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Expression of Recombinant CTLA-4 and PD-L1 Proteins Fused with Thioredoxin, and Determination of Their Ligand-Binding Activities

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Abstract

Background: The use of chimeric proteins that selectively interact with various immune cell receptors to treat oncology patients has increased. One effective way to obtain recombinant proteins is to use the *E. coli* expression system. However, in eukaryotic protein production in *E. coli*, several difficulties arise that can be solved by fusing the target protein with thioredoxin. Thioredoxin can enhance solubility, but its large size can lead to an erroneous assessment of protein solubility, folding, and activity. The present study examined the ligand-binding activity of PD-L1, and CTLA-4 receptors fused with thioredoxin.

Methods: The de novo synthesized genes of the extracellular domains of the PD-L1 and CTLA-4 were cloned into the pET28 and pET32 expression plasmids and used to transform *E. coli* BL21 cells. Purified recombinant proteins were characterized by western blotting, LC-MS/MS spectrometry, and ELISA.

Results: Amino acid sequence comparisons of the recombinant proteins obtained by LC-MS/MS with the SwissProt database resulted in the highest comparison scores from 4950 to 13396. The binding efficiencies of recombinant human B7-1 Fc to rCTLA-4 and rTrx-CTLA-4 proteins in ELISA did not differ significantly. Similar results were obtained with recombinant rhesus monkey PD-1 hFc against rPD-L1 and rTrx-PD-L1.

Conclusions: Recombinant proteins specifically reacted with recombinant human B7-1 Fc and recombinant rhesus monkey PD-1 hFc. The fusion of thioredoxin with recombinant proteins through linkers slightly affected the activity of the extracellular domains of CTLA-4 and PD-L1.

Keywords: Chimeric Protein, CTLA-4, PD-L1, Protein Refolding, Recombinant Protein, Thioredoxin.

Introduction

Activation or inhibition of T-lymphocytes by immunological checkpoints is a central process in immune system function. The main purpose of immunological checkpoints is to ensure a balance between the generation of immunity and maintenance of a normal physiological state of the immune system. In maintaining the physiological balance of the immune system, membrane proteins play significant roles in transferring extracellular signals and inducing intracellular signaling. Disruption of signaling mediated by outer membrane receptors leads to various diseases, including cancer and autoimmune diseases (1).

Based on inhibitory immunological checkpoints, individual tumors are considered by the body as its own, despite genetic changes and antigenic differences. Cancer cells can evade the immune system by activating inhibitory T cell checkpoints. In this regard, the study of the

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inhibitory properties of immunological checkpoints has attracted increasing research interest (2, 3).

Programmed cell death receptor 1 (PD-1), ligands for PD-1 (PD-L1) and PD-2 (PD-L2), and antigen 4 associated with cytotoxic T lymphocytes (CTLA-4) are of great interest. Interest in CTLA-4 is attributed to the receptor being a homolog of the CD28 receptor and having greater affinity for the B7 receptor than CD28 has (4). The interaction of CD28 and B7 receptors leads to increased T cell proliferation and cytokine production. CTLA-4 is essentially a competitor to the CD28 receptor, thereby interfering with the generation of the CD28:B7 signal that stimulates immunity. In addition, CTLA-4 and B7 binding can produce inhibitory signals that counteract the stimulatory signals from CD28:B7 and TCR: MHC binding (5). PD-L1 is the central mediator of cancer cell evasion from immunity and the only biomarker that allows the prediction of the effectiveness of the blockade of immunological checkpoints (6). In addition, PD-L1, like CTLA-4, can bind the B7.1 (CD80) receptor on dendritic cells and prevent the development of antitumor immunity (7).

Currently, there is an increase in the number of treatments that target the immune system through immune checkpoints for a range of diseases, including autoimmune and infectious diseases, and cancer (8). Traditionally, immunotherapy is based on the use of monoclonal antibodies or chimeric proteins that selectively interact with receptors on various immune system cells (9). In addition, chimeric proteins can simultaneously bind and activate different receptors on different cell types, leading to desirable effects. Undesirable side effects can also be derived from the interference of the chimeric protein with biochemical reactions and modulation of the interaction of a ligand with a specific receptor (10, 11).

