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Development of polyclonal heavy chain antibodies targeting programmed death ligand-1

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Abstract

Programmed death ligand-1 (PD-L1, CD274 and B7-H1) has been described as a ligand for immune inhibitory receptor programmed death protein 1 (PD-1). With binding to PD-1 on activated T cells, PD-L1 can prevent T cell responses via motivating apoptosis. Consequently, it causes cancers immune evasion and helps the tumor growth; hence, PD-L1 is regarded as a therapeutic target for malignant cancers. The anti-PD-L1 monoclonal antibody targeting PD-1/PD-L1 immune checkpoint has attained remarkable outcomes in clinical application and has turned to one of the most prevalent anti-cancer drugs. The present study aimed to develop polyclonal heavy chain antibodies targeting PD-L1via *Camelus dromedarius* immunization. The extra-cellular domain of human PD-L1 (hPD-L1) protein was cloned, expressed, and purified. Afterwards, this recombinant protein was utilized as an antigen for camel immunization to acquire polyclonal camelid sera versus this protein. Our outcomes showed that hPD-L1 protein was effectively expressed in the prokaryotic system. The antibody-based techniques, such as enzyme-linked immunosorbent assay, western blotting, and flow cytometry displayed that the hPD-L1 protein was detected by generated polyclonal antibody. Due to the advantages of multi-epitope-binding ability, our study exhibited that camelid antibody is effective to be applied significantly for detection of PD-L1 protein in essential antibody-based studies.

Introduction

In the normal state of the body, many pathogens and cells that have been removed from the normal growth system are identified and killed by components of the innate and acquired immune systems.[1] Immune checkpoints are inhibitory pathways of the immune system preventing autoimmunity via maintaining the self-tolerance.[2] Numerous inhibitory immune checkpoints have been characterized. One of the best described receptor and ligand is the programmed death protein 1 (PD-1), also known as CD279, and its ligand named programmed death ligand-1 (PD-L1, B7-H1 and CD274).[3] Interaction of PD-1 with PD-L1 restricts T cell activity; thereby, controls immune system excessive stimulation and inhibits autoimmune reactions.[4] In fact, PD-L1 with molecular weight of 40 kDa, is a trans-membrane protein and plays an important role in suppression of adaptive immune reactions during pregnancy, autoimmunity, tissue allograft, and cytotoxic T cells activity.[5] Nevertheless, surface expression of PD-L1 is often increased via tumor cells to induce local immune inhibition and weaken the endogenous anti-tumor immune response.[6] Thus, human PD-L1 (hPD-L1) targeting antibodies have been established and expanded for improving immunological responses against cancer. The PD-L1/PD1 blocking antibodies therapeutic application has fundamentally improved cancer treatments.[7] For example, the United States Food and Drug Administration has approved antibodies such as Pembrolizumab (Keytruda®), Nivolumab (Opdivo®), Durvalumab (imfinzi®), and Avelumab (Bavencio®) used for treatment of several kinds of cancer.[8]

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In the early 1990s, a type of antibody called heavy chain antibody (HCAb) was discovered by Hamers-Casterman.[9] Unlike common mammalian antibodies having two heavy and two light chains, in the serum of camels, in addition to the common antibodies, there are heavy chain antibodies not having light chains and CH1 domain.[10] At the beginning of 21st century, the discovery of HCAbs family, beside the molecular technologies, displayed new perspectives for the antibody-bioengineering field.[11] Significantly, HCAbs display 80.00% sequence homology with variable domain of human heavy chain fragments and consequently demonstrate low immunogenicity. Also, due to the increased hydrophilicity and single-domain nature, they show efficient refolding.[12] Regarding unique properties of HCAbs, development of HCAbs against recombinant PD-L1 was the main aim of the current study. For developing HCAbs against PD-L1, the extra-cellular domain of human PD-L1 was cloned in pET-26b plasmid and expressed in *Escherichia coli BL-21(DE3)* and a *Camelus dromedarius* was immunized with this pure recombinant PD-L1.

Materials and Methods

Materials. Isopropyl-β-D-thiogalactoside (IPTG) and imidazole were purchased from DNAbiotech Co., Tehran, Iran. All chemicals were purchased from Sigma-Aldrich Company (St. Louis, USA).

