA pool enriched for CTLA-4-targeting sequences was subjected to NGS. The 1,623 NGS-derived sequence clusters were divided into five categories. There were 28 clusters in the $\geq 7,000$-read category, with sequence-read numbers exceeding 50% of the total reads (Table S1A). Quadruplex-forming G-rich sequences (QGRS) prediction of these 28 clusters suggested a probable G-quadruplex structure within aptCTLA-4 (http://bioinformatics.ramapo.edu/QGRS/analyze.php) (Table S1B). M-fold structure-prediction software predicted that the aptCTLA-4 sequence formed a complex hairpin-bulge folding structure (Figure 1B). 3D-structure predictions and docking simulations further suggested that nucleotides 39-40, 54-57, and 60-61 of aptCTLA-4 bind to amino acids 98LDDS101, 88TYMMNGE94, and 133LMYPPPY139 of CTLA-4 protein (Figure 1C). Of note, the MYPYPYY sequence of CTLA-4 was shown to be the major binding region that interacts with B7 proteins.32 Peptide competition assays further suggested that aptCTLA-4 might have the same binding sites with B7-1 and B7-2, the two known ligands for CTLA-4 protein (Figure S1). On the other hand, the analyses with circular dichroism (CD) spectroscopy did not support the existence of a G-quadruplex within aptCTLA-4. The positive peak of aptCTLA-4 resided at 273 nm instead of 263 nm for a parallel or 295 nm for an anti-parallel G-quadruplex (Figure S2). Nevertheless, we continued our further analyses of aptCTLA-4 based on the results of structural prediction, docking simulation, and peptide competition assays.

aptCTLA-4 Binds to Human CTLA-4 Protein with Good Binding Affinity

We first confirmed the binding between aptCTLA-4 and human CTLA-4. To this end, we incubated 20 nM Alexa Fluor 647-labeled aptCTLA-4 with human CTLA-4-GFP-overexpressing or GFP-overexpressing HEK293T cells, and analyzed fluorescence intensity by flow cytometry. The shift of fluorescence intensity with the
treatment of aptCTLA-4 suggested that aptCTLA-4 was able to recognize CTLA-4-expressed HEK293T cells (Figures 2A–2C). This was further supported by the evidence that CTLA-4 knockdown could reverse the shift of fluorescence intensity induced by aptCTLA-4 or anti-CTLA-4 antibody treatment (Figure S3). Confocal microscopy demonstrated the accumulation of overexpressed CTLA-4-GFP protein on the HEK293T cell membranous region, where it was co-localized with aptCTLA-4 (indigo) and phycoerythrin (PE)-labeled anti-CTLA-4 antibody (red) (Figure 2D). The binding assay showed that aptCTLA-4 possessed good binding affinity toward CTLA-4 protein, with the dissociation constant ($K_d$) being 11.84 nM (Figure 2E). Taken together, our data implicated the potential functionality of aptCTLA-4.

**aptCTLA-4 Binds to Mouse CTLA-4 Protein and Promotes In Vitro T Cell Activity**

Because tumor microenvironment studies are often carried out in murine syngeneic tumor models, we examined the sequence homology between human and mouse CTLA-4 proteins. The analysis revealed that human and mouse CTLA-4 sequences exhibit 76% homology at the amino acid level (Figure S4A). We subsequently incubated fluorescein isothiocyanate (FITC)-labeled aptCTLA-4 with mouse T lymphocytes, which were co-stained with PE-labeled anti-mouse CTLA-4 antibody. The confocal microscopy results showed that aptCTLA-4 could recognize mouse CTLA-4 protein (Figure S4B).

We then examined the effect of aptCTLA-4 on immune cells. The carboxyfluorescein succinimidyl ester (CFSE)-labeled lymphocytes derived from the BALB/c mouse strain were co-cultured with irradiated lymphocytes derived from the C57BL/6 mouse strain, which served as T cell activators. These cells were incubated with aptCTLA-4 or random sequences for 72 hr and then analyzed by flow cytometry (Figure 3A). The experimental protocol and dosage of aptCTLA-4 at 200 nM for cell treatment was based on the study from Santulli-Marotto and colleagues. The results showed that aptCTLA-4 treatment increased lymphocyte proliferation rates up to 21%, whereas the change of proliferation rate in the random sequences control group was about 9% (Figure 3B). The data suggested that aptCTLA-4 binds to CTLA-4 and promotes T cell activity.
**aptCTLA-4 Is Relatively Stable in Serum and Suppresses In Vivo Tumor Growth**