In vivo immunological checkpoint studies face the problem that the cost of CTLA-4, PD-1, and PD-L1 proteins available from commercial suppliers is very high. Currently, commercial human recombinant CTLA-4 and PD-L1 are fused with the Fc domain of antibodies produced in eukaryotic cells and yeast (12). In *E. coli*, only the extracellular domains of CTLA-4 and PD-L1 are produced (13, 14). *E. coli*, a highly effective expression system widely used in biotechnological production, is characterized by a high rate of biomass growth under various cultivation conditions (15). However, depending on the properties of the protein of interest, it is difficult to predict the expression, solubility, activity, and folding of *E. coli*-expressed proteins (16). To address these problems, the target protein was fused with thioredoxin (17, 18).

This study examined the ligand-binding activity of thioredoxin-fused human CTLA4 and rPD-L1 receptors. To study the binding activities of the thioredoxin-fused receptors, the genes of extracellular domains of CTLA4 and PD-L1 were synthesized de novo. The rTrx-CTLA4 and rTrx-PD-L1 were analyzed by MS-MS spectrometry and enzyme-linked immunosorbent assay (ELISA) using recombinant human B7-1 Fc and rhesus monkey (*Macaca mulatta*) PD-1 hFc proteins.

Materials and Methods

Bacterial strain, plasmids, and antibodies

E. coli DH5α and BL21 (DE3) cells (Novagen, Cambridge, UK) were used for cloning and expression. *E. coli* were cultivated in LB medium. Genetic constructs were generated using pGEM-TEasy (Promega, Madison, USA), pET28, and pET32 (Novagen Cambridge, UK) plasmids. Mouse monoclonal antibodies against His-tag conjugated with horseradish peroxidase (Thermo Scientific Waltham, Massachusetts, USA), Super Signal West Dura Extended Duration Substrate (Thermo Scientific), recombinant human B7-1 Fc, and recombinant rhesus PD-1 hFc proteins were used to analyze recombinant proteins by western blotting and ELISA.

DNA and oligonucleotide design

The PD-L1 and CTLA-4 extracellular fragment nucleotide sequences were obtained from the PubMed NCBI reference sequence: NP_001300958.1 and AAL07473.1. The DNA sequences were codon-optimized for

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expression in *E. coli*. Oligonucleotides were designed using DNAWorks software (http://helixweb.nih.gov/dnaworks/) and synthesized in the Laboratory of Organic Synthesis of Republican State Enterprise "National Center for Biotechnology," which functions under the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan. The overlapping oligonucleotides were 60 bp in length. All oligonucleotides were examined for AT and GC content, presence of large repeats, and priming errors.

Gene synthesis and cloning of the sequences encoding the extracellular domains of CTLA-4 and PD-L1

To obtain the DNA of rPD-L1 and rCTLA-4 genes, two-step PCR was performed. The reaction was carried out in a 25 µL volume containing 2.5 µL of 10× Phusion GC buffer, 1 µL of oligomix, 1 µL of outer primers, 20 µL of mQ H_2O, and 0.5 µL of Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). The first and second steps of PCR thermal cycling were as follows: initial denaturation at 98 °C for 1 min, followed by 30 cycles of 98 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and final extension at 72 °C for 10 min. The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Germantown, Maryland, USA) and cloned into a pGEM-TEasy plasmid. Three positive clones were selected and sequenced using an ABI PRISM® 310 Genetic Analyzer (Thermo Fisher Scientific). The resulting genes included BamHI, EcoRI, NcoI, and XhoI restriction sites for subsequent cloning.

The synthesized DNA fragments were cloned into T/A multi-copy vector pGEM, generating pGEM/CTLA-4 and pGEM/PD-L1 recombinant vectors. The constructs were used to transform chemo-competent *E. coli* DH5α cells, which were plated on LB Amp+ agar for the rapid identification of bacterial colonies containing vectors by blue-white selection. Recombinant constructs from 10 positive bacterial colonies were then isolated and sequenced. For pGEM/CTLA-4, three sequences were correct. For pGEM/PD-L1, all five clones had the correct sequences. The resulting genes were cloned into pET28 and pET32 expression vectors using the appropriate restriction enzymes. The EcoRI and XhoI restriction sites were cloned into the pET28 vector, and NcoI and XhoI into the pET32 vector. The obtained expression vectors were used to transform competent *E. coli* BL21 (DE3) cells, which were cultured on LB agar. After PCR screening, single positive clones from each transformation were inoculated into LB broth tubes and cultured.