Cloning and expression of PD-L1. The extra-cellular domain of hPD-L1 was obtained from UniProt database (Q9NZQ7, amino acids from 19-238) and the codon was optimized for expression in *E. coli BL-21* and synthesized in pET-26b plasmid (pET26b-PD-L1). The recombinant construct was transformed into *E. coli BL-21(DE3*) using heat shock and 0.10 M $CaCl_2$. Expression of recombinant PD-L1 was induced by 0.10, 0.50, and 1.00 mM IPTG cultured in 300 mL Luria-Bertani broth medium containing kanamycin being incubated for 5 hr at 37.00 ˚C. Then, the pellet was collected at 8,000 *g* for 15 min, re-suspended in the binding buffer (8.00 M urea, 10.00 mM imidazole, 20.00 mM Tris-HCL, and 500 mM NaCl with the pH of 8.00), and sonicated for 20 cycles with 1 min rest. The supernatant was collected at 8,000 *g* for 15 min and loaded on the packed chromatography column with Ni-NTA resin (Sigma). The column was washed with 8.00 M urea, 20.00 mM imidazole, 20.00 mM Tris-HCL, and 500 mM NaCl with the pH of 6.30, and the recombinant protein was eluted from the column using phosphate-buffered saline (PBS) with 500 mM imidazole. The urea was gradually removed using dialysis into gradient urea buffer (6.00, 4.00, 2.00 and 1.00 M).[13] This method has been proved to be effective for solubilization and renaturing of recombinant protein being expressed in *E. coli.*[14]

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. The purity and existence of His tag at the C-terminal of recombinant PD-L1 were evaluated by 15.00% SDS-PAGE and western blotting. For western blotting analysis, protein bands from the SDS-PAGE gel were transferred into nitrocellulose membrane. The membrane was blocked with 2.00% skimmed milk at the room temperature (RT) overnight. The blocking was removed and anti-His horseradish peroxidase (HRP) conjugated antibody (1:1,000) was added and incubated at RT for 2 hr. The membrane was washed 5 times with PBS with tween 20 (PBST; 0.05 % [v/v]). Western blotting was developed with 3,3'-diaminobenzidine (DAB; Sigma).[15]

Camel immunization. A female *C. dromedarius* (6 - 7 months) was immunized with 200 µg of pure recombinant PD-L1 protein six times every other week. Complete and incomplete Freund's adjuvants (equal volume of adjuvant and protein was mixed and subcutaneously injected) were used for the first and subsequent injections, respectively. Before and after each injection, sera were collected and stored at – 20.00 ˚C. Immunization process was checked after the last injection by enzyme-linked immunosorbent assay (ELISA). Briefly, recombinant PD-L1 was coated (1.00 µg mL^{-1}) on 96-well plate overnight at 4.00 ˚C. The wells were blocked with 2.00% skimmed milk and incubated at RT for 2 hr. Then, blocking was removed and diluted sera were added serially to the wells being incubated at RT for 1 hr. The wells were washed 10 times with PBST and ready to use 3,3',5,5'-tetramethylbenzidine (TMB) was added to the wells being incubated for 15 min at dark. Then, 2 N H_2SO_4 was added and absorbance was measured at 450 nm (Agilent/BioTek, Santa Clara, USA).[16]

Western blotting using HCAbs. Purified PD-L1 protein on 15.00% SDS-PAGE gel was transferred into nitro-cellulose membrane. The membrane was blocked with 2.00% skimmed milk and incubated at 4.00 ˚C overnight. Camel sera (1:2,000) before and after the last injection (one week after the last injection) were added and incubated for 2 hr at RT. After washing of the membrane with PBST for five times, rabbit anti-camel (being developed in our lab; 1:2,000) antibody was added and incubated for 2 hr at RT. The membrane was washed and incubated with goat anti-rabbit HRP conjugated antibody (1:1,000) for 1 hr at RT. After washing of the wells with PBST for 10 times, DAB was used as a western blotting developer.[17]