Although short serum half-life has been considered as a shortcoming for aptamer in terms of therapeutics, this is a manageable issue.\(^{10}\) To evaluate the feasibility for a subsequent in vivo animal study, we first investigated the stability of aptCTLA-4 with in vitro serum incubation. aptCTLA-4 was incubated with serum for 0, 6, and 24 hr at 37°C, respectively. The gel electrophoresis results revealed that aptCTLA-4 remained nearly intact after a 24-hr serum incubation period (Figure 3C). The data suggested that even before structural or base modification, aptCTLA-4 is relatively stable.

We then investigated whether aptCTLA-4 is biologically functional. We first performed in vivo studies using a mouse syngeneic tumor model created by subcutaneously inoculating C57BL/6 mice with TC-1 murine lung tumor cells. After inoculated tumors had reached a size of ~6 mm (long axis), a single shot of aptCTLA-4 at the dosage of 0.2 mg/kg was administered intraperitoneally. The results showed that aptCTLA-4 effectively inhibited in vivo tumor growth and the body weight of mice remained stable throughout the experimental period (Figures 4A–4C). Direct toxicity of aptCTLA-4 against TC-1 cells was excluded by in vitro cell proliferation assays, which further suggested that the anti-tumor effect of aptCTLA-4 is through immune modulation mediated by its interaction with CTLA-4 (Figure 4D). Nevertheless, this low dose at one shot did not work with the aggressive cell type Lewis lung (Figure S5). We thus escalated the dosage of aptCTLA-4 to 2 mg/kg and adjusted the dosing schedule to one shot daily in 4 to 5 successive days. This protocol amendment was based on the study from Allison and colleagues when they developed anti-CTLA-4 antibody.\(^{27}\) With this modified dosing protocol, the aptCTLA-4 effectively suppressed in vivo tumor growth in both CT26 (BALB/c) and Lewis lung (C57BL/6) mouse syngeneic tumor models (Figure S6).

We next compared anti-tumor effects of aptCTLA-4 with an anti-mouse CTLA-4 mAb using the CT26 mouse syngeneic model. The data showed that aptCTLA-4 had identical tumor-suppressive effects to the anti-mouse CTLA-4 mAb but led to a lesser degree of body weight loss (Figures 5A–5C). Moreover, aptCTLA-4 did not induce measurable liver or renal toxicities, as represented by stable aspartate aminotransferase (GOT), alanine aminotransferase (GPT), and blood urea nitrogen (BUN) levels in groups with and without aptCTLA-4 treatment (Figure S7). On the other hand, the flow cytometry analyses showed that aptCTLA-4 treatment significantly increased the number of tumor-infiltrating lymphocytes (CD45\(^+\)) and the percentage of cytotoxic T lymphocytes (CTLs) (CD45\(^+\) and CD8\(^+\)) (Figure 5D). This was further verified by an immunohistochemistry study, which indicated the increase of CD8\(^+\) cells in tumors treated with aptCTLA-4 (Figure 5E). These preclinical results suggested a translational potential for aptCTLA-4.

**DISCUSSION**

aptCTLA-4 is a novel DNA aptamer that promotes T cell proliferation and inhibits tumor growth. The developmental pipeline adopted an integrated high-throughput technology, and aptCTLA-4 was functionally validated in both cell and animal models.
In the current study, we adopted a DNA aptamer selection system using cell-based SELEX. Although RNA aptamers have more diverse 3D conformations and stronger intra-strand RNA-RNA interactions that may increase binding affinity and specificity, RNAs are vulnerable to nuclease-mediated degradation, an unavoidable problem in a natural biological environment.\(^{10,37}\) Sequence or structural modifications are almost always needed to improve RNA aptamer stability. Commonly used methods include substitutions of the 2'-O-methyl motifs and/or changes of the phosphodiester backbone to the boranophosphate or phosphoro-