Expression of pET28/CTLA-4, pET32/CTLA-4, pET28/PD-L1, and pET32/PD-L1

Competent *E. coli* BL21 (DE3) cells were transformed with the expression vectors using a MicroPulser (Bio-Rad, Hercules, CA, USA) under the following conditions: 100 ng of plasmid per 50 µL of cell suspension at 2.5, 25, and 200 Ω. Electroporation duration was 5.0 ms. To determine the expression, selected transformant colonies were cultured in 5 mL of LB broth containing ampicillin and incubated for 16 h at 37 °C with stirring at 200 rpm. LB medium containing antibiotics (50 mL) was added to the overnight bacterial cultures and incubated until the mid-logarithmic growth phase (absorbance at λ= 600 nm, OD600= 0.6) at 37 °C with shaking at 200 rpm. In the middle of the logarithmic phase, 0.2 mM isopropyl-β-D-1-galactopyranoside (IPTG) was added and incubated at 37 °C with stirring at 200 rpm. Before adding the IPTG and every two hours thereafter, 5 mL of the cell culture were collected for analysis by SDS-PAGE. Cells were precipitated by centrifugation at 6,000×g for 7 min at 4 °C and lysed by sonication. To determine the optimal cultivation temperature, transformants were incubated at 37, 24, and 16 °C after adding IPTG.

Cell lysis and chromatographic purification

To purify proteins, cell pellets were resuspended in TNE buffer (20 mM NaCl, 20 mM HEPES, 0.1 mM PMSF, pH 7.5) and lysed using an OMNI Ruptor 4000 ultrasonic disintegrator (Kennesaw, Georgia, USA). After

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centrifugation, the pellet was resuspended in buffer A (20 mM Tris-HCl, pH 8.0, containing 1 M urea, 500 mM NaCl), incubated for 30 min at room temperature, and centrifuged at 20,000×g for 30 min. The pellet was dissolved in buffer B (20 mM Tris-HCl, pH 8.0, containing 8 M urea, 500 mM NaCl, 20 mM imidazole, 10 mM 2-mercaptoethanol) and sonicated. The proteins were further purified using Ni2+-NTA (His-trap) columns (GE Healthcare, Uppsala, Sweden). The columns were washed and equilibrated with buffer B. Clear protein solution was loaded into the prepared columns. A linear 8–0 M urea gradient was run through 30 mL at 0.5 mL/min to allow refolding of the proteins (19, 20). The refolded proteins were eluted with a linear 20–500 mM imidazole gradient.

Western blotting

The purified proteins were separated by 12% SDS-PAGE and blotted onto nitrocellulose membranes using an immunoblotting device (Bio-Rad). Membranes were blocked by incubation in 1% BSA solution overnight at 4 °C and then washed three times with PBS-Tween buffer (137 mM NaCl, 10 mM Na_2HPO_4, pH 7.4, 5% Tween-20). The membranes were incubated in a solution of anti-6His-tag monoclonal antibody conjugated with horseradish peroxidase at 1:5,000 dilution for 1.5 h at 37 °C. The membranes were washed three times with TBS-Tween and TBS buffer and visualized using the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific).

NanoLC and tandem mass spectrometry (nanoLC-MS/MS)

Purified protein samples were fractionated using 12% SDS-PAGE and stained with Coomassie blue. The gel fragments containing the protein bands were excised and divided into 1×1 mm fragments. One hundred µL of 100 mM NH_4HCO in acetonitrile (1:1) were added to the fragments and incubated at 37 °C for 30–40 min. After processing to reduce disulfide bonds and the sizes of the gel pieces, 2 µL of 100 ng/µL trypsin and 50 µL of 50 mM ammonium bicarbonate were added for protein digestion. The soluble peptide mixture was desalted using a Ziptip Micro-C18 kit (Millipore, Billerica, Massachusetts, USA). The peptides were separated by HPLC and analyzed by LC-MS/MS on an Acclaim™ PepMap™ RSLC column (Thermo Fisher Scientific). Mascot software was used to search the spectra in the SwissProt database.