ELISA using camel anti-PD-L1 antibody. To evaluate if camel polyclonal antibody is able to detect PD-L1, home-made ELISA was designed. One µg mL^{-1} of PD-L1 protein was coated onto 96-well plate overnight at 4.00 ˚C. Then, wells were blocked with 2.00% skimmed milk and incubated at RT for 2 hr. Serially diluted camel sera (before and 7 days after the last injection; 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, and 1:12,800) were added to the wells being incubated at 37.00 ˚C for 1 hr. The wells were washed with PBST for five times and incubated with rabbit anti-camel antibody (1:2,000) at 37.00 ˚C for 1 hr. After washing of the wells with PBST (five times), goat

anti-rabbit HRP conjugated antibody (1:1,000) was added to the wells being incubated for 1 hr at RT. The wells were washed with PBST (10 times) and TMB was added to the wells being incubated for 15 min at dark. Then, 2 N H_2SO_4 was added and signal intensity was measured at 450 nm (Agilent/BioTek).

Flow cytometry analysis. The A431 and HEK293 cells were purchased from the Pasteur Institute (Tehran, Iran). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; DNA Biotech, Tehran, Iran). Culture media were finally supplemented with 2.00 mM L-glutamine, penicillin (20.00 U mL^{-1}), streptomycin (20.00 mg mL^{-1}), and 10.00% (by volume) fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, USA) and cells were incubated at 37.00 ˚C with 5.00% CO_2. For flow cytometry analysis, 3.00 × 10^5 A431 and HEK293 cells were transferred into 1.50-mL tube and washed two times with PBS. About 2.00 µg (100 µL) of camel antibody was added to the tubes being incubated for 1 hr on ice. After washing with PBS (two times), 1.00 µg of rabbit anti-camel antibody was added and incubated on ice for 1 hr. The cells were washed and incubated with goat anti-rabbit fluorescein isothiocyanate conjugated antibody (2.00 µg) for 1 hr on ice. The cells were washed and analyzed using Partec Cyflow (Sysmex, Kobe, Japan).

Results

Expression and purification of recombinant PD-L1. To express and purify hPD-L1 protein, the DNA-coding sequence of PD-L1 was cloned into the pET26b expression vector by the *Nde*I and *Xho*I cloning sites and transformed to *E. coli* Top10F'. Colony polymerase chain reaction with T7 universal primers confirmed the transformation. Final recombinant pET26b-PD-L1 transformed to expression host *E. coli BL21 (DE3)*. By adding three different concentrations of IPTG, gene expression was induced and incubated at 37.00 ˚C for 3 hr (Fig. 1A).

The outcome of the 15.00% SDS-PAGE exhibited the expression of our recombinant PD-L1 corresponding to approximately 27 kDa. According to the existence of His-tag at the C-terminal of PD-L1, purification was performed using Ni-NTA affinity chromatography. Purified protein was evaluated with 15.00% SDS-PAGE (Fig. 1B) and confirmed by western blotting via anti-His tag (Fig. 1C). The final yield of expressed protein was 2.50 mg L^{-1} of medium.

Identification of PD-L1 in western blotting. To evaluate the binding capacity of camelid sera comprising polyclonal anti-PD-L1 antibodies to their ligand, PD-L1 proteins, semi-dry western blotting technique was employed. There was no protein band in sera before immunization. However, sera after the last injection could detect PD-L1 protein in the western blotting (Fig. 1D).

Identification of PD-L1 in ELISA. Checking the camelid humoral immune response was performed using harvested blood samples. Absorbance was measured at optical density of 450 nm. After the 2nd injection, the antibody titer displayed a substantial rising trend (Fig. 2). Results indicated success in immunization process. With decreasing of the sera dilution, signal intensity was reduced and even stayed high compared to the un-immunized sera.

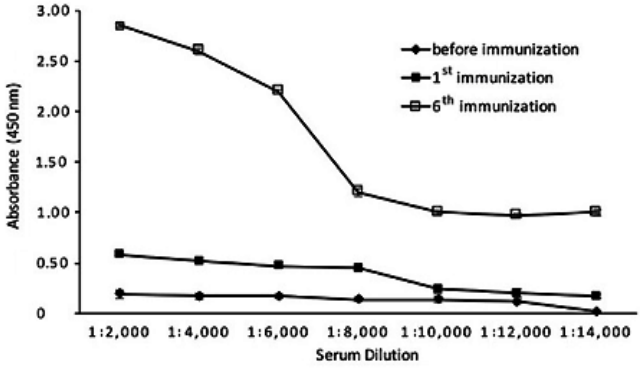

Fig. 2. Camel immune response against recombinant programmed death ligand-1 antigen after six subcutaneous injections being monitored by enzyme-linked immunosorbent assay.