In our previous study,54 the observed differential treatment responses to aptCTLA-4 in TC-1, Lewis Lung, and CT26 models may be partly attributable to differences in their neoantigen landscapes and antigen presentation. TC-1 cells were transformed from C57BL/6 mouse lung epithelial cells with HPV-16 E6 and E7.55 The aptCTLA-4 identified in the current study possesses good binding affinity and fair serum half-life without further modifications. Although the half-life of aptCTLA-4 can be theoretically shorter than an antibody, our study showed that aptCTLA-4 had identical suppressive effects between a functional PD-L1-targeting aptamer, the aptPD-L1, and an anti-mouse PD-L1 mAb were also reported in our previous study.54 The observed differential treatment responses to aptCTLA-4 in TC-1, Lewis Lung, and CT26 models may be partly attributable to differences in their neoantigen landscapes and antigen presentation. TC-1 cells were transformed from C57BL/6 mouse lung epithelial cells with HPV-16 E6 and E7.55 The alloantigen presented may thus augment the aptCTLA-4 anti-tumor effect observed. Clinically, about 20% of solid tumors respond to ipilimumab, and it is hypothesized that this subset of patients presents...
distinct neoantigens that are recognized as non-self and elicit a sustained anti-tumor immune response.\textsuperscript{5} Our results in part support these clinical observations and genomic studies.

In summary, our data support the translational development of DNA aptamers with therapeutic applications. Further investigations following the current proof-of-concept study may determine the role of aptCTLA-4 in immuno-oncology.
10% FBS. All cell lines were cultured at 37°C in a humidified 5% CO₂ environment.

**Cell-Based SELEX and NGS**

The cell-based SELEX was carried out based on the protocol reported by Sefah et al., with modification. In the first round (R1) of positive selection, CTLA4-expressing HEK293T cells at a cell density of 80%–90% confluence were incubated with a ssDNA library composed of 10¹⁵ molecules in SELEX buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 5 mM MgCl₂) at 4°C for 30 min with rotation. The cells were washed twice with SELEX buffer to remove unbound ssDNAs. Subsequently, the cells were suspended in 100 µL of SELEX buffer, lysed by heating at 95°C, and then cooled at 4°C. Total lysates were centrifuged at 500 × g for 5 min. The supernatant containing the eluted ssDNAs was incubated with HEK293T cells in the eppendorf tube at 4°C for 30 min with rotation for negative selection. After centrifugation, the supernatant was collected for PCR amplification using 9-forward primers and biotin-labeled 9 reverse primers (200 nM each) in PCR buffer (10 mM Tris-HCl, pH 8.9, 50 mM NaCl, 1 mM MgCl₂, 10 mM betaine, and 1% DMSO) containing 200 nM each of dNTP and 2 U of Taq DNA polymerase. The PCR amplicons were captured with streptavidin-coated magnetic beads (1X SSC buffer incubation at 37°C for 3 hr with rotation) and eluted with heating at 95°C for 2 min. After the 12th round of the cell-based SELEX, the eluted ssDNAs enriched for CTLA4-targeting sequences were subjected to NGS on an Illumina MiSeq system (Illumina, San Diego, CA). The data were processed using Galaxy and FASTA aptamer software.

**Flow Cytometric Analyses**

The specificity of isolated aptamers was first evaluated by flow cytometry. A total of 1 × 10⁸ GFP and CTLA4-GFP-overexpressing HEK293T cells were resuspended in 50 µL of PBS. The cells were then incubated with 20 nM Alexa Fluor 647-labeled selected aptamers for 30 min at 4°C. Next, cells were washed and resuspended in 0.5 mL of 4% paraformaldehyde (PFA), and analyzed by flow cytometry (4-color FACSCaliber; BD Biosciences, San Jose, CA, USA). A total of 20,000 cells were collected for each analysis.

**Determination of Dissociation Constants**

The 2-fold serial diluted aptCTLA-4 (starting at 125 nM) was incubated with CTLA4-GFP-overexpressing HEK293T cells for 30 min at 4°C. After PBS washing, the cell-bound aptCTLA-4 was eluted and quantified by RT-qPCR. The K_d values were calculated by nonlinear regression of the relationship, Y = B_max × X/(K_d + X), using the equation: one site-specific binding, saturating binding from GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). B_max is the maximal binding at equilibrium, K_d is the ligand concentration that binds to half the receptor sites at equilibrium, and B_max is the maximum number of binding sites.