ELISA

Ten and five µg/mL of rCTLA-4, rTrx-CTLA, rPD-L1, and rTrx-PD-L1 in 0.1M $NaHCO_3$ buffer (pH = 9) were immobilized in Nunc-Immuno™ MicroWell™ "Maxisorp" 96-well microplates (Nalge International, NY, USA) by incubating for 60 min at 37 °C. The microplates were blocked with 1% (w/v) BSA in PBS at 37 °C for 60 min. Recombinant human B7-1 Fc or rhesus monkey PD-1 hFc (10 µg/mL) dissolved in PBS was added to the immobilized recombinant proteins and incubated at 37 °C for 60 min. The microplates were washed with PBS-Tween and the peroxidase-conjugated secondary antibody solution was added and incubated at 37 °C for 60 min. The microplates were washed with PBS-Tween and PBS, and bound peroxidase activity was measured by adding 100 µL/well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Thermo Fisher Scientific). After 15 min of incubation at room temperature, the reaction was stopped by adding 2 M H_2SO_4 and the absorbance was measured at 492 nm on a microplate reader.

Results

Expression and purification of rCTLA-4 and rPD-L1 extracellular fragments

Transformed *E. coli* BL21 (DE3) cells were incubated in 50 mL of LB broth at various temperatures, times, and IPTG concentrations. The greatest protein expression was observed at 0.2 mM IPTG and 37 °C. At this temperature and IPTG concentration the greatest protein expression was observed 2 h after the IPTG addition (Fig. 1). The molecular weights of the proteins were ~ 20, ~ 34, ~31, and ~ 43 kDa

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for rCTLA-4, rTrx-CTLA-4, rPD-L1 and rTrx-PD-L1, respectively (Fig. 2).

Using 8 M urea for denaturation and Ni2+-NTA chromatography for purification, we obtained highly purified proteins. Protein refolding was implemented in His-trap columns using an 8–0 M urea linear gradient. Proteins were eluted with a 20-500 mmol/L imidazole linear gradient (Fig. 3). Positive results were obtained when the proteins were refolded by dialysis. The refolding by serial dilutions resulted in proteins that did not react in ELISAs with recombinant B7-1Fc or PD-1 hFc.