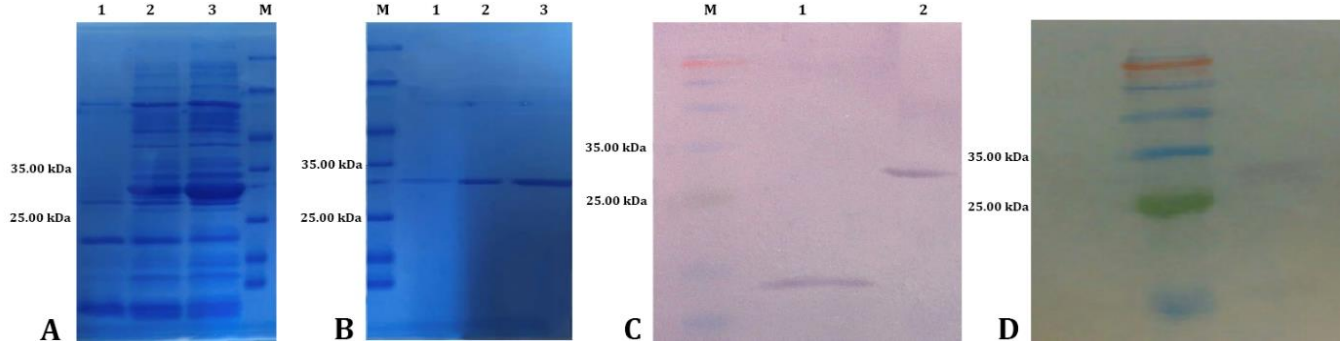

Fig. 1. A) Protein expression was induced with different concentrations of isopropyl-β-D-thiogalactoside (IPTG) and evaluated by 15.00% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). M: Protein marker, and lanes 1, 2 and 3: 0.10, 0.50 and 1.00 mM concentrations of IPTG, respectively. **B)** The SDS-PAGE on purified programmed death ligand-1 (PD-L1). M: Protein marker, and lanes 1, 2 and 3: 0.10, 0.50 and 1.00 mM concentrations of IPTG, respectively. **C)** Western blotting analysis. M: Protein marker; lane 1: Purified programmed death protein 1 as a control; lane 2: Purified PD-L1 protein. **D)** Western blotting technique was used to detect interaction of programmed death ligand-1 proteins and camelid antibodies on nitrocellulose membrane.

Camelid antibodies binding capacity assessment. The binding capacity of the camelid anti-PD-L1 antibodies was assessed at cellular level via flow cytometry. Outcomes displayed that camelid antibodies were able to detect and bind PD-L1 expressed by cancerous cells. Treated A431 cells with camelid sera comprising polyclonal HCAbs exhibited positive signals and had no cross-reaction with HEK293 as negative cells, being represented in the graph by the red and blue lines, respectively (Fig. 3).

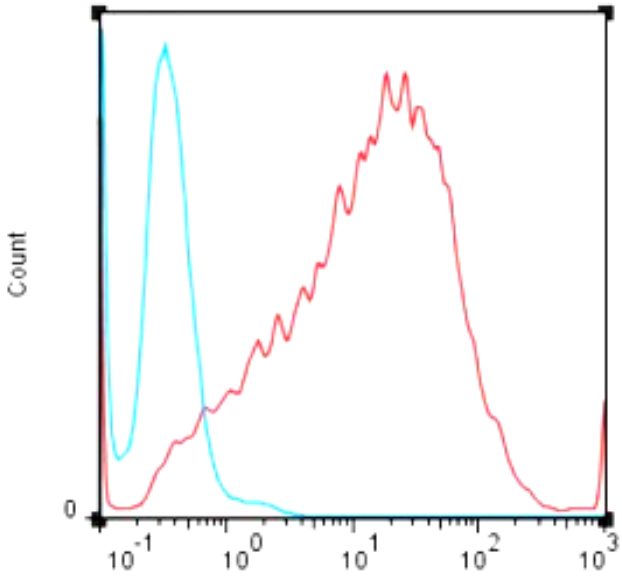

Fig. 3. Camelid sera comprising polyclonal antibody accomplished in flow cytometry assay. Camelid antibodies were able to detect programmed death ligand-1 antigen expressed on A431 cells. Blue line: HEK293 cells; Red line: A431 cells being treated with immunized camelid sera.