**Mouse In Vitro Lymphocyte Proliferation Assay**

For the preparation of mouse spleen cells, the spleen was cut into pieces and passed through a steel mesh using a plunger. Cells were subsequently flushed into a Petri dish containing PBS, and then washed with collagenase in red blood cell lysis buffer (10 mM Tris-HCl, 10 mM KCl, 10 mM MgCl₂, 2 mM EDTA, and 2% NP-40). After centrifugation at 1,800 rpm, the supernatant was removed and the pellet was suspended and cultured in RPMI containing 10% FBS. This procedure yielded approximately 5 × 10⁶ to 1 × 10⁷ cells per spleen. A total of 1 × 10⁵ lymphocytes was labeled with 2 µM CFSE (Thermos Fisher, Waltham, MA, USA) at 37°C for 20 min, and then seeded into 96-well plates. CFSE-labeled lymphocytes were mixed with unlabeld, allogeneic lymphocytes and then treated with CTLA4 aptamers (200 nM) or random sequences control (200 nM). After a 72-hr incubation, cell proliferation was examined by flow cytometry.

**Tumor Immunotherapy Study**

C57BL/6 mice were subcutaneously inoculated with mouse TC-1 (3 × 10⁴) or Lewis lung (1 × 10⁵) cancer cells. BALB/c mice were subcutaneously inoculated with CT26 (2 × 10⁵) cancer cells. Tumor volumes were measured beginning on day 4 or 5 after tumor inoculation according to the relationship (L × D²)/2, where L is the long dimension and D is the short dimension. The aptCTLA-4, anti-mouse CTLA-4 antibody (9H10; Bio-cell), isotype antibody (Syrian Hamster immunoglobulin G [IgG]; Bio-cell), random sequences, or PBS was administered intraperitoneally once the long axis of tumors had reached ~6 mm, as measured by digital caliper.

**Confocal Image**

aptCTLA-4 was incubated with CTLA4-GFP-expressed HEK293T cells or mouse lymphocytes in culture medium (DMEM+10% FBS) at 37°C for 30 min. After washing by PBS and fixing the cell by 4% PFA for 10 min, the samples were blocked in PBS containing 1% normal goat serum and 2% BSA. PE-conjugated anti-human CTLA-4 antibody (1:400) and PE-conjugated anti-mouse CTLA-4 antibody (1:400) antibodies (eBioscience, San Diego, CA, USA) were prepared in 1% normal goat serum and 2% BSA in PBS. After PBS washing for three times, samples were stained by DAPI (Invitrogen) for cell nucleus identification. Images of cross-sections were acquired using a Zeiss LSM710 confocal microscope (Carl Zeiss Microimage, Thornwood, NY, USA).

**CD Spectroscopy**

CD spectra experiments were performed with Jasco J-815 CD Spectropolarimeter (JASCP, USA). Aptamers (10 µM) were prepared in SELEX buffer (pH 7.5), denatured at 95°C, and then kept at 25°C overnight prior to CD spectra experiments. CD spectra data were obtained from 200 to 350 nm at a step size of 1 nm, a 0.2-s time per point, and a bandwidth of 1 nm. Each spectrum was an average of three scans at room temperature and was corrected with buffer baseline.

**Blood Biochemistry Analyses**

The amount of GOT, GPT, and BUN was detected by Fuji Dri-Chem 4000i (Fujifilm, Tokyo, Japan) under technical support of the Taiwan Mouse Clinic.
Flow Cytometry Analyses and Immunohistochemical Staining of the Tumors

Tumors were digested with collagenase, and cells in the tumors were isolated following the procedures built by Zabel et al. Antibodies against mouse CD45 and CD8 and relative isotype control antibodies (BioLegend) were used to stain these isolated cells. Formalin-fixed, paraffin-embedded tissues were rehydrated and subjected to an antigen-retrieval process in citrate buffer (pH 6.0). A primary antibody against mouse CD8 (Thermo Scientific) was used at the concentration of 1:100 dilution.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.omtn.2017.08.006.

AUTHOR CONTRIBUTIONS

B.T.H. conceived the work, designed and performed the experiments, and analyzed the data. W.Y.L. designed and performed the experiments and analyzed the data. Y.C.C. conceived the work and designed the experiments. J.W.W. performed the experiments. S.D.Y. advised the experiments. B.T.H. conceived the work, designed and performed the experiments, and analyzed the data.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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