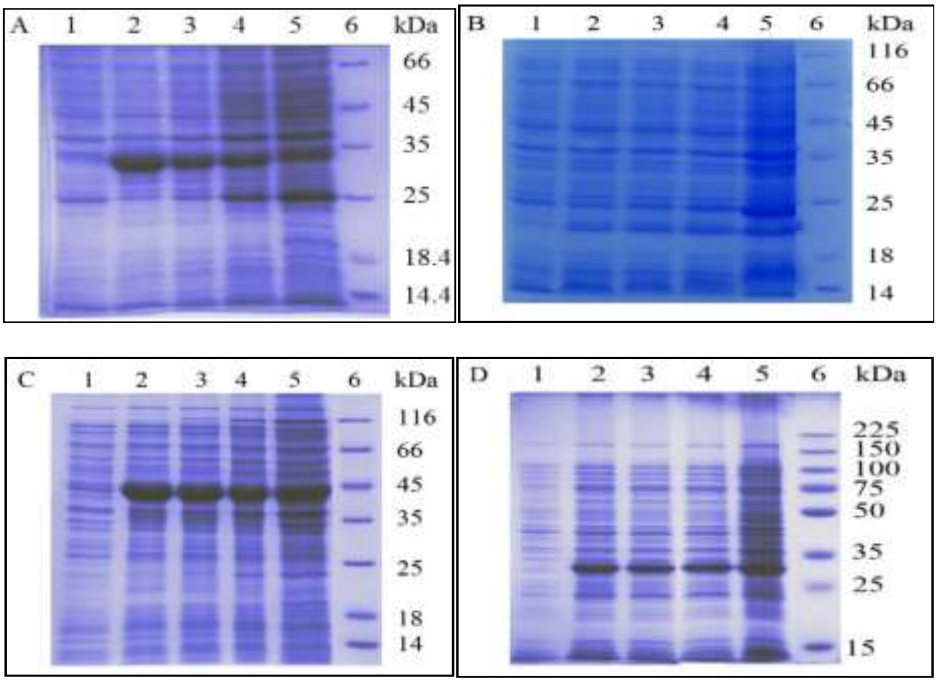

Fig. 1. SDS-PAGE of expressed proteins (A) rTrx-CTLA-4, (B) rCTLA-4, (C) rTrx-PD-L1, (D) rPD-L1. Lane 1—*E. coli* culture without IPTG; Lane 2—protein expression 2 h after IPTG addition; Lane 3—protein expression 4 h after IPTG addition; Lane 4—protein expression 6 h after IPTG addition; Lane 5—protein expression 12 h after IPTG addition; Lane 6-molecular-weight markers.

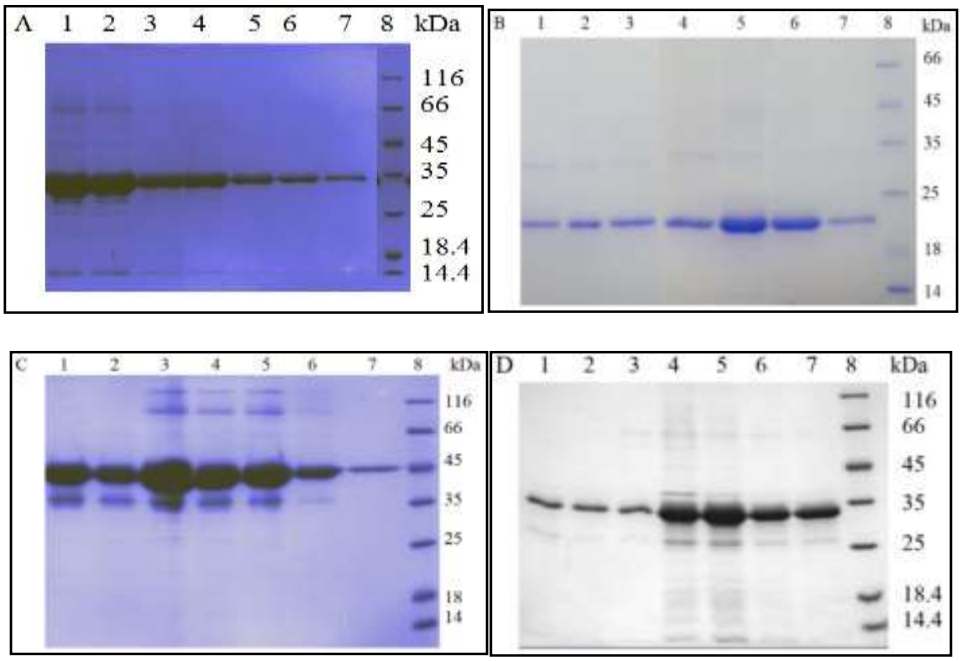

Fig. 2. SDS-PAGE of purified (A) Trx-CTLA-4, (B) rCTLA-4, (C) rTrx-PD-L1, and (D) rPD-L1 proteins expressed in *E. coli* BL21 (DE3) cells. Lanes 1-7 – purified recombinant protein fractions; Line 8 – molecular-weight markers.

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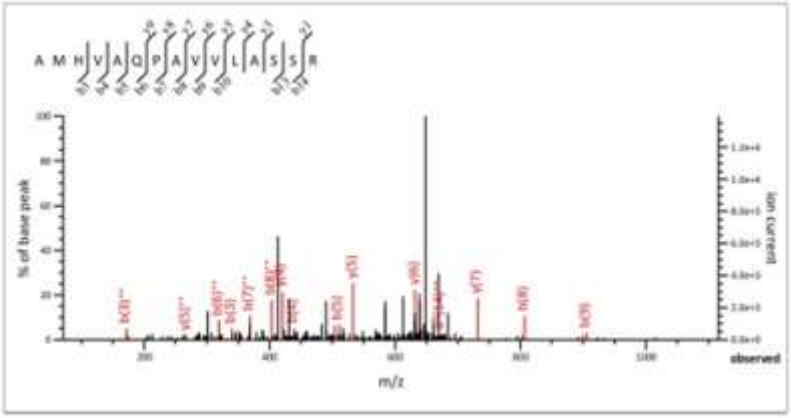

Fig. 3. MS/MS spectra of fragmented peptides derived from trypsin-digested Trx-CTLA-4.