Discussion

One of the major mechanisms of immune evasion for tumor cells is immune checkpoint pathways, principally against T cells being specific for tumor antigens.[18] Numerous cancer cells have over-expression of PD-L1 to block anti-tumor immune responses of T cells.[19] The monoclonal blocking antibodies being able to inhibit PD-L1 activity and rescue T cells from inhibition have attracted attention in immunotherapy.[20] Interruption of immune checkpoint interactions via monoclonal antibodies is promising in the treatment of cancers and has replaced chemotherapy as a standard of care for metastatic tumors.[21] For example, checkpoint blockade pembrolizumab has been widely explored in several malignancies.[22] Since the discovery that camelids generate functional antibodies lacking light chains, investigations have proposed the camelid HCAbs utilization for drug delivery, inflammatory and neurodegenerative diseases therapies, tumors monitoring and treatment, passive immunotherapy or biosensors production.[23] As a substitute for full-sized antibodies, camelid-derived HCAb against PD-L1 was developed in the present study. Referred to camelid HCAbs (immunoglobulin [Ig] G2 and IgG3), these molecules have about 90 kDa, and the antigen recognition site is shaped via a single domain, named variable domain of camelid heavy-chain-only antibody. The percentage of HCAbs in the total IgG of these animals represents up to 75.00% of all serum IgGs, demonstrating the significance of these antibodies in the camelid's immune protection.[10]

Upon ligating its receptor, PD-L1 has been reported to decrease T cell receptor-mediated proliferation and cytokine production. The PD-L1 expression was found to be abundant in many murine and human cancers.[24] Preliminary data propose that PD-L1 recognition utilizing polyclonal antibody and immunohistochemistry in formalin-fixed and paraffin-embedded tissues may predict clinical response to PD-1/PD-L1 therapy.[25] To decide about eligibility of a patient for PD-1/PD-L1 immunotherapies, immunohistochemical investigation of PD-L1 expression utilizing normally processed histological sections is crucial and quantitative recognition of its expression could be advantageous for checking the therapy responses.[26] Thus, numerous companies have developed various polyclonal antibodies against PD-L1 protein aiming the extra-cellular or intra-cellular domains. These commercially PD-L1 polyclonal antibodies vastly used for detection of PD-L1 and tumor diagnosis via immunohistochemistry, immunofluorescence, flow cytometry, and western blotting analyses. Based on promising outcomes in this study, PD-L1 polyclonal antibody can employ for predictive and/or prognostic tests and diagnostic pathology in tumors via immune blotting (western blotting), immune imaging (single-photon emission computerized tomography and positron emission tomography), sandwich ELISA, flow cytometry and immunohistochemistry.

In the current study, extra-cellular domain of PD-L1 was cloned and expressed in *E. coli BL-21 (DE3)*. Protein was purified using nickel affinity chromatography and then injected to a female camel to immunize against PD-L1. It has been identified that using recombinant protein results in efficient immunization.[27] Immunization results showed that camel immunized successfully against PD-L1. Camel polyclonal antibodies efficiently detected PD-L1 protein through ELISA as well as western blotting. In addition, this polyclonal antibody identified PD-L1 protein on the surface of A431 cell line (PD-L1 expressing cell) in flow cytometry assay.

In summary, the data presented here exhibited HCAbs easy expression and purification as well as high specificity and affinity to PD-L1, making them valuable reagents for PD-L1 recognition. Our study showed that recombinant expressed PD-L1 is able to stimulate camel immune system.

Camelid sera comprising polyclonal heavy-chain anti-PD-L1 antibodies were effective to detect PD-L1 protein and can be utilized in antibody-based assays, such as flow cytometry, western-blotting, and ELISA, as the cellular and histological laboratory approaches. Additional study can develop these selected HCAbs application scope.

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Conflict of interest

No competing financial interests exist.

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