Analysis of recombinant proteins

LC-MS/MS confirmed the identity of rCTLA-4, rTrx-CTLA-4, rPD-L1, and rTrx-PD-L1. At the spectra of rCTLA-4, the Mascot report with the highest score of 4,950 corresponded to CTLA-4. Representative MS/MS spectra of the rCTLA-4 AMHVAQPAVVLASSR peptide and its fragmentation ions are shown in Figure 3. At the spectra of rPD-L1, the Mascot report with the highest score of 13,396 corresponded to PD-L1. The MS-MS spectra of the PD-L1 DLYVVEYGSNMTIEC peptide and its fragmentation ions are shown in Figure 4. Western blot analysis of rCTLA-4, rTrx-CTLA-4, rPD-L1, and rTrx-PD-L1 with anti-His-tag monoclonal antibodies showed protein bands with molecular masses of 20, 34, 31, and 43 kDa, respectively (Fig. 5).

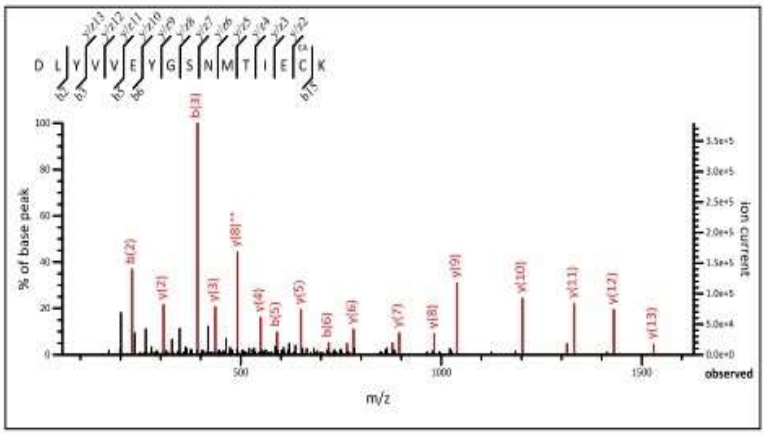

Fig. 4. MS/MS spectra of fragmented peptides derived from trypsin-digested rTrx-PD-L1.

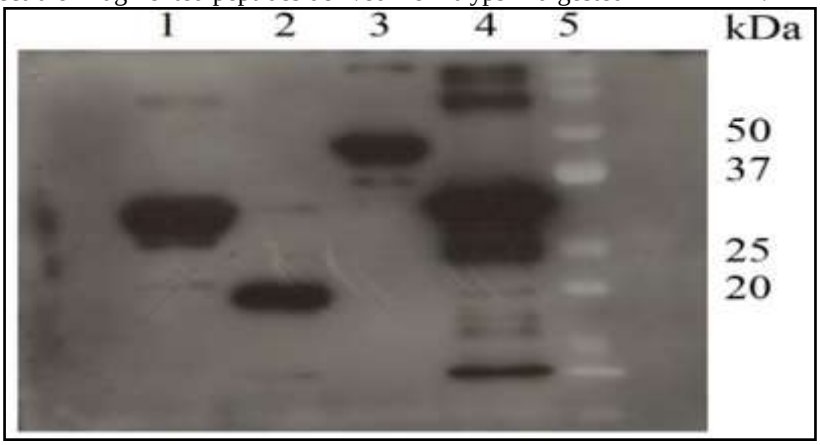

Fig. 5. Western blot of recombinant proteins with anti-6His-tag monoclonal antibodies. Lane 1, rTrx-CTLA-4; Lane 2, rCTLA-4; Lane 3, rTrx-PD-L1; Lane 4, rPD-L1; Lane 5, molecular markers.

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ELISA was used to determine the binding activity of rTrx-CTLA-4, rCTLA-4, rTrx-PD-L1, and rPD-L1 to recombinant B7-1Fc and PD-1Fc. rCTLA-4 and rPD-L1 were immobilized in the wells of an immunological plate at 10 and 5 μg/mL concentrations. The 50% effective concentration (EC_{50}) of recombinant B7-1Fc binding to rCTLA-4 immobilized at 10 μg/mL was 75 ng/mL, while that of rCTLA-4 immobilized at 5 μg/mL was 150 ng/mL (Fig. 6A). When determining the binding activity of rTrx-CTLA-4 at 10 and 5 μg/mL, the EC_{50} values of recombinant B7-1Fc were 150 and 1,250 ng/mL, respectively (Fig. 6B). The EC_{50} of bindings of the recombinant PD-1 hFc rhesus monkey to rPD-L1 were 600 and 1,250 ng/mL, and those of rTrx-PD-L1 were 900 and 1,800 ng/mL (Figs. 6C and 6D).

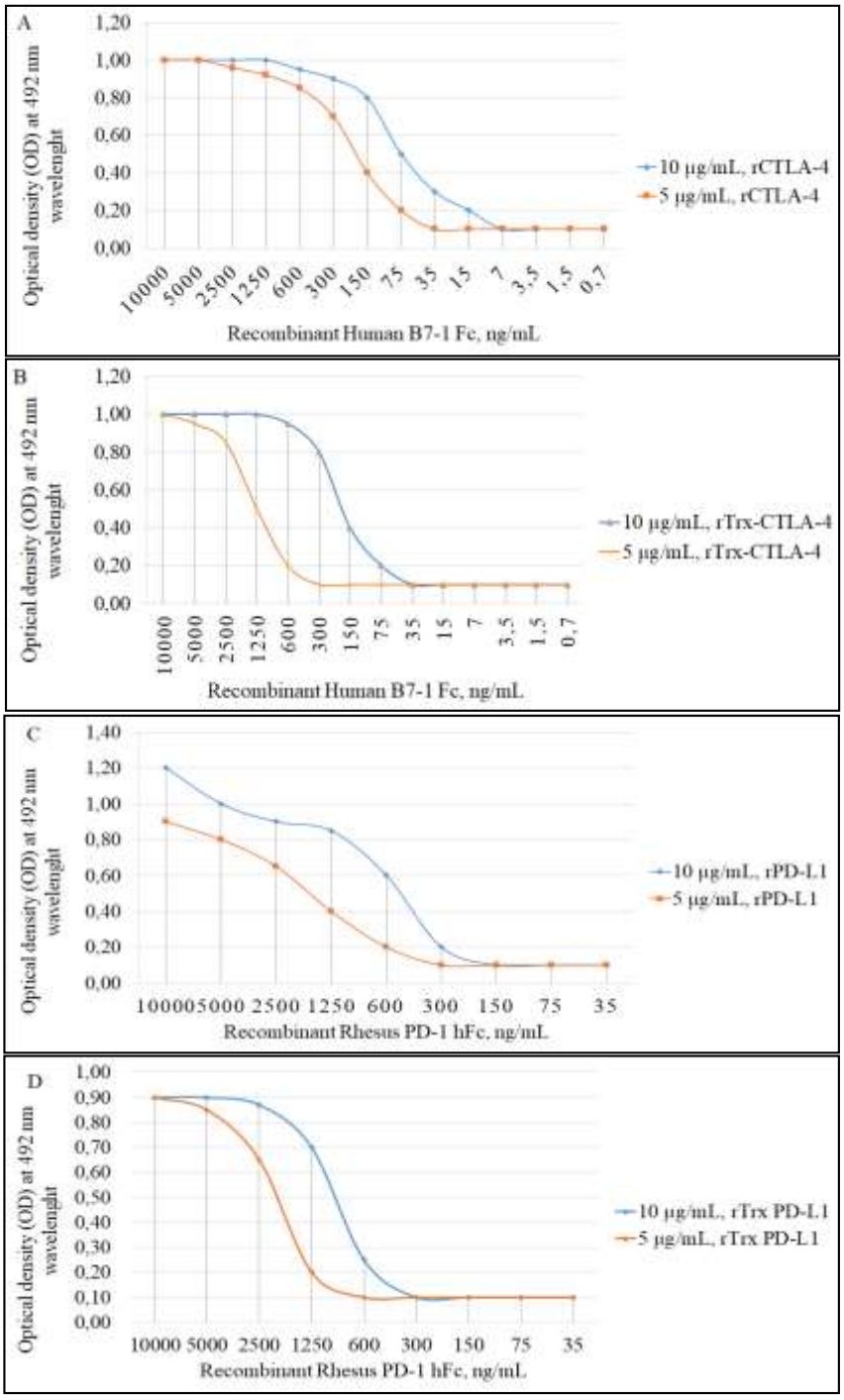

Fig. 6. ELISA to assess the binding of recombinant CTLA-4 and PD-L1 proteins to recombinant B7-1Fc and PD-1 hFc proteins.

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When rTrx-CTLA-4 and rTrx-PD-L1 were used in the binding assays, 50% decreases in the effective concentrations of the commercial hFc B7-1Fc and PD-1 preparations were observed. Presumably, the decreases in the CTLA-4 and PD-L1 concentrations were due to combining the proteins with thioredoxin.

Discussion

CTLA-4 and PD-L1 receptors are potential targets for immunotherapy in several diseases; their blockage leads to an increase in immune system activity (21, 22). Blocking these receptors with antibodies activates T cells and improves antitumor immune responses (23–25). Nevertheless, when studying the mechanism of treatment effectiveness with anti-PD-1, anti-PD-L1, and anti-CTLA-4 blockers, tumor cells capable of evading the action of blockers were observed (26, 27).

Strategies to study such effects include the development of chimeric proteins, in which proteins with different functions are fused into a multifunctional molecule. An example is a multispecific molecule that can bind simultaneously to T-lymphocytes and a tumor cell antigen. Various bispecific fusion proteins have also been reported (9). To obtain chimeric proteins and determine their functions, de novo gene synthesis and the use of bacterial expression systems are effective strategies. Bacterial expression systems have significant advantages over other systems, such as ease of control, low cost, and high productivity (28). Nevertheless, the prediction and control of the specificity and biological function of bifunctional proteins obtained in bacterial expression systems remain urgent problems (29). Several studies have shown that *E. coli* can produce insoluble extracellular domains of human CTLA-4 and PD-L1 (13, 14).

In our study, the functional rCTLA-4, rTrx-CTLA-4, rPD-L1, and rTrx-PD-L1 were produced in *E. coli*. To purify rCTLA-4, rTrx-CTLA-4, rPD-L1, and rTrx-PD-L1 we used the protocols of isolation from inclusion bodies; denaturation with urea and refolding using an urea gradient on His-trap columns. Additionally, gradual dilution and dialysis methods have been used to refold recombinant proteins (30). However, the dilution method used for refolding human recombinant proteins did not yield the expected results. According to Xu et al. (2006), the reason for this negative result is the use of unmodified eukaryotic proteins (20). Based on the example of our studies with rTrx-CTLA-4, we conclude that not all human proteins, even in modified forms, refold correctly by the dilution method.

Positive refolding results were obtained by dialysis, but the method was inferior to refolding using the urea gradient in terms of efficiency and duration. In the ELISA, recombinant B7-1Fc and rhesus monkey PD-1Fc reacted with rCTLA-4, rTrx-CTLA-4, rPD-L1, and rTrx-PD-L1. These results imply correct refolding of the resulting recombinant extracellular domains of CTLA-4 and PD-L1.

Recombinant chimeric proteins are widely used in protein engineering, biotechnology, and healthcare applications. The most intensively developing direction for recombinant chimeric proteins is their expression in *E. coli*. However, when obtaining chimeric proteins using *E. coli*, difficulties arise due to incorrect protein refolding. The fusion of thioredoxin with proteins through marker peptides, which also act as linkers, had no affect extracellular domain activities. This method enabled the correct folding of extracellular domains of human CTLA-4 and PD-L1, which specifically reacted with human B7-1 Fc and rhesus monkey PD-1 hFc, respectively. For correct recombinant protein folding, applying the urea gradient on His-trap columns was the most effective method.

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